



## Research article

## Synthesis, characterization, and evaluation of quaternary ammonium-based polymerizable antimicrobial monomers for prosthodontic applications

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## ABSTRACT

The present study aims to synthesize and characterize two quaternary ammonium (QAM) based monomers such as - dimethyl-hexadecyl-methacryloxyethyl-ammonium iodide (DHMAI) and 2-dimethyl-2-dodecyl-1-methacryloxyethyl ammonium iodine (DDMAI) and assess their cytotoxicity and antimicrobial properties. The study also aims to incorporate the optimized concentration of these monomers as copolymerizing monomers into conventional Polymethyl methacrylate (PMMA) denture base resin and evaluate their suitability for prosthetic applications. DHMAI and DDMAI monomers were synthesized through a Menshutkin reaction and their chemical structure was characterized using FT-IR and <sup>1</sup>H-NMR spectroscopy. Cytotoxicity was determined using Methyl Thiazolyl Tetrazolium (MTT) assay whereas antimicrobial activity was assessed using the agar-disc diffusion method. Subsequently, optimized concentrations of DHMAI or DDMAI, based on the cytotoxicity results, were added to conventional PMMA resin. Antimicrobial activity, cytotoxicity, surface hardness, and water sorption of PMMA denture base resin incorporated with DHMAI or DDMAI were evaluated. FT-IR and <sup>1</sup>H-NMR results confirmed the structure of monomers and copolymerization of DHMAI and DDMAI with PMMA resin. DHMAI and DDMAI monomers were found to be cytocompatible with mouse fibroblast cells up to a concentration of 5 µg/mL and 20 µg/mL respectively. In addition, incorporation of DHMAI or DDMAI at 5 µg/mL and 20 µg/mL respectively into PMMA denture base material did not affect their cytocompatibility. PMMA denture base resin incorporated with DHMAI or DDMAI significantly reduced the adhesion of microbes. Further, an increase in the surface hardness and a reduction in the water sorption was observed. Hence DHMAI and DDMAI can be considered as potential candidates for imparting antimicrobial activity to polymeric denture base materials.

## 1. Introduction

Ever since PMMA was introduced to dentistry during the late 1940s, it has been widely used for the fabrication of various prosthesis such as complete dentures, partial dentures, and maxillofacial prosthesis. In addition, it also used for denture repair, relining, rebasing and for the fabrication of special trays, orthodontic retainers, occlusal splints, and

obturators, etc [1]. Despite the introduction of various alternative materials, PMMA remains the choice of material for complete and partial denture fabrication due to its favorable properties such as biocompatibility, insolubility in oral fluids, lifelike appearance, and easy to process, repair, and polish. Notwithstanding the popularity of this material, denture prosthesis made from PMMA exhibits inferior mechanical characteristics such as low flexural and impact strength, low surface hardness,

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high water sorption and allergic reactions due to the presence of un-polymerized monomer. In addition, the surface of dental prostheses made from PMMA material inherently do not possess any antimicrobial activity. Hence the surfaces of the denture made from this material harbor various microorganisms, mainly the fungi such as *Candida albicans* (*C. albicans*) [1, 2]. These microorganisms form a biofilm, subsequently leading to the development of an oral infection called denture stomatitis (DS). DS is characterized by inflammation and erythema of the oral mucosa underneath the denture-bearing areas [3, 4]. It is common in elderly population and immunocompromised patients, causing health complications such as infectious endocarditis, malodour, pulmonary candidiasis, and aspiration pneumonia [5, 6]. Epidemiological studies reported the prevalence of DS to be 78% [4]. To overcome this problem, attempts have been made to impart antimicrobial activity to PMMA denture base materials by incorporating antimicrobial additives. Antimicrobial additives such as chlorhexidine, QAM-compounds, silver, and titanium dioxide nanoparticles have been investigated. However, the antimicrobial action was not long-lasting due to the leaching or cross-reactivity with the oral tissues [1, 4]. Alternatively, in recent years, research focusing on chemically copolymerizing antimicrobial agents to PMMA resin networks is gaining attention. Such antimicrobial agents are uniformly distributed throughout the bulk of the material and provide long-lasting antimicrobial activity without leaching out from the host material [4,7]. Copolymerizing antimicrobial monomers such as MDPB (12-Methacryloyloxy -Dodecyl -Pyridinium -Bromide), MUPB (Methacryloyloxy Undecyl Pyridinium Bromide), QAM (Quaternary Ammonium Monomer), DMADDM (Dimethyl Amino Dodecyl Methacrylate), TBAEMA (2-Tert-Butyl Amino Ethyl Methacrylate), and AAM (Acryl Amide Monomer) have been investigated and were reported to be effective in inhibiting the microbial growth [7, 8, 9, 10].

Most polymerizable monomers are based on QAM salt that can covalently bond with the methacrylate group of the matrix-forming monomers [7]. Such polymerizable antimicrobial agents, in general, consist of a methacrylate group to immobilize the bacterial agents, and QAM salts for antimicrobial action. The antimicrobial activity of these monomers seems to be dependent on their chain length. In general, longer chain length imparts superior antimicrobial activity, especially for monomers with chain lengths varying between C5 to C18 [10], [11], [12].

Novel QAM salt-based copolymerizing antimicrobial monomers, DHMAI, and DDMAI have recently been investigated for use with dental

restorative resins [11, 13]. These monomers were synthesized through a Menshutkin reaction in which different alkyl iodides such as iodo-hexadecane, and iodododecane were combined with polymerizable tertiary amine namely 2-dimethylamino ethyl methacrylate (DMEMA). The resultant polymerizable monomers such as DHMAI, and DDMAI with different alkyl chain lengths [C16, and C12 respectively] exhibited superior antimicrobial properties [11]. Their antimicrobial activity is attributed to the contact killing through electrostatic interaction between the cationic/positively charged QAM-based antimicrobial monomer, and a negatively charged bacterial cell membrane causing cellular membrane damage following cell lysis, and death [1, 6, 10].

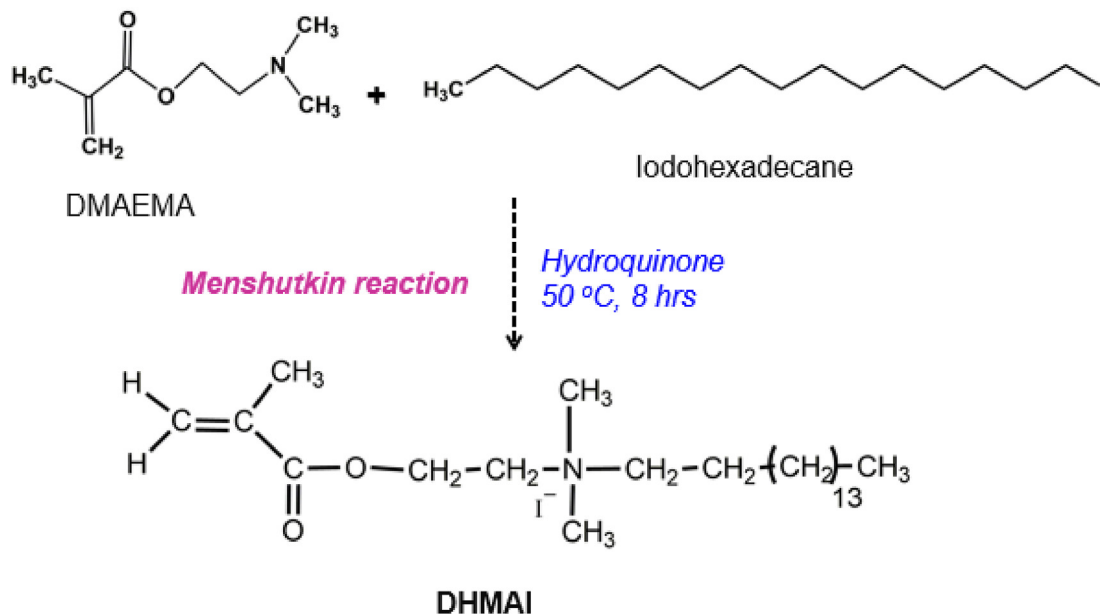
Although extensive research in the evaluation of various antimicrobial monomers was reported, their use in resin-based materials for the fabrication of complete denture prostheses has not been widely reported. In this regard, the use of DHMAI and DDMAI at different concentrations as copolymerizing liquid with PMMA based denture base resin increased their mechanical properties [14]. However, it is not clear if such an addition also imparts antimicrobial activity to the denture bases. Hence the present study aims to synthesize these antimicrobial monomers, incorporate them as polymerizable monomers into heat-activated PMMA based denture base resins and evaluate its biocompatibility, antimicrobial properties, surface hardness, and water sorption.

