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Dure-E- Shahwar  
University of the Punjab

Naureen Shahzadi  
University of the Punjab

Saba Zia Butt  
University of the Punjab

Maqsood Ahmad  
The Islamia University of Bahawalpur Pakistan

Mohammad Saleem  
University of the Punjab

Nadeem Irfan Bukhari  
University of the Punjab

Khalid Hussain (✉️ hussain_761@yahoo.com)  
University of the Punjab  https://orcid.org/0000-0001-9627-8346

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Anti-inflammatory activity of a novel steroid isolated from *Ziziphus jujube* (L.) Gaertn. var *Hysudrica* Edgew.

Dure-E-Shahwar¹,², Naureen Shahzadi¹, Saba Zia Butt¹, Maqsood Ahmad³, Mohammad Saleem¹, Nadeem Irfan Bukhari¹, Khalid Hussain¹

¹Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan

²Department of Pharmaceutical Chemistry, Lahore College for Women University, Lahore, Pakistan

³Institute of Chemistry, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

**Corresponding authors**

Khalid Hussain (Email: hussain_761@yahoo.com) and
Mohammad Saleem (saleem2978@hotmail.com)
ABSTRACT

Inflammation is a hallmark in autoimmune diseases and cancer. The present study was carried out to evaluate anti-inflammatory potential of a novel steroid isolated from chloroform extract of stem bark of Ziziphus jujube (L.) Gaertn. Var. hysudrica Edgew. (Family: Rhamnaceae). The dried material was extracted by maceration at room temperature using methanol, acetone, ethyl acetate, dichloromethane, and chloroform. All extracts were subjected to in vitro screening studies employed to assess their anti-inflammatory activity and extract that exhibited significant biological activity was subjected to column chromatography that yielded a crystalline compound. The structure of compound was elucidated using X-ray crystallography. The isolated compound as well as most active extract were also investigated for anti-inflammatory activity using carrageenan-induced paw oedema model. Moreover, quantitative polymerase chain reaction was also done to evaluate inhibitory action on inflammatory biomarkers. Results revealed that chloroform extract showed higher anti-inflammatory activity in all in vitro models as compared to other extracts \( (p < 0.05) \). The isolated compound-2 was found to be a novel steroid and showed comparable anti-inflammatory effects with dexamethasone. In addition, downregulation in concentrations of inflammatory biomarkers (e.g., TNF-α, IL-1β, NF-κβ, and COX-2) was also noted. Histopathological analysis showed intact structure of paws in all treatment groups. On the basis of findings, it is concluded that chloroform extract of Ziziphus jujube and isolated compound-2 possess good anti-inflammatory attributes and can be developed as drug to manage inflammatory conditions in future. Furthermore, long clinical trials and efficacy studies of compound-2 are still required to be elucidated.

Keywords: Inflammation, steroids, Ziziphus jujube, oedema, dexamethasone

Introduction
The plant under investigation, *Zizyphus jujuba* (L.) Gaertn. var. *hysudrica* Edgew., is a hybrid of *Zizyphus mauritiana* and *Zizyphus spina-christi*. The plant is well-known due to its medicinal properties and edible fruit (Azam-Ali et al., 2006). The plant is reported to have a number of alkaloids possessing cyclopeptide moiety (Tschesche et al., 1977; Tschesche et al., 1973; Khokhar et al., 1993; Khokhar et al., 1996). Many plants of genus *Zizyphus* have been investigated for anti-inflammatory activity (Huanger et al., 1990; Azdu et al., 2001; Ganachari and Shiv, 2004; Shiv et al., 2004; Waggas and Al-Hasni, 2009; Siddharth et al., 2010; Asgarparah and Haghighat, 2012). However, there is no scientific study which report anti-inflammatory attributes of *Zizyphus jujuba* (L.) up till now, though the plant is expected to have anti-inflammatory compounds.

