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In vitro antimicrobial screening of *Manihot esculenta* Sao Pedro Petro Extract and Identification of Active Compounds

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Abstract: Sao Pedro Petro cassava tubers (*Manihot esculenta* Ctro) are one of the cassava varieties, generally cultivated in tropical and subtropical areas. This plant has the potential to contain antimicrobial bioactive compounds. This research aimed to demonstrate the antibacterial activity of each fraction and subsequently identify the specific active fraction for in-depth analysis of its bioactive compounds. The evaluation of antimicrobial activity and the identification of bioactive compounds were conducted using the well-diffusion method and UPLC-QTOF MS (Ultra Performance Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry). Results indicated that the ethanol, n-hexane, and *n*-butanol fractions exhibited no inhibitory effects on the tested microorganisms. In contrast, the ethyl acetate fraction displayed the highest level of antimicrobial activity (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 1023). Further analysis of the ethyl acetate fraction revealed key bioactive compounds, including Coixol (phenol-group), Trigonelline and 2,6-Dimethyl quinoline (alkaloid-group), and Chebuloside-II (terpenoid-group). In summary, this pioneering research represents the first-ever exploration of the antimicrobial potential of cassava tubers, specifically focusing on the Sao Pedro Petro variety. The study not only underscores the antimicrobial properties of *Manihot esculenta* tubers but also identifies specific bioactive compounds within them, providing valuable insights into their potential therapeutic applications.

Kata kunci: *antimicrobial activity*, cassava, *Manihot esculenta* Sao Pedro Petro, UPLC-QTOF MS, *well-diffusion method*

***Abstrak*:** *Umbi singkong Sao Pedro Petro (Manihot esculenta Ctro adalah salah satu varietas singkong, umumnya dibudidayakan di daerah tropis dan subtropis. Tanaman ini memiliki potensi mengandung senyawa bioaktif antimikroba. Penelitian ini bertujuan untuk mengevaluasi aktivitas antibakteri masing-masing fraksi dan selanjutnya mengidentifikasi fraksi aktif spesifik untuk analisis mendalam senyawa bioaktifnya. Evaluasi aktivitas antimikroba dan identifikasi senyawa bioaktif dilakukan dengan metode difusi sumur dan UPLC-QTOF MS (Ultra Performance Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry). Hasil menunjukkan bahwa fraksi etanol, n-heksana, dan n-butanol tidak menunjukkan efek penghambatan pada mikroorganisme yang diuji. Sebaliknya, fraksi etil asetat menunjukkan tingkat aktivitas antimikroba tertinggi (Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 27853, dan Candida albicans ATCC 1023). Analisis lebih lanjut terhadap fraksi etil asetat mengungkapkan senyawa bioaktif utama, termasuk Coixol (gugus fenol), Trigonelline dan 2,6-Dimetil kuinolin (gugus alkaloid), dan Chebuloside-II (gugus terpenoid). Penelitian ini mewakili eksplorasi pertama mengenai potensi antimikroba dari varietas Sao Pedro Petro. Penelitian ini tidak hanya menggarisbawahi sifat antimikroba dari umbi Manihot esculenta tetapi juga*

mengidentifikasi senyawa bioaktif spesifik di dalamnya, sehingga memberikan wawasan berharga mengenai potensi aplikasi terapeutiknya.

Keywords: *aktivitas antimikroba, manihot esculenta sao pedro petro, metode difusi sumur, singkong, UPLC-QTOF MS*

INTRODUCTION

Cassava is the second-largest agricultural food product in Indonesia beside of rice, and has great penitential for both food and industry (Boukhers *et al*. 2022; Udoro *et al*. 2021). While extensive research has been conducted on the biological and antimicrobial attributes of *Manihot mulfida* cassava (L) (Muiruri *et al*. 2021), there is a growing interest in exploring its potential as a medicinal resource. Extracts derived from methanol and hexane, obtained from both the tubers and leaves of *Manihot mulfida* (L), have exhibited antimicrobial properties against various pathogens, including *Staphylococcus aureus*, *Candida albicans*, Bacillus cereus, and *Escherichia coli*, which are known to cause human infections (Fabri *et al*. 2015). Ethno-pharmacological insights have further confirmed the biological potential of *Manihot mulfida* (L), particularly in combating *Candida* species and Cryptococcus neoformans (Tavares De Oliveira *et al*. 2019; Aguirre *et al*. 2021), making it a potential candidate for treating infections, especially in immunocompromised HIV patients (Salazar *et al*. 2022).

