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Chemical Prospection of *Qualea grandiflora* Mart. Fruit and Stem Extracts and Their *in vitro* and *in vivo* Antiplasmodial Activity

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Qualea grandiflora Mart. ("pau-terra") is a medicinal plant symbolic from Cerrado biome. *Qualea* species reportedly have antiparasitic properties, but their active compounds have yet to be identified. In this study, we investigated the antiplasmodial activity of *Q. grandiflora* fruit and stem extracts against *Plasmodium falciparum*. The polar extracts of both fruits and stems showed promising *in vitro* antiplasmodial activity against sensitive and resistant *P. falciparum* strains (half-maximal inhibitory concentration (IC_{50}) = 1.2 and 4.2 ng mL^{-1}, respectively), low cytotoxicity against human hepatic cells ($IC_{50}^{HepG2} \geq 400$ ng mL^{-1}), and a noteworthy selectivity index (SI ≥ 322 and ≥ 96, respectively). The fruit ethanolic extract revealed significant oral efficacy at 100 mg kg^{-1} in a mouse model of *P. berghei* malaria (100% reduction in parasitemia on day 5 post-infection). To investigate the chemical composition of the extracts, we developed a method involving liquid chromatography-high resolution quadrupole-time of flight mass spectrometer. The chemical profiling of active extracts indicated 32 compounds, including gallotannins, ellagic acid derivatives and flavonoids. The common component in the active polar extracts was found to be ellagic acid (EA), which may contribute favorably to the *in vitro* and *in vivo* inhibitory activity observed in this study.

Keywords: *Qualea grandiflora*, *Plasmodium falciparum*, ellagic acid

Introduction

Over 200 million cases of malaria, an infectious tropical disease caused by *Plasmodium* spp., were reported worldwide in 2017,[1] leading to an estimated death toll of 435,000 people. Despite numerous prevention and control measures, malaria remains one of the world's main public health problems.

Various species of *Plasmodium*, e.g., *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*, are known to cause malaria infection in humans. *Plasmodium* protozoans, transmitted through the bites of *Anopheles* spp. mosquitoes have a complex life cycle, and despite the existence of antimalarial drugs such as quinolones, antifolates and artemisinin derivatives, all the known types of treatment have reportedly encountered parasite resistance, including the gold-standard treatment for malaria and artemisinin-based combination therapies (ACTs).[2-7]

Natural products are a rich source of antimalarial compounds.[6,8] *Qualea grandiflora* Mart., commonly known in Brazil as "pau-terra" or "pau-terra-do-cerrado," belongs to the family Vochysiaceae and is one of the most common trees of the Brazilian cerrado biome. In folk medicine, the stem bark and leaves are used to treat inflammatory diseases and gastrointestinal disorders.[9-11] Moreover, *Q. grandiflora* stem and leaf extracts have shown several pharmacological properties, including antiulcerogenic, antibacterial, analgesic and anticonvulsant activity.[9,12]

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Previous studies of *Qualea* species (*Q. multiflora*, *Q. paraensis*, *Q. grandiflora*) have described the antiparasitic properties of the plants' extracts.[13] Recently, fractions of ethyl acetate extract of leaves have shown antiparasitic activity against *Trypanosoma cruzi*, *T. brucei gambiense* and *P. falciparum*.[14] However, to the best of our knowledge, there are no reports of the antiplasmodial properties of *Qualea grandiflora* Mart. fruit and stem extracts.

In this work, we investigated the *in vitro* and *in vivo* antiplasmodial activity of *Q. grandiflora* stem and fruit extracts. We then identified the active components of the extracts using ultra-high-performance liquid chromatography (UHPLC) coupled with high-resolution mass spectrometry (LC-HRMS). Our findings indicate that *Qualea grandiflora* Mart. fruit and stem extracts are alternative sources of natural products with *in vitro* and *in vivo* antiplasmodial properties.

Experimental

General experimental procedures

The solvents used were purchased from J.T. Baker (Phillipsburg, USA), all solvents were of analytical and HPLC grades. The LC-HRMS system was composed of UHPLC, 1260 Infinity II system (Agilent, Barueri, SP, Brazil), and it was equipped with a high-resolution mass spectrometer (HRMS) containing a quadrupole time-of-flight mass analyzer (QTOF, Impact HD) with an electrospray ionization (ESI) source (Bruker Daltonics, Bremen, Germany).

Plant material

The fruits and stems from *Qualea grandiflora* were collected in October 2015 in the cerrado at Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil. The species (voucher No. 8854) were identified by Maristela Imatomi and deposited at the Herbarium of the Botany Laboratory (HUFSCar) at the same University.

Extraction and sample preparation

Air-dried (40 °C) stems (2.0 kg) were extracted with ethanol. Fruits (1.8 kg) were directly extracted with ethanol as well. The concentrated ethanolic extracts from fruits (QgEfr) and stems (QgEs), 15 g of each, were dissolved in ethanol and distilled water (1:3) for liquid-liquid partition. The extracts were partitioned, and the polar extracts obtained were used for antimalarial assays as well for chemical fingerprinting.

The ethyl acetate (EtOAc) extract from fruits (QgEAfr) and stems (QgEAs), and hydroalcoholic extracts from stems (QgHs) were concentrated for the LC analysis. The samples were prepared using 1 mg mL^{-1} of extracts (QgEAfr, QgEAs and QgHs) with Milli-Q water (Massachusetts, USA) and methanol (8:2 v/v), and 1 µL of each solution were injected into the LC-HRMS system.

