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Original Research Article

Efficacy of *Nitraria retusa* L. Fruits Aqueous and Methanol Extracts as Antioxidant and Anti-inflammatory Activities on Carrageenan-Induced Paw Edema in Rats.

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ABSTRACT

The aim of this study was to assess the anti-oxidant and anti-inflammatory activities of aqueous and methanol extracts of *Nitraria retusa* against carrageenan induced paw edema in rats. The extracts were studied for antioxidant activity using Diphenyl-2-picryl-hydrazyl assay. Male Wister rats were divided into six groups including positive and negative controls. Group 1 (normal saline) as negative control, group 2 (150 mg/kg acetylsalicylic acid) as positive control, group 3 (100 mg/kg methanolic extract), group 4 (200 mg/kg methanolic extract), group 5 (100 mg/kg aqueous extract), group 6 (200 mg/kg aqueous extract). The results showed that aqueous and methanolic extract at doses of 100 and 200 mg/kg significantly decrease paw edema ($P<0.05$). Also, the results revealed that methanol extract at 200 mg/kg is more effective in inhibiting paw edema ($P<0.005$) than other doses and is equal to efficacy of acetylsalicylic acid. This finding indicated that *N. retusa* has an anti-inflammatory activity against carrageenan induced paw edema in rats. DPPH assay showed that methanol extract of *N. retusa* has high antioxidant activity (958.2±178.3 µg/mL) compared to aqueous extract (1249.3±233.5 µg/mL). In conclusion, *N. retusa* fruit extracts have an antioxidant activity and anti-inflammatory activity against carrageenan induced paw edema.

Keywords: *Nitraria retusa*, Diphenyl-2-picryl-hydrazyl, Paw edema, Inflammation

Introduction

Inflammation is the body's reaction to tissue damage brought on by toxins, germs, trauma, or other noxious substances.[1] Granulocytes (like neutrophils) and monocytes, which can later differentiate into macrophages, are drawn to the damaged tissues through chemotaxis, amplify inflammatory responses, and start phagocytosis in response to a variety of stimuli, including damaged cells, pathogens, and cytokines like interleukin-6 (IL-6), IL-1, and tumor necrosis factor alpha (TNF-).[1] Inflammation plays a key role in many diseases, some of which are becoming more common and severe. Chronic inflammatory diseases contribute to more than half of deaths worldwide.[2] Non-steroidal anti-inflammatory medications (NSAIDs) are among the most widely used medicines due to their effectiveness in treating pain and inflammation, validating their inclusion in the WHO's model list of essential medicines. NSAIDs, on the other hand, have been linked to gastrointestinal, cardiovascular, hepatic, renal, cerebral, and pulmonary problems in many placebo-controlled trials.[3] Natural compounds derived from medicinal plants, which have their own therapeutic potential, as well as drugs based on natural products, have applications in the healthcare industry. These drugs have helped to improve people's health by controlling a variety of physiological processes. In terms of effectiveness, natural materials greatly surpass manufactured drugs in the pharmaceutical industry.[4]

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Approximately 250,000–500,000 plant species on earth, 20,000 have medicinal characteristics and function as a medications source.[5] There are around 363 species of medicinal plants in Jordan that are utilized in traditional medicine and have a wide range of pharmacological activities.[6] *N. retusa* is a plant that belongs to the family Zygophyllaceae, commonly known as "Gharqad" in Mediterranean countries.[7] Humans and birds eat its fleshy red fruits, locals use its wood for fuel, its bitter fleshy leaves are used as a poultice to reduce swellings, and its ashes are used to dry fluids secreted by infected wounds.[8] Several previous studies revealed the activity of *N. retusa* as antimicrobial and anti-inflammatory agents.[9, 10, 11] Phytochemical studies have shown that *N. retusa* contain a variety of compounds and plant-derived components, flavone O-glycosides and flavanols compounds, as well as flavone C-glycosides, which have been shown to protect against oxidative stress.[12] The present study aims to evaluate the antioxidant and efficacy of *N. retusa* methanolic and aqueous extracts against carrageenan-induced paw edema in an experimental rat model.

