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Antibacterial and antihemolytic assessment of *Allium Sativum* through optimized protein fraction extraction

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ABSTRACT

Background: Garlic (*Allium sativum*), a widely utilized plant in the Mediterranean region, has long been renowned for its therapeutic properties, primarily attributed to its bioactive compounds such as phenolic compounds and flavonoids. However, the potential of garlic proteins remains largely unexplored. **Aims:** This study aimed to investigate the *in vitro* biological activities of the protein fraction extracted from this plant, optimize the extraction process for the garlic protein fraction (GPF), and elucidate its antibacterial and antihemolytic effects. **Material and Methods:** A Box-Behnken design was employed, using Design Expert 10 Software, to optimize the extraction process of GPF, involving water maceration followed by precipitation using ammonium sulphate. The antibacterial activity of GPF was evaluated against *Staphylococcus aureus* (FRI 137) and *Enterococcus faecalis* (ATCC49452) using the well diffusion method. The antihemolytic activity of GPF was evaluated using human erythrocytes. Additionally, the antihaemolytic activity was evaluated using human erythrocytes. **Results:** The highest extraction yield of 46.57 mg/mL was obtained under the following conditions: pH of 6.22, maceration time of 102 minutes, and temperature of 24.53°C. Remarkably, GPF exhibited significant antibacterial activity, evidenced by inhibition zones of 20 ± 0.5 mm against *Enterococcus faecalis* and *Staphylococcus aureus,* two targeted bacterial strains. The minimum inhibitory concentration (MIC) values were determined as 9.36 ± 0.11 mg/mL and 18.72 ± 0.2 mg/mL, while the minimum bactericidal concentration (MBC) values were found to be 37.45 ± 0.13 mg/mL and 18.72 ± 1.08 mg/mL for the respective strains. Furthermore, the investigation of GPF's anti-hemolytic activity on human erythrocytes demonstrated an inhibition rate of 83.77 ± 1.05% at a GPF concentration of 0.25 mg/mL. **Conclusions:** These findings underscore the significant biological activities associated with the protein fraction of *Allium sativum*. The protein fraction derived from *Allium sativum* exhibits notable antibacterial and antihemolytic properties, shedding light on its potential therapeutic applications.

Keywords: *Allium sativum*, Garlic protein, antibacterial properties, hemolysis, RSM.

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1 Introduction

The rise in antibiotic resistance can be attributed to various microbial characteristics. Consequently, the need for effective antibiotic treatments and the implementation of technical advancements to combat microbial resistance have become essential. The development of synthetic drug substances and the emergence and proliferation of highly pathogenic and resistant microbes, have driven interest in the exploration and application of herbal remedies (Upadhyay et al., 2014). Certain medicinal plants offer a promising alternative therapy for reducing and eliminating antibiotic-resistant infectious

organisms. Among these, *Allium Sativum* L., a robust bulbous medicinal plant of the Alliaceae family (Ali-Delille, 2013), is widely known for its therapeutic properties in addressing various infections and physiological disorders (Singh & Singh, 2008). This efficacy is attributed to its rich composition of sulfur-containing bioactive components, including essential oils (Berthet, 2014), cysteine sulfoxides (especially alliin), carbohydrates, lipids (Szychowski et al., 2018), liposoluble vitamins (K, D and A), and soluble water vitamins (B, C) (Melguizo-Rodríguez et al., 2022).

The sulfur compounds in *Allium sativum* are synthesized through the enzymatic conversion of γ-Glutamyl-Peptides (GSPCs) into Alkyl-l-Cystein Sulfoxides such as alliin, isoalliin and methiin, by γ-Glutamyl-Transpeptidase and oxidase (Lawson et al., 2005). These bioactive constituents have been extensively studied, with research focusing on antibacterial activity (Ali-Delille, 2013; Benkeblia, 2004), hypoglycemic activity, anticoagulant activity, anti-inflammatory, antioxidant, with beneficial effects against cardiovascular diseases, cancers, and immune system disorders (Foroutan-Rad et al., 2017). Despite the wealth of research on these properties, the protein fraction of *Allium sativum*, which constitutes 6% of its total composition and notably rich in sulfur amino acids (Trudel, 2005), remains relatively underexplored. Advances in food proteomic and peptides research now present an opportune moment to investigate these primary metabolites, evaluate their structural stability, refine extraction techniques, and assess their biological activities (Ezeorba et al., 2023). Studies also suggest that garlic is a source of bioactive proteins with diverse therapeutic and pharmacological properties, making it a valuable functional food ingredient (Kovarovič et al., 2019). Recent research indicates that all garlic varieties contain approximatively 6.38 to 9.5 % protein (Tahir et al., 2022), with extraction methods aligning with standard protocols for other plants (Bar et al., 2022).

