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Extraction, Milk-Clotting Activity Measurements and Purification of *Solanum dubium* Fresen (Gubbain) for Cheesemaking

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Abstract: This investigation was carried out in order to extract a milk clotting enzyme from *Solanum dubium* Fresen (locally known as Gubbain), determine its milk clotting activity, purify and characcterize the enzyme for cheesemaking. The fruits, coats and seeds of *Solanum dubium* were separated from each other, carefully cleaned and then coarsely powdered using an electric grinder. The enzyme was extracted using four methods and the activity was determined. The proteolytic activity of the enzyme was measured and purification was carried out with ammonium sulphate (0-90% saturation). Results indicated that *Solanum dubium* seed extracted with distilled waster had the highest milk clotting activity and lowest coagulation time, while both seed and fruit extracted with 5% NaCl had the lowest activity and no clot was observed after 5 minutes. Ammonium sulphate saturation range of 40-50% for the seed extracted with distilled water gave the highest activity (91.06 U/ml), yield (27.59%) and purification fold (1.88). The partially purified enzyme was chromatographed in a column of Sephadex G-100 and the purification exhibited two peaks of proteolytic activity.The milk-clotting activity of the extract decreased with increasing pH, incubation temperature and NaCl concentration and increased with increasing $CaCl_2$ concentration.

Key words: *Solanum dubium* · Milk-clotting activity · Extraction · Purification · Characterization

INTRODUCTION

Bovine chymosin prepared from the abomasum of young calves is an acid protease almost exclusively used for the manufacture of cheese all over the world. The large increase in cheese consumption besides the increase of calf rennet's price encouraged investigators to search for other sources as substitutes for calf rennet such as animal, microbial and vegetable rennet. Much research interest has been directed towards discovering a milk-clotting enzyme which would satisfactorily replace calf rennet in cheese manufacture and numerous enzyme preparations of animal [1], plant [2] or microbial[3] origin have been isolated and studied. Some plants have been reported to have milk-clotting properties such as *Carica papaya* [4], *Cyana cardunculus* [5], *Cyana scolymus* [6], *Capparis spinosa* [7], *Fagopyrum esculentum* [8], *Thaumatococcus daniellii*[9], *Jacaratia corumbensis* [2] and *Albizia julibrissin* [10].

If animal rennet is not available or slaughter of calves for chymosin is not feasible or the cheese is only for vegetarians, vegetable rennet becomes very important and the use of vegetable rennet for cheese making could contribute to improving the nutrition of those populations, whereas restrictions are imposed against the use of animal rennet [2, 6].

Some plants of the family Solanaceae such as *Solanum dubium*, *Solanum innacum*, *Solanum esculentum*, *Solanum macrocarpon* and *Solanum melongena* have been tried for the extraction of milk-clotting enzymes [11-15].

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Solanum dubium is a well known wild plant found in most regions of the Sudan belonging to the family Solanaceae and quite often used for milk coagulation, especially in rural areas. This plant is grown widely in many places in northern, central and western states of the Sudan. The plant can be described as a bushy pubescent herb [15].

This study aimed to select an appropriate method for extraction of milk clotting enzyme from *Solanum dubium* seed and fruit and to purify and characterize the enzyme in order to use for cheese making.

MATERIALS AND METHODS

Materials: The plant material used in this study was collected from Shambat area, Khartoum North, Sudan. The fruits, coats and seeds of *Solanum dubium* were separated from each other, carefully cleaned, washed several times with distilled water and then coarsely powdered using an electric grinder. Skim milk and salt were purchased from the local market, while cow's milk was obtained from the University of Khartoum Farm. Chemicals were obtained from: 1- BDH Chemicals Ltd. Poole, England; 2- E. Merck Ltd. Bumbai, India; 3- Oxoid Ltd. Basing Stock Hants, England; 4- Aldrich Company, England; and 5- Pharmacia, Uppsala, Sweden.

Extraction of *Solanum dubium* Fruit and Seed: The crude enzyme was extracted by the following methods:

Freezing and Evaporating under Reduced Pressure (Freeze-drying): Coarsely powdered yellow fruits and seeds (100 g each) were macerated in a conical flask for 24 hours using distilled water with occasional shaking for the first three hours and solutions were then filtered. The filtrate was spread on a shallow basin surrounded by a freezing mixture under vaccum for water evaporation within two hours.

Drying in a Current of Warm Air: Extract from seeds and fruits (100 ml each) was spread on a shallow basin and exposed to a current of warm air (50°C).

