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Article · December 2021

DOI: 10.26855/ijfsa.2021.12.025

International Journal of Food Science and Agriculture, 2021, 5(4), 762-774
http://www.hillpublisher.com/journals/jsfa/
ISSN Online: 2578-3475
ISSN Print: 2578-3467

Antioxidant Potential of Fermented Milk Supplemented with Various Aqueous Herbal Extracts

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How to cite this paper: Kanik, Birbal Singh, Jyoti B. Dhar, Gauri Jairath, Rinku Sharma, Devi Gopinath, Gorakh Mal. (2021) Antioxidant Potential of Fermented Milk Supplemented with Various Aqueous Herbal Extracts. *International Journal of Food Science and Agriculture*, 5(4), 762-774.
DOI: 10.26855/ijfsa.2021.12.025

Received: October 19, 2021
Accepted: November 16, 2021
Published: December 27, 2021

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Abstract

The objective of this study was to investigate the *in vitro* antioxidant activity of fermented milk of indigenous hill cattle (*Himachali Pahari Cow*) supplemented with various aqueous herbal extracts. The probiotic *Lactobacillus rhamnosus* GG (LGG) fermented milk supplemented with 1% aqueous herbal extracts of fruits of *harad* (*Terminalia chebula*), *baheda* (*Terminalia bellerica*), *arjuna* (*Terminalia arjuna*), and *amla* (*Phyllanthus emblica*) were evaluated for antioxidative activity. Fermented milk containing various aqueous herbal extracts, each @ 1.0%, and corresponding *in vitro* digested samples were centrifuged and analysed thereafter for estimation of total phenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ferric reducing antioxidant power (FRAP), and O-pthaldialdehyde (OPA) activity. TPC (mg TAE/100 ml) was significantly (P<0.05) higher in fermented milk containing aqueous *baheda* extract in undigested (26.43±1.16), pepsin digested (36.53±0.30) and overnight digested (33.98±0.26) samples. Lowest TPC was found in undigested sample of fermented milk containing aqueous *arjuna* extract. FRAP (mg $FeSO_4$ equivalent/100ml) was significantly (P<0.05) higher in fermented milk containing aqueous *harad* extract in undigested (247.46±1.38), pepsin digested (266.21±0.38) and overnight digested (235.64±0.62) samples. DPPH (%) activity was found to be highest in undigested and pepsin-digested fermented milk containing aqueous *baheda* extract (26.40±0.94 and 39.66±0.44) while in overnight digested sample DPPH activity was lowest in fermented milk containing aqueous *amla* extract (27.31±0.48). Highest OPA (mg TE/ml) value was found in fermented milk containing aqueous *harad* extract in undigested milk (4.95±0.04), pepsin digested (8.08±0.15) and overnight digested milk (9.13±0.29). Based on the results described above, we speculate that herbal extracts in milk fermented by probiotic LGG had better antioxidant activity.

Keywords

Himachali Pahari cow milk, *Lactobacillus rhamnosus* GG, Fermented milk, Aqueous herbal extracts, Plant polyphenols, Antioxidant activity

1. Introduction

With the increasing health concern, foods are not only intended to satisfy hunger and provide essential nutrients for humans, but also to thwart nutrition-related diseases and improve consumer's health [1-2]. Cow milk is an important source of nutraceuticals as it contains several substances such as lactose, proteins, lipids, amino acids, creatinine, and urea etc. Moreover, milk also provides calcium, phosphorus, riboflavin, vitamin A, ascorbic acid and thiamine etc. [3].

India is known for its dairy products and is the largest producer of milk, second only to the United States in terms of cow's milk [4]. Milk is an essential source of potent bioactive compounds. Many of these compounds are released during fermentation of milk. Fermented milk products are consumed worldwide due to digestibility and bioavailability of proteins, minerals, etc. and several inactive peptides are activated during the fermentation of milk. Biological active peptides have been identified in the amino acid sequences of native milk proteins. Due to their physiological and physico-chemical versatility, milk peptides are considered to be very important components of natural foods and nutraceuticals. Fermented milk products are easily digestible to consumers with milk allergies and lactose in-tolerance. Consumption of fermented milk has been highly effective against many health conditions, such as diarrhoea, arthritis, asthma, biliary conditions, constipation, stomach flu, hay fever, gastroesophageal reflux disease, hypertension, high cholesterol, etc. [5].

Medicinal plants rich in natural antioxidants and phenols are widely used in food production as in view of important nutritional and therapeutic properties and reduction of oxidative degradation of lipids. In addition, the quality and nutritional values of foods considered to be functional, such as herbal yogurt, can also be enhanced [6]. Medicinal plants and their extracts have a long history of use as natural remedies for human health problems, including metabolic disorders such as insulin resistance, obesity, diabetes, etc. [7]. Since 1960s, there has been an increased interest in "natural health" which has stimulated interest in the natural remedies, i.e., herbs, phytochemicals and their preparations. A large portion of today's natural food market consists of herbal functional foods [8]. Several medicinal plants, such as *Withania somnifera* and *Pueraria tuberose*, have been used to improve the therapeutic value of milk [8].

Terminalia is a tree having three important species with medicinal properties. *T. bellerica* ("baheda"), *T. arjuna* ("arjun") and *T. chebula* ("*harad*") are used in medicine. In traditional ayurvedic medicine, *T. arjuna* was used to balance the three "humors": *kapha*, *pitta*, and *vata*. In India, the bark of *T. arjuna* has been primarily used as a heart remedy for more than 3,000 years [9]. Presently, *T. arjuna* is used against cardiovascular diseases (CVDs) including heart attack and associated chest pain, heart failure, high blood pressure, and high cholesterol [10-11].

T. chebula is known as the "King of Medicines" in Tibet and is one of India's primary ayurvedic herbs due to its remarkable healing powers [12], and presence of a large number of different phyto-constituents. The fruit of *T. chebula* is recognized for its health benefits in the digestive system. This is commonly used as a natural colon cleanser (such as Jon Barron's Colon Corrective Formula) and is used to treat constipation, high fever, ulcers, digestive disorders and hemorrhoids, tumors, ascites (abdominal swelling), enlargement of the liver or spleen, bacteria, colitis, and food poisoning [13]. The fruit is also a good source of vitamin C and minerals such as selenium, manganese, potassium, iron and copper, as well as antibacterial and anti-inflammatory properties [14].

T. bellerica is used to protect the liver and to treat respiratory conditions, including cough, sore throat and respiratory tract infections. In traditional ayurvedic medicine, *T. bellerica* has been used as a "health-harmonizer" in combination with *E. officinalis* and *T. chebula*. The formulation is also used to reduce cholesterol and to prevent death of heart tissue [15].

