ORIGINAL ARTICLE

Milk Clotting, Rennet Properties of Latex from Medicinal Plants and Its Nutritional Analysis

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ABSTRACT Rennet is protease enzyme that acts as a milk coagulant for the production of cheese in the dairy industries. Generally, the rennet used is from animal sources which are of less availability and high in cost. Hence an alternative source of rennet from plants and microbes were needed. Lattices are collected from the plants *Artocarpus heterophyllus*, *Carissa carandas*, *Carica papaya* and *Ficus Carica* homogenized and stored in 0-5 °C. Phytochemical analyses of lattices were done in methanol, chloroform and aqueous solvents for the selected plant species. The lattices in methanol solvent showed good result in phytochemical analyses compared to the other solvents. Antioxidant activities of the collected lattices were determined by DPPH assay. The milk clotting property of the crude latex was then analyzed and the cheese was extracted. The percentage of yield, total solid, moisture content and chemical composition of resultant cheese samples were also compared with the standard. It is concluded that the yield obtained from the rennet of *Carissa carandas* and *Carica papaya* showed best results comparatively which would be used in dairy industries with consistent efficiency of clotting.

Keywords: *Artocarpus heterophyllus*, *Carissa carandas*, *Carica papaya*, *Ficus Carica*, Plant Rennet, Lattices, Milk clotting, Cheese

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INTRODUCTION

Milk is the essential beverage that occupies a unique position among many foods as it plays a major role during the first part of the lives of humans and all mammals. It has all the necessary nutrients that help in the growth and development of the young organisms that includes vital protein, minerals etc. which has the valuable fat products in health. Generally, milk and milk products have been considered as an important part of a balanced diet of every individual due to the rich constituents of nutrients compared to other foods or drinks.[1] Cheese is one of the products obtained from the milk and currently cheese production is about 19×10^6 tons per annum in North, Europe, South America and Oceania. Globally, 35% of total milk is used for cheese production approximately.[2] Cheese processing is the most commonly used methods of milk preservation involving complex process of concentrating protein along with a variable fraction of fat and minerals by eliminating a significant amount of water and lactose.[3]

Rennet is a type of protease enzyme used for clotting of milk which causes the milk proteins (caseins) to clump together. They change the liquid milk proteins into a semi –firm gel usually called curd and is cut into small pieces to promote the expulsion of the whey.[2] Rennet can be extracted from various sources animal, plant and microbial sources. Currently, in dairy industries, Calf rennet which consists of over 90% chymosin is commonly used for the curdling of milk. There are many steps involved in the extraction and subsequent purification of calf rennet from the tissues of animal stomach which makes demand for cheese production. In Mediterranean countries, rennet from the animal source is widely used to produce various cheeses including Protected Designation of Origin (PDO) cheeses. Being prepared from plant source, it can also meet the demands relating to religious priorities.[4]

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Plant rennet also plays an alternative role as a coagulant in the cheese technology. The selection of plant coagulant is necessary due to the increasing global demands of cheese with reduced supply of calf.[5] Proteases from plants are used in milk coagulation and cheese-making process especially in Mediterranean countries, Middle East, West Africa and Southern Europe.[3] Plant latex is a rich source of various hydrolytic enzymes that are responsible for their diverse health applications[6] and it's a natural source of pharmaceuticals.

Plant lattices of *Artocarpus heterophyllus* and *Ficus carica* belonging to the family *Moraceae, Carissa carandas* of family *Apocynaceae* and *Carica papaya* of family *Caricaceae* (Figures 1-4) have the milk clotting properties which is used as an alternative source of animal rennet.

Based on the above criteria, the present study was designed to evaluate the milk clotting properties of lattices collected from the four different plants *Artocarpus heterophyllus, Carissa carandas, Carica papaya* and *Ficus Carica* in bovine milk using Amul cheese as standard and the nutrient analysis of the cheese formed was also done. The phytochemical content was analyzed in the crude latex of selected plants along with it antioxidant activity was also determined.

