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The flavonoid fustin exerts anti-inflammatory effect in a model of carrageenan-induced paw oedema

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PRELIMINARY COMMUNICATION

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ABSTRACT

Carrageenan-induced paw oedema is a widely used model of acute inflammation. The objective of this study was to assess the effect of the flavonoid fustin on carrageenan-induced acute paw inflammation in rats. Thirty male Wistar rats were distributed equally between three groups – control, F10, and F20. In the course of 1 week, animals were treated once daily by intragastric gavage as follows: control group – with distilled water (10 mL kg^{-1}) containing 50 µL Tween 80; groups F10 and F20 – with 10 mL kg^{-1} suspensions containing fustin in two doses (10 mg kg^{-1} and 20 mg kg^{-1}, respectively) and 50 µL Tween 80. After the treatment period, carrageenan was injected in the left hind paw and paw oedema was evaluated 0, 30, 60, 120, 180, 240, and 300 min after the injection using a plethysmometer. In the control group, paw

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oedema increased gradually and peaked at the 180th minute. Fustin treatment reduced the oedema in all time intervals and the effect was significant on the 30th and 60th minute after the injection. The present study indicated that fustin could suppress acute inflammation.

KEYWORDS

fustin, anti-inflammatory, carrageenan, paw, oedema, rats

1. INTRODUCTION

Carrageenan is a high-molecular weight food additive, derived from red seaweed species. At present, its main application is in experimental medicine. λ-Carrageenan induces an acute inflammatory response manifested as oedema when injected in the hind paw of experimental animals. Carrageenan-induced paw oedema is a well-known experimental model of acute inflammation used to assess the anti-inflammatory potential of bioactive compounds (Fehrenbacher et al., 2012).

Flavonoids are major polyphenol compounds, which have been shown to prevent inflammation (Bae et al., 2017). *Cotinus coggygria*, known as "European smoketree", is a medicinal plant whose extracts are characterised by a high content of tannins and flavonoids (sulfuretin, fisetin, fustin, quercetin, butin, and myricetin). Studies show that *C. coggygria* extracts exert *in vitro* and *in vivo* wound-healing, anti-inflammatory, antibacterial, cytotoxic, antioxidative, hepatoprotective, and antidiabetic effects (Antal et al., 2021).

Fustin is a major flavonoid in *C. coggygria* heartwood. Available data indicate that it exerts a neuroprotective effect (Park et al., 2007; Jin et al., 2009; Cho et al., 2012). However, there are no data about the effect of this polyphenol on acute inflammation. To explore its anti-inflammatory potential, further *in vivo* studies are needed.

The objective of this study was to explore the anti-inflammatory potential of the flavonoid fustin isolated from *C. coggygria* heartwood in a model of carrageenan-induced acute inflammation of the left hind paw in rats.

2. MATERIALS AND METHODS

2.1. Fustin isolation

The method of fustin isolation is a modified version of the method described by Novakovic et al. (2019).

2.1.1. Plant material and extraction. The *C. coggygria* heartwood was collected at Deliblatska Peščara (Deliblato Sand), Vojvodina province, Serbia, in May 2021. Plant material was identified by Professor Milan Veljic, Faculty of Biology, University of Belgrade, and voucher specimen BEOU 17422 was deposited at the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Belgrade, Serbia. The heartwood was air-dried and milled to fine powder. Wood powder, 1 kg, was extracted three times with 10 L of methylene chloride/methanol 1:1 for 24 h at

room temperature to give 76 g of the crude extract, which was subjected to fractionation by Si gel column chromatography CC.

2.1.2. Isolation and identification of fustin. For column chromatography (CC) Merck silica gel (Si gel) (particle size 0.063–0.200 mm), methanol, and methylene chloride were used. Analytical TLC was performed on aluminium plates precoated with Merck silica gel 60 F254 (0.25 mm thickness). The NMR spectra were obtained on a Bruker Avance III 500 (500 MHz for ^{1}H; 125 MHz for ^{13}C), in CD_3OD as solvent. Chemical shifts (d) were expressed in ppm and coupling constants (*J*) in hertz (Hz). Semipreparative reversed phased HPLC was performed on an Agilent Technologies 1100 Series HPLC-DAD and Zorbax Eclipse XDB C18 column (150 × 9.4 mm i.d., 5 µm particle size) was used.

