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Research Article

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Characterization of Azolla Pinnata Used as Antioxidant in Poultry Diet and Optimization of The Method of Analysis of Its Caffeic Acid Content by LC-MS/MS

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Abstract: In a trial to determine the active ingredients of Azolla pinnata, both GC-MS/MS and LC-MS/MS were used. The result of GC-MS/MS analysis demonstrated that, 9-Octadecenoic acid was the most predominant compound followed by 3,4,2',4',6'-Pentamethoxychalcone and 5-Hydroxy-7-methoxyflavone. Using LC-MS/MS to determine liquid soluble compounds revealed that, Caffeic acid, chlorogenic acid, coumaroylquinic acid, and dicaffeoylquinic acid were the predominant compounds. Validation of LC-MS/MS analytical method was performed using Caffeic acid standard solution at different concentrations which resulted in determination of recovery percentage, limit of detection, limit of quantitation and building of standard curve which then was used to measure Caffic acid content in the examined Azolla pinnata extract. This method meets validation requirements for the quantitative analysis of Caffeic acid.

Keywords: Azolla, Phenolic compounds, Caffeic acid, LC-MS/MS, validation, antioxidant

1. Introduction

The primary sources of protein in a chicken's feed are typically corn and soya bean, which form the main components of their dietary intake [1]. In recent times, research efforts have shifted towards identifying cost-effective, safe, and high-quality substitutes for feed raw materials, which are currently in short supply. One promising alternative is Azolla (Azolla pinnata), an aquatic plant rich in protein that thrives in freshwater environments. This plant exhibits rapid growth, with its biomass doubling within a span of two to three days [2]. This has been extensively utilized as a substitute feed resource for farm animals, including poultry, as it contributes to reducing production expenses and consequently enhancing overall profitability [2, 3 and 4]. Studies have shown that Azolla meal can be used to substitute up to 5-10% of protein sources in poultry diets without negatively impacting the birds' health or performance [3]. The protein content found in Azolla has been determined to fall between 21 and 30% [4] containing almost all essential amino acids. Furthermore, Azolla serves as an abundant source of carotene and various growth-promoting elements including calcium, phosphorus, magnesium, potassium, iron, and copper.

In addition to its use as an antioxidant in poultry nutrition due to its high levels of phenolic compounds and flavonoids [3 and 5], Azolla serves another important function. Antioxidants play a crucial role in safeguarding the liver against

damage induced by chemicals. They achieve this by impeding the production of free radicals through the stabilization of cellular antioxidant systems [6].

The liver plays a crucial role in the body by managing metabolism, secretion, storage, and detoxification processes. Damage to the liver often results in disruption of these essential functions. Oxidative injury, which occurs when there is an imbalance between oxidative forces and antioxidant defense mechanisms, has been linked to various health conditions. These include atherosclerosis, diabetes, cancer, and liver cirrhosis [6].

Determination of nutritional composition of Azolla gives an informative output to enable good utilization of this feed source in the formulation of optimum diet. Also, characterization of phenolic and flavonoid content of this feed material gives very important data that facilitates understanding and estimating its mode of action. Several studies have reported the nutritional content of Azolla including Crude Protein, Ether Extract, Crude Fiber, Ash content, Moisture Content [7, 8 and 9], Phenolic compound and flavonoids [10, 11 and 12] in Azolla, but it is crucial to determine the nutritional and chemical content of the source under study to be able to judge its ability for improving the performance and general condition of the target species.

Mass spectrometry is considered as one of the most sophisticated approaches that can determine and identify active ingredients present in feed materials. This technique is valuable for quickly examining the components of complex mixtures, verifying compound identities through product ion scanning, identifying specific product ions (precursor ion mode) or charged fragments from neutral losses (neutral loss mode), and categorizing organic substances [13].

Caffeic acid is one of the effective active ingredients present in Azolla that has antioxidant activity [14]. The mode of action of Caffeic acid is due to the presence of -OH group in its structure [15]. Amount of Caffeic acid in Azolla differs according to the species [16] and its presence in valuable amounts in feed materials gives it a highly protective efficiency as it helps in liver and intestine protection by antioxidants scavenge free radicals and maintain the oxidative/antioxidative balance [17].

