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AROMATHÉRAPIE EXPÉRIMENTALE

Antioxidant, Anti-inflammatory, and Analgesic Activities of *Citrus reticulata* Blanco Leaves Extracts: An *In Vivo* and *In Vitro* Study

Activités antioxydantes, anti-inflammatoires et analgésiques des extraits des feuilles de *Citrus reticulata* Blanco: étude in vivo et in vitro

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Abstract Citrus species are cultivated and consumed widely. Citrus have been investigated for their pharmacological activity and human health. Their beneficial effects include antibacterial, analgesic, anti-inflammatory, and antitumoral effects. This study was designed to evaluate the analgesic effect and the antioxidant and anti-inflammatory activities of *Citrus reticulata* Blanco leaves extracts (ECR) in cell and animal models. Antioxidant, anti-inflammatory, and antinociceptive activities were evaluated in mice using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition, xylene-induced ear edema, formalin assay and acetic acid-writhing response. Expression of anti-inflammatory genes was measured in lipopolysaccharide (LPS)-treated Huh7 cells. ECR showed a significant DPPH radical scavenging activity. No behavioral changes or deaths were observed in mice at doses less than 2,000 mg/kg body weight. Different concentrations of methanolic and aqueous extracts (100–500 mg/kg body wt.) reduced the duration of linking behavior in the second phase of the formalin chemical nociception assay and decreased the number of acetic acid-induced writhing responses in mice, indicating significant analgesic activity. ECR also diminished xylene-induced ear swelling in mice, suggesting an *in vivo* anti-inflammatory action. No toxicity of ECR in the range of 0.1–10 μg/ml was observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Cell treatment with LPS-induced oxidative/nitrosative stress as assessed by flow cytometry as the fluorescence of 2′,7′-dichlorofluorescein. This effect was significantly inhibited in a dose-dependent manner by ECR. Administration of ECR caused a dose-dependent inhibition of cytochrome P450 2E1, inducible nitric oxide synthase, tumor necrosis factor α, and interleukin-6 expression in LPS-treated cells. The present study demonstrates that extracts of *Citrus reticulata* leaves are safe, having antioxidant, anti-inflammatory, and analgesic effects both *in vivo* and *in vitro*.

Keywords Analgesic activity · Anti-inflammatory · Antioxidant · *Citrus reticulata* · Huh7 cells

Résumé Les citrus sont cultivés et consommés largement. Les citrus ont été étudiés pour leur activité pharmacologique et leurs avantages sur la santé humaine. Ces bienfaits incluent l'activité antibactérienne, analgésique, anti-inflammatoire et antitumorale. Cette étude a été conçue pour évaluer l'effet analgésique et l'acticité antioxydante et anti-inflammatoire des extraits des feuilles de *Citrus reticulata* Blanco (ECR) dans des modèles cellulaires et animaux. Les activités antioxydante, anti-inflammatoire et antinociceptive ont été évaluées chez des souris en utilisant l'inhibition du radical DPPH, l'œdème de l'oreille induit par le xylène, test de formaline et *writhing reponse* par l'acide acétique. L'expression des gènes anti-inflammatoires a été évaluée dans les cellules Huh7 traitées par le lipopolysaccharide (LPS). L'ECR a montré une activité antiradicalaire vis-à-vis du 1,1-diphenyl-2-picryl-hydrazyl (DPPH). Aucun changement de comportement ni de décès n'ont été observés chez les souris à des doses inférieures à 2 000 mg/kg de poids corporel. Les différentes concentrations des extraits aqueux et méthanoliques (100–500 mg/kg poids corporel) ont réduit la durée de léchage dans la deuxième phase de l'essai de la nociception chimique de formaline et ont diminué le nombre de torsions induites par l'acide acétique chez les souris, ce qui indique une activité antalgique significative. L'ECR a également diminué l'œdème de l'oreille induit par le xylène chez les souris, ce qui suggère une action anti-inflammatoire in vivo. Aucune toxicité de l'ECR dans la

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gamme de 0,1–10 µg/ml n'a été observée avec le bromure de 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT). Le traitement des cellules avec du LPS induit le stress oxydatif/nitrosatif tel qu'évalué par la cytométrie en flux en tant que fluorescence de 2′,7′-dichlorofluorescéine. Cet effet a été significativement inhibé par l'ECR de manière dose-dépendante. L'administration d'ECR a provoqué une inhibition dose-dépendante du cytochrome P450 (CYP) 2E1, de l'oxyde nitrique synthase inductible (iNOS), du facteur de nécrose tumorale (TNF)α et de l'expression de l'interleukine (IL)-6, dans les cellules traitées par le LPS. La présente étude démontre que les extraits des feuilles de *Citrus reticulata* ne sont pas dangereux, et possèdent des effets antioxydants, anti-inflammatoires et analgésiques in vivo et in vitro.

Mots clés Activité analgésique · Anti-inflammatoire · Antioxydante · *Citrus reticulata* · Cellules Huh7

Abbreviations

CYP : cytochrome
DCF : 2′,7′-dichlorofluorescein
DCFH-DA : 2′,7′-dichlorodihydrofluorescein diacetate
DPPH : 1,1-diphenyl-2-picrylhydrazyl
ECR : *Citrus reticulate* Blanco leaves extracts
GAE : gallic acid equivalents
IL : interleukin
iNOS : inducible nitric oxide synthase
LPS : lipopolysaccharide
MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB : nuclear factor kappa B
NSAIDs : nonsteroidal anti-inflammatory drugs
RNS : nitrogen species
ROS : reactive oxygen
TNF : tumor necrosis factor

Introduction

Excess of reactive oxygen species not adequately balanced by antioxidant defense systems may cause oxidative stress and result in damage of many biological molecules, including proteins, fatty acids or deoxyribonucleic acid. Oxidative stress is considered a pathogenic mechanism contributing to aging and to the development of different chronic diseases and frequently is associated to inflammation and pain [1]. Inflammation is a frequent finding in cardiovascular diseases, cancer or neurodegenerative diseases [2]. Although nonsteroidal anti-inflammatory drugs are widely available, their use may result in undesirable effects, such as gastrointestinal, renal or cardiovascular damage [3]. Effects on the inflammatory response of extracts and molecules obtained from edible medicinal plants, with none or little toxicity, have been tested in recent years. Bioactive components such as flavonoids, steroids, polyphenols, coumarins, terpenes, and alkaloids have shown a wide range of antioxidant, anti-inflammatory, or analgesic properties [4,5].

