A new acetophenone derivative from flowers of Helichrysum italicum (Roth) Don ssp. italicum

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A new acetophenone derivative from flowers of *Helichrysum italicum* (Roth) Don ssp. *italicum*

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ABSTRACT

A new acetophenone derivative named gnaphaliol 9-*O*-propanoate (**1**) was isolated from the chloroform fraction of EtOH extract of *Helichrysum italicum* ssp. *italicum* flowers along with the five known acetophenones 12-acetoxytremetone (**2**), 13-(2-methylpropanoyloxy)toxol (**3**), [2,3-dihydro-2-[1-(hydroxymethyl)ethenyl]-5-benzofuranyl]-ethanone (**4**), 1-[2-[1-[(acetyloxy) methyl]ethenyl]-2,3-dihydro-3-hydroxy-5-benzofuranyl]-ethanone (**5**) and gnaphaliol (**6**). The structures of compounds **1**–**6** were elucidated by extensive spectroscopic methods including 1D- (^{1}H and ^{13}C) and 2D-NMR (DQF-COSY, HSQC, HMBC, TOCSY and ROESY) experiments as well as ESIMS analysis. The isolated compounds were investigated for their cytotoxicity, anti-inflammatory and antioxidant properties. Biological assays on human colonic epithelial cells showed that compound **2** possessed antioxidant effects reducing reactive oxygen species (ROS) production.

1. Introduction

The genus *Helichrysum* (family Asteraceae, tribe Inuleae) consists of a few hundred species widespread throughout the world, but particularly distributed in the Mediterranean region [1], where plays an important role in traditional medicine. Particularly, its flowers and leaves are the most used parts in the treatment of health disorders such as allergies, colds, cough, skin, liver and gallbladder disorders, inflammation, infections and sleeplessness [2]. One of the species with more reported traditional uses is *Helichrysum italicum* (Roth) G. Don, a species rich in active compounds such as acetophenones, flavonoids and phloroglucinol derivatives [2]. Extracts obtained from *H. italicum* have been reported to possess remarkable anti-inflammatory and antioxidant properties [2–4]. These activities have been attributed to the presence of flavonoids such as 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside, kaempferol-3-glucoside, naringenin-glycoside (three glycosyl-flavonoids), gnaphaliin (a methoxyflavone), pinocembrin (a flavanone) and tiliroside (a flavonol acyl-glucoside) which have been found active in assays of antioxidant and anti-inflammatory activities [5,6]. *H. italicum* also contains non-flavonoid phenolics (pyrones, phloroglucinols, acetophenones) that are involved in its biological activities; indeed, arzanol, a pyrone-phloroglucinol etherodimer isolated from the aerial parts of *H. italicum* (ssp. *microphyllum*) has been demonstrated to exert a potent antioxidant activity protecting animal cells from lipid peroxidation [7,8].

In order to further identify the chemical compounds responsible for the above mentioned properties of *H. italicum*, and as a continuation of our previous research on this important Mediterranean medicinal plant [9], we performed a phytochemical analysis on the flowers of *H. italicum* ssp. *italicum* collected in Southern Italy, which resulted in the identification of six

Abbreviations: BHT, 2,6-Di-*tert*-butyl-4-methylphenol; DCFH-DA, 2-7-dichlorofluorescein diacetate; FBS, fetal bovine serum; LPS, lipopolysaccharides; NR, neutral red; PBS, phosphate buffered saline; ROS, reactive oxygen species.

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acetophenones, including one with a new chemical structure. The biological profile of some of these compounds has been investigated in assays of cytotoxicity, inflammation and inhibition of oxidative stress in cell culture.

2. Experimental

2.1. General experimental procedures

Optical rotations were determined on a Jasco P-1010 digital polarimeter. UV spectra were obtained on a Jasco 7800 UV–Vis spectrophotometer. IR measurements were obtained on a Bruker IFS-48 spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrometer (BrukerBioSpinGmBH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI Cryo Probe at 300 K. All 2D-NMR spectra were acquired in CD_3OD (99.95%, Sigma Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, and HMBC spectra. The NMR data were processed using UXNMR software. Exact masses were measured by a Voyager DE mass spectrometer. Samples were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDITOF) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18–39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard. ESIMS was obtained on Applied Biosystem API-2000 mass spectrometer. Merck Silica gel (70–230 mesh), deactivated with 15% H_2O, was used for column chromatography. Normal-phase HPLC was performed with a TSP SpectraSeries P100 instrument equipped with rheodyne injector and a refractive index detector, using a hypersil silica column (Thermo, 250 × 4.6 mm, flow rate 1.5 mL/min). Thin-layer chromatography (TLC) was performed on plates coated with silica gel 60 F_{254} Merck, 0.25 mm.

