Camel Milk Casein Hydrolysates and its Fractions Having Antioxidative and Antibacterial Properties Against Selected Bacteria

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ABSTRACT:

This study was designed to demonstrate bioactive peptide production from camel milk casein (CMC) and to assess their antioxidative and antibacterial capacities. CMC was subjected to enzymatic hydrolysis employing pepsin (P), trypsin (T) and pepsin-trypsin combination (PT). The resultant peptides formed by peptic, tryptic and peptic+ tryptic hydrolysates of camel milk casein (P-CMC) (T-CMC) (PT-CMC) respectively, were confirmed by the SDS-PAGE and further analyzed for their antioxidant and antibacterial activity against Gram +ve bacterial strains (Staphylococcus aureus, Bacillus subtilis) and Gram -ve bacterial strains (Escherichia coli, Salmonella enteritidis). In contrast to T-CMC and PT-CMC, the EC_{50} value of P-CMC hydrolysate was found significantly closest to that of standard ascorbic acid. Moreover, the P-CMC hydrolysate revealed substantially higher antimicrobial activity as compared to T-CMC and PT-CMC hydrolysates. On this basis, peptides of the selected P-CMC hydrolysate were purified into fractions (F1-F5) by HPLC for further authentication. All the fractions, except F4, also confirmed its significant antibacterial efficacy against the tested bacteria. Hence, it was concluded that P-CMC hydrolysates and its purified peptides have significant antioxidant potential and may be an effective approach in the production of potent antimicrobial peptides.

Keywords: antibacterial, antioxidant, bioactive peptides, camel milk casein

INTRODUCTION:

Camel milk is an efficient source of dietary proteins (casein and whey) that play a crucial role in disease prevention and contribute to overall well-being through their physicochemical attributes (Ayyash et al., 2020; Kaskous, 2017; Mirmiran et al., 2017; Yehia et al., 2020). In recent years, extensive in vitro research has been carried out on these milk proteins to identify, synthesize, and characterize their peptides having biological activity. However, these peptides need enzymatic hydrolysis for their release and activation (FitzGerald et al., 2004; Gobbetti et al., 2002). Previous research has proved that Camel milk casein (80% of total protein) is highly susceptible to proteolysis, leading to the production of a variety of bioactive peptides with diverse biological activities as compared to whey protein activities (Jrad et al., 2014). Moreover, casein derived peptides have been shown to affect the heart, brain, alimentary canal, and body's defense mechanism (Shahidi & Zhong, 2019; Silva & Malcata, 2005). Due to their anti-thrombotic, antioxidative, immunoregulatory, immunosuppressant, and anti-proliferative capacity and these peptides exert their effects by free radical scavenging activity (Korhonen & Pihlanto, 2006; Silva & Malcata, 2005).

There are multiple methods for the generation of biofunctional peptides of casein i.e. either through enzymatic hydrolysis or fermentation of milk with proteolytic prepared cultures or via microbial fermentation. However, the bioactive peptides are commonly produced through enzymatic hydrolysis of whole proteins. Synthesis of these peptides can be confirmed by Gel electrophoresis (SDS-PAGE) which is one of the simplest techniques for identification of low molecular weight peptides. These plant, microbial origin, or gastrointestinal tract (GIT) enzymes can be used singly (pepsin and trypsin) as well as in combinations (chymotrypsin, Pancreatin, trypsin, and thermolysin) (Shivanna & Nataraj, 2020).

The radical scavenging activities has been shown by various casein hydrolysates in previous studies (Chiu & Kitts, 2004; Díaz & Decker, 2004; Elias et al., 2008), particularly towards the superoxide anion, hydroxyl radical, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Although there are research studies available on fermented milk, whole milk, total protein hydrolysate or some specific protein such as lactoferrin (Al-Ayadhi & Elamin, 2013; Al-Shamsi et al., 2018; Ayyash et al., 2018; Habib et al., 2013; Zhu et al., 2016) of camel milk. However, less information is available on the antioxidant potential of camel milk casein-derived hydrolysates using GIT enzymes (pepsin, trypsin, and pepsin followed by trypsin). Consequently, there is a persistent need to assess these antioxidant peptides.

