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RESEARCH ARTICLE

Assessment of bioactive components of essential oils for antimicrobial activity in the dairy food matrix

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Abstract: Essential oils as a whole have been widely investigated for their antimicrobial properties against spoilage microorganisms and pathogens. However limited information is available about the inhibitory activities of components of EO in foods and more specifically in dairy food matrices. The widespread components of common EOs were evaluated for their antibacterial activity in media and skim milk. Amongst the EO components, trans-cinnamaldehyde and eugenol were the most effective on the basis of disk assay and minimum bactericidal concentrations in milk. The MBC of these EOs was unaffected by the conditions of the incubation temperature of milk. However the MBC was decreased in paneer slurry against *L. monocytogenes* and *E. coli* O157:H7 when incubated at 7 °C than 37 °C. In the dairy spread, trans-cinnamaldehyde and eugenol were effective in inhibiting TBC, coliform, and yeast & mold count at 0.26 mg/g without affecting sensory properties. The microbial load in paneer made from EO added to milk (10 mg/ml) significantly inhibited microbial growth during storage. In addition eugenol has shown antioxidant activity in milk through ABTS radical scavenging assay.

Keywords: Cinnamaldehyde, Eugenol, Channa-based dairy spread, Essential oil

Introduction

Consumers nowadays demand minimally processed foods that are nutritionally rich, free of chemical preservatives and microbiologically safe for human consumption. As a result, research has focused on reducing the intensity of heat treatment, developing alternatives to heat processing techniques, and replacing conventional chemical preservatives with natural ones that can preserve the food product freshness without compromising food safety (Ait-Ouazzou et al. 2011, Badola et al. 2018, Mitropoulou et al. 2022).

Essential oils (EOs) are aromatic and volatile hydrophobic compounds that can be extracted from any part of the plant. For several decades, EOs have been known for their antimicrobial activity and are now widely used in cosmetics, pharmaceuticals, food preservation, and food additives (Rota et al. 2004, Chen et al. 2014). Due to their safe application and the image of being natural many EOs currently generally as recognized as safe (GRAS). Chemically, EOs are complex natural mixtures that are composed of more than 20-80 individual constituents, each of which is present at significantly varying concentrations (Saraiva et al. 2021). The major widespread constituents of EOs thymol, linalool, citral, p-cymene, carvacrol, eugenol, etc. Numerous studies have investigated the antimicrobial activity of EOs as a whole against foodborne pathogens and these properties are attributed mainly to constituents of EO (Mith et al. 2014, Cho et al. 2020). However, the chemical composition of whole essential oils is highly varying and is dependent on several factors such as environmental conditions, extraction method, and stage of harvesting. Concerning this challenge, few studies have explored the use of purified major constituents of EO in food preservation (Ait-Ouazzou et al. 2011). Since the constituents in an EO have different chemical structures and may have different chemical properties such as solubility, volatility, and oxidative stability which in turn affect the antimicrobial properties of the whole EO (Chen et al. 2014). The major individual compounds can be easily procured in large quantities and according to desired specifications (Das et al. 2021). Moreover, a reproducible control of spoilage and pathogens in foods can be achieved with a steady supply of essential oils with major compounds at the same concentrations tested (Ju et al. 2020). The compounds selected in this study are the major constituents of commonly available EOs. Studying microbial sensitivity in liquid and solid food matrices as models might facilitate optimization for the final application of essential oil constituents (Gutierrez et al. 2009).

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This study was carried out to evaluate the antibacterial activity of widespread constituents of EOs eugenol, carvacrol, cinnamaldehyde, menthol, thymol, vanillin, and p-cymene in skim milk against pathogenic and spoilage bacteria. Further, paneer and chhana-based dairy spread were used as dairy product food models to assess the application potential of those effective EO components *in situ*.

Materials and Methods

Essential oils

Eugenol, carvacrol, cinnamaldehyde, menthol, thymol, vanillin, and p-cymene were purchased from Sigma-Aldrich, St. Louis, USA.