E. officinalis ('*amla*') is one of the most important plants in the traditional ayurvedic medical system as well as in other traditional system using its immunomodulatory, anti-inflammatory, anti-ulcer, hepatoprotective and anti-cancer properties. The fruits are rich in vitamin C and also in phenolic compounds, including gallic acid, ellagic acid, quercetin, kaempferol, geranin, furosin, corilagin, gallotanins, emblicanins, flavonoids, glycosides and proanthocyanidins. Roots contain glycosides and tannins. In Indian medicine system, *E. officinalis* is used as diuretic, laxative, liver tonic, digestion, restorative, anti-pyretic, refrigerant, hair tonic, prevention of ulcer and common cold, fever; either alone or in combination with other herbs [16].

However, the antioxidant activity of herbal supplemented fermented milk has not been previously prepared and evaluated. Hence, the present study was conducted with the aim to improve the quality of milk obtained from indigenous cow breed known as '*Himachali Pahari cow*' distributed in 7 districts of Himachal Pradesh including Chamba, Mandi, Kullu, Kangra, Sirmour, Kinnaur & Lahaul Spiti, and registered as a unique species with distinct features/qualities [17].

2. Materials and methods

2.1. Materials

Acrylamide, bisacrylamide, N,N,N′,N′-tetramethylenediamine and ammonium persulphate were obtained from Sisco Research Laboratories (Mumbai, India). 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 4, 6 tripyridyl-s-triazine (TPTZ), tannic acid (ACS grade) and O-phathaldialdehyde (OPA) were purchased from Sigma (St. Louis, MO, USA). Molecular markers were obtained from HIMEDIA (Mumbai, India). The F254 TLC silica gel coated aluminium plates were purchased from Merck KGaA (Germany) and used for analyzing antioxidant activities of fermented milk containing various aqueous herbal plant extracts. All other chemicals and reagents were of high purity analytical grade**.**

2.2. Milk samples were collected from surrounding area of Palampur

The pH and total titratable acidity of milk were determined, and then milk containers were stored in a freezer at -20°C for further use [17].

2.3. Collection of herbal plant material

The bark of *T. arjuna* (arjuna) and fruits of *T. bellerica*, *T. chebula* and *E. officinalis* were collected from the surrounding areas of Palampur and processed as described in [17].

2.4. Water extraction of herbal plants

10 g dried powder of *T. chebula, T. bellerica, T. arjuna* and *E. officinalis* was extracted in distilled water as described in [17].

2.5. Preparation of starter culture

Starter culture was prepared by using probiotic grade *Lactobacillus rhamnosus* GG (347) bacteria, purchased from National Collection of Dairy Cultures (NCDC), ICAR-NDRI Karnal (India) using the method described by [17].

2.6. Preparation of fermented milk containing herbal water extracts

Fermented milk was prepared from fresh boiled milk of Himachali Pahari cow as per the method described by [17].

2.7. *In vitro* enzymatic digestion of fermented milk containing aqueous herbal extracts

In vitro enzymatic digestion protocol described by [18] with modifications was used. Undigested and digested samples were centrifuged at 12,000 rpm for 30 min. Supernatant harvested was stored at -20°C for further analysis [17].

2.8. TPC assay

The TPC was determined as described by [19] with minor modifications. An aliquot of 10 µl was taken from different fermented milks, and *in vitro* digested samples. In case of aqueous herbal extracts, 5 µl aliquots were taken. The absorbance was recorded at 765 nm on spectrophotometer (Electronic India D-5). Results were calculated using calibration curve for tannic acid and expressed as mg/100 ml fermented milk equivalent to tannic acid.

2.9. DPPH antioxidant activity assay

DPPH inhibition was determined as described by [20]. An aliquot of 5 µl was taken from the milk, aqueous herbal extract, fermented milk containing aqueous herbal extracts and *in vitro* digested samples. An aliquot of 10 µl of fermented milk served as control. Absorbance was recorded at 517 nm. The percentage of DPPH free radical scavenging activity (% inhibition) was calculated using following equation:

$$\% \text{ inhibition} = \frac{\text{Abs (blank)} - \text{Abs (test)}}{\text{Abs (blank)}} \times 100$$

2.10. FRAP antioxidant activity assay

FRAP was determined as described by [19] with slight modifications. An aliquot of 5 µl from milk, aqueous herbal extract, different types of fermented milk and its digested sample and 10 µl aliquot in case of control fermented milk was taken. The absorbance was recorded at 595 nm using spectrophotometer. Results were calculated using calibration curve for $FeSO_4$ and expressed as mg $FeSO_4$ equivalent/100 ml fermented milk.

2.11. Thin Layer Chromatographic (TLC) analysis using DPPH

The TLC was done for the radical scavenging activity of antioxidant peptides, which might not dissolve in the liquid-based assay [21]. A visible yellowish spot on purple background indicated the presence of antioxidant activity.

2.12. Milk protein proteolysis by OPA

Proteolytic activity using OPA (O-pthaldialdehyde) assay was determined as per [22] with minor modifications. An aliquot of 10 µl was taken each from milk, herbal water extract, fermented milk containing various aqueous herbal extracts and its *in-vitro* digested samples to which 2 ml of OPA reagent was mixed. The mixture was incubated at ambient temperature for 2 min. and the absorbance was recorded at 340 nm on spectrophotometer.

2.13. Milk protein profile by SDS-PAGE

The protocol of Laemmli [23] was followed for Sodium Dodecyl Sulphate Poly-acrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was run in a vertical slab gel electrophoresis apparatus. The resolving gel was 15% and thickness was 1.5 mm. An aliquot of 10 µl of each sample, and molecular weight markers 10 to 245 kDa (HiMedia) were loaded, and electrophoresed at 70 V and 30 mA till the tracking dye reached the bottom of the separating gel.

2.14. Statistical analysis

The statistical analysis was done by using SAS 9.2 statistical package. Results were presented as means and standard error of means. A *P-value* of 0.05 ($p<0.05$) was considered statistically significant.

3. Results and discussion

Four different herbal-supplemented fermented milks along with control fermented milk used in the study were evaluated for their antioxidant activities and protein profile by SDS-PAGE.

3.1. pH and acidity of *Himachali Pahari* cow milk

In present study, the mean pH and acidity of 8 fresh milk samples was observed to be 6.66±0.09 and 0.21±0.01% lactic acid, respectively. The pH of milk is influenced by hygienic and climatic conditions. The pH of indigenous healthy cattle milk was found to vary between 6.14-6.54 [24] according to lactation period. Any change in the pH would destabilize the proteins and result in precipitation and gelation. The determination of acidity of milk is a rapid measure to understand the stability of milk during heat processing. In indigenous cattle, significantly higher values of acidity (0.31%) were reported in early stage of lactation [24]. When the lactic acid increased, the pH decreased from about 6.8 to about 4.6 [25].

