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Essential oils enriched Dant-Kanti-Gandush (oil-pulling) inhibits inter-kingdom biofilm formation on orthodontic fixtures and ameliorates cariogenic virulence factors of oral pathogens

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Orthodontic fixtures provide conducive niche for microbial colonization and inter-kingdom biofilm formation, exacerbating oral hygiene challenges. Conventional mouthwashes, though effective, are associated with adverse effects and potential antimicrobial resistance. Oil-pulling is an Indian traditional method of oral detoxification. This study evaluates blend of six essential oils in Dant-Kanti-Gandush (referred as DKG) from *Syzygium aromaticum*, *Mentha piperita*, *Eucalyptus globulus*, *Zanthoxylum armatum* and *Ocimum sanctum*, mixed with coconut and sesame carrier oils, as a potential oil-pulling formulation. Gas chromatography–mass spectrometry confirmed phytochemical composition of DKG. Antimicrobial assays demonstrated MIC_{50} of DKG ranging from 0.10%(v/v) to 0.45%(v/v) against *Streptococcus pyogenes*, *Streptococcus mutans*, *Proteus mirabilis* and *Candida albicans*. DKG delayed the exponential phase and perturbed the growth of these pathogens. At ≥ 1.0×MIC_{50}, DKG impaired cariogenic traits of *S. mutans* with reduced biofilm formation, acid production and lower survival under acidic stress. DKG inhibited biofilm formation, hyphal transition and depleted total ergosterol levels in *C. albicans*, thereby disrupting cell wall integrity. Scanning electron microscopy on orthodontic fixtures revealed that DKG reduced microbial density, disrupted bacterial aggregation and fragmented hyphae in cross-kingdom biofilms of *S. mutans* and *C. albicans*. These findings highlight plant-based DKG as an anticariogenic alternative for maintaining oral health in individuals with orthodontic fixtures.

Keywords Oil-pulling, Essential oil, Dant-Kanti-Gandush, Orthodontic fixtures, *S. mutans-C. albicans* cross-kingdom biofilms, Cariogenic, Oral health

Abbreviations

DKG	Dant-Kanti-Gandush
MIC	Minimum inhibitory concentration
SEM	Scanning electron microscopy
EPS	Extracellular polymeric substance

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OD	Optical density
GC-MS/MS	Gas chromatography-mass spectrometry
UHPLC	Ultra-High-Performance Liquid Chromatography

Dental caries, commonly known as tooth decay or cavity formation, are one of the most prevalent global health issues, affecting individuals of all ages[1]. According to the WHO Global Oral Health Status Report (2022), nearly 3.5 billion people are affected by oral diseases, with 3 out of 4 people living in middle-income countries. Specifically, about 2 billion people suffer from caries of permanent teeth, and 514 million children are affected by caries of primary teeth. Interestingly, oral cavity harbors a complex microbiome, consisting of approximately 700 bacterial species that form biofilms on surfaces like teeth, gingiva, and prosthetic devices[2,3]. *Streptococcus mutans* along with fungal yeast, *Candida albicans*, are key contributors to dental caries and biofilm formation lays the foundation for their development[4] *S. mutans*, initiates biofilm development by adhering to salivary pellicles on tooth surfaces, aided by extracellular polymeric substances (EPS), mainly glucans produced by glucosyltransferases[5]. These glucans facilitate bacterial aggregation and biofilm maturation, making the biofilm highly resilient and structured. Additionally, *Candida albicans*, another key pathogen involved in biofilm formation, produces organic acids such as pyruvate and formate, which lower the pH of the surrounding environment, further promoting enamel demineralization. This acidic environment created by *S. mutans* and *C. albicans* enhances bacterial survival and accelerates tooth decay[6,7]. *Streptococcus pyogenes* has been associated with dental plaque while *Proteus mirabilis*, is commonly linked to urinary tract infections, has also been isolated from the oral cavity[8,9].

Orthodontic surfaces provide niche for bacterial adhesion and biofilm development, they contribute to increased bacterial load, enamel demineralization, and inflammation of the gingiva[10]. Therefore, preventing biofilm formation is essential in the management of dental caries and preserving oral health, particularly in individuals with orthodontic fixtures, where biofilm control is challenging yet crucial for long-term oral health maintenance.

Oil pulling, also known as *Gandusha* or *Kavala Graha* in Ayurvedic medicine, is a traditional Indian remedy practiced for centuries to maintain oral hygiene and overall health. The technique involves swishing edible oils such as coconut, sesame, or sunflower oil in the mouth for several minutes to facilitate oral detoxification and enhance oral hygiene. It is believed that oil pulling works by emulsifying and trapping bacteria within the oil, effectively reducing microbial load, dissolving plaque biofilms, and promoting gum health. Unlike commercial mouthwashes, oil pulling is safe, accessible, and free from side effects such as staining, unpleasant aftertaste, or allergic reactions, making it a favorable adjunct to conventional oral hygiene practices[11,12]. Coconut oil contains lauric acid, which has shown antimicrobial properties, significantly reducing the levels of *Streptococcus mutans*, a key bacterium responsible for dental caries. Studies have demonstrated that oil pulling with antimicrobial oils not only reduces plaque accumulation but also promotes gum health by combating harmful bacteria and supporting oral hygiene. In orthodontic settings, essential oils such as eucalyptol have been shown to reduce biofilm thickness and bacterial colonization on orthodontic fixtures, including brackets and arch wires[13]. Other essential oil components like limonene and linalool disrupt bacterial cell membranes, inhibiting the growth of *S. mutans*, further highlighting their role in managing biofilm-related complications[14,15].

The current study demonstrates the antimicrobial activity of essential oils of Dant-Kanti-Gandush (DKG) against oral pathogens, including *S. mutans*, *P. mirabilis*, *S. pyogenes*, and *C. albicans.* DKG is a combination of essential oil extracted from clove (*Syzygium aromaticum*), peppermint (*Mentha piperita*), eucalyptus (*Eucalyptus globulus*), prickly ash (*Zanthoxylum armatum*), and basil (*Ocimum sanctum*). DKG could disrupt the cross-kingdom colonization and biofilm formation of *S. mutans* and *C. albicans* on orthodontic braces. These findings suggest that DKG may serve as a safe, natural adjunct for managing dental caries and biofilm-associated oral infections, reinforcing the role of Ayurveda as a complementary and alternative medicine.

