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Characterization by LC-MS and evaluation of the antioxidant capacity of particle size fractions from *Manihot esculenta* leaves

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Abstract

This study examined how the particle size fractionation of *Manihot esculenta* leaves influences their phytochemical composition and antioxidant activity. Of the three fractions (ground, sieved, and residue), only the residual fraction was analyzed by LC-MS in this study, while for the ground and sieved fractions had already been obtained under identical conditions. The residue fraction contained only three major metabolites (rutin, gallic acid, and caffeic acid), while the ground fraction exhibited greater diversity, including hydroxycoumarins and nicotiflorin. The sieved fraction, although lacking hydroxycoumarins, was rich in phenolic compounds. Antioxidant activity, as measured by spectrophotometry using the DPPH radical, was highest in the sieved fraction, followed by the ground fraction, while the residual fraction exhibited low activity. These results highlight the key role of particle size in metabolite diversity, bioactivity, and extraction optimization.

Keywords: Particle size fractionation; Phytochemical composition; Antioxidant activity; Phenolic compounds

1. Introduction

Extraction is a key step in the processing of plant-derived organic compounds. Its efficiency and selectivity depend on several physicochemical parameters, among which particle size plays a major role (**Azmir et al., 2013**). This parameter refers to the size of the particles obtained after grinding and sieving plant material. Depending on the intensity of the grinding, the plant material is reduced to particles of varying sizes, which are then separated by sieving into different particle size fractions. With a view to optimizing the extraction of specialized metabolites (formerly known as secondary metabolites), some studies favor the use of the total ground material, while others focus more on the sieved fraction, which is believed to offer better access to bioactive compounds.

A previous study conducted on *Manihot esculenta* leaves showed, via LC-MS analysis, that the ground material contained six major bioactive compounds: scopoletin, scopolin, rutin, nicotiflorin, caffeic acid, and gallic acid. In contrast, the sieved fraction exhibited a more limited chemical profile, notably characterized by the absence of hydroxycoumarins such as scopoletin and scopolin (**Ouattara et al., 2025**). However, the so-called residue fraction, corresponding to the particles retained by the sieve during the fractionation process, has not yet been subjected to phytochemical investigation. Furthermore, no comparative study has evaluated the antioxidant activity of the different particle size fractions (ground material, sieved material, and residue) using the DPPH radical scavenging assay.

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With this in mind, the present study aims to characterize, via LC-MS, the chemical composition of the residue fraction of *Manihot esculenta* Crantz (Euphorbiaceae) leaves and to determine the antioxidant activity of the ethanolic extracts derived from the ground material, the sieved material, and the residue. This approach will allow us to evaluate the influence of particle size on the diversity of the specialized metabolites extracted and to examine the correlation between the chemical composition of the fractions and their antioxidant potential.

2. Materials and methods

2.1. Plant material

The plant material used consists of leaves of *Manihot esculenta*, collected in February 2024 at the Nangui ABROGOUA University campus in Abidjan, Côte d'Ivoire. Botanical identification was performed by comparing the samples with specimens from the herbarium of the National Center for Floristics (CNF) at Félix HOUPHOUËT-BOIGNY University (Cocody, Abidjan). After harvesting, the leaves were carefully cleaned and then dried in three stages: 48 hours out of direct sunlight in a well-ventilated room, seven days in an air-conditioned room maintained at 18 °C, and finally three days in an oven set to 45 °C.

2.2. Preparation of particle size fractions from *Manihot esculenta* leaves

Once drying was complete, the plant material was ground into a powder using an electric grinder (Nutri Blitzer GS 158-V1457) to obtain a uniform powder. The ground material was then mechanically sieved using a 1-mm mesh sieve. The fraction that passed through the sieve, collected below, constituted the sieved fraction, while the fraction retained on the sieve was designated as "residue".

2.3. Soxhlet extraction

A Whatman filter paper containing 5 g of plant powder was placed in the extraction chamber, connected at the top to a condenser and at the bottom to a flask with a ground-glass stopper containing 300 mL of ethanol. After extraction, the extracts were vacuum-filtered and then concentrated using a rotary evaporator (BÜCHI) set at 40 °C. The ethanolic extracts obtained from the *Manihot esculenta* leaf residue were stored in the dark in opaque vials. These extracts were used for phytochemical screening via colorimetric assays as well as for LC-MS analysis. Furthermore, the ethanolic extracts derived from the ground material, the sieved material, and the residue were used to evaluate antioxidant activity via the DPPH assay.

2.4. Phytochemical screening using color reactions

A preliminary phytochemical screening was conducted on extracts from the leaves of *Manihot esculenta* to identify the main chemical constituents. The ethanolic extracts were subjected to specific colorimetric reactions, in accordance with standardized protocols described in the literature (Békro et al., 2007).

