ISSN: 0067-2904

Inhibition of the Hyaluronidase Enzyme Produced by *Staphylococcus* Bacteria Using Vasicine Alkaloids of the *Vasica* Plant

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Received: 22/6/2023 Accepted: 26/8/2023 Published: 30/10/2024

Abstract

Hyaluronidase is an enzyme that breaks down primarily hyaluronic acid, thereby disrupting the structural integrity of the extracellular matrix (ECM) found in connective tissues, which is considered a virulence factor. The aim of this research was to study the effect of a plant-active compound (vasicine) that was separated from a plant sample as an inhibitor agent for the hyaluronidase enzyme that was isolated from *Staphylococcus aureus*, which is considered a virulence factor. 120 samples were randomly isolated from infected burns. The isolates showing highly hydrolyzed zones were identified. Cell growth as well as hyaluronidase activity are measured spectrophotometrically by the turbidity reduction assay. The results showed that 100 out of 120 clinical samples grew bacteria when cultured on blood agar medium. Also, it was found that of the 100 isolates, 56 (56%) belonged to *S. aureus* and had an inhibitory zone of hyaluronidase of 5–26 mm, and the inhibitory zone was increasing over time.

Keywords: vasicine, inhibitor, hyaluronidase and *S. aureus*.

تثبيط إنزيم هيالورونيداز الذي تنتجه بكتيريا المكورات العنقودية الذهبية باستعمال قلويدات فازيسين لنبات الحلق السبع الشجيري

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الخلاصة

الهيالورونيداز هو إنزيم يقوم بتكسير حمض الهيالورونيك بشكل أساسي مما يؤدي إلى تعطيل السلامة الهيكلية للمصفوفة خارج الخلية (ECM) الموجودة في الأنسجة الضامة والتي تعتبر عامل ضراوة. الهدف من هذا البحث هو دراسة تأثير المركب الفعال للنبات (فازيسين) الذي تم استخلاصه من عينة النبات كعامل مثبط لأنزيم هيالورونيداز المعزول عن المكورات العنقودية الذهبية والذي يعتبر عامل ضراوة. تم عزل 120 عينة عشوائياً من الجروح والحروق والكسور المصابة. تم التعرف على العزلات التي أظهرت مناطق عالية التحلل بالماء. يتم قياس نمو الخلايا وكذلك نشاط الهيالورونيداز بطريقة طيفية بواسطة مقايسة تقليل التعكر. أظهرت النتائج أن 100 من أصل 120 عينة سريرية تنمو للبكتيريا عند زراعتها على وسط أجار الدم. كما وجد أنه من بين 100 عزلة فإن 56 (56٪) عزلة تنتمي إلى المكورات العنقودية الذهبية يمكن أن تكون منتجة للإنزيم مع منطقة تثبيط 4.5 سم. كان النمو يتزايد مع مرور الوقت.

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1. Introduction

Hyaluronidase plays a significant role in infections, particularly in the spread and dissemination of bacterial and other microbial pathogens within the body. Hyaluronidase is an enzyme that specifically targets hyaluronic acid, a major component of the extracellular matrix (ECM) found in connective tissues. The enzyme breaks down hyaluronic acid, thereby disrupting the structural integrity of the ECM [1].

In the context of infection, many pathogenic microorganisms produce hyaluronidase as a virulence factor. By degrading hyaluronic acid, these microbes can overcome physical barriers, invade tissues, and facilitate the spread of infection. Hyaluronidase allows bacteria and other pathogens to break down the ECM, promoting tissue invasion and the establishment of infection.

The mechanism of hyaluronidase-mediated spread involves creating gaps in the ECM, allowing bacteria or other pathogens to move through the tissues more easily. This enzyme helps the microorganisms penetrate host barriers, such as the skin, blood vessels, and connective tissues. Consequently, the spread of infection can occur more rapidly and extensively throughout the body [2].

Hyaluronidase is commonly produced by several pathogenic bacteria, including Streptococcus pyogenes (group A Streptococcus), Staphylococcus aureus, Clostridium perfringens, and some strains of Streptococcus pneumoniae. It also plays a role in the pathogenesis of certain fungal and viral infections [3], [4].

