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Antihypertensive Activity of Fermented Milk Containing Various Aqueous Herbal Extracts

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Abstract

The present study was conducted with the objective to enhance the Angiotensin converting enzyme (ACE, EC 3.4.15.1) inhibitory activity of indigenous cattle milk through supplementation with various herbal extracts. The fermented milk supplemented with aqueous extracts of fruits of harad (*Terminalia chebula*), baheda (*Terminalia bellirica*), amla (*Phyllanthus emblica*) and bark of arjuna (*Terminalia arjuna*) were evaluated for ACE inhibitory activity. Maximum (37.92±0.72%) ACE inhibitory activity was observed in pepsin-digest of fermented milk containing aqueous harad extract, whereas minimum (25.37±0.59%) ACE inhibitory activity was observed in fermented milk containing aqueous extract of amla. Undigested samples of fermented milk containing aqueous arjuna extract exhibited significantly higher ACE inhibitory activity (19.70±0.58%), followed by amla (17.00±0.70%), and baheda (16.59± 0.94%). Fermented milk containing aqueous harad extract had significantly lower value (14.97±0.62%). No significant difference was found between fermented milk containing aqueous extracts amla and extracts of baheda. Based on the results described above, we surmise that herbal supplemented fermented milk had better antihypertensive activity.

Keywords

ACE (Angiotensin Converting Enzyme) Activity, Fermented Milk Containing Various Aqueous Herbal Extracts, Antihypertensive Activity

1. Introduction

Indigenous cattle breed of Himachal Pradesh, India known as '*Himachali Pahari cow*' is distributed in 7 districts including Chamba, Mandi, Kullu, Kangra, Sirmour, Kinnaur & Lahaul Spiti, and registered as a unique species with distinct features/qualities. Cattle milk proteins possess beneficial attributes including opoid, immunomodulatory, antimicrobial and antioxidant activities. Angiotensin converting enzyme (ACE) plays a key role in blood pressure control by producing the vasoconstrictor angiotensin II. Significant goal of hypertension prevention is to inhibit ACE with natural inhibitors to avoid the side-effect of synthetic medicines [1]. Hypertension is a significant risk factor for heart failure, stroke, Myocardial infarction, atherosclerosis, kidney diseases and peripheral arterial disease as well as chronic kidney disease [2-5]. Six classes of drugs (angiotensin II receptor blockers, ACE inhibitors, diuretics, calcium channel blockers, α-adrenergic antagonist, and β-blockers) are active for anti-hypertension [6]. ACE inhibitors are more commonly used because they protect the target organ while having no negative effects on glycolipid metabolism.

ACE is a metalloproteinase also known as kinase II which contains two zinc-catalytically active sites and can simultaneously affect the renin-angiotensin (RAS) system and the Kallikrein/kininine (KKS) system [7]. Under the combined action of renin and ACE, inactive angiotensin-I is hydrolyzed to angiotensin-II with successful vasoconstrictor action in the RAS system, resulting in elevated blood pressure. The KKS is an endogenous blood pressure system in which the

ACE inhibits the antihypertensive effect by bradykinin deactivation. The role of ACE in both systems eventually led to a higher blood pressure [8-12].

Angiotensin I-converting enzyme (ACE) is a key enzyme in regulation of blood pressure through two different reactions in the renin-angiotensin-aldosterone system (RAAS) and the kinin nitric oxide system (KNOS). For this, many synthetic ACE inhibitors, such as captopril, enalapril, fosinopril, lisinopril, and ramipril were identified and used for the treatment of hypertension. However, these synthetic inhibitors have side effects including coughing, taste disturbance and skin rash [13-14]. Thus, one of the major challenges to today's world healthcare sectors is to identify ACE inhibitors from natural resources.

The assay of ACE inhibitory activity is based on specific binding of TNBS to the primary amine of His-Leu dipeptide produced by hydrolytic cleavage from Hip-His-Leu by ACE, forming TNP-His-Leu (TNP-HL) by desulfitation, followed by formation of a yellow complex with sulfite detected at 420 nm [15].

Animal proteins, vegetable proteins, and algae are the key sources of food-derived ACE inhibitory peptides that have been identified. A source of ACE inhibitory peptides for animal protein products is milk protein, which includes casein, lactalbumin, and lactoglobulin [16-17].

