

IN VITRO WOUND-HEALING ACTIVITY OF *ZIZIPHUS*
JUJUBA AND ITS ANTI-INFLAMMATORY EFFECTS IN
RATS

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Abstract

Jujube (*Ziziphus jujuba*) has a long history of use in traditional medicine. It has many pharmacological properties, including neuroprotective, antioxidative, hepatoprotective, anti-cancer, anti-inflammatory, and immunomodulatory. Thus, this study aimed to evaluate the anti-inflammatory activity of the ethanolic extract of *Ziziphus* (EEZ) fruit and its wound-healing effect. The anti-inflammatory activity of EEZ was assessed in vivo by carrageenan-induced paw edema test. NIH-3T3 mouse embryonic fibroblast cells were used to evaluate the wound healing effect of EEZ. The effect of the extract on wound-healing was examined by in vitro scratch test. Also, the level of C-reactive protein (CRP) in the serum of rats was determined using the CRP latex test kit. The ethanol extract of jujube fruit induced wound closure in NIH-3T3 fibroblast cells for 18 h (88.5%) at 1 µg/mL concentration. Also, EEZ significantly inhibited carrageenan-induced edema in rat paws. However, a statistically significant decrease in CRP levels was observed in rats pre-treated with EEZ. As a result, *Z. jujuba* fruit extract had anti-inflammatory and wound healing effects, which might be strongly related to its phytochemical composition.

Key words: *Ziziphus jujuba* Mill., jujube, wound healing, paw edema, anti-inflammatory

Introduction. For thousands of years, many countries have been using plants as a source of traditional medicine. The chemical diversity of plants has

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made them one of the primary sources for the isolation of active compounds. Inflammation is generally treated by opioids and nonsteroidal anti-inflammatory drugs (NSAIDs). Both classes, like renal damage, gastrointestinal disturbances, and respiratory depression, produce severe side effects. Traditionally used plants show different uses. Though different types of anti-inflammatory agents are available in the market, to overcome their side effects, searching for new effective agents from plant sources is still progressing [1].

Wound-healing is a complex process involving many cells consisting of four phases: hemostasis, inflammation, proliferation, and remodelling. The hemostasis phase begins the wound healing process by involving platelets. During the inflammatory phase, fibroblasts function as cytokine secretions and growth factors to activate the body's defense system. During the proliferation and remodelling phases, fibroblasts are essential for granulating and reorganizing extracellular matrix tissues [2].

Jujube (*Ziziphus jujuba* Mill.), belonging to Rhamnaceae family, is a spiny plant distributed in many countries worldwide. It bears fruits of various shapes and sizes of great medicinal and nutritional value. Jujube fruit is rich in proteins, minerals, vitamins, organic acids, and carbohydrates. It also contains phytochemical components such as polyphenols, flavonoids, terpenoids, anthocyanins, alkaloids, and carotenoids [3]. It is well known that the dried fruits of *Z. jujuba* are applied as an anticancer, refrigerant, styptic, pectoral, sedative, stomachic, liver, tonic, and as immune response enhancer [4].

Based on the above findings, the present work investigated the in vitro wound healing activity of the ethanolic extract of *Ziziphus* (EEZ) fruit and its anti-inflammatory effect on the carrageenan-induced paw edema model in rats.

Material and methods. Plant material. The fruits of *Zizyphus jujuba* were collected when they were ripe from Sidi Bel Abbès, Algeria, in September 2011. The fruits were identified by the botanists in the department of Environmental Sciences, Djillali Liabes University, Sidi Bel Abbès and the voucher specimen was deposited under the code of FJ SBA 2012. The pulps of the fruit were separated from the seeds and left in the shade at room temperature. The edible part was kneaded to prepare the ethanolic extract.

Extraction. The phenolic compounds were extracted from the freshly prepared pulp fruit jujube (PFJ). The sample of PFJ (50 g) was mixed with 80% ethanol and extracted by ultrasound treatment for 20 min. The alcoholic phase was evaporated under reduced pressure using a rotavapor at 45 °C. The ethanolic extract of *Ziziphus* (EEZ) was stored at –20 °C in the dark until needed.

Phytochemical screening. The presence of phytochemicals was analyzed in the EEZ. For the screening of alkaloids, saponins, tannins, glycosides, phenols, carbohydrates, proteins, and flavonoids, the method established by KHUDA et al. [5] was used.