3.2. Antioxidant activities and total phenolics in *Himachali Pahari* cow boiled milk and aqueous herbal plant extracts

Our study revealed that *Himachali Pahari* cattle boiled milk have antioxidant activities as well as a source of total phenolics (Table 1). Antioxidant activity in cow, buffalo and goat milk, and milk products may be due to presence of antioxidant compounds such as aromatic amino acid residues (tyrosine, phenylalanine, tryptophan), and free sulfhydryl groups. In addition, the antioxidant activity of milk could also be due to the natural antioxidants, such as α-tocopherol, carotenoids, conjugated linoleic acid, casein and lactoferrin occurring naturally in whey, and the microorganisms and their activities present in milk [26].

Total phenol and antioxidant activities in aqueous herbal extracts are presented in Table 1. The content of phenolic compounds ranged quite markedly, from a low of 279.11±3.99 mg TAE/100ml for the *baheda* fruit extract to a high of 829.96±3.03 mg TAE/100ml for the *harad* fruit extract samples. Total phenols were significantly ($P<0.05$) highest in *harad* fruit extract. DPPH and FRAP antioxidant activities were observed in the aqueous extracts of all four plants screened. The greatest significantly ($P<0.05$) DPPH antioxidant efficacy was recognized from the *baheda* fruit. FRAP values were significantly ($P<0.05$) higher in the *arjuna* bark extract. Although, all plant extracts are rich source of total phenolics and antioxidants. There is increasing interest in the use of nutraceuticals as alternative therapeutics for preventing diseases and ageing [27-28]. The relation between TPC and antioxidant activity is not directly associated which may be due to use of different concentration of extract for assay and the dose-dependent effect of the extracts [29].

Table 1. Antioxidant activities and total phenolics in milk and aqueous herbal extracts

Sample	DPPH (% inhibition)	FRAP (mg $FeSO_4$ equivalent/100ml)	TPC (mg TAE/100ml)	OPA (mg TE/ml)
Milk (n= 8)	4.51^{e}±0.21	36.31^{e}±0.94	74.40^{e}±0.60	15.26^{d}±0.11
Harad fruit (n= 8)	93.70^{b}±0.31	$2,842.19^{b}$±9.72	829.96^{a}±3.03	20.40^{a}±0.21
Baheda fruit (n= 8)	95.75^{a}±0.31	$2,78.73^{c}$±10.38	279.11^{d}±3.99	8.59^{b}±0.15
Amla fruit (n= 8)	93.19^{b}±0.15	$1,966.21^{d}$±6.32	303.65^{c}±0.89	5.21^{c}±0.05
Arjuna bark (n= 8)	91.80^{c}±0.17	$2,905.68^{a}$±9.52	428.48^{b}±4.03	6.15^{c}±0.05

Values with different superscripts within column are statistically significant (a, b, c, d = $P<0.05$); n=number of samples analysed.

3.3. TPC of fermented milk containing aqueous herbal plant extracts

The effects of supplementation of aqueous herbal extracts in fermented milk and *in vitro* digestion on TPC are presented in Table 2. Effect of supplementation of herbal plant extracts in rising the TPC content of fermented milk was significant (P <0.05) in comparison to control samples. No significant effect on TPC was noticed for undigested fermented milk containing aqueous extracts of the *arjuna*. However, gastric (pepsin) and overnight duodenal (trypsin and pancreatin) digested fractions of fermented milk containing aqueous extracts of the arjuna exhibited an overall appreciably higher antioxidant activity in comparison to undigested fermented milk (Figure 1).

Table 2. Total phenol contents (TPC) of fermented milk containing aqueous herbal plant extracts

S. No.	Sample	TPC (mg TAE/100ml)				
		Control	*Harad*	*Baheda*	*Amla*	*Arjuna*
1	Fermented milk (UD) (n= 8)	1.95^{Dc}±0.27	16.25^{Bc}±0.22	26.43^{Ac}±1.16	13.45^{Cc}±0.24	2.34^{Dc}±0.13
2	Pepsin digest (PD) (n= 8)	13.99^{Da}±0.48	35.23^{Aa}±0.37	36.53^{Aa}±0.30	27.15^{Ca}±0.44	30.16^{Ba}±0.41
3	Overnight digest (OD) (n= 8)	6.83^{Eb}±0.35	32.51^{Bb}±0.27	33.98^{Ab}±0.26	19.03^{Db}±0.29	27.69^{Cb}±0.28

Different upper-case letters correspond to significant differences between the groups (P≤ 0.05); Different lower-case letters correspond to significant differences within the same group (P ≤ 0.05).

Our study revealed that undigested fermented milk containing aqueous baheda extract exhibited significantly higher TPC value (26.43±1.16 mg TAE/100 ml) followed by *harad* (16.25±0.22 mg TAE/100 ml) and *amla* (13.45±0.24 mg TAE/100 ml) as compared at P<0.05 level of significance. No significant effect on total phenols (2.34±0.13 mg TAE/100 ml) was observed in the fermented milk containing aqueous *arjuna* extract.

A significant increase in TPC (P<0.05) was observed in the pepsin digested fermented milk containing aqueous *harad*, *baheda*, *amla* and *arjuna* extract as compared to control with maximum increase in *baheda* (36.53±0.30 mg TAE/100 ml) and minimum being in fermented milk containing aqueous *amla* extract (27.15±0.44mgTAE/100ml) (Figure 1). However, among the different fermented milk containing aqueous herbal extracts no significant difference was observed between fermented milk containing aqueous *harad* and *baheda* extracts (Figure 1).

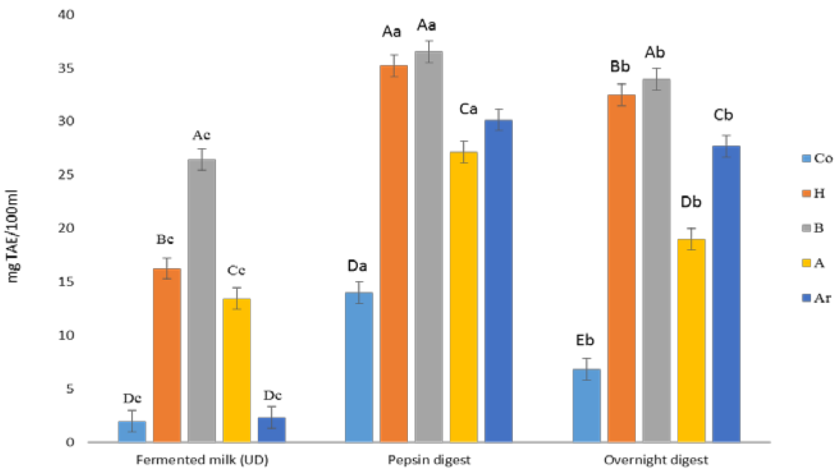

Figure 1. TPC of fermented milk containing aqueous herbal plant extracts (Co- Control, H- *Harad*, B- *Baheda*, A- *Amla*, Ar- *Arjuna*). Different upper-case letters correspond to significant differences between the groups (P ≤ 0.05). Different lower-case letters correspond to significant differences within the same group (P ≤ 0.05).

