Anti-inflammatory and analgesic activities of flavonoid and saponin fractions from *Zizyphus lotus* (L.) Lam.

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Abstract

The effect of the flavonoid and saponin fractions from the leaves and root bark of *Zizyphus lotus* (200 mg/kg) was evaluated on carrageenan-induced paw edema in rats and on acetic acid-induced algesia in mice. In addition, two methanolic extracts from the plant (1 mg/ear) were tested on oxazolone-induced contact-delayed hypersensitivity (DTH) in mice. The effect of the different fractions was also evaluated *in vitro* on the nitrite production induced by lipopolysaccharide (LPS) in RAW 264.7 macrophages. The results showed that both the flavonoid and saponin fractions significantly inhibited paw edema, algesia, and nitrite production without affecting cell viability. Furthermore, the methanolic extracts of the leaves and root bark of *Z. lotus* were found to significantly inhibit the DTH induced by oxazolone, with the extract obtained from root bark being more active than that obtained from leaves.

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1. Introduction

Many plants used in traditional medicine have the potential to provide pharmacologically active natural products. For example, several recent pharmacological studies (Nunes et al., 1987; Adzu et al., 2001; Borgi et al., 2007) have focused on the various species of *Zizyphus* (Rhamnaceae), including *Zizyphus lotus*, a species commonly known as ‘sedra’ in Tunisia, where it is used in traditional medicine as a pectoral demulcent to treat throat and broncho-pulmonic irritations (Le-Floc’h, 1983). In a previous study, we reported on the anti-inflammatory and analgesic activities of *Z. lotus* root bark (Borgi et al., 2007). In the present study, we have gone on to investigate the effects of the flavonoid and saponin fractions of the root bark and leaves of *Z. lotus* on both carrageenan-induced paw edema in rats and the algesia induced in mice by the intraperitoneal administration of acetic acid. We have also examined the effects of the extracts on nitrite production in RAW 264.7 macrophages. In addition, methanolic extracts from the root bark and leaves were tested on oxazolone-induced contact-delayed type hypersensitivity (DTH) in mice. Finally, the cytotoxicity of *Z. lotus* fractions on different cells was evaluated.

2. Materials and methods

2.1. Reagents

Carrageenan (BDH Chemicals Ltd Poole England), and acetylsalicylic acid (AAS) were from Adwya Laboratory, Tunisia. RAW 264.7 macrophages, lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma), Nω-nitro-L-arginine methyl ester (1.-NAME) and DMEM medium all from Invitrogen, Langley, OK, USA. 96-well microplates, oxazolone, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Griess reagent and acetic acid were purchased from Sigma.

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2.2. Plant material

*Z. lotus* (L.) Lam. was collected in Cherahil-Monastir, Tunisia, in 2005 and identified in the Botanic laboratory of the Faculty of Pharmacy in Monastir. A voucher specimen (No. 0269) has been deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, in Monastir, Tunisia.

2.3. Preparation of extracts

The root bark (RB) and leaves (L) were dried, coarsely powdered, and exhaustively extracted in a Soxhlet apparatus with methanol for 8 h to yield the methanolic extract (ME); ME-RB: 25.27% and ME-L: 15.28%. In order to obtain total oligomers flavonoids (TOF), the powdered root bark and leaves were macerated separately for 24 h in a mixture of acetone/water (2:1). Each extract was then filtered and the acetone was evaporated under low pressure. Tannins were removed from the aqueous phase through precipitation with NaCl for 24 h at 5 °C and the supernatant was then extracted with ethyl acetate, concentrated, and precipitated with an excess of chloroform. The precipitate containing TOF was separated by means of filtration (Paris and Moyse, 1976a,b,c). The yield of TOF-RB and TOF-L fractions was 0.12% and 0.13%, respectively. The presence of flavonoids was verified by the reaction of ‘cyanidine’ in the presence of hydrochloric acid and of magnesium. After release of hydrogen, a coloring of orange with the red purple indicates the presence of hydrochloric acid and of magnesium. After release of hydrogen, a coloring of orange with the red purple indicates the presence of hydrochloric acid and of magnesium. After release of hydrogen, a coloring of orange with the red purple indicates the presence of hydrochloric acid and of magnesium. After release of hydrogen, a coloring of orange with the red purple indicates the presence of hydrochloric acid and of magnesium. After release of hydrogen, a coloring of orange with the red purple indicates the presence of hydrochloric acid and of magnesium.

The saponin fraction (S) was obtained as follows: powdered root bark and leaves were macerated separately in petroleum ether. After 24 h, each defatted plant material was macerated in MeOH for another 24 h and then the filtrate was concentrated under low pressure. Ethyl ether was added to the methanolic extract and kept at −5 °C for 24 h. A precipitate was obtained under low pressure and was separated by means of filtration (Boiteau et al., 1964). The yield of S-RB and S-L fractions was 2.19% and 0.34%, respectively. The presence of saponins was verified as follow: after dissolution of the active ingredients in hot water, the tubes containing the solutions are agitated during 15 s (2 agitations a second). The development of persistent foam which lasts more than 15 min indicates the presence of the saponins (Boiteau et al., 1964).

