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Exploring antifungal potential: The flavonoid composition of *Machaerium villosum* extracts against *Cryptococcus neoformans*

Explorando o potencial antifúngico: A composição de flavonóides do extrato de *Machaerium villosum* contra *Cryptococcus neoformans*

Exploración del potencial antifúngico: La composición en flavonoides de lo extracto de *Machaerium villosum* contra *Cryptococcus neoformans*

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Abstract
The escalating prevalence of fungal infections, coupled with the limitations and adverse effects associated with existing antifungal drugs, necessitates the exploration of alternative therapeutic approaches. The objective of this study was, therefore, to conduct a chemical analysis and assess the biological potential of the hydroethanolic extract obtained from the leaves of *Machaerium villosum*. Thus, this study investigates the extract from this plant, which belongs to the Fabaceae family, known for its rich flavonoid content. Employing UHPLC-ESI-IT-MS/MS, the extract was characterized, revealing the presence of various flavonoids, including glycosylated derivatives of kaempferol and quercetin, along with organic acids and fatty acid derivatives. The total flavonoid content was quantified at 45.7 mg/g. Subsequent antifungal evaluation unveiled significant activity against *Cryptococcus neoformans*, with a minimum inhibitory concentration (MIC) of 16 µg/ml and fungicidal action at 256 µg/ml. The observed efficacy against *C. neoformans* aligns with the documented antifungal properties of flavonoids, which disrupt membrane integrity and impede crucial cellular processes. The findings suggest that *M. villosum* extract, particularly its flavonoid constituents, holds promise as a potential source for developing new antifungal therapies.
Keywords: Jacarandá; Antifungal; Flavonol.

Resumo

A crescente prevalência de infecções fúngicas, juntamente com as limitações e efeitos adversos associados aos medicamentos antifúngicos existentes, torna necessária a exploração de abordagens terapêuticas alternativas. O objetivo deste estudo foi, portanto, realizar uma análise química e avaliar o potencial biológico do extrato hidroetanólico obtido das folhas de *Macherium villosum*. Assim, este estudo investiga o extrato dessa planta, que pertence à família Fabaceae, conhecida por seu rico conteúdo de flavonoides. Utilizando UHPLC-ESI-IT-MS/MS, o extrato foi caracterizado, revelando a presença de vários flavonoides, incluindo derivados glicosilados de kaempferol e quercetina, juntamente com ácidos orgânicos e derivados de ácidos graxos. O teor total de flavonoides foi quantificado em 45,7 mg/g. Avaliações antifúngicas subsequentes revelaram atividade significativa contra *Cryptococcus neoformans*, com uma concentração inibitória mínima (CIM) de 16 µg/ml e ação fungicida a 256 µg/ml. A eficácia observada contra *C. neoformans* está alinhada com as propriedades antifúngicas documentadas dos flavonoides, que perturbam a integridade da membrana e impedem processos celulares cruciais. Os achados sugerem que o extrato de *M. villosum*, especialmente seus constituintes flavonoides, apresenta potencial como fonte para o desenvolvimento de novas terapias antifúngicas.

Palavras-chave: Jacarandá; Antifúngico; Flavonol.

Resumen

La creciente prevalencia de infecciones fúngicas, junto con las limitaciones y efectos adversos asociados a los medicamentos antifúngicos existentes, hace necesario explorar enfoques terapéuticos alternativos. El objetivo de este estudio fue, por lo tanto, llevar a cabo un análisis químico y evaluar el potencial biológico del extracto hidroetanólico obtenido de las hojas de *Machaerium villosum*. Así, este estudio investiga el extracto de esta planta, que pertenece a la familia Fabaceae y es conocida por su rico contenido de flavonoides.Empleando UHPLC-ESI-IT-MS/MS, se caracterizó el extracto, revelando la presencia de varios flavonoides, incluyendo derivados glicosilados de kaempferol y quercetina, junto con ácidos orgánicos y derivados de ácidos grasos. El contenido total de flavonoides se cuantificó en 45,7 mg/g. La posterior evaluación antifúngica reveló una actividad significativa contra *Cryptococcus neoformans*, con una concentración mínima inhibitoria (CIM) de 16 µg/ml y una acción fungicida a 256 µg/ml. La eficacia observada contra *C. neoformans* se alinea con las propiedades antifúngicas documentadas de los flavonoides, que perturban la integridad de la membrana e impiden procesos celulares cruciales. Los hallazgos sugieren que el extracto de *M. villosum*, especialmente sus componentes flavonoides, presenta promesa como fuente potencial para el desarrollo de nuevas terapias antifúngicas.

Palabras clave: Jacarandá; Antifúngico; Flavonol.

