Journal of Food Science

ORIGINAL ARTICLE

Health, Nutrition, and Food

Comprehensive Analysis of Bioactive Peptides From Spleen Amaranth *(Amaranthus dubius)* and Multi-Functional Bioactivity Evaluation

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Received: 16 September 2024 | **Revised:** 7 August 2025 | **Accepted:** 9 August 2025

Keywords: *Amaranthus dubius* | anti-bacterial | anti-inflammatory | antioxidant and anti-thrombotic activity | bioactive peptides | enzymatic hydrolysis | FTIR

ABSTRACT

Amaranthus dubius, commonly known as spleen amaranth, is a valuable nutritional source rich in protein, vitamins, and minerals, especially in regions such as India. This study investigated the protein content of leaves, stems, and seeds, with the seeds showing the highest protein concentration. Protein isolates from seeds were enzymatically hydrolyzed using proteolytic enzymes. The resulting hydrolysates were characterized by FTIR, DSC, and SEM techniques to analyze their structural properties. The peptides were further purified using molecular weight cut-off (MWCO) filters and assessed for various bioactivities. Seed protein isolates demonstrated significant antioxidant properties, including DPPH (56.65 ± 0.027 μg AAE/mg), ABTS (31.37 ± 0.955 μg AAE/mg), FRAP (23.986 ± 0.031 μM/mg), and Fe^{2+} chelation (32.03 ± 0.020%). Additionally, membrane stabilization activity was highest in seed isolates (25.56 ± 0.045%). Among the hydrolysates, chymotrypsin-derived fractions larger than 50 kDa showed the strongest DPPH activity, whereas alcalase hydrolysates in the 10–50 kDa range exhibited the highest ABTS scavenging activity. The highest ferric reducing power was observed in alcalase >50 kDa fractions, and Fe^{2+} chelating activity was greatest in <3 kDa chymotrypsin hydrolysates. The most potent anti-inflammatory effect was observed in <3 kDa alcalase hydrolysates, whereas antithrombotic activity peaked in 10–50 kDa chymotrypsin fractions. Antibacterial activity was notable against *E. coli* in <3 kDa alcalase fractions and *S. aureus* in 10–50 kDa chymotrypsin fractions. These multi-functional bioactivities highlight the potential of *Amaranthus dubius* seed proteins and peptides for applications in food, nutraceutical, and pharmaceutical industries. This study underscores the promising health benefits of *A. dubius* proteins and encourages further research into their therapeutic uses.

1 | Introduction

The global challenge of ensuring food security, health promotion, and sustainable agriculture amid a rapidly growing population has driven the scientific exploration of underutilized, nutrient-dense crops. Among these, the *Amaranthus* genus, comprising 93 accepted species (POWO 2024), has emerged as a promising plant owing to its exceptional nutritional composition and environmental resilience. Several *Amaranthus* species produce edible seeds and leaves, both of which are rich in protein, highly nutritious, gluten-free, proteinaceous grains, dietary fiber, vitamins, minerals, and bioactive phytochemicals (Baraniak and

Abbreviations: A > 50KDa, Alcalase > 50 KiloDalton; ABTS, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ADA, *Amaranthus dubius* alcalase hydrolysate; ADC, *Amaranthus dubius* chymotrypsin hydolysate; ADL, *Amaranthus dubius* leaves; ADS, *Amaranthus dubius* stem; AD seed, *Amaranthus dubius* seed; ADT, *Amaranthus dubius* trypsin hydrolysate; C > 50KDa, Chymotrypsin > 50 KiloDalton; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DSC, Differential scanning calorimetry; DTA, Differential thermal analysis; FICA, Ferrous ion chelating ability; FRAP, Ferric reducing antioxidant power; FTIR, Fourier-transform infrared spectroscopy; HRBC, Human red blood cells; NSAIDs, Non-steroidal anti-inflammatory drugs.; T > 50KDa, Trypsin > 50 KiloDalton; TGA, Thermogravimetric analysis.

Kania-Dobrowolska 2022; Martínez-López et al. 2020; Zhu 2023; Venskutonis and Kraujalis 2013; Aderibigbe et al. 2020; Zhang et al. 2023; Martínez-Villaluenga et al. 2020; Malik et al. 2023). *Amaranthus* is classified as a pseudocereal because of its grain-like seeds, which are used like true cereals (Graziano et al. 2022; Nimbalkar et al. 2012). Pseudocereals have made major contributions to the human diet in a few specific locations and may have emerged as significant new crops. Pseudocereals may be useful for human nutrition in individuals with allergies to conventional cereals (Fletcher 2016).

Among the *Amaranthus* species, *Amaranthus dubius* is traditionally consumed in subtropical and tropical regions, particularly India and Africa. Its edible leaves and seeds are recognized for their nutritional value, serving as a source of food, fodder, and famine relief in resource-scarce environments (Fletcher 2016; Ruth et al. 2021; Rodriguez et al. 2011). However, further research is necessary before it can be utilized for industrial purposes, particularly for the identification and validation of its protein. Although the protein composition of amaranth seeds has been extensively studied, there is still limited research on the protein profiles of its leaves and stems. Amaranth seeds are rich in proteins, particularly the essential amino acid lysine, which is often deficient in other cereals, making them a valuable alternative protein source (Nimbalkar et al. 2020). As interest in sustainable, alternative proteins grows, amaranth protein, a by-product of grain processing, could be valuable for food applications such as meat analogs and emulsions (Bojórquez-Velázquez et al. 2018; Zhu 2023). Hydrolyzed amaranth proteins offer health-promoting peptides, but limited research has restricted their use as a sustainable food source (Zhu 2023).

Recent advances in food science have focused on the development of nutraceuticals and functional foods using bioactive peptides and small sequences (2–20 residues) of amino acids derived from food proteins, which exhibit diverse health-promoting properties. These peptides are typically inactive within the parent protein sequence and must be released via digestive enzymes, chemical hydrolysis, proteolytic enzymes, food processing by curing, and fermentation to express their biological functions (Agrawal et al. 2016; Cruz-Casas et al. 2021). Specific proteases such as trypsin, chymotrypsin, and mixtures of several non-specific proteases have been employed to synthesize more steady and potent bioactive peptides (De Castro and Sato 2015).

The intake of foods containing bioactive compounds like antioxidants and antithrombotics may contribute to lowering the risk of cardiovascular diseases, among the most prevalent global health issues (Sabbione, Ibanez, et al. 2016). Additionally, protein hydrolysates can reduce inflammation by influencing the activity of inflammatory mediators (La Manna et al. 2018). Antimicrobial peptides (AMPs) are promising alternatives to antibiotics, offering broad-spectrum activity with fewer side effects. However, their clinical application still requires extensive evaluation (López-García et al. 2022).

Protein hydrolysates, particularly those derived from plant-based proteins, have been shown to possess a broad range of bioactivities, including antihypertensive, antimicrobial, hypocholesterolemic, antithrombotic, antioxidant, antiadipogenic, antidiabetic, antiproliferative, immunomodulatory, and anticancer (Montoya-Rodríguez et al. 2015; Agrawal et al. 2016; Agrawal et al. 2019; Delgado et al. 2016; Delgado et al. 2011; De Castro and Sato 2015; Ayala-Niño, Rodriguez-Serrano, Jimenez-Alvarado, et al. 2019; Akbarian et al. 2022). To date, several biologically active peptides have been extracted from numerous sources of grain protein, likely from finger millet (Agrawal et al. 2019), pearls millet (Agrawal et al. 2016), rice (Yan et al. 2015), soy, pea, wheat (Rudolph et al. 2017), and foxtail millet (Ji et al. 2020). Considering amaranth, many researchers work on grain of amaranth bioactive peptides such as *Amaranthus hypochondriacus* (Ayala-Niño, Rodriguez-Serrano, Gonzalez-olivares, et al. 2019; Sabbione, Ibanez, et al. 2016; Montoya-Rodríguez et al. 2015; Moronta et al. 2016; Suárez and Añón 2019; Silva-Sanchez et al. 2008), *A. cruentus* (Soares et al. 2015), *A. caudutus* (Taniya et al. 2020), and *A. mantegazzianus* (Tironi and Anon. 2010), while *A. viridis* (Famuwagun et al. 2020) and *A. dubius* (Rodriguez et al. 2011) isolate bioactive peptides from leaves. The novelty of the present study lies in its systematic investigation of bioactive peptides derived from enzymatic hydrolysates of *Amaranthus dubius* seed proteins. Although previous studies have examined the phytochemicals, phenolic acids, and nutritional and antinutritional components of *Amaranthus dubius* seeds, their peptide profiles and associated bioactivities remain largely underexplored.

This study was driven by the hypothesis that proteolytic hydrolysis of *A. dubius* seed proteins can generate low-molecular-weight peptides with distinct functional properties. Furthermore, such peptides could serve as natural health-promoting constituents for the development of nutraceuticals or functional foods, especially in regions facing nutritional insecurity. The main objective of this study was to extract and hydrolyze proteins from *A. dubius* seeds using specific proteolytic enzymes and to characterize the resultant hydrolysates and peptide fractions for their physicochemical and bioactive properties. The workflow includes enzymatic hydrolysis, followed by fractionation using molecular weight cut-off (MWCO) membranes and comprehensive structural and functional profiling of hydrolysates using Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and differential scanning calorimetry (DSC) as shown in Figure 1.

The present work not only addresses a significant gap in the current understanding of *Amaranthus dubius* protein biochemistry but also contributes to the broader goal of identifying sustainable, plant-based protein sources that are suitable for integration into health-oriented food systems. This aligns with the urgent global need for accessible and ecologically sound protein alternatives, especially in light of projected population growth and climate-induced agricultural constraints (Wood and Tavan 2022). This study contributes to a better understanding of *A. dubius* seed protein hydrolysates and their bioactivities, opening new avenues for the application of underutilized crops in health and food security strategies.

