RESEARCH PAPER

LC-MS/MS-Based Phytochemical Profiling and Tentative Identification of Flavonoids and Polyphenols in Echinochloa frumentacea Seeds

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Abstract

Introduction: Echinochloa frumentacea (barnyard millet) is an underutilized cereal known for its rich nutritional and phytochemical composition. It has gained attention as a potential functional food due to the presence of bioactive compounds such as flavonoids and polyphenols. However, detailed characterization of these compounds using advanced analytical techniques remains limited. The present study aimed to investigate the phytochemical profile of E frumentacea seeds using LC-MS/MS analysis.

Materials and Methods: The seeds of E. frumentacea were shade-dried, powdered, and extracted using ethanol through maceration. The crude extract was subjected to LC-MS/MS analysis using a high-resolution ESI-Q-TOF system operating in positive ionization mode. Chromatographic separation was achieved on a C18 column using a water-methanol mobile phase with formic acid and ammonium formate. The mass spectral data were analyzed based on m/z values, retention time, and fragmentation patterns for tentative identification of phytoconstituents.

Results: The LC-MS chromatogram revealed multiple peaks across a retention time range of 1-40 minutes, indicating a complex phytochemical composition. Prominent ions were observed at m/z 287.0788 and 301.0903, corresponding to flavonol-type compounds such as kaempferol and quercetin derivatives. Fragmentation of m/z 301 produced characteristic ions at m/z 287 and 161, supporting flavonoid identification. High-intensity peaks at m/z 337.2949 and 339.3143 indicated the presence of methoxylated flavonoids. Additionally, high molecular weight ions at m/z 520.3622 and 522.3871 suggested flavonoid glycosides. The relative peak area analysis showed that compounds at RT 40.35 min (15.86%), m/z 337.29 (6.20%), and m/z 339.31 (5.64%) were among the most abundant constituents.

Discussion: The detection of flavonol cores, methoxylated derivatives, and glycosylated flavonoids highlights the chemical diversity of E. frumentacea. Methoxylation and glycosylation are known to enhance stability, solubility, and biological activity of flavonoids, suggesting potential functional relevance. The observed fragmentation patterns

further strengthen the tentative identification of these compounds. However, the study is limited to qualitative profiling and requires further validation through advanced spectroscopic techniques.

Conclusion: The LC-MS/MS analysis demonstrated that E. frumentacea seeds are a rich source of structurally diverse flavonoids and polyphenols. The presence of flavonol derivatives, methoxylated flavonoids, and glycosides indicates significant nutraceutical potential. Further studies involving quantitative estimation and biological evaluation are necessary to confirm their therapeutic relevance.

Keywords: Echinochloa frumentacea, Barnyard millet, LC-MS/MS, Flavonoids, Polyphenols, Phytochemical profiling

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1. Introduction

Millets are a group of small-seeded cereal crops that have gained considerable attention in recent years due to their exceptional nutritional composition, environmental resilience, and potential health benefits. Among these, *Echinochloa frumentacea* (barnyard millet) is an ancient crop cultivated predominantly in Asia, particularly in India, China, and Japan, and is known for its adaptability to diverse agro-climatic conditions, including drought-prone and marginal soils [1, 2]. Its short growth cycle, low input requirements, and tolerance to environmental stress make it an important crop for sustainable agriculture and food security in the context of climate change [1,3].

Barnyard millet is recognized as a nutritionally rich cereal, containing significant amounts of proteins, dietary fiber, essential amino acids, vitamins, and minerals such as iron, calcium, and magnesium [2,4]. In addition to its nutritional value, it is gluten-free, making it a suitable dietary alternative for individuals with celiac disease and gluten intolerance [2]. Recent studies have also highlighted its role as a functional food due to the presence of various bioactive phytochemicals that contribute to its health-promoting properties [5].

Phytochemicals, particularly phenolic compounds and flavonoids, are secondary metabolites widely distributed in plant-based foods and are known for their antioxidant, anti-inflammatory, antidiabetic, and cardioprotective activities [6]. Millets, including *E. frumentacea*, have been reported to contain diverse classes of flavonoids such as quercetin, kaempferol, catechin, apigenin, and luteolin, which contribute significantly to their biological activities [5, 7]. These compounds play a crucial role in scavenging free radicals, reducing oxidative stress, and preventing chronic diseases such as diabetes, cancer, and cardiovascular disorders [6, 8].