1. Introduction

Antibiotics have ushered in a significant transformation in modern medicine. Infectious diseases that were once fatal can now be effectively treated with drugs capable of eliminating their causative agents (Machado et al., 2019). However, the consumption, production, and disposal of antibiotics pose environmental challenges, contributing to pollution and fostering the development of microbial resistance (Tannus, 2011). Despite the undeniable benefits of antibiotics, their usage may lead to undesirable effects, jeopardizing patient safety and potentially resulting in long-term consequences affecting various systems, including the nervous, renal, gastrointestinal, hepatic, and cardiovascular systems (Tavares, 2014). The annual global accounts revealed the prevalence of various fungal infections among individuals, including skin, hair, and nail infections (approximately 1 billion), recurrent vulvovaginal candidiasis (approximately 134 million), esophageal candidiasis (approximately 1.3 million), invasive candidiasis (approximately 750,000), and HIV/AIDS-related cryptococcal meningitis (approximately 223,100) (Limper et al., 2017; Bongomin et al., 2017; Rajasingham et al., 2017).

There has been a noticeable rise in infections caused by *Candida* species among patients in intensive care units, correlating with a mortality rate of 30 to 40%. In Brazil, data reveals a higher frequency of *Candida albicans* infections in immunosuppressed patients, particularly those with human immunodeficiency virus (HIV). This underscores the heightened susceptibility to *Candida* sp. infections in situations of compromised immune systems (Santos Jr et al., 2005).

Ketoconazole and fluconazole are recommended for treating *Cryptococcus neoformans* and *Candida* sp., but their use carries risks such as hepatitis, liver necrosis, and nausea. Notably, *Paracoccidioides brasiliensis*, *Zygomycetes*, *Fusarium* sp., and *Aspergillus* sp. exhibit resistance to fluconazole (Melo et al., 2012). The number of drugs available to treat fungal diseases is limited, and the development of new drugs has declined, while the number of resistant pathogens has grown (Derengowski,

2011; Machado, 2019). In general, current drugs have limitations such as toxicity, interactions, and restricted efficacy, especially in immunocompromised patients. This necessitates complex and prolonged treatments due to the immune system's inability to effectively eradicate the infection. These aspects indicate the need for studies in the search for new antifungal therapies that are less toxic to the environment and human health and that are biodegradable, as is the case with secondary metabolites from plants and microorganisms.

Among the classes of natural products, studies have indicated that flavones show activity against human pathogenic fungi such as *Cryptococcus neoformans*, *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *Aspergillus fumigatus* (Aboody & Mickymaray, 2020). Flavonols such as kaempferol, quercetin, rutin, and isoquercitrin exhibit action against *Candida albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *Cryptococcus neoformans*, *Trichosporon beigelii*, and *Trichophyton rubrum* (Aboody & Mickymaray, 2020; Yhiya et al., 2015). Ivanov et al. (2020) reported the antifungal action of luteolin, quercetrin, isoquercetrin, and rutin with an MIC of 37.5 µg/mL (Ivanov et al., 2020). These data highlight the therapeutic potential of molecules from natural products with antifungal action. In this sense, plants from the Fabaceae family are recognized in the literature for containing a high content of flavonoids (Lima et al., 2018), and within this family, there is the genus *Machaerium*. Studies show that this genus contains alkaloids, triterpenes, steroids, saponins, and mainly flavonoids. This genus comprises approximately 130 species, and only 11% have been studied from a phytochemical point of view, these include *M. kuhlmannii* Hoehne, *M. nictitans* (Vell.) Benth., *M. vestitum* Vog., *M. pedicellatum* Vog., *M. acutifolium* Vog., *M. opacum* Vog., *M. scleroxylon* Tul, *M. pedicellatum* Vog., *M. floribundum* Benth., *M. hirtum* Vell., *M. eriocarpum* Benth, *M. amplum* Benth., and *M. villosum* Vogel (Yhiya et al., 2015; Tahira et al., 2021; Tahira, 2022). This last species is popularly known as "Jacarandá Paulista," and according to the literature, there is a study on the inhibitory action of the heartwood extract against *Schistosoma mansoni* in mice, an investigation of the genetic structure of the plant, reforestation of the Atlantic Forest, and evaluation of the nutrients in its composition (Garcia et al., 2010; Yhiya et al., 2015; Gilbert et al., 1970; Giudice-Neto et al., 2014; Almeida & Viani, 2020). In terms of chemistry, the aqueous and ethanolic extracts of the leaves contain flavonols, and have antioxidant potential (Santos et al., 2009; Higa et al., 2006). However, to date, there have been no reports on the chemical study of the hydroethanolic extract of the leaves of this species correlated with its antifungal potential against human pathogenic fungi.

2. Methodology

2.1 Botanical stage

The collection, identification, and preparation of *Machaerium villosum* leaves' exsiccate were conducted by the taxonomist Prof. Dr. Ângela Lúcia B. Sartori of the Federal University of Mato Grosso do Sul, UFMS - Campo Grande. Plant material collection took place in November 2019 under the coordinates 21º5'47"S and 56º34'52"W. The exsiccate is deposited in the Herbarium of the Federal University of Mato Grosso do Sul Foundation, and its registration in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) is under the code A17D490.