Crude extract was chromatographed on a Si gel CC column (750 × 45 mm), with methylene chloride/methanol (gradient elution - from 97/3 to 60/40). This step was repeated seven times to obtain high amounts of fustin. Column chromatography was monitored by TLC, and the fractions with similar Rf values were combined. Fustin was found in fractions eluted with methylene chloride/methanol approximately 80:20. Pure fustin was isolated from these fractions by reversed phase semi-preparative HPLC using water/acetonitrile system, 254 nm for detection and the following program: 0–20 min, 20–37% CH_3CN; 20–21 min, 37–50% CH_3CN; 21–27 min, 50% CH_3CN; and 27–30 min, 50–100% CH_3CN. Fustin was purified up to 98% on reversed phase semipreparative HPLC using the following program: 0–20 min, 25–40% CH3CN (Rt = 5.1 min).

2.2. Experimental design

2.2.1. Experimental animals. In the current investigation, thirty male Wistar rats weighing between 150 and 200 g were included. They were housed in plastic cages at a constant temperature of 20–25 °C, under a 12-h light/dark cycle and had unrestricted access to water and food.

All procedures concerning the animal treatment and experimentation were carried out in accordance with the national and international laws and policies (EU directive 2010/63/EU for animal experiments) and were approved with Protocol № 23/April 15th, 2021 by the Bulgarian Food Safety Agency (Permission № 305/June 28th, 2021).

2.2.2. Treatment. The rats were distributed equally between three groups: control, F10, and F20 ($n = 10$ per group). The animals received daily oral treatment through an orogastric tube over the course of one week as follows: the control group received distilled water in a total volume of 10 mL kg^{-1} containing 50 µL Tween 80 (diluent), F10 and F20 animals received 10 mL kg^{-1} suspensions containing 50 µL Tween 80 and fustin in two doses – 10 mg kg^{-1} and 20 mg kg^{-1}, respectively.

2.2.3. Induction of hind paw oedema. To produce an acute inflammation, 1 mg of carrageenan (Cat. № 22049 of Sigma-Aldrich) dissolved in 100 µL of saline was administered subcutaneously in the left hind paw of the experimental animals (Albertini et al., 2007). Using a digital plethysmometer LE7500 (Panlab, Barcelona), paw volume (mL) was measured 0, 30, 60, 120, 180, 240, and 300 min after the injection. The difference in paw volume before and after the injection correlated with the severity of the inflammation.

Paw oedema (mL) was calculated using the following formula:

$$\text{Paw oedema (mL)} = (V_s – V_0):$$

V_s: paw volume measured at the corresponding time interval after carrageenan injection;
V_0: initial paw volume.

Inhibition of the paw oedema (%) was calculated using the formula:

$$(V_C – V_T)/V_C \times 100:$$

V_C: mean paw oedema volume of the animals from the control group;
V_T: mean paw oedema volume of the animals from each treated with fustin groups.

2.3. Statistical analysis

GraphPad Prism 7.00 statistical software (San Diego, California) was used to analyse the data. The following analyses were performed: one-way ANOVA, followed by Dunnett's multiple comparisons test, and Student's *t*-test to compare two independent groups. Results were presented as mean ± SEM. A value of $P < 0.05$ indicated a statistical significance.

3. RESULTS AND DISCUSSION

The results are shown in Table 1 and Fig. 1.

Subplantar carrageenan injection induced an acute inflammation of the left hind paw of rats from all experimental groups. In the control group, carrageenan-induced oedema increased gradually and peaked at a value of 0.60 ± 0.06 mL at the 180th minute after the injection and then started to decline. Compared to the control group, fustin treatment in F10 and F20 groups reduced paw oedema at all time intervals, with the effect being statistically significant at the 30th minute (60.8% inhibition at a dose of 10 mg kg^{-1} and 47.8% inhibition at a dose of 20 mg kg^{-1}, $P < 0.0001$ vs. control group) and the 60th minute (58.3% inhibition at a dose of 10 mg kg^{-1}, $P < 0.0001$ vs. control group; 44.4% inhibition from a dose of 20 mg kg^{-1}, $P < 0.001$ vs. control group). In addition, Student's *t*-test analysis revealed that paw oedema in F10 and F20 groups did not differ significantly on the 30th ($P < 0.5658$) and on the 60th min ($P < 0.4702$).

