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RESEARCH ARTICLE

Characterization and Proteolytic Activity of *Arum palaestinum* extract

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ABSTRACT

Medicinal plants have been utilized to treat a wide range of diseases and have constituted a primary source of bioactive compounds for pharmaceutical development. Plant proteases are essential in regulating metabolic pathways that influence gene expression and remove unfavourable metabolites. The current study aims to evaluate the *in vitro* proteolytic and bioactivity of the methanolic leaf extract of Arum palaestinum. The plant extract's TPC, antioxidant, anti-inflammatory, and anti-hemolytic activities were determined using colorimetric methods, and the proteolytic activity was assessed using casein as a substrate. The investigations revealed that the extracts possess anti-inflammatory properties of 90–95%, with IC_{50} = 1.1133 mg/mL, and anti-hemolytic activity of 90–95% with IC_{50} = 0.2149 mg/mL. The extract exhibited good antioxidant activity of 71.30 % with IC_{50} = 0.1975 mg/ml. The result also showed that the raw extracts contain phenolic compounds (65.48 mg GAE/g). The extract's proteolytic effects on casein as a substrate were observed at pH 7.0 and 50 °C. The raw extract showed the most significant activity toward casein as a substrate. The Lineweaver-Burk reciprocal plot indicated a Km score of 0.667 mg/mL and Vmax = 5.0 μmol/min for the proteases in the extracts. The plant extract demonstrates potent antioxidant, anti-inflammatory, and anti-hemolytic properties, which could be associated with the polyphenolic compounds. The extract also showed potential as a source of proteolytic enzymes and could be harnessed as a source of proteases for industrial uses. This plant can further be explored as a source of potent compounds for drug development and industrial applications.

Keywords: *Arum palaestinum*, Antioxidant, Anti-inflammatory activities, Proteases, Phenolic compounds

Introduction

Medicinal plants have been used to treat many diseases since the dawn of mankind. They are a great source of biologically active compounds. Bioactive compounds found in seedlings are vital for different physiological activities, including growth, disease resistance, and pest resistance.[1] Bioactive compounds can exert their action on various specific physiological and biochemical processes of organisms, and it is possible to detect bioactive compounds in organism after consuming plant-based diets.[2] Various plant secondary metabolites such as polyphenols, plasmin, kallikreins, thrombin, trypsin, chymotrypsin, bovine Factor Xa, metalloproteinases, and human Hageman factor fragments have been shown to regulate viral proteases both in animals and in humans. Plant–derived proteases account for almost 43% of all characterized proteases, and nearly half of them are stored in plant seedlings.[3]

Proteases are some of the major industrial enzymes used in the production of household (food, medicine and detergent), furniture (leatherette), and in the removal of toxic wastes, pollutants and contaminants from the environment (bioremediation). They are crucial to the digestive system due to their ability to hydrolyze polypeptide chains in proteins to liberate amino acids for metabolic processes in the body.[4]

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Plant proteases have been employed in various fields of human endeavours (biotechnology, medicine, food industries, etc.) due to their ability to withstand extreme temperatures, resist changes in pH, and high solubility in aqueous condition, as well as substrate specificity.[5] Notwithstanding their involvement in many regulatory processes, there is a paucity of research in the literature concerning their regulatory duties and responsibilities. Most of the reported plant proteases have been centred on their utility as additives in food industry processes, and have been greatly harnessed in food production due to their low cost.[6]

Proteases are present copiously in plant bioactive phytoconstituents, polyphenols and carotenoids and have been shown to exhibit potent antimicrobial and antioxidant activities and exert potent inhibitory activity against low-density lipoprotein (LDL) and influence deoxyribonucleic acid (DNA) oxidation.[7] Generally, synthetic antibiotics are used clinically in the fight against pathogenic microbes. However, better protection against these invading organisms can be enhanced when the body's innate defence system is deployed. The body's defenses, in combination with antibiotics, provide maximum and better protection against microbial infections, and some proteases have shown promise in inhibiting the attachment of microorganisms to body tissues.[2,8]

