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RESEARCH ARTICLE

Effect of interaction of herb (*Pueraria tuberosa*) components with milk constituents on properties of milk

Sawale Pravin Digambar[1](✉), N Veena[2], RRB Singh[3] and Sumit Arora[3]

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Abstract: Milk is a carrier which has been effectively used to deliver phytochemicals for targeted health benefits in the traditional Indian systems of medicine, particularly Ayurveda. The model system consisted of cow milk fortified with aqueous extract of *Pueraria tuberosa* (@ 0.4%) on the basis of sensory evaluation by using 9-point hedonic scale. Effect of addition of herb on compositional and storage stability of control and experimental milk was investigated. The interactive effect of milk proteins with *Pueraria tuberosa* extract (@0.4%) was also studied by SDS-PAGE and urea-PAGE electrophoresis. The addition of *Pueraria tuberosa* to milk resulted in no change in composition, increase in phenol content and decrease in pH (during successive period of storage) as compared to control. Electrophoretic pattern of sodium caseinate and whey protein concentrate containing 0.4% herb extract showed that the band width changed in terms of height and raw volume. It can be concluded that addition of *Pueraria tuberosa* to milk at 0.4% concentration altered the functional properties of milk which could be due to interaction of components of *Pueraria tuberosa* with milk constituents.

Keywords: Milk, *Pueraria tuberosa* extract; Compositional analysis; Electrophoresis

Introduction

Pueraria tuberosa commonly known as Vidarikand and Indian Kudzu, belongs to *Fabaceae* family. The plant's tuber has very widely used as an active component in various formulations of Indian system of medicine (Ayurveda). It has been used as an aphrodisiac, cardiotonic, diuretic, galactogogue, hypolipidemic, immune booster, anti-inflammatory, antioxidant, antiageing and spermatogenic in various Ayurvedic formulations. The major active components in the tuber of *Pueraria tuberosa* are isoflavonoids viz. puerarin, genistein, daidzein, tuberosin and flavanoids (Maji et al. 2014).

Milk is one such carrier which has been effectively used to deliver phytochemicals for targeted health benefits of the traditional Indian system of medical sciences (Veena et al. 2015; Sawale et al. 2020). Addition of herbs or its extracts to milk and subsequent processing treatments however poses a definite challenge as possibilities exist for varying degree of interactions among the major and minor biomolecules of milk and bioactive compounds of herbs (Sawale et al. 2019). Such interactions could have a beneficial effect but sometimes it may also lead to certain practical difficulties if they modify properties of foods.

The isoflavonoids of *pueraria tuberosa* could interact with milk proteins viz., bovine serum albumin (Cao and Liu 2009), casein micelle (Xi and Guo 2008) and â-lactogobulin as has been reported in case of certain food and drug preparation containing soya isoflavonoids. Unavailability of such data on stability of bioactive molecules of *Pueraria tuberosa* in model dairy food systems and their interaction with milk constituents is a determinant to establish efficacy of their use as nutraceutical in functional dairy foods. Hence, the present study was aimed to investigate the effect of herb (*Pueraria tuberosa*) in an herb-milk model system.

Materials and Methods

Materials

Raw cow milk was obtained from the Livestock Research Centre of the ICAR-National Dairy Research Institute, Karnal, India. A freeze-dried hot water extract of herb (*Pueraria tuberosa)* was procured from National Botanical Research Institute (NBRI), Lucknow, Uttar Pradesh, India. Sodium caseinate and whey protein concentrate was purchased from Thomas Baker Pvt. Ltd., Mumbai, India. Molecular weight markers (205 KDa to 3.5 KDa) were purchased from Genei, Bengaluru, India. All other reagents used in this study were of analytical grade.

[1]College of Dairy Technology, Warud (Pusad), Maharashtra Animal and Fishery Sciences University, Nagpur, India
[2]DSLD College of Horticultural Engineering and Food Technology, UHSB, Devihosur, Haveri, Karnataka, India
[3]ICAR-National Dairy Research Institute, Karnal, Haryana, India

Sawale Pravin Digambar (✉)
College of Dairy Technology, Warud (Pusad)-445204, MAFSU, Nagpur, India.
Email: pravins92@gmail.com

Preparation of hot water extract of herb

The tubers of *P. tuberosa* were bought from local market and authenticated. They were deposited in the departmental herbal drug museum of the Pharmacognosy Division, NBRI, Lucknow for future reference. Figure 1 represents the procedure for preparation of hot water extract of herb. The coarse air-dried (40–50°C), powdered tuber (500 g each) of *P. tuberosa* was extracted with hot water by heating on a boiling water bath. The respective extracts were pooled, filtered, concentrated at reduced temperature (below 55°C) by rotary evaporation (Büchi, USA), and lyophilized (Freezone 4.5, Labconco, USA) under high vacuum (133 × 104 mbar) at "40 ± 2°C to yield the hot water (112.0 g) extract with 22% yield.

