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Use of Artichoke (*Cynara scolymus*) Flower Extract as a Substituteto Rennet in the Manufacture of Camel Milk Cheese

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ABSTRACT

Background: The present study aims to improve coagulation abilities of camel milk using enzyme extracts from artichoke.
Methods: The properties of purified extracts were studied in this research and a cheese was manufactured with artichoke extracts.
Results: It showed an extraction yield of 38.83% and optimum coagulation conditions of the artichoke enzyme extract were: pH=5; temperature= 55°C; $CaCl_2$ concentration= 0.01M and optimal enzyme concentration= 1%. A fresh cheese made from camel milk with a particular nutritional quality, consistency. The artichoke proteases displayed chymosin-like properties and thus hold the best potential for use as a milk coagulant in cheese production.

Key words: Camel milk cheese, Clotting activity, Plant enzymes, Proteolytic activity.

INTRODUCTION

Camel milk is popular for its traditionally anti-infective (Morrin *et al.,* 2021), anti-cancer (Krishnankutty *et al.,* 2018), anti diabetic (Sboui *et al.,* 2022; Ashraf *et al.,* 2021) proprieties and more generally as a restorative agent in convalescent patients. This could be attributed to some of its components as antimicrobial factors such as Lactoferrin, Lysozyme, Lactoperoxidase and Immunoglobulin (Konuspayeva *et al.,* 2004).

Camel milk has been used fresh or fermented in different regions of the world but rarely processed into cheese due to the low amount of ê-casein (Farah, 1993) and the high content of salts and enzymes inhibiting the growth of bacterial flora (Ramet, 2003). It also have particular properties which make coagulation difficult, especially using conventional bovine rennet. In spite of the above difficulties, satisfactory cheese have been made when cheese-making procedures were adapted to camel's milk particular characteristics (Kamoun, 1990; Ramet, 1993). In general, bovine chymosin is the most used enzyme in cheese-making procedure. However, the availability of calf rennet has become limited due to the worldwide increase in cheese manufacture, coupled with the reduction in the slaughter of calves due to their low meat production, has led to the search for milk-clotting enzymes alternatives, as appropriate rennet substitutes (Rolet-Répécaud *et al.,* 2013; Shah *et al.,* 2014; Leite Júnior *et al.,* 2017). Proteases from plant sources offer a high potential as non-animal rennet alternate in production of cheese, food (*e.g.*, production of novel dairy products, meat tenderizers and protein hydrolyzates production) and medicine (*e.g.*, digestive and anti-inflammatory agents) (Katsaros *et al.,* 2010; Huang *et al.,* 2011). An increasing interest toward milk clotting agents obtained from plants in cheese industry have been noticed because of their easy availability and simple purification processes (Shah *et al.,* 2014; Ben Amira *et al.,* 2017). Furthermore, the use of plant proteases in cheese manufacturing promotes the greater acceptability by the vegetarians, some ethnic groups and exigency of banning genetically engineered food (Grozdanovic *et al.,* 2013) or ban on recombinant calf rennet in some European country (Roseiro *et al.,* 2003). For years, plant extracts have been used as milk coagulants in traditional cheeses which are mainly produced in Mediterranean countries, West Africa and Asia (Rosario *et al.,* 2003). The extracts of the flowers of two other Cynara species, *C. humilis* and *C. scolymus*, have also been claimed to be effective as rennet substitutes (Verissimo *et al.,* 1998). Likewise, Sidrach *et al.* (2005) have purified the proteinases of *C. scolymus*, a species that contains three main proteinases (cynarases A, B and C) with milk clotting activity and gave satisfactory results. Recently, Bueno-Gavilá *et al.* (2020) determined the proteolytic activity of *C. scolymus* flower extracts on bovine casein and evaluated the suitable proteolysis conditions of pH, temperature, substrate concentration, enzyme concentration and hydrolysis time. This study showed that the proteases present in artichoke flower extracts showed a high proteolytic activity on bovine casein and the casein hydrolysates obtained contained

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bioactive peptides with antioxidant and antihypertensive activity in vitro. All these results indicate that it would be possible to use proteases from the artichoke flower in the cheese manufacturing process, which would also entail the use of an abundant agricultural residue.

