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Antibacterial Potential of Protein Solution Separated from Bovine Serum by SDS Treatment

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study explores the separation of antibacterial proteins from bovine serum using a simple and novel method developed. The salt out protein precipitate was prepared from the acid extract of the bovine serum using 30% ammonium sulphate solution. One volume of the precipitate is treated with two volumes of each concentration of SDS solution (1 – 10%) separately in refrigerator temperature. After a week of treatment, two layers are formed. The lower layer is frozen SDS where the upper one is unfrozen protein solution. The upper layer formed by each percentage of SDS was carefully collected and its protein content is also measured. The maximum protein content (90%) has been recovered in the liquid layer formed by 3 % SDS. The λ-max of pooled test protein

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solution is 276 nm. The Minimum Inhibitory Concentration (MIC) of this protein was checked against seven human pathogenic bacteria and it is ranged from 4 to 1µg. In contrast, there is no heamolysis even at the higher concentration (33 µg) of the test solution. A single mild and faint band with approximate Molecular Weight of 45 kDa is observed in SDS-PAGE. This method of antibacterial protein preparation from cattle serum could lead a new insight in pharmaceutical industries for the development of protein based natural antibiotics.

Keywords: Antibacterial protein; bovine serum; SDS; λ-max; MIC.

1. INTRODUCTION

Studies have demonstrated the rapid development of Multi- Drug Resistant (MDR) bacterial strains which is a major global challenge in treating infectious diseases [1]. Several compounds from natural sources alternative to classical antibiotics combat the adverse effects [2,3]. Nevertheless, it is tiresome to identify and isolate the specific active compounds since they are highly complexed nature. The protein antibiotics specially termed as antimicrobial proteins or peptides (AMPs) found in all forms of life play a crucial role in many physiological processes including immune response. These natural AMPs are cationic molecules consisting less than 100 amino acid residues with amphipathic properties [4]. They are potential to be used as suitable alternatives to overcome bacterial resistance by their direct killing capacity using diverse mechanisms with the basic principle of ionic interactions [5]. Hence, they have been paid more attention for the past few decades [6] as AMPs are supposed to be the next generation of antibiotics. However, we argue that the reported literatures have certain limitations for their cost effective large scale preparation. It remains only as reverie because of lack of knowledge on their bulk availability in natural sources.

In this line, to our knowledge, for the first time, we have substantiated the presence of antibacterial proteins (ABPs) in the heart tissue of goat and chicken which are easily available from the slaughter houses [7-9]. Subsequently we have precipitated crude ABPs from acid extract of kidney, liver, heart and gills of common carps, *Labeo rohita* and *Catla catla*. It has strong bactericidal activities against many clinical bacteria at the lowest concentration of 16 µg [10,11]. Significant quantities of ABP precipitates were also prepared from the heart tissues of slaughter house animals (chicken, goat and cow) [12]. We have also reported the antibacterial effects of 10% TCA precipitate prepared from acid extract of a wild mushroom *Ganoderma lucidum* at very lowest volume against human pathogenic bacteria with less hemolytic activities [13]. In recent past, the bactericidal potential of salt out protein precipitate prepared from bovine heart tissue extract against clinical isolates were studied [14].

Further, there is scarcity of information on AMPs in cattle serum. Hence, a preliminary study is made for identification and quantification of ABPs in bovine serum as it is cheaply available in slaughter houses. In addition, it was intended to develop a simple and novel method to dissolve the salting out protein precipitate using SDS and to purify the bioactive proteins to a possible extent. Our study might be crucial for developing cost effective method for the preparation of protein based natural antibiotics.

2. MATERIALS AND METHODS

The principle of this experiment is to purify the antibacterial protein solution from salt out precipitate acquired from acid extract of the bovine serum.

2.1 Blood Collection and Serum Preparation

The blood samples were collected from jugular veins of three healthy individual bovines in a local veterinary hospital, Thanjavur, India after getting consents from the owners of the animals. The samples were allowed to clot for an hour and the clear fluid was transferred in to a clean and sterile container. Then it was centrifuged at 3000 rpm to obtain clear serum in the laboratory and the as prepared serum was stored in refrigerator for further experiments.