The most prevalent secondary metabolites derived from plants are phenolic compounds, which are made up of one or more aromatic rings joined to one or more hydroxyl groups.[9] They possess anti-inflammatory, antioxidant, anti-microbial, anti-mutagenic, antilipidemic, and anti-depressant qualities.[9,10] By scavenging free radicals, phenolic substances can lower oxidative stress and potentially slow the progression of several diseases.[11] Furthermore, they can prevent and lessen inflammation, which helps to lessen the discomfort and tissue damage brought on by inflammation. Chronic inflammation results in the long-term release of inflammatory mediators and the activation of detrimental signal transduction pathways, which can cause several illnesses, including cancer, arthritis, and allergies.[12] Additionally, phenolic compounds may engage in a variety of interactions to prevent the degradation of RBC membranes.[13]

Arum palaestinum Boiss belongs to the Araceae family, *native* to Lebanon, Syria, and Palestine. It is commonly referred to as black calla lily or Solomon's lily, with bright, beautifully colored flowers and broad green leaves. It has applications in the treatment of burns, gingivitis, cancers, and skin ulcers.[14] Its use in the treatment of prostate cancer, gout, and kidney disease has been reported.[15] It has been shown to contain important phytochemicals such as alkaloids, proanthocyanidins, and flavonoids, rich in chlorophyll, anthocyanins, oxalates, amino acids, and fats.[16] This study aimed to determine the physicochemical properties and proteolytic activity of *Arum palaestinum* leaf extracts on casein, as well as to characterize the kinetic parameters of its proteases.

Materials and methods

Preparation and collection of plants

Samples of *Arum palaestinum* (*A. palaestinum*) were purchased from the local market (March 2022) in AL Aghwar Shamaliyah District (Longitude and latitude coordinates 35.6085061, 32.6101751), Jordan.

The plant was recognized by Professor Salah Al-Quran from the Department of Biological Sciences at Mu'tah University, Jordan. The dried plant sample (13.5 g) was homogenized in 70% methanol using a mechanical blender for five hours at 25 °C. The resultant solution was filtered employing Whatman No. 1 filter paper and cotton sheets. The filtrate was subjected to centrifugation for 10 to 15 minutes at 3000 revolutions per minute (rpm). Following this, the supernatant was obtained and evaporated to dryness to yield 2.8 g of the raw extract.[17] Voucher samples, labelled as HW2022-AL, were securely stored at the biochemistry laboratory at Mutah University, Jordan.

Determination of total protein content

Slight modifications were made to the assay previously reported by Gerrano *et al.* (2019) to determine the total protein content in the current study. The raw plant extracts' total protein content was estimated using the Biuret technique in this study. The calibration curve was established using bovine serum albumin (BSA) as a standard, with a concentration ranging from 0.07 to 2.4 mg/mL.[18]

Evaluation of total phenolic content (TPC)

The total phenolic content of the plant extract was determined using the Folin-Ciocalteu assay. In this procedure, 0.5 mL of 10% Folin-Ciocalteu reagent was mixed with 0.1 mL of raw plant extract in phosphate buffer (pH 7.0) and 2.5 mL of Na_2CO_3 (mM). After being vortexed, the mixture was incubated at 25 °C for 30 minutes. To generate a calibration curve, gallic acid was employed at a concentration of 0.01 to 0.5 mg. Milligrams of Gallic acid equivalents per gram of extract solution were the unit of expression for the TPC.[19]

Evaluation of antioxidant activity

A colorimetric assay was employed to evaluate the plant extract's capability to neutralize the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical. One millilitre of plant extracts was mixed with 100 mL of a 60 μM DPPH solution in methanol at 25 ˚C. In comparison to a neutral solution (DPPH in methanol), the absorbance of the solution was measured at 517 nm following incubation in the dark for 30 minutes. A calibration curve was generated using gallic acid, and the IC_{50} was determined using an IC_{50} calculator.[20] The percentage antioxidant activity of the extract was computed from the following formula:

$$DPPH\ Scavenging\ activity\ (\%) = \frac{Ac - As}{Ac}\ x\ 100$$

Where As represents the absorbance of the test sample, and Ac represents the absorbance of the blank (control).

