Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(2): 61-67
Received: 01-01-2020
Accepted: 05-02-2020

Rita Mehla
Dairy Chemistry Division, ICAR-National Dairy Research Institute, Karnal, Haryana, India

Rajesh Bajaj
Dairy Chemistry Division, ICAR-National Dairy Research Institute, Karnal, Haryana, India

Corresponding Author:
Rita Mehla
Dairy Chemistry Division, ICAR-National Dairy Research Institute, Karnal, Haryana, India

Strawberry polyphenols-casein interaction: Impact on antioxidant activity and α-glucosidase inhibitory activity

Rita Mehla and Rajesh Bajaj

Abstract
The objective of the present study was to evaluate the effect of binding of strawberry polyphenols with caseins/its different fractions (α_s, β, and κ-casein) at different polyphenols concentration (0.5 to 5 mg/ml) as well as at different pH using protein precipitation technique. The polyphenols precipitation was observed to be ranged from 58% to 72% and found to be utmost with β-casein (72.7%), moderately lesser with κ- casein and least with whole casein at 0.5 mg/ml concentration. A decrease in DPPH radical scavenging activity from 29.95% to 30.28% was observed due to interactions of strawberry polyphenols (0.5 mg/ml) with casein and its different fractions (10 mg/ml). An additive effect was found in antioxidant activity determined by ORAC-FL assay in polyphenol- α_s casein, polyphenol- β casein, polyphenol- κ casein and polyphenol- whole casein with the polyphenol concentration ranged from 0.5 mg/ml to 2.5 mg/ml. Binding of strawberry polyphenols with casein and its different fractions at different concentrations caused a decrease in the α-glucosidase inhibitory activity of polyphenols. The change in pH of protein-polyphenol solution did not affect the α-glucosidase inhibition activity, demonstrating that no significant ($p>0.05$) variation takes place in polyphenols binding with whole casein, α_s-casein, β-casein, and κ-casein either at pH 5.5 or 6.6.

Keywords: Polyphenols, casein, DPPH, α-glucosidase, ORAC-FL

Introduction
Plant polyphenols or phytochemicals are composed of a wide range of bioactive components (Altemimi *et al*. 2017) [1]. These are the plant-derived chemicals formed during the secondary metabolism of plants (Gallo *et al*. 2013) [9]. Amongst phenolic compounds, phenolic acid and flavonoids contribute to 29.7% and 58.9% of total ingested flavonoids in the diet, respectively (Manach *et al*. 2004, Ramos 2007 and Xiao *et al*. 2011) [12, 14, 20]. Plant polyphenols are becoming an interesting area of research due to their potential therapeutic properties like anticarcinogenic, antioxidant, antidiabetic, antimicrobial, antiatherogenic and anti-inflammatory (Shahidi *et al*. 2018) [18]. Plant derived phytochemicals are highly linked with the antioxidant activities, α-amylase and α-glucosidase inhibitory activities, which play an important role in management of hyperglycemia (Rasouli *et al*. 2017) [15]. These beneficial attributes have enforced the interest of dairy industries/researchers to use polyphenols as functional ingredients in milk and milk products. However, polyphenols can interact with milk proteins which might affect its functionality as well as structural integrity.
The binding forces involved in polyphenols and milk proteins are mainly observed to be noncovalent (Elikoglu *et al*. 2018) [8]. The structural integrity of both polyphenols and proteins is the main factor explaining the type and strength of binding interactions involved. Apart from this, environmental factors such as pH, temperature, and concentration also might affect the extent of binding of polyphenols to milk proteins (Ekoglu *et al*. 2018) [8]. Therefore to incorporate these functional ingredients in milk and milk products, it is essential to understand the interactions of polyphenols with milk proteins to envisage the optimal processing conditions and final product characteristics. Hence the present study was aimed at the study of polyphenols protein interaction and their effect on functional properties i.e. antioxidant activity and α-glucosidase inhibitory activity.

Material and Methods
Materials
Strawberry fruit pulp was obtained from M/S Delta nutritive Pvt. Ltd., Mumbai. Raw buffalo milk was collected from Livestock Research centre, National Dairy Research Institute, Karnal, Haryana. All other reagents were purchased from Sigma Aldrich chemicals Pvt. Ltd., Bangalore.

Methods

Preparation of whole casein

Fresh Buffalo milk was skimmed by centrifugation (Sorvall, Model RC 6 Plus, Thermo Scientific) at 4000 rpm / 30 min at 4 ºC. The skimmed milk was diluted in 1:1 with distilled water. The pH of skimmed milk was adjusted to 4.6 with 1N HCl at 20 ºC with continuous slow stirring. Then the mixture was held at room temperature for half an hour for complete separation of whey. Precipitated casein was filtered and washed several times with excess of water.

