Comparison of Phytochemical Profile and Bioproperties of Methanolic Extracts from Different Parts of Tunisian Rumex roseus

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The genus Rumex (Polygonaceae) is distributed worldwide and the different species belonging to it are used in traditional medicine. The present study aimed at the evaluation of the phytochemical profile and the biochemical properties of methanolic extracts from different parts (roots, stems, and leaves) of Rumex roseus, a wild local Tunisian plant traditionally used as food.

The phytochemical analysis on the extracts was performed using standard colorimetric procedures, HPLC-DAD, and HPLC-DAD-ESI-MS; then, several in vitro cell-free assays have been used to estimate their antioxidant/free radical scavenging capability (TAC-PM, DPPH, TEAC, FRAP, ORAC, SOD-like activity, and HOCl-induced albumin degradation). Additionally, anti-inflammatory effect of these extracts was evaluated in an in vitro model of acute intestinal inflammation in differentiated Caco-2 cells. The results showed that the methanolic extracts from stems and, especially, leaves contain substantial amounts of flavones (apigenin and luteolin, together with their derivatives), while the extract from roots is characterized by the presence of tannins and quinic acid derivatives. All the extracts appeared endowed with excellent antioxidant/free radical scavenging properties. In particular, the extract from roots was characterized by a remarkable activity, probably due to its different and peculiar polyphenolic composition. Furthermore, both Rumex roseus roots and stems extracts demonstrated an anti-inflammatory effect in intestinal epithelial cells, reducing TNF-α-induced gene expression of IL-6 and IL-8. In conclusion, R. roseus methanolic extracts have shown to be potential sources of bioactive compounds to be used in the prevention and treatment of pathologies related to oxidative stress and inflammation.

Keywords: Rumex roseus, antioxidant activity, anti-inflammatory activity, Caco-2, interleukins, HPLC/ESI-MS.

Introduction

The craze for natural remedies and the growing distrust in chemical drugs have considerably renewed the interest in medicinal plants in general and particularly in plants based functional foods.1,2,3 That is the case of Rumex species, polygonaceous plants that have been recently attracted the interest of the scientific community thanks to their significant phytochemical potentials.4 Some Rumex species have exhibited healthy effects and have been used as traditional foods and herbal remedies, and numerous articles deal about the important activity of their extracts5,6,7. The therapeutic effects of Rumex species are entrusted to their richness and diversity in...
secondary metabolites, mainly polyphenols. Indeed, these species are characterized by the presence of several flavonoids (luteolin, apigenin, epicatechin, etc.), phenolic acids (caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acids, etc.) and tannins, particularly in their roots. These bioactive molecules are reported as effective therapeutic agents against oxidative stress and inflammatory diseases. Following on our previous study, thanks to which Rumex roseus (RR) was added to the functional food list due to its excellent antioxidant power, the aim of the present work was to assess the chemical composition of methanolic extracts obtained from different parts of RR (roots (RRR), stems (RRS) and leaves (RRL)) and to evaluate their antioxidant and anti-inflammatory properties. 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. Finally, their anti-inflammatory properties were investigated in an in vitro Caco-2 model of acute intestinal inflammation.

Results and Discussion

Phytochemical Characterization

Methanolic extracts from RR organs gave the following yields after vacuum concentration (see also Table 1): leaves (RRL), 11.6%; stems (RRS), 8.7%; roots (RRR), 7.1%. In order to evaluate the phytochemical composition of the different RR parts, several in vitro tests were carried out (Table 1). Results coming from the evaluation of the flavonoid composition showed RRL as the richest extract (RRL: 44.28 ± 0.44; RRS: 13.20 ± 0.74; RRR: 10.81 ± 0.04, expressed as µg QE/mg of dry extract) in fact, as reported in literature the leaves of Rumex species are particularly rich in flavonoids compared to the other plant parts. As regarding tannins (total, hydrolysable and condensed) RRR were the richest organ of the plant, with condensed tannins far more abundant than hydrolysable ones (Table 1), thus confirming that this particular subclass of polyphenols could be considered as one of the peculiar characteristics of Rumex roots, as already has been reported in the literature for 7 different Rumex species.