MATERIALS AND METHODS

Chemicals

All the chemicals, reagents and enzymes used in the study was procured from Himedia Lab Ltd., Mumbai, India, Amul Cheese used as reference was obtained commercially.

Figure 1: ***Artocarpus heterophyllus***

Figure 2: ***Carissa carandas***

Figure 3: ***Carica papaya***

Figure 4: ***Ficus carica***

Latex Collection and Extraction

The latexes are extracted from the unripe fruit and bark samples of selected medicinal plants *Carissa carandas, Artocarpus heterophyllus*, *Carica papaya* and *Ficus Carica* (Figure 5) from Yercaud adivaram and around Kolli hills in cool climate during morning.

Phytochemical Analysis

The phytochemical analysis was done in the crude latex of all the selected plants using the three solvents - Polar solvent (Water), Non polar (Chloroform), and Bipolar (Methanol). For the further phytochemical analysis, 5ml of latex of each plant were extracted and added to 20ml of water, chloroform and methanol.

Preliminary phytochemicals analysis was carried out for all the extracts as per standard methods described by Brain and Turner 1975 and Evans 1996. Polar, non-polar and bipolar extract of *Carissa carandas*, *Artocarpus heterophyllus*, *Carica papaya* and *Ficus carica* obtained by the above method was subjected to qualitative analysis of Alkaloids, Flavonoids, Steroids, Terpenoids, Anthraquinones, Phenolic groups, Saponins, Tannins, Glycosides, Oils and gums as described by the method of Trease and Evans (2002), and also as specified in the book of Practical Pharmacognosy.

Detection of Alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrates were used to test the presence of alkaloids.

Mayer's Test: Filtrates were treated with Mayer's reagent. Formation of a yellow cream precipitate indicates the presence of alkaloids.

Wagner's Test: Filtrates were treated with Wagner's reagent. Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

Detection of Flavonoids

Lead Acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

H_2SO_4 Test: Extracts were treated with few drops of H_2SO_4. Formation of orange colour indicates the presence of flavonoids.

Detection of Steroids

2 ml of acetic anhydride was added to 0.5 ml of the extracts, each with 2 ml of H_2SO_4. The colour changed from violet to blue or green in some samples indicate the presence of steroids.

Detection of Terpenoids

Salkowski's Test: 0.2 g of the extract of the whole plant sample was mixed with 2 ml of chloroform and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the inner face was indicating the presence of terpenoids.

Detection of Anthraquinones

Borntrager's Test: About 0.2 g of the extract was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heated. Formation of pink color indicates the presence anthraquinones.

Detection of Phenols

Ferric Chloride Test: Extracts were treated with few drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenol.

Lead Acetate test: Extract was treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of phenol.

Detection of Saponins

About 0.2 g of the extract was shaken with 5 ml of distilled water. Formation of frothing (appearance of creamy miss of small bubbles) shows the presence of saponins.

Figure 5: Latex Samples Collected from (a) *Artocarpus heterophyllus*, (b) *Carissa carandas*, (c) *Ficus carica*, (d) *Carica papaya*

(a) (b) (c) (d)

Detection of Tannins

A small quantity of extract was mixed with water and heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green color formation indicates the presence of tannins.

Detection of Carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrate was used to test the presence of carbohydrates.

Detection of Oils and Resins

Test solution was applied on filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and resins.

Antioxidant Activity of the Lattices

The efficacies of the lattices from the selected plants were studied under *in vitro* conditions by 1-diphenyl-2-picrylhydrazyl (DPPH).

