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Effect of *Beta vulgaris* root extracts in Rayeb milk on its microbiological, chemical and nutritional composition

Abeer, F. Ahmad[1*]; Afaf, O. Ali[1]

[1]Regional Center for Food and Feed, Agricultural Research Center, Giza, Egypt

[*]Corresponding author E-mail: Abir_fouad76@yahoo.com

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Abstract

The purpose of this study was to examine the use of beetroot (*Beta vulgaris* L.) extracts as natural colorant and antibacterial additives in Rayeb milk. Citric acid: ascorbic acid (2:1) (BCA) and citric acid (0.2%) (BC) were used as extraction solvents of beetroot. Antibacterial activity; minimum inhibitory concentration (MIC), minerals content (calcium, iron, phosphorus, potassium, and selenium), and phytochemical screening were determined in BCA and BC extracts. Moreover, the antibacterial potential, microbiological analysis, and pH were estimated for Rayeb milk supplemented with beetroot extracts after storage for different periods; 0 time, 5, and 10 days. Sensory evaluation was also carried out after 0 and 10 days of storage of Rayeb milk samples. Results of disc diffusion assay showed that both beetroot extracts were more effective against *Pseudomonas aeruginosa*, and less effective against *Escherichia coli*. The lowest MIC was determined for both extracts. Rayeb milk supplemented with extracts showed antibacterial potential toward the tested bacteria. Addition of beetroot citric acid: ascorbic acid (2:1) extract to Rayeb milk; led to a decrease in its total bacterial count, and increase in number of Lactic acid bacteria (LAB). Colored RBCA extract had more acceptable flavor and color. Results of the current work suggested that beetroot extracts could be used as natural additives to Rayeb milk with several advantages including; their antibacterial potency, positive effects on the phytochemical and chemical composition of Rayeb milk.

Keywords: Rayeb milk, Beetroot extracts, Antibacterial potency, MIC, Natural color, Natural flavor

1. Introduction

Color and flavor are two main aspects that determine the consumer acceptance of foods. Recently; due to increasing people's awareness, the application of synthetic colorants in food is decreasing with the existence of several possible health risks, or verified harmful effects. Consumers are in need of more natural products which are safer and beneficial for health. Thus; there is a trend

towards replacement of synthetic colorant by natural products (Chandrasekara *et al*., 2012).

Beetroot plant is a member of the Chenopodiaceae family which includes silver beet, sugar beet and fodder beet. Beetroot is rich in several nutrients, vitamins and phytochemicals; hence it is an ideal vegetable crop for humans. Beetroot has attractive color along with its nice flavor. Beet root pigment is used commercially as a food dye. It is used as an additive in ice-cream; sweets, yoghurt and other confectionary, in addition, it is a good flavoring agent with no known allergic side effects (Mbaeyi-Nwaoha and Onyinyechi, 2012). Consumption of beetroot which is a rich source of antioxidants, can contribute to protection from age-related diseases. It also inhibits cervical ovarian and bladder cancer cells *in vitro*. Ravichandran *et al*., (2013) added that red beet can also be used as an antioxidant.

Beetroot is considered as a source of valuable water soluble nitrogenous pigments called betalins; which were one of the most important natural colorants (Sri Vidhya and Radhai Sri, 2018). Betalains include two main groups; the red betacyanins and the yellow betaxanthins. In addition to their red colors; betalains possess several desirable biological activities including, antioxidant, anti-inflammatory, hepatoprotective, and anti- tumor properties (Velićanski *et al*., 2011). According to Koubaierab *et al*., (2014), betalains were used as additives in the food industries; owing to their natural colorant properties, high solubility in water, and non- toxic effects.

The nutritional and healthy profits of fermented milk "Rayeb" are various. It is a good source of protein; energy (calories), vitamins and minerals. As a fermented product; Rayeb milk also have therapeutic effects, and may be responsible for reduced incidences of lactose intolerance (Ebringer *et al*., 2008). In the current work; citric acid: ascorbic acid (2:1), and citric acid (0.2%) were used as extraction solvents of beetroot. The prepared extracts were analyzed to assess their antibacterial potential, phytochemical and chemical compositions. The aims of the current study were to formulate Rayeb milk with beetroot extracts as additives; and to determine the effects of these extracts on the sensory, microbiological and chemical quality of Rayeb milk.

2. Materials and methods

2.1. Collection of samples

2.1.1. Milk: Skimmed milk (protein 4.16%, ash 0.74% and total solid (TS) 16.74%) was purchased from Faculty of Agriculture, Cairo University, Giza, Egypt.

