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Evaluation of Antibacterial and Antibiofilm Activity of Essential Oils Against Isolated Bacteria from Milk Samples

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The consumption of food contaminated with bacteria or their toxins can result in foodborne infections or illnesses. This study investigated the antibacterial and antibiofilm activity of essential oils against bacteria isolated from milk samples collected in Pune, Maharashtra, India. Twenty-five raw milk samples were collected from local vendors and examined for the presence of biofilm-forming microorganisms. The isolated bacteria were identified and characterized using morphological and biochemical tests, revealing that 20% of the samples were contaminated with Acinetobacter spp. and 28% with E. coli. The antibacterial activity of the essential oils was evaluated using the disc diffusion method, and the minimum inhibitory concentration (MIC) was determined using 96-well plates. The minimum bactericidal concentration (MBC) was also assessed by inoculating assay mixtures from wells exhibiting no microbial growth onto sterile nutrient agar medium. Biofilm formation and disruption were evaluated using crystal violet assay and biofilm disruption assay, respectively. The results demonstrated that the Cinnamon Bark and Oregano essential oils exhibited significant antibacterial and antibiofilm activity at the lowest MIC value of 0.02-0.04 μg/ml against the isolated bacteria. The findings suggest that essential oils could be potential natural alternatives to conventional antibiotics for controlling bacterial contamination and biofilm formation in milk and dairy products. Further research is needed to explore the practical applications of essential oils in the dairy industry and to ensure their safety and efficacy as natural antimicrobial agents.

Keywords: Acinetobacter spp.; Antibacterial; Biofilm; E. coli; Minimum inhibitory concentration.

Although milk is a key component of human nutrition, it may also act as a haven for several foodborne diseases[1]. These bacteria pose a serious risk to public health because they can contaminate milk at various points during production, processing, and storage. Foodborne infections are a major global health hazard that arise from eating food infected with microorganisms or their toxins[2]. Every year, millions of people worldwide contract these diseases, highlighting the urgent need for improved food safety protocols. As a result, the food sector has prioritized maintaining food safety a top priority[3].

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Essential oils (EOs) are a class of secondary metabolites that are extracted from aromatic and therapeutic plants. These complex and volatile chemicals are widely used in many industries, such as fragrances, cosmetics, and even food applications[4]. However, the potential of EOs extends beyond their well-known applications. Their varied chemical makeup, which is affected by extraction methods, plant genetics, and geographic origin, provides novel possibilities for their use in food safety. Foodborne diseases and a wide range of other bacteria can be efficiently inhibited from growing by EOs, as demonstrated by numerous studies[5].

The purpose of this study was to examine EOs' potential of EOs as a natural substitute for bacterial contamination control in milk. We investigated their efficacy against bacteria isolated from raw milk samples obtained from local vendors in Pune, Maharashtra, India, with a focus on their antimicrobial and antibiofilm qualities[6]. The objective of this study was to enhance food safety and reduce the hazards associated with bacterial contamination of milk by assessing the antibacterial and antibiofilm properties of essential oils against milk-borne bacteria[7]. Antibiotics were once used to treat bacterial contamination in milk. Antibiotic-resistant bacteria have emerged because of the overuse of antibiotics, raising concerns regarding their efficacy and possible health problems related to antibiotic residues in milk[8].

The transmission of microorganisms resistant to drugs (MDR) is a serious hazard to both human and animal health. The increasing occurrence of multidrug-resistant bacteria in the food chain, especially in the dairy industry, indicates the need for alternative antimicrobial medications. Because of their antibacterial properties, essential oils (EOs) have attracted considerable attention[9]. This study investigates the antibacterial and antibiofilm activity of ten essential oils (EOs): Cajeput (EO1), Aniseed (EO2), Cedarwood (EO3), Eucalyptus (EO4), Tea tree (EO5), Cinnamon Bark (EO6), Bergamot (EO7), Citriadore (EO8), Palmorosa (EO9), and Oregano (EO10) against isolated bacteria Escherichia coli and Acinetobacter spp. obtained from milk samples. The results of this study could help in the development of novel strategies to prevent bacterial contamination and growth of biofilms in milk, thereby protecting the quality and safety of food.