Vasicine is an alkaloid found in the *Adhatoda vasica* plant, also known as Vasaka. This plant has been traditionally used in Ayurvedic medicine for its potential therapeutic properties. Some studies have investigated vasicine's effects on the respiratory system and its potential anti-inflammatory, anti-asthmatic, and bronchodilatory activities. However, it's important to note that the research is still preliminary and that vasicine has not been established as a standard treatment for any specific disease [5]. The presence of hyaluronidase in infections can be clinically significant, as it contributes to the severity of the disease and the ability of pathogens to invade and disseminate. In some cases, hyaluronidase inhibitors may be used as therapeutic agents to hinder the spread of infection by inhibiting the enzymatic activity of hyaluronidase [6].

Overall, hyaluronidase is an important enzyme involved in the pathogenesis of various infections, facilitating the spread of microbial pathogens by degrading hyaluronic acid and compromising the integrity of the extracellular matrix [7]. Understanding the role of hyaluronidase in infections is crucial for developing strategies to prevent and control the spread of infectious diseases [8]. In this study, an experiment was carried out to determine the vasicine-isolated effect on the hyaluronidase enzyme activity of bacteria that produce it.

2. Materials and methods

2.1 Sampling

One hundred and twenty medical samples were taken from patients at Al-Yarmouk Educational Hospital in Baghdad, Iraq. The specimens included burn swabs.

2.2 Isolation of bacteria:

Bacteria were first isolated as pure colonies on blood agar and Mannitol salt agar, and they were examined microscopically using the Gram's stain technique [9]. Tests were then carried

out on all the bacterial isolates to determine their cultural, physiological, and morphological properties [10], [11].

2.3 primary screening of hyaluronidase

The primary screening of hyaluronidase activity typically involves the detection of its ability to break down hyaluronic acid. This can be done using a variety of methods, such as the following:

1. Hydrolysis zone test: This is a common screening test used in microbiology laboratories to identify bacteria that produce hyaluronidase.
2. Gel electrophoresis: Hyaluronidase can be detected by running a sample of the enzyme through a gel electrophoresis system.
3. Spectrophotometric assay: This method measures the ability of hyaluronidase to break down hyaluronic acid by monitoring the absorbance of light at a specific wavelength.

Overall, the primary screening of hyaluronidase activity is important for identifying potential sources of the enzyme and for characterizing its enzymatic properties [12–14].

2.4 Extraction and isolation of hyaluronidase

Shake-flask fermentation was used to further screen the isolates that showed high production. The chosen isolates were subcultured onto nutrient agar slants, where they were incubated for 24 hours at 37 °C. Each slant's contents were put into a 250 mL Erlenmeyer flask containing 50 mL of nutritional broth. The flasks were incubated for 48 hours at 37 °C with a rotary shaker spinning at 150 rpm. The clear supernatant from the centrifugation of the fermentation broth in each flask at 8000 rpm and 4 °C was utilized for the crude hyaluronidase assay. The experiment was carried out in triplicate, and the average results were taken into consideration [15].

2.5 Enzyme purification

The S. aureus was grown in thin tryptic soy broth for an overnight at 37 °C, and then it was centrifuged at 8,000 X g for 30 minutes using a cool centrifuge. Take the extracellular hyaluronidase-containing supernatant. Hyaluronidase is concentrated by ethanol precipitation. The supernatant was cooled with dry ice and mixed with ethanol (95%) to obtain the final concentration of 33% ethanol, then centrifuged at 8,000 X g for 30 minutes using a cool centrifuge. The precipitate that had the majority of the enzyme activity was then suspended in 10 ml of sodium phosphate buffer (pH 6) at 0.05 M NaCl. The dialysate was run against a phosphate buffer [16].

2.6 Optimum conditions for hyaluronidase production

The optimum conditions for hyaluronidase production may vary depending on the source of the enzyme, but here are some general factors that can affect hyaluronidase production:

1. Media: BHIB, nutrient broth, and BHIBSA are used; the best are BHIB.
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 temperatures between 37 °C.
4. Incubation periods: The effect of different incubation periods of the selected isolate of *Staphylococcus aureus* on hyaluronidase production was studied.

Optimization of these factors is typically required to achieve the highest possible levels of enzyme production [17].