Recently, we tested the use of plant extracts as clotting agents substitutes in cheese made from camel milk known as hard to coagulate milk. Results revealed that a fresh cheese with satisfactory physicochemical, microbiological and nutritional quality could be obtained from camel milk using an enzymatic extract of fig (*Ficus carica*), kiwi (*Actinidia deliciosa*), pineapple [*Ananas comosus* (L.) *Mer*] and ginger rhizomes (*Zingiber officinale*) (Fguiri *et al.,* 2021a and b). Yet, further research was recommended in order to improve camel milk clotting proprieties and enhance the outcome of the cheese making process by proposing substitutes for commercial rennet. Hence, the objective of the present study was to evaluate the camel milk-clotting activities of artichoke flower extract and to establish the most adequate conditions to obtain fresh cheese made from camel milk in terms of temperature, pH, $CaCl_2$ concentration and enzyme concentration compared to camel calf rennet.

MATERIALS AND METHODS

Samples

Mature artichoke flowers (*Cynara scolymus L.)* from fresh, well-developed flower heads are purchased from a local market (Medenine, Tunisia). The variety used in our study is the cultivar called 'Violet d'Hyères'. This variety is exploited in Tunisia during the autumn and winter periods till the second half of February. Camel chymosin FAR-M was obtained from CHYMAX® M. 1000 International Milk Clotting Units (IMCU) ml 1, Chr. Hansen A/S, Hørsholm, Denmark was used as control. Fresh camel milk was collected from she-camels (*Camelus dromadarius)* belonging to the Arid Land Institute camel herd (IRA Medenine, Tunisia, in 2021). Samples were brought to the laboratory in an isotherm container and were analyzed and processed upon arrival.

Preparation of the enzymatic extract

The flowers were dried for about three weeks at a room temperature not exceeding 25°C and shielded from light, scattered on filter paper according to the process used by Tsouli (1974). Extraction is performed on 10 g of dried flowers using a sodium acetate buffer solution at 0.1 M, pH 5 adjusted with boric acid at 0.2% depending on the optimized process in the laboratory. After macerating for 24 h under gentle agitation, then freezing and thawing, the solution obtained is centrifuged at 1000 rpm for 45 min at 4°C (Thermo Electron LED GmbH Am Kalkberg, Germany). The recovered supernatant goes through 2 successive filtrations, on filter paper, then a vacuum filtration on a 0.4 microns membrane. The crude, enzymatic extract is adjusted to enzymatic stability pH at 5 (Laurent 1974; Tsouli 1974).

Characterization of the enzyme extract

Extraction yield and characteristics

The extraction yield (EY) constitutes the ratio between the weight of the extract obtained and the total weight of artichoke powder prepared for the extraction. This yield is given as a percentage according to the following formula:

$$EY\ (\%) = \frac{\text{Extracted weight}}{\text{Total weight}}$$

pH and dry matter of enzymatic extract were determined using International standard methods (AFNOR, 1993). The protein content was determined according to the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as standard.

Optimization of pH, temperature and CaCl2 concentrations

Optimum pH and temperature were determined according to the method of Kunitz (1947). To determine the optimal pH for the enzyme activity; 1ml of plant extract was added to 10 ml of camel milk at different pH values (5; 5.6; 6.2; 6.6; 7.5 and 8.5) at 30°C and flocculation time (Tc) were measured. In order to determine the optimum temperature, the purified enzyme solution was incubated with casein solution at various temperatures ranging from 30 to 60°C for 10 min in a controlled temperature water bath and the flocculation time was noted. The optimal $CaCl_2$ concentration was prepared by dissolving 12 g of skimmed milk powder in 100 ml of $CaCl_2$ solution with a calcium ion concentration range of 0.01 to 0.09 M, added in tubes containing 10 ml of milk at pH= 6.6 and at T= 30°C, the flocculation time was then determined.

