Inhibitory Effect on Nitric Oxide Release in LPS-Stimulated Macrophages and Free Radical Scavenging Activity of Croton Linearis Jacq. Leaves

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Abstract: Oxidative stress is an important component of many diseases including cancer, along with inflammatory and neurodegenerative processes. Natural antioxidants have emerged as promising substances to protect the human body against reactive oxygen and nitrogen species. The present study evaluates the inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 murine macrophages and the free radical scavenging activity of Croton linearis Jacq. leaves. UPLC-QTOF-MS analysis identified 18 compounds: nine alkaloids with a morphinane, benzylisoquinoline or aporphine nucleus, and nine O-glycosylated-flavonoids with quercetin, kaempferol and isorhamnetin as the aglycones. The crude extract (IC50 21.59 µg/mL) and the n-hexane fraction (IC50 4.88 µg/mL) significantly reduced the NO production in LPS-stimulated macrophages but with relatively high cytotoxicity (CC50 75.30 and CC50 70.12 µg/mL, respectively), while the ethyl acetate fraction also showed good activity (IC50 40.03 µg/mL) without affecting the RAW 264.7 cell viability. On the other hand, the crude extract, as well as the dichloromethane and ethyl acetate fractions, showed better DPPH and ABTS free radical scavenging activities. Considering the chemical composition and the activity observed for Croton linearis leaves, they may be considered a good source of antioxidants to combat oxidative damage-related diseases.

Keywords: Croton linearis; nitric oxide release; alkaloids; flavonoids; antioxidants

1. Introduction

Oxidative stress has emerged as one of the important factors in the pathogenesis of different diseases such as cancer and diabetes, autoimmune, cardiovascular and neurodegenerative diseases, inflammatory processes, etc. [1,2]. For this reason, interest in the use of natural antioxidants to protect the human body against diseases mediated by reactive oxygen species (ROS), reactive nitrogen species (RNS) and free radicals has increased [3–5].

Plants are susceptible to environmental stress and have developed numerous defense systems, resulting in the formation of potent antioxidants [6]. In fact, it is well-accepted in the literature that plants are a natural reservoir of antioxidant bioactive compounds [7].
Among the compounds that are produced by plants, the phenolic compounds (characterized by one or more hydroxyl groups bound to one or more aromatic rings) such as phenolic acids, flavonoids, anthocyanidins and tannins are the major contributors to the antioxidant activity of plant extracts [8,9]. These compounds act by various mechanisms, including binding metal ions, scavenging reactive oxygen and nitrogen species, converting hydroperoxides to non-radical species, absorbing UV radiation and deactivating singlet oxygen [2].

*Croton linearis* Jacq. (Euphorbiaceae) is a small shrub endemic to the Bahamas, Florida and the West Indies, growing mostly in rather dry environments and on rocky land near the coast, in sandy and gravelly soils and open areas [10]. In Cuba, it is a native plant distributed along the southern coast of the eastern region, where the coastal and pre-coastal xeromorphic shrub predominates [11,12]. Ethnopharmacological information refers to its use mainly to relieve pains related to menstrual periods, childbirth and rheumatism, and to treat fever and colds [11,13]. A few studies for this species have shown the presence of isoquinoline alkaloids as the main compounds [14,15], while a diterpene [16], two flavonoids [15,17] and volatile compounds [18] have also been reported. In addition, studies have revealed its antiparasitic potential against *Leishmania* sp. [18], *Trypanosoma* sp. [15] and *Plasmodium* sp. [19], as well as larvicidal activity [20]. According to this phytochemical and pharmacological evidence and considering the dry and harsh environmental conditions of its habitat, it was recently mentioned as one of the medicinal plants growing in the Siboney-Jutíci Ecological Reserve (located in the southeast part of Cuba) of interest for its possible but not yet explored antioxidant potential [21].

Considering the absence of investigations regarding the antioxidant properties of *C. linearis* Jacq. leaves, the aim of this study was to evaluate the extract and its fractions for radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and the nitric oxide (NO) inhibitory activity in LPS-activated RAW 264.7 murine macrophages. ultra-performance liquid chromatography-quadrupole time of flight-mass spectrometry (UPLC-QTOF-MS) was used for the tentative identification of potentially bioactive compounds present in the most active fractions.