MATERIALS AND METHODS

Milk Sampling

We collected 25 raw milk samples were collected from local vendors (Pune, Maharashtra, India). The samples were collected in sterile snap cap milk collection vials, placed in ice-cooled containers, and processed within 24 h of collection.

Isolation, Identification and Characterization of Bacteria from Milk sample[10]

Twenty-five distinct milk samples were collected from local vendors in Pune. The milk samples were then serially diluted. Nutrient agar plates from tubes 10^{-6}, $10^{"7}$, and 10^{-8} dilution were spread out and then incubated for 24 hours at 37º C. The suspected colonies were subsequently subcultured on MacConkey agar and purified for 24–48 h at 37 °C. Suspected isolates underwent staining, morphological characterization, and various biochemical tests, including glucose fermentation and assays for catalase, oxidase, citrate, and nitrate reduction.

Antimicrobial Activities of essential oils against Isolated bacteria[11]

The antibacterial activity of the essential oils was evaluated against isolated bacteria using the Muller Hinton Agar (MHA) medium and disc diffusion method. Sterile Muller Hinton agar plates were equally covered with 0.1 ml of bacterial culture. Each MH agar plate contained 20 µL of essential oil placed at the center. Ampicillin antibiotic was used to prepare the Positive Control. A disc-containing solvent was used as the negative control, and it was placed directly on a Muller Hinton agar plate containing the test organisms. The plates were incubated for twenty-four hours at 37 °C. The antimicrobial inhibitory zones formed around the discs were measured in millimeters (mm). Each test was repeated thrice. Antibiotic discs were used as positive controls, while disc without oil and disc with DMSO solvent (dimethyl sulfoxide) were used as negative controls.

Minimum inhibitory concentration[13]

Using 96-well plates, the Minimum Inhibitory Concentration (MIC) was determined. In an additional assay, which consisted of 100 µL of

freshly prepared MHB, 20 µL of diluted bacterial culture (containing 10^8 CFU/mL of bacteria) and 80 µL of EO concentrations ranging from 25 to 0.02 µg/mL were applied to an mtp plate. Direct two-fold dilutions of each essential oil were prepared by using an organosulfur solvent DMSO (Dimethylsulfoxide). For 24 hours, the plate was incubated at 37ºC. We observed the plates. Using a Citation 5 reader, the OD was measured at 630 nm and compared with that of the control wells. For each oil, the MIC was determined at the lowest dilution that did not exhibit visible growth. The growth control (negative control) consisted of growing the microorganisms in 100 µL MHB culture medium with 100 µL DMSO 2.5% Tween 20 was put into 12th well. MIC was defined as the lowest concentration of EO at which no visible growth (no white pellet) of the pathogen was observed compared with the control. Gentamicin, the positive control, consisted of growing the microorganisms in 100 µL MHB culture medium with 100 µL antibiotic concentrations ranging same as EO.

Minimum Bactericidal concentration[14]

The minimum bactericidal concentration (MBC) was determined by inoculating the assay mixtures from wells that exhibited no microbial growth on the surface of sterile nutrient agar medium. After incubation for 24 hours at 37º C. If there was microbial growth on the medium, it meant that the essential oil had bacteriostatic activity; if there was no growth, it meant that the essential oil sample had bactericidal activity.

