

Unraveling the Adaptive Chemical Traits of *Rhamnidium elaeocarpum* in Response to Fire and Environmental Factors in Pantanal Wetlands

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Abstract

We conducted a study on the effects of fire on *Rhamnidium elaeocarpum*, a widely distributed woody species found in the Pantanal wetlands, using LC-MS metabolomics, total phenolic and tannin content analysis, and thermogravimetric behavior. We sampled individuals from four different groups and found that recent fires had no significant impact on the species' phenolic metabolism, except for those specimens whose aerial parts had died during the 2020 megafire, which showed a compound decline due to leaching. The high levels of phenolics in *R. elaeocarpum* suggest that the species has a biochemical tolerance to the stress caused by seasonal fires. Metabolomic profiling revealed the presence of proanthocyanidin oligomers, which protect against oxidative stress and post-fire environmental disturbances. However, the passage of fire also led to a high incidence of toxic karwinaphthopyranone derivatives, which could be a concern for the species' medicinal use. Finally, the thermogravimetric analysis showed that the species is thermotolerant, with an intrinsic relationship between the secondary compounds and thermotolerance. Our study enhances our understanding of the impact of fire on woody species' metabolism, although distinguishing whether chemical traits are an adaptive feature or a response to surrounding environmental factors is challenging.

Introduction

Global transformations have led to widespread fires, causing severe impacts on plant communities, society, and the economy (Garcia et al. 2021). Even humid landscapes are not spared due to the prolonged dry periods (Garcia et al. 2021; Libonati et al. 2020). The Brazilian Pantanal, the most extensive wetland in the world, has experienced increased fire incidence and severity (Bowman et al. 2020). In 2020, the Pantanal suffered from the longest and most severe drought in the last 60 years (SPEI - Standardized Precipitation-Evapotranspiration Index 2020), which, combined with high temperatures and reduced soil moisture, led to the largest forest fire ever recorded in the area (Libonati et al. 2020). The National Institute for Space Research (INPE 2022) and ALARMES alert system reported that about 3.9 million hectares of the biome were affected by fire in 2020, with more than 15.000 fires registered, three times more than the previous year (Marques et al. 2021).

Fire significantly alters plant community composition and structure, favoring more fire-tolerant species and restricting vulnerable ones (Agee et al. 2002; Stellmes 2013). Specific physiological and anatomical adaptations such as bark thickening, rapid growth, protection of photosynthetic structures, and improved germination power have been linked to fire events (Lukac et al. 2010). However, the ability of plant communities to recover after a fire event is influenced by the frequency, intensity, and duration of fires (Tanentzap et al. 2014). This variability in ecological resilience to fire highlights the importance of adaptive traits in plants to a specific fire regime and the potential threat posed by changes in stress patterns (Bradstock 2008, Keeley et al. 2009, Keeley et al. 2011).

Considering the various strategies that plants develop to survive fire events, highlighting the biochemical mechanism is vital since it is amongst the least elucidated in fire ecology. Plant secondary metabolism

protects against biotic and abiotic stresses (Seigler, 1998). Several metabolites are involved in this process, varying according to their physiological role (Wallis et al. 2011). For instance, phenolics are stress bioindicators of changes due to their abundance in specific environmental conditions (Diaz-Guerra et al. 2018; Ganthaler et al. 2017). Cannac et al. (2009) observed an increased phenolic biosynthesis in *Pinus laricio* in response to prescribed fires. The relationship between phenolic biosynthesis and fire was also reported in thermotolerant species in the Pantanal riparian forests in Brazil. Da Silva et al. (2021) recorded higher concentrations of total phenolic and tannin in resistant species compared to thermosensitive ones. Additionally, phenolics, mainly tannins, have low thermal conductivity (Celzard et al. 2011; Huss et al. 2019; Tributsch and Fiechter 2008), which could have important roles during a fire event.

Tannin is a crucial defense mechanism against herbivores, particularly in post-fire environments, where it plays a significant role (Santacruz-García et al., 2015). In addition to providing mechanical resistance, reducing flammability, and preventing damage from microorganisms, tannins are the second most abundant class of phenolic compounds (Khanbabaee et al., 2001). They are oligomeric products formed by flavan-3-ol units, such as catechin and galocatechin, via the flavonoid biosynthetic pathway. The molecular size is essential to their biological activities, the large number of hydroxyls in tannins' structure and their conjugated bonds make them potent antioxidant agents, protecting cells from free radical damage (Lin et al., 2018; Asif et al., 2015).

The Pantanal wetlands represent a suitable scenario to analyze the impact of fire events on the secondary metabolites. In addition to the need to expand knowledge about the relationship between fire and wetlands, since an increasing occurrence of extreme events is expected due to climate change and land use (Garcia et al. 2021). We hypothesized that secondary metabolites accumulate more in response to different fire events, and chemical profiles contribute to its thermotolerance. Therefore, in this study, we sought to: (a) evaluate the effect of fire events on total phenolic and tannin accumulation; (b) verify if there is an accumulation of phenolic compounds in older plants; (c) discover the chemical response according to fire events; (d) determine whether thermogravimetric properties change in individuals subjected to more recent fire events; (e) estimate the extent to which secondary metabolites influence the thermal stability of this specie. In addition to expanding the insufficient chemical knowledge of the species, the study adds to the discussion about the effect of fire on ecophysiological behavior, contributing to the understanding of plant resistance and resilience mechanisms against environmental stresses.

Methods And Materials

Study area and Imagery. The study was conducted in *capões* forests located at *São Bento* Farm (19°28'49.26" S e longitude 57°00'57,38" W) in the Pantanal subregion Abobral, in the city of Corumbá, State of Mato Grosso do Sul, Brazil. The average annual rainfall is about 1070 mm, with a yearly mean temperature of 25.5 °C (Machado et al. 2012). The climate is seasonal and characterized by a rainy period between October and March. The maximum point of the flooding regime occurs in April, when the

flatlands fields are flooded (Hamilton et al. 1996; Macedo et al. 2014). During floods, there is a high accumulation of organic matter, increasing the production of biomass during the dry season, which becomes a potential fuel for fires (Heinl et al. 2006, 2007; Parolin and Wittmann 2010), increasing the chance of natural or man-made fires (Cochrane 2003; Heinl et al. 2006, 2007). Soils have a high base content due to the deposition of calcareous shells and their color darkens towards the center due to the gradual accumulation of organic matter (Cunha et al. 1985).

The study had a time-lapse of 21 years (1999 to 2020) on regional fire episodes. We used the information collected by Souza (2017) from 1999 to 2016. For the following years, we obtained images from the Landsat 8 OLI sensor with 30 m of spatial resolution, considering only the bands B3, B6 and B7, as the composition highlights the passage of fire in the image. Data was downloaded from INPE's catalog. The satellite images recorded the intense fires in 2019 and 2020 and areas free of fires for an extended period, the last being observed in 2009.

Selected species and Sampling. We chose the species *Rhamnidium elaeocarpum*, due to its high cover value, abundance, and dominance in capões (Rivaben et al., 2021). In addition to occurring in environments with frequent fire (Bortolotto et al., 2018), remarkable regrowth is observed after passing the fire. The species is highly recommended for mixed reforestation used for degraded areas restoration (Lorenzi 2008).