Recent research has demonstrated that barnyard millet possesses a considerable amount of total phenolic and flavonoid content, which is strongly associated with its antioxidant capacity [9]. The presence of polyphenols, tannins, and flavonoids in millet grains has been linked to improved metabolic health and immune function, further emphasizing their nutraceutical potential [8, 10]. However, the complexity and diversity of these phytochemicals require advanced analytical techniques for accurate identification and characterization.

Liquid chromatography-mass spectrometry (LC-MS/MS) has emerged as a powerful and reliable tool for the profiling and identification of phytochemicals in plant extracts due to its high sensitivity, selectivity, and ability to provide structural information through fragmentation patterns [11]. This technique allows for the detection of both low and high molecular weight compounds, including flavonoids and their glycosylated derivatives, thereby facilitating comprehensive phytochemical analysis.

Despite the recognized nutritional and pharmacological importance of *E. frumentacea*, there is limited literature focusing on detailed LC-MS/MS-based phytochemical profiling of its seed extract. Most previous studies have primarily focused on total phenolic and flavonoid estimation or general antioxidant activity, with insufficient emphasis on compound-level identification and structural characterization [9, 12]. Therefore, there is a need for systematic investigation using advanced analytical approaches to better understand the phytochemical composition of this underutilized millet.

In this context, the present study aims to perform LC-MS/MS-based phytochemical profiling of the ethanolic seed extract of *E. frumentacea*, with a particular focus on the identification of flavonoids and polyphenolic compounds. The findings of this study are expected to provide scientific insights into the chemical composition of barnyard millet and support its potential application as a functional food and nutraceutical resource.

2. Materials and Methods

2.1 Chemicals and Reagents

Analytical grade ethanol (≥99.9%) was procured from Merck (India). HPLC-grade methanol, acetonitrile, formic acid, and ammonium formate used for LC-MS analysis were obtained from standard commercial suppliers and were of LC-MS grade. Dimethyl sulfoxide (DMSO) was used for sample preparation. All reagents and solvents were used without further purification.

2.2 Plant Material Collection and Authentication

Indian barnyard millet (*E. frumentacea*) seeds were collected in October 2025 from Garhwal Himalayas, Uttarakhand, India. The collected plant material was thoroughly cleaned to remove extraneous matter and stored at room temperature under conditions protected from direct sunlight and excessive humidity until further processing. The plant specimens were submitted to the Raw Materials Herbarium and Museum Division (RHMD), CSIR-National Institute of Science Communication and Policy Research (CSIR-NISCPR), New Delhi, for botanical identification and authentication. The authentication was carried out and a voucher specimen was deposited in the herbarium for future reference.

2.3 Preparation of Plant Extract

The collected seeds were thoroughly washed with distilled water to remove adhering impurities and shade-dried at room temperature (25 ± 2ºC) until a constant weight was achieved. The dried seeds were then pulverized into a coarse powder using a mechanical grinder and stored in an airtight container until extraction [13, 14]. Approximately 50 g of the powdered plant material was subjected to maceration with 500 mL of ethanol (1:10 w/v) in a closed container for 72 hours at room temperature (25 ± 2ºC) with intermittent shaking to facilitate efficient extraction of phytoconstituents. The extraction process was repeated twice under identical conditions to ensure maximum recovery of bioactive compounds, and the combined extracts were pooled. The extract was filtered using Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure using a rotary vacuum evaporator at a controlled temperature (below 45°C) to avoid thermal degradation of thermolabile compounds. The concentrated extract was further dried to constant weight to obtain a semi-solid crude extract. The percentage yield of the extract was calculated with respect to the initial dry weight of the plant material and was found to be 13.48% (w/w). The dried extract was stored in an amber-colored airtight container at 4ºC until further analysis [15, 16].