Citrus species are cultivated in many countries, particularly in the Mediterranean region [6], being fruits widely consumed, which are also used as food supplement and additives [7]. Infusion of citrus leaves is used in view of their medicinal properties [8], and it has been reported that flower, fruit, pulp, peel and leaf of different *Citrus* species have beneficial pharmacological activities including antibacterial, vascular protectant, antispasmodic, analgesic, antipyretic, anti-inflammatory and antitumoral effects [9–12] These therapeutic properties are attributed to ingredients such as vitamin C, essential oil, flavonoids and other bioactive components. Among these components, *Citrus* flavonoids seem to play an important role for human health and have beneficial effects in the prevention of human diseases [13].

Citrus reticulata Blanco, known as mandarin, is a plant that belongs to the genus *Citrus* of the Rutaceae family. Different studies from this species have shown an antifungal activity of their essential oils [14], a high inhibitory effect of flavonoid extracts on fatty acid synthase [15], an apoptotic effect of extracts on cancer gastric cells [16], a decrease of intestinal tumor growth in mouse models [17] and a suppression by extracts of nitric oxide production in RAW 264.7 macrophage cells [18,19]. The main active ingredients are flavonoids, with reported antioxidant and anti-inflammatory properties in different experimental models [20,21]. Generally, the leaves of different *Citrus* are used as by-products [22]. It is important to exploit the perennial foliage properties of *Citrus reticulata* and its availability in pharmaceutical preparation and food, in order to replace synthetic compounds, which are being restricted due to their potential risks and toxicity. However, information on the beneficial effects and possible toxicity of *Citrus reticulata* extracts is scarce. In this respect, anti-inflammatory capacity of peel has been described in rats [23], and antagonist effects of leaves on angiotensin II activity have been shown in mice [8]. No study has focused on the anti-inflammatory and analgesic effect of *Citrus* leaves.

This research was designed to examine the content of phenolic and flavonoid compounds, the acute toxicity, the analgesic effect, and the antioxidant and anti-inflammatory activities of leaves extracts from *Citrus reticulata* in *in vivo* (mice) and *in vitro* (Huh7 cells) experimental models.

Materials and methods

Chemicals

Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/F12 medium, fetal calf serum, gentamycin and Trizol reagent and were obtained from Life Technologies (Carlsbad, CA, USA). TaqMan® Gene Expression Assays and High-Capacity cDNA Archive Kit were provided by Applied Biosystems (Weiterstadt, Germany). Aspirin and indomethacin were provided by the Research and Development Center of SAIDAL (Algiers–Algeria). All other chemicals used in the study were obtained from Sigma Chemical Co. (St. Louis, MO).

Plant material and preparation of crude extracts

To have representative samples, the leaves of *Citrus reticulata* were collected from 15 trees located at three different gardens in Bejaia (North Algeria). Leaves were dried for 2 weeks in free air and in dark, and powdered. The powder was conserved in glass bottle at ambient temperature until use.

Citrus reticulata leaves powder was soaked in 80% aqueous-methanol with a ratio of plant material and extracting solvent of 1:10 w/v, under agitation overnight at 4 °C. The extract was filtered on filter paper, and then, on sintered glass, to obtain the first filtrate. This procedure was repeated on the residue using 50% aqueous-methanol under agitation for 4 h to obtain the last filtrate. The first and the last filtrates were combined, then methanol was removed under reduced pressure on a rota vapor below 45 °C. The resulting extract was considered the methanolic extract.

Water extraction was carried out according to the method of Gülçin et al. [24]. Briefly, 25 g of citrus leaves powder was placed in a beaker glass and mixed with 500 ml of boiling water followed by magnetic stirring for 15 min; the extract was then filtered over Whatman filter paper and evaporated as mentioned above. Dried crude extracts were weighed and stored at 4 °C for further experiments.

Total phenolic content

The amount of total phenolic compounds in the citrus extract was determined with the Folin–Ciocalteu's reagent according to Li et al. [25]. Briefly, 200 µl of appropriate dilution of each extract was added to 1 ml of 1:10 diluted Folin–Ciocalteu's reagent. After 4 min, the reaction mixture was neutralized with 800 µl of saturated sodium carbonate (75 g/l). The absorbance was measured at 765 nm after 2 h incubation at room temperature. Total phenolic content was determined in triplicate. Gallic acid (20–140 mg/l) was used for the standard calibration curve. Results were expressed as milligrams of gallic acid equivalents per gram dry matter.

Total flavonoid content

Total flavonoid compound was measured by an aluminium chloride colorimetric assay [26]. Briefly, 1 ml of each extract was added to equal volumes of a solution of 2% $AlCl_3$. The mixture was vigorously shaken, and absorbance was read at 430 nm after incubation in dark at room temperature for 10 min. Quercetin (1–40 mg/l) was used as standard for calibration curve. Total flavonoid compounds of extracts were expressed as mg quercetin equivalent/g dry matter.

Determination of DPPH radical inhibition

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical inhibition was measured according to Cuendet et al. [27] with slight modifications. Briefly, 50 µl of various dilutions of each extract or standards was mixed with 1250 µl of a 0.004% methanol solution of DPPH. After an incubation period of 30 min in dark at room temperature, the absorbance of the samples was read at 517 nm. Ascorbic acid, quercetin and rutin were used as standards. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity. All tests were run in triplicate and the mean values calculated. The ability to scavenge the DPPH radical was calculated by using the following equation:

$$Scavenging\ effect\% = \frac{A_c - A_e}{A_c} \times 100$$

where Ac is control absorbance and Ae is absorbance in the presence of extracts.

Animals

Swiss albino female or male mice weighing 18–23 g, provided by the animal house of Research and Development Center of SAIDAL, were housed in transparent plastic cages and were acclimatized for 7 days under standard laboratory conditions at 25 ± 2 °C and 50 ± 10% room humidity, with a 12/12-h light/dark cycle and free access to food and water. All animal studies were approved by the local Animal Ethics Committees in accordance with the policies and procedure details in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23 1985). Oral treatments were given in a volume of 10 ml/kg to mice. All the test compounds were solubilized in physiological saline.

Evaluation of acute toxicity

In order to determine acute toxicity, the study followed the method of Mota et al. [28]. Animals were kept fasting

overnight. Different concentrations of aqueous and methanolic extracts of *Citrus reticulata* (500, 1000, and 2000 mg/kg body wt.) were administered by oral gavage to six groups of mice (n = 10 each), the control group receiving normal saline solution (0.9% NaCl). No food or water was given up to 4 h after experimental treatment. Mice were closely observed during the first 4 h after the administration of the treatments, and then once daily during the following 14 days.