Chromatographic Analyses (HPLC-DAD and HPLC-ESI-MS)

Chromatographic analyses performed on stems, leaves and roots from R. roseus allowed to have a look on the distribution of different secondary metabolites in RR organs (Figure 1 and Table 2). While RRR methanolic extract is characterized by a relatively simple metabolic pattern comprising flavan-3-ols and various phenolic acids for a total of nine metabolites identified, RRS and RRL extracts showed a more diverse profile. In fact, ca. 20 different peaks were detected and tentatively identified, all belonging to the class of polyphenols and in particular those of flavanols and derivatives, phenolic acids, and derivatives and two different flavones (apigenin and luteolin) together with many derivatives (Figure 1 and Table 2). Interestingly, RRR extract is characterized by the presence of many quinic acid derivatives as CFQA (caffeoylferuloylquinic acids) and dCQA (di-cafeoylquinic acids), while pCoQA (p-coumaroylquinic acids) are detected only in RRS extracts. All these compounds were not found in RRL extracts (Table 2, Figure 2).

The different distribution of secondary metabolites within a given plant is a very well-known phenomenon and it has to do, among other things, with the specific function of a particular organ in plant organism.

Antioxidant Activity

All plant parts have shown excellent antioxidant power, although, roots appeared to be the most active RR organ compared to leaves and stems for most tests

Table 1. Extraction yields and phytochemical analysis results from Rumex roseus methanolic extracts. Data were expressed as means ± SD of three different experiments.

| Plant part | Yields | Flavonoids | Total Tannins | Hydrolysable tannins | Condensed tannins |
|------------|--------|------------|---------------|----------------------|------------------|
|            |        | µg QE/mg de[a] | µg TAE/mg de[a] | µg TAE/mg de[a] | µg CE/mg de[a] |
| RRR        | 7.1    | 10.81 ± 0.04  | 121.40 ± 4.64[ab] | 40.70 ± 7.34[ab] | 112.80 ± 19.64[ab] |
| RRS        | 8.7    | 13.20 ± 0.74[bc] | 23.70 ± 3.5  | 18.30 ± 1.5  | 11.30 ± 2.6  |
| RRL        | 11.6   | 44.28 ± 0.44[bc] | 43.90 ± 3.3[bc] | 26.40 ± 0.6[bc] | 12.30 ± 5.5  |

[a] QE: quercetin equivalents; CE: catechin equivalents; TAE: Tannic acid equivalents; de: dry extract. [b] *P < 0.05 vs. RRL; [bc] **P < 0.05 vs. RRS; [c] ***P < 0.05 vs. RRR.
Figure 1. HPLC-DAD chromatograms of *Rumex roseus* methanolic extracts. (a) RRR = roots; (b) RRS = stems; (c) RRL = leaves. A) chromatogram extracted at 280 nm; B) chromatogram extracted at 330 nm; C) chromatogram extracted at 350 nm. Peaks are numbered as reported in Table 2.
such as: TAC-Pm, TEAC, FRAP, SOD and ORAC (Table 3).

However, the antioxidant activity of RRR was higher in a statistically significant way than RRS and RRL in TAC-

PM, TEAC and FRAP assays and, than RRS only in DPPH and SOD assays, while no statistical difference among the extracts was observed in the ORAC assay (Table 3). These differences are related to the different chemical composition of RRR, RRS and RRL (Table 2, Figure 2) and also to the different chemical environment of each assay.

Similar to our findings, the roots of *R. obstifolius* were also more active than its leaves for the ABTS, DPPH and FRAP assays. Moreover, the significant excellence of the underground organ of RR for this antioxidant activity could be explained by its richness in tannins, mainly condensed tannins, as well as by their content in diCQA and CFQA (Table 2, Figure 2). In fact, the therapeutic potentials of proanthocyanidins are diverse such as cardioprotective, antidiabetic, anticancer, antimicrobial, neuroprotective, antioxidant and anti-inflammatory. Furthermore, chlorogenic acids are well-known for their antioxidant properties demonstrated in cell-free, cell-based, and animal-based assays.

