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Research Article

OCIMUM BASILICUM L. ESSENTIAL OIL COATED BIOMATERIAL SURFACES PREVENT BACTERIAL ADHESION AND BIOFILM GROWTH

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ABSTRACT

Objective: Biomaterials associated infection is the most common issue associated with the biomaterial implants regardless of its form or function. Bacteria form colonies and this result in the formation of biofilm on the surface, making the infection unreceptive to antibiotics and host defense mechanisms. The implant is removed as an outcome. Medicinal plants have widespread usage for their active biomolecules, and the study of their antimicrobial activities has gained widespread importance.

Methods: In this study, the essential oil of *Ocimum basilicum* L. (OB) is coated on biomaterial surfaces to study their efficacy in preventing bacterial colonization and biofilm formation. The essential oil is coated on polymethylmethacrylate and polystyrene substratum surfaces. Gram-positive bacteria, including *Staphylococcus aureus* and *Staphylococcus epidermidis,* and Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa,* are allowed to adhere and grow for 1 hr, 3 hrs, and 24 hrs.

Results: The number of bacteria adhering to the coated surfaces is significantly less (**p<0.01) compared to uncoated surfaces, at the measured instances of time. The zone of inhibition of the essential oil is observed for both Gram-positive and Gram-negative bacteria. Maximum inhibition was observed for *S. aureus* (30±1.2 mm diameter) compared to *S. epidermidis* (28±0.8 mm diameter), *E. coli* (25±1.1 mm diameter), and *P. aeruginosa* (21±0.6 mm diameter).

Conclusion: The study reveals potent bacteriostatic effects of OB essential oil on both Gram-positive and Gram-negative bacteria. Thus, OB. essential oil serves to be a promising coating on the implant surfaces for preventing bacterial adhesion and biofilm growth.

Keywords: Biomaterials, Bacterial adhesion, Biofilm, *Ocimum bacilicum* L., Antibacterial coating.

INTRODUCTION

Biomaterials are either natural or artificial in nature that is biocompatible and implanted into the human body to repair or substitute the failing tissue or organ. Biomaterials are integrated into implants and the biological response to these biomaterial implants is extremely complex in nature. For instance, a recent estimate of total hip replacements across the globe counts to a million per year more than 2,50,000 knee replacements are done every year and about 10% of the patients hospitalized across the globe are using biomaterial implants and other medical devices. Diverse natural and synthetic materials are used in the fabrication of biomaterials, and the understanding of the characteristics of these biomaterials becomes extremely important. Biomaterial-associated infection (BAI) is the most common issue associated with any biomaterial implant regardless of its form or function. Bacteria forms colonies and results in biofilm formation on the surface of the biomaterials, and this makes the infection unreceptive to antibiotics and host defense mechanisms. The design and composition of the biomaterial also play a vital role in influencing these infections. BAI sources many complications that restrict the use of biomaterial implants and other medical devices [1,2]. The occurrence of BAI strongly relies on the site of the implant and more frequently in instances of revision surgery and trauma [3-5]. The process that begins with the adhesion of individual bacteria and progresses toward the maturation of a biofilm (Fig. 1).

The host compromises to cope with the microorganisms owing to the presence of a foreign biomaterial in the body. There are diverse methods by which the microorganisms can invade the host and elicit BAI with permanent implants. In 1987, Anthony Gristina explained the concept of "race for the surface," describing the possible interactions of the microorganisms and the host cells with the substratum surfaces [6]. The biomaterial surface is covered by host tissue cells and is made less susceptible to the formation of bacterial colonies when the tissue cells win the race. In contrast, bacterial colonization and biofilm formation occur when the bacteria win the race and the bacterial virulence factors hamper the normal functions of tissue cells. Thus, the "race for the surface" decides the fate of the implants. Bacteria cannot trigger the biofilm-related phenotype ahead of attaching firmly to the substratum surface (Fig. 1a). Once they firmly attach and the phenotype is changed, an extracellular polymeric matrix is formed that protects the microorganisms from host immune cells and antibiotics. The biomaterial surface is covered by the host tissue cells and is made less susceptible to the formation of bacterial colonies when the tissue cells win the race (Fig. 1b). A window of chance exists from the period of concrete attachment and phenotypic variations when the anti-biofilm methodologies are adopted (Fig. 1c). Perioperative contamination is the best-documented route that allows the direct contamination of the implanted biomaterial during the process of surgery.

