Studies on Antimicrobial Effect of Mentha arvensis (pudina) and Ocimum sanctum (tulsi) in Cow Milk

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Abstract

Milk samples (raw cow and buffalo milk, processed milk) were collected from different areas of Shyamnagar city, North 24 Parganas, India. Adulterations (urea, cane sugar, starch, glucose, formalin, and coloring agent) of different milk samples were analysed. Microbial count was measured before and after the addition of Mentha arvensis (pudina) and Ocimum sanctum (tulsi) extract. The investigation was undertaken to explore the possibilities of utilizing Pudina and tulsi extracts in different type of milk samples to improve the shelf life of milk and improve the health benefits. Mentha arvensis (Pudina) and Ocimum sanctum (Tulsi) leaves extract were prepared through the mortar pestles. The extracts were mixed in milk samples by taking its proportion 2g, 3g and 5g in 10 ml milk samples. The Lactobacillus or NCDC-167 (BD4) culture was propagated in 90 ml sterile de Man-Rogosa Sharpe (MRS) broth and Yeast and mold cultures were propagated in 90 ml sterile Dextrose broth. Enumeration of lactobacillus, yeast and mould count of milk were determined by using pour plate method by using the Man-Rogosa Sharpe (MRS) agar for Lactobacillus and Dextrose agar for yeast and mould count. From the observation it was concluded that after adding the Pudina and Tulsi extract the Lactobacillus and yeast and mould count were decreased, with increase the level of both extractions the microbial counts were decreased largely. Pudina and tulsi may have the similar function to decrease the bacterial count due to their antimicrobial properties.

Keywords: Mentha extract, Ocimum extract, cow milk, Lactobacillus, yeast.

INTRODUCTION

Milk in its natural form has high food value. It supplies nutrients like proteins, fat, carbohydrates, vitamins and minerals in moderate amounts in an easily digestible form. Due to its nutritive value, milk is significant to young and old people [1]. Milk has distinct physical, chemical and biological characteristics, which justifies its high quality for consumption. This makes milk essential for human consumption as a complete food supplement in various parts of the world. Milk can be obtained from different animal species, such as goats, cows and camels, buffaloes and processed are available in the market for example full fat milk, low fat milk and flavored milk include bananas milk, strawberry milk, chocolate milk and coffee milk. When different flavored milk is provided, more people and especially children choose milk, and drink more amount of milk and so get more calcium. However, cow's milk remains the most preferred type of milk. Generally, all types of milk are composed of the same kind of constituents, but in different concentrations. The chemical state of some elements and mineral usually found in milk is very important due to their absorption in the intestine and biological utilization i.e., transport, assimilation in cells and their conversion into biologically system forms [2]. From ancient time food adulteration has been seen but it is becoming concern to all. Dairy or dairy products are

most targets for adding adulterants like starch, flour, urea, sugar, vegetable oils, detergents and even lethal chemical formalin [3, 4]. A group of unscrupulous producers are also using harmful chemicals in milks and dairy products intentionally for extra financial benefits [5]. These used adulterants, chemicals or preservatives in raw or processed milk causing various dangerous diseases [6]. Urea, hydrogen peroxide, antibiotics, caustic soda, carbonates and bicarbonates adulterated milks are also very harmful for adolescent girls and pregnant women [7]. Milk is highly perishable and spoiled within five hours after milking by bacterial contamination from different sources like utensils, water supply, hands, cloths, air, floor etc [8]. Contaminated milk is good source of various foods borne pathogens [9].

The use of herbs in combination with different food has become regular practice to conserve the functional as well as nutritional attributes from herb. Many food items in the market available by different company are popular due to their acceptability and functionality viz. Herbal beverages, Arjuna ghee, curd [10].