In this context, the current study aimed to investigate the antibacterial and anti-hemolytic activities of the protein fraction derived from *Allium sativum* L. To achieve this, the present study was initiated by optimizing the extraction process of the protein fraction from *Allium sativum*. Subsequently, this study was conducted to assess the anti-hemolytic activity of this fraction on human erythrocytes and examine its antibacterial potential against two reference strains: *Staphylococcus aureus* (FRI137) and *Enterococcus faecalis* (ATCC49452)."

2 Material and Methods

2.1 Extraction of total proteins

Fifty grams of fresh garlic cloves were crushed, and the resulting material was soaked in 100 mL of Tris-HCl buffer (0.1 M and pH 6) for 2 hours at room temperature. The resulting solution was then filtered using Whatman No. 1 filter paper. The filtrate underwent ammonium sulphate precipitation at a saturation level of 60%, following a modified protocol base on Duong-Ly & Gabelli, (2014). The obtained mixture was refrigerated and centrifuged at 3000 rpm (EZ Swing -3K) for 20 minutes. The resulting pellet was subjected to overnight dialysis at 4 °C against Tris-HCl buffer (0.1 M; pH = 6) as described by (Burian et al., 2017).

2.2 Optimization of total protein extraction conditions

The optimization of protein extraction conditions was conducted using the Box Behnken Design approach, implemented in Design Expert 10 software. The study aimed to identify the optimal values for three critical factors influencing the protein extraction process from garlic cloves. These factors, along with their respective ranges, were: maceration time (X_1) ranging from 1 to 3 hours; maceration temperature (X_2) °C: ranging from 4 to 25 °C; and the pH of the extraction buffer (X_3): ranging from 5.5 to 8. Preliminary experiments were conducted to establish the minimum and maximum values for these parameters.

A total of 17 experiments were performed, each applying a unique combination of the specified parameter values within the extraction protocol. The mathematical model utilized for analysis was described by the following equation:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j$$

With: Y: response or quantity of interest; Xi: level of factor i; X_j: level of factor j; β_0, β_j, β_{ij}, β_{ii}: are the coefficients of the polynomial (Groupy & Greighton, 2006).

2.3 Statistical analysis

Experimental responses were analyzed using analysis of variance (ANOVA) to evaluate the effects of the selected factors and determine the optimal values. Additionally, response surface plots were generated to visualize and assess the combined effects of the various factors.

2.4 Antibacterial assay

2.4.1 Preparation of the bacterial inoculum

Two bacterial strains were utilized in this study: the Gram-Positive *Staphylococcus aureus* (FRI 137) and the Gram-Negative *Enterococcus faecalis* (ATCC 49452). A modified version of the protocols outlined by Chergui et al. (2016) and Sellem et al. (2017) was employed to recover the target strains. The bacteria were rapidly thawed and inoculated into tubes containing 5 mL of nutrient broth. The mixture was homogenized and incubated at 37 °C for 24 hours. Following incubation, the bacterial suspensions were standardized to an

optical density (OD) of 0.08 at a wavelength of 625 nm, corresponding to a concentration of 10^7 colony-forming units per milliliter (CFU/mL) as described by Barefoot et al., (1983).