Based on the observed patterns of fire history, the study categorized four groups: The first group, named No fire (n= 14), comprised individuals sampled in capões where the last fire had occurred in 2009. The second group, Fire 19 (n= 10), consisted of individuals sampled in capões who had experienced fires in 2019. The third group, Fire 20 (n= 13), included individuals collected from capões that had fires in 2020. The fourth and final group, APD 20 (n= 10), consisted of individuals whose aerial parts had died during the 2020 fire event.

Extraction of samples. The powdered samples (20 mg) were extracted with 2 mL of methanol:deionized water (7:3) for 10 min on an ultrasound bath (Cristofoli, 42 kHz, 2.5 L) and, subsequently, the samples were centrifuged for 5 min and the methanolic extract was obtained that was applied for the determination of total phenolic and tannin contents. From this sample, we separated 1 mL, added 10 mg of hide powder (SIGMA), and placed the product in a shaker for 60 min, followed by centrifugation for five minutes.

We followed the method of Herald et al. (2012) with modifications to evaluate the total phenolic content (TPC) and total tannin content (TTC). In a 96-well plate, we added 50 µL of deionized water to all wells, followed by 50 µL of the methanol extract, and using serial dilution, we performed five different concentrations. Subsequently, 50 µL of *Folin-Ciocalteu* reagent diluted in deionized water was added (1:1 v/v). After six minutes, we added 100 µL of 75 gL⁻¹ sodium carbonate (Na₂CO₃) water solution, except for blank or control sample wells. The plate was incubated in the dark at room temperature for 90 min, and the absorbance at 765 nm was measured with a spectrophotometric microplate reader

(SpectraMax®Plus384, Molecular Devices, Sunnyvale, CA, USA). The blank reagent was prepared at 50% methanol, using the same method described above, and the standard was a gallic acid aqueous solution ($1 \text{ mg/m}^{-1} \text{ L}$). Gallic acid was used as a standard a concentration range of $2\text{--}125 \text{ } \mu\text{g mL}^{-1}$ to generate a calibration curve (average $R^2=0.9989$) and the concentrations of phenolic and tannin were expressed in mg g^{-1} of gallic acid equivalent.

Statistics analysis from total phenolic and tannin contents. To verify the differences in groups and if DBH (diameter at breast height) influences the TPC and TTC, we performed a generalized linear model (GLM). On the R platform (RDevelopment Core Team, 2021), the distributions were verified by the *fitdistrplus* package, models, and interactions by the GLM function. For both, the Gaussian family was used. Their graphical presentations were generated using the *vis-a-vis* package. Differences were considered significant with $P < 0.05$.

Thermogravimetric analyses. We used pulverized plant and dried methanol extract to analyze the species' thermal resistance and behavior of secondary metabolites under high temperatures. The extracts were obtained by the powder samples (40 mg) extracted in methanol and ultrapure water 7:3 (v/v) in an ultrasonic bath for 10 min, followed by centrifugation for 5 min. Finally, the supernatant was collected and left to dry at room temperature for 36 h. To evaluate the thermal behavior, we obtained the TGA/DTG curves by a TGA-Q50 instrument (TA Instruments, New Castle, DE, USA) and added approximately 7-9 mg of samples in a platinum crucible. The analysis was performed at a heating rate of $10 \text{ } ^\circ\text{C min}^{-1}$ from room temperature to $900 \text{ } ^\circ\text{C}$ under a synthetic air atmosphere with a purge flow rate of 60 mL.min^{-1} in the oven and 40 mL.min^{-1} of nitrogen atmosphere on balance.

HPLC-DAD-ESI-MS/MS analyses. We extracted 40 mg of powdered plant material in methanol and ultrapure water 7:3 (v/v) with 1% formic acid. Then, these materials were extracted in an ultrasonic bath for 10 min and centrifuged for 5 min. The supernatant was filtered by syringe filters (Millex PTFE $0.22 \text{ } \mu\text{m}$, Millipore) for further analysis.

For these analyses, a Shimadzu LC-20AD HPLC chromatograph coupled to a diode array detector and a mass spectrometer ESI-QTOF MicroTOF-Q III, Bruker Daltonics, Billerica, MA, USA) were used. The analyses were performed by monitoring the UV wavelength between 240 and 800 nm and mass spectrometer operating in negative ion mode. The chromatographic column was a Kinetex C-18 ($2.6 \text{ } \mu\text{m}$, $150 \times 2,1 \text{ mm}$, Phenomenex). The mobile phase consisted of ultrapure water (solvent A) and acetonitrile (solvent B), both added formic acid 0.1% (v/v). The gradient elution profile was the following: 0-2 min, 3% B (isocratic); 2-25 min, 3-25% B (linear gradient); 25-40 min, 25-80% B (linear gradient); 40-43 min, 80% B (isocratic); 43-44 min, 80-3% B (linear gradient), and added the time to equilibrate the chromatographic column and system (5 min). The flow rate was 0.3 mL/min and the injection volume was $4 \text{ } \mu\text{L}$ for each sample. The oven temperature was 50°C during the analyses and all samples were randomized for the injection of the system. The quality control (QC) sample consisted of a pool produced by an aliquot of 2 mg of each sample (powdered plant material) and extracted with the same method applied for the samples. The QC was injected after every six samples.

Data alignment was performed by Metalign software (Lommen, 2009) and entries were reduced by Msclust (Tikunov et al. 2012). We evaluated the reproducibility of the equipment by comparing replicates and the QC samples. We also evaluated the variations of ions between the groups and the pattern of metabolites by Partial Least-Squares Discriminant Analysis (PLS-DA) and Volcano plot, respectively, using the Metaboanalyst 5.0 platform (Pang et al. 2021). For these analyses, data were log-transformed and autoscaled. The chemical constituents from the samples were annotated based on UV, accurate mass, MS/MS data and compared to data reported in the literature.

Results

When interpreting the results, it is essential to consider the time between the fire histories in the study area. In 2009, all the capões in the region were burned, indicating a significant fire event. However, in 2019 and 2020, the fire incidents were more localized, affecting different capões. That allowed us to observe and compare the impact of fire on the chemical composition of *Rhamnidium elaeocarpum* for a minimum of two years.

Total phenolic and tannin contents. We recorded high contents of phenolics and tannins from the samples, independent of fire occurrence, on *Rhamnidium elaeocarpum*. The mean total phenolic content (TPC) and total tannin content (TTC) of the bark samples from individuals in the No fire group were 185.91 ± 54.21 mg/g and 47.94 ± 23.62 mg/g, respectively. The results obtained from the Fire 19 and Fire 20 groups were similar to those of the No fire group. The mean TPC and TTC values for individuals in the Fire 19 group were 181.47 ± 32.62 mg/g and 39.97 ± 15.39 mg/g, respectively. For individuals in the Fire 20 group, the mean TPC and TTC values were 192.84 ± 30 mg/g and 42.25 ± 23.36 mg/g, respectively. However, individuals in the APD 20 group showed significantly lower TPC (22.08 ± 11.73 mg/g) and TTC (10.41 ± 8.27 mg/g) concentrations. In other words, the individuals whose aerial parts died during the 2020 wildfire experienced a substantial reduction in their phenolic metabolite content.