2.7 Hyaluronidase activity assay:

Hyaluronidase activity is measured spectrophotometrically by a turbidity reduction assay using HA sodium salt as a substrate [18]. When 1 mL of HA at 70 mg/mL was incubated with 1 mL of the enzyme sample in the presence of NaCl (0.05 M) and sodium phosphate buffer (0.05 M) at pH 7, there was an enzymatic reduction in turbidity. Then the mixture was incubated for 30 minutes, and 2.5 mL of an acidified protein solution (1% w/v) of bovine serum albumin fraction (BSA) was added to 0.5 M sodium acetate buffer at pH 3.1. After incubation for 10 minutes at 37 ℃, a reduction in turbidity was detected by measuring the absorbance at 600 nm. The blank was an uninoculated culture broth. The quantity of enzyme that results in a decrease in turbidity at 600 nm (A600) under particular assay conditions was referred to as "one unit of hyaluronidase activity."

2.8 Collection and drying of Adhatoda vasica leaves

The leaves of *A. vasica* F: (Acanthaceae) were collected from several locations at the Baghdad University gardens. The plant leaves were cleaned with fresh tap water, dried at room temperature for two to three weeks at 22–25 ℃, and then ground into powder using an electric mill.

2.9 Crude extraction and vasicine separation

Crude leaf extract was made using 80% methanol, dried leaf powder (200 g), and a Soxhlet apparatus with 600 ml of the solvent. Each extraction was done independently at 50 °C for 36 hours with a 1:3 extraction ratio. The extract was then concentrated using a rotary evaporator after being filtered via Whatman No. 4 filter paper. In a refrigerator set to -4 °C, the extract was stored in a glass jar. By extracting the leaves of A. vasica with 80% methanol in a soxhlet system, the vasicine alkaloid was obtained. The methanolic extract was then treated for 15 minutes with aqueous 2% H2SO4 at room temperature, followed by three separate defatting steps using chloroform and a separator funnel. Crude vasicine was produced by basifying the aqueous layer with liquid ammonium hydroxide and extracting it with chloroform. This vasicine was then refined through crystallization and recrystallization using a solution of ethanol and diethyl ether (1:1) [19].

2.10 High performance liquid chromatography

The effective compounds (vasicine) were examined using high-performance liquid chromatography (HPLC). Depending on the condition of the HPLC (Shimadzu LC-2010 ATH) chromatogram of vasicine, the column of examination is 250*4.6 mm, 5 micron C18, flow rate 1.5 ml/min, wave length λ 280 nm, mobile phase (80%–15%–5%–H2O–methanol–acetomatral), oven 40 ºC, volume injection 20 µl, and weight 10 g/100 ml. The results agree with this condition.

2.11 Minimum Inhibitory Concentration (MIC) of vasicine

Studies have reported MIC values of vasicine ranging from 126.7 µg/mL to 10.416 µg/mL against bacteria, including *Staphylococcus aureus*. In one study, the MIC of vasicine against *S. aureus* was found to be 325.5 µg/mL [20].

2.12 Minimum Bactericidal Concentration MBC determination

The MBC stands for minimum bactericidal concentration, which is the lowest concentration of an antimicrobial agent that kills 99.9% of the initial inoculum of a specific microorganism. MBC determination is an important step in determining the effectiveness of an antimicrobial agent and is typically performed following the determination of the minimum inhibitory concentration (MIC).

Overall, the MBC determination is an important tool for determining the effectiveness of antimicrobial agents and is used to guide appropriate dosages for clinical use.

2.13 Inhibitory Assay

The method adopted was described by Adu et al. (2013) [21], with a slight difference. Briefly, 2 ml of the crude enzyme extract and various concentrations of vasicine (0.2, 0.4, 0.6, 0.8, and 1 ml) of 10% w/v of plant crude in 1.8 ml of hyaluronic acid solution were added. This reaction mixture was mixed and incubated for 10 minutes at 37 °C before being stopped by adding 2 ml of 5% tris-HCl. The procedure was then repeated without an inhibitor [22, 23].

2.14 Statistical analysis

The results of this study were analyzed by a one-way Anova test to find out if there was an effect of the active compound on hyaluronidase enzyme inhibition.

3 Result and Discussion

3.1 Collection of samples

Results showed that 100 out of 120 clinical samples grew bacteria when cultured on blood agar medium. Also, it was found that of the 100 isolates, 56 (56%) belonged to S. aureus [24]. This result, like in the [25] study, is 53%, and in the [26] study, it is 53.93%.