Additionally, cassava's antimicrobial properties have been attributed to the presence of terpenoid compounds, which exhibit antibacterial efficacy against several Gram-positive bacteria (Sinaga, Fajar, *et al*. 2023; Mayanti *et al*. 2022). Furthermore, the pioneering work of Dr. M Goenawan, Head of the Pulmonary Tuberculosis Hospital in Cisarua Bogor, in using raw cassava of the Sao Pedro Petro (SPP) variety for cancer treatment since 1962 has garnered attention (Widiastuti *et al*. 2019). The gradual consumption of cassava has been found to trigger the absorption of medicinal substances and enzymes, as highlighted in previous studies (Wooding & Payahua 2022; Widiastuti *et al*. 2018; Widiastuti *et al*. 2019). Subsequent research by Widiastuti *et al*. (2018) revealed the inhibitory effects of SPP cassava tuber extract from Cisarua, Bogor, on P-388 murine leukemia cancer cells, emphasizing the cytotoxic potential of cassava tubers (Widiastuti *et al*. 2018).

Compounds like Quercetin-3-O-rutinoside have been isolated from SPP cassava tuber extract, further adding to its medicinal potential (Widiastuti, Salam, Lesmana, *et al*. 2019; Feduraev *et al*. 2022). In light of these fascinating discoveries, it becomes crucial to further investigate the antimicrobial properties inherent in cassava tuber extract, particularly from the SPP variety, and to examine its potential as an alternative medicinal resource (Aguirre *et al*. 2021; Tsumbu *et al*. 2011; Muiruri *et al*. 2021). Therefore, this study aims to assess the potency and antibacterial activity of SPP cassava tuber extract against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. Additionally, the study seeks to identify active compounds within the extract using Ultra Performance Liquid Chromatography (UPLC) coupled with a Quadrupole Time of Flight Mass (QTOFMS) detector, a form of spectrometry (Salazar *et al*. 2022). Notably, previous research has highlighted the presence of phenolic compounds and glycosides in this cassava species, known for their diverse activities. This study marked the inaugural exploration of the antimicrobial potential of SPP cassava tuber extract, specifically the SPP variety and identified active compounds that contribute to its medicinal properties.

MATERIALS AND METHODS

Study area

Sao Pedro Petro is one of the varieties of *Manihot esculenta* Crantz. The SPP tubers were planted in Cisarua, Bogor district, and the species was identified by the Head of the Botany laboratory in the Department of Biology at Universitas Pakuan, Bogor. This study was conducted at the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Bogor, Indonesia.

Extraction of Sao Pedro Petro Tubers

The cassava tuber was peeled and washed first, and then we recorded its weight, which was 115 kg. It was then blended until it achieved a smooth consistency. The cassava tube was macerated using ethanol for 24 hours. This process was repeated three times. The ethanol extract was later dissolved in 1.500 mL of distilled water and transferred to a separating funnel. Two layers were formed, with the n-hexane fraction above the water fraction. The water fraction obtained was then mixed again with ethyl acetate. A higher dielectric constant value dissolved compounds with more polar properties than compounds that dissolved in n-hexane. The same thing happened in the last fractionation with the *n*-butanol solvent. The fraction obtained was then concentrated using a rotary evaporator so that it had a gel texture (Sinaga *et al*. 2022; Widiastuti *et al.* 2023).

Antimicrobial Activity

The research continued with testing the antibacterial activity of SPP cassava tuber extract against *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 1023. The assessment of antibacterial activity was ascertained through the Kirby-Bauer technique, employing oxytetracycline as

a positive control and sterile phosphate buffer as a negative control. Each extract was formulated to a final concentration of 500ppm in 10% DMSO, while the bacterial suspension concentration was standardized to 0.5 McFarland units. A solution of 100µL from the extract, positive and negative controls was intricately deposited into the well atop a surface layer of 100µL bacterial suspension-enriched Mueller-Hinton agar (MHA). Incubation at 37°C for 24 hours ensued, culminating in the observation and measurement of the ensuing clear zone. The experimental procedure was executed thrice (Sinaga *et al*. 2023a; Sinaga *et al*. 2023b).