2.2 Preparation of Acid Extract

To 5ml of serum sample, an equal volume of 10% acetic acid was mixed and allowed to stand for an hour. The mixture was boiled at 100°C for about 10 minutes and centrifuged at 17000 rpm for 10 minutes. The filtrate was collected in a

separate sterile vial and stored in refrigerator until use [15].

2.3 Salting Out Protein Precipitation

The acid extract was mixed with equal volume of 30% ammonium sulphate solution and allowed to stand at room temperature for about 30 minutes. After precipitation it was centrifuged at 6000 rpm for 10 minutes and the supernatant fluid was discarded. The precipitate was dissolved with 10 ml of sterile water and stored in refrigerator for further analysis [14].

2.4 SDS Separation of ABPs from Salt Out Precipitate

A trial was made by our own hypothetical procedure to dissolve the as prepared salt out protein precipitates and to purify them using SDS by adopting the freezing and anti-freezing nature of SDS and certain proteins respectively at 2-8^{0}C. Ten serial concentrations of SDS (1 to 10%) were prepared. One volume of protein precipitate and two volumes of each percentage of SDS (1:2) were mixed individually. The mixtures were agitated well in a rotary shaker for 30 minutes for easy disassociation of proteins from the precipitate. Then the mixtures were kept in refrigerator (2-8^{0}C) for a week to form two layers; a bottom frozen layer (SDS with some proteins) and an upper liquid layer (detergent free proteins). The protein liquid layer was carefully transferred as quick as possible in to sterile vials before the frozen SDS layer is getting melted and its protein concentration was measured in each. Based on the recovered protein contents of the liquid layers separated from each percentage of SDS solution, the optimum SDS percentage which gives highest protein recovery was identified.

2.5 Protein Estimation

The proteins concentration of ammonium sulphate precipitates and the protein solution purified from the precipitate by SDS treatment were estimated adopting the procedure of Lowry et al. [16] using BSA as standard. Then these three protein solutions were pooled as test protein solution for further analysis.

2.6 Determination of Absorption Spectra

The wave length at which the test protein solution had maximum absorption (Absorption peak pick- λ max) was determined by scanning the wave lengths of 200 – 800 nm using Double Beam UV-Vis Spectrophotometer (300 – Cary) following the instructions of the manual.

2.7 Antibacterial Assay (MIC)

Seven pure bacterial cultures; *Escherchia coli, Proteus mirabilis, Staphylococcus aureus, Klebsiella pneumonia, Salmonella typhi, Salmonella paratyphi 'A'* and *Pseudomonas aeruginosa* kindly supplied by Microbiology laboratory, KAPV Medical College, Tiruchirappalli, India were sub cultured and confirmed by routine biochemical tests.

The minimum inhibitory concentrations (MICs) of salt out precipitates and SDS purified test protein solution were determined in triplicate against the organisms by microdilution method using resazurin as an indicator of cell growth as described by Sarker et al. [17]. The turbidity of test organisms was adjusted to obtain 5×10^5 CFU/ml approximately by comparing the turbidity to 0.5 Mac Farland standards. Then, they were diluted in to 200 times by 1% peptone water. A sterile 96 micro well plates were prepared under aseptic conditions and was labelled as per the protocol designed. A volume of 200µl of test protein was pipette out into the first column of the plate after estimating its amount. To all other wells, 100µl 1% peptone water was added. A serial dilution was performed and ensured that each well has 100µl of protein solution in serially descending concentration. Then 10µl of bacterial suspension was added to each well to achieve a concentration of 5×10^6 CFU/ml. Finally, to each well, 10 µl of resazurin indicator solution (270 mg resazurin diluted in 40 ml of distilled water) was added. The plates were well covered and incubated for 24 hours at 37ºC. The colour change from purple to pink or colourless and retaining of purple colour inferred the bacterial growth and their inhibition respectively.

2.8 Minimum Hemolytic Concentration (MHC)

To check the cytotoxicity of the test protein solution its MHC (Minimum Hemolytic Concentration) was carried out in micro well plates according to Zhu et al. [18] with little modification in triplicate. The human blood sample was gifted by a clinical laboratory in Tanjore, Tamilnadu, India. The red blood cells were sedimented and repeatedly washed with 0.9% Nacl. The cells were made in to 4% suspension with normal saline. All the test wells of three rows (for triplicate) were filled with 100µl

of normal saline. The first wells of each row were filled with 100µl of the test protein solution and serially diluted to ensure that each first well had 50µl of the protein solution and 50% of the descending volumes serially (33 to 1 µg). Then 100 µl of 4% hRBCs was added in all wells of each row. The erythrocytes of the same concentration alone and 10% SDS with the erythrocytes were used as 0% and 100% hemolytic controls respectively. After 2 hours of incubation the formation of brown colour was recorded as occurrence of haemolysis where the retention of original red colour as absence of haemolysis.

