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FULL PAPER

CHEMISTRY & BIODIVERSITY

Evaluation of Antioxidant, Anti-Inflammatory and Antityrosinase Potential of Extracts from Different Aerial Parts of *Rhanterium suaveolens* from Tunisia

Sabrine Chelly,[a] Meryam Chelly,[a] Cristina Occhiuto,[b] Francesco Cimino,[b] Mariateresa Cristani,*[b] Antonina Saija,[b] Maria Sofia Molonia,[b] Giuseppe Ruberto,[c] Valeria D'Angelo,[b] Maria Paola Germanò,[b] Laura Siracusa+,[c] Hanen Bouaziz-Ketata+,[a] and Antonio Speciale+[b]

[a] Toxicology-, Microbiology- and Environmental Health Laboratory (RL 17ES06), Faculty of Sciences, University of Sfax, 3000 Sfax, Tunisia

[b] Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali, Università di Viale Annunziata, 98168, Messina, Italy, e-mail: mcristani@unime.it

[c] Istituto di Chimica Biomolecolare del Consiglio Nazionale delle Ricerche (ICB-CNR), Via Paolo Gaifami, 18, 95126 Catania, Italy

The genus *Rhanterium* (Asteraceae) is a widely distributed medicinal plant throughout western North Africa and some *Rhanterium* species are used in folk medicine. The aim of research was to investigate methanolic extracts from different parts (flowers, leaves, and stems) of Tunisian *Rhanterium suaveolens* as potential sources of bioactive products useful for healthy purposes. In particular, were analyzed the phenolic composition of these extracts and their antioxidant, anti-inflammatory, and anti-tyrosinase properties.

The phytochemical analyses were performed using standard colorimetric procedures, HPLC-DAD and HPLC-DAD-ESI-MS. Then, several in vitro cell-free assays have been used to estimate the antioxidant/free radical scavenging capability of the extracts. Moreover, *in vitro*, and *in vivo* anti-melanogenesis activities of these extracts were tested, respectively, with the tyrosinase inhibition assay and the Zebrafish embryo model. Finally, the anti-inflammatory potential of these extracts in an *in vitro* model of acute intestinal inflammation in differentiated Caco-2 cells was evaluated.

The *R. suaveolens* extracts under study appeared particularly rich in flavonols and hydroxycinnamic acids and all extracts appeared endowed with good antioxidant/free radical scavenging properties, being the flower extracts slightly more active than the others. Moreover, *R. suaveolens* flowers extract was able to inhibit in vitro tyrosinase activity and exhibited bleaching effects on the pigmentation of zebrafish embryos. Furthermore, all extracts showed good anti-inflammatory activity in intestinal epithelial cells as demonstrated by the inhibition of TNF-α-induced gene expression of IL-6 and IL-8.

R. suaveolens aerial parts may be considered as a potential source of whitening agents, as well as of agents for the treatment of disorders related to oxidative stress and inflammation.

Keywords: *Rhanterium suaveolens*, anti-inflammatory activity, HPLC/ESI-MS, tyrosinase activity, interleukins.

Introduction

Among plants scientifically proven to act as therapeutic agents used in phytotherapy and aromatherapy, those belonging to the genus *Rhanterium* (Asteraceae) deserve a special mention.[1–3] According to the literature, phenolics-rich extracts of plants from this genus possess remarkable antioxidant activities, as reported for *R. adperessum*, *R. epapposum* and Algerian and Tunisian *R. suaveolens*,[3–5] while a powerful

+ These authors share senior authorship.

antimicrobial activity is addressed to the corresponding essential oils.[2,6–8] The composition of hydro-alcoholic extracts from *R. suaveolens* collected in Tunisia was recently studied in detail,[9] revealing the presence of numerous cinnamic acids and their derivatives, and of the flavonols kaempferol and quercetin. In particular, quercetin is the most common dietary flavonoid found in fruits, vegetables, seeds, coffee, and tea, frequently present as glycoside, rhamnoside, galactoside and rutinoside, and is characterized by strong anti-inflammatory and antityrosinase activity.[10,11]

Although, the extracts from the whole aerial part of *Rhanterium suaveolens* (Rs) was already studied,[9,12] to the best of our knowledge no study has been carried out on those obtained from different Rs organs (flowers, stems, and leaves).

The aim of this study was to evaluate the phytochemical composition and the antioxidant, anti-inflammatory and anti-melanogenesis activities of three methanolic extracts obtained from Rs flowers (RSF), stems (RSS), and leaves (RSL). Phytochemical analyses of these extracts were carried out by means of standard colorimetric procedures, HPLC-DAD, and HPLC-DAD-ESI-MS; the antioxidant/free radical scavenging capability was measured using *in vitro* cell-free assays. In addition, *in vitro* and *in vivo* anti-melanogenesis activities of these Rs extracts were tested respectively with the tyrosinase inhibition assay and the Zebrafish embryo model. Finally, their anti-inflammatory properties were investigated in an *in vitro* model of acute intestinal inflammation using differentiated Caco-2 cells.

Results and Discussion

Determination of R. suaveolens Phenolic Compounds

Extract yields were calculated from residues weights obtained after solvent extraction and vacuum concentration of filtrates. The highest value (11.3%) was recorded for RSL, followed by that of RSF (10.7%) and RSS (10.2%).

Phytochemical screening performed on various Rs aerial parts revealed remarkable amounts of phenolic compounds, particularly flavonoids (*Table 1*). According to our determinations, the amount of polyphenols (evaluated as μg GAE/mg of dry extract) peaked in Rs flowers (200 ± 12 μg GAE/mg), followed by stems (158 ± 11 μg GAE/mg) and leaves (98 ± 13 μg GAE/mg); all values are far higher than those reported in literature for the same species collected in Tunisia.[9] Results coming from the evaluation of the flavonoid content showed RSF as the richest extract (RSF: 47.85 ± 0.70 QuE/mg, RSS: 30.70 ± 1.68 QuE/mg, RSL: 24.43 ± 1.04 μg QuE/mg); as also reported in literature, the flowers of *Rhanterium* species are particularly rich in flavonoids compared to the other plant parts.[9,13] Even, regarding tannins, we observed that RSF was the organ richest in total and in hydrolysable tannins (*Table 1*), instead the organ richest in condensed tannins was RSL.