Identification Results of Secondary Metabolites using UPLC-QTOFMS

Subsequently, the extract used in the study was a crude extract and the identification of compound content within the extract was conducted through testing with UPLC-QTOFMS. A sample weighing 0.2 grams was dissolved in 10 ml of 70% UPLC grade methanol before being injected into the instrument. The data were presented in two chromatogram readings employing the positive and negative ESI detectors.

RESULTS AND DISCUSSION

The Yield Exract

The yield of each extract was 0.0096% for the n-hexane fraction, 0.0061% for the ethyl acetate fraction, and 0.1568% for the *n*-butanol fraction. Secondary metabolite compounds in cassava roots, which had anti-microbial properties, were separated based on their polarity with ethyl acetate, n-hexane, and *n*-butanol solvents. The compounds were able to dissolve in solvents that had relatively the same polarity value. The polarity criteria of a solvent could be seen from the dielectric constant and dipole moment. Polar solvents had a larger dielectric constant than non-polar solvents. In the initial extraction using ethanol, which was quite polar with a dielectric constant value of 24.30, all components of secondary metabolites with relatively the same polarity properties dissolved and were in the extract. After being concentrated, the extract was dissolved again in water to form a solution phase, which was then separated gradually with the most non-polar solvent, namely n-hexane. This non-polar solvent bound secondary metabolites that had non-polar properties with similar dielectric constant values.

Result of Microbial Inhibitory Activity

Analysis was conducted on pathogenic microbes, namely *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. These three types of microbes were the most common pathogens in food, thus requiring avoidance as contaminants (Okla *et al*. 2021). The results of the antibacterial activity test can be seen in Table 1.

The cassava tuber extract was expected to possess inhibitory power against the tested microbes. The positive control utilized was oxytetracycline, a tetracycline derivative compound derived from *Streptomyces rimosus* (Biswas *et al*. 2022). It was alkaline, had a yellow and bitter color, and displayed low solubility in water. Its antibacterial properties exhibited a very broad spectrum, making it a common positive benchmark in microbial inhibition tests. This antibiotic functioned by inhibiting protein synthesis in the 30s ribozyme. Meanwhile, sterile phosphate buffer served as the negative control, lacking antimicrobial activity and used for sample dilution in the analysis. The ethyl acetate extract fraction displayed a clear zone against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. Conversely, the *n*-butanol extract results only led to clear zones forming on *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The ethanol extract fraction and n-hexane did not generate clear zones against any of the three microbe types. Clear zones with a diameter of <5 mm were classified as weak, 5-10 mm as medium, and >10 mm as strong. Consequently, the ethyl acetate extract fell under the strong antimicrobial category, displaying a diameter of 13.84 mm against *Staphylococcus aureus*, a clear zone diameter of 11.05 mm against *Pseudomonas aeruginosa*, and a clear zone diameter of 10.37 mm against *Candida albicans* (Okla *et al*. 2021; Rosa *et al*. 2019).

Clear zones failed to manifest in the ethanol, n-hexane, and *n*-butanol extract fractions against *Candida albicans* isolates. However, inhibition by the ethyl acetate fraction was observed in the medium inhibition category with a diameter of 7.10 mm. This fungus could generate substantial, thick-walled tubes or spores termed chlamidiospores. These spores exhibited resistance to penetration by antimicrobial compounds, thereby enabling their survival. In contrast, gram-positive bacteria have thicker peptidoglycan layers than gram-negative bacteria. Consequently, some antibacterial compounds only target gram-negative bacteria. Gram-negative bacteria possess a thin peptidoglycan cell wall with a porin protein in the outer membrane, which functions as a channel for active compounds to enter and exit. This attribute renders the cell wall more susceptible to damage, and the lipid content increases permeability (Lee *et al*. 2008; Nielsen-Marsh *et al*., 2009). The emergence of clear zones was linked to the class of antimicrobial compounds within the extract. Ethyl acetate exhibited polarity properties aligned with flavonoid compounds, terpenoids, and other antimicrobial compounds (Eruygur *et al*. 2019; Chhetry *et al*. 2022). These compounds were subsequently subjected to identification using the UPLC-QTOFMS instrument.