2 | Materials and Methods

2.1 | Materials

2,4,6-tripyridyl-S-triazine (TPTZ), ascorbic acid, sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), 2,2′-azino-bis(3-

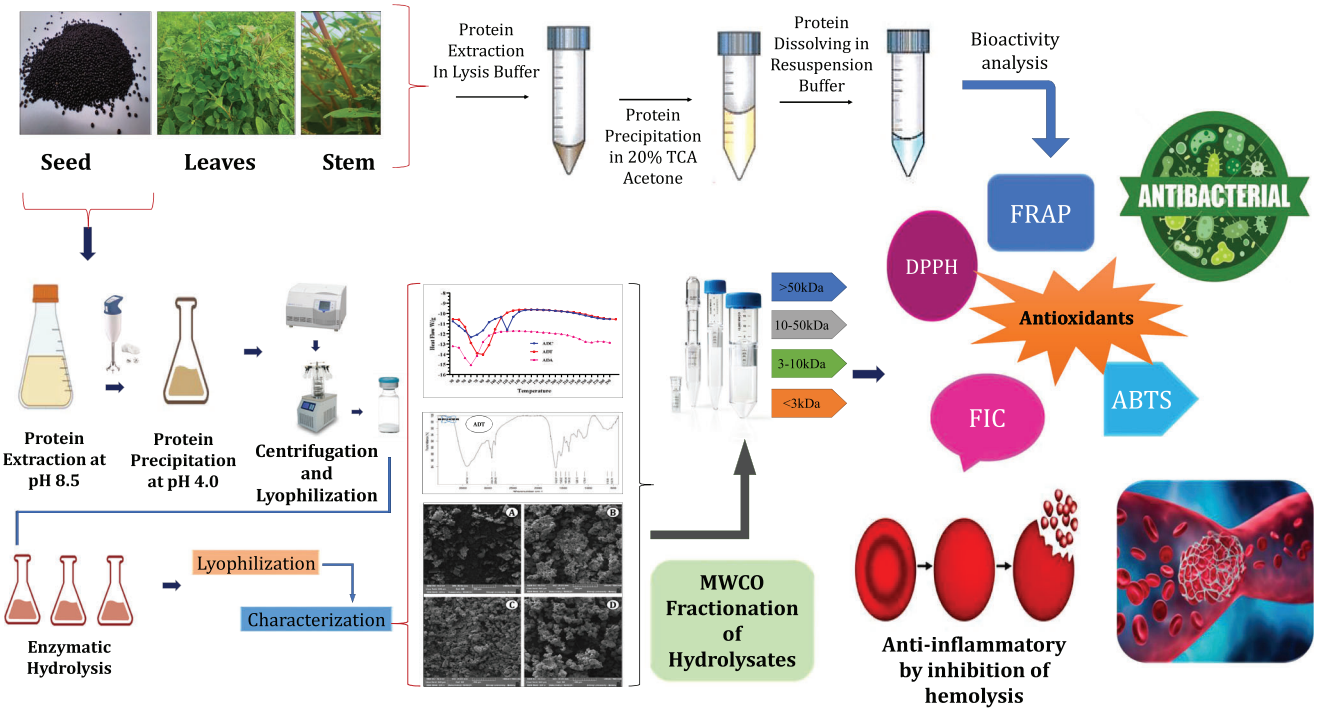

FIGURE 1 | Graphical representation of isolation of proteins, hydrolysis, characterization, and bioactivity evaluation of *Amaranthus dubius*.

ethylbenzothiazoline-6-sulfonic acid) (ABTS), sodium phosphate monobasic, ethylenediaminetetraacetic acid disodium salt dihydrate (Na2EDTA), sodium dodecyl sulphate (SDS), tris-HCl, sodium chloride, ferric chloride ($FeCl_3$), sodium phosphate dibasic, ferrous sulphate ($FeSO_4$), trichloroacetic acid (TCA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), nutrient broth were obtained from HiMedia Laboratories Private Limited, Mumbai, India. Acetone, ethanol, methanol, hydrochloric acid (HCl), potassium persulfate, dimethyl sulfoxide (DMSO), Folin-cocatteau reagent, and glacial acetic acid were procured from Sisco Research Laboratories Pvt. Ltd. (SRL)—India. Streptomycin, acetyl salicylic acid (aspirin), microbes *Escherichia coli* (NICM 2832), and *Staphylococcus aureus* (NICM 2654). Trypsin, chymotrypsin, alcalase, heparin, fibrinogen, and thrombin were procured from Sigma-Aldrich Chemicals Private Limited, Bangalore, India.

2.2 | Plant Material Collection

Amaranthus dubius Mart. ex Thell. was collected from Kolhapur, Maharashtra, India. The sample was identified and processed in the research laboratory and was subsequently stored at −80°C.

Fresh specimens of *Amaranthus dubius* Mart. ex Thell. were collected during June 2024 from Nimshirgaon, Kolhapur, Maharashtra, India (N16.784, E74.509). For a comprehensive analysis, samples of leaves, stems, and seeds were carefully harvested. The collected material was immediately shifted to the research laboratory of the Botany Department, Shivaji University, Kolhapur, India, in insulated containers containing ice packs to preserve the biochemical integrity. The samples were subjected to taxonomic identification and authentication. Subsequently, they were washed with water, soaked (for leaves and stems), and then frozen at −80°C. Seeds were isolated and stored at room temperature (25°C) in a glass container until further use for experimental analyses.

2.3 | Extraction of Proteins

2.3.1 | Trichloroacetic Acid/ Acetone Precipitation

Proteins were extracted from the fresh morphological parts of *Amaranthus*, that is, leaves, stem, and seeds. A modified trichloroacetic acid/acetone precipitation method was used (Niu et al. 2018).

Leaves, stems, and seeds, each of 1 g fresh weight, were ground in a chilled mortar in 15 mL of the extraction solvent (1% SDS, 2 mM Na2-EDTA, 20 mM DTT 0.1 M Tris-HCl (pH 6.8), and before use, 2 mM PMSF added). Seeds were initially pre-crushed using a mixer grinder in the presence of liquid nitrogen. This helped make the seeds brittle, allowing efficient subsequent grinding in a chilled mortar to obtain a fine powder suitable for extraction. The homogenate was centrifuged at ten thousand rpm for 5 min, and the supernatant was collected. Protein extract was mixed with 20% cold TCA/acetone (1:1, v/v; final 10% TCA, 50% acetone), kept on ice for 5 min, then centrifuged (REMI Cooling centrifuge C-24) at ten thousand rpm for 3 min. The clear supernatant was then discarded. The precipitate protein was centrifuged, allowed to air dry for one to three minutes, and then dissolved in molecular-grade water after being twice cleaned with 10 mL of 80% acetone.

2.3.2 | pH Precipitation Method

Protein extraction was performed as described by Agrawal et al. (2016) with minor modifications. First, amaranth seeds were

ground into flour and defatted with hexane (1:2 w/v) for 4 h at 25°C. After soaking defatted flour in 1:15 w/v distilled water, it was homogenized for an hour at 25°C. The pH of the solution was then raised to 8.5 using 0.5 M NaOH. It was then homogenized again for 30 min and left for 5 h. After subsequent centrifugation (REMI Cooling centrifuge C-24) of the solution at 7000 rpm for 10 min, the pH of the obtained supernatant was changed to 4.5 using 1 M HCL. The solution was centrifuged again at 7000 rpm for 10 min after 30 min, and the pellet was cleaned with 10 mL of distilled water. The gathered protein was freeze-dried and kept in a −20°C deep freezer (Blue Star) for analysis.

2.4 | Quantification of Isolated Protein

Isolated proteins were quantified using Lowry et al. (1951). BSA was used as a standard. 20–100 μg/mL working standards were used to plot a standard curve. One hundred microliters of BSA working standard or test samples were added to a tube. The tube with 100 μL of deionized water served as a blank. 450 μL of Reagent I (1% NaK Tartrate in H_2O, 0.5% $CuSO_4$. 5 H_2O in H_2O, 2% Na_2CO_3 in 0.1 N NaOH, at a ratio of 48:1:1) was incubated for 10 min, followed by 50 μL of Reagent II (1:1 Folin-Phenol [2N] and water), and kept in the dark for 30 min. Optical density was measured at 660 nm, and the protein content was expressed as mg/100 mg fresh weight using a standard curve.

2.5 | Enzymatic Hydrolysis

2.5.1 | Trypsin Hydrolysis

Trypsin hydrolysis was performed on 25 mg protein in 10 mL water (pH 9.0) at 37°C, with constant stirring (120 rpm) for 4 h at an [E:S] Enzyme: Substrate ratio of 1:25. The enzyme was deactivated at 80°C for 20 min, and hydrolysates were centrifuged (ten thousand rpm, 4°C, 15 min). The supernatant was then lyophilized for future use (Agrawal et al. 2016; Agrawal et al. 2019; Awosika and Aluko 2019).

2.5.2 | Chymotrypsin Hydrolysis

Similar to trypsin hydrolysis, chymotrypsin hydrolysis was conducted at pH 8.0, followed by constant stirring (120 rpm) for 4 h. The enzyme was inactivated, and the hydrolysates were processed similarly (Awosika and Aluko 2019).

2.5.3 | Alcalase Hydrolysis

Protein extract (25 mg) was suspended in water (pH 10) and stirred at 37°C for 1 h. Alcalase (100 μL/100 mg) was then added, and the mixture was incubated at 37°C for 4 h. Enzyme activity was halted at 85°C for 10 min, followed by freeze-drying (Delgado et al. 2011).

2.6 | Antioxidant Activity

2.6.1 | DPPH Radical Scavenging Ability

DPPH activity was assessed by a modified method of Kumaran and Karunakaran (2006). 75 μL of sample was added to the 96-well microplate, and 225 μL of 0.1 mM methanolic DPPH solution was added. The microplate was kept at least 30 min in the dark. The optical density was recorded at 517 nm to assess the DPPH scavenging activity of each fraction, compared to a blank solution (DPPH only). Ascorbic acid was used as the standard, and enzymes alone were used as the enzyme control. DPPH radical scavenging ability was expressed as μg AAE/mg using a standard ascorbic acid calibration curve.

2.6.2 | ABTS Scavenging Activity

The ABTS scavenging potential of the test sample was assessed by Re et al. (1999). In this assay, 10 μL (10 mg/mL) of the sample was mixed with 290 μL of ABTS reagent. A 1:1 ratio of 7 mM aqueous ABTS and 2.45 mM potassium persulfate was mixed and incubated in the dark for 12–16 h. The ABTS working mixture was prepared by diluting the reagent to achieve an absorbance of 0.7±0.02 at 734 nm. After 10 min of incubation, scavenging activity was assessed by recording the absorbance at 734 nm. The standard was ascorbic acid, and enzymes alone were used as the enzyme control, and the sole blank control was ABTS. Results were reported as μg AAE/mg using a standard ascorbic acid calibration curve.

2.6.3 | Ferric Reducing Antioxidant Power (FRAP)

The capacity to reduce Fe^{3+} ions was evaluated by slight alterations to the FRAP method described previously (Yan et al. 2015). The reaction mixture contains 290 μL of FRAP reagent (100 mL of 0.3 M acetate buffer, 10 mL of 10 mM TPTZ, and 10 mL of 20 mM $FeCl_3$) and 10 μL of sample. The reaction mixture had been left at 37°C for 15 min, and the optical density at 595 nm was recorded. Ferric reduction was calculated using an ascorbic acid linear calibration curve, while the enzyme control was done only using the studied enzymes, and μM ascorbic acid equivalents were used to express the antioxidant activity based on the capacity to reduce ferric ions.