## 2. Material and methods

### 2.1. Synthesis, and characterization of antimicrobial monomers, and modified PMMA resin

#### 2.1.1. Synthesis of 2-Dimethyl-hexadecyl-methacryloxyethyl-ammonium iodide (DHMAI)

DHMAI monomer was synthesized through Menshutkin reaction in which the reaction between a tertiary amine, and alkyl halide results in the formation of a QAM salt. 0.06 mol of DMAEMA (9.43 g), 0.05 mol of iodo-hexadecane (14.81 g), and 0.05% of hydroquinone (0.025 g) were mixed in a 50 mL round bottom flask. Subsequently, the flask was placed in a silicone oil bath under a nitrogen atmosphere at 50 °C with constant stirring for 8 h (Scheme 1). At the end of the reaction time, the resultant white paste was washed 4 to 5 times with diethyl ether by centrifugation method. Subsequently, it was dried in a rotary evaporator at 40 °C for 2 h [11].



Scheme 1. Synthesis of DHMAI.

### 2.1.2. Synthesis of 1-2-dimethyl-2-dodecyl-1-methacryloxyethyl ammonium iodine (DDMAI)

DDMAI monomer was synthesized through Menshutkin reaction. 0.06 mol of DMAEMA (9.43 g), 0.05 mol of 1-Iodododecane (Dodecyl iodide) (14.81g), and 0.05% of hydroquinone (25 mg) were taken in a 50 mL round bottom flask. Subsequently, the flask was placed in a silicone oil bath under a nitrogen atmosphere at 50 °C with constant stirring for 16 h (Scheme 2). At the end of the reaction, a white paste was formed which was washed 4 to 5 times with diethyl ether followed by drying in a rotary evaporator at 40 °C for 2 h [11].

### 2.1.3. Characterization of antimicrobial monomers, and modified PMMA resin

FT-IR and <sup>1</sup>H-NMR Spectroscopy were used to characterize the chemical structure of the synthesized monomers. FT-IR spectra of the monomers were recorded on a Shimadzu IR Spirit (QATR-S) spectrometer in the range of 4000–400 cm<sup>-1</sup>. Briefly, the procedure involved the addition of a small amount of synthesized monomer with potassium bromide powder. Both the powders were thoroughly mixed in an agate mortar using a pestle. Subsequently, the powder was pressed into a pellet form, and the FT-IR was recorded. FT-IR spectra of DHMAI, DDMAI, and

### 2.2. Cytotoxicity of antimicrobial monomers (DHMAI and DDMAI)

Cytotoxicity of antimicrobial monomers (DHMAI and DDMAI) was analyzed using MTT assay (Mosmann, 1983). Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), and 1% antibiotic-antimycotic solution was used to culture L929 mouse fibroblast cells. Cells were incubated at 37 °C, and 5% CO<sub>2</sub> in a humidified environment for at least three consecutive passages before being utilized in the experiments. At a seeding density of 5,000 cells per well, cells were seeded onto 96 well microtiter plates. After adherence, they were treated with different concentrations of antimicrobial monomer (DHMAI or DDMAI), viz., 1, 2, 5, 10, 20, 40, 60, 80, and 100 µg/mL concentrations. Methyl methacrylate (MMA) was used as a positive control. MTT reagent (1 mg/mL) was added to the wells after 24 h and incubated at 37 °C for 4 h. Later, dimethyl sulfoxide (DMSO) was used to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech). The percentage viability of the cells after treatment with the test compounds in triplicates was calculated in comparison to the untreated cell control. Cytotoxicity was calculated using the following formula [15].

$$\text{Cytotoxicity (\%)} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the test compound})}{\text{Absorbance of the control}} \times 100$$

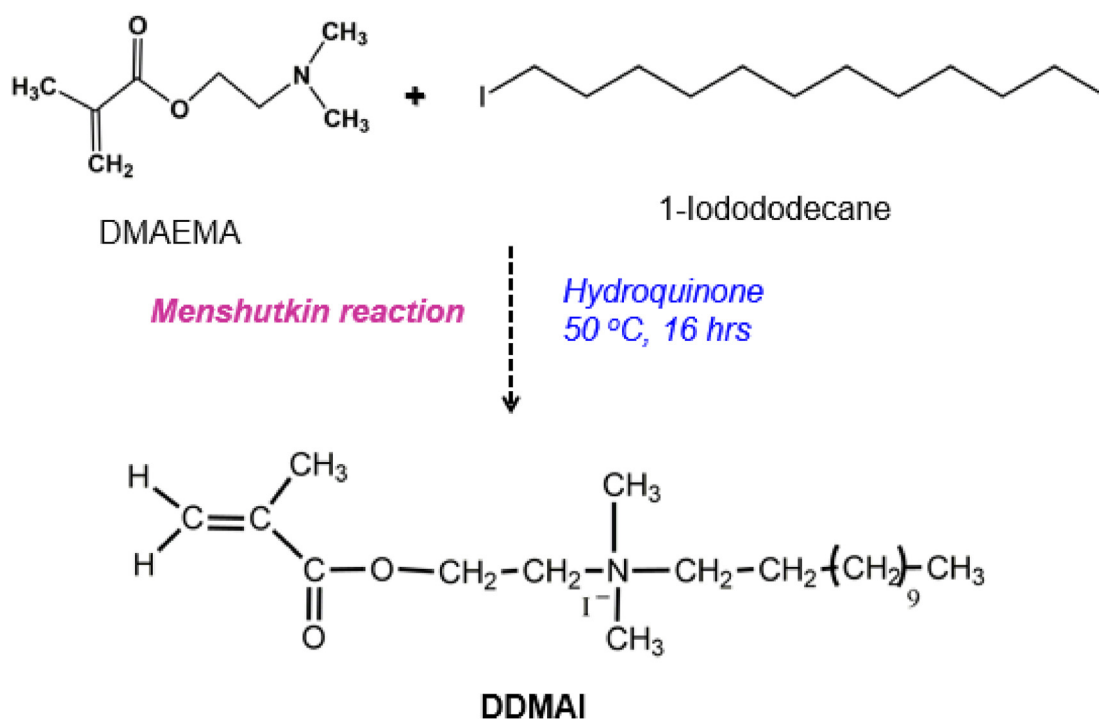
modified PMMA resin incorporated with DHMAI or DDMAI were recorded. Pure PMMA powder was considered as control.

The <sup>1</sup>H-NMR spectra of DHMAI, DDMAI, and modified PMMA resin incorporated with DHMAI or DDMAI, and control (PMMA) were recorded with the help of NMR spectrometer (Bruker Avance III 400 MHz) using deuterated dimethyl sulfoxide (DMSO d<sub>6</sub>) as a solvent.

### 2.3. Antimicrobial activity of monomers

#### 2.3.1. Disc diffusion method

The antimicrobial activity of unpolymerized monomers was evaluated using an agar-disc diffusion test against the standard strains of



Scheme 2. Synthesis of DDMAI

*Candida albicans* (ATCC 90028), *Staphylococcus aureus* (ATCC 25923), and *Streptococcus mutans* (ATCC 25175) [1]. *C. albicans* was cultured on Sabouraud's Dextrose Agar (SDA) whereas *S. aureus*, and *S. mutans* were cultured on Nutrient -Agar, and -Sheep Blood -Agar (SBA) respectively. A loopful inoculum of each microorganism was transferred to 2 ml of Mueller Hinton Broth to make up to 0.5 McFarland Standard. Lawn cultures with a sterile cotton swab were prepared on Mueller Hinton Agar (MHA) for *S. aureus*, MHA with Methylene blue for *C. albicans*, and -SBA for *S. mutans*. Subsequently, 6 mm diameter sterile Whatman filter paper discs (No.1) were placed on the media, loaded with 10 µg of different concentrations of DHMAI (1, 2, or 5 µg/mL) or DDMAI (5, 10, or 20 µg/mL), and incubated at 37 °C for 24 h for bacteria, and 48 h for fungal growth respectively. The zone of inhibition was measured using a caliper and was reported in millimeters (n = 3) [4].