2.2. Chemicals

Hydrogen peroxide (H_2O_2), iron(II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red, NR), 2-7-dichlorofluorescein diacetate (DCFH-DA), lipopolysaccharides (LPS) from *Escherichia coli* serotype O111:B4, 2,6-Di-*tert*-butyl-4-methylphenol (BHT), dexamethasone, dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich (Milan, Italy). All reagents for cell culture were obtained from Sigma (Milan, Italy), Bio-Rad Laboratories (Milan, Italy) and Microtech Srl (Naples, Italy). All solvents (analytical, deuterated and HPLC grade) were obtained from Carlo Erba Reagenti (Milan, Italy).

2.3. Plant material

Flowers of *H. italicum* ssp. *italicum* were collected in July 2007 in San Potito Sannitico loc. Sardarulo (CE, Southern Italy). A voucher specimen (N° NAP# 23/06) has been deposited at the Herbarium Neapolitanum (NAP), Department of Biological Sciences of University "Federico II" of Naples (Italy).

2.4. Extraction and isolation

Air-dried and powdered flowers of *H. italicum* (235 g) were soaked with EtOH (3500 mL) three times at room temperature for 12 h. The ethanol extracts, concentrated under vacuum, afforded 18 g of a glassy material and were subjected to a Kupchan's partition procedure [10] modified as follows. The ethanol extract was dissolved in 10% aqueous methanol and partitioned against *n*-hexane. The water content (% v/v) of the MeOH was adjusted to 20 and 40% and partitioned against $CHCl_3$. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-butanol (*n*-BuOH). Three extracts were obtained as follows: *n*-hexane (0.85 g), $CHCl_3$ (2.45 g) and *n*-BuOH (5.15 g). All the extracts were submitted for biological testing (data not shown). The chloroform extract, which was shown to be the most active, was further purified to yield compounds **1**–**6** (Fig. 1). Particularly, the $CHCl_3$ extract was dissolved in $CHCl_3$ and then submitted to column chromatography on silica gel 60 Merck (70–230 mesh, deactivated with 15% H_2O), eluting with $CHCl_3$/MeOH (from 100:0 to 0:100 gradient), to afford 15 fractions of 250 mL each. The fractions 1–15, estimated by thin-layer chromatography (TLC) (eluent system petrol/EtOAc (1:1 vv), spray reagent $Ce(SO_4)_2$ in H_2SO_4), were opportunely gathered on the basis of their similar TLC

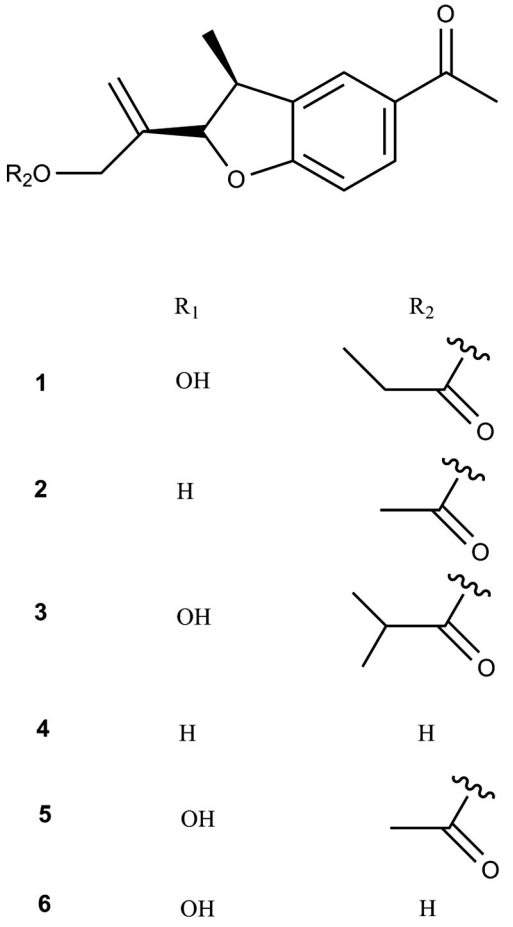

Fig. 1. Compounds isolated from *Helichrysum italicum* ssp. *italicum* flowers.

behavior giving the eight fractions A–H, that were submitted for biological testing. Fractions A, C, D and E, which showed to be the most active, were further purified by normal phase HPLC on a silica column to yield compounds **1**–**6** as described below.