A series of *in vitro* and *in vivo* models are available to screen out the therapeutic potential of different plant extracts. *In vitro* models are based on various types of anti-inflammatory mechanisms, so four types of models were employed to evaluate different types of extracts of stem and bark of plant for anti-inflammatory activity and select the more potent extracts for further isolation studies. *In vitro* activity needs to be confirmed using *in vivo* models which can further be used to explore mechanisms of action at molecular level. Such studies provide active compounds as well as scientific evidence to traditional medicinal use. Therefore, the active extract and isolated compound were aimed to be investigated for *in vivo* anti-inflammatory activity and attributable molecular mechanism of action responsible for biological effects.

Inflammation is involved in cardiovascular diseases, diabetes, respiratory diseases, autoimmune diseases, and cancers, and the reduction of inflammation lowers risk of such diseases (Michels da Salvia et al., 2019; Yousuf et al., 2019; Milenkovic et al., 2019; Cutolo et al., 2019; Needham et al., 2019). So, there is a tremendous need of finding new anti-inflammatory compounds that are devoid of side effects associated with the currently available drugs. For such purposes, the plants being used traditionally to relieve pain and inflammatory conditions, and/or belonging to particular taxa can be explored. Hence, an unexplored plant of genus *Zizyphus* has been selected in the present study to isolate anti-inflammatory compound(s).

### Materials and methods

#### Plant material

The stem bark was collected from Lahore, Pakistan, in March 2015. The plant was identified by Prof. Dr. Zaheer-u-Din Khan, Department of Botany, Govt. College University, Lahore, Pakistan. A voucher specimen was deposited at Herbarium of Govt. College University, Lahore, vide reference No. 3571. The material was chopped into small pieces, shade-dried, and pulverized.

#### Chemicals and solvents

The chemicals and solvents used in present study included potassium persulfate, sodium phosphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, trypsin, casein, Tris HCl, perchloric acid, sodium chloride, and carrageenan (Sigma-Aldrich), methanol, acetone, dichloromethane, chloroform and n-hexane (E-Merck, Germany), ethyl acetate (Daejung, Korea) and aspirin (gifted by Highnoon Laboratories Limited, Pakistan). Other materials included normal saline (Surge Laboratories), gum acacia, propylene glycol, and dexamethasone (Merck, Germany).

#### Instruments

The instruments used in present study included a thermostatic oven (U10 Memmert®, Germany), incubator (MIR-153®), centrifuge machine (ScieNovo Lab®), water bath (Thermo Fischer Scientific®) double beam UV/Visible spectrophotometer connected with Fujitech computer and equipped with U.V probe 2.2.1 operating system (Model-2550, Shimadzu Corporation®, Kayoto, Japan), Single Crystal X-Ray Diffractometer of Bruker D8 Venture Dual Source (Micro Focus Cu, Mo) with high-efficiency photon II detector attached with an Oxford Cobra device having temperature range 80-400°K, qPCR SYBR Select Master Mix (Applied Biosystems Thermo Scientific®, USA) and Real-time PCR machine, QuantStudio3 (Applied Biosystems Thermo Scientific®).

#### Extraction
The plant material was extracted using methanol, acetone, ethyl acetate, dichloromethane, and chloroform. The material (500 g) was macerated for 7 days at room temperature separately using 2 L of each solvent. Each extract was filtered and dried under reduced pressure at 40°C.

In vitro anti-inflammatory screening

Inhibition of protein denaturation

The protein denaturation inhibition was determined using the method of Williams et al. (2008). The test sample (1mL) was transferred to a test tube containing 5 mL egg albumin (0.2%, W/V in 0.05 M tris buffer saline, pH 6.8) and a control (methanol) was also prepared. Both test tubes were incubated for 20 min at room temperature. Afterward, the test tubes were heated at 70 ± 5°C for 5 min in a thermostatic oven and cooled immediately in ice-cold water to stop reaction. The absorbance of resulting mixture was taken at 660 nm against distilled water as a blank. The inhibition of protein denaturation was calculated using the following formula:

\[
\text{% Inhibition of albumin denaturation} = \left( \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100
\]

