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Pharmacological Evaluation of the Anti-inflammatory Activity of a Citrus Bioflavonoid, Hesperidin, and the Isoflavonoids, Duartin and Claussequinone, in Rats and Mice

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Abstract—Pretreatment of rats with hesperidin (50 and 100 mg kg^{-1}, s.c.) reduced the paw oedema induced by carrageenan by 47 and 63%, respectively, within 5 h. The effect was equivalent to that produced by indomethacin (10 mg kg^{-1}, p.o.), although unrelated to the administered dose, particularly at high doses. At 100 mg kg^{-1} hesperidin decreased the rat paw oedema induced by dextran by 33%, without influencing the histamine-induced paw oedema. Hesperidin also inhibited pleurisy induced by carrageenan, reducing the volume of exudate and the number of migrating leucocytes by 48 and 34%, respectively, of control values. Equal doses of duartin and claussequinone were ineffective in all the above tests. Pretreatment of mice with hesperidin (100 mg kg^{-1}, s.c.) reduced acetic acid-induced abdominal constriction by 50%, but did not affect the tail flick response. Hyperthermia induced by yeast in rats was slightly reduced by hesperidin. No lesions of the gastric mucosae were detected in rats pretreated with hesperidin. The results indicate that hesperidin obtained from citrus cultures may present a potential therapeutical use as a mild anti-inflammatory agent, being also useful as a precursor of new flavonoids endowed with such activity.

Approximately 4000 different flavonoids have been chemically identified in plant extracts worldwide, making those widespread compounds important constituents of the natural human diet (Middleton 1988). Pharmacological activities have been attributed to some flavonoids, particularly those related to their anti-inflammatory and analgesic properties (Gábor 1975; Viswanathan et al 1984; Thirugnanasambatham et al 1990; Ferrandiz & Alcaraz 1991). Hesperidin, a citrus bioflavonoid, was shown to decrease the inflammatory reaction when administered subcutaneously to rats (Martin et al 1953; Salgado & Green 1956) and, for many years, it was included in a general formulation to treat peripheral vascular diseases (Jaeger et al 1988). The mechanism of action of this flavonoid, however, is still unknown. Hesperidin is an abundant and inexpensive byproduct of citrus cultivation. It is isolated in large amounts from the discarded rinds of the ordinary orange *Citrus aurantium* L. In view of the potential use of hesperidin, we have re-evaluated its anti-inflammatory, analgesic and antipyretic activities. Two related isoflavonoids, duartin and claussequinone, isolated from *Machaerium villosum* Vog and *Cyclolobium clausseni* Benth were also screened for comparison.

Materials and Methods

Animals

Male or female albino rats (Wistar) and mice were used in the experiments. Four to six hours before oral treatment, food was withdrawn from all animals with water freely available. The same conditions were maintained for all animals treated with the tested flavonoids.

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Drugs

Hesperidin (3′,59-dihydroxy-4′-methoxy-7-O-rutinosyl flavanone) was isolated from rinds of *Citrus aurantium* L., the commonest orange used by the juice industry. Duartin (7,3′-dihydroxy-2′,4′,8-trimethoxy-isoflavan) was isolated from *Machaerium villosum* Vog (Kurosawa et al 1968) and claussequinone (7-hydroxy-4′-methoxy-isoflavanquinone) (Oliveira et al 1975) from *Cyclolobium clausseni* Benth (Fig. 1). The compounds were diluted in saline after dispersion in 0·1% Tween 80 (Riedel). Other drugs used were κ-carrageenan, dextran, histamine dihydrochloride, diphenhydramine (Sigma Chem. Co., USA), phentanyl (Janssen), and dipyrone sodium (Hoechst). All drugs were diluted in physiological saline. Indomethacin (Indocid, MSD) was diluted in sodium bicarbonate (5%).

