ISSN: 0067-2904

Impacts of Peganin Alkaloids from *Peganum harmala* as Inhibitors for Hyaluronidase Enzyme Produced by *Staphylococcus aureus*

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Received: 12/11/2023 Accepted: 27/2/2024 Published: 28/2/2025

Abstract

Hyaluronidase is an enzyme that primarily breaks down hyaluronic acid, thereby disrupting the structural integrity of the extracellular matrix (ECM) found in connective tissues, which is considered a virulence factor. Peganum is a genus of plants in the family Nitrariaceae that contains a number of alkaloids, including harmaline and harmine, which have been used in traditional medicine. This study aimed to investigate the effects of a plant-active compound (peganin) isolated from a plant sample (Peganum harmala) as an inhibitor of hyaluronidase. In total, 120 skin samples were randomly isolated from infected burns. Isolates with highly hydrolyzed zones were identified. The Turbidity Reduction Assay was used to assess cell growth and hyaluronidase activity spectrophotometrically. The results showed that 100 out of 120 clinical samples grew bacteria when cultured on a blood agar medium. Also, it was recognized that, out of the 100 isolates, 56 (56%) belonged to *Staphylococcus aureus* bacteria and had an inhibitory zone of hyaluronidase with a range of 5-26mm; the inhibitory zone was increasing over time.

Keywords: Peganin; inhibitor; *S. aureus*; hyaluronic acid.

تأثير البيجانين القلويدي لنبات الحرمل كمثبط لإنزيم الهيالورونيداز المنتج بواسطة المكورات العنقودية

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الخلاصة

الهيالورونيداز هو إنزيم يقوم بتكسير حمض الهيالورونيك في المقام الأول وبالتالي تعطيل السلامة الهيكلية للمصفوفة خارج الخلية الموجودة في الأنسجة الضامة والتي تعتبر عامل فوعة. البيجانين هو جنس نباتات من عائلة

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Nitrariaceae، يحتوي على عدد من القلويدات، بما في ذلك الهارمالين والهارمين، والتي تم استخدامها في الطب التقليدي. يهدف هذا البحث إلى دراسة تأثير المركب النشط النباتي (البيجانين) المفصول من عينة نباتية كعامل مثبط لإنزيم الهيالورونيداز المعزول من بكتيريا المكورات العنقودية الذهبية. تم عزل 120 عينة عشوائياً من المصابين بالحروق. وقد تم التعرف على العزلات ذات المناطق شديدة التحلل المائي. يتم استخدام اختبار تقليل التعكر لتقييم نمو الخلايا ونشاط الهيالورونيداز طيفيًا. أظهرت النتائج أن (100) من أصل 120 عينة سريرية نمو البكتيريا عند زراعتها على وسط أكار الدم. كما تم اكتشاف أنه من بين 100 عزلة، 56 (56٪) تنتمي إلى المكورات العنقودية الذهبية ولديها منطقة مثبطة من الهيالورونيداز تتراوح بين 5–26 ملم، وكانت المنطقة المثبطة تتزايد مع مرور الوقت.

Introduction

The genus Peganum is classified within the plant family Nitrariaceae and encompasses a variety of blooming plants [1]. *P. harmala*, the most prominent species within this genus, possesses a rich historical background of utilization in traditional medicinal and religious contexts across many areas of Asia and North Africa [2]. *P. harmala* is known to possess a diverse array of psychoactive alkaloids, notably harmaline and harmine, which have been scientifically demonstrated to exhibit therapeutic attributes, including anti-inflammatory and anticancer qualities [3]. Nevertheless, the ingestion of this particular plant may result in potentially hazardous adverse reactions, necessitating prudence throughout its usage. *P. harmala* has been employed not only for its therapeutic properties but also for its application as a natural dye and its examination as a potential biopesticide [4]. Exploration of its possible applications across several disciplines remains a subject of keen interest among scholars. In general, the genus Peganum exhibits considerable intrigue owing to its extensive historical utilization and promising prospects for future investigations in medicine and agriculture [5].