Materials and methods

Procurement of test article, microbial strains and growth conditions

The test article, a blend of essential oil present in Dant-Kanti-Gandush (internal batch# PRF/CHI/0424/0288) was supplied by Herbal Chemistry Department, Patanjali Research Foundation, Haridwar, India. The study employed specific microbial strains, which were sourced from Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology (Chandigarh, India). The bacterial strains used included *Streptococcus mutans* (MTCC 497), *Streptococcus pyogenes* (MTCC 442), and *Proteus mirabilis* (MTCC 1429), as well as the fungal strain *Candida albicans* (MTCC 183). Among the four pathogens tested in the study, *Streptococcus mutans* (MTCC 497) is the clinically isolated oral pathogen strain, initially collected from carious dentine (decayed tooth material)[16–18]. The clinical isolate MTCC 497 is also syn-classified as *S. mutans* Clarke (ATCC 25175) at American Type Culture Collection, USA or NCTC 10449 at National Collection of Type Cultures, UK or DSM 20523 at German Collection of Microorganisms and Cell Cultures, Germany. Bacterial species were cultured in Brain Heart Infusion (BHI) broth (HiMedia, India), while the yeast (*Candida albicans*) was cultured in Yeast Extract Peptone Dextrose (YPD) broth (HiMedia, India). Single isolated colonies of each microorganism were inoculated in their respective media and incubated at 37 °C for 24 h. For routine maintenance, these strains were sub-cultured onto Brain Heart Infusion agar and Yeast Extract Peptone Dextrose agar and preserved as glycerol stocks at − 80 °C for long-term storage.

Gas Chromatography-Mass spectrometry (GC-MS/MS) analysis of Dant-Kanti-Gandush essential oil (DKG)

GC-MS/MS (7000D GC/MS triple quad with 7890B GC system, Agilent, USA) was performed using mass hunter software to identify, quantify, and characterize chemical compounds present in DKG. Separation was

carried out using Agilent HP-5MS capillary column (30 m x 0.25 mm, 0.25 µm). Helium was used as carrier gas at a flow rate of 1 mL/min. The temperature of the split injector was maintained at 280 °C and the split ratio was 20:1. The column temperature was initially set at 60 °C without hold, then ramped at 5 °C/min to 100 °C (held for 1 min), followed by an increase at 3 °C/min to 160 °C (held for 3 min), and finally ramped at 10 °C/min to 280 °C, where it was held for 1 min. The GC-MS temperature was 230 °C and ionization potential was 70 eV.

Antimicrobial effects of Dant-Kanti-Gandush essential oil (DKG)

Antibacterial activity of DKG was evaluated using the disk diffusion method. The optical density (OD) of an overnight-grown culture was adjusted to 0.06 at 600 nm, and the bacterial culture was swabbed onto Muller Hinton Agar, whereas *Candida albicans* yeast cells were swabbed on Yeast Peptone Dextrose agar. Sterile disks carrying varying concentrations of DKG (5% (v/v), 25% (v/v), 50% (v/v), and 100% (v/v)) were placed on the agar plates. A sterile disk impregnated with sterile water was included as a negative control and placed at the center of the plate. The plates were incubated at 37 °C for 24 h.

Microbroth-dilution assay

The antibacterial activity of DKG was assessed using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines. The Minimum Inhibitory Concentration (MIC) test was conducted in 96-well tissue culture microplates, each containing 100 µL of growth media. The stock solution of DKG was initially diluted to 10% (v/v) by adding sterile autoclaved water, and this was transferred to the first well. Two-fold serial dilutions were subsequently performed, resulting in concentrations ranging from 5.0% (v/v) to 0.02% (v/v). The microbial inoculum (OD 0.06–0.08 at 600 nm) was added to each well, except for the blank control wells, containing only the medium with respective DKG concentration. The plates were incubated at 37 °C for 24 h. Absorbance values at OD_{600} (Infinite 2000 Pro microplate reader, Tecan Group Ltd., Switzerland) were used to evaluate DKG-mediated growth inhibition in percentage with reference to untreated condition. Using the inbuilt option of Prism 8.0.2 software (GraphPad Software Inc., MA, USA), minimum inhibitory concentration (MIC) of DKG inhibiting 50% (MIC_{50}) and 90% (MIC_{90}) of microbial growth were determined by plotting non-linear regression curve fit. The experiments were repeated in three biological replicates.

Growth kinetic assessment in oral pathogens

The microbial cultures were adjusted to an optical density (OD) 0.06–0.08 and treated with different concentrations of DKG (0.25× MIC_{50}, 0.50× MIC_{50}, 1.0× MIC_{50}, 2.0× MIC_{50} and 4.0× MIC_{50}) in 96-well microtiter plate. Growth kinetics of the bacterial and yeast cultures were recorded every two hours while their incubation at 37 °C for 24 h in Infinite 2000 Pro microplate reader (Tecan Group Ltd., Switzerland). All experiments were performed in independent triplicate to ensure consistency and reproducibility. The recorded absorbance, after blank correction, was plotted using a non-linear regression curve fit function.

Assessment of biofilm formation

Biofilm biomass assessment was performed by crystal violet staining method and scanning electron microscopy. Actively growing *S. mutans* culture adjusted at optical density of 0.1 at 600 nm in BHI media (supplemented with 1% sucrose) statically established biofilms on the sterile cover slips in the 6-well plate under aerobic incubation in an incubator with 5% CO_2 at 37 °C[19]. Biofilm in *C. albicans* was developed statically on the sterile cover slips in the 6-well plate in YPD broth (supplemented with 50 mM glucose). Subsequently while setting the biofilms, DKG was added at different concentrations (0.5× MIC_{50}, 1× MIC_{50} and 2× MIC_{50} to the cultures. For dual-species co-culturing, equal volumes (1:1) of the above cultures in their respective media were added to either sterile coverslips in a 6-well plate or elastomeric ligatures and allowed to develop biofilm statically under aerobic incubation in an incubator with 5% CO_2 at 37 °C[19]. The supernatant was aspirated after 72 h of incubation and the tightly adhered cells forming biofilms were washed twice with PBS to remove planktonic bacterial cells. Biofilm was fixed with 2.0% formaldehyde for 20 min, followed by crystal violet (0.1%) staining for 30 min. Once dried, the stained biofilms on cover slips were photographed under an TS2 inverted brightfield microscope (Nikon, Japan). The surface area covered by the biofilm was evaluated using ImageJ software (US National Institutes of Health, USA). Elastomeric ligatures were imaged under stereomicroscope (Leica microsystems, Germany). Crytal violet stain retained by the biofilms was further solubilized in 95% (v/v) ethanol and measured the optical density at 570 nm (Infinite 2000 Pro microplate reader, Tecan Group Ltd., Switzerland) to quantify biofilm biomass. Obtained absorbance values were blank corrected and represented as absorbance in bar graph or used to assess percentage change with respect to the untreated[17–19]. The biofilms were examined for morphological and structural changes in the presence of DKG using scanning electron microscope (FlexSEM1000, Hitachi, Japan).

Biofilm metabolic activity

Colorimetric alamar blue assay assesses the biofilm susceptibility to DKG by determining the metabolic activity of the biofilm[20]. Coverslips and elastomeric ligatures with biofilms formed by *S. mutans* and *C. albicans*, either alone or in co-culture conditions were thoroughly washed by phosphate buffer saline and incubated at 37 °C for 2 h with 1x alamar blue reagent prepared in the culture media. Reduction of resazurin in alamar blue assay, indicates the metabolic activity and viability of cells that was measured via fluorescence at an excitation/emission of 560/590 nm Infinite 2000 Pro microplate reader, Tecan Group Ltd., Switzerland). The obtained arbitrary fluorescent units were expressed as a percentage change in metabolic activity compared to the untreated.