LC-HRMS

For the analysis, a CortecsTM C18+ (2.7 µm particle size; 10 × 0.21 cm) (Waters, Milford, MA, USA) analytical column was used with a mobile phase composed of water (A), acetonitrile (B), and 0.1% v/v of formic acid was added to both solvents. A linear gradient of 5 to 100% B in 20 min was used at a flow rate of 0.2 mL min^{-1}, with temperature of 40 °C.

The ionization experiments were carried out at positive [M + H]$^+$ and negative [M − H]$^-$ modes. The parameters used for mass spectrometry ionization source were as following: nebulizer, 3.0 bar; dry gas flow, 8.0 L min^{-1}; dry heater temperature, 180 °C; capillary voltage, 4500 V; end plate offset, 500 V; collision cell energy, 5 eV; and full-MS scan range, *m/z* 100-1000. The acquisition was obtained in auto MS/MS mode (number of precursors: 3) in experiments with collision energy of 20, 25, 30, 35 and 40 eV for all *m/z* analyzed range. The Data Analysis 4.0 software and Bruker Smart Formula (Bruker Daltonics, Bremen, Germany) were used as tools for compounds identification.

In vitro assay in *Plasmodium falciparum*

A culture of *Plasmodium falciparum*, 3D7 strain, was maintained in complete Roswell Park Memorial Institute (RPMI) medium, supplemented with Albumax II®. This culture was synchronized using the sorbitol method,[15] and the resulting ring-stage culture was adjusted to 2% hematocrit and 0.5% parasitemia using fresh medium and human O+ red blood cells (RBCs).

In a 96-well plate, 20 µL of two-fold serial dilutions of each compound were prepared, to a concentration 10 times higher than the final desired concentration. Each compound was tested in duplicate, and compounds were tested in two independent experiments for result confirmation. A volume of 180 µL of the prepared culture was then distributed in each well, and positive (untreated parasitized RBCs) and negative (non-parasitized RBCs) growth controls were included in each plate. The plate was then incubated at 37 °C and 5% CO$_2$, for 72 h. After the incubation, the plate fluorescence was read in a spectrophotometer SpectraMax® Gemini™ microplate readers (Molecular Devices,

California, USA), using the SYBR Green I method[16] and the intensity data were analyzed in OriginPro software,[17] resulting in a sigmoidal dose response curve from which the IC_{50} values were obtained.

In vitro cytotoxicity evaluation

A culture of hepatoma cells (HepG2) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum until it reached confluency. For cytotoxicity evaluation, the confluent culture flask was trypsinized for deadhesion and the suspended cells were counted, followed by distribution on a 96-well plate to a final concentration of 5×10^6 cells *per* well. The plate was then incubated for 24 h for cell adhesion. In a separate 96-well plate, 20 μL of serial dilutions of the compounds were prepared and transferred to the cell culture plate. After another 24 h of incubation, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability in each well. Briefly, 20 μL of a 5 mg mL^{-1} solution of MTT was added to each well, followed by a 3-h incubation period. The formazan crystals formed were solubilized in 100 μL of dimethyl sulfoxide (DMSO), and the plate absorbance was read in the SpectraMax® Plus 384 microplate readers (Molecular Devices, California, USA). Intensity data were normalized and plotted using the OriginPro software,[17] and the resulting curves were analyzed to determine the half-maximal inhibitory concentration (IC_{50}^{HepG2}) for each compound.

In vivo assay in *Plasmodium berghei*

The suppressive test was performed as described.[18] The *P. berghei* NK65 strain was obtained as a donation from New York University and maintained through weekly blood passages. For the experiments, the use of animals is in agreement with ethics and animals care which was approved by the Ethics Committee for Animal Use of Universidade Federal do Estado de São Paulo (UNIFESP) under CEUA No. 6630080816. The mice were inoculated i.p. (intraperitoneal) with 1×10^5 infected erythrocytes, kept together for about 24 h, then randomly distributed into groups of five *per* cage. The mice were treated daily for three consecutive days with compounds freshly diluted in distilled water and administered orally at 100 mg kg^{-1}; the control groups received either the drug vehicle or the antimalarial chloroquine (CQ) administered at 20 mg kg^{-1}. On days 5 and 7 after the parasite inoculation, blood was taken from the tail of each mouse and used to prepare thin smears that were methanol-fixed, Giemsa-stained, and examined microscopically (1000×) to determine parasitemia.

The inhibition of parasite growth was determined in relation to parasitemia in the untreated mice, considered to be 100% parasite growth. Compounds reducing the parasitemia by > 40% were considered active, between 30 and 40% partially active, and by less than 30% were considered inactive. The experiments were performed twice.

Results and Discussion

The *in vitro* assays indicated that the six *Q. grandiflora* extracts were active against both sensitive (3d7) and resistant (k1) *P. falciparum* strains (IC_{50} values between 1.2 and 7 ng mL^{-1}) (Table 1). The inhibitory activity evaluated showed good correlation between the sensitive and resistant strains, indicating that the active compounds in the fractions showed no cross-resistance underlying the resistance mechanism of the k1 strain. Moreover, all the extracts produced very low toxic effects on human hepatic cells in assay conditions ($IC_{50}^{HepG2} \geq 400$ ng mL^{-1}), showing significant selectivity indices (SI ≥ 58) (Table 1). In view of this finding, we assessed the *in vivo* activity of the most potent and selective extract, the fruit ethanolic extract (QgEfr, $IC_{50} = 1.2$ ng mL^{-1} and SI ≥ 322), in *Plasmodium berghei*-infected mice.

