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Activity based insights into the seed proteins of *Datura alba* from Pakistan

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Abstract: **Background**: There are fifteen species of *Datura*, a perennial shrub-like herb, with *Datura alba* (*D. alba*) being the most important medicinal plant because of its therapeutic and aesthetic uses. Pharmaceutical goods use fresh seeds, fresh leaves, roots, dried leaves, dried mature seeds and fruits of *D. alba*. Seed proteins are the plant component most often overlooked for use in complementary and alternative medicine, even though all plant parts have nearly identical therapeutic properties and effects. This results from a deficiency of knowledge on the components of proteins. **Objectives**: The current study is to extract proteins from *D. alba* plant seeds in order to show data that will identify few major enzymes in the whole protein extract. **Methods**: The protein chemistry techniques, such as gel electrophoresis and chromatography, have been applied for seed protein analysis. After extraction of seed proteins in PBS (pH 7.4) buffer, the chromatography using DEAE Sephacryl (macroprep) resin, SDS-PAGE, zymography and biochemical tests were performed. **Results**: There were three distinct peaks identified by the chromatographic separation. Protein bands were visible in the reduced and non-reduced SDS-PAGE analysis, while the results of substrate zymography indicated the presence of proteases and phospholipases. Strong antioxidant activity and antibiofilm activity on MRSA were also observed in the *D. alba* seeds proteins. **Conclusion**: Enzymatic and biochemical activities were observed with the protein extract obtained from the seeds of *D. alba*. Fractions of the proteins were also active and showed separation of protein components on the basis of ionic strength.

Keywords: Assays; Biochemical; *Datura alba*; Protein; Seeds

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INTRODUCTION

The Solanaceae family includes plants in the genus *Datura*, which is a wild shrub, widely grown throughout the equatorial regions of the world, including Pakistan. There are typically nine recognized species of *Datura*, while the literature records show nine to fifteen species, which can be confusing (Buchholz *et al*., 1935; Palazón *et al*., 2006). The plant is also known by the names thornapple, locoweed, devil's weed, stinkweed and angel's trumpet. These names usually relates to the morphological, phytochemical and pharmacological traits of the plant (Krenzelok, 2010). One species that is frequently observed growing in waste areas is *Datura alba*, a variant of *Datura fastuosa*, which is found in Pakistan (Nasir and Nasir, 1985). *Datura alba*, commonly known as Safed Datura, is frequently regarded as a variant of *Datura metel* due to the variation in blossom color (Aggrawal, 2014). This annual plant has a height range of 3.5 to 4.0 feet and can reach upto 6 feet or more on good soils. It is bushy, silky and foetid. When the seeds have dried, they are kidney-shaped, about 4-5.5 mm long, compacted and brown (Soni *et al*., 2012; Gupta, 2008; Singh *et al*., 2023; Neha *et al*., 2022; Krenzelok, 2010). Hyossyamine and scopolamine, two tropane alkaloids, make up the majority of its active components. The chemical composition of several *Datura* species have been uncovered by studies for example, the seeds of *Datura metel*, *Datura stramonium*, *Datura innoxia* and *Datura alba* contain 20.73, 16.20, 13.90 and 21.353 mg of crude protein, respectively (Sharma *et al*., 2021; Oseni *et al*., 2011; Ayuba *et al*., 2011; Hussain *et al*., 2011; Al-Snafi, 2017). Although the entire plant has therapeutic potential, the leaves and seeds are the most commonly utilized components (Uddin *et al*., 2012). When topically administered to albino rat burn wounds at different phases of the healing process, ointment made from the alcoholic extract of *D. alba* leaves had considerable prohealing action (Priya *et al*., 2002). It was also reported by Gnanamani and colleagues that the *D. alba* leaf extract showed antibacterial activity against eight pathogens associated with burn injuries (Gnanamani *et al*., 2003). *D. alba* leaf extracts with right concentration of alkaloids in laboratory settings, exhibit extremely strong toxicity against two significant insect pests on stored rice, black ants and aphids (Ali *et al*., 2012; Kuganathan *et al*., 2008). *D. stramonium* seeds extract was also found to be a good source of noteworthy natural antioxidants that also possess anticancer properties (Iqbal *et al*., 2017). *Datura* seed proteins also seem highly appealing, given that these have been shown to exhibit anti-cancer action against