Polyphenol Profiles and Content (HPLC-DAD and HPLC-ESI-MS)

Chromatographic analyses performed on methanolic extracts from different Rs plant parts (flowers, leaves, and stems) revealed the presence of 31 metabolites that were tentatively identified by means of their UV/VIS and mass spectral data (*Table 2* and *Figure 1*). In these extracts we have found two subclasses of polyphenols, namely hydroxycinnamic acids (caffeic acid, chlorogenic acid and their derivatives) and glycosides derivated from the two flavonols quercetin and kaempferol (*Table 2*). Our results are totally in agreement with the literature, since quercetin and chlorogenic acids were often detected as the main compounds in the aerial parts of plants belonging to the Asteraceae family, such as *Anthemis nobilis*,[14] *Achillea*

Table 1. Phytochemical analyses of *Rhanterium suaveolens* extracts. Data were expressed as means ± SD of three different experiments.

	Polyphenols	Flavonoids	Total Tannins	Condensed tannins	Hydrolysable tannins
	μg GAE/mg de[a]	μg QuE/mg de[a]	μg TAE/mg de[a]	μg CE/mg de[a]	μg TAE/mg de[a]
RSF	$200 \pm 12^{ab[b]}$	47.85 ± 0.70	59.7 ± 6.6^{a}	14.9 ± 4.4	59.5 ± 3.5^{ab}
RSS	158 ± 11^{a}	30.70 ± 1.68	56.6 ± 4.8^{a}	15.7 ± 2.5	45.0 ± 0.3^{a}
RSL	98 ± 13	24.43 ± 1.04	41.9 ± 6.3	25.8 ± 1.8^{bc}	33.9 ± 4.3

[a] QuE: quercetin equivalents; GAE: gallic acid equivalents; CE: catechin equivalents; TAE: Tannic acid equivalents; de: dry extract. [b] $^{a}P < 0.05$ *vs.* RSL; $^{b}P < 0.05$ *vs.* RSS; $^{c}P < 0.05$ *vs.* RSF.

Table 2. Content of flavonoids and hydroxycinnamic acids identified in *Rhanterium suaveolens* methanolic extracts by HPLC-DAD. Results are presented as average value of three replicates and expressed as mg individual compound over 100 mg extract (see Experimental Section and Ref. [12] for details).

Peak No.	Rt, min	Compound[a]	RSF	RSS	RSL
1	7.10	pCoQA isomer 1	0.092	0.096	0.071
2	9.51	pCoQA isomer 2	0.034	0.005	0.049
3	10.42	FQA isomer 1	0.034	0.327	0.194
4	10.80	CQA*	0.243	0.374	0.245
5	11.68	Caffeic acid*	0.226	0.069	0.035
6	15.18	FQA isomer 2	0.307	0.301	0.219
7	16.05	FQA isomer 3	0.453	n.d.	n.d.
8	17.08	FQA isomer 4	0.159	n.d.	n.d.
9	20.91	quercetin 3-*O*-galactoside*	27.363	4.817	6.390
10	21.41	Caffeic acid trimer derivative	0.592	1.550	0.604
11	22.49	diCQA isomer 1	7.477	7.434	3.493
12	22.94	CSA	0.152	n.d.	0.060
13	23.48	diCQA isomer 2	3.678	0.190	0.415
14	24.83	kaempferol 3-*O*-rutinoside*	0.508	0.321	0.648
15	25.37	quercetin pentoside isomer 1	2.243	n.d.	n.d.
16	25.73	diCQA isomer 3	1.657	3.101	1.237
17	26.15	quercetin pentoside isomer 2	10.235	8.947	4.888
18	27.18	CFQA isomer 1	0.170	0.474	0.207
19	28.20	CFQA isomer 2	1.620	1.369	0.958
20	28.76	CFQA isomer 3	1.467	2.014	0.993
21	29.66	CFQA isomer 4	0.928	0.273	0.241
22	31.22	CFQA isomer 5	0.410	1.003	0.590
23	32.06	CFQA isomer 6	0.285	0.744	0.372
24	34.72	diFQA isomer 1	0.601	0.354	0.286
25	35.42	diFQA isomer 2	0.296	0.045	0.048
26	35.85	Caffeic acid dimer derivative**	0.488	0.111	0.313
27	36.75	triCQA	1.288	3.655	2.238
28	39.04	Caffeic acid eptamer derivative**	1.069	0.116	0.427
29	39.74	diCFQA isomer 1	0.190	0.500	0.316
30	39.93	diFCQA isomer 2	0.578	1.955	1.282
31	40.81	triFQA	0.100	n.d.	0.016

[a] pCoQA: *p*-coumaroylquinic acid; FQA: feruloylquinic acid; CQA: chlorogenic acid; diCQA: di-caffeoylquinic acid; CSA: caffeoyl-lshikimic acid; CFQA: caffeoylferuloylquinic acid; diFQA: diferuloylquinic acid; triCQA: tricaffeoylquinic acid; diCFQA: dicaffeoylferruloylquinic acid; triFQA: triferuloylquinic acid. n.d. – compound not detected. *compound identity confirmed with the use of the corresponding analytical standard. **troublesome mass spectra.

millefolium,[15] *Golden thistle*,[16] and *Rhanteuim suaveolens* from Tataouin.[9] Qualitatively, chromatograms coming from the extracts of flowers, leaves and stems of Rs are apparently characterized by similar profiles (*Figure 1*), while the total polyphenol abundance order was RSF > RSS > RSL. In particular, the total amount of flavonols (especially quercetin 3-*O*-galactoside and quercetin pentoside) was significantly higher in RSF extract than in RSS and RSL extracts, both showing similar levels of these polyphenols. Levels of hydroxycinnamic acids, similar in RSF and RSS extracts, were higher in these extracts than in that from RSL (*Table 2*, *Figure 2*). The extracts from RSF was characterized by the presence of quercetin 3-*O*-galactoside (peak 9) and a diCQA isomer (peak 13), while two CFQA isomers (peaks 20 and 22), triCQA (peak 27) and a diFCQA isomer (peak 30) were the compounds characterizing the RSS extract (*Figure 1* and *Table 2*). These promising results prompted us to evaluate their antioxidant and anti-inflammatory potential for possible use of extracts from aerial parts of this species in natural medicine.

