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Antimicrobial Insights into a Partially Purified Enzyme Protein Derived from *Myrica esculenta*

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ABSTRACT

Of all industrial enzymes, proteases are the most significant resource of our life. Though new proteases with better physiochemical properties for industrial usage are constantly being researched, the most widely implemented plant proteases are papain, bromelain and ficin. In this study, research was conducted for extracting and employing the required plant protease enzyme from *Myrica esculenta* leaves (using ammonium sulphate) and evaluating its antibacterial and anti-inflammatory properties towards a handful of pathogenic microbial strains. For this investigation, the total protein content of the collected material was assessed using the colorimetric technique (Bradford test), which was followed by polyacrylamide gel electrophoresis. The partly purified fraction of the recovered enzyme protein had a molecular weight of 31 kDa. The specific partially purified enzyme activity of dialysed *M. esculenta* protease was 35.20 U/ml. A variety of pH values (4.5, 7.5 and 8.5) was used to calibrate the enzyme's efficiency for maximum activity. In this study, the partially purified enzyme protein remained actively functional at pH 4.5. Further, the antimicrobial potential was examined against gram-positive and gram-negative bacteria using the disc diffusion method, and also its anti-inflammatory activity against specific antigen. The results of antimicrobial susceptibility and anti-inflammatory activity of the partially purified enzyme protein of leaves were observed at pH 4.5 against bacterial strains and specific antigens were evaluated. This finding demonstrated that the partially purified enzyme protein of *M. esculenta* leaves appeared to be a viable source for the extraction of proteolytic enzymes.

Key words: *Myrica esculenta*, plant-derived protease, enzymatic activity, protein purification, antimicrobial activity

INTRODUCTION

Almost every plant has protease enzymes. Numerous sectors, particularly food, cleaning products, leather and medicine, incorporate plant proteases (Alomrani *et al*., 2021). Their significant attributes, particularly their diverse temperature variability, pH, prolonged stability under challenging conditions, substrate specificity and improved dispersion, have resulted in their use in an abundance of scientific disciplines and investigations (Yadav *et al*., 2021). Protease enzymes within plants were progressively easier to separate, attributable to their subsequent improvement. Alkaline proteases are more frequently utilized in multiple industrial sizes than neutral and acidic proteases (Ammasi *et al*., 2020). Protease manufacturing processes, on an industrial scale, generate approximately 60% of commercially available enzymes worldwide (Arya *et al*., 2021). Nevertheless, protease's affordability does not yet fulfil industries' entire needs. Such circumstances attract some researchers to persist in exploring fresh approaches for producing protease, particularly by utilising inexpensive fundamental ingredients. Plants are now employed in new protease enzyme research since they are more affordable and easier to acquire than microbes and mammals (Benmrad *et al*., 2018). Furthermore, the broad spectrum of proteases, which can be distinguished by specific characteristics that involve specificity of action

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and thermal stability, has brought awareness to the search for sources that are affordable on a global scale. The origins for certain of the various industrial procedures that employ proteases to help break down proteins involve plants. Proteases, including papain, bromelain and ficin are employed in an assortment of industrial and medicinal procedures (Choudhary *et al*., 2022). Some of this happens because papain proteases have recently been utilised in the beverage and cheese-making sectors (Chen *et al*., 2021). One of the biggest categories of proteolysis enzymes, plant-derived proteases, involves itself in an assortment of plant regulation systems. Despite being the most significant group of proteases, regulating potential and functioning are not well understood, and recognisable is a lack of identification. The overwhelming majority of plant proteases which have been determined and extracted are often used in an abundance of food industry processes. As a result, deploying the proteases in industry is economically feasible. In addition, the hunt for novel plant-based proteases is also ongoing. By comprehending their biological purposes, scientists endeavour to find efficient and affordable industrial solutions (Razzaq *et al*., 2019). Several enzymes in our bodies neutralise microorganisms during infections. The enzyme's localised administration alongside antibiotics could result in greater comfort than antibiotics alone. Proteolytic enzymes, on the other hand, might decrease bio adhesive owing to their mucolytic action (Zhou *et al*., 2018). *Myrica esculenta* is a prevalent tropical plant that is considered to have therapeutic properties. It is additionally admired for its commercial importance and adaptability (Sood and Shri, 2018; Suryawanshi *et al*., 2020).The key aims of this investigation were extracting, somewhat purifying, and examining the protease enzyme from *M. esculenta* leaves, as well as to explore the enzyme's impacts on certain pathogenic bacteria and anti-inflammatory and anti-biofilm action against pathogens.

