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Isolation and *in vitro* antioxidant potential of tryptic hydrolysates of *Balanites aegyptiaca* (L.) Delile seed proteins

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Abstract
The present finding investigates the antioxidant potential of trypsin hydrolysates fractions. Among the hydrolysate fractions, 120 min hydrolysate was identified as most potent for antioxidant properties. Enzymatic hydrolysis conditions significantly affect the bio-activities of hydrolysates. The trypsin hydrolysates of 120 min showed max. SOD activity than FRAP and DPPH radical scavenging activity. HPLC profiles confirm the presence of bioactive peptides in the 120 min of trypsin hydrolysate fraction, highlighting trypsin enzyme-specific generation and potential application as natural antioxidant supplements in functional foods and therapeutics.

Keywords: Functional food, hydrolysate, natural antioxidants, nutraceuticals, protein yield

1. Introduction
Oxidative stress, which are naturally generated by reactive oxygen species (ROS) and free radicals during cellular respiration and various physiological processes, have been associated in the etiology of numerous chronic conditions, including neurodegenerative disorders and metabolic diseases [1, 2]. Persistent oxidative imbalance not only intensifies vascular dysfunction but also increases the risk of cardiovascular complications including heart failure, stroke, and premature mortality [3]. Natural antioxidant compounds are crucial for neutralizing ROS and free radicals, thereby preventing oxidative damage to cellular components, and maintaining cellular homeostasis [4, 5]. Given the growing global prevalence of oxidative stress-related disorders, there is an urgent need to identify natural substances that exhibit effective antioxidant properties.

Plant-derived proteins are increasingly being recognized for their potential health benefits, particularly in mitigating oxidative damage and managing health complications associated with oxidative stress [6, 7]. Compared with synthetic and animal-based proteins, plant proteins are often considered safer and more suitable for human consumption [8]. However, the extraction and purification of proteins from plant sources pose significant challenges, including low solubility, susceptibility to denaturation, the existence of interfering components such as tannins and polyphenols, and difficulties in obtaining high yields and purities [9, 10]. Protein hydrolysates, which are produced through enzymatic hydrolysis and are composed of short-chain peptides and free amino acids, exhibit improved intestinal absorption, lower osmotic pressure, and enhanced bioavailability [11]. Their nutritional and functional characteristics make them promising candidates for addressing global nutritional demands and contributing to the development of sustainable and affordable protein-based food systems [12, 13]. Recent researches have confirmed the antioxidant properties of protein hydrolysates derived from various plant sources including legumes, nuts, fruits, vegetables, and whole grains [14, 15]. Enzymatic hydrolysis not only enhances the functional attributes of plant proteins but also promotes the release of bioactive peptides with significant pharmacological potential. However, proteolytic enzymes exhibit site-specific cleavage patterns and the nature and bioactivity of the resulting peptides are highly dependent on the type of enzyme used [16, 17].

Balanites. aegyptiaca (L.) Delile, generally termed as the “Desert Date”, is a drought-tolerant species belonging to the Balanitaceae family.

It is widely distributed across Northern Africa, West Asia, and parts of the Middle East [18]. Despite the well-documented traditional uses, nutritional composition and pharmacological possessions of *B. aegyptiaca* [19, 20, 21], research on the isolation and functional evaluation of its seed protein isolates remains limited. Owing to their great protein content, their seeds represent a promising source of active substances with potential health-promoting properties [22, 23, 24]. Therefore, the present study was designed to evaluate the *in vitro* antioxidant activities of protein hydrolysates generated by trypsin and characterize the peptide profiles of the active hydrolysate fractions using High-Performance Liquid Chromatography (HPLC).

2. Materials and Methods

2.1 Materials

The fruits of *B. aegyptiaca* were collected from naturally occurring populations in the Bhilwara District of Rajasthan, India. The hydrolytic enzymes used in this study trypsin (EC 3.4.21.4; activity >2500 U/mg) was from HiMedia. All other chemicals used in this study were of analytical grade, including 2, 2-diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), o-phthaldialdehyde (OPA), sodium dodecyl sulfate (SDS), ascorbic acid, potassium persulfate, sodium borohydride, ferric chloride ($FeCl_3$), bromophenol blue, and β-mercaptoethanol, all of which were purchased from Himedia.