### Table 2. Secondary metabolites tentatively identified in *R. roseus* plant parts (by HPLC-DAD). Results are presented as average value of three replicates and expressed in mg over 100 mg dry extract.

| Peak No. | Rt (min) | Compound[a] | Biochemical subclass | RRR | RRS | RRL |
|----------|---------|-------------|----------------------|-----|-----|-----|
| 1        | 4.85    | gallic acid* | organic acids        | 0.0152 | 0.0232 | 0.0159 |
| 2        | 8.89    | EGC*        | flavan-3-ols         | n.d. | 0.0132 | n.d.    |
| 3        | 10.10   | catechin*   | flavan-3-ols         | n.d. | 0.0190 | n.d.    |
| 4        | 11.64   | epicatechin*| flavan-3-ols         | n.d. | 0.1826 | 0.3680  |
| 5        | 12.10   | caffeic acid* | cinnamic acids      | 0.0232 | 0.0589 | 0.4093  |
| 6        | 13.25   | EGCG        | flavan-3-ols         | 0.1153 | 0.0285 | 0.0534  |
| 7        | 16.39   | pCoQA (MW = 338) | cinnamic acids       | n.d. | 0.0724 | n.d.    |
| 8        | 16.81   | luteolin di-glycoside (MW = 610) | flavones | n.d. | 0.0319 | 0.3702  |
| 9        | 18.33   | ferulic acid* | cinnamic acids      | 0.0233 | 0.0458 | 0.1246  |
| 10       | 20.59   | apigenin glycoside (MW = 432) | flavones | n.d. | 0.0037 | 0.0704  |
| 11       | 21.46   | luteolin 7-O-glucoside* | flavones | 0.0534 | 0.1986 | 0.9740  |
| 12       | 25.85   | apigenin di-glycoside (MW = 594) | flavones | n.d. | 0.0774 | 0.4479  |
| 13       | 26.36   | diCQA (MW = 516) | cinnamic acids       | 0.0934 | n.d. | 0.1996  |
| 14       | 27.08   | luteolin hexoside-pentoside (MW = 580) | flavones | n.d. | 0.0418 | 0.1996  |
| 15       | 27.52   | methyl luteolin hexoside-deoxyhexoside (MW = 608) | flavones | n.d. | 0.0770 | 0.4046  |
| 16       | 32.21   | CFQA (MW = 530) | flavones | 0.1342 | n.d. | n.d.    |
| 17       | 32.45   | apigenin derivative | flavones | n.d. | n.d. | 0.0785 |
| 18       | 33.20   | luteolin deoxyxide (MW = 432) | flavones | n.d. | 0.0107 | 0.0491 |
| 19       | 33.79   | methyl luteolin glycoside (MW = 462) | flavones | n.d. | 0.0578 | 0.1885  |
| 20       | 36.09   | luteolin | flavones | 0.0662 | 0.1547 | 0.8049  |
| 21       | 37.84   | methyl luteolin (MW = 300) | flavones | n.d. | 0.0172 | 0.0588 |
| 22       | 38.19   | apigenin pentoside (MW = 402) | flavones | n.d. | 0.0251 | 0.1201 |
| 23       | 40.57   | apigenin* | flavones | n.d. | 0.0541 | 0.2959 |
| 24       | 41.02   | methyl luteolin isomer (MW = 300) | flavones | n.d. | 0.0274 | 0.0871 |
| 25       | 42.93   | p-coumaric acid derivative | flavones | 0.1328 | n.d. | n.d.    |

[a] EGC: Epigallocatechin; EGCG: Epigallocatechin gallate; pCoQA: p-coumaroylquinic acid; diCQA: dicafeoylquinic acid; CFQA: caffeoylferuloylquinic acid; n.d.: compound not detected. *Peak identity confirmed with the use of the corresponding analytical standard.
Table 3. Antioxidant[a] evaluation of Rumex roseus methanolic extracts. Data were expressed as means ± SD for three different experiments.

| Plant part | TAC-Pm | DPPH | TEAC | FRAP | SOD | ORAC |
|------------|--------|------|------|------|-----|------|
|            | nmol TE/mg de | μmol TE/mg de | μmol TE/mg de | μmol Fe²⁺ E/mg de | nmol QE/mg de | mmol TE/g de |
| RRR        | 1.39 ± 0.08ab[b] | 0.39 ± 0.12b | 0.54 ± 0.022ab | 1.30 ± 0.03ab | 5.00 ± 1.9b | 8.93 ± 2.92 |
| RRS        | 0.38 ± 0.06 | 0.19 ± 0.04 | 0.32 ± 0.098 | 0.34 ± 0.01 | 2.80 ± 0.6 | 5.44 ± 2.09 |
| RRL        | 0.63 ± 0.06b | 0.26 ± 0.05 | 0.35 ± 0.077 | 0.70 ± 0.11b | 4.70 ± 1.9 | 5.77 ± 1.95 |