Xylene-induced ear edema

The *in vivo* anti-inflammatory activity of extracts was evaluated in xylene-induced mouse ear edema. The study was conducted according to the method of Xu et al. [29]. Briefly, 0.03 ml of xylene was applied to the anterior and posterior surfaces of the right ear to eight groups of mice (n = 6 each). The left ear was considered as control. One hour before xylene application, the aqueous and methanolic extracts of *Citrus reticulata* were administered orally (100, 250, and 500 mg/kg body wt. in physiological saline) to the treated groups; reference group received indomethacin (50 mg/kg body wt.) and the control group received saline. Thirty minutes after xylene application, mice were killed by cervical dislocation and a 7-mm-diameter disc from both ears was removed with a metal punch and weighed. Ear edema was calculated by subtracting the weight of the untreated left ear section from that of the treated right ear section, and was expressed as edema weight. Inhibition percentage was expressed as a reduction in weight with respect to the control group.

Percent inhibition = (Δ control–Δ treated/Δ control) × 100

Formalin-induced nociception in mice

The antinociceptive activity of *Citrus reticulata* was assessed using the formalin assay [28]. Testing or data recording were performed by an observer unaware of the treatment in each experiment. Eight different groups of mice (n = 6 each) were treated orally with saline (control) extracts of *Citrus reticulata* leaves (100, 250, and 500 mg/kg body wt.) and aspirin (5 mg/kg body wt.). After 60 min of oral treatment, 20 µl of 1% formalin was injected into the subplantar space of the right hind paw and then the animals were placed in a transparent plastic observation cage; the amount of time that the animal spent licking the injected paw was considered as indicative of pain. Two distinct phases of intensive licking activity were identified: an early phase and a late phase (0–5 and 15–45 min after formalin injection, respectively). These phases represented neurogenic and inflammatory pain responses, respectively [28]. Per cent inhibition of nociception during first and second phases was determined by comparing the paw licking time with the respective control group.

Acetic acid-induced writhing in mice

The abdominal writhing responses induced by intraperitoneal injection of 0.8% acetic acid (10 ml/kg body wt.), including contraction of the abdominal muscle and stretching of the hind limbs, were measured according to procedures described by De la Puente et al. [30]. Mice were divided into eight different groups (n = 6 each), which were pretreated orally with saline (negative control) or different doses of extracts of *Citrus reticulata* (100, 250, and 500 mg/kg body wt.) or (5 mg/kg body wt.) of aspirin (positive control). After 30 min of the oral treatment, acetic acid was administered through intraperitoneal route. Immediately after the challenge, mice were individually placed into open transparent plastic cages, and the abdominal writhing responses were counted cumulatively during 30 min. Per cent inhibition of the number of writhing movements was calculated according to:

Per cent Inhibition = (number of writhes control–number of writhes test/number of writhes control) × 100

and compared with negative control.

Cells, cell culture, and treatment protocols

To establish the anti-inflammatory and antioxidant capacities of *Citrus reticulata* leaves in an *in vitro* model we used Huh7 cells treated with LPS. Huh7 cells were grown at 37 °C with a 5% CO_2 atmosphere in DMEM/F12 medium, supplemented with 10% fetal calf serum and 50 mg/mL gentamycin. In order to prevent phenotypic drift, the cultures were used for only 8–10 weeks before reverting to frozen stocks from an early passage. The medium was supplemented with or without LPS (20 µg/ml) and different concentrations of the aqueous extract of *Citrus reticulata* leaves (0.5, 1, 5, and 10 µg/ml) for 16 h to evaluate its effect on oxidative/nitrosative stress.

Cell viability in cell culture

The cell viability was assessed by the mitochondrial function, measured by MTT reduction activity for measuring cell proliferation and cytotoxicity as previously reported [31]. Briefly, cells were seeded in a 24-well plate and incubated with the different treatments. After that, the cells were treated with 0.5 mg/mL MTT for 2 h at 37 °C. Subsequently, the media were aspirated and the cells were lysed with dimethyl sulfoxide, where after the absorbance was read at 560 nm, with background subtraction at 650 nm, using a microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Generation of reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (ROS/RNS) production in cultured cells were analyzed by flow cytometry. The ROS

and RNS production were assessed as the fluorescence of 2′,7′-dichlorofluorescein (DCF), which is the oxidation product of DCFH-DA with a sensitivity for H_2O_2/NO-based radicals [32]. Briefly, cell monolayers were washed twice with phosphate buffered saline (PBS) and incubated with 5 μM for DCFH-DA for 30 min at 37 °C, then washed twice, resuspended in PBS and analyzed on a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Fluorescence of 10,000 cells was assessed using the Cell Quest software (Becton Dickinson Biosciences) [33].

Quantitative real-time PCR

Total RNA was obtained by using a Trizol reagent. First-strand cDNA was synthesized using High-Capacity cDNA Archive Kit. For gene expression assays, cDNA was amplified using multiplex real-time PCR reactions on a StepOne Plus [34] (Applied Biosystems). TaqMan primers and probes were derived from the commercially available TaqMan® Gene Expression Assays (Applied Biosystems; Table 1). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method [35]. The cycle number at which the transcripts were detectable (C_t) was normalized to the cycle number of glyceraldehyde-3-phosphate dehydrogenase detection, referred to as ΔC_t (PCR efficiency was determined by TaqMan analysis on a standard curve for targets and endogenous control amplifications that were highly similar).

Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical evaluation of differences was carried out by one-way analysis of variance and Newman–Keul's post-hoc test. A value of $p < 0.05$ was considered as being statistically significant.

Results

Total phenolic and flavonoid content

The content of total phenols and flavonoids in *Citrus reticulate* Blanco leaves extracts (ECR) is shown in Table 2. Data obtained indicate that total phenolic and flavonoid content was higher in the methanolic extract compared with the aqueous extract (+31% and +109%, respectively).

Determination of DPPH radical inhibition

As shown in Table 3, the half inhibitory concentration (IC_{50}) value for methanolic extracts of *Citrus reticulata* for 50% of DPPH was higher than the aqueous extract, while the value for quercetin, used as standard in this assay, was 1.92 μg/ml. Quercetin showed a free radical scavenging activity significantly higher than that of methanolic extract (66 folds) and the aqueous extract (56 folds).

Evaluation of acute toxicity

The acute toxicity test of ECR by oral administration in mice did not record any behavioral changes or deaths in mice at doses of 500, 1000, and 2000 mg/kg body wt. during the 14 days observation; as the result, the LC50 was higher than 2000 mg/kg.

Table 1 Primers and probes used for the quantitative reverse transcription PCR.