2.5. HPLC-ESI-Q-TOF-MS Analysis

The analysis of the fractions by HPLC-ESI-QTOF-MS was performed using an Agilent LC-MS system, combining an Agilent 1260 Infinity liquid chromatograph and an Agilent 6530 QTOF-MS mass spectrometer equipped with an ESI source. Data were acquired in positive ionization mode. Chromatographic separation was performed on a Waters Sunfire® C18 analytical column (150 mm × 2.1 mm, 3.5 µm) at a flow rate of 250 µL/min. The elution gradient consisted of two mobile phases: phase A, consisting of water acidified with 0.1% formic acid, and phase B, consisting of acetonitrile. The elution program, with a total duration of 40 min, included: a transition from 0 to 5% B between 0 and 5 min, a gradual increase from 5 to 95% between 5 and 15 min, then from 95 to 100% between 15 and 25 min, a hold at 100% B from 25 to 30 min, a decrease from 100% to 0% between 30 and 32 min, followed by a hold at 0% B from 32 to 41 min. The injection volume was set at 5 µL. All data were processed and interpreted using Agilent Mass Hunter Workstation software (**Kouamé et al., 2025**).

2.6. Assessment of antioxidant activity using the in vitro DPPH assay

The antioxidant activity of *Manihot esculenta* leaf extracts was assessed using the method described by Brand-Williams et al. (1995), adapted with minor modifications (Tanoh et al., 2019; Etekpo, 2024).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was dissolved in absolute ethanol to obtain a working solution at a concentration of 0.03 mg/mL. Serial dilutions of each plant extract were prepared in the same solvent, ranging from 0.001953 mg/mL to 1 mg/mL, in a 2-fold dilution series (1; 0.5; 0.25; 0.125; 0.0625; 0.03125; 0.015625; 0.007812;

0.003906; and 0.001953 mg/mL). In dry, sterile test tubes, 1 mL of each extract solution was mixed with 2 mL of the DPPH solution. After homogenization by shaking, the mixtures were incubated in the dark for 30 minutes at room temperature to prevent any photochemical degradation of the radical. The absorbance of each reaction mixture was then measured at 517 nm using a UV-Vis spectrophotometer (Spectro AL 800), with a blank consisting of 2 mL of DPPH and 1 mL of absolute ethanol. Ascorbic acid (vitamin C), prepared under the same conditions as the extracts, was used as a positive control. The percentage of DPPH reduction (PR) is calculated using the formula:

$$PR(\%) = (1 - \frac{A_e}{A_b}) \times 100$$; where A_e is the absorbance of the extract and A_b is the absorbance of DPPH.

The median reducing concentration (CR_{50}), corresponding to the extract concentration required to reduce the DPPH radical by 50%, was determined by graphical interpolation of the dose-response curves using Microsoft Excel 2016.

3. Results and Discussion

3.1. Qualitative Phytochemical Profile

The results of the preliminary phytochemical screening using color reactions, conducted to detect the presence of several families of metabolites in the ethanolic extract obtained from the leaf waste of *Manihot esculenta*, are presented in Table 1.

Table 1 Phytochemicals identified in the ethanolic extract derived from the leaf residue of *Manihot esculenta*

Compound families	**Reagents**	**Residue of *Manihot esculenta***
Polyphenols	F_eCl_3 (2%)	+
Tannins	$FeCl_3$ (1%)	+
Flavonoids	Mg + HCl $_{concentrate}$ (Shinoda test)	+
Coumarins	NaOH (10%)	+
Terpenes/Sterols	Acetic anhydride + H_2SO_4	+
Sugars	Molisch	+
Reducing compounds	Fehling	-
Proteins	NaOH (20%) + $CuSO_4$	+
Note: (+): Present; (-): Absent		

Qualitative phytochemical analysis of *Manihot esculenta* waste reveals a rich and diverse phytochemical composition, characterized by the predominant presence of specialized metabolites with high biological potential. The detection of polyphenols, including tannins, flavonoids (flavanones and flavanonols identified by the characteristic pink-to-red coloration of a positive Shinoda test), and coumarins highlights remarkable antioxidant and protective properties, suggesting potential applications in the nutraceutical or cosmetic sectors. From a nutritional standpoint, although the Molisch test confirms the presence of sugars and the copper sulfate test that of proteins, the absence of reducing compounds (negative Fehling's test) indicates that the available non-reducing carbohydrates are predominantly in the form of complex polymers, such as starch. Furthermore, the presence of terpenes and sterols reinforces the value of these residues for animal feed and, subject to appropriate processing to reduce the content of cyanogenic glucosides, for human consumption. These results demonstrate that cassava residues are not merely lignocellulosic waste but a valuable biomass within a circular economy. It should also be noted that the ethanolic extract derived from *Manihot esculenta* leaf waste exhibits a qualitative profile of specialized metabolites comparable to that of extracts obtained from pulped and sieved materials, as reported by Ouattara et al. (2025).