Bacterial strains

L. monocytogenes ATCC 15313, *E. coli* O157:H7, *S. aureus* MTCC 3160, *E. faecium* NCDC 211, *B. cereus* NCDC 66, *B. subtilis* NCDC 215, *S. typhi* NCDC 13 and *E. faecalis* NCDC 223 were used in this study. These bacterial cultures are part of the National Collection of Dairy Cultures, NDRI, Karnal. All bacterial strains were maintained at 4 °C on tryptic soy broth (TSB) medium (Himedia, India).

Disk diffusion assay

As a preliminary step, the agar disc diffusion technique was performed to screen antibacterial activity against test bacterial strains (Rota et al. 2004). A 15 ml of TSA agar (Himedia, India) was poured into sterile Petri plates and allowed to solidify and then overlaid with 5 ml of TSA soft agar (Agar 0.75%) inoculated with 10^6 CFU/ml of each tested bacterium. The paper disk impregnated with 15 µl of EO was placed on the surface of the agar. The plates were incubated overnight at the appropriate temperature, and the diameter of the resulting zone of inhibition was measured in millimeters.

Minimum bactericidal concentration (MBC) test

The minimum bactericidal concentration (MBC) in sterilized skim milk was determined using the microdilution method (Cava-Roda et al. 2012). Briefly, 100 µl of sterilized milk was dispensed into the wells of the 96-well flat bottom plate. A 100 µl of sterilized milk containing EO was loaded into the first well, and subsequent serial double dilutions were carried out to obtain concentrations ranging between 32-0.0125 mg/ml. To each well, another 100 µl of sterilized skim milk spiked with a tested bacterium (10^6 CFU/ml) was added and mixed for 30 seconds in a microplate shaker. The microplate was incubated for 24 h at an appropriate temperature without shaking. After incubation 25 µl sample from each well was spot inoculated on a TSA plate and growth was observed after incubation at 37 °C for 24 h. The concentration of compound at which no visible growth was observed was defined as MBC of EO.

Challenge studies

Sterilized milk, 30 ml without or with different concentrations of EO was transferred to sterilized tubes with cap. The test bacterium was inoculated at 1% from cell suspension containing 10^6 CFU/ml. Subsequently samples were transferred to sterilized 100 ml sample bottle and capped. For the survivor counts serially diluted samples were pour plated on tryptic soy agar and after 37 °C/24-48 h the CFU/g was recorded.

A portion of sterilized paneer (32 g) was blended with sterile water (16 g) to obtain slurry by eliminating natural microbiota. Subsequently, samples were transferred to sterilized 100 ml sample bottles with a cap. The samples were experimentally inoculated with test bacterial strains (10^6 CFU/ml) and desired concentration of EO was added and MBC was determined as described above.

Activity against natural microflora in paneer and chhana based dairy spread

For paneer, EO was added to raw milk at a level of 10 mg/ml and mixed at 15000 rpm/5 min using Ultra-Turrax T25 (IKA Labortechnik). The temperature of the milk was increased to 90 °C with no hold and the coagulation of milk was done at 70 °C by the addition of citric acid solution (1% w/v). The coagulum was collected in a muslin cloth and pressed for 15 min under 1.5-2 kg/cm^2 pressure. Following this coagulum was immersed in chilled water for 1 h which was then packed in LDPE films and stored at 7 °C. Similarly, the control paneer was prepared from milk without EO addition. The strict hygiene was observed during paneer production. The microbiological analysis was conducted at regular intervals. Pour plate methods were performed for total bacteria with plate count agar, coliform with VRBA, yeast and mold with potato dextrose agar and *Staphylococcus* sp. with Baird Parker agar with egg yolk and potassium tellurite as supplement. Subsequently, CFU/g was calculated using dilution factors.

