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DEVELOPMENT, MICROBIAL QUALITY ASSESSMENT AND ANTIOXIDANT POTENTIAL OF HERBAL MILK FORTIFIED WITH *NIGELLA SATIVA* AND *TERMINALIA CHEBULA*

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ABSTRACT

Food industries have rather high demand for the products that meet the consumer demand for a healthy life style for which functional food fortified with the plant ingredients plays important role. Flavoured milk is becoming an integral part of market milk industry because it has good consumer acceptance as a refreshing and nourishing milk beverage. The present study was undertaken to develop the functional product herbal milk aim to incorporate *Nigella sativa* and *Terminalia chebula* these herbs in milk as a supplementary and to study the efficient combination to increase the acceptability of the milk and also study its microbiological and antioxidant properties. In the microbial aspect, all the samples have a normal microbial count (SPC), within the standard acceptable level of coliforms count and yeast and moulds. The standard plate count (cfu/ ml) of herbal immunobooster milk samples were $4.78 \times 10^{-6,}$ 3.67×10^{-6}, 3.52×10^{-6} and 3.63×10^{-6} for control treatments T1, T2, and T3. Coliform count (cfu/ml) of immunobooster milk samples were 2.37×10-1, 2.50×10^{-1}, 1.67×10^{-1}, and 2.37×10^{-1} and yeast and mould count (cfu/ml) of herbal immunobooster milk samples were 3.6×10^{-1}, 2.5×10^{-1}, 2.0×10^{-1}, and 2.3×10^{-1} for control and treatments. The results of DPPH free radical scavenging activity of herbal immunobooster milk samples were IC_{50} values of TC, T1, T2, T3 and L-ascorbic acid were found 82.7, 71.8, 42.3, 44.4, and 39.4 mg/ml. The data with respect to $ABTS^{+}$ free radical scavenging activity of herbal immunobooster milk samples were IC_{50} values of TC, T1, T2 and T3 and Buttylated Hydroxyl Toluene (BHT) were found 85.8, 58.6, 46.8, 57.5, and 40.5 mg/ml and the positive standard (BHT) analysis of data revealed a significant difference ($P<0.05$) between control and a treatment indicating the addition of herbal powder, jaggery and palm sugar at different level produces significant. Therefore, enrichment of herbal milk recipes may be considered for

optimization and development towards producing new functional herbal milk may be recommended for its health beneficial effects like antioxidants agents.

KEYWORD: Herbal milk, Physicochemical, Microbial analysis, Antioxidant activity.

INTRODUCTION

Functional foods have emerged as a novel category of health-promoting products, often based on traditional ingredients. According to the American Dietetic Association, functional foods include enriched or fortified foods, while nutraceuticals are isolated compounds added to foods to improve health beyond normal dietary intake (Ross, 2000). Consumer interest in functional foods, particularly those containing probiotics and antioxidant-rich compounds, has increased due to their health benefits, such as enhanced immunity, improved lactose digestibility, blood glucose management, and reduced risk of diseases like colon cancer and inflammatory bowel disorders (Douaud, 2007; Yadav et al., 2007). Milk is a nutrient-rich, naturally white fluid containing essential proteins, fats, carbohydrates, vitamins, and minerals, making it vital for human nutrition from infancy to adulthood (Dilek Özdemir & Deren Tahmas Kahyaoğlu, 2019; Heck et al., 2009). Its high nutritional value, digestibility, and amino acid composition make milk an ideal medium for producing functional beverages, especially when combined with plant-based ingredients (Ramos et al., 2017; Lazareva et al., 2007).

The use of plant additives in dairy products has gained attention for their functional properties. Wild and medicinal plants are rich in vitamins, minerals, and bioactive compounds that enhance digestion, cardiovascular function, and overall well-being (Swati Sethi *et al.,* 2021). Among these, *Nigella sativa* (black cumin) seeds are known for their essential oils, saponins, polyphenols, and thymoquinone, which confer immunomodulatory, anti-inflammatory, antioxidant, antimicrobial, and other therapeutic effects (Franco-Ramos *et al.,* 2020; Hadi *et al.,* 2016). *Terminalia chebula*, a widely used medicinal herb in India, is rich in tannins, flavonoids, sterols, and polyphenols, providing antimicrobial, antioxidant, anticarcinogenic, antidiabetic, anti-aging, and antiviral properties (Assie *et al.*, 2016; Fraga-Corral *et al.*, 2020; Trinh *et al.*, 2020). Its bioactive compounds also exhibit selective cytotoxicity against cancer cell lines.

Antioxidant compounds, including flavonoids and polyphenols, play a crucial role in neutralizing free radicals, preventing oxidative stress, and reducing the risk of degenerative diseases such as cancer, cardiovascular disorders, diabetes, and inflammatory conditions (Beckman, 2000; Panche *et al.,* 2016; Rafieian-Kopaei *et al.*, 2013). Considering these health benefits, the present study was undertaken to develop a functional herbal immunobooster milk incorporating *Nigella sativa* and *Terminalia chebula*. Hence, study aimed to optimize the combination of these herbs in milk and evaluate the products microbiological and antioxidant properties to enhance acceptability and nutritional value.