Materials and Methods

Collection of plant sample

The fruits of *N. retusa* were collected during July 2022 from Azraq wetland reserve, Jordan. The plant species was authenticated by Prof. Sawsan Oran (Department of Biological Sciences, Jordan University) (Voucher specimen number: NR1)

Preparation of aqueous and methanolic extracts of N. retusa

All fruits were thoroughly washed in distilled water three times then shade-dried for 7 days. The dried fruits were powdered by a mechanical grinder and then 100 g of powder separately placed into 1000 ml each in absolute (MeOH) or distilled water, for 72 hours accompanied by occasional stirring at 25ºC. The resultant solutions were sieved and filtered through Whatman N° 1 filter paper. The process was repeated three times and after each 72 hours period, the filtrates were concentrated by evaporation using a rotary evaporator

(Büchi 011, Flawil, Switzerland) at 60ºC (for MeOH) under reduced pressure and aqueous extracts were lyophilized using a Martin Christ Beta 2–8 lyophilizer (Germany). The methanolic concentrates were air-dried until constant weights were achieved. The final masses of the extracts were recorded and used to calculate the yield. The residues from the extracts were collected and stored at 4°C.[13]

Phytochemical analysis of the aqueous and methanolic extracts of N. retusa

Determination of total phenolic compounds

The total soluble phenolic compounds in the different extracts of *N. retusa* were determined with Folin-Ciocalteu reagent using gallic acid as a standard.[14] Extracts were diluted to the concentration of 1 mg/mL in distilled water and 0.5 mL of the extract was mixed with 2.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of NaHCO3 (7.5%). After 30 min at RT in dark, the absorbance was measured at 760 nm versus blank sample on spectrophotometer (ISKRA, MA9523-SPEKOL 211). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. For the calibration of a standard curve, gallic acid concentrations (Sigma-Aldrich, USA) ranging from 0.03 to 0.5 mg/mL are utilized. In these tests, the unit (mg gallic acid equivalent/g) was used as an equivalent for quantifying total phenol concentration.

Determination of total flavonoids

The aluminum chloride colorimetric method was used to determine total flavonoid concentration.[15] 3 mL MeOH l, 0.2 mL potassium acetate (1 M), 0.2 mL $AlCl_3$ (10%), and 5.6 mL distilled water were combined with 1 mL diluted fruit extract (1 mg/mL). The blank prepared by the same procedure excluding the extract. For 30 minutes, the mixture was incubated at 25°C. At 415 nm, the absorbance was measured. The calibration curve was made with quercetin (10-200 ug/mL). TFC was measured in milligrams of quercetin equivalent (QE) per gram of extract.

Antioxidant activity determined by 2,2-diphenyl-1-picrylhydrazyl(DPPH) assay

This method depends on the reduction of the radicals resulting in a color change from oxidized purple to reduced yellow. Principally Diphenyl-2-picryl-hydrazyl (DPPH) undergoes reduction in MeOH solution, in the presence of a hydrogen-donating compound due to the formation of the non-radical form DPPH-H. This change in color can be quantitatively measured using a spectrophotometer at 515–520 nm. In contrast to other radical scavenging assays, a DPPH radical is stable and can provide reproducible spectroscopic values. A DPPH solution (0.2 mM) was diluted with MeOH and then mixed with test extracts as well as ascorbic acid with a DPPH solution in a concentration ratio of 1:1 using a 96-well plate (so that a final concentration range 6.25-200 µg/mL was obtained for test treatments); the treated solution was incubated one hour isolated from light. Finally, a change in absorbance at 517 nm wavelength was measured using microplate reader (Bio-Tek Instrument, USA). Ascorbic acid was the robust and classical standard radical scavenging reference agent for comparison purposes. The calculation of the DPPH radical scavenging activity inhibition was determined by the following equation where A represents photometric absorbance: in % = (A control – A sample) / A control x 100% .[16]