3.2 Primary screening of the isolates:

Figure 1 shows the larger inhibition zones (mm) of staph 49, staph 53, staph 57, staph 69, and staph 96, respectively, upon treatment with hyaluronic acid as a substrate solution around the colony, then selected for secondary screening by the shake flask fermentation method and further for hyaluronidase activity. Results showed that 5 (8.9%) out of 56 isolates of S. aureus were able to produce enzymes [27].

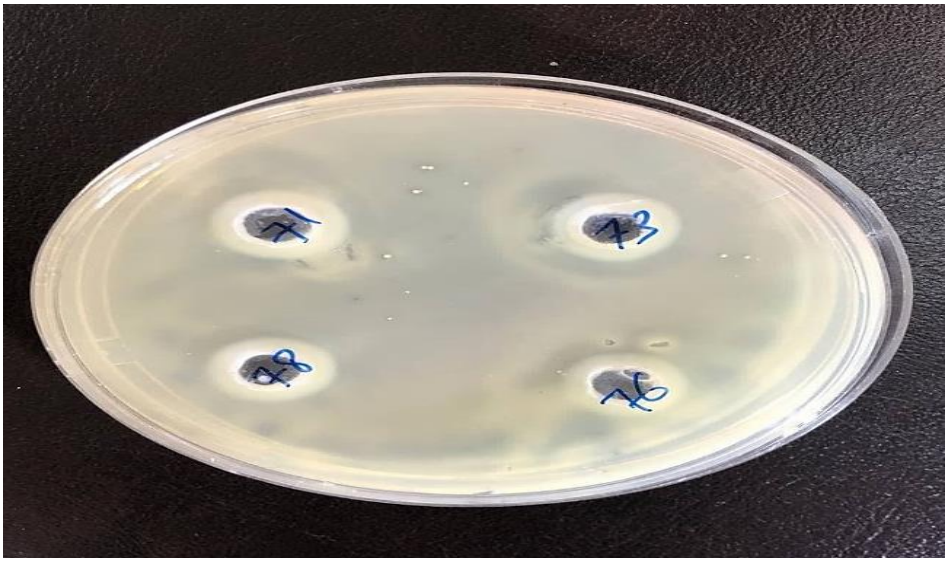

Figure 1: Diameter of the hydrolysis zone around S. aureus isolates showing hyaluronidase production at 37 °C after 24 hours

Table 1: Diameters of clear zones around colonies of staph 53 grown on brain and heart serum albumin for 24 hours at 37 °C

Isolate	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 100 isolates; 56 (56%) belonged to S. aureus bacteria, which produced a zone of clearance (Figure 1), while 44 isolates showed no hyaluronidase activity. Isolation staph 53 gave the highest inhibition zone (26) mm.

Table 2: Activities of hyaluronidase produced by *S. aureus* after 24 h of incubation at 37 °C, pH 7, in brain heart broth

Number of isolates	Enzymatic activity(U/ml)
staph 49	6.321
staph 53	6.700
staph 57	9.222
staph 69	6.465
staph 96	6.512

Quantitative estimation of hyaluronidase production was performed by the hyaluronidase plate assay method. The hyaluronidase concentration was calculated using a standard curve, where the diameter of the clearance zone was directly proportional to the logarithmic concentration of the hysA enzyme. *S. aureus* isolate no. staph 53 showed the highest level of hyaluronidase production (6.7 U/ml).

Table 3: Purification steps of hyaluronidase produced from a Staph 53 isolate

Purification Step	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Yield (%)
Crude Extract	50	6	0.070	85.7	300	1	100
Concentration by sucrose	18	7	0.044	159	126	1.8	42
Gel Filtration Chromatography (by Seph acryl S-300)	24	9	0.03	300	48	1.8	38
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	Significant	Non-Significant	Significant

Data presented as Chi-square (χ^2) goodness of fit. ^{NS} Non- Significant At a probability ($p \leq 0.05$), **Statically significant at $p \leq 0.05$.

The results showed that this ratio gave a specific activity of concentration by sucrose of 159 U/mg protein (Table 3), indicating an observable increase in the specific activity compared to that of the crude extract (85.7 U/mg protein). Upon dialysis of crude hyaluronidase, the results indicated in Table 3 showed an increase in the activity of hyaluronidase when the specific activity reached 300 U/mg protein.

