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# Bioactive compounds from *Vellozia pyrantha* A.A.Conc: A metabolomics and multivariate statistical analysis approach

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ABSTRACT

The chemical composition of *V. pyrantha* resin (VpR) and fractions (VpFr1–7 and VpWS) were assessed by LC-MS and NMR. Twenty-eight metabolites were identified, including 16 diterpenoids, seven nor-diterpenoids, one fatty acid, one bis-diterpenoid, one steroid, one flavonoid, and one triterpenoid. The pharmacological potential of VpR, VpFr1–7, and isolated compounds was assessed by determining their antioxidant, antimicrobial, and cytotoxic activities. VpFr4 ($IC_{50}$ = 205.48 ± 3.37 μg.mL$^{-1}$) had the highest antioxidant activity, whereas VpFr6 ($IC_{50}$ = 842.79 ± 10.23 μg.mL$^{-1}$) had the lowest. The resin was only active against *Staphylococcus aureus* (MIC 62.5 μg.mL$^{-1}$) and *Salmonella choleraesius* (MIC and MFC 500 μg.mL$^{-1}$), but fractions were enriched with antibacterial compounds. *V. pyrantha* resin and fractions showed great cytotoxic activity against HCT116 ($IC_{50}$ = 20.08 μg.mL$^{-1}$), HepG2 ($IC_{50}$ = 20.50 μg.mL$^{-1}$), and B16-F10 (12.17 μg.mL$^{-1}$) cell lines. Multivariate statistical analysis was used as a powerful tool to pinpoint possible metabolites responsible for the observed activities.

## 1. Introduction

The Velloziaceae family comprises 250 monocotyledonous angiosperms distributed amongst seven genera: *Acanthochlamys*, *Barbacenia*, *Barbaceniopsis*, *Nanuza*, *Talbotia*, *Vellozia*, and *Xerophyta*. Species of Velloziaceae frequently occur in tropical regions of the globe, including Brazil, several countries in Africa, the Arabian Peninsula, and China. In Brazil, the genus *Vellozia* is concentrated in the rupestrian fields of Bahia and Minas Gerais, Brazil [1]. Several species of *Vellozia* have been described in these regions in the last few years, including species from Minas Gerais, such as *V. dracaenoides* [2], *V. fontellana* [3], *V. albohexandra* [4], *V. mellosilvae* [4], and species from Bahia, such as *V. inselbergae* [5] and *V. pyrantha* [6,7].

The chemical composition of *Vellozia* species has been widely studied, and flavonoids [8–16] and diterpenes [17–26] are the major compounds present. However, they are rarely described simultaneously in the same species. A few examples include the isolation of the flavonoids quercetin 3-*O*-methyl ether and 6-*C*-methylquercetin 3-*O*-methyl ether from the aerial parts from *V. dasypus* [8], the isolation of the eight flavonoids from the leaves of *V. epidendroides* and *V. lilacina* [15], the isolation of four diterpenoids from the roots of *V. gigantea* [18], and the isolation of five cleistanthane diterpenoids from *V. pyrantha* resin [17].

*Vellozia pyrantha* A.A.Conc. is endemic to the campo rupestre regions of Brazil and exhibits an exciting feature to flower and produce adventitious shoots in response to fire [6,27]. This species was first described by Conceição [6], which showed that this species was mistakenly identified as *V. sincorana* L.B.Sm. & Ayensu. This is the first large-scale metabolite profiling study of any *Vellozia* species. Thus, we aimed to assess the chemical composition of *V. pyrantha* resin and fractions by liquid chromatography–mass spectrometry (LC-MS) and to assess the

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antioxidant, antimicrobial, and cytotoxic activity of *V. pyrantha* resin crude extract (VpR), fractions (VpFr1-VpFr7), and isolated compounds. Multivariate statistical analysis was used as a powerful tool to pinpoint possible candidate metabolites for the observed activities and it included partial least squares-discriminant analysis (PLS-DA), variable importance in projection (VIP), hierarchical cluster and corelation analyses.

## 2. Results and discussion

### 2.1. Chemical characterization of Vellozia pyrantha resin (VpR), fractions, and isolated compounds

*Vellozia pyrantha* is endemic to the State of Bahia, northeastern Brazil, where local inhabitants have used it to ignite fires in rudimentary stoves and lamps [6]. However, this species was very recently characterized taxonomically [7]. Over the years, this species was mistaken for *V. sincorana* because of their morphological similarities [7]. Nevertheless, the chemical composition of *V. pyrantha* has yet to be investigated in depth. Thus, we conducted a study to investigate the metabolite profile of *V. pyrantha*, aiming at discovering potential bioactive compounds produced in the resin. *V. pyrantha* resin crude extract (VpR) was fractionated in a chromatographic column using hexane, acetate, and methanol in increasing polarities as eluents to produce seven fractions (VpFr1-VpFr7). Curiously, a white solid (VpWS) precipitated at the tip of the chromatographic column during the beginning of the fractionation and it was collected separately for further analysis. The fractionation aimed to produce enriched fractions with compounds of different natures (polarities), thus allowing us later on to correlate the chemical composition of the resin and its fractions with their antioxidant, antimicrobial, and cytotoxic activities.

The initial chemical characterization of the resin was performed by nuclear magnetic resonance (NMR) (Fig. 1a). The $^{1}$H NMR spectrum of VpR showed an abundance of peaks in three major regions: 7.65–6.60 ppm, 2.34–2.15 ppm, and 1.35–0.95 ppm. The first region (7.65–6.60 ppm) is characteristic of hydrogen atoms directly attached to carbons with $sp^2$ hybridization, such as in aromatic rings and double bonds. Similarly, the second region (2.34–2.15 ppm) is characteristic of hydrogens belonging to methyl groups directly attached to carbons with $sp^2$ hybridization, whereas the third region (1.35–0.95 ppm) is usually attributed to hydrogens belonging to methyl groups attached to an aliphatic carbon chain. Based on the chemistry of the *Vellozia* genus [17,18,20], these results suggest that the resin is composed of diterpenoids, mainly with the cleistanthane core skeleton. The presence of flavonoids was ruled out, at least as major componets, since no peaks between 14 and 12 ppm were observed. Peaks in this regions are usually attributed to the hydroxyl groups in the A-ring of flavonoids that form an intramolecular hydrogen bond with the carbonyl group at C-4 [28].

The $^{1}$H NMR spectrum of VpWS showed a pair of doublets at 7.06 and 6.97 ppm (1H, 8.1 Hz) attributed to H-11 and H-12 (Supplementary Tables 1, Fig. 1b and Fig. 2). The $^{1}$H NMR spectrum suggested the presence of five methyl groups: 2.28 ppm (s, 3H, H-17), 1.11 ppm (t, 7.6 Hz, 3H, H-16), 1.20 ppm (s, 3H, H-20), 0.95 ppm (s, 3H, H-18), and 0.93 ppm (s, 3H, H-19) (Supplementary Tables 1 and Fig. 1b). The $^{13}$C NMR spectrum of VpWS showed six peaks between 150 and 120 ppm, confirming the presence of an aromatic ring in the compound (Supplementary Tables 1 and Fig. 1c). Additionally, fourteen peaks were observed at the aliphatic region of the spectrum. After comparision of the NMR data of VpWS with the literature, this compound was identified as the diterpenoid cleistanthan-8,11,13-triene (**28**) [17].