2.9 SDS-PAGE Analysis

20 µl of test protein solution was subjected to SDS-PAGE on 12% mini gel according to the standard protocol [19] without heating the sample. The electrophoresis was run along with mid -range marker proteins. The gel was fixed and the bands were visualized after coomassie blue staining using UV lamp. Finaly the approximate molecular weight of the test protein was determined manually.

3. RESULTS

From the results, it is evident that the bovine serum is a rich source of AMPs. Crude low molecular weight proteins were prepared from three samples of bovine serum separately by acid extraction followed by salt out precipitation. The levels of so prepared proteins are 6.6, 6.7 and 7.1 mg / ml of serum; A, B & C respectively with mean value of 6.8 mg / ml (Table.1).

Table 1. Total amount of salt out proteins and SDS separated proteins

Serum sample	Salt out protein (µg/ml serum)	% of SDS treated	Amount of separated proteins/ml of serum (µg) (After SDS Treatment)	Percentage of separated protein concentrations (After SDS Treatment)
A	6563	1	345	5%
	(6.6 mg)	2	5160	79%
		3	5169	79%
		4	4266	65%
		5	2827	43%
		6	2100	32%
		7	1758	27%
		8	1055	16%
		9	853	13%
		10	438	7%
B	6715	1	104	2%
	(6.7 mg)	2	5643	84%
		3	6498	97%
		4	4098	61%
		5	4230	63%
		6	3013	45%
		7	2005	30%
		8	1052	16%
		9	940	14%
		10	365	5%
C	7142	1	619	9%
	(7.1 mg)	2	6234	87%
		3	6711	94%
		4	3866	54%
		5	3571	50%
		6	3178	44%
		7	2880	40%
		8	2240	31%
		9	1050	15%
		10	510	7%
Mean Value	(6.8 mg/ml)			90% (by 3% SDS Treatment)

3.1 SDS Treatment of Salt out Precipitate and Recovery of ABPs

The separation of antibacterial proteins (ABPs) from the salt out precipitates was endeavoured by SDS treatment based on the hypothesis of that two different temperatures required for freezing of SDS and proteins. Two volumes of each concentration of SDS solution (1 – 10%) were mixed with one volume of the precipitate separately and kept in refrigerator for few days after shaking well. Two layers; the lower frozen (SDS) and the upper unfrozen (protein) layers have been formed (Fig. 1). The upper layer formed by each percentage of SDS was carefully collected and the recovered protein content was calculated. Table 1 represents the total amounts of salt out protein precipitates; 6563, 6715 and 7142 µg/ml of respective serum samples (A, B & C). The highest percentages of proteins recovered after SDS treatment from these samples are 79%, 97% and 94% respectively. Table 1 transparently reveals that 3% SDS favours the separation of maximum amount of proteins from salt out precipitates. The mean value of maximum recovered proteins is 90% as shown in the Table 1.

3.2 Absorption Spectra

The pooled protein solution purified by 3% SDS was checked for its purity by scanning its absorption spectra (λ-max). The maximum OD value, 0.198 was observed in 276 nm. It is noted that the value is very closer to 280 nm which is typical for wide varieties of proteins (Fig. 2).

Table 2. Comparison of MIC of salt out protein precipitate and SDS purified protein solution of bovine serum

Organisms	MIC (µg)	
	Salt out protein precipitate	SDS purified protein
E.coli	8	4
P. mirabilis	4	1
S. areus	4	1
K. pneumonia	16	4
S.typhi	8	2
S. Paratyphi A	4	1
P. aeruginosa	4	1