Preparation of different fractions of caseins

Prepared buffalo casein was separated into different fractions like α_s, β, and κ-casein following procedure described by Burr 2001 [6].

Preparation of strawberry polyphenols extract

Strawberry polyphenols extract was prepared as per the procedure given by Cossu *et al*., 2009. Strawberry fruit pulp was mixed with distilled water in 1:3. Then the mixture was boiled for 30 min. and cooled at room temperature. After centrifugation, supernatant obtained was frozen and concentrated (Lyophilizer, Hanil Science Industrial). Concentrated polyphenol extract was stored at -20 ºC for further use.

Preparation of stock solutions

To study the protein polyphenol interaction by precipitation method stock solutions of 60 mg/ml of whole casein and its different fractions were prepared in 10mM tris-HCl buffer 9 (pH 7.4).

Preparation of protein-polyphenol solutions at different concentration of polyphenols

Solutions of whole casein and its different fractions (α_s, β, and κ-casein) were prepared from the stock solution (60 mg/ml) to get the final concentration of 10 mg/ml. The strawberry polyphenols extract was added to the whole casein and its different casein fractions solution ranged from 0.5 to 5.0 mg/ml. Then the solutions were vortexed for 1 min. and kept uninterrupted at room temperature for 15 min. The solutions were centrifuged at 4025g /10 min and supernatants were used for further analysis.

Preparation of protein-polyphenol solutions at different pH

Polyphenols protein reaction solutions were prepared with final protein concentration 10 mg/ml and polyphenols concentration at 0.5 mg/ml. Two sets of reaction mixtures were prepared. In one set of samples pH, 6.6 was adjusted in the final reaction mixture and in another set, the pH of the final reaction mixture was adjusted at 5.5. On the addition of polyphenols to protein solutions (α_s, β, and κ-casein), solutions were vortexed and kept uninterrupted for 15 min. at room temperature. The solutions were centrifuged at 4025g / 10 min and the supernatants were used for further analysis.

Effect of protein- polyphenols interaction on total phenolic content

Total phenolic content of supernatants was estimated by the Folin-Ciocalteau method given by Zhang *et al*. 2006.20 µl of sample / gallic acid was added to 100 µl Folin-Ciocalteau's reagent (2N Folin Cioalteau's solution diluted with distilled water upto 1:10). Then mixed and vortexed for 3 min. then added 80 µl sodium carbonate solution (7.5% w/v) and incubated at room temperature for 30 min. A calibration curve of gallic acid ranged from 10- 100 µl was also prepared.

Effect of protein- polyphenol interaction on antioxidant activity

Antioxidant potential of the protein polyphenols reaction mixtures was evaluated by DPPH method described by Brand Williams *et al*. 1995 and ORAC-FL assay given by Zulueta *et al*. 2009 [22].

DPPH radical scavenging activity assay

100 µl of sample / standard solution was added to 3.9 ml of freshly prepared DPPH solution (6.925 mg/L) in methanol, incubated at 37ºC/ 30 min and absorbance was measured at 515 nm using spectrophotometer (Specord 200, Analytik Jena AG, Germany). A calibration curve of Trolox (25-250 µg/mL) was prepared and results were calculated as% DPPH scavenging activity = [(A_{515nm} blank - A_{515nm} sample)/ A_{515nm} blank] x 100 and expressed as trolox equivalent (TE) values i.e µg TE/g of sample.

Oxygen Radical Absorbance Capacity- Fluorescein (ORAC-FL) Assay

25 µl of sample / standard solution was added to microplate, sealed and followed by incubation for 30 min at 37 ºC. After incubation fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken after every 90 sec to determine the background signal. After 3 cycles, 25 µl (240 mM) of AAPH (2, 2'-azobis (2-methylpropionamidine) dihydrochloride was added manually. The Fluorescent measurements were taken up to 90 minutes. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as : $AUC = 1 + \sum_{i=1}^{i=90} fi/f0$.

Where, f_0 is the fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to a sample was measured as difference of sample AUC and blank AUC. ORAC-FL value was calculated from the slope of Trolox curve. Results were expressed as µM of Trolox equivalent.