The chhana based dairy spread was manufactured from cow's milk by following the flow chart described in (Amitraj et al. 2016). The mixture after adding all the ingredients (chhana, maltodextrin, skimmed milk powder, whey powder, emulsifying salt, emulsifier, EMC, edible salt mixed with a known amount of water) the EO was added and mixed for 40-45 seconds using a domestic blender. The mixture after preheating to 70 °C was immediately homogenized without pressure and heat treated at 80 °C for 5 minutes. When the product was still hot packed in polystyrene cups with air-tight screw caps and stored at 5 °C. Batches without EO were prepared similarly. Total aerobic count, coliform, and yeast & mold counts were determined at different intervals by the plate count technique.

Sensory evaluation

The EO-added products were subjected to sensory evaluation by a panel of judges. The changes caused by the incorporation of EO were assessed through the difference-from-control where only product without EO addition was used as control. Each panelist compared the smell and taste, and the results were quantified on 9 points hedonic scale for paneer. For the spread, the organoleptic scheme consisted of flavor (45 points), body and texture (35 points), spreadability (10 points), and color (10 points) (Amitraj et al. 2016).

Antioxidant activity

The antioxidant activity was determined by ABTS (2, 2-azino-bis 3-ethyl benzothiazoline-6-sulphonic acid) assay as described by (Re et al. 1999). Before use, the absorbance of ABTS solution was adjusted to 0.7±0.02 at 734 nm (Specord-200, Analytik Jena, Germany). Three ml of ABTS radical solution was added with 30 µl sample and absorbance was read at 734 nm at 1 min interval for 6 min.

$$\text{Inhibition (\%)} = 1 - \frac{Absorbance\ (sample)}{Absorbance\ (control)} \times 100$$

The total antioxidant activity of experimental samples determined from the standard curve and expressed as Trolox equivalent antioxidant capacity (TEAC).

Statistical analysis

Experiments were performed in triplicates and the results were analyzed with SPSS software for analysis of variance (ANOVA) followed by Tukey's comparison test to determine the significant differences ($P < 0.05$) amongst the mean values.

Results and Discussion

The preliminary screening of EO for antibacterial activity was carried out against different spoilage and pathogenic microorganisms by disk diffusion method. Figure 1 shows the the diameter of inhibition zone including diameter of paper disk (6 mm diameter of the disc). By generating an inhibition zone, each EO components demonstrated inhibitory effects against the microorganisms under study. Cinnamaldehyde and eugenol showed the largest inhibition zone values, which ranged from 12 to 33 mm. The diameter of the zone that was generated by menthol, vanillin and *p*-cymene was less than 12 mm. However the disk diffusion used in study is not highly quantitative and inappropriate for hydrophobic compounds like EO which lack uniform diffusion in the media (Ait-Ouazzou et al. 2011).

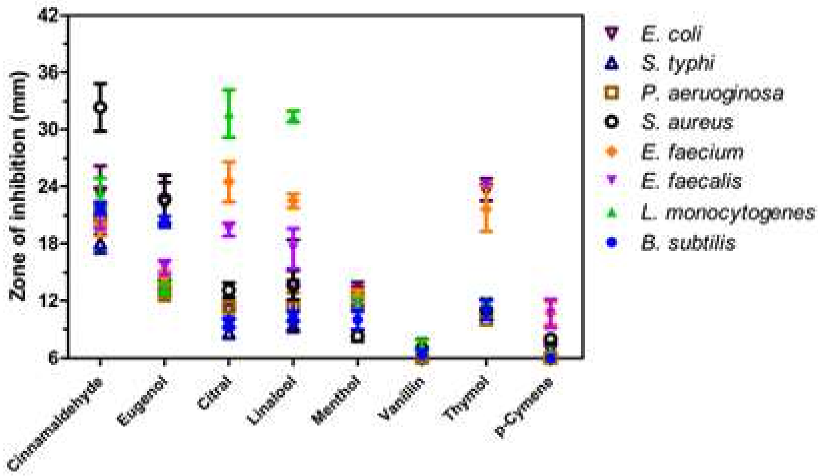

Fig 1: Antibacterial activity of EO components using paper disc diffusion method

Values are given as mean ± SD (n=3).