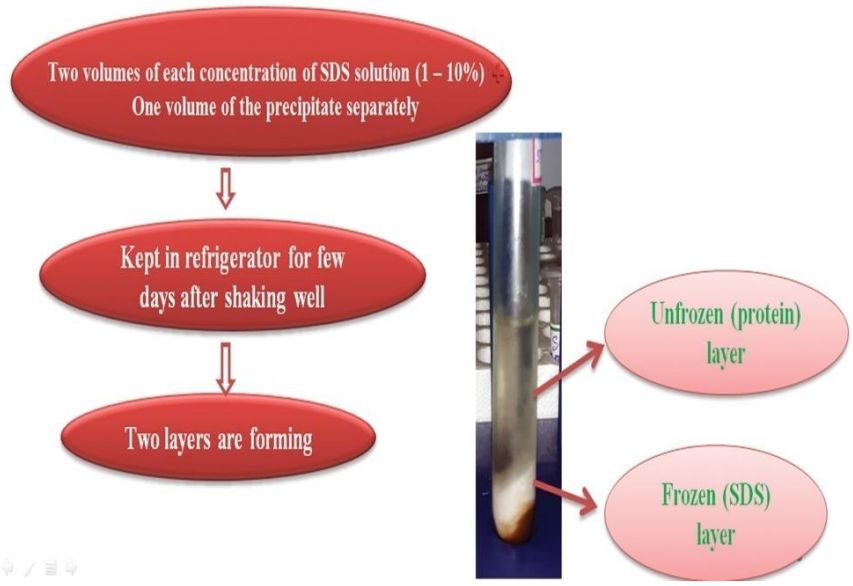


Fig. 1. Formation of SDS and protein layers after SDS treatment

3.3 Antibacterial and Hemolytic Activity

The MICs of both salt out precipitate and SDS purified proteins were performed against seven clinical isolates as mentioned in the methodology. Fig. 3 reveals the antibacterial efficacy of the test protein solution against these bacterial strains. The precipitate has inhibited the growth of all test organisms effectively. The growth of four organisms; *P. mirabilis*, *S. aereus, S.paratyphi A* and *P. aeroginosa* has been subdued at 4µg whereas the growth inhibition of others found in between the concentrations of 8 and 16 µg (Fig. 3). Similarly, the MICs of SDS purified protein was also checked for the same organisms and results showed the same trend as that of the crude precipitate (Fig. 3). However, it exhibited more efficient activities on the organisms to compare the precipitate i.e. the MIC values are significantly lower than that of the precipitates (Table 2). As shown in the Fig. 4, there is no hemolysis even at the higher concentration (33 µg) of the test protein subjected.

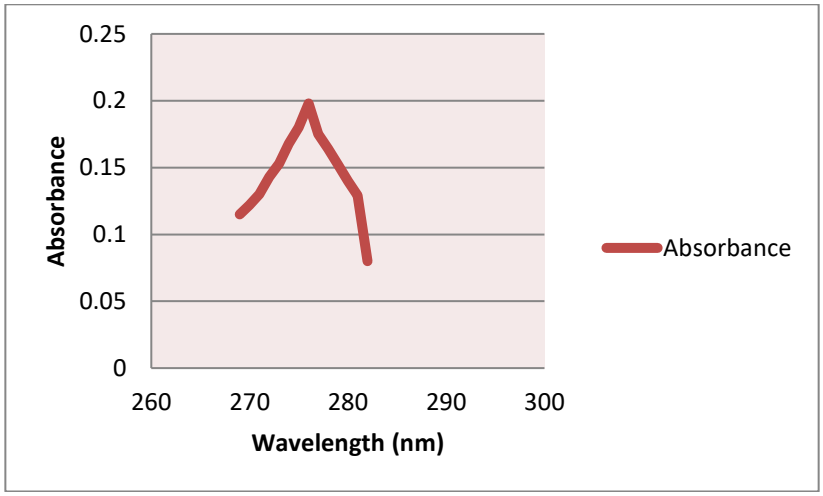
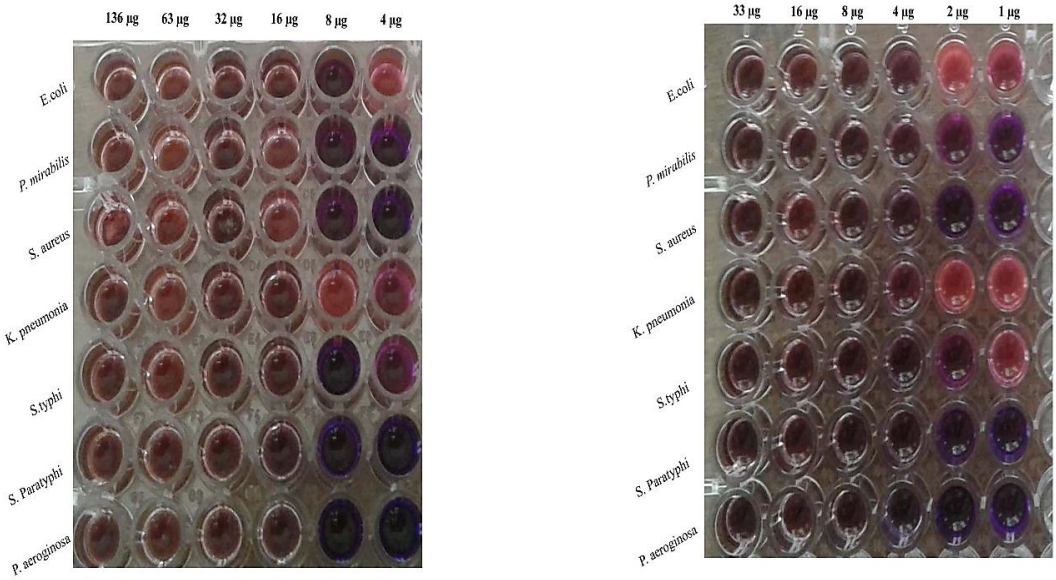


