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Identification of diterpenos flexibiliene from *Stillingia loranthaceae*, using LC-MS^2 and Molecular Networking

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Abstract: The genus Stillingia (Euphorbiaceae) is represented by 30 species distributed in the America and islands of the Pacific. In Brazil, seven species are distributed between Caatinga and Atlantic Forest, four of which are predominantly Caatinga. Only four species of Stillingia were studied chemically. Diterpenes with rare flexibilane skeletons have been reported from the roots of S. sanguinolenta. These compounds demonstrated interesting pharmacological activities. The use of hyphenated techniques, such as LC-MS2, coupled with bioinformatics techniques such as Molecular Networking, are able to rapidly identify substances from complex biological extracts. Thus, the objective of the study was the identification of flexibilene diterpenes, using LC-MS2 and Molecular Networking, of root bark of S. loranthaceae. The botanical identification was carried out in the Herbarium Alexandre Leal Costa at the Biology Institute of UFBA. The hexane extract (HE) from the root bark was analyzed by LC-MS2, and the data were used to generate a molecular network in GNPS site. It was possible to observe a cluster represent this diterpene skeleton in the molecular network. This data associated to MS/MS fragmentation approach suggested the presence of several new flexibilene diterpenes and known compounds (tonantzitlolone A-C) already identified from other Stillingia species.

Keywords: Flexibilene diterpene, Molecular Networking, LC-MS/MS and *Stillingia loranthaceae*.

1. Introduction

Recently was identified of *S. sanguinolenta* the substances tonantzitlolones A and B have considered rare diterpenes with cyclic flexibilene backbone[1]. These compounds showed interesting cytotoxic activities against human cancer kidney and breast cell lines[1]. The unusual backbone and interesting pharmacological activities have been raising the number of studies of this genus[2]. Recents studies show synthesis of these diterpenes and pharmacological activities of antiviral[3], cytotoxic[4] activities and inhibition of the enzyme kinesin 5[5-6].

Studies using hyphenated techniques, such as LC-MS / MS, allow analysis of the chemical profile of complex matrices without procedures to isolate their substances[7]. Another advantage of these techniques is the high sensitivity that allows the analysis of substances with low concentrations in complex matrices such as natural products and it may be improve the identification processes of substances that were difficult to identify and isolate[8].

The development of new analytical techniques with new bioinformatics approaches, such as Molecular Networking, that have emerged as a tool capable of visualizing the chemical spaces present in the samples analyzed by MS / MS[9] experiments. The use of this tool, makes the analysis of several spectra generated in an LC-MS / MS analysis quicker and easier to visualize[10].

This work suggested the presence of several new flexibilene diterpenes and known compounds (tonantzitlolone A-C) already identified from other Stillingia species.

2. Results and Discussion

The hexane extract was subjected to chromatographic analysis using C18 column and ACN: H2O (0.1% formic acid) as eluents. The generated data were analyzed using the GNPS site platform to obtain Molecular Networking.

Based on the cosine similarity, the MS2 spectra of the substances were grouped into clusters. The generated molecular networking had 318 nodes (148 after blank removal). The intensities of the lines between the nodes were related to the cosine values, indicating how much greater the thickness the greater the degree of similarity between the nodes.

It was observed the presence of a cluster in the Molecular Networking of the extract of the root bark referring to the flexibilenos diterpenes. Data from the literature show characteristic losses of the diterpene skeleton generating a common ion to skeletons with m / z 333 (Fig. 1)[3]. This ion can be observed in the spectra of MS2 of the nodes present in this cluster indicating if they are compounds with the skeletal flexibilene (Fig 2). Three nodes had precursor ions m/z 447 [M- H_2O + H], m/z 505 [M- H_2O + H] and m/z 463 [M- H_2O + H] tonantzitlolone substances A, B and C, respectively. After injection of the extract into the LC-HRMS it was possible to verify the molecular

formulas of the ions and to suggest that they be these substances (Fig. 3). In addition to these substances, it is possible to observe the presence of other substances with *m/z* not yet reported in the literature, these being novel compounds.

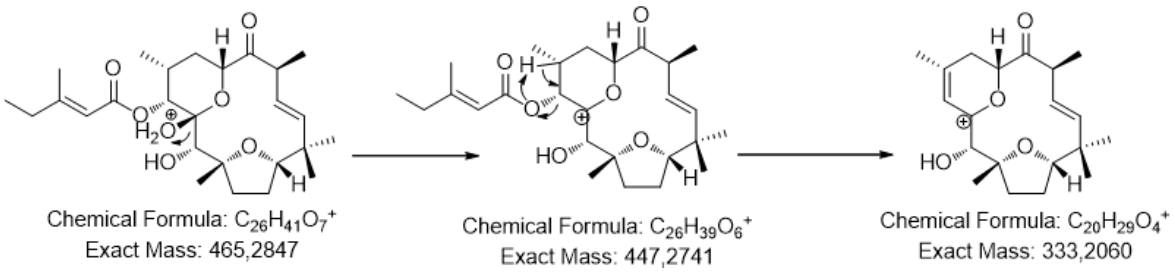

Figure 1. Proposed fragmentation mechanism for tonantzitlolones A.