Enzymes are of utmost importance in the context of infections because they significantly contribute to the interplay between invading pathogens and the immunological response of the host [6]. Microorganisms produce a diverse array of enzymes that play a significant role in the initiation, development, and resolution of infections [7]. Moreover, host enzymes play a crucial role in the activation of immune defense mechanisms aimed at countering infections. Understanding the impact of enzymes on infection offers valuable insights into the intricate dynamics between microorganisms and the host immune system [8].

The process by which hyaluronidase facilitates the dissemination of bacteria or other pathogens entails the formation of discontinuities within the extracellular matrix (ECM), thereby enhancing the ability of these microorganisms to traverse tissues with greater ease [9]. This enzyme facilitates the ability of microbes to breach host barriers, including but not limited to the skin, blood vessels, and connective tissues. Hence, rapid and extensive dissemination of infection might ensue across the entirety of the organism [10-12].

Hyaluronidase is frequently produced by several pathogenic bacteria such as Streptococcus pyogenes (also known as group A Streptococcus), *Staphylococcus aureus*, Clostridium perfringens, and certain strains of Streptococcus pneumoniae. Furthermore, it is implicated in the pathogenesis of specific fungal and viral infections [13-15]. This study aimed to investigate the effect of peganine on hyaluronidase activity in *Staph* sp.

Materials and methods

Sampling

A total of 120 patients at Al-Yarmouk Educational Hospital in Baghdad, Iraq, were enrolled for the collection of swabs of medical samples from the skin burn areas. The Ethics Committee at the College of Science/ University of Baghdad approved the study protocol (Reference: CSEC/0623/0048; June 8, 2023).

Isolation of bacteria

Bacterial colonies were initially isolated in a state of purity on blood agar and mannitol salt agar (MSA), followed by microscopic examination using the Gram stain technique. The cultural, physiological, and morphological features of all bacterial isolates were assessed using symmetry tests [16, 17].

Primary screening of hyaluronidase

The initial assessment of hyaluronidase activity commonly involves the identification of its capacity to degrade hyaluronic acid [18, 19]. This goal can be achieved through the use of the following methods:

1. The hydrolysis zone test is a screening method widely employed in microbiology laboratories for the identification of bacteria capable of producing hyaluronidase.
2. Gel electrophoresis is a technique that can be employed to identify the presence of hyaluronidase by subjecting a sample of the enzyme to an electrophoretic separation on a gel matrix.
3. The spectrophotometric test is a technique used to quantify the enzymatic activity of hyaluronidase by monitoring the absorbance of light at a specified wavelength. This method assesses the capacity of hyaluronidase to degrade hyaluronic acid.

In general, the main evaluation of hyaluronidase activity holds significance in the identification of prospective enzyme sources and the characterization of enzymatic characteristics [20-22].

Extraction and isolation of hyaluronidase

The shake flask fermentation method was employed to conduct an additional screening of isolates that exhibited a high level of output. The selected isolates were sub-cultured on nutrient agar slants and incubated for 24 hours at 37°C. Each slant was placed in a 250mL Erlenmeyer flask containing 50 mL of nutritional broth. The flasks were incubated for 48 hours at 37°C on a rotary shaker at 150 rpm. The clear supernatant from the centrifugation (at 8000rpm and 4°C) of the fermentation broth in each flask was used for the crude hyaluronidase test. The experiment was performed thrice, and the average results were used [23].

Enzyme purification

S. aureus in thin tryptic soy broth was grown overnight at 37°C and then centrifuged at 8000Xg for 30 minutes using a cold centrifuge. Extracellular hyaluronidase-containing supernatant was collected. Hyaluronidase was concentrated by ethanol precipitation. The supernatant was centrifuged at (8000Xg), using a cooling centrifuge for 30minutes, and then chilled with dry ice and mixed with ethanol (95%) to obtain a final concentration of 33% ethanol. The precipitate, which contained the majority of the enzyme activity, was then suspended in 10

ml of sodium phosphate buffer (pH6) containing 0.05M NaCl. The dialysate was run against phosphate buffer [24].