Fig. 2. Absorption spectra of the SDS separated protein solution from salt out precipitates



Salt out precipitate solution **SDS separated protein solution**

Fig. 3. Minimum inhibitory concentrations of salt out protein precipitates and SDS Separated proteins of bovine serum against human pathogenic bacteria

Interpretation:

Pink colour - Bacterial Growth

Blue/ Brown colour - Inhibition of Bacterial Growth

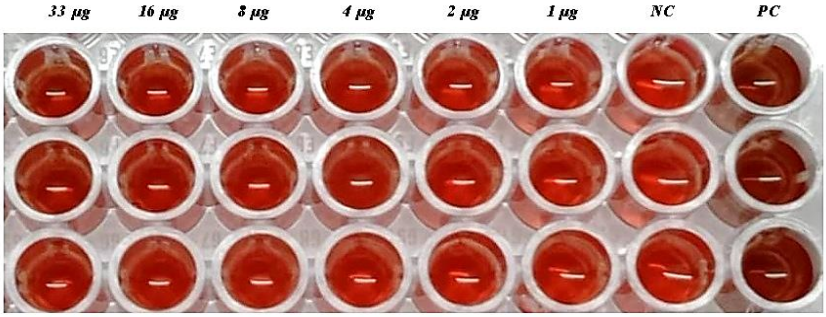


Fig. 4. Minimum Hemolytic Concentration (MHC) of SDS separated protein of bovine Serum

Interpretation:
Red Color - Absence of Haemolysis
Brown Color - Haemolysis
Controls:
NC - Negative Control (0% Haemolysis)
PC - Positive Control (100% Haemolysis)

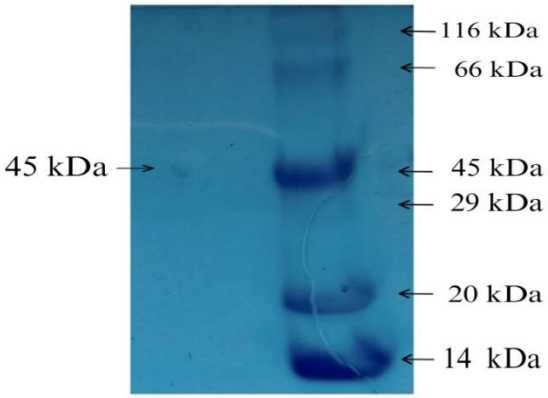


Fig. 5. SDS-PAGE analysis of the SDS separated protein of bovine serum

3.4 SDS PAGE Analysis

The SDS PAGE analysis of the SDS separated test protein solution has produced a single mild and faint band with approximate Molecular Weight of 45 kDa as seen in the Fig. 5.