Antioxidant Activities of Rs

In order to evaluate antioxidant effect of extracts from Rs aerial parts, a group of *in vitro* tests was carried out. Although only in two of these assays (TEAC and FRAP) the difference reached a statistical significance, RSF extract appeared to be endowed with a higher antioxidant power than RSS and RSL extracts, showing

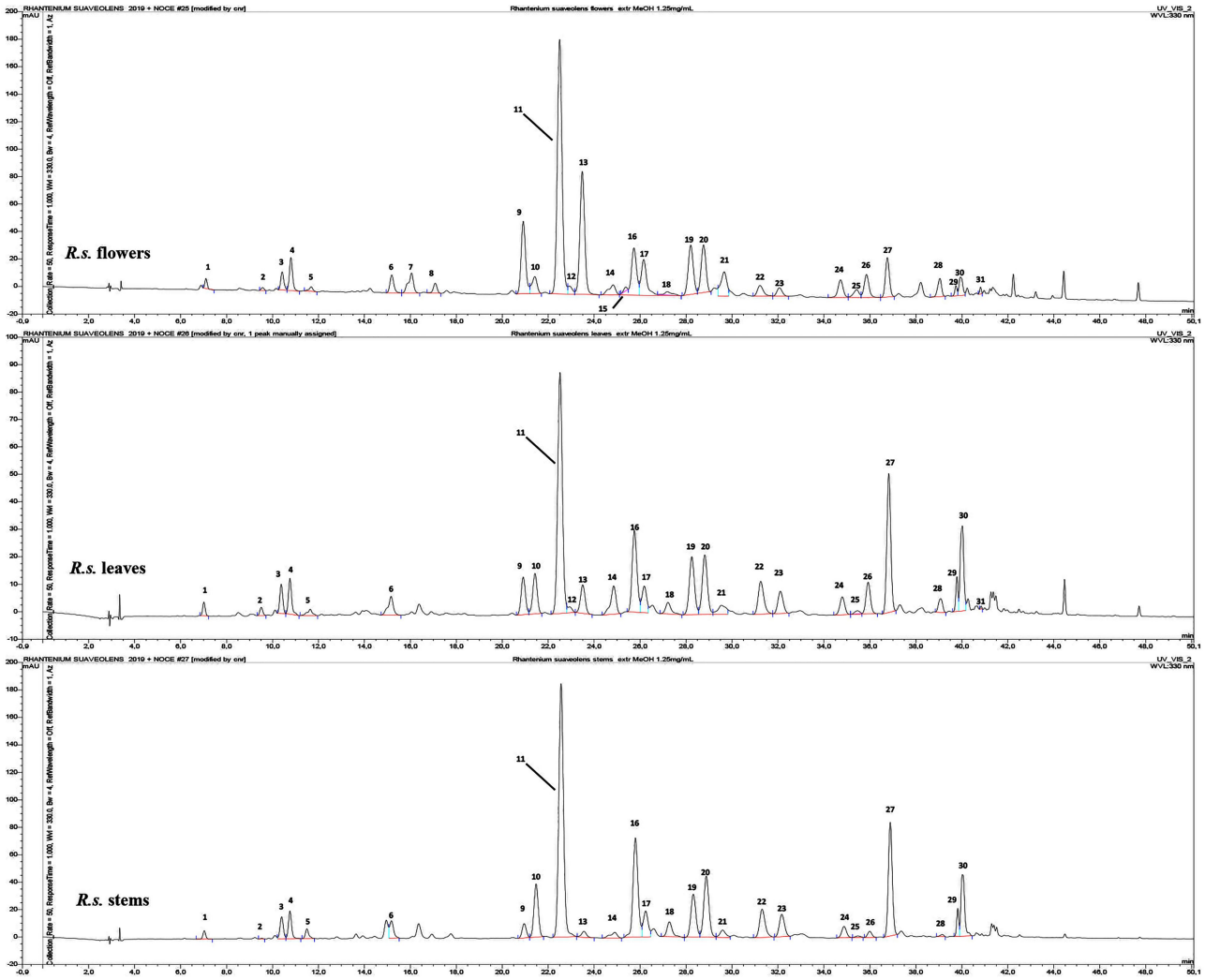

Figure 1. HPLC-DAD chromatograms, visualized at 330 nm, of methanolic extracts from *Rhanterium suaveolens* flowers, stems, and leaves. Peak numbers refer to *Table 2*.

the last two extract a similar behavior (*Table 3*, *Table 4*, *Figure 3*). These results could be related not only to the different quantitative content of bioactive compounds, but also to the chemical complexity of the investigated matrices (*Table 2*). Remarkably detected in all Rs organs, quercetin and chlorogenic acids could

Table 3. Antioxidant[a] evaluation of *Rhanterium suaveolens* methanolic extracts. Data were expressed as means ± SD of three different experiments.

	TEAC	FRAP	TAC-Pm	ORAC
	μmol TE/mg de	μmol Fe^{2+} eq/mg de	nmol TE/mg de	mmol TE/g de
RSF	$0.90 \pm 0.062^{a[b]}$	2.64 ± 0.02^{ab}	1.23 ± 0.20	8.6 ± 1.7
RSS	0.69 ± 0.094	2.13 ± 0.02^{a}	0.97 ± 0.06	6.2 ± 0.6
RSL	0.62 ± 0.068	1.91 ± 0.04	1.01 ± 0.08	7.4 ± 1.8

[a] TEAC: Trolox equivalent antioxidant capacity; FRAP: ferric reducing antioxidant power; TAC-Pm: Total antioxidant capacity-Phosphomolybdate; ORAC: Oxygen radical absorbance capacity; TE: Trolox equivalents; Fe^{2+} E: ferrous equivalents; de: dry extract.
[b] $^{a}P < 0.05$ *vs.* RSL; $^{b}P < 0.05$ *vs.* RSS; $^{c}P < 0.05$ *vs.* RSF.