The $^{1}$H NMR spectrum of VpWS also showed a set of minor peaks suggesting the presence of a second diterpenoid. First, a pair of doublets was observed at 7.00 and 6.95 ppm (1H, 8.1 Hz), which were attributed to H-11 and H-12. Then, further inspection of the minor peaks indicated the presence of an additional set of methyl groups: 2.29 ppm (s, 3H, H-17), 1.13 ppm (t, 7.6 Hz, 3H, H-16), 1.05 ppm (s, 3H, H-20), 1.05 ppm (s, 3H, H-18), and 0.97 ppm (s, 3H, H-19) (Supplementary Tables 2 and Fig. 1b). The main difference between the major and minor components was the presence of double dublets at 6.81 and 6.05 ppm (dd, 3.0 e 10 Hz, 1H), which were attributed to H-7 and H-6, respectively (Supplementary Tables 2 and Fig. 1c). The $^{13}$C NMR spectrum also showed a set of minor peaks between 150 and 120 ppm, suggesting a second cleistanthane-like diterpenoid in the sample (Supplementary Tables 2 and Fig. 1c). After comparision of the NMR data of VpWS with the literature, the minor component of the mixture was identified as the diterpenoid cleistanthan-6,8,11,13-tetraene (**25**) [4]. Later on, this compound was obtained pure (Supplementary Figs. 2–19). VpWS is composed mainly of cleistanthan-8,11,13-triene (**28**, 90%) with a minor presence of cleistanthan-6,8,11,13-tetraene (**25**, 10%) (Supplementary Figs. 2–19).

A third cleistanthane diterpenoid was isolated, and its $^{1}$H NMR spectrum showed a pair of doublets at 7.25 and 7.11 (1H, 8.1 Hz) attributed to H-11 and H-12 (Supplementary Table 3). The $^{1}$H NMR spectrum suggested the presence of five methyl groups: 2.33 ppm (s, 3H, H-17), 1.22 ppm (t, 7.4 Hz, 3H, H-16), 1.05 ppm (s, 3H, H-20), 1.01 ppm (s, 3H, H-18), and 0.91 ppm (s, 3H, H-19) (Supplementary Table 3). The $^{13}$C NMR spectrum showed six peaks between 150 and 120 ppm, confirming the presence of an aromatic ring in the compound (Supplementary Table 3). Additionally, fourteen peaks were observed at the aliphatic region of the spectrum. After comparision of the NMR data with the literature, this compound was identified as cleistanthan-8,11,13-trien-7-one (**2**) (Supplementary Figs. 2–19) [17].

This study provides the first investigation of the chemical composition of a *Vellozia* species by a metabolomics approach. For that, high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) was applied. Twenty-eight metabolites were identified, including 16 diterpenoids, seven norditerpenoids, one fatty acid, one bis-diterpenoid, one steroid, one flavonoid, and one triterpenoid (Supplementary Table 4). The diterpenoids cleistanthan-8,11,13-trien-7-one (**2**), cleistanthan-6,8,11,13-tetraene (**25**), and cleistanthan-8,11,13-triene (**28**) are amongst the diterpenoids identified in VpR and its fractions (VpWS, VpFr1-VpFr7), highlighting the powerfulness of the metabolomics approach to identify the compounds present in the samples.

Initially, one-way ANOVA and the post-hoc Fisher's LSD (Least Significant Difference) test were applied to the identified metabolites to compare the chemical profile of VpR and its fractions (VpFr1-VpFr7). All metabolites showed statistically significant differences amongst the samples, except for 7-oxo-cleistanthan-8,11,13-trien-19,20β-olide (**16**). Then, partial least squares discriminant analysis (PLS-DA) was applied to assess further differences in the chemical profile of VpR and its fractions (VpFr1-VpFr7). The goodness of fit ($R2 = 0.9304$) and the predictability of the model ($Q2 = 0.9028$) values suggest that this is a PLS-DA model with strong predictive power (Supplementary Fig. 1). The Kaiser criterion, also known as the eigenvalue-one criterion, is usually used to ensure that a given component can be used to explain the distribution of the samples in the PLS-DA plot. Both principal components of the PLS-DA analysis have met the Kaiser criterion. Principal component 1 (PC1) explained 49.8% of the total variation (eigenvalue of 4.98), whereas principal component 2 (PC2) explained 24.9% (eigenvalue of 2.49) (Fig. 3a).

There is a clear separation between VpR and its fractions (VpFr1-VpFr7). It is possible to identify three main clusters in the PLS-DA plot: the first encompassing VpR and fractions VpFr1 to VpFr3, the second encompassing VpFr4 to VpFr6, and the third encompassing only VpFr7. Thus, using the concept that “proximity means similarity”, it is clear that fractions VpFr1 to VpFr3 have a similar chemical composition amongst them and that the chemical composition of fractions VpFr4 to VpFr6 differs from the chemical composition of fractions VpFr1 to VpFr3. It is also clear that VpFr7 has the most distinct chemical composition amongst VpR fractions (Fig. 3a). The results provide evidence that VpR fractions have differences in their chemical composition resulting from the fractionation of VpR.