Table 1. MBCs of EO components in skim milk (mg/ml)

Essential oil	*B. cereus*	*B. subtilis*	*L. monocytogenes*	*E. faecalis*	*E. faecium*	*S. aureus*	*P. aeruginosa*	*S. typhi*	*E. coli*
Cinnamaldehyde	20	4	4	10	4	4	4	6	4
Eugenol	30	8	4	4	8	4	15	9	8
Thymol	15	10	4	8	2	20	25	10	4
Citral	25	20	20	15	10	4	40	20	20
Linalool	50	20	20	50	10	20	40	20	10
Vanillin	50	20	20	50	50	20	20	20	15
p-Cymene	50	5	50	50	40	50	40	50	40
Menthol	50	20	50	50	50	50	50	50	50

To obtain more precise data on the antimicrobial activity of EO component, MBC values were obtained in skim milk against each test bacterium and are shown in Table 1. Based on the MBC values cinnamaldehyde and eugenol, out of eight EO components, had the strongest antibacterial effect aganist *B. subtilis, L. monocytogenes, E. fecalis, E. fecium, S. aureus, P. aeruginosa, S. typhi* and *E. coli,* with MBC values ranging 4-10 mg/ml. Thymol and citral had moderate antibacterial activity as measured by MBC values ranging from 4-25 mg/ml. Vanillin, *p*-cymene, linalool and menthol, on the other hand had lower antibacterial activity owing to higher MBC values (20-50 mg/ml). These results are in agreement with other published reports (Friedman et al. 2002, Ananda Baskaran et al. 2009, Yuan et al. 2019) which emphasize the stronger activity of cinnamaldehyde and eugenol against a wide range of microorganisms including food borne pathogens and spoilage bacteria. Thus, further research was conducted on cinnamaldehyde and eugenol, which exhibited the strongest antibacterial activity in this study.

Table 2 depicts the MBC values that were observed in paneer and semi-skimmed milk at different storage temperatures. The concentrations of 4-12 mg/ml eugenol and 12 mg/ml cinnamaldehyde in skimmed milk were sufficient for complete bactericidal activity against *L. monocytogenes* and *E. coli*. The storage temperature conditions did not influence the MBC of these EOs. These finding in this regard confirm the observations of Cava et al. (2007) that storage temperature had no effect on the MBC of clove and cinnamon EOs in skim milk against *L. monocytogenes.* On the other hand MBC of eugenol for *E. coli* O157:H7 in paneer decreased to 80 mg/ml at 7 °C from 100 mg/ml at 37 °C. In a previous study the bactericidal activity of LC-EO against *L. monocytogenes* increased when the storage temperature of tofu was decreased from 37 °C to 7 °C (Liu and Yang, 2012). This suggests that various dairy products that require low temperatures could benefit from the use of EO in conjunction with refrigeration temperature.

The results of time kill assay using eugenol and cinnamaldehyde in sterilized milk is shown in Figure 2. At respective MBC concentrations of eugenol and cinnamaldehyde the target bacteria were completely inactivated at 1 hour and 3 hour incubation, respectively. Eugenol appeared faster in killing *L. monocytogenes* and *E. coli* than cinnamaldehyde. However the MBC values of eugenol and cinnamaldehyde are 5 times higher in paneer than skimmed milk indicating food matrix effect on the antibacterial efficacy. The higher MBC values of EO up to 10 times in food than in vitro are recurrent in several studies (Cava-Roda et al. 2012). Nevertheless addition of EO to food product at concentration equal to MBC might mainly negatively impact the sensory scores of the food product (Saraiva et al. 2021). Therefore this study also highlight the importance of evaluating efficacy in simultaneous studies in vitro and in real foods to arrive at the balance between sensory scores and microbiological quality.