Fraction A (0.055 g), eluted with $CHCl_3$/MeOH (90:10), was subjected to HPLC (*n*-hexane-EtOAc, 70:30) to give 7.0 mg of **2** (t_R 2.5 min) and a mixture of compounds **2** and **4** (6.0 mg, t_R 6 min).

Fraction C (0.150 g), eluted with $CHCl_3$/MeOH (85:15), was subjected to HPLC (*n*-hexane-EtOAc, 50:50) to afford 0.7 mg of **3** (t_R 4.5 min), 3.6 mg of **1** (t_R 5.5 min) and 1.6 mg of **4** (t_R 9.0 min).

Fraction D (0.019 g), eluted with $CHCl_3$/MeOH (85:15), was subjected to HPLC (*n*-hexane-EtOAc, 50:50) to give 6.7 mg of compound **5**; t_R 7.5 min. Fraction E (0.545 g), eluted with $CHCl_3$/MeOH (85:15), was subjected to HPLC (*n*-hexane-EtOAc, 35:65) to give 2.7 mg of **6**; t_R 11 min.

Gnaphaliol 9-*O*-propanoate (**1**). Pale-yellow powder. $[\alpha]^{25}_D$-13.09 (*c* 0.29, $CHCl_3$); UV (EtOH) λmax(log ε) 275 (4.0); IR ν^{KBr}_{max} cm^{-1}: 3430 (>OH), 2920 (>CH), 1680 (C═O), 1650 (C═C); ^1H and ^{13}C NMR (CD_3OD, 600 MHz) see Table 1; ESI/MS (positive mode) 329 ($[M + K]^+$), 313 ($[M + Na]^+$), 291 ($[M + H]^+$). HR-MALDITOF-MS ($[M + Na]^+$) 313.1057 ($C_{16}H_{18}O_5Na$; calcd. 313.1052). For experiments on cell cultures, compounds **1**, **2** and **5** were dissolved in DMSO.

2.5. Cell culture

The murine monocyte/macrophage J774 cell line and a conditionally immortalized human colonic epithelial cell line (HCEC) were used. J774 macrophages, obtained from ATCC (LGC Standards, Milan, Italy), were grown (at 37 °C in a 5% CO_2 atmosphere) in Dulbecco's modified Eagles medium (DMEM 4.5 g/l glucose without phenol red) supplemented with 2 mM glutamine, 25 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), penicillin (100 μ/ml), streptomycin (100 μg/ml), 10% fetal bovine serum (FBS) and 1.2% sodium pyruvate. HCEC, supplied by Fondazione Callerio Onlus (Trieste, Italy), were routinely maintained at 37 °C in a 5% CO_2 atmosphere in 75 cm^2 polystyrene flasks in Dulbecco's modified Eagle's medium (DMEM, 1.0 g/l glucose) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM Hepes, 2 mM L-glutamine and 1 mM sodium pyruvate. The medium was changed every 48-h. The inflammatory response in macrophages was induced by LPS from *E. coli* serotype O111: B4 (1 μg/ml). The acute inflammatory response in macrophages required an LPS incubation time of 18-h. The oxidative stress in HCEC was induced by Fenton's reagent (H_2O_2/Fe^{2+} 2 mM, time of incubation 3-h).

Table 1
^{13}C and ^1H NMR data (*J* in Hz) of compounds **1** (600 MHz, δ ppm, in CD_3OD).