### 2.3.2. Preparation of PMMA resin with antimicrobial monomers

Modified PMMA resin samples were prepared by adding different concentrations of DHMAI at 1, 2, or 5 µg/mL or DDMAI at 5, 10, or 20 µg/mL to the MMA liquid. MMA liquid with or without antimicrobial monomers was later mixed with PMMA powder at a powder: liquid ratio of 2.5:1 by weight, and allowed to reach the dough stage. The resultant dough was used to prepare the samples using the compression molding technique, and the curing of the specimens was carried out in a water bath at 70 °C for 8 h. Prepared samples were assessed for cytotoxicity, antimicrobial activity, surface hardness, and water sorption.

### 2.3.3. Cytotoxicity of modified PMMA resin

Disc-shaped specimens (1mm diameter × 1mm thick) of control and test specimens were prepared using the compression molding technique as explained above. The specimens were cleaned ultrasonically using distilled water for 20 min and exposed to ultraviolet light for 20 min. Subsequently, the discs were placed in test tubes with 10 mL of artificial saliva and incubated at 37 °C. At regular intervals of time [1 day, 1 week, and 1 month], incubating medium was pipetted, and the cytotoxicity was assessed using the MTT assay as described earlier [4].

## 2.4. Antimicrobial activity of modified PMMA resin

### 2.4.1. Disc Diffusion method

The antimicrobial activity of control and test specimens was measured using the agar-disc diffusion test as explained above. Disc-shaped specimens of 15 mm diameter and 1mm thickness were prepared using the compression moulding technique. Prepared samples were sterilized using ethylene oxide (ETO). Subsequently, the specimens were placed on the lawn cultures of respective microorganisms and incubated as mentioned above. The zone of inhibition in millimeters was measured using a caliper at the end of the incubation period (n = 3) [1].

### 2.4.2. Microbial adherence test

Control and test specimens of 10 × 10 × 3 mm were prepared using the compression molding technique. Prepared specimens were placed in the test tubes containing 10 mL of Tryptic Soy Broth (TSB) inoculated with each microorganism, and incubated at 37 °C for 24 h. At the end of the incubation time, the specimens were retrieved and rinsed with saline to dislodge loosely adhered microorganisms. Subsequently, specimens were immersed in 10 mL of 1 mol/L NaCl, vortexed vigorously for 1 min, and left to stand for 9 min, followed by a brief vortex for 1 min to collect the adherent cells. The solution thus obtained was serially diluted (10<sup>-1</sup> to 10<sup>-3</sup>), and 10 µL of each dilution was transferred to plates containing an SBA with the help of a 4 mm diameter inoculation loop, and a semi-quantitative streaking technique was followed. Plates were incubated for 24 or 48 h at 37 °C, and the number of colony-forming units (CFU) were calculated [4].

### 2.4.3. Field emission scanning electron microscopy (FESEM)

The adherence of microorganisms onto the surfaces of control and test specimens was analyzed using scanning electron microscopy. PMMA samples retrieved from the microbial adherence test were further washed with phosphate buffer solution (PBS) (pH = 7) followed by immersion in 2.4% Glutaraldehyde solution at 4 °C for 2 h. Subsequently, the specimens were retrieved, washed with PBS, and dehydrated using a series of ethanol washes (70, 95, and 100%) for 10 min. These specimens were sputter-coated with gold, mounted onto aluminum stubs, and a scanning electron microscope (FESEM, Carl Zeiss) was used to examine the samples [16].

Similarly, FESEM was also used to observe the surface morphology of control and PMMA denture base resin incorporated with DHMAI or DDMAI.

### 2.5. Surface hardness of modified PMMA resin

Surface hardness of disk-shaped (14 mm diameter and 4 mm thick) control and test specimens was measured using a microhardness tester (Vickers hardness tester, Tescol, India). 25 g of load was applied on the surface of each specimen for 30 s. After the dwell time, load was removed, and the area of the indentation was measured. Load divided by the area of indentation was calculated and presented as Vickers Hardness Number (VHN). Hardness on each specimen was measured at 8 different places, and an average hardness was reported [17].

### 2.6. Water sorption of modified PMMA resin

Disk-shaped (5 mm diameter and 0.5 mm thick) control and test specimens were prepared and immersed in distilled water for 24 h before the test. Later, the samples were desiccated under vacuum at 37 °C for 23 h and again stored in another desiccator containing freshly dried silica gel at 23 °C for 1 h until a constant weight was observed (m1). After the measurement of weight, the samples were immersed in distilled water at 37 °C and stored for 7 days. After 7 days, samples were retrieved from the water, surface water blotted with tissue paper, and the weight (m2) and volume (V) of the samples were measured. Water uptake (µg/mm<sup>3</sup>) was determined using the following equation [1].

$$W_{sp} = \frac{m_2 - m_1}{V}$$

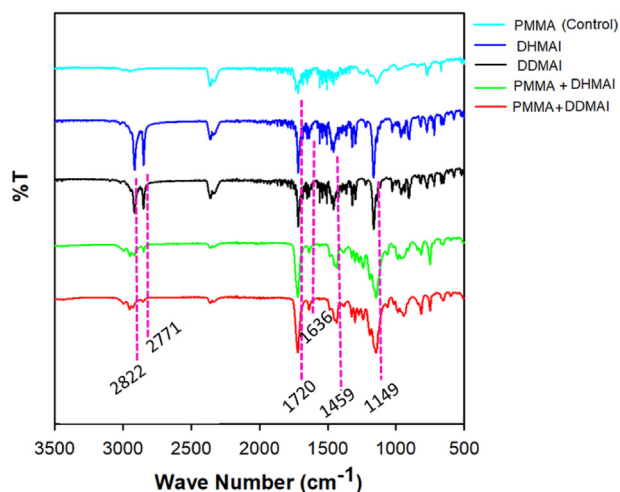
### 2.7. Statistical analysis

The cytotoxicity of DHMAI and DDMAI monomers, cytotoxicity, surface hardness, and water sorption data of PMMA with and without antimicrobial monomers, were analyzed using One-Way ANOVA followed by Tukey post hoc test. Cytotoxicity of modified PMMA at different time intervals, and antimicrobial activity (Zone of inhibition) of DHMAI, and DDMAI were analyzed using Two-Way ANOVA followed by Tukey post hoc test at a 5% level of significance (α = 0.05).

## 3. Results

### 3.1. FT-IR spectra analysis

The chemical structure of synthesized monomers (DHMAI, and DDMAI), and modified PMMA resins were characterized using FT-IR spectroscopy [Figure 1]. The characteristic band around 1,720 cm<sup>-1</sup> and 1,636 cm<sup>-1</sup> in FT-IR spectra revealed the presence of methacrylate groups. The characteristic peaks appeared at 2,822, and 2,771 cm<sup>-1</sup> represent C-H stretching of -N(CH<sub>3</sub>)<sub>2</sub> groups, 1,720 cm<sup>-1</sup> represent C=O, and C-O-C stretching of acrylate groups, 1,459 cm<sup>-1</sup> represent



**Figure 1.** FT-IR spectra of PMMA, DHMAI, DDMAI, PMMA modified with DHMAI, and PMMA modified with DDMAI monomer.

$\text{CH}_2$  bending, and  $1,149\text{ cm}^{-1}$  represents C–N stretching. FT-IR spectrum of PMMA (Control) indicated the presence of its characteristic functional groups. Because of the presence of ester carbonyl group stretching vibration, a sharp peak at  $1,720\text{ cm}^{-1}$  was observed. Broad peaks between  $1,260$  and  $1,000\text{ cm}^{-1}$  are due to C–O (ester bond) stretching vibration, whereas broadband between  $950$  and  $650\text{ cm}^{-1}$  is due to C–H bending. From **Figure 1**, it is also evident that characteristic peaks present in DHMAI, DDMAI monomers, and PMMA are also present in the copolymer prepared by adding these monomers with PMMA. Thus, FT-IR spectra analysis confirms the copolymerization of PMMA with DHMAI and DDMAI monomers.