Gene	Genbank	Assay ID	Amplicon size	Dye
CYP2E1	NM_000773.3	Hs00559367_m1	71	FAM™
iNOS	NM_000625.4	Hs00167248_m1	74	FAM™
IL-6	NM_000600.3	Hs00174131-M1	95	FAM™
TNF-α	NM_000594.3	Hs00174128_m1	80	FAM™
GAPDH	NM_002046.3	4326317E	122	VIC™

Abbreviations: CYP2E1, cytochrome P450 2E1; iNOS, inducible nitricoxide synthase; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Gene = gène; Genbank = Genbank; Assay ID = Assay ID; Amplicon = Amplicon; Size = taille; Dye = marqueur fluorescent; CYP2E1 = CYP2E1; iNOS = iNOS; IL-6 = IL-6; TNFα = TNFα; GAPDH = GAPDH; CYP2E1: cytochrome P450 2E1 = CYP2E1 : cytochrome P450 2E1.
iNOS: inducible nitricoxide synthase = iNOS : oxide nitrique synthase inducible; IL-6: interleukin-6 = IL6 : interleukine-6; TNFα: tumor necrosis factor alpha = TNFα : facteur de nécrose tumorale alpha; GAPDH: glyceraldehyde-3-phosphate dehydrogenase = GAPDH : glycéraldéhyde-3-phosphate déshydrogénase; FAM™ = FAM™; VIC™ = VIC™.

Table 2 Extraction yield, total phenolic and flavonoid content of *Citrus reticulate* leaves.

Extract	Extraction yield (%)	Total phenols (mg GAE/g DM)	Flavonoids (mg QE/g DM)
Methanolic	20.90 ± 0.95	21.45 ± 0.32	1.38 ± 0.13
Aqueous	23.16 ± 0.38	16.39 ± 0.32*	0.66 ± 0.02*

Abbreviations: GAE, gallic acid equivalents; QE, quercetin equivalents, DM, dry matter.
Values are means ± standard error of the mean.
*$p < 0.05$ compared to the methanolic extract.
Extract = Extrait; Extraction yield (%) = rendement d'extraction (%); Total phenols (mg GAE/g DM) = phénols totaux (mgEAG/g MS); flavonoids (mg QE/g DM) = flavonoïdes (mg EQ/G MS); methanolic = méthanolique; aqueous = aqueux; GAE: gallic acid equivalents = EAG: équivalents acide galique; QE: Quercetin equivalents = EQ: équivalents quercetine; DM: dry matter = MS: matière sèche; *$p < 0.05$ compared to the methanolic extract = *p <0,05 par rapport à l'extrait méthanolique.

Table 3 DPPH scavenging activity of *Citrus reticulata* extracts and standards.

		IC_{50} (µg/ml)
Extracts	Methanolic	128.20 ± 1.10*
	Aqueous	107.33 ± 0.97*,**
Standards	Quercetin	1.92 ± 0.09
	Ascorbic acid	2.89 ± 0.07
	Rutin	4.62 ± 0.10

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; IC_{50}, half inhibitory concentration.
Values are means ± standard error of the mean. IC_{50} values are concentration of extract or standard necessary to decrease the initial concentration of DPPH by 50%. *$p < 0.001$ compared to quercetin; **$p < 0.05$ compared to methanolic extract.
IC_{50} (µg/ml) = IC_{50} (µg/ml); extracts = extraits; methanolic = méthanolique ; aqueous = aqueux; standards = standards; quercetin = quercétine; ascorbic acid = acide ascorbique; rutin = rutine; *$p < 0.001$ compared to quercetin = *p < 0,001 par rapport à la quercétine; **$p < 0.05$ compared to methanolic extract = **p < 0,05 par rapport à l'extrait méthanolique.

Xylene induced ear edema

The results presented in Figure 1 show that the methanolic extract did not demonstrate any significant anti-inflammatory activity; only the different concentrations of

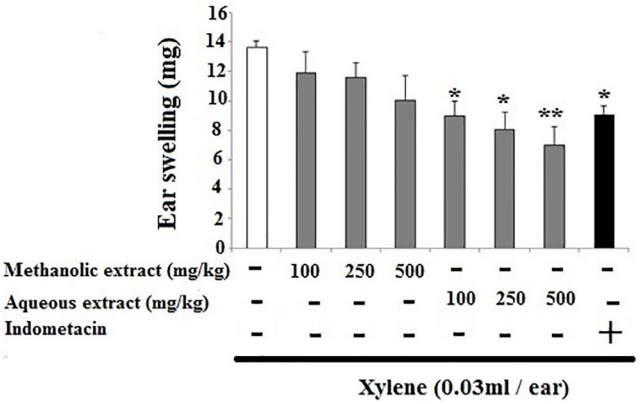

Fig. 1 Effect of *Citrus reticulata* Blanco leaves extract and indomethacin on xylene-induced mouse ear swelling The increase in weight caused by xylene represents the ear swelling that was measured by subtracting the weight of the untreated left ear section from that of the treated right ear sections Each bar represents the mean ± standard error of the mean ($n = 6$). *$p < 0.05$; **$p <$ 0.01 versus control Ear swelling (mg) = œdème de l'oreille (mg); methanolic extract (100, 250, 500 mg/kg) = extrait méthanolique (100, 250, 500 mg/kg); aqueous extract (100, 250, 500 mg/kg) = extrait aqueux (100, 250, 500 mg/kg); indometacin = indométacine; xylene (0.03 ml/ear) = xylène (0,03 ml/oreille)

the aqueous extract (100, 250, 500 mg/kg body wt.) and indomethacin (50 mg/kg) exerted a significant reduction in ear swelling induced by xylene (–39%, –41%, –49%, and –21%, respectively), with a significant dose-dependent effect of ECR.

Formalin-induced nociception in mice

The time spent licking the injected paw was recorded in the early phase (0–5 min) and late phase (15–45 min) after the subcutaneous injection of formalin (Fig. 2). No statistically significant differences were found in the total amount of licking during the early phase between the extracts, aspirin and vehicle-treated groups; however, during the late phase all concentrations of aqueous and methanolic extracts resulted in a significant decrease of the time spent by mice in licking the hind paw (aqueous extract, 100: –7%, 250: 81%, 500: –98%; methanolic extract, 100: –70%, 250: –81%, 500: –100%). At higher doses of aqueous and methanolic extracts the antinociceptive effect was similar to that produced by the positive control aspirin (–100%).