Position	1	
	δ_C	δ_H
1	–	–
2	89.3	5.20 (*d*, *J* = 6.1)
3	72.0	5.32 (*d*, *J* = 6.1)
3a	130.6	–
4	128.2	8.14 (*d*, *J* = 1.7)
5	132.4	–
6	132.9	8.04 (*dd*, *J* = 1.7, 8.0)
7	110.8	7.00 (*d*, *J* = 8.0)
7a	165.0	–
8	140.1	–
9	65.3	4.84 (*d*, *J* = 13.3) 4.74 (*d*, *J* = 13.3)
10	115.5	5.51 (*s*) 5.46 (*s*)
11	198.8	–
12	26.3	2.60 (*s*)
$COCH_2CH_3$	175.6	–
$COCH_2CH_3$	28.0	2.43 (2H) (*q*, *J* = 7.3)
$COCH_2CH_3$	8.9	1.17 (*t*, *J* = 7.3)

2.6. Cytotoxicity assay

Cellular viability was assessed by the neutral red (NR) assay which is based on the ability of viable cells to incorporate and bind the supravital dye NR in the lysosomes [11]. After incubation with the compounds **1**, **2** and **5** (1–100 μM) at 37 °C for 24 h, macrophages (3×10^4 cells per well seeded in a 96-well plate) or HCEC (1×10^4 cells per well seeded in a 96-well plate) were incubated with NR dye solution (50 mg/ml) for 3 h, and then lysed by adding 1% acetic acid. The absorbance was measured at a wavelength (λ) of 550 nm using a multiwell reader (Perkin-Elmer Instruments). Treatments were compared with 20% DMSO and the results are expressed as percentage of cell viability.

2.7. Nitrite measurement in J774 macrophages

Nitrites, stable metabolites of nitric oxide, were measured in macrophages medium as previously described [12]. Macrophages (2.5×10^5 cells per well seeded in a 24-well plate) were incubated with the compounds **1**, **2** and **5** (1–30 μM) or 2,6-Di-*tert*-butyl-4-methylphenol (BHT, 300 μM, used as a positive control) for 30 min and subsequently with LPS (1 μg/ml) for 18 h. At the end of the incubation, the supernatants were collected for the nitrite measurement. The nitrite concentration in the samples was measured by the Griess reaction, adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_2PO_4; vol. 1:1) to 100 μl samples. The absorbance was measured at a wavelength (λ) of 550 nm using a multiwell reader (Perkin-Elmer Instruments). The nitrite concentration was calculated by comparison with absorbance of a standard curve of sodium nitrite prepared in culture medium.

2.8. Detection of reactive oxygen species (ROS) generation in HCEC

Generation of intracellular reactive oxygen species (ROS) was estimated by a fluorescent probe, DCFH-DA [13] which diffuses readily through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent DCFH. DCFH is then rapidly oxidized to form highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is paralleled to the amount of ROS formed intracellularly. For experiments, HCEC were plated in a 96 multiwell plate at the density of 1×10^4 cells/well. After 48-h of culturing, the medium was removed and the cells were treated for 24-h at 37 °C with compounds **1**, **2** and **5** (1–100 μM) or dexamethasone (1 μM, used as a positive control). Then, the cells were rinsed and incubated for 30 min with 100 μM DCFH-DA in Hanks' Balanced Salt Solution (HBSS) containing 1% FBS. Finally,

cells were rinsed and incubated with the Fenton's reagent (H_2O_2/Fe^{2+} 2 mM) for 3-h at 37 °C. The DCF fluorescence intensity was detected using a fluorescent microplate reader (Perkin-Elmer Instruments), with the excitation wavelength of 485 nm and the emission wavelength of 538 nm.

2.9. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA, USA). All values are expressed as mean ± SEM. The one-way analysis of variance (one-way ANOVA), followed by Tukey's multiple comparison tests was used for analysis of multiple treatment means, and the Student's t-test was used to compare a single treatment mean with a control mean. A p value < 0.05 was considered statistically significant.

3. Results and discussion

The EtOH extract of *H. italicum* flowers was fractionated in turn with *n*-hexane, H_2O, $CHCl_3$ and *n*-BuOH. The $CHCl_3$ extract was analyzed using column chromatography on Si gel and normal-phase HPLC, affording one new (**1**) and five known (**2**–**6**) acetophenones, which were identified by spectroscopic analysis. Gnaphaliol 9-*O*-propanoate (**1**) was obtained as pale-yellow powder. The HRMALDITOF mass spectrum of **1** (*m/z* 313.1057 $[M + Na]^+$, calcd for $C_{16}H_{18}O_5Na$, 313.1052) supported a molecular formula of $C_{16}H_{18}O_5$. The ESI-MS spectrum showed a major ion peak at *m/z* 313 $[M + Na]^+$.