Separation of puerarin by high performance thin layer chromatography (HPTLC)

Dried hot water extracts in 10 mg/ml concentration was prepared for analysis. Puerarin (1 mg/ml, as marker compound) was used as standard. A Camag HPTLC system (Muttenz, Switzerland) comprising of a Linomat 5 automatic applicator, Camag TLC scanner 3 and win-CATs version 4 software was used. Precoated silica gel-60 F_{254} plates (0.2 mm thickness, Merck) on aluminium sheets were used as adsorbent layers. 2 µl of standard and 10 µl of sample solutions were applied and the plate was developed using ethyl acetate: methanol: water (10:1:1) as developing solvent. The plate was visualized under UV at wavelength of 254 nm and 366 nm. The presence of puerarin was simultaneously identified in the hot water extracts.

Preparation of *Pueraria tuberosa*-milk model

Lyophilized extract of *Pueraria tuberosa* was crushed using pestle and mortar with a small quantity of milk and then the mixture was added to bulk milk. Herb extract was added @ 0.4% in milk was observed to be an optimum level based on preliminary sensory evaluation for colour, flavour and consistency by 9-point hedonic scale. The fortified milk was then subjected to pasteurization treatment (63ºC/30 min) before further analysis.

Compositional analysis

Total solid, protein, lactose and ash content of control and experimental milk samples were determined as per the AOAC (2000) method. Fat content was determined by the Gerber method (IS 1981). Total phenolic content of milk samples was analyzed by Folin-Ciocalteu method (Kahkonen et al. 1999).

Interaction studies by electrophoresis

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to know the interaction between the milk protein and herb extract. SDS-PAGE was run for both control and *Pureria tuberosa* added milk samples, but the visibility of bands was poor and overlapping each other because of the interfering substances in the milk. Hence, sodium caseinate and whey protein concentrate were used to study the interactive effect. Sodium caseinate (2.4%) and whey protein concentrate (0.7%) (with or without *Pueraria tuberosa* extract @ 0.4%) were analyzed by the Urea-PAGE in polyacrylamide gels was performed according to the method of Andrews (1983) and by SDS-PAGE was performed by standard method of Laemmli (1970) with direct staining using Coomassie Brilliant Blue G-250.

The gels were analysed by using ImageAide gel analysis software (Spectronics Corporation ImageAide for Windows) and the densitograms were then drawn. The results were expressed in terms of height and raw volume (area under the curve) of each band in the lane.

Storage study

Storage of control and experimental milk samples were done at refrigerated temperature (6-8°C) for 5 days and samples were analyzed on every day of storage for pH and acidity. The pH and acidity of control and experimental milk sample was determined (IS 1981).

Statistical analysis

The entire experiments were replicated three times. All statistical analyses were performed using SYSTAT 6.0.1 software. Results are presented in means ± standard error of mean (SEM), and statistical significance was set at $p<0.05$. The t-test was used to determine the main effects of treatments.

Results and Discussion

Identification of peurarin by HPTLC

Figure 1 shows the HPTLC chromatograms of the *Pueraria tuberosa* hot water extract and peurarin standard visualized under UV at 254 and 366 nm. The identity of puerarin in hot water extract of herb was confirmed by comparison of its spectrum and retardation factor (R_f) with the authentic standard. Since the health benefits of the nutraceuticals or functional foods containing different botanicals is due to the presence of the phytoconstituents, it is important to have a biological marker and also to be able to associate that biological marker with the quality of life (Veena et al. 2014). Tubers of *Pueraria tuberosa* are rich in various isoflavonoids including puerarin. The Puerarin is the major isoflavanoid present in *Pueraria tuberosa* and demonstrated to have antioxidant and immunomodulatory activity (Pandey et al. 2007). Puerarin present in Indian Kudzu, possess a cardioprotective activity and give rotection against stress induced myocardial ischemia (Verma et al. 2009).