2.2 Obtaining the extract

The chemical study conducted is of an experimental and analytical scale.

M. villosum leaves were dried in a ventilation oven at 40 °C for 7 days, ground in a knife mill, and subjected to a percolation extraction process using ethanol and water in a 70:30 (v/v) ratio as the extracting solvent for 24 h. The flow rate was maintained at 1.0-2.0 mL/min, following the method described by Prista **(**1995**)**. The solvent was eliminated in a rotary evaporator at 45 °C. The resulting extract was transferred to an amber bottle and kept in an exhaust hood until the solvent was eliminated.

2.3 High-performance liquid chromatography with photodiode array detector (HPLC-PDA)

The extract (2.0 mg) was solubilized in 5 mL of methanol, followed by filtration through a 45 µm membrane. HPLC-PDA analyses were carried out on an Agilent 1260 chromatograph equipped with a 60 mm flow cell (model 1260) coupled to a UV detector. Chromatographic separation used a Zorbax Eclipse Plus C-18 column (4.6 x 150 mm - particle size 3.5 µm) at a constant temperature of 45 ºC, a flow rate of 1 mL.min^{-1}, and an injection volume of 3 µL. The mobile phase composition was 0.1% acetic acid in water (A) and acetonitrile (B) in gradient mode.

2.4 High-performance liquid chromatography coupled to a mass spectrometer

Mass spectra were analyzed on an LCQ FLEET mass spectrometer (UHPLC-PDA-ESI-IT-MS^n, Thermo Scientific®), equipped with a direct sample insertion device via continuous flow injection analysis (FIA). Chromatographic separation used a UHPLC-PDA system, RP18 reverse-phase column, Acquity UPLC® BEH C18 (2.1 x 50 mm 1.7 µm) in gradient mode. The mobile phases were acidified water (A) and acetonitrile (B) acidified with 0.1% formic acid. Matrices were studied in electrospray ionization (ESI) mode, and the MS^2 stage fragmentations were carried out on an ion-trap (IT) interface. Xcalibur software (Thermo Scientific®) was used to acquire and process the spectrometric data.

2.5 Determination of total flavonoids

The quantification of total flavonoids was carried out utilizing the reaction with aluminum chloride, according to Tahira et al. (2022). A solution of aluminum chloride at a concentration of 50 mg mL^{-1} was employed. A rutin standard curve was established using 10 dilutions, ranging from 3.0 mg mL^{-1} to 60 mg mL^{-1}, derived from a stock solution of 0.1 mg mL^{-1}. The extracts were prepared at a concentration of 1 mg mL^{-1}. For the determination of total flavonoids, 0.5 mL of the sample and 0.5 mL of the aluminum chloride solution were combined in microtubes. Following a 15 min reaction period, 200 µL aliquots from each microtube were transferred to a 96-well plate for measurement at 420 nm. The absorbance values of the samples were extrapolated onto the rutin standard curve, thereby obtaining the rutin equivalent mass values. The calibration curve for determination was derived from the equation of a straight line expressed as y = 0.0169x, where y represents the absorbance at 420 nm, and x denotes the rutin concentration in µg, with an R^2 value of 0.999**.**

2.6 Quantification of flavonoids

To establish calibration curves, quercetin was dissolved in HPLC-grade methanol (2 mg.mL^{-1}) and filtered through a 0.45 µm syringe filter. The resulting solutions were diluted across a concentration range of 2—1600 µg.mL^{-1}, generating ten standard solutions for each compound. These solutions were subjected to triplicate analysis using HPLC–DAD-UV, with an injection volume of 3 µL. The column temperature was maintained at 45°C, and the chromatographic method employed a solvent gradient of 0.1% acetic acid in H_2O (solvent A) and acetonitrile (solvent B) with the following proportions: 0–6 min (10% B); 6–7 min (10–15% B); 7–22 min (15% B); 22–23 min (15–20% B); 23–33 min (20% B); 33–34 min (20–25% B); 34–44 min (25% B); 44–54 min (25–50% B); 54–60 min (50–100% B). Calibration curves were constructed by plotting average peak areas against the concentration of each analyte, resulting in the equations y=14682x+89.77; R2=0.9991 and y=16733x–58.90; R^2=0.9990 for quercetin. Limits of detection (LOD) and quantification (LOQ) were calculated as 0.252 µg.mL^{-1} and 0.185 µg.mL^{-1}, respectively, for gallic acid and quercetin.

To quantify quercetin derivatives, the extract was solubilized in HPLC-grade methanol (2 mg·mL^{-1}) and filtered through a 0.45 µm syringe filter. The resulting sample underwent triplicate analysis by HPLC–DAD-UV, utilizing the same parameters as those employed for the calibration curve.