The TPC in overnight digested fermented milk containing aqueous herbal extracts was found to be significantly higher as compared to control (P<0.05). The maximum increase was observed in fermented milk containing aqueous *baheda* extract (33.98±0.26mgTAE/100ml) and lowest in fermented milk containing aqueous *amla* extract (19.03±0.29 mg TAE/100 ml).

Overall observation of the result exhibited maximum TPC value in fermented milk containing aqueous *baheda* extract (UD, PD and OD) followed by *harad*. The pepsin digested and overnight digested samples exhibited higher TPC in fermented milk containing aqueous *arjuna* extract, whereas undigested fermented milk containing aqueous *amla* extract exhibited significantly higher TPC in comparison to fermented milk containing aqueous *arjuna* extract (Figure 1).

Higher TPC value in the samples supplemented with *baheda* and *harad* may be attributed to higher phenolic content present in the extracts [30]. The improvement in the total phenolic content in milk samples has been reported on supplementation with cinnamon powder [31] and garlic extract [6].

3.4. DPPH antioxidant activity of fermented milk containing aqueous herbal plant extracts

DPPH antioxidant activity of undigested and digested hydrolysates was determined *in vitro* as per method described by [20] with slight modifications. The DPPH radical scavenging activity of the undigested samples and their hydrolysates were measured as function of its percentage inhibition.

The effects of supplementation of aqueous herbal extracts in fermented milk and *in vitro* digestion on DPPH antioxidant activity are presented in Table 3. DPPH antioxidant activity of fermented milk was found to increase after supplementation of aqueous herbal extracts. Significantly ($P < 0.05$) higher DPPH activity was noticed with *baheda*. It was found that the gastric (pepsin) digested fractions of fermented milk containing aqueous herbal extracts displayed an overall significantly ($P < 0.05$) higher antioxidant activity as compared to other samples. Overnight duodenal (trypsin and pancreatin) digested fermented milk also showed a greater percentage of inhibition (Figure 2).

Table 3. DPPH assay of fermented milk containing aqueous herbal plant extracts

S. No	Sample	DPPH (% inhibition)				
		Control	*Harad*	*Baheda*	*Amla*	*Arjuna*
1	Fermented milk (UD) (n= 8)	$3.86^{Eb}\pm0.24$	$23.14^{Bb}\pm0.22$	$26.40^{Ab}\pm0.94$	$14.91^{Cc}\pm0.50$	$7.30^{Da}\pm0.33$
2	Pepsin digest (PD) (n= 8)	$18.20^{Da}\pm0.50$	$31.13^{Ba}\pm0.59$	$39.66^{Aa}\pm0.44$	$25.35^{Cb}\pm0.33$	$8.86^{Ea}\pm0.34$
3	Overnight digest (OD) (n= 8)	$1.19^{Dc}\pm0.20$	$13.88^{Bc}\pm0.44$	$14.09^{Bc}\pm0.38$	$27.31^{Aa}\pm0.48$	$4.85^{Cb}\pm0.28$

Different upper-case letters correspond to significant differences between the groups ($P \leq 0.05$) ; Different lower-case letters correspond to significant differences within the same group ($P \leq 0.05$).

Undigested fermented milk containing aqueous *baheda* extract demonstrated a substantially higher DPPH value (26.40±0.94%), followed by *harad* (23.14±0.22%) and *amla* (14.9l±0.50%) at the significance level of $P<0.05$. Fermented milk containing aqueous *arjuna* extract exhibited significantly lower value (7.30±0.33%).

A substantial increase in DPPH ($P<0.05$) was observed in pepsin digested fermented milk containing aqueous *harad*, *baheda*, and *amla* extracts as compared to control. The maximum antioxidant activity was observed in fermented milk containing aqueous *baheda* extract (39.66±0.44%), whereas, minimum was observed in fermented milk containing aqueous *arjuna* extract (8.86±0.34%) (Figure 2).

The DPPH activity was found to be significantly higher as compared with control ($P<0.05$) in overnight digested fermented milk samples containing aqueous herbal extracts. The highest increase was in the fermented milk containing aqueous *amla* extract (14.09±0.38%) and lowest found in fermented milk containing aqueous *arjuna* extract (4.85±0.28%). Nevertheless, no significant difference was found between fermented milk containing aqueous *harad* and *baheda* extracts (Figure 2).

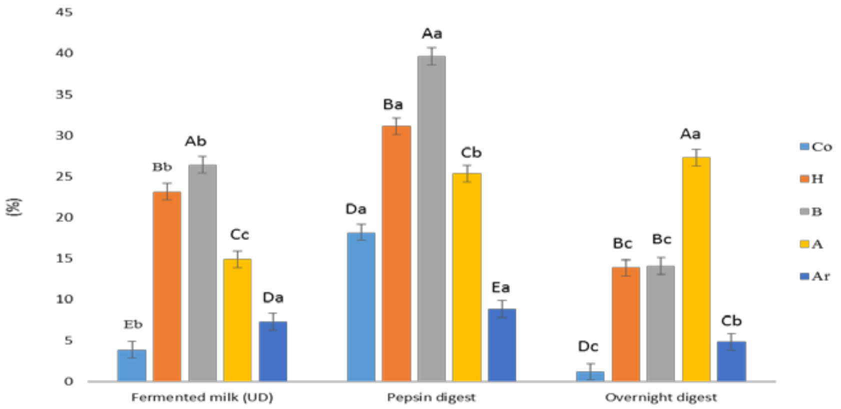

Figure 2. DPPH assay of fermented milk containing aqueous herbal plant extracts (Co- Control, H- *Harad*, B- *Baheda*, A- *Amla*, Ar- *Arjuna*). Different upper-case letters correspond to significant differences between the groups ($P \leq 0.05$). Different lower-case letters correspond to significant differences within the same group ($P \leq 0.05$).

Overall observation shows maximum DPPH value in fermented milk containing aqueous *baheda* extract (UD and PD) followed by *harad*, *amla* and *arjuna* samples. The overnight digested samples demonstrated higher DPPH activity in fermented milk containing aqueous *amla* extract, whereas, fermented milk containing aqueous *baheda* extract and it is *in vitro* digested samples exhibited significantly higher antioxidant activity in comparison to samples containing aqueous *harad* extract. Fermented milk containing aqueous *arjuna* extract and its digested samples exhibited least antioxidant activity (Figure 2).