Inflammation is a protective, immune-mediated response of the body to harmful stimuli. At tissue level, acute inflammation is manifested with five cardinal symptoms: rubor (redness), dolor (pain), calor (elevated temperature), tumor (oedema) and functio laesa (loss of function)

Table 1. Inhibition of the left hind paw oedema (%) in rats, treated with fustin at doses of 10 mg kg^{-1} (F10) and 20 mg kg^{-1} (F20)

Group	Paw oedema inhibition – 30th min	Paw oedema inhibition – 60th min	Paw oedema inhibition – 120th min	Paw oedema inhibition – 180th min	Paw oedema inhibition – 240th min	Paw oedema inhibition – 300th min
F10	60.8%	58.3%	25.5%	16.6%	11.8%	16.3%
F20	47.8%	44.4%	4.3%	15.0%	19.6%	0%

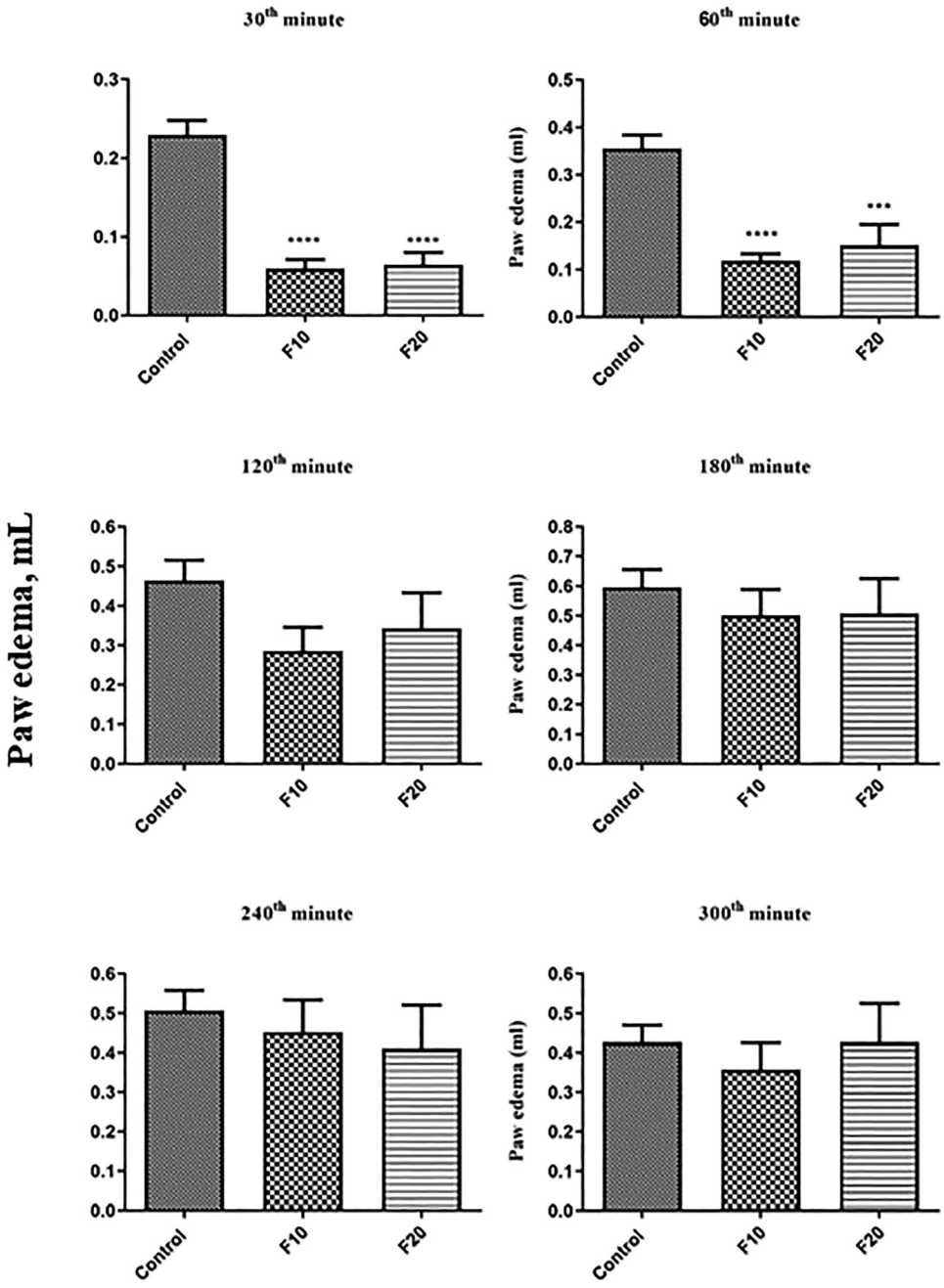

Fig. 1. Left hind paw oedema (ml) on the 30th, 60th, 120th, 180th, 240th, and 300th minute after carrageenan injection in rats, treated with fustin at doses of 10 mg kg^{-1} (F10) and 20 mg kg^{-1} (F20); ***: $P < 0.001$ vs. control group, ****: $P < 0.0001$ vs. control group

(Chen et al., 2017). Carrageenan-induced paw oedema is an easy, cheap, and reproducible model of acute inflammation, which serves as an important screening tool for the possible anti-inflammatory activity of different compounds. This study revealed that subplantar carrageenan injection in rats induced acute inflammation of the left hind paw and increased its volume in all subsequent time intervals, with a peak value at the 180th min. These results are in accordance with available studies since carrageenan-induced oedema maximum is at 3–5 h after its injection (Fehrenbacher et al., 2012).

In addition, this experiment examined the effect of the flavonoid fustin on acute carrageenan-induced paw oedema. The results showed that fustin treatment at doses of 10 mg kg^{-1} and 20 mg kg^{-1} reduced the oedema gradually and this effect was the highest and significant at the 30th and 60th min after the carrageenan injection.