2.2. Defatting *B. aegyptiaca* seed powder

The fruits of *B. aegyptiaca* were carefully cleaned to eliminate all extraneous materials, such as dust, soil particles, stones, and any immature, damaged, or defective seeds. Following this initial cleaning, the seeds were manually cracked to separate the hard outer shells from internal kernels. To mitigate the natural bitterness of the kernels, they were immersed in boiling water for 40 minutes. After boiling, the kernels were thoroughly dried to eliminate any remaining moisture, and subsequently ground into a fine powder using a mechanical grinder. The powdered material was then passed through a No. 20 mesh sieve to ensure a uniform particle size. The resulting powder was defatted using n-hexane in a Soxhlet extraction system according to the method described by the Association of Official Analytical Chemists (AOAC). The residual hexane was removed from the defatted extract using a rotary evaporator.

2.3. Preparation of *B. aegyptiaca* protein isolates by Alkaline extraction–acid precipitation (AE–AP) method

Protein extraction was performed using alkaline extraction followed by acid precipitation at the isoelectric point, as per the method by Wang [25] with slight alterations. Initially, the defatted seed powder was suspended in distilled water at a ratio of 1:10 (w/v) and the pH was adjusted to 8.5 using 0.1 N NaOH. The mixture was agitated at room temperature for 2 h and subsequently centrifuged at 12, 000 g for 20 min. The pH of the collected supernatant was adjusted to 4.5 (the isoelectric point of *B. aegyptiaca* protein) using 0.1 N HCl. The precipitated proteins were separated by centrifugation at 12, 000 × g for 15 min, re-dissolved in distilled water, and neutralized to pH 7.0 using 0.1 N NaOH. The final protein product, representing the protein isolate was freeze-dried and stored at -20°C for further use.

2.4. Quantification of protein content

The protein content of *B. aegyptiaca* protein isolates and defatted seed powder were quantified using the Bradford assay [26].

2.5 Tryptic hydrolysis of *B. aegyptiaca* protein isolates

Following protein isolation, enzymatic hydrolysis of *B. aegyptiaca* protein isolates was performed using trypsin under optimal conditions, with an enzyme-to-substrate ratio of 1:10 (w/w). The protein isolates were initially dissolved at various concentrations (1, 2, 5, 10, 20, and 50 mg/ml) in appropriate buffer systems: 10 mM Tris-HCl buffer (pH 8.0) for trypsin. Hydrolysis reactions were conducted in a temperature-controlled shaker incubator (Tanco, India) with continuous agitation and allowed to proceed for up to 3h, with aliquots collected at specific time intervals (0, 30, 60, 120, and 180 min). The enzymatic activity was terminated by heating the reaction mixture at 100°C for 5 min. The hydrolysed samples were subsequently centrifuged at 4, 000 × g for 10 min at 4°C to separate the insoluble fractions. The supernatants, representing the protein hydrolysate were collected, freeze-dried, and stored at -20°C until further analysis. These hydrolysate samples were subsequently subjected to detailed *in vitro* analyses to evaluate their antioxidant activities.

2.6 Extent of proteolysis using OPA (o-Phthaldialdehyde) assay

The degree of proteolysis of *B. aegyptiaca* protein isolates was assessed using the o-phthaldialdehyde (OPA) assay, following the method described by Salami [27], with slight modifications. In this assay, 10 µl of each protein hydrolysate was added to 1.0 ml of the freshly prepared OPA reagent. The reaction mixture was gently mixed and incubated at room temperature (25.0 °C ± 0.5°C) for 2 min to allow for a complete reaction. The absorbance was recorded at 340 nm using a UV-Visible spectrophotometer (India). The extent of proteolysis was determined by following formula

Extent of proteolysis = (AHP − AUHP)

(AHP: absorbance of hydrolysed protein; AUHP: absorbance unhydrolysed protein)

