Revista Fitos

RESEARCH ARTICLE

Cytotoxicity assessment and molecular diversity through mass spectrometry analysis of *Costus spicatus*

https://doi.org/10.32712/2446-4775.2025.1851

Guarneire, Gracimério José[1]
https://orcid.org/0000-0002-8699-7491

Lima, Nerilson Marques[2,3*]
https://orcid.org/0000-0001-9669-0306

Silva, Giovanna Duarte [1]
https://orcid.org/0009-0007-4066-3270

Balbino, Naará da Silva[2]
https://orcid.org/0009-0002-0823-0874

Andrade, Teresinha de Jesus Aguiar dos Santos[4]
https://orcid.org/0000-0002-2415-9222

Tabai, Beatriz Joia[6]
https://orcid.org/0000-0001-7011-5792

Rodrigues, Rebeca Rocha[1]
https://orcid.org/0009-0009-4374-3238

Asghar, Salva[2]
https://orcid.org/ 0009-0000-7722-4609

Cardoso, Samuel Gouveia Silva Neres[1]
https://orcid.org/0009-0006-2635-0318

Carli, Alessandra de Paula[1]
https://orcid.org/0000-0002-7956-8947

Castro, Sandra Bertelli Ribeiro de[5]
https://orcid.org/0000-0002-5535-0919

Alves, Caio Cesar de Souza[6]
https://orcid.org/0000-0001-9765-8527

[1]Federal University of Jequitinhonha and Mucuri Valleys, *Campus* JK, Institute of Sciences, Engineering and Technology, Centro, CEP 39803-371, Teófilo Otoni, MG, Brazil.

[2]Federal University of Goiás, Institute of Chemistry, Praça Doutor Pedro Ludovico Teixeira, 11, Setor Central, CEP 74001-970, Goiânia, GO, Brazil.

[3]Federal University of Alfenas, Institute of Chemistry, Rua Gabriel Monteiro da Silva, 700, Centro, CEP 37130-001, Alfenas, MG, Brazil.

[4]Federal Institute of Maranhão, Nucleus of Applied Research to Sciences (NIAC), *Campus* Presidente Dutra, Rua Adalberto de Macedo, s/n, Ui Joana Lima de Mac. Paulo Falcão. CEP 65630-000, Presidente Dutra, MA, Brazil.

[5]Federal University of Juiz de Fora, UFJF, *Campus* Governador Valadares, Rua São Paulo, 745, Centro, CEP 35010-180, Governador Valadares, MG, Brazil.

[6]Federal University of the Jequitinhonha and Mucuri Valleys - Mucuri *Campus*, Faculty of Medicine - FAMMUC. Rua Cruzeiro, 01, Jardim São Paulo, CEP 39803-371, Teófilo Otoni, MG, Brazil.

∗ Corresponding author: nerilsonmarques@gmail.com.

Abstract

Costus spicatus Swartz exhibits notable biological properties due to its rich content of flavonoids and saponins, suggesting promising anti-inflammatory, antinociceptive, and antimicrobial properties. This study investigated the cytotoxic effects and molecular diversity of *Costus spicatus* extracts. Cytotoxicity assays revealed varying sensitivities across leaf, stem, and rhizome extracts, with ethanolic extracts generally exhibiting higher potency. Tandem mass spectrometry analysis identified eleven metabolites, including

polyphenols like quercetin and rutin. Higher levels of polyphenols were observed in leaf and stem extracts compared to rhizomes. Among these plant parts, the leaves emerged as the richest source of flavonoids, constituting 45.83% of the total flavonoid content. Hierarchical clustering analysis highlighted dissimilarities between extract types. Overall, *Costus spicatus* extracts displayed cytotoxic effects against tested cell lines, with ethanolic extracts showing greater potency. This comprehensive analysis provides valuable insights into the potential medicinal applications of *Costus spicatus* and its molecular composition.

Keywords: Cytotoxicity; Costaceae; mass spectrometry; polyphenols; flavonoids; Hierarchical Clustering Analysis.

Introduction

Costus spicatus Swartz, a medicinal plant commonly referred to as "cana-do-brejo" in the northeastern region of Brazil, is an important member of the Costaceae family found in wet coastal forests. Traditionally, the rhizomes of this plant are used for various purposes, including as a diuretic, hypoglycemic agent, and treatment for cutaneous ulcers, infections, inflammation, urethritis, bladder and urethral complaints, as well as for expelling kidney stones[1,2].