2.4 LC-MS/MS Analysis

2.4.1 Instrumentation and Chromatographic Conditions

Phytochemical profiling of the ethanolic extract of *E. frumentacea* seeds was carried out at the Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh, using a Bruker Daltonik Impact II electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer coupled with a Bruker Elute UHPLC system (Bremen, Germany) [17-19]. Chromatographic separation was achieved on a reversed-phase C18 column (100 x 2.1 mm, 1.8 µm particle size) maintained at 30°C. The mobile phase consisted of water and methanol in a ratio of 90:10 (v/v), supplemented with 0.1% formic acid and 5 mM ammonium formate [20-22]. The analysis was performed under isocratic elution conditions at a flow rate of 0.3 mL/min, with a total run time of 20 minutes for each sample. The autosampler temperature was maintained at 8°C, and an injection volume of 3 µL was used [23]. All solvents employed were of LC-MS grade and were filtered prior to use. Each sample was analyzed under identical experimental conditions to ensure reproducibility of chromatographic separation and mass spectral data.

2.4.2 Mass Spectrometry Conditions

Mass spectrometric detection was carried out using an electrospray ionization (ESI) source operated in positive ion mode (ESI+), as this mode is suitable for the ionization of flavonoids and polyphenolic compounds [21, 24]. The mass spectrometer was operated over a scan range of m/z 50-1000 to allow detection of both low and

high molecular weight phytoconstituents [17]. The capillary voltage was maintained at approximately 4.5 kV. Nitrogen was used as both nebulizing and drying gas, with a nebulizer pressure of 2.0 bar and a drying gas flow rate of 8 L/min. The drying gas temperature was set at 200°C to ensure efficient desolvation of ions. Data acquisition was performed in full scan mode along with auto MS/MS (data-dependent acquisition) to obtain fragmentation patterns of the detected ions [25, 26]. These fragmentation spectra were used for the tentative identification of compounds based on characteristic product ions and neutral loss patterns. All mass spectrometric parameters were optimized to achieve maximum sensitivity and stable ionization of analytes. Instrument control and data acquisition were performed using Compass Data Analysis software.

2.4.3 Sample Preparation for LC-MS

The dried extract was accurately weighed and dissolved in a minimal volume of dimethyl sulfoxide (DMSO) to ensure complete solubilization. The solution was then diluted with LC-MS grade acetonitrile to obtain a final concentration of 1 mg/mL. The mixture was vortexed and sonicated for 10 minutes to facilitate complete dissolution of phytoconstituents. The prepared solution was centrifuged at 4000 rpm for 5 minutes to remove insoluble particulate matter. The clear supernatant was carefully collected and further filtered through a 0.22 µm syringe filter to ensure removal of fine particulates and to protect the LC column. A 3 µL aliquot of the filtered sample was injected into the LC-MS system for analysis. All sample preparation steps were performed under controlled conditions to minimize contamination and ensure reproducibility of the analytical results [17, 27].

2.5 Data Analysis and Compound Identification

The acquired LC-MS/MS data were processed using Compass Data Analysis software. Chromatographic peaks were detected and integrated based on retention time (RT), mass-to-charge ratio (m/z), and peak intensity obtained from the total ion chromatogram (TIC). Tentative identification of phytoconstituents was carried out by analyzing accurate mass measurements, isotopic distribution patterns, and MS/MS fragmentation spectra [28, 29]. The observed m/z values were compared with previously reported data and available literature on flavonoids and polyphenolic compounds. Fragmentation patterns were carefully examined to identify characteristic product ions and neutral losses, which are diagnostic for specific classes of phytochemicals.

For flavonoids, typical fragmentation pathways such as Retro-Diels-Alder (RDA) cleavage, loss of carbon monoxide (CO), and loss of small neutral molecules (e.g., CH3, H_2O) were considered. The presence of product ions at m/z 287 from a parent ion at m/z 301 was interpreted as a characteristic fragmentation pattern of quercetin-like flavonols. Similarly, ions detected at m/z 287 were attributed to kaempferol-type structures based on their mass and fragmentation behavior. High molecular weight ions observed in the range of m/z 520-522 were interpreted as flavonoid glycosides, supported by the presence of fragment ions corresponding to the loss of sugar moieties (e.g., 162 Da for hexose units). Methoxylated flavonoids were inferred from mass shifts of + 14 Da, indicating methyl substitution on the flavonoid backbone [30, 31].

The relative abundance of detected compounds was estimated using peak area percentage obtained from the chromatographic data. Major peaks were identified based on their higher area percentages, indicating dominant phytoconstituents in the extract. The identification of compounds in this study was considered tentative and corresponds to Level 2/Level 3 confidence according to the Metabolomics Standards Initiative (MSI).