Furthermore, research has shown that peptides produced from the camel milk casein, inhibited the growth of selected species of gram-negative and positive bacteria (Muhialdin & Algboory, 2018; Niaz et al., 2019). Among these pathogens, S. aureus and Enterococcus species currently pose a severe pandemic threat and likewise, Gram-negative pathogens have become troublesome by creating a period similar to the pre-antibiotic era (Golkar et al., 2014; Rossolini et al., 2014). In such a time of antibiotic resistance crisis, milk-derived antimicrobial peptides are gaining attraction as a safe and effective alternative to antibiotics.

However, data are scarce regarding the assessment of antibacterial and antioxidant activities of the camel milk casein-derived hydrolysates and its fractions purified using reverse phase-high performance liquid chromatography (RP-HPLC) techniques to illustrate the actions of

GIT enzymes. Therefore, this research was intended to explore the efficacy of hydrolysates and purified fractions of camel milk casein in the reduction of oxidative stress as a naturally occurring alternative to antimicrobial agents in an era of antibiotic resistance crisis resulting from the overuse and misuse of these medications.

MATERIALS AND METHODS:

Chemical and reagents

Enzymes included pepsin (EC 3.4.23.1) and trypsin (EC: 3.4.21.4) were obtained from Sigma-Aldrich Chemical Co., India. The reagent, 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) was also purchased from Sigma-Aldrich Chemical Co., India. Four types of bacteria used: Staphylococcus aureus (ATCC 25923), Escherichia coli (ATCC 25922) Bacillus subtilis (ATCC 6051) Salmonella enteritidis (PT4 (P125109), were sourced from, Institute of Microbiology, University of Veterinary & Animal Sciences, Lahore, Pakistan. Rest of the chemicals were of analytical grade from renowned companies.

Physico-Chemical analysis of Camel milk

Fresh samples of camel milk were obtained from healthy Camels (*Camelus dromedarius*). The milk was collected in sterile glass bottles, and its pH was recorded at room temperature. Each milk sample underwent comprehensive analysis for various physicochemical properties, including color, taste, percentage of water, proteins, lipids, carbohydrates, and percent casein yield.

Camel milk casein powder preparation

The isolation of camel milk casein powder (CMCP) followed the method outlined by (Ibrahim et al., 2018), with slight alterations. Briefly, raw milk temperature was brought to 37°C and immediately defatted by centrifugation at 5000 ×g for 20 minutes at 4°C. The fat was separated by passing the supernatant through three layers of gauze. Subsequently, the skimmed milk's pH was adjusted to 4.6 by adding 10% acetic acid. Casein precipitation occurred, and the separation from whey protein was attained through centrifugation at 5000 ×g for 10 minutes at 4°C. The obtained casein (CN) precipitates underwent washing with distilled water to remove any residual whey. Casein confirmatory test was conducted to ensure its purity (Patni & Bosmia, 2015). The acquired Casein was freeze dried and stored at -20°C for subsequent analysis.

Preparation of Camel Milk Casein Hydrolysates (CMCHs)

The camel milk casein hydrolysates (CMCHs) were prepared by dissolving the CMCP (at 5% total solid) in phosphate buffer and pH was adjusted (2.0 for pepsin and 7.8 for trypsin). The protein solution underwent 3 treatments using proteolytic enzymes, T1 (pepsin), T2 (trypsin) and T3 (pepsin followed by trypsin) at 37^{o}C with a ratio of 1:10 (w/w). The peptic and tryptic samples were subjected to protein hydrolysis for 2 hours while in the case of peptic followed by tryptic hydrolysis, the substrate underwent initial pepsin hydrolysis for 2 hours, succeeded by trypsin hydrolysis for an additional 2 hours. The enzymes were inactivated in all three treatments, by heating the mixtures at 85°C for 15 minutes. The hydrolysates were centrifuged at 10,000 × g for 10 min at 4°C, after which supernatant was collected and filtered (Mudgil et al., 2020). Thereafter, all the samples were preserved at -20°C until further analysis.