Post-operative contamination occurs when the microbes spread through blood circulation from the infections sourced elsewhere in the host [7,8]. The bacterial counts are significantly higher during the periods of activity, in the presence of more personnel in the operation theater [9]. Perioperative bacterial contamination is reduced with the use of sophisticated, ventilated operation theaters, allowing 20 changes of air/hrs [10]. Microorganisms can reach the biomaterial surface as early as during implantation and interact with the bare substratum surfaces. Microorganisms adhere to the substratum surface because of the interactions between cell surface structures and specific molecular groups on the substratum surface. Most proteins in conditioning films are capable of reducing microbial adhesion, but fibronectin and fibrinogen are shown to promote the adhesion of certain *Staphylococcus*

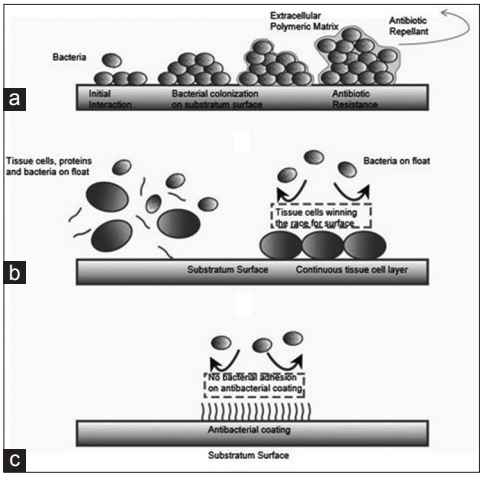

Fig. 1: (a-c) Race for the surface

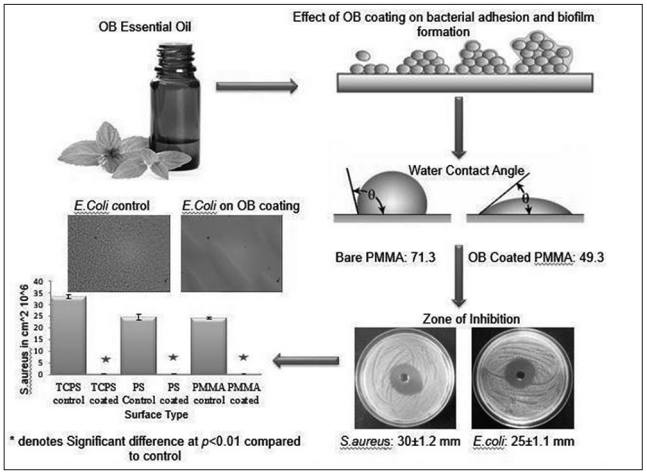

Fig. 2: Schematic diagram of the experimental methodology showing *Ocimum basilicum* L. extract as a coating to implant surfaces to prevent bacterial adhesion and biofilm growth

epidermidis and *Staphylococcus aureus* strains [11]. Microbial adhesion mechanisms in post-operative infections or hematogenous infections are unclear. Biomaterial implants containing metallic parts are not colonized easily by the host cells and thus are generally not totally integrated with host tissue. Microbes form colonies on the uncovered metal surfaces. After adhering to the substratum surface, the microorganisms subsequently grow, producing an extracellular polymeric substance that results in the formation of biofilms. This prevents the antibiotics and host immune cells to breakthrough and kills the microorganisms [12,13].

Biofilm eradication is extremely complex since the bacteria encased within the biofilms normally call for 500-5000 times higher doses of antibiotics than the microorganism present in the body fluids [14]. The implant is finally removed as an outcome of BAI. Usually, *S. epidermidis* and *S. aureus* are the most commonly isolated microorganisms from the infected biomaterial surfaces. *Escherichia coli* and *Pseudomonas aeruginosa* are the other organisms isolated from the implant surfaces [15]. *S. epidermidis* sources about 50% of BAI, especially in catheters and joint replacements. *S. aureus* sources 23% of infections in prosthetic joint replacements [16]. *P. aeruginosa* is the source for about 12% of the urinary tract infections, 10% of bloodstream infections, and 7% of hip-joint infections, acquired during hospitalization [17]. *S. epidermidis* is the widespread cause of post-hospitalization infections. Bacterial virulence also plays a significant role in BAI pathogenesis. *S. aureus* and *P. aeruginosa* infections frequently progress a lot forcefully than the infections sourced by *S. epidermidis*. *S. aureus* appears more commonly in severe infections within 4 weeks post-surgery, in comparison to *S. epidermidis* [18]. The lacks of genes producing tissue-damaging toxins make *S. epidermidis* exhibit low virulence in comparison to *S. aureus* or *P. aeruginosa*. The formation of biofilm is the only virulent factor in infections sourced by *S. epidermidis,* and thus, the sourced infections are generally sub-acute [19-21].

