SUPPLEMENTARY MATERIAL

Evaluation of antioxidant and anti-inflammatory activity of green coffee beans methanolic extract in rat skin

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Abstract

Since a long time caffeine has been used in pharmaceutical and cosmetic preparations due to its favorable effects on the skin. The aim of this study was to evaluate the topical anti-inflammatory activity (carrageenan-induced paw oedema) of an ointment prepared using a methanolic extract from green beans of *C. robusta via* histology. Results showed that the treatment with the ointment reduced the paw oedema within 3 and 5 hours post-carrageenan administration. The immunohistochemical evaluations of αSMA, Langerin and S100 gave further support to the morphological analysis. Finally, the methanolic extract from green beans of *C. robusta* proved to possess elevated free radical scavenger capability by DPPH assay, which may contribute to the observed anti-inflammatory activity.

Experimental section

Plant Materials

Green beans of *Coffea robusta* L., were obtained by "Zi Caffè s.p.a." from Marsala (Sicily, Italy), a local broker and industrial coffee roaster. The seeds were refrigerated until analysis. Voucher specimens are deposited at the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, under accession number n° 11/09.

Preparation of extract

One hundred g of green seeds were extracted for 4 hours with methanol, using a Soxhlet apparatus. The methanol extract was concentrated in a rotary evaporator under reduced pressure at temperature of 40 °C (yield 2.00%).

Determination of total phenolic compounds

The total phenolic content of coffee beans methanol extract was determined by Folin-Ciocalteau method, referring to calibration curve of gallic acid, phenol compound used as a standard (Wynn, 2018; Germanò et al., 2005).

Analysis of the polyphenolic content by HPLC-MS

Instrumentation: The analyses were carried out using a Shimadzu HPLC-MS system (Milan, Italy) as previously reported (Miceli et al., 2017).

Sample preparation: 20 mg of green coffee were dissolved in 1 mL of MeOH.

Chromatographic and detection conditions: Analyses were carried out on a Ascentis Express C18, 15 cm × 4.6 mm I.D. with particle size of 2.7 μm (Supelco, Bellefonte, PA) and chromatographic and detection conditions were previously reported (Miceli et al., 2017).

Animals

Male Wistar rats (200-250 g) were obtained from Harlan (Milano, Italy). The animals were housed in standard cages at room temperature (22 ± 4 °C) with food and water *ad libitum*.

Anti-inflammatory activity

Oedema was induced in rats injecting 25 µl of 1% (w/v) carrageenan suspension in saline (0.9%) into the sub plantar region of the right hind paw according to the a slightly modified method by Winter et al. (1962) and percentages of inhibition were calculated according to Germanò et al., 2008. The extracts of coffee beans were applied topically in an ointment form, soon after the carrageenan was injected. The ointments were prepared using methanol extract (140 and 420 mg/g of drug) and vaseline, resulting in concentration of 7% and 21% (w/w), and a ointment containing caffeine (Sigma, Milano, Italy) in the same concentration, which were then topically applied (0.3 g) on the paw of rats.

Tissue preparation

Skin plantar samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) 0.1 M (pH 7.4) for 12–18 h, dehydrated in graded ethanol, cleared in xylene, embedded in Paraplast® (McCormick ScientificLLC, St. Louis, MO, USA) and cut into 5-10 µm sections.

Light Microscopy

For analysis of cell components and tissue structures, some serial sections (5 µm thick) were stained with the routinary histological stain: Mallory's trichrome Ignesti mod. (Lauriano et al., 2012).

Immunoperoxidase method

The immunohistochemical investigations were carried out using indirect method of peroxidase as previously reported by Kuciel et al. (2014). Serial sections were incubated overnight at 4◦C in a humid chamber with primary polyclonal antibody S100 (1:100; Sigma-Aldrich). The sections were then incubated with a goat anti rabbit IgG-peroxidase conjugate (1:100, Sigma-Aldrich, St. Louis, MO, USA) and then dipped in 0.02% 3-3'diaminobenzidine (DAB, Sigma-Aldrich) with 0.015% hydrogen peroxide.

Immunofluorescence

Serial sections, after rehydration, were rinsed several times in PBS and blocked in 10% normal goat serum for 1 h (Lauriano et al., 2017; Zaccone et al., 2015). Primary antibodies αSMA in mouse (Sigma-Aldrich, Milan, Italy; 1:200), and Langerin/207 in goat (Santa Cruz Biotechnology, Dallas,

TX, USA; 1:100) were diluted in a permeabilizing solution (PBS, 0.2% Triton X-100, 0.1% sodium azide - PBST) and placed on the slides, then incubated overnight at r.t. Sections were treated, for 2 h in the dark at r.t. with fluorescent labeled secondary antibodies, Alexa Fluor 488 Donkey anti-mouse IgG FITC conjugated and Alexa Fluor 594 Rabbit anti-goat IgG TRITC conjugated (MolecularProbes, Invitrogen, Eugene, OR, USA; 1:100). The images were acquired using a Zeiss LSMDUO confocal laser scanning microscope with META module (CarlZeiss Micro Imaging GmbH, Germany). Each image was rapidly acquired in order to minimize photodegradation. Digital images were cropped and the figure montage prepared using Adobe Photoshop7.0 (Adobe Systems, San Jose, CA, USA).