Table 1. Results of antimicrobial activity test of SPP Extracts

Fraction	Inhibition zone (mm)		
	***Staphylococcus aureus* ATCC 6538**	***Pseudomonas aeruginosa* ATCC 27853**	***Candida albicans* ATCC 1023**
Ethanol	-	-	-
n-hexane	-	-	-
ethyl acetate	13.84 ± 0.07	11.05 ± 0.14	10.37 ±0.05
n-butanol	8.89 ± 0.12	7.88 ± 0.09	-
oxytetracycline	22.22 ± 0.05	20.48 ± 0.05	10.29 ± 0.06

Identification Results of Secondary Metabolites with UPLC-QTOFMS

The results of the analysis on the inhibition of cassava tuber extract using organic solvents revealed the most prominent clear zone formation in the ethyl acetate extract. Subsequently, the identification of compound content within the extract was conducted through testing with UPLC-QTOFMS. A sample weighing 0.2 grams was dissolved in 10 ml of 70% hypergrade methanol before being injected into the instrument. Data were presented in two chromatogram readings employing positive ESI and negative ESI detectors. The summarized analysis data results were presented in Figure 1. The identified compounds fell into several classes of active compounds known for their antimicrobial properties, including alkaloids, phenols, and terpenoids. Numerous active compounds like tannins, flavonoids, glycosides, saponins, alkaloids, terpenoids, and other phytoconstituents found in spices and herbal ingredients exhibit antimicrobial effects.

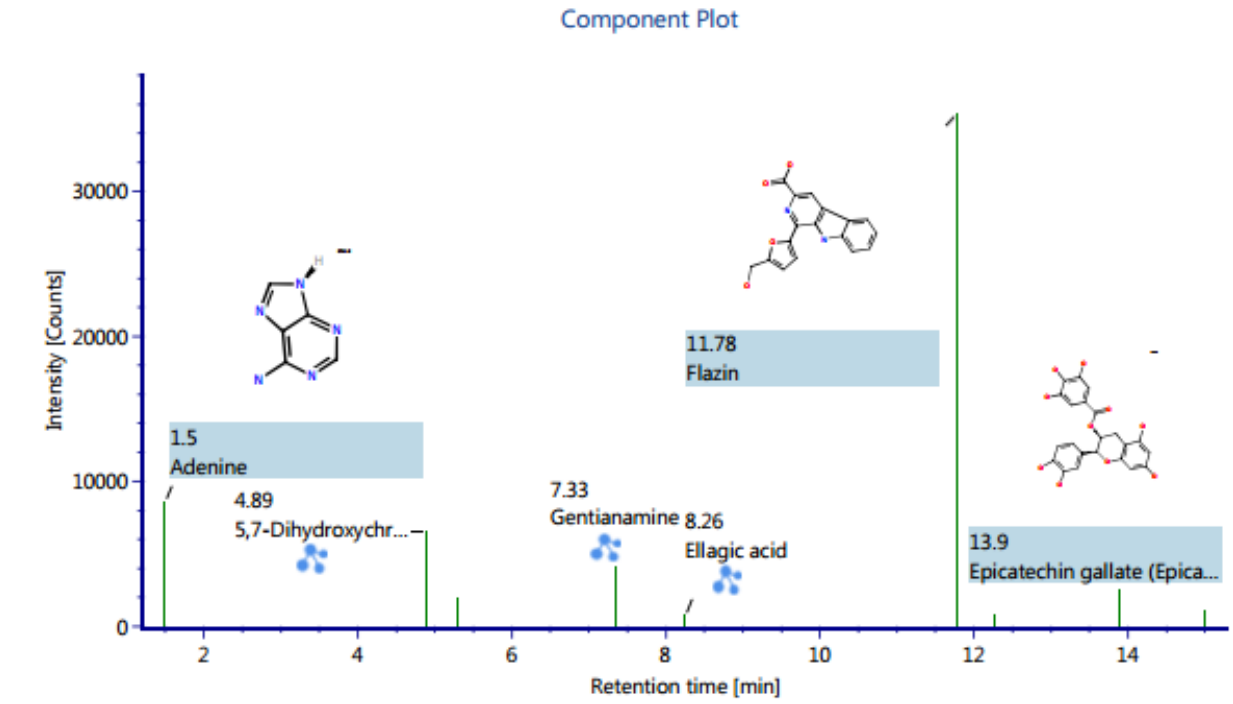
The previous study demonstrated the antibacterial effects of alkaloid compounds against both gram-positive and gram-negative bacteria (Han *et al*. 2021). These compounds induced cell lysis and alterations in bacterial morphology. As depicted in Figure 1, the ethyl acetate extract revealed the identification of at least five compounds in substantial amounts: adenine, flazin, epicatechin gallate, isosalsoline, and 1-Carbomethoxy-β-carboline. Among these compounds, there were phenolic groups including coixol and epicatechin gallate (Epicatechin-3-O-gallate).