The phytoconstituents present in medicinal herbs are extremely effective against pathogens that stimulated the interest of using the extracts from these medicinal plants as a possible coating on biomaterial surface to induce surface modification. Sweet basil, botanically called *Ocimum basilicum* L. (OB) is a medicinal herb, native to the Asian continent [22,23]. The medicinal plant has found profound use as antispasmodic, digestive and tonic agent [24-26]. Extracts from the plant are also used to treat diverse ailments including nausea, migraine, depression, dysentery, insomnia, and other gastrointestinal disorders [27]. These extracts are also used extra-orally, in treating acne, snake bites and dermal pathologies [28]. Conversely, the antimicrobial potency of sweet basil has not been well discussed in the literature. Antimicrobial resistance has turned out to be a very serious issue in recent times and the pathogens have developed multiple drug resistance owing to the indiscriminate usage of commercial antibiotics [29-31]. Antibiotics also impose serious side effects including hypersensitivity and allergies in the long run. Studies on identifying potent alternative to these antibiotics to combat bacterial infections is under the limelight [32,33].

This study is focused on studying the extent of bacterial colonization and biofilm formation on the biomaterial substratum surfaces coated with OB essential oil, as an effective measure to combat BAI (Fig. 2).

METHODS

Procurement of OB essential oil

The essential oil of OB along with Gas Chromatography/Mass Spectrometry (GC/MS) report was obtained from Aromatics International, USA. The essential oil was stored at 4°C.

Biomaterial surfaces and their characterization

Commonly used biomaterials including polymethylmethacrylate (PMMA) and polystyrene (PS) were procured from Industrial Insulation, Chennai, India, and used as substratum surfaces. The substratum surfaces were rinsed in ethanol and washed with sterile water before experimental usage. Tissue culture Polystyrene (TCPS) well plates were used as a control surface and were procured from Nest Biotech., China. Sessile drop method based water contact angle measurements were performed to determine the wettability of the surfaces at room temperature. Averaging five droplets on one sample helped in obtaining each value.

Bacterial growth conditions and harvesting

American Type Culture Collection (ATCC) strains of *S. aureus, S. epidermidis, E. coli,* and *P. aeruginosa* were used for this *in vitro* study. Bacterial strains were purchased from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The specifications including strain designation, bio-safety level, and product format are listed in Table 1.

Bacteria were cultured overnight at 37°C aerobically from a frozen stock on blood agar and maintained at 4°C. One bacterial colony was inoculated in 10 ml Tryptone Soy Broth (TSB), procured from Hi media, Mumbai, for each experiment and was cultured for 24 h. Centrifugation was done for 5 m at 3000 rpm for bacterial harvesting. The harvested bacteria were suspended at a concentration of 10^7 bacteria/ml, in TSB.

Bacterial adhesion and biofilm growth on OB essential oil coated surfaces

The substratum surfaces including PMMA and PS were kept inside the TCPS well plates and filled with 1 ml of OB essential oil. They were then allowed for surface adsorption for 10 m at 37°C. TCPS well

plates were used as a control. After the period of surface adsorption, the unadsorbed essential oil was removed from the TCPS wells. Each TCPS well was added with 1 ml of bacterial suspension and allowed for adhering and aerobic growing at 37°C for different time instances including 1 hr, 3 hrs, and 24 hrs. The adherent bacteria in the absence of OB essential oil coating were considered the control. Unbound bacteria were removed by washing the wells with sterile phosphate buffer saline containing 10 mM potassium phosphate and 0.15 M NaCl at a pH value of 7.0. A phase contrast microscope was used to capture the images and ImageJ® software was used to identify the number of bacteria adhering per cm^2. Experiments were performed in triplicate with bacterial strains. The mean and standard deviation value of the obtained data were calculated. A statistical analysis was performed using one-way analysis of variance followed by Tukey's HSD *post hoc* test. A significant difference at $p<0.01$ was measured compared to control. The presence of biomolecules adhered to the surface of PS and PMMA were determined by Fourier Transform Infrared Spectroscopy (FTIR) analysis. For this, the OB oil coated PS, PMMA substratum surfaces were placed on sample holder, and the FTIR spectrum was recorded in the range 4000-500/cm using Bruker™ FTIR spectrometer.