2.7 Antifungal assy

The antifungal activity test involved standard strains obtained from the American Type Culture Collection (ATCC). The standards included strains such as *Candida albicans* (SC5314), *Aspergillus fumigatus* (ATCC 16913), *Cryptococcus neoformans* (H99) e *Fusarium oxysporum* (ATCC 48112). These strains were utilized to determine the antifungal activity by the broth microdilution method, as described in documents (CLSI, 2017a; CLSI, 2017b).

The extract was solubilized in a mixture of PBS with the addition of a 10 % Tween 20 (Carlo Erba, Italy) solution, and subsequently diluted in RPMI 1640 medium buffered with 0.165M MOPS (both from Sigma-Aldrich), resulting in a final concentration of 4096 µg/ml. The solutions were then sterilized through filtration using a 0.22 mm Millipore filter (MA01730, USA) and employed as stock solutions. Standard antibacterial and antifungal drugs, including amphotericin B, and fluconazole, were used for comparative analyses.

Briefly, serial dilutions (1:2) of the extract were carried out in RPMI 1640 medium buffered with 0.16 M MOPS at pH 7.0 in 96-well microplates. Each well was then inoculated with fungi, reaching final concentrations of 0.5-2.5 x 10^3 CFU/mL for yeasts and 5 x 10^4 CFU/mL for filamentous fungi and final concentrations from 2 to 1024 µg/ml of the extract. Each experiment was triplicated and incubated at 35 °C for 24-72 hours depending on the fungal species. Following the incubation period, the Minimum Inhibitory Concentration (MIC) was determined visually and defined as the lowest extract concentration inhibiting 90% of fungal growth. Moreover, fungal cells treated with concentrations hindering growth were sub-cultured on Sabouraud dextrose agar for 48 hours at 35ºC to ascertain the Minimum Fungicidal Concentration (MFC). The MFC is defined as the lowest concentration diminishing the viability of over 99.9% of the initial inoculum.

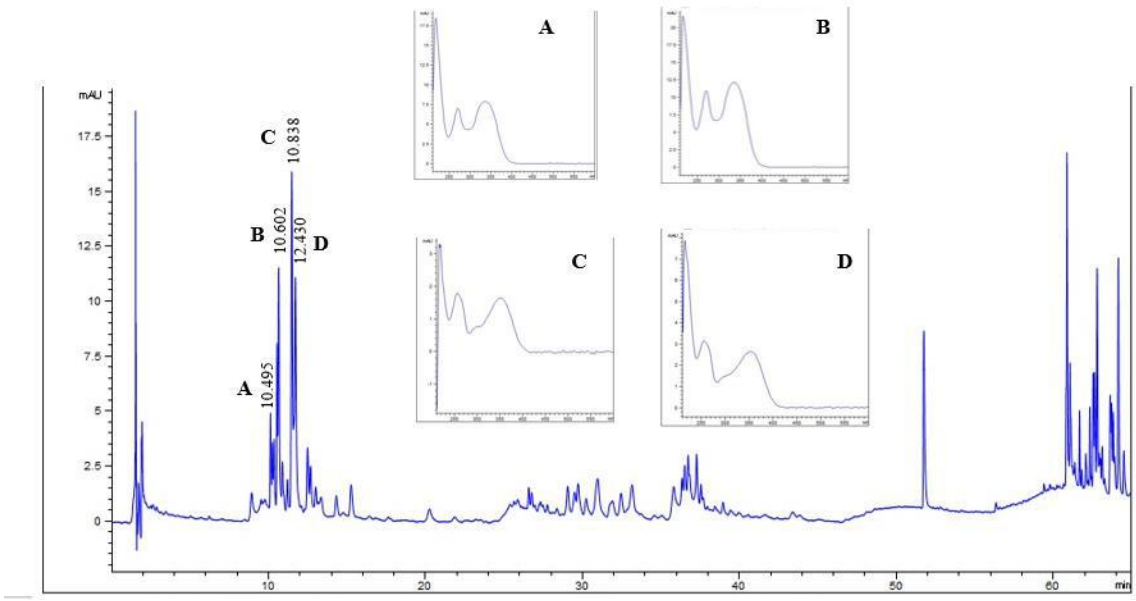
3. Results and Discussion

Invasive fungal infections pose significant challenges for hospitalized and immunocompromised patients. *Candida*, *Aspergillus*, *Cryptococcus*, and *Pneumocystis* are responsible for over 90% of deaths from these infections (Brown et al., 2012). The United States Food and Drug Administration (FDA) acknowledges the necessity for new antifungals, emphasizing *Candida* and *Aspergillus* on its list of qualified pathogens (Food and Drug Administration). The severity and associated costs underscore the urgency for effective prevention and treatment strategies (Brown et al., 2012).