3.2. Phytochemical composition by LC-MS

The total ion chromatogram (TIC) obtained in ESI^+ mode (Figure 1) confirms the phytochemical richness of *Manihot esculenta* residue. A series of polar peaks can be observed between 0 and 2 min, consistent with the presence of sugars and proteins. LC-MS analysis in ESI^+ mode of the *Manihot esculenta* extract revealed that a major compound elutes at

approximately 9.7 min, with UV absorbance between 210 and 260 nm, indicating the presence of a phenolic-type aromatic chromophore (Figure 2). The mass spectrum (Figure 3) reveals a quasi-molecular ion $[M+H]^+$ at m/z 340, corresponding to a neutral mass of approximately 339 Da, consistent with the molecular formula $C_{17}H_{25}NO_6$. MS/MS fragmentation analysis highlights characteristic ions at m/z 248, 236, 220, and 204, reflecting successive losses of the amide side chain. Other fragments at m/z 177, 161, 149, and 133 reflect the presence of a methoxylated aromatic ring, typical of hydroxycinnamic acid derivatives. These results indicate that the major compound is likely a hydroxylated and methoxylated phenolic amide, whose aromatic fragmentation pattern corresponds to natural structures found in cassava roots. Thus, the dominant peak observed at 9.7 min most likely corresponds to a natural phenolic amide of the formula $C_{17}H_{25}NO_6$, derived from a hydroxycinnamic acid and linked to an aliphatic amine chain, representative of the specialized phenylpropanoid metabolites typical of *Manihot esculenta*. The cluster of peaks eluting between 12 and 16 min corroborates the presence of nonpolar compounds such as sterols and terpenes identified during the preliminary screening. Overall, this molecular fingerprint highlights the complexity of the extract and validates the potential for the valorization of this residue. To further characterize the chemical composition of the *Manihot esculenta* leaf waste, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was performed. This analytical approach, recognized for its sensitivity and structural power, enabled the putative identification of the main metabolites based on their retention times (RTs), molecular masses, precursor ions, and characteristic fragments (m/z), in comparison with data from the scientific literature. Table 2 presents the identified compounds, notably rutin, gallic acid, and caffeic acid, along with their corresponding analytical parameters and associated bibliographic references.

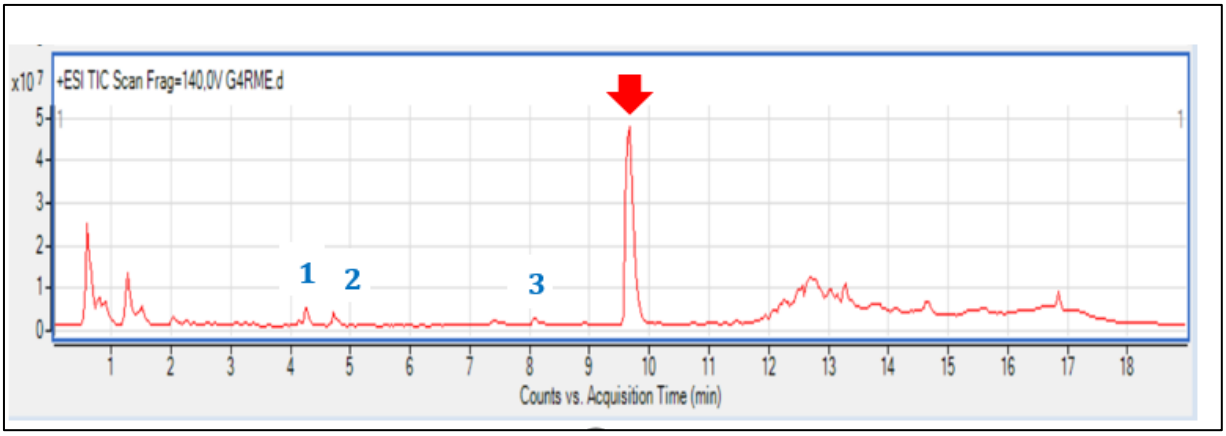

Figure 1 MS chromatographic profile from *Manihot esculenta* leaf residue

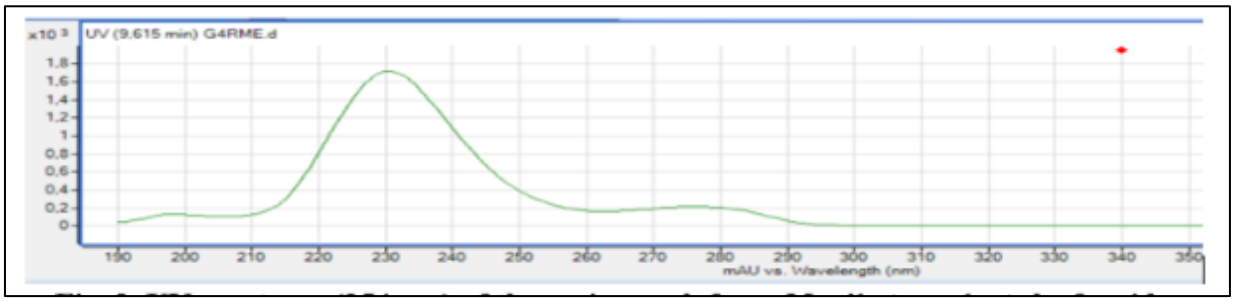

Figure 2 UV spectrum (254 nm) of the major peak from *Manihot esculenta* leaf residue