Menthol (*Mentha arvensis*) belongs to the family *Libeaceae* is a common edible and aromatic perennial herb which is cultivated throughout the India. Common name is Pudina. The physical-chemical properties of menthol are melting point 43 °C, freezing point is 27-28 °C and boiling point is 212 °C. Molecular formula $C_{10}H_{20}O$ and molecular weight is 156.27 g/mol. The aromatic leaves widely used for flavoring foods and beverages. It is an erect aromatic herb that grows up to 60 cm height with suckers. The stem is cylindrical, and the leaves are simple and opposing type. It is used as a contraceptive, carminative, antiseptic ulcer agent and has been given to treat indigestion, skin diseases, cough, and colds in folk medicine. In beverages *Mentha* is used as a cooling and flavoring agent [11]. Mint leaves have anti-bacterial. Chew mint leaves to fight harmful bacteria in the mouth, teeth and tongue. The mint leaves are useful in the treatment of diabetes, diarrhea, fevers, hypertension, jaundice, nausea, pain, respiratory and urinary tract infections [12].

Ocimum sanctum is a grassy annual plant originated from Iran, Afghanistan and India [13, 14, 15]. Some of the phytochemicals of medicinal importance present in *Ocimum sanctum* have already been identified [16]. Some of these phytochemicals have been shown to possess useful biological activities belonging mainly to phenolic, flavonoid, and carotenoid compounds [17]. The ability of this plant to be used in traditional medicine in the treatment of headaches, cough, diarrhea, constipation, warts, kidney malfunctions, nasal polyps and ulcers has also been reported [13,14,18]. Further, its action as insecticide, nematicide, fungicide and antimicrobial compound also has been reported [17, 19, 20, 21, 22, 23]. *Ocimum sanctum* or tulsi has been the pillar of Ayurvedic holistic health system in India. Since time immemorial, various parts of the plant have been used extensively in the treatment of several systemic diseases like upper respiratory infections, bronchitis, skin diseases, malaria etc. *Candida albicans*, *Staphylococcus aureus*, enteric pathogens, *Klebsiella*, *E. coli* and *Proteus* are the microorganisms against which the antimicrobial property of tulsi has been tested [24]. Tulsi has demonstrated anti-gonorrheal efficacy against multi resistant strains of *Neisseria gonorrhoea* and clinical isolates of beta-lactamase producing methicillin resistant *Staphylococcus aureus* [25].

MATERIAL AND METHODS

Collection of the samples:

Raw milk samples were collected from different cow sheds of Shyamnagar city, North 24 parganas. The samples were collected from nearby areas in the morning to be transported easily without any delay. The samples were collected in 100 ml screw capped sterilized bottles (borosil). All the possible precautions were taken to avoid external contamination at the time of collection of sample and during processing. The different samples were cow milk, buffalo milk, Amul double toned milk, Amul creamed milk, Amul slim and trim milk. They were designated as Sample 1, Sample 2, Sample 3, Sample 4 and Sample 5 respectively for the ease of working.

v) Test for formalin: 10 ml of milk samples was taken in test tubes, and added 5 ml of concentrated sulfuric acid, and the ferric chloride was added slowly along the side of the test tube, so that it forms a layer at the bottom, without mixing with the milk. Development of violet or blue color ring at the junction of the two liquids indicates the presence of formalin.

vi) Test for coloring agents: 10 ml of milk samples was taken in test tube and added few drops of hydrochloric acid. Development of pink color indicates the presence of coloring agents in milk.

Methylene Blue reduction Test:

10 ml of milk sample was taken in sterile test tubes. Methylene blue solution of 1ml was added to 10 ml raw milk samples (without addition to control sample). The test tubes were stoppered with cotton. The tubes (both control and experimental) were incubated at 37 ºC. Next each of the test tubes was allowed to keep at room temperature in an undisturbed position. Test tubes were checked every 30 min of interval for any change of color. Time of decolorization of the methylene blue dye was noted in case of test sample.

Isolation of *Lactobacillus*:

Lactobacillus was isolated from milk samples by Man-Rogosa Sharpe (MRS) broth, Petri plates were incubated at 37 °C for 48 h in anaerobic condition. Colonies differ in morphology, pigmentation; shape and size. Initially all of the isolates were examined for Gram staining and catalase production. The single colony of *Lactobacillus* was isolated by observing their colony morphology and by some biochemical tests and the culture was maintained at 4 °C in MRS broth at pH 5.5.

