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Scavenging Activity on Reactive Oxygen Species with Biological Relevance by *Varronia curassavica*

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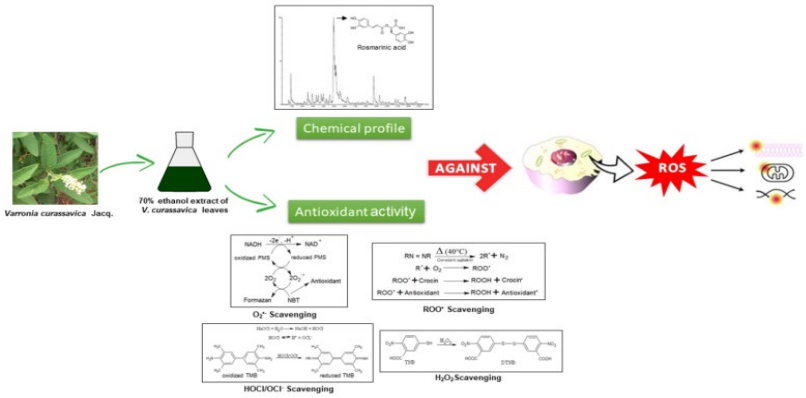
Varronia curassavica Jacq. is a medicinal plant found in Brazil used as anti-inflammatory. Here, we investigated the *in vitro* antioxidant activity of 70 % ethanol extract of *V. curassavica* leaves on synthetic radicals ($ABTS^{\bullet+}$/$DPPH^{\bullet}$) and reactive oxygen species ($O_2^{\bullet-}$, $ROO^{\bullet}$, $HOCl/OCl^-$, H_2O_2), besides its *in vitro* cytotoxicity. The extract was characterized by UPLC-ESI-QToF-MS^E and the annotated compounds were one hydroxybenzoic acid, five phenylpropanoids, and three glycosylated quercetin derivatives, being the main compound rosmarinic acid or its isomer. The antioxidant activity was very promising in all tests, highlighting on the capture of $O^{2\bullet-}$, which EC_{50} value was three times lower than Trolox. This activity may be due to the presence of the major compounds, all phenolic compounds. The extract also presented low cytotoxicity. Thus, the extract from *V. curassavica* leaves has great potential as an antioxidant.

Graphical abstract



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1. Introduction

Varronia curassavica Jacq. (Boraginaceae), a shrub known as erva-baleeira, is a medicinal plant and a non-conventional food plant found in Brazil, mainly in the Atlantic Forest and South of the Amazon Rainforest [1, 2]. According to molecular and morphological data, *V. curassavica* is the accepted botanical name, while *Cordia verbenaceae* DC. and *Cordia curassavica* (Jacq.) Roem. & Schult (Boraginaceae) are synonyms for the species [3]. Phytochemical studies on *V. curassavica* leaves identified flavonoids (quercetin, rutin, 7,4'-dihydroxy-5'-carboxymethoxy isoflavone, 7,4'-dihydroxy-5'-

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methyl isoflavone, brickellin, and artemetin), phenolic acids (rosmarinic acid, gallic acid, chlorogenic acid, and caffeic acid), triterpenes (cordialin A and B) [4, 5,6,7], as well as monoterpenes and sesquiterpenes, such as *α*-humulene, (*E*)-caryophyllene, spathulenol, *α*-pinene, *β*-gurjunene, and alloaromadendrene in essential oil (EO) [8].

Its leaves are used in folk medicine to prepare hydroethanolic extracts or aqueous preparations for topical or oral administration due to its anti-inflammatory activity. The hydroethanolic extract and EO of *V. curassavica* leaves showed anti-inflammatory effects in topical and oral administration in mice and rats [8, 9]. The hydroethanolic extract presented a protective effect on the gastric mucosa of rodents associated with low toxicity. This is a possible advantage because non-steroidal anti-inflammatory agents produce gastrointestinal adverse reactions [10]. The EO and extracts of its leaves also presented antimicrobial and analgesic activities [4]. Furthermore, hydroethanolic extracts of *V. curassavica* leaves have demonstrated antioxidant activity against the synthetic free radical $DPPH^{\bullet}$ [11].