Evaluation of inflammation induced by carrageenan. Female Wistar albino rats (150–200 g) were maintained under the following conditions: temperature ($23 \pm 2^\circ\text{C}$), humidity ($55\% \pm 5\%$), and light (12 h light/dark cycle), and had access to food and water ad libitum. To induce acute paw inflammation, 50 μL of 1% carrageenan was subcutaneously injected into the right hind paw. The control group was injected 50 μL of phosphate buffered saline instead of 1% carrageenan. EEZ was administered intraperitoneally at a 500 mg/kg dose 15 min before carrageenan injection. Diclofenac was a standard anti-inflammatory drug (100 mg/kg). After inducing acute inflammation, paw edema was measured hourly for up to 6 h using calipers and compared with the corresponding starting point observation. All experimental procedures were approved by the Oran 1 ABB University Animal Ethics Committee (No: 45/DGLPAG/DVA.SDA.14), and complied with the EU Directive 2010/63/EU for animal experiments.

CRP analysis. Five milliliters of blood were drawn from each rat and centrifuged at 3000 rpm for 15 min. The content of C-reactive protein (CRP) in the serum of rats was determined using the CRP latex test kit.

Cell culture assays/Cell viability assessment. Potential cytotoxic effects of the extract was investigated by MTT assay on embryonic mouse fibroblast (NIH-3T3) cell line as previously described in detail [6]. The cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated overnight. The next day, the cells were treated with different concentrations of the extract (0, 1, 5, 10, 50, 100, 200 $\mu\text{g/mL}$) and incubated for 24 h. Formazan formation was spectrophotometrically quantified at 520 and 620 nm using a microplate reader. Cell viability was calculated and revealed as a percentage (%) of control. The Graph-Pad Prism software was used to calculate the IC_{50} concentrations. Afterwards, graphics were created.

In vitro wound healing assessment. The wound healing assay is based on observing cell migration in a “wound” that is created in a cell monolayer. Then, the rate of wound closure and cell migration can be quantified by photography with an inverted light microscope [7]. For the in vitro wound healing assessment of the extract, NIH-3T3 fibroblast cells were seeded into (5×10^4 cells/well) 12-well plates and after 24 h incubation, wells were scratched with a sterile 200 μL pipette tip. After scratching cells were washed with PBS and treated with various concentrations (1, 10, 50 $\mu\text{g/mL}$) of the extract for 18 h. Cell migration was observed under an inverted phase-contrast microscope. Data were analyzed using the ImageJ software and calculations for the wound closure percentage (WC%) were performed using the following equation: $\text{WC}(\%) = [(A_0) - A(t)/A_0] \times 100$, where A_0 is the area at time zero (0) and $A(t)$ is the area after incubation time (t).

Statistical analysis. Statistical analysis and data processing were performed by using GraphPad Prism 8.0.2. Comparisons of the treatments among groups were analyzed by one-way ANOVA with post-hoc Tukey’s test. Signifi-

cance was accepted as $p < 0.05$.

Results and discussion. The natural world has been a resource of curative agents for thousands of years, and an inspiring number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine [8]. *Ziziphus jujuba* is a plant with a long history of consumption as a fruit and a medicinal plant, with various pharmacological effects. This plant has been widely used in traditional medicine and modern phytotherapy [3]. This study evaluated the anti-inflammatory and wound-healing potential of ethanol extract of *Z. jujuba* (EEZ) fruits using in vivo and in vitro models, respectively.

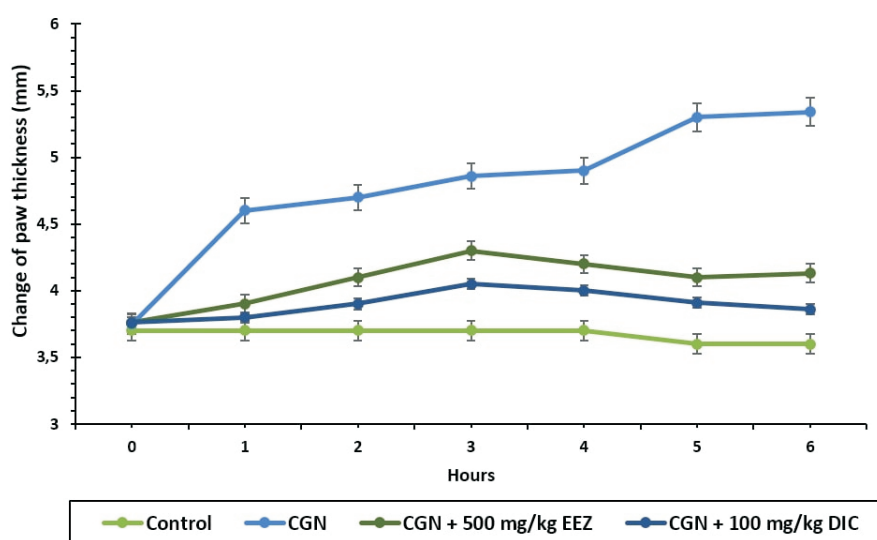


Fig. 1. Effect of ethanolic extract of *Ziziphus jujuba* (EEZ) fruit on carrageenan-induced paw edema. Data are presented as mean $\pm$ SD. CGN: Carrageenan, DIC: Diclofenac (standard drug)