Compositional analysis

Fat, protein, lactose, ash and total solids content of experimental milk did not differ significantly (P>0.05) compared to control (Table 1). However, significant difference (P<0.05) in phenolic content was observed between control and *Pueraria tuberosa* added milk. Significant difference was also found between aqueous solution of *Pueraria tuberosa* extract (@0.4%) and *Pueraria tuberosa* added milk (Table 1). After pasteurization also concentration of polyphenol increased significantly (p<0.05) in *Pueraria tuberosa* added milk. The present result was corroborated with the study of Gad and Abd El-Salam (2010). They reported that the addition of green tea, rosemary extract, to skim milk significantly increased the phenol content and antioxidant activity of skim milk and they were increased on heat treatment (65°C/30 min) of skim milk. It could be due to excess phenolic compounds released as the breaking of the bonds between polyphenols and milk protein in the complexation compound (Rohn et al. 2004). Decrease in phenolic content of *Pueraria tuberosa* added milk in comparison to an aqueous solution of the extract itself could therefore be related to the polyphenol present in *Pueraria tuberosa* which might have interacted with milk protein and chelated metals.

Electrophoresis (Urea-PAGE and SDS-PAGE)

To investigate the interactive effect of milk proteins with *Pueraria tuberosa* extract, sodium caseinate and whey protein concentrate (WPC) were used for urea-PAGE and SDS-PAGE and change in band intensity was measured using Image Aide gel analysis software. Figure 2 shows the urea-PAGE patterns of sodium caseinate and WPC containing 0.4% herb extract. The electrophoretic pattern of with and without addition of herb extract did not show any difference in band pattern i.e there was no difference in mobility based on charge of the proteins, but the intensity (width) of band differed. Table 3 represents the effect of *Pueraria tuberosa* on band intensities of sodium caseinate and WPC in terms of height and area under curve (raw volume). From Table 3, it could be observed that the height as well as raw volume decreased in sodium caseinate+*Pueraria tuberosa* extracts and WPC+*Pueraria tuberosa* extract as compared to sodium caseinate and WPC lanes, respectively. Therefore it could be inferred that the addition of aqueous extract of *Pueraria tuberosa* to milk and subsequent pasteurization led to formation of protein-polyphenol (isoflavons) complexes. Brown and Wright (1962) studied the tea polyphenol/milk protein system. They concluded that the possibility of hydrogen bond formation by performing electrophoresis on membrane filters in the presence of urea which is well known agent for breaking hydrogen bonds. The absence of complex formation in the presence of urea would suggest a mechanism involving hydrogen bonds. They also reported that the protein patterns are unchanged by the presence of the tea polyphenols. In the presence of the urea there was no precipitation of α-lactalbumin or β-lactoglobulin on addition of

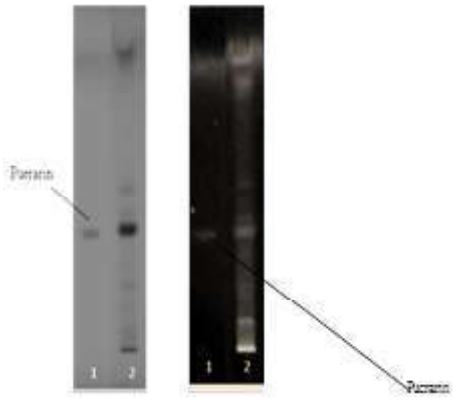

Fig.1 HPTLC Profiles of hot water extracts of *Pueraria tuberosa* using Puerarin as standard visualized under A) UV (λ at 254 nm); B) UV (λ at 366 nm) (Track 1 - Puerarin standard; Track 2 - Hot water extract (Solvent

Table 1: Compositional parameter of control and milk added with *Pureria tuberosa*

Constituents	Control	Milk added with PT	Aqueous solution of PT (0.4%)
Fat (%)	3.80±0.26[a]	3.73±0.30[a]	
Total Solids (%)	13.14±0.08[a]	13.33±0.06[a]	
Protein (%)	3.35±0.03[a]	3.36±0.02[a]	
Lactose (%)	3.93±0.06[a]	3.99±0.10[a]	
Ash (%)	1.12±0.02[a]	1.123±0.02[a]	
Phenol content (µg gallate eq/ml)	141.56±1.686[a]	181±16.22[b]	265.55±0.33[c]

Results are expressed as Mean±SEM (n=3). The values with same superscripts (a) in each row did not differ significantly (p>0.05), PT- *Pueraria tuberosa*