Anti-inflammatory and antioxidant actions may be associated with the regulation of reactive oxygen species (ROS: $O_2^{\bullet-}$, $OH^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$, H_2O_2, $^1O_2$, HOCl) which are important in metabolic regulation and immunologic defenses against infections. On the other hand, they may cause damage to biomolecules due to ROS overproduction - effects of oxidative stress [12]. Reactive species include reactive nitrogen species (RNS) such as nitric oxide radical ($NO^{\bullet}$) and peroxynitrite ion ($ONOO^-$) [13]. ROS are directly involved in pathologies such as Alzheimer's and Parkinson's diseases, cancer, multiple sclerosis, Down syndrome, and diabetes mellitus, and are associated with the aging process [14, 15]. In inflammation sites, neutrophils are found in abundance, and they suffer various metabolic changes which result in ROS formation. In normal conditions, these events are vital for human survival, but when they occur excessively, they may be related to the degenerative process of chronic inflammation and several pathologies [16].

Therefore, we investigated the *in vitro* antioxidant activity of 70 % ethanol extract of *V. curassavica* leaves against synthetic radicals and ROS of biological relevance. In addition, the extract was chemically characterized through UPLC-ESI-QToF-MS^E analysis for the annotation of its main components, phenolic compounds.

2. Results and Discussion

Total phenolic compounds and total flavonoids contents in the 70 % ethanol extract were determined as 14.5 ± 0.2 μg EAG·100 μg^{-1} (14.5 %) and 3.8 ± 0.1 μg EQ·100 μg^{-1} (3.8 %), respectively. The phenolic compounds content in the 70 % ethanol extract is similar to the determined for extracts of some species with high content of phenolic compounds (tannins and flavonoids) as *Stryphnodendron adstringens* (Mart.) Coville, *S. polyphyllum* Mart., *Dimorphandra mollis* Benth. and *Vittis vinifera* L. [17, 18].

UPLC-ESI-QToF-MS^E analysis, negative mode, allowed the annotation of compounds **1-9** in the extract (**Table 1**), based on the mass fragmentation pattern, accurate mass (chemical formula), and comparison with literature data, as well as the Lamiaceae family due to the chemical composition similarity [19–24]. The annotated compounds include one hydroxybenzoic acid, five phenylpropanoids, and three glycosylated quercetin derivatives, being the main peak observed in TIC (Rt = 4.97 min) attributed to rosmarinic acid or its isomer. Rosmarinic acid was previously identified in the leaves of the species [1].

Compound **1** presented in the MS^1 spectrum a deprotonated molecule signal at *m/z* 197.0456 which matches with the molecular formula of syringic acid. MS^2 fragment ion signal at *m/z* 179 corresponds to the loss of water molecule (- 18 Da) and at *m/z* 135 to the following loss of carbon dioxide (- 44 Da) [20]. Compound **2** was annotated as caffeic acid hexoside. Its MS^1 spectrum showed the signal of deprotonated molecule at *m/z* 341.0868 and the MS^2 spectrum showed a fragment ion at *m/z* 179 indicative of caffeoyl moiety, besides a neutral loss of 161 Da correspondent to a hexose moiety [24]. Caffeic acid was previously identified in *V. curassavica* leaves [1].

Compounds **3** and **4** were annotated as isomers of quercetin *O*-(hexosyl-(-*O*-(coumaroyl))-*O*-deoxyhexosyl-*O*-hexoside))-*O*-deoxyhexoside and their deprotonated molecule signals were observed at *m/z* 1063.2871 and 1063.2853, respectively. Both MS^2 spectra showed characteristic ion signals at *m/z* at 917 (-146 Da), 771 (-146 Da), 609 (-162 Da), 301 (-308 Da) [21]. Spectrum of compound **5** presented a deprotonated molecule signal at *m/z* 463.0888. Fragment ion signal recorded at *m/z* 301 corresponds to the loss of a sugar moiety yielding deprotonated quercetin. Thus, it was annotated as quercetin hexoside [23]. Quercetin was identified in *V. curassavica* leaves in other studies [1].

Compounds **6** and **7** showed signals of the deprotonated molecule at *m/z* 359.0776 and 359.0779, respectively, and the MS^2 fragment ions were observed at *m/z* 197, 179, and 161. Thus, the compounds were annotated as rosmarinic acid isomers according to literature data [1].