Cytotoxicity test

The cytotoxicity of *N. retusa* fruit extracts on periodontal ligament fibroblasts (PDL) (Cell Culture Research Laboratory, Collage of Pharmacy, Jordan University) was evaluated by MTT assay.[17] PDL cells were seeded in a 96-well plate at an initial seeding density of 2 $x10^4$ cells/well/200 µL of DMEM and were cultured for 12 h. The cells were then treated with different concentrations of *N. retusa* fruit extracts (31.25, 62.5, 125, 250, and 500 µg/ml), five different concentrations of Doxorubicin as positive control were generated at 200, 100, 50, 25, 10 µg/ml. and were incubated for 48 h at 37^0C and 5% CO_2. Post incubation, the spent medium was removed and 20 µL of 5 mg/mL of MTT reagent was added to the cells and incubated for 2 h in the CO_2 incubator. The formazan crystals were solubilized with 100 µL of DMSO and absorbance at 570 nm was determined using a microplate reader. The cells treated with DMEM alone were considered as negative control and 100% viable. The percentage of cell viability is calculated using the formula:

% of viability = Mean absorbance of test sample /Mean absorbance of negative control × 100

Percentage of cell viability is plotted against concentrations of test samples. Three sets of experiments are performed in triplicate.

Animals

Adult healthy male Wistar rats weighing 200-250 g were used for this study. Animals were kept under uniform standard management condition at the institutional laboratory animal facility in Jordan University. The temperature is maintained at 23 ± 1°C with a cycle of 12 hr light and 12 hr darkness. Animals were given standard rat feed. Clean water was provided *ad libitum*. Ethical approval with reference number 87-2022 was obtained for the study.

Ethical clearance

All experimental protocols were approved under the Department of Biology, Jordan University, Jordan, and all experiments were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

Toxicity study of Nitraria retusa fruits extracts using male albino rats.

Six groups of (n= 5) overnight-fasted male rats (150-200 g) were used for toxicity study for each extract. Groups 1 to 3 received orally 200, 400, and 800 mg/kg body weight of the *N. retusa* aqueous extract dissolved in 1 mL distilled water, respectively. Groups 4 to 6 received orally 200, 400, and 800 mg/kg.b.w of the *N. retusa* methanolic extract, respectively, using animal oral gavage feeding needles. Control group received 1 mL orally normal saline. The symptoms of toxicity and mortality were observed in each group after 24 hrs. The survived animals were observed for any signs of delayed toxicity for two weeks.[18]

Anti-inflammatory activity

Carrageenan-induced paw edema and extract treatment

Thirty-six male rats were used to study the anti-inflammatory activity of methanol and aqueous extracts of *N. retusa* fruits against carrageenan induced acute paw edema. The animals are divided into six main groups each comprising six rats. Table 1 presents different groups used in this part of study. The animals were divided into control and experimental groups. The animals received different doses of each plant extract (100 and 200 mg/kg body weight). Plant extract doses were administrated to animals orally by gavage feeding needles. Positive control group received 150 mg/kg body wt. acetylsalicylic acid. The animals were kept for 1 hour after the injection of plant extracts, vehicle or acetylsalicylic acid. After 1 hour, 0.1 ml of 1% carrageenan was subcutaneously injected into the hind paw. The left paw is to be served as reference to non-inflamed paw for comparison. The paw thickness was measured by digital caliper at 0h, 3hr and 5hr after carrageenan injection.[19] The carrageenan-induces edema was evaluated as the percentage inhibition of paw thickness using the following formula:

% inhibition: $((C_t-C_0)\ c-(C_t-C_0)\ t)/\ (C_t-C_0)\ c*100$

Where C_t = average paw thickness after carrageenan treatment at time t, C_0 = average initial (basal) paw thickness for each group, c= control group.[20]

Statistical analysis

Data were presented as means ± SD. The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) with Fisher's LSD test using Graph Pad Prism version 9. $P < 0.05$ was considered significant.