Optimum conditions for hyaluronidase production

The optimum conditions for hyaluronidase production may vary depending on the source of the enzyme; however, there are some general factors that can affect hyaluronidase production.

1. Media: brain heart infusion broth, nutrient broth and brain heart infusion broth, and bovine serum albumin are used, the best being brain heart infusion broth
2. pH: The pH range for optimum hyaluronidase production can vary, but it is typically around pH 7.
3. Temperature: hyaluronidase production is typically highest at temperature of 37°C.
4. Incubation period: The effect of different incubation periods of the selected *S. aureus* isolate on hyaluronidase production was studied.

Optimization of these factors is typically required to achieve the highest possible levels of enzyme production [25, 26].

Hyaluronidase activity assay

Utilizing HA sodium salt as a substrate in a turbidity reduction test, hyaluronidase activity was evaluated spectrophotometrically [27, 28]. When 1mL of HA at 70mg/ml was incubated with 1mL of enzyme sample in the presence of 0.05M sodium phosphate buffer with 0.05M NaCl at pH 7, the turbidity was reduced by enzymes. A decrease in turbidity was detected by measuring the absorbance at 600nm after the mixture had been treated for 30 min with 2.5mL of acidified protein solution (1% $w.v^{-1}$) of bovine serum albumin fraction (BSA) in 0.5M sodium acetate buffer (pH 3.1). The blank was a culture broth that was not infected. The quantity of enzyme that decreased turbidity at 600nm (A600) under specific conditions was used to define one unit of hyaluronidase activity.

Isolation of Peganum harmala crude alkaloids

Peganum harmala seeds were procured from the local markets in Baghdad. The seeds were ground into a coarse powder which was extracted with ethanol at a concentration of 80%, which included diluted HCL (1N). Unwanted elements were eliminated by shaking with chloroform. Excess ammonia was added to precipitate the free alkaloids, which were subsequently filtered and separated. Using a modified version of Dragendorff's reagent (potassium bismuth solution), the extracted fraction containing alkaloids was detected [29].

High performance liquid chromatography

The effective compounds (peganin) were examined using high-performance liquid chromatography (HPLC) [30], based on the HPLC (Shimadzu LC-2010 ATH) chromatogram of peganin, with a 250*4.6 mm, 5 micron, C18 column, flow rate1 of.5 ml/min, wavelength of 280 nm, mobile phase (80%-15% -5%- H_2O-methanol –acetomatral), oven 40 °C, volume injection 20 µL, and weight 10g /100 ml. These results were consistent with this condition.

Minimum inhibitory concentration (MIC) of peganin

The minimum inhibitory concentration (MIC) of peganin refers to the lowest concentration of an extract or compound derived from Peganum that can inhibit the growth of a particular microorganism, typically a bacterium or fungus [31, 32]. The MIC values for the peganin against *S. aureus* ranged from 156.2 to 20.000 µg/ml.

Minimum bactericidal concentration (MBC) of peganin

The MBC (minimum bactericidal concentration) of peganin refers to the lowest concentration of an extract or compound derived from Peganum that can completely kill a particular microorganism, typically a bacterium [33]. Numerous studies have been conducted to examine the antibacterial efficacy of peganin alkaloids against diverse bacterial strains. The broth dilution method was employed in these studies to determine the MBC. The MBC values for the various peganin samples ranged from 156.2 to 20,000 μg.ml-1. The aforementioned investigations indicated that peganin alkaloids are promising natural antibacterial agents. However, additional investigations are required to ascertain the effectiveness and safety of employing peganin alkaloids in this specific application.