Identification of *Lactobacillus* species:

Identification of the isolated bacteria as *Lactobacillus* species was performed according to their morphological, cultural, and physiological and biochemical characteristics by the procedures as described in Bergey s Manual of Systematic Bacteriology [26]. The tests carried out were Gram reaction and production of catalase.

i) Gram reaction: After the isolation the smear of samples was made on glass slide, air dried and heat fixed. The smear was then covered with crystal violet for 30 min. Then the slides were washed with distilled water for a few seconds, the smear was then covered with iodine solution for 30 sec. Then the iodine solution was washed with 95 % (v/v) ethyl alcohol until no more color floats from the smear, and washed with distilled

water and drained. After that the Safranine was applied in the smear for 30 sec. Then the slides were washed with distilled water and air dried and ovserved under the microscope.

ii) Catalase test: Catalase test was performed by adding few drops of 3 % hydrogen peroxide (w/v) to a test-tube containing overnight culture of the isolate.

Isolation of yeast and mould:

Yeast and mould were isolated from milk samples. Cultures were propagated in 10 ml sterile Dextrose broth. Plates were incubated at 37 °C for 48 h in anaerobic condition.

Yeast and mould identification:

i) Lactophenol cotton blue (LPCB) staining: The single colony of *Lactobacillus* was isolated by observing their colony morphology and Lactophenol cotton blue (LPCB) staining. 1 to 2 drops of Lactophenol cotton blue solution (LPCB) were taken on a clean glass slide, and transfer a pin-head size amount of growth from a colony with a sterile loop. Mix the content with the inoculating loop, and put a cover slip over the preparation. Then the preparation examine under the microscope [27].

Effect of Pudina (*Mentha arvensis*) and Tulsi (*Ocimum tenuiflorum*) in milk samples:

i) Collection of *Mentha* and *Ocimum* leaves: Fresh *Mentha* and *Ocimum* leaves were purchased from local market of Shyamnagar, 24 pgs (N).

ii) Preparation of *Mentha* and *Ocimum* leaves extract: The *Mentha* and *Ocimum* extract (*Mentha arvensis*) were prepared as shown in the following flow chart:

Sorting (not injured)

⇓

Washing

⇓

Grind in mortar pestles

⇓

Cold storage (5 ˚C)

Extraction of *Mentha* and *Ocimum* leaves

iii) Chemicals: Analytical (AR) or guaranteed grade (GR) reagents were used in the chemical analysis.

iv) Treatment details: The *Mentha* and *Ocimum* leaves extract were mixed in milk samples by taking its proportion 2 g, 3 g and 5 g as per following treatments combinations.

- T1 – 10 ml of milk samples.
- T2 – 10 ml of milk samples + 2g of *Mentha* or *Ocimum* extract.
- T3 -10 ml of milk samples +3g of *Mentha* or *Ocimum* extract.
- T4- 10 ml of milk samples+ 5g of *Mentha* or *Ocimum* extract.

- **Microbiological analysis of Milk after adding Pudina (*Mentha arvensis*) and Tulsi (*Ocimum sanctum*) extract**

The milk samples were examined for the lactobacilli count and yeast and mould count.

- ***Lactobacillus* culture:** The NCDC-167 (BD4) culture was propagated in 10 ml sterile de Man-Rogosa Sharpe (MRS) broth.
- ***Lactobacillus* count:** Enumeration of *lactobacilli* count of milk was determined by using pour plate method [28].
- **Yeast and mould culture:** Yeast and mold cultures were propagated in 10 ml sterile Dextrose broth.
- **Yeast and mould count:** Enumeration of yeast and mold count of milk was determined by using pour plate method [28].

Sensory Evaluation of *Mentha* and *Ocimum* extracts mixture milk

40 panelists were allowed to taste the sample for sensory evaluation.

RESULTS AND DISCUSSION

Methylene Blue reduction Test (MBRT)

Table 1: Methylene blue reduction test in cow milk

Milk samples	Methylene blue reduction test	Classification of the milk samples
Sample1	3 h	Fair quality
Sample2	Less than 2 h	poor quality
Sample3	8 h	Good quality
Sample4	8 h	Good quality
Sample5	8 h	Good quality

Identification of *Lactobacillus* species

i) Gram staining: The short, rod shaped bacterial cells appeared purple, so the cells of the given samples are considered as Gram positive.

