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Buffalo (*Bubalus bubilis*) colostrum and milk fat globule membrane fractions are potent antioxidants

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ABSTRACT

Colostrum is the first liquid food for the new born baby, secreted after the parturition. After few days of maturation the colostrum turns to milk. The cream or fat fraction of milk consists of fat droplets composed primarily of triacylglycerols that are secreted from the apical surface of the mammary cells surrounded by cellular thin membranes membrane, the fat globule membrane (FGM), formed by proteins which have been suggested to be cholesterolemia-lowering factors, inhibitors of cancer cell growth, vitamin binders, antioxidant, bactericidal, suppressors of multiple sclerosis. FGM was isolated from buffalo colostrum and milk, the fat content was > 2-fold higher in the former than in the latter. The colostrum fat globule membrane (CFGM) fraction also showed significantly higher protein content than milk fat globule membrane (MFGM) extract. Further, the SDS-PAGE separation of the total proteins from the CFGM and MFGM showed differential banding pattern. Antioxidative property of the two FGM extracts was deliberated based on their free radical scavenging ability and reducing power. CFGM was found to exhibit a relatively higher antioxidant and reductive capacity than the MFGM fractions. The results obtained in the present study showcases the superior beneficial health effects of the CFGM over the MFGM.

1. INTRODUCTION

Milk is a complex biological fluid used as a food by all the mammalians in nature and also in the manufacture of a wide range of dairy products. Cows are the largest milk providing animal in the world. Milk from other animal species such as buffalo, ewes, goat and camel are essential to the human diet in various parts of the world. Globally, buffalo milk represents the second largest quantity of milk produced, with more than 97 million tons/year [1]. Buffalo milk is also one of the compositionally richest milks [2]. Compared to other animals fat constitutes the main fraction of buffalo milk, with almost twice the fat content of bovine milk (7.4–8.8% (w/w) vs 3.6– 4.7% (w/w)) [3, 4] and this fat is responsible for the high energetic and nutritive value of buffalo milk. Colostrum, the lacteal secretion vital for mammalian newborn is a complex physiological fluid loaded with free oligosaccharides, glycoproteins, gangliosides, phagocytes, antimicrobial and immune-enhancing components which are unique compared to normal milk [5, 6]. The core of the milk fat globule (MFG) is mainly composed of triacylglycerols (TAG); esters of fatty acids and glycerol constitute total 98% of milk lipids. MFGs originate near the basal region of the alveolar lumen of secretory cells as small droplets of fat. They migrate through the cytoplasm, gradually increasing in size, as the synthesis of TAG proceeds. MFG are secreted from the apical surface of the cell, surrounded by a thin biological bilayer membrane called the Milk Fat Globule Membrane (MFGM) [7, 8]. Milk is a complex biological fluid used as a food by all the mammalians in nature and also in the manufacture of a wide range of dairy products. Cows are the largest milk providing animal in the world. Milk from other animal species such as buffalo, ewes, goat and camel are essential to the human diet in various parts of the world. Globally, buffalo milk represents the second largest quantity of milk produced, with more than 97 million tons/year [1]. Buffalo milk is also one of the compositionally richest milks [2]. Compared to other animals fat constitutes the main fraction of buffalo milk, with almost twice the fat content of bovine milk (7.4–8.8% (w/w) vs 3.6– 4.7% (w/w)) [3, 4] and this fat is responsible for the high energetic and nutritive value of buffalo milk. Colostrum, the lacteal secretion vital for mammalian newborn is a complex physiological fluid loaded with free

