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Interpreting the production, characterization and antioxidant potential of plant proteases

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Abstract

Cheese has become an important food item that may provide great nutritional benefits to consumers all over the world. Rennet is the most important milk coagulant obtained from calf stomach but nowadays due to lesser availability of ruminant stomach, higher rennet prices, religious concerns (Islamism; Halal / Haram) or the ban of recombinant calf rennet has given rise to the need for other substitutes than rennet. The present study was conducted to develop vegetative extracts of *Citrus aurantium* flowers, fig, pineapple, and melon extract as vegetative coagulants as an economical, easily available and halal source and their evaluation on basis of pH, dry matter protein content, milk clotting, and proteolytic activities potential in comparison with rennet and acid. The protein contents of CAFE, fig latex and bromelain were much greater than melon extract. The trend in milk clotting activity (MCA), proteolytic activity (PA) and MCA/PA ratio was rennet > acid > bromelain > CAFE > fig latex> melon extract. ORAC assay for antioxidant potential of extracts showed the following trend: fig latex 592 µM (TE)>CAFE 566 µM (TE)>bromelain 130 µM (TE)> melon extract 120 µM (TE) using Trolox as standard. These plant extracts proved a better substitute for animal rennet in the cheese industry.

Keywords: plants; vegetative extracts; milk coagulation; antioxidant potential.

Practical Application: Economical and easily available commercial plant proteases as rennet replacers.

1 Introduction

Cheese is consumed all over the world as a significant human food item because of its nutritional value and sensory attributes. Cheese production has been enhanced (2-3% annual increase in production) over the last two decades due to its high consumption globally (González-Velázquez et al., 2021; Gulzar et al., 2020). Europe has the highest level of per capita cheese consumption i.e., 20.44 kilograms of cheese followed by the U.S. and Canada consuming 17.9 and 15 kilograms of cheese per capita in the year 2021 (Racovita et al., 2021). Cheese production has been regarded as a dynamic process comprising the steps like thermal treatment, homogenization, and coagulation of milk. Out of these steps, milk coagulation is one of the most critical steps in cheese-making and the choice of milk clotting factors may greatly attribute to flavor, yield, and texture of cheese. Calf rennet contains chymosin as the principal milk clotting protease and is used as the renowned milk clotting source for centuries but higher rennet prices, religious concerns (Islamism; regarding halal or haram clarity), vegetarian diet concerns or ban of recombinant calf rennet (especially in Germany, Netherlands and France) created a need to search for other protease milk clotting substitutes (Ozdemir et al., 2021).

Recently, plant proteases are valued as significant milk clotting substitutes for calf rennet (Farias et al., 2020). These proteases are present in almost every tissue of any plant type with one general rule that all these are proteolytic enzymes either in form of aspartic, serine or cysteine proteases with milk clotting ability under specific processing conditions (Ben Amira et al., 2017).

Citrus aurantium commonly known as bitter orange contains proteolytic enzymes which are obtained from the generative portion of flowering plants (Khan et al., 2019). The soluble protein in *C. aurnatium* flower extract is 85% in respect of total protein content, but it depends on pH, extraction procedure and ionic strength of the solution (Shah et al., 2014; Khan et al., 2021). Likewise, another plant *Ananas comosus* (pineapple) also contains proteolytic enzymes that can be used as milk clotting agents. This milk coagulation activity of bromelain obtained from pineapple has gained significant importance due to its stability over a wide range of pH which provides firm textural properties in cheese and it is also effective over the entire gastrointestinal tract (Banerjee et al., 2018). *Ficus carica*, commonly known as fig is an indigenous plant to the Mediterranean and western Asia. The sticky fluid with a milky appearance in figs is called latex and its role in pathogen protection is a renowned physiological phenomenon in plants (Raskovic et al., 2016). Recent advances have led to the utilization of fig latex from fig fruit or fig latex in milk coagulation. Moreover, the sarcocarp of melon fruit (*Cucumis melo* L.) has higher serine protease concentrations known as cucumisins. There is one recent study on its proteases that have been studied for their

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milk coagulation activities which are comparable to rennet due to their activity against casein (Khan et al., 2019).

According to the ideal pH, proteases can generally be divided into alkaline (basic) proteases (8.0-11.0), neutral (about 7.0), and acidic (2.0-5.0) proteases. The majority of neutral proteases are derived from plants; however, they can also be categorized into cysteine, serine, aspartic, and metalloproteases (which have a metal ion cofactor in the catalytic site) based on the amino acid residues involved in the catalytic site. It depends on the type of vegetable protease present in the plant, but their diversity and activation characterize the nature of proteolytic activities. Many vegetable proteases preferentially hydrolyze the Phe105-Met106-casein bond, while others hydrolyze alternative locations (Varghese & Georrge, 2020).

The present study was conducted to compare the milk clotting potential of *C. aurnatium* flowers, pineapple, fig and melon, to evaluate them based on protein content, milk clotting and proteolytic activities as well as antioxidant potential, to understand the enzymatic activity and the kinetics working behind the milk clotting ability of these plants. Acid treatment and rennet treatment were used as controls to check which coagulants express the activities towards either of both controls. This study will also provide a comprehensive account to compare the coagulation potential of these plant extracts which can be employed as commercial milk coagulants in the future.

2 Materials and methods

2.1 Samples

Fresh *C. aurnatium* flowers were collected from nurseries of Sargodha, Pakistan during citrus bloom season, pineapple (*A. comosus*) and melon (*C. melo*) were collected from the local market, and crude fig latex was obtained from the fig plants. Commercial bovine milk (5 liter) was purchased from the local market of Faisalabad, Pakistan, acetic acid was purchased from New Chemical Centre, Lahore, Pakistan and animal rennet was procured from the commercial chemical market of Faisalabad, Pakistan. Milk samples were standardized composition typically related to cow milk and fat content was reduced to 1% with help of the homogenizer. All treatments were analyzed in triplicates. All chemical reagents used were from Sigma Aldrich, USA.