Glycolytic pH drop assay

Using a pH-drop experiment, the impact of DKG on *S. mutans* acidogenicity was assessed. The actively growing *S. mutans* culture was harvested, washed and resuspended in the buffer containing 50 mM potassium chloride (KCl) and 1 mM magnesium chloride ($MgCl_2$) in the presence of DKG at different concentrations (0.25× MIC_{50}, 0.50× MIC_{50}, 1.0× MIC_{50}, 2.0× MIC_{50} and 4.0× MIC_{50}). Glucose was added to a final concentration of 55.6 mM, and the initial pH of the mixtures was adjusted to 7.2–7.4 using 1.0 M potassium hydroxide. Change in the pH was recorded (Laboholic Microprocessor pH meter, India) at 20-minute intervals over a total duration of 100 min[21,22]. The experiment was conducted in triplicate to ensure reproducibility.

Acid tolerance assay

Acid tolerance is defined as the ability of bacteria to survive in acidic environments, a critical characteristic of *S. mutans*, which employ both constitutive and acid-inducible mechanisms (Matsui et al., 2010). The effect of DKG on the acidurity of *S. mutans* was assessed by exposing bacteria to an acidic pH of 5.0. Actively growing *S. mutans* bacterial cells were harvested to resuspend in TYEG (Tryptone Yeast Extract Glucose) broth (pH 5.0) in the presence of DKG at sub-inhibitory and inhibitory concentrations of DKG (0.5%, 1%, and 2% (v/v)) at 37 °C for 6 and 24 h[22]. The untreated control group contained no DKG. After incubation, viability of *S. mutans* was determined from all treatment conditions by plating the bacterial cells on Brain heart infusion agar plates. The experiment was independently repeated three times to ensure reproducibility (15).

Hyphal growth assessment in *C. albicans*

Candida albicans cultured in Yeast Peptone Dextrose (YPD) medium supplemented with 10% Fetal Bovine Serum to induce hyphal formation[23]. Yeast culture adjusted to an optical density of 0.1 at 600 nm was inoculated in hyphal-inducing media (YPD medium containing 10% Fetal Bovine Serum) in the presence of DKG (0.5× MIC_{50}: 0.05% (v/v), 1× MIC_{50}: 0.10% (v/v), and 2× MIC_{50}: 2.0% (v/v)) at 37 °C with shaking for 6 h and 24 h. After incubation cell morphology was observed and photographed using a Zeiss Observer.Z1 microscope (Carl Zeiss, Jena, Germany) (16).

Ultra-high performance liquid chromatography (UHPLC) coupled with diode array detection (UHPLC-DAD) based ergosterol detection

From an overnight-grown culture, a 100 mg pellet of untreated yeast cells and cells treated with DKG (at 0.5x, 1.0x and 2.0x MIC_{50}) was dissolved in 0.25 mL of methanol and homogenized by adding sterile stainless-steel balls for 10 min. This solution was then centrifuged for 5 min at 10,000 rpm and 100 µl of supernatant was transferred in injecting vials and injected into the system. Ergosterol standard (of 1 mg/mL) in methanol was used to prepare 1000 ppm standard solution. 0.1 ml of this standard solution was diluted to 10 ml to prepare 10 µg/mL working stock. Ergosterol content of the treated and untreated *C. albicans* was analyzed on Prominence-XR UHPLC system (Shimadzu, Japan) equipped with Quaternary pump (Nexera XR LC-20AD XR), DAD detector (SPD-M20 A), Auto-sampler (Nexera XR SIL-20 AC XR), Degassing unit (DGU-20 A 5R) and Column oven (CTO-10 AS VP). Separation was achieved using a Shodex C18-4E (5 µm, 4.6*250 mm) column subjected to isocratic elution with a flow rate of 1.0 mL/min. The mobile phase was used for the analysis consisted of the ratio of methanol: acetonitrile (80:20). 50 µL of standard and test solutions were injected and wavelength was set at 280 nm.

Scanning electron microscopy on orthodontic fixtures

The dual-species biofilms were formed using the same method as described in the above section, with the only difference being that clean orthodontic brackets and rings (Koden orthodontic brackets, provided by Dental Clinic and Research Centre, Patanjali Ayurved Hospital, Haridwar, India) replaced the coverslips in the six-well plate. Subsequently, DKG (1.0% (v/v)) was added to the co-cultures and plates were aerobically incubated at 37 °C for 72 h in 5% CO_2 (17). Orthodontic brackets and rings were thoroughly washed to remove planktonic cells, fixed in formaldehyde and air dried before imaging under SEM scanning electron microscope (FlexSEM1000, Hitachi, Japan) to observe the effect of DKG on dual-species biofilms.

RESULTS

Gas Chromatography-tandem mass spectrometry (GC-MS/MS) analysis of Dant-Kanti-Gandush essential oils (DKG)

The oil-pulling formulation, DKG consists of 90% (v/v) *Sesamum indicum* (sesame) oil and 8% *Cocos nucifera* (coconut) oil. The oil base, constituting 98% of the formulation, is supplemented with a 1.5% essential oil blend from *Syzygium aromaticum*, *Mentha piperita*, *Eucalyptus globules*, *Zanthoxylum armatum* and *Ocimum sanctum*, in a ratio of 2:3:8:1:1, respectively (Table 1). The essential oil blend of Dant-Kanti-Gandush (hereafter, called as DKG) was subjected to detailed microbiological testing, and GC-MS/MS analysis for phytochemical profiling. The chromatograph generated demonstrated prominent peaks, which were subsequently identified by matching mass fragmentation data with the National Institute of Standards and Technology (NIST, USA) library (MS Search 2.2). Notably, the identified phytometabolites, D-limonene, eucalyptol, linalool, menthol and eugenol were further validated and quantified against their respective reference standard (Fig. 1A). The quantified content percentage (w/w) for D-limonene, eucalyptol, linalool, menthol and eugenol were 5.64%, 21.79%, 3.34%, 5.55%, and 3.32%, respectively (Fig. 1B).