2.6.4 | Fe^{2+} Ion Chelation Assay

Fe^{2+} was monitored by measuring the formation of a red complex with ferrozine at 562 nm. It was studied by the method described by Adjimani and Asare (2015) with minor alterations. The iron chelators (75 μL) at working concentrations (2-10 μg/mL) and test samples/enzyme control were mixed with 75 μL of ferrous sulphate (0.1 mM) and 150 μL of 0.25 mM ferrozine to initiate the reaction. After carefully mixing the resultant concoction, it was incubated for 10 min. The optical density (O.D.) of the mixture solution was checked at 562 nm. The chelator's ability to bind ferrous iron decreases with increasing absorbance at 562 nm. The standard utilized was Na_2EDTA.

The percentage inhibition was calculated by the formula

$$\text{Percentage inhibition of ferrozine fe2} + \text{complex formation} = \frac{(A0 - A1)}{A0} \times 100 \quad (1)$$

where, A1 = optical density of sample, A0 = optical density of control

2.7 | Anti-Inflammatory Activity

2.7.1 | HRBC Membrane Stabilization Activity

2.7.1.1 | Preparation of Red Blood Cells Suspension (RBCs). 5 mL of fresh human blood was collected from an individual who had not been taking NSAIDs for the two weeks before the experiment. As an anticoagulant, EDTA was used to prevent blood clotting. The blood sample was spun for 10 min at 3000 rpm. Use an identical amount of regular saline (0.9% NaCl) to wash twice. The blood volume was measured and then diluted by an isotonic buffer (10 mM sodium phosphate buffer, pH 7.4, including 154 mM NaCl) as a 10% v/v solution (Mane et al. 2022).

2.7.1.2 | Hemolysis Assay by Hypotonic Solution. Human erythrocyte hemolysis caused by a hypotonic solution was used to measure the membrane-stabilizing activity (Mane et al. 2022) of the protein isolates. 125 µL of sample/enzyme control was mixed with 1 mL of a hypotonic mixture (50 mM NaCl in phosphate buffer, pH 7.4) in a 2 mL tube. 125 µL of RBC suspension was added, and the mixture was kept for 10 min. The control contained 1.125 mL of hypotonic solution and 125 µL of RBCs. Acetylsalicylic acid (ASA) was used as a positive control. All samples were centrifuged at 2500 rpm for 5 min, after which the absorbance was recorded at 540 nm.

The percentage inhibition calculated by

$$\%\ \text{Inhibition of haemolysis} = \frac{(A0 - A1)}{A0} \times 100 \qquad (2)$$

where, A1 = optical density of sample, A0 = optical density of control

2.8 | Antithrombotic Activity

The antithrombotic activity was assessed by the method of Zhang et al. (2008), which involved some modification in the fibrinogen concentration. The fibrinogen (0.025%) was prepared in 0.05 M Tris–HCl buffer (pH 7.2) comprising 0.12 mM NaCl. A thrombin from bovine plasma (12 IU/mL) and protein and peptide samples were prepared in distilled water. A sample blank was prepared by mixing 140 µL of fibrinogen solution with 40 µL of sample/enzyme control, and the optical density was measured. To start the thrombin-catalyzed coagulation of fibrinogen, 10 µL of thrombin solution was added to the well. Following a 10 min incubation period, the sample's absorbance was measured once more. The sample solution was replaced with 40 µL of Tris–HCl buffer (pH 7.2, 0.05 M) to assess the absorbance of the control and the control blank. A positive control was heparin (20-100 µg/mL). The following formula was used to analyze the inhibitory effects:

$$\text{Inhibitory effect of coagulation of fibrinogen}\ (\%) = \frac{(C - CB) - (S - SB)}{(C - CB)} \times 100 \qquad (3)$$

where,

CB (control blank): The initial optical density of the negative control of inhibition.

C (control): Optical density of the negative control following a 10-min thrombin incubation period.

SB (sample blank): The initial optical density of the sample.

S (sample): Optical density of the sample following a 10-min thrombin incubation period.

2.9 | Antibacterial Activity

In this assay, the 96-well plate method was employed (Magdum et al. 2024). *Escherichia coli* (NICM 2832) and *Staphylococcus aureus* (NICM 2654) were tested against streptomycin and amaranth seed protein isolates, hydrolysates, and ultra -fractions.

2.9.1 | Bacterial Suspension Preparation

Bacterial inocula (prepared suspensions of bacterial cultures used for inoculation) were adjusted to the desired optical density before use and were ready by overnight incubation in 25 mL of nutrient broth at 37°C. Prepared suspensions of bacterial cultures used for inoculation were adjusted to the desired optical density before use.

2.9.2 | Preparation of 96 Well-Plates

In a 96-well sterile plate, 280 µL of nutrient broth (NB), 10 µL of test sample or standard, and 10 µL of bacterial suspension were added per well. The plates were incubated at 37°C for 16 h and then analyzed using a Multiskan Sky spectrophotometer at a wavelength of 600 nm. NB alone served as the blank, and NB with bacterial suspension as the control.

$$\%\ \text{Inhibition of bacterial growth} = \frac{(A0 - A1)}{A0} \times 100 \qquad (4)$$

Where, A0 = Absorbance of control (Absorbance at 16 h—Absorbance at 0 min), A1 = Absorbance of sample (Absorbance at 16 h—Absorbance at 0 min)

2.10 | Characterization of Protein Isolates and Their Hydrolysates

2.10.1 | Fourier-Transform Infrared Spectroscopy (FTIR)

Differences between the amaranth protein isolates and their hydrolysates were determined using FTIR spectrophotometry. 1 mg of lyophilized powder of each isolate and trypsin and chymotrypsin hydrolysates were subjected to analysis. Functional group frequencies were analyzed using an Alpha Bruker FT-IR spectrometer (4000–400 cm^{-1}) with samples on KBr pellets.

2.10.2 | Scanning Electron Microscopy (SEM)

A scanning electron microscope (TESCAN VEGA-3 SBU) was used to analyze the morphological characteristics of the amaranth protein isolates and their hydrolysates. Samples were placed on stubs made up of aluminum with carbon tape and coated with gold under 10 Pa vacuum for 60 s using a Quorum SC7620 sputter coater. The following parameters were used to operate the electron microscope: 10 kV accelerating voltage, a 25–26 mm sample distance, and a 0° tilt angle. The samples were inspected at a magnification of 500X.

2.10.3 | Differential Scanning Calorimetry (DSC) -Differential Thermal Analysis (DTA) -Thermogravimetric Analysis (TGA)

Thermal analysis (TGA-DSC) was performed using a TA Instruments SDT Q600. Protein hydrolysates were analyzed by DSC through the methods by Condés et al. (2009). Lyophilized powder of samples was prepared and loaded with 4–5 mg and incubated for at least 30 min at 25°C before testing. Runs were accomplished using a heating rate of 10°C/min. The reference was a double-empty, sealed capsule. All experiments were carried out in triplicate.

2.11 | Fractionation of Hydrolysates Through Molecular Weight Cutoff Filters

Ultrafiltration was used to purify the protein hydrolysates, as earlier reported (Agrawal et al. 2016). After hydrolysis, ultrafiltration is the initial stage of purification of peptides. Using a molecular weight cut-off (MWCO) of 3 kDa (kilodalton), 10 kDa, and 50 kDa, the lyophilized amaranth protein hydrolysates were solubilized in deionized water and fractionated using an ultrafiltration membrane (Amicon Ultra Centrifugal Filter Units, Merck Millipore). Initially, the 50 kDa membrane was used to filter the protein hydrolysates, producing a retentate with MW > 50 kDa. A 10 kDa membrane was then used to filter the 50 kDa permeate, yielding two fractions of 10 kDa retentate with MW 10–50 kDa. A 3 kDa membrane was then used to filter the 10 kDa permeate, producing two fractions: 3 kDa retentate with MW 3–10 kDa and 3 kDa permeate with MW <3 kDa.

2.12 | Statistical Analysis

Experimentations were conducted in triplicate, with results were reported as mean ± SE. Data were analysed using GraphPad Prism 5.0 and Microsoft Office Excel, with significance set at $p < 0.05$.

3 | Results and Discussion

3.1 | Extraction of Proteins Using TCA-Acetone Precipitation Method and Quantification of Proteins

TCA-acetone precipitation enhances the protein yield and effectively removes contaminants such as salts and phenolics, thereby improving the sample quality for analysis. (Niu et al. 2018). Yadav et al. (2020) compared the three protein extraction methods. Tris-HCl (50 mM, pH 7.5) provided the highest protein yield and best SDS-PAGE resolution. 1.5 M Tris–HCl (pH 8.8) was less efficient, and the PVPP-containing buffer increased crude protein yield but reduced soluble protein after TCA-acetone precipitation. Despite lower yield, 20% TCA-acetone precipitation was preferred for its ability to remove contaminants and inactivate degradative enzymes, resulting in high-quality protein pellets suitable for analysis.

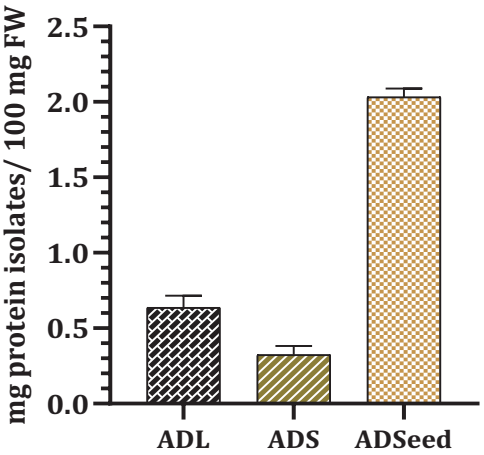

FIGURE 2 | Quantification of *Amaranthus dubius* proteins by the Lowry Method (ADL- *A. dubius* leaves, ADS- *A. dubius* stem, ASDeed- *A. dubius* seeds). The results were described as mean value ±SE ($p < 0.05$).