MATERIALS AND METHODS

Ammonium sulphate, casein acid hydrolysate, along with other high-analytical-grade chemicals, were bought from Sigma, Merck and other vendors. The fresh leaves of *M. esculenta* were collected in the month of April 2024 from the Derayakhal Lansdowne (1700 m asl), Pauri Garhwal, Uttarakhand. Forty grams of the leaves samples were ground and macerated using liquid nitrogen. Following a fine powdering process, the suspensions were dissolved in 0.1 M phosphate buffer (pH 7.0). The homogenized liquid was passed through filters following centrifugation for 10-15 min.
To determine the antimicrobial potential of the enzyme protein using the following microbial strains were used: *E. coli* (MTCC 1687), *Staphylococcus aureus* (MTCC 3160), *Pseudomonas aeruginosa* (MTCC 1688) and *Salmonella typhi* (MTCC 3220). The standard bacterial strains were purchased from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India.
Two grams of *M. esculenta* powdered leaf were suspended in phosphate buffer (pH 4.5, 7 and 7.5) and 0.1% Tween 20 detergent and protease cocktail inhibitor followed by centrifugation at 10000 rpm for 10 min at 4°C. Ammonium sulphate precipitation was performed using leaf extract in a stirrer (4°C). A series of six saturation percentages (30-80%) were attained through nocturnal ammonium sulphate precipitation. Centrifugation was carried out at 11000 rpm for 35 min at 5°C subsequent precipitation. In order to eliminate excessive salt from the extract, the precipitating protein component was dialyzed in desalting columns utilizing Sephadex G-25 Medium at 4°C.
Protein concentration was determined by the Bradford colorimetric method. The standard used was bovine serum albumin (BSA) protein amount in the extracts (mg/ml). The extract of protein was placed into a DEAE cellulose column. An arrangement of an ultraviolet detector, a visualization recording device, and a pump model (Biorad) was implemented for performing ion exchange column chromatography. Protein elution from the column was carried out with a gradient of 0.05 M to 0.5 M NaCl. The gradient was operated at an average flow rate of 1 milliliter per min for 150 min. Optical density (OD) of all fractions was determined at 280 nm by applying a UV-Vis spectrophotometer.
BSA digestion was placed for one hour at 37°C and pH 4.5/7/8.0. The molecular weight of the partially purified enzyme protein was calculated using Tris-glycine (pH 8.2) buffer on SDS-PAGE.

The resulting samples were placed in every well at a 1:1 dilution via loading preparation buffer. The samples were electrophoresed in a 12% polyacrylamide gel, labelled with Coomassie Brilliant Blue, and their molecular weight was determined through contrasting them with a middle-range protein marker (having a flow rate of 0.500 µl/min and 200 psi pressure). After filter-sterilizing the samples, 20 µl of enzyme plant protein one was injected with 1 mg/ml (BSA) as a usual procedure, and the Biorad gel documentation system was used to photograph the gel.

An assay standard curve was plotted using tryosine (100 µg Tyrosine/ml; 0.100 g of Tyrosine in 0.10 N Hydrochloric Acid 60 ml, HCL). Three more dilutions were prepared from the above stock solution using distilled water to contain 25.0, 40.0 and 75 µg of tyrosine per ml. The absorbance of the Tyrosine solutions at 25, 40 and 75 micrograms in a Spectrophotometer were determined using 0.006 N HCl as a blank. A plot of absorbance versus tyrosine concentration was prepared. The slope from the data generated was calculated.

In this study, the protease activity of the enzyme using the substrate implemented was casein. Casein substrate (5 ml) solution was placed in test tubes (3 tubes for sample pH 4.5, 7 and 8 partially purified enzyme), and additionally prepared two tubes was taken as the blank. After that, the test tubes were then placed in a bath of boiling water at 37°C for 5 min. After adding 1 ml of enzyme solution, the test samples were well combined and incubated for 10 min at 37°C. Each test tube received 5 ml of trichloroacetic acid, whereas the blank test tube contained 1 ml of enzyme solution. The samples were mixed, incubated for 30 min at 37°C, and then centrifuged for 10 min at 4°C at 10,000 rpm. Five ml of sodium carbonate and 1 ml of Folin-Ciocalteu reagent were added to 2 ml of the supernatant. The spectrophotometric measurements were taken at 660 nm. Enzyme activity was computed at each saturation percentage to figure out how much active enzyme was accessible. The largest proportion of protease activity in acidic, basic and neutral protein enzymes was found. After performing a protease assay for samples, enzyme activity levels for each sample were estimated using the standard formula (Banik *et al*., 2018).