Characterization of CMCHs by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The CMCHs were characterized by SDS-PAGE using the protocol established by Laemmli (1976) with slighter modifications described (Al-Shamsi et al., 2018), using a gel system consisting of a 15% resolving gel and a 4% stacking gel. The gel was subjected to an initial run at 80 V for 30 minutes, followed by a subsequent run at 120 V for 1.5 hours. The gel was stained using Coomassie Brilliant Blue R-250 stain solution. The molecular weight marker BSM0441 (Bio Basic Inc., 20 Konrad Cres, Markham Ontario L3R 8T4 Canada), was incorporated into the gel for calibration and reference purposes.

Antibacterial activity assay

Two Gram +ve bacteria (Staphylococcus aureus ATCC 25923 and Bacillus subtilis ATCC 6051) and two Gram -ve bacteria (Escherichia coli ATCC 25922 and Salmonella enteritidis PT4 (P125109)) were used as indicator strains in the current study. The bacterial pathogens were provided by, Institute of Microbiology, University of Veterinary & Animal Sciences, Lahore, Pakistan.

The peptides resulting from enzymatic hydrolysis, in both crude form (CMCHs) and various fractions (F1-F5) obtained after RP-HPLC, were employed for assessing their antibacterial efficacy using the well diffusion method, as defined by the method (Lajnaf et al., 2020). LB agar plates were prepared, and a homogenous spread of fresh microbial culture was applied to the plates. Wells of 10 mm were created using a sterilized borer, and 100 µL of each enzymatic hydrolysate was introduced. Aerobic conditions were provided to the plates for 24 hours at 37ºC, and the zones of inhibition were measured. Ciprofloxacin and Amoxicillin served as positive controls, while a buffer containing casein (without enzymatic lysate) was employed as the negative control.

Antioxidant activity by DPPH assay

The antioxidant potential of CMCHs was evaluated by radical-scavenging ability using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, following the method (Lajnaf et al., 2021) with slight modifications. A standard solution of 0.096% DPPH, was prepared and kept at room temperature under dark for 30 minutes. Working solutions of CMCHs (peptic, tryptic, and peptic followed by tryptic) and ascorbic acid were prepared by mixing 100 µL of the sample with 100 µL of dimethyl sulfoxide (DMSO) in the initial well, followed by twofold serial dilution to achieve final concentrations ranging from 1000 to 15.62 µg/mL. For the antioxidant assay, 10 µL of the CMCHs and standards (ascorbic acid) from the working solution plate along with 190 µL of DPPH solution, were added to the corresponding wells of the assay plate. The contents were gently mixed for the measurement of absorbance at 517 nm. DPPH was used as blank and 1% DMSO as negative control.

The percentage DPPH radical scavenging activity, termed as effective concentration (EC_{50}) was calculated as a decrease in absorbance from the following equation:

$$\text{DPPH radical scavenging activity (\%)} = 100 - \frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \times 100$$

RP-HPLC for peptide separation

The peptides generated from peptic hydrolysates of camel milk casein (P-CMC) were the ones with the greatest antibacterial and antioxidant activity. These were purified by High-

Performance Liquid Chromatography (HPLC) (Agilent 1100 series system), using the method defined by Liu (Liu et al., 2020). The separation was carried out on a silica-based reversed-phase C-18 column. A 10-fold dilution of the peptide sample was reconstituted in Milli-Q water. Peptides were separated under a constant flow rate of 0.6 mL per minute, the temperature of column was 35°C and the wavelength was 215 nm. The column was conditioned with a mobile phase of eluent A (0.1 percent (V/V) trifluoroacetic acid (TFA) in acetonitrile) and eluent B (0.1 percent (V/V) TFA in Milli-Q water), with detection at over 20 minutes, a linear gradient of solvent A was applied, resulting in the collection of distinct fractions F1-F5 (1.5 mL each).

Statistical Analysis

All results were presented as means ± standard (SD) of triplicate determinations. One-way ANOVA was applied using SAS 9.4 to explore the effect of treatment on the antioxidant and antimicrobial properties against pathogenic bacteria. Post hoc analysis (Duncan Multiple Range Test) was applied to check the significant effect at alpha 0.05.

RESULTS:

Physico-Chemical analysis of Camel milk

Camel milk, characterized as a white opaque liquid, exhibited an average pH range of 6.55-6.67 and a specific gravity of 1.029. The chemical composition of camel milk is delineated as follows: water content at 87.34 ± 1.05%, protein at 3.20 ± 0.11%, fat at 3.14 ± 0.65%, ash at 0.52 ± 0.02%, and lactose at 3.53 ± 0.07%. The isolation of moist casein from 1L of camel milk yielded 57.2g, equivalent to 17g of dried casein.