DPPH Radical Scavenging Activity

The DPPH free radical which is purple in color is reduced to a corresponding hydrazine when it reacts with hydrogen donors and changes to yellow color. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490 nm. The reagents used are 2-Diphenly 1-picryl hydrazyl solution (DPPH, 100 µM): 22 mg of DPPH was accurately weighed and dissolved in 100ml of methanol. From this stock solution, 18 ml was taken and diluted to 100 ml using methanol to obtain 100 µM DPPH solutions. The test solutions were prepared by dissolving 21 mg each of the extracts in dimethyl sulfoxide (DMSO) separately to obtain solutions of 21 mg/ml concentrations. Each of these solutions was serially diluted separately to obtain lower concentrations. 10 mg each of ascorbic acid and rutin were weighed separately and dissolved in 0.95 ml of DMSO to get 10.5 mg/ml concentrations. These solutions were serially diluted with DMSO to get lower concentrations. 200 ml of DPPH solution, 10 ml of each of the test sample and the standard solutions were added separately in 96 well microtiter plates. The final concentration of the test and standard solutions used were 100, 80, 60, 40, and 20 µg/ml. The plates were incubated at 37 ºC for 30 minutes and the absorbance of each solution was measured at 517 nm, using a micro plate reader.

Milk Sample Collection and Cheese Preparation

Milk samples were collected from the cow in the cattle field located in Rasipuram. It was collected in the early morning and immediately transferred at 4 ºC to the laboratory. The fat, protein, casein, lactose content analyses were observed. 500 ml of milk sample is boiled at 60 ºC and it is separated into two separated conical flasks as 250 ml each. Later 2 ml of each crude latex along with the pinch of $CaCl_2$ is added to the respective conical flask of warm milk. After 2 minutes of incubation, coagulation of milk occurs and the whey is removed using filter paper. Cheese extracted was collected and weighed.

Nutrient Analysis Cheese

The prepared cheese of total solid, moisture content, total carbohydrate, total protein, total fat and yield was analyzed by the Official Methods of Analysis (AOAC, 1990).

Determination of Yield

The yield of cheese was calculated from the following equation:

$$\text{Yield}(g/100\ g \text{ of milk}) = (W1 \times 100)/(W2 + W3)$$

where, W1 was the weight of the cheese prepared, W2 was the weight of the milk and W3 was the weight of the enzyme used.

Total Solids (TS) and Moisture Content

A known weight of grated cheese is dried at a constant temperature (102 ± 2 °C) to a constant weight. The weight after drying is the weight of total solids and is expressed as % by weight. Moisture (% cheese) = 100 – TS.

Moisture content of cheese samples was determined in triplicate using nutritional analysis methods as described above for food. 3 grams of samples were weighed into pre-dried and weighed moisture dish with tight-fit cover. Samples were partially dried and weighed on a steam bath prior to oven combustion at 105 ºC for 8 hrs. Moisture content was determined by difference and expressed as a percentage of the initial weight of cheese product.

Total Fat

One gram of cheese sample was first hydrolyzed with 4 M hydrochloric acid and placed in a water bath (70-80 ºC) with stirring them frequently for about 30-40 minutes. After cooling to room temperature it is subjected for extraction with 25 mL of petroleum ether. During extraction, upper ether layer was taken into clean dried weighed flasks and dried them in a water bath at 80 º C until a constant weight obtained.

% of Total fat = (Wt. of Fat/Wt. of sample) * 100

Total Carbohydrate

Total carbohydrate was estimated by the anthrone method described in the book written by Sadasivam and Manickam (2008). Briefly, 1mL aliquot of appropriately diluted sample or standard solutions of glucose (0-100 µg/ml) was taken to which 4 mL of the anthrone reagent was added and kept them in boiling water bath for 10 minutes. Cool to room temperature and measured the optical density of blue colour at 620 nm against blank.

Total Protein

Protein concentration in the enzyme extract was determined using Folin Ciocalteu reagent as per the procedure of Lowry *et al.* (1951), Crystalline Bovine Serum Albumin used as standard protein for preparation of standard curve. The absorption of the blue color developed was measured at 660 nm using spectrophotometer.