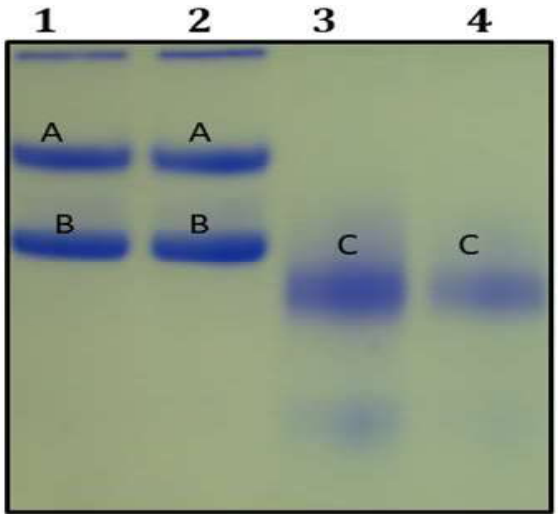

Fig 2. Urea-PAGE pattern of sodium caseinate and WPC added with *Pueraria tuberosa* separated on 12% gel. Lane 1 - Sodium caseinate; Lane 2 - Sodium caseinate+ *Pueraria tuberosa* herb extract, Lane 3 - WPC, Lane 4 - WPC+ *Pueraria tuberosa* herb extract. (A -β –Casein, B - α-Casein, C - β-Lactoglobulin)

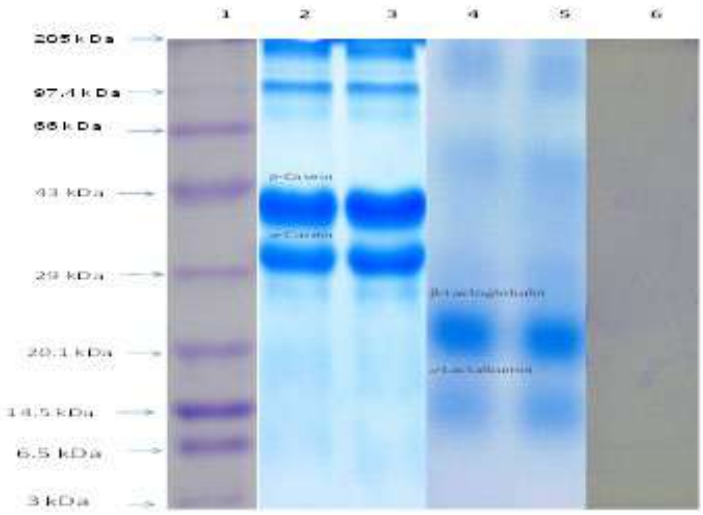

Fig. 3 SDS-PAGE patterns of Sodium caseinate and WPC added with *Pueraria tuberosa* separated on 15% gel. Lane 1 - Molecular weight standards ranging from 205 KDa to 3.5 KDa, Lane 2 - Sodium caseinate, Lane 3 - Sodium caseinate + *Pueraria tuberosa* extract, Lane 4 - WPC, Lane 5 - WPC + *Pueraria tuberosa* extract, Lane 6 - *Pueraria tuberosa* extracts

Table 2: Effect of storage period on pH and titratable acidity of control and *Puraria tuberosa* added milk

Parameter	Types of milk	Storage days (at 6–8ºC)					
		0	1	2	3	4	5
pH	Control	6.72 ± 0.03^{aA}	6.67 ± 0.05^{bA}	6.54 ± 0.03^{cA}	6.32 ± 0.03^{dA}	6.24 ± 0.03^{eA}	6.09 ± 0.03^{fA}
	Milk added with PT	6.65 ± 0.05^{aB}	6.48 ± 0.03^{bB}	6.30 ± 0.03^{cB}	5.12 ± 0.03^{dB}	6.091 ± 0.03^{eB}	5.99 ± 0.03^{afB}
Acidity (% lactic acid)	Control	0.135 ± 0.001^{A}	0.151 ± 0.002^{Al}	0.168 ± 0.001^{Ac}	0.17 ± 0.001^{Ad}	0.178 ± 0.001^{Ae}	0.20 ± 0.001^{Af}
	Milk added with PT	0.153 ± 0.001^{B}	0.178 ± 0.001^{Bl}	0.182 ± 0.003^{Bc}	0.19 ± 0.003^{Bd}	0.12 ± 0.001^{Be}	0.25 ± 0.0005^{B}

Results are expressed as Mean± SEM with different superscripts in each row (a, b, c, d, e, f) and in each column (A, B) differ significantly ($P<0.05$) ($n=3$). PT- *Pueraria tuberosa*

Table 3: Effect of *Pueraria tuberosa* on band intensities of sodium caseinate and WPC by urea-PAGE

Track No	Lane 1 Sodium caseinate		Lane 2 Sodium caseinate+ *Pueraria tuberosa*		Lane 3 WPC		Lane 4 WPC+ *Pueraria tuberosa*	
	Height	Volume	Height	Volume	Height	Volume	Height	Volume
1	71.254	982551.00	67	804078.25	45.170	4364145.61	27.67	1914389
2	77.55	1320863.75	72	1123163				

the tea infusion and in all cases none of the brown colour was seen to move with the protein. Membrane filter electrophoresis in phosphate buffer (pH 6.7) and 7 M with respect to urea indicates that the milk protein/tea polyphenol interactions are at least initiated by the formation of hydrogen bonds. In a similar study, Chapon et al. (1961) studied beer polyphenol and protein interactions. It was concluded that beer polyphenols formed complexes with proteins through the formation of hydrogen bonds.