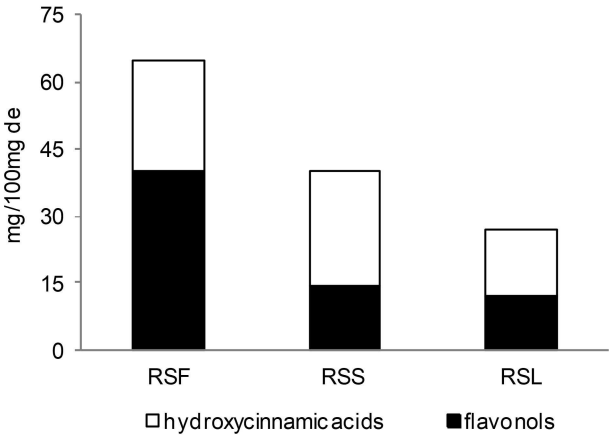

Figure 2. Main chemical subclasses of polyphenols identified and quantified by HPLC/DAD in RSF, RSS and RSL methanolic extracts. de: dry extract.

Table 4. Evaluation of scavenging activity against superoxide radical ($O_2^{\bullet-}$) and nitric oxide radical (NO) and protective effect against β-carotene bleaching (βCB) of the *Rhanterium suaveolens* methanolic extracts under study.

	$O_2^{\bullet-}$	NO	βCB
	SC_{50} (C.L.) mg/mL[a]	SC_{50} (C.L.) mg/mL[a]	IC_{50} (C.L.) mg/mL[a]
RSF	1.65 (1.10–2.48)	0.637 (0.506–0.802)	0.54 (0.43–0.68)
RSS	1.92 (1.38–2.68)	0.830 (0.692–0.995)	0.55 (0.38–0.81)
RSL	1.96 (1.25–3.07)	0.997 (0.811–1.226)	0.78 (0.52–1.17)

[a] IC_{50}: half maximal inhibitory concentration; SC_{50}: half maximal scavenging concentration; C.L.: 90% confidence limits.

explain the important plant antioxidant power. In fact, quercetin constitutes an excellent antioxidant potential thanks to its interesting power for trapping free radicals and its important transition metal ions binding capacity forming more stable complexes.[10] In this regard, this flavonol has been shown to protect against tissue damage and low-density lipoprotein (LDL) oxidation induced by free radicals.[10] This strong antioxidant power of quercetin is attributed to its catechol group in B ring, its 2,3 double bond in C ring, and its OH group at 3 and 5 positions.[17] Moreover, quercetin demonstrated an ABTS radical scavenging capacity 6 times better than that of Trolox.[17] Besides quercetin, chlorogenic acids have been shown to be the principal antioxidants for LDL.[18] These phenolic acids are characterized by an excellent capacity of scavenging free radicals such as $O^{2-\cdot}$ and NO, a marked inhibiting power against linoleic acid peroxidation,[18] and a significant ferrous ion reduction activity.[19] Finally, one cannot forget that also condensed tannins are known to be endowed with several pharmacological effects, including antioxidant and free radical scavenging activity.[20,21] Otherwise, there is a relation between oxidative stress and inflammation related to reactive oxygen species (ROS) increase, which generates NF-kB activation and pro-inflammatory cytokines production.[22] In fact, the occurrence of various inflammatory diseases was described often correlated with ROS production and proteins denaturation. According to Opie,[23] inflammatory tissue damage could be linked to cell protein constituent's denaturation. Therefore, phenolic extract ability to inhibit protein degradation signifies its anti-inflammatory potential in general.[24] Thus, in order to test Rs extracts anti-inflammatory power, an SDS/PAGE experiment was carried out to evaluate Rs protective effect against BSA denaturation induced by HOCl. Indeed, BSA defines the major plasmatic protein characterized as an accessible marker for screening inflammatory diseases and a target for HOCl.[25] Thereby, our results revealed that albumin characteristic band intensity was reduced by HOCl at a concentration of 177 mM, reflecting almost total protein degradation, whereas Rs extracts protected BSA from degradation. According to the literature, hypochlorous acid could lead to organic chloramines formation by interacting with side chains amino functions of amino acids. These chloramines have an oxidation capacity proved by their decomposition into chloramide radicals.[25] Thus, the presence of various bioactive compounds in Rs methanolic extracts could contribute to scavenge chloramide radicals in order to inhibit BSA degradation. Interestingly, quercetin has an important scavenging free radicals capacity evidenced by multiple transfers of hydrogen atoms or electrons[10] and was ranked first among out of 15 flavonoids tested *in vitro* for their protective power against HOCl inducing BSA denaturation.[26]

Anti-Inflammatory Effects on Intestinal Epithelial Cells Treated with TNF-α

With the aim to assess the anti-inflammatory effects of the three parts of Rs, we used an *in vitro* experimental model of intestinal inflammation consisting of intestinal epithelial Caco-2 cells exposed to TNF-α. This cytokine increases the transcription of proinflammatory genes mainly through the activation of NF-kB