Effect of protein-polyphenols interaction on α-glucosidase activity

α-glucosidase inhibitory activity was assessed as per the protocol described by Apostolidis *et al*. 2006 with some modifications. 500 µl of sample was added to 1ml α-glucosidase solution (1.0 U/ml) prepared in 0.1 M phosphate buffer pH 6.90 and incubated at 25 ºC / 10 min. After incubation substrate solution (p-nitrophenyl-D-glucopyranoside) in o.1 M potassium phosphate buffer pH 6.90 was added. Further incubation was done for 5 min. Absorbance was measured at 405 nm before and after incubation and also compared with the blank. The percentage inhibition activity was estimated as% inhibition = (Control absorbance) - (Sample absorbance) / (Control absorbance) *100.

Statistical analysis

Data obtained was statistically analyzed at 5% level of significance by one way ANOVA. Data presented as mean ± standard deviation.

Result and Discussion

Strawberry polyphenols-proteins (whole casein, α_s, β- and κ-casein) interaction study was conducted in a model system by protein precipitation method. The amount of polyphenol

precipitation, antioxidant and α-glucosidase inhibitory activity was estimated for all the four systems: - polyphenol-whole casein, polyphenol - α_s casein, polyphenol - β casein and polyphenol- κ casein

Effect of change in concentration of polyphenol extract

The amount of polyphenol precipitation with casein and its different fractions as indicative of their binding capacity is presented in Fig.1 (a). At 0.5 mg/ml of polyphenol concentration, the level of precipitation was ranged from 58 to 72.7% and maximum precipitation was observed with the β-casein fraction, whereas κ-casein exhibited the comparatively lower and whole casein exhibited the least binding with polyphenol. As the polyphenol concentration was further increased to 2.5 mg/ml, it resulted in 69.2% polyphenol precipitation with β-casein and followed the similar trend of polyphenol precipitation i.e. β-casein > α_s casein> κ casein. As polyphenol concentration was further increased to 5 mg/ml, non significant (p>0.05) difference was observed in the polyphenol precipitation.

Results presented in Fig. 1 (b) and (c) showed the antioxidant activity of polyphenol- α_s casein, polyphenol- β casein, polyphenol- κ casein and polyphenol- whole casein consequently with control samples estimated by DPPH and ORAC-FL assay respectively. DPPH and ORAC values presented the total antioxidant activity of all the antioxidative components present in model system based on polyphenol-whole casein, polyphenol- α_s casein, polyphenol- β casein and polyphenol- κ casein, consequently with control samples i.e. whole casein, α_s - casein, β - casein and κ- casein and PP extract.

The presence of casein/ its different fractions in combination with polyphenol decreased the DPPH radical scavenging activity up to 30.28% and 29.95% in comparison with polyphenol alone at 0.5 mg /ml (Fig. 1(b)). On the other hand, DPPH radical scavenging activity was minimum with β-casein –polyphenol as compared to other fractions and remained almost unchanged in polyphenol-whole casein solution.

Similar results were observed with an increase in polyphenol concentration up to 2.5 mg/ml for DPPH radical scavenging activity of polyphenol- α_s casein, polyphenol - β casein and polyphenol- κ casein and polyphenol- whole casein. Further, an increase in polyphenol concentration from 2.5 to 5.0 mg/ml exhibited the DPPH radical scavenging activity of polyphenol – whole casein, polyphenol - κ-casein similar to that of control whereas polyphenol- α_s casein and polyphenol-β casein exhibited the lower values.

An additive effect was shown on antioxidant activity estimated by ORAC-FL assay in polyphenol- α_s casein, polyphenol- β casein, polyphenol- k casein and polyphenol-whole casein with the polyphenol concentration ranged from 0.5 mg/ml to 2.5 mg/ml. Conversely, the trend for a decrease in ORAC values for casein/its different fractions along with polyphenol was similar as indicated in DPPH radical scavenging activity (Fig 1(c)).

DPPH and ORAC values represent the total of values of antioxidant activity of all the antioxidative components present in the sample. Variation in the performance of polyphenol- α_s casein, polyphenol- β casein, polyphenol- κ casein and polyphenol- whole casein in DPPH and ORAC assay might be linked with the different mechanism associated with these two assays. ORAC was performed in phosphate buffer (pH nearer to polyphenol- α_s casein, polyphenol- β casein, polyphenol- κ casein and polyphenol-whole casein), determining the tendency of antioxidant to donate hydrogen to quench radicals and it is sensitive to protein (Schaich 2006) [16].