The aim of this study is to highlight additional studies on Azolla in order to better understand active compounds that have performance and health-promoting properties, as well as potential mechanisms of action in poultry. Additionally, the objective is to develop and validate an efficient, secure, and precise analytical method for Caffeic acid detection. This method should offer high sensitivity, accuracy, and reliability, enabling both qualitative and quantitative assessment of Caffeic acid.

2. Materials And Methods

One Kilogram of Azolla pinnata was purchased from private farm in El Sharkeya governorate and was botanically characterized at National Research Center (NRC), Egypt. LC-Grade methanol and formic acid were purchased from Merck, Mumbai, India. Water was obtained from a Milli-Q System. Phenomenex Luna C18, 2.1 x 150 mm, 5 µm column was used for of LC separation.

spectrometry analysis of Azolla pinnata extract:

I. Sample preparation according to [18]:

Five-gram samples were added to 75 ml of methanol and were soaked for 24 hours in dark place. The samples then were centrifuged at 10,000 rpm for 5 min and supernatant was collected and injected in LC-MS/MS.

II. GC-MS/MS analysis of extracted samples according to [19]:

The methanol extract underwent GC-MS analysis using a device fitted with a VF-5 ms fused-silica capillary column measuring 30 m in length, 0.25 mm in diameter, and 0.25 mm in film thickness. The detector employed an electron ionization system with 70 eV ionization energy. Helium gas (99.99%) served as the carrier gas, flowing at a steady rate of 1.51 mL/min. The injector and mass transfer line temperatures were maintained at 200ºC and 240ºC, respectively. The oven temperature was programmed to increase from 70ºC to 220ºC at 10ºC/min, held steady for 1 min, then raised to 300ºC at 10ºC/min. Samples were diluted and 2 mL aliquots were manually injected in split-less mode, with a 1:40 split ratio and a mass scan range of 50e600 AMU. The entire GC-MS analysis lasted 60 min.

III. LC-MS/MS analysis of extracted samples according to [18]:

A full scan was performed at one spectrum per second in the m/z range of 100-1000, followed by MS/MS fragmentation of the most intense precursor ions (only single charge) at different collision energy values.

- **Calibration, tuning, optimization, method elaboration and batch submission of Caffeic acid:**

Simple and specific liquid chromatography–tandem mass spectrometry (4000 QTRAP LC–MS/MS) method was used for detection of phenolic compounds in azolla extraction. The MS optimization was performed by direct infusion with the Pump HARVARD APPARATUS 11PLUS at a flow-rate of 0.2 mL/min. with an isocratic mobile phase composed of 5mM ammonium acetate: Methanol (30:70 v/v).

The compound optimization procedure was established by following the instructions outlined in the users' manual, which included activating compound optimization for both negative and positive ion modes. Compound-specific parameters were configured using the designated software (Analyst®) as per the manual's guidelines. To initiate a batch and acquire data, an acquisition method was developed for both LC and MS in accordance with the manual's instructions. This method incorporated parent masses, daughter masses, DP, CE, and CXP. The analysis utilized a Phenomenex Luna C18 column (2.1 x 150 mm, 5 µm) with an isocratic mobile phase consisting of 5mM ammonium acetate and methanol (30:70 v/v), flowing at a rate of 0.5 ml/min [20 and 21].

- **Determination of Signal to Noise ratio, LOD and LOQ for Caffeic acid compound [20 and 21]:**

1. LC-MS/MS analysis was conducted on a 50 ng/ml reference solution of Caffeic acid, utilizing all relevant parameters.
2. The Signal to Noise (S/N) ratio was determined according to the instructions in the users' manual. The standard solution underwent sequential dilution to achieve an S/N ratio of 3, which was designated as the Limit of Detection (LOD), and an S/N ratio of 10, which was established as the Limit of Quantitation.

- **Building calibration curves for Caffeic acid:**

To generate standard curves for Caffeic acid, various concentrations (10, 25, 50 and 100 ng/ml) were introduced into the MS/MS system after passing through the LC separating column. These curves were then used to evaluate linearity and accuracy.