Determination of the Anti-inflammatory activity of plant extract

A test solution (0.5 mL) was employed in this test, which contained 0.45 mL of 0.5% w/v aqueous solutions of BSA and 0.050 mL of the plant raw extract. Following transfer to test tubes, the test solution was first placed in test tubes and then incubated at 37 ˚C for 20 minutes, followed by an additional 10 minutes at 70 ˚C. A total of 2.5 ml of phosphate buffer with a pH of 7 was then added to each tube after the samples had been removed and allowed to cool. A spectrophotometer was employed to measure the test sample's absorbance at 660 nm. A 100% denatured protein was employed as a reference sample, and 0.05 mL of distilled water was substituted for the test sample following the aforementioned procedure. The percentage by which the plant extract inhibited protein denaturation was calculated from the following formula:[21]

$$Percentage\ of\ anti-inflammatory = 100 - (AT - AP/Ac)\ x\ 100\%$$

Where AP represents the absorbance of the product control, AT represents the absorbance of the test solution, and Ac represents the absorbance of the test control. The AAT Bioquest IC_{50} calculator was employed to determine the IC_{50} (https://www.aatbio.com/tools/ic50-calculator).

Anti-hemolytic activity of the extract

One mL of 4% blood suspension was mixed with the plant extract with a concentration ranging from 0.1 mg to 2 mg. The reaction medium volume was adjusted to 1.25 mL using PBS (phosphate-saline buffer) of pH 7.4. 0.25 mL of H_2O_2 solution was added to the mixture, and it was incubated for 10 minutes at 37 ˚C. After the mixture was incubated at room temperature for an additional two hours, it was centrifuged at 3000 rpm for 10 minutes. The supernatant's absorbance at 540 nm was measured using a spectrophotometer.[22] The percentage of the extract's anti-hemolytic activity was computed from the following equation:

$$Percentage\ of\ anti-hemolytic\ = \frac{Ac\ (As - Aa)}{Ac} x100$$

Where As represents the sample absorbance, Ac represents the control positive absorbance, and Aa represents the control negative absorbance. The AAT Bioquest IC_{50} calculator was employed to calculate the IC_{50}.

The proteolytic enzyme activity of the extract

The Fahmy method was employed to measure proteolytic activity, using casein as the substrate and tyrosine as the standard reference.[23] Briefly, 1 mL of 1% casein solution was prepared by dissolving casein in 1.5 mL of 0.1 M phosphate buffer (pH 7). The enzymatic reaction was initiated by adding 1 mL of the plant extract supernatant to 0.5 mL of this casein solution.[21] The mixture was incubated at 37 ˚C for one hour with intermittent agitation. The reaction was stopped by adding 2 mL of 4% trichloroacetic acid (TCA), followed by centrifugation at 3000 rpm and 37 ˚C for 20 minutes to remove precipitated proteins. The absorbance of the supernatant was measured at 750 nm after adding 5 mL of sodium carbonate and 1 mL of Folin reagent. Tyrosine standard solutions (0.1 to 1 mg/mL) were used to generate a calibration curve for quantification.[24]

To ensure the accuracy and reproducibility of the proteolytic activity measurements, each assay was performed in triplicate to account for experimental variability. Further, the tyrosine standard curve was constructed using a wide concentration range (0.1–1 mg/mL) with multiple replicates per concentration, and the linearity of the calibration curve was confirmed with a correlation coefficient (R^2) exceeding 0.99, ensuring precise quantification of released tyrosine. The addition of 4% trichloroacetic

acid effectively stopped the enzymatic reaction by precipitating unreacted proteins. Immediate centrifugation following incubation minimized post-reaction hydrolysis and ensured clear separation of soluble peptides for accurate absorbance measurement.

The temperature effect on the extract proteolytic activity

To determine the optimal temperature for protease activity, the enzymatic reaction was performed at temperatures ranging from 25 ˚C to 90 ˚C. One milliliter of the plant extract was mixed with 3 mL of 1% casein solution and incubated for one hour at each temperature point. Activity was measured as described above to assess temperature dependence. [25]

pH effects on the extract's proteolytic activity

The enzyme activity was tested across a pH range of 3.6 to 10 using three different buffer systems: acetate buffer (acidic), phosphate buffer (neutral), and sodium carbonate buffer (alkaline). The reaction mixtures were incubated at 50 ˚C for one hour before measuring activity, allowing determination of the optimal pH. [26]