2.4.2 Antibacterial activity test

Twenty milliliters of nutrient semi-solid agar were inoculated with 20 µL of standard suspensions of the target strains. Subsequently, four wells were created in each agar, and each well was loaded with 70 µL of one of the following solutions: sterile distilled water (negative control), protein extract "E," trypsin enzymatic preparation "Try," or pepsin enzymatic preparation "Ps." The enzymatic preparations were obtained by mixing 1 mL of the protein extract, neutralized at pH = 7, with 1 mL of a 1 mg/mL enzyme solution following the protocols described by Chen et al., (2003) and Benkerroum et al., (2000). The agars were then incubated at 37°C for 24 hours. The inhibitory effect was determined by observing the formation of an inhibition zone larger than 2 mm around the wells containing the protein extract (Yildirim & Yildirim, 2001).

2.4.3 Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of GPF were determined for the two target bacterial strains. Serial dilutions of the extract were prepared using a two-fold dilution method (broth dilution method). To each dilution, a standardized bacterial suspension, prepared from the inoculum of the target strains, was added. After incubation under the same conditions, the MIC was determined as the lowest concentration of the extract that completely inhibited visible bacterial growth. To determine the MBC, aliquots from tubes showing no growth were subcultured onto solid medium. The MBC was defined as the lowest concentration of the extract at which no colonies were observed.

2.5 Anti-hemolytic activity

2.5.1 Preparation of erythrocytes

The erythrocyte suspension was prepared following the protocols described by Ebrahimzadeh et al. (2010) and Alinezhad et al. (2012). Heparinized blood samples were centrifuged at 1500 rpm for 10 minutes at 4°C. The resulting erythrocyte pellet was resuspended in Phosphate-buffered Saline (PBS) buffer (0.2 M, pH 7.4) at a 1:10 (v/v) ratio. The suspension underwent an additional centrifugation step at 1500 rpm for 10 minutes at 4°C. This washing step was repeated three times under the same conditions to ensure thorough removal of plasma and any contaminants. Finally, the washed erythrocytes were suspended in PBS buffer at a 1:1 (v/v) ratio and stored at 4°C.

2.5.2 Screening of anti-hemolytic activity

The prepared mixture was incubated at room temperature for 5 minutes. Subsequently, 0.5 mL of a 0.1M hydrogen peroxide (H_2O_2) solution, prepared in PBS was added to induce hemolysis. The final solution was then incubated for 2 hours at 37°C. Afterward, the mixture was centrifuged at 1000 rpm for 10 minutes, and the absorbance of the resulting supernatant was measured at 540 nm.

To ensure complete hemolysis, a control solution was prepared by mixing 2 mL of 4% erythrocyte suspension with 2 mL of H_2O_2. Ascorbic acid was employed as the standard for comparison under identical experimental conditions.

The percentage inhibition of hemolysis was calculated using the following equation:

$$\% \text{ of inhibition of hemolysis} = (Ac - Ae / Ac) * 100$$

Where:

- Ac: absorbance obtained after total hemolysis.
- Ae: absorbance obtained in presence of extract.

3 Results and discussion

The optimization study was used in order to increase the performance of extraction. The Box Behnken design of experiments takes account of interaction effect between all the selected factors in opposite with the One Factor at a Time then it perfectly represents the optimal conditions for the extraction procedure (Savic et al., 2022). In the current work, three factors were selected: maceration time (X_1), temperature (X_2), and pH (X_3) and the used software Design Expert generated 17 extraction experiments distributed with the chosen levels, including three center points. Table 1 represents the distributed experiments with their different levels of the studied factors: X_1, X_2, X_3 and the obtained responses (protein concentrations) for each of the 17 experiments. Also, in the same table, the predicted responses for the same experiments can be observed. The standard error for each response is defined as the variability between the observed values and the predicted responses. This variability is quantified through the Mean Squared Error (MSE), which is a measure of model fit quality and highlights the significance of the combined effect of the factors. To statistically evaluate the model, an ANOVA was performed (as shown in Table 2), revealing that the experimental protein concentrations closely matched the predicted values. This confirms the correct application of the extraction conditions, particularly concerning the studied factors. However, in some experiments, the standard error was significant, probably due to residual factors in the model. On the other hand, the lack of adjustment is not significant

Table 1. Optimizing Protein Fraction Extraction from Garlic using Box-Behnken Experiment Plan and Response Surface Methodology (RSM)