Metabolic profiling investigations have unveiled the presence of flavonol diglycosides in the leaves extracts, specifically identified as tamarixetin 3-*O*-neohesperidoside, kaempferide 3-*O*-neohesperidoside, quercetin 3-*O*-neohesperidoside, along with tamarixetin 3-O-*β*-D-glucopyranoside, kaempferide 3-O-*β*-D-glucopyranoside, quercetin 3-O-*β*-D-glucopyranoside, tamarixetin, kaempferide, and quercetin[3] Furthermore, steroidal saponins exhibiting a mild hemolytic effect have been isolated from the rhizome's extracts[4]. Various other compound classes such as saponins, phenolic acids, tannins, alkaloids, and triterpenes have also been identified in *Costus* genus[5-8].

Notably, the methanol extract derived from the leaves of *Costus spicatus* has displayed anti-inflammatory properties in the carrageenan test, attributed to its interference with the synthesis of inflammatory mediators. This suggests the extract's potential as an antinociceptive and anti-inflammatory agent in rodents[9]. Moreover, flavonol glycosides isolated from the leaf's extracts have demonstrated inhibitory activity on nitric oxide production, implying a potential anti-inflammatory effect by inhibiting certain macrophage functions involved in the inflammatory process[10]. Additionally, reports indicate that the phytochemical extract of *C. spicatus*, with a majority chemical composition of secondary metabolites from the flavonoid class, possesses antinociceptive properties possibly through interaction with the opioid system[11]. Furthermore, antimicrobial activities have also been documented[12].

Evaluating metabolic coverage to detect bioactive constituents and identify metabolites linked to pharmacological properties necessitates the use of modern analytical tools. In this context, Mass spectrometry (MS) is an essential tool for screening bioactive molecules in medicinal plants, enabling the identification of metabolites responsible for pharmacological properties. Given that the isolation and structural elucidation of chemical components from complex matrices is a time-consuming and labor-intensive process, metabolomic approaches using advanced analytical technologies have become crucial for discovering new bioactive compounds. MS stands out as the technique of choice due to its ability to analyze a wide range of chemical species with diverse physicochemical properties, even in trace amounts, from complex metabolite mixtures.

However, the large volume of spectral data generated by MS requires robust tools for data exploration and organization to effectively profile the metabolome of target species[13-15].

Considering the pharmacological properties and metabolite diversity attributed to *Costus spicatus*, this study investigated the cytotoxic effects and the chemical profile of *C. spicatus* extracts.

Experimental

Plant material and MS analysis

Leaves, stems, and rhizome of *C. spicatus* were extracted using ethanol and water. For metabolite fingerprinting, the samples were injected into a Thermo LTQ mass spectrometer using an electrospray ionization source (ESI) in positive and negative ionization mode (ESI (±)). The metabolite fingerprinting was obtained in positive and negative ionization mode (ESI (±)). The ESI source conditions were set as follows: the capillary temperature was 275 °C; Source Voltage: 4 kV; Sheath Gas Flow Rate: 9 arb; Aux Gas Flow Rate: 5 arb; Capillary Voltage 38 V; Tube Lens Voltage: 80 V; Sweep Gas Flow Rate: 0 arb; Flow Rate: 10 uL/min. For MS^n analysis, the collision energy for compound fragmentation was 20, 25, and 30 eV. The mass range in the full MS scanning experiments was *m/z* 100-1200.

Structural annotation of metabolites and chemometric analysis

The structural annotation was performed based on MS^n fragmentation patterns. Tandem mass spectrometry data combined with chemometrics tools such as hierarchical cluster analysis (HCA) was applied as a fast and simple method to discriminate extracts with cytotoxic effect and trace bioactive metabolites. Structural annotation of the metabolites was carried out using *in silico* fragmentation tools like Sirius 5.5.7 and GNPS platform tools.

Cell viability assay

The ethanol and aqueous extracts obtained from the leaves, stem, and rhizome were evaluated for cytotoxic activity in human breast cancer cell line MDA-MB-231.