To examine the anti-inflammatory effects of EEZ in vivo, we assessed the degree of inflammation relief after intraperitoneal treatment with EEZ in a carrageenan-generated paw edema rat model. As indicated in Fig. 1, subcutaneous injection of carrageenan (CGN) for 6 h significantly increased the size of edema in CGN group compared to the control group, which exhibited standart paw size. The treatment with EEZ fruit extract (CGN+EEZ) or diclofenac (CGN+DIC) mainly reduced the edema size after the 3rd hour. Diclofenac (100 mg/kg of body weight) and 500 mg/kg b.w. EEZ suppressed paw edema of rats at the 6th hour by 3.86 ± 0.08 mm and 4.13 ± 0.11 mm, respectively, compared with those of the untreated control group. The inhibitory activity of EEZ in rats was examined by measuring the CRP levels. Changes in acute phase proteins reflect the presence and intensity of inflammation. The level of CRP in the serum shows the intensity of the inflammatory process. Carrageenan injection dramatically increased CRP

levels in CGN group compared to the control. However, a statistically significant decrease of this parameter was observed in rats pre-treated with EEZ ($p < 0.05$) or DIC ($p < 0.001$) in comparison with CGN group. EEZ significantly reduced CRP levels in the serum of inflamed rats by 68.4%, while DIC caused a 75.1% reduction.

Inflammation is a broad and ancient medical term referring to classic signs and symptoms, including pain, edema, hyperemia, warmth, and loss of function [9]. Although there are currently available anti-inflammatory drugs, there is a need for safer alternatives. Despite the effectiveness of the medications presently used in managing inflammation, such as nonsteroidal anti-inflammatory drugs, opioids, and corticosteroids, some critical adverse reactions may halt their use [10]. Hence, medicinal plant extracts could be a potential lead anti-inflammatory drug candidate source. The EEZ fruit extract significantly inhibited the development of inflammation quite close to the reference drug group (DIC) ($p < 0.05$). Therefore, this could be partly caused by the decrease in the release of inflammatory mediators by phytochemical compounds in the extract.

Jujube fruit contains antioxidative phytochemicals, including phenolics and flavonoids, and polysaccharides with immunomodulatory potential. A couple of studies have demonstrated that crude mixtures containing jujube extract can alleviate symptoms of nasal or gastrointestinal inflammation in mice [11]. Some polysaccharides obtained from jujube by ultrasonic-assisted extraction reportedly suppressed pro-inflammatory cytokine production in activated RAW 264.7 macrophage cells [12].

We evaluated the potential cytotoxic and wound healing effects of EEZ in mouse embryonic fibroblast (NIH-3T3) cell lines. Cells were incubated with EEZ at 0–200 $\mu\text{g}/\text{mL}$ concentrations range for 24 h, then cell viability was determined by MTT experiments. The percentages of survival of NIH-3T3 cells are presented in Fig. 2. The half-maximal inhibitory concentration (IC_{50}) of EEZ against NIH-3T3 cells was calculated as $> 200 \mu\text{g}/\text{mL}$. Furthermore, the MTT assay showed that EEZ extract has a proliferative effect on fibroblast cells at 1, 5 and 10 $\mu\text{g}/\text{mL}$ doses; however, at higher doses than 50 $\mu\text{g}/\text{mL}$ EEZ led to a significant decrease in cell viability compared to control ($p < 0.05$).

Since ancient times, people have used plants and preparations to accelerate wound-healing. The scratch assay has been proven as a valuable and inexpensive tool to obtain first insights into how plant extracts can positively influence the formation of new tissue [7]. The wound healing assay demonstrated in Fig. 3 and 4 that control cells closed 67.3% of scratched wounds within 18 h. EEZ 1 and 10 $\mu\text{g}/\text{mL}$ induced wound closure for 18 h (88.5% and 76.4%, respectively), compared to the control. On the other hand, EEZ inhibited wound closure at concentrations of 50 $\mu\text{g}/\text{mL}$ (59.3%) compared to the control.