Synthesis of Camel Milk Casein Hydrolysates (CMCHs)

Camel milk casein (CMC) was hydrolyzed with proteolytic enzymes pepsin, trypsin and pepsin followed by trypsin for 2 h each, and then lyophilization for storage till further use.

Characterization of CMCHs by SDS-PAGE

The CMCHs were examined for reducing SDS-PAGE for peptide formation. Notably, two large bands were manifested by whole casein solution prepared in water (C_W) and in sodium acetate buffer (C_A). The results of this analysis indicated complete hydrolysis of CMC into respective short chain peptides formed by pepsin, trypsin and pepsin followed by trypsin treatments. The results were obvious by the presence of deeply stained bands at the bottom of the chromatogram (Figure 1).

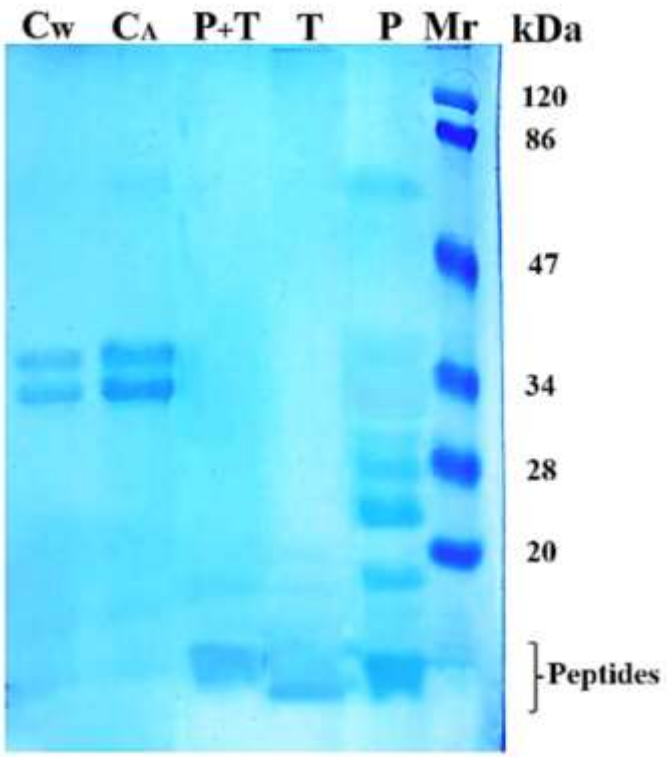


Figure 1: Coomassie brilliant blue R-250 stained polyacrylamide gel showing the proteolytic hydrolysis of camel milk casein. C_W: Casein prepared in water, C_A: Casein prepared in sodium acetate buffer, P+T: Casein hydrolyzed by pepsin followed by trypsin enzyme, T: Casein hydrolyzed by trypsin, P: Casein hydrolyzed by pepsin, M: Molecular weight marker.