Inhibitory assay

The methodology employed was elucidated by [34] with some modification. A total volume of 0.2 mL of crude enzyme extract was combined with varying concentrations of peganin (0.2, 0.4, 0.6, 0.8, and 1 mL) derived from a 10% w/v plant crude solution. This mixture was then added to 1.8 mL of hyaluronic acid solution. Subsequently, the reaction mixture was thoroughly mixed and incubated at a temperature of 37 °C for a duration of 10 minutes. The reaction was halted with the addition of 2 mL of a 5% tris-HCl acid solution, and subsequently, the technique was repeated in the absence of the inhibitor [35, 36].

Statistical analysis

The results of this study were analyzed using one-way ANOVA to determine whether the active compound had an effect on hyaluronidase enzyme inhibition [37].

Results and Discussion

Collection of samples

The results showed that 100 out of 120 clinical samples grew bacteria when cultured on blood agar medium. Also, it was found that, from the 100 isolates, 56 (56%) isolates belonged to *S. aureus*. They gave a distinguishing golden yellowish color on (MSA) medium, indicating the presence of *Staph. aureus, which was* confirmed by morphological testing of gram-positive (+) cocci that are regularly forming aggregates or tetramers [38]. This result of S. aureus percentage was similar to the findings reported in [39], which was 53%, and in [40], which was 53.93%.

Primary screening of isolates

Table 1 shows larger inhibition zones of isolates (49, 53, 57, 69, and 96 mm) upon treatment with hyaluronic acid as a substrate solution around the colony. These bacteria were then selected for secondary screening using the shake flask fermentation method and further tested for hyaluronidase activity.

Table 1: Diameters of clear zones around colonies of *S. aureus* grown on brain heart serum albumin for 24 hours. at 37°C**.**

Isolate No.	Diameter of clear zone (mm)
staph 49	16
staph 53	26
staph 57	20
staph 69	17
staph 96	18

Hyaluronidase production was detected in the 100 isolates; 56 (56%) isolates belonged to *Staph. aureus* bacteria, whereas they produced a zone of clearance (Figure 1), while 44 isolates showed no hyaluronidase activity. Hyaluronidase production was quantitatively estimated using the hyaluronidase plate test. Isolation no. 53 yielded the highest inhibition zone (26 mm).

Table 2: Specific activity of hyaluronidase produced by *S. aureus* after 24h. with incubation at 37 C° and pH 7 on brain-heart infusion agar.

Isolate No.		**Specific activity (u\mg)**
1	**staph 49**	**71.82**
2	**staph 53**	**97.101**
3	**staph 57**	**97.101**
4	**staph 69**	**75.174**
5	**staph 96**	**87.906**

Quantitative assessment of hyaluronidase production was performed using the hyaluronidase plate assay. Based on a standard curve, the diameter of the clearing zone was proportional to the logarithmic concentration of hyaluronidase. *S. aureus* isolation no. 53 produced the highest specific activity of hyaluronidase (97.101u\mg).

Table 3: Purification steps of hyaluronidase produced from *Staph. aureus* no.53.

Purification Step	**Volume (ml)**	**Activity (U/ml)**	**Protein Conc. (mg/ml)**	**Specific Activity (U/mg)**	**Total Activity (U)**	**Purification Fold**	**Yield (%)**
Crude Extract	**50**	**9**	**0.049**	**183.67**	**450**	**1**	**100**
Concentration by Ammonium sulfate	**35**	**9.5**	**0.044**	**215.91**	**332.5**	**1.18**	37.8
Dialysis	**10**	**10**	**0.03**	**333.33**	**100**	**1.81**	**40**
Gel Filtration Chromatography (by Seph acryl S-300)	**30**	**5**	**0.02**	**250**	**150**	**1.36**	**33.3**
P-Value	**<0.0001***	**0.727^{NS}**	**0.395^{NS}**	**<0.0001***	**<0.0001***	**0.819^{NS}**	**<0.0001***
Significance	**Significant**	**Non-Significant**	**Non-Significant**	**Significant**	**Significan**	**Non-Significant**	**Significant**

Data are presented as chi-square (χ^2) goodness of fit. ^{NS}, not statistically significant ($p \leq 0.05$); *, statistically significant at $p \leq 0.05$.