Anti-inflammatory activity

Rat paw oedema. Female rats, 150–200 g, were treated with either the vehicle (Tween 80 + saline, s.c.) or the flavonoids (25–100 mg kg^{-1}, s.c.) 30 min before injection of 0·1 mL

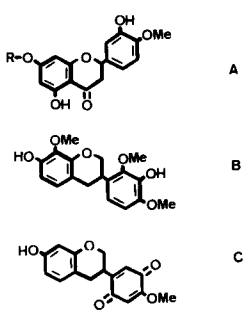

FIG. 1. Chemical structures of the flavonoids: hesperidin (A), duartin (B), claussequinone (C).

carrageenan (1%), dextran (1%) or histamine (0·1%) in the right hindpaw; the contralateral paw was injected with an equal volume of saline. The paw volumes were determined hourly by plethysmography (Winter et al 1962) for 5 h and the swelling calculated as the difference between the two paws as a percentage of the initial volume. Positive controls were obtained in rats treated with either indomethacin (10 mg kg^{-1}, p.o.) or diphenhydramine (60 mg kg^{-1}, p.o.) given 30 min before.

Pleurisy induced by carrageenan. Rats were pretreated with either the flavonoids (100 mg kg^{-1}, s.c.), the vehicle (Tween-saline 0·1%) or indomethacin (10 mg kg^{-1}, p.o.). Thirty minutes afterwards all animals received an intrapleural injection of 0·25 mL carrageenan (0·2% in saline) on the right side of the thorax. The animals were anaesthetized with ether 3 h later and bled. The pleural exudate was collected and the pleural cavity washed with 1·0 mL saline containing heparin (10 int. units mL^{-1}) (Vinegar et al 1973). The number of migrating leucocytes was determined in an electronic cell counter.

Analgesic activity. The antinociceptive effect was determined in mice using the tail-flick test in hot water (55°C) (Janssen et al 1963; Grotto & Sulman 1967). The responses were elicited every 30 min in the hour before and 2 h after treatment with either hesperidin (100 mg kg^{-1}, s.c.) or the vehicle. Mice treated with phentanyl (0·2 mg kg^{-1}, s.c.) were employed as positive controls. Abdominal constrictions were induced by 1% acetic acid (0·1 mL/10 g, i.p.) in mice pretreated with saline or one of the test compounds (Koster et al 1959). The number of abdominal constrictions was measured over 30 min at 5 min intervals, and animals treated with indomethacin (10 mg kg^{-1}, p.o.) were used as positive controls.

Antipyresis. Antipyretic action was determined in 45-day-old male rats injected with 15% brewer's yeast in saline (1·0 mL/100 g, s.c.) (Winder et al 1961). Eighteen hours later the animals presenting an increase in the rectal temperature of about 1°C were randomly treated with either the vehicle, hesperidin (100 mg kg^{-1}, s.c.) or sodium dipyrone (100 mg kg^{-1}, p.o.), and the effects were measured every hour for 5 h.

Ulcerogenic activity. Young male rats, 200–250 g, fasted for 24 h, received tap water (1 mL/100 g) by gavage 30 min before treatment with either the vehicle, hesperidin (100 mg kg^{-1}, s.c.) or indomethacin (10 mg kg^{-1}, s.c.). The gavage was repeated after 3 h and the animals were killed by deep ether anaesthesia on the 6th hour. Following removal of the stomach, the mucosa was exposed by sectioning the lesser curvature and the degree of gastric irritation (ulcer index) and the number and severity of ulcers per cm^2 were determined according to a weighed criteria as formerly described (Aguwa & Mittal 1981; Macaubas et al 1988; Gamberini et al 1991).

Statistical analysis.
All data were presented as means ± s.e.m. Statistical differences among control and treated groups were determined by analysis of variance followed by the Scheffé method. The differences were considered significant at $P < 0·05$.