Anti-proteinase activity

The activity was determined by following method of Oyedapo and Famurewa (1995). A reaction mixture was prepared by mixing 1 mL of trypsin (0.06 mg/mL), Tris HCl buffer (20 mM, pH 7.40), and a test sample. The reaction mixture was incubated at 37±1°C for 10 min and then, 1 mL casein solution (0.80%, W/V) was added and again incubated for 20 min. The reaction was stopped by adding 2 mL perchloric acid (70%, V/V) and centrifuged at 2500 rpm for 10 min. The absorbance of supernatant was recorded at 210 nm against buffer as a blank. The same procedure was repeated for aspirin (standard) and control, and activity was calculated by following formula:

\[
\text{% Antiproteinase activity} = \left( \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100
\]

Membrane stabilization activity

Three milliliters of horse blood were centrifuged at 3000 rpm for 10 min and cell pellet was dissolved in normal saline equivalent to the volume of supernatant. The RBCs solution was reconstituted with isotonic phosphate buffer solution (137 mM sodium chloride, 2.70 mM potassium chloride, 10 mM disodium hydrogen phosphate, and 2 mM potassium dihydrogen phosphate, pH 7.40) to get final concentration (40%, V/V).

The membrane stabilization activity was carried out as per protocol of Sadique et al. (1989). Briefly, reaction mixture (containing 1 mL test sample, 4 mL isotonic phosphate buffer, and 0.10 mL RBCs suspension) was incubated at room temperature for 20 min and then hemolysis was induced with heat (water bath at 54±2°C for 20 min) and hypotonic solution. The mixture was cooled immediately, centrifuged at 1300 g for 5 min and hemoglobin release was measured in supernatant at 540 nm against distilled water as a blank. The control (methanol) and aspirin (standard) solution tubes were also prepared. In the latter, a test tube containing 1 mL sample, 2 mL isotonic buffer solution (pH 7.40), 2 mL hyposaline (0.36%), and 0.1 mL 40% RBCs suspension was incubated at room temperature for 20 min. The mixture was centrifuged at 1300 g for 5 min and supernatant was analyzed at 540 nm against distilled water as blank. The hemoglobin release was calculated using the following formula:

\[
\text{% Inhibition of hemolysis} = \left( \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100
\]

Column chromatography

A glass column (40 cm long and 3 cm internal diameter) was packed with 70 g silica gel (Kiesel gel 60 H). Chloroform extract (15 g) dissolved in 10 mL chloroform was loaded on column and yielded 22 fractions by following the elution with solvent system (hexane and dichloromethane (100 mL); 10% decrease in hexane every time). Based on TLC profile, fractions 8, 9, and 10 were pooled and dried to get FA (1.73 g). FA was subjected to re-column through a glass
column (10 cm long and 1.5 cm diameter), packed with 20 g silica gel (Kiesel gel 60 H). The elution was carried using 10 ml of each solvent system comprising hexane, ethyl acetate and methanol (starting from 8:1:1 and then ending at 1:8:1 ratio). Eight sub-fractions were obtained that were left at room temperature for 30 days. The sub-fraction 8 yielded crystals which were named as compound-2. The compound-2 was purified through recrystallization by dissolving in hexane.

Characterization of compound-2

For qualitative group testing, sample solution in hexane was applied on a TLC plate and derivatized by reagents such as ceric ammonium nitrate, natural product reagent, Dragendorff’s reagent, and anisaldehyde reagent (Harborne, 1998). The compound was also tested using Salkowski and Liebermann-Burchard tests (Harborne, 1998). The crystals were subjected to X-Ray crystallography to elucidate the chemical structure.

In vivo anti-inflammatory activity of chloroform extract and isolated compound

Animals

Swiss rats, weight (180-200g), obtained from the Animal House, Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan, were housed in animal transit room for 7 days to acclimatize with standard conditions; 12 h light and dark cycle and 22–25°C room temperature.