Through the General Linear Models (GLM), we observed that the contents of total phenolic and tannins differed only for the *APD 20* group (Fig.1). Furthermore, the concentration of phenolic compounds increased with an increasing DBH of individuals in the No fire group ($p = 0.03$), indicating more compounds in older trees (Fig.1). For the low molecular weight phenolics, we observed that the No Fire and Fire 19 groups ($p = 0.02$ and 0.01) had a higher concentration of compounds as the DBH increased; however, there was no statistical separation between the groups (see Figure in Supplementary Material S1). On the other hand, the concentration of tannins did not present a significant difference between the groups and the relationship between the accumulation of compounds and DBH.

Annotation of the constituents from samples. After processing the data from the metabolomics analysis, we detected 55 peaks and annotated 23 of the totals. The main class of metabolites observed in the study was proanthocyanidins, found as dimers from *R. elaeocarpum* (Fig.2, Table 1). Furthermore, we highlight peaks **41-42** and **49-50**, which were putatively annotated as derivatives of karwinaphthopyranones, dimeric anthracenones.

TABLE 1. COMPOUNDS OF *RHAMNIDIUM ELAEOCARPUM* EXTRACT BY HPLC-DAD-
MS/MS

Peak	RT (min)	UV (nm)	MF	[M-H] ⁻	MS/MS	Compound	Reference
1	1.2	-	C ₁₂ H ₂₂ O ₁₁	341.1094		Hexoside dimer	
2	1.5	-	C ₆ H ₈ O ₇	191.0197	111	Citric acid	Peng, et al., 2018.
3	4.5	270	C ₃₀ H ₂₆ O ₁₄	609.1250	547, 474, 305	Prodelphinidin dimer type B	Baldivia et al., 2018
4	4.5	270	C ₁₅ H ₁₄ O ₇	305.0667	261, 219	Gallocatechin	Ali et al., 2021; Sakakibara et al., 2003
5	5.2	276	C ₃₆ H ₃₆ O ₁₈	755.1829	-	Flavonol derivative	
6	6.2	275	C ₃₀ H ₂₆ O ₁₃	593.1301	423, 305	Procyanidin-prodelphinidin dimer B type	Gomes et al., 2021
7	7.9	270	C ₁₉ H ₂₆ O ₁₃	461.1307		NI	
8	8.7	280	C ₃₆ H ₃₆ O ₁₇	739.1880	435, 273	Procyanidin dimer type B hexoside isomer	Martini et al., 2018
9	9.0	272	C ₂₁ H ₂₄ O ₁₁	451.1246	313, 166 161	Epicatechin-3-O-hexoside isomer	Martini et al., 2018
10	9.3	276	C ₁₅ H ₁₄ O ₇	305.0667	261, 219	Epigallocatechin	Sakakibara et al., 2003
11	9.7	277	C ₃₆ H ₃₆ O ₁₇	739.1880	569, 407	Procyanidin dimer type B hexoside isomer	Martini et al., 2018
12	10.2	280	C ₃₀ H ₂₆ O ₁₃	593.1301	423, 305	Procyanidin-prodelphinidin dimer type B	Gomes et al., 2021
13	11.1	280	C ₅₁ H ₄₈ O ₂₂	1011.2564	-	NI	
14	11.3	280	C ₁₃ H ₂₄ O ₉	323.1348	298, 165	NI	
15	11.5	278	C ₅₉ H ₆₂ O ₃₃	649.1592*	-	NI	
16	11.6	278	C ₃₆ H ₃₆ O ₁₆	723.1931	553, 407, 255	Procyanidin type B deoxyhexoside	Barnaba et al., 2018, Rue et al. 2018
17	12.6	278	C ₃₀ H ₂₆ O ₁₂	577.1351	407, 289	Procyanidin dimer type B	Martini et al., 2018
18	13.0	279	C ₂₁ H ₂₄ O ₁₀	435.1310	289, 271, 203	Epicatechin deoxyhesoxide	
19	13.2	279	C ₂₁ H ₂₂ O ₁₁	449.1089	-	NI	
20	14.1	279	C ₁₆ H ₂₀ O ₁₀	371.0984	249, 189	NI	
21	15.2	279	C ₁₆ H ₂₀ O ₁₁	387.0933	-	NI	
22	16.0	280	C ₂₇ H ₃₂ O ₁₆	611.1618	-	NI	
23	16.4	278	C ₁₇ H ₂₂ O ₁₀	385.1140	-	NI	
24	16.7	278	C ₃₄ H ₄₄ O ₂₁	787.2324	-	NI	
25	17.0	282	C ₂₃ H ₃₉ O ₁₃	523.2396	-	NI	
26	17.5	278	C ₂₇ H ₃₄ O ₁₆	613.1802	-	NI	
27	17.7	278	C ₁₇ H ₂₄ O ₉	531.0828	-	NI	
28	17.8	278	C ₁₈ H ₂₅ O ₂₀	561.0945	-	NI	
29	18.4	280	C ₄₇ H ₅₈ O ₂₉	1085.2978	-	NI	
30	18.6	278	C ₂₈ H ₄₀ O ₁₃	583.2396	-	NI	
31	19.3	279	C ₂₉ H ₃₀ O ₁₆	597.1480	-	NI	
32	19.6	278	C ₄₁ H ₅₀ O ₂₄	925.2622	-	NI	
33	20.8	280	C ₄₅ H ₅₄ O ₂₈	1041.2729	-	NI	
34	20.9	280	C ₂₈ H ₃₅ O ₁₆	627.1931	-	NI	
35	21.0	282	C ₄₆ H ₅₃ O ₂₇	1037.2780	-	NI	

36	21.1	280	C ₅₄ H ₅₂ O ₂₃	1067.2827	-	NI	
37	21.3	280	C ₆₄ H ₇₆ O ₄₀	742.1962*	-	NI	
38	22.9	282	C ₄₈ H ₅₆ O ₂₇	1063.2936	-	NI	
39	24.4	280	C ₁₉ H ₃₄ O ₁₀	421.2079	-	NI	
40	31.5	270	C ₁₈ H ₃₃ O ₅	329.2333	-	NI	
41	35.8	278, 429	C ₃₀ H ₂₆ O ₈	513.1555	495, 471, 410, 57	karwinaphthopyranone derivative	Rojas-Flores et al., 2014
42	36.0	278, 429	C ₃₀ H ₂₆ O ₈	513.1555	495, 471, 410, 57	karwinaphthopyranone derivative	Rojas-Flores et al., 2014
43	36.5		C ₃₀ H ₂₄ O ₈	511.1411		NI	
44	36.7		C ₁₈ H ₃₀ O ₃	293.2112	-	NI	
45	36.8		C ₃₄ H ₆₀ O ₁₆	723.3809	-	NI	
46	37.1		C ₃₀ H ₂₈ O ₈	515.1741	-	NI	
47	37.3		C ₃₄ H ₄₄ O ₉	595.2913	-	NI	
48	37.9	-	C ₁₈ H ₃₂ O ₃	295.2279	277, 171	(±)9-hydroxy-10E,12Z-octadecadienoic acid (9-HODE)	Yuan et al., 2013
49	38.4	268, 413	C ₃₂ H ₃₂ O ₈	543.2024	528, 513	karwinaphthopyranone derivative	Rojas-Flores et al., 2014
50	38.8	268, 413	C ₃₂ H ₃₂ O ₈	543.2024	528, 513	karwinaphthopyranone derivative	Rojas-Flores et al., 2014
51	40.9	-	C ₃₇ H ₁₆ O ₃	507.1027	-	NI	
52	41.3	-	C ₁₈ H ₃₀ O ₂	277.2173	-	Fatty acid	
53	42.5	-	C ₁₈ H ₃₂ O ₂	279.2330	-	Fatty acid	
54	43.7	-	C ₁₆ H ₃₂ O ₂	255.2330	-	Fatty acid	
55	44.2	-	C ₁₈ H ₃₄ O ₂	281.2486	-	Fatty acid	