Materials methods

Materials requirements

Toned milk, Herbal immunobooster powder, Palm sugar, Jaggery, Cardamom, Stabilizers, Mixer Grinder, Autoclave, Hot air oven, pH meter, Weighing Balance, Utensils, Laminar airflow chamber, Poiseuille's flow apparatus, Moisture analyser, Muffle furnace, Violet Red Bile Agar, Plate Count Agar, Potato Dextrose Agar.

Study area

This study was carried out the laboratory of the Department of Rural Development Science, Arul Anandar College (Autonomous), Karumathur, Madurai from November, 2022 to March, 2023.

Method of Preparation of Herbal immunobooster milk

The herbal immunobooster powder was prepared by mixing ingredients like Black cumin seed 12 g and Yellow Myrobalan seed 12 g, and made into fine powder. From this combination required quantity of herbal immunobooster powder was collected and used in the treatment as immunobooster powders. Herbal immunobooster milk was prepared using 1 L of toned milk per treatment, following a modified method of Palthur et al. (2014). Milk was preheated to 40 °C, filtered, and heated to 65 °C before adding palm sugar, herbal powder, stabilizer, and cardamom as per treatment. The mixture was homogenized at 2000 psi, pasteurized at 63 °C for 30 min, cooled to 5 °C, and stored under refrigeration and the results are presented in Table 1 and Fig.1

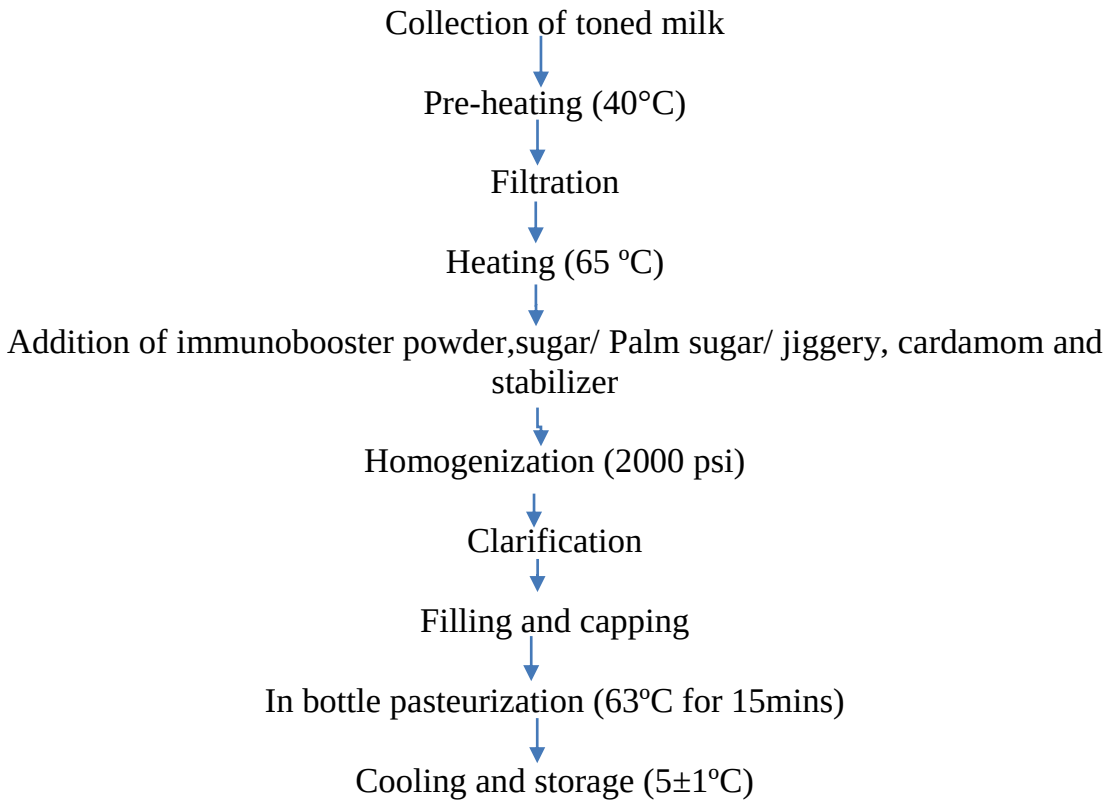

Fig. 1 Flow diagram for preparation of herbal immunobooster milk

Treatment details

Table 1: Treatment combination for the preparation of herbal immunobooster milk as detailed below;

Trials	Toned milk (Lts)	Sugar (%)	Palm sugar (%)	Jaggery (%)	Herbal Immunobooster powder (%)	Stabilizer (%)	Cardamom (Pieces)
TC-Control	1	5				0.2	4
T1	1	5			2.4	0.2	4
T2	1		7		2.4	0.2	4
T3	1			7	2.4	0.2	4

TC Control - 1000 ml milk + 50 gm Palm sugar + 2 gm stabilizer + cardamom 4pieces
T1- 1000 ml milk + 50 gm sugar + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces
T2 - 1000 ml milk + 70 gm Palm sugar + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces
T3 - 1000 ml milk + 70 gm Jaggery + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces

Microbiological analysis

All herbal immunobooster milk samples underwent microbiological analysis for Standard Plate Count (SPC), Coliform Count (CC), and Yeast & Mould Count (YMC). Samples were serially diluted in sterile saline (up to 10^{-7}), with 1 ml of appropriate dilutions used for SPC (10^{-4}–10^{-6}) and 10^{-1} dilutions for coliform and yeast/mould counts.

