Clotting Activities of Partially Purified Extracts of *Moringa oleifera* L. on Dromedary Camel Milk

Mezgebu Abate Terefe[1]*, Ameha Kebede[2], and Misrak Kebede[2]

[1]Department of Biology, College of Natural and Computational Sciences, Haramaya University, Ethiopia
[2]Department of Biology, Haramaya University, Ethiopia

Abstract: Processing camel milk into shelf stable value added milk products is not yet well developed. Unlike the milk of cows and small ruminants, camel milk does not readily coagulate by rennet due to its inherent properties. Therfore, experiments were conducted to evaluate the clotting activities of partially purified *Moringa oleifera* extract on camel milk and to identify the optimum pH, temperature and concentration of the partially purified extract that would result in strong curd of camel milk. These included three temperature points (55, 60 and 65°C), three pH values(4.5, 5 and 5.5) and five levels of partially purified *Moringa oleifera* extracts obtained from seed and leaf samples (0, 10, 20, 30 and 40%). The results revealed that temperature, pH, and concentrations of partially purified seed and leaf extracts of *Moringa oleifera* had significant ($p<0.05$) effect on the clotting activities of camel milk. The highest camel milk clotting activity and curd firmness were observed at pH 5, temperature of 65°C and partially purified extract concentration of 10% for both seeds and leaves, while the lowest values were recorded at pH 5.5, temperature of 55°C and a partially purified extract concentration of 40%, respectively. An increase in camel milk clotting activity was observed with a decrease in milk pH from 5.5 to 4.5. Camel milk clotting activities increased with increasing temperature. However, it decreased with increase in partially purified extract concentration for both seed and leaf extracts. Therefore, the capability of the partially purified extract of *Moringa oleifera* seeds to coagulate camel milk and to form firm curd combined with its high ratio of milk clotting to proteolytic activity could make it a useful rennet substitute in the dairy industry.

Keywords: Ammonium sulfate fractionation; Curd firmness; Milk-clotting activity; Partially purified extract

1. Introduction

Dromedary camels are generally known to produce more milk of high nutritional quality for a longer period of time in hostile environments (Khan and Iqbal, 2001). Camel milk is widely consumed fresh in traditional pastoral systems of several countries. However, processing of the camel milk into more shelf stable value added milk products is not yet well developed and thus camel milk products are not common (Mehaia, 2006). Unlike the milk of cows and small ruminants, camel milk does not readily coagulate by rennet due to its inherent properties (low total solids content of the coagulum, especially casein, and inadequate rennet action). However, possibilities of cheese-making from camel milk were reported by Khan *et al.* (2004), Mehaia (2006), and Ahmed and Elzubeir (2011) using coagulants of animal origin.

Milk coagulation is the basic step in the manufacture of most types of cheeses and calf rennet has been the most widely used milk-clotting enzyme preparation (Mohamed *et al*., 2009a). However, the last decades have witnessed a population explosion that led to an increased demand for cheese production and consumption. On top of this, the price of calf rennet was greatly increased along with the reduced supply of natural calf rennet (Mohamed *et al*., 2010). In addition, the use of animal rennet is limited by religious and safety considerations, change in diet (vegetarianism), or fear of consumption of foods containing genetically engineered products (Roseiro *et al*., 2003). All these factors have necessitated the search for new proteases with high specific milk-clotting activities and low general proteolytic activities to be used as a rennet substitute. Accordingly, interest has been directed towards discovering a milk-clotting enzyme which would satisfactorily replace calf rennet in cheese manufacturing; and numerous enzyme preparations of animal, microbial, and plant origin have been studied (Jacob *et al*., 2011).

Microbial rennets produced by genetically engineered bacteria have proven to be suitable substitutes for animal rennet, but increasing attention has been directed towards natural rennet extracted from plants such as *Ananas comosus, Carica papaya, Calotropis procera, Ficus carica, Calm viscera, Cynara cardunculus* (Roseiro *et al*., 2003), *Cynara scolymus* (Sidrach *et al*., 2005), and *Solanum dubium* (Mohamed *et al*., 2009 a, b) among others. Unfortunately, most of these plant rennets were found to be inappropriate because they possess high general proteolytic activity, which leads to the production of short peptides that are responsible for the defect in flavor and texture of cheese (Anusha *et al*., 2014). An exception to this general rule is represented by the aqueous extract of *Cynara cardunculus* flowers containing two aspartic acid-type proteases, named cardosin A and B (Verissimo *et al*., 1995), which has been used for years for the manufacture of sheep milk cheese in several areas

*Corresponding Author. E-mail: mezgebuabate16@gmail.com
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of Portugal and Spain. However, the flowers of *Cynara cardunculus* are not used for the production of cow-milk cheeses as they tend to produce bitter taste because of the formation of several peptides identifiable in the digests of isolated bovine β- and α-casein (Macedo *et al.*, 1996). Thus, the search for a rennet substitute from plant sources having high ratio of milk clotting to general proteolytic activity was highly needed to overcome the above mentioned problems.