3. Results

3.1 LC-MS Chromatographic Profile

The LC-MS analysis of the ethanolic extract of *E. frumentacea* seeds, performed in positive electrospray ionization mode (ESI+), revealed a complex chromatographic profile characterized by multiple peaks distributed across the retention time (RT) range of 1-40 minutes. The total ion chromatogram (TIC) indicated the presence of a diverse range of phytoconstituents with varying polarities and molecular weights (fig. 1). Numerous peaks were detected throughout the chromatographic run, among which several peaks exhibited relatively higher abundance. The most intense peak was observed at RT 40.35 min with an area percentage of 15.86%, followed by peaks at RT 26.59 min (8.34%), RT 22.13 min (6.20%), and RT 27.82 min (5.64%). These peaks correspond to the major components of the extract based on their relative abundance in the chromatogram.

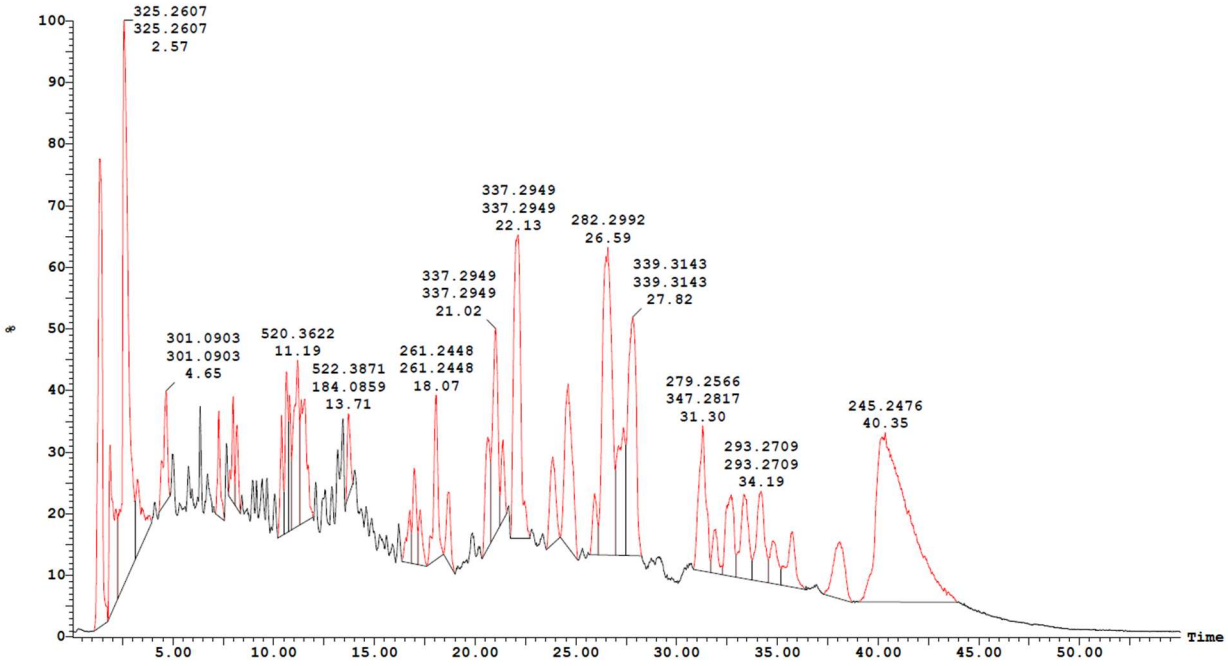

Figure 1. Total ion chromatogram (TIC) of the ethanolic extract of *E. Frumentacea* seeds obtained using LC-MS analysis in positive electrospray ionization (ESI+) mode

Table 1. LC-MS Profile of *E. frumentacea* Seed Extract

S. No.	RT (min)	m/z (ES+)	Area (%)	Tentative Identification	Compound Class
1	3.24	287.0788	1.33	Kaempferol-like compound	Flavonol
2	4.65	301.0903	1.36	Quercetin-like compound	Flavonol
3	7.99	291.1175	0.69	Flavonoid derivative	Flavonoid
4	11.19	520.3622	2.50	Flavonoid glycoside	Glycosylated flavonoid
5	11.53	520.3622	2.32	Flavonoid glycoside	Glycosylated flavonoid
6	13.71	522.3871	0.72	Flavonoid glycoside	Glycosylated flavonoid
7	18.07	261.2448	2.05	Polyphenolic compound	Polyphenol
8	21.02	337.2949	3.07	Methoxylated flavonoid	Flavonoid
9	22.13	337.2949	6.20	Major methoxylated flavonoid	Flavonoid
10	27.82	339.3143	5.64	Flavonoid derivative	Flavonoid
11	34.19	293.2709	2.12	Polyphenolic compound	Polyphenol
12	38.10	307.2890	1.86	Flavonoid-range compound	Flavonoid
13	40.35	-	15.86	Major unidentified compound	Unknown
14	26.59	-	8.34	Major unidentified compound	Unknown