Proteins were quantified using the Lowry assay, a sensitive method suitable for detecting protein concentrations in the 5—100 µg range (Shen 2019). Niu et al. (2018) reported that TCA-acetone precipitation yielded protein concentrations of 4.82 ± 0.07 µg/mg in maize roots, 4.13 ± 0.11 µg/mg in leaves, and 5.80 ± 0.13 µg/mg in embryos. Amaranth, quinoa, and millets contain approximately 13.4–16.5%, 12.1–14.5%, and 7–11% protein, respectively, in their seeds or grains (Balakrishnan and Schneider 2022). As shown in Figure 2, the highest protein content was observed in seeds (2.035 ± 0.052 mg/100 mg), followed by the leaves (0.635 ± 0.084 mg/100 mg), and the lowest was observed in the stem (0.324 ± 0.057 mg/100 mg). When we consider the extraction in phosphate buffer, the protein yield is higher (i.e., 2–4-fold higher than the experimental results). This is because during extraction for downstream analysis, the protein sample must be free from interfering substances, and must be washed repeatedly with organic solvents, which may be the reason for the lower yield.

3.1.1 | Antioxidant Activity

A proton-donating chemical, like an antioxidant, scavenges radicals and decreases the absorbance when DPPH comes into contact with it (Zhang et al. 2008). Figure 3a illustrates the ability of leaf, seed, and stem protein isolates from *A. dubius* to scavenge DPPH radicals. The highest level of DPPH scavenging activity was discovered in protein isolates from seeds (56.65 ± 0.027 µg AAE/mg protein isolates) as compared to the leaves (31.08 ± 0.019 µg AAE/mg protein isolates) and stem (45.78 ± 0.031 µg AAE/mg

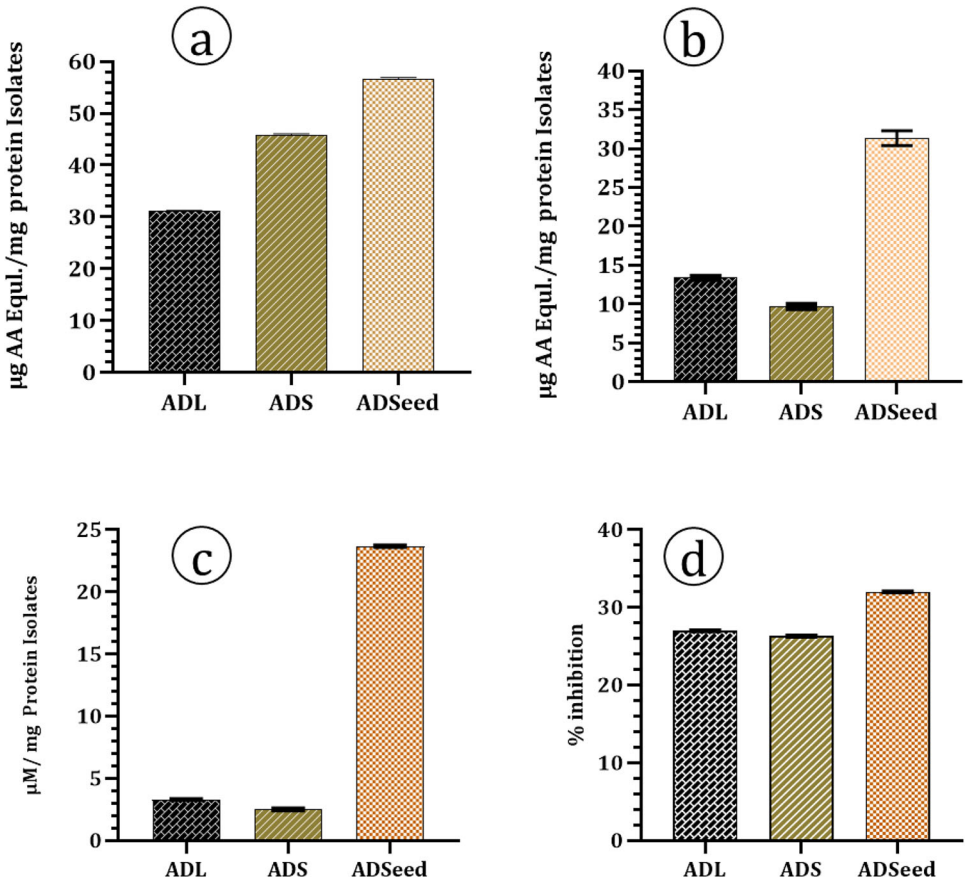

FIGURE 3 | Antioxidant activity of leaves, stems, and seeds protein isolates from *Amaranthus dubius*. (a) DPPH Scavenging Activity ($p < 0.05$), (b) ABTS inhibiting activity, (c) ferric reducing antioxidant power, (d) ferrous ion chelation activity ($p < 0.05$). The results were reported as mean value ± SE ($p < 0.05$).

protein isolates). The optical density of the free radical gradually decreased in the seed protein isolates, indicating effective free radical quenching.

The addition of antioxidants to the preformed chromophore radical cation reduces absorbance, depending on the capability of the test sample and exposure duration. As a result, the degree of discoloration linked to antioxidant capacity depends on concentration and time (Sabbione, Ibanez, et al. 2016). The antioxidant potential of proteins and peptides is because of their ablity to chelate reactive molecules or contribute electrons or hydrogen. Considering the ABTS inhibitory activity of protein isolates from leaves (13.38 ± 0.135 µg AAE/mg protein isolates) and stem (9.72 ± 0.192 µg AAE/mg protein isolates), in comparison to the seeds, it showed the highest ABTS radical scavenging activity (31.37 ± 0.955 µg AAE/mg protein isolates), as shown in Figure 3b.

As shown in Figure 3c, protein isolates from seeds, like DPPH and ABTS showed the highest ferric-reducing antioxidant power (23.986 ± 0.031 µM/mg protein isolates), and stem protein isolates (2.527 ± 0.051 µM/mg protein isolates) had the lowest.

The reactive oxygen species (ROS) are produced as by-products of cellular metabolic processes, including mostly mitochondrial electron transport. Moreover, ROS are produced as crucial intermediates in metal-catalyzed oxidation reactions. By gaining or losing electrons, the transition metal ion Fe^{2+} can sustain the production of free radicals. Hence, by chelating metal ions with chelating agents, it is possible to reduce the creation of reactive oxygen species (Sudan et al. 2014).

In Figure 3d, seed protein isolates (32.03% ± 0.020%) showed the highest percent inhibition of ferrozine-Fe^{2+} complex formation, and the lowest was found in stem protein isolates (26.31% ± 0.034%).

3.1.2 | Anti-Inflammatory Activity

The membrane stabilization activity of *Amaranthus dubius* leaves, stems, and seeds protein isolates was mentioned in Figure 4. Seed protein isolate had more membrane stabilizing activity (i.e., 25.56 ± 0.045%). Compared to the standard drug, the studied protein isolates did not have as much membrane stabilization potential. It is predicted that compounds with the ability to stabilize membranes will greatly shield cell membranes from harmful materials. (Gambhire Manoj et al. 2009). As the erythrocyte membrane is similar to the lysosomal membrane, the HRBC membrane stabilization assay was utilized for screening. When the lysosomal membrane is damaged, components of the active neutrophil, such as bactericidal enzymes and proteases, are released into the extracellular space, where they cause more tissue inflammation and damage (Oladele et al. 2011). In this experiment, stress conditions were a hypotonic solution

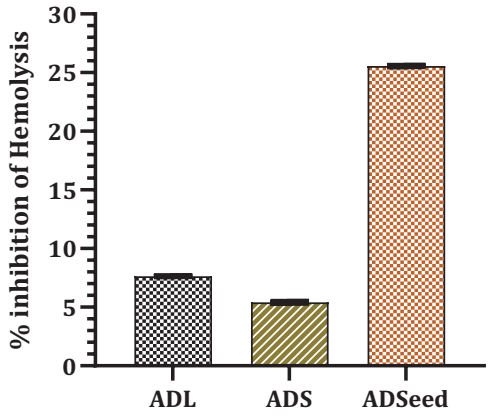

FIGURE 4 | Anti-inflammatory activity using HRBC membrane stabilization assay of protein isolates from *Amaranthus dubius* leaves, stem, and seeds. The results were described as mean value ± SE ($p < 0.05$).

containing 50 mM of NaCl salt in phosphate buffer, causing the rupture of the RBC membrane. The membrane-stabilizing molecules may be coupled to the RBC membrane and prevent lysis and hemoglobin release. Anti-inflammatory plant proteins represent a promising avenue for developing natural therapies for chronic inflammatory diseases. Their ability to modulate inflammatory pathways with fewer side effects compared to traditional pharmaceuticals makes them an attractive option for both nutritional and therapeutic applications (Liu et al. 2022; Zaky et al. 2022; Chakrabarti et al. 2014).

3.2 | Extraction of Proteins Using pH Precipitation Method and Quantification of Proteins

The process of extraction occurs because the interactions of the protein with the aqueous environment are altered by changes in pH or hydrophobicity, or because metals or salts bind to functional groups in the protein, triggering protein denaturation, aggregation, and leaving the solution (Stone 2017). The precipitated proteins were then collected, and after freeze-drying, quantification was carried out by the Lowry method, and it was 377.5 ± 0.25 mg/g. Das et al. (2021) reported extraction efficiency at varying pH levels. Maximum protein recovery (90.08%) was achieved at pH 4.5, the isoelectric point of *Amaranthus hypochondriacus* protein isolate, due to reduced solubility and effective precipitation. Protein yield dropped markedly from 85.42% at pH 9 to 55.56% at pH 12, highlighting the adverse effect of highly alkaline conditions on protein recovery. Rodríguez and Tironi (2020) reported a 78.40 g/100 g protein content in the protein isolate from *Amaranthus mantegazzianus* flour. Experimental results ranged from 75.8% at intermediate pH levels (extraction pH 8.5, precipitation pH 5.0) to a maximum of 83.4% when using a higher extraction pH of 9.0 and a lower precipitation pH of 4.5, indicating that this combination enhances protein recovery. Amaranth grains show genetic diversity in protein content, typically ranging from 12% to 17.8% across species and genotypes (Zhu 2023).

After protein hydrolysis, net recovery of hydrolysate after freeze-drying/lyophilization was 5.7 ± 0.17 mg for ADC, 4.7 ± 0.11 mg for ADT, and 4.6 ± 0.9 mg for ADA. The freeze-dried hydrolysates were subsequently dissolved in deionized water to prepare 1 mg/mL stock solutions for further analysis.