Compounds **8** and **9** showed deprotonated molecule signals at *m/z* 717.1450 and 685.1548, respectively. The fragment ions at *m/z* 529 and 321 were observed for compound **8**, which indicate two successive losses of danshensu units, typical for salvianolic acid B. Compound **9** showed the same fragmentation pattern of the compound **8** (danshensu units losses) and 32 Da less than salvianolic acid, suggesting the loss of two oxygen moieties (deoxy). It was thus annotated as *bis*-deoxy-salvianolic acid B [19].

EC_{50} values of the 70 % ethanol extract and standards in the $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, HOCl/OCl^-, H_2O_2, and $ROO^{\bullet}$ scavenging assays are presented in **Table 2**. EC_{50} value of 70 % ethanol extract in the $DPPH^{\bullet}$ assay (13.4 μg/mL) was approximately five times higher than ascorbic acid (2.7 μg/mL). In a comparative study of antioxidant activity ($DPPH^{\bullet}$) of different extracts from *V. curassavica* leaves, 25 and 50 % ethanol extracts showed lower EC_{50} values (27.4 and 29.0 μg/mL, respectively) than ethanol extract (82.5 μg/) [11]. EC_{50} value (8.2 μg/mL) of 70 % ethanol extract was 2-10 times higher than ascorbic acid (2.6 μg/mL), Trolox (4.0 μg/mL), and quercetin (0.7 μg/mL) in the $ABTS^{\bullet+}$ scavenging assay. EC_{50} value of 70 % ethanol extract was converted to Trolox equivalent antioxidant capacity per gram of extract (TEAC value for extract = 2,271.0 μmol/g) to compare the results with literature data. An evaluation of $ABTS^{\bullet+}$ scavenging of several extracts from *V. curassavica* and the TEAC values for the 25 % and 50 % ethanol extracts were 1,031.0 and 584.0 μmol/g, 2 - 4 times lower than 70 % ethanol extract. Thus, the 70 % ethanol extract was more active in the scavenging $DPPH^{\bullet}$ assay than 25 % and 50 % ethanol extracts but in scavenging $ABTS^{\bullet+}$ assay, the 25 % and 50 % ethanol extracts showed lower EC_{50} values.

Table 1. Phenolic compounds annotated in the extract by UPLC-ESI-QToF-MS^E analysis in negative mode.

Peak	Rt (min)	Deprotonated molecule Experimental	Deprotonated molecule Calculated	Product Ions *m/z*	Empirical Formula	Error (ppm)	Tentative identification
1	2.53	197.0456	197.0450	179, 153, 135	$C_9H_9O_5$	3.0	syringic acid [20]
2	3.50	341.0868	341.0873	179, 135	$C_{15}H_{17}O_9$	1.5	caffeic acid hexoside [24]
3	3.78	1063.2871	1063.2931	917, 755, 609, 463, 301	$C_{48}H_{55}O_{27}$	5.6	quercetin *O*-(hexosyl-(-*O*-(coumaroyl))-*O*-deoxyhexosyl-*O*-hexoside))-*O*-deoxyhexoside isomer [21]
4	3.88	1063.2853	1063.2931	917, 755, 609, 463, 301	$C_{48}H_{55}O_{27}$	7.3	quercetin *O*-(hexosyl-(-*O*-(coumaroyl))-*O*-deoxyhexosyl-O-hexoside))-*O*-deoxyhexoside isomer [21]
5	4.27	463.0888	463.0877	301, 300	$C_{21}H_{19}O_{12}$	2.4	quercetin hexoside [23]
6	4.59	359.0776	359.0767	197, 159	$C_{18}H_{15}O_8$	2.5	rosmarinic acid isomer [20]
7	4.97	359.0779	359.0767	197, 179, 161,135	$C_{18}H_{15}O_8$	3.3	rosmarinic acid isomer [20]
8	5.20	717.1450	717.1456	519, 321	$C_{36}H_{29}O_{16}$	0.8	salvianolic acid B [19]
9	6.24	685.1548	685.1557	503, 339, 321	$C_{36}H_{29}O_{14}$	1.3	*bis*-deoxy-salvianolic acid B [19]

Antioxidant activity may be classified according to the EC_{50} values of DPPH$^{\bullet}$ assay: < 10.0 µg/mL (very strong), 10.0 - 50.0 µg/mL (strong activity), 50.0 - 100.0 µg/mL (moderate), 100.0 - 250.0 µg/mL (weak activity), and > 250.0 µg/mL as inactive [25]. Therefore, the 70 % ethanol extract displayed strong antioxidant activity in DPPH$^{\bullet}$ assays.