Animal grouping

The rats were randomly segregated into 5 groups (n=6), which were designated as group-I (un-treated and uncompromised, control), group-II (disease control), group-III (dexamethasone-treated), group-IV (extract-treated), and group-V (compound-treated).

Study protocol

The study was carried out according to protocol approved by Animal Ethical Committee, University of the Punjab, Lahore, Pakistan (AEC/PUCP/1105-A) and guidelines of UK Animal Scientific Procedure Act, 1986. Group-I and -II received normal saline (1 mL/kg) orally, whereas group-III, -IV, and -V received dexamethasone (1 mg/kg, p.o), chloroform extract (500 mg/kg, p.o), and compound-2 (1 mg/kg, p.o), respectively. After 1 h of dose administration, acute inflammation was induced in sub-plantar region of right-hind-paw of animals of all groups, except group-I, by injecting 0.1 mL carrageenan suspension (1% carrageenan in 2% gum acacia in normal saline). Then, volume was measured at 0, 1, 2, 3, 4, and 5 h, and inhibition of inflammation was determined using following equation:

\[ \text{Inhibition} \% = \left[ 1 - \frac{d_I}{d_C} \right] \times 100 \]

Where “dt” is the difference in paw volume in drug-treated group and “dc” is change in paw volume in control group.

Collection of blood and paw tissue

After last reading, blood of all animals was collected by cardiac puncture under chloroform anesthesia and paw tissues were collected and preserved in neutral buffered formalin (10%). The blood samples were used to quantify pro-inflammatory biomarkers like TNF-α, IL-1β, NF-κB, and COX-2, whereas paw tissues were subjected to histopathological examination.

Determination of pro-inflammatory biomarkers

Primers

The primers (Forward/Reverse) used for various pro-inflammatory biomarkers are given in Table 1.
### Pro-inflammatory biomarker

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>5’-ATGGGCTCCCTCTCATCAGT-3’</td>
<td>5’-GCTTGGTGTTGCTACGAC-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-GTCCTCTGCAAAGTCAGGTC-3’</td>
<td>5’-CAGGGAGGGAAACACACGTT-3’</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>5’-CTGACTCCGCCCTTCTAA-3’</td>
<td>5’-CTCCACCAGCTATTTCAATGGT-3’</td>
</tr>
<tr>
<td>COX-2</td>
<td>5’-ATGCTACCATCTGGCTTCGG-3’</td>
<td>5’-TGGAAACAGTCGCTCGTATC-3’</td>
</tr>
</tbody>
</table>

### Quantitative polymerase chain reaction (qPCR)

A reaction mixture was prepared by combining 10 µl master mix, RNase-free water as required, and cDNA template +150-400 nM reverse and forward primers. Then, reaction mixture (20µl) was transferred to a microplate and placed in qPCR instrument. Thermal cycler temperature was set at 20˚C for 2 min for 40 cycles duration, annealing was done for 15 sec at 60˚C followed by extension for 1 min at 72˚C. Finally, results were calculated as threshold cycles (CT) and relative quantification (ΔCT) (Table 1).

### Histopathological studies

The tissues were dehydrated and embedded in paraffin for section cutting. Hematoxylin and Eosin were used to stain 4-5µm thick tissue sections and pathological changes were examined by placing prepared slides under light microscope.

### Statistical analysis

The samples and standards were analyzed in triplicate and results were presented as mean ± standard deviation. one-way ANOVA with LSC Post-Hoc was used to analyse multiple comparisons. A *p* value ≤ 0.05 was considered as a significant difference.

### Results and discussion

#### Extraction

The yield of methanol extract (54 g) was higher as compared to other extracts. Subsequently acetone extract was 33 g, while ethyl acetate, dichloromethane, and chloroform yielded equal amounts of extracts (19 g). These results showed material contained polar compounds in higher amounts. Heat-assisted extraction produces more yield however higher temperature can change chemical nature of compounds. Therefore, extraction at room temperature is considered better to extract chemical compounds with no or minimal change. Keeping it in view, material was extracted at room temperature using maceration in present study.