2.2 Preparation of extracts

Citrus aurnatium flowers extract (CAFE)

CAFE was prepared by blending citrus flowers with five parts of the cold buffer of 20 mmol/L of Tris-HCl having pH 7.2 in a food blender (Vitamix A3300, Ascent Series, USA) for three intermittent periods of 15 to 30 seconds. The flower blend was filtered with a cheesecloth and centrifuged at 7500 rpm, 10 °C for 30 minutes. The aqueous phase was separated while the remaining pallet debris settled down. Then the aqueous extract was kept in a refrigerator at 4 °C till use (Salehi et al., 2017).

Preparation of melon extract

Fresh melons were purchased from the local market during the fruiting season. Melon mesocarp slices were homogenized in the blender without using any buffer to make melon juice. The homogenized melon juice was centrifuged at 5000 rpm for 30 minutes at 4 °C in a Beckman centrifuge (ThermoScientific CL10, Centrifuge, USA) to remove suspended particles. Fresh extracts were maintained at 4 °C or frozen at -40 °C and lyophilized with a freeze drier (Labconco 700401000, Freeze dryer, Labconco Corporation, USA) (Mazorra-Manzano et al., 2013).

Preparation Ficus carica extract (Fig latex)

Fig latex was used to prepare plant extracts that included the fig latex enzyme. No further processing was applied to the crude fig latex (Siar et al., 2020).

Preparation of Pineapple extract (Bromelain)

The crude bromelain extraction was carried out by peeling and cutting 500 g of *A. comosus* into smaller pieces, which were grounded in mortar and pestle to extract the filtrate. After that, the filtrate was mixed with 0.1 M phosphate buffer and centrifuged at 3500 rpm for 15 minutes. Then it was incubated at 4 °C and filtered using Whatman filter paper (150 mm) to get the crude bromelain enzyme (Vergara-Alvarez et al., 2019).

2.3 Characterization of extracts

The pH, dry matter and protein content of CAFE, fig latex, bromelain and melon extract were determined by following the method of AOAC (AOAC International, 2006).

2.4 Milk clotting activity (MCA)

The MCA was calculated with slight modification in the method of Mazorra-Manzano et al. (2013) and Salehi et al. (2017). The MCA of vegetative coagulants derived from CAFE, melon extract, fig latex and bromelain was compared with acid and rennet. 1 mL of each extract was mixed with 10 mL of pasteurized low-fat milk containing 0.02 g per 100 mL of CaCl2 and incubated at 35, 45, 55, 65 and 75 °C, respectively and MCA of plant extracts was assessed. The clotting time "t" was defined as the interval between the addition of coagulant and the occurrence of milk clotting (seconds). The experiment was conducted using vegetative coagulants at different clotting times and temperatures. Soxhlet units (U) were used to measure the amount of protein in 1 mL of extract needed to coagulate 1 mL of low-fat milk at a different time and temperature treatments (Equation 1).

$$MCA(U) = \frac{2400}{t} \times \frac{S}{E} \quad (1)$$

Where t= clotting time in seconds; S = volume of milk in mL; E= volume of extract in mL

2.5 Proteolytic activity (PA)

The PA of vegetative coagulants from CAFE, bromelain, fig latex, and melon extract was measured by slight modification in the method of Mazorra-Manzano et al. (2013), Nasiri et al. (2020). The PA of vegetative coagulants was compared with that of acid and rennet by using bovine serum albumin (BSA)

or casein as a substrate. In brief, 450 µL of 1 g/100 mL protein substrate solution (100 mmol/L phosphate buffer, pH 7.0) and 50 µL of each extract were mixed individually and incubated at 50 °C for 60 minutes. 500 µL of 50 g/L trichloroacetic acids (TCA) was added after incubation to terminate the reaction. The samples were maintained on ice for the same amount of time as the control samples and TCA was added before incubation. The mixture was vortexed (Mini Vortex MV 1), refrigerated for 30 minutes, and then centrifuged (Eppendorf model 5417R, Massachusetts, USA) at 20,800 g for 20 minutes. After centrifugation, 100 µL of TCA extract, 200 µL of 0.2 N NaOH, and 100 µL of phenol reagent (Folin-Ciocalteu phenol solution/water 1:2) were combined to quantify the amount of soluble nitrogen for 15 minutes at 35 °C. The optical density (OD) was measured using a Cary 50Bio spectrophotometer (Varian, Palo Alto, CA, USA) at 280 nm. The amount of protein that enhanced absorbance by one unit at 280 nm was defined as one unit of enzyme activity (U) under the conditions stated.

2.6 MCA to PA ratio (MCA/PA)

MCA to PA ratio of vegetative coagulants from CAFE, fig latex, bromelain and melon extract was compared with acid and rennet by simply dividing the values of MCA to PA.

2.7 Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples of CAFE, fig latex, bromelain and melon extracts were analyzed by SDS-PAGE for protein profiling. All chemicals and instruments used in electrophoresis were purchased from Bio-Rad (Richmond, Virginia, USA). Samples were suspended in 0.25 M Tris-HCL buffer of 6.8 pH containing 7.5% glycerol, 2% SDS and 5% beta-mercaptoethanol. Then it was heated for 10 minutes at 100°C. The electrophoresis run at 90V at room temperature for 6 hours was performed and Coomassie Brilliant Blue G-250 dye was used to dye the gels and then analyzed with a laser densitometer as the method described by Akasha et al. (2016).