The results showed that this ratio gave a specific activity of 215.91 U/mg protein by ammonium sulfate (Table 3), indicating an observable increase in the specific activity compared to that of the crude extract (183.67U/mg protein). Upon dialysis with crude hyaluronidase, the

results indicated in Table 3 showed an increase in the activity of hyaluronidase, when the specific activity reached 333.33 U/mg protein.

Optimum conditions for hyaluronidase production

Due to its highest production of hyaluronidase, S53 isolate of *Staph. Aureus* was used to determine the optimum production conditions [41].

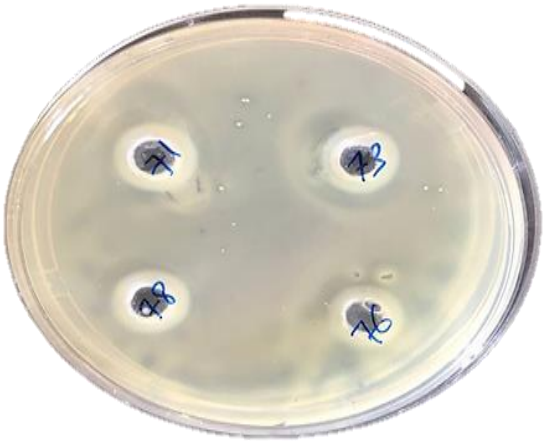

Figure 1. Specific activity of hyaluronidase produced by the five S. aureus isolates.

Isolate no. 53 was found to be the most prolific in producing the enzyme, with the highest enzyme specific activity estimated at 96 U/mg protein. The difference in the enzymatic activity of the different isolates may be due to the difference in the sources of the isolated samples and the difference in the expression rate of the genes encoding the enzyme.

The hyaluronidase produced from Staph. aureus

Effects of medium composition

The effects of the medium composition are shown in Figure 2.

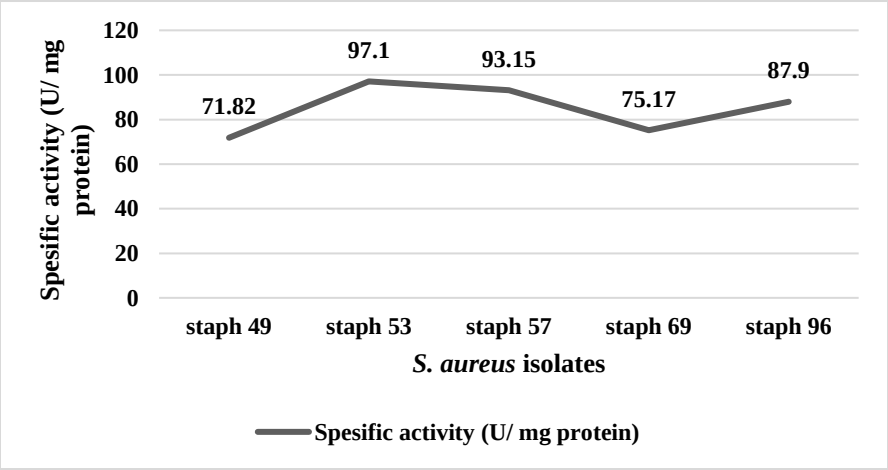

Figure 2- Hyaluronidase production by *S. aureus* cultured in different media with incubation at 37 °C and PH 7 for 24h.