2. Materials and Methods

2.1. Material and Reagents

All solvents, including *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol and dimethyl sulfoxide (DMSO), and ethanol (Emsure grade) were purchased from Acros Organics (Geel, Belgium) or Fisher Scientific (Leicestershire, UK) and were analytical grade. Lipopolysaccharide (LPS) from *Escherichia coli* (0128:B12), tamoxifen, resazurin sodium salt (7-hydroxy-3H-phenoxazin-3-one-10-oxide), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) was sourced from Gibco (New York, NY, USA). A Griess reagent kit, L-NAME (N5-(imino(nitroamino)methyl)-L-ornithine methyl ester monohydrochloride) and ascorbic acid (99%) were purchased from Molecular Probes (Eugene, OR, USA), Alexis Biochemicals (New York, NY, USA) and Fluka (Germany), respectively. UPLC-grade solvents, such as acetonitrile (ACN) and formic acid, were purchased from Biosolve (Valkenswaard, The Netherlands). Water was dispensed by a Milli-Q system from Millipore (Bedford, MA, USA) and was passed through a 0.22 µM-membrane filter before usage.

2.2. Samples

*C. linearis* samples, before processing, were collected in the Siboney-Jutíci Ecological Reserve, Santiago de Cuba, Cuba, following the guidance from previous studies’ authors [15,19]. After processing, the samples consisted of ethanol crude extract (CEL) and four fractions: *n*-hexane (HF), dichloromethane (DCM), ethyl acetate (EAF) and *n*-butanol (BF). The crude extract (CEL) was obtained by percolation with ethanol 95%, while its
fractions (HF, DCM, EAF and BF) were obtained by liquid-liquid fractionation. The extract and fractions were vacuum concentrated until dry at 40 °C (with a Kirka–Werke rotary evaporator) and then stored at −20 °C for further analyses.

2.3. Free Radical Scavenging Activity

The antioxidant activity of the crude extract and its fractions was determined using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). Stock solutions were prepared by dissolving all samples in absolute ethanol at 1 mg/mL. Afterward, the samples were serially diluted to concentrations ranging from 500 to 31.125 µg/mL.

DPPH assay: This was carried out according to previous laboratory protocols [22]. Briefly, 1.5 mL of DPPH solution (0.1 mM in ethanol) was mixed with 0.25 mL of the samples (CLF, HF, DCM, EAF and BF) at different concentrations, shaken and incubated in the dark at room temperature for 30 min. After that, the absorbance was measured against a blank (ethanol) at 517 nm using a spectrophotometer (T60 UV-Visible Spectrophotometer/PG-instruments, UK). The percentage of scavenging activity was calculated using the equation: % scavenging activity = [(A0 − As)/A0] × 100, where A0 is the absorbance of the control (DPPH or ABTS solution without sample), and As is the absorbance of the tested sample (DPPH or ABTS plus sample).

ABTS assay: The ABTS radical scavenging capacity of the extract and its fractions was also measured following previous laboratory protocols [23]. The ABTS radical was produced by incubating a solution of ABTS (7 mM) with a solution of potassium persulfate (2.45 mM) at room temperature for 12 h. The ABTS solution (3 mL) was mixed with the samples (1 mL) at different concentrations, and the absorbance was measured after 90 min at 734 nm using a spectrophotometer (T60 UV-Visible Spectrophotometer//PG-instruments, UK). A solution of 50 µL of distilled water and 3 mL of diluted ABTS was used as the absorbance blank. The ABTS scavenging activity was calculated using the same equation as above.

In both assays, the mean inhibitory concentration (IC50) values of the extract and fractions were determined using a non-linear regression curve. Ascorbic acid was used as the positive control (from 31.125 to 500 µg/mL). All the experiments were done in triplicate and the results are presented as the mean ± standard deviation.

2.4. Cell Viability Assay

The effect of the extract and its fractions on the RAW 264.7 cell viability was determined by a resazurin dye reduction assay [24]. Briefly, 200 µL of cell suspension (5 × 10^5 cells/mL) was added to a 96-well plate and incubated in the same conditions mentioned above. After 24 h, the cells were washed twice with 200 µL of DPBS, then fresh DMEM without fetal calf serum (FCS) was added. The samples at different concentrations (8–256 µg/mL) were added to each well and incubated at 37 °C, with 5% CO2. Then, 50 µL of resazurin solution (2.2 µg/mL) was added, and the fluorescence was measured after 4 h (λexcitation 550 nm, λemission 590 nm) using a TECAN GENios microplate reader (Männedorf, Switzerland). Two independent experiments were performed, and the samples were tested in triplicate. The results were expressed as the percentage of reduction in cell growth/viability compared to control wells, and the IC50 was determined. Tamoxifen was included as a positive control (at 2–64 µg/mL).