2.8 Lipid extraction

Lipid extraction from CAFE, fig latex, bromelain and melon extracts was carried out by using the Folch method with slight modifications. The extracts were homogenized in chloroform: methanol solution (2:1) and filtered or centrifuged to obtain an aqueous phase. The debris was rinsed again with fresh chloroform: methanol solution to have efficient aqueous phase extraction. Then 0.9% NaCl solution was added and vortexed for 2 minutes. Then this solution was again centrifuged at a lower speed of 2000 rpm to separate into two phases. The upper phase was then removed, and the lower chloroform phase with the lipids layer was separated by evaporating chloroform. The final last layer of residue was dissolved in 5 mL of chloroform and stored at -20°C (Sheng et al., 2011).

2.9 Free fatty acid (FFA) determination

Oil samples were analyzed by following the method described by D'Alessandro & Antoniosi (2016). 1 mL of oil was mixed with 10 mL of iso-propyl alcohol and a few drops of phenolphthalein indicator were added. The titration was done against 0.1 N NaOH until the pale pink color of the last longing for 10 seconds appears. That endpoint value of the burette represented the acid value of oil directly and free fatty acids (FFA) will be determined through a simple relation represented as; FFA % = Acid Value/2.

2.10 Phosphate analysis

Phosphate analysis was performed with a slight modification in the spectrophotometric method described by Lu et al. (2019). The standard curve from the blank and standard solution of diphosphate (with different concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mL) was used for comparison. Samples of lipid extracts were measured at different concentrations of 0.5 and 1.0 mL in triplicate. The methanolic extract samples were taken in test tubes for evaporation and drying in the water bath and 10% $MgNO_3$ was added to each test tube and vortexed. Then the samples were dried at 100 °C in a hot air oven. Later dried tubes were put over a strong flame till brown fumes ceased to come out of the tubes and tubes were cooled down for 15 minutes. 0.5 N HCl was added to all tubes and vortexed to mix. The tubes were reflex tubes in boiling water for 15 minutes and add reagent (mixture of ammonium molybdate and ascorbic acid solutions). The test tubes were incubated for 20 minutes at 45 °C and the reading of each test tube including blank and standard solutions was measured by a spectrophotometer at 820 nm.

2.11 Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay technique was used after further modifications in the method of Zulueta et al. (2009). The fluorescent probe was fluorescein, while the peroxyl radical generator was AAPH. Fluorescein (40 L, 75 nM), sample (20 L), and AAPH (140 L, 12 mM) made up the final reaction mixture. Every reagent was made in a 75 mM phosphate buffer solution with a pH of 7.4. Black 96-well Eppendorf Microplate 96/U-PP plates were used for the ORAC test. A fluorescent microplate reader (Synergy Biotek HTX-MX, Multi-mode microplate reader, Massachusetts, USA) with an excitation wavelength of 485 nm and emission wavelength of 520 nm was run in kinetics mode for three hours to calculate the fluorescence intensity of vegetative coagulants the microplate reader measured the fluorescence intensity of the mixture after every minute with 5 seconds of agitation. The data were expressed as Trolox equivalent (TE) after creating a Trolox standard curve.

2.12 Statistical analysis

The data for each parameter were analyzed statistically by using a Completely Randomized Design (CRD) (Montgomery, 2017) and the significant differences comparisons were performed by Duncan's Multiple Range (DMR) Test (SAS 9.1 Statistical Software).

3 Results and discussions

3.1 Characterization of vegetative extracts

Plant proteases have been used as alternatives to chymosin in the manufacturing of cheese. The MCA of these enzymes is highly specific and can affect the yield as well as biochemical properties during cheese ripening (Mazorra-Manzano et al., 2013). The physical parameters of vegetative extracts examined during our current study are shown in Table 1.

Table 1. pH, dry matter, and protein contents in various fruit extracts.

Extracts	pH	Dry Matter (mg/L)	Protein (mg/L)
Melon extract	5.14 ± 0.04[a]	112.13 ± 3.12[a]	50.02 ± 0.01[c]
CAFE	4.28 ± 0.02[c]	91.56 ± 2.12[b]	85.98 ± 4.5[a]
Bromelain	4.12 ± 0.04[d]	83.25 ± 2.47[c]	81.28 ± 6.38[a]
Fig latex	4.59 ± 0.05[b]	84.85 ± 5.97[c]	72.98 ± 2.78[b]

Different small alphabets show significant differences among different treatments ($P < 0.05$).

Table 1 showed significant ($p<0.05$) differences in pH, dry matter, and protein content of plant extracts. The highest pH and dry matter content were found in the melon extract as compared to the other three extracts. The higher pH and dry matter of melon extract were because melon extract was obtained from the fresh sarcocarp part of the melon with no further processing. Our result regarding melon extract was in agreement with the study of Rizzello et al. (2016) who had observed higher pH and dry matter from fresh fruits was higher without any processing treatments thus melon had higher pH and dry matter than the other three coagulants extracted from different processing conditions. Moreover, Fundo et al. (2018) reported that fresh fruit juice showed higher pH and dry matter in pure form but processing conditions affected the pH and dry matter. The highest protein content was in CAFE and bromelain than fig latex and melon. According to a study by Nasiri et al. (2020), higher protein content in CAFE was due to its protein content during the blooming period of flowers which increases its protein content range from 20 to 35 mg/mL, but protein content varies with season and type of fruit, type of citrus species and extraction method while protein may contribute to increasing in dry matter of total weight depending upon the type and source of the sample, extraction process, pH and ionic strength of the solution. The bromelain extract was in purified form and it showed lower pH and dry matter due to processing conditions. Ke et al. (2021), reported that bromelain protein content was lower in saturated form but increased two times upon the purification of the plant but it also depends upon their purification processes. Fig latex has been investigated in many studies, from raw fig latex to crude ficin and purified ficin enzyme but most studies focused on extracted ficin and fig fruit. Raskovic et al. (2016) reported that unripen fig latex had lower protein content, but its protein content increased during the ripening phase. Thus, latices of laticiferous plants are rich sources of protein so fig latex collected in summer is one of the plant lattices with the highest protein content. The study of pH and dry matter content of such plants with coagulating enzymes is reported by Nasiri et al. (2020) that such characterization of vegetative extracts depends upon the action of several factors like plant type, origin, and extraction methods thus these plant extracts show variation in pH upon further treatments.