Many researchers have used BHI medium alone or supplemented with 0.5% yeast extract to produce hyaluronidase. The BHI medium was used to produce and purify the enzyme in large

quantities from *S. aureus* [42, 43]. In addition, [27] indicated the use of BHI medium supplemented with 0.5% yeast extract to produce and purify the enzyme in large quantities from *Streptococcus* bacteria, while yeast extract was added at a rate of 0.2% to Todd-Hewitt broth to produce the enzyme from Group A *Streptococci*; the difference in the components of the medium, such as carbohydrates, proteins, and inorganic materials leads to a difference in the production of enzymes from microorganisms [44].

Effects of pH of the medium

The results of different incubation pH values, which ranged 4-9, are shown in Figure 3.

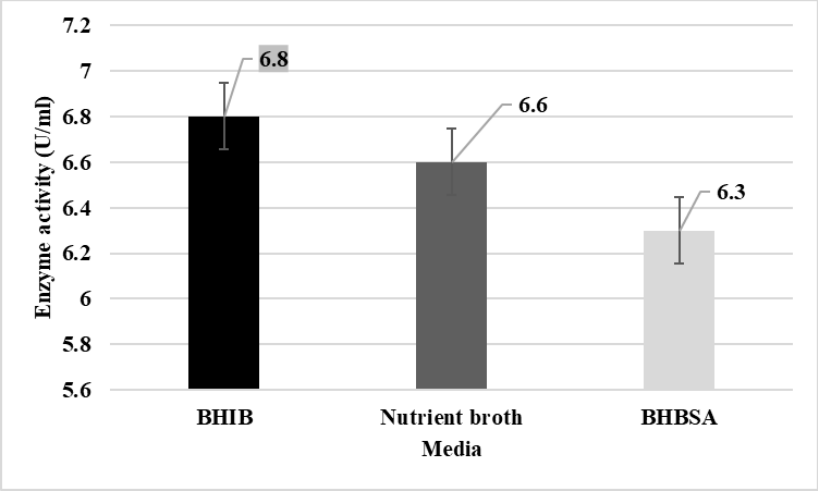

Figure 3- Hyaluronidase production by *S. aureus* cultured in BHIB medium at different pH values and incubated at 37 °C. for 24h.

The best result was observed with a pH value of 7. Previous studies [45, 46] reported that the best production of hyaluronidase enzyme from *S. aureus* bacteria was achieved at pH 7.2.

Effects of incubation temperature

The results of different incubation temperatures, which ranged 30-55°C, are shown in Figure 4

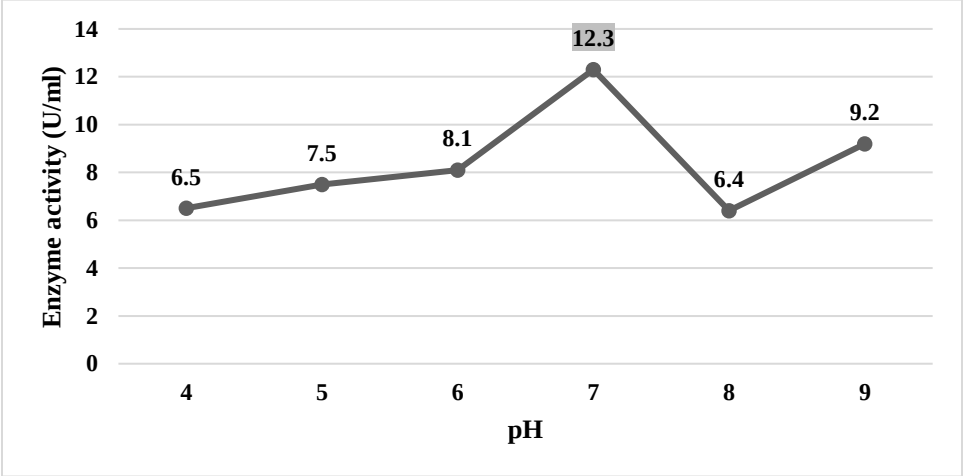

Figure 4- Hyaluronidase production by *S. aureus* cultured in BHIB medium at different temperatures and incubated at pH 7. for 24h.