Std	Run	Factors			Protein concentration (mg/mL)	Predicted values	Standard error
		Maceration time (X_1): (h)	Temperature (X_2): (°C)	pH (X_3)			
3	1	1	25	6.75	28.775	27.99	0.785
15	2	2	14.5	6.75	51	50.93	0.07
2	3	3	4	6.75	41.33	41.01	0.33
9	4	2	4	5.5	60	59.01	0.99
1	5	1	4	8	42.55	41.79	0.76
10	6	2	25	5.5	66.02	62.98	3.04
11	7	2	4	8	62.346	62.02	0.326
7	8	1	14.5	8	40.92	40.15	0.77
17	9	2	14.5	6.75	52.36	51.72	0.64
12	10	2	25	8	56.224	56.63	-0.406
13	11	2	14.5	6.75	55.2	53.91	1.29
4	12	3	25	6.75	40.704	40.53	0.174
14	13	2	14.5	6.75	58.673	54.88	3.79
6	14	3	14.5	5.5	61.938	50.88	11.05
5	15	1	14.5	5.5	46.02	45.24	0.78
16	16	2	14.5	6.75	54.77	54.61	0.16
8	17	3	14.5	8	45	44.08	0.92

compared to the pure error, as evidenced by an F-value of 1.08 (Table 2). There is a 45.18% likelihood that this misfitting F-value may occur due to random noise.

The response surfaces depicting the influence of maceration time and extraction temperature on the protein level (Figure 1 – A) displayed a convex shape, indicating appropriate selection of variable ranges. The highest protein concentration was achieved at a maceration time of approximately 2 to 2.5 hours and a temperature of 25°C, with a fixed pH of 6.75.

Similarly, the response surface showing the variation of protein concentration with maceration time and pH (Figure 1 – B) exhibited a wavy form, signifying the significant impact of these two factors on the protein level. The maximum level of 60 mg/mL was observed at pH levels close to 6.5 – 7 and a maceration time between 2 to 2.5 hours. Furthermore, the pH-temperature variation was studied, resulting in a response surface with a more or less concave form, and maximum protein concentrations were observed at pH levels around 6.5 and 7, along with peak temperatures at 20°C and 25°C (Figure 1 – C).

Based on these findings, it is feasible to obtain an optimal protein level of 46.57 mg/mL using the extraction process employed in this study, with the following parameters: extraction pH of 6.22, maceration time of approximately 1 hour and 42 minutes, and a temperature of 24.53°C. This conclusion is supported by the minor difference between the experimental protein concentration obtained under optimal conditions (45.82 mg/mL) and the predicted value (46.57 mg/mL). The literature contains limited studies specifically addressing garlic proteins in bibliography, and with most focusing were about other compounds as Allicin, Alliin, phenolic and flavonoids bioactive molecules. The optimized maceration was followed, by salting-out using ammonium sulfate, as an essential step to isolate the protein fraction. Wang et al. (2011) performed an extraction of Alliinase (54KDa garlic enzyme), using ammonium sulfate precipitation, an essential step to ensure a selective fractionation of bioactive proteins, Although, when this investigation was compared with others, it was observed that the same parameters had been selected by Lee et al. (2016) during the optimization of extraction of the sulfur-amino acid Cycloalliin from garlic bulbs, using Principle Components Analysis (PCA). It was also revealed that time, pH and temperature, were studied as factors during the extraction of protein fraction from sunflower meal produced in Bulgaria (Ivanova et al., 2012). Regarding the optimization procedure, a significant increase in protein level was observed (46.57 mg/mL) in comparison of previous studies: 7.87% (Aouadi et al., 2000).