Addressing the demand for new clinical candidates, the structural diversity inherent in natural product chemistry, along with their biological properties, provides a promising avenue for developing novel antifungal agents, such as flavonoids. The isoflavonoid Vatacarpan exhibited activity against *C. albicans* with a Minimum Inhibitory Concentration (MIC) of 1 µg/ml (Santana et al., 2015). Additionally, (*E*)-6-(2-carboxyethenyl) apigenin, derived from *Mimosa caesalpiniifolia*, inhibited *C. krusei* with an IC_{50} of 44 nM (Silva et al., 2019).

To assess the classes of natural products in the extract, it underwent HPLC-PDA analysis at wavelengths of 215, 225, and 352 nm. The latter wavelength revealed greater diversity and intensity of peaks. Moreover, flavonoids derived from flavones and flavonols were identified in the ultraviolet spectra (Figure 1), with bands I showing absorption between 300 and 380 nm and bands II in the region of 240 - 280 nm (Andersen & Markham, 2006; Beelders, 2011). According to the ultraviolet spectra, peaks with retention times of 10.495 and 10.602 min correspond to flavones, while peaks at 10,838 and 12,430 min are flavonols. The absorption maximum in band I for flavonols is in the region of 348-354 nm, while for flavones, it is between 256-259 nm (Vihakas, 2014; Santos et al., 2005).

Figure 1 - Chromatogram of the analysis by High-Performance Liquid Chromatography with Photodiode Array Detector (HPLC-PDA) at 352 nm.



Source: Authors (2022).

To gain further insight into the characteristics of the substances present in the extract, analyses were conducted using UHPLC-ESI-IT-MS/MS. Through the examination of mass spectra and comparison with literature data, twenty-one compounds were putatively identified, encompassing three organic acids (2, 3, and 21), twelve flavonols (eight derived from kaempferol and four from quercetin), sucrose, and two fatty acids (19 and 20) (Table 1).

Table 1 - Secondary metabolites identified through UHPLC-ESI-IT-MS/MS analysis of the hydroethanolic extract of *M. villosum* leaves.

N °	Tr (min)	$[M-H]^-$	Íons fragments *(m/z)*	Molecular Formule	Identified compound
1	0.50	341.15	179, 161, 143, 131, 119, 113	$C_{12}H_{22}O_{11}$	Sucrose
2	0.51	191.02	173; 155; 127; 111	$C_7H_{12}O_6$	Quinic acid
3	0.60	195.00	177, 159, 129, 99	$C_6H_{12}O_7$	Gluconic Acid
4	1.92	595.37	505, 475, 385, 355, 313	$C_{27}H_{32}O_{15}$	Naringenin-*C*-dihexose
5	1.94	387.01	207, 163, 145, 372, 136	$C_{21}H_{24}O_7$	medioresinol
6	2.0	593.38	503, 473, 383, 353	$C_{27}H_{30}O_{15}$	6, 8-*C*-dihexosylapigenin
7	2.34	741.35	609, 301, 179, 151	$C_{32}H_{38}O_{20}$	Quercetin-*O*-pentose-deoxyhexose-hexose
8	2.40	885.22	739, 593, 431, 285	$C_{39}H_{50}O_{23}$	Kaempferol-*O*-deoxyhexose -hexose-deoxyhexose- deoxyhexose
9	2.66	739.19	593, 575, 285	$C_{33}H_{40}O_{19}$	Kaempferol-*O*-deoxyhexose-hexose-deoxyhexose
10	2.71	725.24	575, 431, 285	$C_{32}H_{38}O_{19}$	Kaempferol-*O*-pentose-hexose-deoxyhexose
11	2.88	301.00	273, 256, 179, 151	$C_{15}H_{10}O_7$	Quercetin
12	2.99	609.35	447, 463, 301, 271, 255	$C_{27}H_{30}O_{16}$	Quercetin-*O*-deoxyhexose- *O*-hexose (isomer 1)

13	3.10	609.35	447, 463, 301, 271, 255	$C_{27}H_{30}O_{16}$	Quercetin-*O*-deoxyhexose-*O*-hexose (isomer 2)
14	3.63	593.38	447; 285; 255; 227	$C_{27}H_{30}O_{15}$	kaempferol-*O*-deoxyhexose-hexose
15	4.15	579.14	447, 285, 257	$C_{26}H_{28}O_{15}$	Kaempferol-*O*-hexose-*O*-pentose
16	6.38	285.00	255, 241, 229, 151 e 107	$C_{15}H_{10}O_6$	Kaempferol
17	6.55	769.32	593, 447, 285	$C_{38}H_{38}O_{18}$	Kaempferol-*O*- ferulloyl-deoxihexose-hexose
18	7.21	759.40	593, 447, 285	$C_{35}H_{36}O_{19}$	Kaempferol-*O*-acyl-deoxyhexose-hexose
19	9.71	295.22	277, 251, 171	$C_{18}H_{32}O_3$	9-hydroxy-10,12-actadecadienoic acid
20	10.39	297.00	155, 171, 183, 253, 279	$C_{18}H_{34}O_3$	Oleic acid epoxide
21	18.69	169.78	125	$C_7H_6O_5$	Gallic acid

Source: Authors (2023).