A temperature spectrum of 25, 30, 40, 50, 60, 70 and 80°C was utilized in evaluating the proteolytic activity of the partially isolated enzyme from *M. esculenta*. A 1% (w/v) casein solution was incorporated with 120 ìl of enzyme extract, and the resultant mixture underwent incubation for one hour. Activity levels of the partly purified extract were evaluated at different temperatures (25 to 80 °C) with the objective of figuring out a suitable temperature for protease. To test the protease's heat durability, a partly purified crude extract was incubated for 1 h at different temperatures in 50 mM phosphate buffer (pH 7). The amount of residual activity was assessed. Using phosphate buffer, the optimum pH for the protease was established at 4.5, 7 and 8.5.

The hydrolytic efficiency of 120 ìl of enzyme on 3 ml of casein solution at 35°C was tracked to figure out the detrimental effect of time on enzyme activity. The incubation intervals were: 15, 30, 45, 60, 75 and 90 min. To find the ideal period for maximal enzyme activity, a plot of relative enzyme activity against time was created.

Media were prepared for inoculation, and they were autoclaved at 15 lbs pressure for 20 min to test their antibacterial activity. The bacterial cultures were then seeded using a spread plating approach. Fifty µl of each microorganism's suspension was applied to an agar plate after the microbial suspension's concentration was adjusted to the 2.0 McFarland. Whatman No. 1 filter paper was used to create discs with a diameter of 6 mm. They were further autoclaved and were air-dried for an hour at 80°C. Using flamed forceps, discs were put on the agar surface and extracts of *M. esculenta* were added upon it (Abd-ElKhalek *et al*., 2020). The Petri dishes were then incubated for 24 h at 37°C for bacterial strains, respectively, and finally the diameter (mm) of the inhibition zone surrounding each disc was measured. A distinct zone of growth inhibition (mm) suggested the presence of antibacterial agent (Bimantara *et al*., 2022).

A modified version of the standard method was used to test the anti-inflammatory efficacy. The protocol was adhered to in execution to hinder albumin denaturation. Any anti-inflammatory medicaments can aid in preventing protein or albumin denaturation, which serves as antigens and triggers autoimmune diseases. Therefore, the reaction

mixture comprising corresponding volumes of diverse quantities of partially purified protease (8.8-35.2 g/ml) using two standard vaccines (Hepatitis B vaccine and typhoid vaccine) were tested against 1% Bovine serum albumin. A small quantity of 1 N HCl was utilized to change the pH. The sample was first incubated at 37 °C for 20 min and then it was heated to 51°C for another 20 min. At last, the percentage of suppression of protein denaturation was calculated (Poveda *et al*., 2022; Jha *et al*., 2023). Bacteria were raised on 12-well polystyrene tissue culture plates employing Christensen *et al.*'s technique, with minor changes. Long-term cultures were dispersed using fresh media (OD600 of 0.2) and bacterial suspension (3 ml) was mixed to each well of the tissue culture plate. The negative control wells comprised the equivalent amount of sterile medium. Plates were maintained at 30°C for seven days, and the development of biofilm was observed each day. For the assessment of biofilm formation, a designated set of wells was utilized each day. Bacterial cultures were aspirated and removed; the wells were rinsed three times using 3 ml of pH 7.4, PBS. After being allowed to air dry, biofilms were dyed for 20 min using 3 ml of 0.1% crystal violet. After the dye disappeared, the wells were cleaned using five sterile PBS washes. After letting the wells dry, three milliliters of ethanol were incorporated to disintegrate the pigmented biofilms. The optical density (OD) at 595 nm was determined using a microtiter-plate reader on 200 ìl from each well with the goal to quantify biofilm formation (Maali *et al*., 2020; Arnaouteli *et al*., 2021). Experiments were conducted three times and on three separate occasions. All experiments were carried out in triplicate and average along with standard deviation were presented.