Table 1. *In vitro* antiplasmodial and cytotoxic activity of *Q. grandiflora* extracts against *P. falciparum* and HepG2

| Extract | $IC_{50} \pm$ SD / (ng mL^{-1}) | | | Selectivity index (SI)[a] |
| | *P. falciparum* | | HepG2 | |
	3d7	k1		
QgEs	4.5 ± 0.5	4.2 ± 0.5	≥ 400	≥ 96
QgEfr	1.2 ± 0.7	1.2 ± 0.7	≥ 400	≥ 322
QgHs	7 ± 1	7 ± 1	≥ 400	≥ 58
QgHfr	3 ± 2	3 ± 2	≥ 400	≥ 150
QgEAs	3 ± 2	5 ± 2	≥ 400	75
QgEAfr	7.0 ± 0.1	6 ± 1	370 ± 19	97
Artesunate	11 ± 0.008 nM	7 ± 0.002 nM	279 ± 24	25.36

[a]Selectivity index = IC_{50}^{3d7} / IC_{50}^{HepG2}. IC_{50}: half-maximal inhibitory concentration; QgEs: ethanolic extracts from stems; QgEfr: ethanolic extracts from fruits; QgHs: hydroalcoholic extracts from stems; QgHfr: hydroalcoholic extracts from fruits; QgEAs: ethyl acetate extract from stems; QgEAfr: ethyl acetate extract from fruits.

Five infected mice were treated orally with 100 mg kg^{-1} of the ethanolic extract for three consecutive days after infection (Figure 1). Parasitemia was evaluated on days 5 and 7 post-infection. The antimalarial drug CQ was used as positive control (20 mg kg^{-1}). QgEfr extract reduced parasitemia by 100% on day 5 post-infection, similar to the CQ control, and by 83% on day 7 (Figure 1).
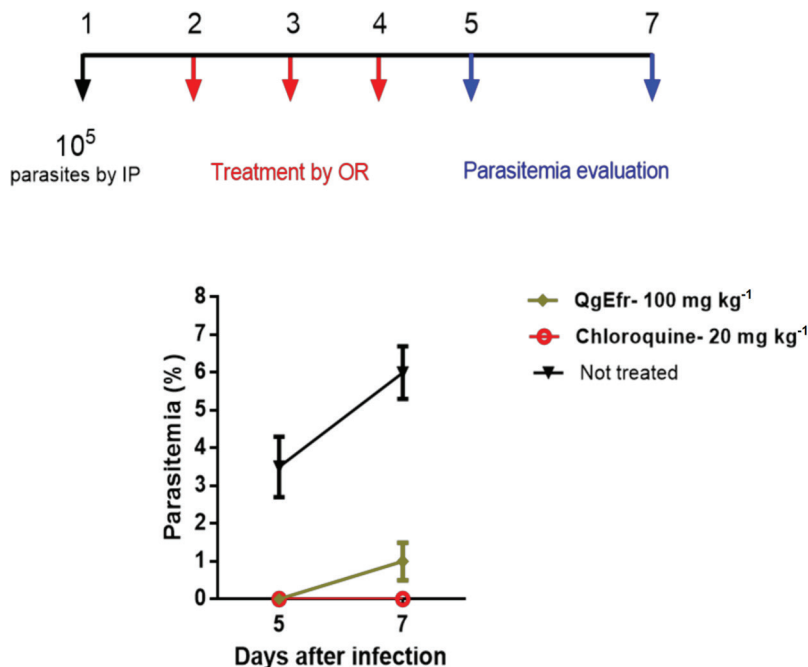
Figure 1. *In vivo* inhibitory activity evaluation of *Qualea grandiflora* fruit ethanolic extract (QgEfr, 100 mg kg⁻¹) and chloroquine (CQ, 20 mg kg⁻¹) against *P. berghei*. Summary of percentage of survival parasite after treatment with QgEfr extract and chloroquine (control). QgEfr extract reduced parasitemia by 100% on day 5 post-infection, similar to the CQ control. On day 7 QgEFr reduced parasitemia by $(83 \pm 12)\%$ and CQ control maintained 100% of reduction.

To assess the chemical profile of the active polar extracts of *Q. grandiflora* fruits and stems, we analyzed the samples by LC-HRMS and optimized chromatographic conditions. The MS/MS data in negative ion mode $[M - H]^-$ provided product ion spectra. To identify the compounds, we investigated each spectrum and compared it to free compound screening libraries online.[19] In addition, we used data published in the literature[20-28] to compare the exact mass and MS/MS fragmentations patterns, enabling us to identify 32 compounds from several chemical classes, including gallotannins, ellagic acid derivatives and flavonoids (Table 2).