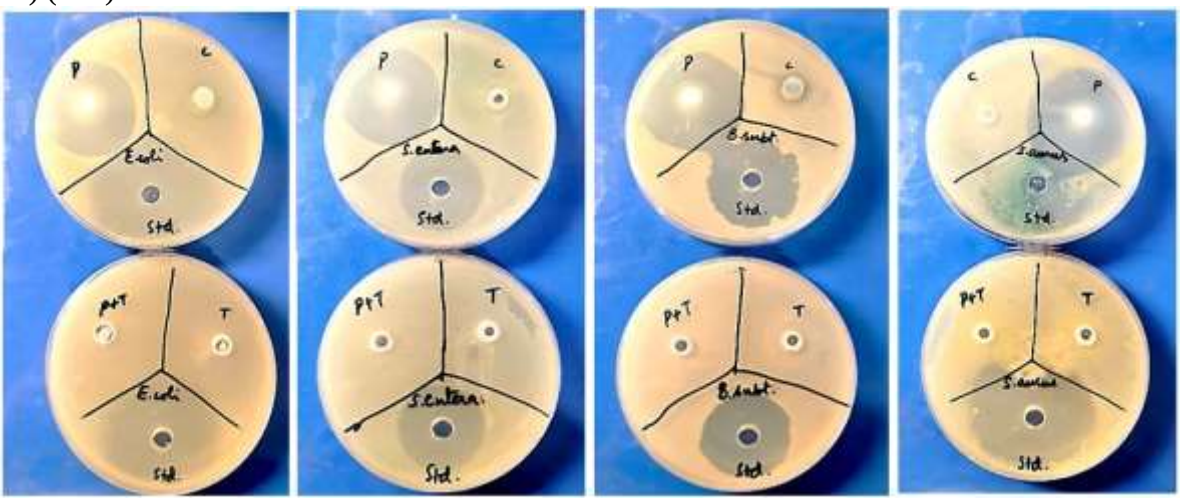
Antibacterial activity assay of CMCHs

The antimicrobial potential of CMCHs was investigated against gram +ve and gram -ve bacteria. The peptic hydrolysates of camel milk casein (P-CMC) demonstrated significantly higher antibacterial activity as compared to tryptic hydrolysates of camel milk casein (T-CMC) and peptic followed by tryptic hydrolysates of camel milk casein (PT-CMC). However whole camel milk casein (CMC) showed lesser antibacterial activity against gram +ve and gram-ve bacteria in comparison to P-CMC (Table 1, Figure 2).

Table 1: Antimicrobial activity (zone of inhibition in mm) of whole camel milk casein and its hydrolysates (CMCHs) (Mean ± SE).

Samples	Gram negative		Gram positive	
	E. coli	***S. enteritidis***	***B. subtilis***	***S. aureus***
Positive reference*	21±0.15[a]	18±0.10[b]	25±0.21[b]	25±0.17[b]
Camel Milk Casein	4±0.06[c]	2.4±0.06[c]	1.60±0.15[c]	1.30±0.10[c]
Pepsin Hydrolysate of Camel Milk casein	19±0.15[b]	22±0.21[a]	27±0.15[a]	28±015[a]
Trypsin Hydrolysate of Camel Milk casein	NI	NI	NI	NI
Pepsin+Trypsin Hydrolysate of Camel Milk casein	NI	NI	NI	NI
P value	<0.0001	<0.0001	<0.0001	<0.0001

*Ciprofloxacin and Amoxicillin were used as positive reference standards for gram-negative and gram-positive bacteria, respectively against camel milk casein and its enzymatic hydrolysates. NI: no inhibition. Different superscript letters within a column mark significantly different value ($P < 0.05$) (n=3).



A **B** **C** **D**

Figure 2: Inhibition zones of *E. coli* (A), *S. enteritidis* (B), *B. subtilis* (C), and *S. aureus* (D) in response to casein (C) and peptic (P) hydrolysate, compared with standard, as well as against tryptic (T) and peptic + tryptic (P+T) hydrolysates, alongside the standard.

Antioxidant activity by DPPH assay

The mean EC_{50} value of ascorbic acid (21.82 ± 0.1581%), for the assessment of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, served as the reference. A statistically significant difference ($p < 0.001$) in the mean EC_{50} values of the tryptic hydrolysates of camel milk casein (T-CMC) (33.05 ± 1.46%) and peptic followed by tryptic hydrolysates of camel milk casein (PT-CMC) (33.25 ± 0.98%) as compared to ascorbic acid was observed. Conversely, no significant difference ($p > 0.05$) for the mean EC_{50} value of the P-CMC (22.84 ± 0.59%) in comparison with ascorbic acid was found. Besides, a significant difference ($p < 0.001$) was noted in the mean EC_{50} value of the P-CMC as compared to those of the T-CMC and PT-

CMC. However, the EC_{50} values' comparison of the T-CMC and PT-CMC were found insignificant (Table 2).

Table 2: % scavenging activity of ascorbic acid and different Camel Milk Casein Hydrolysates: P-CMC (Pepsin Hydrolysate of Camel Milk casein), T-CMC (Trypsin Hydrolysate of Camel Milk casein), PT-CMC (Pepsin+Trypsin Hydrolysate of Camel Milk casein) at various concentrations (15.62-1000 µg/mL) (Mean ± SE).