3. Results and Discussion

Recent research suggests Azolla may offer additional benefits beyond its basic nutritional value. Specifically, Azolla is increasingly recognized as a potential source of bioactive compounds, including phenolics and flavonoids, which possess potent antioxidant properties [4]. These antioxidant compounds are believed to play a crucial role in poultry health by scavenging free radicals and mitigating oxidative stress, ultimately contributing to improved performance and product quality [3].

However, a thorough understanding of the specific phenolic and flavonoid profiles of Azolla and their influence on poultry health requires the application of modern and sophisticated analytical techniques as traditional methods for characterizing these bioactive compounds often lack the sensitivity and specificity necessary for a comprehensive analysis [22]

In this study; (Table 1) illustrated the active ingredients determined in Azolla pinnata sample measured by GC-MS/MS. It is clear from the obtained data that, the tested samples were rich in many flavonoids and phenolic substances, with 9-Octadecenoic acid (Z) found to have the highest amount together with 3,4,2',4',6'-Pentamethoxychalcone and 5-Hydroxy-7-methoxyflavone, respectively. This was concluded by comparing the obtained peak area of all detected ingredients (Table 1 and Figure 1). The highest area percentage value was observed for 9-Octadecenoic acid, and this is consistent with the results of [23] study, which stated that the presence of this compound is mainly found in Azolla extract. Also, these findings was confirmed by that stated by [24, 25, 26 and 27] who found that 9-Octadecenoic acid, 3,4,2',4',6'-Pentamethoxychalcone and 5-Hydroxy-7-methoxyflavone are among the predominant active compounds of Azolla pinnata extract which have hepatic protection activity through its antioxidant effect.

Table 1: Name, peak area and retention time (RT) of detected active ingredients in tested A. pinnata analyzed by GC-MS/MS

Name	RT	Peak Area
7,2',3'-Trimethoxyflavanone	9.00	2.89
2'-Hydroxy-2,3,4'-trimethoxychalcone	10.26	5.16
3,4,2',4',6'-Pentamethoxychalcone	12.37	20.86
5-Hydroxy-7-methoxyflavone	13.45	15.03
9-Octadecenoic acid (Z)	14.49	39.17
3,4-Dimethoxy-2'-(acetyl)oxy-5'-methylchalcone	16.55	9.40
Kaempferol 3-methyl ether	18.78	0.81
4',6'-Dimethoxy-2'-hydroxy-4-methylchalcone	20.44	0.40
3',4',5,7-Tetrahydroxy-3-methoxyflavone	21.52	6.28