In their study, Loghmanifar et al. (2022) extracted allicin from a garlic preparation in water using a sonication-assisted extraction method. Using Design Expert Software to optimize

Table 2. Analysis of variance (ANOVA) for the experimental results obtained by the total protein extraction

Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	1538.55	9	170.95	19.00	0.0004
X_1- maceration time	207.11	1	207.11	23.02	0.0020
X_2-temperature	0.6102	1	0.6102	0.0678	0.8020
X_3-pH	113.10	1	113.10	12.57	0.0094
$X_1 X_2$	0.1943	1	0.1943	0.0216	0.8873
$X_1 X_3$	36.73	1	36.73	4.08	0.0830
$X_2 X_3$	38.66	1	38.66	4.30	0.0769
X_1^2	994.13	1	994.13	110.51	< 0.0001
X_2^2	40.62	1	40.62	4.52	0.0712
X_3^2	340.54	1	340.54	37.85	0.0005
Residual	62.97	7	9.00	-	-
Lack of Fit	28.22	3	9.41	1.08	0.4518
Pure Error	34.76	4	8.69	-	-
Cor Total	1601.52	16	-	-	-

the parameters, they found that the optimal extraction time was 10 minutes and the extraction temperature was 30°C.

Garlic extract is widely recognized for its antimicrobial properties (Hasan et al., 2012), and is considered as medicinal remedy, according to Ezeorba et al. (2023) . Garlic contains numerous proteins with diverse biological activities, including immunomodulatory, anticancer, antimicrobial and antioxidant effects.

In the present study, the protein extract (37.45 mg/mL) exhibited significant antibacterial activity against the two bacterial strains tested, resulting in a significant inhibition zone of 20 mm for both strains. This observation underscores the potent inhibitory effect of the garlic protein fraction on these pathogens. However, no inhibitory effect was noticed around the deposition point of Try solution. This can be attributed to the sensitivity of the active proteins to trypsin, which likely inactivated them at this site (Figure 2). This result offers insights into the structural composition of the active sites within the extracted proteins, particularly the presence of basic amino acids, as evident from Trypsin's hydrolysis at this level (Nabavi et al., 2013).

Interestingly, a notable 20 mm inhibition zone around the well containing the P solution exclusively in the *Enterococcus faecalis* culture, as shown in Figure 2 – A. This observation can be attributed to the resistance of active proteins to the enzymatic action of pepsin, which cleaves neutral amino acids on the left side of peptide bonds (Ghasemi, 2007). Conversely, in the *Staphylococcus aureus* culture, as displayed in Figure 2 – B, no inhibition zone was detected around the well containing the P solution. This lack of antibacterial activity is likely due to pepsin's hydrolytic action, which exhibits high proteolytic activity on the proteins active against this strain, thereby confirming the presence of neutral amino acids in these proteins (Cui et al., 2013).

Identical antimicrobial activity was reported by Cahayani et al. (2019) using an aqueous extract of garlic cloves, against *Staphylococcus aureus* and *Corynebacterium Diphteriae* strains, employing the Kirby-Bauer disk diffusion test. Similarly, Barbu et al. (2023) demonstrated the antimicrobial effect of garlic extract, against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Candida parapsilosis,* using the same technique, with inhibition zones ranging from 20 to 30 mm, consistent with the results of the current study. On the other hand, previous studies have identified a 13 KDa antimicrobial protein in garlic cloves, named Alliumine, which possesses antibacterial, antifungal and antiproliferative properties. Using a comparable method to the present study, chymotrypsin and trypsin were utilized to confirm the studied activities, as proteolytic enzymes responsible on the disappearance of inhibition zones (Xia & Ng, 2005).

To complete the antibacterial assessment, the results point out that the MIC values of the peptide fraction were determined to be 9.36 ± 0.11 mg/mL and 18.72 ± 0.2 mg/mL, against *Staphylococcus aureus* (FRI137) and *Enterococcus faecalis* (ATCC49452), respectively. Those of MBC values were found to be 37.45 ± 0.13 mg/mL and 18.72 ± 1.08 mg/mL. In the context of biological activities, particularly antibacterial properties, most studies have focused on using allicin and secondary metabolites derived from garlic. This study, however employed a similar procedure to evaluate the antibacterial activity of the peptide fraction.