In view of stronger activity of cinnamaldehyde and eugenol the effectiveness were evaluated in chhana based dairy spread. The table 3 shows the sensory evaluations of chhana based dairy spread with and without EO. With the addition of EO at 0.26 mg/g the scores of sensory were not affected significantly. However EO at a concentration of 0.52 mg/g caused the negative effect on the flavor and overall acceptability ($P<0.05$) of spread. Overall the cheese spread with EO at a concentration of 0.26 mg/g was acceptable concerning the sensory scores.

As shown in Figure 3 the growth of total plate count, mold and yeasts were inhibited as a result of addition of EO components. In control, the counts were found to increase with progress in the storage period. The addition of eugenol or cinnamaldehyde (0.26 mg/g) caused the total plate count, yeast and mold counts to decrease significantly ($P<0.05$). For instance on week 6 the count of total plate count, molds and yeast in control was 3.97 and 1.86 log CFU/g, respectively. The total plate count in cinnamaldehyde and eugenol added spread samples reached 2.39 and 2.61 log CFU/g, respectively. Eugenol incorporated spread samples showed yeast and mold count 1.3 log CFU/g and cinnamaldehyde was more effective to prevent the growth of yeast and molds with counts below 1 log CFU/g throughout the storage period ($P<0.05$). According to previous published reports the essential oils as antimicrobial agents suppress the growth of microorganisms including spoilage causing yeast and mold (Balaguer et al. 2013, Ju et al. 2020, Mitropoulou et al. 2022). Makhal et al. (2014) reported that the addition of thymol to cottage cheese at 40 ppm increased the shelf life by decreasing the psychrotrophs and yeast and mold counts. Likewise, Badola et al. (2018) observed that curry leaf EO (0.15 ml/kg) and Clove bud EO (0.25 ml/kg) were effective to keep the microbiological counts within the limits of FSSAI in Indian traditional confection, burfi. Overall the addition of cinnamaldehyde and eugenol can enhance the microbiological quality of chhana-based dairy spread and improve the shelf life.

When added to milk EO exhibited protective effect on paneer during storage against various microbial populations (Figure 4). According to earlier reports, the typical concentration for spices and herbs used in food system ranges from 0.05 to 0.1% (Licon et al. 2020). TBC of control samples increased from initial counts of 3.02 to 7.21 log CFU/g after 21 days, but in cinnamaldehyde and eugenol containing samples 3.3 and 6.49 log CFU/g, respectively ($P<0.05$). Similarly, yeast and mold counts compared to control there was a significant reduction in cinnamaldehyde and eugenol treated paneer samples ($P<0.05$). Coliform counts in paneer was inhibited by eugenol and remained below 1 log CFU/g in cinnamaldehyde containing samples. The final increments of *Staphylococcus* sp counts in cinnamaldehyde treated samples was 1.69 log CFU/g was significantly less than eugenol and control samples 4.5 log CFU/g. There are reports in the literature on application EO as an ingredient to milk and to control microbial counts in the product such as cheese (Zantar et al. 2014, Hachana