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human cancer cells, along with those of other plant species (Abozaid *et al*., 2014). It is known that the proteins from plant seeds are a significant source of nutrients for both human and animal diets and also these proteins are involved in several physiological processes, such as stress response, seed germination and play a critical role in the growth and development of plant embryos. Proteins that are created during seed development and accumulate in the seed tissues include enzymes, storage proteins and structural proteins, among many other types of proteins found in plant seeds (Marcone, 1999). Seed proteins are extracted from many members of the family Solanaceae, like tomato (Piyakina and Yunusov, 1995), tobacco (Rossi *et al*., 2013) and other plant species like Amaranth (Bojórquez-Velázquez *et al*., 2019), Chia seeds (Sandoval-Oliveros and Paredes-López, 2013), Chickpea (Singh *et al*., 2016), Radish seeds (Terras *et al*., 1992) and Soybean seeds (Natarajan *et al*., 2005). While much research has been done to identify the beneficial components (protein, oil and alkaloids) in the plant parts of *Datura* species (Uddin *et al*., 2012; Qureshi *et al*., 2011), nothing is known about the protein content of *Datura alba* leaves, seeds, roots, or shoots. Among the several platforms available for protein extraction, seeds offer the benefit of inclosing proteins in a covering and offering a stable environment that prevents protein degradation. Therefore, the current study focuses on the *D. alba* seed proteins extraction followed by analysis of their biological activities.

MATERIALS AND METHODS

Extraction of protein from seeds

Pods of *D. alba* were collected from the surrounding area of the University of Karachi. and identified by the taxonomist. The seeds were removed from the pods and air-dried under a fume hood. The seed defatting and afterwards its protein extraction steps were optimized for the sample to obtain the maximum protein yield. The seeds were finely ground and steeped in n-hexane in a ratio of 1 gm powder to 9 mL for defatting for 24 hours. After drying the defatted seed powder, the proteins were extracted in 2X PBS buffer a slight modification of the method of Habiba et al., 2021 (0.27 mM NaCl, 0.005 mM KCl, 2.93 µM KH2PO4, 20.28 µM Na2HPO4, pH 7.4) in a 1:4 ratio of seed powder to buffer for 16 hours at 4°C with constant stirring. The mixture was then centrifuged at 6000 rpm for 30 minutes at 4 °C to collect the supernatant. The seed crude extract was precipitated with 70% ammonium sulfate and dialyzed overnight (16 hours) to obtain the whole protein extract.

Chromatography

The crude extract of the seed protein (~ 40 mg) was subjected to DEAE Sephacryl (macroprep) 10 mL, Bio-Rad) column attached to FPLC (Amersham) equilibrated with PBS buffer with 2X concentration, pH 7.4. The elution was performed with the same buffer added with 0.5 M NaCl pH 7.4 at 1 ml/min, flow rate. The eluent was monitored at 280 nm. Each peak was manually collected into separate tubes. The collected peaks were labelled as peak 1, peak 2 and peak 3 (P1, P2 and P3).

Protein quantification

Following the manufacturer's instructions (Thermo Fisher Scientific) for the kit, the amount of protein was determined in microtiter plates using the bicinchoninic acid (BCA) assay (Smith *et al*., 1985) using bovine serum albumin (BSA) as the standard protein. The absorbance was measured with a Beckman Coulter 96-well plate reader at 562nm.

SDS-PAGE electrophoresis

In order to observe protein bands, electrophoresis was performed in accordance with the Laemmli protocol (Laemmli, 1970) with minor modifications based on the lab conditions. Chromatographic fractions and whole protein (~15 µg each) were subjected to 12 % polyacrylamide gel electrophoresis. Using molecular weight protein markers (Molequle-on), the relative electrophoretic mobility of the separated bands was compared for apparent molecular mass.

Gelatin zymography

A 12 % SDS PAGE gel with a final concentration of 0.3 % gelatin was used for protease zymography. Samples (10 µg) were prepared under non-reducing and non-denaturing conditions. Following the run, the gel was placed on an orbital shaker and treated with 2.5 % Triton X for 40 minutes at room temperature. Afterwards, deionized water was used to wash the gel, and incubation was performed in Tris buffer (20 mM TrisHCl, 1 mM NaCl, 10 mM $CaCl_2$, pH 8.0) at 37°C overnight. Coomassie blue staining was applied to the gels at room temperature, followed by destaining with deionized water to visualize the activity.

Phospholipase A_2 (PLA_2) zymography

A 12 % gel was performed using 10 µg of each sample under non-reducing conditions. Following the run, the gel was rinsed with 2.5 % Triton X-100 for at least 40 minutes at room temperature and it was then incubated for three hours in reactivation buffer to activate the enzyme. Finally, it was placed on a 2 % agar plate added with 5% egg yolk in Tris buffer (20 mM TrisHCl, 1 mM NaCl, 10 mM $CaCl_2$, pH 8.0) and left to incubate for the entire night at 37 °C. The gel was examined the following day for clear translucent bands caused by the activity of phospholipase A_2.