### 3.2. NMR spectra analysis

The chemical structure of DHMAI monomer was further confirmed by  $^1\text{H-NMR}$  spectra (**Figure 2**). Following corresponding peaks in the

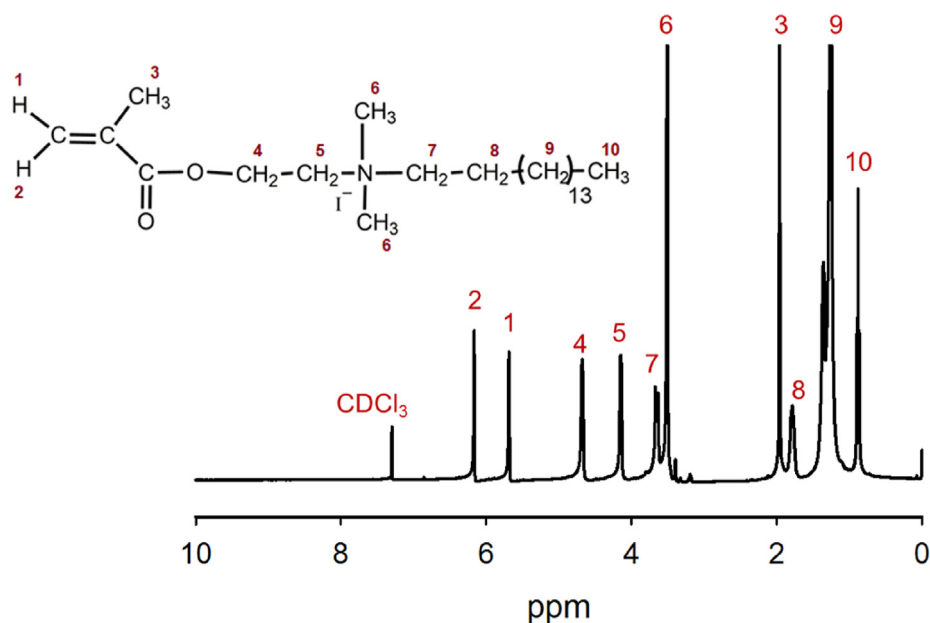
spectra, their peak position, and its multiplicity were observed:  $^1\text{H-NMR}$  ( $\delta$  ppm):  $6.12$  (1H,  $\text{CH}_2 = \text{C}(\text{CH}_3)$  trans, s),  $5.64$  (1H,  $\text{CH}_2 = \text{C}(\text{CH}_3)$  cis, s),  $4.62$ – $4.64$  (2H, N +  $\text{CH}_2\text{CH}_2\text{OC}(\text{O})$ , t),  $4.09$ – $4.11$  (2H, N +  $\text{CH}_2\text{CH}_2\text{OC}(\text{O})$ , t),  $3.61$ – $3.64$  (2H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_{13}\text{CH}_3$ , t),  $3.46$  (6H, N +  $(\text{CH}_3)_2$ , s),  $1.91$  (3H,  $\text{CH}_2 = \text{C}(\text{CH}_3)$ , s),  $1.73$ – $1.76$  (2H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_{13}\text{CH}_3$ , m),  $1.21$ – $1.31$  (26H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_{13}\text{CH}_3$ , m),  $0.82$ – $0.85$  (3H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_{13}\text{CH}_3$ , t). These NMR spectra findings are in accordance with the previously reported study [11].

Similarly, the chemical structure of DDMAI monomer was also confirmed by  $^1\text{H-NMR}$  spectra (**Figure 3**). Following characteristics peaks, peak position, and its multiplicity were observed:  $^1\text{H-NMR}$  ( $\delta$  ppm):  $6.16$  (1H,  $\text{CH}_2 = \text{C}(\text{CH}_3)$  trans, s),  $5.68$  (1H,  $\text{CH}_2 = \text{C}(\text{CH}_3)$  cis, s),  $4.67$  (2H, N +  $\text{CH}_2\text{CH}_2\text{OC}(\text{O})$ , t),  $4.14$  (2H, N +  $\text{CH}_2\text{CH}_2\text{OC}(\text{O})$ , t),  $3.63$ – $3.66$  (2H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_7\text{CH}_3$ , t),  $3.50$  (6H, N +  $(\text{CH}_3)_2$ , s),  $1.95$  (3H,  $\text{CH}_2 = \text{C}(\text{CH}_3)$ , s),  $1.76$ – $1.79$  (2H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_7\text{CH}_3$ , m),  $1.25$ – $1.35$  (14H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_7\text{CH}_3$ , m),  $0.86$ – $0.89$  (3H, N +  $\text{CH}_2\text{CH}_2(\text{CH}_2)_7\text{CH}_3$ , t). The characteristic features observed in NMR spectra for DDMAI are similar to those reported earlier [11].

The  $^1\text{H-NMR}$  spectra of modified PMMA resin incorporated with DHMAI or DDMAI were also recorded (**Figure 4**). **Figure 4**, shows  $^1\text{H-NMR}$  spectra of PMMA modified with DHMAI, where the signals of PMMA and DHMAI were overlapped resulting in a broad spectrum compared to DHMAI spectra. Similarly, in  $^1\text{H-NMR}$  spectra of PMMA modified with DDMAI, signals of PMMA, and DDMAI were overlapped leading to broad spectra compared to DDMAI spectra (**Figure 5**).

### 3.3. Disc diffusion method

Results of the disc diffusion test of antimicrobial monomers were shown in **Figure 6**. DHMAI and DDMAI monomers showed antimicrobial activity in a dose-dependent manner. DHMAI showed significant antimicrobial activity against *S. aureus* at  $5\text{ }\mu\text{g/mL}$  concentration showing a zone of inhibition of  $20 \pm 0.6\text{ mm}$  ( $P = 0.06$ ) whereas the zone of inhibition for *S. mutans*, and *C. albicans* was found to be  $18 \pm 0.6\text{ mm}$  ( $P = 0.01$ ), and  $19 \pm 00\text{ mm}$  ( $P < 0.0001$ ) respectively. DDMAI showed significant antimicrobial activity against *S. aureus* at  $20\text{ }\mu\text{g/mL}$  concentration, showing a zone of inhibition of  $19 \pm 0.6\text{ mm}$  ( $P = 0.01$ ) whereas the zone of inhibition for *S. mutans*, and *C. albicans* was found to be  $16 \pm 00$



**Figure 2.**  $^1\text{H-NMR}$  spectra of DHMAI monomer.

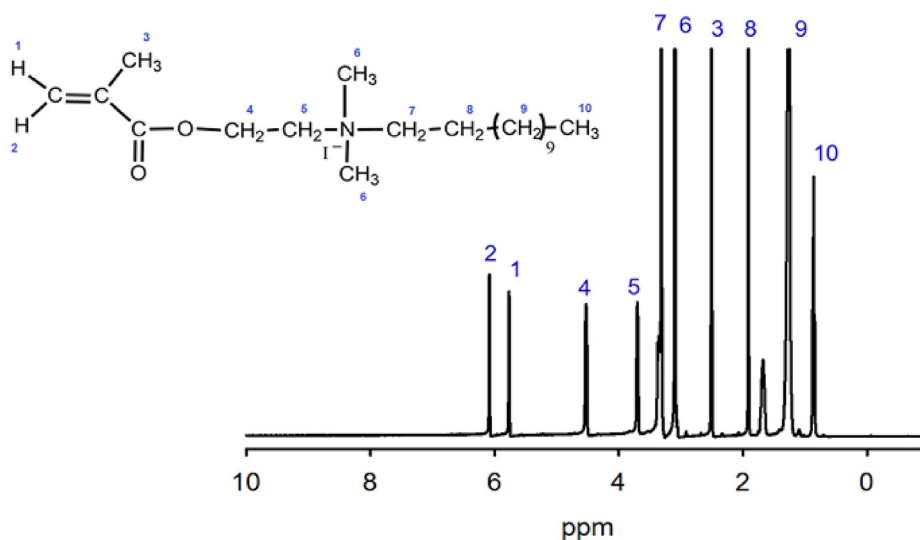


Figure 3.  $^1\text{H-NMR}$  spectra of DDMAI monomer.