2.4. Animals

For studying the anti-inflammatory and analgesic activities, adult Wistar rats (150–200 g) and Swiss albino mice (18–22 g) were obtained from the Pasteur Institute (Tunis, Tunisia). For experiments on oxazolone-induced hypersensitivity in mice, animals were obtained from Harlan InterFauna Ibérica (Barcelona, Spain). All animals were fed a standard diet *ad libitum* and allowed free access to drinking water. Animals fasted overnight before any experiments, all of which were conducted in accordance with the guidelines established by the European Union on Animal Care (CEE Council 86/609). Housing conditions and all *in vivo* experiments were approved and followed by the Institutional Ethics Committee of the Faculty of Pharmacy (University of Valencia, Spain).

2.5. Anti-inflammatory activity

2.5.1. Carrageenan-induced rat paw edema

Wistar rats were divided into groups of six animals each. Edema was induced by injecting 0.05 ml of 1% carrageenan subcutaneously into the sub-plantar region of the left hind paw (Winter et al., 1962). Flavonoid (TOF) and saponin (S) fractions of the root bark (RB) and leaves (L) of *Z. lotus* (200 mg/kg) were administered intraperitoneally (*i.p.*). The reference group received acetylsalicylic acid (ASA, 300 mg/kg, *i.p.*) and the control group received a solution of 9% NaCl (2.5 ml/kg, *i.p.*). All drugs were administered 15 min before the injection of carrageenan. The hind paw volume was measured with a plethysmometer (model 7150, Ugo Basile, Comerio, Italy) at 0 h (before carrageenan injection) and then again at 0.5, 1, 2, 3, 4, 6, and 24 h.

2.5.2. Oxazolone-induced delayed type hypersensitivity in mice

In accordance with the method previously described by Young and De Young (1989), female mice (*n* = 6) were sensitized through topical application to the shaved ventral abdomen of 150 µl of a 3% (w/v) solution of oxazolone in acetone on day 1. Challenge was performed on day 6 by applying 10 µl of 1% oxazolone to the inner and outer surfaces of both ears. Root bark (ME-RB) and leaf (ME-L) methanolic extracts (1 mg/ear) and dexamethasone (0.025 mg/ear) were applied topically (10 µl) to the inner and outer surface of right ear on days 6, 7, and 8 after challenge. The thickness of each ear was measured with a micrometer (Mitutoyo Series 293, Kawasaki, Japan) on days 7, 8, and 9 after challenge just before drug application. The edema was calculated for each ear as the difference in thickness before treatment and 24, 48, and 72 h after challenge. The activity was expressed as the inhibition percentage of ear thickness with reference to the control group.

2.6. Cytotoxicity assay

The cytotoxicity of *Z. lotus* extracts was determined with the aid of the colorimetric assay (Mosmann, 1983). Cells were exposed to the extracts (50–1000 µg/ml) in a microplate for 30 min (rat peritoneal neutrophils) or 1 day (RAW 264.7 macrophages) and then 100 µl/well of a 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added. The plates were then incubated at 37 °C until blue deposits were visible. The coloured metabolite was dissolved in dimethyl sulfoxide (100 µl per well). Absorbance was measured at 490 nm with a L asbestos MUSIK MCC/340 plate reader (Helsinki, Finland). Results are expressed as absolute absorbance readings with a decrease indicating a diminution in cell viability. Controls received vehicle only and correspond to 100% viability.
2.7. Inhibition of nitrite production in RAW 264.7 macrophages

RAW 264.7 macrophages were cultured in a DMEM medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% foetal bovine serum. Cells were removed from the tissue culture flask with a cell scraper and resuspended until a final relation of 1×10⁶ cells/ml was reached. The cells were then co-incubated in a 96-well culture plate (200 µl) with 1 µg/ml of lipopolysaccharide (LPS) at 37 °C for 24 h in the presence of extracts (50 and 100 µg/ml), DMSO (control), or nitro-L-arginine methyl ester (L-NAME) as a reference drug. The supernatants were used directly to measure nitrite production by a microplate reader (Bas et al., 2007). Nitrite production was measured as the index of NOS activity.

2.8. Antinociceptive activity

Swiss mice were selected 1 day prior to the assay and were divided into different groups of six animals each. The control group was treated with 10 mg/kg, i.p. of 9% NaCl. The positive control (reference) group received acetylsalicylic acid (ASA, 200 mg/kg, i.p.) while the remaining groups were given TOF-RB, TOF-L, S-RB, and S-L of Z. lotus (200 mg/kg, i.p.). Fifteen minutes later, 1% acetic acid (10 ml/kg. i.p.) was injected into each animal and the amount of writhing during the following 30 min was quantified. A significant reduction in writhing as compared to the control group was considered to be an antinociceptive response (Koster et al., 1959).

2.9. Data analysis

Data are presented as the mean±standard error (s.e.m.). Statistical analysis was performed using Student’s t-test. The significance of difference was considered to include values of P<0.05.