3.2 Milk Clotting Activity (MCA)

The MCA of rennet, acid and plant extracts is shown in Figure 1. The time taken by vegetative coagulant to coagulate milk showed a significant difference as compared to rennet and acid.

Rennet and acid showed higher MCA at a lower temperature while vegetative coagulants required a higher temperature for their MCA. CAFE, acid and rennet showed higher MCA at acidic pH while melon and bromelain showed higher MCA at basic pH. This study was in accordance with Khan et al. (2019) that MCA of CAFE extracts showed maximum MCA at the optimum temperature, time and pH treatments and it required a higher quantity of the vegetative extract to coagulate the same quantity of milk while acid and rennet coagulated milk with lower quantity. Moreover, Mazorra-Manzano et al. (2013) also reported that a higher vegetative extracted amount of melon, ginger and kiwi was required to coagulate milk at their specific pH, time and temperature treatments (Figure 2).

Figure 1. Milk coagulation under the action of acid, rennet, CAFE, bromelain, fig latex, and melon juice.

The MCA of rennet and acid was higher than plant extracts. Rennet has chymosin and pepsin which shows higher activity depending on time, temperature and pH treatments (Table 2).

The higher MCA of rennet was due to its action upon k-casein and making protein and fat matrix complex but long-term exposure or higher rennet amount generated the brittle and grainy texture of curd. The acid required a higher temperature than rennet to act upon the k-casein protein linkage to coagulate the milk. The rennet and acid showed lower activity on lower time, temperature and pH treatments. Freitas et al. (2016) reported that MCA is time and temperature dependent thus upon the higher temperature (above 70°C) delayed or no coagulation occurs as protein denaturation occurs at higher rates and structural changes, which leads to the brittle and grainy texture of milk and low temperature provides less activity of the enzymes to act as a coagulant which gives the low curdling or compact mass of curd. Although the rennet and plant protease specifically hydrolyze the k-casein, the optimum time, temperature and pH of every coagulant varies. MCA of plant extracts was usually affected by the factors like pH, temperature, and the kind of protease present in crude extracts (Lemes et al., 2016; Gurumallesh et al., 2019). The kind of protease determines the optimum pH, time and temperature for MCA and its stability in a plant extract (Lee et al., 2022). Therefore, CAFE and bromelain showed higher coagulation activity at higher temperatures and coagulation was

Table 2. The MCA of vegetative coagulants.

Vegetative coagulants	MCA (U/mg)
Acetic acid	178.4 ± 0.02[b]
Rennet	183.4 ± 0.04[a]
Melon extract	1.28 ± 0.02[f]
CAFÉ	2.01 ± 0.06[d]
Bromelain	3.33 ± 0.09[c]
Fig latex	1.49 ± 0.01[e]

Different small alphabets show significant differences among different treatments ($P < 0.05$).

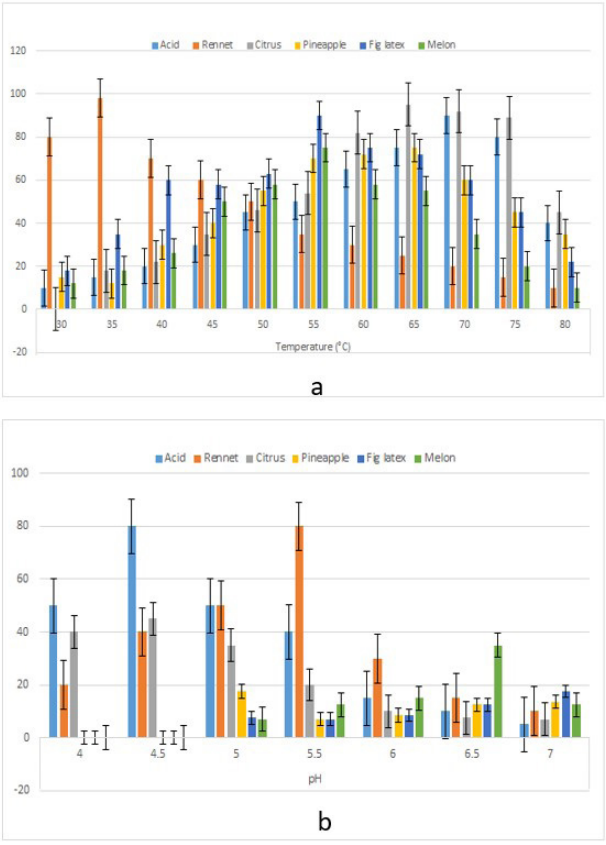

Figure 2 (a) Effect of temperature on MCA (b) Effect of pH on MCA.

noticed to be decreased after certain higher temperatures, which is due to the inhibition of enzyme activity for both CAFE and bromelain (Mazorra-Manzano et al., 2018). Fig latex showed higher MCA at the same temperature as rennet and melon but tended to decrease by increasing the temperature. The melon extract showed maximum activity at higher temperatures. Mazorra-Manzano et al. (2018) reported that melon showed higher activity at a higher temperature, but it led to weaker gel and less curdling while no curdling was observed at lower temperatures and pH treatments. Thus, several studies were done on MCA based on time and temperature treatments. One study by Tian et al. (2022) reported that higher clotting activity of *Cynara sps.* was observed at the start of the experiment, which decreased with the passage of time. But in some cases, there was no activity at the start and after some time sudden increase in the activity was noticed implying that this activity of plant extract was due to the different nature of enzymatic proteins in plants. Hence, more research is needed to determine the nature of the proteases before using them in rennet replacement. In the cheese industry, before employing any prospective applications of vegetative extracts it must be considered what type of cheese has to be made and what qualities of proteases are present in new plant-based sources of vegetative coagulants. Because of the low MCA in crude extracts, large quantities of vegetative extracts are required to coagulate milk, as in the case of melon extract. Thus, better protease activity in crude flower extracts of the plants made them useful in cheese-making.