RT: retention time; MF: molecular formula, NI: Not identified; * Double charge.

Compound 1 revealed the chemical formula C₁₂H₂₂O₁₁, with no absorption in the UV spectra, and was defined as hexoside dimer. **Compound 2** demonstrated an ion at m/z 191.0197 and a fragment ion at m/z 111, corresponding to the loss of one CO₂ and two water molecules [M-H-44-18-18]⁻; this fragmentation profile matches citric acid following Peng et al. (2018).

Compounds 4 and 10, molecular formula C₁₅H₁₄O₇, showed an intense ion at m/z 305.0667, which yielded the fragment ion at m/z 219 by the loss of a unit of C₃O₂ [M-H-88]⁻. Although they have similar fragmentation patterns, the large discrepancy in retention time between them indicates to be isomers. According to Sakakibara et al. (2003), we have a pattern for the given characteristics, where the compound with the shortest retention time corresponds to galocatechin, in our case, peak 4. Epigallocatechin has a longer retention time, represented by peak 10 in this study. The **compound 18**, is epicatechin *O*-deoxyhexoside, has molecular formula C₂₁H₂₄O₁₀ and deprotonated ion at m/z 435.1310. We observed the product ion at m/z 289, yielded by loss of deoxyhexose [M-H-146]⁻, followed by the loss of a water molecule [M-H-146-18]⁻ and A ring [M-H-68]⁻.

Compound 5, chemical formula $C_{36}H_{36}O_{18}$, reveals a UV spectrum compatible with the characteristic bands of flavonols. However, the second peak corresponding to the 330-350 nm range showed low intensity in the UV spectrum.

Compound 3 exhibited ions m/z 609.1250 and **compounds 6 and 12** m/z 593.1301, which correspond to $C_{30}H_{26}O_{14}$ and $C_{30}H_{26}O_{13}$, characterizing respectively as prodelphinidin dimer type B and procyanidin-prodelphinidin dimer type B, reported by Martini et al. (2018). The peaks showed fragment ions at m/z 305 yielded from Quinone Methide (QM) reactions with losses of 304 and 288 u ; thus, prodelphinidin and procyanidin units were confirmed. The fragment ions m/z 423 observed for **peak 6 and 12** are products of the retro-Diels-Alder (RDA) reaction $[M-H-152-H_2O]^-$. The **compounds 8 and 11**, molecular formula $C_{36}H_{36}O_{17}$ and m/z 739.1880 were annotated as isomers of procyanidin dimer type B hexoside with different fragments. **Compound 8** showed a product ion at m/z 435 yielded by two consecutive RDA fissions $[M-H-152-152]^-$, followed by hexose loss $[M-H-162]^-$. **Metabolite 11** had a loss of 152 u by RDA with subsequent elimination of a water molecule $[M-H-152-18]^-$, generating the ion m/z 569, followed by loss of hexose $[M-H-162]^-$ that resulted in the fragment m/z 407. **Peak 16** is a derivative of the **compounds 8 and 11**, with a deoxyhexose moiety. **Peak 17**, is relative to a procyanidin dimer type B, which exhibited a deprotonated ion at m/z 577.1351, compatible with the molecular formula $C_{30}H_{26}O_{12}$. The fragmentation pattern matches two common pathways of procyanidins; the fragment m/z 407 is the product of RDA fission, followed by the loss of a water molecular $[M-H-152-18]^-$ and QM reaction produced fragment m/z 289. The fragmentation pathways of procyanidins annotated in the study are consistent with previously reported data (Hammerstone et al. 1999, Karonen et al. 2004, Hellström et al. 2007, Sui et al. 2016).

Compounds 41-42 and 49-50 exhibited identical UV spectra with bands at approximately 278 and 429 nm, compatible with anthracenones, from the deprotonated ions at m/z 513.1555 and m/z 543.2024, and indicate the molecular formula $C_{30}H_{26}O_8$ and $C_{32}H_{32}O_8$ respectively. We putatively annotated them as derivatives karwinaphthopyranones, compatible with the structure identified by Rojas-Flores et al. (2014). However, confirmation is not possible due to the lack of information on fragmentation patterns in the literature. The compounds are formed by dimeric hydroxyanthracenones, and are characteristic of genera of the Rhamnaceae family (Piñeyro-Lopez and Waksman 2000, Rojas-Flores et al. 2014, Yussim et al. 1995).

Through the MS/MS spectrum, we annotated **compound 48**, deprotonated ion at m/z 295.2279 and molecular formula $C_{18}H_{32}O_3$, as 9-HODE, classified as an oxidized derivative of linoleic acid, and based on the typical fragmentation pattern as reported by Yuan et al. (2012). The ion m/z 277 resulted from the loss of H_2O , followed by cleavage between the carbon-carbon bond adjacent to the hydroxyl group, generating the main fragment at m/z 171. **Compounds 52, 53, 54, and 55** were annotated as fatty acids due to the strong interaction between the metabolites and the stationary applied here, with the chemical formulas $C_{18}H_{30}O_2$, $C_{18}H_{32}O_2$, and $C_{18}H_{34}O_2$, respectively, in addition to not having UV spectra.

Metabolomics analyses. Partial least squares discriminant analysis (PLS-DA), which recognizes patterns through supervised classification, showed distinctions only between the compounds of the APD 20 group and the others (Fig. 4). PLS-DA explained 61.6% of the data variability and components 1 and 2 explained 55.9 and 5.7%, respectively.

The volcano plot showed some qualitative differences between determined groups, demonstrating the concentration of metabolites according to the fire occurrence. Comparing *No Fire* and *Fire 20* (Fig. 5), we obtain 12 compounds with statistical significance and fold-change of great magnitude. At the same time, the analysis of *Fire 19* and *Fire 20* groups (Fig. 6) showed nine compounds of interest.

The ion intensities of secondary metabolites varied considerably among individuals, irrespective of their group, indicating a high degree of heterogeneity within the samples. However, certain compounds, such as gallocatechin and karwinaphthopyranone derivatives, were significant in individuals from the *Fire 20* group, as demonstrated in both volcano plots (Fig. 5 and Fig. 6). There were no significant interactions observed between the other groups.