RESULTS AND DISCUSSION

The protein amounts in crude extracted plant material were measured employing the Bradford technique (Table 1). The protein concentration against the leaves of *Myrica esculenta* using different pH values were calculated, based on the standard curve. A maximum amount of protein was found to be in the *M. esculenta leaves* (0.741 mg/ml, acidic) and low concentrations (0.0036 mg/ml, basic, and 0.0034 mg/ml, neutral), relatively. In addition, SDS and NATIVE PAGE studies were performed (Fig. 1) related to protein extracted from a different range of pH values. These studies revealed that protein extracted at pH 4.5 showed its molecular weight, i.e., 31 kDa, and the rest of them do not show any significant effect. The findings suggest that the protein's stability and functionality were influenced by the pH during extraction. Further investigation into the biochemical properties of the proteins, particularly at the optimal pH level, could provide insights into their potential applications in disease models. Serial dilutions of standard (BSA) were prepared for analyzing the protein content in the sample of acidic, basic and neutral protein extracted

Table 1. Estimation of protein content in *Myrica esculenta* using Bradford method

Samples	Concentration (mg/ml)	OD
Standards	1.00	1.852
	0.5	0.992
	0.25	0.575
	0.125	0.298
	0.0625	0.186
	0.0321	0.093
	0.0016	0.072
Acidic protein	0.741	1.408
Basic protein	0.036	0.121
Neutral protein	0.0034	0.061

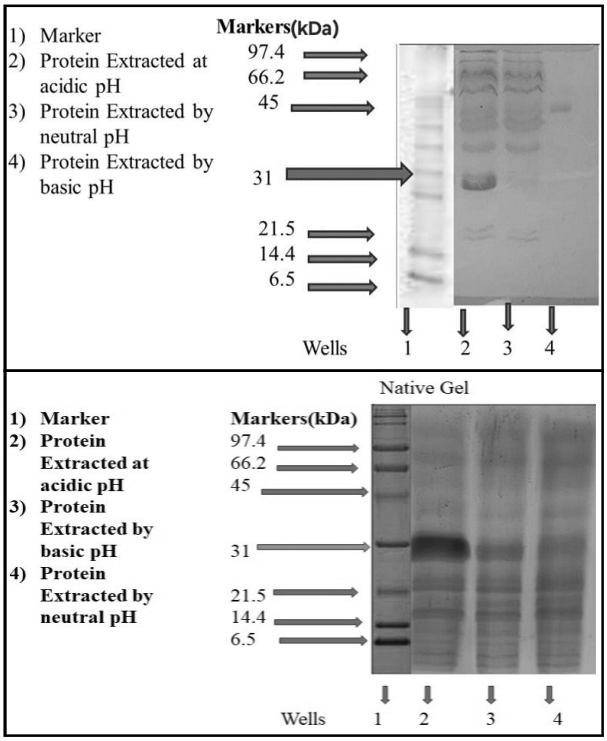


Fig. 1. SDS PAGE and NATIVE PAGE analysis.

from *M. esculenta.* Values were expressed in the form of mg/ml.

The current work aimed at extracting protease from *M. esculenta* using an acknowledged, established technique with minor modifications. Several tests, including ammonium sulfate precipitation, protease assay, dialysis and SDS page, were implemented to obtain and purify the protease enzyme. The ammonium sulphate precipitation was performed in different concentrations ranging from 30 to 80%, to find the ideal concentration for the extraction of protease in high concentration. The presence of the protease was confirmed using casein as a substrate. Once the presence was established, substantial purification was carried out overnight using phosphate buffer. Ultimately, SDS-PAGE and ion exchange chromatography were employed in determining the molecular weight of the enzyme located within the samples. Finally, SDS-PAGE and ion exchange chromatography were primarily utilized to estimate the molecular weight of the protease retrieved from the samples. The proteases turned out to be 31 kDa, displayed in Fig. 1, in which the data compilations illustrated the envisioned molecular weight of the protease that was retrieved from the leaves. Recent research revealed that *M. esculenta* crude extract might represent an intriguing supplier of purified proteases.

The proteolytic efficiency of partly purified extracts was assessed, and the proteolytic enzymes in the leaves were investigated using SDS-PAGE and NATIVE PAGE for the purpose of making plant proteases in *M. esculenta* accessible. The protease enzyme encountered in leaves had a disinfecting impact.