Hypertension is an important factor in cardiovascular disease. Angiotensin-I-converting enzyme (ACE) inhibitors such as synthetic drugs are commonly used to regulate hypertension. ACE-inhibiting food-borne peptides may be a viable alternative to synthetic drugs. A variety of plant-based peptides have been investigated for their potential ACE inhibitor activity using in vitro and in vivo. These plant based peptides can be generated by extraction of solvents, enzymatic hydrolysis with or without novel methods of food processing, and fermentation. ACE-inhibiting activities of peptides can be affected by their structural features, such as chain length, composition and sequence. ACE-inhibiting peptides should have gastrointestinal stability and enter the cardiovascular system to demonstrate their bioactivity [18].

The lassi, fermented milk product containing angiotensin-I-converting-enzyme (ACE)-inhibitory peptides, was made by using *Lactobacillus acidophilus* NCDC-15 and the incubation period and simmering effect was also optimized for production of ACE-inhibitory peptides. The biological activity was measured in the supernatant of the fermented milk after centrifugation. The milk fermented by *L. acidophilus* has a high ACE-inhibitory activity because it contained the sequences of β-casein (β-CN) fragment [19].

Nutrition plays an important role in the prevention of cardiovascular disease (CVD) such as atherosclerosis, coronary heart disease, stroke and heart failure [20-22]. According to the World Health Organization (WHO), these diseases are the highest cause of global death (WHO). Hypertension is the most important risk factor for CVDs, a condition in which blood vessels have a persistent increase in pressure. Blood pressure medications, particularly angiotensin-I-converting enzyme inhibitors (ACE; EC 3.4.15.1) are commonly used to control blood pressure in the renin-angiotensin system [22].

Terminalia is a tree species with medicinal properties. Three species of terminalia are *Terminalia bellerica* ("Baheda"), *Terminalia arjuna* ("Arjun") and *Terminalia chebula* ("Harad") used in medicine. *Emblica officinalis* ('Amla') is one of the most important plants in the traditional Ayurvedic medical system as well as in other traditional immunomodulatory, anti-inflammatory, anti-ulcer, hepatoprotective and anti-cancer systems. The fruits are rich in vitamin C and also in phenol reservoirs, including gallic acid, ellagic acid, quercetin, kaempferol, geranin, furosin, corilagin, gallotanins, emblicanins, flavonoids, glycosides and proanthocyanidins.

Over the past few decades, numerous studies for the treatment of hypertension have been conducted to determine whether blood pressure reduction in middle age reduces the risk of stroke and of CHD. For stroke, the overview provides direct and highly significant evidence that both fatal and non-fatal strokes are prevented within just a few years of blood pressure lowering [3]. The present study was aimed to investigate the *in vitro* antihypertensive activity of herbal supplemented fermented milk prepared from indigenous hill cattle milk.

2. Materials and methods

2.1. Collection of Milk

Milk samples of indigenous cattle 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.

2.2. Collection of herbal plant material

The bark of *Terminalia arjuna* (arjuna) and fruits of *Terminalia bellerica* (baheda), *Terminalia chebula* (harad) and *Emblica officinalis* (amla) were collected from the surrounding areas of Palampur. The bark and fruits were dried in hot air oven by maintaining temperature at 37°C. The dried samples were ground to powdered form. The powdered samples were stored in air tight containers at room temperature for further use.

2.3. Water extraction of herbal plants

Dried powdered of *Terminalia chebula*, *Terminalia bellerica*, *Terminalia arjuna* and *Emblica officinalis* (10 g) was

suspended in 100 ml of distilled water in separate flasks and incubated overnight in a water bath at 70°C. Filtered the samples through whatman filter paper no.1 and filtrate was used as herbal water extract and stored at -20°C till further use.

2.4. Preparation of starter culture

Starter culture was prepared by using *Lactobacillus rhamnosus* (347) bacteria, purchased from National collection of dairy culture (NCDC), NDRI Karnal. The lyophilized *Lactobacillus rhamnosus* (347) bacterium was re-activated by inoculating the bacterium in MRS (De Man, Rogosa and Sharpe) broth and following the incubation in incubator at 37°C for 24 hours. It was again re-inoculated for 3-4 times after that colony inoculated in skim milk. The cultured skim milk 5 g was introduced in 100 ml boiled milk of indigenous cattle and mixture was incubated for 6-7 hours at 41°C until pH 4.5 and stored in refrigerator at 4°C.

2.5. Preparation of fermented milk containing herbal water extracts

Fermented milk was prepared by adding 10 ml of various herbal water extracts into 85 ml of fresh boiled milk of indigenous cattle and 5 g of starter culture. The mixture was mixed thoroughly followed by incubation at 41°C until pH was reduced to 4.5. The same procedures were carried out to prepare control fermented milk by using distilled water in place of herbal water extract. Samples were refrigerated at (4°C) till further use.