Characterization

Ellagic acid (**1A**, **2F** and **3Hi**) and 14 ellagic acid derivatives (**2A**, **3A**, **6A**, **7A**, **13A**, **3F**, **7F**, **8F**, **9F**, **11F**, **1Hi**, **2Hi**, **4Hi** and **5Hi**) were identified from *Q. grandiflora* fruit and stem extracts. Ellagic acid was identified in the ethyl acetate (EtOAc) extracts of stems (QgEAs) (**1A**) and fruits (QgEAfr) (**2F**), as well as in hydroalcoholic extract of stems (QgHs) (**3Hi**). The peaks pertaining to compounds **1A**, **2F** and **3Hi** indicated the $[M - H]^-$ ions with *m/z* values of 300.9991, 300.9997 and 300.9965 ($C_{14}H_6O_8$), respectively, with fragment ions characteristic of ellagic acid, such as those at *m/z* 283.9954, 273.0041, 257.0083, 245.0084, 229.0137, 201.0190, 185.0244, 173.0242, 157.0290, 145.0295, and 129.0345 (Table 2).[20,21]

The ellagic acid derivatives were identified based on the abundant ion fragments of MS² spectra with *m/z* values varying from 299.9883 to 299.9919. Compounds **2A** (QgEAs), **1Hi** (QgHs) and **11F** (QgEAfr) displayed similar $[M - H]^-$ ions at *m/z* 461.0731, 461.0679 and 461.0733 ($C_{21}H_{18}O_{12}$), respectively. Furthermore, we observed the loss of 146.0588 Da related to a hexose and of 15.0231 Da pertaining to a CH_3 group (Figure 2). The rhamnose moiety was suggested for the hexose since the compound 3'-*O*-methyl-4-*O*-(rhamnopyranosyl) ellagic acid has reportedly[11,21,22] been identified in *Q. grandiflora* stems.

The ellagic acid derivatives **3A** (QgEAs), **2Hi** (QgHs) and **9F** (QgEAfr) exhibited $[M - H]^-$ ions at *m/z* 447.0574, 447.0522 and 447.0573 ($C_{20}H_{16}O_{12}$), respectively, and the fragmentation ions 132.0435 Da (removal of a pentose moiety) and 15.0229 Da (loss of a CH_3 group), suggesting they were methyl-pentopyranoside ellagic acids (Figure 2). Compounds **6A** (QgEAs) and **4Hi** (QgHs) with $[M - H]^-$ ions at *m/z* 433.0414 and 433.0376, respectively, showed the loss of pentose in both compounds (133.0505 Da).

The peak (retention time (t_R) = 7.02 min) related to **13A** (QgEAs) produced the deprotonated ion *m/z* 629.0787 ($C_{28}H_{22}O_{17}$), and the MS² spectra showed the elimination of a hexose moiety $[M - h - 162]^-$, a galloyl $[M - h - 152]^-$, and a methyl unit $[M - h - 15]^-$, allowing for the assignment of a methyl-galloyl-glucopyranosyl ellagic acid.[22,23] Compound **5Hi** (QgHs) displayed a $[M - H]^-$ ion

Table 2. Secondary metabolites identified from QgEAfr, QgEAs and QgHs of *Q. grandiflora*. The exact mass of compounds was assigned based on MS experimental data

Cpd	t_R / min	Formula	[M – H]$^-$	Error / ppm	MS^2 products ions	Identification
1A	6.42	$C_{14}H_6O_8$	300.9991	−0.4	283.9954; 273.0041; 257.0083; 245.0084; 229.0137; 201.0190; 185.0244; 173.0242; 157.0290; 145.0295; 129.0345	ellagic acid
2A	8.04	$C_{21}H_{18}O_{12}$	461.0731	−1.0	315.0143; 299.9912	methyl-*O*-rhamnopyranosyl ellagic acid
3A	7.74	$C_{20}H_{16}O_{12}$	447.0574	−1.1	315.0139; 299.9910; 270.9881	methyl-*O*-pentopyranoside ellagic acid
4A	4.28	$C_{20}H_{22}O_{12}$	453.1030	1.3	169.0147; 139.0386; 124.0165	methoxy-hydroxyphenol-*O*-galloyl-hexoside
5A	5.63	$C_9H_{10}O_5$	197.0455	1.3	140.0126; 124.0167	ethyl gallate
6A	7.91	$C_{19}H_{14}O_{12}$	433.0414	−0.3	299.9910; 244.0010	pentopyranosyl ellagic acid
7A	8.30	$C_{15}H_8O_8$	315.0148	−0.2	299.9909; 216.0062; 160.0169	methyl ellagic acid
8A	1.06	$C_{13}H_{16}O_{10}$	331.0664	2.2	169.0139; 151.0033; 125.0235	galloyl-hexose
9A	1.28	$C_7H_6O_5$	169.0138	1.8	124.0161	gallic acid
10A	2.96	$C_{34}H_{24}O_{22}$	783.0664	2.9	300.9982; 275.0187; 249.0398	pedunculagin
11A	3.65	$C_{20}H_{20}O_{14}$	483.0767	2.8	301.0002; 271.0449; 211.0240; 169.0139; 125.0245	digalloyl-hexose
12A	4.99	$C_{27}H_{24}O_{18}$	635.0868	3.5	465.0654; 313.0554; 300.9976; 275.0202; 211.0242; 169.0139; 125.0240	trigalloyl-hexose
13A	7.02	$C_{28}H_{22}O_{17}$	629.0787	−0.5	477.0630; 315.0141; 299.9909	methyl-galloyl-glucopyranosyl ellagic acid
1F	4.13	$C_{34}H_{24}O_{22}$	783.0690	−0.4	481.0635; 301.0001; 275.0212	pedunculagin
2F	12.23	$C_{14}H_6O_8$	300.9997	−2.4	283.9973; 273.0015; 257.0085; 245.0094; 229.0150	ellagic acid
3F	18.26	$C_{16}H_{10}O_8$	329.0311	−2.4	314.0078; 298.9841	di-*O*-methyl ellagic acid
4F	16.02	$C_{24}H_{24}O_{13}$	519.0325	−0.9	461.0731; 315.0153	isorhamnetin acetyl-hexoside
5F	8.39	$C_{20}H_{18}O_{14}$	481.0630	−1.3	421.0405; 300.9999; 275.0203; 249.0413	HHDP-hexose
6F	1.32	$C_{13}H_{16}O_{10}$	331.0680	−0.9	271.0468; 211.0258; 191.0196; 169.0151	galloyl-hexose
7F	19.40	$C_{17}H_{12}O_8$	343.0470	0.5	328.0235; 313.0000; 303.1469	tri-*O*-methyl ellagic acid
8F	11.13	$C_{21}H_{10}O_{13}$	469.0066	−2.6	425.0158; 301.0003; 169.0150	valoneic acid dilactone
9F	15.49	$C_{20}H_{16}O_{12}$	447.0573	−0.9	315.0151; 299.9919	methyl-*O*-pentopyranoside ellagic acid
10F	7.90	$C_{21}H_{20}O_{10}$	431.1007	2.7	269.0455; 125.0235	apigenin-*O*-hexoside
11F	16.04	$C_{21}H_{18}O_{12}$	461.0733	−1.6	315.0155; 300.0279	methyl-*O*-rhamnopyranosyl ellagic acid
12F	7.89	$C_{27}H_{22}O_{18}$	633.0742	−1.3	301.0002; 275.0211; 169.0113	HHDP galloyl hexose
13F	9.82	$C_{41}H_{28}O_{26}$	935.0784	1.3	783.0674; 633.0720; 301.0004; 275.0191; 249.0386	di-*O*-galloyl-lactonised valoneoyl-hexose
14F	9.23	$C_{34}H_{26}O_{22}$	785.0841	1.0	615.0644; 483.0780; 419.0746; 300.9976; 275.0215; 249.0368	di-*O*-galloyl-HHDP-hexose
1Hi	8.0	$C_{21}H_{18}O_{12}$	461.0679	−2.8	315.0113; 299.9884; 270.9860	methyl-*O*-rhamnopyranosyl ellagic acid
2Hi	7.6	$C_{20}H_{16}O_{12}$	447.0522	−2.7	315.0111; 299.9877; 270.9859	methyl-*O*-pentopyranoside ellagic acid
3Hi	6.4	$C_{14}H_6O_8$	300.9965	4.9	283.9935; 245.0073; 229.0132; 173.0241; 161.0233; 145.0290; 117.0342	ellagic acid
4Hi	6.3	$C_{19}H_{14}O_{12}$	433.0376	3.0	299.9885; 271.9932	pentopyranosyl ellagic acid
5Hi	6.5	$C_{21}H_{18}O_{13}$	477.0631	−3.1	315.0122; 299.9883; 270.9874	methylellagic acid-*O*-glucopyranoside