RESULTS AND DISCUSSION

Collection of Latex from the Selected Plants and Phytochemical Analysis

Qualitative analysis of aqueous extracts of *Artocarpus heterophyllus*, *Carissa carandas*, *Carica papaya* and *Ficus Carica* exhibited abundant results in alkaloids, flavonoids, tannins and moderate in amino acid, carbohydrates and low concentration in saponin, oil, steroid, gums & mucilage. In bipolar solvents, phytochemical tests like alkaloids, flavonoid, tannin, amino acid, carbohydrates were positive. In chloroform extracts, tests like alkaloid, flavonoid, protein, tannin and gums & mucilage shown positive results and the chlorogenic compounds were not deducted for all the selected plant extracts (Table 1).

Antioxidant Activity of the Lattices

DPPH Radical Scavenging Activity of Latex from Selected Plants

The activity of DPPH radical scavenging of latex from *Artocarpus heterophyllus, Carissa carandas, Carica papaya* and *Ficus carica* values were compared with standard Rutin was presented in Table 2 and Figure 6. The percentage of inhibition in DPPH with different concentration of latex from *Artocarpus heterophyllus, Carissa carandas, Carica papaya* and *Ficus carica* like 20, 40, 60, 80, and 100 µg/ml were observed in 79.99, 61.52, 41.39, 32.59, 20.77; 86.76, 68.48, 45.13, 40.21, 30.95; 41.33, 53.72, 69.00, 76.39, 84.10 and 36.18, 48.01, 60.15, 71.58, 80.60 respectively. The percentage of inhibition in DPPH in different concentration latex from

Table 1: Phytochemical Analysis of *Artocarpus heterophyllus*, *Carissa carandas*, *Carica papaya* and *Ficus carica* Latex Samples

S. No.	Name of the Test	*Aqueous*				*Methanol*				*Chloroform*			
		AH	CC	CP	FC	AH	CC	CP	FC	AH	CC	CP	FC
1	*Alkaloids*	+++	+++	+	ND	+++	+++	++	+	++	+++	ND	ND
2	*Flavonoids*	+++	+++	++	+	+++	+++	+++	+++	+	+	ND	ND
3	*Phenolic compounds*	+	ND	+ +	++	+	+++	+++	+++	ND	ND	ND	+
4	*Glycosides*	+	ND	+	ND	ND	ND	++	+	ND	ND	ND	ND
5	*Triterpenoids*	ND	+++	+	+	+++	++	+++	++	+++	+++	+	+
6	*Amino acid*	+	+++	ND	+++	++	+++	ND	ND	ND	ND	ND	ND
7	*Protein*	++	ND	ND	ND	+	++	+	ND	+++	+++	ND	ND
8	*Carbohydrates*	++	++	+	++	+++	ND	++	++	ND	ND	ND	ND
9	*Tannin*	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+	+
10	*Saponins*	++	+	+++	+	ND	ND	++	+	ND	+	ND	ND
11	*Oil*	+	++	ND	++	ND	+	ND	ND	ND	ND	ND	ND
12	*Steroid*	+	+	+ +	+	+++	+++	+++	+++	ND	ND	ND	ND
13	*Gums and mucilage*	+	+	+	+	+++	+++	++	+	++	++	+	ND
14	*Chlorogenic compounds*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