To *in vitro* assay, the cells were maintained in culture bottles containing RPMI-1640 medium (Roswell Park Memorial Institute) supplemented with 10% (v/v) fetal bovine serum - FBS (Gibco), 1% (*v/v*) non-essential amino acids, and 0.5% (*v/v*) streptomycin/penicillin. The cells were incubated in a 37°C humidified atmosphere with 5% CO2 until reaching >85% confluence for the MTT cytotoxicity assay.

Cytotoxicity of the extracts was assessed by the MTT colorimetric method [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide]. *C. spicatus* extracts were prepared by dissolution in sterile dimethyl sulfoxide (DMSO) (20 mg/mL) and subsequently diluted to different concentrations. The cell suspension was prepared in supplemented RPMI medium. 100 µL/well of the cell suspension, at a density of 2 x 10^5 cells/mL, were seeded in a sterile 96-well plate and 10 µL of the tested extracts at different concentrations (333; 100; 33.3; and 10 µg/mL) were added and incubated for 48 hours in a 37°C, 5% CO_2 incubator. After the test period, the supernatant was removed and 100 µL of supplemented RPMI medium and 10 µL of MTT solution were added, and the cells were incubated for 4 hours. Subsequently, the supernatant was again removed, and

100 µL of DMSO was added. Optical density reading was measured at 570 nm using a microplate reader (Biochrom).

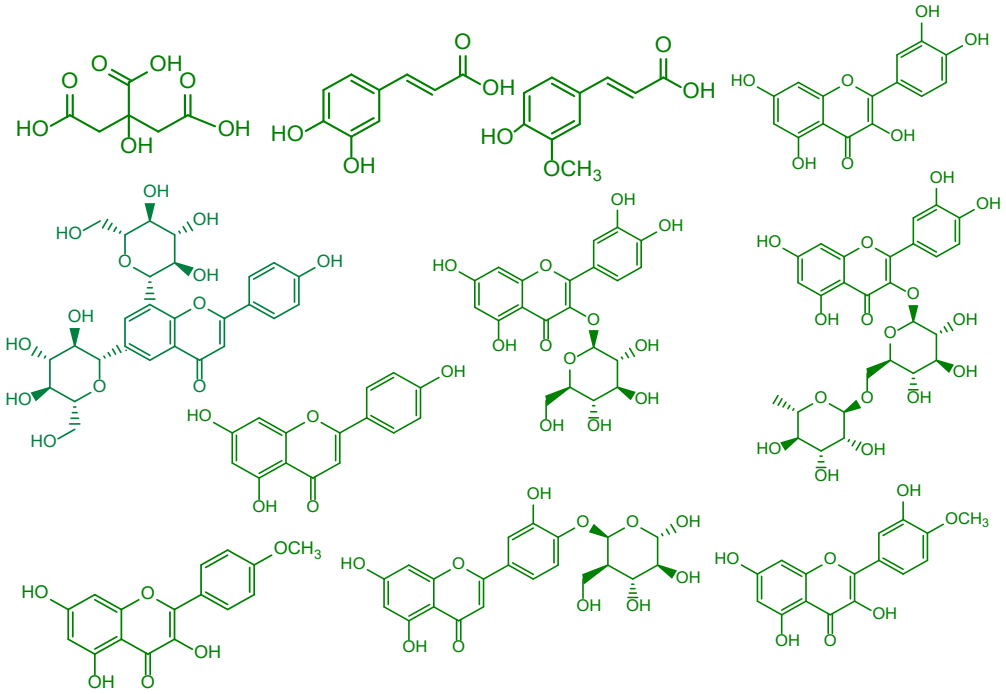
Results and Discussion

Mass spectrometry-based untargeted metabolomics approaches for structural annotation

The putative metabolite annotation was performed through molecular structure searches in natural products databases and chemotaxonomic data from genus *Costus*.