Acetic acid-induced writhing in mice

As shown in Figure 3, the treatment of mice with ECR (100, 250, 500 mg/kg body wt.) produced a dose-dependent significant reduction of acetic acid-induced abdominal writhing

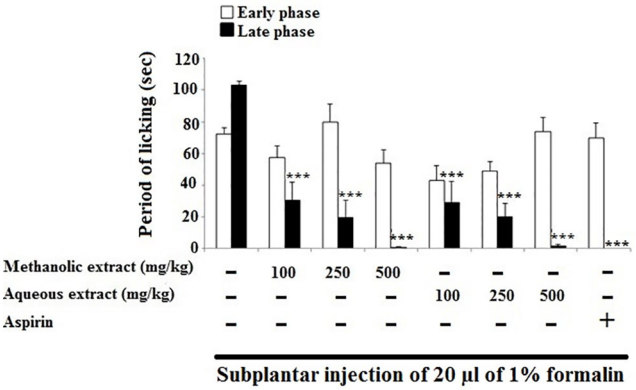

Fig. 2 Antinociceptive effect of *Citrus reticulata* Blanco leaves extract (100–500 mg/kg body wt.) and aspirin on the formalin test induced paw licking response in mice Each bar represents the mean ± standard error of the mean of eight groups for six mice ***$p < 0.001$ compared to control Period of licking (sec) = pèriode de léchage (sec); methanolic extract (100, 250, 500 mg/kg) = extrait méthanolique (100, 250, 500 mg/kg); aqueous extract (100, 250, 500 mg/kg) = extrait aqueux (100, 250, 500 mg/kg); aspirin (5 mg/kg) = aspirine (5 mg/kg); early phase = phase précoce; late phase = phase tardive; subplantar injection of 20 µl of 1% formalin = injection sous-plantaire de 20 µl de 1 % formol

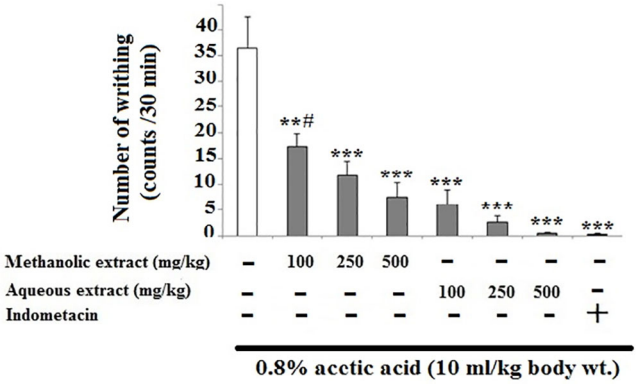

Fig. 3 Antinociceptive effects of *Citrus reticulata* Blanco leaves extract (100–500 mg/kg body wt.) and aspirin on acetic acid-induced abdominal writhing in mice Each bar represents the mean ± standard error of the mean ($n = 6$) **$p < 0.01$; ***$p < 0.001$ versus control; $^{\#}p < 0.05$ compared to aspirin Number of writhing (counts/30 min) = nombre de crampes (comptage/30 min); methanolic extract (100, 250, 500 mg/kg) = extrait méthanolique (100, 250, 500 mg/kg) = aqueous extract (100, 250, 500 mg/kg) = extrait aqueux (100, 250, 500 mg/kg); aspirin = aspirine; 0.8% acetic acid (10 ml/kg body wt.) = 0,8 % acide acétique (10 ml/kg p. corporel)

in mice (aqueous extract: –83%, –93% and –99%; methanolic extract: –23%, –68%, and –79%, respectively). A similar reduction of the writhing number by the aqueous extract and aspirin was observed (5 mg/kg) (99%).

Huh7 cell viability

Cell viability was assessed by the MTT test. Incubation for 24 h with LPS and/or 0.5, 1, 5, and 10 µg/ml of *Citrus reticulata* aqueous extract did not significantly decrease cell viability (Fig. 4). Accordingly, these concentrations of aqueous ECR were used for *in vitro* experiments.

ROS/RNS generation in LPS-treated Huh7 cells

We investigated ROS and RNS generation by flow cytometry using DCFH-DA. Analysis of histograms in which the fluorescence, detected with the green fluorescence (FL1-H) channel, was plotted against the relative number of events and quantification of the corresponding fluorescence intensity indicated that LPS induced a significant increase in ROS and RNS production as compared with unstressed control cells (+73%) (Fig. 5). Supplementation of LPS-treated cells with aqueous ECR at 0.5, 1, 5, and 10 µg/ml significantly decreased ROS and RNS production in a dose-dependent manner (–19%, –24%, –44%, and –48%, respectively, vs. LPS-treated cells).

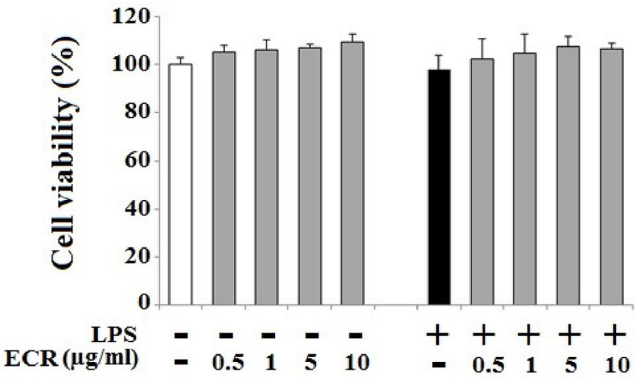

Fig. 4 Effect of *Citrus reticulata* Blanco leaves aqueous extract (ECR) on cell viability in Huh7 cells. Cells were incubated with lipopolysaccharide (LPS) and 0.5, 1, 5, and 10 µg/ml of aqueous citrus extract. Cell viability was determined by the MTT assay. Data represent means ± standard error of the mean from four separate experiments Cell viability (%) = viabilité cellulaire (%); LPS = LPS; ECR (0.5, 1, 5, 10 µg/ml) = ECR (0,5, 1, 5, 10 µg/ml)

Expression of oxidative/nitrosative stress and inflammation-related genes in LPS-treated Huh7 cells

To investigate the contribution of ECR antioxidant and anti-inflammatory capacities to the reduction of ROS/RNS production at a molecular level, we assessed the effects on mRNA expression of cytochrome (CYP) 2E1, tumor necrosis