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oligosaccharides, glycoproteins, gangliosides, phagocytes, antimicrobial and immune-enhancing components which are unique compared to normal milk [5, 6]. The core of the milk fat globule (MFG) is mainly composed of triacylglycerols (TAG); esters of fatty acids and glycerol constitute total 98% of milk lipids. MFGs originate near the basal region of the alveolar lumen of secretory cells as small droplets of fat. They migrate through the cytoplasm, gradually increasing in size, as the synthesis of TAG proceeds. MFG are secreted from the apical surface of the cell, surrounded by a thin biological bilayer membrane called the Milk Fat Globule Membrane (MFGM) [7, 8]. The MFGM contains many bioactive compounds which are involved with several biological functions and health benefits, such as neonatal gut maturation, antibacterial infection, inhibition of colon development and lowering cholesterol absorption [9, 10]. MFGM has an emulsifying and stabilizing effect in milk and prevents the aggregation and enzymatic degradation of the MFG [9, 11]. These studies advanced the understanding of mammary function and milk secretion. Recently, articles on bovine mammary proteomics have been published, conducting a survey of proteins expressed in MFGM, and showing developmental changes during lactation phases [12, 13]. Considering the nutraceutical and biological significance of these proteins, currently there is an increased interest in MFGM [14]. Despite MFGM proteins making up only 1–4% of the total milk protein content, it is a protein family that shows a greater complexity than other fractions in milk [15, 16]. The significance of MFGM proteins has been surveyed and examined by researchers, especially their bioactivities [17, 18]. Because of the functional and nutritional features of MFGM proteins, many components in mammalian milk have been examined [19, 20]. The different bioactive compounds on the MFGM are still being researched, but there are a variety of beneficial compounds such as lactoferrin, IgG, secretary IgA, sialic acid, phospholipids, and gangliosides. Several of these constitutents are the key in antiviral and antimicrobial mechanisms that counter infections in the gut**.** Beneficial properties have been reported for even the membrane encasing the milk-fat. Adipophilin (milk synthesis), butyrophilin (multiple sclerosis suppression), cluster of differentiation (glycoprotein receptors), fatty acid binding protein (FABP; cell growth-inhibitor, anti-cancer factor, as selenium carrier), lactadherin (epithelialization, cell polarization, cell movement and rearrangement, synaptic activity, antiviral activity in gut), mucin (strong antiviral properties), xanthine oxidase/reductase (bactericidal agent) are the major FGM proteins [21]. Vitamin E and carotenoids, constituents of FGM, are antioxidative in nature [22]. Antioxidants can function by a number of mechanisms. Different enzymes can prevent the formation of radicals or scavenge radicals or hydrogen peroxide and other peroxides. Other enzymes catalyse the synthesis or regeneration of non-enzymatic antioxidants. In milk, antioxidant enzymes- superoxide dismutase and catalase have also been found. The FABP shows immunostimulating, opioid and antioxidant peptides. Because of MFGM's phospholipid enrichment and unique biochemical nature, we hypothesized that diets which include MFGM as a fat source might offer better protection against an exaggerated inflammatory response [23, 24, 25]. Compared to MFGM very little literature is available on colostrum fat globule membranes (CFGM). The limited work carried out so far on CFGM, from human and bovine sources, has focussed on the structural and developmental proteomes [26, 27, 28]. In the present study the FGMs were isolated from buffalo milk and colostrum. The antioxidant and reducing power assays were performed using different concentrations of milk and colostrum FGMs. CFGM sample exhibited higher antioxidant and reducing power than the MFGM extract.

2. MATERIALS AND METHODS

Colostrum and milk samples were collected from local healthy buffaloes (*Bubalus bubilis*) after parturition and stored at -20 ^{0}C. Creams from both the samples were obtained by centrifugation at 4000 rpm for 15 min at 4 ^{0}C.

2.1. Isolation of colostrum and milk fat globule membranes

The FGM was extracted from cream as described by Basch *et al.* [29]. The cream was washed twice separately with phosphate buffer saline (PBS) (0.01M Na_2HPO_4/NaH_2PO_4; 0.9% NaCl; pH 7.2) (4500 x*g*, 10 min, 4 ^{0}C) followed by water wash, once using double distilled water. Washed fat globules of both the samples were suspended in distilled water (1:1 (v/v)) and allowed to crystallize for 20 h at 4 ^{0}C. Globules of both the samples were churned at 4 ^{0}C in a blender to separate the fat and serum fractions. Both the milk and colostum sample fractions were warmed at 45 ^{0}C for 30 min, in order to melt the fat. The fat fractions were washed with distilled water for recovery of the residual serum. The total serum was centrifuged twice (5000 x*g*, 15 min, 4 ^{0}C) to remove traces of fat. We performed MFG protein delipidation with 4 volumes of ice cold acetone at 4 ^{0}C for 20 min at 8000 x*g*.

2.2 Protein estimation

Quantitative protein estimation of MFGM and CFGM samples was performed as described by Lowry *et al.* [30] using BSA as standard. To 1 ml sample, 5 ml of solution C [(Solution 'A' 50ml (2% $NaCO_3$ in 0.1 NaOH) + 1 ml of reagent 'B' (50mg $CuSO_4$ dissolved in H_2O containing 100 mg of potassium sodium tartarate)] was added and incubated at room temperature for 10 min. Folin-Ciocalteu reagent (0.5 ml) was added and mixed thoroughly. After incubation for 30 min at room temperature, colorimetric measurements were carried out at 660 nm.

2.3 SDS-PAGE analysis

Fifty micrograms of the CFGM and MFGM proteins were separated by SDS-PAGE following the method of Webber and Osborn [31] in a 1-mm thick, 12% separating polyacrylamide gel under reducing conditions. The medium range standard protein ladder was obtained from Genei, Bangalore were used. Coomassie blue–R 250 staining was carried out to visualize the protein banding pattern.