2.7 Assessment of *in vitro* Antioxidant Activities

2.7.1 DPPH radical scavenging assay

The DPPH scavenging activity of *B. aegyptiaca* protein hydrolysates was assessed following the method of Hatano [28] with slight modifications. The reaction mixture (total volume: 3 ml) consisted of 0.5 ml of hydrolysate sample, 0.5 ml of 0.5 M acetic acid buffer (pH 5.5), 1.0 ml of 0.2 mM DPPH solution in ethanol, and 1.0 ml of 50% (v/v) aqueous ethanol. The reaction mixture was thoroughly mixed and incubated at room temperature (approximately 25°C) for 30 min in the dark. Following incubation, absorbance was measured at 517 nm using a UV-Visible spectrophotometer. The DPPH radical scavenging activity was calculated as the percentage reduction in absorbance compared to that of a control sample that did not contain hydrolysate. Ascorbic acid was used as a positive control. The scavenging activity was calculated using the following equation:

DPPH radical scavenging activity (%) = [(Ac − As) / Ac] × 100
(Ac: absorbance of the control (50% ethanol without DPPH); As: absorbance of the sample)

2.7.2 *Ferric reducing power (FRAP) assay*
The FRAP activity of *B. aegyptiaca* protein hydrolysates was assessed using the method developed by Benzie and Strain [29], with minor modifications. Briefly, 30 µL of each hydrolysate sample was mixed with 3.0 ml freshly prepared FRAP reagent. The reaction mixture was incubated at 37°C for 30 min in a temperature-controlled water bath to facilitate reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). After incubation, the absorbance of the resulting solution was measured at 593 nm using a UV–Visible spectrophotometer. The FRAP value was determined by calculating the difference in absorbance between the sample and reagent blank. The results were expressed as millimoles of ferrous sulphate equivalents (mM $FeSO_4$) per gram of hydrolysate.

2.7.3 *Superoxide radical scavenging activity (SOD)*
The superoxide radical scavenging activity of *B. aegyptiaca* protein hydrolysates was evaluated based on the inhibition of nitroblue tetrazolium (NBT) reduction, as described by Jain [30], with minor modifications. Superoxide anions were generated in a non-enzymatic reaction system involving phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide (NADH). The reaction mixture, with a total volume of 750 µl, consisted of 2.5 µl of hydrolysate sample, 185 µl of phosphate buffer (0.1 M, pH 7.4), 187.5 µl of NADH solution (468 µM), 187.5 µl of NBT solution (150 µM), and 187.5 µl of PMS solution (60 µM). The mixture was incubated at room temperature for 5 min to facilitate the formation of chromogenic formazan via the reduction of NBT by superoxide anions. Absorbance was measured at 560 nm using a UV-Visible spectrophotometer, and the scavenging activity was expressed as a percentage inhibition of superoxide radical formation relative to the control, calculated using the DPPH equation.

2.8. HPLC analysis
The *B. aegyptiaca* protein hydrolysates, which demonstrated the most pronounced antioxidant activities, were subsequently subjected to metabolic profiling using reverse-phase high-performance liquid chromatography (RP-HPLC). This analysis was performed using an Agilent HPLC system equipped with a semi-preparative C18 Luna column (250 mm × 4.6 mm; Phenomenex, Torrance, CA, USA) at ambient temperature. The mobile phase was comprised of solvent A (deionized water with 0.1% trifluoroacetic acid [TFA]) and solvent B (80% acetonitrile in water with 0.1% TFA). Peptide separation was achieved using a programmed linear gradient: 0% solvent B for the initial 16 min, a gradual increase from 0% to 50% B over the subsequent 83 min, followed by a ramp from 50% to 100% B over 9 min. This was maintained at 100% B for an additional 16 min, after which the system was re-equilibrated to 0% B for 5 min. The flow rate was maintained at 0.8 ml/min throughout the analyses. The eluted peptides were detected at 280 nm using an inline UV detector and chromatographic data were recorded for further peptide characterization.

2.9 Statistical Analysis
The experimental results are presented as mean values ± standard deviation from three independent replicates. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) to determine significant differences among means. Differences were considered statistically significant at a confidence level of $p \leq 0.05$.