Increase in antioxidant activity on supplementation with herbal extract has been reported in goat milk yogurt fortified with 2% beet root and 2% ginger extract and cow milk yogurt supplemented with 2% ginger extract [32]. DPPH is an appropriate method for investigation of the total antioxidant activity of milk [33-35].

3.5. FRAP antioxidant activity of fermented milk containing aqueous herbal plant extracts

The effect of supplementation of aqueous herbal extracts in fermented milk and *in vitro* digestion on FRAP are presented in Table 4. Effect of supplementation of herbal plant extracts in increasing the FRAP content of fermented milk was significant (P <0.05) in comparison to control samples.

Undigested fermented milk containing aqueous *harad* extract had a far higher FRAP value was observed in fermented milk samples (247±0.46 mg $FeSO_4$ equivalent/100ml), followed by *baheda* (238.44±0.76 mg $FeSO_4$ equivalent/100 ml) and *amla* (169.01±0.93 mg $FeSO_4$ equivalent/100 ml) at P<0.05. Fermented milk containing aqueous *arjuna* extract exhibited lower value (25.54 ±0.61 mg $FeSO_4$ equivalent/100 ml).

Table 4. FRAP assay of fermented milk containing aqueous herbal plant extracts

S. No.	Sample	FRAP (mg $FeSO_4$ equivalent/100ml)				
		Control	*Harad*	*Baheda*	*Amla*	*Arjuna*
1	Fermented milk (UD) (N= 8)	8.90^{Eb}±0.37	247.46^{Ab}±1.38	238.44^{Ba}±0.76	169.01^{Cc}±0.93	25.54^{Db}±0.61
2	Pepsin digest (PD) (N= 8)	13.00^{Ea}±0.51	266.21^{Aa}±0.38	239.20^{Ba}±1.68	220.45^{Ca}±1.00	61.99^{Da}±1.18
3	Overnight digest (OD) (N= 8)	8.73^{Eb}±0.32	235.64^{Ac}±0.62	104.49^{Cb}±0.62	174.01^{Bb}±1.06	23.24^{Db}± 0.69

Different upper-case letters correspond to significant differences between the groups (P≤ 0.05) ; Different lower-case letters correspond to significant differences within the same group (P ≤ 0.05).

Pepsin digested fermented milk containing aqueous *harad*, *baheda*, *amla* and *arjuna* extracts showed a substantial increase in FRAP (P<0.05) compared to control. Maximum FRAP value was observed in fermented milk containing aqueous *harad* extract (266.21±0.38 mg $FeSO_4$ equivalent/100ml) and minimum FRAP value was observed in fermented milk containing *arjuna* extract (61.99 ±1.18 mg $FeSO_4$ equivalent/100 ml).

The overnight digested fermented milk samples containing aqueous herbal extracts exhibited significant increase in FRAP activity as compared to control (P<0.05) (Figure 3). The maximum FRAP activity was observed in fermented milk containing aqueous *harad* extract (235.64±0.62 mg $FeSO_4$ equivalent/100ml) whereas, the lowest activity was observed in fermented milk containing aqueous *arjuna* extract (23.24±0.69 mg $FeSO_4$ equivalent/100ml).

The overall highest FRAP value was observed in fermented milk containing aqueous *harad* extract (UD, PD and OD) and lowest FRAP value was observed in fermented milk containing aqueous *arjuna* extract (UD, PD and OD) (Figure 3).

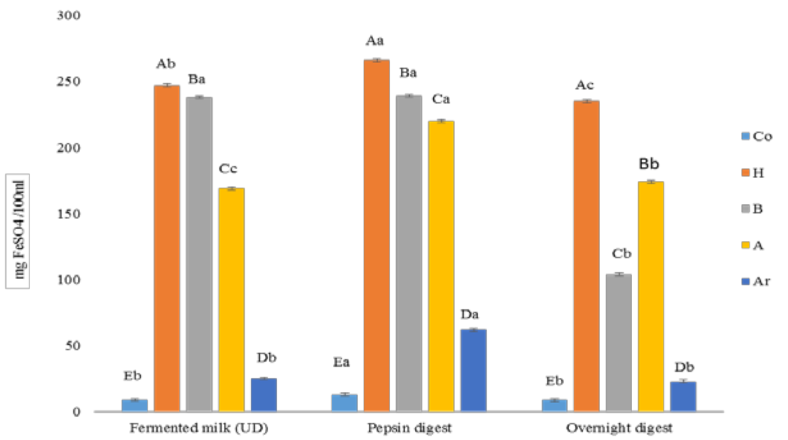

Figure 3. FRAP assay of fermented milk containing aqueous herbal plant extracts (Co- Control, H- *Harad*, B- *Baheda*, A- *Amla*, Ar- *Arjuna*). Different upper-case letters correspond to significant differences between the groups (P ≤ 0.05). Different lower-case letters correspond to significant differences within the same group (P ≤ 0.05).

The biologically active compounds such as chebulagic acid, gallic acid and ellagic acid make *T. chebula* highly powerful antioxidant, which may be responsible for its immunomodulatory activity [36-38]. Various fermented milk supplemented with aqueous herbal extracts comprising good source for antioxidants. *Terminalia chebula* also showed high phenolic compounds and FRAP radical-scavenging activities [39].

3.6. Detection of antioxidant activity on TLC plates using DPPH

For detecting antioxidant activity, fermented milk containing aqueous herbal extracts and it is *in vitro* digested samples were applied onto TLC plates. The appearance of yellow spot on TLC plates indicated reduction of DPPH radicals. The TLC analysis of antioxidant activity in fermented milk containing aqueous herbal extracts and it is *in vitro* digested samples after 30 min. and after 24 hrs have been shown in Figure 4.