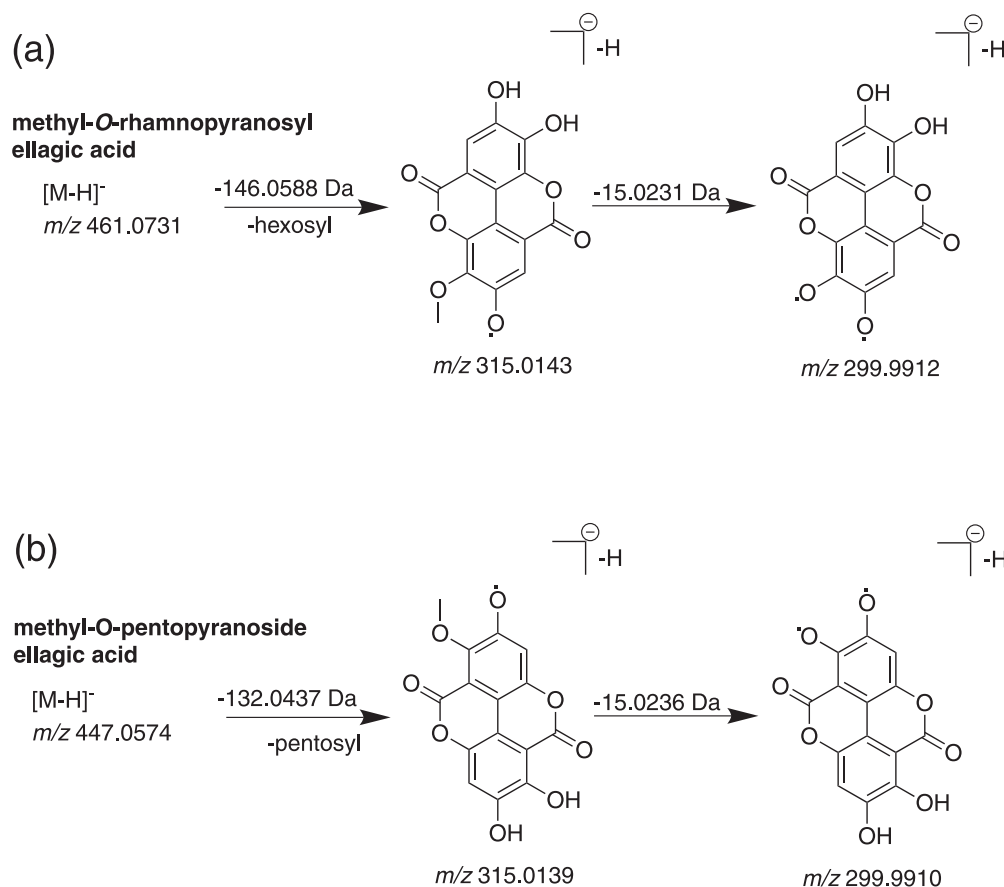Cpd: compound; t_R: retention time; HHDP: hexahydroxydiphenoyl.