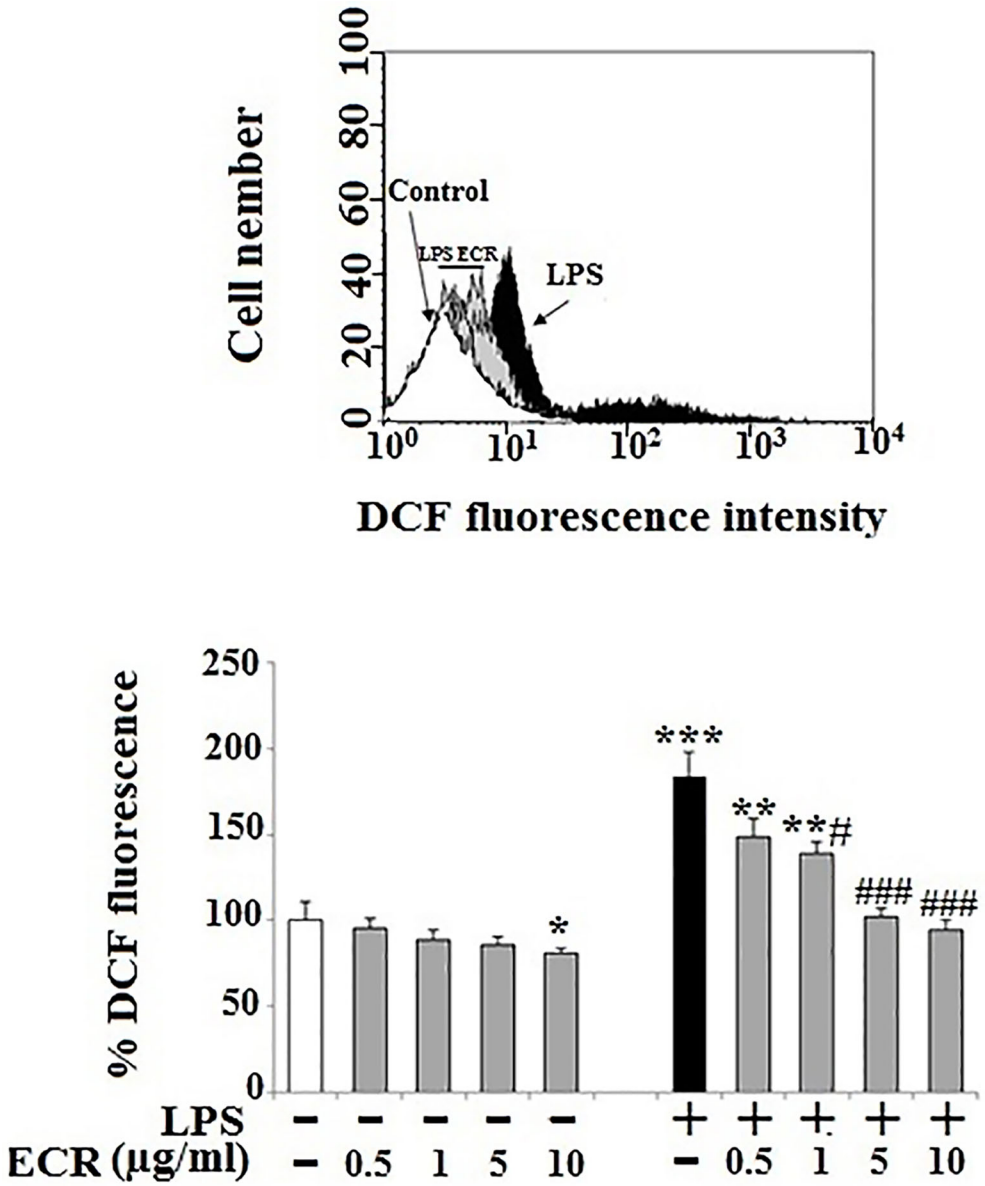

Fig. 5 Effect of *Citrus reticulata* Blanco leaves aqueous extract on intracellular reactive oxygen species and reactive nitrogen species generation in Huh7 cells measured by flow cytometry with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) Cells were incubated with lipopolysacharride and 0.5, 1, 5, and 10 µg/ml of aqueous citrus extract. (A) Representative histograms of DCF fluorescence. The fluorescence (FL1-H) is plotted against the number of cells. (B) Fluorescence intensity as percentage of control values. Data represent means ± standard error of the mean from four separate experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control cells, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus LPS-treated cells Cell number = nombre de cellule; DCF fluorescence intensity = intensité de fluorescence DCF; control = contrôle; LPS ECR = LPS ECR; LPS = LPS % DCF fluorescence = % fluorescence DCF; LPS = LPS; ECR (0.5, 1, 5, 10 µg/ml) = ECR (0,5, 1, 5, 10 µg/ml)

factor (TNF)-α, inducible nitric oxide synthase (iNOS) and interleukin (IL)-6. As shown in Figure 6, LPS treatment caused a significant upregulation of these oxidative and proinflammatory genes (CYP2E1: +33%, iNOS: +53%, TNF-α: +188%, and IL-6: +168%, vs. untreated cells) that was effectively attenuated by treatment with aqueous ECR (CYP2E1, 0.5: –15%, 1: –25%, 5: –35%, 10: –37%; iNOS, 0.5: –63%, 1: –59%, 5: –71%, 10: –71%; TNF-α, 0.5: –45%, 1: –49%, 5: –47%, 10: –46%; and IL-6, 0.5: –10%, 1: –12%, 5: –23%, 10: –26%, vs. LPS-treated cells). Inhibition of iNOS and TNF-α gene expression by aqueous ECR was significantly stronger for stressed LPS cells when compared with unstressed control cells (iNOS, 0.5: –35%, 1: –43%, 5: –50%, 10: –67% and TNF-α, 5: –34%, 10: –42% vs. untreated control cells).