Standard Plate Count (SPC)

Preparation of media

Microbiological analysis of herbal immunobooster milk was performed as per IS standards. Standard plate count was done using plate count agar (IS: 5402, 1969) with appropriate dilutions (10^{-4}–10^{-6}), incubated at 37 °C for 24–48 h, and colonies were counted. Coliforms were estimated following BIS SP: Part XI (1981), and yeast and mould counts were determined on Potato Dextrose Agar as per IS: 5403 (1969).

Antioxidant activities

DPPH (1, 1- diphenyl – 2 – picrylhydrazyl hydrate radical scavenging activity)

DPPH radical scavenging activity of herbal immunobooster milk was measured using the method of Blois (1958). Samples (0.1 ml) were mixed with 1 ml of 0.2 mM DPPH in methanol, incubated 20 min at 28 °C in the dark, and absorbance was measured at 517 nm. Vitamin C served as a positive control, and antioxidant activity was expressed as IC_{50}, the concentration (mg/ml) required to inhibit 50% of DPPH radicals.

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100$$

$ABTS^+$ Scavenging effects (2, 2-Azino-bis-3-ethyl benzthiazoline-6-sulphonic acid)

The antioxidant activity of herbal immunobooster milk was assessed using the ABTS•+ method (Re et al., 1999). ABTS•+ radicals were generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating 12–16 h in the dark. Flavored milk samples at different concentrations were added to 1 ml ABTS•+ solution, and absorbance was measured at 734 nm after 6 min. BHT was used as the standard, and percent inhibition was calculated. All assays were performed in triplicate.

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100$$

Statistical Analysis

The results were expressed as the mean ± SD. All statistical analyses were performed using SPSS version 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Student's t-test was performed to determine any significant for *in vitro* antioxidant assay was carried out using one-way analysis of variance (ANOVA) and Duncan test. *P* value < 0.05 was considered statistically significant.

RESULT

In this study, herbal immunobooster milk was prepared using toned milk with sugar (control) and also with herbal immunobooster powder, jaggery, palm sugar and a combination of both at different percent levels were incorporated along with cardamom as a flavouring and stabilizer agent. The results product was analyzed for physicochemical, microbiological, and antioxidant activities. The data obtained were subjected to statistical analysis to compare different parameters of control and treatments.

Microbial analysis

Standard Plate Count

Table 2. shows the results of microbial quality analysis of herbal immunobooster milk samples. The standard plate count (cfu/ ml) of herbal immunobooster milk samples were $4.78 \times 10^{-6,}$ 3.67×10^{-6}, 3.52×10^{-6} and 3.63×10^{-6} for control treatments T1, T2, and T3 respectively. The results of the samples were ranged from 3.52×10^{-6} to 4.78×10^{-6} and results are presented in Fig 2. Analysis of data showed no significant difference between control and treatments indicating that the addition of herbal immunobooster powder, jaggery and palm sugar at different level produces no significant change in the standard plate count because of maintenance of strict hygienic measures during the process of manufacture. A similar result recorded by Saxena and Rai (2013) reported the value of SPC was higher the standard plate count indicates the improper hygienic condition. Holm *et al.* (2004) suggested that high standard plate count can be influenced by poor storage temperature, long storage period after milking, health and hygiene of the cow, environment where milking is done as well as procedures used in cleaning and sanitizing the milking and storage equipment.

Table: 2 Microbial analyses of herbal Immunobooster milk

Microbial analysis	TREATMENTS			
	TC	T1	T2	T3
Standard Plate Count (cfu/ ml)	4.78×10^{-6}	3.67×10^{-6}	3.52×10^{-6}	3.65×10^{-6}
Coliform Count (cfu/ ml)	2.37×10^{-1}	2.50×10^{-1}	1.67×10^{-1}	2.33×10^{-1}
Yeast and Mould Count (cfu/ml)	3.66×10^{-1}	2.50×10^{-1}	2.00×10^{-1}	2.33×10^{-1}

a- score values of flavored milk

[b]-mean of six assays; ± - standard deviation** significant at $p < 0.05$

TC Control - 1000 ml milk + 50 gm Palm sugar + 2 gm stabilizer + cardamom 4pieces

T1- 1000 ml milk + 50 gm sugar + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces

T2 - 1000 ml milk + 70 gm Palm sugar + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces

T3 - 1000 ml milk + 70 gm Jaggery + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces.