Hydrolysate Concentration (µg/mL)	**Standard (Ascorbic Acid)**	**P-CMC**	**T-CMC**	**PT-CMC**	**P value**
15.62	39.15 ± 0.53^{a}	37.84 ± 1.26^{b}	1.73 ± 1.78^{c}	35 ± 0.11^{a}	<0.005
31.25	63.02 ± 0.07^{a}	61.47 ± 0.59^{c}	48.67 ± 1.08^{b}	48.7 ± 0.60^{c}	<0.005
62.5	64.16 ± 0.17^{c}	69.09 ± 0.06^{d}	6.06 ± 0.04^{b}	58.34 ± 0.56^{a}	<0.005
125	68.95 ± 0.39^{a}	70.08 ± 0.12^{b}	4.05 ± 0.08^{b}	64 ± 0.03^{a}	<0.005
250	71.14 ± 0.06^{b}	76.15 ± 0.03^{b}	72 ± 0.14^{b}	71 ± 0.07^{a}	<0.005
500	74 ± 0.23^{d}	85.09 ± 0.05^{c}	0.09 ± 0.04^{b}	76 ± 0.12^{a}	<0.005
1000	84.13 ± 0.03^{d}	89.02 ± 0.02^{b}	6.04 ± 0.04^{a}	81.01 ± 0.04^{c}	<0.005
EC_{50} (µg/mL)	21.82 ± 0.16^{c}	22.84 ± 0.59^{a}	33.05 ± 1.46^{a}	33.25 ± 0.98^{b}	<0.005

Different superscript letters within a row mark significantly different value ($P < 0.05$) (n=3).

RP-HPLC for peptide separation

The peptic hydrolysates of camel milk casein (P-CMC) exhibiting notable anti-oxidant and antibacterial activity against various microbes, underwent further analysis using (RP-HPLC) for their peptide separation. The chromatogram revealed 5 distinct peaks (F1-F5) within the time interval of 3 to 12 minutes, corresponding to different peptide fragments. The presence of peaks at diverse time intervals indicated the generation of shorter peptides during the hydrolysis of the P-CMC (Figure 3).

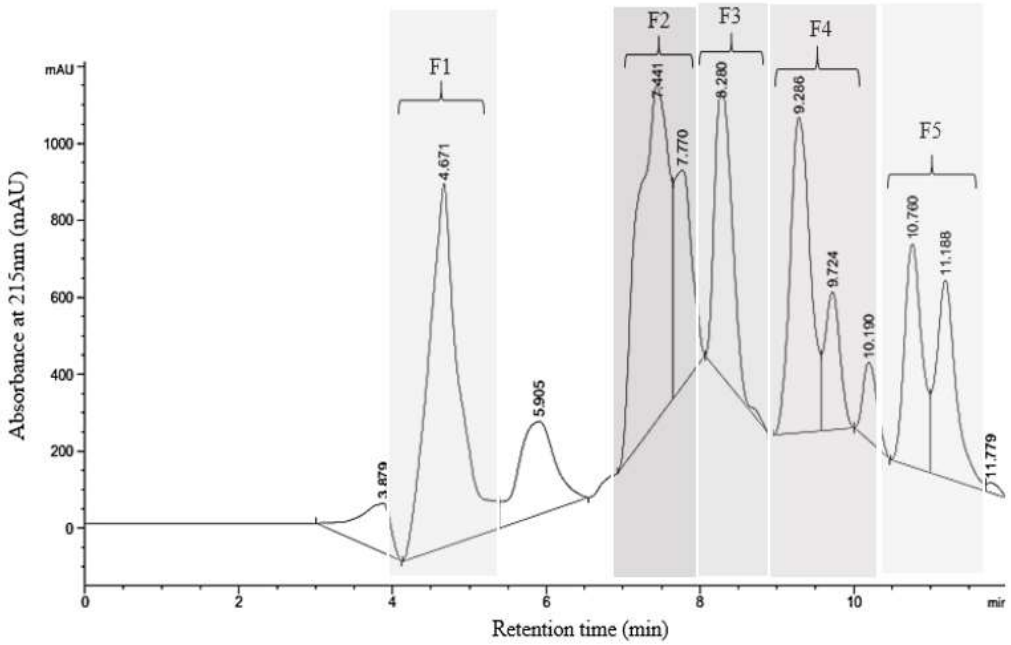


Figure 3: RP-HPLC profile of peptides from Peptic hydrolysates of camel milk casein showing fractions (F1-F5) as indicated on HPLC chromatogram.