Evaluation of Km and Vmax

The Km (Michaelis constant) represents the substrate concentration at which the reaction rate is half of the maximum velocity (Vmax). It reflects the enzyme's affinity for the substrate—a lower Km indicates higher affinity. The Vmax (Maximum velocity) represents the highest reaction rate achieved by the enzyme when the substrate is saturating, indicating the catalytic capacity of the enzyme. Generally, the proteolytic activity was measured at varying casein substrate concentrations (0.1 to 2.0 mg/mL) under optimal assay conditions. Initial reaction rates (velocity) were recorded, ensuring the reaction proceeded under steady-state conditions without substrate depletion or product inhibition. To derive Km and Vmax, the reciprocal of substrate concentration (1/[S]) was plotted against the reciprocal of reaction velocity (1/V), generating a straight line. The y-intercept corresponds to 1/Vmax, and the x-intercept corresponds to $^{-}$1/Km. This double-reciprocal plot allows for accurate estimation of kinetic parameters. [27] All assays were conducted in triplicate to minimize random error. Reaction rates were confirmed to be linear over the measurement intervals to ensure initial rate conditions. The selected substrate concentrations spanned below and above the estimated Km to improve the accuracy of parameter estimation.

Statistical analysis

The results are expressed as the average value (mean) plus or minus the standard deviation (SD) (mean $\pm$ SD, n = 3). Each experiment or measurement was repeated three times independently. This repetition helps ensure the reliability and reproducibility of the data. The "n = 3" indicates the number of replicates used to calculate the mean and standard deviation. The software used to perform the calculations and organize the data was Microsoft Excel 2016.

Results and discussion

This study employed the Biuret test to quantify the protein content of the raw extract of *A. palaestinum*, and the result is shown in Table 1. The result showed that the extract's protein content was 1.28 mg/mL. The Folin-Ciocalteu assay method also showed that the extract contained a significant number of phenolic compounds (65.48 mg GAE/g) in comparison to gallic acid, as indicated by the total phenolic content Table 1. However, a previous study reported different TPC values for various *Arum species,* ranging from 27.49 to 60.07 mg GAE/mg dry material, which is slightly lower than our finding (65.48 mg GAE/g). [28] The antioxidant capacity results from the DPPH free radical scavenging assay indicated that the extract exhibits 81.50% antioxidant activity, with a half maximal inhibitory concentration (IC_{50}) of 0.1975 mg/mL, as shown in Table 2. Researchers utilized the antioxidant activity of *A. palaestinum* extracts to assess the plant's antioxidant activities, as shown in previous studies conducted by Khalaf *et al.* [29] and Ahmad *et al.* [30] The IC_{50} value of 0.06975 mg/mL at the tested concentrations was lower than the current finding. Their findings showed remarkable free radical scavenging activity at 39.0%, which was lower than the current finding. The variations in the antioxidant activities of these plant samples could be attributed to variations in geographical locations, climatic conditions, methods of processing plant materials (grinding) and storage conditions. Sometimes, the plant parts used for extraction and genetics could influence the phytoconstituents of the plant under study. The plant extract exhibited potent anti-inflammatory activity of 90.1% and an IC_{50} value of 1.1133 mg/mL, as shown in Table 2. According to previous studies, it was stated that the anti-inflammatory activity of A. palaestinum *was about* 65.5%. This finding was less than the value

Table 1. The content of total protein, total phenols and the yield (%) in *Arum palaestinum* extract.

Extract	Total protein (mg/ml)	Total phenol (mg GAE/g)	Yield (%)
Arum palaestinum	1.28 ± 0.017	65.48 ± 0.016	20.74± 0.011

Mean ± SD, n = 3.

Table 2. The biological activities (%) and IC_{50} values (mg/mL) of *Arum palaestinum* extract.

	Antioxidant	Anti-inflammatory	Anti-hemolytic
Activity (%)	81.5 ± 3.421	90.1 ± 4.58	84.3 ± 6.572
IC_{50}(mg/mL)	0.1975	1.133	0.2149

Mean ± SD. n = 3.