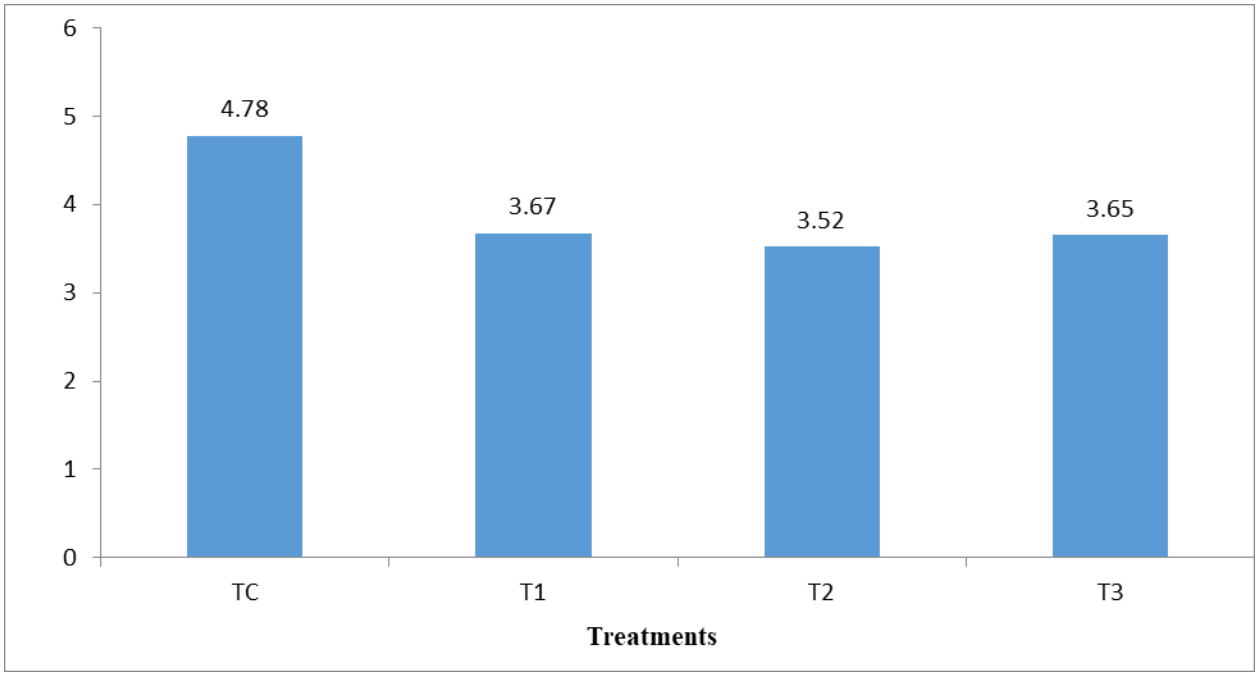

Fig. 2: Standard plate count (cfu/ ml) of herbal immunobooster milk samples Coliform Count.

Figure 3, shows the results of Coliform count (cfu/ml) of immunobooster milk samples were 2.37×10-1, 2.50×10^{-1}, 1.67×10^{-1}, and 2.37×10^{-1} for control and treatments T1, T2, and T3 respectively. The results of the samples were ranged from 1.67×10^{-1} to 2.50×10^{-1}. Analysis of the data revealed no significant difference between control and treatment indicating that the addition of immunobooster powder, jaggery

and palm sugar at the different level does not produce any significant change in the coliform count and also due to proper handling and aseptic measures during the preparation of immunobooster milk. Saxena and Rai (2013) and Fadaei (2014) observed the value of coliform count was 5.14 log cfu/ml. Gurmessa (2014) reported the value of coliform count was in the range of 6.4 to 6.1 log cfu/ml. All samples were completely free from yeast and mould. It is evident from that yeast and mould count in experimental samples were 100% negative. A similar result recorded by Kumar *et al.* (2015).