Thermogravimetric behavior. The fire groups exhibited similar combustion patterns, with an average temperature of 193.7 °C at which the thermal decomposition of barks began (Fig. 6A). The first curve indicated a loss of about 9.17% of moisture content, followed by a significant loss of biomass, with the second thermogravimetric curve resulting in an initial average loss of 48.08%, and a subsequent reduction of 31.60%. The coefficient of variation for the curves ranged from 0.03 to 0.14%. Similarly, the TGA graph of dry extracts of *R. elaeocarpum* showed two intense peaks of biomass loss in the second curve at average temperatures of 223.42 °C and 468.44 °C, indicating a clear separation (Fig. 6B).

Discussion

Our findings show that *Rhamnidium elaeocarpum* bark contains high levels of total phenolic and tannin in all sampling groups, except APD 20 (as illustrated in Fig. 1). This indicates that the species' overall phenolic metabolism remains unaffected by recent fire events. It is worth noting that the region has experienced low-intensity fire for a considerable time, suggesting that the plant has developed a biochemical tolerance to the physiological stress caused by fire. While da Silva et al. (2021) reported high levels of phenolics in thermotolerant species from Pantanal wetlands, our research demonstrates that this feature is not necessarily directly linked to fire.

In 2020, *R. elaeocarpum* specimens were subjected to the most severe and prolonged fire event in the Brazilian Pantanal during the development of this study (Libonati et al., 2021), resulting in damage to the species. The APD 20 group, in particular, experienced bark decomposition, which may have caused the observed leaching of biomass phenomenon, explaining the significant difference in total phenolic levels compared to the other groups. When leached or released from decomposing plant residues, phenolics can play a significant ecological role through allelopathic action by affecting other organisms' germination, development, and reproduction. Therefore, due to their effects, phenolics are considered one of the most important and common plant allelochemical classes (Li et al. 2010; Ooka and Owens 2018).

The absence of chemical defense variability in plants under stress caused by the environment or other organisms would imply a possible population reduction. Therefore, high chemical diversity allows plants to respond to various environmental conditions, as Santacruz-García et al. (2021) reported in a recent study on six woody species from the Chaco, including a Rhamnaceae family species with wide variations in total phenolic and tannin concentrations. Our results presented a high standard deviation value, suggesting a high intrapopulation variability, which can be attributed to adaptation, according to Valares (2011).

The metabolism analysis showed a higher accumulation of proanthocyanidin oligomers in *R. elaeocarpum*, without significant differences among groups (Fig.4 and Table 1). This result indicates that the biosynthesis of these compounds has not been induced by fire injury for at least six months since the fire event. The biochemical response of the species may be an exaptation by environmental factors other than fire. For instance, adaptations developed against large herbivores are the same to provide fire resistance in woody species (Jaureguiberry and Díaz 2015) and drought conditions (Hodaei et al. 2018). Hence, although the proanthocyanidin oligomers synthesis in *R. elaeocarpum* is not directly related to fire events, it may be related to the defense against herbivores and must have been helpful against flame damage.

Moreover, proanthocyanidins and flavan-3-ol monomers play critical ecological functions in environments with recurrent fires due to their excellent antioxidant capacity, resulting from their numerous hydroxyl groups (Dumanović et al., 2021; Hasanuzzaman et al. 2020; Saini et al. 2018). Thus, we can assume that the prior presence of these compounds acted as a protective barrier against heat transfer during fires (Celzard et al. 2011; Huss et al. 2019; Tributsch and Fiechter 2008).

The volcano graphs (Fig. 6 and 7) indicate that only individuals from the Fire 20 group exhibited noticeable changes in their chemical profile. The results suggest that *R. elaeocarpum* can maintain metabolic balance even under environmental disturbances without significant quantitative changes (Fig. 4). However, the metabolic pathway is altered due to the effects of fire, leading to the accumulation of different compounds. Additionally, the accumulation of compounds increased with age, particularly in individuals with higher DBH (Fig. 1). This is expected given the plant's exposure to multiple fire events and other stresses throughout its life. Notably, galocatechin was the most significant metabolite accumulated due to fire events (Fig. 6 and 7) and age, as shown in Supplementary Material S2. This compound's antioxidant properties protect the plant from post-fire environmental stress and herbivores (Massad et al., 2015). Therefore, it is plausible to hypothesize that this compound helps defend against pathogens and increases sunlight exposure after the fire has passed.

The passage of fire also led to a high incidence of karwinaphthopyranone derivatives, as observed in Figs. 6 and 7. These metabolites have been extensively studied in *Karwinskia*, a genus closely related to *Rhamnidium* (Hauenschild et al., 2016; Richardson et al., 2004). Some hydroxyanthracenones have been shown to exhibit antimicrobial and antifungal activities (Salazar-Aranda et al., 1998; Salazar et al., 2006) and toxic properties (Bermudez et al., 1986; Martínez et al., 1997; Piñeyro-Lopez and Waksman, 2000;

Rojas-Flores et al., 2014). This finding serves as a warning to the Pantanal population who have been using *R. elaeocarpum* barks for food and therapeutic purposes (Bieski et al., 2012; Bortolotto et al., 2018; Pott and Pott, 1994).

Based on the thermogravimetric analysis, *R. elaeocarpum* maintained its stability up to an average temperature of 193.7 °C (Fig. 8A), classifying the species as thermotolerant according to the criteria established by da Silva et al. (2021). This result suggests an excellent resistance to the local fire regime, typically involving low-intensity fires. We believe this adaptation is linked to the high concentrations of proanthocyanidins in the bark. The TGA graph of the dry extract (Fig. 8B) clearly shows that the second curve splits and indicates two significant biomass losses, a characteristic behavior of condensed tannins (Lisperguer et al., 2016). We hypothesize that these losses are mainly due to polymer breakdown and subsequent degradation of proanthocyanidin monomers.

Conclusion

Our findings indicate that fire stress and post-fire conditions act as modulators in the plant community, altering the metabolic balance with a lasting qualitative impact for at least two years. The high concentration of phenolic in the bark of *R. elaeocarpum* contributes to its thermotolerance, protecting against seasonal damage and aiding in resilience in post-fire environments. We also observed a tendency for metabolite accumulation in older individuals, indicating the species' chemical defense against successive environmental stresses. In addition, the metabolomics results highlighted antioxidant compounds such as proanthocyanidins, which protect against damage by reactive oxygen species in metabolic processes. However, the responses observed after different fire histories cannot be attributed solely to fire but also to a combination of drought, high solar incidence, and common pathogenic responses in post-fire environments, describing an adaptive trait known as exaptation. We would also like to caution communities in the Pantanal wetlands who use *R. elaeocarpum* for medicinal purposes about the presence of karwinaphthopyranone derivatives, which are considered toxic. Further qualitative studies on metabolism responses are necessary to understand chemical defense mechanisms.