The mechanisms behind the described acute anti-inflammatory effect could be sought in the pathogenesis of carrageenan-triggered inflammation. Available evidence indicate that carrageenan injection induces a biphasic response. The first phase occurs shortly after the injection (the first 1–2 h) and it is characterised by production of serotonin, bradykinin, and histamine (Crunkhorn and Meacock, 1971; Vinegar et al., 1987). These chemicals act as potent vasodilators, which contribute to increased vascular permeability and oedema. Studies have shown that flavonoids could down-regulate mast cells and inhibit histamine release. Moreover, some studies indicate that they could act as bradykinin antagonists (Calixto and Yunes, 1991; Yun-Choi et al., 1993). Fustin, as a typical flavonoid, might suppress extravasation of fluid and prevent oedema through these mechanisms.

The second phase occurs 3–6 h after carrageenan injection and it is characterised by local infiltration of neutrophils in the inflamed tissue area, induction of oxidative stress with production of reactive oxygen species (ROS), and release of prostaglandins (PGs), leukotrienes, and cytokines (interleukins such as IL-1β, IL-6, IL-10, and TNF-α) (Crunkhorn and Meacock, 1971; Vinegar et al., 1987). Compared to the control group, fustin treatment at both doses was not associated with statistically significant suppression of the paw oedema in the time intervals after the 60th min (second phase).

Since fustin treatment predominantly suppressed the first phase of carrageenan-induced inflammation in this experiment, it is important to clarify the reasons behind this finding. According to several studies, COX-2 achieves maximal expression one hour after carrageenan injection and after that it continues to produce PGs in the second phase, although in smaller amounts (Nantel et al., 1999). In this study, fustin exerted maximal anti-oedematous effect 30 and 60 min after carrageenan injection. This might indicate fustin could achieve an effect, similar to that of the non-steroidal anti-inflammatory drugs by blocking PG production rather than affecting other mediators related to inflammation. However, COX-2 expression and PG levels were not evaluated in this study.

4. CONCLUSIONS

In conclusion, fustin treatment mitigated the carrageenan-induced left hind paw oedema. The present study indicated that fustin administration could suppress acute inflammation. Further studies are necessary to elucidate the mechanisms behind the described acute anti-inflammatory effect.

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