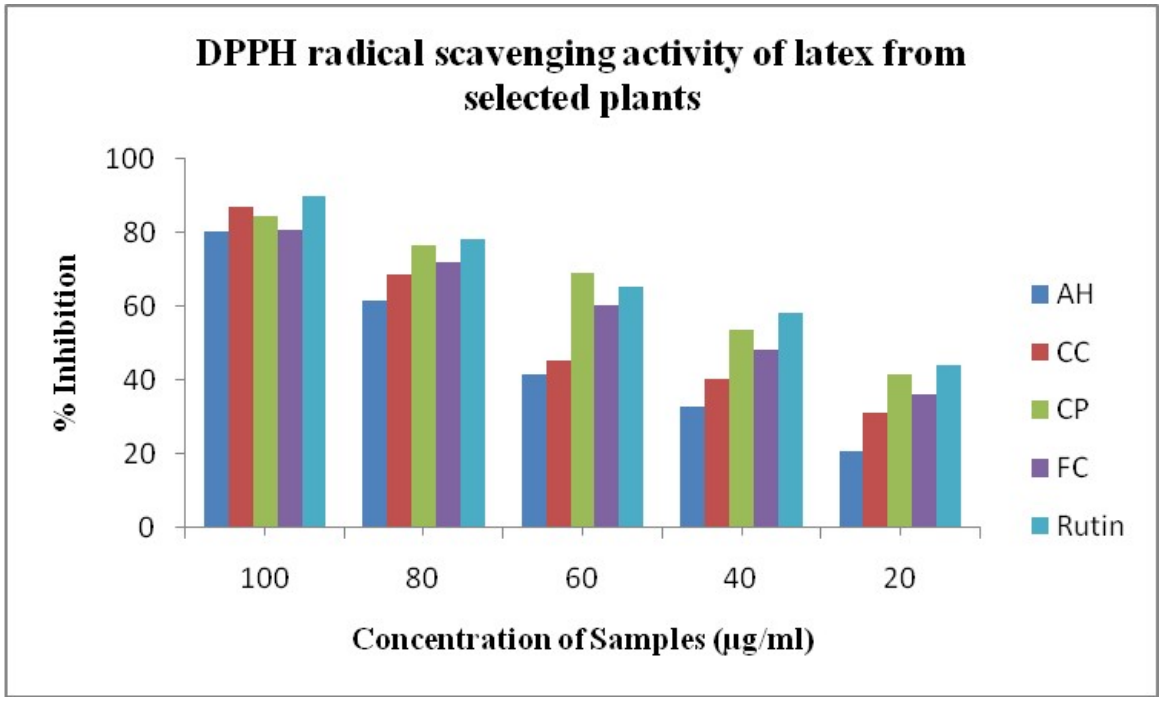
Note: AH-*Artocarpus heterophyllus,* CC-*Carissa carandas,* CP-*Carica papaya,* FC-*Ficus carica.*

Table 2: DPPH Activity of the Selected Plant

Concentration (µg/ml)	% of Inhibition				
	A. heterophyllus	*C. carandas*	*C. papaya*	*F. Carica*	Rutin
IC_{50} Value (µg/ml)	63.72	53.83	32.45	43.45	29.22

Note: AH-*Artocarpus heterophyllus*, CC-*Carissa carandas,* CP-*Carica papaya*, FC-*Ficus carica.*

Figure 6: DPPH Radical Scavenging Activity of *Artocarpus heterophyllus, Carissa carandas, Carica papaya, Ficus carica* and *Rutin*



these plants values were compared with standard Rutin like 20, 40, 60, 80, and 100 µg/ml were observed in 44.16, 58.22, 65.19, 78.05, 89.67 respectively. The IC_{50} values for DPPH scavenging activity for latex from selected plants and Rutin were 63.72 µg/ml, 53.83 µg/ml, 32.45 µg/ml, 43.45 µg/ml and 29.22 µg/ml. The higher inhibition activity was recorded dose dependent manner.

Nutritional Analysis of Cheese Sample from the Plant Coagulant

Yield

The yield of cheese products was determined on the basis of weight of coagulated milkproduct. Cheese yield produced from each of the crude enzyme of *Artocarpus heterophyllus, Carissa carandas*, *Carica papaya* and *Ficus carica* was expressed in percentage. However, *Artocarpus heterophyllus, Carissa carandas*, *Carica papaya* and *Ficus Carica* gave the yield of cheese (26.44%, 30.52%, 26.19% and 22.54%).

Total Solids and Moisture Content

Loss of weight and moisture in cheese during storage was observed. The weight loss incheese during ripening has been attributed mainly to the loss of moisture. The uptake of salt also affects the loss of moisture.[9] Cheese samples during ripening in brine confirm the Donnan equilibrium which controls the partition of ions between the curd and the brine.[2] Total solids were found to be 51.9% in *Artocarpus heterophyllus*, 55.7% in *Carissa carandas,* 51.6% in *Caricapapaya* and 56.9% in *Ficus Carica*. Highest total solids and minimum moisture content was present in Amul cheese (60.3 and 43.65%).