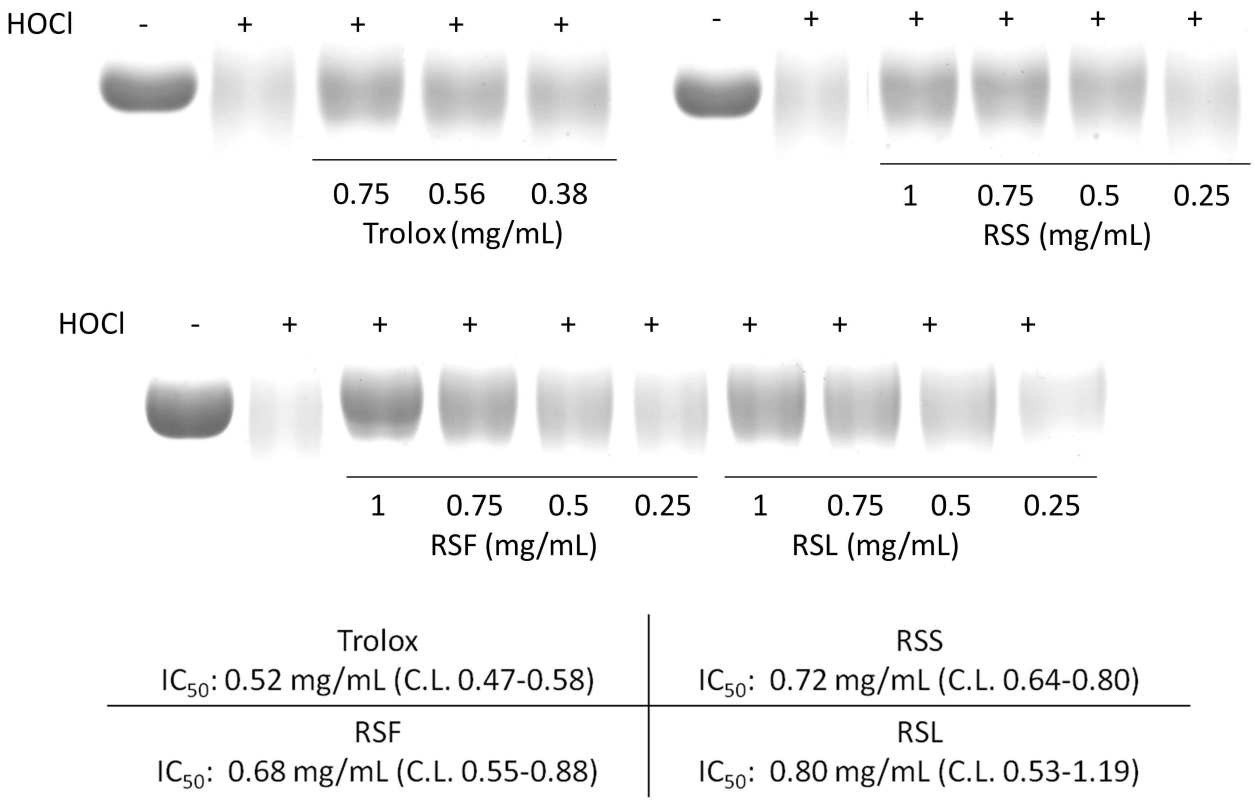

Figure 3. Inhibition of HOCl induced albumin degradation. BSA has been incubated in absence or in presence of 177 mM HOCl and with increasing amounts of the extracts, and then, subjected to SDS-PAGE, and developed with Coomassie Blue. Quantitative analysis was performed by gel densitometry using ImageJ software. Trolox was used as positive/standard control. Smearing of the parent protein band is proportional to the degree of BSA degradation. Image is representative of three independent experiments, and results are expressed as IC_{50} values (mg extract/mL) and 90% C.L.

pathway, thus promoting the expression of a multitude of inflammatory cytokines (*e.g.*, IL-6 and IL-8),[27,28] present in elevated concentrations in Inflammatory Bowel Diseases (IBDs), and supporting inflammation.[29] Furthermore, several drugs for IBDs hamper NF-kB pathway.[30]

Firstly, the biocompatibility of the extracts was tested on NIH/3T3 fibroblasts that were exposed to the extracts (up to 70 μg/mL) for 24 h. RSF and RSS did not show toxicity on these cells, thus Caco-2 were then treated for 24 h with RSF and RSS at 35 and 70 μg/mL. RSL, instead, induced significant toxicity at the higher concentrations, thus in the inflammatory model it was used at lower, non-toxic, doses (15–30 μg/mL). Successively, Caco-2 were challenged with TNF-α for 6 h.

TNF-α exposure induced a significant increase in IL-6 and IL-8 gene expression compared to control (*Figure 4*). This overexpression was moderately and dose dependently inhibited by pretreatment with all the extracts. At lower concentrations, the RSL extract showed a better activity in comparison with the others, although the higher concentration tested was 30 μg/mL, due to its toxicity. The extracts from RSF, and especially RSS, at the doses of 35 and 70 μg/mL, induced a strong and dose-dependent protective effect. The only treatment Rs extracts, without any exposure to TNF-α, however, did not altered the basal levels of the expression of the two cytokines (*Figure 4*).

Being present in various Rs organs as major polyphenols, quercetin as well as chlorogenic acids could explain the anti-inflammatory potential proven by plant aerial parts. In fact, chlorogenic acids have been proven to be excellent anti-inflammatory agents in Caco-2 and RAW 264.7 cells, by reducing the levels of IL-8 and IL-6, respectively.[31,32] Besides, Shin et al.[33] had shown the anti-inflammatory power of these phenolic acids *in vivo* against colitis induced by sodium dextran sulfate in mice. Regarding quercetin, its anti-inflammatory activity has been extensively