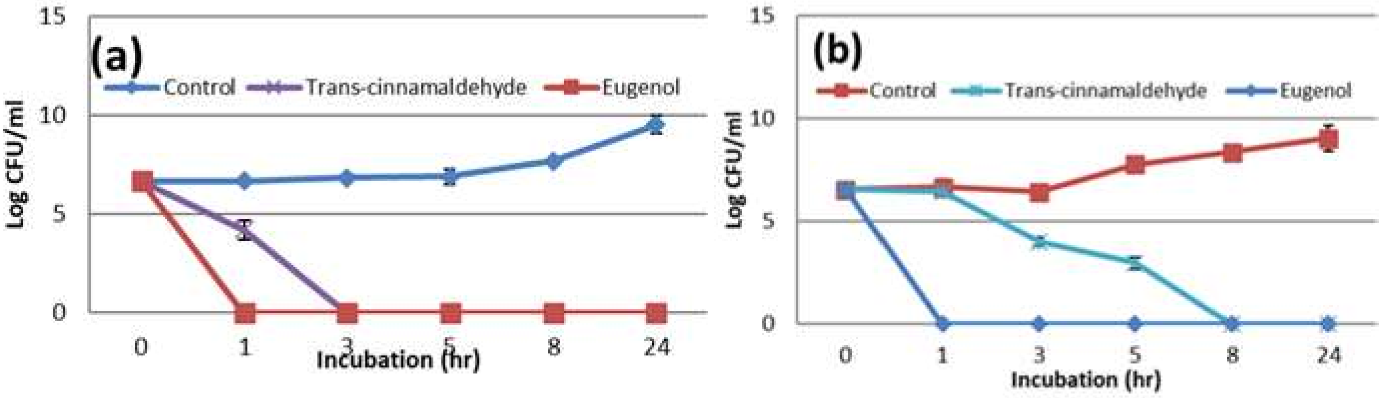

Fig 2: Time kill curves obtained after treating EO at MBC concentrations in skim milk(a) E. coli O157:H7, b) L. monocytogenes)

Table 2: Minimum bactericidal concentrations (mg/ml) in milk and paneer incubated at 7 and 37 °C

EO	Test organism	7 °C for 14 days		37 °C for 24 h	
		Milk	Paneer	Milk	Paneer
Cinnamaldehyde	*L. monocytogenes*	12	12	12	20
	E. coli O157:H7	12	80	12	100
Eugenol	*L. monocytogenes*	12	12	12	20
	E. coli O157:H7	4	80	4	100

Table 3: Sensory scores of chhana-based dairy spread incorporated with EO components

Parameter	Control	Eugenol (mg/g)		Cinnamaldehyde (mg/g)	
		0.26	0.52	0.26	0.52
Flavor	42.00±0.71[a]	40.00±0.71[a]	32.25±1.64[b]	39.25±2.48[a]	35.50±0.50[b]
Body and texture	33.0±0.31[a]	32.5±0.41[a]	32.0±0.50[a]	32.0±0.25[a]	32.2±0.12[a]
Colour and appearance	8.2±0.24[a]	8.1±0.03[a]	8.2±0.02[a]	8.3±0.13[a]	8.1±0.21[a]
Spreadability	7.5±0.50[a]	7.5±0.13[a]	7.4±0.30[a]	7.4±0.20[a]	7.4±0.15[a]
Overall acceptability	90.7±1.76[a]	88.1±1.28[a]	79.85±2.46[b]	89.95±3.06[a]	83.2±0.98[b]

Superscript in the treatment column indicates significant difference with control ($P<0.05$)
Values are given as mean ± SD (n=3)