Results

Anti-inflammatory activity

Rat paw oedema. The volume of the rat paws immediately after injection of 0·1 mL carrageenan was 1·15 ± 0·01 mL (n = 20). In control vehicle-treated rats, the oedema induced by carrageenan was progressive reaching a maximum after 3 h. At this time the volume of the injected paw was 32% (0·41 mL) greater than the contralateral paw injected with saline. Previous treatment of the rats with 25 mg kg^{-1} hesperidin did not influence the paw oedema, but at 50 and 100 mg kg^{-1}, hesperidin reduced the paw oedema by 47–63% from the 1st to the 5th hour after carrageenan injection, compared with the vehicle-treated rats (Fig. 2). The magnitude of the effect was comparable with that induced by indomethacin (10 mg kg^{-1}, p.o.). At doses of up to 100 mg kg^{-1}, duartin and claussequinone did not influence the paw oedema induced by carrageenan in rats (Fig. 3).

The oedema induced by dextran in the rat paw was maximum after 60 min, when the volume of the injected paw was 60% greater than that of the contralateral paw. This effect persisted up to 3 h afterwards. Pretreatment of rats with hesperidin (100 mg kg^{-1}, s.c.) reduced the paw oedema

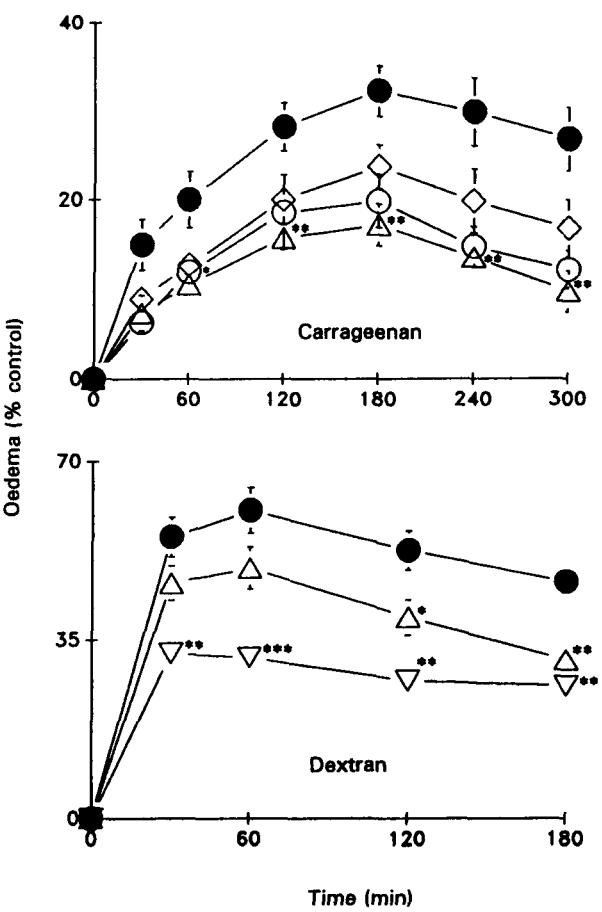

FIG. 2. Effect of pretreatment with the vehicle (●), hesperidin (◇ 25, ○ 50, or △ 100 mg kg^{-1}, s.c.), or diphenhydramine (▽ 60 mg kg^{-1}, p.o.) on the rat paw oedema induced by carrageenan, or dextran. Symbols are means ± s.e.m. (five animals) of the volume difference between the paw injected with either phlogistic agent and the contralateral paw injected with saline. The paw volumes determined immediately after injection of the phlogistic agents were taken as 100%. $*P < 0·05$, $**P < 0·01$, $***P < 0·001$.