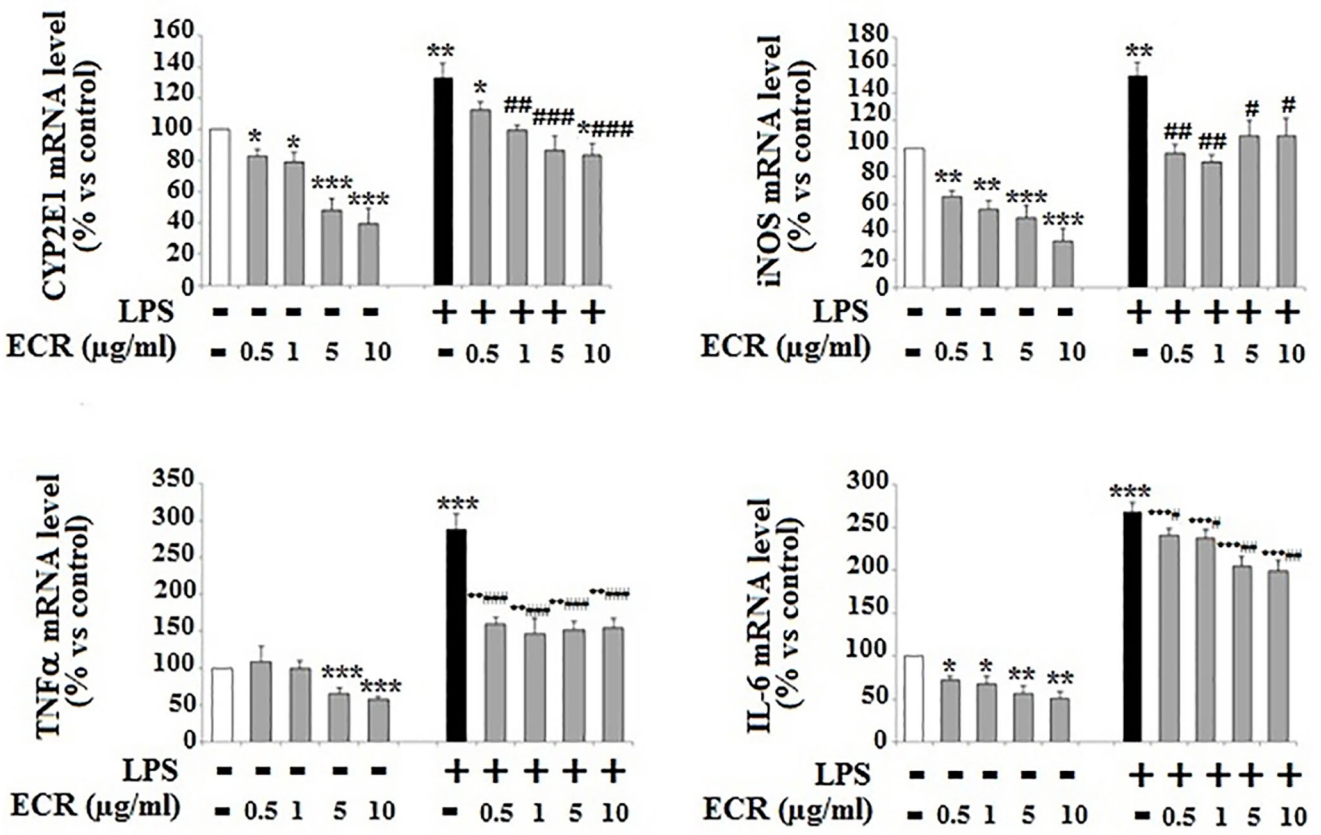

Fig. 6 Effect of *Citrus reticulata* Blanco leaves aqueous extract on mRNA level of CYP2E1, iNOS, TNF-α, and IL-6 as quantitated by real time polymerase chain reaction analysis in Huh7 cells Cells were incubated with LPS and 0.5, 1, 5, and 10 µg/ml of citrus extract. Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. Data, normalized against GAPDH, are presented as percentage of control values and represent means ± standard error of the mean from four separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus control cells, $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus LPS-treated cells. CYP2E1, cytochrome P450 2E1; iNOS, inducible nitricoxide synthase; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; LPS, lipopolysacharride; GAPDH, glyceraldehyde 3-phosphate dehydrogenase CYP2E1 mRNA level (% vs control) = niveau de RNAm CYP2E1 (% vs contrôle); LPS = LPS; ECR (0.5, 1, 5, 10 µg/ml) = ECR (0,5, 1, 5, 10 µg/ml) iNOS mRNA level (% vs control) = niveau de RNAm iNOS (% vs contrôle); LPS = LPS; ECR (0.5, 1, 5, 10 µg/ml) = ECR (0,5, 1, 5, 10 µg/ml) TNFα mRNA level (% vs control) = niveau de RNAm TNFα (% vs contrôle) LPS = LPS; ECR (0.5, 1, 5, 10 µg/ml) = ECR (0,5, 1, 5, 10 µg/ml) IL-6 mRNA level (% vs control) = niveau de RNAm IL-6 (% vs contrôle); LPS = LPS; ECR (0.5, 1, 5, 10 µg/ml) = ECR (0,5, 1, 5, 10 µg/ml)

Discussion

Results obtained show an effect of solvent extraction on the concentration of total phenolic and flavonoid compounds in ECR, indicating that methanol is a more efficient solvent to extract phenolic and flavonoid compounds compared to boiling water. Our data are in agreement with differences reported between the concentration of phenolic and flavonoid compounds of methanol, ethanol and hot water extracts from *Citrus aurantium* [36]. The effect of extraction solvents on the concentration of black and black mate tea polyphenols has also been investigated [37], showing that 80% methanol was more efficient than water for extracting total phenolics. Water extracts have a quantity of impurities (organic acids, glucides and soluble proteins), which may affect the determination of polyphenols [38].

The scavenging model of DPPH free radical is often used as an indicator to test antioxidant activity of plant extracts [39]. In our study, the methanolic extract showed a lower DPPH-free radical scavenging activity compared to the aqueous extract. However, the standards, quercetin, ascorbic acid and rutin exhibited a higher DPPH radical scavenging effect than both ECR. Our results differ from a previous report that the DPPH-free radical scavenging activity of *Citrus aurantium* Bloom methanolic extract was higher than that of boiling water [36]. It has been reported that the radical

scavenging activity of citrus extracts depends on the amount of polyphenolic and flavonoid compounds [40]. However, in our study, the methanolic extract scavenging activity was lower than that of the aqueous extract, despite the fact that the last contains the lowest amount of polyphenols and flavonoids. This could be explained by the presence of other compounds in the aqueous extract, in addition to polyphenols and, also, to their specific antioxidant activity. Moreover, synergy existing between different compounds should be taken into consideration for the biological activity [41].

To test the analgesic effects of extracts, the formalin-induced paw licking and acetic acid-induced writhing methods were used [42]. The test of formalin employs a chemical nociceptive stimulus that elicits a spontaneous behavior indicative of pain in two phases. The early phase (0–5 min after formalin injection) is due to a direct effect on nociceptors in the paw and reflects central pain. Substance P and bradykinin participate in this phase, which is believed to be noninflammatory pain, while the late phase (15–45 min after formalin injection) is an inflammatory response with a release of prostaglandins and nitric oxide [43,44]. Centrally acting drugs are antinociceptive in both phases, while peripherally acting drugs inhibit only the second phase [19]. In our work, the inhibitory effect of formalin-induced licking time by the extracts was observed only in the late phase. Similarly to nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, which suppress only the second phase [45], ECR seems to act independently from the central system; this antinociceptive activity may be due to the influence of compound extracts on peripheral mechanisms with inhibition of the production or action of inflammatory mediators.

ECR was also able to reduce the number of acetic acid-induced writhes in mice in a dose-dependent manner, similarly to aspirin. Administration of acetic acid leads to stimulation of local peritoneal receptors f and stimulates peripheral nociceptive neurons indirectly, by inducing the release of endogenous mediators, which excites pain endings [46]. TNF-α and interleukins released by resident peritoneal macrophages and mast cells mediate the release of eicosanoids, and the increase in prostaglandin levels contributes to the onset of inflammatory pain by the activation of chemosensitive nociceptors, increasing capillary permeability [28,29]. Results from the writhing test are in agreement with those obtained from the formalin test, suggesting a peripherally mediated analgesic activity of ECR to achieve pain relief.