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

In vitro enzymatic digestion protocol described by [23] with modifications. Yogurt sample (10 ml) was taken. Undigested and digested samples were centrifuged at 12,000 rpm for 30 min and harvested supernatant was stored at -20°C for analysis.

2.7. ACE (Angiotensin converting enzyme) inhibitory activity in fermented milk containing aqueous herbal extracts

ACE assay was estimated using the method that is described by [24] with some modifications.

The assay mixture contained 540 µl for control B, 580 µl for Control C, 530 µl for Control A of 0.1 M sodium borate buffer (pH 8.3), 200 µl of 5 mM HHL (Hippuryl-histidyl-leucine**)**, 40 µl of ACE enzyme and 10 µl of diluted sample (Sample was diluted 10 times with sodium borate buffer pH 8.3). The reaction was terminated after incubation at 37 °C for 60 min, through the addition of 300 µl of 1M HCl. After stopping the reaction, 600 µL of pyridine was added followed by 300 µl of benzene sulphonyl chloride (BSC) and the solution was mixed before cooling down on ice. The absorbance was measured at 410 nm.

3. Results and Discussion

Angiotensin converting enzyme (ACE) inhibitory activity in fermented milk containing aqueous herbal plant extracts

The effect of different fermented milk containing aqueous herbal extracts and its *in vitro* digestion on ACE are presented in Table 1. Angiotensin-converting enzyme (ACE) inhibitory activity can be measured using the substrate hippuryl-L-histidyl-L-leucine (HHL).

Undigested samples of fermented milk containing aqueous arjuna extract exhibited significantly higher ACE inhibitory activity (19.70±0.58%), followed by amla (17.00±0.70%), and baheda (16.59±0.94%) at the significance level of $P<0.05$. Fermented milk containing aqueous harad extract exhibited significantly lower value (14.97±0.62%). Nevertheless, no significant difference was found between fermented milk containing aqueous extracts amla and extracts of baheda (Table 1).

Table 1. ACE inhibitory activity of fermented milk containing aqueous herbal plant extracts

S. No.	Sample	ACE (% inhibition)				
		Control	Harad	Baheda	Amla	Arjuna
1	Fermented milk (UD) (n= 8)	10.52^{Dc}±0.84	14.97^{Cc}±0.62	16.59^{Bc}±0.94	17.00^{Bc}±0.70	19.70^{Ac}±0.58
2	Pepsin digest (PD) (n= 8)	25.77^{Db}± 0.59	37.92^{Ab}±0.72	28.60^{Cb}±0.95	25.37^{Db}±0.59	34.54^{Bb}±0.36
3	Overnight digest (OD) (n= 8)	33.06^{Da}±0.71	48.58^{Aa}±0.47	49.79^{Aa}±0.23	45.34^{Ba}±0.62	41.16^{Ca}±0.95

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 ($P \leq 0.05$); n= number of samples.