Antibacterial activity of OB essential oil

Nutrient agar plates were prepared afresh for the experimental study. The agar plates were inoculated with the cultured bacterial suspension and incubated for 40 m at 37°C. The nutrient agar plates were punched with 6 mm diameter holes and filled with 150 µl of OB essential oil. The agar plates were incubated for 24 h at 37°C. The zone of inhibition (ZOI) was measured to assess the antibacterial potency.

RESULTS

The phytoconstituents present in OB essential oil as obtained from GC/MS analysis is shown in Table 2.

Table 1: ATCC strains and their product description

Bacteria	Strain designation	Biosafety level	Product format
S. aureus	NCTC 8532	1	Freeze dried
S. epidermidis	FDA strain PCI 1200	1	Freeze dried
P. aeruginosa	Genomic DNA from *P. aeruginosa* strain PAO1-LAC (ATCC® 47085™)	1	Dried
E. coli	Genomic DNA from *E. coli* strain Crooks (ATCC® 8739™)	1	Dried

FDA: Food & Drug Administration, DNA: Deoxyribonucleic acid, ATCC: American Type Culture Collection, *S. aureus: Staphylococcus aureus, P. aeruginosa: Pseudomonas aeruginosa, S. epidermidis: Staphylococcus epidermidis, E. coli: Escherichia coli*

Table 2: Chemical compositions of OB essential oil as obtained from GC-MS analysis

Compound	Percentage
Linalool	62.22
Geraniol	5.23
Terpinen-4-ol	1.46
Eugenol	3.02
1,8-cineole	3.51
Trans-b-ocimene	0.76
d-limonene	0.36
b-myrcene	0.42
b-elemene	1.15
Germacrene D	1.92
y-cadinene	1.65
t-cadinol	1.77
Geranyl acetate	0.53

GC-MS: Gas chromatography - mass spectrometry, OB: *Ocimum basilicum*

It was found that Linalool is the major component (62.22%) present in the essential oil. It also includes other main components including monoterpenes such as trans-b-ocimene (0.76%), d-limonene(0.36%), b-myrcene (0.42%), sesquiterpenes such as y-cadinene (1.65%), b-elemene (1.15%), a-trans-bergamotene (3.71%), sesquiterpenols such as t-cadinol (1.77%), epi-cubenol (0.34%), esters such as geranyl acetate (0.53%), phenols such as Eugenol (3.02%) and oxides including 1,8-cineole (3.51%). The water contact angles for different surfaces are shown in Fig. 3. The substratum surfaces coated with OB essential oil were found to be hydrophilic in comparison to the uncoated surfaces.

The result became evident from the water contact angle measurements made for bare PMMA and PS that were found to be 71.33±1.53 and 68.67±1.52, respectively, in comparison to the OB essential oil coated PMMA and PS surfaces that measured 49.33±1.67 and 57.66±1.38, respectively. The FTIR spectra of PS (Fig. 4) show the presence of characteristic peaks at the corresponding wave numbers. The band at 3683.38/cm is due to the O-H stretching of H-bonded alcohols and phenols.