Total Protein

The ripening of cheese is accompanied by partial protein degradation.[10] Proteolytic enzymes such as rennin are responsible for the formation of nitrogenous products ofintermediate size, such as proteases, peptones, polypeptides, peptides and free amino acids. Enzymes of

micro-organisms act on these and other substances to form products like amino acids, amines, fatty acids, esters, aldehydes, alcohols and ketones.[2] Cheese serves as a store house of essential amino acids, having similar proportion of essentialamino acids that is present in milk except the methionine and cysteine. The minimum decline recorded for salted samples may be due to the inhibition of micro-organisms and enzyme activity. Highest protein content was found in Amul cheese (25.0%) followed by *Artocarpus heterophyllus and Carissa carandas* (19.11% and 23.76%) and *Carica papaya* and *Ficus Carica* (19.76% and 18.3%)

Total Fat Content

According to Talib *et al.* (2009) during ripening, gradual decrease in fat content due to breakdown of fat, salt uptake and continuous loss of degraded components of cheese was observed. Previous studies have indicated that calcium chloride may inhibit lipolysis in cheese.[12] However, within the range 0.5%-3.0% (w/w) of $CaCl_2$ investigated nostatistical influence of on lipolysis.[13] Highest fat content was found in Amul cheese (30.8%) followed by *Carica papaya* (25.33%), *Ficus Carica* (22.18%), *Artocarpus heterophyllus* and *Carissa carandas* (24.41 and 27.33%).

Total Carbohydrate Content

Lactose is one of the basic nutrients consumed by lactic acid producing micro-organisms. Lactose remaining in the curd is converted into lactic acid. Lactic acid inhibits the growth of undesirable micro-organisms. It is very important in production of acid flavour in the cheese. It determines the smoothness of the body of the cheese.[14] In addition to lactose, milk contains small amounts of glucose, galactose, and other saccharides.[15] When milk is coagulated, greater percentage of the lactose is present in the whey and the remaining inthe curd. For this reason, cheese that is prepared from the curd is low in carbohydrates.[16] (Figure 7). Highest

Figure 7: Cheese Extracted from *Carissa carandas*, *Artocarpus heterophyllus*, *Carica papaya* and *Ficus Carica*