3.3 Optimum conditions for hyaluronidase production

Due to its highest production of hyaluronidase (Figure 2), the staph 53 isolate of *S. aureus* was used to detect the optimum production conditions [28], [29].

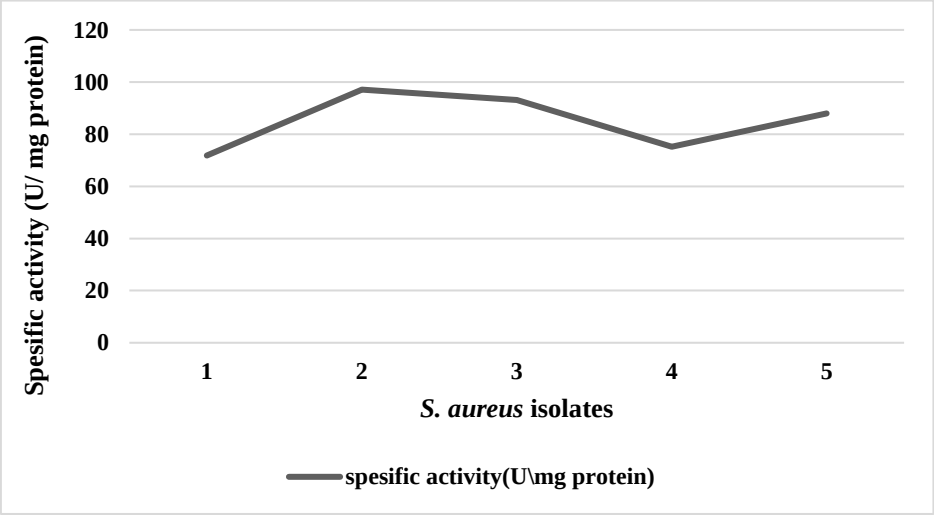

Figure 2: Specific activity of hyaluronidase produced by five isolates of *S. aureus*

Isolate staph 53 was characterized by being the most prolific in producing the enzyme; it had the highest enzyme-specific activity estimated at 96 units/mg protein. The difference in the enzymatic activity of the different isolates may be due to the difference in the sources of isolating samples and to the difference in the expression rate of the genes encoding the enzyme.

3.4 The hyaluronidase produced from S. aureus

I) Effect of medium compositions:

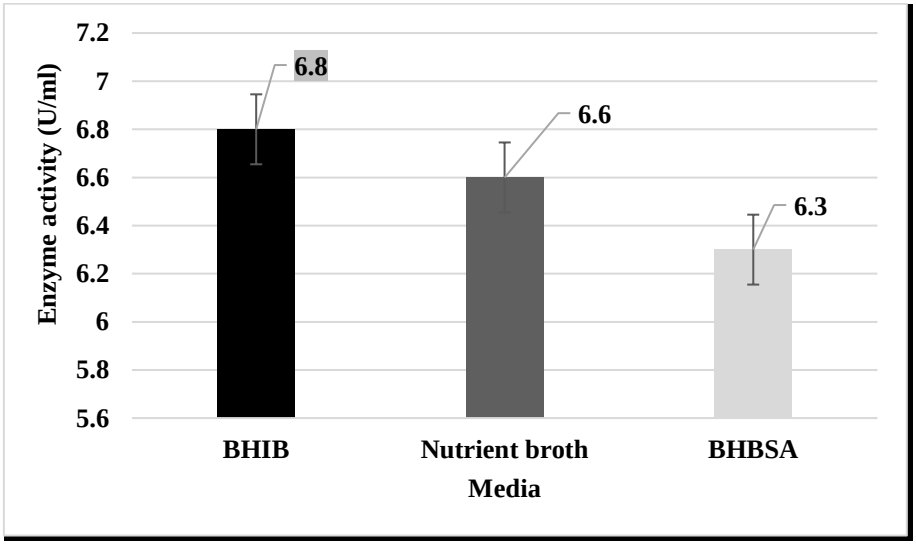

Figure 3: Hyaluronidase production by *S. aureus* cultured on different media with incubation at 37 ˚C and PH 7 for 24 h

Table 4: Significant differences in the ability of S. aureus to produce hyaluronidase on different media

Media	Enzyme activity (U/ml)	Standard Deviation	F-Value	P-Value	Significance
BHIB	**6.8**	**0.6**			
Nutrient broth	6.6	0.2	0.126	0.886^{NS}	Non- Significant
BHBSA	6.3	1.8			

Data presented as One-way ANOVA. ^{NS} At a probability ($p \leq 0.05$).