3.2 Identification of Flavonoid and Polyphenolic Compounds

The LC-MS/MS analysis (Table 1) suggested the presence of multiple phytoconstituents belonging to flavonoid and polyphenolic classes based on their mass spectral characteristics and fragmentation patterns. Prominent ions were detected at m/z 287.0788 (RT 3.24 min) and m/z 301.0903 (RT 4.65 min), which fall within the characteristic mass range of flavonol aglycones (fig. 2). The ion at m/z 301.0903 exhibited diagnostic fragment ions at m/z 287.0729 and 161.0722 (fig. 3). The fragment at m/z 287 corresponds to the loss of a neutral CO group (-28 Da), which is a well-established fragmentation

pathway of flavonols, while the fragment at m/z 161 is indicative of Retro-Diels-Alder (RDA) cleavage of the flavonoid C-ring. These fragmentation features are consistent with a quercetin-like structure. Similarly, the ion at m/z 287.0788 was tentatively assigned to a kaempferol-type flavonoid based on its molecular ion and typical flavonol fragmentation behavior.

High-intensity peaks observed at m/z 337.2949 (RT 21.02 and 22.13 min) and m/z 339.3143 (RT 27.82 min) were attributed to methoxylated flavonoid derivatives. The mass shift of +14 Da relative to flavonol cores suggests methyl substitution on the hydroxyl groups of the flavonoid backbone, a modification known to enhance lipophilicity and stability. The relatively higher peak areas of these ions indicate that methoxylated flavonoids are among the major constituents of the extract.

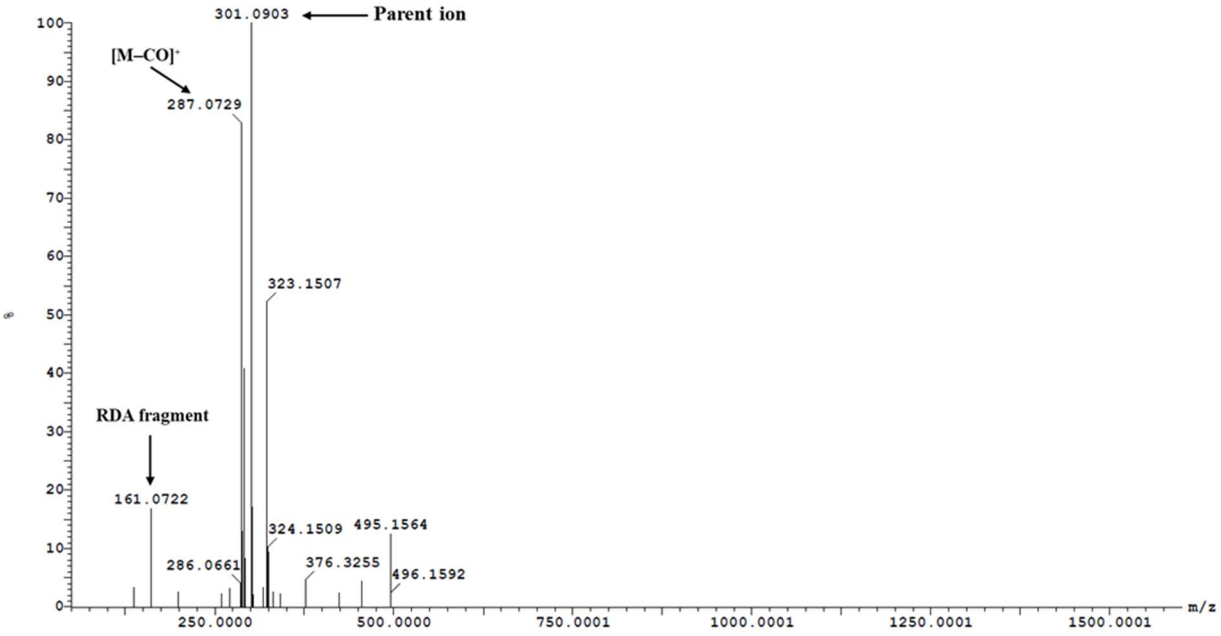

Figure 2. MS/MS Spectrum of the iron at m/z 301.0903 obtained in positive electrospray ionization mode (ESI+) corresponding to quercetin-like flavonoid