Table 2. Scavenging activities of extract and standards are expressed as EC_{50} ± SEM* (µg/mL).

Samples	DPPH$^{\bullet}$	ABTS$^{\bullet+}$	$O_2^{\bullet-}$	HOCl/OCl$^-$	H_2O_2	ROO$^{\bullet}$
extract	13.4 ± 0.3	8.2 ± 0.64	196.7 ± 1.8	3.6 ± 0.0	301.8 ± 4.3	10.4 ± 0.2
ascorbic acid	2.8 ± 0.1^a	2.6 ± 0.1^a	56.2 ± 1.27^a	2.6 ± 0.1^a	57.9 ± 0.4^a	0.4 ± 0.0^a
quercetin	**	0.7 ± 0.0^{a,b}	21.2 ± 0.6^{a,b}	0.4 ± 0.0^{a,b}	***	1.3 ± 0.0^{a,b}
trolox	**	4.0 ± 0.1^{a,c}	590.9 ± 14.7^{a,b,c}	2.2 ± 0.1^{a,b,c}	***	2.7 ± 0.1^{a,b,c}

Values followed by different superscript letters indicate a significant difference ($p < 0.05$). *SEM: standard error of the mean; **Standard not used in the assay; ***No effect at the concentration used. [a]Significant differences with the extract. [b]Significant differences with ascorbic acid. [c]Significant differences with quercetin

These results showed 70 % ethanol extract activity on synthetic free radicals, but they are not present in organisms. Thus, we evaluate the antioxidant activity of ROS with biological relevance [13, 26]. EC_{50} value of 70 % ethanol extract in the $O_2^{\bullet-}$ scavenging assay (196.7 µg/mL) was four and ten times higher than the values of ascorbic acid (56.2 µg/mL) and quercetin (21.2 µg/mL), respectively. On the other hand, the EC_{50} value of Trolox (590.9 µg/mL), was three times higher than the EC_{50} value of 70 % ethanol extract. The antioxidant activity of 25 and 50 % ethanol extracts of *V. curassavica* leaves were also evaluated on $O_2^{\bullet-}$ scavenging assay [9] and their EC_{50} values (286.0 and 373.0 µg/mL, respectively) were higher than the EC_{50} value of 70 % ethanol extract.

EC_{50} value of 70 % ethanol extract (3.6 µg/mL) in the HOCl/OCl$^-$ assay was similar to the EC_{50} values of ascorbic acid and Trolox (2.6 and 2.2 µg/mL, respectively) which emphasizes the efficacy of 70 % ethanol extract to scavenge the HOCl/OCl$^-$.

Among the reference compounds used in the H_2O_2 scavenging assay, only ascorbic acid scavenged H_2O_2; the compounds were tested at concentrations up to 100 µM for quercetin and 2.5 mM for Trolox due to solubility restrictions. The 70% ethanol extract showed EC_{50} value of 301.8 µg/mL which was five times higher than EC_{50} for ascorbic acid. Considering that an extract is a complex mixture of compounds, this assay demonstrates the great potential of 70 % ethanol extract as antioxidant. The ability of ascorbic acid, quercetin, Trolox and extract to scavenge ROO$^{\bullet}$, that is, to inhibit crocin bleaching, is shown in **Table 2**. Comparing the values of the slope for standards and extract, the order of decreasing antioxidant capacity was ascorbic acid > quercetin > Trolox > extract.

EC_{50} values (**Table 2**) follow the same order of efficiency as the values of slope in the competitive assay, which are demonstrated in **Table 3**. This means that the lower EC_{50} value corresponds to the more efficient scavenging ROO$^{\bullet}$, and the greater the slope value of the reaction rate regression line, the higher the antioxidant activity (**Table 3**). EC_{50} extract value (10.4 µg/mL) was 4-20 times higher than the EC_{50} of Trolox (2.7 µg/mL), quercetin (1.3 µg/mL) and ascorbic acid (0.4 µg/mL). For comparison, the antioxidant capacity value of the extract was expressed in Trolox equivalents, which was obtained by dividing the slope of the extract by the slope of Trolox (2.4/8.3 = 0.3).