Dant-Kanti-Gandush demonstrated remarkable antimicrobial activity against oral pathogens

This study tested the anti-microbial activity of DKG, a blend of essential oils, against few common oral pathogens associated with dental caries and periodontal infections, such as *Streptococcus mutans*, *Proteus*

S. No.		English name	Scientific name	Plant Part	Form	Qty. (g)
1	Essential oils	Clove	*Syzygium aromaticum*	Buds	Oil	0.20
2		Peppermint	*Mentha piperita*	Leaves	Oil	0.30
3		Eucalyptus	*Eucalyptus globules*	Leaves	Oil	0.80
4		Prickly ash	*Zanthoxylum armatum*	Seeds	Oil	0.10
5		Basil	*Ocimum sanctum*	Leaves	Oil	0.10
6	Carrier oils	Coconut	*Cocos nucifera*	Endosperm	Oil	8.00
7		Sesame	*Sesamum indicum*	Seeds	Oil	90.00
8		Flavour	-	-	-	0.50

Table 1. Composition of Dant-Kanti-Gandush.

mirabilis, *Streptococcus pyogenes*, and *Candida albicans*. Initial screening by disc diffusion demonstrated the potential antimicrobial activity of DKG, tested at 50% (v/v) and 100% (v/v) against these oral pathogens. DKG against *Streptococcus pyogenes* showed a zone of inhibition of 10 mm ± 0.40 at 50% (v/v) and 11.5 mm ± 0.54 at 100% (v/v). The bacterial lawn of *Proteus mirabilis* exhibited < 10 mm and 12.1 mm ± 0.75 zone at 50% (v/v) and 100% (v/v), respectively. DKG against *Streptococcus mutans* showed < 10 mm and 10.8 mm ± 1.16 of clear zone at 50% (v/v) and 100% (v/v), respectively. Most prominent clearance with zone diameters of 11.8 mm ± 1.83, 19 mm ± 2.96 and 25 mm ± 2.28 at 25% (v/v), 50% (v/v) of DKG, respectively was observed against *Candida albicans* (Fig. 2A). Carrier oils of Dant Kanti Gandush, sesame oil and coconut oil, were also tested in these experiments with minimal effects (Data not shown).

Minimum inhibitory concentrations (MIC) corresponding to MIC_{50} and MIC_{90} were determined using broth microdilution method to evaluate the DKG concentrations that inhibited 50% and 90% of visible microbial growth, respectively. For *Streptococcus pyogenes*, DKG exhibited MIC_{50} and MIC_{90} at 0.10% (v/v) and 0.17% (v/v), respectively (Fig. 2B). MIC_{50} and MIC_{90} for DKG against *Proteus mirabilis* were evaluated to be 0.29% (v/v) and 1.59% (v/v), respectively (Fig. 2C). MIC_{50} and MIC_{90} for DKG against *Streptococcus mutans* were evaluated as 0.45% (v/v) and 1.70% (v/v), respectively (Fig. 2D). DKG demonstrated MIC_{50} and MIC_{90} against *Candida albicans* at 0.11% (v/v) and 0.64% (v/v), respectively (Fig. 2D).

Dant-Kanti-Gandush delayed exponential growth phase of the oral pathogens

The growth kinetics in Fig. 3 (A-D), demonstrated a time-dependent anti-microbial activity of DKG over a 22 h incubation period. The untreated controls from all four pathogens tested exhibited continuous logarithmic growth up to 22 h, whereas DKG exposure retarted the growth progression profiles. In *Streptococcus pyogenes*, a delay in the onset of the exponential phase was evident at 1.0× and 2.0× MIC_{50} of DKG compared to the untreated control (Fig. 3A). Similarly, in *Proteus mirabilis*, DKG not only delayed the exponential phase by 4 h at 0.25× MIC_{50} of DKG, but the pathogen also showed bacteriostatic growth at 0.5× MIC_{50} and complete inhibition with DKG at 1.0× and 2.0× MIC_{50}. (Fig. 3B). A pronounced inhibition in growth dynamics was observed in *Streptococcus mutans*, where half-maximal growth was achieved in less than 4 h. DKG delayed the onset of lag phase and exponential growth phase in *S. mutans* and pushed the half-maiximal growth to 8 h and 16 h at 0.5× MIC_{50} and 1.0× MIC_{50}, respectively. At the highest dose of 2.0× MIC_{50}, no growth progression was observed (Fig. 3C). *Candida albicans* attained half-maximal growth by 8 h. DKG at 1.0× MIC_{50} and 2.0× MIC_{50} delayed the exponential phase and achieved the half-maximal growth by 10 h and 13 h (Fig. 3D). Collectively, DKG limited the microbial proliferation rate and even inhibited the pathogens to achieve usual population density and biomass required for achieving stationary phase.

Dant-Kanti-Gandush suppressed cariogenic properties of *S. mutans*

S. mutans contribute substantially to oral biofilm formation, enamel demineralization posing risks to oral and overall health. DKG was evaluated for its impact on the biofilm forming potential of *S. mutans*. Untreated samples exhibited dense biofilm formation, whereas DKG treatment at 0.5× MIC_{50} and above showed a dose-dependent significant disruption in the biofilms formed (Fig. 4A). ImageJ analysis revealed a significant reduction in the surface area covered by biofilm in the presence of DKG. The surface area covered reduced by ~ 28%, ~ 31% and ~ 61% at 0.5×, 1.0× and 2.0× MIC_{50} of DKG (Fig. 4B). The absorbance of solubilized-crystal violet stain that was extracted from the biofilms indicating total biofilm biomass demonstrated a dose-dependent reduction with DKG (Fig. 4C). The viability and metabolic activity of biofilms was also assessed with alamar blue dye. There was a significant reduction in the metabolic activity of biofilms formed by *S. mutans* in the presence of 1.0× and 2.0× MIC_{50} of DKG (Fig. 4D). The effect of DKG on *S. mutans* acidogenicity was determined by monitoring glycolytic pH drop (Fig. 4E). In untreated cultures, pH decreased from 7.01 ± 0.03 to 3.89 ± 0.12 within 100 min, indicating acid production capabilities of *S. mutans*. However, DKG treatment significantly delayed the pH drop, with terminal pH values of 5.30 ± 0.61, 5.95 ± 0.61, and 6.95 ± 0.00 at 0.5×, 1.0×, and 2.0× MIC_{50}, respectively (Fig. 4E). Higher concentrations of DKG notably impeded acid production. An exponential decay curve was generated on the obtained pH values to calculate Tau (τ) constant. The decay rate (τ^{-1}) evaluated by fit function for untreated (41.14), 0.5×MIC_{50} (83.30), 1.0×MIC_{50} (74.36) and ambiguous results for 2.0×MIC_{50} indicated inhibited acidogenicity potential in *S. mutans* in the presence of DKG targeted. In addition, the acid tolerance capability of S. *mutans*, when evaluated in the presence of DGK, on the contrary showed notable reduction in the number of viable *S. mutans* colonies at 6 h and 24 h under acid stress. At 6 h, viable counts calculated