3.3 Proteolytic Activity (PA)

PA of vegetative coagulants from CAFE, fig latex, bromelain and melon extract was compared with acid and rennet by using BSA and casein as substrates (Table 3). The results showed that the PA of rennet and acid was higher at a lower temperature while vegetative coagulants showed higher PA at a higher temperature. The acid and rennet showed higher PA on BSA and casein substrates as they require less unit of enzyme than vegetative coagulants to initiate proteolysis of protein to coagulate milk. The lowest PA was observed in melon (Mazorra-Manzano et al., 2018). Afsharnezhad et al. (2019) reported that the lower PA of vegetative coagulants in proteolytic enzyme systems was due to several biological processes such as the blooming period of plants flowers and fruits including development, pollination, defense, and senescence. Our findings are consistent with earlier research by Moreno-Hernández et al. (2017), who revealed that melon and fig had higher PA due to higher protein content, and it also depends on the kind of plant tissue from which the protease was extracted, the coagulation behavior of the protease, and the concentration and types of bond linkage they express during PA. A higher protease concentration was required in a lesser quantity of extract to coagulate milk. Nasiri et al. (2020) reported that the blooming period of flowers of plants had higher protein content and it increases upto85 mg/g protein content and it solemnly depends upon the nature of the protein, soil conditions in which the plant grows and genetic type of the plant.

The PA of acid and rennet depends on protease types, such as cysteine, aspartic and serine, etc. in the rennet and acidic nature of acetic acid. In food processing, a kind of protease (such as cysteine, serine, and aspartic) and its specificity is crucial to determine how it must be used (Mazorra-Manzano et al., 2018).

Table 3. PA of vegetative coagulants.

Vegetative coagulants	PA (U/mg)	
	Substrate	
	BSA	Casein
Acetic acid	0.06 ± 0.32[d]	0.15 ± 0.12[e]
Rennet	0.09 ± 0.12[c]	0.28 ± 0.22[d]
Melon extract	0.36 ± 1.02[a]	0.91 ± 1.04[a]
CAFE	0.09 ± 1.04[c]	0.35 ± 0.07[c]
Bromelain	0.12 ± 0.06[b]	0.35 ± 0.12[c]
Fig latex	0.10 ± 0.01[b]	0.54 ± 0.91[b]

Different small alphabets show significant differences among different treatments ($P < 0.05$).

High levels of PA in plant extracts can cause excessive milk coagulation, progressive hydrolysis of the protein chain and occasionally non-specific bitter-tasting peptides, as a result, it is necessary to treat milk at the right pH, time, and temperature to improve coagulation. These effects of plants on PA are similar to those reported by Ben Amira et al. (2017) that PA of CAFE and bromelain is sometimes not dependent on APs and this abrupt shift in PA at different pH, time or temperature was unaffected by most of the inhibitors demonstrating that enzymes other than APs are responsible for PA at neutral pH. Gomes et al. (2019) reported that in some cases, PA of CAFE and bromelain depend on the APs that peptide of approximately 14 kDa which utilize such substrate at pH 7 thus exhibit a shift of PA at different pH, time and temperature treatments. Therefore, APs present in these plants must be needed to screen out these enzymes, understand their roles, and assess their potential applications in biotechnological processes. The melon extract showed lower peptide reaction and PA while the highest protease reaction was observed in the bromelain and fig latex PA depending on the time and temperature conditions. Afsharnezhad et al. (2019) evaluated that such plants protein fragments correlate to para-casein (f1-105) in characteristics, which is produced by hydrolysis of k-casein at Phe105-Met106 peptide bond during milk coagulation.

However, before plant proteases on their structural properties and inhibition studies to an enzyme on basis of their PA, it must be purified and characterized. This must be the first step to study before providing any reports regarding the presence of proteases in plant extracts. There is a need to elucidate roles and specify potential biotechnological uses to conduct more research on how these proteins interact with different organs and metabolic processes have advanced our understanding of plant physiology.

3.4 MCA to PA ratio

MCA to PA ratio was measured by simply dividing milk clotting activity by PA (Table 4).

The selection of a suitable plant protease that is better for MCA depends on MCA/PA ratios with low use of coagulant and such optimization of coagulation parameters is necessary to keep the ripening stage in control. Rennet and acetic acid showed higher MCA/PA than vegetative coagulants on basis of BSA and casein (Table 4). Bromelain and CAFE showed higher MCA/PA than fig latex and melon extract. Thus rennet and acid studies are in good agreement with Mazorra-Manzano et al. (2018) findings that a higher MCA/PA ratio of rennet and acid was 500 times greater when utilizing BSA as the substrate and such protease with higher MCA/PA ratio a better ability to form curd with higher yields and develop less bitterness during cheese processing whereas low ratio leads to lower curd recovery with weak curd firmness and release of bitter peptides that may affect sensory properties in cheese production that decrease in MCA/PA may be due to enzyme denaturation. The melon extract showed lower MCA/ PA but is still suitable to be used as a substitute for Mazorra-Manzano et al. (2013) reported that melon extract has pepsin and trypsin-like characteristics as it has shown the MCA/PA are similar to aspartic pepsin (with MCA/PA in range of 2 to 50) and higher MCA/PA than trypsin in some cases depending on treatment conditions. In conclusion, the bromelain with a high MCA/PA ratio among four plant extracts proved best option as an animal rennet alternative.