2.5. Nitrite Determination

Nitric oxide (NO) production by RAW264.7 macrophages from ATCC (American Type Tissue Culture Collection, USA) was determined by measuring the accumulation of nitrite, an indicator of NO, in the supernatant after 24 h of lipopolysaccharide (LPS) treatment with or without the samples (CEL, HF, DCM, EAF and BF) using the Griess reagent [24]. The RAW 264.7 cells (5 × 10^5 cells/mL in a 96-well plate) were activated by incubation in a medium containing 100 ng/mL of lipopolysaccharide (LPS) alone (control),
and treated simultaneously with different concentrations of the samples dissolved in DMSO and further diluted in culture medium (from 8 to 64 ug/mL). The concentration of DMSO in the experiment did not exceed 0.5%. After 24 h of incubation, 150 µL of culture supernatant was collected and transferred to a 96-well plate filled with 130 µL of demineralized water. After adding 20 µL of Griess reagent, samples were incubated for 30 min while protected from light, and the absorbance was measured at 540 nm using a TECAN GENios microplate reader (TECAN Group Ltd., Männedorf, Switzerland). A standard calibration curve was set up by diluting the nitrite standard solution of the kit. The percentage of NO inhibition was calculated based on the ability of each sample to inhibit NO production by RAW 264.7 macrophages compared with the control (cells treated with LPS without samples). L-NAME (100 µM) was used as a reference control to suppress NO production in LPS-stimulated RAW 264.7 macrophages. Two independent experiments were carried out in triplicate and the results are presented as the mean ± standard deviation.

2.6. UPLC-QTOF-MS Analysis

The most active fractions were subjected to a fingerprint analysis by UPLC-UV-HRMS using a Xevo G2-XS QTOF spectrometer (Waters, Milford, MA, USA) coupled with an Acquity LC system equipped with MassLynx version 4.1 software (Waters, Milford, MA, USA). For analysis, 5 µL of each sample was placed on a HSS T3 column (100 mm × 2.10 mm, 1.8 µm, Waters, Milford, MA, USA). The mobile phase consisted of H₂O + 0.1% formic acid (A), and acetonitrile (ACN) + 0.1% formic acid (B), and the gradient was set as follows (min/B%): 0.0/3, 1.0/3, 17.0/100, 19.0/100, 21.0/3 and 25.0/3. Full scan data were recorded in ESI (−) and ESI (+) MS/MS modes from m/z 50 to 1500. UV detection was performed at 210 and 254 nm. The conditions used for the ESI source were as follows: capillary voltage, 1.0 KV in ESI+, 0.8 kV in ESI--; sampling cone, 40 V; source temperature, 120 °C; desolvation temperature, 550 °C; cone and desolvation gas flow rates, 50 L/h and 1000 L/h, respectively. Nitrogen was used as cone and desolvation gas.

2.7. Data Processing

The UPLC-MS raw data were converted to abf files (Reifycs Abf Converter) and processed with MS-DIAL version 4.90 (https://doi.org/10.1038/nmeth.3393/ accessed on 16 July 2022) for mass signal extraction between 50 and 1500 Da from 0 to 25 min [25]. The MS1 and MS2 tolerance levels were set to 0.05 in the centroid mode. The optimized detection threshold was set to 8000 for MS1 and 5000 for MS2. Then, MS-FINDER software version 3.52 (https://doi.org/10.1021/acs.analchem.6b00770/ accessed on 16 July 2022) was used for in silico fragmentation predictions. Compounds were tentatively identified according to their similarity score, which was based on a comparison between experimental MS/MS fragments and in silico spectra. The MS1 and MS2 tolerances were set to 0.01 and 0.25 Da, respectively. The natural product databases integrated into MS-FINDER (ChEBI, PlantCyc, UNPD, NPA, KNAPSAck, PubChem, COCONUT and NNPDB) were used for compound identification and to predict the molecular structures of the compounds. The following public databases were also consulted: MassBank of North America (MoNA) (http://mona.fiehnlab.ucdavis.edu/ accessed on 16 July 2022), and NIST Mass Spectrometry Data Center (http://chemdata.nist.gov/).