2.1.2. Beetroot: Beetroot samples were purchased from local markets in Giza governorate, Egypt; then washed with running tap water, chopped to half-slices and then blanched in water (beet roots: water ratio, 1:5) at 90°C for 2 min. without stirring. This step was desirable to inactivate enzymes in reference to De Azeredo *et al*., (2009).

2.1.3. Bacterial strains: *Escherichia coli* 0157: H7, *Staphylococcus aureus* ATCC 20231 were isolated and then serologically identified by Dairy Microbiological Lab., National Research Centre, Giza, Egypt; Whereas; *Streptococcus faecalis*, *Pseudomonas. aeruginosa* ATCC 9027 were obtained from Faculty of Sciences, Cairo University, Giza, Egypt.

2.2. Preparation of beetroot extracts

Two beetroot extracts were prepared according to the procedure described by Sturzoiu *et al*., (2011) with some modifications as follow:

Extract 1: One hundred grams of chopped beetroot were soaked in 500 ml solvent (citric acid 0.2%: ascorbic acid: 0.1%) overnight at 4°C. The solvent was evaporated to 50 ml using rotary evaporator at 40°C; and then stored at -20°C until use. The obtained extract was abbreviated as (BCA).

Extract 2: The same previous steps were repeated; but we used 500 ml of citric acid (0.2%) as an extracting solvent. The obtained extract was abbreviated as (BC).

2.3. Preparation of Rayeb milk

Skimmed milk was heated at 85°C for 10 min. to destroy the pathogenic microorganisms, then transferred into a sterilized container and cooled to 39±1°C. Milk was inoculated with a desirable proportion 2.5% of starter culture (commercial yoghurt). Once the starter was completely mixed; the red beet extract previously pasteurized was incorporated into the Rayeb milk at 4% level in different cups except control. The plastic cups were sterilized before use. Rayeb milk with BAC extract was abbreviated as (RBCA); whereas, Rayeb milk with BC extract was abbreviated as (RBC). The samples were incubated at 41-43°C until complete curd formation/ coagulation of Rayeb (8-12 h) was observed. The Rayeb samples were stored at 4°C until further analysis (Abou-Dobara *et al*., 2018)

2.4. Disc diffusion assay

Antibacterial potency of the beetroot extracts (BCA, BC), and Rayeb milk supplemented with beetroot extracts (RBCA, RBC) was determined using a modified Kirby–Bauer disc diffusion assay (Bauer *et al*., 1966). Briefly; bacteria was grown in 10 ml of fresh nutrient broth media, its concentration was adjusted to 10^8 cells/ ml (Pfaller *et al*., 1988). 100 µl of this bacterial suspension was spread onto Mueller Hinton (MH) agar medium (NCCLS. 1993; 1997) in Petri plates. An aliquot (10 µl) of each sample (beetroot extracts and Rayeb milk) was pipetted onto a sterile paper disc (Whatman No. 1, diameter of 5.5 mm) placed on the agar surface. Plates were inverted and incubated for 18 h at 37°C. Bacterial growth inhibition was determined by measuring the diameter of clear zones around each disc; and recorded as diameter of inhibition zones in mm. These diameters of inhibition zones were measured with slipping calipers according to the National Committee for Clinical Laboratory Standards (NCCLS. 1993).

2.5. Determination of minimum inhibitory concentration (MIC)

MIC of BCA and BC extracts was determined using the disk diffusion assay; and their efficiency in controlling tested bacteria was evaluated according to Mostafa *et al*., (2018). Different concentrations of the effective plant extracts (0-64 µg/ ml) were prepared separately; and then 10 µl of each concentration was loaded over sterilized filter paper discs (8 mm in diameter). MH agar seeded with bacterial suspensions of *P. aeruginosa* (6×10^5 cells/ ml) was poured into sterile Petri plates. The loaded filter paper discs were then placed on the surface of the MH agar plates. Plates were kept at 5°C for 2 h.; and then incubated at 37°C for 24 h. Inhibition zones were measured by Vernier caliper; and recorded according to the different corresponding concentrations of the extracts.

2.6. Microbiological analysis of Rayeb milk

25 ml of Rayeb samples were homogenized in 225 ml sterile diluent (0.1% bacteriological peptone, 0.85% NaCl; pH 7.0 using a stomacher for 30 sec.). Serial dilutions were prepared; 1 ml from each dilution was transferred into duplicates petri plates of nutrient agar media (NA), and then incubated at 30°C for 48 h, to determine the total bacterial viable count as described by Harrigan and McCance, (1998). Yeasts and molds fungal counts were enumerated using Potato dextrose agar (PDA) medium adjusted to pH 3.5 (APHA. 1994); after incubation at 25°C for 4 days.