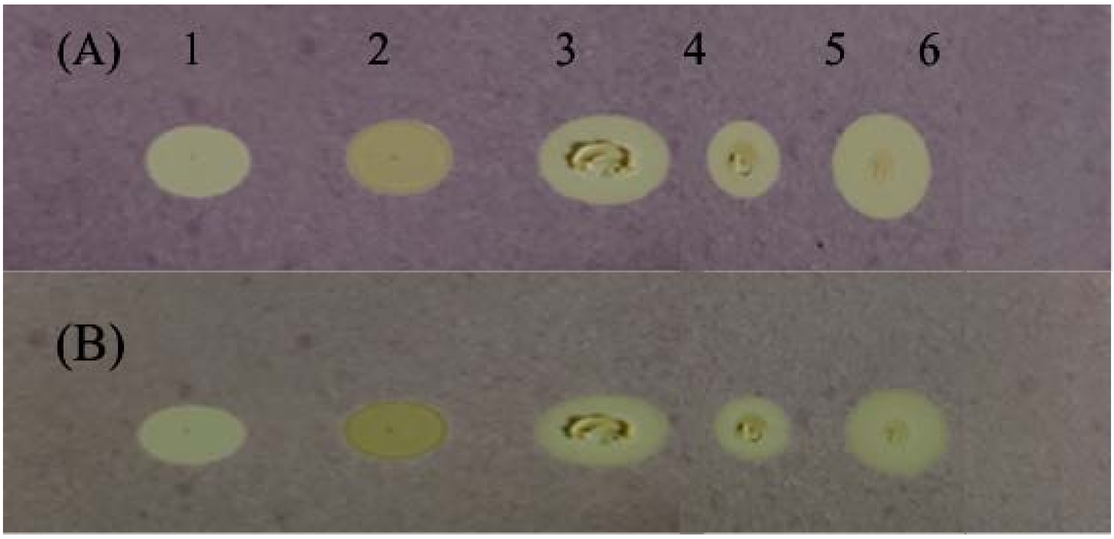

Figure 4. TLC analysis of antioxidant activity in fermented milk containing aqueous *baheda* extract and it's *in vitro* digested samples using DPPH. A: Detection after 30 min DPPH spray; B: Detection after 24 hrs DPPH spray; Lane 1- ascorbic acid (+ve control); Lane 2- aqueous *baheda* extract; Lane 3- fermented milk (with aqueous *baheda* extract); Lane 4- pepsin digest (*baheda*) ; Lane 5- overnight digest (*baheda*); Lane 6- methanol (-ve control).

TLC analysis of antioxidant activity using DPPH exhibited higher antioxidant activity after 30 min. in *harad*, *baheda* and *amla* aqueous extract supplemented fermented milks and their corresponding *in vitro* digested samples whereas in control and *arjuna* aqueous extract supplemented samples the antioxidant activity was observed to be higher after 24 hrs.

The TLC analysis of fermented milk containing aqueous *harad* extract and it is *in vitro* digested samples exhibited noticeably utmost antioxidant activity in overnight digested samples followed by fermented and pepsin digested samples. Similar pattern was observed after 24 hrs, though the antioxidant activity was low as compared to 30 min. The fermented milk containing aqueous *harad* extract also exhibited apparently higher antioxidant activity than the control fermented milk and it is *in vitro* digested samples detected after 30 min. The enhanced antioxidant activity in aqueous *harad* extract supplemented fermented milk may be attributed to higher polyphenolic content of *harad* fruits [40]. The increased antioxidant activity due to supplementation of aqueous *harad* extract may also be attributed to the compounds casuarinin, chebulanin, chebulinic acid and 1, 6-di-O- galloyl –β-D-glucose present in *harad* fruits [41].

The fermented milk containing aqueous *baheda* extract and it is *in vitro* digested samples also exhibited antioxidant activity as shown in Figure 4. The prominent antioxidant in fermented milk containing aqueous *baheda* extract as compared to control which may be attributed to Various antioxidant compounds (termilignan, thannilignan, 7-hydroxy-3′,4′-(methylenedioxy) flavone, anolignan B, gallic acid, ellagic acid, β-sitosterol, arjungenin, belleric acid, bellericoside and cannogenol 3-O-β-D-galactopyranosyl-(1→4)-O-α-L-rhamnopyranoside) found in the fruits of *baheda* [42].

The fermented milk containing aqueous *amla* extract and it is *in vitro* digested samples exhibited prominent antioxidant activity. The antioxidant activity was observed to be higher as compared to control, which might be attributed to high content of ascorbic acid (ranging from 1-100 g of fruit) in *amla* fruits [43].

Moreover, reduction in antioxidant activity in the fermented milk and it is *in vitro* digested samples supplemented with *harad*, *baheda* and *amla* after 24 hrs may be due to loss of active compounds responsible for antioxidant activity or diffusion of the complex formed or due to degradation of DPPH with time.

The fermented milk containing aqueous *arjuna* extract and it is *in vitro* digested samples also exhibited antioxidant activity in fermented milk, overnight digested and pepsin digested samples, respectively. However, the antioxidant activity was observed to be higher after 24 hrs, suggesting that the antioxidant mechanism was slow and complex. Sustained antioxidant activity of fermented milk containing aqueous *arjuna* extract and it is *in vitro* digested samples even after 24 hrs suggests that the active ingredients responsible for antioxidant activity remains stable and were active for longer duration in the samples. It is widely accepted that antioxidants from natural sources such as herbal supplements and botanicals are more superior to those synthesized artificially, because some synthetic antioxidants have been reported to have undesirable mutagenic and carcinogenic activities [44-45]. Thermal processing of different milk types led to increased total phenol, antioxidant, and antimicrobial activities [46-47].

3.7. OPA assay in fermented milk containing aqueous herbal plant extracts

The effect of different fermented milk containing aqueous herbal extracts and it is *in vitro* digestion on OPA are presented in Figure 5. OPA assay has been adapted for the determination of protein content, peptides, and amino acids in our samples. OPA reacts specifically with primary amines above their isoelectric point (pI) in presence of thiols. OPA reacts also with thiols in presence of an amine such as n-propylamine or 2-aminoethanol.

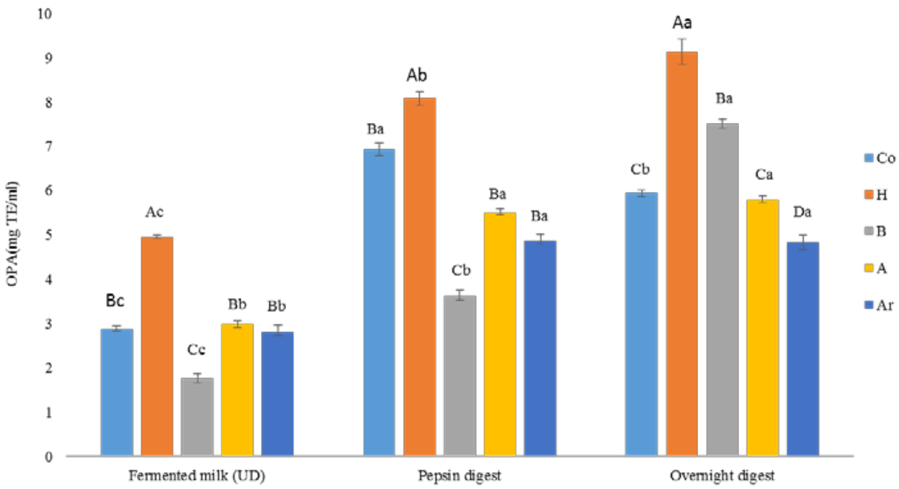

Figure 5. OPA assay of fermented milk containing aqueous herbal plant extracts (Co- Control, H- *Harad*, B- *Baheda*, A- *Amla*, Ar- *Arjuna*). Different upper-case letters correspond to significant differences between the groups (P ≤ 0.05). Different lower-case letters correspond to significant differences within the same group (P ≤ 0.05).