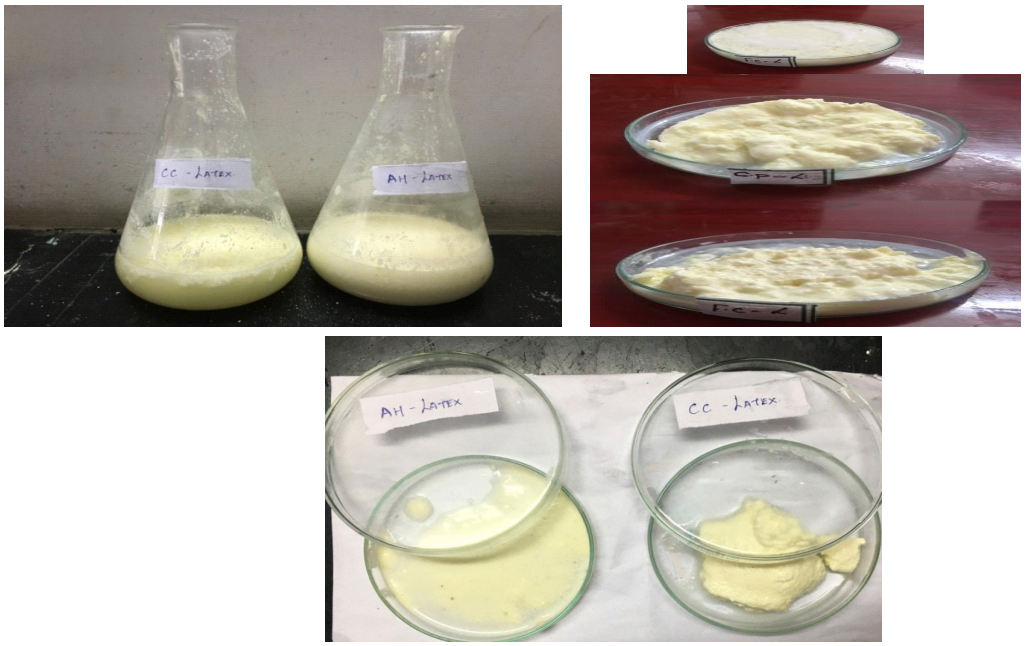
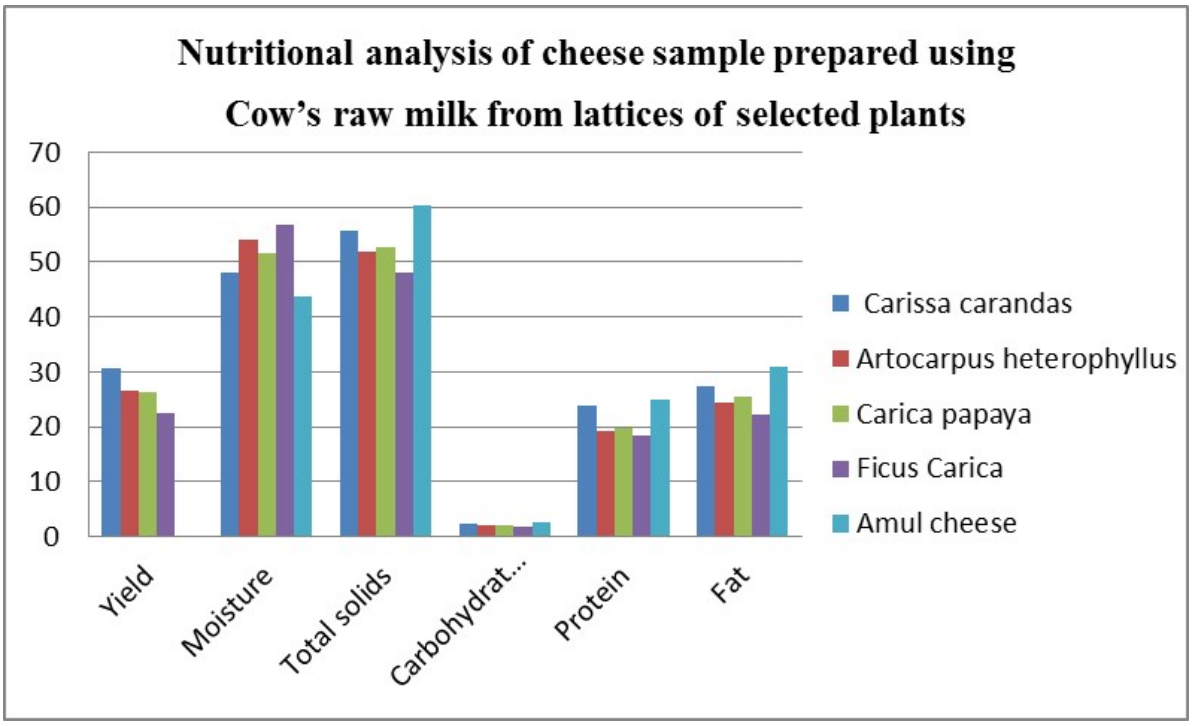


Table 3: Nutritional Analysis of Cheese Sample Prepared Using Latex of Selected Plants

S. No.	Cheese	Yield	Moisture	Total Solids	Carbohydrates	Protein	Fat
1	*CC*	30.52	48.22±1.6	55.7±2.2	2.28±2.19	23.76±0.06	27.33±1.25
2	*AH*	26.44	54.05±3.1	51.9.±1.6	1.99±2.04	19.11±1.4	24.41±0.17
3	*CP*	26.19	51.6±3.1	52.8±1.2	1.90±2.16	19.76±0.06	25.33±1.25
4	*FC*	22.54	56.9±7.2	48.1±1.3	1.65±2.60	18.30±1.2	22.18±0.11
5	Amul cheese	--	41.7±1.1	56.7±1.8	2.5±0.24	23.00±1.8	28.14±0.21

Note: All values are expressed in percentage. Values are expressed as mean ± SEM.