Other studies revealed that an incubation temperature of 37°C was optimal for the production of hyaluronidase by *Staphylococcus* spp., *Streptococcus* spp., and *C. freundii* [24]. Kadhum (2023) [43] discussed suppressed enzyme activity at low and high temperatures due to the inadequacy of these temperatures for the growth of bacterial cells, leading to slow growth. Temperature may also have a negative effect on the dynamic energy of molecules, the speed of reactions, and metabolic processes in the cell.

Determination of optimum incubation time

The results of different incubation periods, ranging 24-96 hours, are shown in Figure 5.

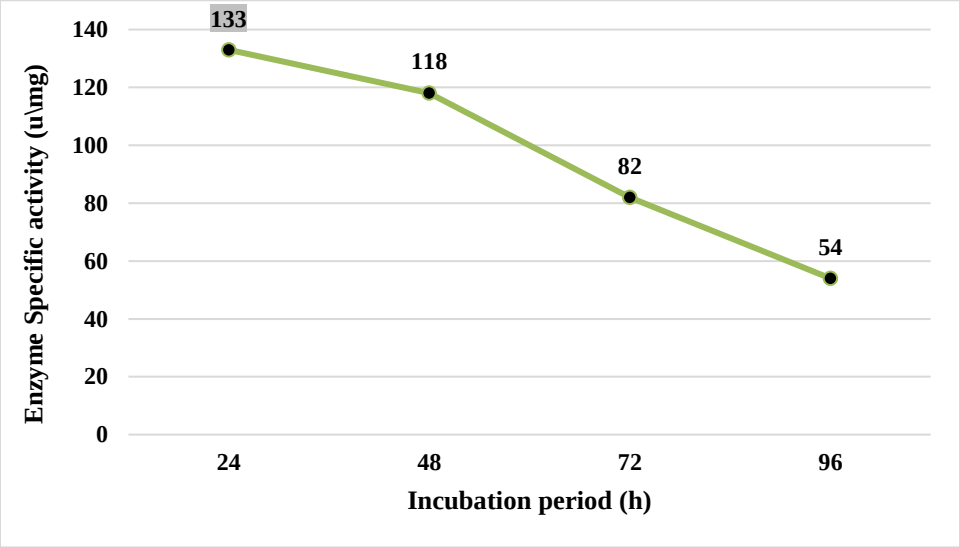

Figure 5: Hyaluronidase production by *S. aureus* cultured in BHIB medium at pH 7 for different periods and incubated at 37 °C. for 24h.

Characterization of peganin

By reagent: Formation of a creamy-white coloured precipitation indicates positive result test, confirming the presence of alkaloids [47]**.**

By High-performance liquid chromatography (HPLC)

1. Determination of the melting point for the separated peganin.
2. One of the fundamental and crucial tests for identifying substances for the characterization of the peganin alkaloid is the measurement of the melting point. The melting point of the drug was 210°C, which is consistent with studies that reported that peganin melting values varied from 204 to 210°C [22].
3. HPLC analysis of peganin

HPLC analysis of the active plant compound (peganin) revealed several peaks that had important bioactive natural chemical compounds [48], as shown in Figure 6.