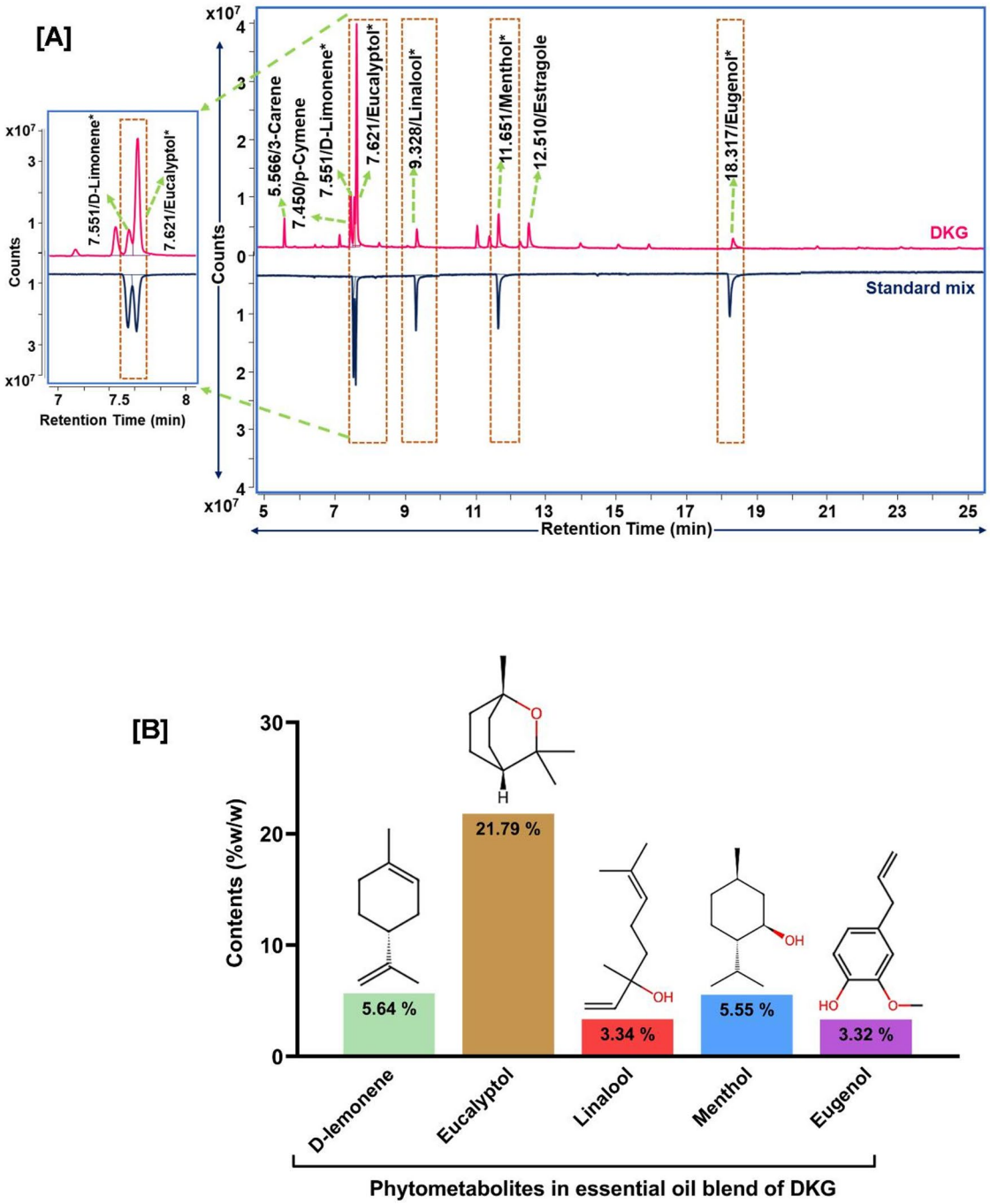


Fig. 1. Gas Chromatography-tandem mass spectrometry (GC-MS/MS) analysis of essential oils of Dant-Kanti-Gandush (DKG). (**A**) Representative chromatograms of DKG and a standard mixture, illustrating the retention times of identified phytometabolites. Major peaks corresponding to key phytocompounds are annotated. (**B**) Quantitative analysis of predominant phytometabolites in DKG, presented as bar graphs alongside their chemical structures. Values represent the relative percentage composition of each compound.

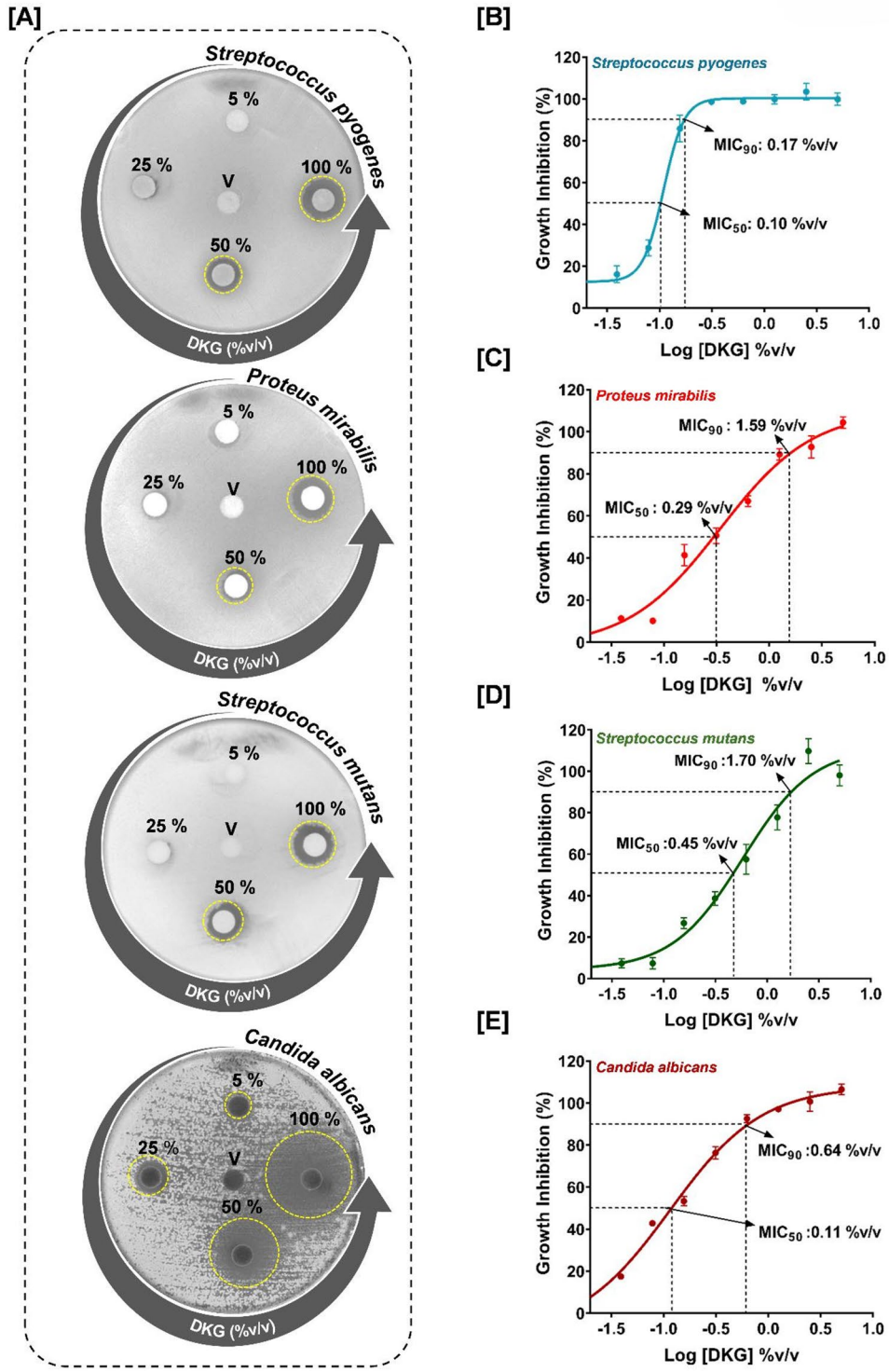


Fig. 2. DKG demonstrated remarkable antimicrobial activity against oral pathogens. (**A**) Antimicrobial activity of DKG against different microbial strains, by the disk diffusion method. The inhibition zones correspond to different concentrations of DKG. (B-E) A dose-response curve was plotted using non-linear regression based on data from broth microdilution assays of DKG against *S. pyogenes* (**B**), *P. mirabilis* (**C**), *S. mutans* (**D**) and *C. albicans* (**E**). MIC_{50} and MIC_{90} values are marked with arrows, indicating the concentrations required to inhibit 50% and 90% of microbial growth, respectively. Data represents the mean from three independent experiments, with error bars indicating mean ± SEM.