3.3 Detection of Glycosylated Flavonoids

In addition to flavonol aglycones, several high molecular weight ions were detected in the range of m/z 520-522 at RT 11.19, 11.53, and 13.71 min (Table 1). These ions are consistent with the presence of flavonoid glycosides (fig. 4). The MS/MS spectra of these ions exhibited characteristic fragmentation patterns corresponding to the neutral loss of sugar moieties. In particular, a mass loss of approximately 162 Da was observed, which is typically associated with the cleavage of a hexose unit (e.g., glucose). The resulting fragment ions correspond to flavonoid aglycones, supporting the presence of glycosylated derivatives.

Furthermore, the appearance of fragment ions in the lower m/z range, consistent with flavonol cores, indicates that these glycosides are likely conjugated forms of flavonoid aglycones such as kaempferol or quercetin derivatives. Glycosylation is a common modification in plant secondary metabolites and is known to enhance solubility, stability, and bioavailability of flavonoids. The detection of these glycosylated compounds reflects the structural diversity and complexity of phytochemicals present in the extract.

3.4 Presence of Additional Polyphenolic Compounds

In addition to flavonoid derivatives, other ions were detected at m/z 261.2448 (RT 18.07 min) and m/z

293.2709 (RT 34.19 min) (Table 1), suggesting the presence of additional polyphenolic constituents. The MS/MS spectra of these ions exhibited fragmentation patterns characteristic of phenolic compounds, including the loss of small neutral molecules such as carbon monoxide (CO, 28 Da) and carbon dioxide (CO2, 44 Da), which are commonly observed in polyphenolic structures. The presence of fragment ions in the lower m/z range further supports their classification as low molecular weight phenolic derivatives.

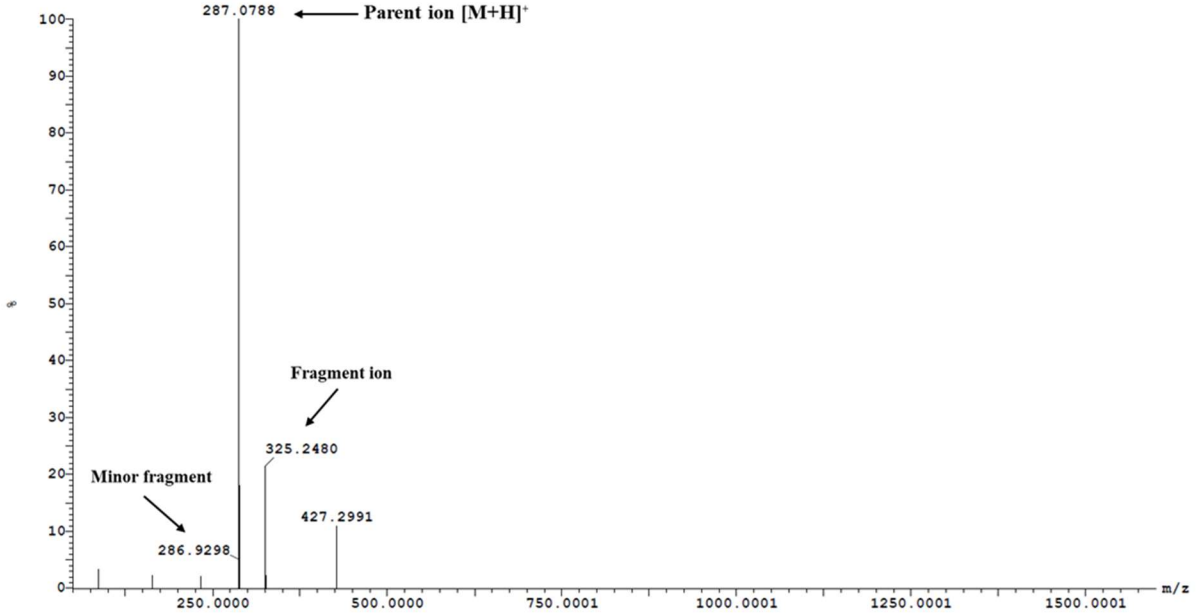

Figure 3. MS/MS Spectrum of the iron at m/z 287.0788 obtained in positive electrospray ionization mode (ESI+) corresponding to kaempferol-like flavonoid