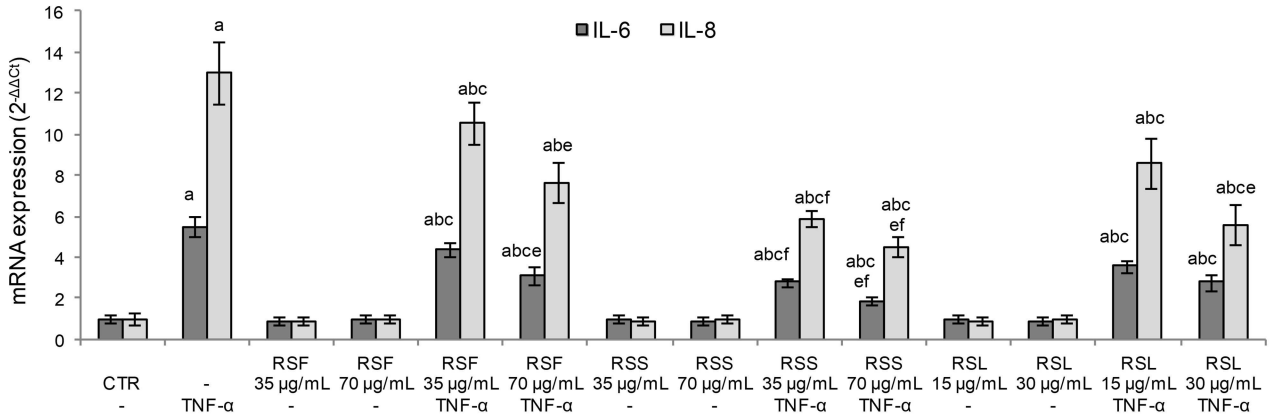

Figure 4. IL-6 and IL-8 gene expression in differentiated Caco-2 cells. The Caco-2 monolayer was pretreated with the extracts (RSF and RSS 35–70 μg/mL; RSL 15–30 μg/mL) for 24 h, and subsequently exposed to 50 ng/mL TNF-α for 6 h. Cultures treated with the vehicles alone were used as controls (CTR). The results, obtained from three independent experiments, are expressed as $2^{-\Delta\Delta Ct}$ (mean ± SD). 18S rRNA was used as housekeeping gene. [a] $P < 0.05$ *vs.* CTR; [b] $P < 0.05$ *vs.* TNF-α; [c] $P < 0.05$ *vs.* cells exposed to the same concentration of the same extract alone; [e] $P < 0.05$ *vs.* cells exposed to the lower concentration of the same extract + TNF-α; [f] $P < 0.05$ *vs.* same concentration of RSF + TNF-α.

reported.[10,17] In addition to quercetin and chlorogenic acids, leaves were also characterized by the presence of condensed tannins.[34] In this regard, tannins from *Diospyros kaki* fruits were proven to prevent from oxidative stress induced by H_2O_2 in Caco-2 cells, and to reduce TNF-α level in THP-1 cells treated with LPS.[35,36]

Anti-Melanogenesis Activity

Tyrosinase Inhibition Assay and Zebrafish Assay

Results of the enzymatic *in vitro* assay showed that RSF exhibited the strongest inhibitory effects on tyrosinase as compared with the other extracts, since it was able to inhibit efficiently L-DOPA oxidation (IC_{50} 61.56 ± 0.66 μg/mL) (*Table 5*).

Table 5. Inhibitory effects of *Rhanterium suaveolens* extracts on tyrosinase-induced L-DOPA oxidation. Data were expressed as means ± SD of three different experiments.

	Tyrosinase inhibitory effects on L-DOPA oxidation IC_{50} μg/mL[a]
RSF	61.56 ± 0.66[ab]
RSS	124.13 ± 1.13
RSL	96.72 ± 2.03[b]
Kojic acid	2.24 ± 0.18

[a] IC_{50}: half maximal inhibitory concentration. [a] $P < 0.05$ *vs.* RSL; [b] $P < 0.05$ *vs.* RSS; [c] $P < 0.05$ *vs.* RSF.

The best *in vitro* inhibitory effects of RSF on melanogenesis was confirmed by an *in vivo* assay on zebrafish embryos (*Figure 5*). RSS and RSL did not show any activity (data not shown), whereas it is evident that treatment with RSF both at 0.5 and 1 mg/mL induced a statistically significant inhibition of melanin accumulation respect to the negative control ($P < 0.05$).

As reported from Lee et al.[37] hydroxycinnamic acids are natural anti-melanogenic substances inhibiting tyrosinase, which catalyzes a rate-limiting step of melanogenesis. Concerning flavonoids, especially those belonging to the group of flavonols, they are also known for their tyrosinase-inhibitory activity and could be useful in pharmaceutical and cosmetic industries as well as in the food field. In particular, in recent articles Fan et al.[38] and Yu et al.[39] demonstrated that quercetin could be a promising tyrosinase inhibitor and may have a potential application for the treatment of pigmentation disorders. Taken together, a synergistic effect of phenolic acids and flavonoids cannot be excluded to justify the whitening efficacy of RSF extract, that is rich in hydroxycinnamic acids and the richest in flavonols, especially quercetin and derivatives.

Conclusions

The results of this study show that all methanolic extracts from flowers, stems, and leaves of *R. suaveo-*