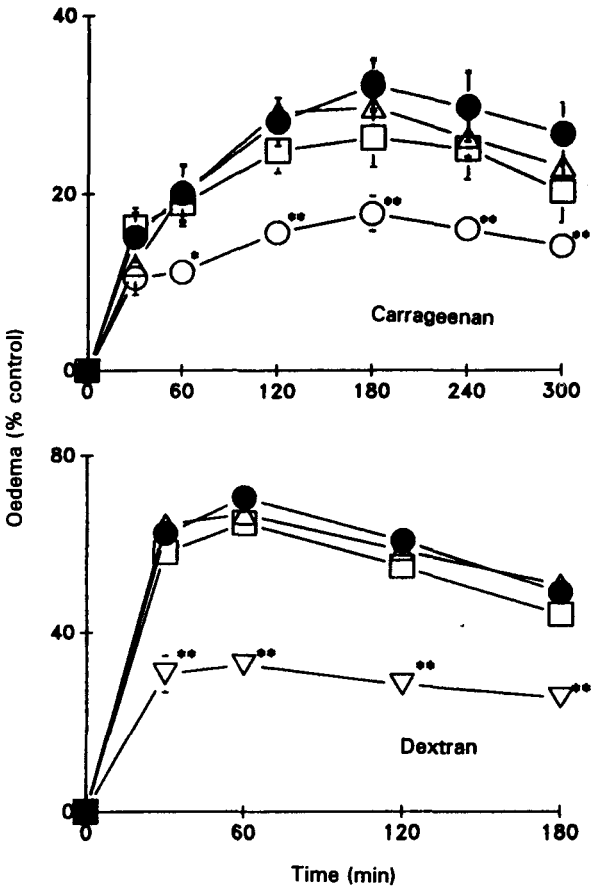

FIG. 3. Effect of pretreatment with the vehicle (●), duartin (△ 100 mg kg^{-1}, s.c.), claussequinone (□ 100 mg kg^{-1}, s.c.), indomethacin (○ 10 mg kg^{-1}, p.o.) or diphenhydramine (▽ 60 mg kg^{-1}, p.o.) on the rat paw oedema induced by carrageenan or dextran. Symbols are means ± s.e.m. (five animals) of the volume difference between the paws injected with phlogistic agents and the contralateral paw injected with saline. $*P<0.05$, $**P<0.01$.

by 25 and 33% after respectively 2 and 3 h injection of dextran, when compared with the control group (Fig. 2). Duartin and claussequinone, both at 100 mg kg^{-1}, had no detectable effect on the paw oedema induced by dextran. Pretreatment with diphenhydramine (60 mg kg^{-1}, p.o.) reduced the oedema induced by dextran by 50% over 3 h (Figs 2, 3).

Pretreatment of rats with hesperidin (100 mg kg^{-1}, s.c.) did not significantly reduce the paw oedema induced by histamine, but diphenhydramine reduced the same paw oedema by 60%.

Pleurisy induced by carrageenan. The volume of the pleural exudate in vehicle-treated rats was 0.61 ± 0.05 mL and the leucocyte count was $7.3 \pm 0.3 \times 10^3$ cells mm^{-3}. Treatment with hesperidin (100 mg kg^{-1}, s.c.) decreased both the pleural exudate (0.32 ± 0.03 mL) and leucocyte migration ($4.8 \pm 0.6 \times 10^3$ cells mm^{-3}). This effect was equal to that obtained in rats pretreated with 10 mg kg^{-1} indomethacin (0.31 ± 0.5 mL and $4.1 \pm 0.5 \times 10^3$ leucocytes mm^{-3}, respectively). No effect was detected in rats pretreated with 100 mg kg^{-1}, of either duartin (0.50 ± 0.03 mL, $6.6 \pm 0.4 \times 10^3$ leucocytes mm^{-3}) or claussequinone (0.60 ± 0.05 mL, $5.7 \pm 0.5 \times 10^3$ leucocytes mm^{-3}).

Analgesic activity

Abdominal constriction induced by acetic acid. Pretreatment of mice with hesperidin (100 mg kg^{-1}, s.c.) reduced by 50% the number of abdominal constrictions counted during 30 min after injection of the acid (from 47 ± 6 to 24 ± 4). The same effect was obtained after pretreatment with indomethacin (10 mg kg^{-1}, p.o.) (25 ± 2 in 30 min) (Fig. 4).

Tail flick. In control mice, the tail-flick latency was 1.5 ± 0.1 s ($n = 10$). In mice previously treated with hesperidin (100 mg kg^{-1}, s.c.) the responses did not change over 2 h. Previous treatment of mice with phentanyl (0.2 mg kg^{-1}, s.c.) increased the tail-flick latency to 3.8 ± 0.1 s; $n = 10$ ($P<0.05$).