The variations in the antioxidant activity in the presence of polyphenols might be due to the reason that on mixing natural source of phenol with casein/ its fractions complexed compounds

can be formed as phenolic compounds act as the polydentate ligand on the protein surface by hydrogen as well as hydrophobic interactions. This kind of interaction depends on the type as well as the molar ratio of both polyphenol and protein (Prigent *et al*. 2003) [13]. Binding of polyphenol to protein depends on the phenolic structure and molecular characteristics (Seczyk *et al*. 2019) [17]. The higher binding of polyphenol with β casein might be due to higher hydrophobicity. β-casein is a proline-rich protein that disrupts its α and β-sheets configuration. The open structure of casein contributes towards its hydrophobicity. The proline residues present in α_{s1}-casein, α_{s2}-casein, β-casein, κ- casein are 17, 10, 35 and 20, respectively (Broyard and Gaucheron 2015) [5].

It has been observed that the binding of polyphenols with protein increases as the number of OH groups increases. β –casein formed the stronger complexes with polyphenols than α_s casein due to the more hydrophobic nature (Hansi *et al*. 2011) [10].

Similar observations were made by Sharma *et al* (2008) [19] in black tea with the addition of milk and sugar. Study revealed that the addition of milk to black tea has reduced the DPPH radical scavenging activity. This effect is caused by the polyphenols protein bindings which unfavourably affect the antioxidant activity of polyphenols. Similar findings were observed by Arts *et al*. 2002, who stated that a decrease in antioxidant activity is related to polyphenol composition as well as on the kind of milk proteins involved in the binding interactions.

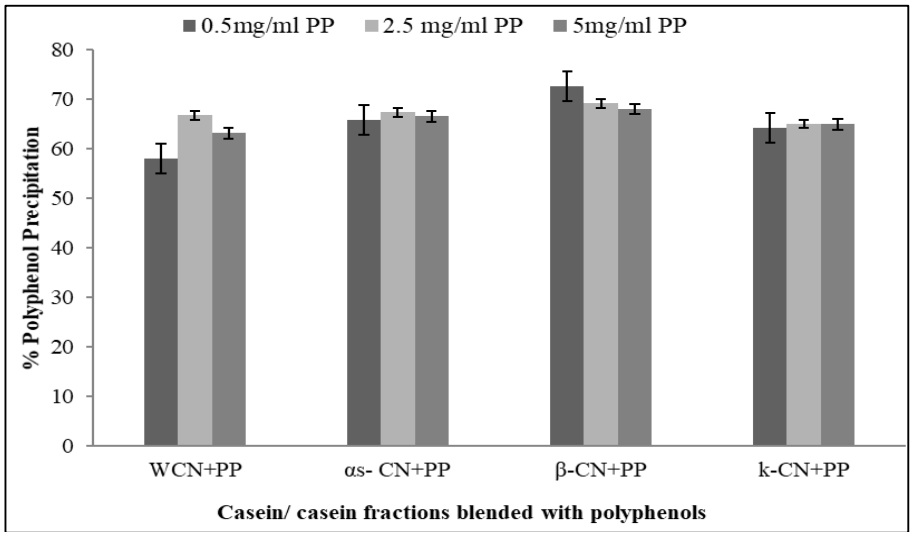
As displayed in Fig 1 (d) α-glucosidase inhibitory activity of polyphenols extract was found to be 25% at 0.5 mg/ml polyphenol concentration and observed to be increased up to 51% and 80% with the increase in polyphenol concentration from 2.5 to 5 mg/ml, respectively. Cheplick *et al*. 2010 observed the α-glucosidase inhibitory activity for water and alcohol soluble extract of strawberry different cultivars and showed the inhibition activity of 2, 4 and 24% at 10µg/ml, 50µg/ml and 100µg/ml for water extract of Northen east variety. In the present study whole casein and its fractions also showed a similar α-glucosidase inhibitory activity of 10% and on binding with strawberry polyphenols at different concentrations resulted in a decrease in α-glucosidase inhibitory activity in comparison with strawberry polyphenols alone. The utmost decrease was with β-casein, subsequently with α_s casein, whereas it was alike for whole casein as well as κ-casein. Moreover, an increase in polyphenol concentration in concert with whole casein and its fractions resulted in progressive augment in α-glucosidase inhibitory activity at 0.5, 2.5 and 5 mg/ml concentration.