Milk-clotting activity

The milk-clotting activity (MCA) of artichoke extract was determined according to the Berridge method (1945) modified by Collin *et al*. (1977) with some modifications. One unit of enzymatic activity or rennet unit (RU) corresponds to the number of units of weight or volume of milk that can be coagulated with 1 ml of coagulant preparation in 100 seconds and at optimal pH and temperature for the plant extract or camel chymosin. The time needed for curd formation was recorded (Tc). MCA was expressed as RU units and calculated as follows:

$$RU = \frac{(10 * V)}{(Tf * Q)}$$

- RU: Unit of coagulating activity or rennet unit.
- V: Volume of substrate (ml).
- Q: Volume of coagulant extract (ml).
- Tf: Flocculation time (sec).

Proteolytic activity

Proteolytic activity was determined by the method of Bergere and Lenoir (1997) using BSA as a substrate. Briefly, 1 ml of 1% protein substrate solution (0.1 M Phosphate buffer, pH 7.0)

was mixed with 1ml of coagulant and incubated for 60 min at 35°C. After incubation, the reaction was stopped by the addition of 1ml of 12% (w/v) trichloroacetic acid. The mixture was vortexed vigorously. The absorbance of the clear filtrate was measured at 280 nm. One unit of the enzymatic activity (U) was defined as the amount of protein that gave an increase of one unit in the absorbance at 280 nm under the described conditions.

Cheese making process

Fresh camel milk was pasteurized at 65°C for 30 minutes then cooled to 40°C. The starter culture (*Lactococcus lactis*) isolated from artisanal fermented milk was then added to decrease pH at 5.5. After about one hour, the enzymatic preparation was then added at the rate of 10% of milk and mixed thoroughly. The mixture was incubated for 24 hours at 37°C. After coagulation the whey was drained to obtain a fresh cheese and kept at 4°C for further analysis.

Cheeses characterization

The physicochemical characteristics were determined using International standard methods (AFNOR, 1993). The protein content was determined according to the Bradford method (Bradford, 1976), using (BSA) as standard. The moisture content (Hm) was calculated according to the following formula:

$$Hm = 100\text{-}DMC$$

Where:
Hm: Humidity (%).
DMC: Dry matter content.

Sensory analysis

Samples of cheese were subjected to sensory evaluation by 42 untrained panelists. Cheese samples were assessed for their taste, color, flavor, acidity and texture. Panellists received a set of two samples, representing cheeses made with artichoke extract and the control made with chymosin. Each sample was evaluated in duplicate. The panellists were asked to drink plain water at the beginning of the sensory evaluation and between samples to try to make the palate conditions similar for each sample. Cheese traits are rated on the basis of 10 cm unstructured lines with 10 points scale (0 = lower intensity, 10 for higher intensity). Scores were the distances (cm) from the left anchor point. At the end of sensory evaluation, panellists were asked to rank overall cheese acceptability.

Statistical analysis

The data obtained were subjected to a statistical analysis of variance (ANOVA) using SAS software (version 9.0) By the GLM procedure. Results are reported as means± standard deviation, which were separated by the comparison test Duncan's multiple at $p \leq 0.05$ (Cochran and Cox, 1992). Triplicate measurements were performed for each analysis.

RESULTS AND DISCUSSION

Characterization of enzymatic extract

The enzymatic extract yield was 38.81%± 8.63 and this yield was higher than that found for cardoon flowers (6%) cited by Zikiou (2013).

The physicochemical characteristics of the enzymatic extracts obtained were showed in Table 1. The pH equal to 5.00±0.01, a dry matter content of around 48.31±7.06 g/l and the total protein concentration is relatively high (17.64 g/l) compared to the result of Nouani *et al.,* (2009) who found 5.6 mg/ml.

Optimum conditions of the enzymatic extract clotting activity

As shown in Fig 1, the temperature for optimal clotting activity of enzymatic extract of artichoke was at 55°C. The effect of temperature proceeds mainly on the secondary phase of coagulation which corresponds to the aggregation step. This is due to the importance of hydrophobic interactions in the aggregation of hydrolysed micelles (Boudjenah, 2012). These results contrast with those reported by Sidrach *et al*. (2005) and Nouani *et al*. (2009) who indicate that the best coagulation activity was observed at 65-70°C using bovine milk.