The results were integrated to study the relationship between the extract and their MCA and PA. The principal component analysis (PCA) showed that total variation was 59% of which the first principal component (PC1) accounted for 36% of variation was dominated positively by MCA while 23% of the variation was accounted for the second principal component (PC2) positively by PA (Figure 3). The MCA cluster represented above showed higher milk clotting in the plant extract concerning lesser time

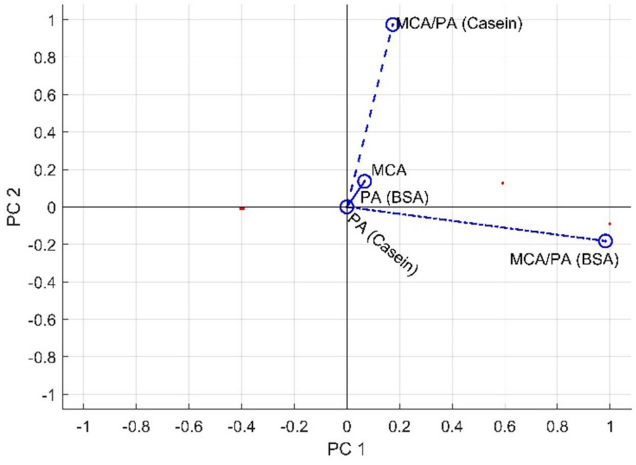

Figure 3. Projection of the MCA, PA and MCA/PA evaluated among four vegetative coagulants in plants defined by principal components (PC1; PC2).

Table 4. MCA / PA ratio.

Vegetative coagulants	MCA (U/mg)	PA (U/mg)		MCA/PA Ratio	
		Substrate		Substrate	
		BSA	Casein	BSA	Casein
Acetic acid	178.4 ± 0.02^{b}	0.06 ± 0.32^{d}	0.15 ± 0.12^{e}	2973.33 ± 0.12^{a}	356 ± 0.18^{b}
Rennet	183.4 ± 0.04^{a}	0.09 ± 0.12^{c}	0.28 ± 0.22^{d}	2037.77 ± 0.32^{b}	655 ± 0.05^{a}
Melon extract	1.28 ± 0.02^{e}	0.36 ± 0.12^{a}	0.91 ± 1.04^{a}	3.55 ± 0.12^{f}	1.41 ± 0.23^{f}
CAFE	2.01 ± 0.05^{d}	0.09 ± 0.32^{c}	0.35 ± 0.07^{c}	22.33 ± 0.41^{d}	5.74 ± 0.25^{d}
Bromelain	3.33 ± 0.01^{c}	0.12 ± 0.13^{b}	0.35 ± 0.12^{c}	27.75 ± 0.11^{c}	9.51 ± 0.03^{c}
Fig latex	1.49 ± 0.31^{e}	0.10 ± 0.14^{b}	0.54 ± 0.91^{b}	14.52 ± 0.13^{e}	2.75 ± 0.19^{e}

Different small alphabets show significant differences among different treatments ($P < 0.05$).

for coagulation. The PCA expressed on the upper right quadrat depends upon the time temperature treatments. Barracosa et al. (2018b) reported that MCA/PA is time-temperature dependent, and pH also has a significant effect on the coagulation cluster of MCA/PA. PA cluster was on the lower right quadrat for extracts when measured by using BSA as a standard and it was lower than the extract on this point and a slight shift of cluster of PA with casein was observed with BSA showed the lower PA. In particular, the shift of PA on basis of BSA and casein was due to the morphological characteristics of plants thus plant extract's protein content and profile showed a shift in PA and there were significant behavioral properties which are dependent on protein content in plants to shift the PA on time temperature treatments (Barracosa et al., 2018a). The MCA/PA trend was observed on basis of casein on the upper right quadrat and with BSA on the lower right quadrat. Thus, explained by Ben Amira et al. 2017 that ratio of MCA/ PA depends upon the capability of plant extracts to show coagulation at optimum time temperature treatments, type of plant and protein content and profiling.

3.5 Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to examine the sample's overall protein profile (Figure 4). The majority of the protein bands blurred, and just a handful could be identified as separate protein bands. The eight protein bands were visible, six of them had MWs ranging from 38 to 91 kDa and the other bands of which were close to 6.4 to 31.4 kDa.

There were multiple additional protein bands observed in extraction and precipitation techniques for the separate analysis of these four vegetative coagulants. Plant tissues contained a diverse array of proteins with a wide range of characteristics and functions Lo Piero et al. (2011). SDS-PAGE did not exhibit high levels of intra-specific variation except at 38 to 91 kDa for vegetative extracts but this diversion accessions based on SDS-PAGE were due to other various sources such as simple sugars, damaged protein, polysaccharides and protein with heterogeneous glycosylation are all soluble substances found in crude aqueous extracts from plants that may interfere protein staining or visualization with SDS-PAGE analysis so its preferable to build broad range based gene pool from diversity center views with maximum variations as interspecific variation was limited (Gali-Muhtasib et al., 2015). More recently, Ben Amira et al. (2017) reported that protein concentration in the initial floral stages of the plant was lower while an increase in protein content was observed in their fruit's protein content depending on factors such as source, plant type, pH or ionic strength in solution but extraction procedures of such extracts contributed towards the limitation of SDS-PAGE to measure the protein variation aspect. The protein content of fresh fig trees started increasing in the floral development stage and higher protein content was observed in the final content of fruit (Akasha et al., 2016). Afsharnezhad et al. (2019) reported that the protein content of lyophilized raw bromelain was higher on a dry basis. Petrova et al. (2020) analyzed the plant protein SDS-PAGE for protein identification and reported that SDS-PAGE has not proved efficient to reflect any clue about the exact quantitative measurement of protein, but these advances are possible by GC-MS analysis that whether the agronomic exert changes or geographic distribution have played a role to contribute to the variation of plant protein profiling in the plant extracts. Therefore, the precise and comprehensive knowledge of agricultural and biochemical data (protein and DNA profiles) is needed to be explored to evaluate the plants for their protein concentration or natural present protein content present in their seed to development of floral stages for better management of extracts protein profile and plant genetic aspects.