Table 1: Treatment groups in assessing the anti-inflammatory effect of the plant extracts.

Group	Treatment
1	Normal saline + 1% carr.
2	150 mg/kg body wt. Acetylsalicylic acid + 1% carr.
3	100 mg/kg body wt. Methanolic extract + 1% carr.
4	200 mg/kg body wt. Methanolic extract + 1% carr.
5	100 mg/kg body wt. Aqueous extract + 1% carr.
6	200 mg/kg body wt. Aqueous extract + 1% carr.

Results and Discussion

Phytochemical analysis and DPPH radical scavenging activity of aqueous and methanolic extracts of N. retusa

The results of this study showed that the MeOH extracts of this plant contained phenolic compounds (17.5±2.92 mg GAE/g), flavonoids (37.3±1.8 mg QE/g) greater than aqueous extract (1.6±0.15 mg GAE/g and 29.3±3.61 mg QE/g) for phenolic compounds and flavonoids, respectively (Table 2). These findings demonstrate the great potential of the plant as antioxidant and anti-inflammatory agent. The majority of flavonoids have been found to have anti-inflammatory, antioxidant, and free radical scavenging properties.[21] A lower IC_{50} value corresponds to a higher scavenging activity on DPPH. The radical scavenging capacities of *N. retusa* fruit extracts were considerably lower than that of the reference. The IC_{50} values of methanolic and aqueous extracts were 958.2±178.3µg/ml and 1249.3±233.5 µg/ml, respectively. The methanolic extract was stronger radical scavenger than aqueous extract that related to high total phenols and flavonoids content compared to aqueous extract.

Toxicity study

Acute toxicity studies showed that all the oral administration doses of the N. retusa aqueous and methanolic extracts used (200, 400, and 800 mg/kg) were safe and non-toxic.

Cytotoxicity test

All *Nitraria retusa* tested aqueous and MeOH extracts were selectively noncytotoxic on PDL fibroblasts in a remarkable similarity to cytotoxic doxorubicin (Table 3).

Evaluation of hind paw edema

The maximum increase in hind paw edema demonstrated by the paw thickness was obtained in rats 5 hours after the injection of carrageenan (Table 4). From the results, it is noted that the pre-treatment of carrageenan induced paw edema in rats with aqueous and methanolic extracts (100 and 200 mg/kg) lead to a significant inhibition of inflammation. Methanolic extract at concentration of 200 mg/kg lead to an inhibition of paw edema more effective ($P<0.005$) than other concentration (100 mg/kg) and other extract concentrations. Moreover, the concentration of 200 mg/kg of methanolic extract caused inhibition equal to acetylsalicylic acid as control. Results showed that the percentage of inhibition of paw thickness in methanolic extracts (100 and 200 mg/kg) were higher than that in aqueous extracts (100 and 200 mg/kg) (Table 5). There are two phases to the development of carrageenan-induced edema. Serotonin, histamine, bradykinin, and substance P are just a few of the mediators that are released, and they cause the initial phase to begin within an hour.[22] However, the late, persistent phase develops after an hour and is mostly brought on by the infiltration of neutrophils at the site of injury. Large levels of pro-inflammatory mediators including prostaglandin (PGE2) and other cytokines like interleukins (IL-1, IL-6, IL-10) and tumor necrosis factor (TNF-) are produced during this period.[23] This study showed that aqueous and methanolic extracts of *N. retusa* have anti-inflammatory activities by reducing inflammation in carrageenan induced edema at two phases. *N. retusa* extracts may play a role in inhibition of inflammatory mediator's synthesis such as histamine and serotonin after 5 hours of treatment. Previous study suggests that *N. retusa* polysaccharide associated with the inhibition of prostaglandin and histamine synthesis, as well as, the inhibition of the increased production of the cyclooxygenase (COX-2).[24]