Antibacterial activity assay of P-CMC purified fractions (F1-F5)

The antibacterial activities of each P-CMC purified fraction (F1-F5) were evaluated against gram-positive and gram-negative bacteria. The effectiveness of all fractions for their antibacterial activity were estimated by zone of inhibition (mm). All the fractions, except F4, were evident against both gram +ve and gram -ve bacterial strains. Standard antibiotics (Ciprofloxacin and Amoxicillin) exhibited clear zones of inhibition, validating the assay, while the Blank (distilled water) displayed no inhibition, confirming the accuracy of the test (Table 3).

Table 3: Antimicrobial activity (zone of inhibition in mm) of Pepsin Hydrolysate of Camel Milk casein purified fractions (F1-F5) (Mean ± SE).

Fractions	**Gram negative**		**Gram positive**		
	E. coli	***S. enteritidis***	***B. subtilis***	***S. aureus***	**P value**
Standard*	20 ± 0.15^{c}	18 ± 0.10^{d}	22 ± 0.12^{b}	24 ± 0.10^{a}	<0.005
F1	18 ± 0.15^{a}	17 ± 0.15^{b}	18 ± 0.15^{a}	18 ± 0.21^{a}	<0.005
F2	20 ± 0.21^{a}	19 ± 0.21^{b}	20 ± 0.10^{a}	20 ± 0.15^{a}	<0.005
F3	18 ± 0.10^{c}	18.5 ± 0.10^{b}	18 ± 0.10^{c}	19 ± 0.15^{a}	<0.005
F4	4 ± 0.10^{b}	2 ± 0.21^{c}	5 ± 0.15^{a}	2 ± 0.21^{c}	0.008
F5	19 ± 0.10^{b}	18 ± 0.23^{c}	20 ± 0.15^{a}	18 ± 0.10^{c}	<0.005

*Ciprofloxacin and Amoxicillin were used as standard for gram-negative and gram-positive bacteria, respectively. Different superscript letters within a row mark significantly different value ($P < 0.05$) (n=3).

DISCUSSION:

Milk-derived bioactive peptides are recognized as efficient constituents of healthy foods. These peptides exhibit multifunctional characteristics, including immune-regulatory, anti-microbial, anti-oxidant, thrombolytic, and antagonistic activities against several toxic agents (Khalesi et al., 2017).

Due to its high levels of nutrients and bioactive component, the camel milk closely resembles with human breast milk, thus making it best alternative to the human milk. In the current study, the physiochemical properties of camel milk were found similar to the previous report, indicating an average water content 87%, proteins 3.4%, fat 3.5%, ash 0.79%, and lactose content of 4.4% (Pak et al., 2019). While the FAO journal reported, camel milk composition with slight variations (Hadef et al., 2019). The contradictions in these studies might have resulted from the fact that milk composition can be affected by several factors like the quality of feed and water, the season of the years' breed, and the health status of animals.

The bioactive peptides are naturally found in inactive form within their parent proteins and can be activated by releasing them from the original sequence of their precursor molecules. Among many methods for the release of these inactive peptides, an enzymatic cleavage using digestive enzymes like trypsin, chymotrypsin, pepsin etc. is one of the commonly used methods nowadays. This step was performed in the current study just like was done previously by Kumar (Kumar et al., 2016). The camel milk casein (CMC) was hydrolyzed by three different treatments using

pepsin (T1), trypsin (T2), and pepsin followed by trypsin enzymes (T3). The resulting hydrolysates like pepsin hydrolysate of camel milk casein (P-CMC), trypsin hydrolysate of camel milk casein T-CMC, and pepsin followed by trypsin hydrolysate (PT-CMC) were identified by the SDS PAGE method. In which the intact casein exhibited a distinct band on the gel while hydrolysates displayed lower molecular weight peptides at the gel's base, indicating successful protein hydrolysis. Similar results were observed in a study involving enzymatic digestion of camel milk proteins (Kumar et al., 2016).