[a] TAC-Pm: Total antioxidant capacity – Phosphomolydate; DPPH: DPPH⁺ radical trapping; TEAC: Trolox equivalent antioxidant capacity; FRAP: ferric reducing antioxidant power; SOD: Superoxide radical trapping; ORAC: Oxygen radical absorbance capacity; TE: Trolox equivalents; Fe²⁺ E: ferrous equivalents; QE: quercetin equivalents; de: dry extract. [b] ²P < 0.05 vs. RSL; ³P < 0.05 vs. RRS; ⁵P < 0.05 vs. RRR.

Although RRR were the bests in antioxidant activity, RRL and RRL also exhibited a noticeable power so that the combination of the different parts may result in a better effect as it is the case of R. roseus aerial parts mixture (stems and leaves) and that of R. crispus and obstusifolius which had showed an interesting antioxidant effect after two by two plant organs combination (leaves, stems, and roots).[11,17]

Anti-Inflammatory Activities

Inhibition of HOCl⁺ Induced Albumin Denaturation

In order to evaluate the anti-inflammatory effect of our extracts, we used an in vitro electrophoretic experimental model (SDS/PAGE) mimicking the physiological conditions that are characterized by remarkable proteins degradation such as albumin. Considered as albumin target molecule, hypochlorous acid (HOCl) was used in our experiment as denaturing agent.[15] In fact, our results showed that HOCl (177 mM) had provoked total protein degradation characterized by a remarkable intensity decrease of BSA characteristic band (62 kDa) (Figure 3). The three RR parts showed significant protection of BSA against HOCl denaturation with the following IC₅₀: 0.42 mg/mL, 0.60 mg/mL, and 0.64 mg/mL of RRL, RRR and RRS, respectively (Figure 3). Our results indicated the RRL extract as the most powerful in this model compared even to the used standard (Trolox). To our knowledge, RRL are the first leaves from Polygonaceae family in general and Rumex genus in particular, which have been tested for this model and have succeeded to prove their anti-inflammatory effect with excellence. Indeed, similar to our results, Cocculus hirsutus leaves (Menispermaceae) were more active than its stems with inhibition percentages of 65.85% and 46.15%, respectively, at a dose of 1 mg/mL, but always these aerial parts are less active than those of RR.[19] In order to better explain the excellence of RRL compared to the other parts, we can refer to the characteristic molecules that distinguish this organ such as luteolin, ferulic acid, caffeic acid. In particular, according to an in silico study conducted to identify the potent leaders of the anti-inflammatory activity, luteolin was classified among 12 compounds which has passed the virtual filtration steps done on 157 compounds naturally isolated from plants and collected from various phytochemical databases.[20] Moreover, according to Siwak et al.,[21] this molecule was ranked as the third just after quercetin and fisetin, when tested among 15 phenolic compounds (flavonoids), thanks to its excellent anti-inflammatory effect proven by its in vitro BSA protection against HOCl denaturation. Similar to our results, HPLC-DAD phytochemical screening of Moroccan date seeds (Phoenix dactylifera) revealed the

![Figure 3. Inhibition of HOCl⁺ induced albumin denaturation. Representative image from three independent experiments. BSA, treated with hypochlorite (177 mM), was exposed to different amounts of RR extracts. Trolox was used as a positive/standard control. Results are expressed as IC₅₀ values (mg dry extract/mL) and 90% C.L.](image-url)
abundant presence of luteolin, caffeic and ferulic acid, to which an excellent BSA anti-denaturing effect is very likely due.\textsuperscript{[22]} Indeed, in this assay the presence of tannins in root extracts may partly explain the observed moderate anti-inflammatory effect when the flexibility of some tannin gives them a preference for binding to some proteins such as gelatin but not for BSA.\textsuperscript{[23]} However, despite their moderate anti-inflammatory effects of stems and roots the three parts of RR together could lead to an excellent anti-inflammatory effect greater than that given by each organ alone.