Several previous studies showed similar results in terms of its anti-inflammatory activity using the carrageenan-induced paw edema model. *Rhus coriaria* fruit methanolic extract has anti-inflammatory activity against carrageenan-induced paw edema in rats.[25] Also, the using of methanolic extract of *Salvia fruticosa* showed a potent inhibitory effect on the production level of pro-inflammatory cytokines in both cellular and animal models.[26-29] Previous study of methanolic and aqueous extracts of *Euphorbia heterophylla* on carrageenan-induced inflammation showed a potential of the use of this plant in the treatment of inflammatory disease conditions.[30] Another study of aqueous extract of *Leea guineensis* to evaluation the anti-inflammatory properties using carrageenan-induced edema seem to support its use by herbalists to treat inflammatory disease condition.[31]

Conclusion

It has been demonstrated that *Nitraria retusa* aqueous and methanol extracts have strong anti-inflammatory and antioxidant properties. Of the two extracts examined, *N. retusa* demonstrated the best anti-inflammatory and antioxidant activities due to the presence of flavonoids and phenols. The usage of herbal products with anti-inflammatory and antioxidant components could be a viable choice given the growing need for alternatives to control the many kinds of pro-oxidative and inflammatory processes.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 3: Cytotoxicity of the of *N. retusa* extracts against normal PDL fibroblasts vs. Doxorubicin (µM).

Plant	PDL fibroblasts IC_{50} value	Doxorubicin
AE	563.2 ± 43.4 µg/mL	NIL
ME	9452.5 ± 1540.8 µg/mL	NIL

NI: Non-Inhibitory within testing dose range

Table 2: Antioxidant activity and phytochemical analysis of *N. retusa* aqueous and methanolic extracts.

Plant extract	Total phenols (GAE mg/g)	Total Flavonoid's (QE mg/g)	DPPH- IC_{50} value
AE	1.6 ± 0.15	29.3 ± 3.61	1249.3 ± 233.5 µg/mL
ME	17.5 ± 2.92	37.3 ± 1.8	958.2 ± 178.3 µg/mL

Results are mean ± SD (n = 3 independent replicates). IC_{50} values (µM) (concentration at which 50% inhibition of DPPH reduction in comparison to non-induced basal incubations) were calculated within testing dose range. AE: aqueous extracts. ME: MeOH extracts. GAE: Gallic Acid Equivalent. QE: Quercetin Equivalent.

Table 4: Effect of *N. retusa* aqueous and methanolic extracts and acetylsalicylic acid on carrageenan-induced paw edema in rats.

S/N	Group	0	3	5
1	1% carrageenan	7.53 ± 0.57	9.06 ± 0.44	9.55 ± 0.64
2	Acetylsalicylic acid (150 mg/kg)	7.54 ± 0.27	8.42 ± 0.48	7.88 ± 0.45**
3	ME (100 mg/kg)	7.56 ± 0.29	8.65 ± 0.42	8.15 ± 0.56*
4	ME (200 mg/kg)	7.51 ± 0.30	8.18 ± 0.48	7.75 ± 0.20**
5	AE (100 mg/kg)	7.55 ± 0.25	8.69 ± 0.49	8.67 ± 0.39*
6	AE (200 mg/kg)	7.52 ± 0.31	8.55 ± 0.61	8.53 ± 0.48*

Values are mean ± S.D of 6 individual rats.*P<0.05, ** P < 0.005

Table 5: percentage of inhibition of paw thickness at 5 hour.

S/N	Group	% inhibition
1	Acetylsalicylic acid (150 mg/kg)	83
2	ME (100 mg/kg)	70
3	ME (200 mg/kg)	88
4	AE (100 mg/kg)	44
5	AE (200 mg/kg)	49

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