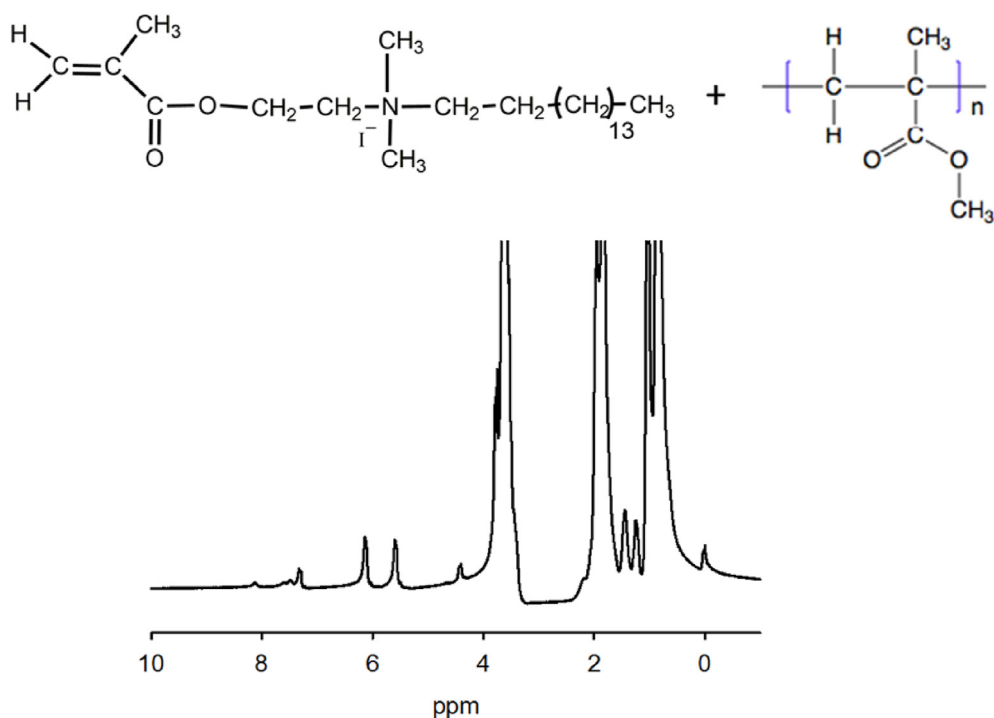


Figure 4.  $^1\text{H-NMR}$  spectra of PMMA modified with DHMAI monomer.

mm ( $P = 0.01$ ), and  $18 \pm 0.6$  mm ( $P = 0.03$ ) respectively at this concentration.

### 3.4. Cytotoxicity

Figure 7, shows cytotoxicity of DHMAI, and DDMAI at 1, 2, 5, 10, 20, 40, 60, 80, and 100  $\mu\text{g/mL}$  concentrations. From Figure 7, DHMAI up to 5  $\mu\text{g/mL}$ , and DDMAI up to 20  $\mu\text{g/mL}$  exhibited significantly less cytotoxicity compared to other concentrations ( $P < 0.0001$ ). This indicates that both DHMAI and DDMAI monomers are biocompatible at lower concentrations. However, the cytotoxicity of these monomers seems to be

dose-dependent indicating that the use of higher concentrations can decrease the cell viability.

Furthermore, based on the cytotoxicity results of unpolymerized monomers, suitable concentrations of antimicrobial monomers were incorporated into PMMA material, and cytotoxicity of the modified PMMA resin was measured. Figure 8, depicts the cytotoxicity of modified PMMA resin (containing DHMAI at 1, 2, or 5  $\mu\text{g/mL}$ , and DDMAI at 5, 10, or 20  $\mu\text{g/mL}$ ). PMMA incorporated with antimicrobial monomer exhibited significantly lower cytotoxicity at DHMAI at 1  $\mu\text{g/mL}$  ( $P = 0.9184$ ), and DDMAI at 5  $\mu\text{g/mL}$  ( $P = 0.5201$ ) concentrations compared to control. Whereas statistically significant difference in cytotoxicity was

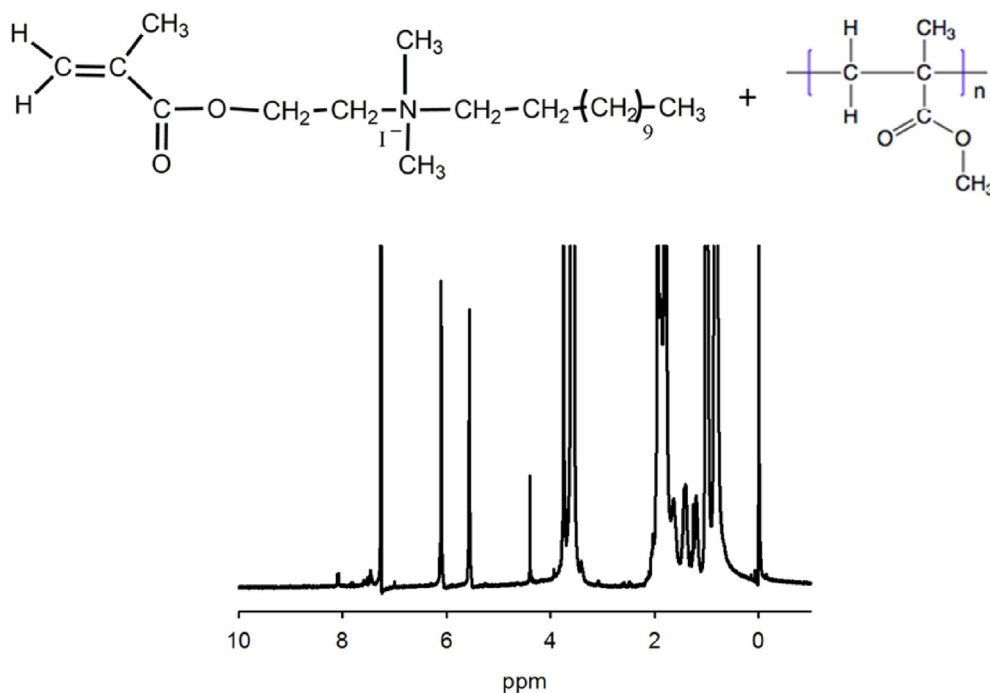


Figure 5.  $^1\text{H-NMR}$  spectra of PMMA modified with DDMAI monomer.

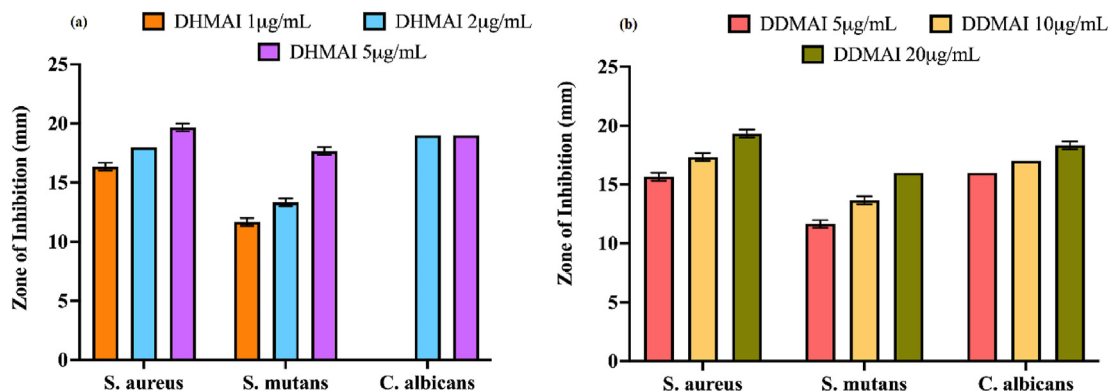


Figure 6. Zone of inhibition of (a) DHMAI (1, 2, or 5  $\mu\text{g/mL}$ ) and (b) DDMAI (5, 10, or 20  $\mu\text{g/mL}$ ) against *Staphylococcus aureus*, *Streptococcus mutans*, and *Candida albicans*.

observed between control and other concentrations of DHMAI (2, or 5  $\mu\text{g/mL}$ ) and DDMAI (10, or 20  $\mu\text{g/mL}$ ) monomer, indicating its higher degree of toxicity towards L-929 cell line.

Figure 9, shows cytotoxicity of modified PMMA resin, and control (PMMA) specimens stored in artificial saliva at regular intervals of time [1 day, 1 week, and 1 month]. From Figure 9, it can be observed that the control group exhibited the lowest cytotoxicity (highest cell viability) at different time intervals compared to the experimental groups. The addition of antimicrobial monomers to PMMA denture base resin increased their cytotoxicity in a dose-dependent manner.