The optimal clotting activity was at pH 5 for artichoke extract (Fig 1). These results are in agreement with those reported by Nouani *et al.* (2009) and Sidrach *et al.* (2005) while Chen *et al.* (2008) reported that the optimum pH value for artichoke proteases was 6. Ramet (1989) reported that all enzymes used in cheese manufacture are acidic proteases, their activity are generally optimal at pH values close to 5.5.

$CaCl_2$ concentration (0.01-0.09 M) has no significant effect on clotting activity (Fig 1). Similar results were mentioned by Castillo *et al.* (2002) who consider that the main effect of $CaCl_2$ is important on aggregation and firming. Thus, it is usually added as a texturing agent. Indeed, the presence of ionized calcium is essential for the achievement of the secondary phase of milk coagulation since Ca_2 + ions neutralize negative casein micelle residues to form a firm curd in the second phase of the coagulation process (Pires *et al.,* 1998).

Proteolytic activity

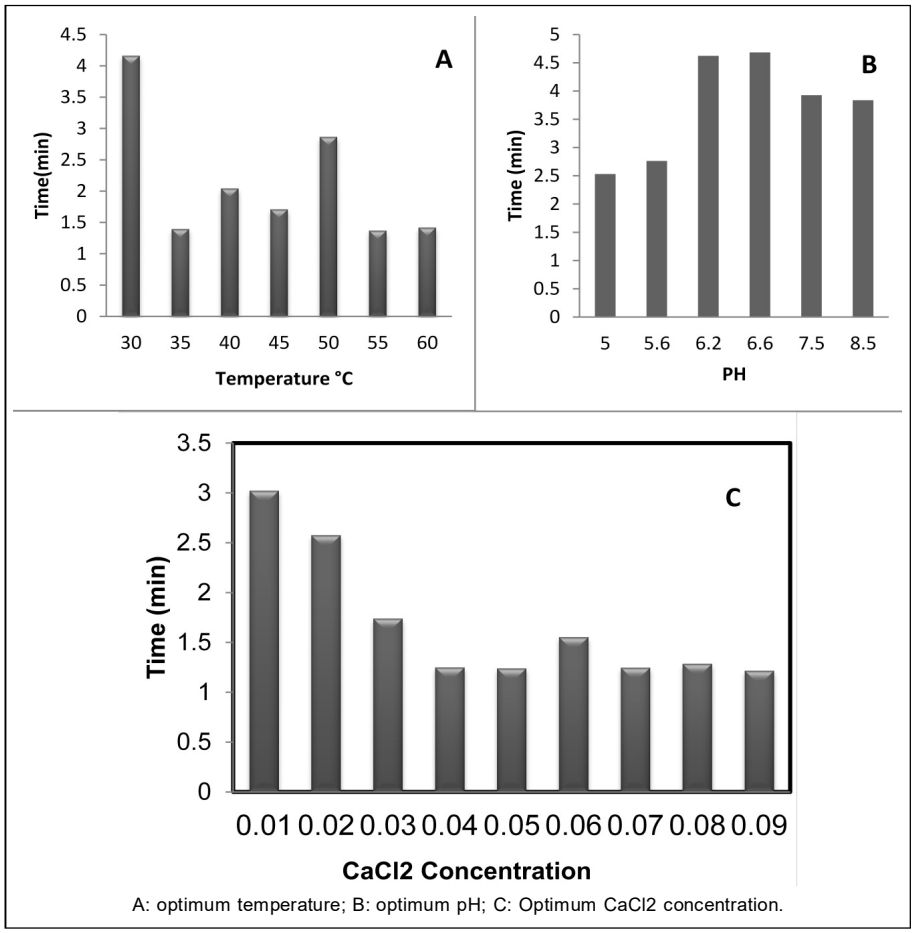
It is certain that the coagulant proteases present a double activity: one very specifically on casein, the other of general

Table 1: Physicochemical characteristics of the enzymatic extract.