Figure 3 represents the SDS-PAGE electrophoretic patterns of sodium caseinate and WPC containing 0.4% herb extract. It is

Table 4: Effect of *Pueraria tuberosa* on band intensities of sodium caseinate and WPC by SDS-PAGE

Track No	Lane 2 Sodium caseinate		Lane 3 Sodium caseinate+ *Pueraria tuberosa*		Lane 4 WPC		Lane 4 WPC+ *Pueraria tuberosa*	
	Height	Volume	Height	Volume	Height	Volume	Height	Volume
1	18.650	114132.84	19.957	125724.06	23.614	1223801.13	73.104	1233715.5
2	129.684	2266318.75	133.645	2388853.5				
3	116.727	1735054.88	118.817	1863118.75				

obvious that native casein was resolved into two major bands (α_s- and β-casein). In addition, some aggregates were also observed. The results are in accordance with the observation of Chobert et al. (2007), who reported that bovine casein separated into two major bands along with some aggregates. In the present study, a slight difference in band intensity was observed. There was no extra band resolved in sodium caseinate + *Pueraria tuberosa* and WPC+ *Pueraria tuberosa* lane as compared to sodium caseinate and WPC lanes. In pure *Pueraria tuberosa* extract (0.4%) (Lane 6), no band was observed. Gel analysis software Image Aide, measured the height and area under curve (raw volume) of each lane of SDS-PAGE gel represented in Table 4. Results revealed that, the band height and raw volume was increased in sodium caseinate+*Pueraria tuberosa* as compared to sodium caseinate alone. Puerarin is an active component in *Pueraria tuberosa* which could have interacted with micelle of identical positive charged head groups and varying tail length, affinity of micelle being more toward greater chain length. Similarly, there was an increase in the height and raw volume in WPC+ *Pueraria tuberosa* as compared to WPC alone. Xi and Guo (2008) reported that puerarin (methanol extract) can bind with blood serum albumin (BSA) at 20-30°C and decrease binding stability with increased temperature and the presence of Cu^{2+} and Fe^{3+} ions increased the binding constants and the number of binding sites of the puerarin–BSA complex.

Storage study

The control and experiment milk samples stored at 6-8°C were evaluated for pH and acidity every day during storage. pH of control and Pueraria tuberosa extract added milk differed significantly (P>0.05) during the entire period of storage. A significant (P<0.05) decrease in pH of milk added with *Pueraria tuberosa* extract and control was noticed during the entire period of storage (Table 2). The decrease in pH of *Pueraria tuberosa* added milk sample was sharper than the control. The acidity of *Pueraria tuberosa* added milk was increased from 0.15 to 0.25% lactic acid (LA) during 5 days of storage (Table 2). A significant increase in titratable acidity was noticed in control as well as *Pueraria tuberosa* added milk samples during storage. Also, significant difference (P<0.05) was observed in titratable acidity between control and *Pueraria tuberosa* added milk throughout the storage period. After 5 days, both the samples were curdled. The results are in accordance with Petrotos et al. (2012) who observed that the rapid drop of pH of olive fruit polyphenol-milk system during fermentation of milk (during successive period storage). A decrease in pH and increase in titratable acidity with increasing storage period was observed in control as well as milk fortified with flaxseed oil, phytosterols and polydextrose (Nagarajappa and Battula 2017). The bioactive compound of *Pueraria tuberosa* might be responsible for growth of bacteria; hence, more drop in pH of milk during storage.

Conclusions

The addition of *Pueraria tuberosa* to milk resulted in no significant change in the proximate composition but increase in phenol content as compared to control. A decrease in pH and increase in acidity was observed in both samples during storage period. The electrophoretic pattern showed that herb components were interacted with the milk proteins. These interactions could alter the different properties of milk. *Pueraria tuberosa* might be responsible for enhance the health benefits due to release of phenol and faster reduction of pH of milk which possibly provide scope for reduction in production time for the fermented products. Furthermore, there is scope to develop *Pueraria tuberosa* fortified dairy products and also need to verify the beneficial effect of interaction of herb components and milk constituents by using animal/ human studies.

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