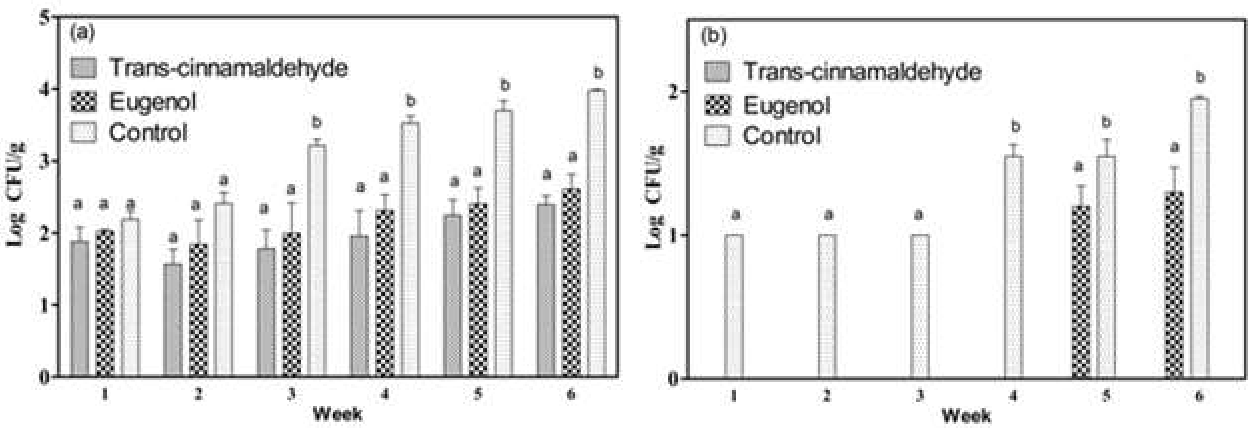

Fig 3: Effect of EO addition (0.26 mg/g) on the microflora of chhana-based dairy spread during storage at 7 °C (a) total plate count (b) yeast and mold count

et al. 2019, Ahmed et al. 2021). Thyme, basil and *M. officinalis* EOs transferrence upto 53% from milk to cheese and up to 3.3 log reduction in microbial counts in cheese has been earlier reported (Licon et al. 2020). The presence of spicy flavor of EO decreased the sensory scores of paneer prepared from milk with EO (10 mg/ml), which were slightly liked on a 9-point hedonic scale. Nevertheless, paneer usually is eaten with condiments or in other dishes and some changes to the EO flavor notes and quantification

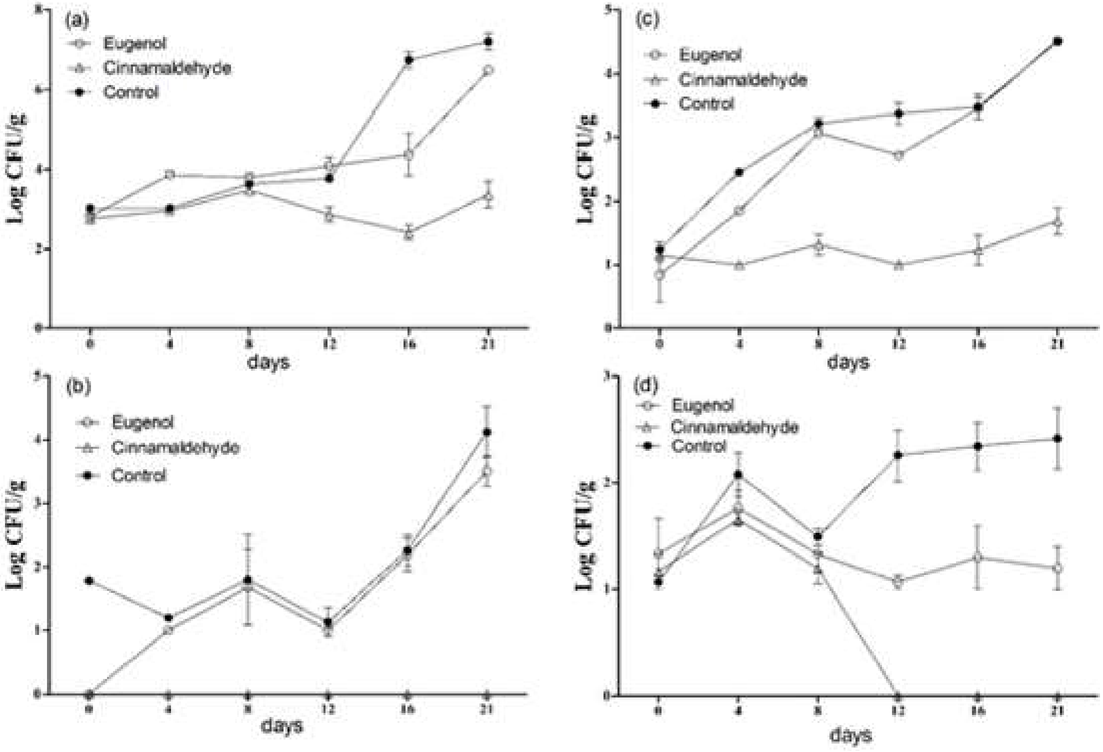

Fig 4: Effect of EO addition on the microflora of paneer made from milk added with EO (1%) during storage at 7 °C. (a) Total plate count (b) coliform count (c) *Staphylococcus* sp. (d) yeast and mold count