Moringa oleifera is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan and it is an important crop in India, Ethiopia, Philippines and the Sudan. All parts of this rapidly-growing tree are edible and have long been consumed by the ancient Romans, Greeks, and Egyptians (Fahey, 2005). It is the most nutrient-rich plant yet discovered as it contains all the essential nutritional elements that are necessary for livestock and human beings (Fahey, 2005). Many parts of this multipurpose tree have been used in various applications such as food for overcoming malnutrion, feed for weight gain and milk production in animals, medicine for the treatment and/or prevention of many diseases, and environment for water purification (Fahey, 2005). Despite the wide spread and huge uses of *Moringa oleifera*, its use as a source of milk clotting enzymes has never been studied extensively. Only one report has recently been published to indicate the presence of caseinolytic and milk-clotting activities from flowers of *Moringa oleifera* (Pontual *et al.*, 2012). This activity has only been detected after the precipitation of the protein with 60% ammonium sulfate (Pontual *et al.*, 2012) which indicated low concentration of the enzyme in the flowers. Moreover, extracts from other parts of this important tree have been tested for their milk-clotting abilities by using different extractants (Amna *et al.*, 2014). Nevertheless, isolation and characterization of milk-clotting enzymes from any part of this tree at different concentrations, pH and temperatures has not yet been reported.

In Ethiopia, although cheese-making from cow milk and consumption have been part of the culture of a substantial proportion of the society, cheese is not traditionally made from camel milk due to long coagulation time requirement, weak curd formation, and low total solids content of the coagulum, especially casein, poor rennet action, and low yield of soft white cheese by conventional processes. This study was, therefore, aimed at assessing the clotting activities of partially purified extracts of *Moringa oleifera* on camel milk and to identify the optimum pH, temperature, and concentration of the partially purified extracts at which strong curd formation of camel milk could be achieved.

2. Materials and Methods

2.1. Study Area

The study was conducted in Animal Genetics and Breeding, Dairy Technology and Molecular Genetics laboratories of Haramaya University which is located at a latitude of 9°25`16`` N, longitude of 42°2`17`` E and at an altitude of 1980 meters above sea level (FAO, 1990). The mean annual temperature was 17° C while the mean minimum and maximum temperatures were 3.8°C and 25°C, respectively.

2.2. Experimental Materials

2.2.1. Milk samples

Camel milk samples were obtained from pastorally managed camels in Shinile (Somali Region), Eastern Ethiopia, and transported in an icebox to the Animal Genetics and Breeding laboratory of Haramaya University.

2.2.2. Collection and preparation of *Moringa Oleifera* seed and leaf samples

Samples of *Moringa oleifera* seeds and leaves were obtained from Tony Farm (i.e. the farm land of Haramaya University) in Dire Dawa, which is located between 9°27`N and 9°49`N latitude and, 41°38`E and 42°19`E longitude above sea level (IDP, 2006), Eastern Ethiopia. All samples of *Moringa oleifera* seeds and leaves were collected and dried at room temperature in the laboratory. Thereafter, each sample was ground using all purpose high speed smashing machine and stored in closed containers in a freezer at -20°C until use (Amna *et al.* 2014).

2.3. Experimental Design

The experiment in this study was carried out in a 2 x 3 x 3 x 5 factorial design. Raw camel milk samples were arranged in test tubes and treated with five concentration levels (0, 10, 20,30 and 40%) of each of the crude extracts of both the seed and leaf samples, three pH levels (4.5, 5 and 5.5)and three temperatures (55°C, 60°C and 65°C). Each treartment was replicated three times. Thus, a total of 90 tests with three replications were carried out using this design. Adjustment of pH was performed by gradual addition of 1M HCl or NaOH. The coagulation temperature was fixed using a thermostatically controlled water bath and a thermometer was used to check the temperature. Milk samples were held at the coagulation temperature (55°C, 60°C and 65°C) for 15 min before crude or partially purified extracts were added.

2.4. Experimental Procedures

2.4.1. Preparation of crude extract

Five percent NaCl solution in sodium acetate buffer (pH 5.0) was used for the extraction of milk-clotting crude extracts from *Moringa oleifera* seed and leaf samples (Amna *et al.*, 2014). *Moringa oleifera* seed and leaf samples, each weighing 50.0 g, were finely ground using all purpose high speed smashing machine and extracted with 0.5 l of extractant for 4 hrs with stirring in a 2 l beaker. The extracts were filtered through cheesecloth and centrifuged at 12,000 g for 20 min. The supernatants

were dialyzed overnight against 0.1 M sodium acetate buffer, pH 5.0 (Amna *et al.*, 2014).