Parameter	
pH	5.00±0.01
Proteins (g/l)	17.64±2.48
DM (g/l)	48.31±7.06
Ash (g/l)	16.85± 0.49

proteolysis likely to appear during the maturing process. Proteolysis is one of the most important phenomena of the maturing process, because it affects not only the flavor of the cheese but also, its appearance and texture. It results in the successive release of peptides followed by amino acids. The latter can be degraded into varied compounds, thus, contributing to the appearance and the flavor.

The result of proteolytic activity using artichoke enzymatic extract compared to camel chymosin was showed in Fig 2. Clearly, artichoke enzymatic extract had higher proteolytic activity compared to animal rennet. In the cheese industry, it is still sought that the conventional coagulating enzymes used have high coagulant activity and low proteolytic activity (Ramet, 1997).



A: optimum temperature; B: optimum pH; C: Optimum CaCl2 concentration.

Fig 1: Optimum condition of clotting activity of enzymatic extract:

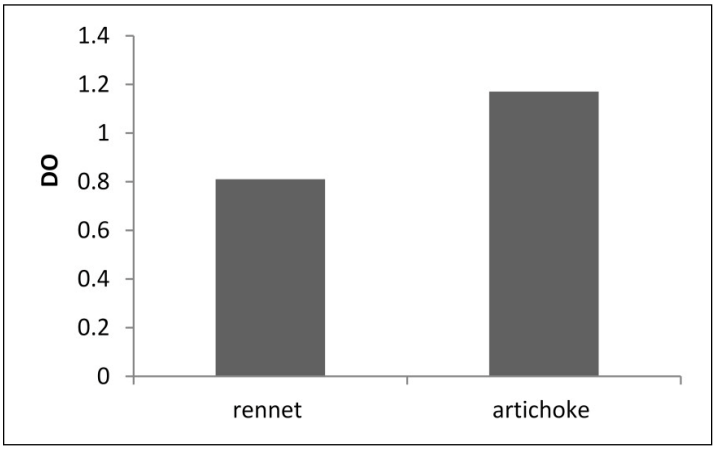


Fig 2: Proteolytic activity of enzymatic extract compared to camel rennet.

The proteolytic activity of the extracts depends on several factors, such as the plant source, concentration and type of protease (Silvestre *et al.,* 2012). Indeed, aspartic proteases with milk-clotting activity have been reported in artichoke (*Cynara scolymus* L.) (Llorente *et al.,* 1997). Also, Bueno-Gavilá *et al.,* (2020) reported high proteolytic activity of Cinarases present in artichoke flower extracts on bovine casein. It was observed in dairy products that these proteases, due to their specificity, produce a higher concentration of hydrophobic peptides in cow milk cheese (Agboola *et al.,* 2004) and goat milk cheese (Tejada *et al.,* 2008) that present a greater inhibitory activity of the angiotensin converting enzyme-I (ACE-I) (Hernández-Ledesma *et al.,* 2008; Alvarado *et al.,* 2010; Bueno-Gavilá *et al.,* 2020). All these results indicate that it would be possible to use proteases from the artichoke flower to obtain hydrolysates rich in bioactive peptides (Bueno-Gavilá *et al.,* 2020).

Cheese curd yield

Artichoke enzymatic extract showed the highest curd yield (20.71%, Fig 3) compared to camel chymosin. This can be explained by the fact that Cheese yield also depends on other factors such as milk quality and composition, heat treatment of milk, type of cheese and the processing methods used (Mazorra-Manzano *et al.,* 2013). Plant coagulants have long been considered as possible substitutes for chymosin in the cheese making process (Fguiri *et al*., 2022), but their potential for such use depends on their catalytic properties, stability and specificity, as these factors can affect cheese yield and sensory properties (Jacob *et al.,* 2011). In the cheese industry, the choice of the coagulating enzyme is a very determining factor. The most appropriate enzyme is the one with the highest clotting activity. The clotting activity is very variable because it is strongly influenced by the state of maturity of the plant and by the conditions of collection and storage (Veisseyre, 1979).