of transferrence make EO usable (Mitropoulou et al. 2022). To summarize the addition of EO to milk followed by paneer making showed transference to paneer and can exhibit preservative effect during storage and a concentration below 10 mg/ml is suggested for paneer.

The oxygenated terpenes and phenylpropanoids are believed to exhibit strong antibacterial activity than hydrocarbons. According to the findings of this study, eugenol and cinnamaldehyde belongs to terpenes and phenylpropanoids had consistently strong activity than hydrocarbons (Cava-Roda et al. 2012). The difference in the antibacterial activity of EO components could be attributed to the distinction in their chemical structure (Cho et al. 2020). The functional groups interact with the plasma membrane and play important role in disruption of cytoplasmic membrane as well as coagulation of cell contents of target microorganisms (Lv et al. 2011, Das et al. 2021). However, the mechanism of antibacterial action of pure compounds of EO is not well established in the literature (Licon et al. 2020).

The Figure 5 shows the antioxidant ability of EO components expressed as Trolox equivalent antioxidant capacity. Only milk had low lowest antioxidant potential which was significantly increased in the presence of EO components. In milk added with eugenol at 0.1 to 1 mg/ml the TEAC increased from 1229.93 to 1438.24 µM respectively, indicate antioxidant activity in a dose dependent manner ($P<0.05$). The only milk antioxidant activity 1033.5 µM TE is reported to be improved by the addition of various plant biomolecules depending on chemical characteristics (Alenisan et al. 2017). The TEAC values resulted by eugenol supplementation are comparatively higher than cinnamaldehyde. Eugenol has been reported to possess considerable radical scavenging, reducing ability and antileishmanial activities than cinnamaldehyde (Sharma et al. 2017). The high antioxidant activities of eugenol is a result of free hydroxyl group on aromatic ring which is absent in cinnamaldehyde.

Conclusions

Amongst different components of EOs investigated in the present study cinnamaldehyde and eugenol exhibited the greatest antibacterial activity. Cinnamaldehyde and eugenol have good potential for antimicrobial application to control microbial counts in dairy products such as chhana based dairy spread and paneer. Eugenol possess better antioxidant activity as compared to cinnamaldehyde. Future studies should investigate different dairy products as strong flavour may make it challenging to use in dairy products. Further the effect of addition of EO on the physico-chemical, textural and sensory properties of dairy products needs a deeper study.

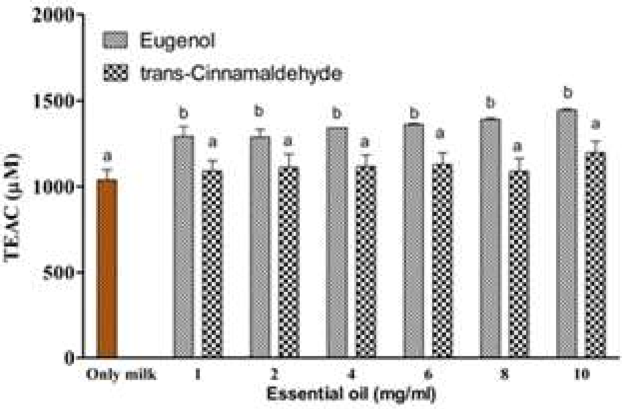

Fig 5: Antioxidant activity of milk with or without EO addition

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