3.3 | Characterization of Protein Hydrolysates

3.3.1 | Scanning Electron Microscopy

SEM was used to examine the microstructure changes that occurred in the protein powder upon hydrolysis. Figure 5 represents the morphology of *A. dubius* protein isolates (AD) and their trypsin (ADT), chymotrypsin (ADC), and Alcalase (ADA) hydrolysates. The results exhibited that the protein had broken down into tiny pieces following enzymatic treatment. ADT, ADC, and ADA displayed a smoother matrix than AD, which displayed masses of packed flake-like particles under the same parameters, as a result of the particle size decrease (Mag = 500 ×; AV = 10 kV). The prior study (Agrawal et al. 2019) also supported the results, which exhibited the structural variations in finger millet (*Eleusine coracana*) protein hydrolysate. These results also supported the study of Alahmad et al. (2023) and Islam et al. (2021). Lyophilization, or freeze-drying, the surface of the particle, which is porous and permits uptake of water, and the formed skin can create a moisture barrier (Dent and Maleky 2022).

3.3.2 | Fourier-Transform Infrared Spectroscopy

FTIR is a valuable tool for monitoring proteolytic reactions. It is useful for identifying structural alterations in peptides and proteins, providing semi-quantitative information related to proteolytic reactions (Kristoffersen et al. 2020). There are noticeable differences between different types of samples. Specifically, the bands coming from samples derived from enzyme-based hydrolysates are not as clearly separated as the amide I and II bands (1700–1500cm^{-1}). According to Böcker et al. (2017), this is probably explained by the fact that these samples are more complicated, consisting of complex blends of many proteins. The backbone of proteins and peptides is made up of repeating amino acid building blocks, which result in a variety of unique infrared absorption bands, or amide bands, that carry structural and chemical information (Kristoffersen et al. 2020). Table 1 displays a list of these regions along with approximate band allocations and wavenumber values. The designations are based on FTIR analyses of proteins and peptides that have been published in the past (Shen et al. 2023; Kristoffersen et al. 2020). Several of these bands are crucial for the maintenance of proteolytic processes. These include bands like the N-terminal (NH_3^+, ~1510 cm^{-1}), the C-terminal (COO−, ~1400 cm^{-1}), the amides I (~1700–1600 cm^{-1}), and II (~1590–1520 cm^{-1}), although alterations can also be seen in other FTIR bands. The large protein molecule begins to lose its secondary structure as it breaks down into smaller peptide fragments, resulting in diminishing bands typical of α-helices bands at 1655 and 1548 cm^{-1}, as shown in Figure 6.

According to Kong and Yu (2007) and Dong et al. (1990), who deconvoluted amide I band frequencies and their secondary structure assignments of studied proteins and their hydrolysates, the amide I band contours that are found in proteins or

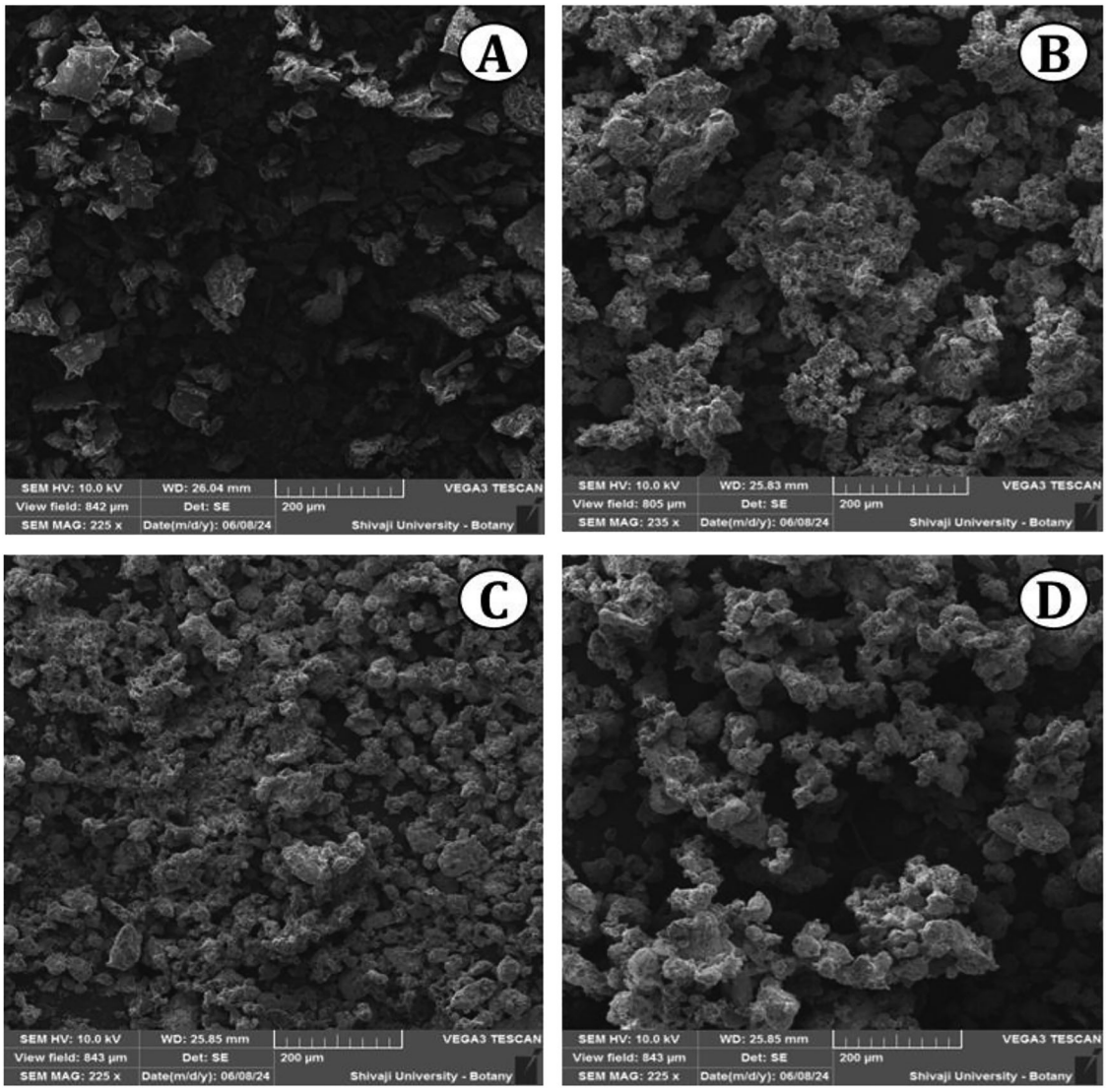

FIGURE 5 | Scanning electron microscopy (SEM) of *Amaranthus dubius* protein isolate and their hydrolysates. A- AD (protein isolates), B- ADT (trypsin hydrolysate), C- ADC (chymotrypsin), and D- ADA (alcalase).

TABLE 1 | Characteristic infrared bands of Amide I and Amide II showing difference wavenumber cm^{-1}.

	Approx. frequency (cm^{-1})					
Sr.No.	**AD**	**ADT**	**ADC**	**ADA**	**Description**	**Designation**
1.	1655.52	1643.37	1645.68	1657.39	C═O stretching	Amide I
2.	1545.3	1545.83	1550.72	1546.14	CN stretching, NH bending	Amide II
	1452.6	1451.94	-	1453.38		

polypeptides are made up of overlapping component bands that symbolize twists, alpha-helices, β-sheets, and random structures. The IR bands observed around 1640–1620 cm^{-1} and 1695–1690 cm^{-1} correspond to β-sheet structures, while a band near 1658–1650 cm^{-1} is indicative of α-helices. Unordered or random coil conformations are typically associated with bands in the 1640–1648 cm^{-1} range. The band frequencies obtained from FTIR of the protein isolates in the amide I band showed a decrease and shift from the α-helix to random coils and β-sheets.

3.3.3 | Differential Scanning Calorimetry- Differential Thermal Analysis- Thermogravimetric Analysis

DSC is a method of analysis that determines molar heat capacity as a function of temperature. When applied to proteins, DSC profiles offer insights into thermal constancy and can function to evaluate structural conformation. The technique is based on the measurement of the thermal transition temperature (also known as the melting temperature; Tm) and the energy needed to

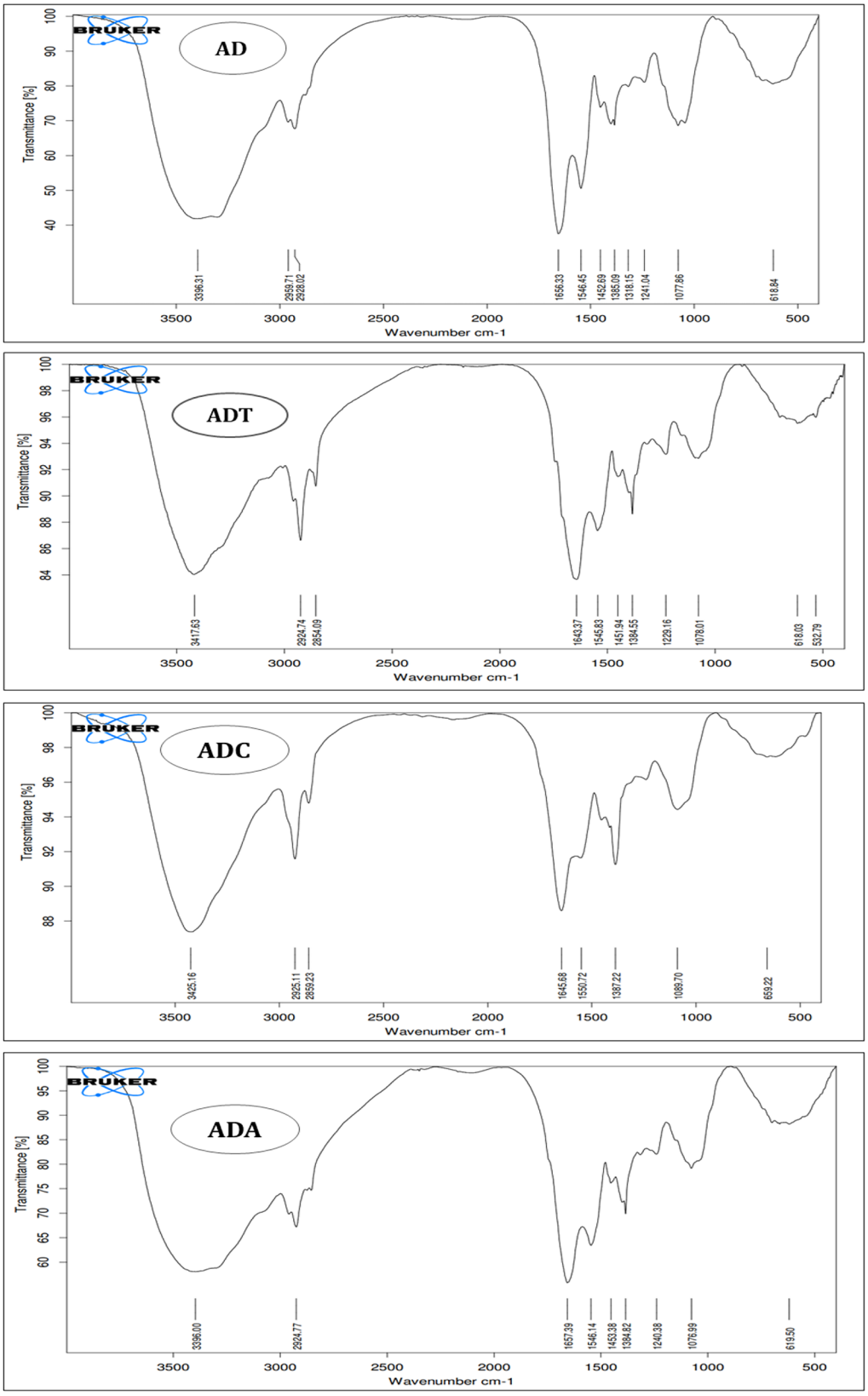

FIGURE 6 | Fourier-transform infrared spectroscopy of *Amaranthus dubius* protein isolate and their hydrolysates. AD-protein isolates, ADT- trypsin hydrolysate, ADC- chymotrypsin, and ADA- alcalase.