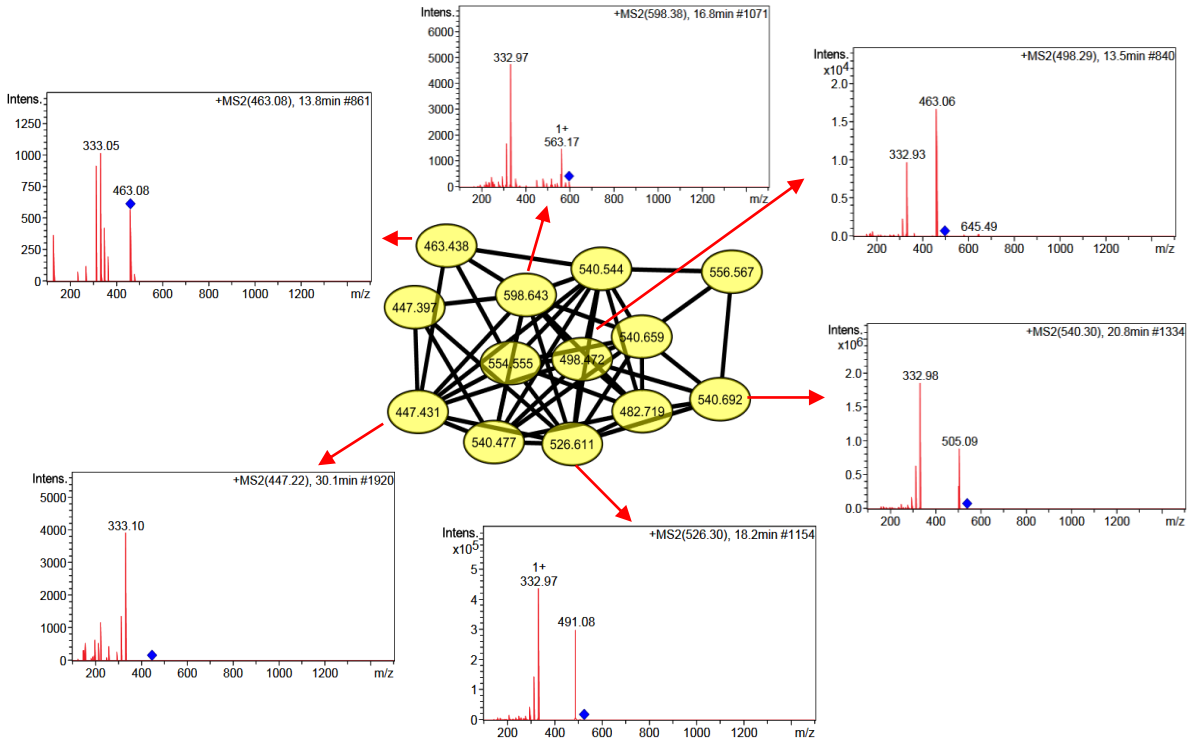

Figure 2. Cluster of flexibilenes diterpenes and MS/MS spectra of some nodes.