The strong peak at 1750.72/cm shows the stretching vibrations of C=O. The value 1472/cm is related to the C-C stretching of aromatic ring structure. The bands at 1250.23/cm are related to the C-N stretching of aromatic amine group. The spectra confirm the presence of OB essential oil coating on PS substratum surface. The FTIR spectra of PMMA (Fig. 5) show the presence of characteristic peaks at the corresponding wave numbers. The band near 3310.72/cm corresponds to O-H stretching H-bonded alcohols and phenols. The peak around 2939/cm is because of C-H stretching and symmetric stretching of methoxy - groups. The

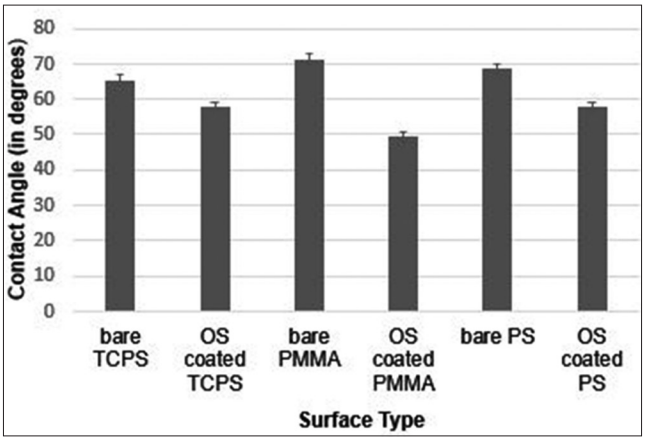

Fig. 3: Water contact angle measurements for different substratum surfaces

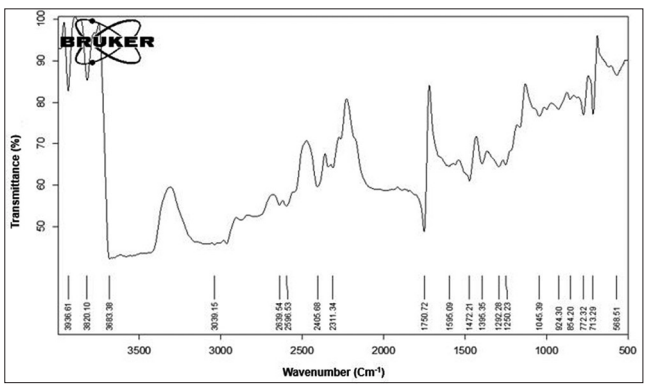

Fig. 4: Fourier transform infrared spectroscopy spectra showing characteristic peaks for *Ocimum basilicum* essential oil coating on polystyrene substratum surface

intense band observed at 1736.38/cm attributes to the antisymmetric stretching vibration of C=O group.

The peak around 1653.69/cm corresponds to N-H bend primary amines. The bands observed between 1258.28 and 1106.01/cm are ascribed to CO stretching mode. The spectra confirm the presence of OB essential coating on PMMA substratum surface.

Adhesion of bacteria and growth of biofilm was monitored on different substratum surfaces at different intervals of time, 1 hr, 3 hrs, and 24 hrs (Figs. 6-8). Quantitative assessment of bacterial adhesion on different substratum surfaces at different time instances is performed.

The results revealed a significant reduction (**p<0.01) in bacterial adhesion on OB essential oil coated substratum surfaces in comparison to uncoated, bare surfaces. Bacterial adhesion was found to be significantly less on OB essential oil coated surfaces in comparison to that on bare surfaces even after 24 hrs of incubation period. All the four bacteria employed in this study (*S. aureus, S. epidermidis, E. coli,* and *P. aeruginosa*) showed similar trends in the number of adherent bacteria on coated surfaces.

The ZOI is measured for all the four bacteria under study (Fig. 9). Maximum inhibition was observed for *S. aureus* (30±1.2 mm diameter) (Fig. 9d) compared to *S. epidermidis* (28±0.8 mm diameter) (Fig. 9c), *E. coli* (25±1.1 mm diameter) (Fig. 9b), and *P. aeruginosa* (21±0.6 mm diameter) (Fig. 9a). Experiments were repeated in triplicate, and the study reveals potent bacteriostatic effects of OB essential oil on both Gram-positive and Gram-negative bacteria.