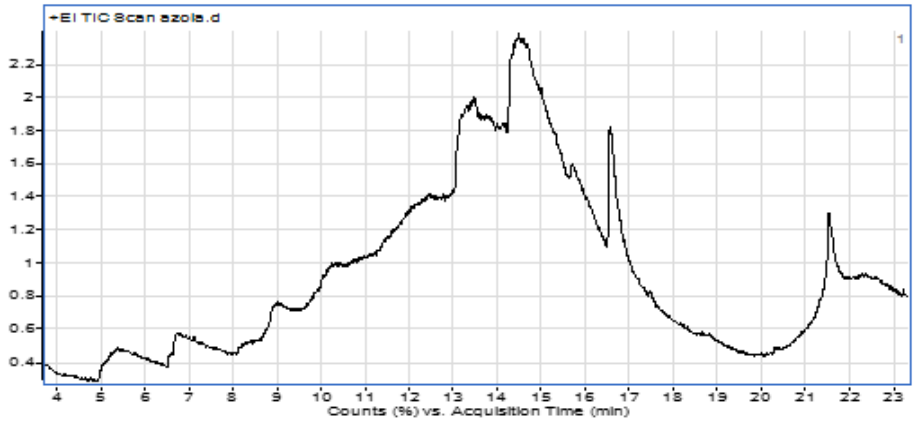


Figure 1: *Chromatogram of the scan of Azolla pinnata sample by GC-MS/MS*

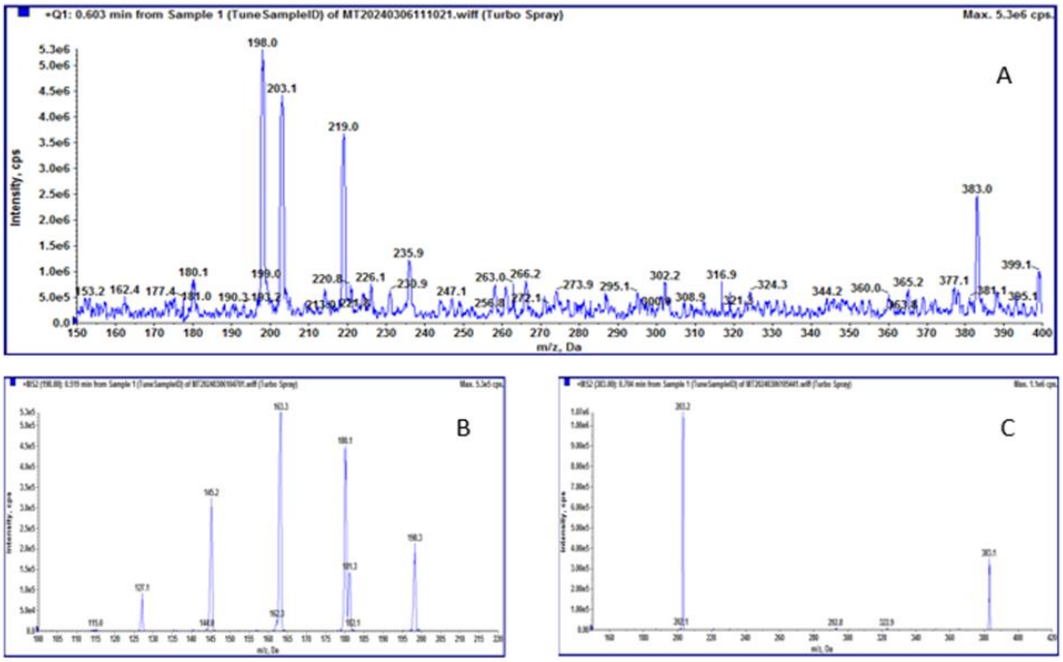


Figure 2: *Result of LC-MS/MS analysis of Azolla pinnata methanolic extract using positive ion mode: A) Chromatogram of scanning using Q1, B) MS2 illustrating parent ion of Glucose (179 m/z) and its fragments, C) MS2 illustrating parent ion of D-Glucose (383 m/z) and its fragments*

Many research work was performed to investigate the most predominant active compound that can be present in methanolic extract of A. pinnata and are soluble in mobile phase and subsequently can be detected by liquid chromatography. In this study, as illustrated in figure 2 A, positive ionization of A. pinnata extract using LC-MS/MS technique revealed the presence of Glucose and D Glucose in remarkable amounts indicated by their peak area. Confirmation and identification of these analytes was assured through the appearance of their daughter fragments that were illustrated in Figures 2 B and 2 C. This finding was explained by that obtained by [28 and 26] who clarified that, the presence of glucose may be present due to fragmentation of glycosides present in the phenolic compounds and flavonoids present in the extract which subsequently resulted in appearance of specific peaks related to Glucose and D-Glucose. Also, [29] concluded that, A, pinnata biomass can give high amount of Glucose when used as a source of energy by fermentation.

Using negative ionization mode on methanolic extract for the screening of phenolic compounds and flavonoids present in the type of fern under study revealed as illustrated in Figure 3A that, there were many active ingredients that could be screened by scanning. Some of these compounds were identified using fragmentation of the obtained parent ions according to [30]. The identified compounds were Caffeic acid, with peak at precursor ion 179.1 and fragment ion 135.1/179.1 (Figure 3B); Dicaffeoylquinic acid, with peak at precursor ion 337.1 and fragment ions 191.1/337.1, 173.1/337.1 and 93/337.1 (Figure 3C), Chlorogenic acid, with peak at precursor ion 353.1 and fragment ions 191.1/353.1, 179.1/353.1 and 161.1/353.1 (Figure 3D) and Coumaroylquinic acid, with peak at precursor ion 515.1 and fragment ions 353.1/515.1, 191.1/515.1, 179.1/515.1, 173.1/515.1 and 135.1/515.1 (Figure 3E). These data were confirmed by [18 and 27] who could state that, Caffeic acid, Dicaffeoylquinic acid, Chlorogenic acid and Coumaroylquinic acid are the most predominant compounds present in A. pinnata extract and have upregulating effect on immunity and hepatic protection related genes.