3. Results
3.1. Protein content and yield of *B. aegyptiaca* seed proteins and MW distribution
The protein content of *B. aegyptiaca* seed protein isolates obtained is 9.33 mg/ml, with a yield of 6.2 g per 100 g of defatted seed powder. In contrast, the direct protein content measured in the defatted seed powder was 5.54 mg/ ml with a yield of 3.6 g /100 g.

3.2. Extent of proteolysis
The extent of proteolysis (measured as DA_{340}) for *B. aegyptiaca* protein are illustrated in Fig 1. Notably, trypsin-treated sample at 5 mg/ml concentration exhibits the highest extent of hydrolysis, with a DA_{340} value of 0.1352. This was slightly higher than the values observed for other concentration of trypsin-treated samples.

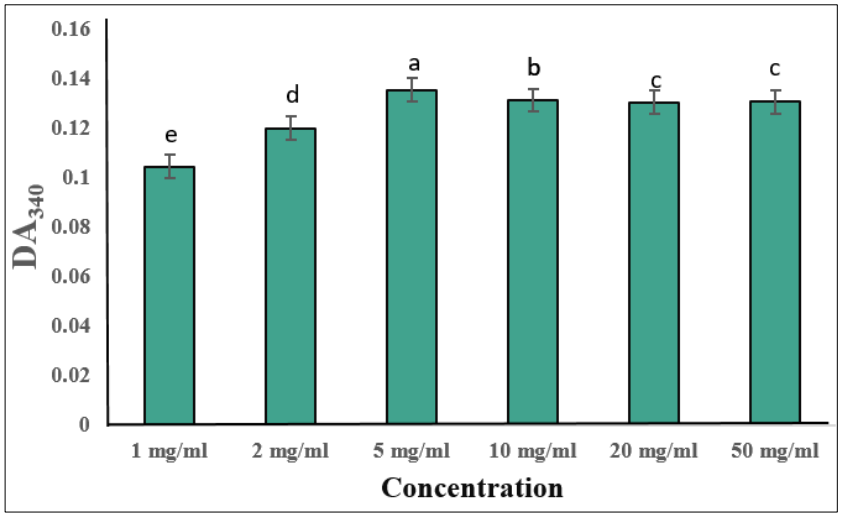

Fig 1: Extent of proteolysis (DA_{340}) of *B. aegyptiaca* tryptic protein hydrolysates

3.3. DPPH radical scavenging activity of *B. aegyptiaca* protein hydrolysate

The DPPH radical scavenging activity of *B. aegyptiaca* protein hydrolysate obtained using trypsin enzyme is presented in Fig 2. The hydrolysates produced with trypsin, exhibited DPPH scavenging activities of 2.72–8.87%. At a protein concentration of 5 mg/ml of trypsin-derived hydrolysate showed comparatively higher DPPH activity than the other concentrations. The obtained DPPH scavenging activity was lower than that of the standard antioxidant ascorbic acid (32.71% ± 0.2%).

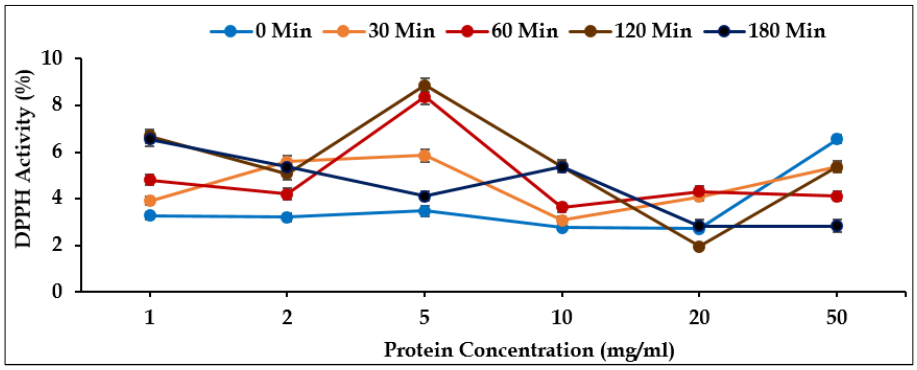

Fig 2: DPPH scavenging activity of B. aegyptiaca protein hydrolysate produced by trypsin (Data analysed the means ± SD for statistical significance; mean values followed by the different subscript in a column are significantly different ($p < 0.05$) based on DMRT).