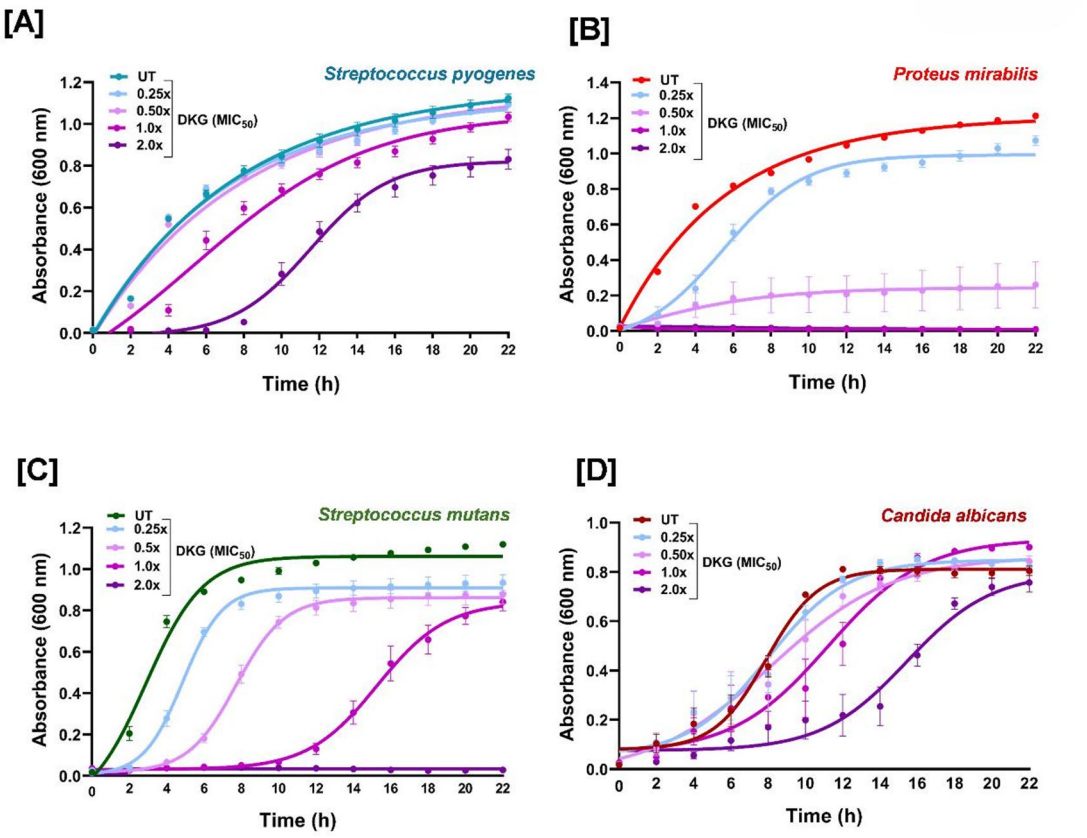


Fig. 3. DKG decelerated the exponential growth phase of oral pathogens. Growth kinetics of microbial strains in the presence of different concentrations (0.25×, 0.5×, 1.0× and 2.0× MIC_{50}) of DKG. Absorbance (OD_{600}) was measured over time to assess the inhibitory effects of DKG on (**A**) *Streptococcus. pyogenes*, (**B**) *Proteus mirabilis*, (**C**) *Streptococcus mutans* and (**D**) *Candida albicans*. Each curve represents microbial growth under varying DKG concentrations, with higher concentrations showing stronger inhibition. Data represents mean from triplicate experiments, with error bars indicating mean ± SEM.

as CFU/mL reduced from 725×10^6 (Untreated) to 329×10^6 (0.5×MIC_{50}) 118×10^6 (1.0×MIC_{50}) and 17×10^6 (2.0×MIC_{50}) (Fig. 4F). Whereas, by 24 h, further reductions were observed from 517×10^6 (Untreated) to 189×10^6 (0.5×MIC_{50}) 4×10^6 (1.0×MIC_{50}). Notably, at 2.0×MIC_{50} no viability of bacteria was observed in the presence of DKG (Fig. 4G).

Dant-Kanti-Gandush inhibited yeast-to-hypha conversion in *C. albicans* and its biofilm formation

Yeast to hyphae transition and biofilm formation are key factors imparting pathogenicity to *Candida albicans*. DKG exposure for 6 h and 24 h notably inhibited the budding and hyphal germination in a dose-dependent manner (Fig. 5A). *C. albicans* biofilm formation also observed a significant inhibition (Fig. 5B). The surface area covered by biofilms was significantly reduced by ~ 45–50% in the presence of DKG (Fig. 5C). Notably, the dose-dependent decrease in crystal violet staining indicating biofilm biomass decreased with DKG (Fig. 5D). Metabolic activity decreased by 20%, 40% and 80% at 0.5×, 1.0× and 2.0× MIC_{50} of DKG (Fig. 5E). Ergosterol is an essential component of fungal cell wall. Disruption in ergosterol biosynthesis or downregulation in ergosterol levels indicate compromised cell membrane integrity[24,25]. In order to test whether DKG could exert similar effect on *C. albicans*, ergosterol levels were evaluated through UHPLC method. The ergosterol content significantly decreased from 100.00 ± 25.27 µg/mg to 33.39 ± 30.67 µg/mg and 19.36 ± 7.50 µg/mg when exposed to DKG at 2.0× MIC_{50} and 4.0× MIC_{50}, respectively (Fig. 5F).