Coixol is commonly found in reeds, while epicatechin is classified as a polyphenol with antioxidant properties. Epicatechin gallate, often referred to as EGCG, can be purified from green tea extract and functions effectively in preserving food ingredients. Phenolic compounds possed antimicrobial properties by altering the permeability of the cytoplasmic membrane or disrupting the cross-links of the cell wall containing peptidoglycan, leading to the leakage of intracellular material. Moreover, phenols denature and deactivate proteins such as enzymes, thus inhibiting the activity and biosynthesis of specific enzymes required in cellular metabolic reactions.

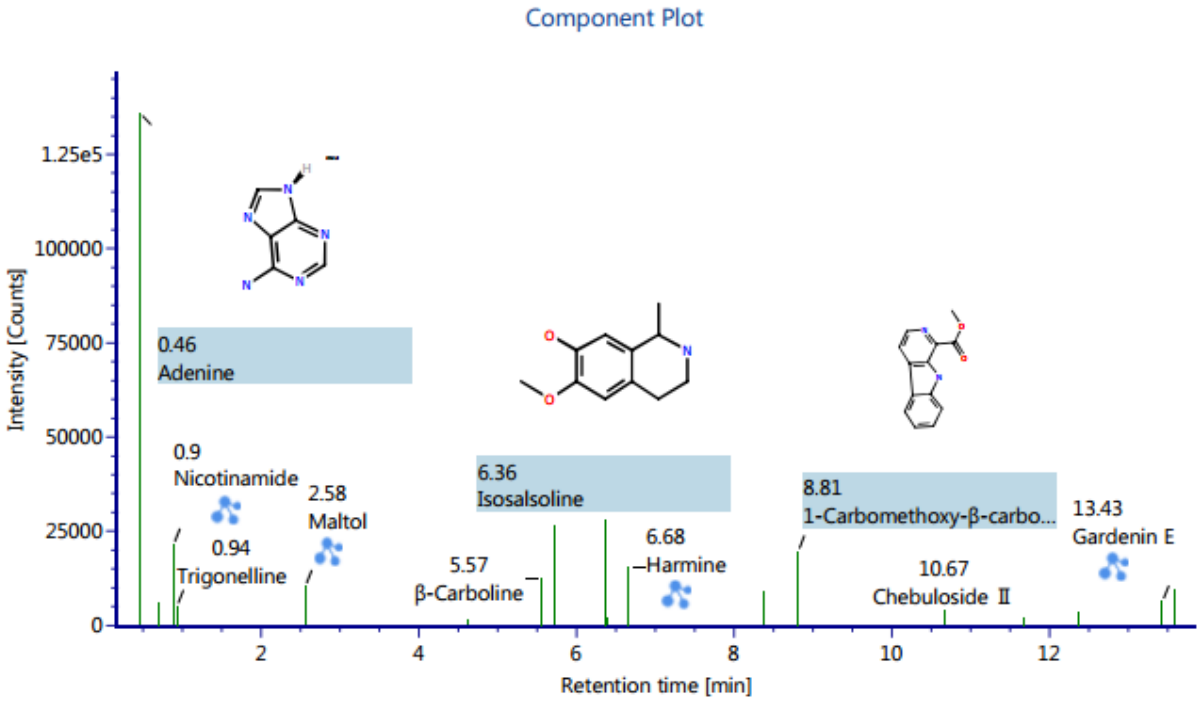
The ethyl acetate extract of Sao Pedro Petro cassava tubers contained secondary metabolites from the alkaloid group, which included Trigonelline, 2,6-Dimethyl quinoline, 4,8-Dimethoxy-1-(2-methoxyethyl)-β-carboline, β-Carboline, and Isosalsoline. Trigonelline, found in coffee beans, had demonstrated antimicrobial effects against L. acidophilus. Quinoline compounds, abundant in curry leaves, had also exhibited antimicrobial properties against *S. thypi*, *E. coli*, *S. aureus*, and *S. epidermis* at a concentration of 20%. Nicotinamide, an isolated compound from natural products, had proven effective in treating diseases caused by *Mycobacterium tuberculosis* (Kemda *et al*. 2017).