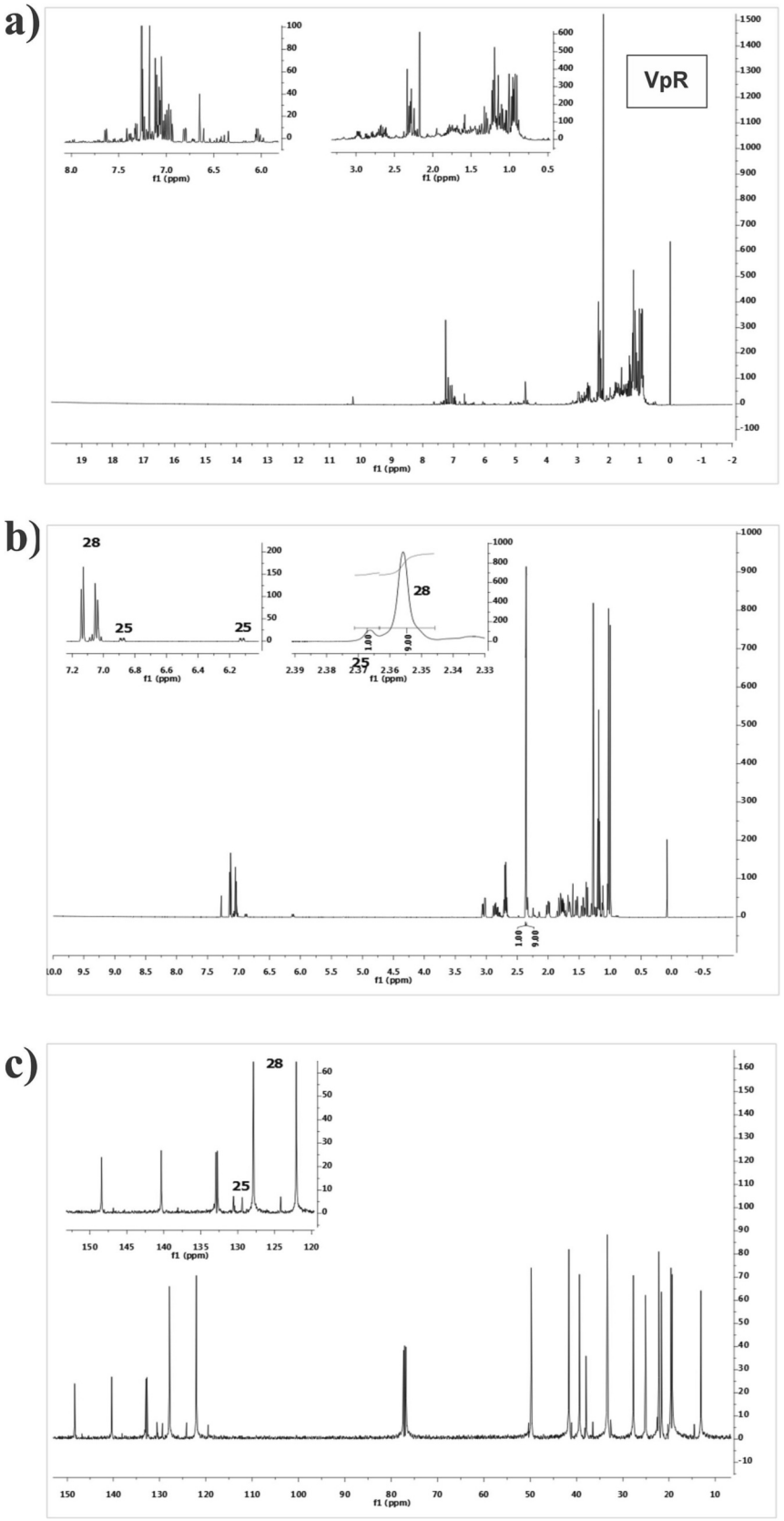


**Fig. 1.** a) $^{1}$H NMR spectrum of *Vellozia pyrantha* resin (VpR), b) $^{1}$H NMR and c) $^{13}$C NMR spectra of the mixture of cleistanthan-6,8,11,13-tetraene (**25**) and cleistanthan-8,11,13-triene (**28**). (500 MHz for $^{1}$H and 125 MHz for $^{13}$C, in $CDCl_3$).

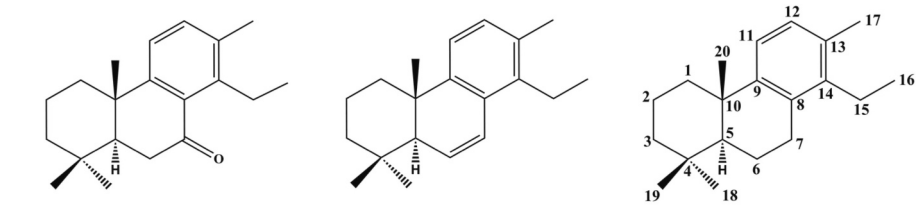


**Fig. 2.** Chemical structures of the cleistanthane diterpenoids isolated from *Vellozia pyrantha* resin (VpR).

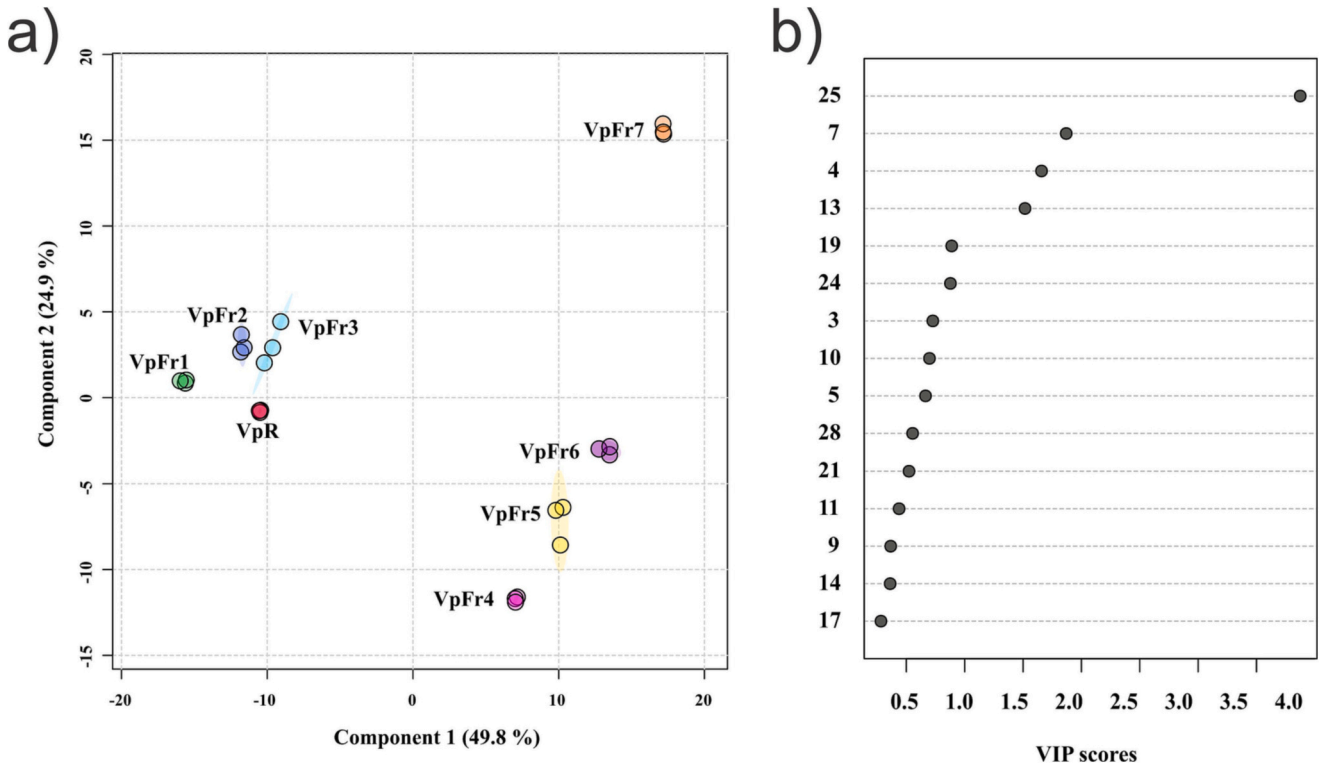


**Fig. 3.** a) Partial least squares-discriminant analysis (PLS-DA) and b) Variable Importance in Projection (VIP) scores based on the compounds identified in *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7). 8,14-dihydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**3**), isopimara-8 (9),15-dien-1,3,7,11-tetraone (**4**) cleistanthan-5,8,11,13-tetraen-7-one (**5**), 8,14$\beta$-dihydroxy-7-oxo-isopimar-15-en-18-oic acid (**7**), cleistanthan-8,11,13-trien-17,19-dioic acid (**9**), 15,16-epoxy-11$\beta$-hydroxy-isopimar-8(9)-en-7-one (**10**), bismagdalenic acid (**11**), (20*R*)-8,20-epoxy-15,16-bis-nor-isopimarane-13,20-diol (**13**), cleistanthan-1,8,11,13-tetraen-3,7-dione (**14**), 12$\beta$-hydroxy-isopimara-8 (9),15-dien-7,11-dione (**17**), isopimar-15-en-7,8,18-triol (**19**), oleic acid (**21**), 7-oxo-cleistanthan-8,11,13-trien-20-al and cleistanthan-8,11,13-trien-3,7-dione (**24**), cleistanthan-6,8,11,13-tetraene (**25**), cleistanthan-8,11,13-triene (**28**).

Variable Importance in Projection (VIP) scores was used to identify major players in the separation observed in the PLS-DA plot. It seems that four metabolites played significant roles in the separation of the groups in the PLS-DA plot: cleistanthan-6,8,11,13-tetraene (**25**), 8,14$\beta$-dihydroxy-7-oxo-isopimar-15-en-18-oic acid (**7**), isopimara-8 (9),15-dien-1,3,7,11-tetraone (**4**), and (20*R*)-8,20-epoxy-15,16-bis-nor-isopimarane-13,20-diol (**13**).