Amylase zymography

A 10 % polyacrylamide gel containing sodium dodecyl sulfate with 0.1 % starch was prepared for amylase activity analysis. Samples (10 µg) were run in a non-reducing sample buffer. Following completion, the gel was rinsed 2.5 % triton X-100for one hour at room temperature and then three times with water for ten minutes. After that, the gel was incubated for three hours at room temperature in tris buffer (20 mM Tris-HCl, 1 mM NaCl and 10 mM

$CaCl_2$). Afterwards, gels were stained with Lugol's solution (1% iodine, 2% potassium iodide) until distinct light colored bands were visible against a dark background (Andrades and Contreras, 2017).

Determination of anti-biofilm activity

The activity assay was conducted with the growth of the MRSA strain (S-4) in 2 mL of BHI (brain heart infusion) media maintained at a 0.5 McFarland index. The culture was diluted (1:100) in the same media and 100 µl was utilized for each well of 96 well plate. The respective concentrations of samples was made in 100 µl. The strain of MRSA was selected on the basis of distinct biofilm formation. 200 µl of media served as a negative control, while pathogen and media, 100 µl each, were taken as a positive control. or positive control 100. After incubation of 24 hours at 37 °C the solution was removed and washed twice with autoclaved deionized water. The plate was placed at 60 °C for 1 hour to fix the attached cells, followed by staining with 1% crystal violet solution for 15 minutes at room temperature. The wells were again washed with sterile water and air-dried for 30 minutes. Acetic acid (30%) was used to re-solubilize the bound dye and absorbance was noted at 595 nm by using Elisa plate reader (Bai *et al*., 2019; Al Atya *et al*., 2016). The well diffusion method was also followed to analyze antimicrobial activity against Methicillin-resistant *Staphylococcus aureus (MRSA)*. Inoculum was spread on Muller-Hinton agar (MHA) to make a lawn using sterile cotton swab. Wells were made with 10 mm borer and labelled as 1 to 4 (crude, P1, P2, P3). Each sample 200 µg, was applied to the respective well. Tigecycline and cefoperazone-sulbactam were used as antibiotic disks. Plates containing bacterial strain was incubated at 37°C for 24 h and observed for zone of inhibition.

DPPH Anti-Oxidant scavenging assay

The DPPH free radical scavenging assay was used to assess the antioxidant activity of protein extracts and fractions from *Datura alba* seeds (Bhardwaj *et al*., 2016). A dark bottle was used to keep a 0.1 mM solution of 2,2-diphenyl-1 picrylhydrazyl in 97% pure ethanol. Furthermore, deionized water was used to prepare many dilutions of lyophilized crude seed extract and fractionated proteins (100, 200, 400 and 600 µg/ml). 1.9 mL of DPPH solution was mixed with 100µl of protein sample. As a standard, ascorbic acid powder (25, 50, 100 and 200µg/ml) was used. For blank or controls, DPPH and 100 µl of water were utilized. For thirty minutes, all reaction mixtures were allowed to sit at room temperature in the dark. 200 µl from each tube were added to a 96-well plate and the absorbance was measured at 517 nm (Petchiammal and Hoper, 2014) using a microplate reader (Epoch 2, BioTek). The sample's increased DPPH radical scavenging activity led to a drop in absorbance when compared to the control. To calculate the percentage of radical scavenging activity, the provided equation was utilized. Every experiment was run in duplicate.

$$\% \text{ Inhibition} = \frac{\text{O.D. (Control)} - \text{O.D. (Sample)}}{\text{O.D. (Control)}} \times 100 \quad (1)$$

RESULTS

A brownish colored protein extract of *D. alba* was obtained as a result of the protein extraction procedure. The first step was to perform the ion exchange chromatography. A representative chromatogram for the fractionation has been shown in fig. 1. The seed proteins were separated into two distinct and one trailing peak. The unbound proteins eluted in peak 1 (P1) within the first 20 minutes. The remaining two peaks P2 and P3 were eluted with the gradient between 25 to 50 minutes of sample injection. All the peaks were collected for further analysis of these fractions.

For protein band profiling, the whole protein extract and fractions were loaded onto the SDS PAGE gel. A 12 % gel was run under non-reducing as well as reducing conditions. The gel analysis (Fig. 2) demonstrated that the seed protein extract of D. alba has protein bands from ~135 k Da to 17 kDa in comparison with the molecular weight marker. The fractionation showed that peak 1 has a range of proteins bands majority of which showed 100-48 kDa range with light intensity bands at the low molecular weight range. Peak 2 presented bands between the range of 100-63 kDa and 35-20 kDa while peak 3 showed presence of protein bands of light intensity around medium range of 35-20 kDa while very light intensity bands at low molecular weight around 17 kDa.