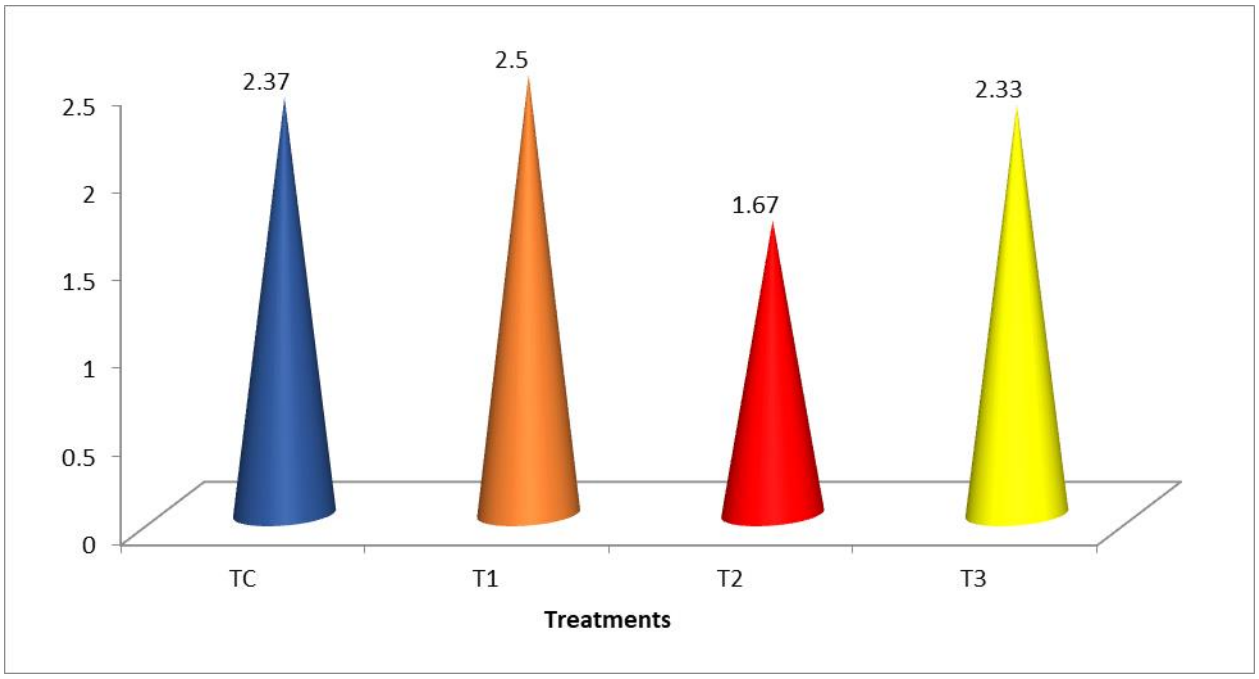

Fig. 3: Coliform count (cfu/ml) of herbal immunobooster milk.

Yeast and Mould Count

The yeast and mould count (cfu/ml) of herbal immunobooster milk samples were 3.6×10^{-1}, 2.5×10^{-1}, 2.0×10^{-1}, and 2.3×10^{-1} for control and treatments T1, T2, and T3 respectively. The results of the samples were ranged from 2.0×10^{-1} to 3.6×10^{-1} and the results are presented in Fig.4. Analysis of the data revealed no significant difference between control and treatment indicating that the addition of immunobooster powder, jaggery and palm sugar at the different level does not produce any significant change in the coliform count and also due to proper handling and aseptic measures during the preparation of herbal immunobooster milk. Yadav *et al.* (2012) reported that the average count of yeasts and moulds of Khoa based Kalajam samples of Kanpur city was recorded $13.33x10^5$ /g with the range of $6.66x10^5$ /g. to $20.00x10^5$ /g. Analysis of data showed significant difference between control and treatments indicating that addition of aspartame tablets and sugar at different level produce significant change in the yeast and mold count due to minimal processing of dietetic flavoured milk during preparation and presence other ingredients along with sugars. The result obtained was similar to the report of Palthur *et al.* (2014).

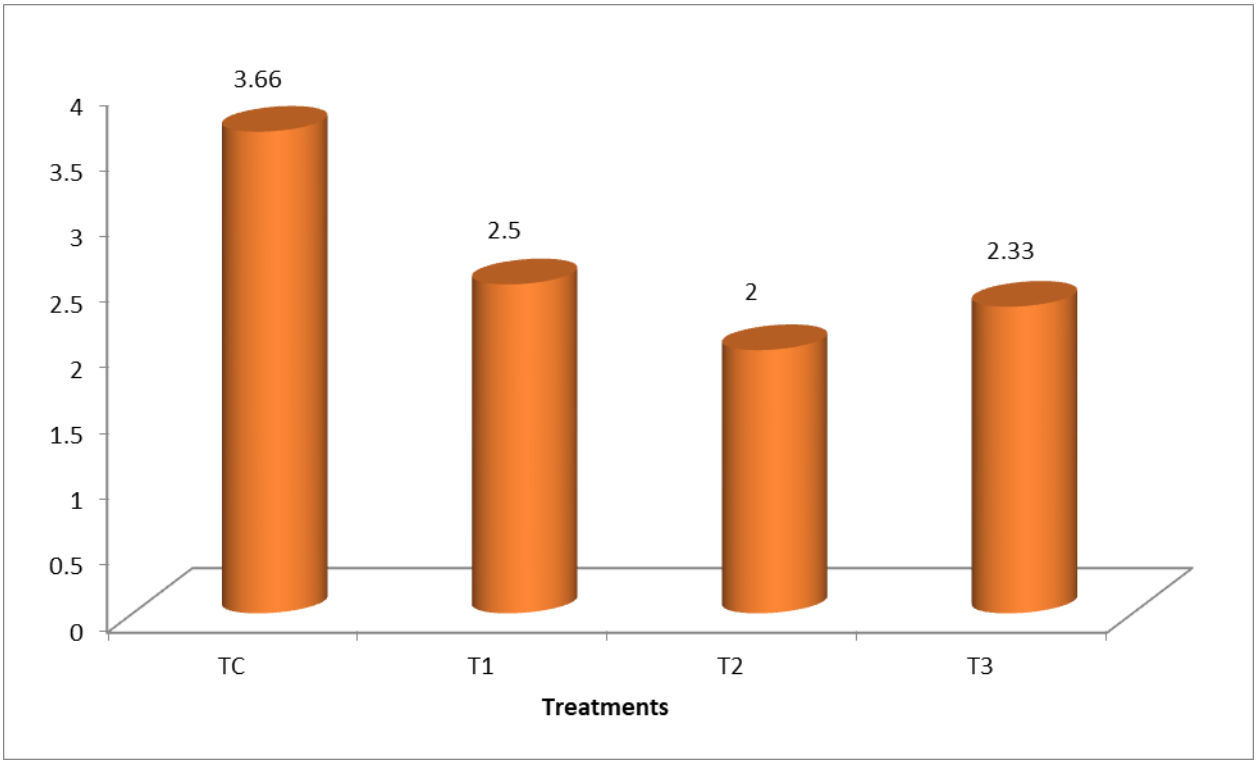

Fig. 4: Yeast and mould count (cfu/ml) of herbal immunobooster milk.