2.4.2. Partial purification of crude extract

Ammonium sulphate precipitation was performed as a single purification step of milk-clotting enzyme from the seeds and leaves of *Moringa oleifera*. Ammonium sulphate fractionation was carried out following the method described by Mohamed *et al.* (2009a). Solid ammonium sulphate was slowly added by stirring the crude extract (50 ml) preparations up to 20% saturation. During the addition of solid ammonium sulphate, the pH of the extract solution was kept at 5.0 by a drop wise addition of either 7% NH_4OH or 0.1 M H_2SO_4, and the mixture was kept in ice for 30 min. The precipitates were then separated from the supernatant by centrifugation at 12,000 g for 20 min at room temperature. Solid ammonium sulphate was further slowly added to the supernatant to 30% saturation and the solution was kept on ice for another 30 min before being centrifuged at 12,000 g for 20 min at 4°C. This process was repeated for each concentration of ammonium sulphate until 60% saturation was obtained. The precipitates collected were dissolved in a small volume (about 10 ml) of 50 mM sodium acetate buffer, pH 5.0, and then dialyzed overnight at 4°C against the same buffer (1.0 liter) with several changes of the dialysis buffer. The dialyzed solutions were further centrifuged to remove any solid particles, and their protein concentration, milk-clotting and proteolytic activities were measured using the methods described below. The most active fractions with the highest yields were pooled and used to characterize the enzyme.

2.4.3. Determination of milk-clotting activities

Camel milk containing 0.15 g of $CaCl_2$ per liter was prepared as suggested by FAO (2001). The pH of the milk samples was adjusted to 4.5, 5.0 and 5.5 as described by Farah and Bachman (1987). Temperature was adjusted to 55°C, 60°C and 65°C using thermostatically controlled water bath and checked periodically using a thermometer. The milk clotting activities (MCAs) were determined according to the methods described by Soledad *et al.* (2007). Briefly, 0, 10, 20, 30 and 40% by volume of the partially purified extract were added into test tubes each containing 10 ml of milk sample and MCAs were determined by rotating the test tubes at regular intervals and checking for visible clot (fine curd) formation on the walls of the test tubes. The formula suggested by Guiama *et al.* (2010) was used to calculate the MCA expressed in Units/ml.

$$MCA\ (Unit/ml) = (100/CT) \times S/E$$

Where: MCA = Milk Clotting Activity (unit/ml), CT = Clotting time (s), S = Substrate (camel milk) volume (ml), E = Enzyme (extract) volume (ml).

All inclusions of partially purified extracts were considered for the curd firmness study. Firmness of the curd samples was measured using Texture Analyzer (Model TA-Plus Lloyd, UK) according to Salvador and Fiszman (2004).

2.4.4. Determination of protein concentration of the extract

Protein concentration in the partially purified extracts was determined using Folin Ciocalteu reagent as per the procedure of Lowry *et al.* (1951); Crystalline Bovine Serum Albumin was used as standard protein for preparation of standard curve. The standard Crystalline Bovine Serum Albumin (500 μg/ml) was serially diluted and the corresponding absorbance was measured at 660 nm using a spectrophotometer for the preparation of standard curve. A similar procedure was employed to determine the absorbance of the unknown sample i.e. the partially purified extract. Then the average protein content of partially purified seed and leaf extract was determined by `TREND` formula obtained for the standard using Microsoft excel.

2.4.5. Determination of the proteolytic activities of extracts

2.4.5.1. Preparation of camel milk casein

Whole casein was prepared following the procedures described by Egito *et al.* (2007), with slight modification of the methods of Maryam *et al.* (2011). Raw camel milk was warmed to 37°C and skimmed immediately by centrifugation at 5000 g for 15 minutes. The pH of the skimmed milk was adjusted to 4.6 with 1N HCl. The solution was mixed at 37°C for 30 minutes and casein was precipitated and separated from whey protein by centrifugation at 5860 g for 15 minutes at 4 °C; and then washed three times with distilled water, lyophilized and stored at -20°C until used. This camel milk casein was used as substrate to determine proteolytic activity.

2.4.5.2. Hydrolysis of camel milk casein

Proteolytic activity was determined according to Silva and Malcata (2005). Camel whole milk casein, 1% (w/v), was subjected to hydrolysis at 30°C in 100 mM phosphate buffer (pH 6.7). The hydrolysis was initiated by addition of 1 ml of each extract to 10 ml of camel whole milk casein solution. The reaction was stopped after 30 min by heating at 100°C for 5 min. The proteolytic activity was quantified by evaluating the soluble peptides in 5% (w/v) trichloroacetic acid (TCA). A 1 ml of each sample was treated with 5% (w/v) TCA at a volumetric ratio of 1:2; the mixture was allowed to settle for 10 min and then centrifuged at 7,500 g for 30 min. The absorbance of the supernatant was measured at 280 nm. An appropriate control was prepared in which the TCA was added without the extract. One unit of proteolytic activity (U) was arbitrarily defined as the amount of enzyme required to cause an increase of 0.1

in absorbance at 280 nm under the assay conditions. Proteolytic activity was calculated as follows:

PA (U/ml) = ∆ Abs280nm x 10 x dilution factor / E x t

Where: PA= Proteolytic Activity; ∆Abs 280nm is the variation of absorbance between assay and control; E is the volume of partially purified extract solution and t is the reaction time.