Soaking in 5% Sodium Chloride and Evaporation in a Current of Warm Air: Coarsely powdered seeds and fruits (100 g) were soaked in 5% sodium chloride overnight at 5-10°C using mustard as a preservative. The solution was filtered and the filtrate was finally spread on a shallow basin and exposed to a current of a warm air at 50°C.

Extraction with Distilled Water: Five grams of the crushed material were shacken with 30 ml distilled water for 15 minutes at room temperature and then filtered. The aqueous filtrate was used for testing its milk-clotting activity [13].

Determination of Milk-clotting Activity: The milk clotting-activity of the enzyme was determined according to the method described by Mohamed and Habbani [13] with slight modifications. One gram of the dry matter was dissolved in 10 ml distilled water, shaked for three hours then filtered. One ml of the filtrate was pipetted into a glass tube containing 10 ml skim milk solution. The tubes were then placed in a water bath at 37°C and continuously examined for the first onest of coagulation. Clotting activity was determined according to the following equation:

$$\text{Activity (U)} = \frac{\text{Volume of extract}}{\text{Clotting time (seconds)}} \times 100$$

The activity is expressed in terms of units (U) which is defined as the volume of the extract required to clot 10 ml of skim milk in 100 seconds at 37°C.

Partial Purification of the Enzyme: The enzyme having highest milk-clotting activity was chosen for further purification studies using the modified method of Otani *et al.* [10]. The supernatant was treated with ammonium sulphate (0-90% saturation). The precipitated enzyme was collected by centrifugation at 3000 rpm for 20 min in a refrigerated centrifuge and dissolved in a minimum amount of distilled water for 24 hrs. The precipitated enzyme was chromatographed on a column (4.5 x 25 cm) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) with 0.2M acetate buffer (pH 4.8) for further purification. Fractions of 5 ml were collected at a flow rate of 1 ml/min and proteolytic activity was determined.

Determination of Protein Content: Protein content of the enzyme was determined colourmetrically according to Ohnishi and Barr [16]. To 8 ml of the sample, 4 ml of lowery reagent (a mixture of two stock solutions: cupric sulphate in sodium potassium tartrate and $Na_2 CO_3$ in NaOH) was added and the solution was allowed to stand for 10 min at room temperature. Phenol reagent (0.4 ml) was pipetted rapidly into the mixture with thorough mixing by a vortex mixture (total volume was 5.2 ml) and after 30 min absorbance was measured by Aminco DW-2 Spectrophotometer at 660 nm against a blank.

Effect of Incubation Temperature, pH, Cacl$_2$ and NaCl Concentrations on Milk-clotting Activity: The milk-clotting activity and coagulation time of the partially purified enzyme were studied as a function of incubation temperature, pH, $CaCl_2$ and NaCl concentrations. One ml of the enzyme extract was added to 10 ml of the substrate and the activity and coagulation time of the extract were determined at varying temperatures, pH values, $CaCl_2$ and NaCl concentrations.

Statistical Analysis: Statistical analysis was carried out using Statistical Analysis Systems (SAS, ver. 9). General Linear Models (GLM) were used to determine the effect of extraction method on the coagulation time and activity of the extract. Means were separated by Duncan multiple range test at P=0.05.

RESULTS AND DISCUSSION

Extraction of the Milk-clotting Coagulant: For the extraction of the coagulant, two parts of the plant were used namely seed and fruit. The results of the effect of extraction method on the enzyme activity are presented in Table 1. Extraction of both seeds and fruits with distilled water gave the highest milk-clotting activity compared to NaCl and freeze-drying. These results are in disagreement with Yousif *et al.* [15], Ahmed *et al.* [11] and Guiama *et al.* [12] who reported higher milk-clotting activity with NaCl. The discrepancy in these results might be the extraction time which was 15 minutes in this study, while it was 14 days and 24 hr repectively in the previous studies.

The comparative response of different milks to enzyme extract has been demonstrated in Table 2. Results indicated that high activity of the enzyme was obtained with cow's milk as compared to other types of milk used. It was clearly seen that S_0 gave highest activity in all types of milk used. This result is in agreement with Guiama *et al.* [12] who reported that coagulation tests are better with skim raw milk than reconstituted skim milk powder.