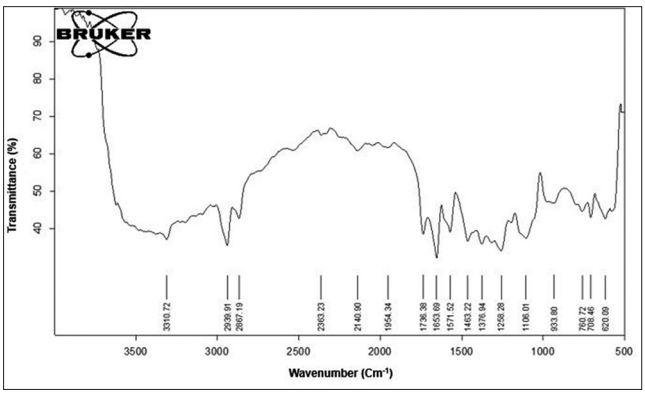

Fig. 5: Fourier transform infrared spectroscopy spectra showing characteristic peaks for *Ocimum basilicum* essential oil coating on polymethylmethacrylate substratum surface

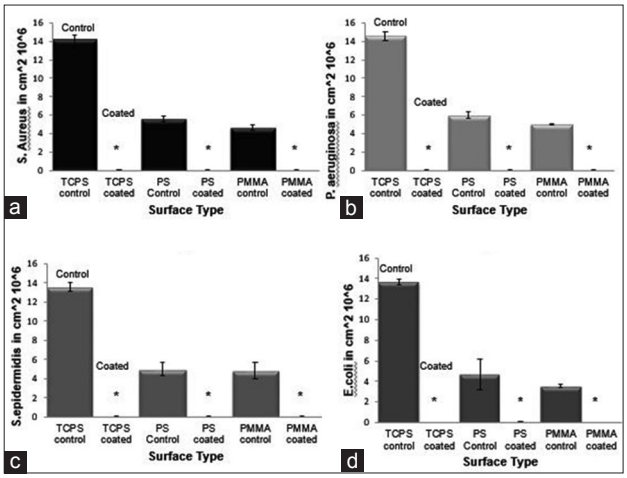

Fig. 6: Number of adherent bacteria ([a] *Staphylococcus aureus,* [b] *Pseudomonas aeruginosa,* [c] *Staphylococcus epidermidis,* and [d] *Escherichia coli*) after 1 hr of growth on different biomaterial surfaces in the absence and presence of OB extract coating. *denotes significant difference at **p<0.01 compared to control. [Sample size: 13, TCPS: Tissue culture polystyrene, PMMA: Polymethylmethacrylate]

DISCUSSION

An unsystematic usage of commercial antimicrobial medications has led to multiple drug resistance in human and plant pathogenic microorganisms. The usage of different types of medicinal plants and herbs in combating drug resistance is widely studied at present [34]. Adigzel *et al*. evaluated the antimicrobial potency of OB extract and identified that the hexane extract exhibited higher antibacterial potency than methanol and ethanol counterparts on the tested bacterial strains [35]. It was also demonstrated that the essential oil of OB had a comprehensive array of phytoconstituents, depending on the

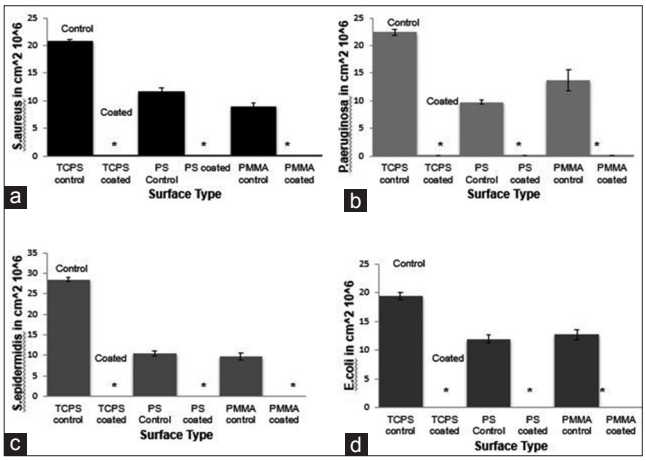

Fig. 7: Number of adherent bacteria ([a] *Staphylococcus aureus,* [b] *Pseudomonas aeruginosa,* [c] *Staphylococcus epidermidis,* and [d] *Escherichia coli*) after 3 hrs of growth on different biomaterial surfaces in the absence and presence of OB extract coating. *denotes significant difference at **p<0.01 compared to control. [Sample size: 13, TCPS: Tissue culture polystyrene, PMMA: Polymethylmethacrylate]