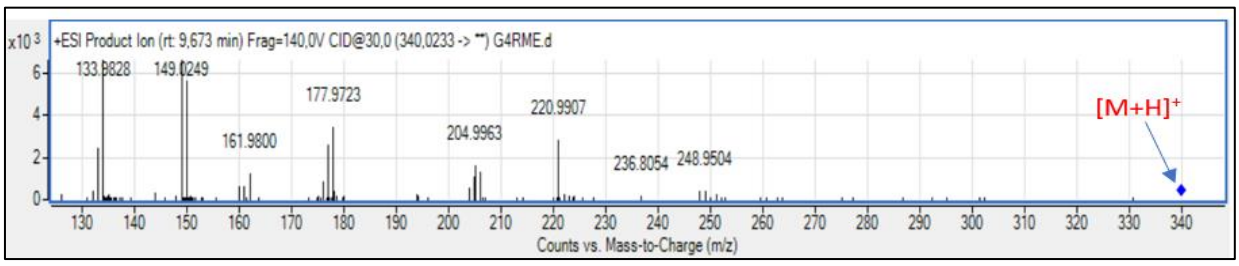

Figure 3 MS/MS spectrum of the main peak derived from *Manihot esculenta* leaf residue

Table 2 Phytochemicals in *Manihot esculenta* leaf residue identified by LC-MS/MS

Peak	Retention time (RT) in minutes	Molecular formula	Molar mass (g/mol)	MS/MS (m/z)	Compound	Sources
1	4.395	$C_{27}H_{30}O_{16}$	610	611; 465; 341; 303; 165	Rutin	**Jampa et *al.*, 2022 ; Prawat et *al.*, 1995**
2	5.145	$C_7H_6O_5$	170	171; 153; 131; 125	Gallic acid	**Scaria et *al.*, 2023**
3	8.276	$C_9H_8O_4$	180	181; 147; 135; 121; 111; 105	Caffeic acid	**Scaria et *al.*, 2023**

-Peak 1 (RT: 4.395 min) is identified as rutin ($C_{27}H_{30}O_{16}$, M = 610 g/mol), a glycosylated flavonoid also known as quercetin-3-O-rutinoside. The main precursor ion observed at m/z 611 $[M+H]^+$ corresponds to the protonated molecule. The predominant fragment at m/z 303 [quercetin + H]$^+$, resulting from the loss of the rutinoside disaccharide (–308 Da), corresponds to the aglycone and constitutes a major diagnostic ion. The fragment at m/z 465 $[M+H–C_6H_{11}O_4]^+$ is explained by the loss of a hexose (–147 Da), while the signal at m/z 341 $[M+H–C_6H_{11}O_5–C_6H_5O_2]^+$ reflects successive fragmentation involving the elimination of a hexose (–163 Da) followed by a diphenolic group (–109 Da). Finally, the ion detected at m/z 165 $[M+H–C_{21}H_{19}O_{11}]^+$ corresponds to the hexosyl residue $C_6H_{12}O_5$. Figures 4 and 5 illustrate, respectively, the MS/MS spectrum of peak 1 and the fragmentation scheme of rutin.