Dant-Kanti-Gandush inhibited biofilm formation of *S. mutans* and *C. albicans* in mono-and co-culture conditions

Dental plaques are tight-structured, multi-species, cross-kingdom biofilms. Their formation not only drives dental caries and other periodontal diseases but also reduces susceptibility to antimicrobial treatments, posing a significant challenge in oral health management. The anti-plaque potential of DKG was evaluated by assessing its efficacy against biofilms formed by *S. mutans* and *C. albicans*, both individually and in a co-cultured interkingdom biofilm model. Scanning electron microscopy (SEM) was performed to morphologically evaluate biofilms

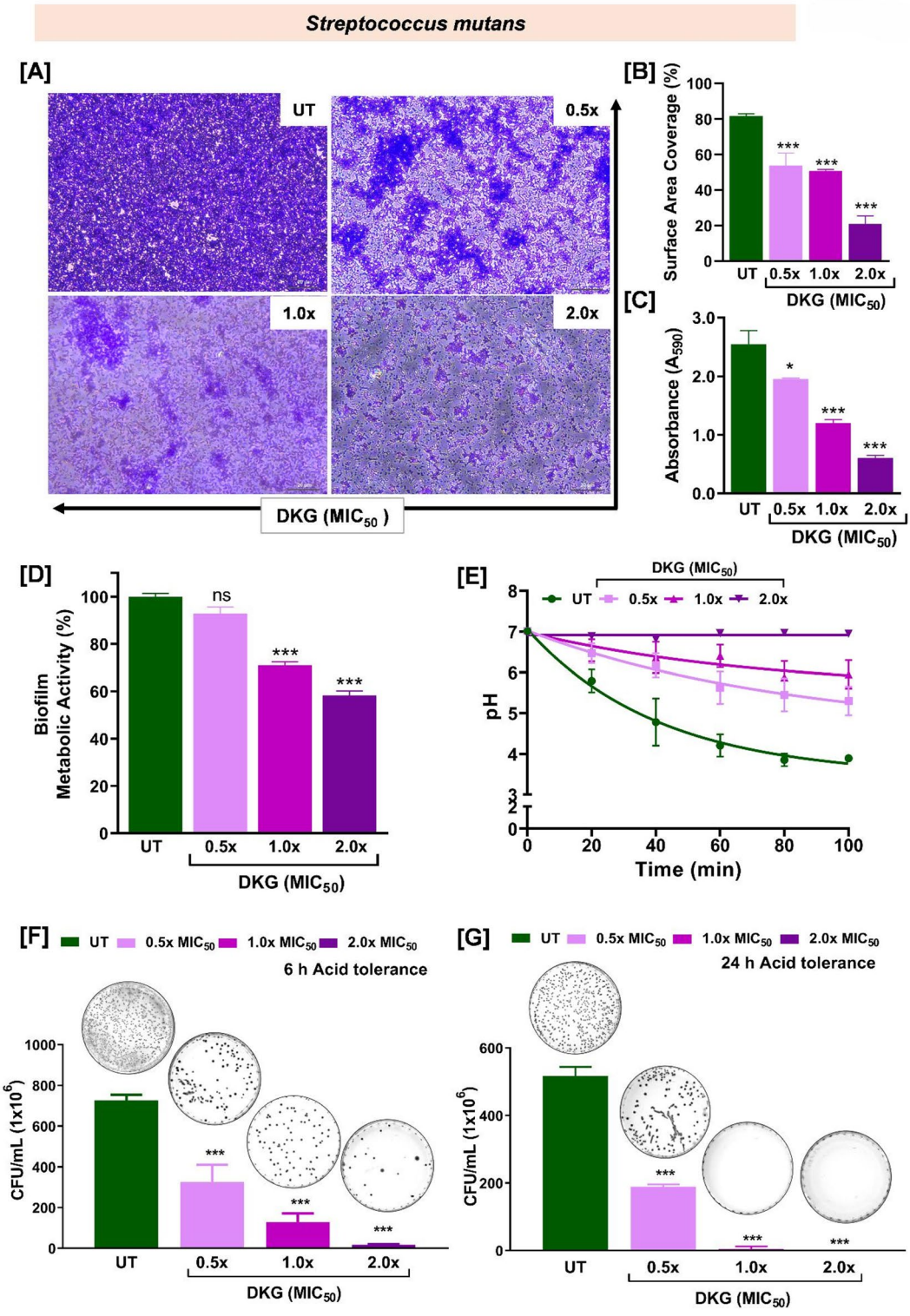
formed on coverslips by indicated oral pathogens. The dense biofilm formed by *S. mutans* showed disrupted and spaced growth in the presence of DKG (Fig. 6A). *Candida albicans* showed a dense growth on the coverslip. However, in the presence of DKG, yeast cells underwent biofilm disruption with deformed yeast structures and even shrinkage (blue arrowheads). This indicated membrane damage or stress confirming the antifungal effect of DKG in suppressing biofilm formed by *C. albicans* (Fig. 6B). The SEM analysis illustrated a stark contrast between the untreated and DKG exposed *S. mutans* and *C. albicans* inter-kingdom biofilms (Fig. 6C). The untreated biofilms illustrate a dense, tight-structured microbial network with extensive interactions between bacterial chains and fungal hyphae (white arrowheads). In contrast, DKG exposure severely disrupted the microbial density (yellow arrowheads), fragmented hyphal structures and disorganized bacterial aggregation.

Dant-Kanti-Gandush prevented cross-kingdom biofilm formation by *S. mutans* and *C. albicans* on the orthodontic fixtures

Orthodontic fixtures provide an ideal niche for microbial adhesion and growth, particularly in the presence of oral saliva[10,26,27]. *S. mutans* and *C. albicans* culture were inoculated on the sterile orthodontic brackets and elastomeric ligature in the presence of their respective growth media. Elastomeric ligatures were incubated in the static co-culture of *S. mutans* and *C. albicans* in the presence and absence of DKG. The biofilm formation was confirmed with crystal violet staining of the elastomeric ligatures that demonstrated a thick dark blue stain picked by untreated (UT) co-cultures. Whereas DKG prevented the biofilm formation that resulted in light crystal violet staining on the ligature. The non-specific binding of crystal violet is shown by the blank control, wherein the ligature was not exposed to any culture medium but stained with crystal violet (Fig. 7A). The crystal violet was further solubilized in ethanol to determine total biofilm biomass on the elastomeric ligature. DKG treatment reduced the total biofilm biomass by nearly 60% (Fig. 7B). Alamar blue assay further indicates that DKG also reduced the metabolic activity by ~ 60% validatng the inhibitory effect of DKG on the cross-kingdom biofilms on the orthodontic ligatures (Fig. 7C). To further corroborate these findings, scanning electron microscopy (SEM) was caried out to visualize the morphological and structural changes in the cross-kingdom biofilms formed on both elastomeric ligature and orthodontic brackets. SEM micrographs captured different locations of elastomeric ligature and orthodontic brackets demonstrated a thick microbial growth, which upon further zooming in showed dense meshwork of hyphal growth by the yeast. *S. mutans* cells appear embedded within the exopolysaccharide (EPS) matrix, reinforcing microbial co-aggregation and resilience. DKG exposure, on the contrary prevented the formation of dense architecture and EPS matrix, thereby inhibiting microbial adherence and biofilm formation. The microbial growth was drastically reduced, with *C. albicans* fragmented hyphae and deformed yeast cells. *S. mutans* appearing sparsely distributed, lysed and ruptured on the surface of both elastomeric ligature (Fig. 8A) and orthodontic brackets (Fig. 8B).