Figure 8: Graphical Representation of Nutritional Analysis of Cheese Sample Prepared Using Cow's Raw Milk



carbohydrate content was found in Amul cheese (2.7%) followed by *Carissa carandas* (2.28%), *Artocarpus heterophyllus* (1.99%), *Carica papaya* (1.9%), *Ficus carica* (1.6%).

The overall Nutritional analysis of cheese sample using latex of *Carissa carandas*, *Artocarpus heterophyllus, Carica papaya, Ficus Carica* was shown in (Table 3) and (Figure 8).

DISCUSSIONS

The yield of cheese produced from the plant rennet of *Carissa carandas, Artocarpus heterophyllus, Carica papaya* and *Ficus Carica* was determined to be high when compared to the yield obtained from other sources like the *Euphorbia tirculli* (20.73%), *E. nivulia* (21.41%), *E. nerifolia* (21.30%), *Pedilanthus tithymaloides* (20.49%) and *Papain* (23.49%). Total solids of *Carissa carandas, Artocarpus heterophyllus, Carica papaya* and *Ficus Carica* was found to be increased when compared with *Euphorbia tirculli* (45.7%), *E. nivulia* (48.1%), *E. nerifolia* (47.9%), *Pedilanthus tithymaloides* (48.3%) and low compared to papain (57.7%) from the previously observed data. Moisture content was found to decrease in the following order Amul cheese < *papain* < *Carissa carandas* < *Caricapapaya* < *Artocarpus heterophyllus* < *FicusCarica* < *P. tithymaloides* < *E. nivulia* < *E. nerifolia* < *E. tirucalli*. Highest protein content was found in Amul cheese (25%) followed by *C. papaya cheese* which is concordance with *Carissa carandas* (23.76%), *Artocarpus heterophyllus* (19.11%), *papain* (15.7%), *E. nivulia* (15.2%), *E. tirucalli* (14.6%), *P. tithymaloides* (13.0%) *and E. nerifolia* (12.1%). Highest fat content was found in Amul cheese (30.8%) followed by *Carissa carandas* (27.33%), *C. papaya* (25.33%), *Artocarpus heterophyllus* (24.41%), *papain* (22.33%), *E. tirucalli* (22.6%), *E. nerifolia* (22.1%), *Ficus Carica* (22.18%), *P. tithymaloides* (21.5%), *E. nivulia* (21.2%), and *papain* (19.5%). Highest carbohydrate content was found in Amul cheese (2.7%) followed by *Carissa carandas* (2.28%), *papain* (2.1%), *Artocarpus heterophyllus* (1.99%), *Caricapapaya* (1.9%), *Ficus Carica* (1.65%), *E. nivulia* (1.2%), *E. nerifolia and P. tithymaloides* have similar (1.0%), and *E. tirucalli* have minimum (0.5%).[17][18]

CONCLUSION

The results of this study proved that the plant lattices of *Artocarpus heterophyllus*, *Carissa carandas, Carica papaya* and *Ficus Carica* have milk clotting properties for the production of cheese in the dairy industries. This milk clotting enzyme (Rennet) from the selected plants would certainly be the substitute of rennet from the animal sources. The results also revealed that nutritional quality of cheese prepared from milk clotting enzymes of plant lattices of *Artocarpus heterophyllus*, *Carissa carandas, Carica papaya* and *Ficus Carica* is almost matched with commercially available Amul cheese and with some of the other cheese produced using plant rennet. Therefore, further investigation is needed to attain the similar quality and nutritional status of cheese produced with Amul

or other commercialized cheeses and it is possible by enriching the essential nutrients like mineral, vitamins, amino acids, probiotics and flavouring agents etc. in cheese samples. These results contributed to the development of new sources of rennet other than the animal sources.

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