#### In vitro anti-inflammatory activity

The results of anti-inflammatory activity of different types of extracts of stem bark using *in vitro* models are given in Fig 1. All extracts were found to be active invariably in all test models. In heat-induced protein denaturation model, activity of chloroform extract was found to be significantly higher as compared to other extracts and aspirin (*p* < 0.05). The anti-inflammatory activity of chloroform extract in anti-proteinase and hypotonicity-induced hemolysis model was found to be comparable to aspirin. However, in heat-induced hemolysis model anti-inflammatory activity of all extracts was found to be significantly lesser than that of standard (*p* < 0.05).
**Fig 1**: *In vitro* anti-inflammatory activity of various extracts of stem bark of *Ziziphus jujube* (L.) Gaertn. var. *hysudrica* Edgew. using inhibition of protein denaturation assay (IPDA), anti-proteinase assay (APA), inhibition of heat-induced hemolysis assay (IHIHA-1), and inhibition of hypotonicity-induced hemolysis assay (IHIHA-2)

**Isolation and structure elucidation**

The chloroform extract showing higher anti-inflammatory activity upon column chromatography gave compound-2. The compound-2 was found to be a steroid using qualitative tests. The chemical structure of compound-2, determined by X-Ray crystallography, is shown in Fig 2. This compound was found to contain a methyl group at position 3 of ring A instead of OH group, present in most of the steroids. Moreover, at position 17, hydroxy methyl group is in ester linkage with carboxyl group.
Fig 2: Structure of compound-2 with and without hydrogen atoms

*In vivo anti-inflammatory activity*

The results of *in vivo* anti-inflammatory activity of chloroform extract and compound-2 using carrageenan-induced inflammatory model are given in Fig 3. The animals of group III (dexamethasone-treated) and group IV (chloroform extract treated) have shown early response, reduction in paw inflammation from 1st h of study, whereas, animals of group V (compound-2 treated) have shown comparatively delayed response, the effect started at 3rd h. The standard drug (dexamethasone), chloroform extract, and compound-2 have shown comparable anti-inflammatory activity ($p < 0.05$). The possible reason for high anti-inflammatory activity of chloroform extract may be due to participation of more than one active moiety present in extract. The disease control group begins to show natural anti-inflammatory response at 5th h of study.
Fig 3: In-vivo anti-inflammatory effect using carrageenan-induced inflammatory model. G-I (Group 1-Normal control); G-II (Group 2-Disease control); G-III (Group 3-Dexamethasone treated); G-IV (Group 4-Chloroform extract of stem bark treated); G-V (Group 5-Isolated pure compound-2 treated).

The results of inhibition of inflammation (%) of groups III, IV, and V are given in Fig 4. In group III, inhibition of inflammation continuously increased from 1\textsuperscript{st} to 5\textsuperscript{th} h, whereas, in group IV inhibition remained constant throughout six hours. The animals of group-V showed inhibition at 3\textsuperscript{rd} h which was then continuously increased till 5\textsuperscript{th} h ($p < 0.05$).

Fig 4: Percentage inhibition of inflammation using the carrageenan-induced inflammatory model. G-III (Group 3-Dexamethasone treated); G-IV (Group 4-Chloroform extract of stem bark treated); G-V (Group 5-Isolated pure compound-2 treated).

The results of estimation of inflammatory biomarkers such as TNF-\(\alpha\), IL-1\(\beta\), NF-\(\kappa\)B, and COX-2 are given in Fig 5. These results showed that group IV and group V had downregulated TNF-\(\alpha\) to a much higher extent as compared to...
Interestingly, group V had downregulated TNF-α to the level of group I ($p < 0.05$), however, groups III, IV, and V possessed almost same tendency to downregulate IL-1β. For NF-κB, group II and IV had possessed same trend of downregulation.