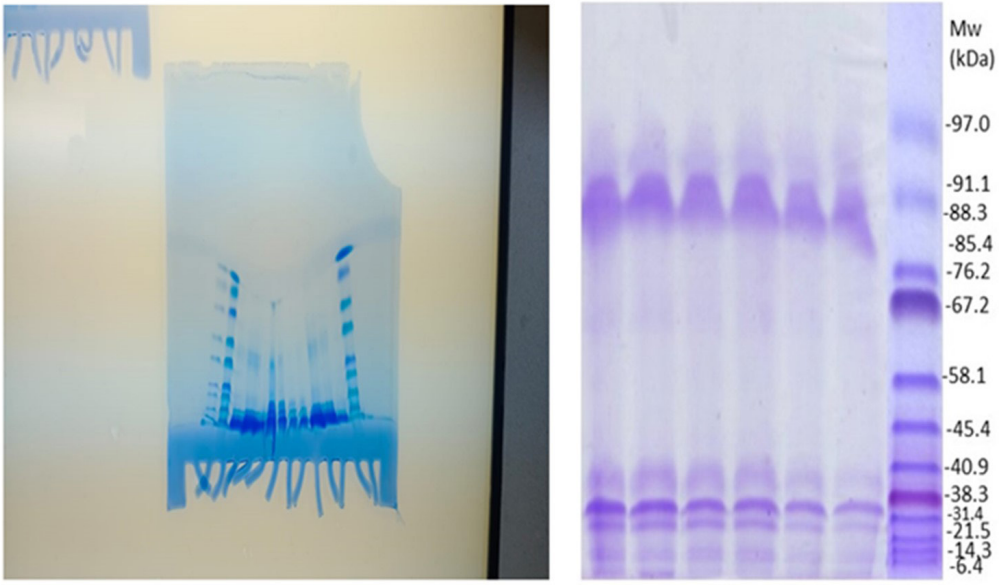

Figure 4. SDS-PAGE analysis of protein in vegetative coagulants.

3.6 FFA determination

The FFA content of oil extracted from CAFE, melon extract, fig latex and bromelain by titration method is shown in Table 5.

The significant difference in FFA content of CAFE oil was due to the extensive processing conditions of CAFE to convert them into an extract and thus they show weak acid tendency (Li et al., 2018) These FFA are considered weak acids and their titration does not have sharp turning points which cause the lower FFA content (Ibanez et al., 2020). The results are inconsistent to the study of Di Pietro et al. (2020) in that the titration system uses solvent for FFA and base, which is independent of the concentration of FFA. Moreover, an automatic determination is practicable without expensive equipment and it needs a potentiometric technique to avoid indicators difficulty regarding high blank and inexact endpoint (Rocha & Zagatto, 2020). Thus, the major factors that affect the fatty acid content are the age of the plant and the length of storage of oil extracted from the plant. The significant difference in titration results was due to difficulty in determining the endpoint in titration because of the lower FFA level in oils.

3.7 Phosphate analysis

The phospholipids analysis of the oil extracted from CAFE, melon extract fig latex and bromelain by spectrophotometry is shown in Table 5. The CAFE has higher phosphate content than fig latex while bromelain and melon extract showed the same phosphate content.

Different small alphabets show significant differences among different treatments ($P < 0.05$).

The phosphate content of the oil extracted from CAFE, melon extract, fig latex and bromelain by spectrophotometry showed a significant difference in the phosphate content used for the determination of phospholipids in the extracts. The higher amount of CAFE is due to its essential oil and crude extracts present in the flower extract and it varies due to floral and genetic and seasonal conditions depending on the genome of the plant (Liu et al., 2013). Wen et al. (2016) indicated that plant trees have multi processes of these phosphate content in signaling different processes like growth, pollen, and melon extract, which affects the phosphate content variation. Another study by Bates et al. (2013) elaborated that seasonal variation, type of plant, and geographical conditions affect plant vascular development and affects phosphate levels. Moreover, phosphate analysis provided an idea about their other beneficiary effects due to other organic compounds and to know what they contribute towards the coagulation of milk.

Table 5. The FFA content in vegetative coagulants.

Vegetative coagulants	FFA content (FFA %)	Phosphate content
Melon extract	0.13 ± 0.010^{b}	0.08 ± 0.005^{a}
CAFÉ	0.08 ± 0.011^{c}	0.04 ± 0.001^{c}
Bromelain	0.14 ± 0.012^{a}	0.05 ± 0.003^{b}
Fig latex	0.14 ± 0.008^{a}	0.04 ± 0.005^{c}

Different small alphabets show significant differences among different treatments ($P < 0.05$).

3.8 ORAC assay

The extract from CAFE, melon extract, fig latex and bromelain were analyzed for their ORAC assay to know about their oxidation potential, which is shown in Table 6 in form of TE and compared at different Trolox concentrations of 6.25, 12.5, 25, 50 and 100 µM.

The higher antioxidant potential was observed in fig latex while melon extract showed the lowest antioxidant potential. ORAC showed slightly lower antioxidant potential in CAFE and bromelain which depends upon the concentration of the sample used and the processing condition of the extract (Phonsatta et al., 2017). Giordano et al. (2022) reported that decreasing pattern of fluorescein absorbance was observed during the ORAC assay of plant extracts in kinetics which indicated antioxidants had a clear oxidation lag time and this increased alkyl chain length exhibited higher ORAC values. ORAC method included both total inhibition time and antioxidant ability to scavenge free radicals into a single quantity in our study.