3.4. FRAP activity of *B. aegyptiaca* protein hydrolysate

Figure 3 shows the FRAP activity of *B. aegyptiaca* protein hydrolysate produced under various enzymatic treatments. The hydrolysates produced using trypsin, exhibited FRAP scavenging activity of 0.108 to 0.96 mM $FeSO_4$/g. At a protein concentration of 5 mg/ml, the trypsin-derived hydrolysate demonstrated higher FRAP activities than other concentrations. The highest FRAP activity (0.96 mM $FeSO_4$/g) was observed in the hydrolysate produced using trypsin after 120 min of hydrolysis.

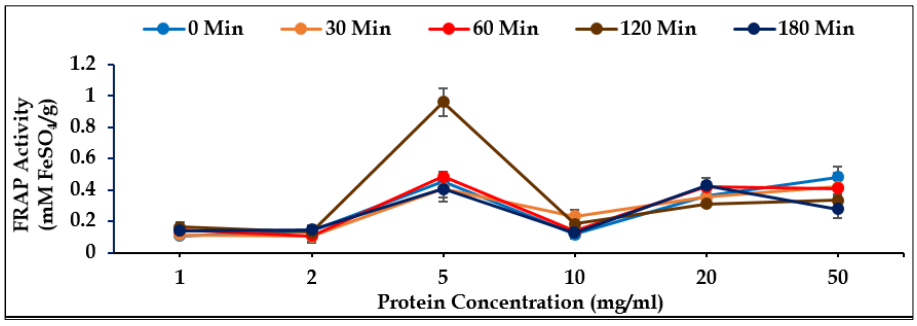

Fig 3: FRAP scavenging activity of B. aegyptiaca protein hydrolysate produced by (Data analysed the means ± SD for statistical significance; mean values followed by the different subscript in a column are significantly different ($p < 0.05$) based on DMRT).

3.5. SOD activity of *B. aegyptiaca* protein hydrolysate

The SOD activity of the *B. aegyptiaca* protein hydrolysate produced by using trypsin enzyme is shown in Fig. 4. The SOD activity ranged from 5.02% to 32.89% for trypsin-derived hydrolysate. At a protein concentration of 5mg/ml, trypsin-derived hydrolysates exhibited significantly higher SOD activity than other concentrations. The highest SOD activity (32.89%) was observed in the hydrolysate produced using trypsin after 120 min of hydrolysis.

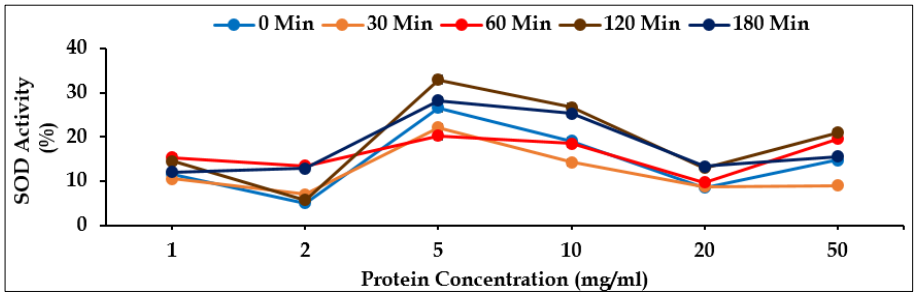

Fig 4: SOD scavenging activity of B. aegyptiaca protein hydrolysate produced by using trypsin (Data analysed the means ± SD for statistical significance; mean values followed by the different subscript in a column are significantly different ($p < 0.05$) based on DMRT).

3.6. HPLC Analysis

The HPLC chromatograms of the protein hydrolysates obtained after 120 min of hydrolysis with trypsin at a protein concentration of 5 mg/ml revealed several distinct peaks, as shown in Fig 5. Five prominent peaks were identified in the hydrolysates derived from trypsin indicating the presence of diverse peptide fractions.