In the samples of undigested fermented milk (UD), fermented milk containing aqueous *harad* extract exhibited significantly higher OPA value (4.95±0.04 mg TE/ml) at P<0.05 level of significance and lower OPA (1.76±0.08 mg TE/ml) value was found in fermented milk containing aqueous *baheda* extract

A considerable increase in OPA (P<0.05) was observed in the pepsin-digested fermented milk containing aqueous *harad* extract as compared to control (Figure 5). The highest OPA value was observed in fermented milk containing aqueous *harad* extract (8.08±0.15 mgTE/ml), whereas lowest OPA value was observed in fermented milk containing aqueous *baheda* extract (3.64±0.12 mg TE/ml).

The OPA in overnight digested samples of fermented milk containing aqueous extracts of herbal species except *arjuna* was significantly higher as compared to the control (P<0.05). Maximum OPA increase (9.13±0.29 mg TE/ml) was observed in fermented milk containing aqueous *harad* extract, while lowest OPA is (4.83±0.16 mg TE/ml) for fermented milk containing aqueous *arjuna* extract.

Overall, it is observed that maximum OPA value was found in fermented milk containing aqueous *harad* extract (UD, PD and OD). Minimum OPA value was found in fermented milk containing aqueous *baheda* extract (UD) (1.76±0.08 mg TE/ml) and pepsin digested sample (PD) (3.64±0.12 mg TE/ml) as compared to the control. Overnight digested fermented milk containing aqueous *arjuna* extract exhibited low OPA value as compared to control sample. Garlic (*Allium sativum*) increased OPA values more for cow-milk yoghurt than for camel- milk yoghurt [6].

3.8. Comparative protein profile of fermented milk by SDS-PAGE.

In the present study, indigenous cattle milk proteins present in fermented milk containing aqueous herbal extracts and *in vitro* digested samples were analyzed by SDS-PAGE. It was noted that when the samples were centrifuged and supernatant was used, no/very faint protein bands were visible. It may be due to that most of the major proteins remain present in the solid part of sample (Residue). The maximum number of proteins bands was detected in samples used without centrifugation.

After pepsin digestion, higher molecular weight proteins degraded into smaller protein bands/peptides and these bands were almost digested after overnight digestion. However, low retention of α-lactoalbumin, β-lactoglobin and casein was observed in the pepsin digested fermented milk supplemented with *harad* and *baheda* extracts (Figures: 6 C and 8 D). SDS-PAGE profile of milk variants such as α-CN, β-CN, k-CN, Lactoferrin (LF), Bovine Serum Albumin (BSA), β-Lactoglobulin (β-Lg) and α-Lactalbumin (α-La) are clearly shown in Figures (6 A, B, C, D, E, F).

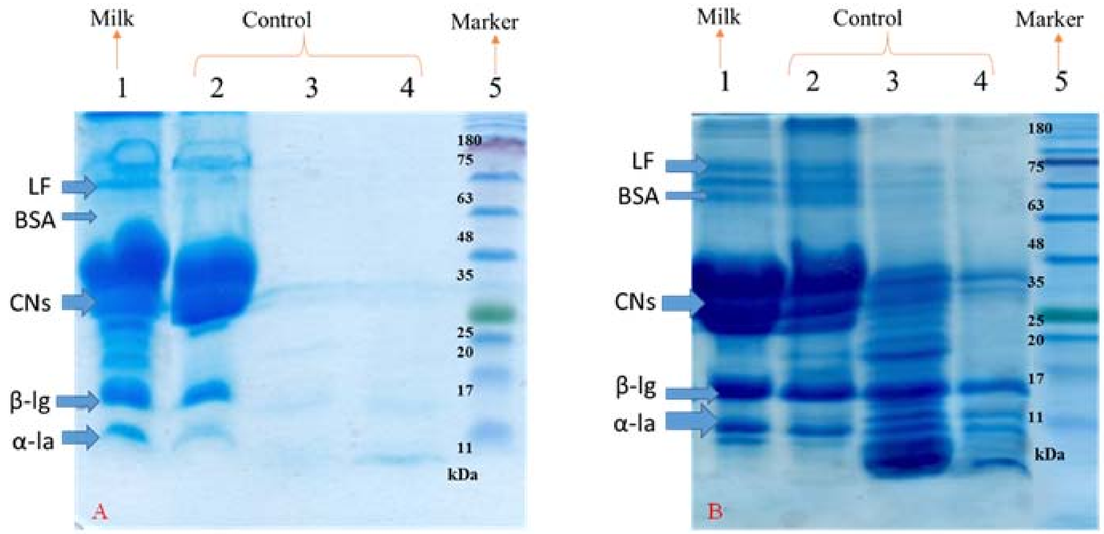

Figure 6A. Control: Lane 1, Lane 2 without centrifugation and Lane 3, Lane 4 used after centrifugation (Supernatant): Lane 1- Milk, Lane 2 - Fermented milk (without aqueous herbal extract) Undigested, Lane 3 - Pepsin Digest, Lane 4 - Overnight Digest, Lane 5 – Marker; Figure 6B. Control: Lane 1, Lane 2, Lane 3 and Lane 4 used without centrifugation: Lane 1- Milk, Lane 2 - Fermented milk (without aqueous herbal extract) Undigested , Lane 3 - Pepsin Digest, Lane 4 - Overnight Digest, Lane 5 – Marker.