The hierarchical cluster analysis provides a clear visual depiction of the concentration of each metabolite across the VpR and its fractions (Fig. 4). Dark red means high concentration in a given sample, whereas dark blue means lower. It is clear, for example, that VpFr1 has five major components 7-oxo-cleistanthan-8,11,13-trien-19,20$\beta$-olide (**16**), oleic acid (**21**), 7-oxo-cleistanthan-8,11,13-trien-20-al (**24**), cleistanthan-6,8,11,13-tetraene (**25**), and cleistanthan-8,11,13-triene (**28**), which includes four cleistanthane diterpenoids (Fig. 4). VpFr4 has three major components 15,16-epoxy-11$\beta$-hydroxy-isopimar-8(9)-en-7-one (**10**), cleistanthan-1,8,11,13-tetraen-3,7-dione (**14**), and 7$\beta$,8$\beta$,13$\alpha$-trihydroxy-15,16-bis-nor-isopimar-18-oic acid (**18**), which includes two isopimare and one cleistanthane diterpenoid (Fig. 4). Finally, VpFr7 has four major components 8,14-dihydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**3**), isopimara-8 (9),15-dien-1,3,7,11-tetraone (**4**), 8,14$\beta$-dihydroxy-7-oxo-isopimar-15-en-18-oic acid (**7**), and 2-oxo-13,14,15,16-tetranor-clerod-3-en-12-oic acid (**22**), which includes three isopimare and one clerodene diterpenoids (Fig. 4).

Amongst the compounds with the highest VIP scores, cleistanthan-6,8,11,13-tetraene (**25**) showed the highest concentration in fractions VpFr1 to VpFr3 and VpR, 8,14$\beta$-dihydroxy-7-oxo-isopimar-15-en-18-oic acid (**7**) and isopimara-8 (9),15-dien-1,3,7,11-tetraone (**4**) showed the highest concentration in fraction VpFr7, whereas (20*R*)-8,20-epoxy-15,16-bis-nor-isopimarane-13,20-diol (**13**) showed the highest concentration in fraction VpFr6. Amongst the isolated compounds, the highest concentration of cleistanthan-8,11,13-trien-7-one (**2**) is observed in VpFr2, cleistanthan-6,8,11,13-tetraene (**25**) across VpFr1 to VpFr3, and cleistanthan-8,11,13-triene (**28**) in VpFr1, which highlight the robustness of the metabolomics approach, since these compounds were isolated from the first fractions of the fractionation column.

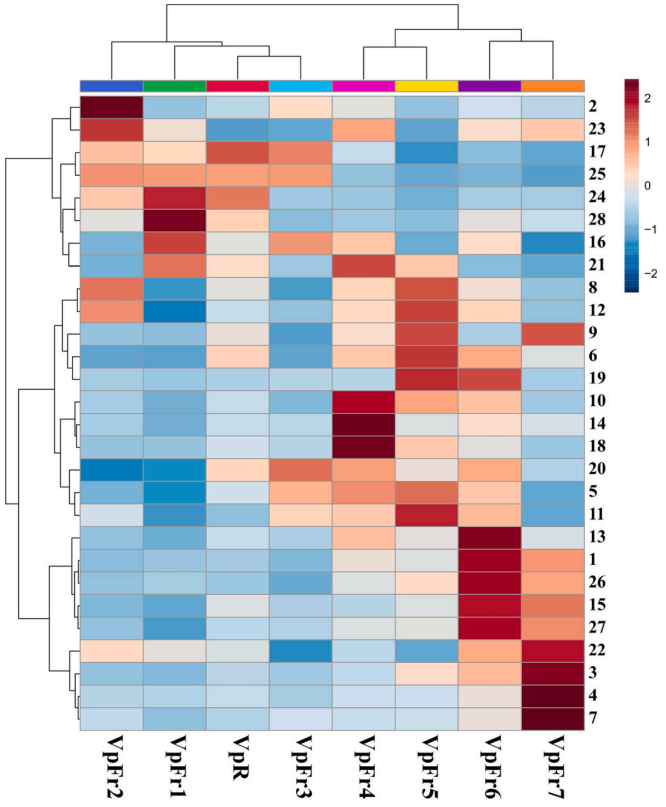


**Fig. 4.** Hierarchical cluster analysis based on the compounds identified in *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7).

### *2.2. Antioxidant, antimicrobial, and cytotoxic activities of Vellozia pyrantha resin (VpR), fractions, and isolated compounds*

Reactive oxygen species (ROS) are a set of oxygen-containing chemical species that readily react with (macro)molecules within a cell, mainly proteins, RNA, and DNA, causing severe oxidative damage to the cell that may lead to its death [29]. Each organism has its endogenous antioxidant mechanism to prevent an imbalance in the ROS levels that might lead to oxidative stress, which might be enzymatic or non-enzymatic [30,31]. Nevertheless, when the biological system's antioxidant mechanisms fail, the damage caused by ROS may cause a series of chronic diseases, such as diabetes, neurodegenerative and cardiovascular diseases, and cancer [32]. Thus, identifying new sources of antioxidant compounds, especially from natural sources such as plants, is imperative.

The antioxidant activity ($IC_{50}$) of *V. pyrantha* samples ranged from 205.48 ± 3.37 μg.mL$^{-1}$ for VpFr4 to 842.79 ± 10.23 μg.mL$^{-1}$ for VpFr6. Thus, VpFr4 had the highest antioxidant activity amongst VpR fractions, whereas VpFr6 had the lowest. VpFr2 and VpFr7 showed $IC_{50}$ values comparable to VpR, whereas the remaining fractions were less active than the VpR (Table 1). VpWS did not show antioxidant activity ($IC_{50}$ > 1000 μg.mL$^{-1}$).

The diterpenoids cleistanthan-1,8,11,13-tetraen-3,7-dione (**14**, 0.69) and 7*β*,8*β*,13*α*-trihydroxy-15,16-bis-nor-isopimar-18-oic acid (**18**, 0.59) showed a positive correlation with the antioxidant activity (Fig. 5a) and might be considered as possible candidates to explain the antioxidant activity of the *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7). Interestingly, they are the major component of VpFr4 which had the highest antioxidant activity amongst VpR fractions. Oleic acid (**21**, 0.49), 8-*C*-methylvelloquercetin-3,5,3′-trimethyl ether (**1**, 0.46), 15,16-epoxy-11*β*-hydroxy-isopimar-8(9)-en-7-one (**10**, 0.41), and 20-hydroxylupan-3-one (**26**, 0.40) might also be significant contributors to the antioxidant activity of the extracts, since they showed moderate correlation with the antioxidant activity (Fig. 5a).

**Table 1**
The antioxidant activity of *V. pyrantha* resin crude extract (VpR) and its fractions (VpWS and VpFr1-VpFr7).

| Samples | Atividade antioxidante ($IC_{50}$, μg mL$^{-1}$) |
|---|---|
| **VpR** | 537.06 ± 8.97 |
| **VpWS** | > 1000 |
| **VpFr1** | 663.20 ± 19.04 |
| **VpFr2** | 518.31 ± 52.68 |
| **VpFr3** | 617.96 ± 3.57 |
| **VpFr4** | 205.48 ± 3.37 |
| **VpFr5** | 743.08 ± 21.09 |
| **VpFr6** | 842.79 ± 10.23 |
| **VpFr7** | 532.89 ± 13.01 |
| **Gallic acid** | 1.98 ± 0.02 |

Few studies are available in the literature in which the antioxidant activity of crude extracts, fractions, or isolated compounds obtained from *Vellozia* species was assessed. In the first study, the antioxidant activity of hydroalcoholic (ethanol:water, 70:30 *v*/v) extracts from leaves and stems of *V. squamata* and the nanoemulsion prepared with them is assessed, and although the authors did not present $IC_{50}$ values, they have claimed that there is a concentration-dependent response to the extracts to the antioxidant activity [34]. In the second study, the antioxidant activity of *V. kolbekii* sheaths and leaf extracts and two isolated flavonoids was assessed. The antioxidant activity ($IC_{50}$) of *V. kolbekii* samples ranged from 35.6 ± 1.2 μg.mL$^{-1}$ for the ethyl acetate extract of the sheaths to 1727 ± 44.7 μg.mL$^{-1}$ for the hexane extract of the leaves. The two isolated flavonoids showed strong antioxidant activity ($IC_{50}$) of 8.4 ± 0.5 and 8.5 ± 0.2 μg.mL$^{-1}$, which is somehow comparable with the positive control used in the study (quercetin, 5.0 ± 0.3 μg.mL$^{-1}$) [12]. Most likely, the antioxidant activity of the different extracts obtained from *V. kolbekii* is attributed to polyphenolic compounds, such as flavonoids, rather than diterpenoids. Diterpenoids, likely flavonoids, might also antioxidant activity. For example, seven diterpenoids were isolated from *Clerodendrum bracteatum* and their antioxidant activity tested in a DPPH-based system. All diterpenoids showed antioxidant activity with $IC_{50}$ varying from 23.23 ± 2.10 to 125.65 ± 6.65 [35]. The antioxidant activity of these compounds might be explained due to the presence of conjugated π-electron system that stabilizes radicals, inactivating ROS [36]. Nevertheless, it seems that our study represents the first time that the antioxidant activity of the resin obtained from any *Vellozia* species is assessed, suggesting that the resin might be a potential source of antioxidant-like compounds.