2.8. Statistical Analysis

One-way analysis of variance (ANOVA) was used to test for differences in mean values between different samples, followed by Tukey’s honestly significant differences (HSD) multiple rank test at p < 0.05. To test for the inhibition of nitric oxide release, a Mann–Whitney test was used. All analyses were performed using Statgraphic® Centurion XV for Windows version 15.2.14 (Stat Point, Inc., Warrenton, VA, USA) software.
3. Results

3.1. Free Radical Scavenging Activity

The free radical scavenging activity was determined using the common DPPH and ABTS methods. Table 1 shows the mean inhibitory concentration (IC\textsubscript{50}) of the samples in these radical scavenging assays. The results show that the \textit{C. linearis} crude extract (CEL) had good antioxidant activity against both radicals: ABTS and DPPH, but especially against the ABTS radical with an IC\textsubscript{50} value lower (IC\textsubscript{50} 105.1 ± 7.43 µg/mL) than the positive control ascorbic acid (IC\textsubscript{50} 182.35 ± 8.07 µg/mL). The dichloromethane (DCM) and ethyl acetate (EAF) fractions showed lower IC\textsubscript{50} values than the crude extract (CEL). Once again, the ABTS radical was the most susceptible. In all cases, but especially for the CEL, DCM and EAF fractions, marked concentration-dependent behavior was observed, allowing for the estimation of the IC\textsubscript{50} values in equations with R-square values over 0.75. In contrast, the \textit{n}-hexane fraction (HF) demonstrated poor activity, with IC\textsubscript{50} values of 406.22 and 426.74 µg/mL for the ABTS and DPPH, respectively, while the \textit{n}-butanol fraction (BF) was not active at the tested concentrations. Similar results were observed for the DPPH radical (Table 1).

Table 1. DPPH and ABTS radical scavenging activity, NO inhibitory effect and cell viability of \textit{C. linearis} leaves' extract and its fractions.

| Sample   | ABTS IC\textsubscript{50} (µg/mL) | DPPH IC\textsubscript{50} (µg/mL) | Nitric Oxide Inhibitory Activity IC\textsubscript{50} (µg/mL) | Cytotoxicity in RAW 264.7 Cells CC\textsubscript{50} (µg/mL) |
|----------|---------------------------------|---------------------------------|-------------------------------------------------|--------------------------------------------------|
| CEL      | 105.10 ± 7.43\textsuperscript{b} | 153.76 ± 23.61\textsuperscript{c} | 21.59 ± 2.97\textsuperscript{b} | 75.30 ± 3.71\textsuperscript{a} |
| HF       | 406.22 ± 25.82\textsuperscript{d} | 426.74 ± 22.00\textsuperscript{d} | 4.88 ± 2.64\textsuperscript{a} | 70.12 ± 7.21\textsuperscript{a} |
| DCM      | 55.93 ± 10.75\textsuperscript{a} | 13.47 ± 3.17\textsuperscript{a} | >64 | >256 |
| EAF      | 35.53 ± 3.33\textsuperscript{a} | 84.23 ± 6.95\textsuperscript{b} | 40.03 ± 2.94\textsuperscript{c} | >256 |
| BF       | >500 | >500 | >64 | >256 |
| AA       | 182.35 ± 8.07\textsuperscript{c} | 28.49 ± 2.91\textsuperscript{a} | ND | ND |
| Tamoxifen | ND | ND | ND | 1.41 ± 0.21 |

Note: Data are expressed as the mean ± standard deviation. Crude extract (CEL), \textit{n}-hexane fraction (HF), dichloromethane fraction (DCM), ethyl acetate fraction (EAF), \textit{n}-butanol fraction (BF) and ascorbic acid (AA). ND: not determined. Different superscripts within the same column indicate significant differences between extracts/fractions (\(p < 0.05\)) according to a one-way ANOVA test followed by Tukey’s test (\(n = 3\)).

3.2. Cell Viability Assay

The effect of the samples on the RAW 264.7 cell viability was measured in a concentration range between 8 and 256 µg/mL. The results are shown in Table 1, highlighting that for CEL and HF, noteworthy cytotoxicity was observed at concentrations above 64 µg/mL, with CC\textsubscript{50} values estimated as 75.30 and 70.12 µg/mL for CEL and HF, respectively. On the other hand, the DCM, EAF and BF fractions did not affect the cell viability at the tested doses (Figure 1). Based on those results, exploration of the inhibitory effect on nitric oxide release in LPS-stimulated macrophages of the extract/fractions was performed at concentrations below 64 µg/mL.