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Declarations

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Figures

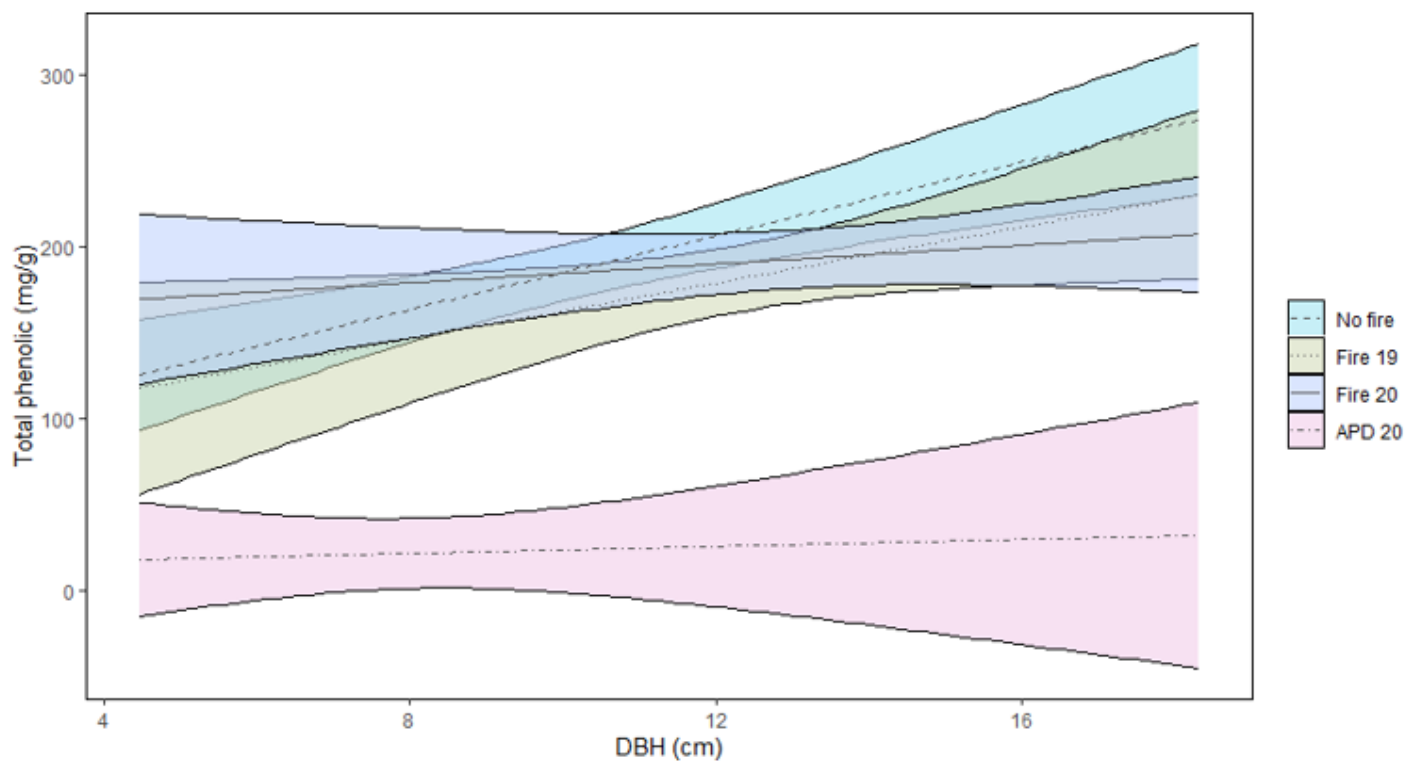


Figure 1

Generalized linear model (GLM) for the concentration of total phenolic in individuals of *Rhamnidium elaeocarpum* in capões of Pantanal wetlands, according to fire history and DBH value ($p = 0.04$; Pseudo $R^2 = 0.85$). The groups are represented by dashed line = No fire; dotted line = Fire 19; continuous line = Fire 20; dotdash line = APD 20. Color areas= confidence interval 95%.

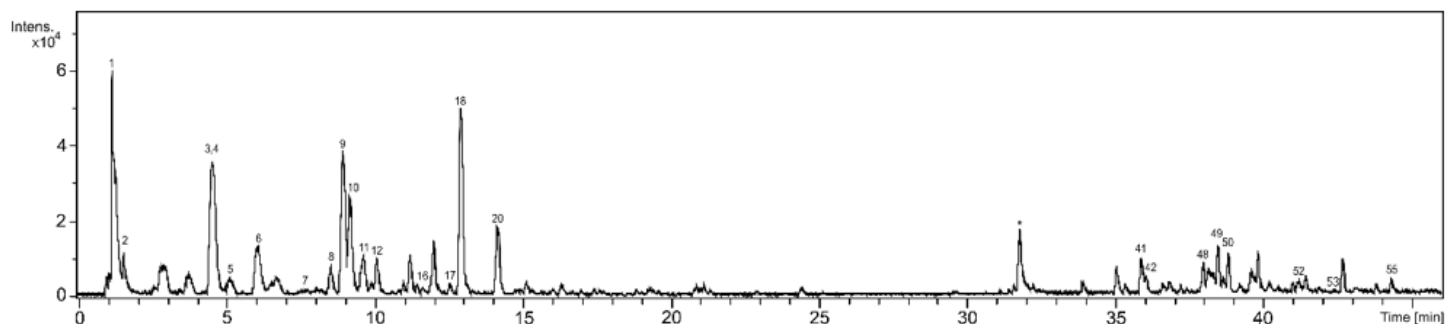


Figure 2

Chromatographic profile of the hydromethanolic extract from *Rhamnidium elaeocarpum* by HPLC-MS/MS obtained by negative ion mode.