Purification of Solanum Crude Enzyme

Fractionation of Solanum Enzyme by Ammonium Sulphate Precipitation: Ammonium sulphate fractionation was used in this study, where the crude extract of distilled water (S_0) was precipitated using different ammonium sulphate concentrations (0-90% saturation). The results sowed that the saturation range of 40-50% gave the highest milk clotting activity, specific activity, total activity, yield (%) and purification fold (Table 3). The degree of saturation with ammonium sulphate greatly affected the enzyme activity, total activity, yield and the purification. Accordingly, the range of 40-50% was selected for potential purification of the milk-clotting enzyme for *Solanum dubium* seeds. These results are in confirmity with the findings of Ahmed *et al.* [11] who reported the highest total acitviy, yield and purification fold with saturation range of 40-55% and Duarte *et al.* [2] who found the best milk-clotting acitivity at saturation of 40-60%. However, Chaiwut *et al.* [4], Demir *et al.* [7] and Otani *et al.* [10] reported the highest milk-clotting activity when the enzyme was precipitated with 50-70% ammonium sulphae saturation.

Table 1: Effect of extraction method on the activity of Solanum dubium

Extraction method	Milk coagulationtime (sec)	Activity(U/ml)
Solanum seed extracted with distilled water (S0)	12.8± 2.48^a	16.10 ± 3.29^a
Solanum fruit extracted with distilled water (F0)	15.7 ± 0.82^a	12.78 ± 0.63^{ab}
Solanum seed extracted with freeze-drying (S1)	43.7 ± 38.66^a	8.00 ± 5.01^a
Solanum fruit extracted with freeze-drying (F1)	23.8 ± 7.47^a	9.3 ± 3.52b
Solanum seed extracted with distilled water and evaporated at 40°C (S2)	20.8 ± 5.85^a	10.21 ± 2.71^{ab}
Solanum fruit extracted with distilled water and evaporated at 40°C (F2)	28.8 ± 13.12^a	8.22 ± 3.54^{bc}
Solanum seed extracted with 5% Nacl (S3)	No clot up to 5 min	5.99±8.41^{bc}
Solanum fruit extracted with 5% Nacl (F3)	No clot up to 5 min	2.02 ± 2.78^c
L.S	NS	***

Means within columns bearing the same letter are not significantly different (P>0.05)

*** = P<0.001

NS = not significant

L.S = Level of significance

Table 2: Effect of extraction method on coagulation time and activity of cow, goat and skim milk

	Cow milk		Goat milk		Skim milk	
Extraction method	CoagulationTime (sec)	Activity (U/ml)	CoagulationTime (sec)	Activity (U/ml)	CoagulationTime (sec)	Activity (U/ml)
S0	10.0 ±0.00[a]	20.0±0.00[a]	13.5±2.12[c]	14. 99±2.38[a]	15.0 ± 0.00[c]	13.5 ± 0.00[a]
F0	15.5± 0.71[a]	12.9 ±0.57[c]	15.0±0.00[c]	13.30±0.00[a]	16.5 ± 0.71[c]	12.13 ± 0.52[a]
S1	14.5 ± 0.71[a]	13.79±0.69[c]	27.5±3.54[a]	7.34±0.94[b]	29.5 ± 0.71[b]	6.79 ± 0.16[b]
F1	15.0 ±0.00b[a]	13.3 ± 0.00[c]	23.50±2.12[b]	8.55±0.77[b]	45.0 ±3.54[a]	2.17 ± 0.08[a]
S2	15.0 ± 0.00[a]	13.3± 0.00[c]	20.0±0.00[b]	10.0±0.00[b]	27.5 ± 3.54[b]	7.34 ± 0.94[b]
F2	16.5 ± 2.12[a]	12.21 ± 1.55[c]	25.0±0.00[a]	8.00±0.00[b]	45.0 ± 0.00[a]	4.44 ± 0.00[c]
S3	12.0 ± 1.41[a]	16.78 ± 1.98[b]	No clot up to 5 min	0.56±0.00[c]	No clot up to 5 min	0.62 ± 0.08[c]
F3	36.0 ± 0.0[a]	5.60 ± 0.0[d]	No clot up to 5 min	0.00±0.00[c]	Noclot up to 5 min	0.45 ± 0.04[c]
L.S.	***	***	***	***	***	***

Means within columns bearing the same letter are not significantly different (P>0.05)

*** = P<0.001

L.S = Level of significance

Table 3: Effect of ammonium sulphate saturation on milk-clotting activity yield and fold of purification in Solanum dubium seeds.