Figure 2. (a) Proposed fragmentation pattern of methyl-*O*-rhamnopyranosyl ellagic acid (**2A** (QgEAs), **1Hi** (QgHs) and **11F** (QgEAfr)); (b) proposed fragmentation pattern of methyl-*O*-pentopyranoside ellagic acid (**3A** (QgEAs), **2Hi** (QgHs) and **9F** (QgEAfr)).

at m/z 477.0631 ($C_{21}H_{18}O_{13}$) and the loss of 162.0509 and of 15.0239 Da suggesting a glucose and a CH_3 moiety, respectively, which correspond to methyl ellagic acid-*O*-glucopyranoside.[24]

Based on the [M − H]⁻ ions at m/z 329.0311 ($C_{16}H_{10}O_8$) and 343.0470 ($C_{17}H_{12}O_8$), followed by the loss of CH_3 groups [M − h − 15]⁻ in MS² spectra, we identified compounds **3F** and **7F** as di-*O*-methyl ellagic acid (**3F**) and tri-*O*-methyl ellagic acid (**7F**), respectively.[21,25] Compound **8F** produced m/z 469.0066 ($C_{21}H_{10}O_{13}$) and the fragment ions at m/z 425.0158, 301.0003 and 169.0150. The diagnostic ion at m/z 169.0150 corresponded to the galloyl unit and the one at 301.0003 to ellagic acid. Thus, we identified the compound as valoneic acid dilactone.[26]

We also identified gallotannins. Six of gallotannin derivatives were found in the stem extracts, QgEAs (**4A**, **5A**, **8A**, **10A**, **11A** and **12A**), together with gallic acid (**9A**), and six gallotannins were identified in the fruit extract, QgEAfr (**1F**, **5F**, **6F**, **12F**, **13F** and **14F**). Gallic acid (t_R = 1.28 min) (**9A**) (QgEAs) was identified based on its [M − H]⁻ ion at m/z 169.0138 ($C_7H_6O_5$), which provided the product ion at m/z 124.0161 through the loss of CO_2 and hydrogen, corresponding to 44.9977 Da.[21]

The gallotannin derivatives **4A**, **8A**, **11A** and **12A** (QgEAs) with ions at m/z 453.1030 ($C_{20}H_{22}O_{12}$); m/z 331.0664 ($C_{13}H_{16}O_{10}$); m/z 483.0767 ($C_{20}H_{20}O_{14}$) and m/z 635.0868 ($C_{27}H_{24}O_{18}$), respectively, produced product ions between m/z 169.0139 and 169.0147, which were attributed to the presence of a galloyl moiety. For the compound **12A**, successive losses of the galloyl group in MS² spectra and decarboxylation [M − H − 44]⁻ produced the characteristic fragments at m/z 465.0654, 313.0554, 300.9976, 275.0202, 211.0242, 169.0139, 125.0240 (Table 2, Figure 3). These data indicated the presence of a sugar core in the structure of compounds **4A**, **8A**, **11A** and **12A**. Those compounds produced the typical loss of hexose units [M − h − 162]⁻. In compound **4A**, the loss of 284.0883 Da pointed to hexose bound to the methyl catechol moiety. According to the fragmentation pathway of compounds **4A**, **8A**, **11A** and **12A**, we identified a methoxy-hydroxyphenol-*O*-galloyl-hexoside, a galloyl-hexose, a digalloyl-hexose and a trigalloyl-hexose, respectively.[20,21] Galloyl-hexoses **8A** and **6F** were identified in the EtOAc extract of stems and fruits, respectively.