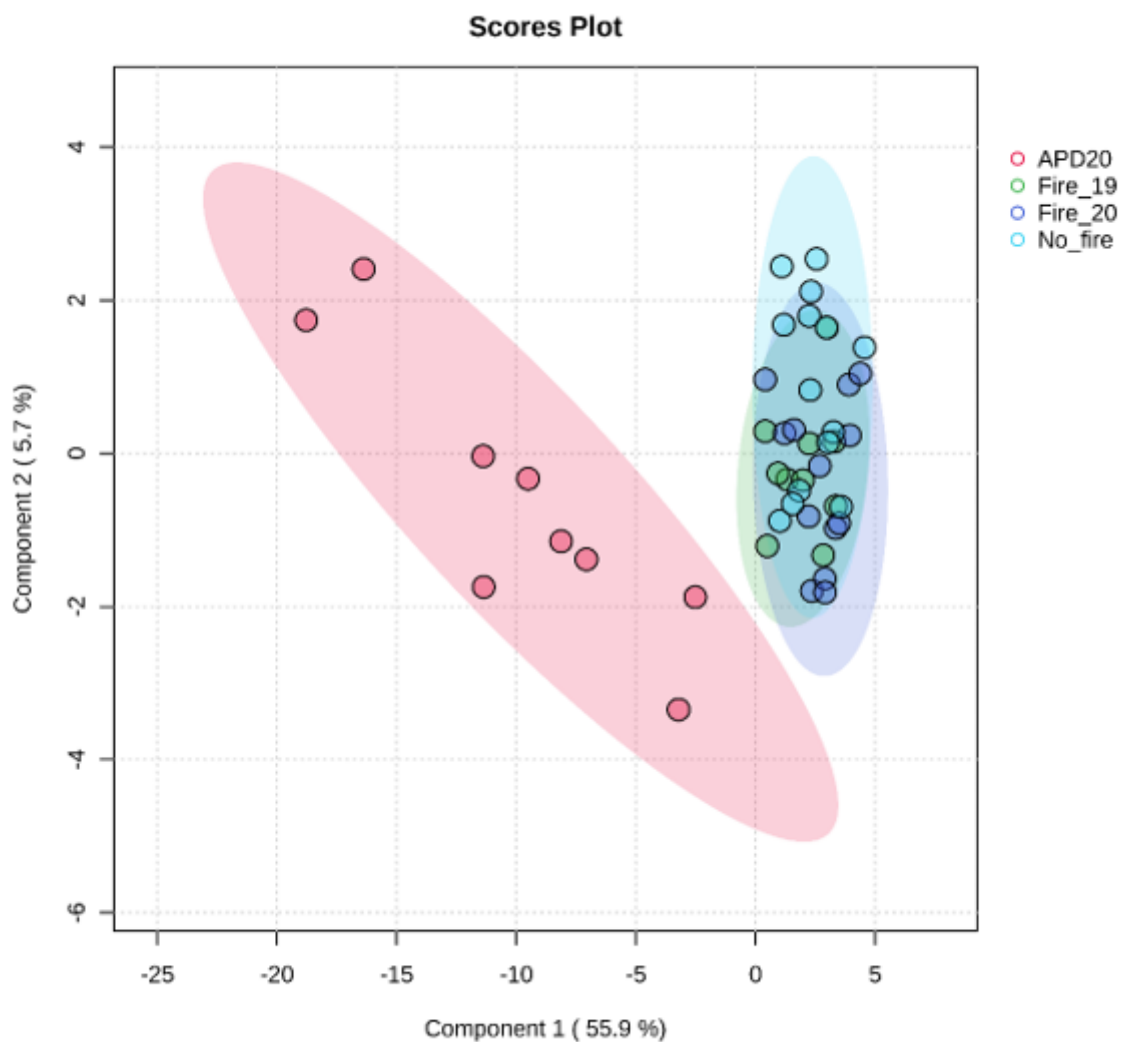


Figure 3

Partial least squares-discriminate analysis (PLS-DA) plot of different metabolites in *Rhamnidium elaeocarpum* groups. PLS-DA score plot for APD 20 (red), Fire 19 (green), Fire 20 (purple) and No fire (blue).

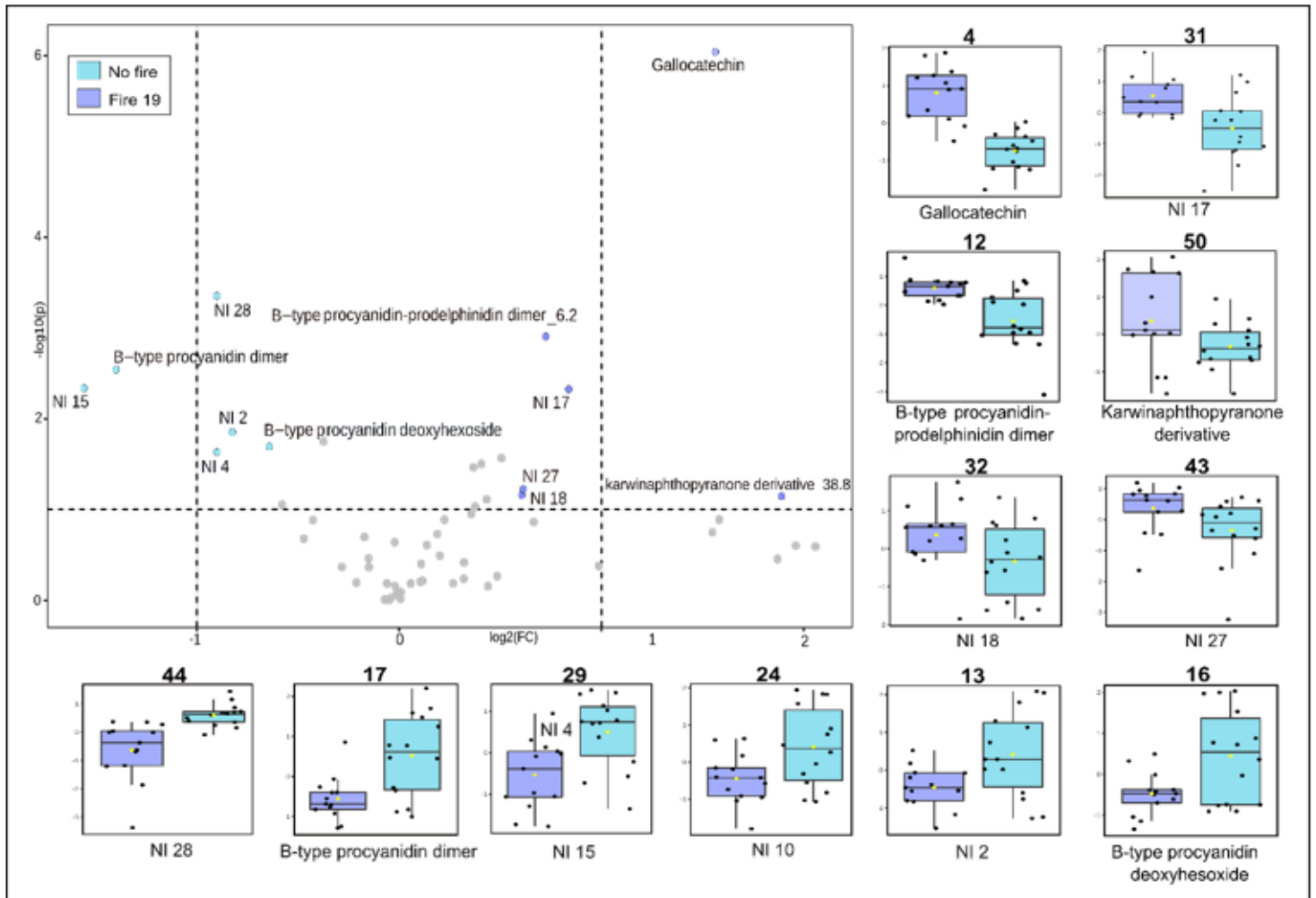


Figure 4

Volcano plot of metabolite concentration changes by fire history, considering the P-value threshold of 0.01 and fold change (FC) threshold of 1.5. Blue/purple circles show metabolites significantly accumulated for No Fire and Fire 20 groups, respectively; symbols in grey show unchanged metabolites. In addition, a box plot with a distribution of the intensities of the ions in *Rhamnidium elaeocarpum*.