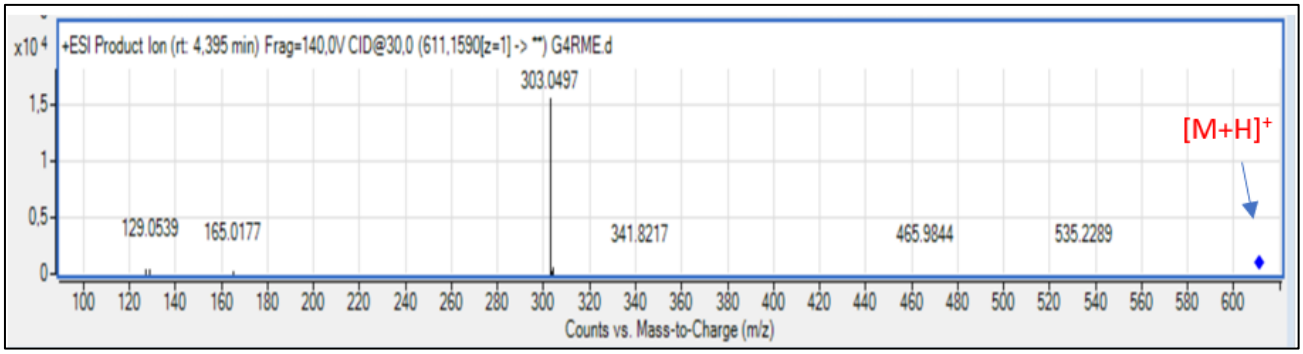

Figure 4 MS/MS spectrum of peak 1 derived from *Manihot esculenta* leaf residue

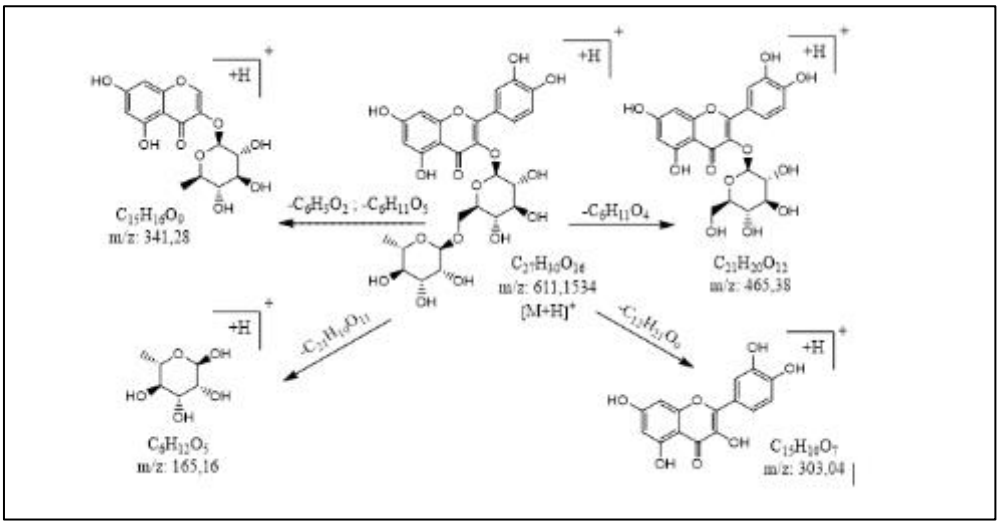

Figure 5 Proposed MS/MS fragmentation of rutin

-Peak 2 (RT: 5.145 min) is identified as gallic acid ($C_7H_6O_5$, M = 170 g/mol), a trihydroxy phenolic acid. The protonated molecular ion is observed at m/z 171 $[M+H]^+$. The fragment at m/z 153 $[M+H–OH]^+$ results from the loss of a hydroxyl group (–17 Da). The signal at m/z 131 is explained by the elimination of the C_2H_2O moiety, accompanied by structural rearrangements. The fragment at m/z 125 $[M+H–CO_2H]^+$ corresponds to the loss of the carboxyl group (–45 Da). Figures 6 and 7 illustrate, respectively, the MS/MS spectrum of peak 2 and the proposed fragmentation scheme for gallic acid.