2.7. Determination of total count of Lactic acid bacteria (LAB)

Lactic acid bacterial count in RBCA and RBC extracts was enumerated on Elliker agar medium after incubation at 35°C for 2 days (Elliker and Anderson, 1956). Moreover; counts of total bacteria, LAB, yeasts and mold fungi were determined in

control Rayeb milk sample, RBCA and RBC, after different periods of storage (0, 5, and 10 days).

2.8. Chemical compositions of different samples

2.8.1. Determination of minerals contents of BCA and BC

Calcium, Iron, Phosphorus, Potassium, and Selenium minerals concentrations were determined in BCA and BC extracts; using Optima 2000 DV inductively coupled plasma spectrometer, (Perkin Elmer). Concentrations were recorded based on calibration curves developed using inductively coupled plasma (ICP) (merk) standard in reference to AOAC. (2012).

2.8.2. pH determination

5 ml of control Rayeb milk, RBCA and RBC were homogenized in 50 ml of distilled water. pH was then determined after storage for; 0, 5 and 10 days using a pH meter (model EAL 920).

2.8.3. Phytochemical screening

Phytochemical detection of; tanins, phenols, alkaloids, flavonoids, steroids, terpenoids, glycosides, and saponin, was carried out to establish the presence of some of these specific chemicals in the BCA and BC extracts. This phytochemical analysis was carried out as described by Harbone, (1998). The components activities were recorded previously by Tiwari *et al*., (2011).

2.8.4. Sensory evaluation

In reference to Taha *et al*., (2011), sensory evaluation of RBCA and RBC was carried out by ten panelists from Regional Centre for Food and Feed (RCFF) after 0 and 10 days of storage. The evaluation scores included: flavor, body and texture, color, and total score.

2.9. Statistical analysis

Statistical analysis of the recorded data was carried out according to Gomez and Gomez, (1984). Treatments means were compared using the least significant test at the 5% level of probability; as outlined by Waller and Duncan, (1969).

3. Results and Discussion

The antibacterial activities of the beetroot extracts were evaluated against some of Gram negative and positive bacteria strains mainly; *S. aureus*, *Strept. faecalis*, *E. coli* and *P. aeruginosa*. Results on investigating the antibacterial activities of beetroot extracts are shown in Table (1).

BCA and BC extracts were more effective against *P. aeruginosa* recording inhibition zones diameters of 20 and 15 mm, respectively. On the other hand; BCA and BC extracts were less effective against *E. coli* with inhibition zones of 12 and 10 mm, respectively.

These results were consistent with those of Koochak *et al*., (2010); who reported that *E. coli* was the most resistant strain among 10 tested bacterial species, when compared with *P. aeruginosa*. They added that *B. vulgaris* ethanolic extract did not show any antibacterial potential against *E. coli*; in contrast to the results of Winkler *et al*., (2005), who revealed that this extract exerted antibacterial potency against *E. coli*.

According to the current results in Table (1); the Gram positive bacteria (S. aureus, *Sterpt. faecalis*) were less sensitive to the extracts; with inhibition zones diameters of (14, 13 mm) for BCA, and (13 and 13 mm) for BC extract, respectively. These results were in agreement with Saani and Lawrence, (2016); who reported that beetroot ethanolic and methanolic extracts had antibacterial activities in descending order against *S. aureus* > *E. coli*. Canadanovic-Brunet *et al*., (2011) attributed this recorded antibacterial potential to the main pigment called betalains present in beetroot.

Table 1: Antibacterial potential of beetroot extracts

Bacterial isolates	Gram reaction	Inhibition zones diameter (mm)	
		BCA extract	BC extract
S. aureus	G^+	14	13
Sterpt. faecalis	G^+	13	13
E. coli	G^-	12	10
P. aeruginosa	G^-	20	15

Where; (BCA) = Beet root citric: ascorbic acid (2:1) extract; (BC) = Beet root citric (2%) extract

Results of phytochemical analysis of the BC and BCA extracts clear in Table (2); reported the presence of phenols, flavonoids and terpenoids. Steroids were found in the BCA extract only. These phytochemicals were well known to possess several bioactivities including; antimicrobial, antidiarrheal, and anthelmintic. Tanins, alkaloids, glycosides, and saponins were not detected in either extracts.