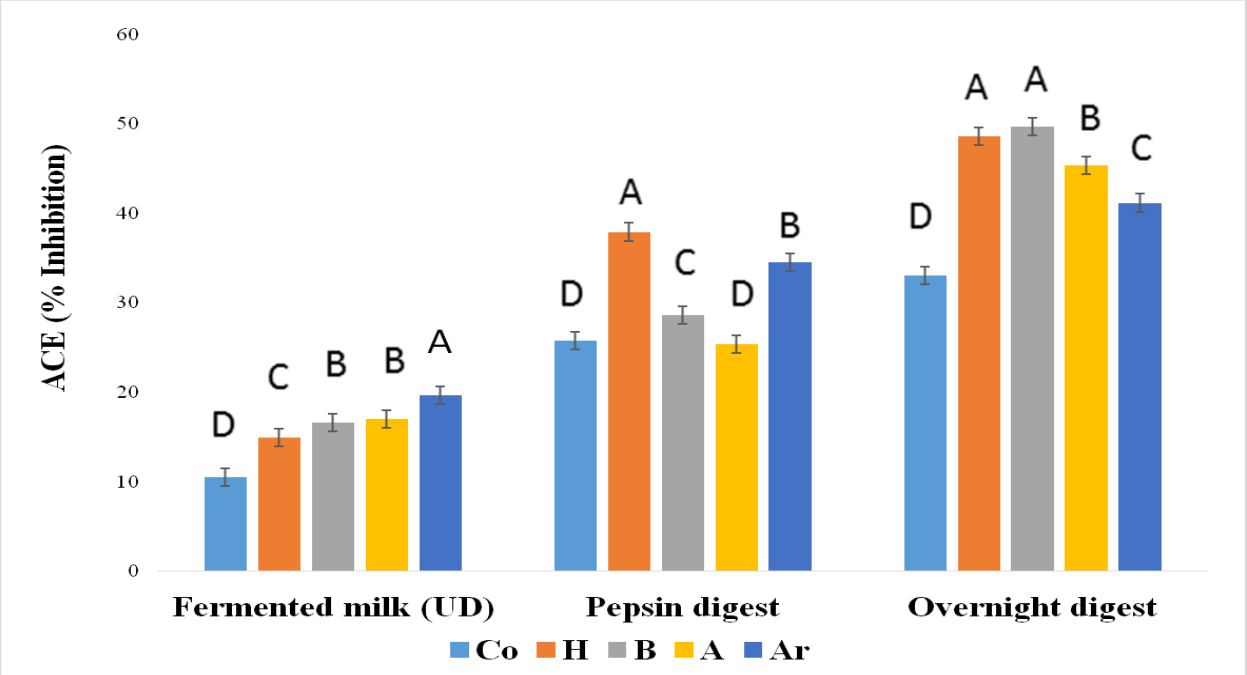

Figure 1. ACE inhibitory activity of fermented milk containing aqueous herbal plant extracts (Co-Control, H-Harad, B-Baheda, A-Amla, Ar-Arjuna). Different upper-case letters correspond to significant differences between the groups ($P \leq 0.05$)

A substantial increase in ACE ($P<0.05$) was observed in pepsin digested fermented milk containing aqueous harad, baheda, amla and arjuna extracts as compared to control. Maximum (37.92±0.72%) ACE inhibitory activity was observed in pepsin-digest of fermented milk containing aqueous harad extract, whereas minimum (25.37±0.59%) ACE inhibitory activity was observed in fermented milk containing aqueous extract of amla (Table 1).

ACE was found to be significantly higher as compared with control ($P<0.05$) in overnight digested fermented milk samples containing aqueous herbal extracts. Highest increase was in the fermented milk containing aqueous baheda extract (49.79±0.23%), and lowest ACE inhibitory activity found in fermented milk containing aqueous arjuna extract (41.16±0.95%). Nevertheless, no significant difference was observed between fermented milk containing aqueous harad and baheda extracts (Table 1).

Overall observation shows maximum ACE inhibitory activity in fermented milk containing aqueous arjuna extract (UD) and in aqueous harad extract (PD). The overnight digested samples demonstrated higher ACE inhibitory activity in fermented milk containing aqueous baheda extract (Figure 1). Plant-origin peptides can be generated by extraction of solvents, enzymatic hydrolysis with or without novel methods of food processing, and fermentation [18]. Several *in vivo* and *in vitro* studies have identified a range of medicinal plants which possess ACE inhibitory activity (Barbosa-Filho et al., 2006). Screening of these plants has identified several groups of natural ACE inhibitors including alkaloids, flavonoids, tannins, phenylpropanoids, proanthocyanidins, fatty acids, and terpenoids [25-26]. Crude extract of *Ipomoea reniformis* has hypotensive, ACE inhibitory and diuretic activities. The extract of *I. reniformis* produced 21.51±3.41, 28.99±2.30, 53.34±0.88 and 61.71±3.37% fall in mean arterial blood pressure of the anesthetized rats at the doses of 0.1, 0.3, 1.0 and 3.0 mg/Kg, respectively. The above plant was found to have serum ACE inhibitory activity, with IC50 value of 422±21.16 µg/mL.

4. Conclusion

Hypertension is a stern menace to human health and food-derived ACE inhibitory peptides can regulate it without any side effect. In the present study, undigested samples of fermented milk containing aqueous arjuna extract exhibited significantly higher ACE inhibitory activity (19.70±0.58%), followed by amla (17.00±0.70%), and baheda (16.59±0.94%). Fermented milk containing aqueous harad extract had significantly lower value (14.97±0.62%). Maximum (37.92±0.72%) ACE inhibitory activity was observed in pepsin-digest of fermented milk containing aqueous harad extract, whereas minimum (25.37±0.59%) ACE inhibitory activity was observed in fermented milk containing aqueous extract of amla. The present study exhibited that herbal extract supplemented fermented milk of the indigenous cattle can act as potential food-derived medicine.

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