The seed proteins were also subjected to zymography analysis to further characterization. Whole protein extract or crude as well as all collected peaks were analysed for protease activity on a 12% SDS PGAE gel copolymerized with gelatin (Fig. 3). The activity of the enzyme was observed at high molecular weight range in the form of transparent bands on a blue gel background (100 kDa), suggesting the presence of metalloproteinases. The crude and three fractions run on the zymography gel all showed activity and at least 3 bands irrespective of the sample either crude or eluted from the ion exchange column under different conditions of sodium chloride containing buffer. The protein-protein interaction of proteases was highlighted as each sample was active in this enzymatic activity.

Phospholipase A_2 gel overlay assay was performed to trace the presence of activity of seed protein extract and fractions. The transparent bands on a slight yellow background represent the activity of the enzyme in this gel overlay zymography. Therefore, after the incubation period, the most prominent activity bands were observed in only two of the samples, which were seed protein extract or crude and P1. The position of the activity band was at low molecular weight positions on the gel (Fig. 4). P2 and P3 did not show any activity in terms of the presence of activity bands at the same position.

The zymography for amylase activity did not show any observable bands. The samples of saliva and the standard α-amylase showed diffused bands at high molecular weight position under same conditions of the zymography (supplementary data Fig. S1). This observation suggested the absence or presence of a very low amount of enzyme in current conditions of protein extraction from seeds.

The fractions of *D. alba* seed proteins were also evaluated against Methicillin-resistant *Staphylococcus aureus (MRSA)* for possible antibacterial activity. The fractions, as well as crude protein extract of the seeds, showed no effect on the bacterial growth (supplementary data Fig. S2). However, three concentrations, 20 ug, 40 ug, and 60 ug of each fraction, were applied to explore the inhibition of *MRSA* biofilm formation (Table 1). Peak 1 was not active against *MRSA* biofilm development, while peak 2 at a concentration of 60 µg showed some anti-biofilm activity. The only sample that was active and showed anti-biofilm activity at all concentrations was peak 3.

On the other hand, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging experiment showed that the activity of all samples increased with increasing concentrations (Fig. 5). The statistical analysis performed with one–way ANOVA has shown significant percentage radical scavenging activity (% RSA) of all three peaks and crude by comparing with the standard values (i.e., <0.0001 P value). The highest concentration of crude protein at 600 µg/mL exhibited 65.57% activity while peak 1 showed 51.08% activity, highest among the three fractions.

DISCUSSION

The extraction and purification of seed proteins has been performed in multiple studies utilizing ion exchange chromatography. The proteins extracted from safflower seeds were analyzed on a Sepharose 6B-100 gel column that separated the proteins into four fractions, with the 1st peak presenting about 60% of the whole extracted protein (Latha and Prakash, 1984). Affinity chromatography has also been performed previously, to purify lectin from *D. innoxia* on a chitin based column. Three fractions were produced during this experiment, with one major and two minor peaks, with a gradient of 100 mM acetic acid. An additional fraction was obtained with a gradient reaching up to 500 mM acetic acid in the study (Petrescu *et al*., 1993). In the current study, a DEAE column has been utilized, which resulted in at least three peaks, with the major peak that was eluted without the gradient, suggesting that the majority of the proteins were in their cationic state at the given chromatography conditions. This unbound peak or peak 1 has an absorbance of 3000 mAu at 280 nm. Similarly the absorbance of peak 2 was approximately 1000 mAu and that of peak 3 at around 500 mAu were the anionic fractions eluted due to the NaCl gradient. On the basis of absorbance peak 1 to peak 2 ratio is 3:1, whereas 2:1 for peak 2 and peak 3 ratio. The buffer system utilized in this study provided reproducibility of the chromatogram and also fulfils the conditions for the biochemical assays to be followed. Furthermore, the sample bands observed on SDS PAGE were better resolved as compared to Tris-HCl or acetate buffers that were used during the optimization steps. The proteins from collected fractions were concentrated by NaCl-acetone method (Nickerson and Doucette, 2020) and the BCA assay (Smith *et al*., 1985) was used for protein estimation. The measured protein fractions were subsequently subjected to further analysis.

The bands reported earlier from such research studies on seed storage proteins under reducing conditions in comparison to our study have shown number of different bands of proteins on SDS PAGE in whole as well as in all collected fractions from chromatography analysis. One such study on the purification of Brazil Nut seed proteins has shown similar SDS PAGE results (Sharma *et al*., 2010).