break the contacts that stabilize the tertiary structure (enthalpy; ΔH) of proteins (Durowoju et al. 2017). The *A. dubius* protein hydrolysates showed denaturation endotherms at different peak temperatures according to their treatment of proteolytic enzymes. These endotherms agree with those obtained earlier by Condés et al. (2009) and Martínez and Añón (1996). The thermogram is shown in Figure 7A, corresponding to trypsin, chymotrypsin, and alcalase hydrolysates. The peak temperature of trypsin hydrolysates was 57.73°C, chymotrypsin hydrolysates showed two endotherms that were 63.57°C and 116.92°C, indicating that the fraction has a higher thermal stability, while Alcalase hydrolysates showed an endotherm at 59.91°C. These suggest that different enzymatic hydrolysis processes introduce conformational changes.

TGA revealed the thermal stability of the protein hydrolysates. TGA curves at a heating rate of 10°C /min are shown in Figure 7C. It exhibits a weight drop in the TGA form. An almost constant plateau is the consequence of a gradual weight decline with two almost abrupt shifts at about 50°C and 250°C. Within the range of 30°C to 300°C, the material has a drop-in weightiness of 51.79% for trypsin hydrolysate, 52.22% for chymotrypsin hydrolysate, and 69.09% for Alcalase hydrolysate.

3.4 | Bioactivity Profiling of Protein Hydrolysates and Their MWCO Fractions

3.4.1 | Antioxidant Activity

Antioxidant activities were studied using the FRAP, DPPH, ABTS, and metal-chelating assays. Digestion significantly enhanced the antioxidant activity of the *Amaranthus dubius* protein isolate, likely due to the release of bioactive peptides during hydrolysis, which can effectively scavenge free radicals. A partially hydrolyzed protein chain has also been suggested to have the ability to donate hydrogen because of increased exposure of its residue amino acids (Agrawal et al. 2019).

Among the protein isolatez and their hydrolysates, trypsin hydrolysate (ADT) showed the highest DPPH scavenging activity (i.e., 57.37 ± 0.128 μg AAE/mg protein hydrolysate), while Ad protein isolate (AD) showed less activity (33.71 ± 0.993 μg AAE/mg protein isolate). A >50 kDa showed the highest activity, 60.05 ± 0.217 μg AAE/mg in chymotrypsin hydrolysate, while the lowest DPPH inhibition activity was recorded at <3 kDa (i.e., 26.13 ± 0.520 μg AAE/mg) (Figure 8).

All the studied protein hydrolysates and their fractions showed promising ABTS activity compared with the protein isolate. Among them, Alcalase hydrolysate showed maximum activity (i.e., 122.03 ± 0.881 μg AAE/mg protein hydrolysate), while the lowest activity (i.e., 65.37 ± 0.081 μg AAE/mg protein isolate) was found in AD protein isolate. The trypsin hydrolysate fraction, 3–10 kDa, Showed the highest activity (i.e., 133.96 ± 0.115 μg AAE/mg), while alcalase hydrolysate was exhibited at 10—50 kDa., which was 134.25 ± 0.667 μg AAE/ mg, as shown in Figure 9.

The protein isolate showed maximum FRAP activity (29.23 ± 0.002 μM), while among hydrolysates, >50 kDa fractions had the highest activity: trypsin (69.23 ± 0.002 μM), chymotrypsin (71.61 ± 0.002 μM), and Alcalase (84.83 ± 0.005 μM). The lowest FRAP activities were found in smaller fractions: 10–50 kDa (40.78 ± 0.002 μM), 3–10 kDa (30.07 ± 0.003 μM), and <3 kDa (33.16 ± 0.001 μM) as shown in Figure 10.

Trypsin and Alcalase hydrolysates showed similar FICA activities, with trypsin having the highest (83.99% ± 0.004%) and ADA (83.87% ± 0.003%). Trypsin hydrolysate at 10–50 kDa had the highest inhibition (90.84% ± 0.008%), while >50 kDa had the lowest (83.66%). Chymotrypsin hydrolysate <3 kDa showed 91.81 ± 0.001%, and >50 kDa had the lowest (85.21 ± 0.001%). Alcalase hydrolysate <3 kDa had 90.71% ± 0.003%, and >50 kDa had 83.95% ± 0.002%. The enzyme controls, trypsin, chymotrypsin, and alcalase showed negligible or no measurable bioactivity in the assays conducted. Results are shown in Figure 11. Similar results were reported previously by Zhuang et al. (2013) and Zhang et al. (2010) for peptides with MW <3 kDa which showed metal chelating ability compared to the other fractions. This may be because Fe^{2+} binds to amino and carboxyl groups in their side chains as a result of peptide cleavage.

Peptides smaller than 20 amino acids have been associated with antioxidant potential, expressing that smaller peptides have superior potential, and contain more free amine groups (Taniya et al. 2020). Antioxidant activity has been known in some protein hydrolysates from rapeseed (Zhang et al. 2008), *Porphyra columbina* (Cian et al. 2012), and *Amaranthus hypochondriacus* (Sabbione, Ibanez, et al. 2016). Antioxidant activity depends on variables such as molecular weight, amino acid content, and sequence, with peptides with more than 20 amino acid residues (molecular weight 4000 Da) exhibiting the highest activity. Moreover, it has been discovered that aromatic rings, free amine acids (Trp, Tyr, and Phe), or imidazole, as well as containing sulphur (Cys and Met), may increase antioxidant activity (Ayala-Niño, Rodriguez-Serrano, Gonzalez-Olivares, et al. 2019). High histidine (His) concentration (levels above 20%) in peptide fractions has greater iron-chelating activity, which was studied previously by Torres-Fuentes et al. (2012).

3.4.2 | Anti-Inflammatory Activity by Membrane Stabilization

The anti-inflammatory activity of *Amaranthus dubius* protein isolate, hydrolysates, and their fractions was evaluated using HRBC membrane stabilization. The protein isolate showed the highest inhibition (18.05% ± 0.02%), while chymotrypsin hydrolysate had the lowest (14.49% ± 0.006%). Trypsin hydrolysate at 10–50 kDa showed the highest activity (29.87% ± 0.001%), and Alcalase hydrolysate at <3 kDa exhibited a remarkable 57.05% ± 0.003% inhibition. The lowest activity was seen in protein isolates and larger fractions, such as >50 kDa, across different hydrolysates (Figure 12). Trypsin, chymotrypsin, and alcalase enzyme controls exhibited little to no detectable bioactivity.

Lower molecular weights and certain amino acids were discovered to be essential for anti-inflammatory actions (Liu et al. 2022). According to Gao et al. (2021) peptides act as signaling molecules and decrease the expression of cyclooxygenase-2 (COX2), inducible nitric oxide synthase (iNOS), TNF-α, and

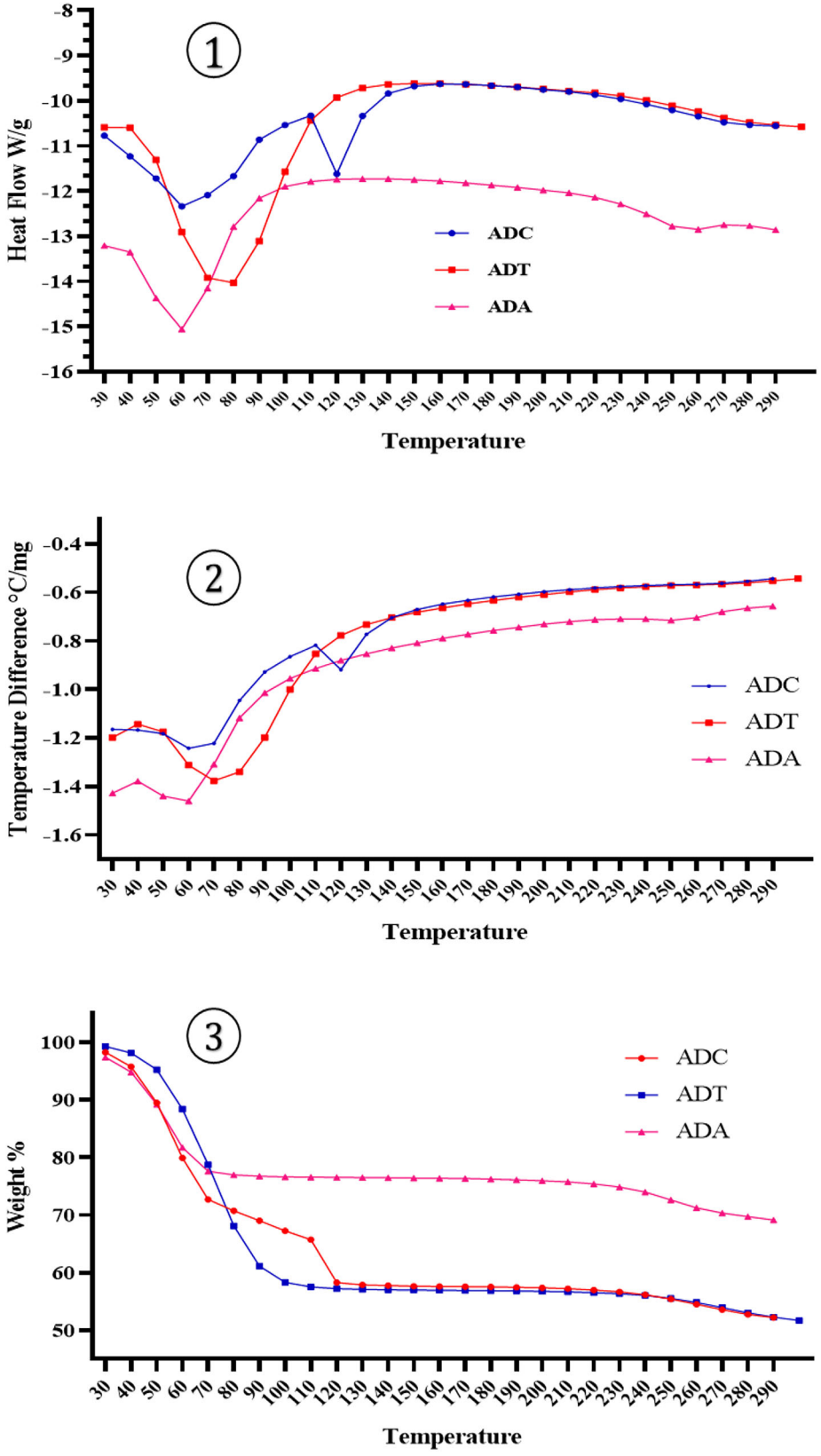

FIGURE 7 | Characterization of *Amaranthus dubius* protein isolates and hydrolysates. 1- differential scanning calorimetry (DSC), 2- differential thermal analysis (DTA), and 3- thermogravimetric analysis (TGA).