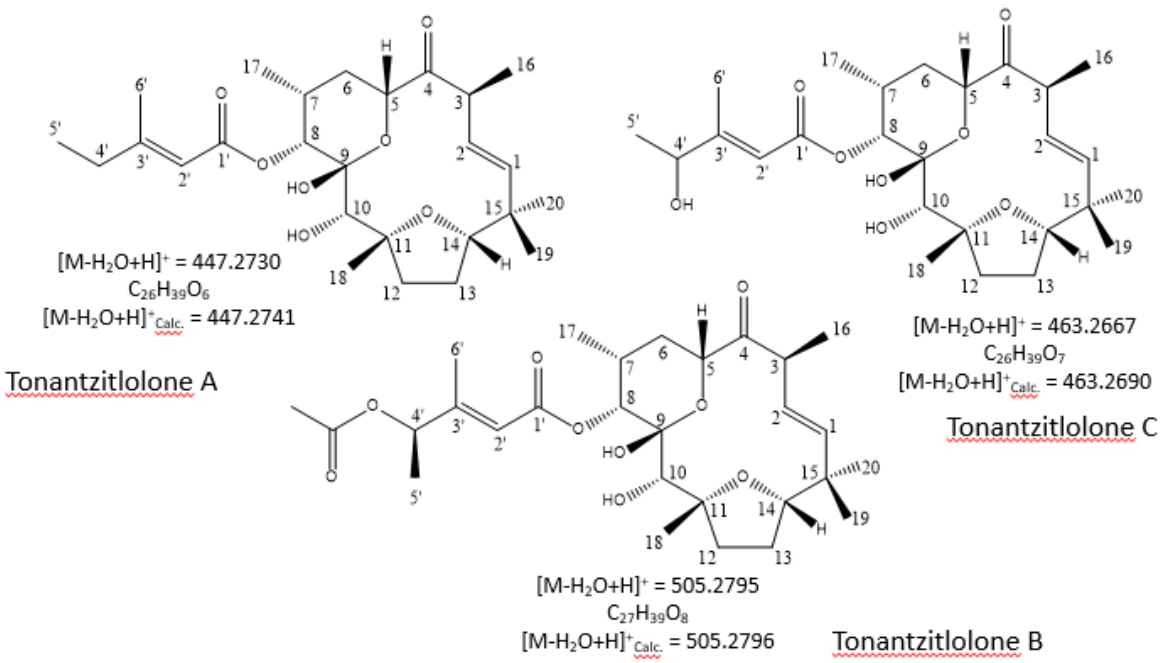

Figure 3. Diterpenes identified in *Stillingia loranthaceae*.

3. Materials and Methods

Material Vegetal

The specimens were collected in Morro do Chapéu, Bahia, Brazil in march 2016. The vouchers specimens were identified by Prof. M. L. S. G., and deposited with the Herbarium Alexandre Leal Costa(ALCB), Institute of Biology, Federal University of Bahia with the registration number 123491.

Analysis of the hexane extract from the root bark by HPLC-IT-MS / MS

1.0 mg of the extract was dissolved in 1.0 mL of ACN and filtered using 0.45 µm PVDF filter. HPLC-IT-MS / MS analysis was performed using a UFLC (Shimadzu) containing two solvent pumps LC-20AD, auto sampler SIL-20AHT, system controller CBM-20A coupled with an Ion-Trap mass spectrometer (AmaZon X).

HPLC experiments were performed using a C18 (Kromasil - 250 mm x 4.6 mm x 5 µm) column with the gradient elution: solvent A = H_2O and formic acid (0.1% v/v); Solvent B = ACN; Elution profile = 0.0 - 70.0 min (85% B); 70.0-80.0 min (85-100% B); 80.0 - 100.0 min (100-100% B); 100.0 - 110.0 min (100-85% B); 110.0-130.0 min (85-85% B), injection volume of 20 µL and flow of 0.6 mL/min. The parameters of the Ion-Trap analysis were: capillary 4.5 kV, ESI in positive mode, end plate offset 500 V, nebulizer 10 psi, dry gas (N2) with flow rate of 6 mL/min and temperature of 250 ºC. CID fragmentation was performed in auto MS/MS mode using advanced resolution mode for MS and MS/MS mode. The spectra (m/z 50-1000) were recorded every 2 sec.

Molecular Networking

The data obtained by HPLC-IT-MS / MS were subjected to a conversion to the mzXML

format using the MSconvert program. This file was submitted to the GNPS platform - GNP (http://gnps.ucsd.edu), where it was submitted to an analysis and generated a Molecular Networking. The Cytoscape 2.8.3 program was used to visualize the generated data[11].

The algorithm used in GNPS compared all possible pairs of MS / MS spectra vectors, considering mass tolerance for fragment peaks (0.5 Da), parental mass tolerance (1.0 Da), minimum number of peaks corresponding to spectral alignment (6) with a minimum cosine score of 0.6. The higher the cosine score between two spectra, the more similar the MS / MS spectra are, and the more similar the molecules[9].

After organizing the spectra based on the similarity of fragmentations, the data were analyzed in Cytoscape. In order to avoid erroneous interpretations of contaminants or analysis noise, the blank injections (mobile phase) were introduced into the spectra network as a group of distinct samples and removed from the network.

4. Conclusions

From the analysis of the data of the hexanic extract of S. loranthaceae root bark, by LC-MS2, Molecular Networking and MS/MS fragmentation approach suggested the presence of several new flexibilene diterpenes and known compounds (tonantzitlolone A-C) already identified from other Stillingia species. Therefore, this extract is promising for the isolation and identification of new diterpenes flexibilenes.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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