Crystal violet assay[15]

A culture of organisms that developed overnight was inoculated into fresh MHB. Each sample was pleased with the culture, which was then incubated for 24 hours at 37ºC. Following incubation, cells were washed with PBS and distilled water. The wells were filled with sterile MHB and essential oils and cultured for 24 hours at 37º C. Following incubation, the contents were washed with distilled water and allowed to dry at room temperature. To stain the biofilms, 0.1% crystal violet was added to each well and incubated for 15 min. The CV solution was discarded and the cells were rinsed three times with D/W and PBS. Ethanol (200 µL) was used to solubilize air-dried biofilms. After visual examination, the plates were

Table 1. Morphological Characteristics of Isolated Organisms A and B

Morphological characteristics	Organism A	Organism B
Size	2-3 mm	1-2 mm
Shape	Circular	Oval
Colour	Greyish White	Creamy white
Opacity	Opaque	Opaque
Surface	Rough	Smooth
Margin	Entire	Entire
Elevation	Convex	Convex
Gram character	Gram negative(rods)	Gram negative (coccobacillus)
Motility	Motile	Non motile

Table 2. Biochemical tests for Isolated Organisms A and B

Biochemical Test	Organism A	Organism B
Sugar tests		
Maltose	+	-
Glucose	+	-
Mannitol	+	-
Sucrose	+	-
Lactose	+	-
Xylose	+	-
IMViC test:		
Indole	+	-
Methyl red	+	-
Voges Proskauer	-	+
Citrate utilization	-	+
Oxidase	-	+
Catalase	+	+
H_2S	-	-
Gelatin hydrolysis	-	-
Nitrate Reduction	+	-
OF (Oxidative-Fermentative	+	_

+ (Positive), -(Negative)

compared with controls. OD was measured using a Citation 5 reader at a wavelength of 630 nm.

Biofilm Disruption Assay[16]

In the same way as the inhibition assay, plates were prepared, but test samples were not added, and they were incubated for 72 hours to find out how essential oils affected the biofilms that had developed throughout that time. Test samples (100 ìg/ml) were added to the wells after the cells were cleaned. After another 24 h of incubation, the biofilms in 24-well plates were measured.

Statistical analysis

ll values were expressed as the mean ± standard error of the mean. $p < 0.05$ was considered statistically significant.

RESULT AND DISCUSSION

Isolation, Identification and Characterization of Biofilm forming Microorganisms from Milk sample[17]

In a study conducted in Pune, Maharashtra, India, 25 milk samples collected from local vendors were examined for the presence of biofilm-forming microorganisms. Recorded results 20% of the examined street vendors' raw milk samples were contaminated with *Acinetobacter spp.* and 28% with *E. coli*. These microorganisms were identified and characterized using a combination of morphological and biochemical tests (Table 1,2).

Characterization of micro-organism was done by morphologically and various biochemical tests. This test gave us confirmatory results for identification of Microorganism. Thus given biochemical test followed by referring Bergey's manual[18] gave confirmation of species as *E. coli* and *Acinetobacter spp.* (Tables1 and 2).

Antibacterial activity

The results obtained against the test organisms indicated that the EOs had varying degrees of antibacterial activity, as shown in Figures 1 and Graph 1. The results of this study indicate that certain EOs may have antibacterial properties against *Acinetobacter spp*. and *E. coli* isolates from milk samples. The most encouraging results were observed with cinnamon bark (EO6)