Table 3. Competitive kinetic slopes and EC_{50} for the crocin bleaching assay.

Samples	EC_{50} (µg/mL)	Slope of regression line
extract	10.4	2.4
trolox	2.7	8.3
quercetin	1.3	20.6
ascorbic acid	0.4	70.4

The antioxidant activity of the 70% ethanol extract of *V. curassavica* may be related to the phenolic compounds annotated by mass spectrometry analysis. For instance, phenolic compounds such as rosmarinic acid, caffeic acid, and quercetin derivatives showed antioxidant and antiglycation activities [7].

The 70 % ethanol extract induced HaCat cell death at 175.0 and 200.0 µg/mL, with EC_{50} value of 163.8 µg/mL (**Fig. 1**). For the HepG2 cell line, significant cell death was not observed at any concentration (15.6 to 500.0 µg/mL). Since the HepG2 cell line is a metabolizer cell, these data indicate that the possible metabolites from the extract were not cytotoxic at the tested concentrations.

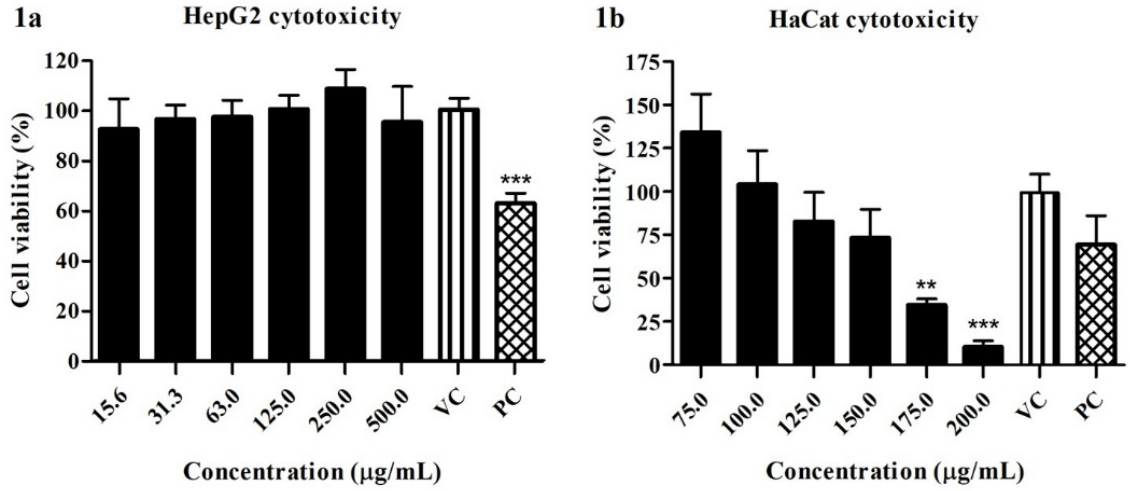

Fig. 1. Effect of *in vitro* exposure of extract on (A) HepG2 and (B) HaCat cell line. Results are expressed as means of three independent experiments ± SEM for each cell line, analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test (**$p \leq 0.01$, the difference with vehicle control (VC: DMSO); ***$p \leq 0.001$, the difference with VC); (PC: positive control) doxorrubicin.

3. Material and Methods

3.1 Plant material

V. curassavica leaves were collected at the Pluridisciplinary Centre for Chemical, Biological and Agricultural Research (CPQBA) of the University of Campinas - UNICAMP (Paulínia, São Paulo, Brazil). The plant material was identified by Dr. Ílio Montanari Júnior (CPQBA-UNICAMP) and a voucher specimen is deposited with the herbarium "Herbário São José do Rio Preto" of the São Paulo State University – Unesp (São José do Rio Preto, São Paulo, Brazil) with the reference number 31235. This study was conducted under the Brazilian National System of Genetic Heritage Management and Associated Traditional Knowledge authorization number ADEE581. Leaves were dried in an oven with air circulation (40 ºC, 7 d) and powdered in a knife mill.

3.2 Extract

Dried and powdered leaves (1000.0 g) were extracted with 70 % ethanol, 35 ºC under occasional stirring. Extraction with 15 L of 70% ethanol was performed in three steps (24, 48, and 48 h). Extractive solutions were filtered, concentrated in a rotary vacuum evaporator, and lyophilized, yielding 211.0 g of dried extract of leaves of *V. curassavica*.