The results were integrated to study the relationship between the different concentrations of Trolox such as 6.25, 12.5, 25, 50, and 100µM. The PCA showed that total variation was 72% of which the first principal component (PC1) accounted for 42% variation was dominated in cluster positively of the upper right quadrat by Trolox concentrations of 6.25, 12.5 and 25 µM and while 30% variation was accounted in a cluster by second principal component (PC2) positively in lower right quadrat by 50 and 100 µM (Figure 5). There were higher ORAC values were observed in vegetative coagulants at 50 and 100 µM while 6.25, 12.5 and 25µM Trolox concentrations showed lower ORAC values.

The higher ORAC values of fig latex and CAFE were due to esterification reactions which are comparable to a study reported by Lorenzo et al. (2018) that esterification reactions may lead to a significant increase in radical scavenging activity, and it was decreased later on due to the difficulty in reaction stability or due

Table 6. ORAC on basis of Trolox concentrations in vegetative extracts.

Vegetative extracts	ORAC µM (TE)				
	Trolox Concentrations				
	6.25 µM	12.5 µM	25 µM	50 µM	100 µM
Fig latex	592.85 ± 1.73^{a}	730.40 ± 1.78^{a}	741.30 ± 0.45^{a}	781.56 ± 0.67^{a}	861.59 ± 2.18^{a}
CAFE	566.28 ± 1.13^{b}	695.42 ± 1.28^{b}	706.86 ± 1.58^{b}	743.01 ± 1.42^{b}	821.54 ± 1.61^{b}
Bromelain	130.82 ± 1.42^{d}	159.25 ± 0.70^{c}	161.17 ± 1.25^{c}	169.99 ± 1.05^{c}	187.44 ± 1.49^{c}
Melon extract	140.54 ± 1.41^{c}	149.67 ± 1.39^{d}	152.43 ± 1.44^{d}	157.23 ± 1.52^{d}	163.24 ± 1.22^{d}

Different small alphabets show significant differences among different treatments ($P < 0.05$).

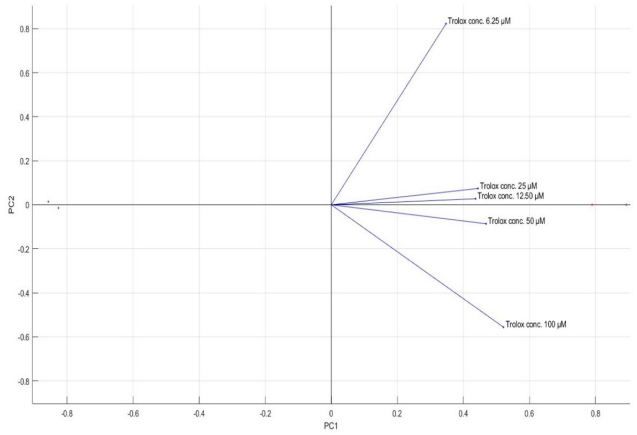

Figure 5. Projection of ORAC on basis of Trolox concentrations (6.25, 12.5, 25, 50 & 100 µM) evaluated among four vegetative coagulants in plan defined by principal components (PC1; PC2).

to inhibition of enzyme or reactive species. The higher ORAC value in plant extract was due to their richness in ascorbic acid (Prior et al., 2016) and thus they react quickly with 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical to give higher ORAC values (Kumari et al., 2018). Although bromelain is also a rich source of ascorbic acid, a lower ORAC value was observed due to processing conditions and purification factors of the extract (De-Melo et al., 2016). The decrease in the ORAC value of melon extract and bromelain was due to the aggregation of lipophilic molecules present in their extracts in the watery phase. This aggregation of lipophilic molecules and watery phase property was due to a decrease in the solubility of antioxidants that results in lower antioxidant potential (Pohl & Kong Thoo Lin, 2018) . The antioxidant capacity of plant extracts in plant protein fractions increased owing to the composition of amino acids such as tryptophan, histidine, lysine, tyrosine, and methionine that have phenolic and indolic groups to act as hydrogen donors which enhance their antioxidant capacity (Zulueta et al., 2009; Karami & Akbari-Adergani, 2019). Thus, higher ORAC values were due to the presence of higher amino groups. In this study, the ORAC method was considered as a reaction to evaluate the vegetative coagulants for their antioxidant potential but different kinetics reaction mechanism of varieties of antioxidants present in plant extract is still to be evaluated in future studies.

4 Conclusion

Plant extracts contain various proteases in an adequate concentration which was used for milk coagulation over broad pH ranges and these extracts represented hydrolyzing capacity against different substrates over a broad range of temperature and pH. These vegetative coagulants represented MCA and PA properties somewhat similar to the rennet and some features were expressed as chymosin-like characteristics. Such vegetative extracts offered new potential sources for milk coagulation in cheese-making as well as other bioprocesses. The current investigation will open new avenues for the economical, easily available, vegetative and halal source of milk coagulation in the cheese industry. The bromelain and CAFE showed the highest potential for milk-clotting during cheese making with MCA/PA ratio more similar to commercial chymosin. The melon extract showed less firmness and curdling properties while fig latex and CAFE had higher antioxidant potential than other vegetative coagulants. The variations in plant coagulants may have an impact on the texture and flavor of cheeses and it may also open possible ways for the production of vegan cheese. Furthermore, we are conducting different miniature cheese development to check the efficiency and properties of the final products. Furthermore, future studies are related to conducting different miniature cheese development strategies to check the efficiency and properties in final products and to have efficient, economical, and easily available vegetative milk clotting enzymes as animal rennet alternatives and their future use in the development of cheese with considerable nutritional importance and capability to work as functional food ingredients on a commercial scale in near future.

Conflict of interest

Authors have no conflict of interest to declare.

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