3.3. Inhibitory Effect on Nitric Oxide Release in LPS-Stimulated Macrophages

The effect of the crude extract and its fractions on NO release in LPS-stimulated RAW 264.7 macrophages was also assessed (Table 1). A significant inhibitory effect on nitric oxide release was detected for the \textit{n}-hexane fraction (HF), followed by the crude extract (CEL) and the ethyl acetate fraction (EAF). The high activity observed for these three samples can be better appreciated in Figure 2 when compared with the nitric oxide inhibition release shown by the positive control L-NAME (100 µM). In all cases, a concentration-dependent relationship was observed. The other samples (DCM and BF) were not active or less active than the positive control (L-NAME) at the concentrations tested.
Figure 1. Effect of crude extract (CEL), n-hexane fraction (HF), dichloromethane fraction (DCM), ethyl acetate fraction (EAF) and n-butanol fraction (BF) on RAW 264.7 murine macrophage cell viability. The effect on the cell viability was determined by the resazurin assay. All values are expressed as the mean ± SD of three replicates. Tamoxifen was the reference control drug for cytotoxicity.

Figure 2. Effect of crude extract (CEL), n-hexane fraction (HF), dichloromethane fraction (DCM), ethyl acetate fraction (EAF) and n-butanol fraction (BF) on nitric oxide production in LPS-stimulated RAW 264.7 macrophages. The extracellular nitrite accumulation was measured by the Griess reaction. * and ** mean significant statistical differences with the untreated group at (p < 0.05) and (p < 0.01), respectively, according to a Mann–Whitney test.

3.4. UPLC-QTOF-MS Analysis

In order to determine the metabolites present in the most active and non-toxic fractions (DCM and EAF), an UPLC-QTOF-MS analysis in the ESI (−) and ESI (+) modes was performed. The chromatograms obtained in both modes for both fractions are represented in Figures S1 and S2 (see supplementary data). From the 23 peaks detected, 18 compounds were tentatively identified, as is shown in Table 2. As can be seen in Table 2, compounds 5, 6, 9, 20, 21, 22 and 23 are common to both fractions, while compounds 1, 2, 7 and 19 appear only in the DCM fraction. Of them, 13 include a nitrogen atom in their structure, thus belonging to the alkaloid family, while the other 10 are polyphenols. Within the alkaloid types, compounds 2, 3, 5 and 7 belong to the morphinan family, compounds 4 and 19 to the aporphine family and compounds 6, 9 and 12 to the benzylisoquinoline family. A fragmentation proposal for two of the alkaloids is presented in Figures 3 and 4. Meanwhile, within the polyphenols, compounds 11 and 13 are O-glucuronide-pentosyl...
derivatives of quercetin, 15, 16 and 20 are O-deoxyhexo-syl-hexoside or O-p-coumaroyl hexoside derivatives of kaempferol and compounds 17, 18, 21 and 22 are O-glucoside or O-p-coumaroyl hexoside derivatives of isorhamnetin. A fragmentation proposal for one of the flavonoid derivatives is presented in Figure 5.

Table 2. Assigned compounds, molecular ions in the ESI (−) or ESI (+) modes and fragment ions of the main components present in the dichloromethane (DCM) and ethyl acetate (EAF) fractions of Croton linearis Jacq. leaves.