4. DISCUSSION

It is interesting to observe that when the mixture of salt out protein precipitate and SDS solution (1-10%) in the ratio of 1:2 kept in the refrigerating temperature for a week after agitating well, the SDS freezes with certain proteins as a bottom layer whereas the SDS free proteins remain as upper layer without freezing. The anti-freezing nature of this protein fraction is appreciable. This temperature difference required for freezing SDS and certain proteins favours the purification of such anti freezing proteins by SDS treatment without any high end techniques. In this protocol the 3% SDS solution is apt to give maximum recovery of such proteins among others (Table 1). The absorption spectra (λ-max - 276 nm) observed to the as purified protein solution is very closer to 280 nm which is the standard λ-max for maximum numbers of proteins and which claims its purity (Fig. 2).

Both the salt out precipitate and the SDS purified protein solution have antibacterial efficacy at microgram levels as shown in the Table 2. However, the SDS purified protein solution has greater bioactive efficacy against human pathogenic bacteria to compare the salt out precipitate even at 1µg of MIC for four test organisms out of seven (Table 2). Hence this protein might have antimicrobial domain. Further, the anti-freezing nature of this protein is lining with a previous report [15]. Moreover, such SDS

freezing dependent protocols for AMP purification is not available to our knowledge best and hence this method could be claimed as simple, cost effective and novel one. Because, in general, AMP purification involves high end expensive techniques such as dialysis, ion exchange, gel filtration and high performance liquid chromotographies etc.,

Further our protocol is expected to give maximum purity of bioactive proteins since the acid extraction step rules out maximum number of bio molecules leaving only acid as well as heat resistant low molecular weight proteins. The 30% Ammonium Sulphate solution was used to salt out the antibacterial proteins (ABPs) from the acid extract following our previous protocol [13,14]. Hence, 30% Ammonium Sulphate solution was used to fractionate the low molecular weight ABPs in this work. Further the protein separation has been achieved using the freezing nature of SDS along with some protein residues in 2-8^{o}C whereas the ABPs do not get freeze.

Obviously the mammalian serum consists albumin and globulins as prime proteins where the globulins play a dynamic role in providing specific immunity as immunoglobulins. Antimicrobial proteins have also been reported for being a part of innate immune system [20]. The protein fraction separated in this study exhibits a potential antibacterial effects on clinical bacteria. Hence, these proteins could also be suggested as acid digested products of serum immunoglobulins or albumins. The recent reports of Jin et al. [21] is also lining with this. They have investigated the antibacterial activities of hydrolysates of porcine blood globulins and albumins. Another reason for the presence of AMPs in serum may be due to a constant secretion, storage and release of AMPs from the blood cells to the plasma for instant elimination of microbial invasion [20].

Though antimicrobial peptides are potential to act against wide range of pathogens, their pharmaceutical applications may confer negative impacts on host cells due to their cytotoxicity. For instance, Fowlicidins (-1 and -2) of chicken displayed potent salt- independent activities against a wide range of bacteria including antibiotic resistant strains. Bur their haemolytic effects do not allow them to use as drug candidates [22]. In contrast, as shown in the Fig.4 there is no heamolysis even at the higher concentration (33 µg) of the test protein separated. Simultaneously it potentially kills the test bacteria even at 1 µg concentration and which is the merit of this protein. The same trend has also been reported in our previous studies [7-14]. Hence the test protein is safe to use as natural antibiotics. In addition, certain serum proteins like albumin inhibit the AMP activities [23] which could also be verified and alleviated.

SDS-PAGE analysis of the protein solution shows a single and faint band with approximate molecular weight of 45 kDa (Fig. 5). The single band formation of this claims that the method used for this study may be specific and more accurate for isolating these kinds of protein based antibiotics. In addition, the molecular weight of the test protein falls in the AMP range (10 – 100 kDa) [8,9]. We have also isolated a 16 and 13 kDa peptides from goat and chicken heart tissues respectively. In chorus, this study reveals that the cattle serum could be considered as a potential source of antimicrobial proteins for their pharmaceutical preparation using our simple, cost effective protocol with safe mode.

5. CONCLUSION

The present communication reveals that the cattle serum could be considered as a potential source of protein based natural antibiotics. Further such protein solution could be pharmaceutically prepared using our simple, cost effective protocol with safe mode. In addition, cattle farms can be set and maintained by pharmaceutical industries for continuous collection of blood without killing the animals. However further deep studies in this regard could be a lead to identify and isolate the exact active proteins for successful pharmaceutical applications.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

CONSENT

As per international standards or university standards, written consent was taken from the owners of the animals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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