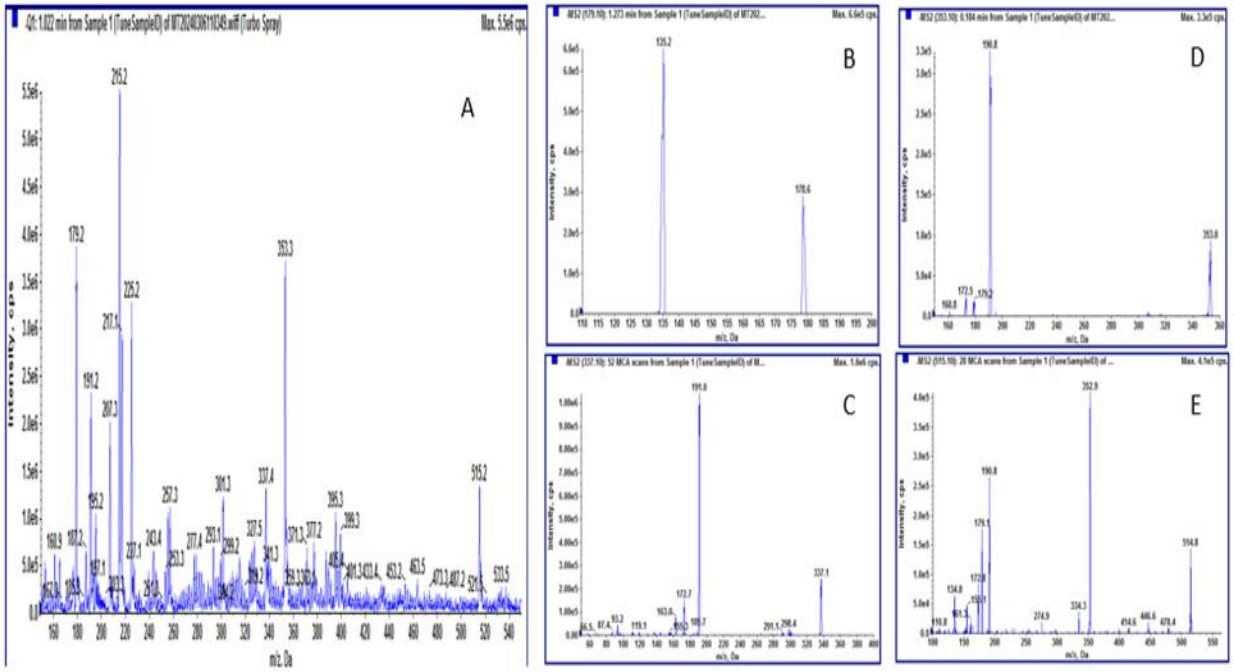


Figure 3: *Result of LC-MS/MS analysis of Azolla pinnata methanolic extract using negative ion mode: A) Chromatogram of scanning using Q1, B) MS2 illustrating parent ion of Caffeic acid (179 m/z) and its fragments, C) MS2 illustrating parent ion of Dicaffeolyquinic acid (337 m/z) and its fragments, D) MS2 illustrating parent ion of Chlorogenic acid (353 m/z) and its fragments and E) C) MS2 illustrating parent ion of Coumaryolquinic acid (515 m/z) and its fragments*

Caffeic acid, a naturally occurring phenolic compound, is garnering increasing attention in animal nutrition due to its potential health benefits. Research suggests it possesses antioxidant, antimicrobial, and immune-modulating properties [27]. However, accurately estimating its concentration in animal feed and food additives is crucial for optimizing its use and ensuring animal health. Here's where the validation of test methods and the power of LC-MS/MS come into play.