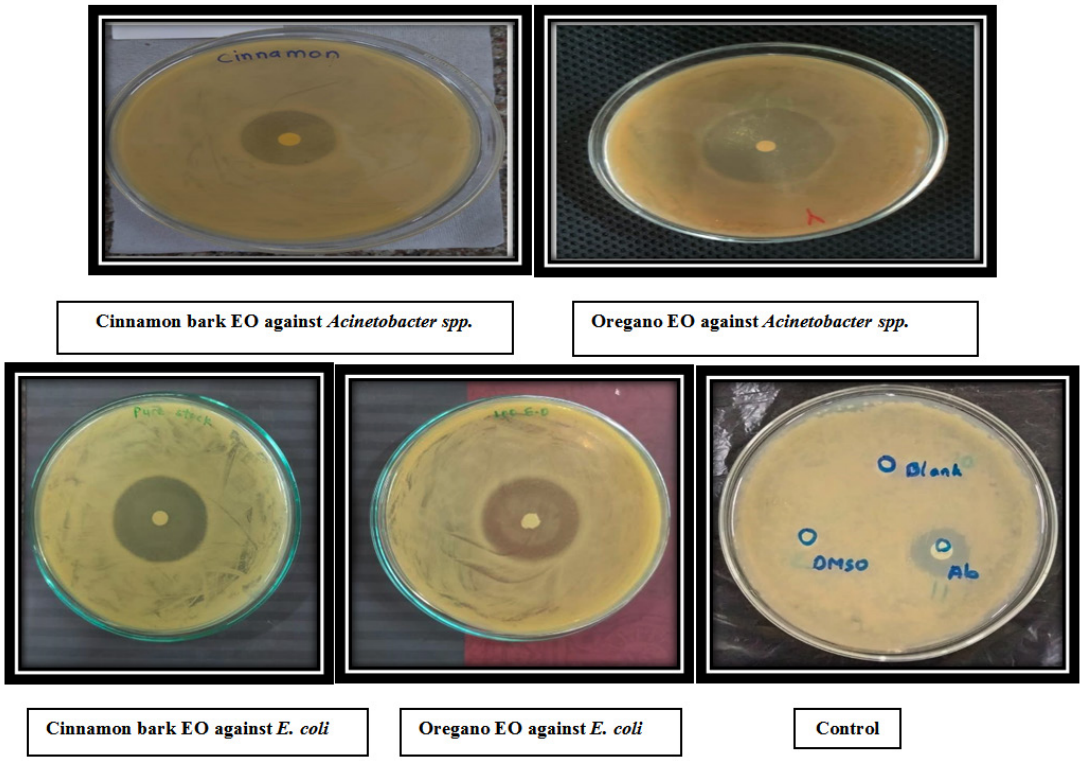


Fig. 1. Antibacterial Activity of Essential Oils against *E. coli* and *Acinetobacter spp.* along with positive (Ampicillin Antibiotic) and negative control DMSO (Dimethylsulfoxide)

and oregano (EO10), where the zones of inhibition for both *E. coli* and *Acinetobacter spp*. ranged from 22 mm to 27 mm.

The other essential oils, including Cajeput (EO1), Aniseed (EO2), Cedarwood (EO3), Eucalyptus (EO4), Tea tree (EO5), Cinnamon Bark (EO6), Bergamot (EO7), Citriadore (EO8), palmorosa (EO9), and oregano (EO10), exhibited little to no antibacterial activity against the test organisms in this study. Each well received the designated volume of oils, along with the placement of the gentamicin disc 5 ìg/disk, which acted as the positive control, negative control of dimethyl sulfoxide (DMSO), and blank discs. This was performed in triplicate, and the average diameter for each EO-bacteria combination was noted (Figure 1).

Minimum inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC)

The lowest concentration of essential oil that visibly prevents the bacterium from growing is known as the minimum inhibitory concentration (MIC). The results reported above revealed the potential of some essential oils, such as cinnamon bark and oregano, as natural preservatives to control food pathogenic and spoilage bacteria. The results showed variable effects of the essential oils and their components on the tested bacterial strains (Table 3). The oils of cinnamon bark and oregano showed strong antimicrobial activities in inhibiting the growth of pathogenic and spoilage bacteria at MICs 0.02 and 0.04 ìg/mL concentration. The essential oils of cinnamon bark and oregano showed bactericidal effects at concentrations 0.02 and 0.04 ìg/mL. The positive control, gentamicin, has a MIC of 4 and 2 g/ml for *E. coli* and *Acinetobacter spp*. Cinnamon and oregano essential oils showed significant bactericidal effects at their respective MICs, effectively preventing the growth of pathogenic bacteria [19,20]. The combination of these oils with other agents, such as oxytetracycline, further reduced MIC values, enhancing their antimicrobial action against resistant strains [21].