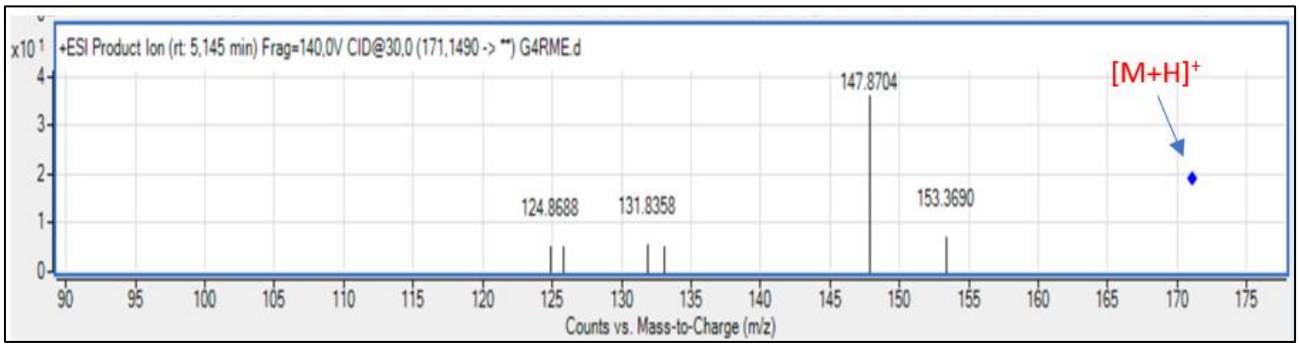

Figure 6 MS/MS spectrum of peak 2 derived from *Manihot esculenta* leaf residue

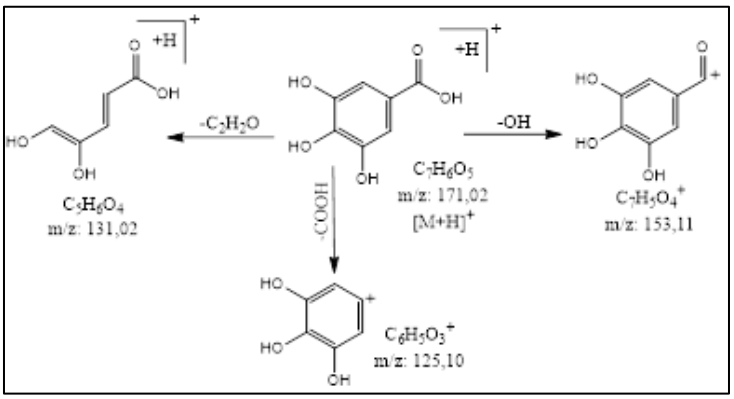

Figure 7 Proposed MS/MS fragmentation of gallic acid

-Peak 3 (RT: 8.282 min) is identified as caffeic acid ($C_9H_8O_4$, M = 180 g/mol), a phenolic acid widely distributed in the plant kingdom. The protonated molecular ion is observed at m/z 181 $[M+H]^+$. The fragment at m/z 147 $[M+H–2OH]^+$ reflects the successive loss of two hydroxyl groups (–34 Da). The signal at m/z 135 $[M+H–CO_2H]^+$ results from decarboxylation corresponding to the elimination of a carboxyl group (–45 Da), while the ion at m/z 121 $[M+H–CO_2H–OH]^+$ stems from decarboxylation (–45 Da) followed by the loss of a hydroxyl group (–17 Da). The fragment at m/z 111 corresponds to the loss of the $C_3H_3O_2$ moiety (–71 Da). Finally, the fragment detected at m/z 105 is explained by decarboxylation associated with the elimination of two hydroxyl groups.

Figures 8 and 9 illustrate, respectively, the MS/MS spectrum of peak 3 and the proposed fragmentation scheme for caffeic acid.

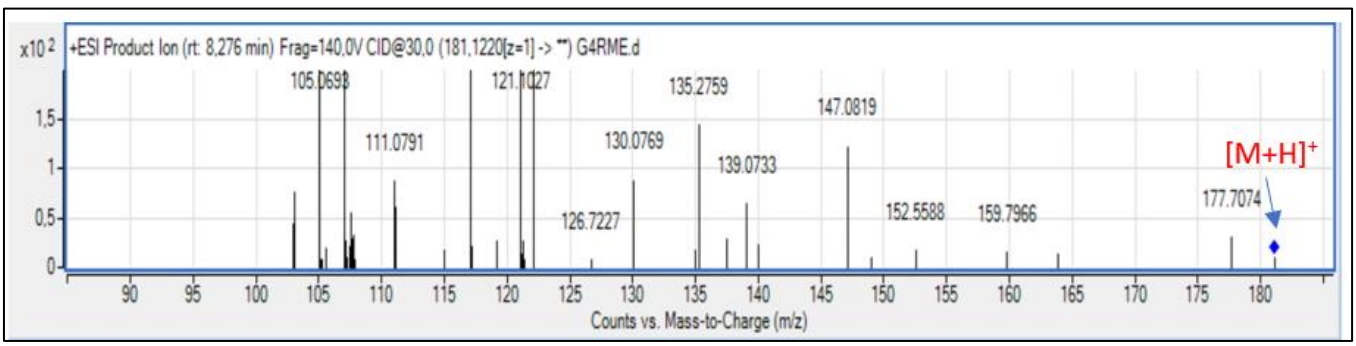

Figure 8 MS/MS spectrum of peak 3 derived from *Manihot esculenta* leaf residue

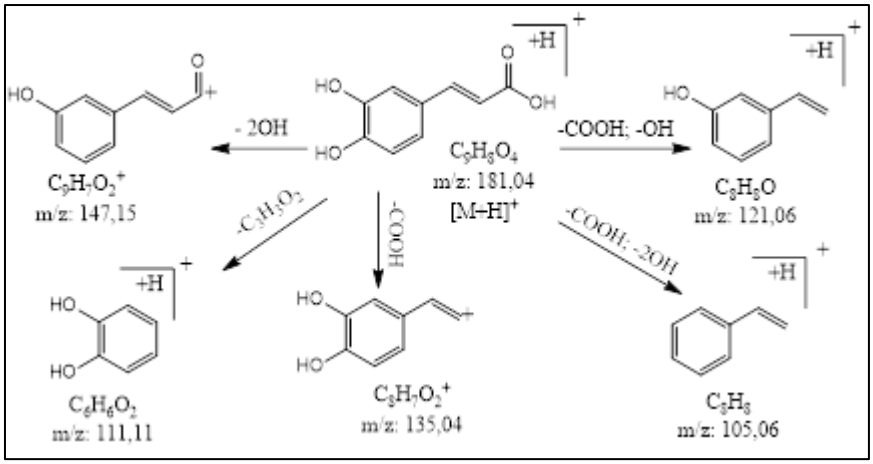

Figure 9 Proposed MS/MS fragmentation of caffeic acid

LC-MS analysis reveals that the ethanol extract of the waste material obtained from the leaves of *Manihot esculenta* contains various phytochemicals, including rutin, gallic acid, and caffeic acid, whose structures are shown below (Figure 10).