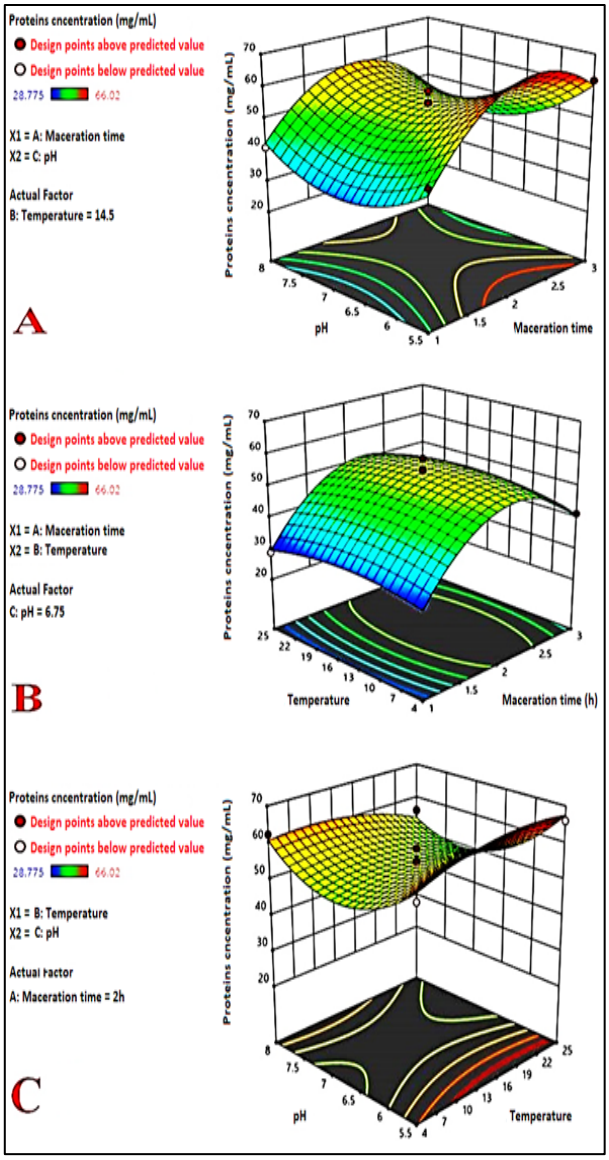

Figure 1. Response surfaces (RS) indicating interaction between selected factors with a protein concentration. **A:** The RS of the interaction between maceration time and extraction temperature; **B:** The RS of interaction between maceration time and pH; **C:** The RS of the interaction between pH and temperatures.

Liaqat et al. (2019) employed the broth dilution method to determine the MIC in their antibacterial tests against foodborne pathogens. Their results similarly demonstrated a stronger antibacterial effect against *Staphylococcus aureus* strains, reflected in lower MIC and MBC values, compared to *Enterococcus faecalis* strains, which exhibited higher MIC and MBC values. Further investigation, into garlic proteins, has identified that Garlic-Lectin-Derived-Peptides (GLDPs) with MW of 12 KDa, as high bactericidal substance against *Staphylococcus aureus* (Li et al., 2024). Previously, Ratnakar et al. (1996) confirmed the antitubercular property (antibacterial effect against the two strains *Mycobacterium tuberculosis* $H_{37}Rv$ and *Mycobacterium tuberculosis* TRC-C1193) of two garlic proteins (43 et 38 KDa respectively) isolated from garlic aqueous extract, with MIC values ranging from 20 to 40 µg/mL and 30 to 60 µg/mL, respectively.

The anti-hemolytic activity was evaluated across various concentrations of the protein extract, resulting in a percentage change in hemolysis inhibition ranging from 23.58 ± 0.02% to 94.24 ± 0.04% (Figure 3 – A). At lower extract concentrations, the percentage of hemolysis inhibition was notably high: 85.33 ± 0.03% - 88.83 ± 0.02% and 94.24 ± 0.02% for extract concentrations of 1 – 0.5 and 0.25 mg/mL, respectively. This activity closely paralleled that of the standard ascorbic acid, which exhibited a hemolysis inhibition range of 84.36 ± 0.03% to 94.65 ± 0.02% (Figure 3 – B). Conversely, at higher extract concentrations (24 – 12 – 6 mg/mL), the percentages of hemolysis inhibition recorded were lower, with 23.58 ± 0.01%, 27.95 ± 0.03% and 55.34 ± 0.05%%, respectively.