A mixture of the culture medium and bacterial suspension was used as the growth control. After 24 h of incubation, to stain the bacteria, 1% 2,3,5-triphenyl tetrazolium chloride aqueous solution was added to each well (20 µL per well). The plates were incubated at 37 °C for 30 min, and the MIC values were visually determined as the minimal concentrations that did not produce a red color. Samples (100 ìL) from the MIC experiment wells with no color change were placed on MH agar plates and incubated for 18–24 h at 37 °C. The minimum bactericidal concentration (MBC) was defined as the lowest concentration at which no bacterial growth was observed.

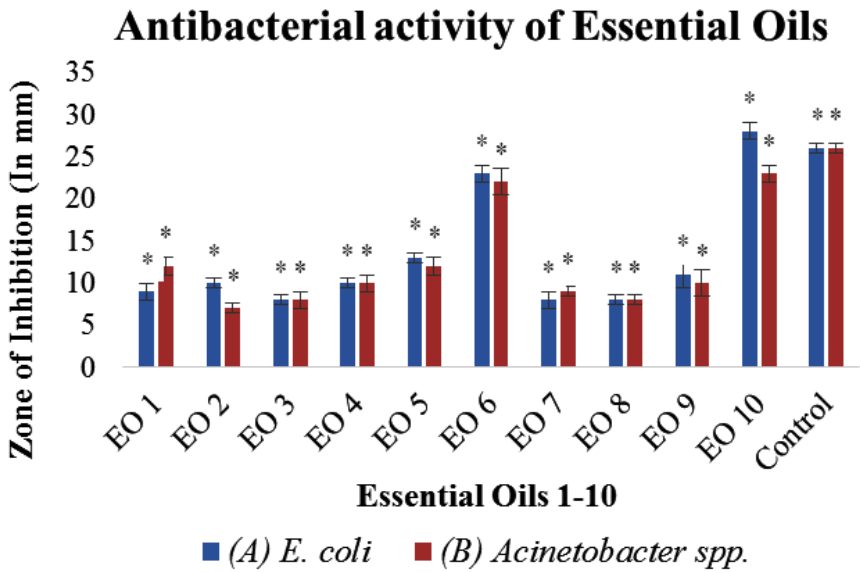
Crystal violet assay

The amount of biofilm produced by the bacteria was quantified by the crystal violet assay. Six of the ten essential oils—Cajeput,

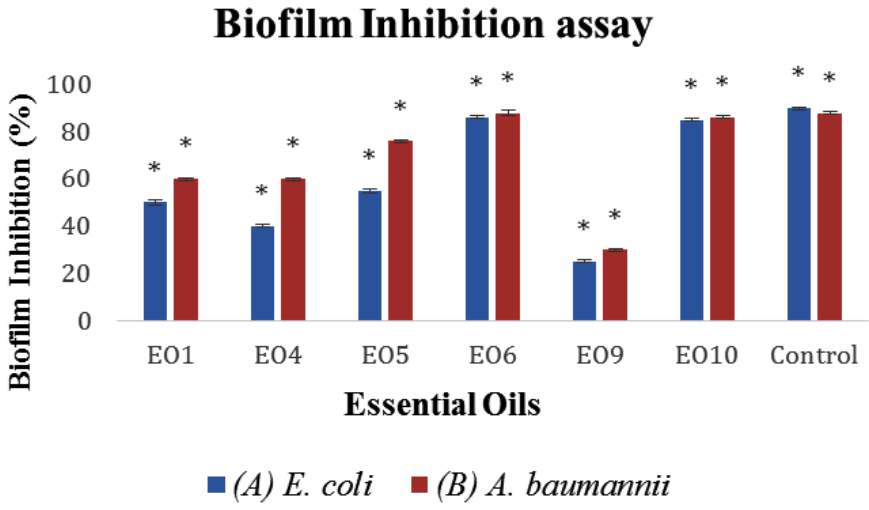
Table 3. Minimum Inhibitory Concentration (MIC) and Sub MIC of 10 Essential Oils: Cajeput (EO1), Aniseed (EO2), Cedarwood (EO3), Eucalyptus (EO4), Tea tree (EO5), Cinnamon Bark (EO6), Bergamot (EO7), Citriadore (EO8), Palmorosa (EO9), Oregano (EO10) against *E. coli* and *Acinetobacter spp.*