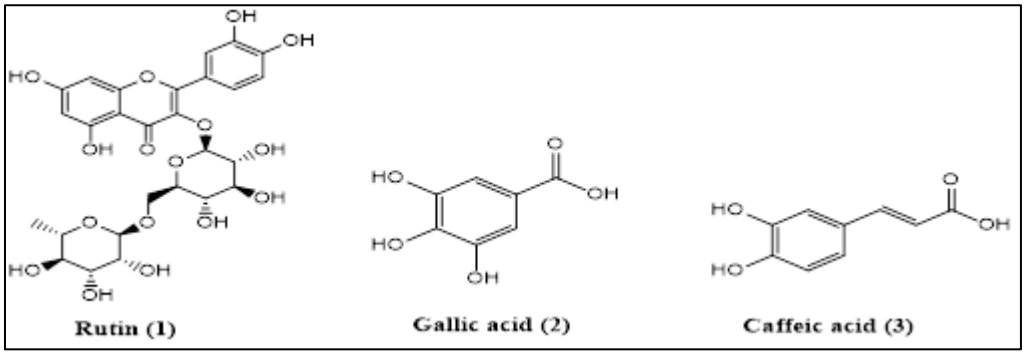

Figure 10 Phytochemicals identified by LC-MS in the ethanolic extract of *Manihot esculenta* leaf residue

In the present study, analysis of the ethanolic extract of material derived from *Manihot esculenta* leaf residue identified three compounds: rutin, gallic acid, and caffeic acid. By way of comparison, **Ouattara et al. (2025)**, in a previous study conducted on the same leaves using LC-MS analysis, had identified six major bioactive compounds in the pulped leaf extract: scopoletin, scopolin, rutin, nicotiflorin, caffeic acid, and gallic acid, whereas the sieved fraction exhibited a more limited phytochemical profile, characterized notably by the absence of hydroxycoumarins such as scopoletin and scopolin. Since this work was conducted under strictly identical experimental conditions, the differences observed between the phytochemical profiles of the different particle size fractions can be attributed to several interdependent factors. On the one hand, the differential tissue distribution of secondary metabolites within leaf tissues, as hydroxycoumarins tend to accumulate preferentially in certain specific cellular compartments such as the epidermis, palisade parenchyma, and vacuoles, with sieving thus inducing a mechanical separation of particles corresponding to distinct anatomical structures, which may lead to qualitative variations in the composition of the extracts. Furthermore, the influence of particle size on extraction efficiency: fine fractions with a higher specific surface area promote solvent penetration and metabolite diffusion, whereas the residue, consisting of coarser particles and lignified tissues, limits solvent access to certain compounds, thereby reducing their extractability. Finally, it is possible that certain metabolites are present in the residue at sub-liminal levels, below the detection limit of the LC-MS method, explaining their apparent absence in the chromatographic profile. Ultimately, these results demonstrate that a variation in particle size distribution, even under otherwise identical experimental conditions, can lead to significant differences in the phytochemical profile of the extracts, thereby highlighting the need to rigorously standardize particle size distribution in comparative phytochemical studies to ensure the reproducibility and reliability of the results.

3.3. *In vitro* antioxidant activity of ethanolic extracts against DPPH

Figure 11 shows the percentage reduction (PR) of the DPPH radical by three ethanolic extracts of *Manihot esculenta* leaves namely, the ground fraction, the sieved fraction, and the residue at five decreasing concentrations (0.500, 0.250, 0.125, 0.062, and 0.031 mg/mL). The evaluation of the antioxidant activity of the three ethanolic extracts of this plant species against the DPPH radical reveals a concentration-dependent relationship for all tested fractions, with percentages reduction (PR) increasing from 0.031 to 0.500 mg/mL. The sieved fraction stands out as the most active, exhibiting the highest PR at 0.500 mg/mL (≈ 86%), and maintaining superiority at all tested concentrations, followed by the ground fraction (≈ 83% at 0.500 mg/mL), while the refuse exhibits the lowest activity (≈ 60% at the same concentration). These differences are largely explained by the phytochemical composition specific to each fraction: the sieved fraction and the ground fraction, which are richer in phenolic compounds and flavonoids, exhibit a more pronounced antioxidant activity by donating hydrogen atoms to the DPPH radical, converting it into stable DPPH-H. The residue, consisting of coarse particles such as veins and stem fragments that are less rich in specialized active metabolites, logically exhibits lower reducing power. Furthermore, the finer particle size of the sieved fraction promotes better extractability of phytophenols during ethanol extraction, which is directly reflected in its superior antioxidant properties. These results thus highlight the importance of particle size fractionation in the valorization of plant extracts and confirm the antioxidant potential of *Manihot esculenta* leaves, whose activity is closely linked to the phenolic compound content of each fraction.