The gallotannins **10A** (QgEAs) and **1F** (QgEAfr) exhibited similar [M − H]⁻ ions at m/z 783.0664 and
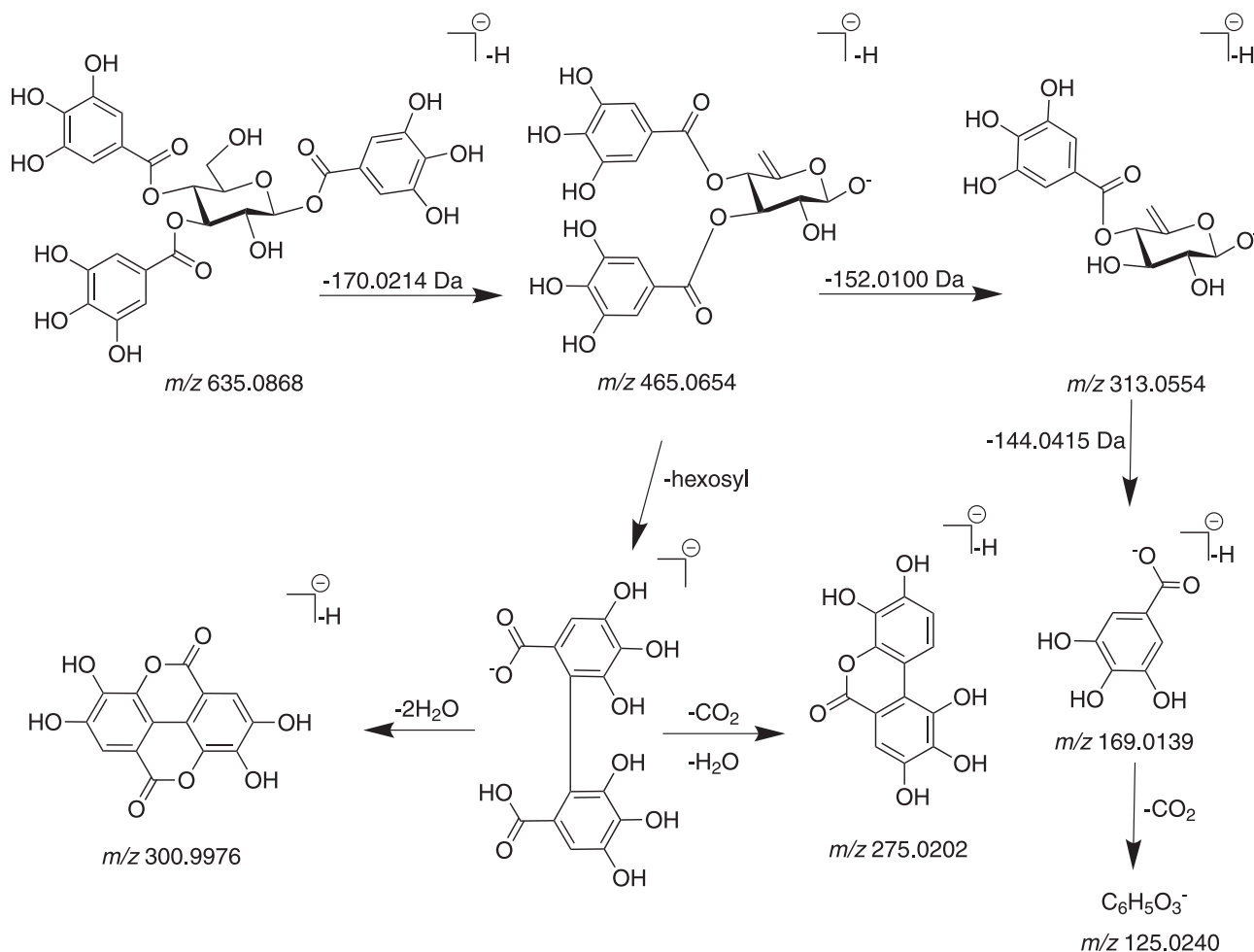
Figure 3. Fragmentation pattern of trigalloyl-hexose **12A** (QgEAs).

783.0690 ($C_{34}H_{24}O_{22}$), respectively, and based on their mass loss observed in the MS^2 spectrum (**1F**) at 302.0055, 180.0634 and 25.9789 Da, we identified them as pedunculagin.[21] The peak (t_R = 5.63 min) of compound **5A** (QgEAs) was identified as ethyl gallate since it exhibited [M − H]$^-$ ions at *m/z* 197.0455 ($C_9H_{10}O_5$), with a loss of $C_3H_5O_2$ corresponding to 73.0288 Da.[20,21]

Compounds **5F**, **12F**, **13F** and **14F**, from fruit extracts (QgEAfr), provided similar product fragments in MS^2 spectra, with a mass loss [M − 162]$^-$ of hexose moiety. Moreover, **12F** showed a [M − H]$^-$ ion at *m/z* 633.0742 ($C_{27}H_{22}O_{18}$), and an evident fragment at *m/z* 169.0113, thereby indicating a galloyl group, which led to the assignment of galloyl-hexahydroxydiphenoyl (HHDP)-hexose.[23] Compounds **5F** and **14F** exhibited [M − H]$^-$ ions at *m/z* 481.0630 ($C_{20}H_{18}O_{14}$) and 785.0841 ($C_{34}H_{26}O_{22}$), respectively, with prominent ions at *m/z* 300.9999 and 300.9976, confirming the presence of the HHDP group in both compounds. The gallotannin **5F** was characterized as HHDP-hexose. Compound **14F** was identified as di-*O*-galloyl-HHDP-hexose isomer based on

its fragment ions of *m/z* 615.0644, 483.0780, 419.0746, 300.9976, 275.0215, 249.0368 and on the loss of H_2O and CO_2 by HHDP, which generated ions at *m/z* 300.9976, 275.0215, and 249.0368.[20]

Compound **13F** showed an [M − H]$^-$ ion at *m/z* 935.0784 ($C_{41}H_{28}O_{26}$), with fragment ions at *m/z* 783.0674, 633.0720, 301.0004, 275.0191, and 249.0386, most of them similar to the fragment ions of compound **14F**. The low abundance of ions at *m/z* 783.0674 was attributed to the loss of a galloyl group (152.0110 Da) from the [M − H]$^-$ ion at *m/z* 935.0784, which is characteristic of di-*O*-galloyl-lactonised valoneoyl-hexose.[20]

The flavonoids in QgEAfr (**4F** and **10F**) were identified as isorhamnetin acetyl-hexoside and apigenin-*O*-hexoside, respectively. In the negative ion mode [M − H]$^-$, the product ion spectra of both flavonoids showed loss of a hexose unit (162.0526 Da). The flavonol exhibited the [M − H]$^-$ ion at *m/z* 519.0325 ($C_{24}H_{24}O_{13}$) with fragment ions at *m/z* 461.0731 and 315.0153, and the loss of 57.9594 Da in the acetyl moiety.[27] The apigenin-*O*-hexoside (**10F**) was identified by its molecular ion [M − H]$^-$ at *m/z* 431.1007,

with fragment ion *m/z* 269.0455 corresponding to aglycone apigenin [M − h − 162]⁻, and the fragment ion *m/z* 125.0235 corresponding to a cleavage in ring C.[28] Glycosylated flavonols and triterpenes have been previously identified in *Q. grandiflora* leaf extracts.[9] Flavanones have been identified in the stems of this species, as well. Flavones, flavanones and dihydroflavonols are usually found in the wood of *Qualea* species.[11] In this regard, this is the first report of the occurrence of flavonol and flavone in the fruits of the genus *Qualea*.