Results showed that BHIB is the best medium for producing the enzyme, giving it the highest enzyme activity estimated at 6.8 units/ml.

Effect of different culture media on the production of hyaluronidase from an isolate of *S. aureus* spp. Many researchers used BHI medium alone or supplemented it with 0.5% yeast extract to produce hyaluronidase. BHI medium was used to produce and purify the enzyme in large quantities from *Staphylococcus aureus* [30]. Also, Tam and Chan (1983) and (1985) [16], [18] indicated the use of BHI medium supplemented with 0.5% of yeast extract to produce hyaluronidase to produce and purify the enzyme in large quantities from Poptostreptococcus bacteria, while yeast extract was added at a rate of 0.2% to Todd-Hewitt broth to produce the enzyme from Group A bacteria.

Rivera and Engleherg (2006) reported that streptococci are affected by differences in the components of the food medium, such as carbohydrates, proteins, and inorganic materials present in the medium, which lead to a difference in the production of enzymes by microorganisms and that enrichment [31].

II) Effect of pH media:

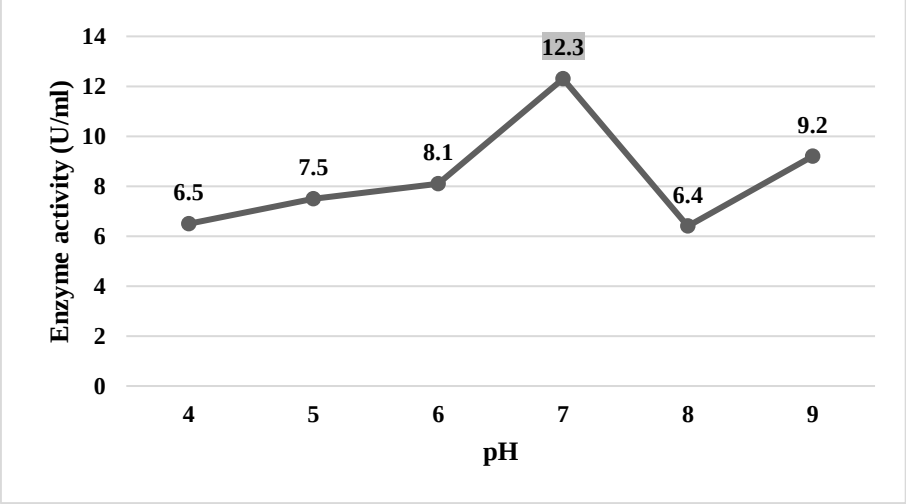

Figure 4: Hyaluronidase production by S. aureus cultured in BHIB medium at different pHs and incubated at 37 °C for 24 h

Table 5: Significant differences in the ability of S. aureus to produce hyaluronidase at different pHs

Ph	Enzyme activity (U/ml)	Standard Deviation	F-Value	P-Value	Significance
4	6.5	0.1	261.4	<0.0001	Significant
5	7.5	0			
6	8.1	0.02			
7	**12.3**	**0.5**			
8	6.4	0.1			
9	9.2	0.03			

Data presented as One-way ANOVA. **Statically significant at $p \leq 0.05$.

Results showed in Table 5 that PH.7 is the best at producing the enzyme by giving it the highest enzyme specific activity estimated at 150 units/Mg proteins.
Skalka (1985) declared that the best production of the hyaluronidase enzyme from the *S. aureus* bacterium was achieved at pH 7.2 [32].

III) Effect of incubation temperature:

Figure 5: Hyaluronidase production by S. aureus cultured in BHIB medium at different temperatures and incubated at pH 7 for 24 h

Table 6: Significant differences in the ability of S. aureus to produce hyaluronidase at different temperatures

Temperature (°C)	Enzyme activity (U/ml)	Standard Deviation	F-Value	P-Value	Significance
30	6.2	0.3	95.036	<0.0001	Significant
37	**11.4**	**0.6**			
40	8.1	0.02			
45	11.3	0.5			
50	6.2	0.3			
55	9.2	0.1			

Data presented as One-way ANOVA. **Statically significant at $p \leq 0.05$.