The anti-inflammatory effect of ECR was studied *in vivo* using the xylene-induced ear edema in mice and indomethacin as standard reference drug. This is a preliminary and simple model for screening potential anti-inflammatory drugs, especially those inhibiting phospholipase A_2 [47]. Xylene acts on sensory neurons that release substance P and calcitonin gene-related peptide CGRP into the periphery, leading to fluid accumulation and edema [29,48]. We found that the methanolic extract induced no significant decrease in ear swelling compared to control, while the aqueous extract resulted in a significant dose-dependent ear section weight decrease.

It is known that LPS causes liver injury, inducing inflammation and oxidative and nitrosative stress in *in vivo* and *in vitro* models [49]. Several studies show that LPS-treated human hepatocyte and hepatoma cell lines such as Huh7 cells are appropriate *in vitro* models to analyze the potential antioxidant and anti-inflammatory activities of different extracts and natural compounds derived from medicinal plants [50,51]. Thus, LPS-treated Huh7 cells were used as an adequate *in vitro* model of oxidative stress and inflammation. Aqueous extract was studied according to its greater DPPH-free radical scavenging activity and *in vivo* antinociceptive and anti-inflammatory capacities in comparison with the methanolic extract. Results obtained showed an absence of toxicity by MTT assay at any of the concentrations studied, and an inhibitory effect of aqueous ECR on oxidative/nitrosative stress and inflammation.

Different studies have demonstrated that LPS triggers the synthesis of ROS/RNS such as superoxide and or nitric oxide, and oxidative damage plays a role in its cytotoxicity [52,53]. By using flow cytometry techniques, we found increases in DCF fluorescence, indicating overproduction of peroxides, nitric oxide and peroxynitrite, when Huh7 cells were incubated with LPS. The increase of emitted DCF fluorescence reached levels significantly higher than those found in nontreated cells. When cells received ECR, a significant concentration-dependent decrease of ROS/RNS generation was observed. Nobiletin from citrus fruit peel has been shown to inhibit LPS-induced increase of DCF fluorescence in RAW 264.7 cells [54]. A similar effect of extracts from *Citrus bergamia* has been previously reported in human NCTC 2544 keranocytes treated with interferon-γ and histamine [10].

Among inflammatory mediators, iNOS, and COX-2 are important enzymes that regulate inflammatory processes. COX-2 catalyzes the rate-limiting step in the formation of prostaglandin E2, a critical pro-inflammatory molecule involved pain and immune response [55]. The inducible isoform of oxide nitric synthase (iNOS) is responsible for the overproduction of nitric oxide in inflammation and is induced in response to interferon-γ, LPS and a variety of pro-inflammatory cytokines [56]. TNF-α is a pleiotropic cytokine that can bind to receptors expressed in most tissues, triggering activation of nuclear factor kappaB (NF-κB), which in turn modulates many pro-inflammatory genes, including IL-6 [57]. Results indicate that mRNA levels of iNOS, COX-2, TNF-α, and IL-6 levels were markedly increased in Huh7 cells, and the effect that was significantly inhibited by ECR, in parallel to the lowering of DCF intensity. A large number of *in vitro* studies have shown anti-inflammatory properties of

fruit polyphenols, including flavones and flavones present in *Citrus* species. Thus, extracts from *Citrus bergamia* fruits containing different flavonoids reduce iNOS expression and nitric oxide concentration in human keranocytes [10], methanolic extracts of young fruits from *Citrus unshiu* inhibit iNOS expression in rat primary astrocytes [58], extracts from *Citrus reticulata* fruits suppress nitric oxide production by inhibiting NF-κB activation in macrophage RAW 264.7 cells [18] and crude methanolic extracts of *Citrus aurantium L*. modulate the expression of iNOS, COX-2, TNF-α, and COX-2 in the same cell type [59]. The citrus polymethoxy flavone nobiletin suppresses the IL-1-induced production of prostaglandin E2 and downregulates COX-2 in human synovial fibroblasts, or inhibits TNF-α and IL-6 expression in LPS-treated J774A.1 macrophages [60]. Nobiletin has also been reported to block activation of MAP kinases pathways, translocation of NF-κB, and the subsequent gene expression of iNOS, TNF-α, and IL-1β in BV-2 microglia cells [61]. Citrus nobiletin also suppresses iNOS expression in IL-1β-treated hepatocytes [62]. Naringenin attenuates LPS/interferon-c–induced activation of glial cells by inhibiting iNOS expression and nitric oxide production [63], and hesperetin and its metabolites suppress LPS-induced NF-κB activation and gene expression of iNOS and COX-2 in RAW 264.7 cells [64]. More recently, it has been shown that flavonoids present in tangerine peel (hesperidin, nobiletin, and tangeretin) are collectively responsible for the inhibition of TNF-α, IL-6, and IL-1β expression in LPS-activated BV-2 microglia cells [52]. Thus our data suggest that mechanisms involved in the anti-inflammatory effect of ECR would be similar to those previously reported in the literature.

An additional interesting finding concerns CYP2E1, a hepatic cytochrome P450 isoform that plays an important role in generation of ROS/RNS [65]. ROS/RNS produced by CYP2E1 and oxidative stress can promote the activation of NF-κB [66], which in turn results in overexpression of iNOS, COX-2, and pro-inflammatory cytokines. Data from our study indicate that treatment with ECR downregulates the expression of CYP2E1, which suggests that inhibition of cytochrome p450 genes may contribute to the protective effect against LPS-induced cell damage. It is known that LPS acts at transcriptional level to increase CYP2E1 mRNA expression in rats [67], and previous studies have demonstrated that inhibition of CYP2E1 by extracts from different medicinal plants may contribute to its free radical scavenging properties [68,69].

In conclusion, the present study showed that ECR possesses anti-inflammatory, analgesic and antioxidant activities both *in vivo* and *in vitro*, supporting previous claims of the traditional use for inflammation and pain. Further phytochemical and pharmacological experiments are required to segregate the active compounds responsible of these biological activities and to identify the molecular mechanisms involved.

Disclosures The authors declare that there are no conflicts of interest. A part of this study was supported by the Pharmaceutical Group Company SAIDAL ethic committee (Algiers, Algeria).

Perspective

This article presents the analgesic, antioxidant and anti-inflammatory activities of *Citrus reticulata* Blanco leaves extracts (ECR) both *in vivo* and *in vitro*. The current research supports previous claims of the traditional use of ECR and may be also helpful to develop novel nonpharmacological therapy for pain and inflammation.

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