Several published research works highlight the significance of this analytical approach. Studies by [31 and 32] emphasize the need for validated test methods to ensure reliable detection and quantification of any analyte. These studies demonstrate that validated methods provide accurate and reproducible results, essential for formulating effective animal diets with targeted levels of Caffeic acid. Also, validating a test method within a specific laboratory environment is paramount for ensuring reliable data. While a published method might be well-established, factors like equipment variations, analyst experience, and even minor lab conditions can influence results. Validation verifies that the method performs as expected, assessing its sensitivity (ability to detect low levels) and specificity (ability to measure the target compound without interference from others) [33 and 34] showcases the advantages of LC-MS/MS for analyzing Caffeic acid and stated that, LC-MS/MS offers superior sensitivity and selectivity compared to traditional methods, allowing distinguishing Caffeic acid from other similar compounds present in a sample. This precise analysis is vital for understanding the true bioavailability of Caffeic acid in animal feed and its potential impact on animal health.

In this study, data illustrated in Figure 4A showed the parameters used in optimization and tuning of Caffeic acid. Parameters illustrated in Figures 4a and 4c were automatically elaborated after using auto tuning utility of the used instrument as clarified in the user's manual. The results of tuning, LC analysis of blank solution and retention time of Caffeic acid using inhouse parameters were illustrated in Figures 5A, B and C. It was clear from the obtained data that, the used parameters gave sensitive and specific results which were indicated by high intensity of the obtained peak in Figure 5A. Also, the method seemed to be specific as indicated by the negative response in the blank solution (Figure 5B) and also indicated the used solvents were free from being contaminated with the compound under investigation. The obtained retention time was 3.75 min as illustrated in Figure 5C. Confirmation of sensitivity, specificity and ability for high recovery rates were clear in the results obtained in Figure 6 as accuracy percent ranged from 98 to 107% and excellent linearity with regression value of 0.9999 demonstrating a good adherence to the linear model.

a

Ionization type	Electrospray (ESI)
Polarity	Negative
Injection volume	20 µl
Column temperature	35 ˚C
Ion spray voltage:	-4500
Temperature	400˚C
Gas	Curtain gas (20 psi),Gas1: (30 psi) Gas2: (30 psi)
Scan mode	MRM
Resolution	UNIT

b

Total time (min)	Flow rate (µl / min)	A%	B%
0	500	30	70
10	500	30	70

A: 5mM ammonium acetate in water **B:** 5mM ammonium acetate in MEOH

c

ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (volts)	CE (volts)	CXP (volts)
Caffeic acid	179.1	135.1	500	-40	-17	-5

Figure 4: *Parameters used in manual tuning and method validation of Caffeic acid: a. LC parameters, b. MS parameters and c. MS/MS Parameters*