Antioxidant activities

Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, and ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS. These free radicals are the major points in lipid peroxidation. The antioxidants may mediate their effect by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions. Several synthetic antioxidants, e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available but are quite unsafe and their toxicity is a problem of concern. Natural antioxidants, especially phenolics and flavonoids, are safe and also bioactive which are capable of absorb and neutralize free radicals, quenching singlet and triplet oxygen or decomposing peroxides.

DPPH (1,1-diphenyl-2-picrylhydrazylhydrate) radical scavenging activity

The table 4. Shows the results of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of herbal immunobooster milk and the positive standard (Ascorbic acid) are presented in Fig. 5. The data with respect to DPPH free radical scavenging activity of herbal immunobooster milk samples were IC_{50} values of TC, T1, T2, T3 and L-ascorbic acid were found 82.7, 71.8, 42.3, 44.4, and 39.4 mg/ml respectively Fig. 5 and 6. The results of the samples were ranged from 39.4 to 82.7 mg/ml. The analysis of data revealed a significant difference ($P<0.05$) between control and a treatment indicating the addition of herbal immunobooster powder, jaggery and palm sugar at different level produces significant. Our work is closely related with the results of Palthur *et al.* (2014a) studied the antioxidant activity by DPPH method of milk prepared by partial substitution of *Ocimum sanctum* powder and found 40% activity. Samaddar *et al.* (2015) observed that the antioxidant activity of Trans-

Cinnamaldehyde and Eugenol enriched flavoured milk were that is 0.1495 and 1.2860 µM of trolox/ml of milk respectively of the product and ultimately enhances the shelf life of the product.

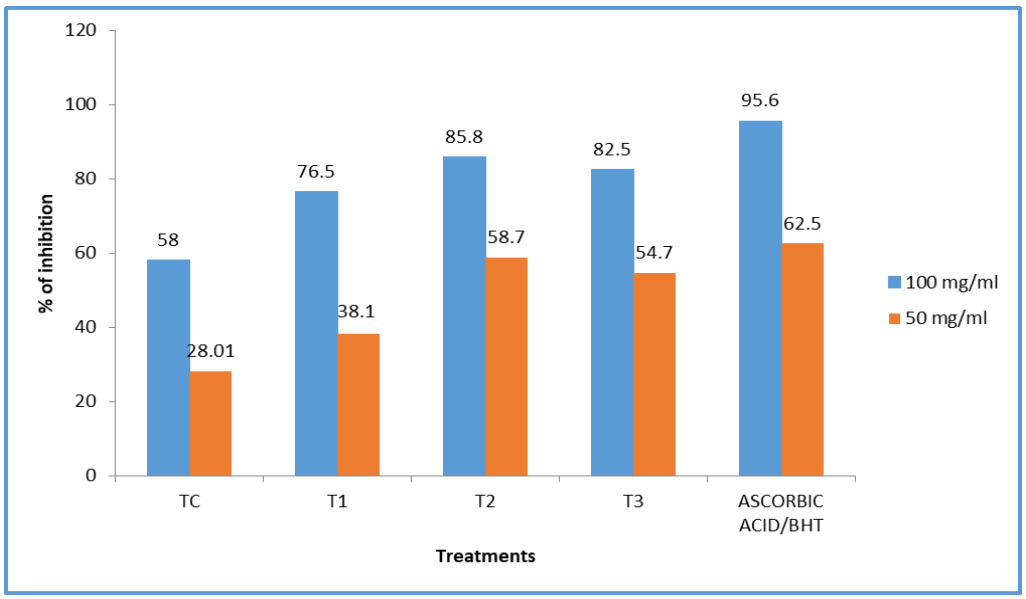

Fig. 5: DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of herbal immunobooster milk.