In 1928, Professor Alexander Fleming discovered the first-ever antibiotic, penicillin, from a rare *Penicillium notatum*. In the subsequent decades, several other antibiotics-like molecules were discovered, but in recent years, the speed of discovery of new and effective antibiotics has faced a worrisome decline. In parallel, the indiscriminate use of antibiotics and the resulting emergence and persistence of antimicrobial resistance has become a general public health concern, especially with the appearance of super-resistant bacteria strains [37]. Plants are well-known sources of such antimicrobial compounds and the demand for plant-based medicines that can be used in parallel with the current antibiotics (synergistically or alternatively) has increased. Thus it is pivotal to identify new sources of antimicrobial compounds from natural sources, such as plants.

*V. pyrantha* resin crude extract (VpR) and its fractions (VpWS, VpFr1-VpFr7) were tested against several bacteria (Gram-positive and Gram-negative) and non-filamentous fungi (Table 2). VpR was only active against *Staphylococcus aureus* (MIC 62.5 μg.mL$^{-1}$) and *Salmonella choleraesius* (MIC and MFC 500 μg.mL$^{-1}$). However, fractionation of VpR seems to have produced factions enriched with antibacterial compounds (Table 2). This might have happened due to the enhancement of the metabolites' synergic effect or simply by removing non-active compounds and enriching the active compounds within each fraction.

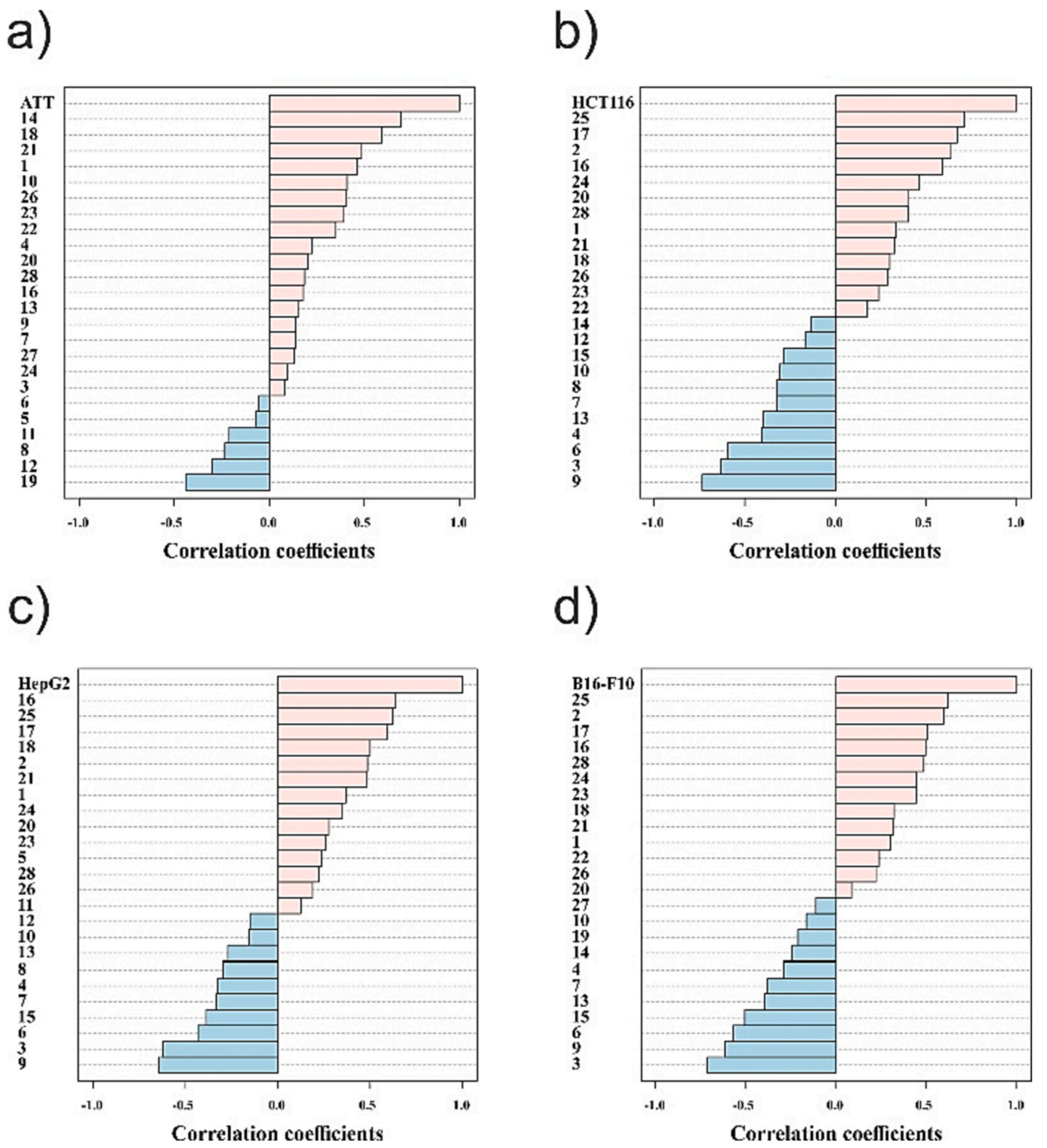


**Fig. 5.** Top 25 metabolites identified in *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7) which correlated with the (a) antioxidant activity and cytotoxic activities against (b) HCT116, (c) HepG2, and (d) B16-F10. Blue bars depict negative correlations, whereas pink bars depict positive correlations. A threshold of 0.5 was set to consider the correlation as strong [33]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nevertheless, this approach has the advantage of increasing the possibility of discovering potential antibacterial compounds present at VpR and its fractions. For example, fractions VpFr6 and VpFr7 showed greater antibacterial activity against *Staphylococcus aureus* than VpR, with MIC of 3.12 and 12.5 $\mu g.mL^{-1}$, respectively. It is essential to highlight that VpFr6 antibacterial activity against *S. aureus* is comparable to the positive control chloramphenicol. Fractions VpFr6 and VpFr7 were selectively active against *S. aureus, Bacillus cereus, and Salmonella choleraesius* (Table 2).