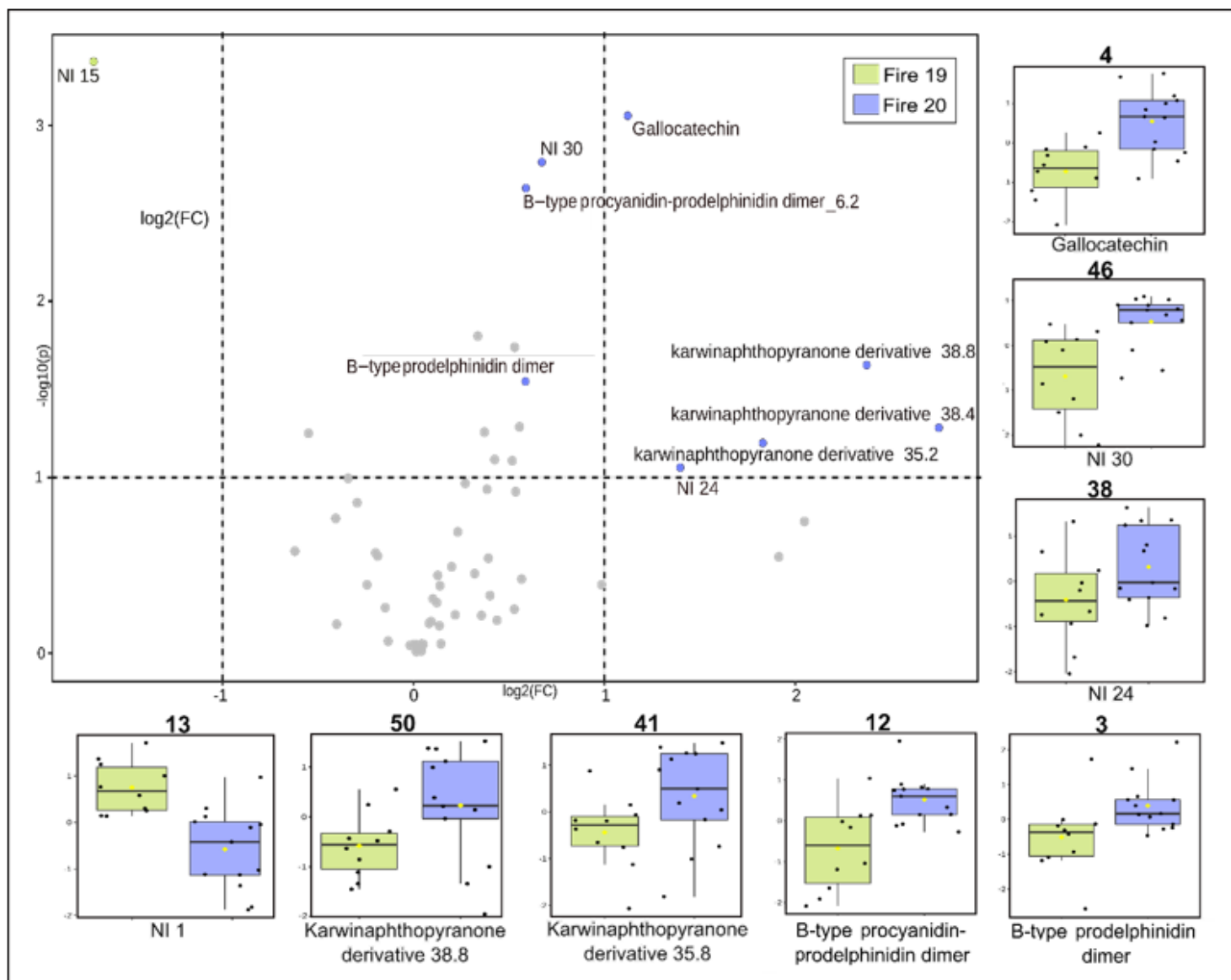


Figure 5

Volcano plot of metabolite concentration changes by fire history, considering P-value threshold of 0.01 and fold change (FC) threshold of 1.5. Green/purple circles show metabolites with significant concentration for Fire 19 and Fire 20 groups, respectively; symbols in grey show unchanged metabolites. In addition, box plot with an average distribution of the intensities of the ions in *Rhamnidium elaeocarpum*.

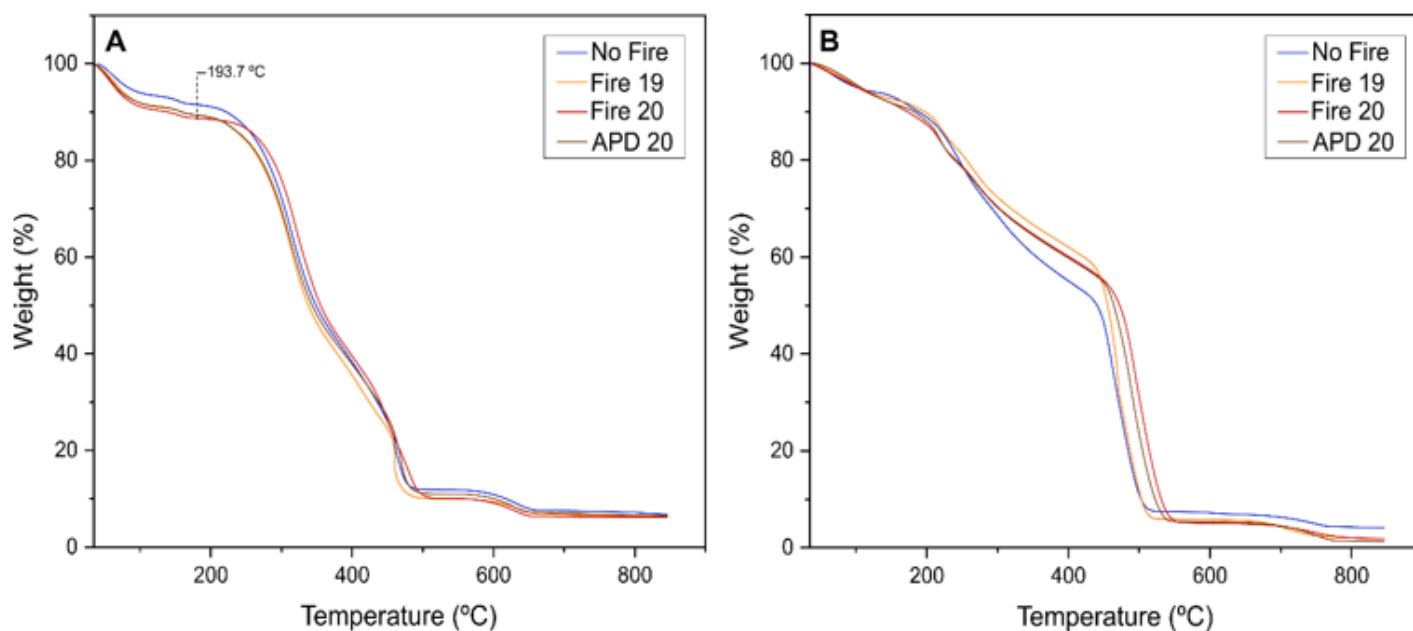


Figure 6

Thermogravimetric analysis (TGA) of plots of *Rhamnidium elaeocarpum* from capões from Pantanal wetlands. A. Thermogravimetric curves of bark powder. B. Thermogravimetric curves of the dry extract. The groups are represented by blue (No fire), orange (Fire 19), red (Fire 20) and brown (APD 20).

Supplementary Files

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