obtained in the current study (90.1%).[31] Therefore, the extract of A. palaestinum can be used as a natural therapeutic agent against inflammation, to reduce the overproduction of inflammatory mediators like radical oxidative species and pro-inflammatory cytokines.[32] The anti-hemolytic potentials of the raw extract of A. palaestinum were investigated with H_2O_2 as the toxicant. The findings indicate a substantial decrease in the hemolysis of red blood cells (RBC) when exposed to H_2O_2 and *A. palaestinum* extract. As shown in Table 2, there was no hemolysis when the RBCs were treated with only the plant extract. The raw extract of A. palaestinum showed 84.3% anti-hemolytic activity with an IC_{50} value of 0.2149 mg/mL. A similar study done by Habash *et al.*[33] reported that the extract of A. palaestinum showed antihemolytic activity in the presence of H_2O_2 with a score of 70.7 %, using ascorbic acid as a control agent. Similarly, the impact of temperature on enzymatic activity was evaluated. The results of the current study revealed that enzyme activity was significant at moderate temperatures, hence an increase in reaction time. Maximum enzyme activity was recorded at 50 ˚C, as shown in Fig. 1. The results are comparable with those reported for *M. officinalis* extracts at the identical temperature.[34] Conversely, a different study indicated that 70 ˚C was optimal for the enzyme activity of *A. palaestinum* extracts, which contradicts the findings of this current study.[35,36] The study indicates that protease activity is markedly affected by pH levels.[37] Proteases extracted from the leaves of *A. palaestinum* showed significant activity at pH 7 against denatured casein, indicating that neutral pH is essential for optimum function, as shown in Fig. 2. However, other studies reported a pH of 8 for the protease enzyme's maximum activity, which was contradicted by the results obtained in this study.[38]

Furthermore, the proteolytic effects of A. palaestinum extract on casein were evaluated. The results show that the K_m score was 0.66 mM, while V_{max} recorded was 5 μmol/min for the extracts of A. palaestinum against casein; the equation of the standard curve was Y = 0.1256 x+ 0.1944; R^2 = 0.6872, as shown in Fig. 3. Also, the results showed a favourable correlation between enzyme proteolytic activity and plant extract (enzyme) concentrations. The reaction activity of raw papain enzyme, as reported by Foda *et al.*,[39] was found to increase with

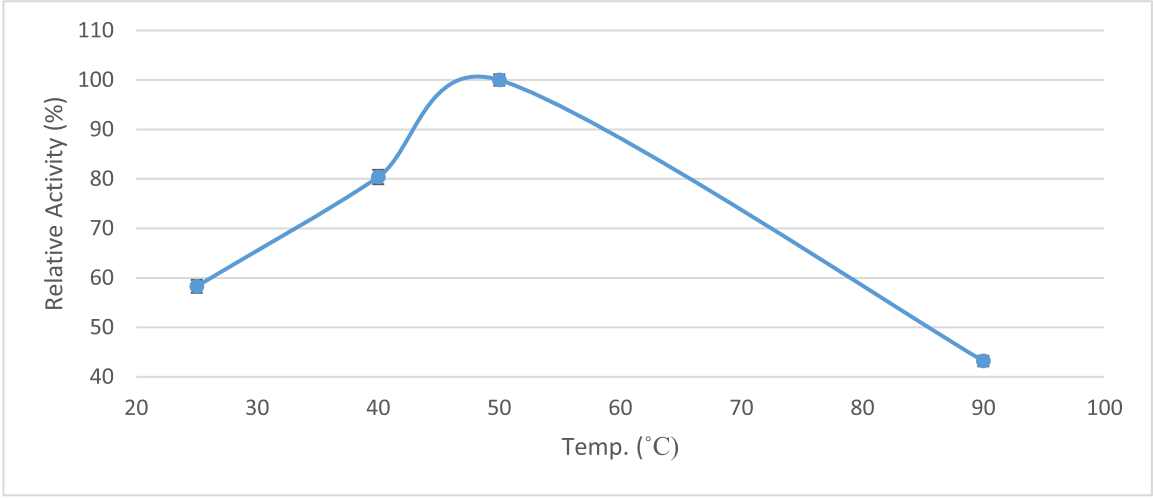

Fig. 1. The relative activity of proteases (%) in the *A. palaestinum* extract across several temperature values (°C). Mean ± SD; n = 3.