3.3 Total Phenolic Compounds Content

Total phenolic compounds content in the extract was determined by the photometric using the Folin-Ciocalteu reagent (Sigma-Aldrich®) [27]. Analytical curve was determined from gallic acid solutions in 70 % ethanol (1.3–20.0 µg/mL). Phenolic compounds content (triplicate) is expressed in micrograms of total phenolic compounds equivalents to gallic acid per 100 µg of dried extract.

3.4 Total Flavonoids Content

Total flavonoids content in the extract was determined according to method A using aluminum chloride as reagent (Sigma-Aldrich®) [28]. Analytical curve was determined from quercetin solutions in 70 % ethanol (0.9–20.0 µg/mL). Flavonoid content (triplicate) is expressed in micrograms of total flavonoids equivalents to quercetin per 100 µg of dried extract.

3.5 Ultra-performance liquid chromatography/electrospray ionization-quadruple time of flight mass spectrometry (UPLC-ESI-QToF-MS^E) analysis

The extract (20 mg, 1.0 mL methanol: water, 95:05 v/v) was submitted to solid phase extraction (Agilent SampliQ® C18; 500 mg; 6 mL; 55 µm). The elution was developed with 4.0 mL of methanol: water 95:05. Eluate was dried, dissolved in methanol (1.0 mL), and filtered through polyvinylidene difluoride (PVDF) membranes (0.22 µm, Millipore®). Analysis was performed on an Acquity UPLC system (Waters®) coupled to a quadrupole/time of flight (Xevo-QTOF, Waters®) system, with a Waters® Acquity UPLC® BEH C18 column (150.0 x 2.1 mm; 1.7 µm). Elution was performed with water 0.1 % formic acid (A) and acetonitrile 0.1 % formic acid (B), under gradient conditions: 0-15 min 2-95 % (B). Flow rate was 0.4 mL/min, injection volume was 5.0 µL and temperature was 40 ºC. ESI negative mode was acquired in the range of 110–1180 Da, the source temperature was 120 °C, desolvation gas temperature was 350 °C, desolvation gas flow was 500 L/h, extraction cone voltage was 0.5 V, and capillary voltage was 2.6 kV. Leucine encephalin was used as the lock mass. Acquisition mode was MS^E. The equipment was controlled by Masslynx 4.1 (Waters® Corporation) software.

3.6 Antioxidant activity

3.6.1 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^•$) Scavenging Assay

$DPPH^•$ (Sigma-Aldrich®, USA) scavenging activity of the extract was evaluated [29]. Extract concentrations were 1.0-

35.0 µg/mL (methanol). Ascorbic acid was used as standard (0.9-5.3 µg/mL). Assays were performed in triplicate and the percentage inhibition of DPPH$^{\bullet}$ was calculated. Inhibition curves and the effective concentration values required to obtain 50 % of antioxidant effect (EC_{50}) were from the values of the percentage inhibition of DPPH$^{\bullet}$ by means of linear regression using OriginPro® 8.0724. Results were expressed as the mean of EC_{50} ± standard error of the mean (SEM).

3.6.2 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) Scavenging Assay

$ABTS^{\bullet+}$ inhibition activity was assessed with modifications. $ABTS^{\bullet+}$ was generated by oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma-Aldrich®, USA) (7 mmol/L) with potassium persulfate (Sigma-Aldrich®, USA) (140 mmol/L) in the dark at room temperature for 12-16 h [30]. $ABTS^{\bullet+}$ stock solution was diluted in sodium phosphate buffer (10 mmol/L, pH 7.0) to an absorbance of 0.750 ± 0.020 at 734 nm. Extract stock solution (DMSO) was diluted in sodium phosphate buffer, 10 mmol/L, pH 7.0 (1.0 to 19.0 µg/mL), the solutions were added to the reaction mixture, incubated for 15 min in the dark at room temperature and the absorbances were measured at 734 nm. Ascorbic acid, quercetin, and Trolox were used as standards. Results were expressed as mean EC_{50} ± SEM.