### 3.5. Antimicrobial activity

The results of disc diffusion test of PMMA discs incorporated with various concentrations of DHMAI or DDMAI monomer did not show any zone of inhibition. However, estimation of CFU using the direct contact test revealed that the incorporation of antimicrobial monomers into PMMA polymer inhibited the adhesion of microorganisms to its surface,

especially *S. mutans*. From Table 1, it can be observed that the addition of both the monomers reduced adhesion of *S. mutans* completely. However, no reduction in the adhesion of *S. aureus* was observed with both the monomers although, the adhesion of *C. albicans* decreased in a dose-dependent manner for both the monomers tested.

The adhesion of microorganisms to the surface of both experimental, and control groups was also observed under FESEM and is presented in Figure 10a to e. Figure 10a shows adherence of *S. aureus* in the control group whereas Figures 10b, and 10c show no adherence of *S. mutans* on modified PMMA resin. Figures 10d, and 10e show adherence of *S. mutans*, and *C. albicans* in the control group respectively.

### 3.6. Surface hardness

Figure 11, shows the surface hardness of PMMA resin with, and without antimicrobial monomers. From Figure 11, it was observed that the addition of antimicrobial monomers increased the surface hardness of PMMA resin. However, the increase in the surface hardness was

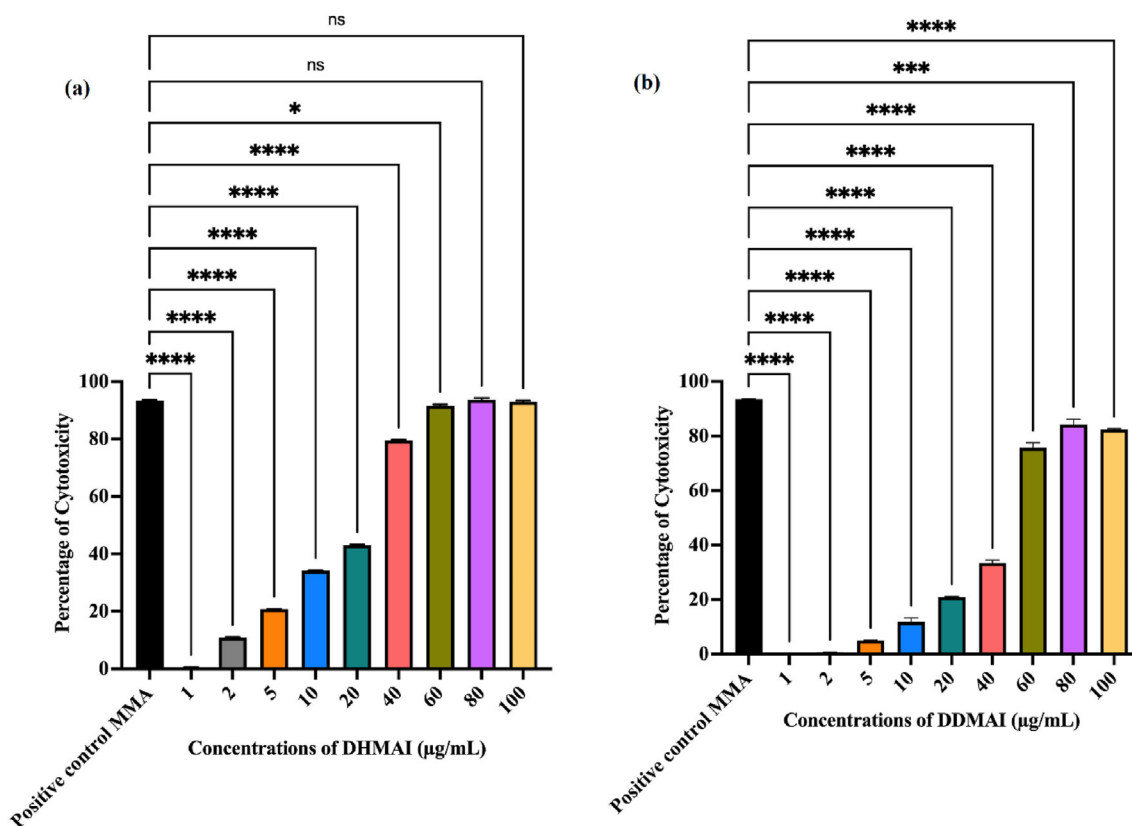


Figure 7. Cell viability of (a) DHMAI, and (b) DDMAI antimicrobial monomer.

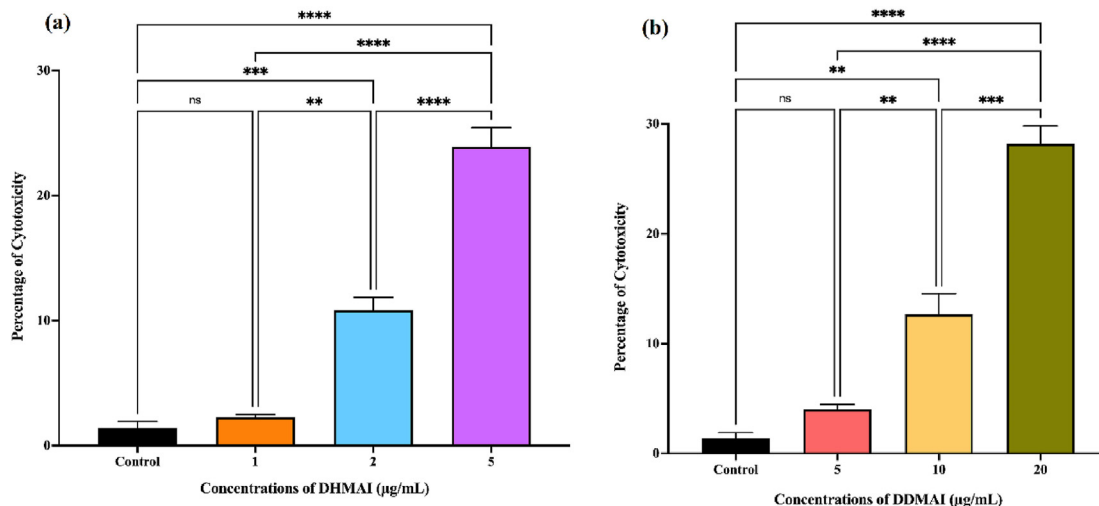


Figure 8. Cell viability of modified PMMA resin containing antimicrobial monomer (a) DHMAI (1, 2, or 5 µg/mL), and (b) DDMAI (5, 10, or 20 µg/mL).

statistically significant only at 5 µg/mL for DDMAI monomer ( $P < 0.05$ ). No significant differences in surface hardness were observed at other concentrations of DHMAI, and DDMAI.

### 3.7. Water sorption

Figure 12, shows results of water sorption of modified PMMA resin. The addition of DHMAI reduced the water sorption of PMMA resin only at 1 µg/mL concentration. In case of DDMAI monomer, both 5 and 10 µg/mL discernibly reduced the water sorption of PMMA resins. However, no statistically significant difference was observed between PMMA (control) and DHMAI at 2 and 5 µg/mL or DDMAI at 20 µg/mL.

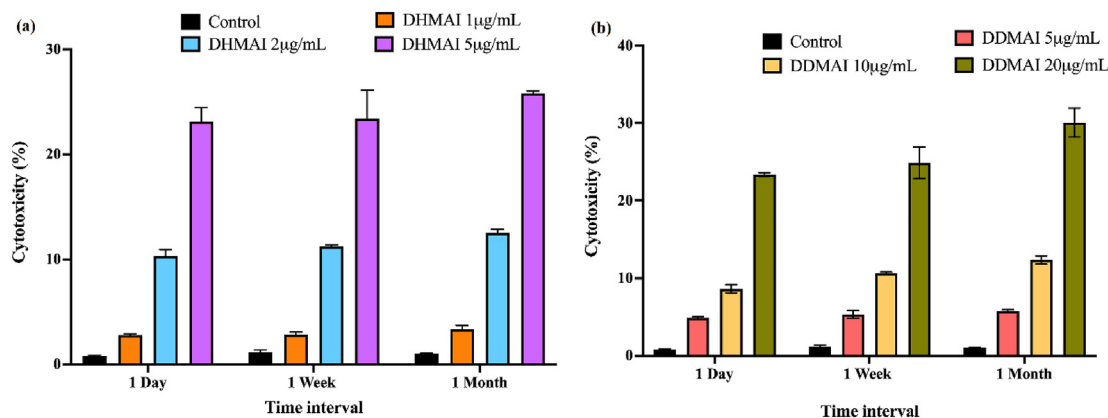
### 3.8. FESEM analysis

The surface morphology of modified PMMA resin incorporated with DHMAI or DDMAI, and control (PMMA), were visualized using FESEM analysis. The microscopic appearance was comparable to the polymeric materials. However, modified PMMA resin samples (Fig. 13b, and c) seem to be more porous compared to the control (Fig. 13a).