The mechanism of action for alkaloids as antibacterials was predicted to involve inhibiting cell wall synthesis, leading to cell death. The mechanism of action for alkaloids as antibacterials was predicted to involve inhibiting cell wall synthesis, leading to cell death. Alkaloids, characterized by the presence of an amine group, exhibit antimicrobial activity through interactions with microbial cell components. The amine group's basic nature facilitates engagement with acidic elements in microbial cells, disrupting cellular functions and ultimately inducing cell death. Additionally, the inclusion of aromatic rings in many alkaloids enables interactions with microbial cell membranes, leading to membrane disruption and the subsequent leakage of cellular contents, contributing to antimicrobial effects. Hydroxyl groups in alkaloids play a role in antimicrobial activity by interfering with microbial cell processes, potentially disrupting enzymatic activities and cell signaling pathways. Moreover, the presence of methoxy groups enhances alkaloids' lipophilicity, facilitating interactions with lipid components in microbial cell membranes and further promoting membrane disruption. Some alkaloids containing quaternary ammonium groups exert pronounced effects on microbial cells by disrupting membranes and interfering with essential cellular processes, adding to their antimicrobial efficacy.

Among the terpenoid group compounds found in the ethyl acetate extract of SPP cassava tubers, Chebuloside-II stood out. Terpenoids, constructed from isoprene units whose number and arrangement dictate their overall structure, exhibit potent antimicrobial properties owing to their lipophilic



(a)



(b)

Figure 1. UPLC-QTOFMS identification results for ethyl acetate extract (a) Negative ESI (b) Positive ESI

nature. The interaction between terpenoids and microbial membranes, facilitated by this lipophilicity, disrupts the structural integrity of membranes and compromises their function. Additionally, the inclusion of functional groups such as hydroxyl or carbonyl further enhances the antimicrobial activity of terpenoids. These functional groups enable interactions with microbial cell components, disrupting cellular processes and interfering with essential functions. Notably, many terpenoids, as integral components of essential oils, leverage their volatile nature to easily penetrate microbial cell membranes, exerting antimicrobial effects. Furthermore, terpenoids play a crucial role in

modulating enzymatic activities within microorganisms, impacting key metabolic processes. This modulation of microbial enzymes contributes to the inhibition of vital pathways, ultimately arresting microbial growth.

The UPLC-QTOFMS identification of compounds within the ethyl acetate extract of cassava tubers revealed a variety of antimicrobial compounds contributing to the formation of clear zones against the tested microbes in this study. However, the study had limitations in specifically identifying the compounds with the greatest role as antimicrobials. In general, alkaloids, phenols, and diterpenoids could function as antioxidants and antimicrobials. Therefore, the direct use of natural products or their extracts without proper quantification should not be relied upon as antimicrobial treatment. Nevertheless, utilizing therapeutic approaches involving natural ingredients and their extracts remained an option for microbial treatment.

CONCLUSION

Clear zones were observed against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* in the ethyl acetate fraction. This observation led to the conclusion that the ethyl acetate fraction exhibited the highest potential as an antimicrobial agent. The active compounds identified from the ethyl acetate fraction using UPLC-QTOFMS (Ultra Performance Liquid Chromatography-Quadrupole Time of Flight Mass Spectrometry) included Trigonelline and 2,6-Dimethylquinolone. These compounds belonged to the alkaloid group and possessed antimicrobial properties. Additionally, new terpenoid compounds with antimicrobial properties, namely Coixol and Chebuloside-II, were identified from the ethyl acetate fraction. These compounds were discovered in plants of the Manihot genus.

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