2.5. Data Analysis

The data were subjected to ANOVA following the procedures described by Gomez and Gomez (1984) using the SAS software, version 9.1. Two-way analysis of variance was carried out to establish the presence or absence of significant difference in the coagulation parameters between treatments. Mean values of MCA treatments and mean values of curd firmness were compared using the Duncan's Multiple Range Test.

3. Results and Discussion

3.1. Clotting Activities of Crude Extracts of *Moringa oleifera Seed* and Leaf

The milk-clotting activities of the leaf and seed extracts of *Moringa oleifera* were compared in order to use the plant part with the highest milk-clotting activity as a source of the enzyme. The results showed that milk clotting activities were detected only in crude extracts of the seeds tested before ammonium sulphate fractionation while the leaf crude extracts showed no activity even when incubated for more than one hour at 65°C (Table 1). This was supported by the findings of Amna *et al.* (2014) who reported that milk-clotting activities were detected only in seed extracts using different types of extractants. High milk clotting activity in *Moringa oleifera* seed extract was expected since most of the proteolytic enzymes in the plants are concentrated in the seeds where it degrades the storage protein during seed germination (Antao and Malcata 2005). Similar to our findings, previous researches had revealed isolation of milk-clotting enzymes from the seeds of numerous plants (Egito *et al.*, 2007; Mohamed Ahmed *et al.*, 2009a, b; Nestor *et al.*, 2012). However, various milk-clotting enzymes had also been extracted from other parts of the plants (Anusha *et al.*, 2014; Beka *et al.*, 2014; Hashim *et al.*, 2011; Guiama *et al.*, 2010; Mazorra-Manzano *et al.*, 2013; Pontual *et al.*, 2012).

Table 1. Comparison between the clotting activities of *Moringa oleifera* seed and leaf extracts.

Moringa parts	Ammonium sulphate precipitation					
	Crude extracts [without $(NH_4)_2SO_4$ precipitation]	20% $(NH_4)_2SO_4$ precipitated extracts	30% $(NH_4)_2SO_4$ precipitated extracts	40% $(NH_4)_2SO_4$ precipitated extracts	50% $(NH_4)_2SO_4$ precipitated extracts	60% $(NH_4)_2SO_4$ precipitated extracts
Leaves	nd	2.3077^d	4.7847^b	12.8219^a	3.5006^c	0.2599^e
Seeds	5.0085^d	35.3616^a	13.1594^d	9.4950^c	6.2895^d	0.6798^e

Note: Means shown by similar superscripts (letters) within a column and within a row are not significantly different at 5% level of significance. Values in the table are means of triplicate samples and expressed in U/ml; nd = not detected.

3.2 The Effect of Partial Purification of Crude Extracts on Milk Clotting Activities

The results showed that the clotting activities of seed extracts significantly decreased ($P \leq 0.01$) with increasing the ammonium sulphate concentration up to 60% saturation, whereas the clotting activities of the leaf extracts significantly increased with increase in ammonium sulphate concentration up to 40% saturation and then showed a decline with further increase (Table 1). Exceptionally, the highest milk-clotting activity for seed and leaf extract was obtained with 20% and 40% ammonium sulphate saturation, respectively (Table 1). This result is comparable with those of Nestor *et al.* (2012) who reported that 20% ammonium sulphate gave the highest milk-clotting activity of *Solanum elaeagnifolium* seed extract. The application of ammonium sulphate at low saturation (20%) in the current study is superior to high concentrations that are usually used for the purification of milk-clotting enzymes from various sources (Mohamed *et al.*, 2009a; Pontual *et al.*, 2012). The results obtained in the current study indicated that the degree of saturation with ammonium sulphate has greatly affected the enzyme activity, yield and total protein as well as the enzyme purity. This procedure not only facilitates the effective removal of the brown-colored materials in the crude seed extract, but also concentrates the enzyme to a workable volume that could efficiently be used for milk coagulation in cheese making industry. Overall, a one-step and cheap purification procedure has been developed to partially purify milk-clotting enzyme from *Moringa oleifera* seeds. Such an economic purification procedure combined with the easy availability of the plant seeds makes large scale preparation of the enzyme possible, allowing a broad study of its various aspects and hence probable applications.