The ^1H NMR spectrum of compound **1** (Table 1) showed signals of three aromatic protons of a typical ABX system at δ 8.14 (d, $J = 1.7$), 8.04 (dd, $J = 1.7$, 8.0), and 7.00 (d, $J = 8.0$), two protons linked to oxygenated carbons at δ 5.32 (d, $J = 6.1$) and 5.20 (d, $J = 6.1$), one alcoholic primary function at δ 4.84 (d, $J = 13.3$) and 4.74 (d, $J = 13.3$), two geminal olefinic protons at δ 5.51 (s) and 5.46 (s), and a signal corresponding to an acetyl group at δ 2.60 (s). Moreover, signals at δ 2.43 (2H, q, $J = 7.3$) and 1.17 (t, $J = 7.3$) were observed. The ^{13}C NMR spectrum (Table 1) displayed signals corresponding to two olefinic carbons at δ 140.1 (C(8)) and 115.5 (C(10)), a carbonyl carbon at δ 198.8 (C(11)) and a hydroxylated carbon at δ 72.0, suggesting the presence of an acetophenone skeleton. In addition a carboxyl carbon at δ 175.6, with two carbon signals at δ 28.0 and 8.9 revealed the occurrence of a propanoid moiety (Fig. 2). A detailed analysis of 2D NMR data of compound **3** identified the acetophenone skeleton of a gnaphaliol derivative [14]. The β configuration of the hydroxyl group at C(9) was established on the basis of the coupling constant value between H-C(9) and H-C(10) ($J = 6.1$), indicative of eclipsed protons and in agreement with literature data [14,15]. The HMBC experiment which showed the correlation between the proton resonances at δ 4.84 and 4.74 (H-C(12)) of gnaphaliol with the carbon resonance at δ 175.6 of the propanoid moiety allowed us to deduce for the compound **1** the structure of gnaphaliol 9-*O*-propanoate as shown in Fig. 1, never reported before. The NMR data of this compound are provided in Table 1.

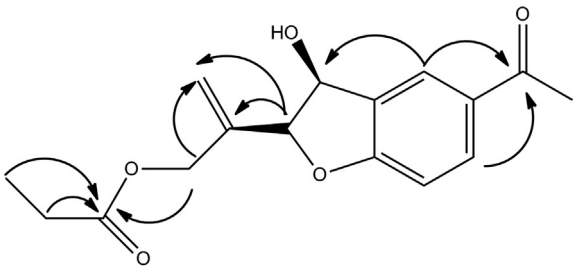

Fig. 2. Key HMBC correlations observed for compound **1**.

Compounds **2** and **4** were respectively identified as acetoxytremetone and 10-hydroxytremetone, previously reported by us [9]. The other known compounds 13-(2-methylpropanoyloxy)toxol (**3**) [16], 1-[2-[1-[(acetyloxy)methyl]ethenyl]-2,3-dihydro-3-hydroxy-5-benzofuranyl]-ethanone (**5**) [17] and gnaphaliol (**6**) [14] were identified by comparing their ^1H and ^{13}C NMR data with those reported in the literature.

The tremetones **2**, **4** and **6** have been already reported in two different varieties of *H. italicum* [7,18]. Interestingly, the tremetones **1**, **3** and **5**, characterized by the presence of a hydroxyl on the benzylic carbon, are reported in *H. italicum* for the first time.