The action of H_2O_2 on erythrocytes is known to induce membrane protein depletion and deformation of cell membranes (Abdellaoui, 2007). Yasmeen et al. (2016) reported similar findings, noting that H_2O_2 exposure leads to lipid membrane peroxidation and increased passive permeability to cations (K+) due to the oxidation of free SH sulfhydryl groups, particularly those containing numerous cysteine residues in membrane proteins. The interaction between the proteins of the extract and those of erythrocytes, which play crucial biological roles in controlling cell morphology, transfer mechanisms, and enzymatic activities, likely contributed to the protection against hemolysis. This interaction may involve membrane amino acids possessing SH groups, preventing their oxidation and inhibiting the passive loss of K+ cations. This, in turn, helps maintain membrane integrity and structure stability.

The protection of erythrocytes in the presence of the proteins from the extract is plausibly mediated indirectly through their ability to compete with membrane proteins in trapping radicals released by H_2O_2. This radical-scavenging action could be instrumental in protecting erythrocytes against hemolysis as radical agents target specific amino acids such as histidine, proline, tryptophan, cysteine, and tyrosine. These interactions lead to oxidation of residues, carbonyl group formation, peptide chain cleavage, and the creation of intra- and inter-chain bi-tyrosine bridges. Such damages are often irreversible and can cause significant functional impairments, with some oxidized proteins form aggregates (Pla, 2000).

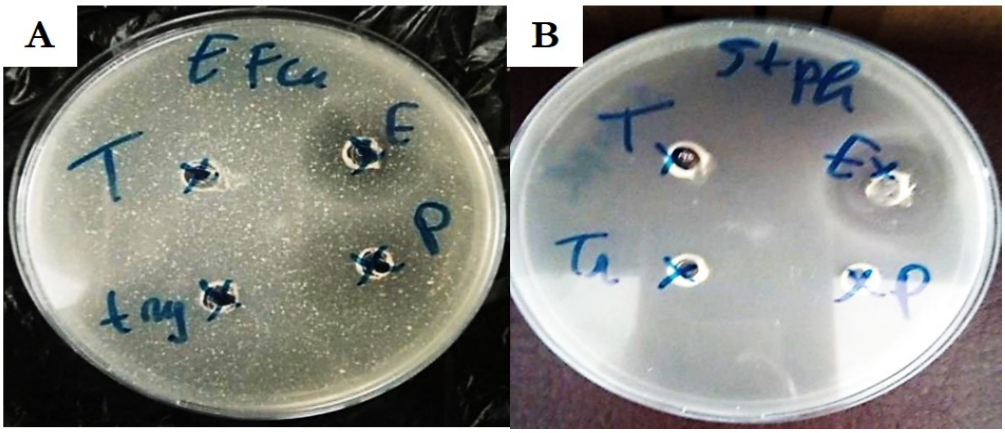

Figure 2. Antibacterial activity result against the two target strains: *Staphylococcus aureus* (Stph) (A) and *Enterococcus faecalis* (EFca) (B) after the well diffusion test

T: Control; **E:** Garlic protein extract; **P:** garlic protein extract added by Pepsin; **Try:** garlic protein extract added by Trypsin.