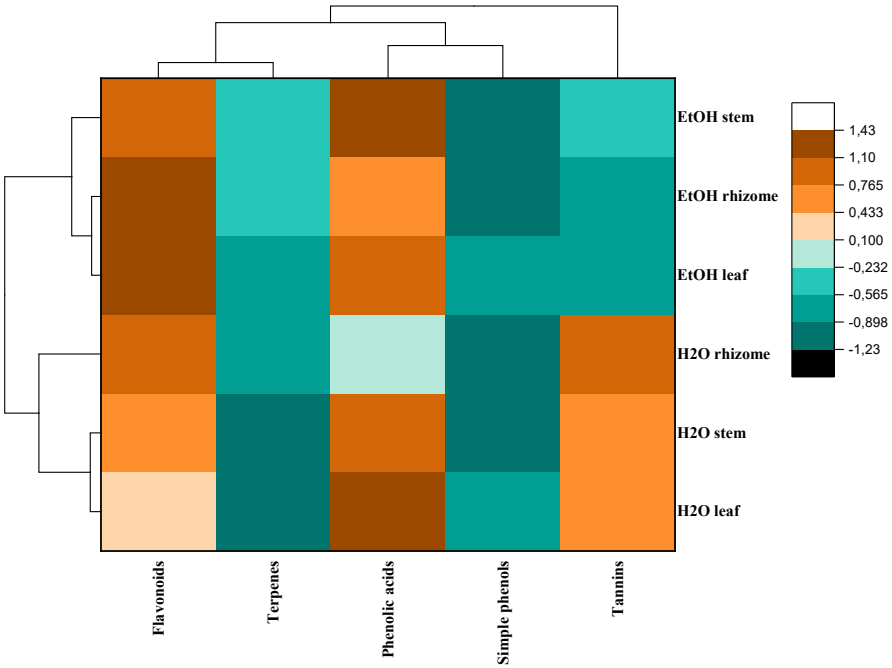
Tandem mass spectrometry data combined with database searches and computer-assisted fragmentation, was used to analyze the molecular diversity and metabolite content of the leaves, stem, and rhizome extracts. Metabolite annotation was based on MS/MS fragmentation patterns. A total of 115 hits were obtained for leaves, 92 hits for stem, and 64 hits for rhizome extract, resulting in the metabolite annotation of eleven metabolites (citric acid, caffeic acid, ferulic acid, caffeic acid derivative, vicenin-2, apigenin, rutin, quercetin 3-*O*-glucoside, luteolin 3-O-glucoside, tamarixetin, kaempferide and quercetin) (**FIGURE 1**).

FIGURE 1: Secondary metabolites annotated by MS^n analysis of *Costus spicatus*.



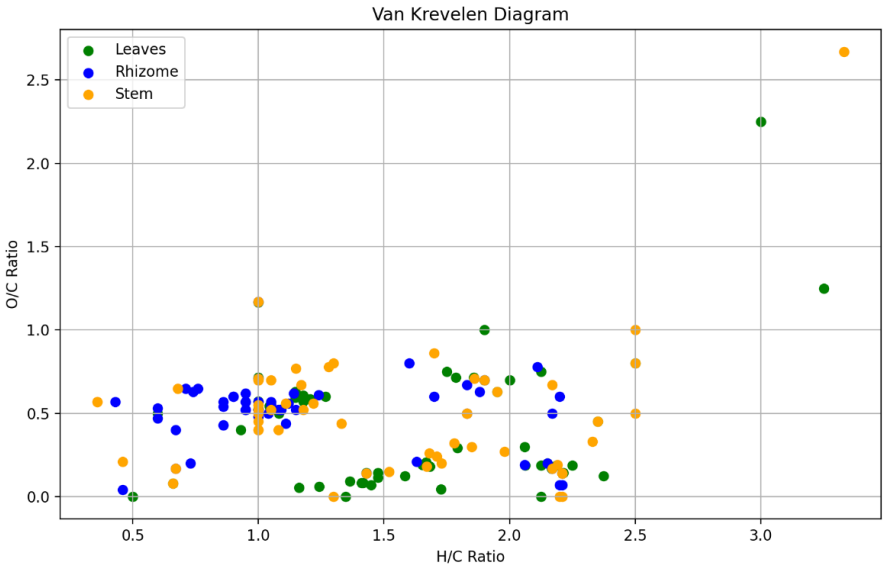
The molecular profiling displayed various secondary metabolite classes including terpenes, steroids, alkaloids, flavonoids, tannins, phenolic acids, and simple phenols. The analysis revealed higher levels of polyphenols in leaves and stem extracts samples (33.6% and 21.3%, respectively) compared to the rhizome (19.2%). Flavonoids were the most abundant class of secondary metabolites, with higher intensity in the ethanolic extracts of leaves and rhizomes and lower intensity in the aqueous extract of leaves. Terpenes and simple phenols were the classes with the lowest content, while phenolic acids showed high abundance in the ethanolic extracts of the stem and in the aqueous extract of leaves. Tannins were predominantly detected in the aqueous extracts (**FIGURE 2**).