There are reports of the positive effects of *Z. jujuba* on treating of burns and wounds. KUMAR et al. [13] investigated the efficacy of the methanolic bark ex-

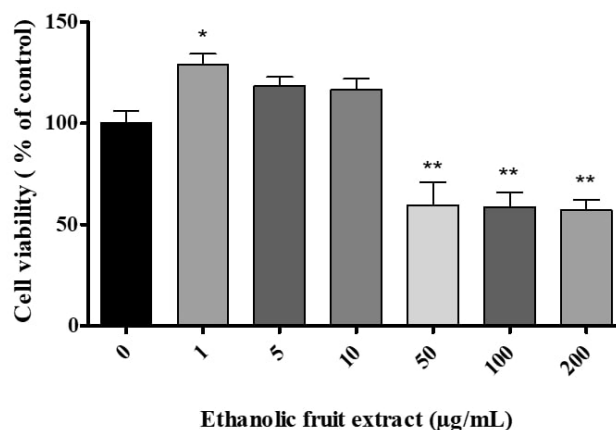


Fig. 2. Cytotoxicity activity of EEZ on NIH-3T3 cells. Cells were treated with extract at indicated concentrations for 24 h. Data were expressed as mean $\pm$ SD ($n = 4$). * $p < 0.05$ significantly increase vs. the control group, ** $p < 0.05$ significantly decrease vs. the control group

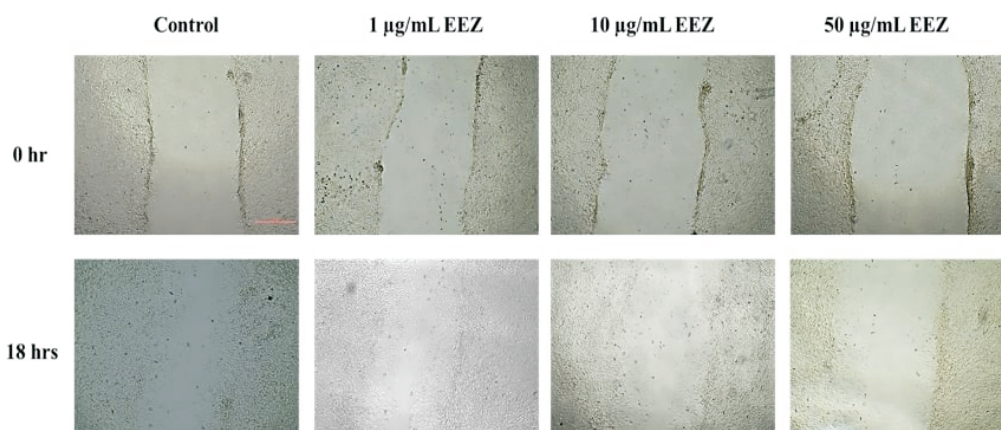


Fig. 3. Analysis of cell migration from the wound healing assay images

tract of *Z. jujuba* for wound healing activity. The outcome showed that the group that received the high extract dose (10%w/w) showed significant wound contraction (98.09%) on the 24th day. Another study reported that water-soluble glucans isolated from jujube fruit improve cell migration and cellular survival, which are important for wound healing [14]. In this study, we found that EEZ induced wound closure in NIH-3T3 fibroblast cells for 18 h (88.5%) at 1 µg/mL concentration. Recent studies with other plant extracts have shown that phytochemical constituents like flavonoids are known to promote wound-healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for

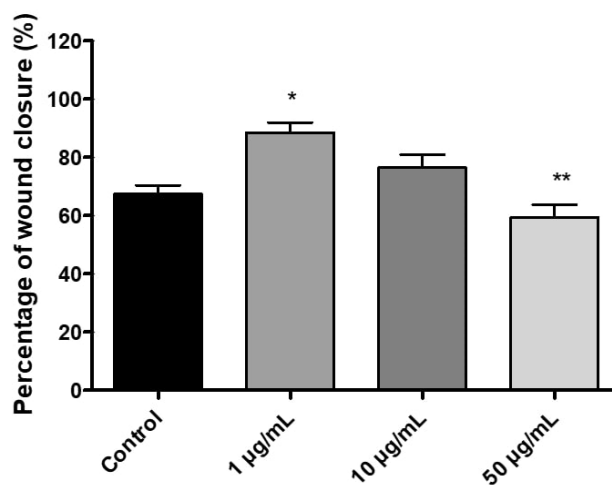


Fig. 4. Percentages of in vitro closure area by the wound healing assay after 18 h of NIH-3T3 cells treated with 1, 10, and 50 µg/mL of EEZ. Scale bar: 500 µm

wound contraction, and increased rate of epithelization [15]. Pharmacological and phytochemical studies have shown that the principal active ingredients in jujube fruit are flavonoid, polysaccharide, and triterpenic acids [16]. According to the results of the phytochemical screening, it was found that the EEZ contains glycosides, tannins, flavonoids, saponins and alkaloids as major secondary metabolites. The wound-healing property of EEZ may be attributed to the phytoconstituents present in the plant and the faster process of wound-healing could be a function of either the individual or the additive effects of the phytoconstituents.

Conclusion. In light of the results of this study, the prepared jujube fruit extract can have anti-inflammatory and wound-healing effects, which are strongly related to its phytochemical composition. EEZ could be considered a choice candidate in pharmaceutical anti-inflammatory drug development.

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