Proteolytic activity of the protease was evaluated using BSA and its Km and Vmax values of the enzyme for BSA were obtained to be 1.04 mg/ml and 0.026 ìmole Tyr/min, respectively. Results on proteolytic activity showed that partially purified enzyme of leaves with 35.2 U/ml activity used 30% ammonium sulphate saturation. Proteolytic enzymes from leaves (an acidic protein) were partially purified to get these outcomes, which were preferable compared with those of basic and neutral proteins. In cell lysosomes, it contained an assortment of hydrolytic enzymes, among the most significant of those called proteases, which possessed both pharmacological and anti-inflammatory properties and had a crucial part in a number of illnesses such as asthma, arthritis and gout.

Effect of temperature, pH and incubation time affected the proteolytic activity in *M. esculenta*. 3 ml of 1% (w/v) casein solution was combined with 120 ìl of enzyme protein, the protease's initial activity was strong between 25 and 37°C, and it was minimal at 90°C. The perfect ambient temperature was found to be 37°C. The activity was enhanced between 25 and 37°C because of the rise in the percentage of the enzyme-substrate complex, which was provoked when the reaction system was heated. There were three possibilities for the reduced activity at 45°C: steric congestion, enzymatic breakdown, or thermal denaturation, all resulting from molecular disturbances that unraveled low-energy bonds and disrupted the three-dimensional arrangement of enzymes. The initial activity level was altered to 100% before incubation at varying temperatures with the aim of ensuring consistency. A partially crude enzyme protease maintained its initial activity between 25 and 37°C after 60 min of incubation at pH 7.0. It achieved around 50% of its activity at 45°C and completely stopped working at 90°C (Fig. 2).

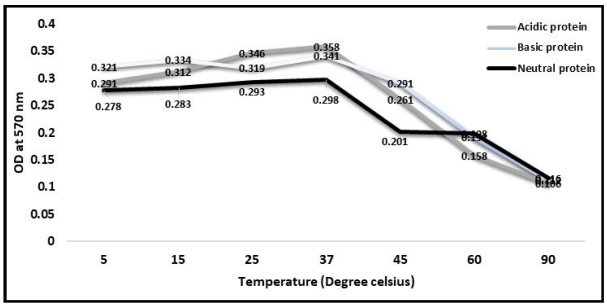
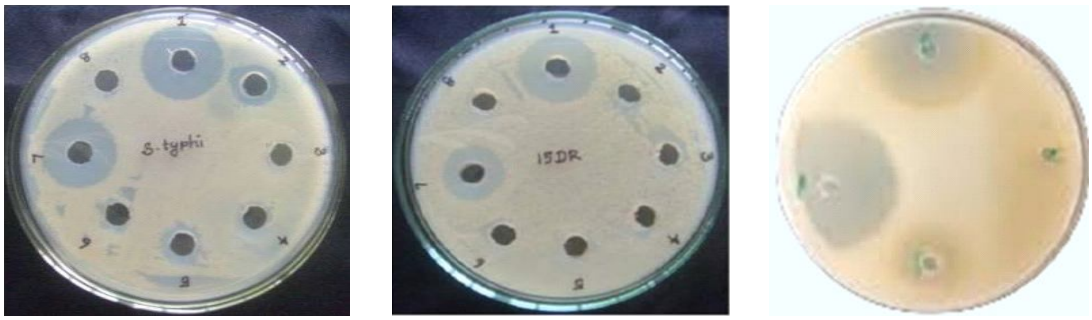


Fig. 2. Impact of temperature on partially purified enzyme.

All of the investigated bacteria, encompassing *Salmonella typhi, Staphylococcus aureus* and *E. coli* that was impervious to them, got impacted by the antibiotic enzyme (Fig. 3a, b and c). Also, there was growth of the *S. typhi*, *S. aureus* and *E. coli* at pH 4. The concentration-dependent nature of the anti-inflammatory activity of the protease from *M. esculenta* was evident.