FIGURE 2: Polar Heatmap graph with Dendrogram of HCA representing sample similarities and abundance of the secondary metabolite classes Flavonoids, Terpenes, Phenolic acids, Simple phenols, and Tannins distributed in the aqueous and ethanolic extracts of the leaves, stem, and rhizome of *Costus spicatus*.



The Van Krevelen diagram (**FIGURE 3**) of ethanolic extracts from *Costus spicatus* highlights the chemical diversity of metabolites across leaves, stem, and rhizome, with distinct clustering patterns reflecting their metabolic profiles. Regions in the diagram correspond to specific metabolite classes: phenolic acids and flavonoids, with high O/C ratios, cluster in the upper-left, while terpenes and alkaloids, with lower O/C and higher H/C ratios, occupy the lower-right. Saponins, with intermediate ratios, are also present. The rhizome shows broader variability, while the leaves and stem form more distinct clusters, showcasing the structural diversity of the plant's secondary metabolites.

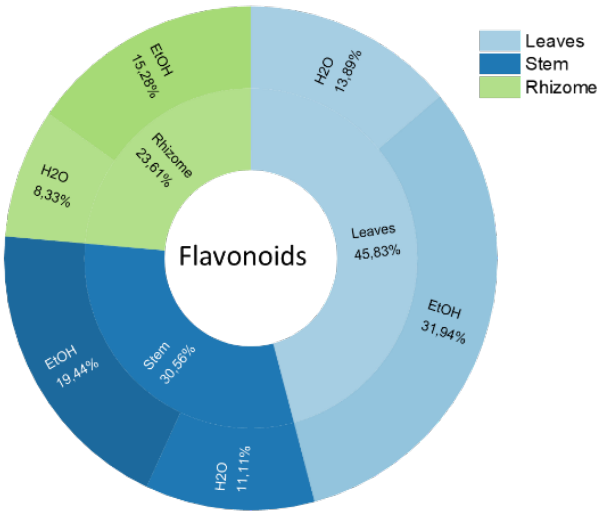
FIGURE 3: Van Krevelen diagram representing the ethanolic extracts of the leaves (green), stem (yellow), and rhizome (blue) of *Costus spicatus*. The O/C (oxygen/carbon) ratio is plotted on the vertical axis, while the H/C (hydrogen/carbon) ratio is on the horizontal axis, highlighting differences in the molecular composition of compounds extracted from these plant parts.



An analysis of the abundance of flavonoids distributed in the leaves, stem, and rhizome of *Costus spicatus* revealed intriguing results. Among these plant parts, the leaves emerged as the richest source of flavonoids, constituting 45.83% of the total flavonoid content. Following closely, the stem exhibited a considerable presence, accounting for 30.56% of flavonoids, while the rhizome displayed a comparatively lower concentration at 23.6%.

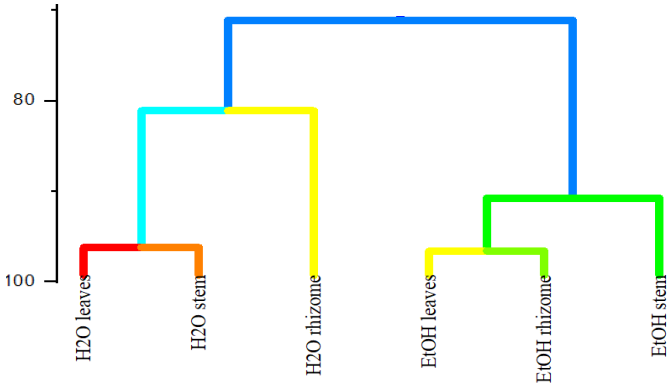
Further examination delved into the variations within different extracts derived from these plant parts. The aqueous extract of the leaves demonstrated modest flavonoid content, measuring up to 13.89%, whereas its ethanolic counterpart revealed a substantially higher concentration, reaching 31.94%. Similarly, the aqueous extract of the rhizome displayed a notable presence of flavonoids, recording at 15.29%, whereas its ethanolic extract displayed a lower concentration at 8.33%. Lastly, the aqueous extract of the stem contained 11.11% of flavonoids, while the ethanolic extract exhibited a slightly higher concentration, measuring at 19.44% (**FIGURE 4**).