The major compounds in VpFr6 are 8-*C*-methylvelloquercetin-3,5,3′-trimethyl ether (**1**), (20*R*)-8,20-epoxy-15,16-bis-nor-isopimarane-13,20-diol (**13**), 5,10-epoxy-7,11-dihydroxy-rosane-7,15-dien-6-one

**Table 2**
Antimicrobial activity of *V. pyrantha* resin crude extract (VpR) and its fractions (VpWS and VpFr1-VpFr7).

| Samples | *Staphylococcus aureus* MIC (MBC) | *Staphylococcus epidermidis* MIC (MBC) | *Bacillus subtilis* MIC (MBC) | *Bacillus cereus* MIC (MBC) | *Escherichia coli* MIC (MBC) | *Salmonella choleraesius* MIC (MBC) |
|---|---|---|---|---|---|---|
| **VpR** | 62.5 (–) | – | – | – | – | 500 (500) |
| **VpWS** | – | – | 500 (–) | – | – | 100 (–) |
| **VpFr1** | – | 100 (–) | 100 (–) | – | – | 50 (100) |
| **VpFr2** | – | – | 100 (100) | – | – | 50 (50) |
| **VpFr3** | – | 25 (–) | 250 (–) | – | – | 100 (–) |
| **VpFr4** | – | 25 (–) | 500 (–) | – | – | 100 (–) |
| **VpFr5** | – | 25 (–) | 500 (–) | – | 100 (–) | 100 (–) |
| **VpFr6** | 3.12 (6.25) | – | – | 250 (–) | – | 100 (100) |
| **VpFr7** | 12.5 (25) | – | – | 500 (–) | – | 50 (100) |
| **Chloramphenicol** | 3.12 (–) | 3.12 (–) | 1.52 (1.56) | 3.12 (6.25) | – | – |
| **Benzylpenicillin** | 0.04 (–) | 2.50 (–) | 0.08 (0.08) | – | – | – |
| **Garamycin** | – | – | – | – | 0.62 (–) | 0.04 (0.08) |

Results are presented as MIC (MBC). MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration. MIC and MBC values are expressed in μg.mL$^{-1}$.

(**15**), 20-hydroxylupan-3-one (**26**), and stigmasterol (**27**), whereas the major compounds in VpFr7 are 8,14-dihydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**3**), isopimara-8 (9),15-dien-1,3,7,11-tetraone (**4**), 8,14*β*-dihydroxy-7-oxo-isopimar-15-en-18-oic acid (**7**), and 2-oxo-13,14,15,16-tetranor-clerod-3-en-12-oic acid (**22**) (Fig. 4). These compounds might act synergically to explain the antibacterial activity of VpFr6 and VpFr7.

Fractions VpFr1, VpFr3, VpFr4, and VpFr5 were highly active against *S. epidermidis*, with MIC varying from 25 to 100 μg.mL$^{-1}$, whereas all the other fractions, except VpFr6 and VpFr7, were active against *B. subtilis*, with MIC varying from 100 to 500 μg.mL$^{-1}$ (Table 2). These fractions have very distinct metabolite profiles, which may indicate that for these bacteria several metabolites are acting synergically.

The antibacterial activity of VpR fractions against *Bacillus cereus* and *Escherichia coli* showed a high degree of selectivity. It is essential to highlight that VpR did not show any activity for both bacteria, but VpFr5 was active against *E. coli*, with MIC of 100 μg.mL$^{-1}$, whereas VpFr6 and VpFr7 were active against *B. cereus*, with MIC of 250 and 500 μg.mL$^{-1}$, respectively (Table 2). The major compounds in VpFr5 are cleistanthan-5,8,11,13-tetraen-7-one (**5**), cleistanthan-8,11,13-trien-17-oic acid (**6**), 8-hydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**8**), cleistanthan-8,11,13-trien-17,19-dioic acid (**9**), bismagdalenic acid (**11**), 7-oxo-cleistanthan-8,11,13-trien-17-oic acid (**12**), and 7-oxo-cleistanthan-8,11,13-trien-19,20*β*-olide (**16**), which most likely are responsible for the activity of VpFr5 against *E. coli* (Fig. 4). To some extent, there is some similarities in the chemical composition of VpFr6 and VpFr7 that might explain their activity against *B. cereus* (Fig. 4). High levels of 8-*C*-methylvelloquercetin-3,5,3′-trimethyl ether (**1**), 8,14-dihydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**3**), 5,10-epoxy-7,11-dihydroxy-rosane-7,15-dien-6-one (**15**), 2-oxo-13,14,15,16-tetranor-clerod-3-en-12-oic acid (**22**), 20-hydroxylupan-3-one (**26**), and stigmasterol (**27**) were observed in of VpFr6 and VpFr7 (Fig. 4). All fractions (VpWS, VpFr1-VpFr7) showed at least a 5-fold increase in the antibacterial activity against *Salmonella choleraesius* compared to VpR, with MIC and MBC varying from 50 to 100 μg.mL$^{-1}$ (Table 2). VpR and none of its fractions were active against *Pseudomonas aeruginosa* (CCT 0090), *Candida albicans* (ATCC 18804), and *C. glabrata* (CCT 0728).

Several studies have reported the antibacterial activity of extracts obtained from *Vellozia* species, as well as the activity of cleistanthane-like compounds. For example, the hydroalcoholic extract obtained from *V. flavicans* leaves showed weak antibacterial activity against *P. aeruginosa* and *S. aureus*, with MIC and MBC varying from 2500 to 5000 μg.mL$^{-1}$. The diterpenoid cleistanthan-8,11,13,15-tetraen-2,3-diol showed antibacterial activity *against S. aureus*, *S. epidermidis*, *B. subtilis*, and *Shigella flexneri,* with MIC of 12 μg.mL$^{-1}$ [39]. Furthermore, the acid 3,5-dihydroxy-cleistanthan-13 (17),15-dien-19-oic showed antibacterial activity *against S. aureus* and *P. aeruginosa*. However, the authors only presented results from a diffusion test on paper disks as indicators of the antibacterial activities of the compound [40].

Although we did not assess the antimicrobial activity of pure compounds cleistanthan-8,11,13-trien-7-one (**2**) and cleistanthan-6,8,11,13-tetraene (**25**) for technical reasons, it is important to highlight that VpWS showed antibacterial activity against *B. subtilis* (MIC of 500 μg.mL$^{-1}$) and *S. choleraesius* (MIC of 100 μg.mL$^{-1}$). Since this sample is composed mainly of cleistanthan-8,11,13-triene (**28**, 90%) with a minor presence of cleistanthan-6,8,11,13-tetraene (**25**, 10%), this result support the hypothesis that *V. pyrantha* is a potential candidate source of cleistanthane-like compounds for follow up studies for the discovery of new antibiotics from natural sources.

Cancer is a disease developed due to the uncontrolled division of abnormal cells. It is currently considered a major epidemic since it is one of the leading causes of death worldwide. Cancer can be developed in any part of the human body, but the lung, colon, liver, breast, and prostate are the most common and lethal types of cancer [41]. Usually, the cancer death rate is higher in developing countries due to precarious access to an efficient health system, which includes good strategies for prevention, detection, and treatment, the high incidence of tobacco consumption, and even prejudice and lack of proper education at some extent, as it is seen for prostate cancer amongst the male population [42]. Plants can produce a wide array of specialized secondary metabolites that can be used as anti-cancer agents. This include paclitaxel isolated from *Taxus brevifolia* [43], artemisinin isolated from *Artemisia annua* [44,45], and atropine isolated from *Atropa belladonna* [46]. Usually, plant-derived compounds possess great antioxidant activity,

which is translated into anti-cancer activity because they act scavenging and deactivating singlet oxygen quenchers and free radical scavengers [47]. However, only very few plant-derived compounds are currently under clinical trial. Thus, it is essential to identify new sources of anti-cancer agents, especially from natural sources such as plants.

*V. pyrantha* resin crude extract (VpR) showed great cytotoxic activity against HCT116 (human colorectal carcinoma), HepG2 (human hepatocellular carcinoma), and B16-F10 (murine melanoma) cell lines (Table 3), with $IC_{50}$ of 20.08 μg.mL$^{-1}$ for HCT116, 20.50 μg.mL$^{-1}$ for HepG2, and 12.17 μg.mL$^{-1}$ for B16-F10.