Results showed in Table 6 that the maximum production of hyaluronidase was obtained when the culture medium was at an incubation temperature of 37 ^{o}C and the specific activity of hyaluronidase reached 168 U/mg protein.

Other studies revealed that the incubation temperature of 37 °C was optimum for the production of the hyluronidase enzyme by *Staphylococcus* spp., *Streptococcus* spp., and *Citrobacter freundii* [16]. Frost (2007) discussed depressed enzyme activity at low and high temperatures due to the inadequacy of these temperatures for the growth of bacterial cells, leading to slow growth. The temperature may also have a negative effect on the dynamic energy of the molecules, the speed of reactions, and metabolic processes in the cell [33].
IV) Determination of the optimum incubation time:
During different incubation periods that ranged between 24 and 96 hours, the results showed

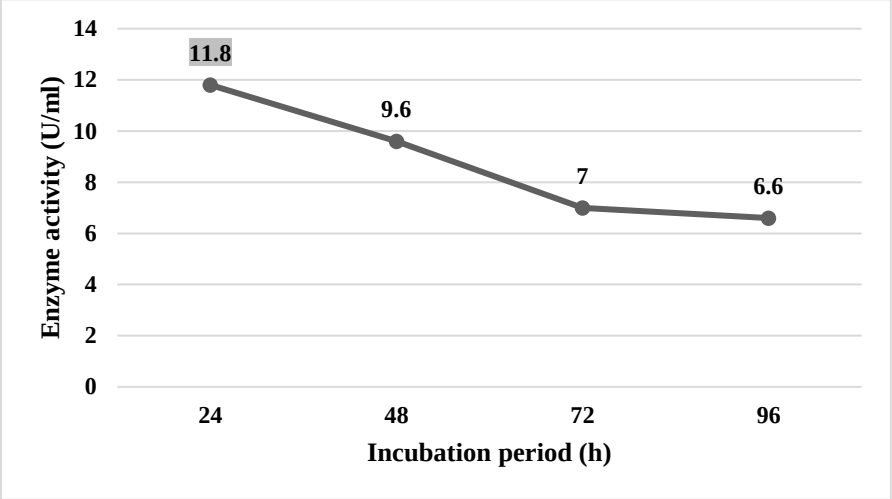

Figure 6: Hyaluronidase production by S. aureus cultured in BHIB medium at pH 7 for different periods and incubated at 37 ˚C for 24 h

Table 7: Significant differences in the ability of S. aureus to produce hyaluronidase for different periods

Incubation period (h)	Enzyme activity (U/ml)	Standard Deviation	F-Value	P-Value	Significance
24	**11.8**	**0.01**	372.820	<0.0001	Significant
48	9.6	.03			
72	7	0.01			
96	6.6	0.4			

Data presented as One-way ANOVA. **Statically significant at p <0.05.

3.5 Characterization of vacicine

1. By reagent The formation of a creamy-white precipitation indicates a positive result in the test and confirms the presence of alkaloids.
2. Determination of the melting point for separated vasicine.
3. One of the fundamental and crucial tests for identifying the substances for the characterization of the vasicine alkaloid was the measurement of melting point. The melting point of the drug was 210 °C, which is consistent with other research that stated that the vasicine melting values varied from 204 to 210 °C [14]. The flowering season and the purity of the vasiline may be to blame for this variance [34].
4. HPLC analysis of vasicine

The analysis of an active plant compound (vasicine) using HPLC revealed several peaks that had important bioactive-natural chemical compounds, as shown in Figure 7 for vasicine.