Table (3) presents the mineral contents of BCA and BC extracts. The BC extract had high contents of K (2407), and P (217) mg/l; whereas BCA extract had high contents of Ca and Fe recording 104.4; 7.6 mg/l, respectively. Kale *et al*., (2018) revealed that K concentration in beetroot was 30.12 mg/100 g; while, Sri Vidhya and Radhai Sri, (2018) reported that beetroot juice contained K and P, in 180 and 10.2 mg/100 ml, respectively. Beetroot juice was high in K content; which could help to regulate the fluid levels and maintain the electrolytes in the human body. Dietary recommended intakes (DRIs) for adults were 4700, 700, and 1000 mg/day of K, P, and Ca, respectively. However; Kumar, (2015) demonstrated that fresh beetroot contained 16, 0.79, 38, and 305 mg/ 100g of Ca, Fe, P, and K, respectively.

Results revealed that the MIC of BCA and BC extracts against *P. aeruginosa* were 40, and 26 µg/ml; respectively. Čanadanović-brunet *et al*., (2011) investigated the antibacterial activity of ethanol extract of beetroot pomace against *S. aureus*; which was one of the most common Gram-positive bacteria responsible for food poisoning and recorded MIC of 0.75 mg/ml. However; weak antibacterial efficacy was recorded against *E. coli* (MIC = 1.5 mg/ml), and *P. aeruginosa* (MIC = 4.5 mg/ml).

Treated Rayeb samples namely; RBCA and RBC, had strong antibacterial effect on *P. aeruginosa*, with diameter of inhibition zones 25 and 23 mm, respectively (Table 4). On the contrary RBCA and RBC recorded less inhibition zones diameters for *E. coli* of 16 and 15 mm, respectively. It is to be mentioned that the untreated Rayeb milk (control) had weak antibacterial potency; due to the presence of lactic acid bacteria (LAB), which acted as probiotics with several health benefits (Ljungh and Wadstorm, 2006).

Thus; the high antibacterial potential of Rayeb milk supplemented with beetroot extracts might be attributed to the presence of peptide produced during fermentation of milk by probiotics, which thus exhibited antibacterial effects. Accordingly; the total antibacterial activities of supplemented Rayeb milk were due to the sum of antibacterial effect of beetroot extracts; in addition to the activity of the indigenous LAB present in the Rayeb milk itself.

Table 2: Phytochemical screening of beetroot extracts

Components of beetroot extracts	BC	BCA	Activities
Tanins	-	-	-
Phenols	++	++	Antimicrobial Antidiarrheal Anthelmintic
Alkaloids	-	-	-
Flavonoids	+	++	Antimicrobial Antidiarrheal
Steroids	-	+	Antidiarrheal
Terpenoids	+	+	Antimicrobial Antidiarrheal
Glycosides	-	-	-
Saponins	-	-	-

Where; (BCA) = Beet root citric: ascorbic acid (2:1) extract; (BC) = Beet root citric (2%) extract

Table 3: Minerals concentrations (mg/ l) of beetroot extracts

Extracts	Components concentration (mg/l)				
	Ca	P	Fe	Se	K
BCA	104.4	151.0	7.6	-	1933
BC	10.44	217.0	-	0.78	2407

Where; BCA = Beetroot citric: ascorbic acid (2:1) extract; BC = Beetroot citric (2%) extract

Table 4: Antibacterial screening of Rayeb milk supplemented with beetroot extracts using the disc diffusion assay

Treatment	Inhibition zones diameter (mm)			
	S. aureus	***Strept. faecalis***	***E. coli***	***P. aeruginosa***
Control (Rayeb milk)	16	15	14	12
RBCA	19	18	16	25
RBC	17	16	15	23

Where; RBCA = Rayeb milk with beet root citric: ascorbic acid (2:1) extract; RBC =Rayeb milk with beet root citric (2%) extract