3. Results and discussion

In a previous study (Borgi et al., 2007), we reported on the anti-inflammatory and analgesic properties of the aqueous and methanolic extracts (200 mg/kg, i.p.) of the root bark of Z. lotus. The results showed that the polar extracts (aqueous and methanolic extracts) were more active than the chloroformic and the ethyl acetate extracts. We also verified the presence of flavonoids and saponins in these extracts, so we direct our research towards the effect of these fractions and their participation in the anti-inflammatory activity. The results obtained in this study indicate that these fractions at the dose of 200 mg/kg administered intraperitoneally present a significant effect against carrageenan-induced inflammation and their effect was more important.

In carrageenan-induced rat paw edema (Table 1), the extracts TOF-RB, TOF-L, S-RB, and S-L (all at 200 mg/kg, i.p.) produced...
a significant reduction of the edema throughout the entire period of observation. Interestingly, the reduction of the edema by 75% and 90% after administration of TOF-RB and S-RB, respectively, was highest at 3 h, whereas at the same time, TOF-L and S-L inhibited the edema by 47% and 65%, respectively. At any rate, these results indicate that the anti-inflammatory effect may be related, at least in part, to the presence of flavonoids and saponins. There have been previous reports on the anti-inflammatory activity of these types of compounds, including several on their possible mechanism of action. In general, flavonoids act as potential inhibitors of cyclooxygenase, lipooxygenase, and nitric oxide synthase as well as being antioxidants (Rao et al., 2005; Rotelli et al., 2003; Havsteen, 2002; Middleton and Kandaswami, 1992; Middleton et al., 2000; Di Carlo et al., 1999). In contrast, saponin mechanisms are related to those of steroids, the release of different enzymes, and the arachidonic acid metabolism (Rios et al., 2000; Safayhi and Sailer, 1997).

In the DTH test, examination of challenged ears showed that oxazolone induced a pronounced inflammatory reaction compared to ears that had not been challenged. Of the extracts assayed, the activity of the ME-RB extract was higher than that of ME-L, although both extracts significantly reduced ear swelling (Table 2). Indeed, the ME-RB extract reduced the edema in all phases of the process by 58%, 36%, and 47% at 24, 48, and 72 h after challenge, respectively, whereas ME-L only reduced the edema by 48% and 35% at 24 and 72 h, respectively. It is worth noting that in this test, only corticosteroids are active because specific inhibitors of the arachidonic acid metabolism have no effect on the inflammatory reaction (Cavey et al., 1990; Zunic et al., 1998; Meurer et al., 1988). These findings suggest that the mechanism of action of Z. lotus saponins may be related to a corticosteroid mechanism.

NO plays an important role in diverse physiological processes including the immune response and inflammation (Ashok et al., 1999). For this reason, numerous plants have been investigated as inhibitors of NO production in inflammatory reactions (Lee et al., 2005; Escandell et al., 2006; Choi et al., 2007). In order to test the anti-inflammatory activity of Z. lotus extracts, we investigated their effects on LPS-induced nitrite production in RAW 264.7 macrophages (Table 3). We first tested the cell viability in the presence of the test extract to determine the toxicity against murine RAW 264.7 macrophages or rat peritoneal neutrophils (PMNL). The results showed that at 50 and 100 μg/ml, Z. lotus extracts were not toxic for cells with viability greater than 95%. The cytotoxicity was observed at high concentration tested (500 and 1000 μg/ml) with viability percentage 90%. Saponins and especially the methanolic extract of the leaves (ME-L) significantly inhibited nitrite production by 50% and 74%, respectively, while the TOF-RB and ME-RB extracts of the root bark only had a moderate effect at 100 μg/ml, whereas at 50 μg/ml both extract induced the nitrite production, especially S-RB, which would be of interest for a future research. These results are in contrast with the in vivo test, in which the extracts obtained from RB were clearly more active, a fact which suggests that the inhibitors of nitric oxide synthase are different from the substances responsible for the anti-inflammatory activity.

In acetic acid-induced writhing in mice, the TOF-RB and S-L fractions (each at 200 mg/kg) reduced the writhing by 81 and 88%, respectively, whereas TOF-L and S-RB only inhibited the writhing by 27% and 47%, respectively (Table 4). This type of writhing in mice has been associated with the liberation of endogenous substances including serotonin, histamine, prostaglandin, and bradykinin (Collier et al., 1968). The results obtained in this test thus suggest that while Z. lotus extracts possess peripheral analgesic properties, this particular activity is probably unrelated to their anti-inflammatory properties.

### 4. Conclusion

In summary, our results have demonstrated that Z. lotus exhibits anti-inflammatory activity, inhibits NO production, and reduces peripheral algesia. These results confirm the potential interest in Z. lotus as a medicinal plant and open the way for future research focussing on the active extracts or fractions. Of special interest are the S-RB fraction as a potential source of anti-inflammatory principles, the ME-RB extract as a possible source of anti-inflammatory agents in DTH reactions, and the TOF-RB extract and S-L fractions as potential sources of analgesic drugs.

### References


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