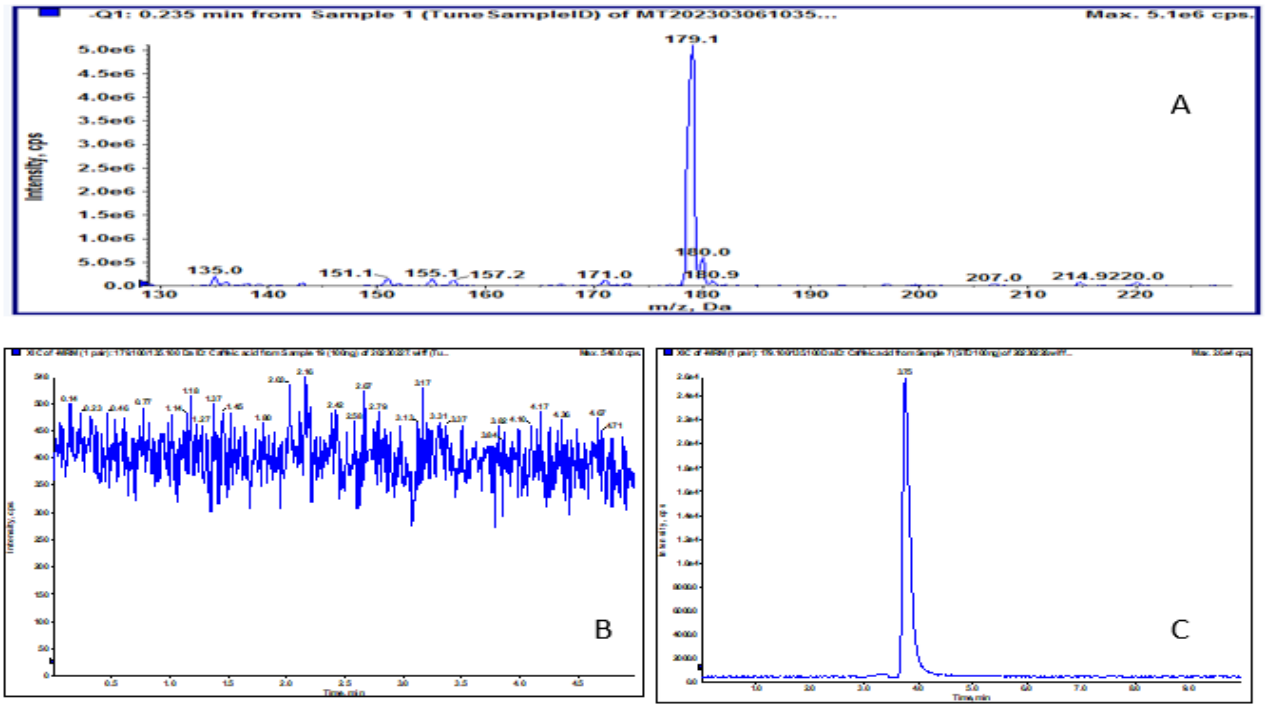
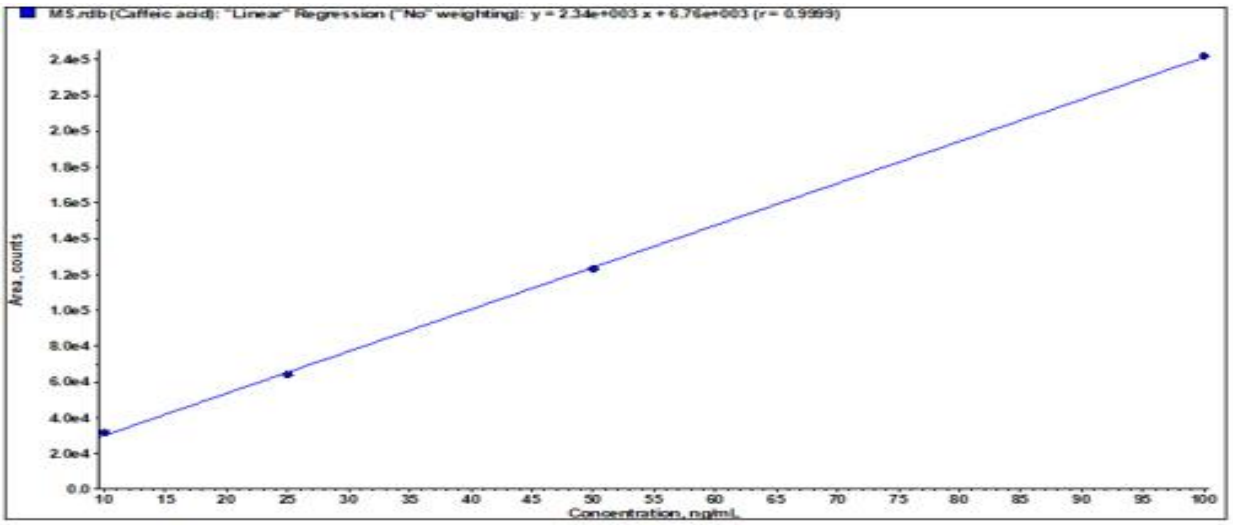


Figure 5: *Result of validation process for Caffeic acid: A) manual tuning B) LC analysis of blank solution C) LC analysis of Caffeic acid showing RT at 3.75 min*