Effect of change in pH

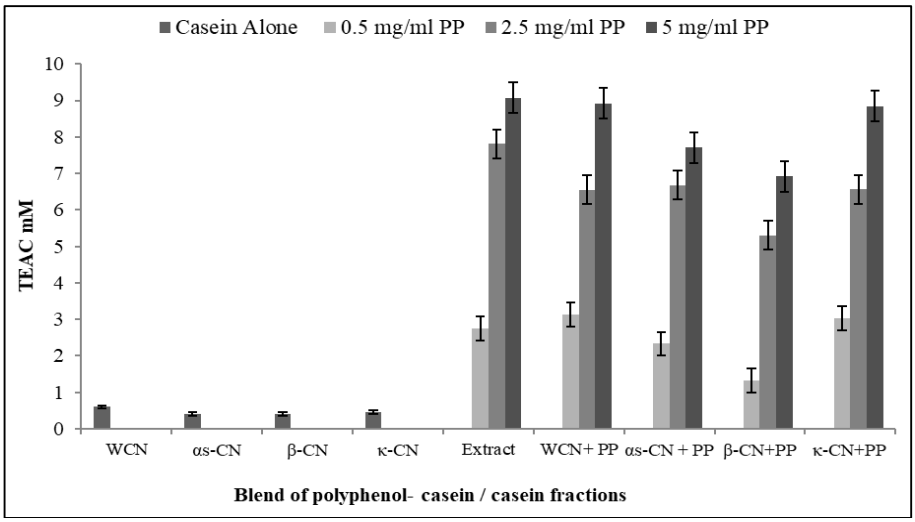
Impact of neutralization on polyphenol precipitation, antioxidant activity (DPPH/ORAC) and α-glucosidase inhibitory activity was measured for whole casein, α_s casein, β-casein, and κ- casein along with polyphenols at 0.5 mg/ml concentration. The native pH of polyphenols extract was observed to be 3.57. The impact of an increase in pH of polyphenols for the blends along with whole casein, α_s casein, β-casein, and κ- casein showed no significant (p<0.05)

difference on the amount of polyphenol precipitation at both pH 5.5 and 6.6 (Fig 2 (a)). Non significant variation ($p>0.05$) was also observed in the DPPH radical scavenging activity (Fig. 2 (b)) and of blends of casein and its different fractions with polyphenols. However, for radical scavenging activity estimated using ORAC assay showed a similar trend for whole casein and κ-casein blended with polyphenols except for α_s casein and β casein (Fig. 2 (c)). Results indicated that change in pH of polyphenols extract from 5.5 to 6.6 did not largely affect the polyphenol precipitation of casein and its fraction.

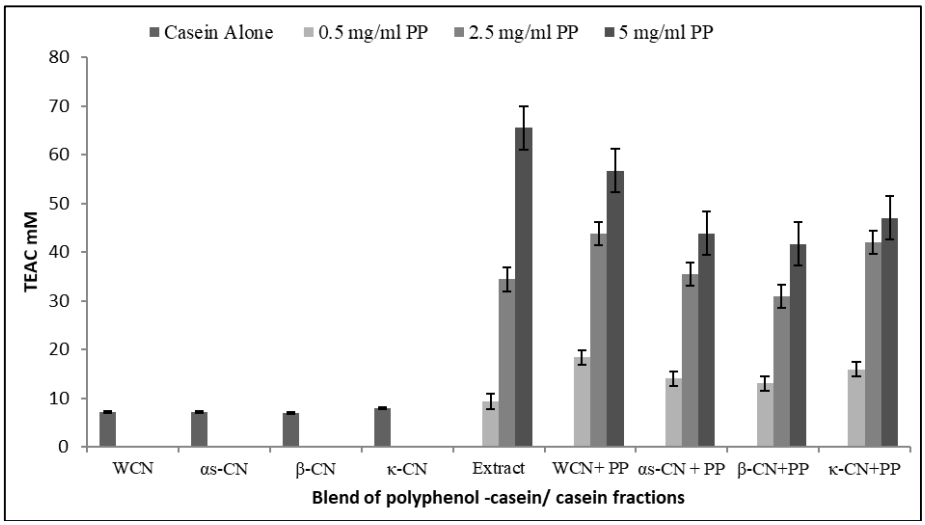
The change in pH of polyphenol extract did not affect the-glucosidase inhibition activity as shown in Fig. 2 (d), demonstrating that no significant ($p>0.05$) variation takes place on polyphenol precipitation by whole casein, α_s-CN, β-casein, and κ-casein either at pH 5.5 or 6.6.