Saturation of ammonium sulpate(%)	Volume (ml)	Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activitya (U/mg)	Totalactivity b (U)	Yieldc (%)	Fold of purificationd
Crude enzyme	25	66.02	0.514	128.44	1650. 5	100.00	1.00
0-10	5	11.68	0.353	33.09	58.42	3.54	0.26
10-20	5	16.40	0.387	42.38	82.00	4.97	0.33
20-30	5	18.99	0.291	65.26	94.95	5.75	0.51
30-40	5	67.71	0.415	163.16	338.55	20.51	1.27
40-50	5	91.06	0.377	241.54	455.3	27.59	1.88
50-60	5	30.36	0.402	75.52	151.80	9.19	0.59
60-70	5	22.96	0.389	59.02	114.80	6. 96	0.46
70-80	5	11.43	0.254	45.00	57.15	3.46	0.35
80-90	5	10.56	0.378	27.94	52.80	3.20	0.22

[a] Specific activity = Enzyme activity/ protein content

[b] Total activity = Enzyme activity x volume of fraction

[c] Yield = Total activity of the fraction/total activity of crude enzyme x 100

[d] Fold of purification = Specific activity of the fraction/specific activity of the crude enzyme

Fractionation of Precipitated Enzyme by Gel Filtration on Sephadex G-100: As a rennet substitute, it is necessary that the enzyme has not only a high milk-clotting activity, but also a low proteolytic activity. Unfortunately, the strong proteolytic activity of rennet substitutes caused curd peptonization and off-flavour, especially a bitter taste which prevented them from being used in cheese making. In this study, the enzyme precipitated by ammonium sulphate at 40-50% saturation yielded 27.59% and 1.88 purification fold.

The partially purified enzyme was chromatographed in a column of Sephatdex G-100 and the purification exhibited two peaks with proteolytic activity (at fractions number 19 and 20) as shown in Figure 1. Results are in agreement with Duarte *et al.*[2] and Chaiwut *et al.* [4] who concluded that two peaks with proteolytic activity were eluted from purification of *Jacaratia corumbensis* and Papaya (*Carica papaya*). However, the findings of Calvo and Fontecha [1] and Otani *et al.* [10] showed only one peak from extract of hygienized kid rennet paste and *Albizia julibrissin* respectively, while Egito *et al.* [17] reported several proteolytic bands in albizia seed extract and one diffuse proteolytic band from sunflower seed extract. The variation in peak number is mainly due to protein content of different values or separation conditions.

Characterization of Purified Enzyme

Effect of pH on Milk-clotting Activity: The pH of the substrate has a tremendous effect on the clotting activity as one might expect. The activity of Solanum extract on a pH range from 5.5 to 8.0 was shown in Figure 2.

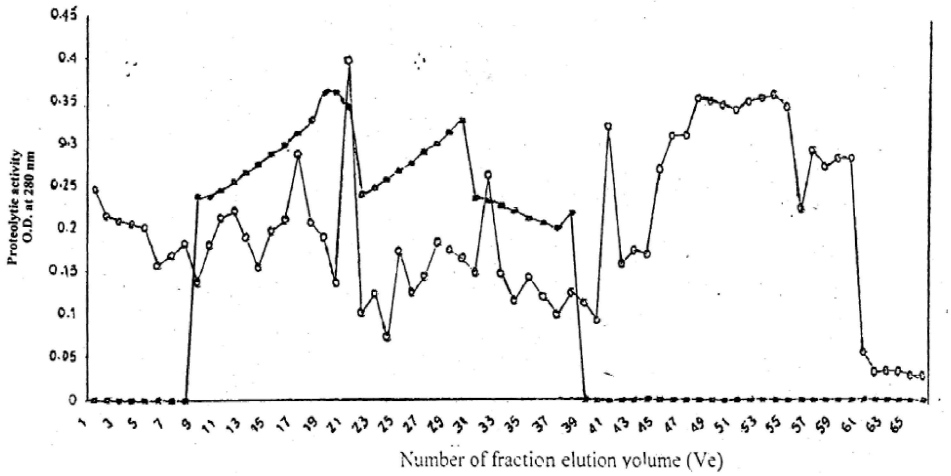

Fig. 1: Purification of milk-clotting enzyme from *Solamim dubium* seed (S_0) by gel filtration on Sephadex G-100 column