## 4. Discussion

Heat-activated PMMA is widely used as denture base material. Although it exhibits excellent aesthetics, and adequate mechanical





**Figure 9.** Cell viability of modified PMMA resin containing antimicrobial monomer (a) DHMAI (1, 2, or 5 µg/mL), and (b) DDMAI (5, 10, or 20 µg/mL) after storage in artificial saliva at different time intervals.

**Table 1.** Colony-forming units per milliliter (CFU/mL) results of control and modified PMMA against *Staphylococcus aureus*, *Streptococcus mutans*, and *Candida albicans* at 10 µL dilution.

Organisms	CFU/mL						
	Control	DHMAI [µg/mL]			DDMAI [µg/mL]		
		1	2	5	5	10	20
<i>S. aureus</i>	10 <sup>5</sup>	10 <sup>5</sup>	10 <sup>5</sup>	10 <sup>5</sup>	10 <sup>5</sup>	10 <sup>5</sup>	10 <sup>5</sup>
<i>S. mutans</i>	5	0	0	0	0	0	0
<i>C. albicans</i>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>

characteristics, a lack of antimicrobial activity leads to microbial colonization on the denture surfaces leading to DS. Several attempts have been made to impart antimicrobial activity to conventional PMMA-based materials either by blending or copolymerizing the antimicrobial agents to the PMMA resin matrix. In recent years, copolymerizing of antimicrobial monomers to biomedical polymers has gained interest due to their long-lasting antimicrobial action. In the present study, two monomers based on QAM salts were synthesized and added to conventional PMMA denture base materials, and their effect on biocompatibility, antimicrobial activity, adherence of microorganisms, hardness, and water sorption of the resultant PMMA polymer has been investigated.

In the Agar-disc diffusion test, a clear inhibitory zone was observed with DHMAI at 1, 2, or 5 µg/mL, and DDMAI at 5, 10, or 20 µg/mL concentrations indicating that these monomers exhibited antimicrobial action against the tested microorganisms in a dose-dependent manner. The antimicrobial activity of these monomers can be attributed to the presence of positively charged QAM salts with negatively charged halogen counter-ions mainly iodine (DHMAI has iodohexadecane, and DDMAI has iodododecane) groups [10, 13, 18]. The mechanism of antimicrobial activity involves contact killing and is attributed to the interaction of positively charged QAM-based antimicrobial monomer, and negatively charged bacterial cell membrane. DHMAI with a C16 alkyl chain and DDMAI with a C12 alkyl chain penetrate the bacterial cell and break the cell membrane, allowing cytoplasmic substances to seep out, resulting in bacterial cell death [11].

The antimicrobial activity of these monomers was also assessed after adding them to PMMA. Interestingly, the addition of these monomers to PMMA did not show any zone of inhibition against any of the microorganisms tested. This may seem to indicate that modified PMMA resin does not exhibit any antimicrobial action. However, it is to be noted that in disc diffusion testing, it is essential for the antimicrobial additive to leach, and diffuse into the agar medium for it to exhibit antimicrobial action [19]. The copolymerization of antimicrobial monomers added to PMMA entrapped them in the PMMA matrix making it difficult for them

to leach out. Pure monomers, on the other hand, could easily diffuse into the agar medium, and hence exhibited antimicrobial action [1].

This was further corroborated by the results of the adherence test in which a significant reduction in the number of CFU was observed especially for *S. mutans*. This indicates that the added monomers are present within and on the surface of the PMMA which prevented the adhesion of microorganisms which is also corroborated by microscopic images of the specimens containing antimicrobial monomers. Both antimicrobial monomers used in the present study significantly reduced the adhesion of *S. mutans* compared to *S. aureus*, and *C. albicans* [13]. In *S. mutans*, Guanylate-binding proteins (GBP) a glycan-binding protein promotes the growth of extracellular polymeric substances (EPS) in biofilm whereas, in *S. aureus*, few enzymes such as proteases, nucleases, teichoic acids (an integral part of the cell wall) play a role, indicating that the EPS production is species-specific. Hence the monomers interfere with different types of proteins differently. Thus, the rate of adhesion, and also inhibition by the test compound may vary among the bacteria [20].

Although, antimicrobial activity is highly desired, for its successful long-term use in clinical conditions, biological compatibility is equally important. Fully polymerized PMMA denture base resins are highly biocompatible. However, the leaching of unpolymerized monomers from the PMMA matrix, especially in self-cure, may lead to adverse reactions such as tissue irritation, allergic reaction, and inflammation [21, 22]. In the present study, cytotoxicity of two polymerizable antimicrobial monomers viz., DHMAI, and DDMAI were investigated after incorporating into PMMA material. DHMAI and DDMAI monomers were found to be cytocompatible in polymerized, and unpolymerized forms. Although dose-dependent cytotoxicity was observed for unpolymerized monomers, the same was not observed upon adding these monomers to PMMA denture base resins. Assessment of cytotoxicity of modified PMMA resin stored in artificial saliva at different time intervals also did not show increased cytotoxicity. For both the monomers, more than 70% of cells were viable even after one month at all the concentrations tested. This suggests that modified PMMA material is cytocompatible, and satisfies the requirements of cytotoxicity according to ISO (1999)10993-5 standard [23].

An increase in surface hardness of PMMA resin was observed with the addition of antimicrobial monomers although it was not statistically significant. An improvement in the hardness could be due to the copolymerization and cross-linking of the monomers with the PMMA matrix. A reduction in the water sorption can be attributed to the hydrophobic nature of the antimicrobial monomers added to PMMA resin. Such hydrophobic nature reduces the tendency of denture base resin to absorb less water and hence superior hardness and less water sorption.

In summary, QAM-based monomers with antimicrobial activity were synthesized and characterized. Optimized concentrations of these monomers were added to conventional heat-activated denture base