Physicochemical characteristics of curd cheese

The physicochemical characteristics of camel cheese with artichoke enzymatic extracts are shown in Table 2.

A significant difference on ash and protein content depending on the type of extract was revealed. The curd obtained with artichoke extract showed the lowest ($P<0.05$) water content over all, although this is still in the range of 60-70% reported for fresh cheese (Lobato- Calleros *et al.,* 2006; Torres-Llanez *et al*., 2006). Proteins content was higher in camel milk cheese with artichoke than with chymosin.

Sensory evaluation of curd cheese

The sensory quality of cheeses varies according to the manufacturing technology and the chemical and microbiological characteristics of the raw material used. The result of sensory evaluation is shown in Table 3.

The adding of enzymatic extract had significant effect on taste, smell, acidity and color of camel cheese. However, camel cheese with artichoke was more acid than with chymosin and that with artichoke have the best texture. The cheeses obtained are characterized by a slightly bitter taste whatever the extract used. Benani (2017) stated that the coagulant agent is characterized by a high proteolytic activity which confers a bitter taste on cheese.

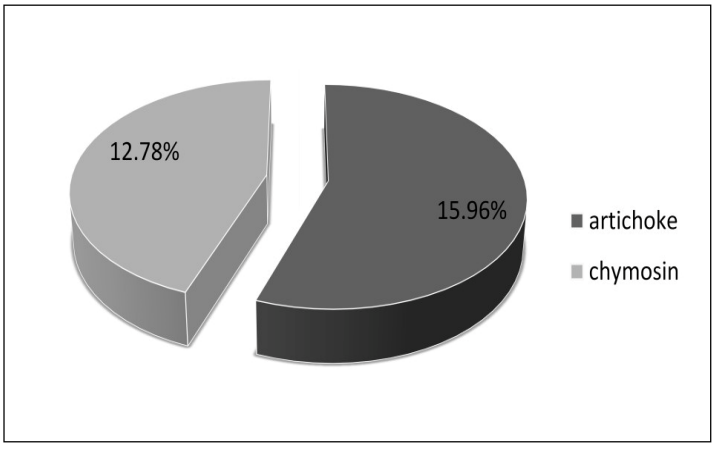


Fig 3: Curd yield (%) with different enzymatic extract (Chymosin, Artichoke).

Table 2: Physicochemical characteristics of camel's cheese.

Type of cheese	pH	Dry matter (%)	Ash (%)	Humidity (%)	Proteins (g/l)	Fat
CMC A	5.47±0.03^{a}	35.66±2.75 ^{a}	2.41±0.35^{c}	64.34±2.75^{b}	31.42±0.13^{a}	16.38±0.70^{a}
CMCChy	6.01±0.02^{a}	35.17±0.43^{a}	5.19±0.32^{a}	64.84±0.43^{b}	28.25±0.47^{b}	13.02±6.78^{a}

a,b means in the same line followed by the same letter are not statistically different $P>0.05$; CMC A: Camel milk cheese with Artichoke; CMC Chy: Camel milk cheese with chymosin.

Table 3: Sensory evaluation of camel curd cheese with different extract.

	Taste	Texture	Odor	Acidity	Colour
CMCA	5.44^{b}±3.86	5.30^{b}±3.81	4.68^{ab}±3.74	5.83^{a}±4.26	6.66^{a}±3.46
CMC Chy	1.70^{c}±1.49	4.64^{b}±2.92	2.54^{b}±2.11	2.91^{ab}±2.87	4.38±3.34

Values with different superscript letters within the same column are different (P<0.05). Values are the mean±standard deviation.

CONCLUSION

The artichoke enzymatic extract showed the highest potential for use as a milk-clotting agent in camel milk cheese making, since it produced a curd with similar characteristics to those obtained when using commercial chymosin.

The textural properties of fresh curds obtained with artichoke extracts were similar to those produced using chymosin. The differences observed between plant coagulants and chymosin may have some effect on the texture and flavor of cheeses, opening the possibility for the production of new cheese varieties with additional plant flavors.

Conflict of interest

All authors have no conflict of interest.

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