3.3. Characterization of Partially Purified Seed and Leaf Extracts of *Moringa oleifera*

3.3.1. Clotting activities of partially purified seed and leaf extracts at varying pH, temperature, and extract concentration

The main effects of temperature, pH and partially purified seed and leaf extract concentration had significant (P<0.01) (Table 2) influence on clotting activity. Moreover, the interaction of temperature, pH and partially purified seed and leaf extract concentration had a significant (P < 0.01) effect on this parameter. The control treatment (untreated camel milk) had no clotting activity compared to milk samples treated with partially purified seed and leaf extract (Table 2).

Clotting activity was increased with increasing temperature, and the effect of temperature on the catalytic activities of partially purified milk clotting extracts of *Moringa* seeds and leaves exhibited a typical activity-temperature relationship. The results (Table 2) showed that the enzyme activity increased as the reaction temperature increased from 55 to 65°C. The activity at 65°C was two-fold higher than that of the activity at 55°C for both extracts. In agreement with this finding, high optimal temperature (70°C) of milk-clotting enzymes from various plants had been reported by Mohamed *et al.* (2009a, b), Pontual *et al.* (2012), and Mazorra-Manzano *et al.* (2013). This indicates that the increase in clotting activity at higher temperature could be attributed to the protein aggregation and molecular rearrangement in the protein structure (Najera *et al.*, 2003). However, an inverse relation was observed between the concentration of partially purified extract and clotting activity, where clotting activity tended to decrease with increasing concentration of partially purified extract (Table 2). On the other hand, clotting time was decreased with increasing concentration of partially purified seed and leaf extract. This result is consistent with the finding of Foltmann and Qvist (1998) who reported linear relationship between enzyme concentration and the reciprocal of clotting time. Significant differences existed in clotting activity among pH profiles and the result showed that the clotting activity increased up to pH of 5.0 and then decreased at pH of 5.5 for all partially purified extract levels (Table 2). This is in agreement with Yonas *et al.* (2014) who reported that the highest clotting activity was observed at pH 5.0.

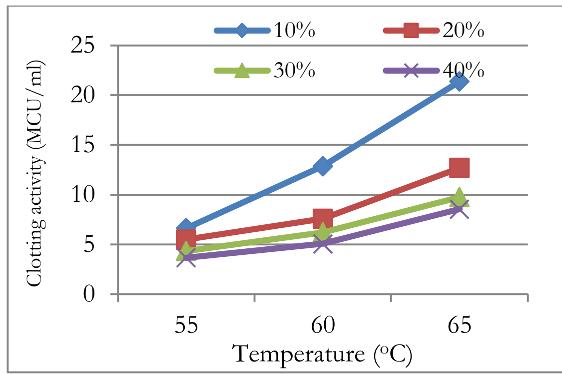
The significant increase in clotting activity in response to the main effect of temperature, pH and partially purified seed and leaf extract concentration could be ascribed to the increased interaction effect. The clotting activity of all partially purified seed and leaf extract concentration increased significantly with the successive increase in temperature (Figure 1). Significant differences were also observed among the interaction effects of temperature with pH and pH with partially purified seed and leaf extract concentration on clotting activity (Figure 2 and 3).

The highest clotting activity was found at pH of 5, temperature of 65°C and partially purified extract concentration of 10% by volume of milk for seed and leaf extracts (35.36 U/ml and 12.88U/ml, respectively), and the lowest was obserbed at pH of 5.5, temperature of 55°C and partially purified extract concentration of 40% by volume of milk for seed and leaf extracts (2.79 U/ml and 0.99 U/ml, respectively) (Table 3). In line with this, Soledad *et al.* (2007) and Mohammed *et al.* (2010) observed that coagulation activity strongly depends on the pH and temperature of milk.

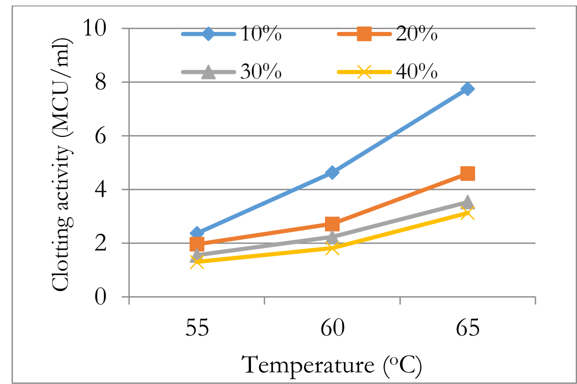
Table 2: Effect of temperature, pH and partially purified *Moringa* seed and leaf extract concentration on clotting activities (MCU/ml).