Table 2: Colony morphology of isolated *Lctobacillus* sp.and yeast and mould in milk samples

Attributes	*Lactobacillus* sp.	Yeast and mould
Configuration	Round	Round
Texture	Dry	Dry
Pigment	White-Creamy	Pale white
Opacity	Opaque	Opaque
Gram's reaction	Positive (+)	--
Cell shape	Rod	Round
Motility	Non motile	Non motile

ii) Catalase test: Bubble formation was not present. The result was negative.

Yeast and mould identification

i) Lactophenol cotton blue solution (LPCB) solution staining: The slide was observed under the microscope, oval and round shape spores were observed.

Bacterial count after adding *Mentha arvensis* (Pudina) and *Ocimum sanctum* (Tulsi) extract:

- ***Lactobacillus* count of cow milk** The lactobacillus count of cow milk simple was prepared from *Mentha* and *Ocimum* extract mixed with cow milk was estimated at 10^{-2} dilution as per the method of pour plate, by Tharmaraj and Shah (2003) was tabulated in [Figure 1] [27].

Yeast and mould count in cow milk: The yeast and mould count of cow milk, simple prepared from *Mentha* and *Ocimum* extract mixed with cow milk was estimated at 10^{-2} dilution as per the method of pour plate, by[Marshall, (1993)] is tabulated in [Figure 2] [28].

- ***Lactobacillus* count in Buffalo milk:** Lactobacillus count of Buffalo milk was estimated at 10^{-2} dilution.

Yeast and mould count in Buffalo milk*:* yeast and mould count of Buffalo milk was estimated at 10^{-2}dilution.

Lactobacillus,* yeast and mould count in Processed milk*: *Lactobacillus,* yeast and mould count of Processed milk was estimated at 10^{-2}dilution.

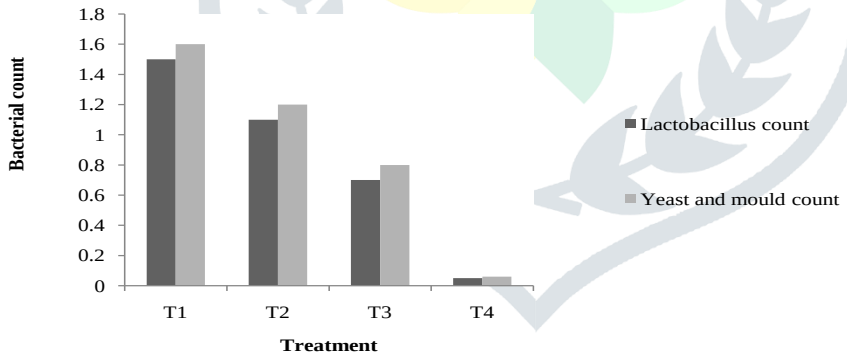

Fig 1: Lactobacillus, yeast and mould count in processed milk after adding *Mentha* extract

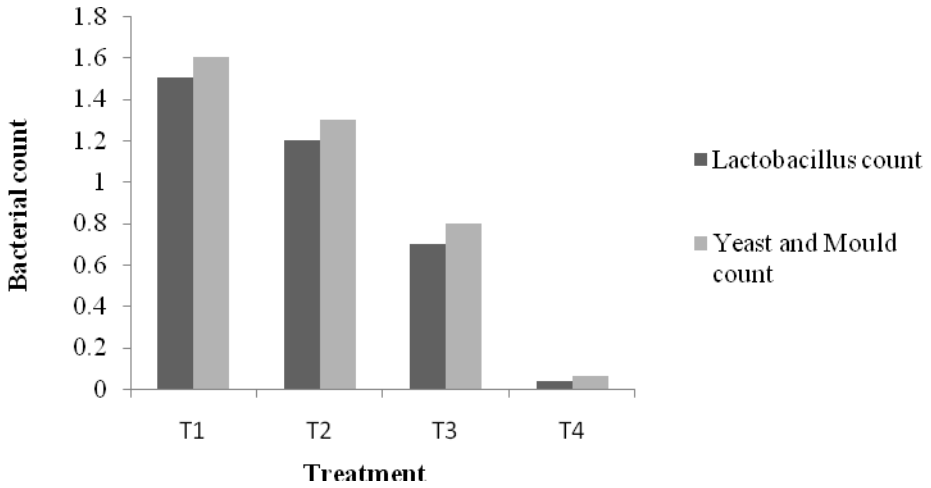

Fig 2: Lactobacillus, yeast and mould count in processed milk after adding *Ocimum* extract

All the experiments were carried out in triplicate.