Sr. No.	EOs	*E. coli*		*Acinetobacter spp.*	
		MIC (µg/mL)	½ MIC (µg/mL)	MIC(µg/mL)	½ MIC(µg/mL)
1.	EO 1	6.25	3.12	3.12	1.56
2.	EO 2	6.25	3.12	6.25	3.12
3.	EO 3	6.25	3.12	12.5	6.25
4.	EO 4	6.25	3.12	12.5	6.25
5.	EO 5	0.78	0.39	0.78	0.39
6.	EO 6	0.02	0.01	0.04	0.02
7.	EO 7	6.25	3.12	25	12.5
8.	EO 8	6.25	3.12	12.5	6.25
9.	EO 9	6.25	3.12	12.5	6.25
10.	EO 10	0.04	0.02	0.04	0.02
11.	Control	4.00	2.00	2.00	1.00

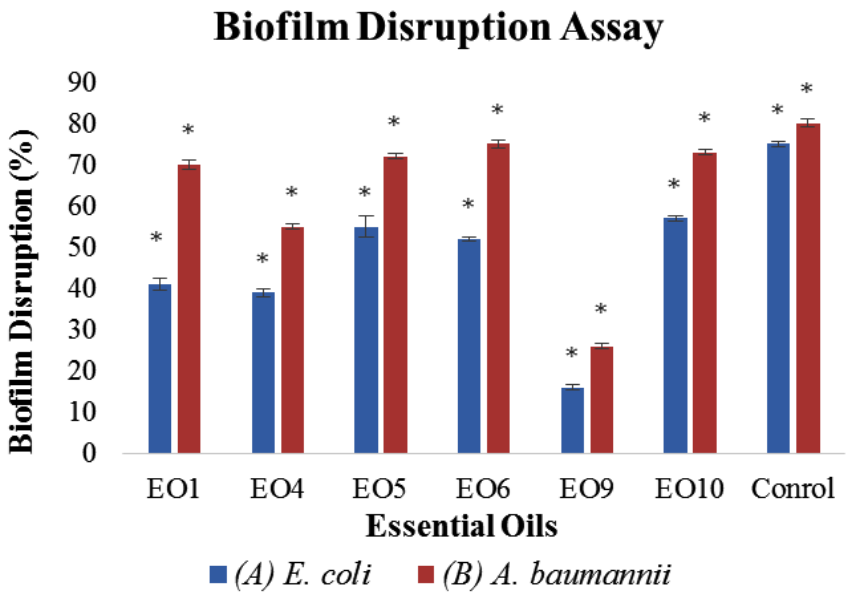
Eucalyptus, Tea Tree, Cinnamon Bark, Palmorosa, and Oregano—showed promising results from the disc disffusion assay and MIC. These oils were examined for antibiofilm efficacy using crystal violet and disruption assays. The essential oils of oregano and cinnamon bark exhibited a maximal inhibitory efficacy of greater than 85%. Gentamicin, the positive control, 4 and 2 µg/ml, had a maximal biofilm inhibitory efficacy of 90% and 88% at its MIC for *E. coli* and *Acinetobacter*