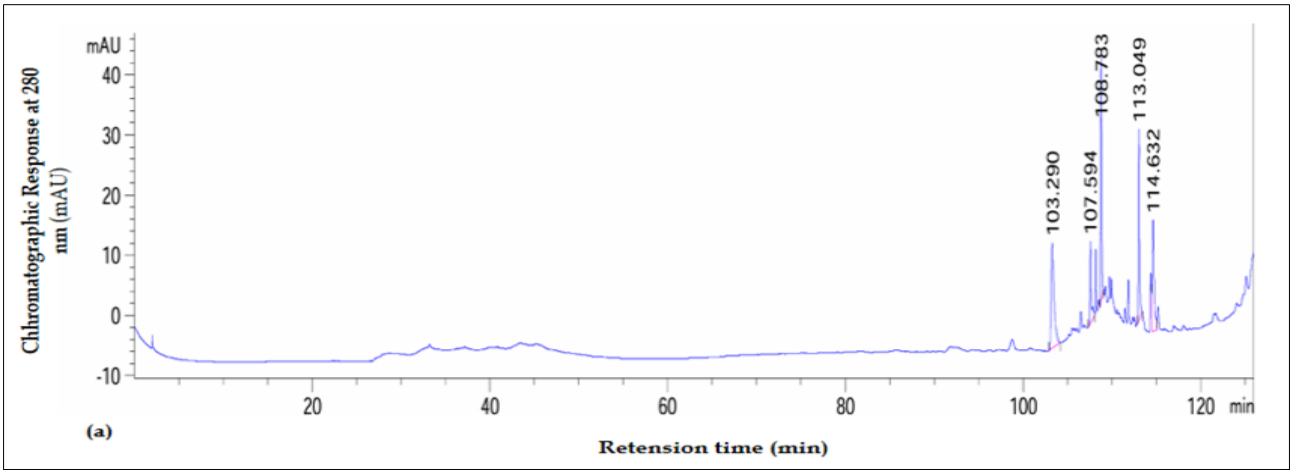

Fig 5: HPLC chromatograms of the *B. aegyptiaca* protein hydrolysates obtained after hydrolysis after 120 min of hydrolysis with trypsin at 5 mg/ml protein concentration.

4. Discussion

The rising demand for functionally active proteins from plants is driven by the need to satisfy increasing global protein requirements, address widespread nutritional deficiencies, and promote economically viable food alternatives [31, 32]. Different protein isolation practices significantly affect the protein yield, purity, and functional properties of the resulting isolates from plant samples. Factors such as pH, temperature, and the specific extraction technique used can influence the amount of extracted protein and its final state [33, 34, 35]. Therefore, optimizing isolation conditions is crucial for maximizing protein yield while minimizing the adverse effects on functional properties. In the present study, the AE-AP method isolation method was evaluated for yield higher as the controlled alkaline and acidic pH shifts, which result in better protein solubilisation and precipitation [36, 37]. Previous studies have similarly demonstrated the effectiveness of pH-shifting methods for maximizing protein yield and facilitating the removal of non-proteinaceous compounds, particularly in seed-based matrices [38, 39, 40].

The use of proteases under controlled conditions facilitates the precise hydrolysis of proteins into smaller peptides or free amino acid residues, optimizing their biological and functional properties while preventing protein degradation [41, 42]. In this study, the considerable proteolysis was achieved using trypsin. This could be because trypsin has a remarkable catalytic ability and tends to break peptide bonds, especially those associated with aromatic amino acids. It is possible that *B. aegyptiaca* seed proteins possess specific amino acid sequences or structural characteristics that make them susceptible to trypsin-mediated hydrolysis, thereby facilitating efficient cleavage at these vulnerable sites [43].