3.6.3 Superoxide Anion Radical ($O_2^{\bullet-}$) Scavenging Assay

$O_2^{\bullet-}$ was produced by the reaction between reduced nicotinamide adenine dinucleotide (NADH, Sigma-Aldrich®, USA), phenazine methosulfate (PMS, Sigma-Aldrich®, USA), and molecular oxygen [31]. $O_2^{\bullet-}$ generated reacts with nitroblue tetrazolium (NBT, Sigma-Aldrich®, USA) reducing it to a blue formazan, whose color intensity is directly proportional to the radical concentration. Test was performed in sodium pyrophosphate buffer (25 mmol/L, pH 8.3), containing PMS (372 µmol/L), NBT (600 µmol/L), NADH (1560 µmol/L) and extract solutions in different concentrations (100.0-500.0 µg/mL, in sodium pyrophosphate buffer, 25 mmol/L, pH 8.3). After 7 min at room temperature, the absorbances were measured at 560 nm to determine the concentration of formazan [32]. Ascorbic acid, quercetin and Trolox were used as standards. As described in item 2.4.2, extract and quercetin were solubilized first in DMSO and then, dilutions were prepared in the buffer. Results were expressed as mean EC_{50} ± SEM.

3.6.4 Hypochlorous Acid ($HOCl/OCl^-$) Scavenging Assay

Antioxidant activity depends on the capacity of the sample to capture $HOCl/OCl^-$, preventing it from oxidizing 3,3',5,5'-tetramethylbenzidine (TMB, Sigma Aldrich, USA). TMB oxidation by $HOCl/OCl^-$ generates a blue compound and its absorbance was monitored at 655 nm [33]. NaOCl was diluted in 10 mmol/L NaOH to produce a standard solution of OCl^-, and its concentration was determined by its molar absorptivity (ε: 350 M^{-1} cm^{-1} at 292 nm) [34]. Extract solutions in dimethylformamide (0.5 to 120.0 µg/mL) were incubated with $HOCl/OCl^-$ (30 µmol/L) for 10 min. TMB (2.8 mmol/L dissolved in 50% dimethylformamide with 0.01 mol/L potassium iodide in 0.8 mol/L acetic acid) was added and this mixture was incubated for 5 min at room temperature in the dark and the absorbance was measured at 655 nm. Ascorbic acid, quercetin, and Trolox were used as standards. Stock solutions of ascorbic acid and Trolox were prepared in sodium phosphate buffer (50 mmol/L, pH 7.4) and quercetin in dimethylformamide. Assay without extract was used as control (100 % reaction) and the absorbance of the reaction medium without HOCl was used as a reading blank. Results were expressed as mean EC_{50} ± SEM.

3.6.5 Hydrogen Peroxide (H_2O_2) Scavenging Assay

H_2O_2 (Merck, German) oxidizes 2-nitro-5-thiobenzoic acid (TNB, Sigma-Aldrich®, USA) to 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), with a decrease in absorbance at 412 nm and increase at 325 nm [35]. TNB solution was prepared in potassium phosphate buffer (50 mmol/L, pH 6.6) [36] and its concentration was determined from its molar extinction coefficient at 412 nm (ε: 13,600 M^{-1} cm^{-1}, [37]; H_2O_2 (Merck®, German) concentration was determined (ε : 80 M^{-1} cm^{-1}, at 230 nm) [38]. Extract solutions were prepared in potassium phosphate buffer (50 mmol/L, pH 6.6), and they were incubated with H_2O_2 (0.3 mmol/L) for 30 min at 37 °C. TNB (53 µmol/L) was added and incubated for 1 h at 37 °C. The absorbance was measured at 412 nm. Catalase (20 units/mL) was used as a standard for H_2O_2 scavenging. Ascorbic acid, quercetin, and Trolox were used as standards. Percent inhibition of TNB oxidation, i.e., percent H_2O_2 scavenge, was calculated from the difference in absorbance between reaction mixtures with and without extract. Results were expressed as mean EC_{50} ± SEM.