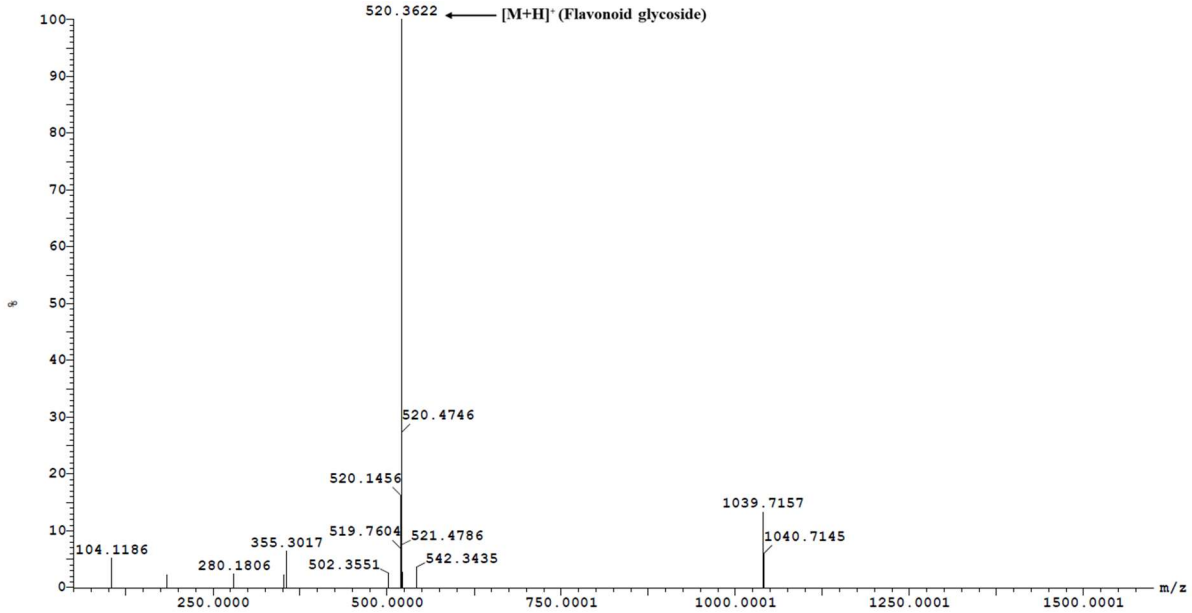

Figure 4. MS/MS Spectrum of the iron at m/z 520.3622 obtained in positive electrospray ionization mode (ESI+) corresponding to a flavonoid glycoside

Although these ions could not be assigned to specific compounds due to limited structural information, their mass spectral behavior is consistent with phenolic acids or related polyphenolic compounds. These constituents contribute to the overall phytochemical diversity of the extract and may play a role in its potential biological activity. However, the identification of these compounds remains tentative and corresponds to Level 2/Level 3 confidence according to Metabolomics Standards Initiative (MSI) guidelines.

3.5 Relative Abundance of Major Compounds

The relative abundance of detected compounds was estimated based on peak area percentage obtained from the total ion chromatogram (TIC) (Table 1). It should be noted that these values represent relative rather than absolute concentrations of the compounds. Among the detected peaks, the highest abundance was observed at RT 40.35 min (15.86%), corresponding to a major yet unidentified compound. This was followed by peaks at RT 26.59 min (8.34%) and methoxylated flavonoid derivatives at m/z 337.2949 (RT 22.13 min, 6.20%) and m/z 339.3143 (RT 27.82 min, 5.64%).

Flavonol-type compounds at m/z 287.0788 (RT 3.24 min) and m/z 301.0903 (RT 4.65 min) exhibited comparatively lower peak areas, indicating moderate relative abundance. Similarly, glycosylated flavonoids detected in the m/z 520-522 range showed lower but appreciable abundance within the extract. Overall, the LC-MS/MS data indicates a diverse phytochemical composition dominated by methoxylated flavonoids and other unidentified constituents, along with the presence of flavonol aglycones, glycosylated flavonoids, and additional polyphenolic compounds.