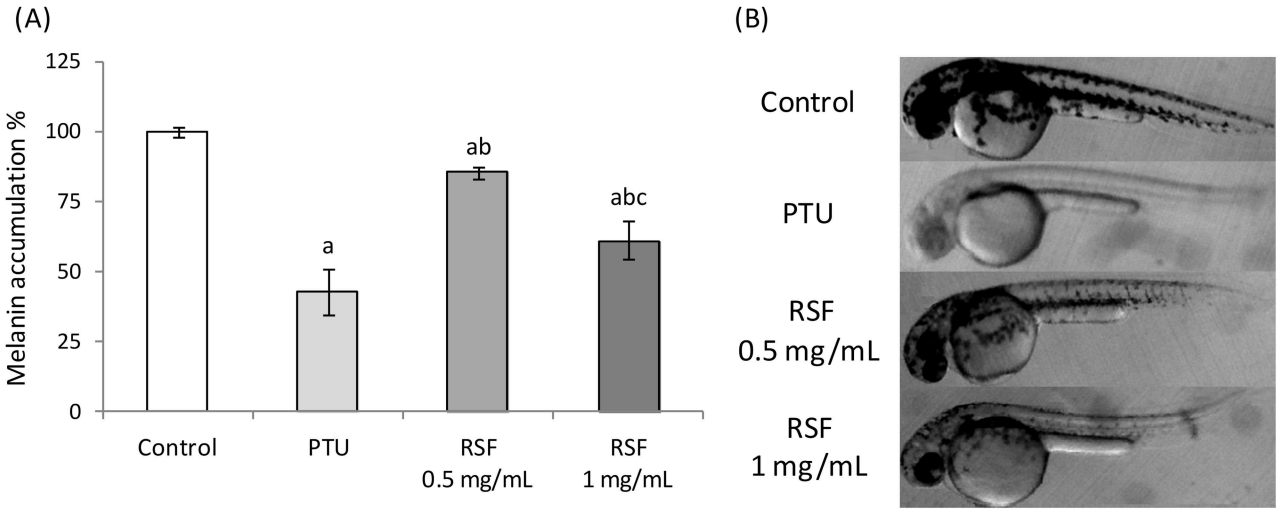

Figure 5. Whitening capability of RSF and of the reference standard PTU (N-phenylthiourea, 0.02 mM). (A) Results are expressed as melanin accumulation after 48 h of exposure in zebrafish embryos in comparison with controls (embryos exposed to the vehicle alone). (B) Representative microscopic pictures of zebrafish embryos treated with RSF (0.5 and 1 mg/mL) or PTU (0.02 mM) after 48 h of exposure. The images were obtained using a stereomicroscope (magnification 20×) equipped with a digital camera. [a] $p < 0.05$ *vs.* control; [b] $P < 0.05$ *vs.* PTU; [c] $P < 0.05$ *vs.* 0.5 mg/mL.

lens are characterized by the presence of several polyphenols, in particular flavonols, hydroxycinnamic acids and tannins, and showed significant *in vitro* antioxidant and anti-inflammatory activities. Furthermore, all extracts exhibited good protective effect in intestinal epithelial cells, as demonstrated by the inhibition of TNF-α-induced overexpression of IL-6 and IL-8. Moreover, RSF was able to inhibit *in vitro* tyrosinase activity and exhibited bleaching effects on the pigmentation of zebrafish embryos.

In conclusion, *R. suaveolens* aerial parts may be considered as a potential source of whitening agents, as well as of agents for the treatment of disorders related to oxidative stress and inflammation.

Experimental Section

Chemicals and Reagents

All solvents were purchased from Merck (VWR International, Milan, Italy); the chemicals from Sigma-Aldrich (St. Louis, MO, USA) and the pure reference standards purchased from Extrasynthese (Lyon, France) and Fluka (Sigma-Aldrich s.r.l., Milan, Italy).

Preparation of Plant Extracts

The aerial part of *Rhanterium suaveolens* was harvested in March 2017 in Sfax, Tunisia (Latitude: 34°44'26'' North; Longitude: 10°45'37'' East, Tunisia). This endemic plant was authenticated by Professor Mohamed Chaieb, Laboratory of Biology, Faculty of Sciences of Sfax, Tunisia. Herbarium voucher specimen (number: 155/2019) was deposited at the Faculty of Science (Sfax, Tunisia). The methanolic extracts from Rs flowers, stems and leaves were prepared as previously described by Chelly et al.[12]

Phytochemical Screening of Extracts

Phytochemical screening was carried out on the extracts to detect and quantify metabolites such as polyphenols, flavonoids, and tannins according to procedures described by: Boussahel et al.[40] for polyphenols; Pękal and Pyrzynska[41] for flavonoids; Tomaino et al.[42] for condensed tannins, Mole and Waterman[43] for hydrolysable tannins, and Russo et al.[44] for total tannins. Results were expressed as mean ± standard deviation (SD) of three different experiments.

Qualitative and Quantitative HPLC Analysis (HPLC/DAD and HPLC/ESI-MS Analyses)

In order to obtain the compositional profiles of different parts of Rs (flowers, leaves and stems), chromatographic analyses using diode array (DAD) and mass spectrometry (ESI-MS) detectors were performed. A

small aliquot of the methanolic extracts described above was re-dissolved in methanol and transferred in a 2 mL HPLC-amber vial immediately prior to analyses. Quantitative results are reported as mg of polyphenol in 100 mg of dry extract.

Chromatographic analyses were carried out on an Ultimate3000 UHPLC focused instrument equipped with a binary high pressure pump, a Photodiode Array detector, a Thermostated Column Compartment, and an Automated Sample Injector (Thermo Fisher Scientific, Inc., Milan, Italy). Collected data were processed through a Chromeleon Chromatography Information Management System v. 6.80. Chromatographic runs were performed using a reverse-phase column (Gemini C18, 250×4.6 mm, 5 μm particle size, Phenomenex Italia s.r.l., Bologna, Italy) equipped with a guard column (Gemini C18 4×3.0 mm, 5 μm particle size, Phenomenex Italia s.r.l., Bologna, Italy). Rs metabolites were eluted using a gradient of B (2.5% formic acid in acetonitrile) in A (2.5% formic acid in water): 0 min: 5% B; 10 min: 15% B; 30 min: 25% B; 35 min: 30% B; 50 min: 90% B; 57 min then, kept for other 7 min, 100% B. The solvent flow rate was 1 mL/min, the temperature was kept at 25 °C and the injector volume selected was 10 μL. Quantification was carried out at 350 nm to detect flavonoid derivatives using kaempferol *3-O*-rutinoside (R2 = 0.9990) and quercetin 3-*O*-galactoside (R2 = 0.9991) as references. Similarly, quantification of hydroxycinnamic acid derivatives was made at 330 nm using chlorogenic acid (R2 = 0.9994) and caffeic acid (R2 = 0.9997) as references. In order to confirm peak assignments, HPLC/ESI-MS analyses were also performed. The HPLC apparatus used was the same described above, whilst ESI mass spectra were acquired by a Thermo Scientific Exactive Plu Orbitra MS (Thermo Fisher Scientific, Inc., Milan, Italy), using the operating conditions described in Chelly et al.[45] Analyses were always carried out in triplicate; results are expressed as mg compound/100 mg dry extract.

Antioxidant Activity

The antioxidant/free radical scavenging capability of the extracts was evaluated by a battery of *in vitro* tests: Trolox Equivalent Antioxidant Capacity (TEAC) assay, Ferric Reducing/Antioxidant Power (FRAP) assay, Total antioxidant capacity – Phosphomolybdate (TAC-Pm) assay, and Oxygen radical absorbance capacity assay using fluorescein assay (ORAC).[46,47] All experiments were carried out in triplicate and results were expressed as mean ± SD of three different experiments.