Graph 1. Antibacterial Activity of Essential Oils Cajeput (EO1), Aniseed (EO2), Cedarwood (EO3), Eucalyptus ((EO4), Tea tree (EO5), Cinnamon Bark (EO6), Bergamot (EO7), Citriadore (EO8), Palmorosa (EO9), Oregano (EO10) against *E. coli* and *Acinetobacter spp.* Error bars and asterisks indicate the SD and statistical significance ($p<0.05$), respectively



Graph 2. Inhibition % of biofilm formation of Bacteria: (A) *E. coli* (B) *A. baumannii* by Essential Oil: Cajeput (EO1), Eucalyptus (EO4), Tea tree (EO5), Cinnamon Bark (EO6), Palmorosa (EO9) and Oregano (EO 10). Error bars and asterisks indicate the SD and statistical significance ($p<0.05$), respectively



Graph 3. Biofilm Disruption Assay (%) of Essential Oil: Cajeput (EO1), Eucalyptus (EO4), Tea tree (EO5), Cinnamon Bark (EO6), Palmorosa (EO9) and Oregano (EO 10) against *E. coli and Acinetobacter spp.* Error bars and asterisks indicate the SD and statistical significance ($p<0.05$), respectively

spp. For each treatment, an overall dose-dependent suppression of biofilm formation was observed. Graph 2 shows the positive control for each type of bacteria. Untreated bacterial cultures served as the positive control. Cinnamon Bark and Oregano both oils showed maximal inhibitory efficacy exceeding 85% against biofilm formation, indicating their strong potential as natural antibiofilm agents[22]. Cajeput and Eucalyptus oils also demonstrated significant biofilm inhibition, supporting their use in combating bacterial infections[23].

Biofilm Disruption Assay

The essential oils of oregano, cinnamon bark and tea tree exhibited a maximal inhibitory efficacy of greater than 50% for *E. coli*. Essential oils of oregano, cinnamon bark, tea tree and cajepot exhibited a maximal inhibitory efficacy of greater than 70% for *A. baumannii*. Gentamicin, the positive control, 4 and 2 µg/ml, had a maximal biofilm inhibitory efficacy of 75% and 80% at its MIC for *E. coli* and *Acinetobacter spp*. For each treatment, there was an overall dose-dependent connection in the suppression of biofilm. The positive controls for each type of bacteria are shown in Graph 3. The positive control is an untreated bacterial culture. Oregano, cinnamon bark, and tea tree oils exhibited over 50% inhibition of *E. coli* biofilm formation[24]. For *A. baumannii*, oregano, cinnamon bark, tea tree, and cajepot oils achieved over 70% inhibition[24]. The essential oils' effectiveness is attributed to their ability to disrupt biofilm formation and enhance antibiotic efficacy[24,25].

CONCLUSION

In this study, 10 essential oils (EOs) Oils Cajeput, Aniseed, Cedarwood, Eucalyptus, Tea tree, Cinnamon Bark, Bergamot, Citriadore, Palmorosa, and Oregano) were tested against isolates of *Acinetobacter spp*. and *E. coli* from milk samples to determine their antibacterial and antibiofilm properties. Consequently, the findings of this study suggest that the essential oils of Cinnamon Bark and Oregano exhibit antimicrobial and antibiofilm properties. Commercial essential oils from cinnamon and oregano have promising antimicrobial and antibiofilm effects against selected food-borne and food spoilage bacteria, which can be attributed to the presence of the principle bioactive constituents. These investigated essential oils and their main active components

could be potential candidates for use as natural alternatives for further application in food preservation to retard or inhibit bacterial growth, for safety, and to extend the shelf life of food products. However, the antimicrobial efficiency and organoleptic impact of these essential oils on foodstuffs need to be evaluated.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Author Contributions

Sanchita Choubey : the major contributor for designed the study and supervised the experiment; Sanchita Choubey, Varada Jamekar and Shreya Chaudhari : carried out the experimental work and manuscript writing; Jyoti Deshpande: reviewed the writing; All authors read and approved the final manuscript.

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