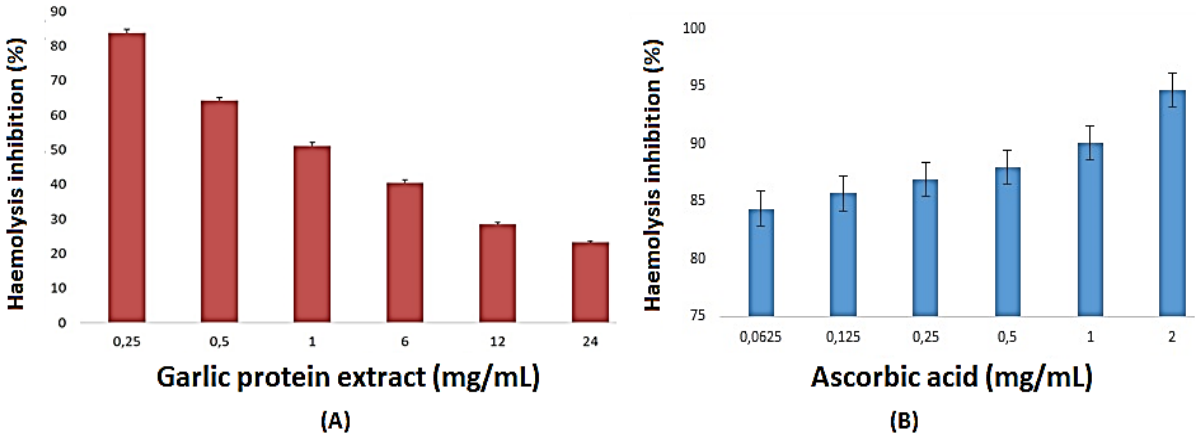

Figure 3. Anti-hemolytic activity of garlic protein extract **(A)** and that of ascorbic acid standard **(B)**

The presence of peroxidase in garlic, as suggested by Osuji et al. (2014), may contribute to the protective effect on erythrocytes. Garlic contains an isoenzyme peroxidase POX with two designated isoforms: POX (1A) and POX (1B). The latter exhibiting high activity at 50°C and 40°C for 40 minutes, with an optimal pH of approximately 5. Based on the findings of the study, the applied parameters during the extraction of garlic proteins (optimal conditions) – maceration time of 1 hour, pH of 6, and temperature of 24.23°C – were comparable to those of the enzyme, thus preserving its activity to reduce H_2O_2 in the reaction medium and protect red blood cells from hemolysis. Furthermore, the inhibition of hemolysis might also be attributed to hydrophobic interactions between the proteins in the extract and erythrocyte membrane lipids, particularly with polyunsaturated fatty acids, shielding them from destruction and peroxidation (Naim et al., 1976).

At higher concentrations of the extract, the anti-hemolytic activity demonstrated an inverse relationship, likely due to the presence of protease-like enzymes in the garlic protein extract. At concentrations of 24, 12, and 6 mg/mL, these enzymes exhibited remarkable activity. However, as the extract was diluted, the enzymatic activity decreased, leading to a progressive increase in hemolysis inhibition. Parisi et al. (2002) identified the presence of cysteine proteases in garlic, which are proteolytic enzymes exhibiting activities even at moderately high temperatures (40°C and 50°C) and for a duration of up to 60 minutes. Azantsa et al. (2019) have concluded that the garlic aqueous extract possesses considerable antioxidant activity in addition of hemolysis inhibition percentage of 87.7% at a concentration of 1mg/mL. In another study, the hydroethanolic extract of the *Asphodelus fistulosis* roots provided potential antioxidant properties and erythrocytes membranes protection with 0,8 mg/mL of concentration (Kitouni et al., 2024). Additionally,

the therapeutic potential of the garlic protein fraction is well-documented. For instance, Hasan et al., (2012) demonstrated the immunomodulatory effects of a 47 kDa garlic protein, which promoted the activation of dendritic cells (*in vitro*), highlighting its relevance in therapeutic applications.

3 Conclusion

In summary, the garlic protein extract displayed two remarkable biological activities: antibacterial and anti-hemolytic. These properties offer the therapeutic potential of garlic and validate its traditional and modern applications in food and medicinal preparations. The optimization process provided a satisfactory protein yield under the conditions of pH 6.22, maceration time of 102 minutes, and a temperature of 24.53°C. Furthermore, contributing to the discovery of intriguing biological activities, and the obtained model's equation demonstrated the importance of the studied factors during the extraction process.

Future research could focus on purifying the bioactive components, leading to the development of natural antibiotics that could be used independently or in conjunction with other conventional molecules. On the other hand, the anti-hemolytic compounds identified in this study hold promise as potent antioxidant agents

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Authors' Contribution: **Achour Chergui:** Conceptualization, Methodology, Supervision and Validation. **Soraya Nezla:** Investigation, Writing and Resources. **Yacine Titouche:** Isolation and Identification of the Reference Target Bacterial Strains. **Ali Imessaoudene:** Software and Validation of Statistical Analysis. **Amina Hadadi:** Visualization and improve of the redaction. **Khalef Lefsih:** Conceptualization, Writing, Review and Editing.

Conflicts of Interest: There are not conflicts and finding.

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