**Anti-Inflammatory Activity in TNF-\(\alpha\)-Challenged Caco-2 Cells**

The pleiotropic cytokine TNF-\(\alpha\) is able to induce a strong cellular inflammatory response through the induction of the NF-kB cell signaling pathway. In fact following activation, NF-kB promotes the transcription of many genes codifying for proinflammatory mediators, such as IL-6 and IL-8, leading to acute inflammatory responses and related to chronic diseases,\textsuperscript{[24,25]} such as Inflammatory Bowel Diseases (IBDs), and actively contributing to gut inflammation.\textsuperscript{[26]} Moreover, many drugs for the treatment of IBDs hinder NF-kB activation.\textsuperscript{[27]}

Thus, the anti-inflammatory properties of these Rumex extracts were studied in a model of TNF-\(\alpha\)-challenged Caco-2 intestinal epithelial cells through the evaluation of IL-6 and IL-8 gene expression. At first, the biocompatibility of the three extracts was evaluated on NIH/3T3 fibroblast cell lines. NIH/3T3 cells were treated with the different extracts (up to 70 \(\mu\)g/mL) for 24 h. RRR and RRS did not show any cytotoxicity at all the concentrations tested (data not shown), whereas only the RRL extract at the highest tested concentration caused a significant decrease (65 \(\pm\) 6\%) in cell viability. Thus, in the Caco-2 model, cells were pre-treated for 24 h with RRS and RRR at 35 and 70 \(\mu\)g/mL, whereas RRL was used at lower concentrations (15 – 30 \(\mu\)g/mL), and then, cells were exposed to TNF-\(\alpha\) at a concentration of 50 ng/mL for 6 h. In this model, cells exposed to TNF-\(\alpha\) showed higher levels of IL-6 and IL-8 gene expression compared to control (Figure 4). In cells not exposed to TNF-\(\alpha\), RRR and RRS extracts (35 and 70 \(\mu\)g/mL) pretreatment had no effect on the basal expression levels of these genes (Figure 4), whereas IL-6 and IL-8 overexpression induced by TNF-\(\alpha\), instead, were moderately and dose dependently inhibited (Figure 4). At both the concentrations used (15 – 30 \(\mu\)g/mL), RRL extract did not show any activity neither under basal conditions nor on TNF-\(\alpha\) challenged cells (data not shown). Thus, the experiment carried out demonstrated an important Caco-2 cells protection against TNF-\(\alpha\) inflammation following their pretreatments with RR roots and stems extracts.

In fact, roots of the genus *Rumex* were often reported to be effective against gastrointestinal diseases.\textsuperscript{[28]} In addition, according to RRR phytochemical screening results, its profile, with condensed tannins, \(p\)-coumaric acid, chlorogenic acids and ECGC,

![Figure 4. IL-6 and IL-8 gene expression. The Caco-2 monolayer was pretreated with the RRS and RRR extracts (35–70 \(\mu\)g dry extract/mL) for 24 h, and subsequently exposed to 50 ng/mL TNF-\(\alpha\) for 6 h. Cultures treated with the DMSO alone were used as controls (CTR). Results are expressed as \(2^{-\Delta\Delta\text{Ct}}\). \(aP < 0.05\) vs. CTR; \(bP < 0.05\) vs. TNF-\(\alpha\); \(cP < 0.05\) vs. same dose of the same extract; \(dP < 0.05\) vs. lower dose of the same extract + TNF-\(\alpha\).](image-url)
may explain the excellence of this organ for this activity. In fact, condensed tannins are recognized to have important anti-inflammatory effects. For example, we previously reported that condensed tannins obtained from grape seeds were able to reduce inflammatory mediators such as COX-2, PGE2, Cyclin D1 in a mouse model of UV-induced skin tumor.\(^{[29]}\) Black tea proanthocyanidins were also reported to prevent from the harmful effects of chemotherapy thanks to their anti-inflammatory effects through inhibition of NF-κB pathway.\(^{[10]}\) Also, p-coumaric acid was reported to possess anti-inflammatory effects, as demonstrated by the reduction of TNF-α expression in a rat model of arthritis.\(^{[30]}\)

However, tight junctions (TJ) are crucial for intestinal epithelium integrity, and some flavonoids such as EGCG,\(^{[31]}\) as well as \(R.\) japonicus extract,\(^{[28]}\) have been reported to exhibit anti-inflammatory activity and protective effects on intestinal TJ barrier functions, ensuring the improvement of disorganized intestinal architecture by regulating the expressions of Zonula Occludens-1, occludin and claudin-2, which may be related to inhibition of pro-inflammatory cytokines, such as TNF-α.\(^{[28]}\)