	Sample Name	Sample Type	File Name	Analyte Peak Area (counts)	Analyte Peak Height (cps)	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
1	Blank	Blank	20230228.wiff	5.01e+003	2.88e+002	0.00	N/A	N/A
2	STD 10 ng	Standard	20230228.wiff	3.17e+004	3.55e+003	10.0	10.7	107.
3	STD 25 ng	Standard	20230228.wiff	6.42e+004	6.94e+003	25.0	24.5	98.0
4	STD 50 ng	Standard	20230228.wiff	1.23e+005	1.29e+004	50.0	49.6	99.1
5	STD 100ng	Standard	20230228.wiff	2.42e+005	2.46e+004	100.	100.	100.
6	AZOLLA R1	Unknown	20230228.wiff	1.20e+005	1.12e+004	N/A	48.2	N/A
7	AZOLLA R2	Unknown	20230228.wiff	1.12e+005	1.09e+004	N/A	44.7	N/A
8	AZOLLA R3	Unknown	20230228.wiff	1.20e+005	1.26e+004	N/A	48.2	N/A
9	SPIKE	Quality Control	20230228.wiff	2.07e+005	1.98e+004	0.00	85.3	N/A

Figure 6: *Chart of linearity and accuracy of Caffeic acid standard*

The software (Analyst®) used in the study determined the Limit of Detection (LOD) and Limit of Quantification (LOQ) as 3 S/N and 10 S/N ratios, respectively. These were essential parameters for completing the method validation protocol. As shown in Figure 7, the analytical curve yielded an LOD of 0.5 ng/ml and an LOQ of 1 ng/ml.

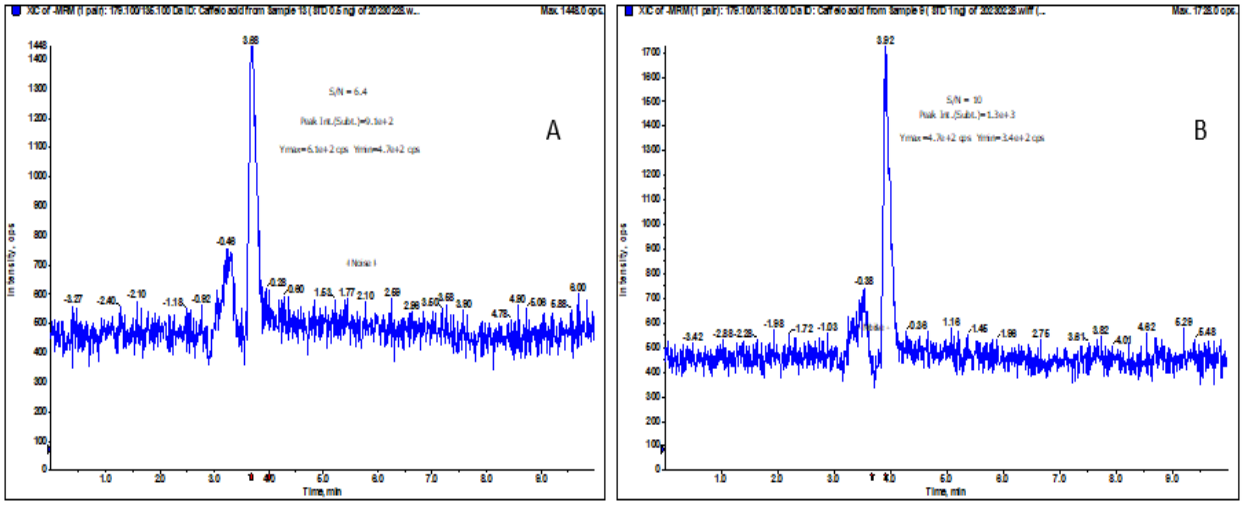


Figure 7: *Result of LC analysis of S/N for of Caffeic acid A) LOQ, B) LOD*

4. Conclusion

Azolla pinnata can be used in animal nutrition to improve immunity and organ protection by the antioxidant activity of its phenolic compounds and flavonoids content. Also, the validated LC-MS/MS technique was approved to be a precise, accurate, specific and reproducible technique that can be relied upon in the analysis of Caffeic acid.

5. Conflict of interest

The authors have no conflict of interest

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