The gelatin zymography results revealed an interesting aspect where the activity observed was distributed in all four samples. This observation may point towards the protein-protein interactions that may have carried the proteases in all fractions despite the polarity differences and gradient conditions. This observation can also be deduced from the SDS PAGE analysis, where in non-reducing conditions, there are diffuse bands at the high molecular weight range with high intensity in crude and peak 1, but with low intensity in peaks 2 and 3. It has been observed that protease activity requires the presence of a small concentration of protein to be visible during protease zymography utilizing gelatin as substrate (Khan *et al*., 2023). The presence of hydrolytic enzymes as well as their isoforms could easily be detected through the polyacrylamide gels copolymerized with respective substrate to determine the activity (Vandooren *et al*., 2013). It has been reported from jack fruit (AqSEJ) seed extract that the protease activity bands were detected at high molecular weight region of the SDS PAGE gel utilizing gelatin as well as casein as substrates. The whole protein extract showed bands from 200 to 14 kDa range (Gangaraju *et al*., 2015). Our results also presented at least three activity bands in gelatin zymography in all samples run under non reducing conditions.

The second enzyme activity that was investigated was of phospholipase A_2. These enzymes are usually low molecular weight proteins. Their role is related to the generation of second messengers, which means that the product of their activity would be involved in signal transduction. Their targets are usually the cell membrane molecules. These are also suggested to be involved in plant defense system against pathogens (Chapman, 1998). Partial purification of a PLA_2 enzyme has been reported recently from soya bean seed (Minchiotti *et al*., 2008).