RRR reduced gene expression of pro-inflammatory IL-6 and IL-8 relatively better than the RRS, this difference was not significant, which could be explained by the presence of common molecules between them, such as cinnamic acid, catechins, and their derivatives. Indeed, Liang and Kitts\(^{[32]}\) and de Magalhães et al.\(^{[33]}\) reported that chlorogenic acids were able to reduce the inflammation induced by TNF-α in Caco-2 cells via the inhibition of NF-κB pathway, with a resulting decrease of IL-6 and IL-8 expressions. Our results are in agreement with Siwak et al.\(^{[21]}\) who had mentioned an inverted ranking for catechin and ECGC which presented moderate activity against hypochlorite-induced protein modifications in a cell-free system, whereas they were the leaders in protecting HeLa cells from hypochlorite cytotoxicity.

Concerning RRL, which presented the best anti-inflammatory activity \textit{in vitro}, it showed a non-significant protection in this \textit{in vitro} cell model. In fact, luteolin which constitutes the characteristic molecule of RR leaves, was previously described for its anti-inflammatory activity \textit{in vitro} in cell-free systems, as well as for its pro-inflammatory one in cell-based assays; at a concentration of 20 μM luteolin protected BSA against HOCl denaturation, whereas in HeLa cells this concentration did not reveal any anti-inflammatory effect but rather it had provoked cell toxicity.\(^{[21]}\) Moreover, Matsuo \textit{et al.}\(^{[34]}\) showed that some flavonoids, such as luteolin and apigenin, are cytotoxic at higher concentrations toward normal cells since, at high intracellular levels, they can increase intracellular ROS levels, and then, exert cytotoxicity.

### Conclusions

The present findings describe for the first time the chemical composition and the antioxidant and anti-inflammatory properties of methanolic extracts from leaves, roots, and stems of Tunisian \(R.\) roseus.

The results show that these extracts are characterized by the presence of several polyphenols, in particular flavanols, flavones, phenolic acids and tannins, and all of them showed good antioxidant activity. Furthermore, the extracts from roots and stems of RR appeared to be able to protect intestinal epithelial cells against inflammation.

In conclusion, we have demonstrated that \(R.\) roseus is a rich source of bioactive ingredients with potential exploitation in phytopharmaceuticals and food industry.

### Experimental Section

#### Chemicals and Reagents

Unless otherwise stated, all reagents and solvents were of analytical grade and used without further purification. Acetonitrile and water were high purity spectroscopic grade solvents obtained from Merck (VWR International, Milan, Italy). Pure reference standards – catechin, epicatechin and epicatechin gallate – were purchased from Extrasynthese (Lyon, France); whilst gallic acid, caffeic acid, ferulic acid, apigenin and luteolin 7-O-glucoside were obtained from Fluka (Sigma-Aldrich, Milan, Italy).

#### Plant Material and Extraction Protocol of Rumex roseus

Leaves, stems, and roots of \(Rumex\) roseus were collected during the early spring of 2017 from Sfax region (Latitude: 34°44′26″ North; Longitude: 10°45′37″ East), Tunisia. A voucher specimen (voucher No. QS-224) authenticated by Prof. Mohamed Chaieb (Faculty of Science, Sfax University), was deposited at the Faculty of Sciences (Sfax, Tunisia). The methanolic extracts were obtained as previously reported by Chelly \textit{et al.}\(^{[11]}\)
Phytochemical Screening of Rumex roseus Extracts

Phytochemical screening was carried out on the three methanolic extracts to detect the presence of flavonoids and tannins by spectrophotometric assays. Flavonoid amount was measured at 425 nm after addition of AlCl₃ solution according to Pękal and Pyrzynska,[35] Methods described by Tomaino et al.,[36] Mole and Waterman[37] and Russo et al.[38] were used to measure condensed tannins, hydrolysable tannins, and total tannins, respectively. In all the tests results were expressed as mean ± SD of three different experiments.