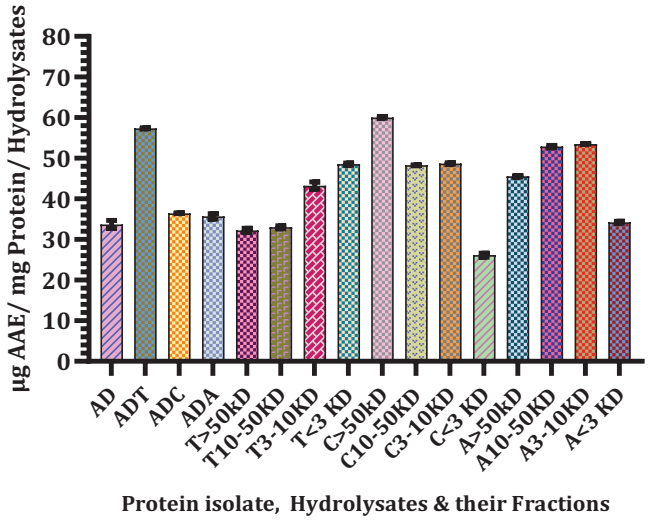

FIGURE 8 | DPPH scavenging activity of *A. dubius* protein isolates, hydrolysates, and their fraction (AD- *A. dubius* protein isolates, ADT- *A. dubius* trypsin hydrolysate, ADC- chymotrypsin hydrolysates, and ADA- alcalase hydrolysates). Results were expressed as mean value ± SE ($p < 0.05$).

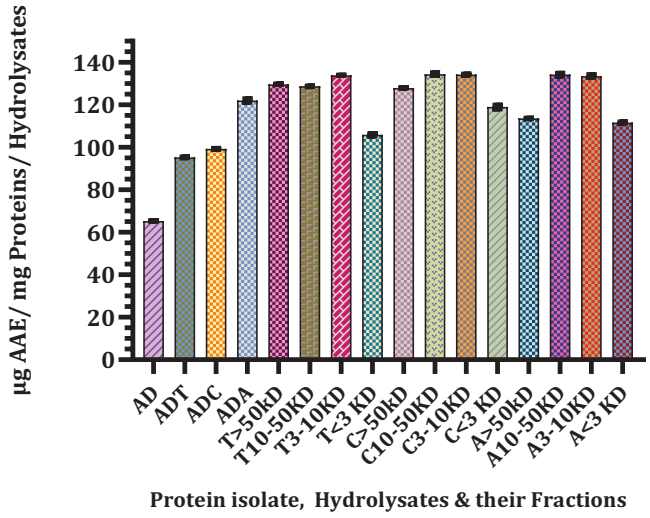

FIGURE 9 | ABTS radical scavenging activity of *A. dubius* protein isolates, hydrolysates, and their fraction (Ad- *A. dubius* protein isolates, ADT- *A. dubius* trypsin hydrolysate, AdC- chymotrypsin hydrolysates, and ADA- alcalase hydrolysates). Results were showed as mean value ± SE ($p < 0.05$).

IL-6 (Rivera-Jiménez et al. 2022; Zaky et al. 2022), p-IkB/IkB, pp65/p65, and p-p38/p38 and increase the expression of heme oxygenase-1 (HO1) and nuclear factor erythroid 2-related factor (Nrf2).

Plant-derived bioactive peptides offer a possible substitute for traditional anti-inflammatory drugs like NSAIDs, with potentially fewer side effects, including reduced gastrointestinal side effects. NSAIDs like aspirin and ibuprofen are commonly linked with gastrointestinal side effects such as stomach ulcers and bleeding. In contrast, plant-derived biologically active peptides are categorized by low molecular weights, which facilitate their

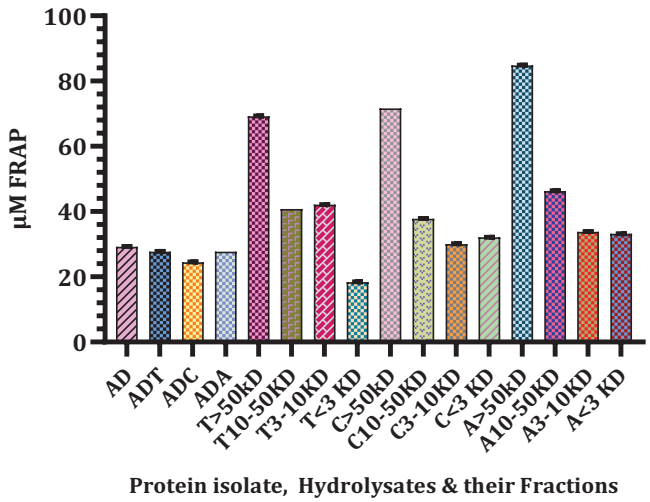

FIGURE 10 | Ferric reducing antioxidant power of *A. dubius* protein isolates, hydrolysates, and their fraction (Ad- *A. dubius* protein isolates, ADT- *A. dubius* trypsin hydrolysate, ADC- chymotrypsin hydrolysates, and ADA- alcalase hydrolysates). Results were described as mean value ± SE ($p < 0.05$).

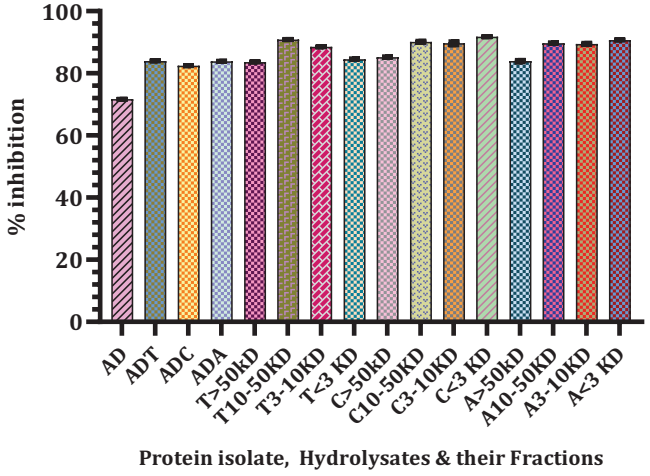

FIGURE 11 | Ferrous ion chelating activity of *A. dubius* protein isolates, hydrolysates, and their fraction (Ad- *A. dubius* protein isolates, ADT- *A. dubius* trypsin hydrolysate, ADC- chymotrypsin hydrolysates, and ADA- alcalase hydrolysates). Results were described as mean value ± SE ($p < 0.005$).

absorption in the intestines without placing a significant burden on the gastrointestinal tract (Liu et al. 2022). This property makes them ideal for individuals with chronic health issues.

3.4.3 | Antithrombotic Activity

ADT (trypsin hydrolysate) showed the highest antithrombotic activity, which was 15.33% ± 0.010% while, AD protein isolate showed the lowest, 0.83% ± 0.002%, antithrombotic activity. When comparing the trypsin hydrolysates, 3–10 kDa hydrolysate showed the highest activity (i.e., 28.11% ± 0.006%), and the lowest activity, 5.43% ± 0.002% was observed in/at <3 kDa. Chymotrypsin at 10–50 kDa exhibited the highest antithrombotic

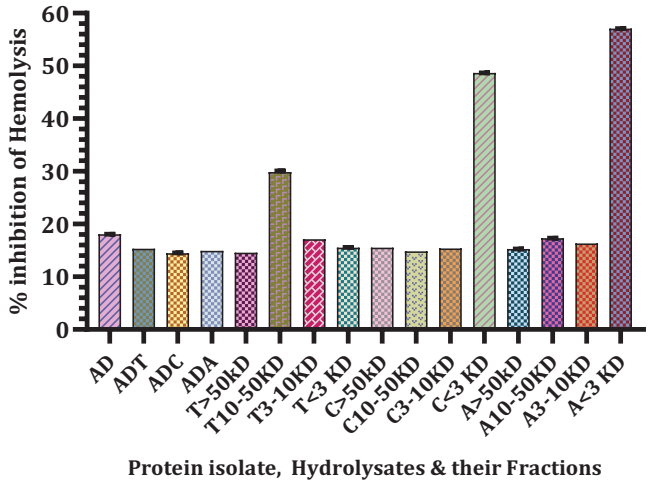

FIGURE 12 | Anti-inflammatory activity by membrane stabilization of *A. dubius* protein isolates, hydrolysates, and their fraction (Ad- *A. dubius* protein isolates, ADT- *A. dubius* trypsin hydrolysate, ADC- chymotrypsin hydrolysates, ADA- alcalase hydrolysates). Results were described as mean value ± SE.