Table 3: Sensory evaluations were done according to 9 point hedonic scale [29].

Sensory Attributes	**Cow milk after adding *Mentha* extract**				**Cow milk after adding *Ocimum* extract**		
Amount of extraction	**Raw milk**	**2g**	**3g**	**5g**	**2g**	**3g**	**5g**
Color	5.1	6.5	9	7	6	5.5	5.1
Flavor	5	6.1	9	7.4	6	5.4	5
Odor	5	6.1	9	6.7	6.1	5.4	5
Mouth feeling	5	6.5	9	5	7	5.2	5
Overall acceptability	5	6.4	9	5	7	5.1	5

Sensory attributes of *Mentha* and *Ocimum* extract milk

DISCUSSION

Methylene Blue reduction Test (MBRT):

The time required for methylene blue reduction test in the samples depends on the microbial content of the milk samples. From the MBRT result (Table 1) it was observed that raw buffalo milk contains much more organisms compared to raw cow milk. Raw cow and buffalo milk contain a higher microbial contamination than the processed milk samples.

Bacterial count after adding Pudina (*Mentha arvensis*) extract:

From the (Table 2) it was observed that the mixed treatments were decreased for l*actobacillus* and yeast count as compared to control milk but count T2, T3 and T4 was not significantly differed whereas control T1 is significantly differ than all of the *Mentha* added samples. This occurs due to the inhibitory action of (*Mentha arevensis*) for the growth of lactobacilli and yeast.

Bacterial count after adding Tulsi (*Ocimum sp.*) extract:

From the (Table 2) it was observed that the mixed treatments were decreased for l*actobacillus* and yeast count as compared to control milk but count T2, T3 and T4 was not significantly differed whereas control T1 is significantly differ than all of the *Ocimum sanctum* added samples. This occurs due to the inhibitory action of (*Ocimum sanctum*) for the growth of *lactobacilli* and yeast.

Sensory Evaluation of *Mentha* and *Ocimum* extracts mixture milk

From the Table 3, it was observed that *Mentha* extracts gave the positive result than the *Ocimum* extracts milk. Highest value observed in 3 g of *Mentha* extracts and 5 g of *Ocimum* extracts gave the lowest value. 3 % (w/v) *Mentha* and 2 % (w/v) level gave desirable result and acceptability by the panel of judges.

Conclusion

From the result of the present study, it was observed that the 2 g *Mentha arvensis* (Pudina) extract in 10 ml milk sample also decreased the microbial count with increase the level of *Mentha* extract the microbial count decreased largely, 2 g *Ocimum sanctum* (Tulsi) extract in 10 ml milk sample also decreased the microbial count and increase the level of *Ocimum* extract the microbial count decreased largely. *Mentha arvensis* and *Ocimum sanctum* may have the similar function to decrease the bacterial count due to their antimicrobial property. But in case of sensory evaluation *Mentha* extracts mixture milk gave better result than the *Ocimum* extract mixture milk. 3 % (w/v) *Mentha arvensis* and 2 % (w/v) *Ocimum sanctum* extract level gave desirable result and acceptability by the panel of judges. So it may be concluded that the *Mentha arvensis* (Pudina) and *Ocimum sanctum* (Tulsi) both can be used to increase the self-life of milk but in case of taste *Mentha arvensis* tasted much better than *Ocimum sanctum.*

Application of research:

The *Mentha arvensis* (Pudina) and *Ocimum sanctum* (Tulsi) extracts mixed milk can provide the great opportunity to dairy industry as well as housewife for better utilization of medicinal attributes of *Mentha arvensis* (Pudina) and *Ocimum sanctum* (Tulsi). This will act as functional food for curative and preventing measures for cardiovascular, immune defense system, upper respiratory infections, bronchitis, skin diseases, malaria and blood system related problems.

ACKNOWLEDGEMENT

I am highly acknowledged to our college authority and UGC for carrying on the research work properly.

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