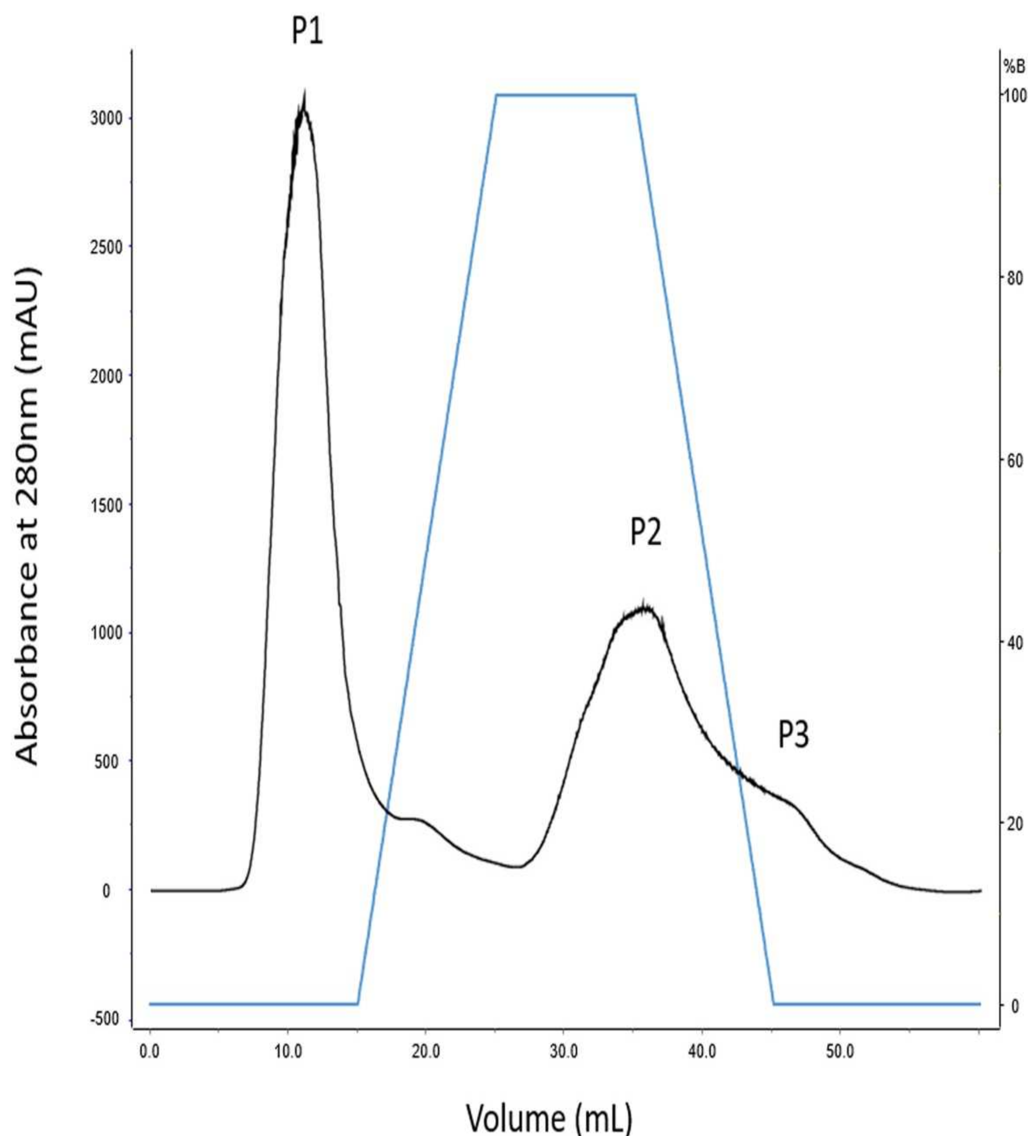

Fig. 1: Separation profile of whole seed proteins from *Datura alba* using DEAE column. The fractions eluted at a wavelength of 280 nm were designated as peak 1 (P1), peak 2 (P2) and peak 3 (P3). The gradient scale is presented at the right with percentage of buffer B (buffer solution with 0.5M NaCl).

Table 1: Activity of *D. alba* on MRSA Biofilm Inhibition.

Fraction	Protein amount (ug)	Biofilm inhibition (%)
Peak 1	20	00
	40	00
	60	00
Peak 2	20	00
	40	48
	60	89.98
Peak 3	20	35.34
	40	74.09
	60	68.87

Fig. 2: 12% SDS–PAGE under reducing and non-reducing conditions of from *D.alba* seed. Whole protein extract 'C' and peaks, P1, P2, P3 obtained from DEAE column separation were analysed. The molecular weight marker (std) with bands of 135 to 11 kDa has been marked. The gel was stained with Coomassie Blue staining.