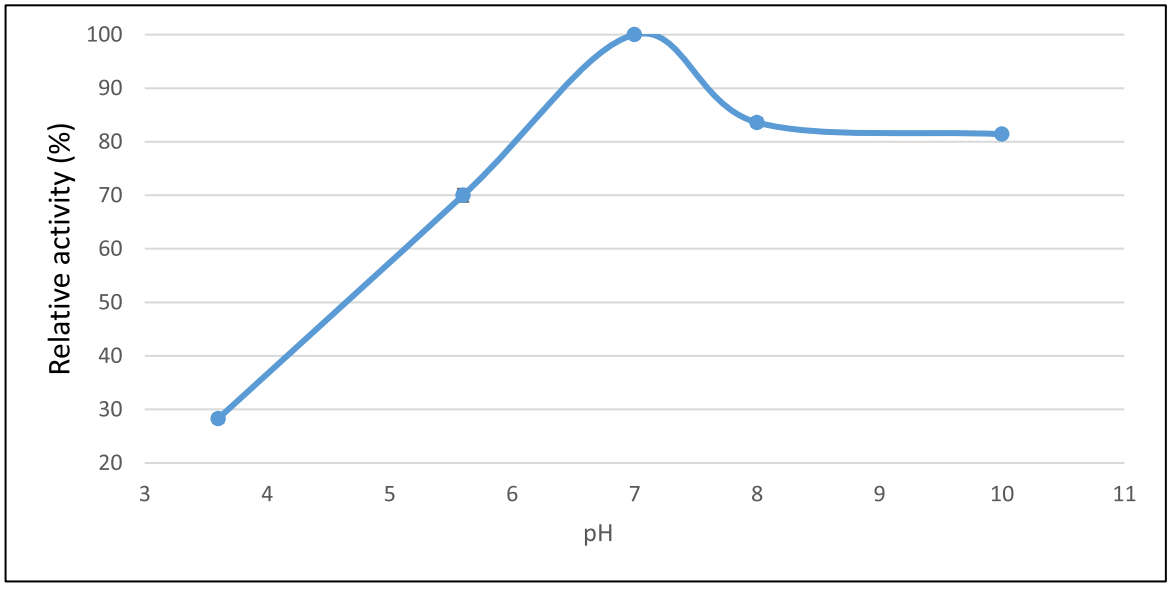

Fig. 2. The relative activity of proteases (%) in the A. palaestinum extract across several pH values. Mean ± SD; n = 3.

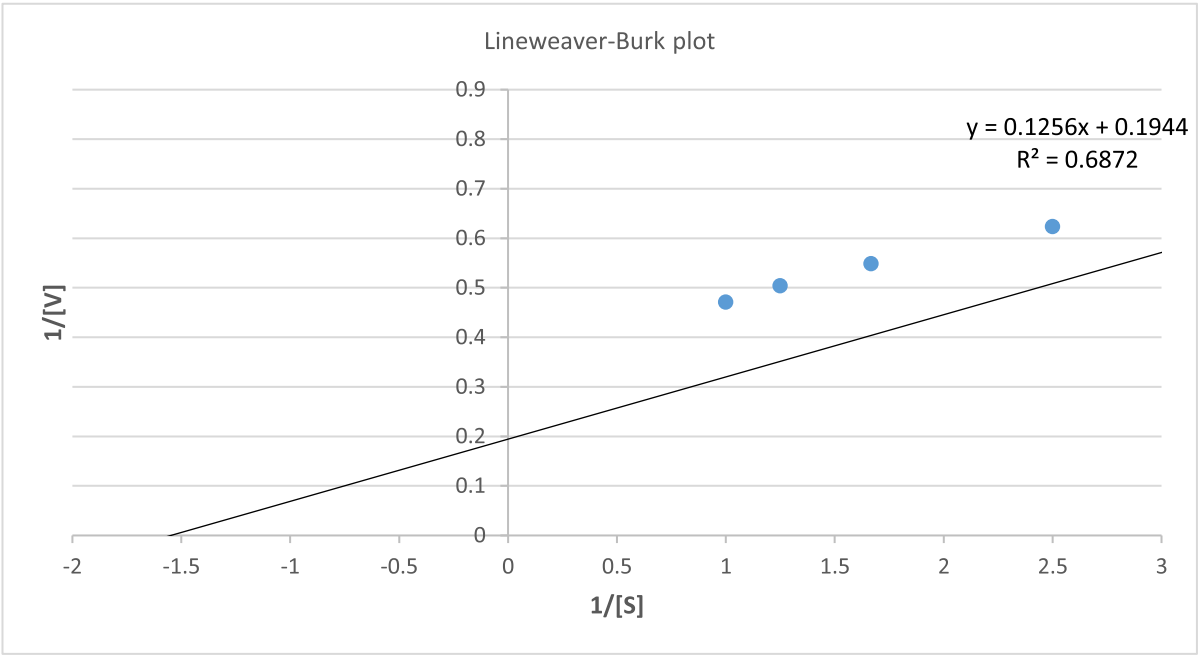

Fig. 3. The Lineweaver-Burk reciprocal plot to assess the Vmax and Km values of casein in the *A. palaestinum* extract. Mean ± SD; n = 3.