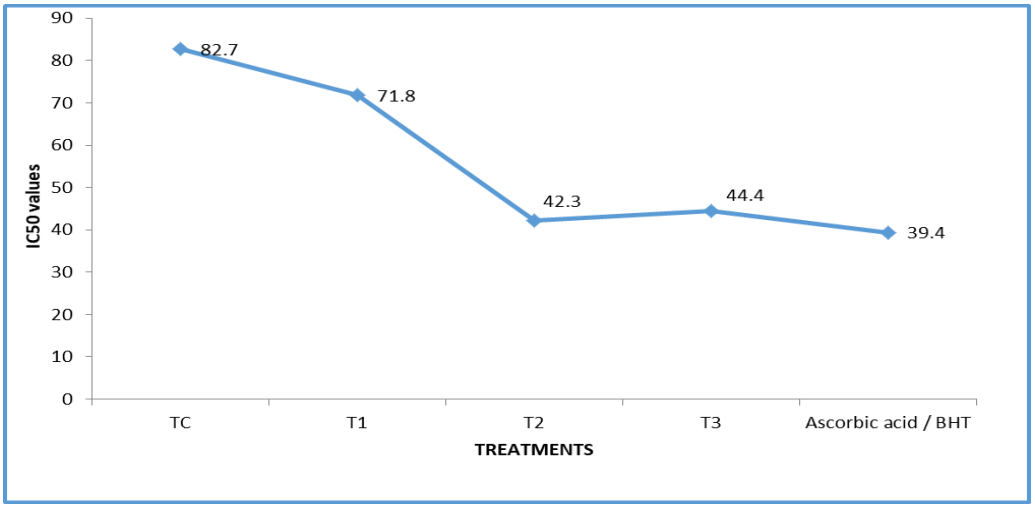

Fig. 6: LC_{50} values of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity of herbal immunobooster milk.

$ABTS^{\cdot+}$ scavenging effects (2,2'-azino-bis-3-ethylbenzthi-azoline- 6-sulphonic acid)

$ABTS^{\cdot+}$ 2,2'-azino-bis-3-ethylbenzthi-azoline- 6-sulphonic acid) radical scavenging activity of herbal immunobooster milk and the positive standard (BHT) are presented. The data with respect to $ABTS^{\cdot+}$ free radical scavenging activity of herbal immunobooster milk samples were IC_{50} values of TC, T1, T2 and T3 and Buttylated Hydroxyl Toluene (BHT) were found 85.8, 58.6, 46.8, 57.5, and 40.5 mg/ml respectively Table 3 and Fig.7 & 8. The results of the samples were ranged from 40.5 to 85.8 mg/ml. The analysis of data revealed a significant difference ($P<0.05$) between control and a treatment indicating the addition of herbal immuno powder,

jaggery and palm sugar at different level produces significant. The method is widely used due to its simplicity, speed, and sensitivity (Mishra *et al.*, 2015). Rashad *et al.* (2020) reported that the $ABTS^{\cdot+}$ radical-scavenging activity of FMPO 300 reaching IC_{50} (57.87 ± 0.34 µg/ml) after 30 days of storage. However, FMPO 150 was IC_{50} (67.60 ± 1.04 µg/ml) compared to FM which was IC_{50} values of 120.41 ± 7.74 µg/m). Lee *et al.* (2020) noticed that the produced fermented milk with the addition of *Cudrania tricuspidata* fruit, which is rich in xanthones and flavonoids.

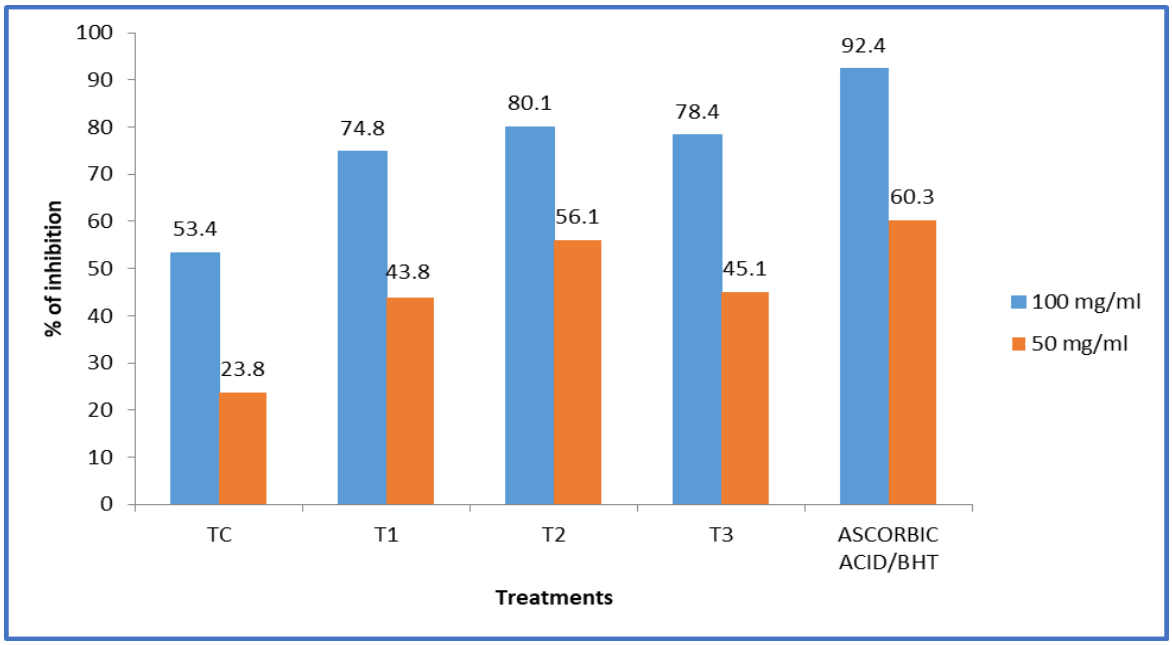

Fig. 7: $ABTS^{\cdot+}$ 2,2'-azino-bis-3-ethylbenzthi-azoline- 6-sulphonic acid) radical scavenging activity of herbal immunobooster milk.