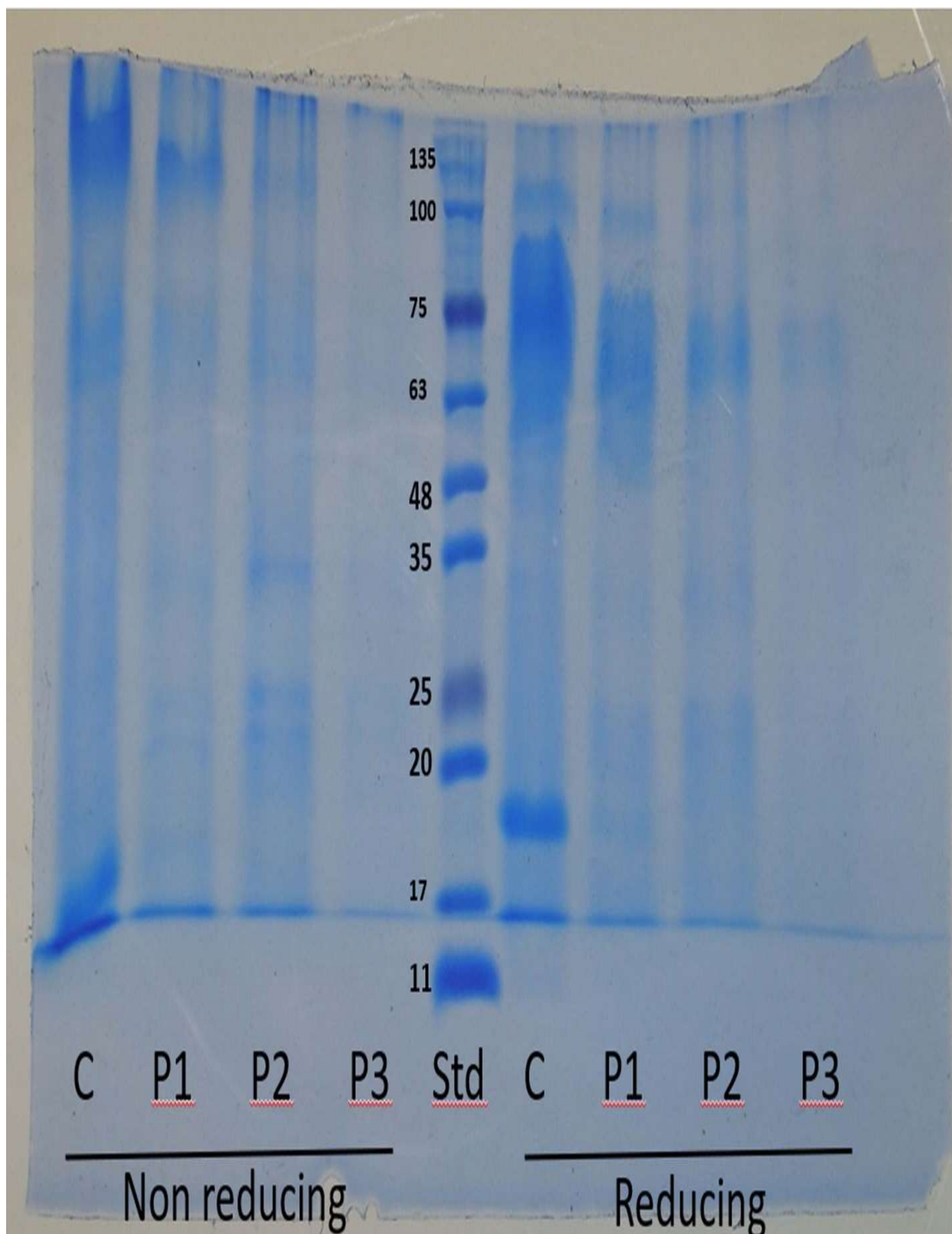

Fig. 3: Representative protease zymography gel utilizing gelatin as a substrate. Crude (C) and fractions of *D. alba* seed protein (P1, P2 and P3) obtained from DEAE column were loaded to the gel. The gel was run under the same conditions as 12% SDS PAGE for band pattern analysis under non reducing conditions.

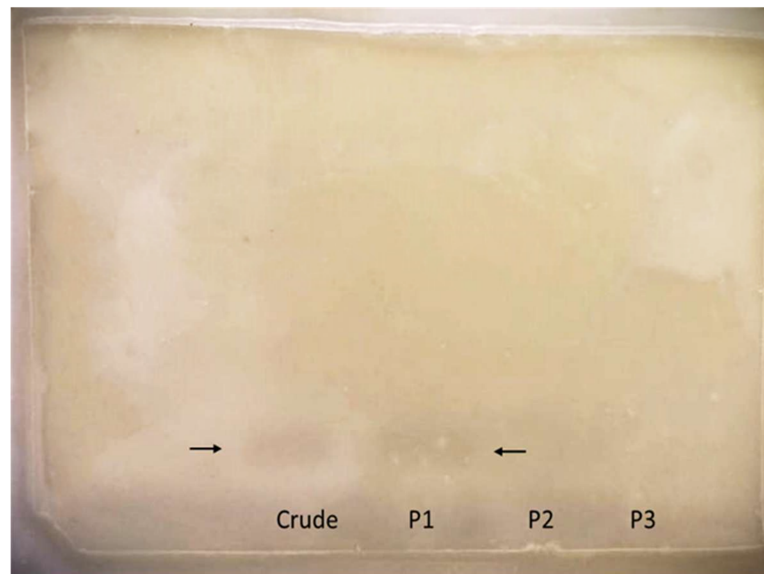

Fig. 4: Representative phospholipase A_2 gel (12 %) utilizing egg yolk agar plate as a substrate. Crude and fractions of *D. alba* seed protein (P1, P2 and P3) obtained from DEAE column were loaded to the gel under non reducing conditions. Transparent bands represents PLA_2 activity (indicated by arrow).

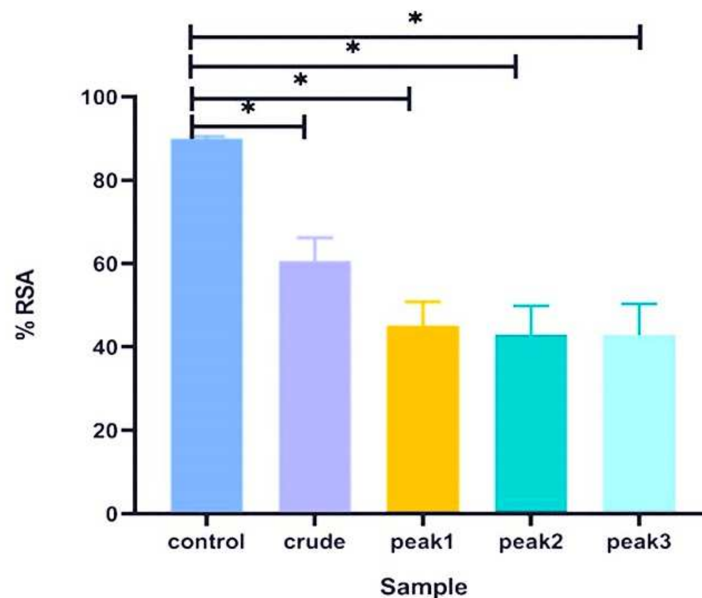

Fig. 5: Statistical analysis of DPPH radical scavenging activity of Datura *alba* seed extracts (standard, crude, peak 1, peak 2 & peak 3). All RSA values are the average of triplicate experiments and represented as mean ±SD and shows significant results on comparison with citric acid as control (P value <0.0001)

In the current study, we have observed the presence of PLA_2 from *D. alba* seeds. Our study showed PLA_2 activity in crude as well as P1 of the *D. alba* proteins, whereas there was an absence of activity in P2 and P3 (Fig. 4). This observation indicates that the phospholipase A_2 enzyme has been eluted in P1. The trace amounts that might be present in P2 or P3 were not enough to show activity. There are a number of plant species that have been reporting the presence of phospholipase A2 enzyme, ranging in molecular weight from 12 to 18 kDa. These enzymes have a conserved calcium binding site and an active site that has an active histidine residue (Mariani and Fidelio, 2019).