FIGURE 4: Flavonoids abundance distributed in the aqueous and ethanolic extracts of the leaves, stem, and rhizome of *Costus spicatus*.



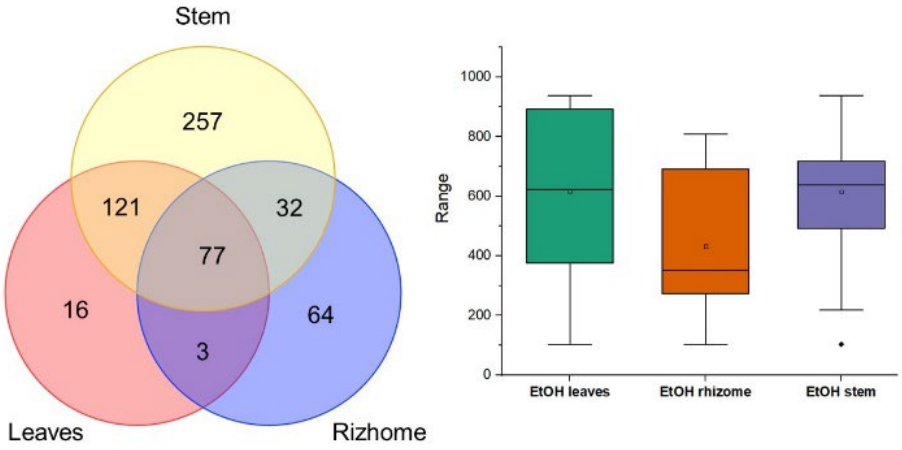
To assess the chemical composition similarity and relationships between sample molecular profiles, HCA and Venn diagrams were employed. HCA grouped ethanolic and aqueous extracts samples together with a 60% similarity, highlighting their dissimilarity. The leaves and stem extract samples exhibited low similarity, whereas the stem and rhizome samples were grouped in the same cluster (**FIGURE 5**).

FIGURE 5: Hierarchical Clustering Analysis (HCA) from ESI (+) mass spectra data representing the similarity between extracts samples (aqueous and ethanolic extracts of leaves, stem, and rhizome) of *Costus spicatus.*



The Venn diagram illustrates the unique and shared compounds detected in aqueous and ethanolic extracts from leaves, stem, and rhizome of *Costus spicatus*. In the rhizome extract, 64 unique ions were identified, whereas leaves and stem extracts exhibited 16 and 257 unique ions, respectively. Notably, all three extracts shared 77 ions. Specifically, 16 ions were shared between stem and leaves, 32 between stem and rhizome, and only 3 between rhizome and leaves. Additionally, box charts demonstrate distinctive ion distributions across the *m/z* range for each extract, providing valuable insights into the chemical composition variations among different plant parts (**FIGURE 6**).