increasing enzyme concentration up to a certain point. However, beyond that point, the activity decreased as a result of inhibition effects or reverse reactions caused by steric retardation and excess enzyme.[40]

Conclusion

The study demonstrated that Arum palaestinum methanol leaf extract contains significant amounts of proteins and polyphenolic compounds, which contribute to its strong antioxidant, anti-inflammatory, and anti-hemolytic activities. The extract exhibited effective proteolytic activity against casein, with optimal enzyme function observed at neutral pH (7.0) and moderate temperature (50 °C). Kinetic analysis revealed a Km value of 0.667 mg/mL and a Vmax of 5.0 μmol/min, indicating efficient enzyme-substrate interaction. These findings suggest that Arum palaestinum is a promising natural source of proteolytic enzymes with potential applications in

pharmaceutical development and various industrial processes. Further exploration of this plant could lead to the discovery of novel bioactive compounds beneficial for health and industry.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images that are not ours have been included with the necessary permission for republication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at Mutah University.

Author's contribution statement

O. M. A. conceived and designed the study, supervised the project, and contributed to the manuscript writing and final revision. S. K. was responsible for initial analysis, and performing all the experiments. O. M. A. contributed to data interpretation and manuscript editing. All authors reviewed the final version of the manuscript. All authors read and approved the final manuscript.

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توصيف ونشاط التحلل البروتيني لمستخلص نبات اللوف الفلسطيني (Arum palaestinum)

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الخلاصة

استخدم الإنسان النباتات الطبية لعلاج العديد من الأمراض منذ القدم، وكانت مصدرًا رئيسيًا للمركبات النشطة بيولوجيًا المستخدمة في الأدوية. تُعد البروتيازات النباتية ضرورية في تنظيم المسارات الأيضية التي تؤثر على التعبير الجيني وتساهم في إزالة المستقلبات غير المرغوب فيها. تهدف الدراسة الحالية إلى تقييم النشاط البروتيني المحلل والنشاطات البيولوجية لمستخلص أوراق اللوف الفلسطيني الميثانولي في المختبر. تم تحديد محتوى المركبات الفينولية الكلية (TPC) والنشاط المضاد للأكسدة، والمضاد للالتهابات، والمضاد لتحلل خلايا الدم الحمراء باستخدام الطرق اللونية، في حين تم قياس النشاط البروتيني المحلل باستخدام الكازين كركيزة. أظهرت النتائج أن المستخلص يمتلك خصائص مضادة للالتهاب بنسبة 90–95% مع قيمة IC50 = 1.1133 ملغم/مل، ونشاطًا مضادًا لتحلل خلايا الدم الحمراء بنسبة 90–95% مع نفس قيمة IC50. كما أظهر المستخلص نشاطًا جيدًا كمضاد للأكسدة بنسبة 71.30%. وأشارت النتائج إلى أن المستخلص الخام يحتوي على مركبات فينولية بتركيز 65.48ملغم/غم. تم تسجيل النشاط البروتيني المحلل للمستخلص باستخدام الكازين كركيزة عند درجة حموضة pH = 7.0 ودرجة حرارة 50 درجة مئوية. وقد أظهر المستخلص الخام أعلى نشاط تجاه الكازين. وبيّن رسم لاينويفر–بورك العكسي أن قيمة Km تساوي 0.667 ملغم/مل ، وقيمة Vmax تساوي 5.0 مايكرومول/دقيقة للبروتيازات الموجودة في المستخلص. تُظهر هذه الدراسة أن مستخلص النبات يمتلك خصائص قوية مضادة للأكسدة، ومضادة للالتهابات، ومضادة لتحلل خلايا الدم الحمراء، والتي قد تكون مرتبطة بمركبات البوليفينول. كما أظهر المستخلص إمكانية أن يكون مصدرًا واعدًا للأنزيمات البروتينية المحللة، ويمكن استغلاله كمصدر للبروتيازات في التطبيقات الصناعية. ويمكن الاستمرار في استكشاف هذا النبات كمصدر جديد لمركبات فعالة لتطوير الأدوية والتطبيقات الصناعية.

الكلمات المفتاحية: اللوف الفلسطيني، مضاد الأكسدة، الأنشطة المضادة للالتهابات.