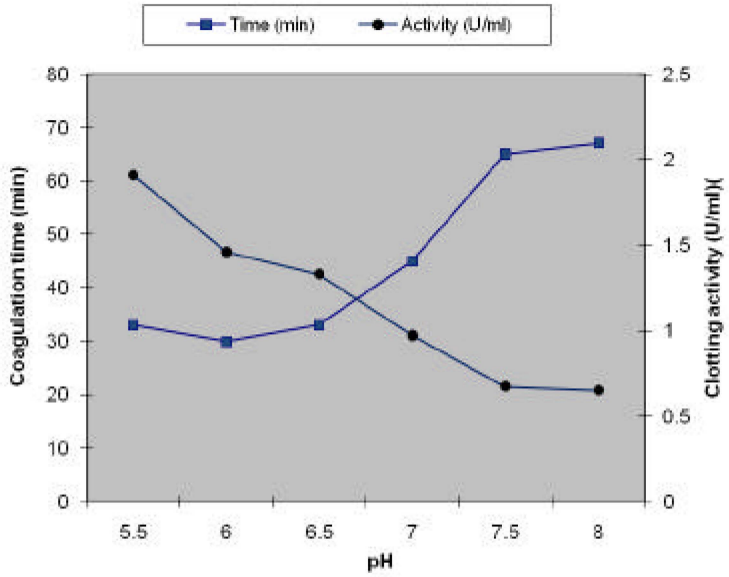

Fig. 2: Effect of pH on coagulation time (min) and milk-clotting activity of *Solanum dubium* extract

Results of the present study showed that the clotting activity decreased and coagulation time increased with increasing pH value, however, the maximum activity was observed at pH 5.5. The findings are similar to Mohamed and Habbani [13] who found that the activity of *Solanum dubium* rennet decreased with increasing pH values. The *Solanum dubium* extract was reported to be stable at pH range of 3.0-12.0 [11]. Other investigators found optimum activity of different extracts at pH 6.5 for *Jacaratia corumbensis* [2], pH 7.5 for hygienized kid rennet paste [1] and pH 7.0 and 8.0 for papaya latex and peel proteases respectively [4]. This discrepancy may be due to the source of milk-clotting enzyme, the plant part used and the extraction or purification methods used.

Effect of Incubation Temperature on Milk-clotting Activity: The incubation temperature influenced the milk-clotting activity of rennet and it is of major importance in determining the optimum concentration of enzyme in cheese making. It is obvious from the data in Figure 3 that Solanum extract was characterized by high thermal resistance. *Solanum dubium* extract exhibited optimum temperature for milk-clotting activity at 60°C, then the activity decreased with increaing temperature. This result agrees with the findings of Duarte *et al.* [2] who reported highest relative acitivity of *Jacaratia corumbensis* seeds at 55°C. However, some results proposed maximum activity of *Solanum dubium* enzyme at 45°C [13]. Ahmed *at al.* [11] reported

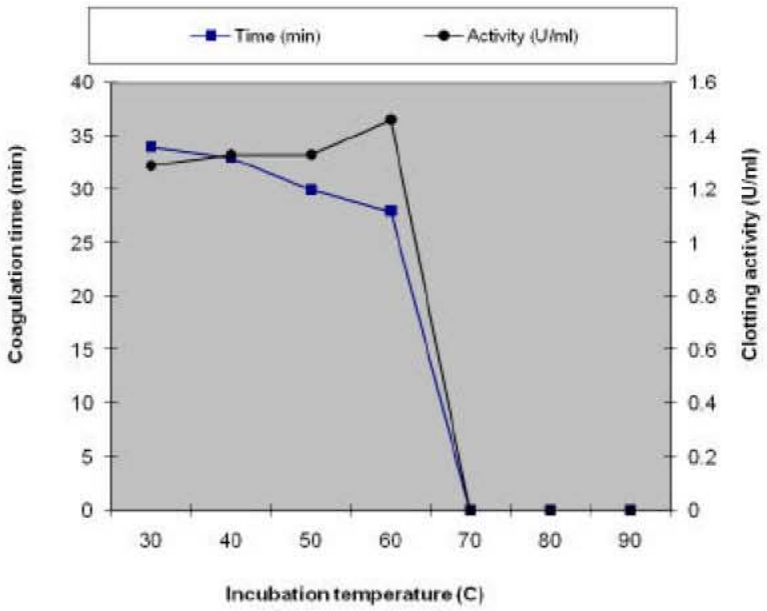

Fig. 3: Effect of incubation temperature (°C) on coagulation time and milk-clotting activity of *Solanum dubium* extract