3.6.6 Peroxyl Radical ($ROO^{\bullet}$) Scavenging Assay (Crocin Bleaching Assay)

Crocin bleaching assay was performed by monitoring the decrease in crocin absorbance (ε: 13,726 M^{-1} cm^{-1}) at 443 nm for 10 min, in competitive kinetics [39]). Reaction is initiated by addition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, Sigma-Aldrich®, USA), which generates peroxyl radicals at a constant rate by thermolysis at 40 °C. Crocin (25 µmol/L, Sigma-Aldrich®, USA) in sodium phosphate buffer (120 mmol/L, pH 7.0) was mixed at 5.0, 10.0, 15.0, 20.0, 25.0 e 30.0 µg/mL) of extract in DMSO. Ascorbic acid, quercetin, and Trolox were used as standard. Ascorbic acid and Trolox solutions were prepared in sodium phosphate buffer and quercetin and crocin solutions, in DMSO. Reaction was started by adding 12.5 mmol/L of AAPH and performed at 40 °C with constant stirring. Rate of crocin bleaching (linear after about 1 min of reaction) was monitored at 443 nm for 10 min. A reaction mixture without crocin was prepared for each extract and standards and used as the reaction blank. Rate of crocin bleaching by the generated peroxyl radical (v_0) decreases in the presence of an antioxidant, as it competes with the crocin for the $ROO^{\bullet}$, and the new bleaching rate (v) is given by:

$$v = v_0 \times \frac{kc[C]}{kc[C]+ka[A]}$$

where: $v_0 = k_1 \times [ROO^{\bullet}] \times [C]$; $kc = k_1 \times [ROO^{\bullet}]$; $ka = k_2 \times [ROO^{\bullet}]$; $[ROO^{\bullet}]$ = concentration of $ROO^{\bullet}$; v_0 = reaction rate between crocin and $ROO^{\bullet}$; k_1 = rate constant for the $ROO^{\bullet}$ (crocin reaction); k_2 = rate constant for the $ROO^{\bullet}$ (antioxidant reaction); [C] = crocin concentration; [A] = antioxidant (extract) concentration.

Decrease in crocin bleaching rate in the presence of an antioxidant can be modeled as follows:

$$\frac{v_0}{v} = \frac{kc[C]+ka[A]}{kc[C]} = 1 + \frac{ka}{kc} \times \frac{[A]}{[C]}$$

from the above equation, coefficient ka/kc, calculated as the slope of the regression line for the v_0/v *versus* [A]/[C] plot, indicates the relative capacity of an antioxidant to interact with ROO˙. By dividing this slope for extract or another pure compound by the slope for a standard antioxidant such as Trolox, the relative antioxidant capacity, of the analyzed compound can be estimated, being expressed in Trolox equivalents.

Another possible way to express crocin bleaching is by determining the percent inhibition [40] and, consequently, EC_{50} values, which can be calculated by this equation:

$$\% \text{ In (or EC)} = \left(1 - \left(\frac{\Delta v}{\Delta v_0}\right)\right) \times 100$$

3.6.7 Cytotoxicity Assay

MTT assay was used to assess the cytotoxicity based on the determination of living cells ability to reduce 3-4(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), resulting in insoluble crystals of formazan [41]. In brief, human hepatoma cells (HepG2) and keratinocyte cells (HaCat) at a density of 2.0 x 10^5 and 1.0 x 10^5 cells/well, respectively, were seeded in into 96-well plates and treated with extract at 15.6 to 500 µg/mL or 1 % DMSO (vehicle control) for HepG2 and at 75 to 200 µg/mL or 0.4 % DMSO (vehicle control) for HaCat, for 24 h. Then, the medium was removed, and cells were incubated with MTT (0.5 mg/mL) for 4 h at 37 ºC. Formazan crystals in cells were solubilized with 100 µL of DMSO. Absorbance was measured at 570 nm using a microplate reader (BioTek®, Epoch™ Microplate Spectrophotometer). Three independent experiments were assessed for each cell line and data were analyzed with a one-way analysis of variance (ANOVA) followed by the Tukey test using GraphPad Prism 5.0 software for comparison between extract and vehicle control.

3.7 Statistical analysis

Data were shown as mean ± standard error of the mean submitted to one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for comparison among extract and standards. The differences were considered significant when $p < 0.05$.

4. Conclusions

In conclusion, it was demonstrated that the 70 % ethanol extract of *V. curassavica* leaves presented several phenolic compounds annotated as glycosylated quercetin derivatives, rosmarinic acid isomers, salvianolic acid derivatives, caffeic acid hexoside, and syringic acid. These compounds may be responsible for antioxidant activity against synthetic radicals and reactive oxygen species with biological relevance.

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Author Contributions

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