| Peak No. | Rt (min) | Precursor Ion (m/z) | Theoretical Mass (m/z) | Accuracy (ppm) | MS/MS Ions | Precursor Ion Formula | Tentative Identification | Extract |
|---------|---------|---------------------|-----------------------|----------------|-----------|-----------------------|--------------------------|---------|
| 1       | 3.09    | 334.1655 [M + H]⁺   | 334.1654              | 0.3            | 318.1709, | C₁₈H₂₃NO₅           | Unknown                  | DCM     |
| 2       | 3.16    | 318.1707 [M + H]⁺   | 318.1705              | 0.6            | 243.1026, | C₁₈H₂₃NO₄           | N-demethyl dihydro-     | DCM     |
|         |         |                     |                       |                | 211.0762, |                        | sinomenine               |         |
|         |         |                     |                       |                | 181.0654 |                        |                          |         |
| 3       | 3.35    | 334.1656 [M + H]⁺   | 334.1654              | 0.6            | 259.0967, | C₁₈H₂₃NO₅           | Sinococuline             | EAF     |
| 4       | 3.73    | 342.1703 [M]⁺       | 342.1705              | −0.6           | 297.1128, | C₂₀H₂₄NO₄⁺          | Magnoflorine             | EAF     |
| 5       | 3.84    | 332.1499 [M + H]⁺   | 332.1498              | 0.1            | 275.0918, | C₁₈H₂₁NO₅           | N-oxide demethyl        | EAF, DCM |
| 6       | 4.04    | 476.1945 [M + H]⁺   | 476.1921              | 5.0            | 326.1244, | C₂₄H₂₉NO₉           | Dauricoside (1)         | EAF, DCM |
| 7       | 4.12    | 330.1717 [M + H]⁺   | 330.1705              | 3.6            | 314.1402, | C₁₉H₂₃NO₄           | Sinomenine               | DCM     |
| 8       | 4.24    | 760.2825 [M + H]⁺   | 760.2817              | 1.1            | 638.2453, | C₃₂H₄₅NO₁₆          | Unknown                  | EAF     |
| 9       | 4.38    | 344.1862 [M + H]⁺   | 344.1862              | 0.0            | 299.1291, | C₂₀H₂₅NO₄           | Laudanidine              | EAF, DCM |
| 10      | 4.45    | 612.2462 [M + H]⁺   | 612.2445              | 2.8            | 478.2087, | C₃₂H₃₇NO₁₁          | Unknown                  | EAF     |
| 11      | 4.68    | 741.1842 [M − H]⁻   | 741.1878              | −4.9           | 609.1470, | C₃₂H₃₈O₂₀          | Quercetin-O-pentosyl-pentosyl-glucuronide | EAF     |
| 12      | 4.78    | 476.1929 [M + H]⁺   | 476.1921              | 1.7            | 358.2010, | C₂₄H₂₉NO₉           | Dauricoside (2)         | EAF     |
Table 2. Cont.

| Peak No. | Rt (min) | Precursor Ion (m/z) | Theoretical Mass (m/z) | Accuracy (ppm) | MS/MS Ions | Precursor Ion Formula | Tentative Identification | Extract |
|----------|----------|---------------------|------------------------|-----------------|------------|----------------------|--------------------------|---------|
| 13       | 4.99     | 609.1456 [M − H]⁻   | 609.1456               | 0.0             | 301.0351, 300.0281 | C_{27}H_{30}O_{16}       | Quercetin-O-pentosyl-glucuronide | EAF     |
| 14       | 5.18     | 952.3251            | 952.3239               | 1.3             | 462.1924, 303.0508 | C_{47}H_{53}NO_{20}      | Unknown                  | EAF     |
| 15       | 5.26     | 593.1503 [M − H]⁻   | 593.1506               | −0.5            | 285.0394, 284.0324 | C_{27}H_{30}O_{15}       | Kaempferol-O-deoxyhexosyl-hexoside (1) | EAF     |
| 16       | 5.38     | 593.1500 [M − H]⁻   | 593.1506               | −1.0            | 285.0399, 284.0326 | C_{27}H_{30}O_{15}       | Kaempferol-O-deoxyhexosyl-hexoside (2) | EAF     |
| 17       | 5.60     | 477.1031 [M − H]⁻   | 477.1033               | −0.4            | 447.0953, 315.0496, 314.0439, 285.0401, 284.0331, 271.0251, 255.0298, 243.0297, C_{22}H_{22}O_{12} | Isorhamnetin 3-O-glucoside | EAF     |
| 18       | 5.68     | 477.1031 [M − H]⁻   | 477.1038               | 1.0             | 315.0496, 314.0441, 285.0410, 271.0253, 255.0296, 243.0297, C_{22}H_{22}O_{12} | Isorhamnetin 7-O-glucoside | EAF     |
| 19       | 5.95     | 356.1870 [M + Na]⁺  | 356.1862               | 2.2             | 325.1547, 310.1197, 294.1272, C_{21}H_{25}NO_{4} | Glaucine                 | DCM      |
| 20       | 6.71     | 593.1284 [M − H]⁻   | 593.1295               | −1.9            | 477.0956, 285.0406, 284.0329, C_{30}H_{26}O_{13} | Tiliroside               | EAF, DCM  |
| 21       | 6.84     | 623.1404 [M − H]⁻   | 623.1401               | 0.5             | 477.1058, 315.0513, 314.0438, C_{31}H_{28}O_{14} | Isorhamnetin-O-p-coumaroyl hexoside (1) | EAF, DCM  |
| 22       | 6.90     | 623.1393 [M − H]⁻   | 623.1401               | −1.3            | 477.1062, 315.0517, 314.0440, C_{31}H_{28}O_{14} | Isorhamnetin-O-p-coumaroyl hexoside (2) | EAF, DCM  |
| 23       | 7.40     | 880.4304 [M + Na]⁺  | 858.4487               | −1.3            | 429.7295, C_{42}H_{47}NO_{17} | Unknown                  | EAF, DCM  |
Figure 3. Peak 4, tentative identification of magnoflorine in the EAF fraction of Croton linearis Jacq. leaves. Hypothetical fragmentation pattern adapted from Jiao et al. [26].