4. Discussion

The present study provides a comprehensive LC-MS/MS-based phytochemical profiling of the ethanolic extract of *E. frumentacea* seeds, revealing a diverse array of flavonoids and polyphenolic compounds [32, 33]. The chromatographic and mass spectral data collectively indicate that the extract is rich in flavonol aglycones, methoxylated derivatives, glycosylated flavonoids, and other phenolic constituents, which are known to possess significant biological activities. The detection of ions at m/z 301.0903 and 287.0788, tentatively assigned to quercetin- and kaempferol-like compounds, respectively, highlights the presence of flavonol cores in the extract [34, 35].

These compounds are well documented for their antioxidants, neuroprotective, and anti-inflammatory properties. The observed fragmentation patterns, including the loss of carbon monoxide (-28 Da) and Retro-Diels-Alder (RDA) cleavage, are consistent with previously reported fragmentation pathways of flavonoids, supporting their tentative identification. In addition to aglycones, the presence of high molecular weight ions in the range of m/z 520-522 suggests the occurrence of glycosylated flavonoids. The characteristic neutral loss of 162 Da observed in the MS/MS spectra corresponds to the cleavage of hexose units, indicating that these compounds are likely flavonoid glycosides [36, 37]. Glycosylation is known to enhance the solubility, stability, and bioavailability of flavonoids, thereby influencing their pharmacokinetic and pharmacodynamic properties.

The identification of methoxylated flavonoids, particularly ions at m/z 337.2949 and 339.3143, further adds to the phytochemical complexity of the extract. Methylation of flavonoids has been associated with increased metabolic stability and improved membrane permeability, which may enhance their biological efficacy. The relatively higher abundance of these methoxylated derivatives suggests that they may contribute significantly to the overall bioactivity of the extract [38]. Furthermore, the detection of additional polyphenolic compounds at m/z 261.2448 and 293.2709 indicates the presence of structurally diverse phenolic constituents. These compounds, although not fully characterized, may act synergistically with flavonoids to exert antioxidant and protective effects. The coexistence of multiple classes of phytochemicals reflects the complex chemical composition of *E. frumentacea* seeds. The relative abundance analysis revealed that a major unidentified compound at RT 40.35 min exhibited the highest peak area (15.86%), suggesting that it may

represent a dominant constituent of the extract [39, 40]. The presence of such unidentified compounds highlights the need for further structural elucidation using advanced spectroscopic techniques such as nuclear magnetic resonance (NMR) and comparison with authentic standards. Overall, the phytochemical profile obtained in this study is consistent with previous reports indicating that millets, including Echinochloa species, are rich sources of bioactive phenolic compounds. The presence of flavonoids and their derivatives may underlie the reported antioxidant and therapeutic potential of barnyard millet. However, it is important to note that the identification of compounds in the present study is tentative and based on LC-MS/MS data. Further confirmation through isolation and structural characterization is required. Despite this limitation, the study provides valuable insights into the chemical composition of E. frumentacea seeds and lays the foundation for future investigations into their pharmacological potential.

5. Conclusion

The present study provides a comprehensive LC-MS/MS-based phytochemical characterization of the ethanolic extract of *E. frumentacea* seeds. The analysis revealed the presence of diverse bioactive constituents, including flavonol aglycones, methoxylated flavonoids, glycosylated derivatives, and other polyphenolic compounds. The identification of quercetin- and kaempferol-like structures, along with glycosylated and methylated flavonoid forms, highlights the chemical complexity and richness of the extract. The observed fragmentation patterns, such as neutral loss of sugar moieties and characteristic flavonoid cleavages, support the tentative assignment of these compounds. The relative abundance analysis further indicates that methoxylated flavonoids and certain unidentified constituents may play a dominant role in the phytochemical profile of the extract.

Overall, the findings suggest that *E. frumentacea* seeds are a promising source of bioactive phytochemicals with potential therapeutic relevance. However, the identification of compounds in this study remains tentative, and further investigations involving isolation, structural elucidation, and biological evaluation are required to confirm their pharmacological potential. This study establishes a scientific basis for future research on barnyard millet and supports its potential application in functional foods and nutraceutical development.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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