3.1 Putative identification of secondary metabolites in the *M. villosum* extract

Sucrose (1): Identified based on the presence of the precursor ion m/z 341 $[M-H]^-$ and fragment ions m/z 179, m/z 161, m/z 143, m/z 131, m/z 119, and m/z 113 (Table 1) (Fraternale et al., 2015; Taylor et al., 2005).

3.2 Organic acids identification

Quinic Acid (2): The mass spectrum displayed the ion of the deprotonated molecule with m/z 191 $[M-H]^-$, and the MS^2 spectrum indicated fragment ions of m/z 173 $[M-H-H2O]^-$, m/z 155 $[M-H-H_2O]^-$, m/z 127 $[M-H-CO]^-$, and m/z 109 $[M-H-H_2O]^-$. These findings suggest the presence of quinic acid (*G*ouveia & Castilho, 201*1*), previously detected in other Machaerium species (Tahira, 2022; Sannomiya et al., 2020; Lopes et al., 2020).

Gluconic Acid (3): Identified from the precursor ion m/z 195 $[M-H]^-$ and fragment ions m/z 177 $[M-H-H_2O]^-$, m/z 159, m/z 129, and m/z 99 (Fraternale et al., 2015; Cádiz-Gurrea et al., 2013).

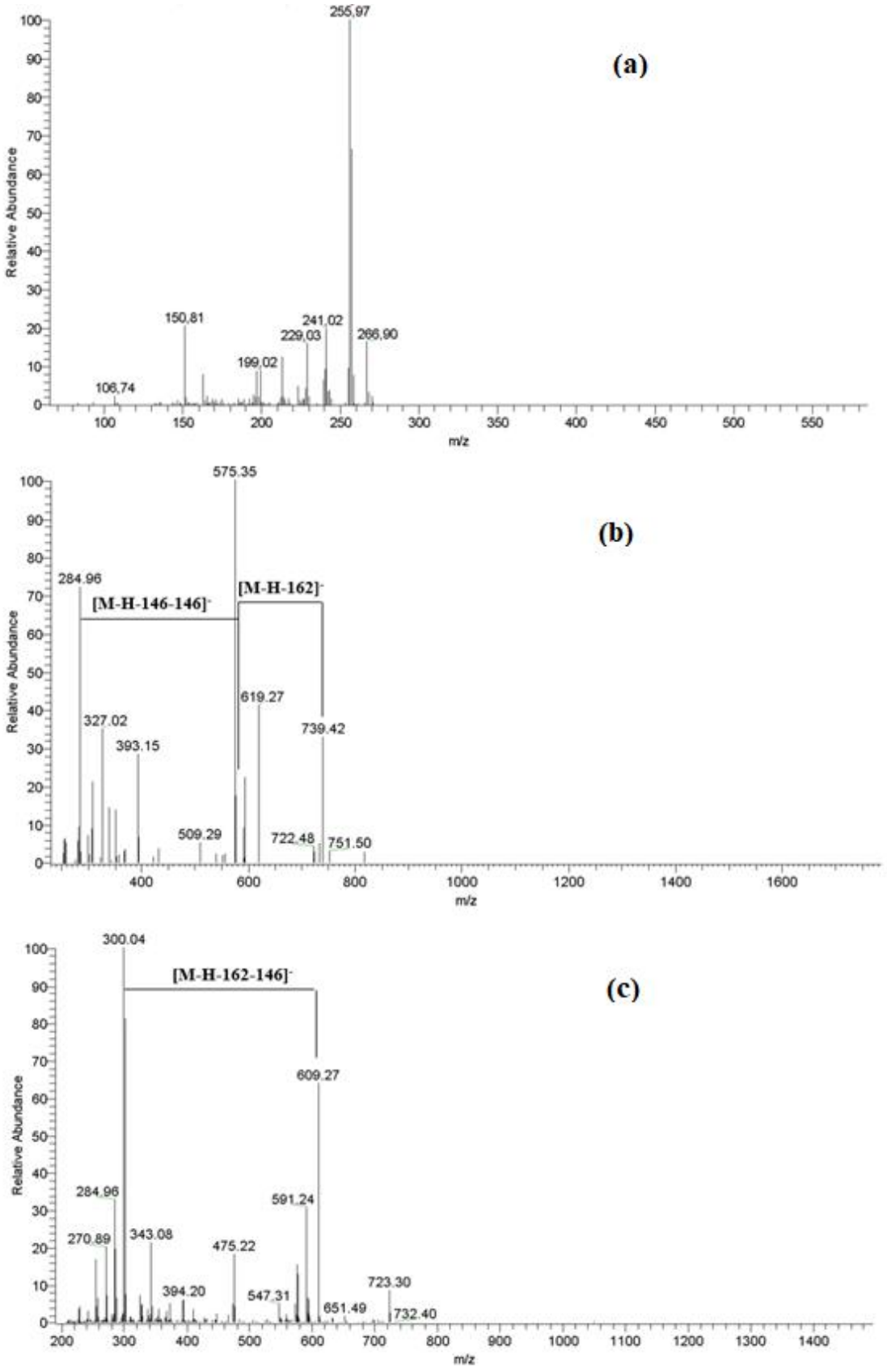
Gallic Acid (21): Identified from the precursor ion m/z 169 $[M-H]^-$ and the fragment ion m/z 125 $[M-H-CO_2]^-$, corresponding to the loss of a CO_2 molecule (Table 1) (Liu & Seeram, 2018; Buzgaia et al., 2021).