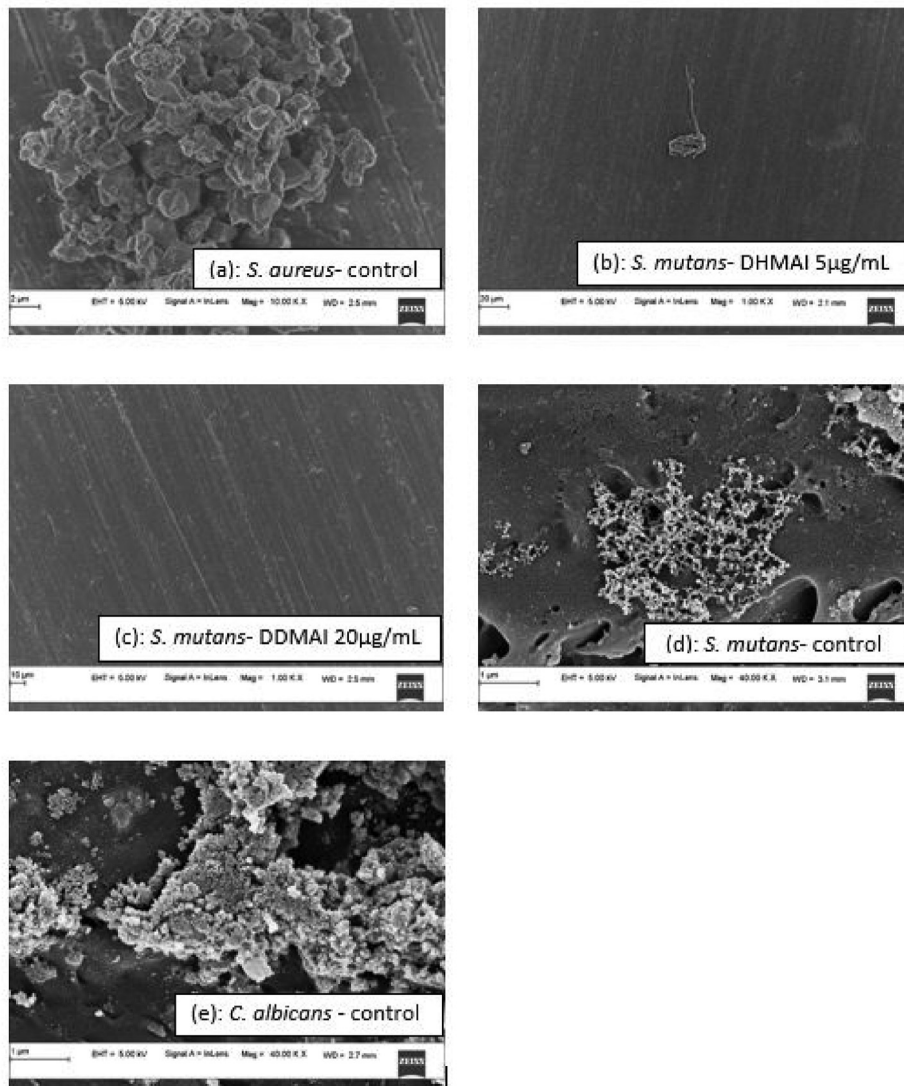


Figure 10. Adherence of microorganisms on control and experimental groups. Scanning electron microscopy images of (a) *S. aureus*-control; (b) *S. mutans*- DHMAI 5 µg/mL; (c) *S. mutans*- DDMAI 20 µg/mL; (d) *S. mutans*-control; (e): *C. albicans* – control.

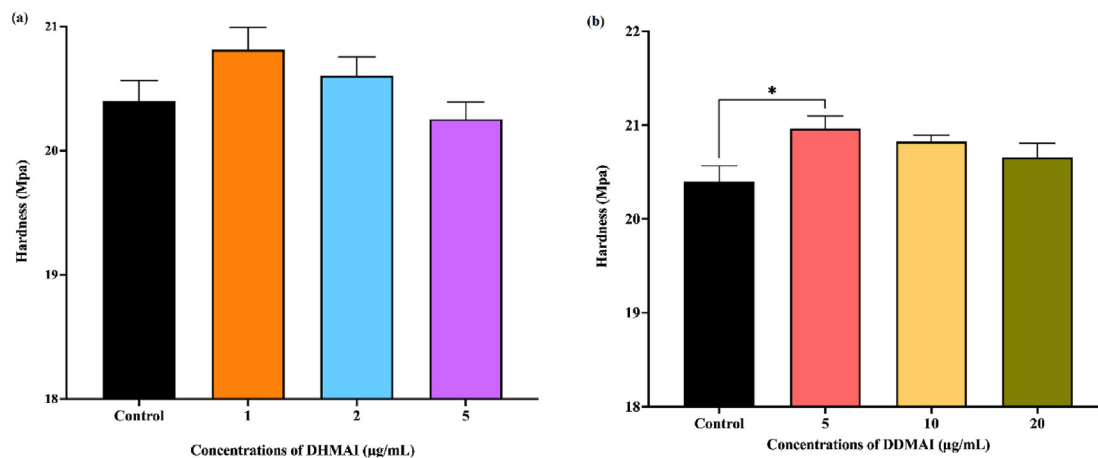
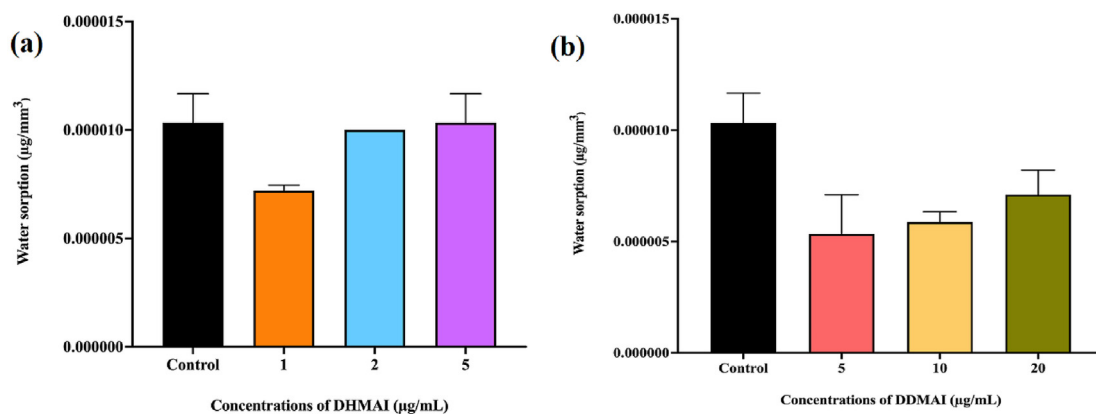


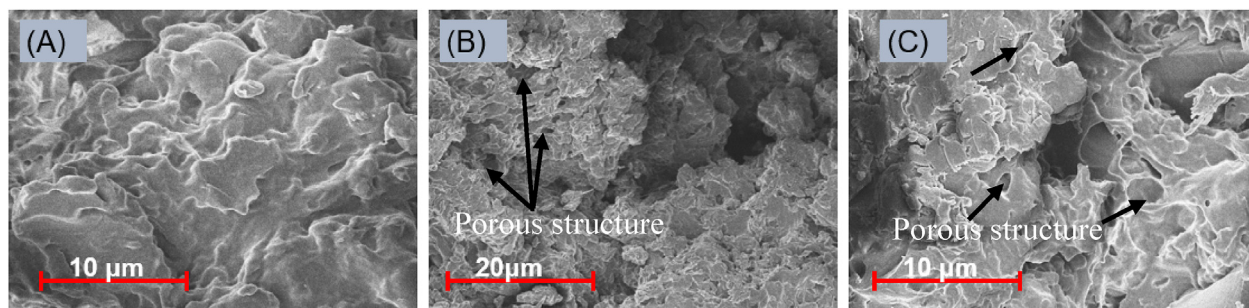
Figure 11. Vickers hardness of modified PMMA resin containing antimicrobial monomer (a) DHMAI (1, 2, or 5 µg/mL), and (b) DDMAI (5, 10, or 20 µg/mL).

materials, and their properties were investigated. The incorporation of antimicrobial monomers imparted antimicrobial action and reduced the

adhesion of microorganisms to the modified denture base material in a dose-dependent manner without significantly affecting other properties.



**Figure 12.** Water sorption of modified PMMA resin containing antimicrobial monomer (a) DHMAI (1, 2, or 5 µg/mL) and (b) DDMAI (5, 10, or 20 µg/mL).



**Figure 13.** FESEM showing surface morphology of (a) PMMA (b) PMMA modified with DHMAI monomer, and (c) PMMA modified with DDMAI monomer.

However, the results of the present study cannot be extrapolated to the clinical performance of the materials due to the difference in the controlled testing conditions of *in vitro* and oral cavity. In addition, the long-term biocompatibility of PMMA denture base material incorporated with antimicrobial monomers as well as long-term antimicrobial activity was not investigated in the present study. Further, the study investigated the effect of antimicrobial monomers with only a single commercially available PMMA material. In this regard, future studies are warranted to analyze the effect of antimicrobial monomers on different types of PMMA denture base materials, and the effect of processing methods to provide a better understanding of their suitability for clinical usage.

## 5. Conclusions

It can be concluded based on the findings of this investigation, that the addition of DHMAI and DDMAI monomers to conventional PMMA-based denture base resins significantly reduced the surface adhesion of microorganism to the denture surfaces. Further, the addition of these monomers, increased surface hardness, and reduced water sorption of the denture base resins. Hence DHMAI and DDMAI can be considered as potential candidates for imparting antimicrobial activity to polymeric denture base materials to combat DS.

## Declarations

### Author contribution statement

Sowmya Rao; Nandish B.T; Kishore Ginjupalli: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Namitha K. Preman; Renjith P. Johnson; Preethishree; Ashwini Prabhu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ranajit Das: Analyzed and interpreted the data.

Jayaprakash K; Vidya Pai: Conceived and designed the experiments.

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### Data availability statement

Data will be made available on request.

### Declaration of interest's statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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