Measurement of the DPPH radical-scavenger activity

The free radical scavenging activity of Coffee beans methanol extract was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) test (Taviano et al., 2018).

Statistical analysis

All values are expressed as mean±standard deviation and were analysed statistically by means of analysis of variance (ANOVA) followed by Student's *t*-test. Value of $P < 0.05$ are regarded as significant.

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Figure S1. LC-PDA chromatogram of the green coffee extract

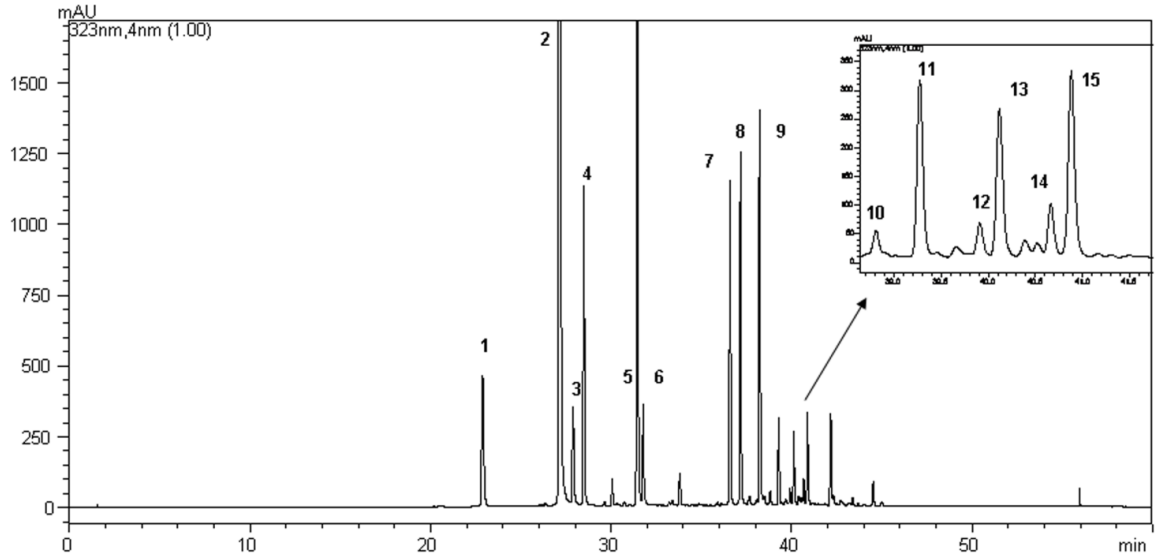

Figure S2. Anti-inflammatory activity of methanolic extract of *Coffea robusta* in the carrageenan-induced paw oedema. [a] Rat paw volume measured before administering carrageenan (*V*0) and 1, 2, 3, 4, and 5 h after (*V*t). Values are expressed as mean±S.D. (*n* = 6). Percentage inhibitions of the carrageenan-induced oedema are indicated as (%).