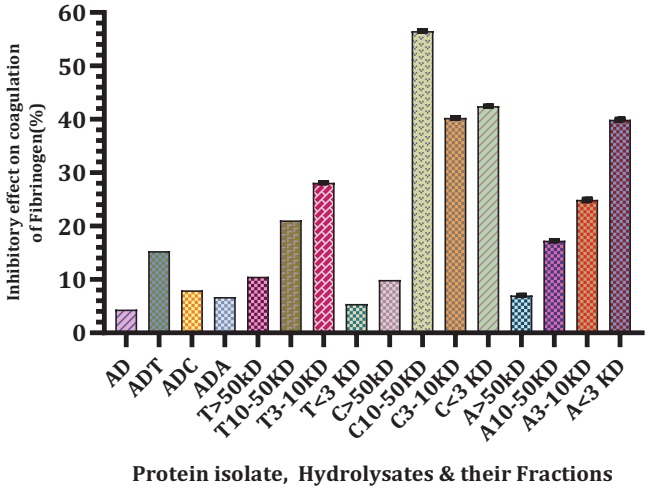

FIGURE 13 | Antithrombotic activity of *A. dubius* protein isolates, hydrolysates, and their fraction (Ad- *A. dubius* protein isolates, ADT- *A. dubius* trypsin hydrolysate, ADC- chymotrypsin hydrolysates, and ADA- alcalase hydrolysates). Results were described as mean value ± SE.

activity, which was, 56.54% ± 0.017% while at 9.90% ± 0.003%, the lowest activity was observed at >50 kDa. Alcalase hydrolysate at <3 kDa has shown the highest antithrombotic activity (i.e., 39.93% ± 0.001%) and the lowest activity was recorded in/at >50 kDa which was 7.02% ± 0.004% (Figure 13). The enzyme controls, trypsin, chymotrypsin, and alcalase showed negligible or no measurable bioactivity in the assays conducted. According to McCarthy et al. (2013), peptides derived from protein hydrolysates can exhibit antithrombotic effects through various mechanisms, including inhibition of platelet aggregation, anticoagulant activity, fibrinolytic effects, and improvement of endothelial function. The specific antithrombotic potency of bioactive peptides can be influenced by factors such as their amino acid sequence, structure, and physicochemical properties.

The process of blood clotting mostly depends on the development of a fibrin clot, which is the outcome of the interaction between fibrinogen and thrombin (Zhang 2016). According to Wu and Xu (2012), the buildup of fibrin in blood arteries typically intensifies thrombosis, as fibrin is the main protein found in blood and is produced by thrombin from fibrinogen. Sabbione, Nardo, et al. (2016) studied amaranth hydrolysate produced by activating an endogenous aspartic protease, and Sabbione, Ibanez, et al. (2016) studied the in vitro antithrombotic activity of amaranth peptides released by simulated gastrointestinal digestion, both studies reported that protein isolates from amaranth do not exhibit antithrombotic activity, while after digestion amaranth hydrolysates can act like an inhibitor of the thrombin enzyme. Heparin and warfarin, traditional anticoagulants, have been prescribed for over fifty years. Both are highly effective and save lives. However, both warfarin and heparin are generic medications with several side effects (Wu and Xu 2012; Bates and Weitz 2006). According to Owens and Mackman (2010), the number of safe and efficient medications for the treatment and prevention of thrombosis is restricted. Antithrombotic peptides prevent platelet aggregation and bind fibrinogen to a particular receptor on the platelet surface. It has also been reported that antithrombotic peptides are found in various food sources, like egg white (Yang et al. 2007), rapeseed (Zhang et al. 2008), peanut protein hydrolysates (Zhang 2016), and velvet beans (Campos et al. 2013).

3.4.4 | Antibacterial Activity

The antibacterial activity (Figure 14a,b) of protein isolate, hydrolysates, and their fractions was assessed by using Gram-positive *Staphylococcus aureus* NICM 2654 and Gram-negative *Escherichia coli* NCIM 2832, screened against the antibiotic streptomycin. The enzymes, trypsin, chymotrypsin, and alcalase showed negligible or no measurable antibacterial activity. Considering *E. coli* growth inhibition, trypsin hydrolysate (ADT) has the highest bacteriostatic activity compared to other hydrolysates and protein isolate, which was 31.13% ± 0.001% inhibition, while protein isolate (Ad) showed the lowest activity, (i.e., 16.09% ± 0.051% inhibition). The trypsin hydrolysate <3 kDa fraction exhibited the highest antibacterial activity, 32.92% ± 0.007% inhibition, while the >50 kDa fraction had the lowest, 26.18% ± 0.015% inhibition activity. As compared to all other chymotrypsin hydrolysates, the 3–10 kDa fraction showed the highest antibacterial activity (i.e., 36.27% ± 0.011% inhibition). Similar results, like trypsin, a remarkable antibacterial activity at <3 kDa fraction was observed in alcalase hydrolysate, which was 37.20% ± 0.008% inhibition and 21.74% ± 0.018% inhibition, the lowest activity was noted in >50 kDa hydrolysate.

For *S. aureus* growth inhibition, it showed that trypsin hydrolysate (ADT) has the highest bacteriostatic activity compared to other hydrolysates and protein isolate, which was 38.07% ± 0.008% inhibition, while protein isolate (Ad) showed the lowest activity (i.e., 30.25% ± 0.011% inhibition). The trypsin hydrolysate <3 kDa fraction and >50 kDa fractions exhibited somewhat similar antibacterial activity, 25.08% ± 0.008% and 30.82% ± 0.017% inhibition. While opposite to these chymotrypsin hydrolysates, 3–10 kDa and 10–50 kDa fractions showed the highest antibacterial activity (i.e., 42.26% ± 0.022%

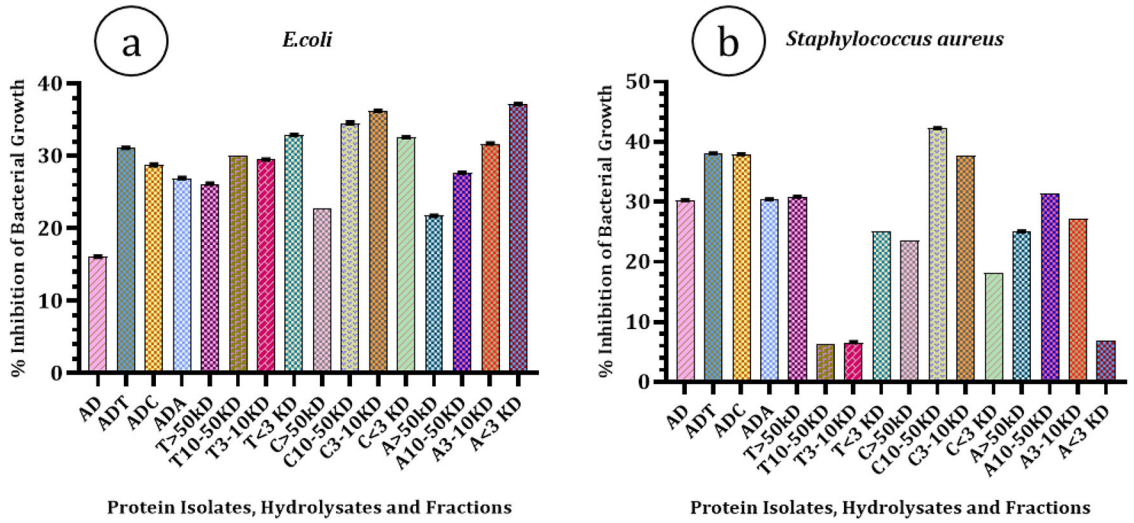

FIGURE 14 | Antibacterial activity of *A. dubius* protein isolates, hydrolysates and their fraction against a. *E. coli* and b. *S. aureus* (Ad- *A. dubius* protein isolates, ADT- *A. dubius* trypsin hydrolysate, ADC- chymotrypsin hydrolysates, and ADA- alcalase hydrolysates). Results were expressed as mean value ± SE ($p < 0.05$).

inhibition). Similar results like chymotrypsin, a remarkable antibacterial activity at 3–10 kDa and 10–50 kDa fractions, were observed in Alcalase hydrolysate, which was 31.39% ± 0.014% inhibition and 27.21% ± 0.012% inhibition.

AMPs are molecules that show potential as next-generation antibiotics due to their ability to combat drug-resistant pathogens. AMPs are key elements of innate immunity, targeting bacteria, viruses, and fungi. Research on AMPs focuses on overcoming microbial resistance and developing effective delivery systems for clinical use, making them a promising tool for future antimicrobial therapies (Moretta et al. 2021). Smaller peptides generated during hydrolysis may exhibit enhanced antibacterial properties by effectively interacting with and disrupting bacterial cell membranes. Their smaller size allows them to penetrate the cell wall, causing membrane depolarization, leakage of metabolites, and cell death. The process occurs in three stages: attraction to the cell wall of bacteria, union with the cell membrane, and peptide incorporation, leading to membrane permeabilization (López-García et al. 2022).

Plants contain large amounts of AMPs, which are produced as a kind of self-defense (Zhang et al. 2021). Cyclotides, defensins, thionins, lipid transfer proteins, snakins, glycine-rich proteins, and hevein-type proteins are some of the groups into which they can be separated (Tang et al. 2018).

Plants offer a rich source of bioactive peptides with various health benefits, challenges related to bioavailability from sustainable sourcing, and economic viability must be addressed to ensure their availability and effective use in the health as well as food industries.

4 | Conclusion

This study highlights the significant potential of *Amaranthus dubius* seeds as a source of bioactive proteins with promising health benefits. This is the first study to report the isolation of proteins from the leaves, stems, and seeds of *Amaranthus dubius*. Among these, seeds were identified as the richest protein source, exhibiting the most potent antioxidant and membrane-stabilizing activities. Furthermore, characterization of protein isolates and their hydrolysates shows significant differences that suggest structural changes. Enzymatic hydrolysis enhanced the functional potential of these proteins, with specific molecular weight fractions showing significant antioxidant, anti-inflammatory, antithrombotic, and antibacterial activities. In particular, alcalase and chymotrypsin hydrolysates in the <3 kDa and 10–50 kDa ranges displayed the most promising bioactivities. These findings highlight the potential of *Amaranthus dubius* seed proteins and their hydrolysates as multi-functional bioactive ingredients. Enzymatic hydrolysis and fractionation significantly enhanced their health-promoting properties, supporting their application in nutraceutical and functional food development.

Author Contributions

Aditya B. Magdum: conceptualization, investigation, writing – original draft, methodology, validation, visualization, software, formal analysis, data curation. **Kapil V. Shinde**: investigation, methodology, validation, visualization, software, formal analysis. **Mahesh P. Mane**: methodology, validation, software, formal analysis. **Ruturaj S. Patil**: methodology, validation, software, formal analysis. **Mansingraj. S. Nimbalkar**: conceptualization, investigation, writing – review and editing, validation, formal analysis, data curation, supervision, resources.

Acknowledgments

The authors are appreciatively thankful to the Head, Department of Botany, Shivaji University, Kolhapur, for the essential laboratory facilities. The author ABM is grateful to the Department of Biotechnology (DBT), New Delhi, India, for financial provision through the DBT BUILDER-SUK program granted to Shivaji University, Kolhapur.

Conflicts of Interest

The authors declare no conflicts of interest.

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