Fig. 3 displays the maximum proteolytic activity of an acidic, partially purified enzyme protein at 35.2 U/ml. In this study, basic and neutral enzyme proteins did not show any effect. The effect of acidic partially purified enzyme protein on standard vaccines and



(a) (b) (c)

Strains	Acidic protein (Inhibition zone, mm)	Basic protein (Inhibition zone, mm)	Neutral protein (Inhibition zone, mm)	Antibiotics (Inhibition zone, mm)
S. typhi	12	8	5	16
S. aureus	11	9	5	17
E. coli	9	7	7	11

Fig. 3. Antimicrobial activity of protease against: (a) *S. typhi,* (b) *S. aureus* and (c) *E. coli.*

these studies claimed that protease at 35.2 U/ml showed inhibition in antigen-specific proliferation as compared to control. In contrast, the acidic sample enzyme protein showed less inhibition in comparison with the hepatitis and typhoid vaccines used as standards (Fig. 4). A partially purified acidic enzyme, exhibiting a specific activity of 35.2 U/ml, demonstrated notable anti-inflammatory activity in human whole blood. This enzyme preparation, stabilized using 1% bovine serum albumin (BSA), effectively inhibited inflammatory responses triggered by typhoid and hepatitis B vaccine antigens. The results suggested potential therapeutic applications of this acidic protease in modulating immune responses. In-depth research was conducted as an intriguing enzyme for the food industry (for accelerating the tenderization of meat and for the manufacturing of disintegrating proteins), biotechnology (for the creation of peptide drugs) and healthcare (for use as an

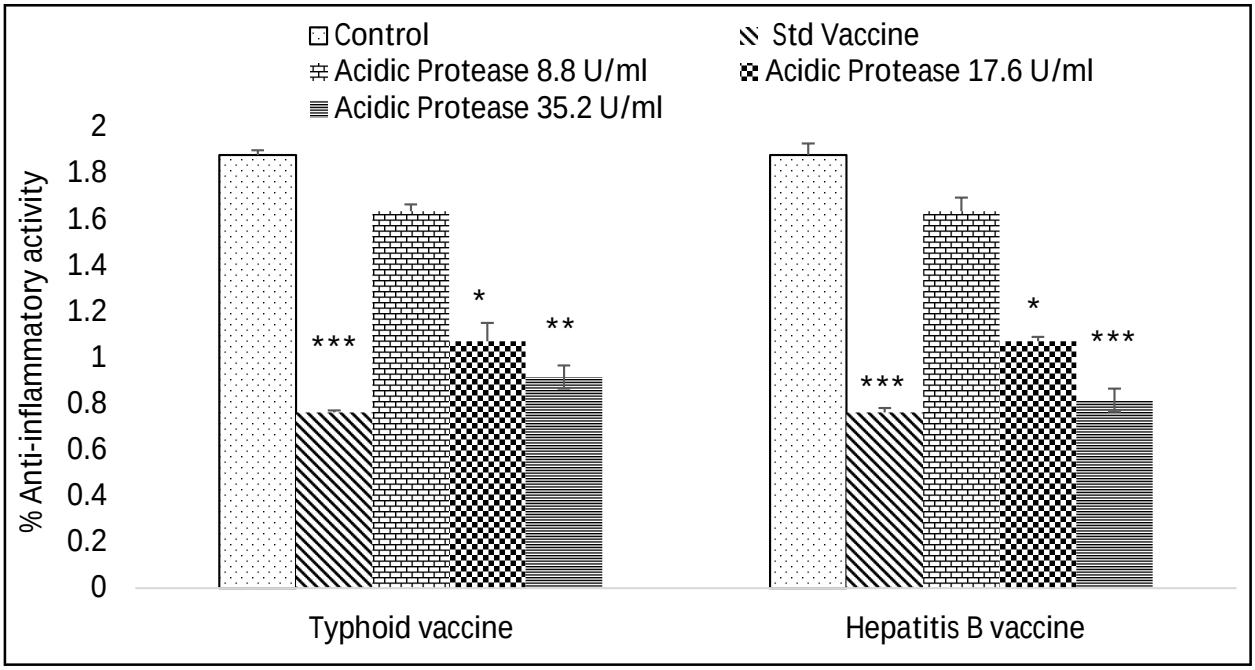


Fig. 4. Anti-inflammatory activity of partially purified enzyme (acidic) using 1% bovine serum albumin against two different vaccines (Hepatitis B and Typhoid vaccine).