Note that most of the abovementioned compounds, e.g., all the gallotannins and gallotannin derivatives and the flavonoids isorhamnetin acetyl-hexoside and apigenin-*O*-hexoside were identified for the first time in *Q. grandiflora* (Table 2). On the other hand, gallic acid and some ellagic acid (EA) derivatives from *Q. grandiflora* stems, such as 3,3'-di-*O*-methylellagic acid-4-*O*-β-D-glucopyranoside, 3-*O*-methylellagic acid-4'-*O*-α-L-rhamnopyranoside, 3,3',4-tri-*O*-methylellagic acid-4'-*O*-β-D-glucopyranoside, and 3,3'-di-*O*-methylellagic acid, have been identified in previous studies.[11,22]

Antiplasmodial activity and chemical profiling

Our findings suggest that *Q. grandiflora* fruit and stems polar extracts contain active components with antiplasmodial activity. These results led us to ascertain the chemical fingerprint of the active extracts in order to identify the key components of the plant's antiplasmodial activity. LC-HRMS proved to be a comprehensive and robust method and an excellent tool for the prospection of known and unknown substances in natural product extracts. This approach combines the separation ability of high-performance liquid chromatography with high-resolution mass spectra from which fragment ions are obtained through different collision energies provided by soft ionization techniques.[29-32]

The LC-HRMS analysis enabled the identification of gallotannins, ellagic acid (EA) derivatives and flavonoids. We identified galloyl-hexose (**8A**, **6F**) and gallotannin pedunculagin (**10A**, **1F**) in both the QgEAs and QgEAfr active extracts, as well as EA analogues (**1A**, **2F** and **3Hi**) and EA bound to sugar moiety (pentose or hexose) (**2A**, **3A**, **9F**, **11F**, **1Hi** and **2Hi**) in the QgEAs, QgEAfr and QgHs extracts. Previous studies[33] found that ellagic acid derivatives exhibited *in vitro* antiplasmodial activity (IC$_{50}$ values ranging from 105 to 330 nM) and *in vivo* activity (median effective dose (ED$_{50}$) activity lower than 1 mg kg⁻¹ day⁻¹, i.p.). Given that EA is the constituent normally identified in active stem and fruit extracts (QgEAs and QgEAfr, respectively), it could be the main component

of the mixture that contributed favorably to the *in vitro* and *in vivo* efficacy of the extracts observed in this study.

The biological activities mainly related to the consumption of ellagitannins in natural sources could be consequence of the EA release as well.[34] Previous studies[35] investigating absorption and bioavailability of bioactive ellagitannins and EA *in vivo* dietary consumption of fruits showed that human ingestion of 237 mL of pomegranate (23 mg free EA) and 500 mg of free EA lead to the same plasma concentration of EA. The EA from natural source was more bioavailable and bioactive than free EA related to anti-inflammatory response. The promising result found on fruit extract (QgEfr) evaluated in *P. berghei in vivo* also may be due to a role of the other constituents with EA that would be further investigated.

Additionally, we identified the presence of isorhamnetin acetyl-hexoside and apigenin-*O*-hexoside (compounds **4F** and **10F**, respectively) in the *Q. grandiflora* fruit ethyl acetate extract (QgEAfr). The *in vitro* antiplasmodial activity of the fruit ethyl acetate extract (QgEAfr) observed here also may be due to the inhibitory property of the apigenin aglycone. The compound isolated from *Melampyrum arvense* L. displayed a significant inhibitory effect against *P. falciparum*, with an IC$_{50}$ value of 15.5 μg mL⁻¹.[36]

Conclusions

To the best of our knowledge, this paper offers the first report of the chemical profile of *Qualea* fruits. Moreover, our findings indicate that both the *in vitro* and *in vivo* inhibitory activity of the fruit ethanolic extract may be related to the presence of ellagic acid, a major component of the mixture. In summary, the collected data provided solid scientific information about the chemical profile of *Qualea* fruits, in addition to the *in vitro* and *in vivo* antiplasmodial activity of polar extracts of *Qualea grandiflora* ("pau-terra") fruits and stems.

Supplementary Information

The MS/MS spectra data of the identified compounds (**1A-13A**; **1F-14F**; **1Hi-5Hi**) are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

Author Contributions

Geraldo S. Gonçalves Neto and Romário P. da Costa carried out chemical experimental procedures, analyzed the samples by LC-HRMS and MS analysis; Camila L. Zanini, Anna Caroline C. Aguiar, Juliana O. de Souza and Guilherme E. de Souza performed the *in vitro* studies; Richele P. Severino, Anna Caroline C. Aguiar, Quezia B. Cass, Fábio C. Cruz and Glaucius Oliva analyzed the data, contributed ideas and with the writing review and editing process of the paper; Lorena R. F. de Sousa and Rafael V. C. Guido conceived the study and wrote the paper. All the authors have read the final manuscript and approved the submission.

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