The antioxidant potential of plant-derived proteins can be effectively evaluated using various *in vitro* analytical assays. The DPPH assay measures the ability of antioxidant compounds to donate protons and neutralize DPPH free radicals, which exhibit a characteristic absorbance maximum at 517 nm [44]. A reduction in absorbance following the interaction between the hydrolysate and DPPH reagent reflects the scavenging potential of the sample, thereby quantifying their DPPH activity [45]. In this study, *B. aegyptiaca* protein hydrolysates obtained using trypsin exhibited highest DPPH radical scavenging activity at a protein concentration of 5 mg/ml, the hydrolysate produced after 120 min of hydrolysis with trypsin. However, the activity levels were not considerable. These results suggest that *B. aegyptiaca* tryptic hydrolysates do not contain a substantial number of peptides capable of donating electrons, which is necessary to react with DPPH radicals and convert them into more stable compounds [46, 47]. The observed variations in DPPH radical-scavenging ability among the different hydrolysates may be attributed to alterations in their amino acid compositions, with decreased DPPH activity often correlated with the presence of hydrophilic amino acids [48, 49].

The FRAP assay is a well-recognized method for assessing the overall reducing power of bioactive compounds, and provides a key measure of their ability to counteract oxidative damage [50, 51]. In the present study, *B. aegyptiaca* protein hydrolysates produced by trypsin enzymatic treatments demonstrated distinct FRAP activity. At a protein concentration of 5 mg/ml, the hydrolysate obtained after 120 min of trypsin hydrolysis exhibited moderate FRAP activity. The obtained FRAP activity observed in trypsin-derived hydrolysate may be attributed to the release of specific amino acids during enzymatic cleavage, which can donate electrons and utilize their charged side chains to effectively reduce transition metal ions [52]. These findings are consistent with previous reports on oilseed protein [53], rice bran [54] and legume seeds hydrolysates [55], further supporting the role of trypsin in enhancing antioxidant potential through metal-reducing mechanisms.

Superoxide anions are highly reactive oxygen species that can impose significant destruction to biological macromolecules and increase the prevalence of oxidative stress-related disorders. Enzyme superoxide dismutase plays a crucial role in cellular antioxidant defence by catalysing the dismutation of superoxide radicals into hydrogen peroxide [56, 57, 58]. In the present study, at a protein concentration of 5 mg/ml, the hydrolysate obtained after 120 min of hydrolysis with trypsin exhibited markedly higher SOD activity. The higher SOD activity in trypsin-derived hydrolysate may be attributed to the presence of abundant negatively charged amino acids, which have been reported to effectively scavenge superoxide radicals [59, 60]. These findings are in agreement with those of previous studies on sesame [61] and hemp seeds [62].

HPLC is an effective tool for identifying and quantifying the amino acid and peptide compositions in hydrolysates generated through enzymatic hydrolysis [63]. In the present

study, several prominent peaks were observed in the chromatograms of hydrolysates derived from trypsin. These distinct elution profiles suggest the presence of distinct amino acids and the formation of various bioactive peptides, which likely contributed to the enhanced antioxidant activities observed in the corresponding hydrolysates. Further investigations are needed to identify and isolate the specific peptide sequences or amino acids involved to clarify the precise mechanisms underlying these bioactivities.

5. Conclusion

This study highlights the significant impact of protein isolation and enzymatic hydrolysis strategies on the bio-functional properties of *B. aegyptiaca* seed proteins. Furthermore, this study demonstrated that hydrolysis conditions significantly influence the active profiles of *B. aegyptiaca* protein hydrolysates. The most potent bioactive hydrolysates, as determined by their antioxidant activities, were obtained after 120 min of hydrolysis with trypsin. Notably, the trypsin-derived hydrolysate demonstrated high extent of SOD like activity. HPLC analysis further confirmed the occurrence of bioactive peptides associated with these functional properties. These results underscore the enzyme-specific generation of bioactive peptides from *B. aegyptiac*a seeds, establishing their potential as natural antioxidant agents for use in functional foods and nutraceuticals. Future research should focus on characterizing specific peptide sequences and evaluating their bioavailability and physiological efficacy through *in vivo* studies to support their therapeutic potential in oxidative stress-related disorders.

6. Author's Contributions: Conceptualization, S.M. and J.A.; methodology, investigation, resources, writing - original draft S.M.; data curation, review and editing, visualization, S.M. A. j., B. K. and J.A.; supervision, J.A. All figures have been made by S.M. All authors have read and agreed to the published version of the manuscript.

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8. Conflicts of interest: The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

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