3.3 Identification of kaempferol derivatives

The peak with a retention time of 6.38 min exhibited the precursor ion *m/z* 285 $[M-H]^-$, indicative of kaempferol (Substance 16, Table 1). The structure was supported by fragment ions *m/z* 255, 241, 229, 151, and 107 in the MS^2 spectrum, consistent with previous descriptions (Li et al., 2016; Chen et al., 2016) (Figure 2-a).

The second-order spectrum derived from the *m/z* 885 $[M-H]^-$ precursor ion (Figure 2-b) revealed the loss of a deoxyhexose, resulting in the loss of 162 Daltons from the precursor ion. This process led to the formation of the *m/z* 739 $[M-H-146]^-$ fragment ion. Subsequently, the *m/z* 739 ion underwent further fragmentation, producing the *m/z* 577 $[M-H-deoxyhex]^-$ fragment upon the loss of a hexose. The stepwise removal of two additional deoxyhexose units from the *m/z* 577 ion ultimately yielded the *m/z* 285 fragment ion, indicating the flavonoid skeleton as kaempferol. Consequently, Substance 8 (Table 1) can be identified as kaempferol-*O*-deoxyhexose-deoxyhexose-hexose-deoxyhexose. Similarly, metabolites 9, 10, 17, and 18 were putatively identified based on mass spectrometry data corresponding to those described by Ding and collaborators (2008), Roriz et al. (2014), Li et al. (2016), and Zhang et al. (2018), respectively.

Figure 2 - Second-order mass spectrum of the *m/z* 285 (a), 885 (b) and 741 (c) precursor ion in negative mode (35 eV).



Source: Authors (2023).

3.4 Identification of quercetin derivatives

The spectrum of the precursor ion *m/z* 741 [M-H]$^-$, eluting at 2.34 min (Figure 2-c), indicates its composition as quercetin-*O*-hexose-deoxyhexose-pentose, designated as Substance 7 (Table 1). The *m/z* 741 precursor ion generates the *m/z* 609 fragment, evidencing the loss of 132 Da, signifying the presence of a terminal pentose. Subsequently, this fragment undergoes successive losses of 146 and 162 units, indicating sequential removal of a deoxyhexose and hexose, respectively. This process results in the formation of the *m/z* 301 fragment, aligning with patterns described by Simirgiotis et al. (2015). Likewise, other glycosylated quercetin derivatives were identified as 11, 12, and 14 (Fathoni et al., 2017; Ding et al., 2008; Kumar et al., 2017; Li et al., 2016).

3.5 Identification of *C*-glycosylated flavone and flavanone

The appearence of a *C*-glycosylated flavone was determined by the existence of the precursor ion *m/z* 593 and its fragment ions: *m/z* 503, *m/z* 473, *m/z* 383, and *m/z* 353. The occurrence of losses of 120 and 90 DA from the precursor ion indicates the loss of *C*-hexosides, suggesting the presence of apigenin-*C*-dihexose, denoted as substance 6 (Parejo et al., 2004). Additionally, flavanone 4, derived from naringenin, was identified using similar methods.

3.6 Identification of fatty acid derivatives

Substance 19, identified from the precursor ion *m/z* 295 [M-H]-, was recognized as 9-hydroxy-10,12-octadecadienoic acid, with fragment ions at *m/z* 277, *m/z* 251, and *m/z* 171 (Grati et al., 2022). The epoxide of oleic acid was derived from the precursor ion *m/z* 297 [M-H]-, with fragment ions at *m/z* 155, *m/z* 171, *m/z* 183, *m/z* 253, and *m/z* 279 (Chintalapudi & Badu-Tawiah, 2020).

The identified substances, along with retention times, precursor ions, and generated fragments (MS^2), are presented in Table 1.

3.7 Evaluation of flavonoid content and antifungal activity

The UHPLC-ESI-IT-MS/MS analysis of the hydroethanolic extract from *M. villosum* leaves revealed the existence of glycosylated derivatives of kaempferol and quercetin. Another specie of *Machaerium* produce quercetin derivatives on its chemical composition (Carvalho et al., 2019). To quantify the content of these compounds, a total flavonoid assay was conducted, yielding a result of 45.7 mg/g of extract, indicating a relatively low flavonoid content in contrast to other *Machaerium* species such as *M. amplum* (63.1 mg/g of extract) and *M. acutifolium* (78.2 mg/g of extract) (Tahira, 2022; Bento et al., 2022).