Antipyretic activity

The basal temperature of untreated rats was 37.8 ± 0.1°C ($n = 15$). Eighteen hours after yeast administration the mean

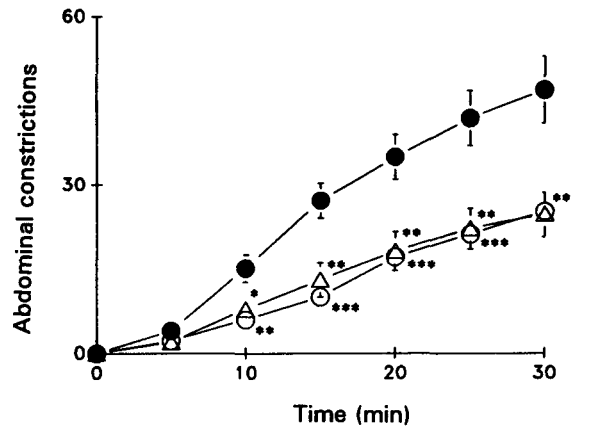

FIG. 4. Abdominal constriction induced by acetic acid (1%, 0.1 mL/10 g, i.p.) in mice pretreated with either the vehicle (●), hesperidin (△ 100 mg kg^{-1}, s.c.), or indomethacin (○ 10 mg kg^{-1}, p.o.). Symbols are means ± s.e.m. (10 animals) of cumulative abdominal constrictions determined at 5 min intervals during 30 min. $*P<0.05$, $**P<0.01$, $***P<0.001$.

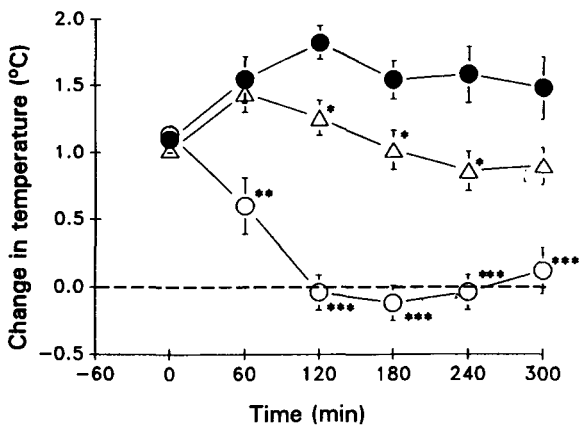

FIG. 5. Effect of the vehicle (●), hesperidin (△ 100 mg kg^{-1}, s.c.) and dipyrone (○ 100 mg kg^{-1}, p.o.), on fever induced by brewer's yeast 15% (1 mL/100 g, s.c.) in rats. Symbols are means ± s.e.m. (five animals) of temperature changes relative to the basal temperature before either treatment. $*P<0.05$, $**P<0.01$, $***P<0.001$.

Table 1. Degree of gastric irritation (ulcer index) and number of ulcers cm^{-2} in gastric mucosa of rats treated with subcutaneous injections of the vehicle, hesperidin (100 mg kg^{-1}) or indomethacin (10 mg kg^{-1}).

Treatment	Ulcer index	Number of ulcers cm^{-2}
Vehicle (5)	2·80±0·12	0
Hesperidin (100 mg kg^{-1}) (5)	3·00±0·15	0
Indomethacin (10 mg kg^{-1}) (5)	12·91±0·59*	3·5±0·29*

Results are means±s.e.m. Numbers in parentheses indicate the number of determinations. *$P<0·01$ compared with vehicle-treated animals.

body temperature of the rats was 38·9±0·04°C, corresponding to a temperature increase of 1·11±0·04°C. In control rats treated with the vehicle, the body temperature increased by 1·82±0·13°C above basal levels. In rats treated with hesperidin (100 mg kg^{-1}, s.c.) however, the fever was gradually lowered to 0·86±0·15°C above normal temperature after 4 h (n=5). Dipyrone (100 mg kg^{-1}, p.o.) administration restored the animal basal temperature after 2 h (Fig. 5).