According to literature data, our ethanolic *H. italicum* extract showed to possess both an anti-inflammatory and antioxidant activities (data not shown). The fractionation of the extract led to the isolation of compounds **1**–**6**. Unfortunately, the amount of compounds **3**, **4** and **6** obtained after HPLC was not sufficient to test their biological effects. The compounds **1**, **2** and **5** were evaluated for their cytotoxicity, anti-inflammatory properties and inhibition of oxidative stress on cell culture. Our results showed that the exposure of J774 macrophages and healthy colonic epithelial cells (HCEC) to various concentrations of compound **1** (C1), compound **2** (C2) and compound **5** (C5) (1–100 μg/ml) resulted in no effect on cell survival (% cell survival in J774 macrophages: control 100 ± 0, C1 1 μg/ml 105.5 ± 1.5, C1 3 μg/ml 108.7 ± 1.33, C1 10 μg/ml 101.6 ± 3.8, C1 30 μg/ml 106.9 ± 3.9, C1 100 μg/ml 92.9 ± 5.6, C2 1 μg/ml 100.6 ± 3.8, C2 3 μg/ml 104.2 ± 3.4, C2 10 μg/ml 99.1 ± 4.3, C2 30 μg/ml 99.2 ± 4.2, C2 100 μg/ml 103.6 ± 6.0, C3 1 μg/ml 97.1 ± 1.7, C3 3 μg/ml 93.7 ± 2.4, C3 10 μg/ml 99.4 ± 3.0, C3 30 μg/ml 94.4 ± 4.2, C3 100 μg/ml 97.3 ± 2.8; % cell survival in HCEC: control 100 ± 0, C1 1 μg/ml 93.6 ± 2.9, C1 3 μg/ml 99.4 ± 4.0, C1 10 μg/ml 98.4 ± 5.4, C1 30 μg/ml 97.7 ± 2.5, C1 100 μg/ml 103.5 ± 6.7, C2 1 μg/ml 99.3 ± 4.4, C2 3 μg/ml 99.8 ± 2.9, C2 10 μg/ml 99.7 ± 3.9, C2 30 μg/ml 94.6 ± 2.2, C2 100 μg/ml 99.7 ± 2.0, C3 1 μg/ml 100.1 ± 4.2, C3 3 μg/ml 106.2 ± 4.2, C3 10 μg/ml 97.8 ± 3.9, C3 30 μg/ml 102.2 ± 4.5, C3 100 μg/ml 95.7 ± 3.0; n = 8). DMSO 20%, used as a positive control, significantly reduced cell viability (% cell survival in J774 macrophages: control 100 ± 0, DMSO 20% 27.4 ± 2.7; $p < 0.001$; n = 8. % cell survival in HCEC: control 100 ± 0, DMSO 20% 34.3 ± 1.4, $p < 0.001$; n = 8) thus to suggest a safe biological profile.

Macrophages play a pivotal role in the inflammatory processes and their stimulation by LPS results in the production of large amounts of nitrites and nitrates which are the stable degradation products of nitric oxide. Therefore, the measurement of nitrite production induced by LPS treatment in macrophages is a well established method to evaluate the anti-inflammatory effect of drugs. We found that compounds **1**, **2** and **5** did not possess anti-inflammatory effects since the increased levels of nitrites induced by LPS were not modified by a pre-treatment with these compounds (1–30 μg/ml, 30 min

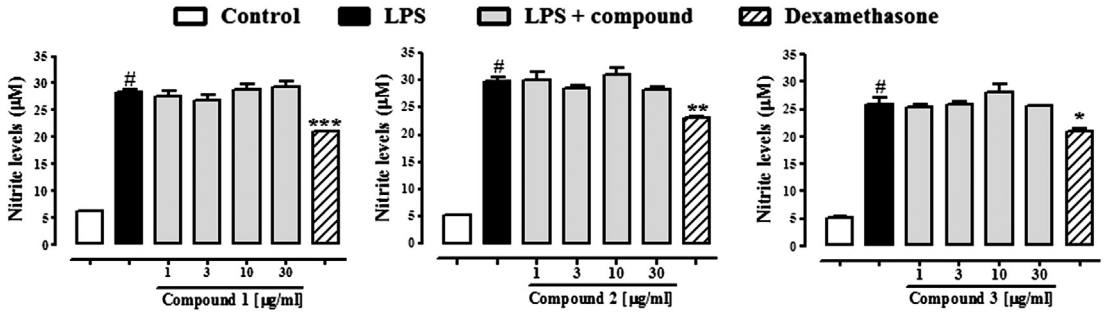

Fig. 3. Effect of compounds **1**, **2** and **5** on nitrite levels in the cell medium of J774 macrophages incubated with lipopolysaccharides (LPS, 1 μg/ml) for 18-h. Compounds **1**, **2** and **5** (1–30 μg/ml) and dexamethasone (1 μM, used as a positive control) were added to the cell media 30 min before LPS challenge (i.e. 18.5-h before nitrites assay). Results are mean ± SEM of six experiments (in triplicates). $^{\#}p < 0.001$ *vs* control; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs LPS alone.