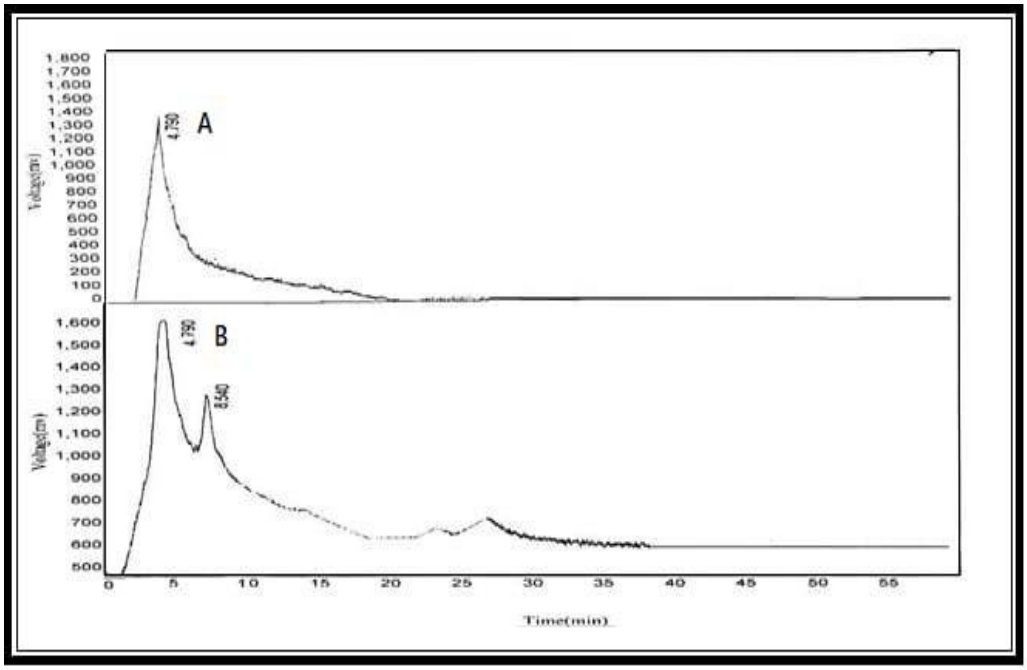

Figure 6: HPLC chromatogram of peganin.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC values of peganin alkaloids (Table 4) were studied in a variety of microorganisms.

Table 4: MIC and MBC of peganin.

MIC concentration µg/ml	MBC concentration µg/ml	Enzyme stop concentration µg/ml
325.5	1.302	651.0

Determining the MIC of peganin, or any specific compound derived from it, requires laboratory tests and studies on various microorganisms. MIC values can vary depending on the specific microorganism tested, form of peganin or compound used, solvent or medium, and other factors [22, 41]. The MIC of peganin against *S. aureus* was found to be 325.5 µg/ml and the MBC was 325.5 µg/ml.

Inhibitory assay

The result of the hyaluronidase inhibitory assay would be the concentration of the tested compound required to inhibit the activity of hyaluronidase, which is an enzyme that degrades hyaluronic acid. Hyaluronidase inhibition is crucial in medical conditions, as it helps maintain the structural integrity of hyaluronic acid-containing tissues such as the skin, joints, and eyes.

Table 5: Inhibitory Assay.

No	Enzyme: extract (Concentration)	Enzyme inhibition activity (%)
1	**1:1**	**83.62**
2	**1:2**	**86.24**
3	**1:3**	**87.55**
4	**1:4**	**87.77**
5	**1:5**	**94.98**

The study suggests that peganin alkaloids may have significant effects on inhibiting hyaluronidase across various domains. Hyaluronic acid breakdown is involved in physiological processes like inflammation, tissue injury, and wound healing. Peganin alkaloids can inhibit hyaluronidase, preserving extracellular matrix, facilitating tissue regeneration, and mitigating inflammatory processes, potentially promoting tissue regeneration and wound healing [49, 50].

Conclusions

Staphylococcus aureus is more prevalent in infections and wounds. An enzyme was synthesized using a medium with 0.5% yeast extract and 0.25 mg hyaluronic acid, pH 7, and 37°C temperature. Ammonium was added at a 50% saturation level. Ion exchange was performed using a DEAE-cellulose column, and gel filtration was performed using a Sephacryl-S300 column. Paganine was found to inhibit hyaluronidase activity with specificity at certain doses. Further investigation is required to understand the mechanisms of inhibition and its potential applications in the pharmaceutical, cosmetic, or industrial sectors. The safety, stability, and efficacy of the extract are crucial for its future advancement and commercialization.

Conflict of Interest

The authors declare no conflicts of interest.

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