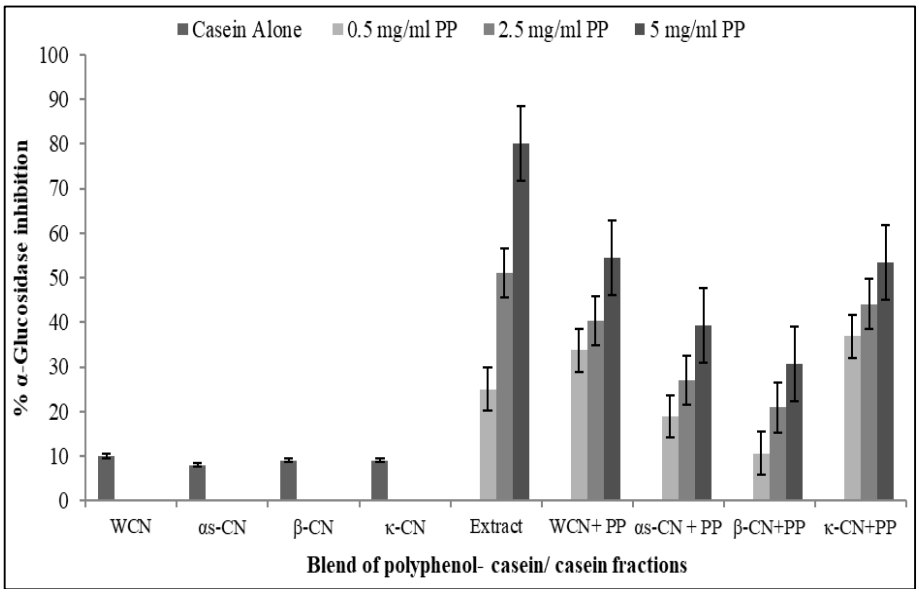
(a)



(b)

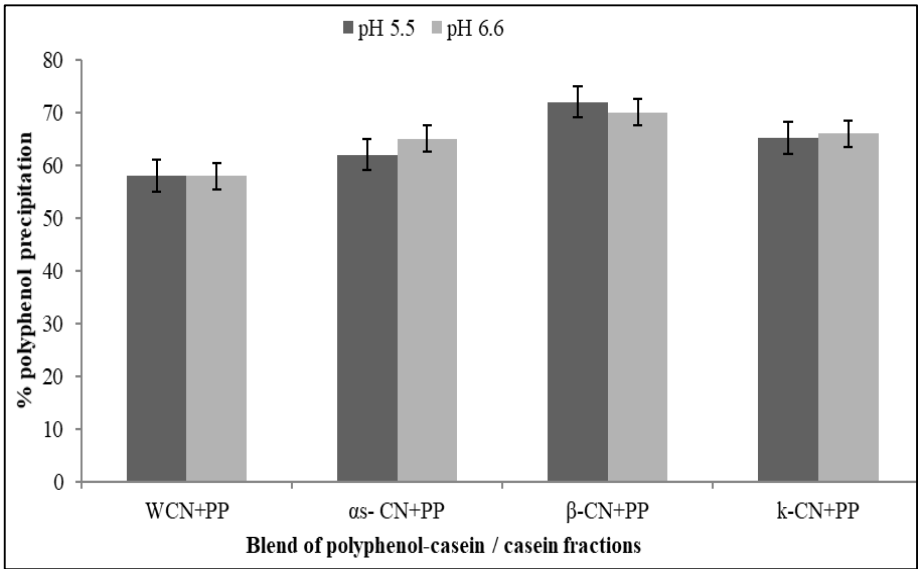


(c)

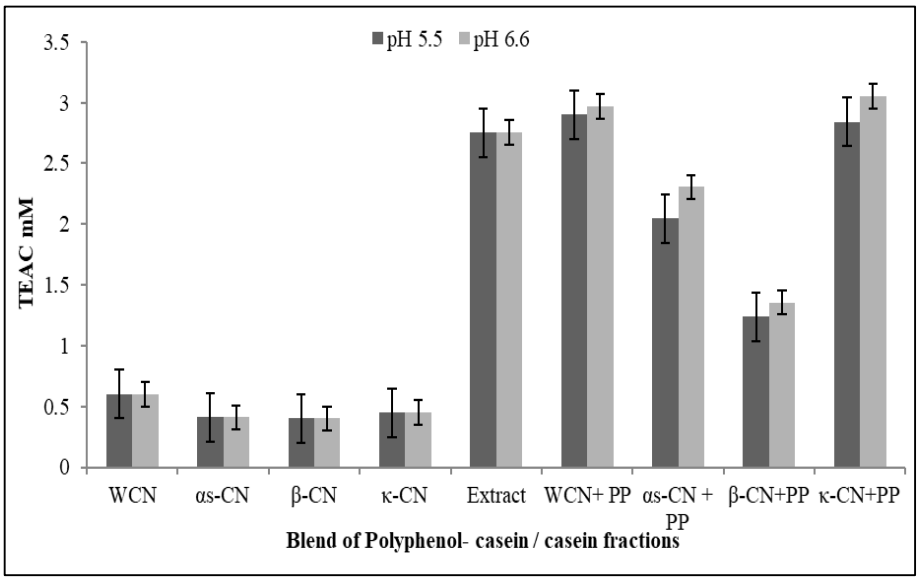


(d)