Treatments	Milk Clotting Activities(MCA)	
	Partially Purified Seed Extract	Partially Purified leaf Extract
Temperature(°C)		
55	4.0244^{c}	1.4389^{c}
60	6.3653^{b}	2.2801^{b}
65	10.4699^{a}	3.8014^{a}
pH		
4.5	6.3269^{b}	2.2774^{b}
5	10.1970^{a}	3.6892^{a}
5.5	4.3357^{c}	1.5537^{c}
PPEC (% inclusion by volume)		
0%	nd	nd
10%	13.6535^{a}	4.9166^{a}
20%	8.5842^{b}	3.0934^{b}
30%	6.7710^{c}	2.4394^{c}
40%	5.7574^{d}	2.0845^{d}

Note: Means shown by similar superscripts (letters) within a column are not significantly different at 5% level of significance. Values in the table are means of triplicate samples and expressed in U/ml; nd = not detected.

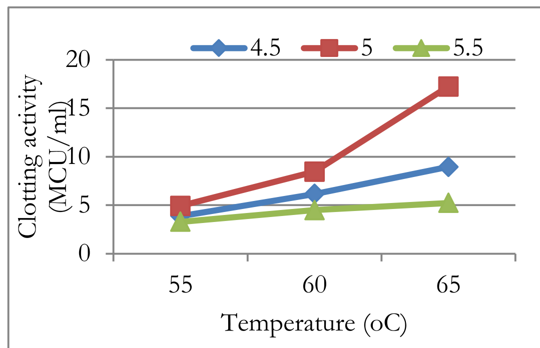


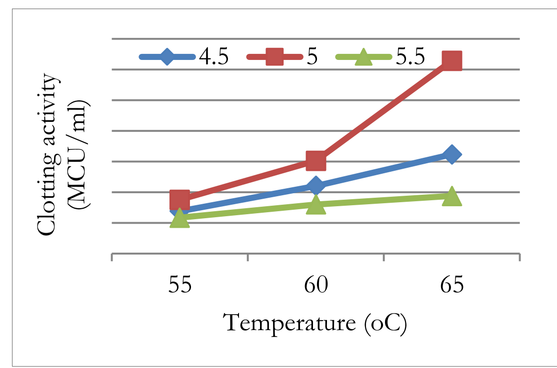
a) Seed partially purified extract



b) Leaf partially purified extract

Figure 1. Interaction effect of temperature and partially purified moringa seed and leaf extract concentration on clotting activities (MCU/ml).

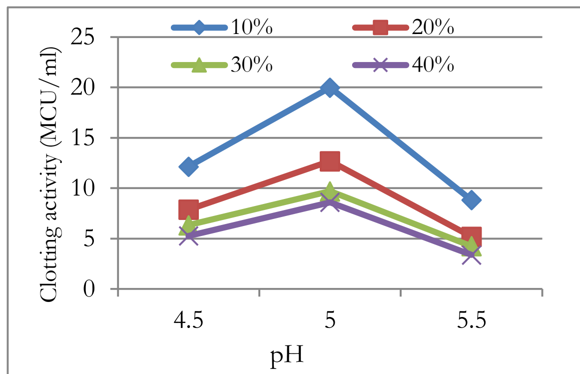




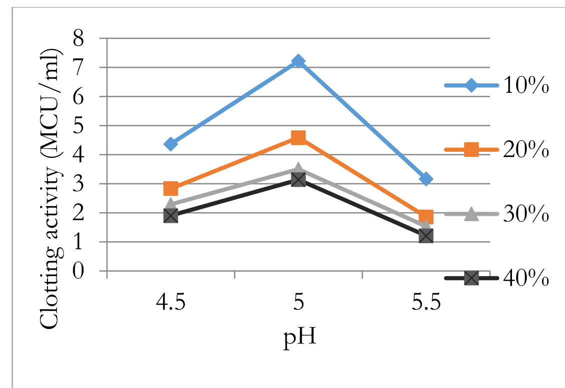
a) Seed partially purified extract

b) Leaf partially purified extract

Figure 2. Interaction effect of temperature and pH on clotting activities (MCU/ml) of partially purified seed and leaf extract.



a) Seed partially purified extract



b) Leaf partially purified extract

Figure 3. Interaction effect of pH and partially purified seed and leaf extract concentration on clotting activities (MCU/ml).

Table 3. Interaction effect of temperature, pH and partially purified *Moringa* seed and leaf extract concentration on clotting activities.