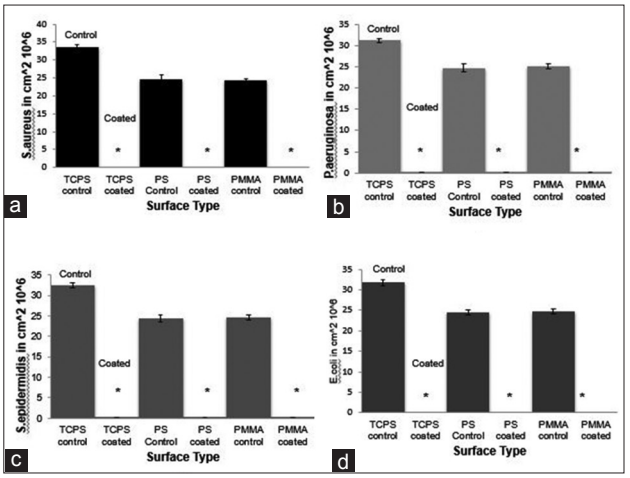

Fig. 8: Number of adherent bacteria ([a] *Staphylococcus aureus,* [b] *Pseudomonas aeruginosa,* [c] *Staphylococcus epidermidis,* and [d] *Escherichia coli*) after 24 hrs of growth on different biomaterial surfaces in the absence and presence of OB extract coating. *denotes significant difference at **p<0.01 compared to control, [Sample size: 13, TCPS - Tissue culture polystyrene, PMMA - Polymethylmethacrylate]

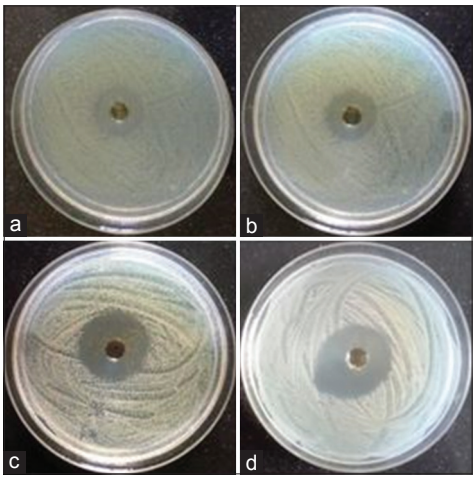

Fig. 9: (a-d) Antibacterial activity of *Ocimum basilicum* essential oil against pathogens

variations in chemotype, leaf color, and flower color, aroma and plant origin [36]. Helal *et al*. studied the potency of essential oil extracted from sweet basil against fungi, bacteria and yeasts and identified that the essential oil showed significant antimicrobial potency against the pathogens [37]. The essential oil was also found to inhibit the formation of conidia in the tested fungi, thereby inhibiting the growth. Runyoro *et al*. demonstrated that the essential oils of OB were extremely potent against *C. albicans, C. tropicalis,* and *C. glabrata* [38]. OB essential also showed excellent antioxidant, antimicrobial, and antitumor activities due to the presence of phenolic acids and aromatic compounds [39,40].

The study of literature revealed that the presence of linalool attributes to the antibacterial activity of OB essential oil [41]. Further studies demonstrated the usage of the plant as a potent antimicrobial against food-poisoning microorganisms [42,43]. The antimicrobial potency of OB essential oil against *Salmonella* strains is also well-documented in the literature [44,45]. OB essential oil was also found to be highly effective against *E. coli*, *S. typhi*, *S. paratyphi*, *P. vulgaris*, and *S. aureus* [46]. Linalool is the major oil constituent in OB L. that has no cytotoxic activity on mammalian cells [47]. An ambiguity exists in understanding the antimicrobial potency of OB essential oil for there is a selection of chemical components in the oil. Specifically, the antibacterial potency of the essential oil does not depend on a single mechanism, but there may be numerous targets in the cell. It was demonstrated that the phytoconstituents present in the essential oil traversed through the cell walls and cytoplasm, thereby disrupting the structure of polysaccharides, fatty acids and phospholipids, bringing cell necrosis [48].

The effectiveness of OB essential oil as a potent antimicrobial coating on substratum surfaces for prevention of bacterial adhesion and biofilm formation is demonstrated in this study. There was a significant reduction in the number of adherent bacteria on the OB essential coated substratum surfaces up to 24 hrs. The antibacterial mechanisms may be attributed to several targets in the cell. Thus, the strategy of OB essential oil coating on biomaterial surfaces could significantly reduce BAI.

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