HPLC/DAD and HPLC/ESI-MS Analyses

Quantitative analyses were carried out on a Ultimate3000 ‘UHPLC focused’ instrument as described in Abidi et al.[39] Quantification was carried out at 280 nm for gallic acid and catechin, epicatechin and epicatechin gallate using calibration curves established with the corresponding analytical standards (gallic acid, correlation coefficient R² = 0.9998; catechin, correlation coefficient R² = 0.9997; epicatechin, correlation coefficient R² = 0.9999; epicatechin gallate, correlation coefficient R² = 0.9999), whilst epigallocatechin gallate was quantified also at 280 nm using epicatechin gallate as reference. Hydroxycinnamic acids were quantified at 330 nm using caffeic acid (correlation coefficient R² = 0.9998) to build the calibration curves. Apigenin derivatives were also quantified at 330 nm using apigenin (correlation coefficient R² = 0.9999) as reference. Luteolin and its derivatives were all quantified at 350 nm using luteolin 7-O-glucoside (correlation coefficient R² = 0.9998) as reference. All analyses were carried out in triplicate; results are reported in mg of compound over 100 mg of dry extract. HPLC-ESI/MS analyses were also performed on the same samples, as described in Abidi et al.[39] Data acquisition was performed using the Excalibur software. Analyses were always carried out in triplicate.

Antioxidant Activity

The antioxidant/free radical scavenging capability of extracts was evaluated by a battery of in vitro tests: Total antioxidant capacity – Phosphomolybdate (TAC-PM) assay, DPPH radical (2,2-diphenyl-1-picrylhydrazyl) scavenging assay, Trolox Equivalent Antioxidant Capacity (TEAC) assay, Ferric Reducing/Antioxidant Power (FRAP) assay, Superoxide radical trapping assay (SOD), Oxygen radical absorbance capacity assay using fluorescein (ORAC).[36,40–42] Results were expressed as mean ± SD of three different experiments carried out in triplicate.

Anti-Inflammatory Activity

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,[18] as described in Abidi et al.[39]

Trolox was used as a positive/standard control. Experiments were carried out in triplicate and the results were expressed as IC₅₀ values and 90% C.L. (mg/mL).

Cell Cultures and Treatments

Cytotoxicity of RR Extracts on NIH/3T3 Cells

The biocompatibility of the extracts on NIH/3T3 fibroblasts (American Tissue Culture Collection, ATCC) was evaluated through the sulforhodamine B (SRB) assay, as described by Vichai and Kirtikara.[43] Semi-confluent NIH/3T3 monolayers were exposed for 24 h to the extracts added to the cell culture medium (up to 70 μg/mL), or to the vehicle alone (DMSO). Results are expressed as percentage of viable cells compared to controls.

Anti-Inflammatory Activity of RR Extracts on Caco-2 Cells

Caco-2 epithelial cells (ATCC), were grown and differentiated to obtain monolayers on the upper side of transwell inserts (0.4 μm pore size; BD Falcon) as previously describe.[44] The differentiated Caco-2 monolayers were pretreated with the Rumex extracts, or with the vehicle alone (DMSO), for 24 h, added to the apical side of the transwell inserts. After 24 h, cells were washed twice with DPBS and exposed for 6 h to 50 ng/mL TNF-α,[44] added in both the apical and the basolateral sides of the inserts.

Cell RNA was extracted using the E.Z.N.A. Total RNA Kit I (OMEGA Bio-Tek VWR), quantified by Quanti-IT™ RNA assay kit QUBIT (Invitrogen, Milan, Italy), and reverse transcribed with the M-MLV reverse transcriptase (Sigma). Gene expression was assessed by real-time PCR with a 7300 Real-Time PCR System (Applied Biosystems, Monza, Italy) coupled with the
Sybr green JumpStart™ Taq Ready Mix kit (Sigma). The specific primers sets used were previously described.[44,45] The fold increase of mRNA expression, compared with the control cells and corrected with 18S rRNA housekeeping gene, was determined using the 2^−ΔΔCt method.[46]

Statistical Analysis

All the experiments were performed in triplicate and repeated three times. Results are expressed as mean ± SD and analyzed by a one-way ANOVA test, followed by Tukey's HSD, using the statistical software ezANOVA (http://www.sph.sc.edu/comd/rorden/ezanova/home.html). Differences considered significant for P < 0.05.

Abbreviations

RRR: Rumex roseus root extract
RRS: Rumex roseus stems extract
RRL: Rumex roseus leaves extract

Author Contribution Statement

Chelly M., Chelly S., Occhiuto C., and Muscarà C. performed the experiments. Chelly M., Cristani M., Speciale, and Siracusa L. analyzed the data and wrote the article. Cimino F., Ruberto G., and Saia A. contributed to the 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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