Concentration of partially purified extract (% inclusion by volume) in 10 ml of camel milk	Temperature (°C)								
	55			60			65		
	pH								
	4.5	5	5.5	4.5	5	5.5	4.5	5	5.5
Seed extract									
0%	nd	nd	nd	nd	nd	nd	nd	nd	nd
10%	6.5080[lmn]	7.5384[jk]	5.8256[nopq]	12.4739[f]	17.0532[c]	9.3480[h]	17.4501[c]	35.3616[a]	11.3230[g]
20%	5.2826[pqrs]	6.7893[klm]	4.3994[stu]	7.0460[klm]	10.6512[g]	5.0519[qrs]	11.2870[g]	20.6011[b]	6.1490[mnop]
30%	4.1332 [tuv]	5.4968[opqr]	3.4135[vw]	6.1785[mno]	7.9485[ij]	4.5067[stu]	8.7048[hi]	15.6775[d]	4.8797[rst]
40%	3.4414 [vw]	4.7495[rst]	2.7886[w]	5.1077[qrs]	6.5911[lmn]	3.5230[vw]	7.2910[jkl]	14.4963[e]	3.8279[uv]
Leaf extract									
0%	nd	Nd	nd	nd	nd	nd	nd	nd	nd
10%	2.33283[pq]	2.69787[m]	2.08046[r]	4.46385[g]	6.07322[d]	3.34827[j]	6.30291[c]	12.87888[a]	4.07066[h]
20%	1.89163[st]	2.43121[op]	1.56906[vw]	2.52961[no]	3.82688[i]	1.80727[tu]	4.07651[h]	7.50265[b]	2.20595[q]
30%	1.47715[wx]	1.96474[rs]	1.21805[y]	2.22733[q]	2.85747[l]	1.61296[v]	3.15502[k]	5.68441[e]	1.75755[tu]
40%	1.23360[y]	1.69312[uv]	0.99340[z]	1.82942[t]	2.36626[p]	1.25844[y]	2.64131[mn]	5.36101[f]	1.38383[x]

Note: Means shown by similar superscripts (letters) within a column or within a row are not significantly different at 5% level of significance. Values in the table are means of triplicate samples and expressed in U/ml; nd = not detected.

3.3.2. Curd firmness of camel milk samples at varying pH, temperature, and extract concentration

Significant ($P<0.05$) differences in gel strength was observed between camel milk samples subjected to different combinations of temperature, pH and partially purified seed and leaf extract concentration levels (Table 4). Milk samples treated with partially purified seed and leaf extract at pH of 5.0, temperature of 65°C and partially purified seed and leaf extract concentration of 10% resulted in strong (firm) curd formation [0.0268732 load at yield (Newton) and 0.00969161 load at yield (Newton)], respectively (Table 5). On the other hand, camel milk samples subjected to partially purified extract at pH of 5.5, temperature of 55°C and partially purified seed and leaf extract concentration of 40% by volume showed the weakest gel strength (0.0021192 load at yield (Newton) and 0.00074755 load at yield (Newton)), respectively. Consistent with the results of this study, Attia *et al.* (2000) and Kherouatou *et al.* (2003) indicated that high gel strength of camel milk (permanent bonds) were created between casein fractions at pH 5.0 and a loose network was formed at low pH (4.4) that led to a pseudo curd formation.

Table 4. Effect of temperature, pH and partially purified *Moringa* seed and leaf extract concentration on curd firmness.

Treatments	Curd Firmness (CF)	
	Partially Purified Seed Extract	Partially Purified Leaf Extract
Temperature(°C)		
55	0.0030584 [c]	0.00108278[c]
60	0.0048374 [b]	0.00171579[b]
65	0.0079567 [a]	0.00286061[a]
pH		
4.5	0.0048082[b]	0.00171380[b]
5	0.0077492[a]	0.00277618[a]
5.5	0.0032950[c]	0.00116921[c]
PPEC(% inclusion by volume)		
0%	nd	nd
10%	0.0103761[a]	0.0036998[a]
20%	0.0065236[b]	0.0023279[b]
30%	0.0051457[c]	0.0018357[c]
40%	0.0043754 [d]	0.0015686[d]

Note: Means shown by similar superscripts (letters) within a column are not significantly different at 5% level of significance. Values in the table are means of triplicate samples and expressed in (Load at yield (Newton)); nd = not detected

Table 5. Interaction effect of temperature, pH and partially purified *Moringa* seed and leaf extract concentration on curd firmness.