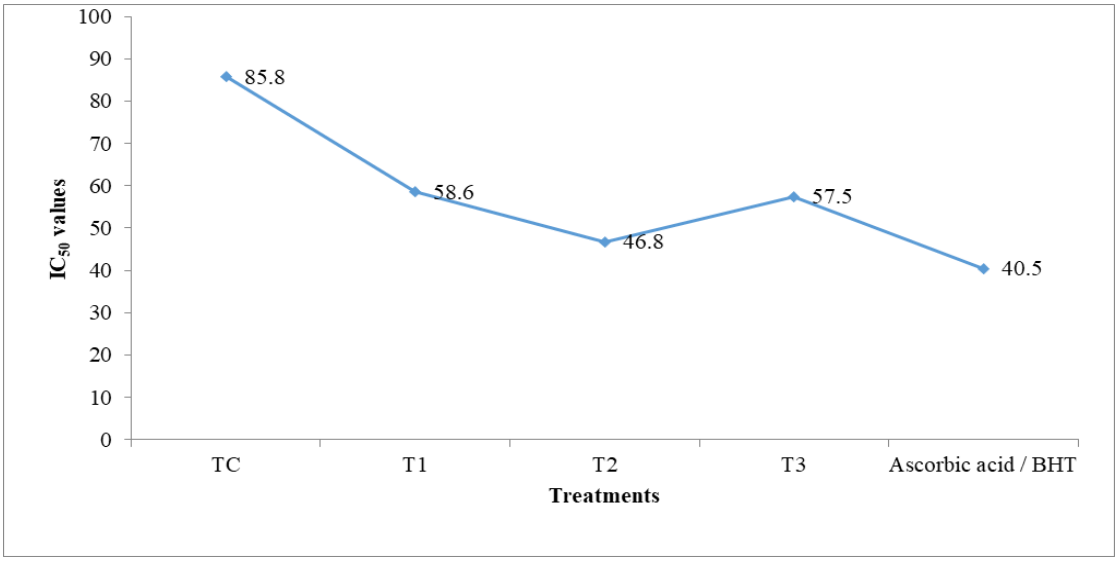

Fig. 8: LC_{50} values of $ABTS^{\cdot+}$ 2,2'-azino-bis-3-ethylbenzthi-azoline- 6-sulphonic acid) radical scavenging activity of herbal immunobooster milk

Table 3. Antioxidant activity of herbal immunobooster milk

S.NO	Milk samples	DPPH scavenging activity (%)		IC_{50} Values mg/ml	$ABTS^{+\cdot}$ scavenging activity (%)		IC_{50} Values mg/ml
		100 mg/ml	50 mg/ml		100 mg/ml	50 mg/ml	
1	TC	58.0 ± 0.3.7c	28.01 ± 0.18	82.7	53.4 ± 0.64	23.8 ± 0.76	85.8
2	T1	76.5 ± 0.32	38.1 ± 0.33	71.8	74.8 ± 0.26	43.8 ± 0.76	58.6
3	T2	85.8 ± 0.71	58.7 ± 0.35	42.3	80.1 ± 0.01	56.1 ± 0.80	46.8
4	T3	82.5± 0.62	54.7 ± 0.37	44.4	78.4 ± 0.49	45.1± 0.33	57.5
5	Ascorbic acid/ BHT	95.6 ± 0.84	62.5 ± 0.35	39.4	92.4 ± 0.84	60.3 ± 0.35	40.5

a- score values of flavored milk

[b]-mean of six assays; ± - standard deviation** significant at $p < 0.05$

TC Control - 1000 ml milk + 50 gm Palm sugar + 2 gm stabilizer + cardamom 4pieces

T1- 1000 ml milk + 50 gm sugar + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces

T2 - 1000 ml milk + 70 gm Palm sugar + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces

T3 - 1000 ml milk + 70 gm Jaggery + 24g herbal immunobooster powder + 2 gm stabilizer + cardamom 4pieces.

CONCLUSION

Finally, it can be concluded that the study developed herbal immune-booster milk and evaluated its microbiological and antioxidant properties. Slight bacterial reductions with higher immunobooster powder indicated its antibacterial effect. Antioxidant activity showed T2 had the strongest DPPH (IC_{50} = 42.3 mg/ml) and ABTS (IC_{50} = 46.8 mg/ml) radical scavenging, close to ascorbic acid and BHT standards. Enriching milk with these herbs enhances its nutritional value and health benefits, making it a promising functional beverage. Therefore, enrichment of herbal milk with these food spices undoubtedly constitutes a good way for improving the nutritional value and health benefits of herbal milk. Such recipes may be considered for optimization and development towards producing new functional herbal milk may be recommended for its health beneficial effects like antioxidants agents.

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Declaration

Consent to participate: Not applicable

Consent for publication: Not applicable

Conflicts of interest: The authors declare to competing interest.

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