VpFr2 and VpFr3 showed greater cytotoxic activity than VpR for HCT116 and HepG2, whereas VpFr2 showed greater cytotoxic activity than VpR for B16-F10 (Table 3). These are quite interesting results since fractionation allowed the enhancement of the cytotoxic activity of the resin and even maintained the original cytotoxic activity for some of the fractions compared to VpR. For example, VpFr1 showed equivalent cytotoxic activity results for all three cell lines compared to VpR. Furthermore, it is important to highlight that all fractions were active, with different degrees, demonstrating the great potential of *V. pyrantha* to produce cytotoxic compounds in its resin (Table 3).

The diterpenoids cleistanthan-6,8,11,13-tetraene (**25**, 0.71), 14$\alpha$-hydroxy-cleistanthan-8,12-dien-7,11-dione (**17**, 0.67), cleistanthan-8,11,13-trien-7-one (**2**, 0.64), and 7-oxo-cleistanthan-8,11,13-trien-19,20$\beta$-olide (**16**, 0.59), showed a positive correlation with the cytotoxic activity against HCT116, whereas the diterpenoids cleistanthan-8,11,13-trien-17,19-dioic acid (**9**, −0.73), 8,14-dihydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**3**, −0.63), and cleistanthan-8,11,13-trien-17-oic acid (**6**, −0.59), showed a negative correlation (Fig. 5b). Most likely the higher level of compounds **25**, **17**, **2,** and **16**, together with lower levels of compounds **9**, **3**, and **6** might explain the cytotoxic activity against HCT116 of the *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7).

The diterpenoids 7-oxo-cleistanthan-8,11,13-trien-19,20$\beta$-olide (**16**, 0.64), cleistanthan-6,8,11,13-tetraene (**25**, 0.62), 14$\alpha$-hydroxy-cleistanthan-8,12-dien-7,11-dione (**17**, 0.59), and 7$\beta$,8$\beta$,13$\alpha$-trihydroxy-15,16-bis-nor-isopimar-18-oic acid (**18**, 0.50) showed a positive correlation with the cytotoxic activity against HepG2, whereas the diterpenoids cleistanthan-8,11,13-trien-17,19-dioic acid (**9**, −0.73) and 8,14-dihydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**3**, −0.63) showed a negative correlation (Fig. 5c). Most likely the higher level of compounds **16**, **25**, **17,** and **18**, together with lower levels of compounds **9** and **3** might explain the cytotoxic activity against HepG2 of the *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7).

The diterpenoids cleistanthan-6,8,11,13-tetraene (**25**, 0.61), cleistanthan-8,11,13-trien-7-one (**2**, 0.59), and 14$\alpha$-hydroxy-cleistanthan-8,12-dien-7,11-dione (**17**, 0.50) showed a positive correlation with the cytotoxic activity against B16-F10, whereas the diterpenoids 8,14-dihydroxy-18-nor-isopimara-4 (19),15-dien-7-one (**3**, −0.71), cleistanthan-8,11,13-trien-17,19-dioic acid (**9**, −0.61), cleistanthan-8,11,13-trien-17-oic acid (**6**, −0.57), and 5,10-epoxy-7,11-dihydroxy-rosane-7,15-dien-6-one (**15**, −0.50) showed a negative correlation (Fig. 5d). Most likely the higher level of compounds **25, 2**, and **17**, together with lower levels of compounds **3**, **9** and **6** might explain the cytotoxic activity against B16-F10 of the *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7). Compounds 14$\alpha$-hydroxy-cleistanthan-8,12-dien-7,11-dione (**17**) and cleistanthan-6,8,11,13-tetraene (**25**) appeared as major contributors of the cytotoxic activity of all three cell lines. In contrast, compounds **3**, **6**, and **9** seems to have a negative effect on the activity of all three cell lines.

We assessed the cytotoxic activity of the isolated compounds and VcWS provide further insights into the identified compounds' contribution to the extracts' cytotoxic activity (Table 3). Compounds **2** and **25** were selectively active against HCT116 with $IC_{50}$ of 18.85 and 12.86 μg.mL$^{-1}$ (70.28 and 45.25 μM), respectively (Table 3), but they were not active against HepG2 and B16-F10, which partially confirms the contribution of compound **25** to the cytotoxic activity of VpR and its fractions. It is important to consider as well that the observed activity might result from the synergism between the identified metabolites. Thus, the cytotoxic activity of the *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7) seems to be a complex trait that depends on the combined action of a few metabolites.

From a structure activity relationship (SAR) perspective, the presence of a double bond between C-6 and C-7 enhanced the activity of compound **25** as compared to compound **2**, which has a carbonyl group at C-7. Furthermore, we also tested the cytotoxic activity of VpWS, a mixture of compounds **25** (minor, 10%) and **28** (major, 90%). VpWS showed $IC_{50}$ of 18.51 μg.mL$^{-1}$ against HCT116, which is comparable with VpR but less active than pure compound **25** (Table 3). Thus, once again, it seems that a double bond between C-6 and C-7 is pivotal for the cytotoxic activity of cleistanthane-like diterpenoids.

Cleistanthane-like diterpenoids have been described as potent cytotoxic agents against cancer cell lines [48–50]. The cleistanthane lactone-derivatives hawaiinolide A, C, and D were isolated from *Paraconiothyrium hawaiiense* and showed potent cytotoxic activity against different cell lines, including A549, T24, HeLa, HCT116, and MCF-7 [48]. Phyllaciduloids B, phyllaciduloids D, and 6-hydroxycleistanthol were isolated from *Phyllanthus acidus* and showed cytotoxic activity against five human cancer cell lines (HL-60, SMMC7721, A-549, MCF-7, and SW480) [49]. However, this is the first report of the cytotoxic activity of cleistanthane-like diterpenoids isolated from *Vellozia* species, providing evidence of the great potential of *V. pyrantha* to produce cytotoxic compounds in its resin.

**Table 3**
Cytotoxic activity of *V. pyrantha* resin crude extract (VpR), fractions (VpWS and VpFr1-VpFr7), and isolated compounds.

| Samples | HCT116 | HepG2 | B16-F10 |
|---|---|---|---|
| **VpR** | 20.08<br>13.91–28.97 | 20.50<br>16.04–26.20 | 12.17<br>9.85–15.02 |
| **VpWS** | 18.51<br>13.50–25.38 | >25 | >25 |
| **VpFr1** | 19.93<br>13.93–28.52 | 21.12<br>17.13–26.03 | 11.16<br>8.31–14.98 |
| **VpFr2** | 14.96<br>10.65–21.03 | 16.44<br>13.04–20.72 | 6.98<br>5.29–9.22 |
| **VpFr3** | 15.95<br>11.87–21.44 | 16.07<br>13.27–19.47 | 14.53<br>10.79–19.56 |
| **VpFr4** | 26.68<br>25.32–28.12 | 19.88<br>9.83–40.18 | 12.87<br>10.76–15.39 |
| **VpFr5** | 27.23<br>25.27–29.33 | 26.85<br>18.86–38.23 | 19.08<br>16.04–22.69 |
| **VpFr6** | 27.70<br>25.06–30.61 | 28.12<br>24.67–32.05 | 16.25<br>11.64–22.68 |
| **VpFr7** | >50 | >50 | >50 |
| **2** | 18.85 (70.28 μM)<br>11.80–27.12 | >25 | >25 |
| **25** | 12.86 (45.25 μM)<br>8.47–17.02 | >25 | >25 |
| **Dox** | 0.06<br>0.05–0.08 | 0.03<br>0.01–0.07 | 0.03<br>0.02–0.05 |

Results are expressed as $IC_{50}$ (μg.mL$^{-1}$). HCT116 (human colorectal carcinoma cell line), HepG2 (human hepatocellular carcinoma cell line), and B16-F10 (murine melanoma cell line). Doxorubicin (DOX) was used as the positive control.