2.4 DPPH (1,1-dipheny-2-picryl hydrazyl) radical scavenging assay

DPPH radical scavenging assay was performed as described by Pulido *et al.* [32] with suitable modifications. To CFGM and MFGM samples (aliquots of 25, 50, 75 and 100 µg in 50 µL methanol) 1 ml of 0.1 mM DPPH in methanol and 450 µl of 50 mM Tris-HCl buffer (pH 7.4) were added. The scavenging of the DPPH radical was determined with respect to a control containing no scavenger. The reaction mixture was incubated at 37 ^{0}C for 30 min, under dark conditions and spectrophotometric measurements were performed at 517 nm. Ascorbic acid (10-100 µg) was used as a positive control. The DPPH scavenging value was calculated by plotting the percentage of DPPH scavenging as a function of the concentration.

Percent inhibition = [Control absorbance –Test absorbance/ Control absorbance] x 100

2.5 Reducing power assay

The method of Shinde *et al.* [33] was used with modifications. MFGM and CFGM at various concentrations (25, 50, 75 and 100 µg) in 2.5 ml distilled water was mixed with equal volumes each of sodium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1% (w/v) in water) in a test tube and incubated for 20 min at 50 ^{0}C. The mixture was cooled using crushed ice and 0.5 ml of trichloroacetic acid (10% (v/v) in water) was added and the total reaction mixture was centrifuged for 10 min, 3000 rpm, room temperature. One ml of the supernatant was collected and an equal volume of water was added with 0.2 ml of 0.1% ferric chloride. The absorbance was read at 700 nm against the blank. Tannic acid was used as the standard. Increased absorbance reading indicates increased reducing power.

2.6 Statistical analysis

Data were analyzed by ANOVA followed by Tukey's multiple range test using the Statistical Analysis System Software. Significant differences were set at a 5% level ($p<0.05$).

3. RESULTS

Approximately 100 ml of cream for a litre of milk and 230 ml of cream for a litre of colostrums was recovered. FGMs were isolated from the creamy layer of both buffalo milk and colostrum. Depending on their lower buoyant density, FGMs fractionated on top during centrifugation and it was thoroughly washed to remove contaminating casein and whey proteins. Further, in order to prevent the interference of lipids during FGM protein analysis, samples were delipidized using ice cold acetone. The recovery of acetone washed dry FGMs were 2 and 8.4 gL^{-1} respectively in milk and colostrum, the protein content was estimated to be 500 µg and 700 µg per mg dry weight of MFGM and CFGM respectively. The proteins in the washed cream were separated by SDS-PAGE (Figure 1). The molecular mass of major bands on the gel was estimated in comparison with the mobility of molecular weight standards. Based on the nomenclature recommended by Mather [21] the following major polypeptide bands corresponding to mucin 1 (Mr, 194 kDa), xanthine oxidase (Mr, 145 kDa), PAS III (Mr, 94kDa), PAS IV (Mr, 78 kDa), butyrophilin (Mr, 67kDa), PAS 6 (Mr, 50 kDa), PAS 7 (Mr, 47 kDa) and FABP (Mr, 15 kDa) were identified with other minor proteins.

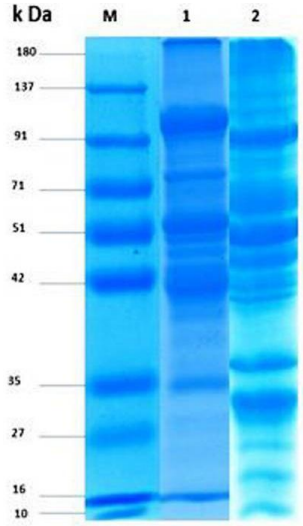

Fig. 1: SDS-PAGE analysis of MFGM and CFGM proteins. Fifty micrograms of the CFGM and MFGM proteins were separated by SDS-PAGE following the method of Webber and Osborn [31] in a 1-mm thick, 12% separating polyacrylamide gel under reducing conditions. Lane M- Standard protein molecular weight markers of medium range (10-180 kDa), lane 1- MFGM proteins, lane 2- CFGM proteins

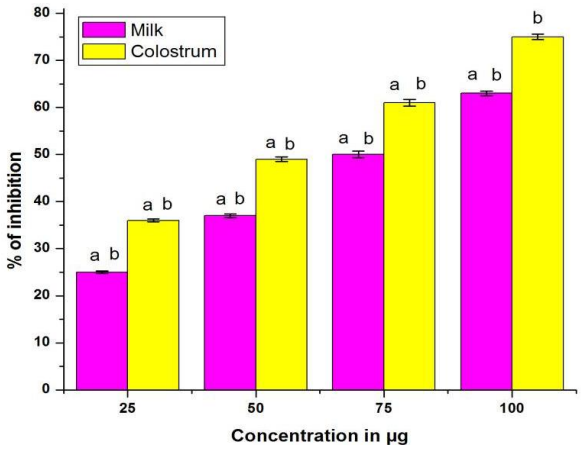

Fig. 2: DPPH assay showing percent inhibition for various concentrations of MFGM and CFGM fractions. Bars indicate ± SE. Means designated with the same letter are not significantly different according to Tukey's multiple range test at $P < 0.05$.