Analysis of the α-amylase activity on the basis of zymography resulted in no observable band of enzyme. The observation could be due to the presence of amounts that are very low, or the zymography utilizing starch as a substrate was not well-suited for the active protein. On the other hand, a heterodimer of 31 kDa inhibitor of α-amylase was reported from kidney bean cultivars. The two monomers were found to be of 17 and 14 kDa (Singh *et al*., 2022). Different peptides have also been reported that exhibit the α-amylase inhibitory activity (Evaristus *et al*., 2018; Siow and Gan 2016). In the present study there was no such assay conducted for analyzing the presence of inhibitors of enzymes such as α-amylase.

Antibacterial activity

To screen for antimicrobial activity, against Methicillin-resistant *Staphylococcus aureus (MRSA)* well diffusion method was followed. All samples showed no zone of inhibition (supplementary data Fig. 1).

The current study also reports the antibiofilm activity against MRSA of the *D. alba* seed proteins. The work conducted on *Datura metel* leaves methanolic extract, which was tested against different pathogens, has a significant effect on biofilm formation by *MRSA*. Concentrations of 50 and 100 mg/mL were applied, and the highest inhibition was observed with a 100 mg/mL concentration, where 90 % of the MRSA, B. subtilis and *E. coli* growth was inhibited. Lower concentrations also showed more than 50 % inhibition of these three bacteria (Prasathkumar *et al*., 2021). These methanolic extracts usually contain certain molecules such as hexadecanol, lucenin, tetradecanol and quercetin (Swamy *et al*., 2017) that may have interfered with the growth of the microorganism. There are other reports where the methanolic extracts were tested for antibacterial (Annadurai *et al*., 2021) and anti-pathogenic activities (Monyela *et al*., 2024).

For radical scavenging or antioxidant activity, the DPPH solutions were prepared in ethanol according to the protocol described in the methods. The one way ANOVA analysis showed significant results in comparison to the standard antioxidant agent (ascorbic acid). *Datura metel* and *Datura inoxia* methanolic extracts of leaves as well as of seeds showed radical scavenging activity (Bhardwaj *et al*., 2016). On the protein side, Chinese leek seeds and Hemp seeds have shown antioxidant activity, suggesting the presence of such polypeptides (Yang *et al*., 2018). The highest activity was present in crude protein extract and among the fractions in peak 1. While the other two fractions showed almost equivalent % RSA. The activity based characterization of plant proteins has remained difficult owing to the complex nature of protein mixture that has been extracted. The number or proportion of inhibitory compounds or complexes in different seed protein extracts and their fractions may play a role (Singh *et al*., 2022; Evaristus *et al*., 2018; Siow and Gan 2016). These may interfere with enzymatic and biological activities based on the relative abundance of such polypeptides that might be different in each peak, leading to different extents of activity. The kind of inhibitory activity out come may also require a concentration threshold as well that has to be surpassed and results in observable effect. The same has been noted in the current study, where more than one fraction showed activity, except PLA2, which was only observed in peak 1.

CONCLUSION

The whole protein extract of seeds from *Datura alba* showed the presence of enzymes and biochemically active components. The absence of α-amylase activity points towards a limitation of utilizing zymography for qualitative assessment of this enzyme or suggests a very low concentration of this enzyme. On the other hand, it also points out that the presence of inhibitor should also be included as a necessary part of such plant protein investigations.

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Authors' contributions

TAH: performed experiments related to the biochemical analysis, acquisition of data and made contributions to drafting the manuscript; LB: performed chromatography experiments, support in trouble shoot, acquisition of data and added to the draft; IS: performed and drafted the work related to the biochemical activity related to the assay of the radical based experiments; SA: performed and drafted the work related to microbial assays and acquisition of data; ND: supervised the microbial assays and supported in trouble shoot and acquisition of data; HW: remained as an expert to designed, drafted and functionally revised the work contributing to the interpretation of data; SFM: conceptualizing the work, design of the work, drafted the work, analysis and interpretation of data and revised it.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

Ethical approval

No ethical approval was required.

Conflict of interest

The authors declare no conflicts of interest.

Supplementary data

https://www.pjps.pk/uploads/2026/05/SUP1778668674.pdf

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