## 3. Conclusions

The chemical composition of *V. pyrantha* resin and fractions (VpWS and VpFr1-VpFr7 was assessed by high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS), nuclear magnetic resonance (NMR), and multivariate statistical analysis. Twenty-eight metabolites were identified, including 16 diterpenoids, seven norditerpenoids, one fatty acid, one bis-diterpenoid, one steroid, one flavonoid, and one triterpenoid. Multivariate statistical analysis was used as a powerful tool to correlate the chemical composition of the

samples with possible candidate metabolites for the observed activities. VpFr4 had the highest antioxidant activity, whereas VpFr6 had the lowest. Compounds **14** and **18** showed a positive correlation with the antioxidant activity. They may be candidates to explain the antioxidant activity of the *V. pyrantha* resin (VpR) and its fractions (VpFr1-VpFr7). Interestingly, they are the major component of VpFr4. The resin was only active against *Staphylococcus aureus* and *Salmonella choleraesius*, but fractions were enriched with antibacterial compounds. *V. pyrantha* resin and fractions showed great cytotoxic activity against HCT116, HepG2, and B16-F10 cell lines. Cytotoxic activity is a complex trait in which synergism is essential. Nevertheless, multivariate statistical analysis pinpointed several metabolites as possible candidates for the cytotoxic activity. Compounds 17 and 25 were pointed out as major contributors of the cytotoxic activity of all three cell lines. In contrast, compounds **3**, **6**, and **9** seems to have a negative effect on the cytotoxic activity. The powerfulness and robustness of the metabolomics approach to identify bioactive metabolites were supported by the isolation of compounds **2**, **25**, and **28** that were selectively active against HCT116.

## 4. Experimental section

### 4.1. Plant material

The resin of *Vellozia pyrantha* A.A.Conc was harvested manually at the Chapada Diamantina National Park in Palmeiras (Bahia-Brazil) and stored at -20 °C until further analysis. The resin is naturally exuded by the plant. This species has been recently identified as a new species and characterized by Conceição, Souza, Carneiro and Menezes [7].

### 4.2. Resin fractionation

*Vellozia pyrantha* resin (**VpR**, 18 g) was subjected to a chromatographic column on silica gel 60 (230–400 mesh), initially using mixtures of hexane and acetate (95:5, 90:10, 80:20, 70:30, 50:50), followed by pure acetate and pure methanol as eluents to produce seven fractions (VpFr1-VpFr7). A white solid precipitated at the tip of the chromatographic column right at the beginning of fractionation. This solid was further characterized as a mixture of cleistanthan-6,8,11,13-tetraene (**25**) and cleistanthan-8,11,13-triene (**28**), in which **28** was the major component (1:9). VpFr1 (5.32 g) was subjected to a chromatographic column on silica gel 60 (230–400 mesh) initially using mixtures of hexane and acetate (95:5, 90:10, 80:20, 70:30, 50:50), followed by pure acetate and pure methanol as eluents to produce 182 fractions (VpFr1_1-VpFr1_182). Fractions VpFr1_70 to VpFr1_90 (35 mg) were subjected to preparative thin-layer chromatography using a mixture of hexane and dichloromethane (70:30) as eluent resulting in the isolation of compounds **2** (6.2 mg) and **25** (8.0 mg).

### 4.3. DPPH radical scavenging assay

The antioxidant activity of the resin (VpR) and its fractions (VpFr1-VpFr7) was assessed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [51]. All measurements were performed in triplicate and results were expressed as $IC_{50}$.

### 4.4. Antimicrobial activity

The antimicrobial activity of the resin (VpR) and its fractions (VpFr1-VpFr7) was assessed as minimum inhibitory concentration (MIC) by using the successive microdilution assay in 96-well plates [52]. The antimicrobial activity was assessed against Gram-positive [*Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (CCT 0096), *Staphylococcus aureus* (ATCC 6538), and *S. epidermidis* (ATCC 12228)] and Gram-negative [*Escherichia coli* (ATCC 94863), *Pseudomonas aeruginosa* (CCT 0090), *Salmonela choleraesuis* (ATCC 14028)] bacteria, as well as against the non-filamentous fungi *Candida albicans* (ATCC 18804) and *C. glabrata* (CCT 0728).

### 4.5. Cytotoxicity assay

Cytotoxicity assay was performed as described by Batista et al. [36]. We used three tumor cell lines MCF-7 (human breast carcinoma), HCT116 (human colon carcinoma), HepG2 (human hepatocellular carcinoma) and HL-60 (human promyelocytic leukemia). The alamarBlue assay was used to assess cell viability. Extracts were tested at a final concentration of 50 μg $mL^{-1}$ and doxorubicin was used as the positive control.

### 4.6. High-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) analysis and compound identification

LC–MS analyses were performed using a liquid chromatography system (Prominence, Shimadzu Co., Japan) coupled to a quadrupole time-of-flight mass spectrometer (microTOF II, Bruker Daltonics, Germany) as described previously [53]. Initially, one milligram of the resin (VpR) and its fractions (VpFr1-VpFr7) were dissolved in HPLC-grade acetonitrile (2.0 mL) to prepare a stock solution (0.5 mg $mL^{-1}$). Full scan spectra were acquired from 50 to 1000 *m/z*.

Data were processed in the XCMS package running on the R platform (3.2.3, R Foundation for Statistical Computing), available at https://xcmsonline.scripps.edu/. Putative metabolite identification was performed by searching the m/z in the Metlin database (http://metlin.scripps.edu) using $[M + H]^+$, $[M + 2H]^{2+}$, and $[M + Na]^+$ as possible adducts and two ppm as maximum error and by comparison with data available in the literature.

### 4.7. NMR analyses

For NMR analysis, samples were dissolved in $CDCl_3$ (10 mg $\mu L^{-1}$) and analyzed using a Varian 500 spectrometer (500 MHz for $^1H$ and 125 MHz for $^{13}C$, Agilent Technologies, Ltd., Santa Clara, CA) [36]. The identification of compounds **2**, **25**, and **28** was performed based on the comparison of their NMR spectra with the literature [17]. NMR spectra were processed on MestReNova (12.0.0, Build 20,080) and ACDLabs 12.0. The chemical structures were all drawn by ChemDraw Ultra (12.0).

### 4.8. Multivariate statistical analysis

Multivariate statistical analysis were performed at MetaboAnalyst 5.0 (http://www.metaboanalyst.ca) using the default settings provided in the platform. Analysis of variance was used to identify statistically significant differences between the samples ($P < 0.05$), followed by Fisher's LSD test. Before MVSA, the metabolite profiling data was normalized by the sum, square root transformed, and scaled by the mean centering method. MVSA encompassed Partial Least Squares-Discriminant Analysis (PLS-DA), cross-validation, hierarchical cluster analysis, heat map, Variable Importance in Projection (VIP) scores, and correlation analysis. Further details can be found at [54].

## Author contribution statement

Conceived and designed the analysis, collected the data; contributed data or analysis tools, and performed the analysis: Luiz A. F. Ribeiro, Iago B. F. dos Santosa, Caline G. Ferraz, Lourdes C. de Souza-Neta, Valdenizia R. Silva, Luciano de S. Santos, Daniel P. Bezerra, Milena B. P. Soares, Leonardo Zambotti-Villela, Pio Colepicolo, Antonio G. Ferreira, Floricéa M. Araújo, Paulo R. Ribeiro.

Wrote the paper: Paulo R. Ribeiro.

All authors have read the final version of the manuscript.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2023.105686.

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