Oxidative stress is a condition in which free radical formation levels are increased, resulting in damage to vital biomolecules (carbohydrates, proteins, lipids, enzymes, and DNA) thus leading to the development of various chronic diseases including atherosclerosis, cancer, diabetes, rheumatoid arthritis, cardiovascular issues, chronic inflammation and other progressive diseases in human (Uttara et al., 2009). Thus, antioxidant peptides can play an important role in the scavenging of free radicals and the prevention of the diseases associated with them. The antioxidant peptides of bovine milk proteins have been studied extensively (Power et al., 2013). While much less research is available on camel milk casein. In the current study, the P-CMC showed a significantly higher reduction of DPPH over the T-CMC and PT-CMC. Similar to our findings, a study using DPPH assay for the assessment of the antioxidant potential of camel milk casein peptic hydrolysates, demonstrated a higher antioxidant ability of this peptide (Ibrahim et al., 2018), The reason for this antioxidant capacity of the peptic hydrolysate might be its ability to donate electron for the reduction of DPPH.

In the era of antibiotic resistance, when scientists are searching for substitutional treatments, antimicrobial peptides have shown their promising effects in this regard as they are abundantly found in nature and the results of preliminary studies of their clinical potential have been encouraging. Many reports have shown that camel milk-derived antimicrobial peptides have inhibited the growth of many pathogens (Rahimi & Kheirabadi, 2012). Consistent with these findings, our study revealed that the intact camel milk casein (CMC) slightly inhibited the growth of bacteria while the pepsin hydrolysate (P-CMC) exhibited significantly strong antibacterial activity through bioactive peptide formation. However, tryptic (T-CMC) and peptic followed by tryptic hydrolysates (PT-CMC) failed to show such activity, possibly due to further degradation of peptides formed during peptic followed by tryptic digestion (Table 1). Similar observations were reported by Jrad, who investigated the antibacterial properties of camel colostrum and milk proteins, finding inhibition of E. coli and L. inoculant growth. He found that antimicrobial activity was present even before digestion but increased post-exposure to proteolytic enzymes. Several factors can influence the antimicrobial activity of the casein hydrolysates including sample loading, structural diversity, hydrophobicity, and specific amino acid composition (Kumar et al., 2016).

As P-CMC showed maximum antimicrobial and antioxidant activity, hence it was recruited for further purification by the HPLC method. Due to the availability of various modes of HPLC, it has become the fundamental technique for the synthesis and identification of peptides and proteins and currently plays a major role in both our understanding of biological processes and in the development of peptide and protein-based antimicrobial agents. In the current study, the P-CMC hydrolysate was purified into five different molecular weight fractions (F1 to F5) by RP-HPLC. All fractions of P- CMC showed significant antimicrobial activity against gram-positive

and negative bacteria, except F4. The results of this study were comparable to that of Kumar (Kumar et al., 2016). The reason for discrepancies in antimicrobial activity between P-CMC hydrolysate and its fractions may stem from differences in peptide size, ionic nature, and concentration.

There are limitations in this research zone due to various shortcomings in molecular methods, cutting-edge technologies, and enhanced products. There is a dire need to emphasize on the development of innovative facilities such as advanced proteomic techniques, genetically engineered enzyme technologies, and microbial fermentation, for the understanding of different effects of bioactive peptides on genetic expression and also for the optimization of the dietary and health impacts of these compounds. In addition, before milk-derived bioactive peptides can be used directly or formulated as chemotherapeutic agents, careful evaluation of their primary beneficial effects on specific diseases must be conducted.

CONCLUSION:

Considering the findings of the current study, it is deduced that camel milk casein serves as a promising reservoir of bioactive peptides after enzymatic hydrolysis with pepsin. The resultant bioactive peptides exhibit noteworthy antioxidant and antibacterial properties. This investigation lays the groundwork for potential utilization of bioactive peptides as indigenous therapeutic entities for addressing diverse bacterial infections in forthcoming medical interventions.

AUTHORS' CONTRIBUTION:

Conceptualization, methodology, and formal analysis were done by SA and MT. TM and GS contributed to data analysis and interpretation. MA and SA worked on protein purification. SA carried out laboratory investigations and prepared an original draft of the manuscript. MT supervised and reviewed the manuscript. The manuscript was approved by all the authors.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors in relation to the research, authorship, or publication of this article.

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