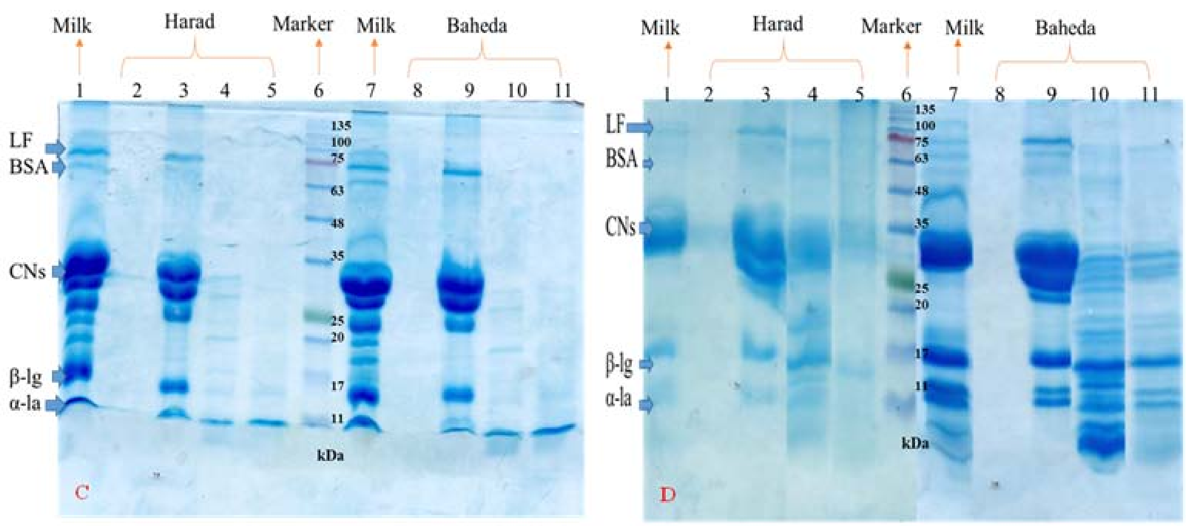

Figure 6C. Lane 1, 2, 3 and Lane 7, 8, 9 without centrifugation and Lane 4, 5 and Lane 10, 11 used after centrifugation (Supernatant): Lane 1- Milk, Lane 2- Aqueous *harad* extract, Lane 3 - Fermented milk (with aqueous *harad* extract) Undigested, Lane 4- Pepsin Digest (*harad*), Lane 5 - Overnight Digest (*harad*), Lane 6- Marker, Lane 7- Milk, Lane 8- Aqueous *baheda* extract, Lane 9- Fermented milk (with aqueous *baheda* extract) Undigested, Lane 10- Pepsin Digest (*baheda*), Lane 11- Overnight Digest (*baheda*). Figure 6D. Lane 1, 2, 3, 4, 5, 7, 8, 9, 10, 11 used without centrifugation: Lane 1- Milk, Lane 2- Aqueous *harad* extract, Lane 3 - Fermented milk (with aqueous *harad* extract) Undigested , Lane 4- Pepsin Digest (*harad*), Lane 5 - Overnight Digest (*harad*), Lane 6- Marker, Lane 7- Milk, Lane 8- Aqueous *baheda* extract, Lane 9- Fermented milk (with aqueous *baheda* extract) Undigested, Lane 10- Pepsin Digest (*baheda*), Lane 11- Overnight Digest (*baheda*).

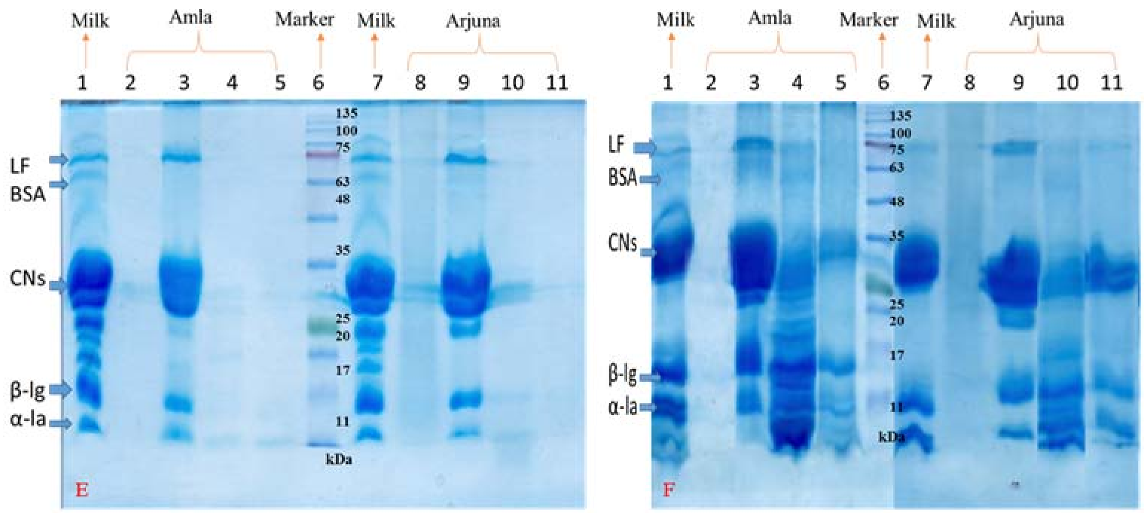

Figure 6E. Lane 1, 2, 3 and Lane 7, 8, 9 without centrifugation and Lane 4, 5 and Lane 10, 11 used after centrifugation (Supernatant): Lane 1- Milk, Lane 2- Aqueous *amla* extract, Lane 3 - Fermented milk (with aqueous amla extract) Undigested, Lane 4- Pepsin Digest (*amla*), Lane 5 - Overnight Digest (*amla*), Lane 6- Marker, Lane 7- Milk, Lane 8- Aqueous *arjuna* extract (*arjuna*), Lane 9- Fermented milk (with aqueous *arjuna* extract) Undigested, Lane 10- Pepsin Digest (*arjuna*), Lane 11- Overnight Digest (*arjuna*). Figure 6F. Lane 1, 2, 3, 4, 5, 7, 8, 9, 10, 11 used without centrifugation: Lane 1- Milk, Lane 2- Aqueous *amla* extract, Lane 3 - Fermented milk (with aqueous *amla* extract) Undigested, Lane 4- Pepsin Digest (*amla*), Lane 5 - Overnight Digest (*amla*), Lane 6- Marker, Lane 7- Milk, Lane 8- Aqueous *arjuna* extract (*arjuna*), Lane 9- Fermented milk (with aqueous *arjuna* extract) Undigested, Lane 10- Pepsin Digest (*arjuna*), Lane 11- Overnight Digest (*arjuna*).

Caseins have a flexible and loose structure that makes them highly sensitive to digestive enzymes [48]. In contrast, the globular structure of whey proteins makes them partly resistant to digestion by pepsin [49-50]. In the present case, the heat treatment applied to milk during yogurt manufacture made these proteins less resistant to digestion than the native form, due to conformational changes [51-53].

4. Conclusion

The supplementation of milk with aqueous herbal extracts improves the quality of dairy products and enhances the nutritional and therapeutical values of fermented milk. Herbal supplemented fermented milk was found to possess the enhanced antioxidant activity as compared to control fermented milk. Maximum total phenolic content value was found in fermented milk containing aqueous *baheda* extract. The protein profile of fermented milk by SDS-PAGE revealed that centrifuged samples contain no or very faint protein bands, whereas prominent proteins bands were detected in samples used without centrifugation.

Practical applications

Himachali Pahari cow milk fermented with LGG increases digestibility and bioavailability of proteins, minerals etc. and also activate many bioactive peptides. Medicinal plants and their extracts have a long history of utilization as natural remedies owing to presence of a wide range of natural bioactive compounds, namely flavonoids, alkaloids, polyphenols etc. Supplementation of aqueous herbal extracts during fermentation of milk increases its nutritional and therapeutic value. Findings of the present study suggest that fermented milk containing various aqueous herbal extracts has enhanced antioxidant activity.

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