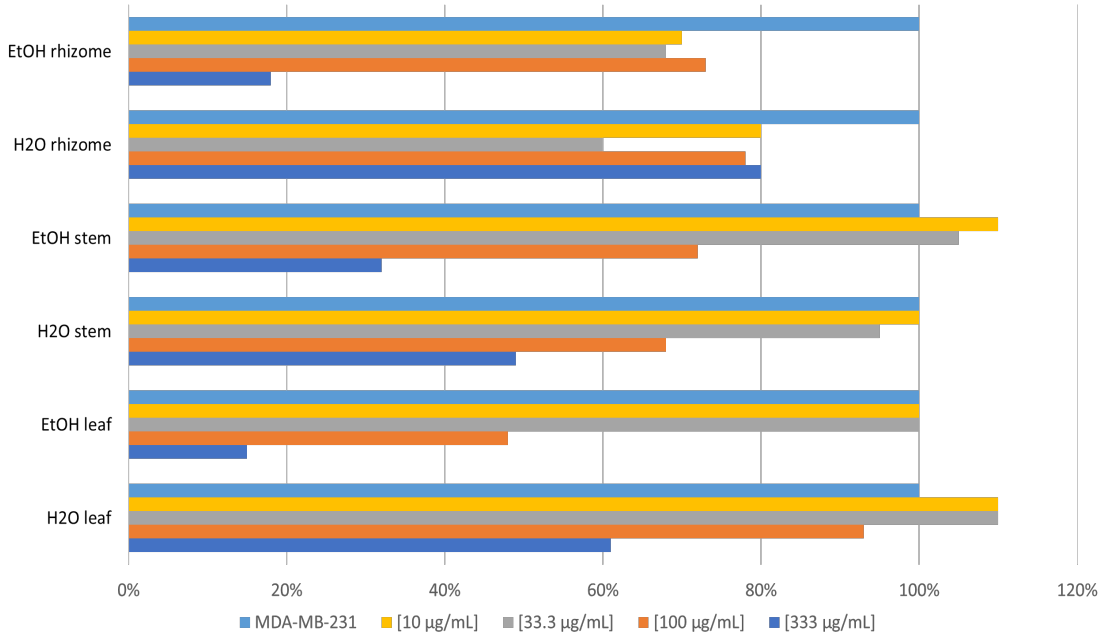
FIGURE 6: Venn diagram displaying the number of compounds detected in the aqueous and ethanolic extracts of the leaves, stem, and rhizome of *Costus spicatus* and box chart highlighting distinctive ion distributions across the *m/z* range for each extract.



Cell viability assessment from *Costus spicatus* extracts

The aqueous leaf extract showed cytotoxic effects with cell viability below 70% only at the highest concentration (333 µg/mL). On the other hand, the ethanolic leaf extract exhibited cytotoxicity at a concentration of 100 µg/mL, with 50% cell viability. The aqueous stem extract had a similar profile, demonstrating cytotoxicity only at concentrations lower than 100 µg/mL, while its ethanolic extract displayed cell viability lower than 70% only at the highest concentration (333 µg/mL). Concerning the aqueous rhizome extract, this sample-maintained cell viability even at high concentrations. However, the ethanolic extract showed cell viability below 20% at a concentration of 333 µg/mL and did not exhibit cytotoxicity at lower concentrations (**FIGURE 7**). The cytotoxic effects noticed in the extracts could be attributed to the wide range of polyphenols, particularly flavonoids and phenolic acids, identified in the bioactive extracts[16].

FIGURE 7: Cell viability of the aqueous and ethanolic extracts of the leaves, stem, and rhizome of *Costus spicatus* using the human breast cancer cell line MDA-MB-231 at concentrations 10, 33.3, 100 and 333 µg/mL.



Conclusion

In conclusion, the cytotoxic effects observed in the leaf and stem extracts of *Costus spicatus* underscore their potential as sources of bioactive compounds with therapeutic applications. The differential cytotoxicity between aqueous and ethanolic extracts suggests varying concentrations of active constituents, with ethanolic extracts generally exhibiting greater potency. Tandem mass spectrometry analysis revealed a diverse array of metabolites across different plant parts, with a notable abundance of polyphenols in leaves and stem extracts compared to rhizomes. The molecular profiling further highlighted dissimilarities between extract types and provided insights into the chemical composition and relationships within *Costus spicatus*, paving the way for further exploration of its medicinal properties.

Funding Sources

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Conflict of Interest

Disclosure statement: No potential conflict of interest was reported by the authors.

Acknowledgment

The authors acknowledge financial support from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Contributors

Study design: GJG; NML; BJT

Data curation: GJG; NML; GSS; NSB

Data collection: BJT; RRR; SA; SGSNC

Data analysis: GJG; NML, NSB; TJASA; BJT; RRR; SA

Writing of the original manuscript: NML; GJG; NSB

Writing the review and editing: APC; SBRC; CCSA; TJASA

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Histórico do artigo | Submissão: 07/04/2025 | **Aceite:** 22/10/2025
Como citar este artigo: Guarneire GJ, Lima NM, Silva GD, Balbino NS, *et a*l. Cytotoxicity assessment and molecular diversity through mass spectrometry analysis of *Costus spicatus*. **Rev Fitos**. Rio de Janeiro. 2026; 20(1): e1851. e-ISSN 2446.4775. Disponível em: <https://doi.org/10.32712/2446-4775.2025.1851>. Acesso em: dd/mm/aaaa.