**Fig 5:** Estimation of regulation of inflammatory markers in rats’ blood samples by qPCR technique. 

- **G-I (Group I- Normal control)**
- **G-II (Group 2-Disease control)**
- **G-III (Group 3- Dexamethasone treated)**
- **G-IV (Group 4-Chloroform extract of stem bark treated)**
- **G-V (Group 5- Isolated pure compound-2 treated)**

The pictures of tissues of animals paws undergone histopathological examination are shown in Figure 6. The examination of stained tissues of control group showed normal skin structure with no infiltration of mononuclear cells and oedema. On the other hand, slides of disease control (group-II) showed severe oedematous condition and pannus formation. The dexamethasone-treated (group-III) was characterized by no inflammation and less pannus formation. The chloroform extract and compound-2 treated groups (group IV and group V) had shown minimal infiltration and no pannus formation.
Acute inflammation is characterized by various events involving excessive production of free radicals, complex enzyme activation, and by release of several pro-inflammatory and inflammatory mediators. To screen out, novel anti-inflammatory compounds carrageenan-induced paw oedema represents a well-known method of acute inflammation. Biphasic oedema is achieved by injecting carrageenan into sub-plantar region of rats paws (Mansouri et al., 2015). The release of bradykinin, histamine, serotonin and to lesser extent prostaglandin production by cyclooxygenase enzyme (COXs) includes all of the events observed around 1 h during early phase of inflammation, whereas, continuous production of prostaglandins, infiltration of neutrophils (Gilligan et al., 1994), the release of nitric oxide, free radicals derived from neutrophils and pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β) are all events occurring in delayed phase inflammation (after 1 h) by carrageenan-induced acute inflammation (Halici et al., 2007). It was suggested by different observations that drugs directly targeting pro-inflammatory gene expression, COX-2 enzyme, and formation of free radicals can control inflammatory state much better as compared to therapeutic agents currently available (Ronchetti et al., 2009).

In present study, administration of chloroform extract (500 mg/kg) and compound-2 from chloroform extract of stem bark of plant (1 mg/kg) showed significant tendency to downregulate concentrations of pro-inflammatory cytokines including TNF-α, IL-1β and COX-2. The anti-inflammatory activity of plant under investigation has been attributed to rich contents of triterpenoids and steroid compounds. The steroid nature of compound-2 further confirms such findings.

Dexamethasone is a well-known anti-inflammatory drug, commonly used as a reference drug in anti-inflammatory studies. Findings of present study suggests that 500 mg/kg of chloroform extract and 1 mg/kg of compound-2 have similar anti-inflammatory activity as shown by dexamethasone, which is further supported by evidence of downregulation of inflammatory markers levels. These results were further corroborated by paw tissue histopathological examination indicated intact architecture of paws with minimal cellular infiltration. Furthermore, higher activity of chloroform extract of stem bark of plant was attributed to triterpenoid/steroids along with some other active moieties. Methanol extract from leaves of *Ziziphus mauritiana*, which is a contributing species of plant under this study has shown *in vivo* anti-inflammatory activity which is reported to be associated with compounds such as n-hexadecanoic acid and its ester L (+) ascorbic acid 2,6 hexadecanolate (Kumar et al., 2017). However, present study is the first report regarding *in vivo* anti-inflammatory activity of chloroform extract and compound-2 from chloroform extract. The activity of stem bark of plant is evidenced by comparable anti-inflammatory effects of oral administration of 500 mg/kg chloroform extract, 1 mg/kg of compound-2, and 1 mg/kg dexamethasone.

The results of present study indicated that chloroform extract of stem bark of *Ziziphus jujube* (L.) at the dose of 500 mg/kg and compound-2 at dose of 1 mg/kg possesses good anti-inflammatory activity.
**Conclusion**

Based on findings of present study, it is concluded that chloroform extract and isolated compound-2 from chloroform extract of stem bark of *Ziziphus jujube* (L.) Gaertn var. *hysudrica* Edgew. possessed promising anti-inflammatory activity by downregulating concentrations of various inflammatory biomarkers. However, further long clinical trials are still required.

**References**


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Fundings

Authors contributions

Competing interests

There is no conflict of interest among authors.