Figure 4. Peak 7, tentative identification of sinomenine in the DCM fraction of Croton linearis Jacq. leaves. Hypothetical fragmentation pattern adapted from Jiang et al. [27].

Figure 5. Peak 21, tentative identification of isorhamnetin-5-coumaroyl hexoside 1 (position of the substituents is arbitrary) in the DCM and EAF fractions of Croton linearis Jacq. leaves. Hypothetical fragmentation pattern adapted from Panighel et al., 2015 [28].
4. Discussion

*Croton linearis* Jacq. is a small shrub endemic to the Bahamas and distributed along the southern coast of the eastern region of Cuba. Its ethnopharmacological use, treating inflammatory and infective diseases, as well as studies reporting the production of alkaloids and polyphenols, led us to explore its inhibitory effect on nitric oxide release in LPS-stimulated RAW 264.7 macrophages. Additionally, the free radical scavenging activity of the *Croton linearis* crude extract and its fractions was explored as a probable mechanism of action associated with some of the reported folk uses. With this purpose, the crude extract (CLE) was partitioned into four fractions, of which the active and RAW 264.7 non-toxic ones (DCM and EAF) were phytochemically characterized by means of UPLC-QTOF-MS analysis, allowing us to tentatively identify 18 compounds: nine alkaloids and nine flavonoid derivatives. Compounds such as laudanidine (peak 9 in Table 2), glaucine (peak 19 in Table 2) and isorhamnetin-O-p-coumaroyl hexoside (peak 21/22 in Table 2) were among those already reported in previous work [15]. Beyond those, we detected compounds belonging to three different alkaloid families (morphinane, aporphine and benzylisoquinoline), and several flavonoid glycosides from three different flavonol aglycones (quercetin, kaempferol and isorhamnetin). Compounds belonging to these three alkaloid families have already been reported in *C. linearis* [15,29], and derivatives of quercetin, kaempferol and isorhamnetin have already been reported, as well [15,17].

Overproduction of reactive nitrogen species (RNS) has been associated with oxidative stress, inflammatory conditions, immune response and other processes [30,31]. Recently, considerable attention has been focused on the use of antioxidants, especially natural antioxidants, to inhibit NO production [32]. NO is a relatively unreactive RNS, but one of its derivatives, peroxynitrite (ONOO\(^-\)), is a powerful oxidant, capable of damaging many biological molecules [30]. NO, meanwhile, acts as a pro-inflammatory mediator associated with acute and chronic inflammation and the release of other pro-inflammatory mediators, such as bradykinins, and histamine [33,34].

The crude extract and the *n*-hexane fraction were the most active inhibitors of NO production in RAW 264.7 macrophages with IC\(_{50}\) values of 21.59 ± 2.97 and 4.88 ± 2.64 µg/mL, respectively; however, they also showed a noteworthy cytotoxic effect at concentrations over 64 µg/mL (Figure 3), limiting their possible future application. On the contrary, EAF exhibited a good inhibitory effect (IC\(_{50}\) 40.03 ± 2.94 µg/mL) on NO production in a concentration-dependent way (R\(^2\) 0.95 ± 0.01) without affecting cell viability, thus emerging as the most promising fraction.

With regard to the phytochemical analysis performed, the EAF fraction contained most of the flavonoids detected, especially those derived from quercetin and kaempferol. Quercetin and kaempferol, and their derivatives, have been reported as important inhibitors of NO release, even in macrophage models [35,36]. Several mechanisms have been reported, with prevalence of the direct inhibition of the inducible nitric oxide synthase (iNOS), cyclooxygenase-2 and reactive C-protein [37], as well as the downregulation of the NF-κB, STAT-1 and MAPK pathways [36]. On the other hand, the DCM fraction contained relatively more alkaloids, and to a lesser extent, flavonoids of the isorhamnetin-O-p-coumaroyl type. Although isorhamnetin has also been reported as an inhibitor of NO release, some studies indicate that it has lower activity compared to quercetin and kaempferol. These observations could explain the higher activity of the EAF fraction compared to the DCM fraction.