β-Carotene Bleaching (βCB) Assay, Superoxide Anion Scavenging Activity (Superoxide Dismutase-SOD Assay) and NO Scavenging Activity

The βCB potential of extracts was measured by the method reported in Abidi et al.[48] The extract scavenging capability against the superoxide anion and against nitric oxide (NO) was measured by the methods described by Tomaino et al.[42] and by Kumaran et al.,[49] respectively. Results were expressed as 50% inhibitory concentration (IC_{50}) or 50% scavenging concentration (SC_{50}) and 90% confidence limits (C.L.), as calculated by the Litchfield and Wilcoxon test. All experiments were carried out in triplicate.

Inhibition of $HOCl^-$ Induced Albumin Denaturation

The ability of the extracts to inhibit the degradation of albumin produced by hypochlorite was evaluated using an electrophoretic method as described by Abidi et al.[48] Quantitative analysis was performed by gel densitometry using ImageJ software. Experiments were carried out in triplicate and results expressed as IC_{50} values (mg extract/mL) and 90% C.L., as calculated by the Litchfield and Wilcoxon test.

Anti-Inflammatory Activity

Cytotoxicity

The biocompatibility of the extracts on NIH/3T3 fibroblasts (American Tissue Culture Collection, ATCC) was examined by the sulforhodamine B (SRBassay, as described by Vichai and Kirtikara.[50] Cells monolayers were treated for 24 h with the extracts (up to 70 μg/mL) or the vehicle (DMSO; controls).

Intestinal Epithelial Cells Inflammation Model

Caco-2 cells (ATCC) were grown and differentiated on the upper side of transwell inserts (0.4 μm pore size; BD Falcon) as previously described.[51] Cells were then pretreated with the extracts, added to the apical side of the transwell inserts, for 24 h. Then, cells were washed with PBS and treated with TNF-α to (50 ng/mL) for 6 h added in both compartments of the inserts.[51]

RNA was obtained using the E.Z.N.A. Total RNA Kit I (OMEGA bio-tek VWR) quantified by Quanti-iT RNA assay kit QUBIT (Invitrogen) and reverse transcripted with the M–MLV reverse transcriptase (Sigma). Gene

expression was assessed by qPCR with an ABI 7300 Real-Time PCR System with the Sybr green JumpStart™ Taq Ready Mix kit (Sigma). The specific primers sets used were already described.[51,52] Fold increase of mRNA expression, compared with the control cells and with 18S rRNA as housekeeping gene, was calculated with the $2^{-\Delta\Delta Ct}$ method.[53]

Anti-Melanogenesis Activity

Tyrosinase Inhibition Assay

The extracts of the Rs were tested for their capability to inhibit the diphenolase activity of tyrosinase according to the method of Ferro et al.[54] Samples were dissolved in DMSO and used for the experiments in the range of concentrations 10–1000 μg/mL. For the assay, a pre-incubation mixture consisted of 0.05 mL of sample, 0.5 mL of L-DOPA solution (1.25 mM) and 0.9 mL of sodium acetate buffer solution (0.05 M, pH 6.8) was maintained at 25 °C for 10 min. Then, 0.05 mL of an aqueous solution of mushroom tyrosinase (333 U/mL) was added last to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm for 2 min.

The extent of inhibition by the addition of samples is expressed as inhibition percentage and calculated as follows:

$$\text{Inhibition}\% = \left(A - \frac{B}{A}\right) \times 100$$

Where A and B represent the absorbance at 475 nm for the blank and *Rhanterium suaveolens* extracts, respectively. Kojic acid (1.25–5 μg/mL) was used as a reference standard. All the experiments were performed in triplicate and repeated three times, The concentrations leading to 50% activity inhibition (IC_{50}) were calculated and results were expressed as mean ± SD.

Zebrafish Assay

Zebrafish embryos of wild type fishes (*Danio rerio*) were purchased in a local pet store and taken in flow through aquaria at 28.5 °C on a 14/10 h (light/dark) photo period.

Embryos were produced by natural mating as described by Kimmel et al.[55] and they were cultured in embryo water (0.2 g L^{-1} Instant Ocean® Salt, Aquarium Systems, USA) at 28.5 °C. Developmental age of the embryos was indicated as hours post fertilization (hpf). All experiments were executed in agreement with the European Directive 2010/63/EU and the ethical guidelines described in the 'National Institutes of Health Guide for Care and Use of Laboratory Animals', According to this Directive, the earliest life-stages of zebrafish (until 120 hpf) are not defined as protected and, therefore, do not fall into the regulatory frame works dealing with animal experimentation. In our experimental procedure, zebrafish embryos survival rate and development were evaluated until 96 h after treatment with RSF, RSS or RSL (72 h of exposure). Embryos were collected at 24 hpf, dechorionated and distributed in 96 well plates (one embryo per well). Test extracts were dissolved in DMSO (0.2%) and then, added to embryo medium (0.5–1 mg/mL). For screening the whitening capability of extracts, embryos were exposed from 24 to 72 hpf and then, observed under a stereomicroscope (48 h of exposure). Ten embryos were used for each treatment. Melanin accumulation, directly correlated to the zebrafish pigmentation areas, was calculated using ImageJ software and expressed as percentage with respect to the negative control (melanin accumulation 100 %). Control group received only DMSO N-Phenylthiourea (PTU; 0.02 mM) was used as reference compound. Results were expressed as mean ± SD.

Statistical Analysis

Results were statistically examined by one-way ANOVA with the ezANOVA software (http://www.sph.sc.edu/comd/rorden/ezanova/home.html). Statistical significance was considered at $P < 0.05$.

Authors Contribution Statement

Chelly S., Chelly M., Occhiuto C., Molonia M.S., D'Angelo V. performed the experiments. Chelly S., Cristani M., Speciale A. and Siracusa L., Germanò M.P. analyzed the data, and wrote the article. Cimino F., Ruberto G., Saija A. contributed samples/reagents/materials/analysis tools and analyzed the data. Speciale A., Siracusa L. and Bouaziz Ketata H. conceived and designed the experiments.

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