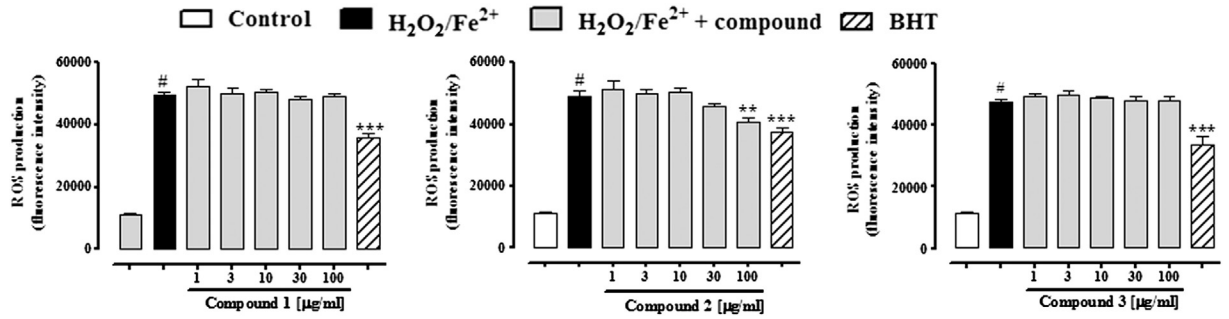

Fig. 4. Effect of compounds **1**, **2** and **5** (1–100 μg/ml) on Fenton's reagent (H_2O_2/Fe^{2+} 2 mM)-induced reactive oxygen species production. Effect observed in human colonic epithelial cells after 24-h exposure to compounds or 2,6-di-*tert*-butyl-4-methylphenol (BHT, 300 μg/ml, used as a positive control). Results are mean ± SEM of six experiments. $^{\#}p < 0.001$ vs control; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs H_2O_2/Fe^{2+} alone.

before LPS) (Fig. 3). By contrast, dexamethasone, used as a positive control, was significantly able to reduce LPS-stimulated nitrite levels (Fig. 3).

One of the approaches to assess the antioxidant activity of molecules is to examine directly their effects on the ROS formation generated by Fenton's reagent (H_2O_2/Fe^{2+}). In our experiments exposure of HCEC to H_2O_2/Fe^{2+} (2 mM) produced a significant ($p < 0.001$) increase in ROS formation (Fig. 4). A pre-treatment for 24-h with compound **2** (1–100 μg/ml), but not with compounds **1** and **5**, reduced the ROS formation as measured by the inhibition of DCF fluorescence intensity. This effect was significant ($p < 0.01$) at the highest concentration tested (100 μg/ml) (Fig. 4). Butyl-4-methylphenol, (BHT, 300 μg/ml), used as a positive control, was significantly able to reduce the ROS formation induced by Fenton's reagent (Fig. 4).

Acetophenones from *H. Italicum* had previously been evaluated for their anti-inflammatory activity in different in vitro and in vivo assays, and some of them were showed to be very effective [19,20]. In fact, Sala et al. demonstrated that 4-hydroxy-3-(2-hydroxy-3-isopentenyl) acetophenone was particularly active in a 12-*O*-tetradecanoylphorbol 13-acetate-induced mouse ear edema test [19] while 4-hydroxy-3-(3-methyl-2-butenyl) acetophenone showed ability to inhibit arachidonic acid metabolism in two different in vitro models [20]. On the other hand, the data presented herein strengthen the observation that acetophenones **1**, **2** and **5** are not responsible for the anti-inflammatory activity of *H. italicum*. By contrast, compound **2**, but not compounds **1** and **5**, seems to be involved in the antioxidant effect of this Mediterranean plant. Interestingly, compound **2**, that here for the first time has been reported to posses antioxidant effect, has previously been shown to exert spasmolytic activity [9].

In conclusion, our results confirm that the *H. italicum* extract and its chemical compounds possess promising pharmacological activities which validate the recognized use of the plant in Mediterranean countries folk medicine. Moreover, if these data will be confirmed in vivo, the chemical compounds here isolated and investigated could represent an option in oxidative stress related diseases.

Conflict of interest

There are no conflicts of interest of all authors with respect to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.09.019.

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