Ulcerogenic activity

Six hours after administration of hesperidin (100 mg kg^{-1}, s.c.) no significant alteration of the gastric mucosae was detected. However, animals pretreated with equi-effective anti-inflammatory doses of indomethacin (10 mg kg^{-1}, s.c.) presented 3·5±0·3 ulcers cm^{-2} (n=5), some as large as 1 mm (Table 1).

Discussion

This study presents pharmacological data favouring a potential use of hesperidin as an inexpensive anti-inflammatory agent or as a lead compound especially for patients with hypersensitivity to the ordinarily used non-steroidal anti-inflammatory agents. Hesperidin is ineffective after oral administration (Salgado & Green 1956), but is active after subcutaneous injection without remarkable changes in the rat behaviour or apparent tissue damage at the site of injection, even after repeated administrations. In contrast, duartin and claussequinone, two widely distributed isoflavonoids, did not exhibit anti-inflammatory activity and were discarded after the initial tests.

When tested on the now classic rat paw oedema induced by carrageenan, hesperidin decreased the paw swelling as effectively as did indomethacin. Similar results were obtained when both agents were tested on pleurisy induced by carrageenan in rats. Those data suggested that the anti-inflammatory activity of hesperidin may be partially related to inhibition of prostaglandin synthesis (Vane 1971; Flower et al 1972). At high doses, hesperidin also reduced the paw oedema induced by dextran, indicating that the flavonoid might also inhibit the release of other inflammatory mediators. The paw oedema induced by dextran has been related to the release of endogenous histamine and 5-hydroxytryptamine (Di Rosa & Willoughby 1971; Pearce 1986; West 1990). It has been shown that hesperitin, the aglucone of hesperidin, inhibited the release of histamine from basophils (Middleton & Drzewiecki 1984), suggesting that this action may account for the anti-inflammatory effect of hesperidin on the dextran-induced paw oedema.

Hesperidin was ineffective on the paw-swelling induced by histamine, suggesting there is no blockade of histaminergic receptors by the flavonoid. Hesperidin pretreatment 30 min before the experiment was effective in all other experiments. In view of the rapid time course of the oedema induced by histamine as compared with that induced by carrageenan or dextran, we also tested the effect of hesperidin injected 60 min before histamine; hesperidin was equally ineffective in that experiment, excluding the need of pretreatment with the flavonoid for periods longer than 30 min.

Hesperidin also exhibited analgesic activity in mice. The effect was obtained in the abdominal constriction test but not on the tail-flick response, which is considered selective for opioid-like compounds in several animal species (Janssen et al 1963). These results indicate that hesperidin exerts an analgesic effect through peripheral, but not central mechanisms. Considering that abdominal constriction is related to sensitization of nociceptive receptors by prostaglandins (Ferreira & Vane 1974), our results re-inforce previous indications of a probable inhibition of prostaglandin release by hesperidin. Hesperidin also decreased the fever induced by yeast in rats. The effect was not as pronounced as that produced by dipyrone, but was consistent with the mild activity of hesperidin already described, being probably related to inhibition of yeast-induced prostaglandin biosynthesis (Dinarello 1989).

Gastric discomfort and ulcers are generally the major side-effects related to the currently employed non-steroidal anti-inflammatory agents. Although our data indicated that hesperidin inhibits the biosynthesis of prostaglandins, the compound did not cause gastric mucosal injury. Indomethacin and hesperidin, at the doses and routes used, were equi-effective on the rat paw oedema induced by carrageenan, but only the former caused severe ulceration of the gastric mucosa. These results favour again an involvement of other mechanisms mediating the anti-inflammatory activity of hesperidin. Thus, besides inhibiting the release of prostaglandins, considered a defensive gastric factor (Glavin & Szabo 1992), hesperidin appears to inhibit histamine release, an effect which would prevent acid secretion and lesion of the gastric mucosa.

In conclusion, our study has indicated that hesperidin exhibits an anti-inflammatory activity, without inducing serious adverse effects. This activity appears to be a characteristic of that flavonoid since related isoflavonoids were ineffective at the same doses. Hesperidin also produced analgesia through peripheral mechanisms, and exerted mild antipyresis. These effects could be related to inhibition of the release of prostaglandins and histamine.

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