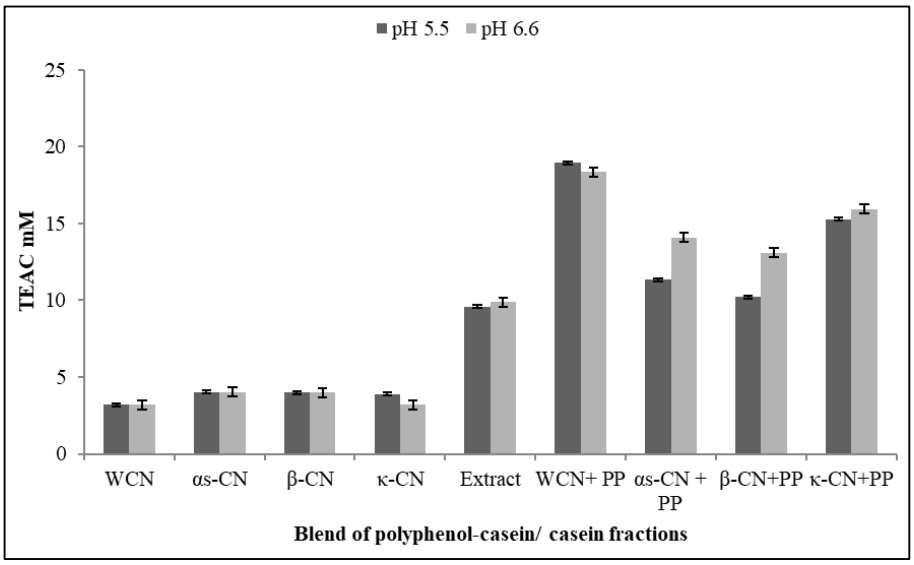
Fig 1: Effect of change in polyphenol concentration (a)% polyphenol binding (b) Antioxidant activity by DPPH (c) Antioxidant activity by ORAC-FL assay (d) α-Glucosidase inhibition.



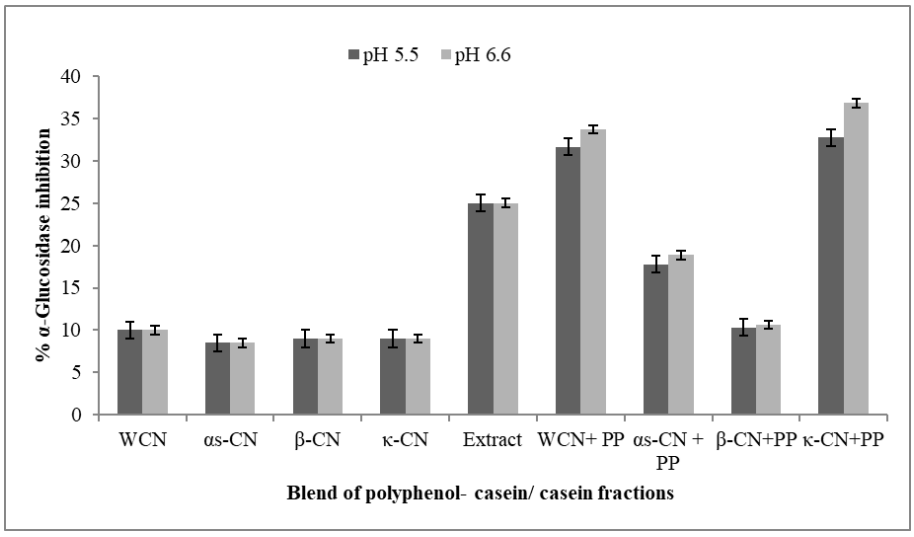
(a).



(b).



(c)



(d)

Fig 2: Effect of change in pH (a)% Polyphenol binding (b) Antioxidant activity by DPPH (c) Antioxidant activity by ORAC-FL assay (c) α-glucosidase inhibition

Conclusion

Present study showed the maximum strawberry polyphenols binding with the β-casein fractions than the others due to its hydrophobic nature. Furthermore, binding of polyphenols with milk proteins (α_s-CN, β-casein, and κ-casein) appears to be an important factor affecting the functionality of protein-rich dairy products.