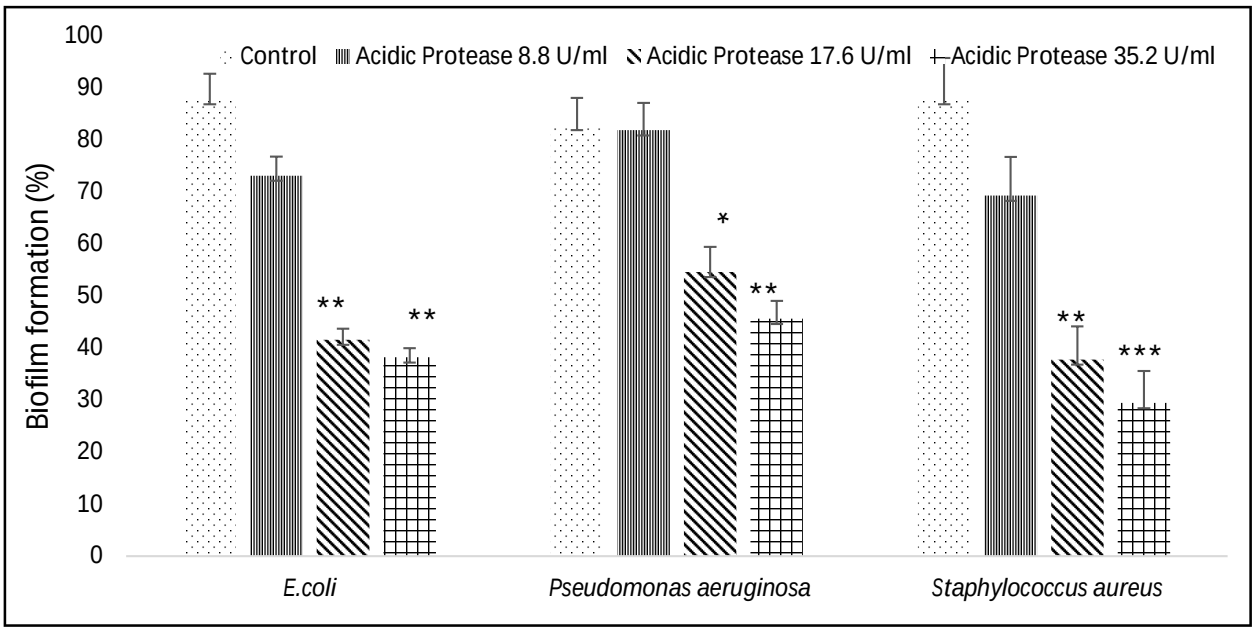


Fig. 5. The inhibition effect of partially purified enzyme against biofilm was quantified by CV staining at 570 nm. All values expressed as mean±SEM (n = 3) and significantly different in comparison to controls (***, P < 0.001) and to protease (P <0.01, *p* < 0.001) by Bonferroni multiple comparison test.

anti-inflammatory, wound-debriding and healing agent).

After confirming the anti-biofilm assessments against *E. coli* biofilm, the enzymatic systems were assessed against the *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm (Fig. 5). The acidic partially purified enzyme protein showed maximum inhibition (35.2 U/ml) in both the *P. aeruginosa* and *S. aureus* biofilm. Non-significant elevation in *S. aureus* biofilm inhibition was observed by basic and neutral protease (data not shown). The society is greatly benefited by the advantageous features of microbial biofilms. EPS, which is a component made up of proteins, lipids, carbohydrates and nucleic acids, contributes to the biofilm. These substances have an assortment of biotechnological applications and are also utilized in the food, pharmaceutical and cosmetics sectors. Another example is the generation of surfactants by the microorganisms that live inside the biofilm. In the commercial sector, biosurfactants are extensively employed as ingredients for phase dispersion, emulsion, wetting and foaming (Arya *et al*., 2021). Through the process of solid-state fermentation, biofilms are also utilized to produce a variety of molecules on an industrial level, notably lactic acid and ethanol. One advantage of this strategy was that it increased the pace of response by concentrating the cell-associated enzymes at the biofilm surface contact.

CONCLUSION

The performance of protease enzymes derived from *M. esculenta* leaves was examined in this study. Most illnesses among people originate with biofilm-induced bacterial infections, which frequently get triggered by *S. typhi, S. aureus* and *E. coli*. Overall development of pathogenic resistance severely hampered the efficacy of a number of antimicrobial medications. Consequently, the majority of the current medications and therapy medicines are not any more effective against bacteria. These illnesses may be treatable by an assortment of plant groupings with distinct therapeutic qualities.

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