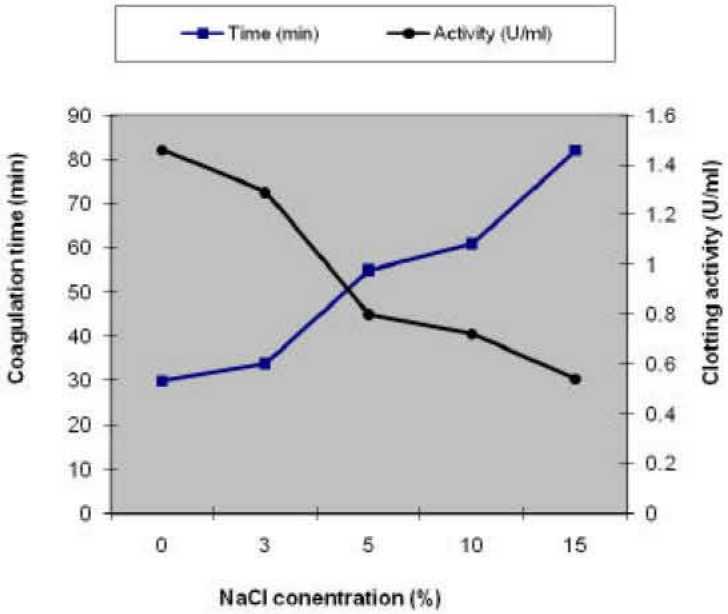

Fig. 4: Effect of sodium chloride concentration (%) on coagulation time and milk-clotting activity of *Solanum dubium* extract

that the activity of enzyme from *Solanum dubium* seeds increased as temperature increased from 20 to 70°C and the acivity rapidly decreased as temperature raised over 80°C.

Effect of Sodium Chloride Concentration on Milk-clotting Activity: Some inhibitors such as hydrogen peroxide, sodium nitrate, cupric sulphate, sorbic acid and sodium chloride affect milk-clotting activity. The present study evaluates one of these inhibitors (Sodium chloride) at different concentrations on milk-clotting activity. The data presented in Figure 4 indicated that with increasing sodium chloride concentration, the clotting activity decreased and coagulation time increased.

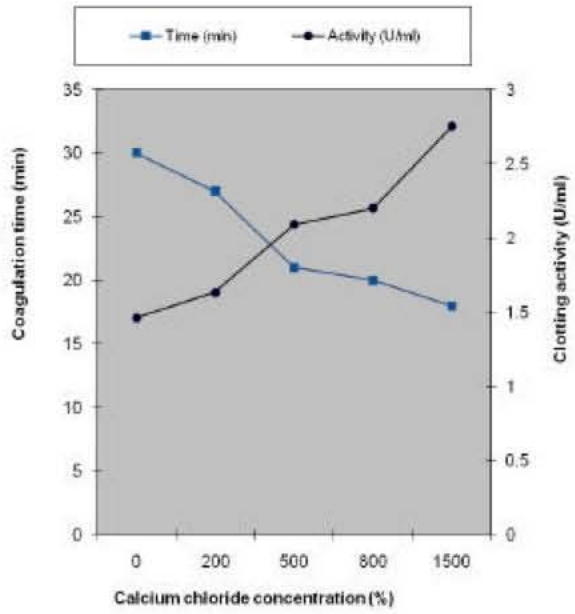

Fig. 5: Effect of calcium chloride concentration (%) on coagulation time and milk-clotting activity of *Solanum dubium* extract

The findings are in agreement with the results obtained by Wahaba *et al.* [18] who found that addition of NaCl to milk resulted in a marked decrease in clotting activity. Shehata *et al.* [19] proposed that the relative milk-clotting activity of bacterial-coagulants decreased as the concentration of NaCl in milk increased up to 15%.

Effect of Calcium Chloride Concentration on Milk-clotting Activity: Calcium chloride is an important factor in cheese making. Addition of calcium chloride to milk prior to curdling was found to favour not only the rate of reaction but also the extraction of clear whey. Data from Figure 5 clearly demonstrated that the clotting activity increased with increasing calcium chloride concentration, while coagulation time decreased. The findings are in agreement with the reports of many investigators who showed that the enzyme activity from various sources increaed with increasing calcium chloride concentration [13, 20].

This study concluded that *Solanum dubium* seed is the part of the plant that gave the highest milk-clotting activity, therefore in the furute research should be focused on obtaining the enzyme from the seeds in the pure form and commercial production of the enzyme for cheese making.

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