Data Availability

The data used to support the results of current study are included within the article.

Conflict of interest: Authors have no conflict of interest

Acknowledgment

I am grateful to Director, National Dairy Research Institute, Karnal, Haryana for providing me the laboratory facility and financial assistance as institutional fellowship to carry out the present investigation.

References

1. Altemimi A, Lakhssassi N, Baharlouei A, Watson D, Lightfoot D. Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. Plants. 2017; 6(4):42.
2. Apostolidis E, Kwon I, Shetty K. Potential of select yoghurt for diabetes and hypertension management. J. Food Biochem. 2006; 30(6):699-717.
3. Art M, Haenen G, Wilms L, Beetstra S. Interactions between Flavonoids and Proteins: Effect on the Total Antioxidant Capacity. J agri food chem. 2002; 50(5):1184-7.
4. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Technol. 1995; 28(1):25-30.
5. Broyard C, Gaucheron F. Modifications of structures and functions of caseins: a scientific and technological challenge. Dairy Sci Technol. 2015; 95:831-862.
6. Burr R. Protein purification from milk. In: Roe, S. (Ed.), Protein purification applications: A practical approach Oxford Univ. Press U.K. 2001, 87-115.
7. Cheplick S, Kwon YI, Bhowmik P, Shetty K. Phenolic-linked variation in strawberry cultivars for potential dietary management of hyperglycemia and related complications of hypertension. Bio. Technol. 2010; 101(1):404-413.

8. Elikoglu S, Erdem Y. Interactions between milk proteins and polyphenols: Binding mechanisms, related changes, and the future trends in the dairy industry. Food Rev Int. 2018; 34(8):665-697.
9. Gallo M, Vinci G, Graziani G, Simone C, Ferranti P. The interaction of cocoa polyphenols with milk proteins studied by proteomic techniques. Food Res Int. 2013; 54:406-415.
10. Hansi I, Bourassa P, Hamdani S, Samson G, Carpentier R, Ali H *et al*. Interaction of milk α- and β-caseins with tea polyphenols. Food Chem. 2011; 126:630-639.
11. Kartsova L, Alekseeva A. Effect of milk caseins on the concentration of polyphenolic compounds in tea. J Analytical Chem. 2008; 63(11):1107-1111.
12. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: Food sources and bioavailability. The Am J Clinical Nutr. 2004; 79:727-747.
13. Prigent SVE, Gruppen H, Visser AJWG, Koningsveld GA, de Jong GAH, oragen AGJ. Effects of Non-covalent Interactions with 5-O-Caffeoylquinic Acid (Chlorogenic Acid) on the Heat Denaturation and Solubility of Globular Proteins. J Agri Food Chem. 2003; 51(17):5088-5095.
14. Ramos S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. The J Nutr Biochem. 2007; 18:427-442.
15. Rasouli H, khodarahmi R, Ghazvini S, Adibi H. Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic compounds: A virtual screening perspective for the treatment of obesity and diabetes. Food and Function. 2017; 8:1942-1954.
16. Schaich K. Developing a rational basis for selection of antioxidant screening and testing methods. Acta Horticulture. 2006; 709:79-94.
17. Sęczyk Ł, Świeca M, Kapusta I, Gawlik-Dziki U. Protein–Phenolic Interactions as a Factor Affecting the Physicochemical Properties of White Bean Proteins. Molecules. 2019; 24(3):408.
18. Shahidi F, Yeo J. Bioactivities of Phenolics by Focusing on Suppression of Chronic Diseases: A Review. Int J Mol Sci. 2018; 19(6):1573.
19. Sharma V, Vijay Kumar H, Jagan Mohan Rao L. Influence of milk and sugar on antioxidant potential of black tea. Food Res Int. 2008; 41(2):124-129.
20. Xiao J, Mao F, Yang F, Zhao Y, Zhang C, Yamamoto K. Interaction of dietary polyphenols with bovine milk proteins: Molecular structure-affinity relationship and influencing bioactivity aspects. Mole. Nutr. Food Res 2011; 55:1637-1645.
21. Zhang Q, Zhang J, Shen J, Silva A, Dennis D, Barrow C. A Simple 96-Well Microplate Method for Estimation of Total Polyphenol Content in Seaweeds. J Appl Phycol. 2006; 18(3):445-450.
22. Zulueta A, Maurizi A, Frígola A, Esteve MJ, Coli R, Burini G. Antioxidant capacity of cow milk, whey and deproteinized milk. Int Dairy J. 2009; 19(6, 7):380-385.