The effect on the NO production of extracts and compounds from other species of the genus *Croton* has been previously studied. Njoya et al., in 2018, reported the ethanolic root extract of *C. zambesicus* (*C. gratissimus*) and the acetone extract of *C. pseudopulchellus* to be good inhibitors of the NO release in LPS-stimulated RAW 264.7 macrophages, with IC\(_{50}\) values of 49.24 ± 0.93 µg/mL and 34.64 ± 0.06 µg/mL, respectively [38]. Recently, the potential anti-inflammatory properties of four South African *Croton* species—*C. gratissimus*, *C. pseudopulchellus*, *C. sylvaticus* and *C. steenkampianus*—was reported. These species demonstrated a high capacity to attenuate NO production with negligible cytotoxicity [39].
Associated with this activity, the phenylpropanoid derivatives obtained from *C. velutinus*, exhibited concentration-dependent suppressive activity on the production of nitrite and IL-1β by macrophages stimulated with LPS and IFN-γ [40].

Independent of the aforementioned mechanisms, the antioxidant capacity stands out as the most common characteristic associated with inhibition of NO release [41,42]. Multiple chemical mechanisms have been proposed for food antioxidants, with phenolic compounds noted such as flavonoids, tannins and phenolic acids [43,44]. In the same way, the presence of those phenolic compounds in extracts and fractions of *Croton* species has been correlated with the antioxidant potential [45,46]. In this regard, both EAF and DCM fractions, as well as the crude extract CEL, showed good antioxidant activity, sometimes with better IC50 values than the positive control, ascorbic acid. However, other metabolites with scavenging capacity, such as alkaloids, may be present, as well. Novello et al., in 2016, reported an indole alkaloid isolated from *C. echioides* with high antioxidant activity (IC50 30.0 ± 0.7 µM) against the DPPH radical [47]. In the present research, we have described the presence of several alkaloids with free phenolic groups, as well as different kinds of flavonoids, which could be responsible for the antioxidant activity observed for the crude extract and its most active fractions (DCM and EAF).

Oxidative stress is implicated in various inflammatory conditions, with ROS and RNS responsible for part of that involvement [30,48]. In that sense, this study revealed the good scavenging capacity of the crude extract and its fractions on ABTS and DPPH radicals; at the same time, it showed strong inhibition of NO production in LPS-stimulated RAW 264.7 macrophages, which was superior when compared to L-NAME (100 µM). This antioxidant capacity, at least in part, suggests the potential of this species to reduce pro-inflammatory molecules generated during inflammation. Several studies have demonstrated the correlation between the antioxidant capacity and anti-inflammatory activity of extracts and compounds from plants [49–51]. Accordingly, they can be considered a good source of antioxidants, which might be beneficial for combating diseases related to oxidative damage. Nevertheless, other antioxidant and anti-inflammatory assays of these extracts must be performed to establish possible mechanisms of action.

5. Conclusions

The effect on nitric oxide release in LPS-stimulated macrophages and free radical scavenging activity of the well-characterized crude extract of *Croton linearis* and its fractions were reported here for the first time. CEL, DCM and EAF fractions were the most active as free radical scavengers, while CEL, HF and EAF fractions showed a strong effect on nitric oxide release in LPS-stimulated RAW 264.7 macrophages. However, CEL and HF showed noteworthy cytotoxicity at concentrations above 64 µg/mL. UPLC-QTOF-MS identification demonstrated the rich arsenal of potentially bioactive components in the DCM and EAF fractions, consisting mainly of alkaloids and flavonoids, which could be responsible for the good antioxidant effects observed. These findings reveal the natural antioxidant potential of this species, as well as its probable anti-inflammatory effect, an activity for which it is used in folk medicine, but with further attention required due to the cytotoxicity observed in some of its fractions. Additional studies will be essential to establish its possible mechanisms of action, safeness and its relationship with the constituents of *Croton linearis* leaves.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/antiox11101915/s1](https://www.mdpi.com/article/10.3390/antiox11101915/s1), Figure S1: UPLC-QTOF-MS chromatogram of AE fraction from *Croton linearis* Jacq leaves: (a) positive ion mode, (b) negative ion mode.; Figure S2: UPLC-QTOF-MS chromatogram of DCM fraction from *Croton linearis* Jacq leaves: (a) positive ion mode, (b) negative ion mode.

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