After the chemical analysis, a broth microdilution test was performed to assess the extract's activity against human pathogenic fungi, including *Candida albicans*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Cryptococcus neoformans*, with Amphotericin B and fluconazole serving as positive controls. The results demonstrated the extract's efficacy solely against *C. neoformans*, exhibiting a MIC of 16 µg/ml (Table 2). Moreover, the extract displayed fungicidal action at 256 µg/ml.

Table 2 - Minimum inhibitory concentration (MIC) of M. villosum leaf extract and antifungals on pathogenic fungi.

Fungi	MIC (µg/ml)		
	Extract	Amphotericin B	Fluconazole
C. albicans (SC5314)	≥1024	0.125	0.25
A. fumigatus (ATCC 16913)	≥1024	0.25	nd*
C. neoformans (H99)	16	0.06	0.25
*F. oxysporum (*ATCC 48112)	≥1024	nd*	nd*

*nd, not determined. Source: Authors (2022).

The observed activity might also be linked to the potential presence of kaempferol in the extract, known for its resistance to microorganisms, particularly fungi. Studies suggest that kaempferol glycosides can act as antibiotics, inhibiting growth or displaying toxicity to fungi at a MIC of 5 µg/ml (Qiu et al., 2020). Previous research highlights the antifungal activity of kaempferol, especially against *Cryptococcus neoformans*, with a MIC ranging from 2 to 32 µg/ml (Tatsimo et al., 2012). Quercetin, quercetin 3-*O*-β-D-galactopyranoside, and apigenin-7-*O*-β-D-glucuronopyranoside isolated from *Oncoba spinosa* showed a MIC 64 µg/ml, 128 µg/ml and 128 µg/ml, against *Cryptococcus neoformans,* respectively *(*Djouossi et al., 2015). Notably, the combination of quercetin with amphotericin B, a common antifungal drug, exhibited an MIC of 0.125 µg/ml. This finding is significant as amphotericin B has inherent toxicity, limiting its application in many patients (Oliveira et al., 2016). Flavonoids demonstrate inhibitory effects on fungal growth by employing various mechanisms, such as disrupting the integrity of the plasma membrane, induction of mitochondrial dysfunction, and the inhibition of key cellular processes such as cell wall formation, cell division, RNA and protein synthesis, and the efflux-mediated pumping system (Abbody & Mickymaray, 2020). The flavonol fisetin demonstrates antifungal properties by effectively restraining the growth of *C. neoformans*, *C. gattii*, *M. gypseum*, *T. mentagrophytes*, *T. rubrum*, and *T. tonsurans*, exhibiting MIC range of 4–128 µg/m^{l}. This investigation also observed reduction in ergosterol levels and identified structural alterations in *C. gattii*.

These findings hold importance, particularly in addressing the challenges posed by the high resistance of the fungal pathogen causing cryptococcosis to conventional treatments. Current treatment options are constrained by the limitations of antifungal drugs, such as amphotericin B, exacerbating the need for alternative approaches in combating *C. neoformans*. The worldwide unavailability of amphotericin B in 42 countries further complicates the treatment landscape (Bermas & Geddes-Mcalister, 2020).

4. Conclusion

The study investigated the hydroethanolic extract of *Machaerium villosum* leaves for its chemical composition and antifungal potential against human pathogenic fungi, including *Candida albicans*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Cryptococcus neoformans*. The chemical analysis revealed the presence of glycosylated derivatives of kaempferol and quercetin, contributing to a total flavonoid content of 45.7 mg/g of the extract. Notably, the extract exhibited selective antifungal activity against *C. neoformans*, with a minimum inhibitory concentration (MIC) of 16 µg/ml and fungicidal action at 256 µg/ml.

The observed antifungal efficacy, particularly against *C. neoformans*, aligns with the documented resistance of kaempferol to microorganisms, highlighting its potential as a natural antifungal agent. The study provides valuable insights into the therapeutic potential of *M. villosum* against fungal infections, emphasizing the need for further exploration of its

bioactive compounds for the development of alternative antifungal strategies. This is particularly relevant given the challenges associated with current antifungal drugs and the growing resistance of fungal pathogens.

The findings underscore the importance of natural products, such as flavonoids, in the quest for novel antifungal treatments. Nevertheless, in prospective investigations, it is imperative to undertake the antifungal assessment of isolated flavonoids from this extract. This evaluation aims to discern their specific contribution to the observed activity and ascertain whether they manifest heightened efficacy in their isolated state. Additionally, *in vivo* studies and clinical trials is warranted to validate and translate these promising *in vitro* results into effective antifungal treatments.

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