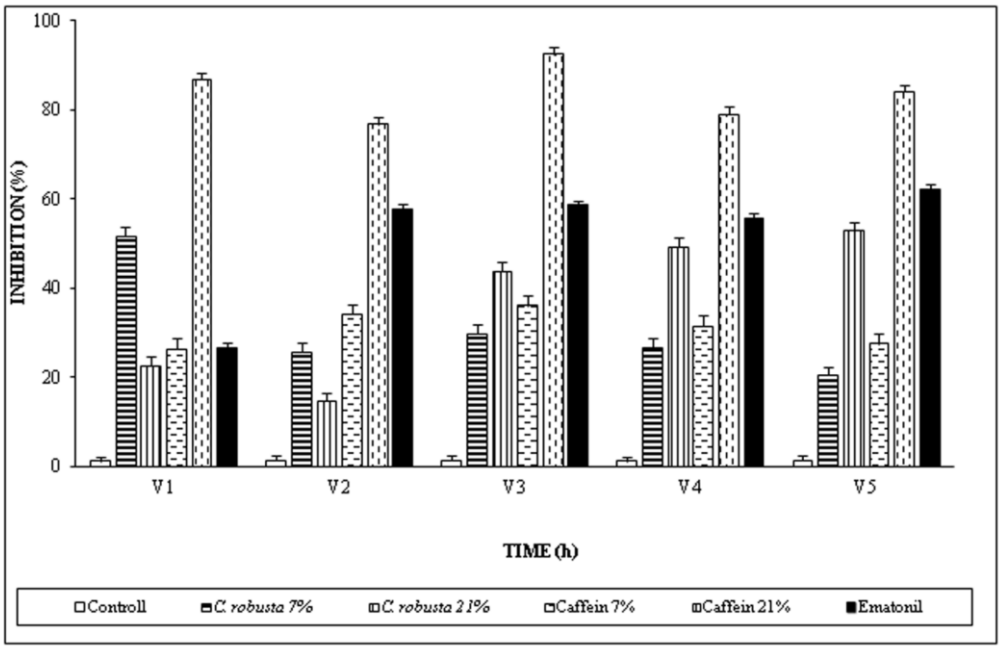

Figure S3. Light microscopy. (a) Normal skin, all epidermal layers (C corneum, G granular, S squamous and B basal) are visible. Dermis (D) is regular in morphology with well arranged collagen fibers and vascular network. (b). Rat skin, 3h oedema. The interlacing of the epidermis and the dermis is radically changed. The basal cells are cubic and the upper layers disorganized, the dermis presents granulation tissue and mast cells (dermal infiltrates-DI). More evident in (c) (5h oedema) in which the disruption of all epidermal layers is shown, some Langerhans cells (L) are detected with histological stain. (d, e) Rat skin 3h oedema with green coffee extract 7% and 21%. In epidermis a restoration of epithelial cells is visible, basal keratinocytes are elongated and some Langerhans cell are detectable, DI visible in outer dermis. (f, g) Rat skin 5h oedema with green coffee extract 7% and 21%. The re-epithelization is enanched, all epidermal layers are well represented, in the dermis less granulation tissue is observed. (h, i) Rat skin, 3h and 5h oedema. S100 immunoperoxidase reactivity shows well recognizable Langerhans cells. Mallory's trichrome Ignesti modified. Magnification100x (a-g). S100 HRP. Magnification 100x (h, i).

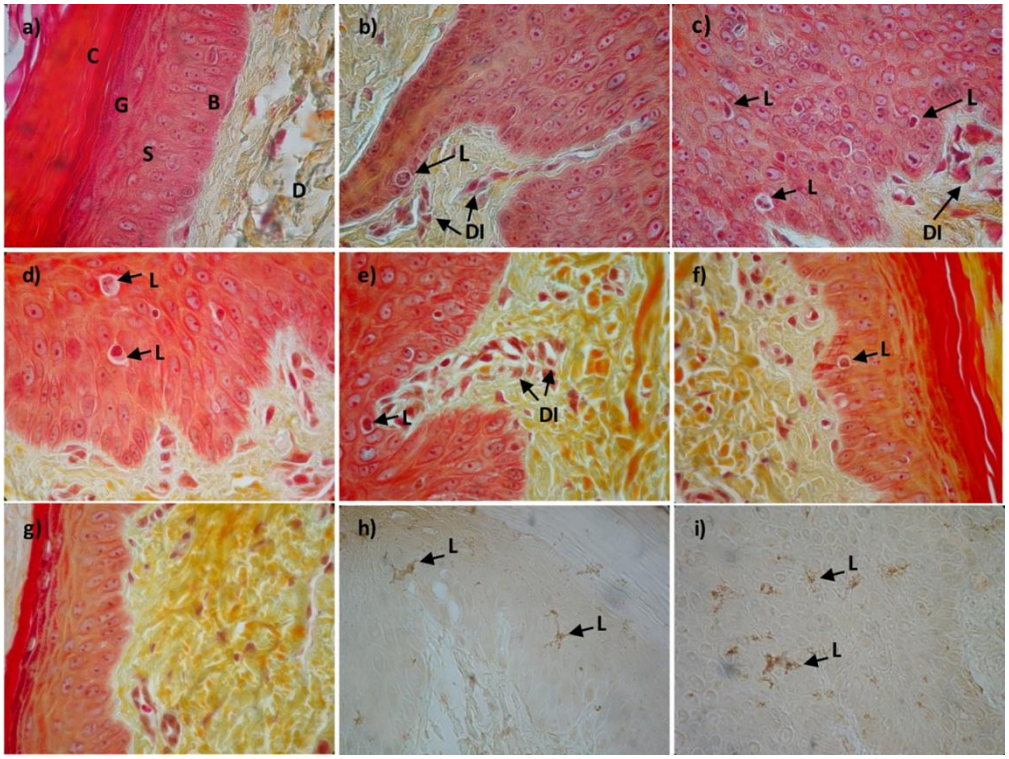

Figure S4. CLSM micrographs with antibodies directed against Langerin (red fluorescent) and αSMA (green fluorescent). (a) 3h oedema. A strong positivity for αSMA is observed, both keratinocytes (K) and in dermis' fibroblast/myofibroblasts (F). The red fluorescence marked Langerhans cells. (b) 5h oedema. More Langerhans cells are detectable between keratinocytes and a large amount of fibroblasts/myofibroblasts overrun the outer dermis. (c) Langerin positive cells decrease during 5h oedema treatment with 7% green coffee and 21% (d). Immunofluorescence for αSMA (c, d) revealed lower marking of fibroblasts in dermis. A marked positivity in keratinocytes stay on both in 7% treatment and above all in 21% treatment. Magnification 40x.