Table (5) demonstrated the total bacterial count; LAB, yeast and mold fungi in control Rayeb milk (untreated), and in supplemented Rayeb milk samples (RBCA and RBC), after different storage periods. Results demonstrated that total viable bacterial count in control was 9×10^6 cells/ml at zero time. This total bacterial count represented the bacteria that makes up the starter culture used for the fermentation of the milk (Streptococcus spp. and Lactobacillus spp.). According to Shah *et al*., (2000), fermented milk must contains viable, live, and abundant cultures of the LAB such as; *Lactobacillus bulgaricus* and *Streptococcus thermophiles*, at a minimum concentration of 10^7 cells/ml. RBAC treatment caused a decrease in total bacterial count from 9×10^6 to 2×10^3 cells/ml after 10 days of storage, while increased count LAB from 5×10^7 to 4×10^{10} cells/ml after same period of storage. Results also revealed that all treated and untreated Rayeb milk samples showed negative growth of mold fungi at zero time, and during different storage periods. After 5 days of storage; yeasts started to appear and gradually increased from 7×10 to 8×10 cfu/ml at the end of storage. Similar results were recorded by Nyambane *et al*., (2014) who reported that yeast populations increased with time in fermented milk. Rayeb milk treated with beetroot extracts had pH ranged from 4.747 to 3.868 after different storage periods (Table 6). pH measurement was important because acidification of Rayeb milk was the key mechanism during its fermentation. Shahraki, *et al*. (2013) reported that declining of pH during fermentation was due to the proto-cooperative action of two strains of bacteria mainly; *Strept. thermophilus* and *L. bulgaricus*. Presence of milk sugars (carbon sources) and milk protein (nitrogen sources) in this rich medium of milk with optimum incubation environment (pH 7); encouraged certain bacterial strains (*Strept. thermophilus*) to grow rapidly. They transformed lactose sugar into lactic acid; acetaldehyde, diacetyl, and formic acid. Accumulation of all these fermentation products corresponded to the increase of acid production during fermentation. Current results were in agreement with Orakwue, (2007), who reported that the pH range was proportional with commercial yoghurt, which ranged between 4.0-4.45.

Table 5: Effect of different storage periods and beetroot extracts on different microorganisms in Rayeb milk

Storage periods	Treatments	Total bacterial count (cells /ml)	LAB (cells /ml)	Yeast (cfu/ml)	Fungi (cfu/ml)
Zero	Control	9×10^6	5×10^7	-	-
	RBC	5×10^6	8×10^7	-	-
	RBCA	10×10^5	10×10^7	-	-
Five days	Control	10×10^4	3×10^8	7×10	-
	RBC	8×10^4	6×10^8	6×10	-
	RBCA	3×10^4	9×10^8	3×10	-
Ten days	Control	8×10^3	5×10^9	8×10	-
	RBC	6×10^3	9×10^9	7×10	-
	RBCA	2×10^3	4×10^{10}	5×10	-

Where; RBCA = Rayeb milk with beet root citric: ascorbic acid (2:1) extract; RBC = Rayeb milk with beet root citric (2%) extract

Table 6. Changes of pH during the storage of control and supplemented Rayeb milk

Storage period	Treatments	pH
Zero	Control	4.747
	RBC	4.685
	RBCA	4.507
Five days	Control	4.146
	RBC	4.036
	RBCA	3.950
Ten days	Control	4.11
	RBC	3.949
	RBCA	3.868

Where; (RBCA) = Rayeb milk with beetroot citric: ascorbic acid (2:1) extract; (RBC) = Rayeb milk with beetroot citric (2%) extract

There was no significant difference between score for flavor of control Rayeb milk, RBCA, and RBC (Table 7). Flavor in all samples slightly decreased at the end of storage; this could be attributed to the development of the acidity, and decrease in the acetaldehyde content (Hebeishy, 2008). Body and texture of all samples were close because of their better consistency. Color of Rayeb milk with RBCA was mostly accepted compared to control and Rayeb milk with RBC.

Table 7: Changes in the organoleptic properties of Rayeb milk affected by supplementation with RBC and RBCA extracts during storage

Treatments	**Flavor**		**Body & Texture**		**Color**		**Total scores**	
	0 time	10 days	0 time	10 days	0 time	10 days	0 time	10 days
Control	19^{a}	17^{b}	9^{a}	9.5^{a}	8^{b}	8^{c}	36	34.5
RBC	19^{a}	18^{ab}	8^{a}	8.5^{a}	9^{ab}	9^{b}	36	35.5
RBCA	19^{a}	18.5^{a}	9^{a}	9.5^{a}	10^{a}	10^{a}	38	38

Where; RBCA = Rayeb milk with beetroot citric: ascorbic acid (2:1) extract; RBC = Rayeb milk with beetroot citric (2%) extract. Different letters in superscripts meant that results were significantly different ($p<0.05$)

Conclusion

Results of the present work suggested that beetroot extracts could be used as natural coloring and flavoring agents in fermented milk products. Rayeb milk with beetroot extract had several health benefits; owing to its minerals content, phytochemical constituents, as well as its proved antibacterial potential. Supplementation of Rayeb milk with beetroot extracts was also advantageous in terms of storage periods. Further studies should be addressed to investigate the possible use of beetroot extracts as additives for other dairy products.

Conflict of interests

The authors declare no conflict of interests.

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