The FGM fractions from both colostrums and milk were assayed for their antioxidant potential using free radical scavenging and reducing power assays. The free radical scavenging assay of samples were measured, showing reduction of DPPH radicals, can be observed by the decrease in absorbance at 516 nm. Figure 2 shows that both CFGM and MFGM extracts displayed antioxidant activities with CFGM displaying >10%

higher free radical scavenging activity than MFGM at all the concentrations studied. Similarly, the CFGM sample exhibited over >10% higher reducing power than MFGM at every concentration tested (Figure 3).

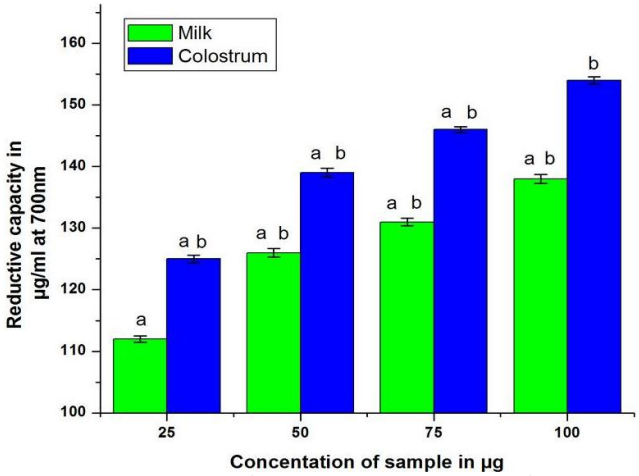

Fig. 3: Reducing capacity for various concentrations of MFGM and CFGM fractions. Bars indicate ± SE. Means designated with the same letter are not significantly different according to Tukey's multiple range test at $P < 0.05$.

4. DISCUSSION

FGM is a complex biopolymer containing various beneficial proteins and lipids. The results of this study also support the hypothesis that dietary FGM proteins offers protection against oxidative stress [35, 36]. FGMs are distributed between intracellular, extracellular and membrane-associated proteins, and they are mainly involved in cell communication and signal transduction, immune function, metabolism, and energy production. Therefore, the consumption of dairy products enriched with MFGM could modulate the pathogenic response [16].

The fat content of colostrum was almost 2-fold higher than the milk and the protein content was also comparatively higher in CFGM. The CFGM exhibited higher scavenging and reductive capacity when compare to MFGM. In the gel, protein bands ranging in molecular weight from 47 to 200 kDa were found to be major FGM proteins and remaining are casein and whey protein except FABP (Mr, 15 kDa) according to Mather [21].

DPPH assay was employed due to its high sensitivity when compared to other available assays like ABTS (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid). In DPPH assay, the highest scavenging value of 75% was found for CFGM at 100 µg and was found to be consistently higher than MFGM, at all the concentrations studied.

Reducing power assay is a popular method used in the assay of the antioxidant activities of various food derived samples and it employs the reduction of Fe^{3+} to Fe^{2+}. The compound's reducing properties are generally associated with the presence of reductones [34], which have an antioxidant action by breaking the free radical chain by donating a proton. Fe^{2+} has been shown to produce oxyradicals and lipid peroxidation. This is because antioxidants are strong reducing agents. The highest reductive capacity obtained was 154 µg ml^{-1} for CFGM at the highest test concentration of 100 µg. The CMFG was found to be a better reducing agent than the MFMG, consistent with the DPPH scavenging activity assay.

Milk, a rich source of bioactive compounds can be used in the functional food production. Present day consumers are in the lookout for foods which offer protection against the lifestyle diseases in addition to their basic nutritional value. An in depth knowledge functional properties of milk compounds is a prerequisite in the design of such foods. Further development of economically viable industrial processes in the production of bioactive milk components is critical challenge to the food technologists [21, 23, 24]. The results obtained in the present study showcases the superior beneficial health effects of the CFGM over the MFGM.

5. CONCLUSION

The present study focused on antioxidant activity of FGM proteins from buffalo colostrum and milk. The study confirms the higher potential of CFGM proteins for free radical scavenging, compared to MFGM proteins. It provides useful information for increasing the commercial value of FGM proteins as an antioxidant. Further analysis of the FGMs will be carried out for its multifunctional properties.

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