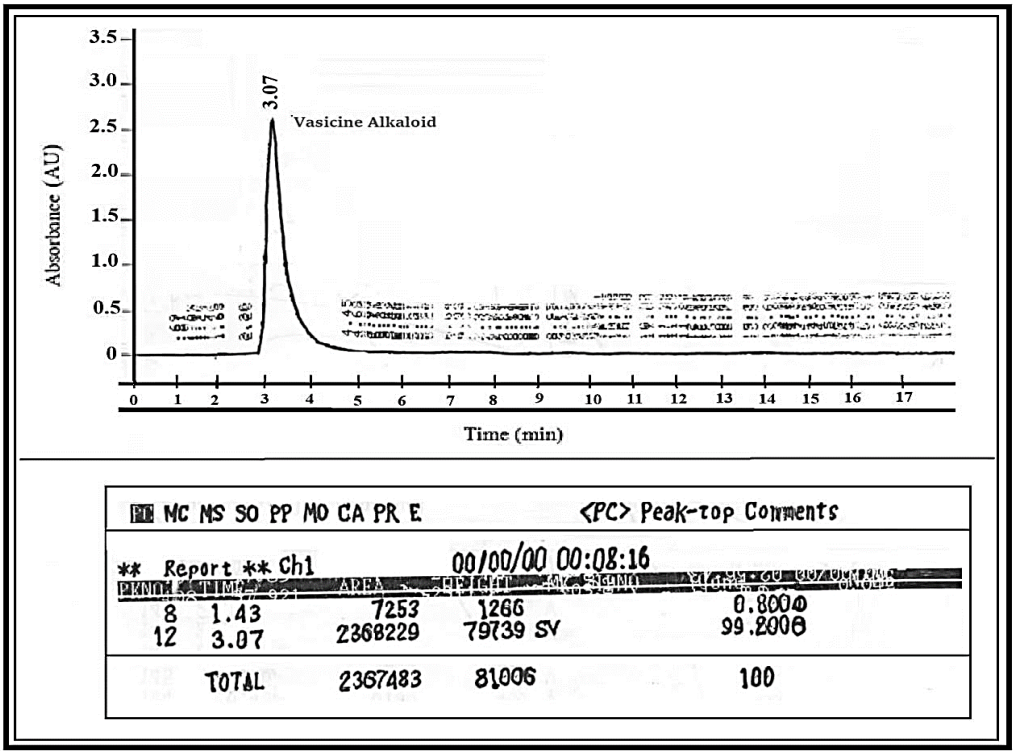

Figure 7: HPLC chromatogram for vasicine

3.6 Minimum inhibitory concentration

Vasicine is an alkaloid compound found in the leaves of *Adhatoda vasica*, a medicinal plant commonly used in Ayurvedic medicine. While vasicine has been shown to have various pharmacological properties, including antimicrobial activity, the MIC of vasicine can vary depending on the microorganism being tested.

Table 8: The minimum inhibitory concentration (MIC) of vasicine

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

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

The MBC is the lowest concentration of an antimicrobial agent, such as an active compound, required to kill bacteria. Usually, a broth dilution assay is used to find out. In this test, the antimicrobial agent is added to a series of test tubes with a set number of bacteria, and the tubes are left to sit for a certain amount of time. After incubation, the tubes are examined for bacterial growth.

3.7 Inhibitory Assay

The result of a hyaluronidase inhibitory assay is the concentration of the tested compound that is needed to stop hyaluronidase, an enzyme that breaks down hyaluronic acid, from doing its job. Hyaluronidase inhibition is important for many medical conditions because it helps maintain the structure of hyaluronic acid-containing tissues like the skin, joints, and eyes.

Table 9: 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

These results show that the best ratio of enzyme to extract for inhibition of the enzymatic activity of hyaluronidase was 1:5. Also, vasicine has a high inhibitory activity (94%).
The inhibition of hyaluronidase by vasicine alkaloids has significant implications in various areas. For instance, hyaluronic acid degradation plays a role in inflammatory processes, tissue damage, and burn healing. By stopping hyaluronidase from working, vasicine alkaloids could help keep the extracellular matrix intact, speed up tissue repair, and reduce inflammation.

4 Conclusion

In conclusion, infection and burn samples were characterized by a high proportion of staphylococcus. The enzyme was produced in a production medium fortified with 0.5% yeast extract and 0.25 mg hyaluronic acid, with a pH of 7, and incubated at 37 °C with ammonium at a saturation of 50%. Ion exchange was performed using a DEAE-cellulose column, followed by gel filtration with a Sephacryl-S 300 column. The vasicine alkaloids exhibit the capacity to inhibit the hyaluronidase enzyme at specific concentrations. Inhibition of hyaluronidase activity offers a promising approach to impede bacterial spread and minimize tissue damage. This research investigates the potential of vasica compounds as natural inhibitors of the hyaluronidase enzyme produced by *Staphylococcus* bacteria. Further research and experimentation are required to determine the precise mechanisms of inhibition and explore the extract's potential in practical settings, such as pharmaceuticals, cosmetics, or other industrial applications. Additionally, it is essential to evaluate the extract's safety, stability, and effectiveness under different conditions to ascertain its viability for future development and commercialization**.**

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