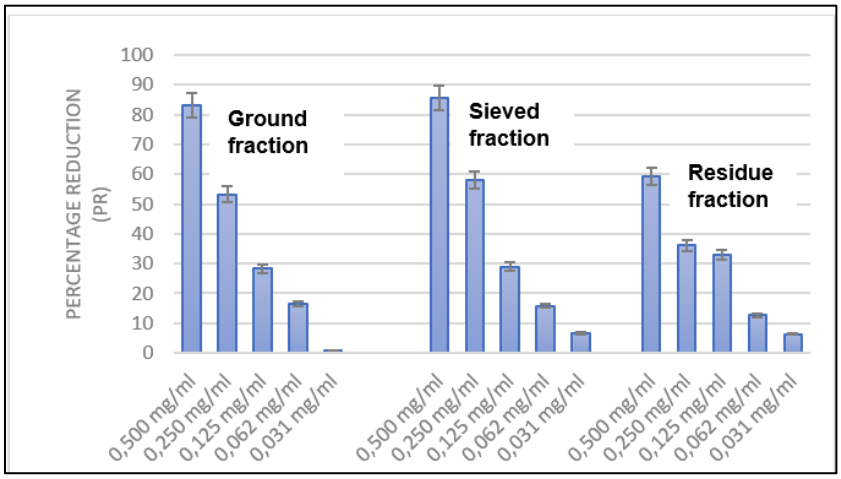

Figure 11 Percentage reduction in DPPH radical activity by ethanolic extracts of *Manihot esculenta* leaves

To evaluate the in vitro antioxidant activity of the ground fraction, sieved fraction, and residue from *M. esculenta* against the DPPH radical, ascorbic acid (vitamin C) was used as a reference antioxidant. Based on the percentages reduction (PR) obtained, the concentrations reducing radical activity by 50% (CR_{50}) were determined graphically from the trend curves plotted in Excel, by solving the linear equation $y = ax + b$ for $y = 50$. The mean CR_{50} values thus obtained for the plant extracts and vitamin C are shown in Table 3.

Table 3 CR_{50} values for *Manihot esculenta* leaf powders and vitamin C

	Ground fraction	**Sieved fraction**	**Residue fraction**	**Vit. C**
CR_{50} (mg/mL)	0.229	0.215	0.403	0.002

Analysis of in vitro antioxidant activity, assessed using the DPPH free radical assay and expressed as the CR_{50} value, reveals a significant variation in scavenging capacity depending on the *Manihot esculenta* fraction studied. It should be noted, however, that a low CR_{50} indicates high antioxidant activity of the extract (Falleh et al., 2006). Vitamin C exhibits the highest activity with an CR_{50} of 0.002 mg/mL, confirming its role as a positive control and its high capacity to donate protons to free radicals (Brand-Williams et al., 1995). Among the plant samples, the sieved material performed best (0.215 mg/mL), followed by the ground fraction (0.229 mg/mL), while the residue exhibited the lowest activity (0.403 mg/mL). This hierarchy demonstrates the decisive influence of particle size: the fineness of the sieved material's particles provides a larger surface area, thereby promoting the solubilization of phenolic compounds and flavonoids known for their antioxidant capacity in cassava leaves (Wootton-Beard et al., 2011). These observations are consistent

with the work of Silva et al. (2007), who emphasize that reducing the size of plant particles optimizes the release of specialized bioactive metabolites through improved disruption of cell walls. Furthermore, recent studies suggest that sieving not only improves access to the compounds but can also concentrate certain essential secondary metabolites compared to raw pulped material (Ouattara et al., 2025). The residue, on the other hand, consisting mainly of fibrous and lignified fragments, has a lower content of extractable antioxidant phytochemicals, which logically results in lower antioxidant activity. Although *M. esculenta* powders are less potent than pure ascorbic acid, their fine fraction constitutes a promising natural source of antioxidants for nutritional or therapeutic applications aimed at combating oxidative stress (Ahuchaogu et al., 2025; Jie et al., 2025).

4. Conclusion

This study aimed to characterize, using liquid chromatography coupled with mass spectrometry (LC-MS), the phytochemical composition of ethanolic extracts derived from the leaves of *Manihot esculenta* and to compare their antioxidant activity according to the type of fraction. The extract corresponding to the residue was found to consist of only three major specialized metabolites (rutin, gallic acid, and caffeic acid), whereas the ground fraction exhibited a broader phytochemical diversity, including scopoletin, scopoline, and nicotiflorin. The sieved fraction, on the other hand, exhibited an intermediate profile, lacking hydroxycoumarins but rich in phenolic compounds. The in vitro DPPH assay revealed maximum antioxidant activity for the sieved fraction, followed by the ground fraction, while the refuse exhibited significantly lower antioxidant activity. Taken together, these results highlight the decisive influence of particle size fractionation on the phytochemical composition and bioactive properties of *M. esculenta* extracts.

Compliance with ethical standards

Disclosure of conflict of interest

This manuscript has been reviewed and approved by all authors, and none of the results presented here have been published elsewhere. The authors have declared that there are no conflicts of interest.

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