Concentration of partially purified extract*	Temperature (°C)								
	55			60			65		
	pH								
	4.5	5	5.5	4.5	5	5.5	4.5	5	5.5
Seed extract									
0%	nd	nd	nd	nd	nd	nd	nd	nd	nd
10%	0.0049458^{lmn}	0.0057288^{jk}	0.0044272^{nopq}	0.0094796^{f}	0.0129597^{c}	0.0071040^{h}	0.0132613^{c}	0.0268732^{a}	0.0086050^{g}
20%	0.0040146^{pqrs}	0.0051596^{klm}	0.0033433^{stu}	0.0053546^{klm}	0.0080944^{g}	0.0038392^{qrs}	0.0085777^{g}	0.0156559^{b}	0.0046730^{mnop}
30%	0.0031411^{tuv}	0.0041774^{opqr}	0.0025941^{vw}	0.0046954^{mno}	0.0060405^{ij}	0.0034249^{stu}	0.0066152^{hi}	0.0119142^{d}	0.0037083^{rst}
40%	0.0026153^{vw}	0.0036094^{rst}	0.0021192^{w}	0.0038816^{qrs}	0.0050090^{lmn}	0.0026773^{vw}	0.0055409^{jkl}	0.0110165^{e}	0.0029090^{uv}
Leaf extract									
0%	nd	nd	nd	nd	nd	nd	nd	nd	nd
10%	0.00175550^{pq}	0.00203020^{m}	0.00156558^{r}	0.00335913^{g}	0.00457022^{d}	0.00251964^{j}	0.00474306^{c}	0.00969161^{a}	0.00306325^{h}
20%	0.00142349st	0.00182953^{op}	0.00118075^{vw}	0.00190358^{no}	0.00287980^{i}	0.00136000^{tu}	0.00306765^{h}	0.00564589^{b}	0.00166002^{q}
30%	0.00111158^{wx}	0.00147851^{rs}	0.00091661^{y}	0.00167611^{q}	0.00215030^{l}	0.00121379^{v}	0.00237421^{k}	0.00427763^{e}	0.00132259^{tu}
40%	0.00092831^{y}	0.00127411^{uv}	0.00074755^{z}	0.00137667^{t}	0.00178066^{p}	0.00094700^{y}	0.00198763^{mn}	0.00403427^{f}	0.00104136^{x}

*Note: * = % inclusion by volume) in 10ml of camel milk); Means shown by similar superscripts (letters) within a column or within a row are not significantly different at 5% level of significance. Values in the table are means of triplicate samples and expressed in [Load at yield (Newton)]; nd=not detected.*

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3.3.3. Proteolytic activity of partially purified seed and leaf extracts

The proteolytic activity of partially purified *Moringa oleifera* seed and leaf extract was determined by using camel whole casein as a substrate and 0.812 and 0.47 Unit/ ml proteolytic activity was recorded, respectively (Table 6). This result is much lower than the value (2.5 Unit/ ml) reported for partially purified *Moringa oleifera* seed extract on cow milk casein by Amna *et al.* (2014). This variation may be due to the inherent characteristics of the substrate (milk). Thus, in the current study, the ratio of milk-clotting activity to proteolytic activity of the partially purified extract was evaluated and compared with partially purified leaf extract (Table 6). Strikingly, partially purified extract from *Moringa oleifera* seeds showed higher ratio of milk clotting activity to proteolytic activity compared to that of leaf extract. The results obtained indicated that the partially purified extract of *Moringa* seeds was highly active compared to leaf extract. This result is also supported by the coagulation behaviour of the enzyme which indicated that the partially purified seed extract greatly coagulated the milk compared to leaf extract and the control sample. Additionally, the partially purified extract from *Moringa oleifera* seeds efficiently clotted the milk and formed a white and firm curd. It has been previously reported that the ratio of milk-clotting activity/proteolytic activity of vegetable rennet was lower, resulting in cheese that was bitter during ripening time (Amna et al. 2014). It is worth to note that in the current study the partially purified milk-clotting enzyme from *Moringa* seeds demonstrated the highest ratio of milk-clotting to proteolytic activity. Consistent with the results of this study Amna *et al.* (2014) reported that partially purified enzyme from *Moringa* seed had higher ratio of milk-clotting activity to protolytic activity.

3.3.4. Protein content of seed and leaf of partially purified extract

The protein contents of the partially purified seed and leaf extracts were found to be 0.88 mg/ml and 0.35 mg/ml, respectively, as determined from the standard curve (Table 6). The differences in protein content of seed extracts were statistically significant. Clearly, this suggest that *Moringa oleifera* seed extract may increase clotting of milk and thereby increase cheese yield. Cheese output is directly related to the amount of milk solids in the milk and more specifically, to the amount of protein. This is because cheese product is formed mainly by the coagulation of proteins in milk, thus the greater protein content, the greater the yield of cheese product (Adedeji and Nwanekezi, 1987).

Table 6. Overall characteristics of partially purified seed and leaf extracts of *Moringa oleifera* L.

Partially purified extracts	Protein contents (mg/ml)	Optimum curd firmness (Load at yield (Newton))	Proteolytic activity (Unit/ ml)	Optimum clotting activity (Unit/ ml)	Ratio of clotting activity to proteolytic activity
Seed	0.88	0.0268732	0.812	35.3616	43.5487685
Leaf	0.35	0.00969161	0.47	12.87888	27.4018723

4. Conclusion

In this experiment we found partially purified seed and leaf extract resulted in clotting activities and curd firmness at different temperatures, pH values, and concentrations on camel milk. Comparison of seed and leaf extract indicated that the seed extract can be used in the cheese industry as an alternative to gastric rennet enzyme due to its high coltting activitiy and specificity in terms of high ratio of milk-clotting to proteolytic activity. In addition, application of *Moringa oleifera* seed extract as camel milk coagulant agent warrants further study on the complete purification and characterization of this promising enzymes along with evaluation of the quality of cheese curd produced by its action.

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