Discussion

Essential oils (EOs) are volatile secondary metabolites produced by plants, responsible for imparting typical aroma, flavor, or both. Essential oils (EOs) have been extensively studied for their therapeutic potential across various diseases. Their pharmacological properties encompass antimicrobial, anti-inflammatory, antitumor, and antioxidant activities[28]. The current study has explored EOs as alternative or adjunctive agents in oral healthcare. Dant-Kanti-Gandush (DKG) oil consists of a 98.0% fixed oil blend of coconut and sesame oil, with 1.5% of essential oils from clove, peppermint, eucalyptus, prickly ash, and basil. DKG is recommended for oil pulling, an ancient Ayurvedic practice, that has recently gained popularity for its natural, cost-effective and health benefits. The process involves swishing oil in the mouth for about 10–20 min. Traditionally, sesame oil or coconut oils are preferred over other edible oils. Sesame oil, known for its antimicrobial properties and plaque-removing ability, contains lignans such as sesamin, sesamolin, and sesaminol, which possess strong antioxidant activity[29,30]. Coconut oil, rich in lauric acid, plays a scientifically recognized role in oral hygiene by exhibiting antimicrobial and antibiofilm activity against plaque-causing and cariogenic bacteria. Additionally, it possesses antioxidant and anti-inflammatory properties[31,32]. However, the combination of five essential oils in DKG serves as an enhancement to the traditional oil-pulling formulation. The present study elucidates the antimicrobial efficacy of this essential oil combination in inhibiting growth, attenuating virulence factors, and disrupting biofilm formation in key oral pathogens, *Streptococcus mutans* and *Candida albicans*. The 1.5% composition of DKG consists of essential oils containing a substantial amount of eucalyptol, D-limonene, menthol, linalool, and eugenol, as identified through GC-MS/MS analysis. Interestingly, MIC determination revealed that this essential oil mix exhibited a remarkably low effective concentration of 0.45% (v/v), 0.29% (v/v), 0.10% (v/v), and 0.11% (v/v) against *S. mutans*, *P. mirabilis*, *S. pyogenes*, and *C. albicans*, respectively. All pathogens under the in vitro conditions are inhibited at < 0.5% (v/v) of essential oil mix present in Dant-Kanti-Gandush (DKG). When treated above MIC_{50}, the essential oil mix significantly inhibited growth dynamics, leading to delayed and reduced exponential growth of these pathogens. The half-maximal growth in the growth curves achieved by all the pathogens in the presence of DKG took longer time (h) compared to the untreated control. This delay in the exponential growth suggests that DKG suppresses the bacterial and yeast proliferation, albeit dose-dependently delays the growth. *S. mutans* are the key pathogen contributing significantly to dental caries and plaque formation. With strong adherence, acid enduring (acidurity) and producing (acidogenicity) properties, *S. mutans* create an environment that promotes enamel demineralization, bacterial colonization, biofilm maturation and persistence[5,19,33,34]. DKG markedly inhibited *S. mutans* biofilm formation and suppressed its aciduric and acidogenic properties, demonstrating its effectiveness in disrupting *S. mutans* metabolism. By reducing acid production and promoting oral pH balance, DKG plays a crucial role in lowering the risk of dental caries and contributing to overall oral health maintenance. *C. albicans*, yeast that also plays a crucial role in oral plaque biofilms by interacting with oral bacteria, albeit enhancing antimicrobial resistance and other oral diseases[7,35,36]. The transition from yeast to hyphal morphology is important for *C. albicans* to adhere to the



host's surface, enhances its ability to invade the host and impart pathogenicity[23,37]. DKG suppressed the hyphal transition ability of *C. albicans* and even reduced the biofilm formation, metabolic activity significantly. The diminishing levels of ergosterol, an important cell membrane component for maintaining membrane integrity, was observed with DKG treatment. Ergosterol also act as a target for many antifungal drugs[24]. Disrupting its levels, DKG compromises the growth and virulence of *C. albicans*, highlighting mechanistic aspects of DKG offering a promising approach to reducing pathogenicity of the yeast and thereby improving oral health.

Several published reports have previously demonstrated that essential oils employ multiple mechanisms to inhibit the bacterial growth[15,38]. Mechanistically, either by cell membrane disruption in the bacteria or interfering with the cell-to-cell communication (quorum sensing), essential oils could even suppress the extracellular polymeric substance (EPS) production, matrix crucial for biofilm formation by the bacteria[39–41]. However, majority of studies have primarily evaluated the antimicrobial activity of essential oils against planktonic

◀**Fig. 4**. DKG suppressed cariogenic potential of *S. mutans.* (**A**) Microscopic analysis of bacterial biofilm formation under different concentrations of DKG. The untreated (UT) sample exhibits dense biofilm coverage, while progressive concentrations of DKG (0.5×, 1.0×, and 2.0× MIC_{50}) concomitantly reduce biofilm formation. The highest concentration (2.0× MIC_{50}) results in significant disruption of biofilm structure. Scale bars represent 20 µm. (**B**) Quantification of the surface area covered by biofilms in (**A**) using ImageJ. (**C**). Absorbance of ethanol-solubilized crystal violet at 590 nm, extracted from *S. mutans* biofilms plotted as bar graph (**D**) Bar graph showing a alamar blue assay-based biofilm metabolic activity in *S. mutans*. (**E**) Glycolytic pH drop assay of *S. mutans*. DKG treatment significantly inhibited the glycolytic pH drop at 0.5×, 1.0×, and 2.0× MIC_{50} concentrations. The decrease in pH values over time was modeled using a one-phase decay fit, and the resulting curve was used to derive τ (tau) values. (**F**-**G**) Effect of DKG on the acid tolerance of bacterial cells. Bar graphs depict the survival percentage of bacterial cells exposed to acidic conditions (pH ~ 5.0) with or without DKG treatment at varying concentrations (0.5×, 1.0×, and 2.0× MIC_{50}). Representative images of bacterial colonies recovered following acid exposure. A dose-dependent reduction in bacterial survival is evident with increasing concentrations of DKG. Error bars represent mean ± SEM; (***) indicate statistically significant differences ($p < 0.001$) from triplicate experiments.

bacterial cultures. Given that biofilms exhibit enhanced resistance due to their protective extracellular matrix, conventional antimicrobial agents often fail to penetrate and eradicate these multi-layers, tightly structured communities. Furthermore, most investigations have focused on single-species biofilms, despite of the fact that oral infections commonly involve complex, inter-kingdom interactions. Addressing these gaps, the current study examines the antimicrobial efficacy of essential oils mix against *S. mutans* and *C. albicans* co-cultured biofilms grown on orthodontic brackets and rings, a clinically relevant surface. This approach provides deeper insights into biofilm susceptibility in realistic conditions, reinforcing the potential of essential oils for combating persistent oral biofilms.

These orthodontic fixtures, being constantly exposed to oral fluids, provide a favorable niche and surface for microbial colonization, making the maintenance of oral hygiene complicated and challenging[10,13,26,27]. Preventive strategies comprising of stringent oral hygiene practices play an important role in mitigating biofilm formation risks and ensure optimal oral health. Oil pulling could be an effective practice for oral health maintenance. Dant Kanti Gandush, containing a combination of essential oils with coconut and sesame oil, offers potential antimicrobial benefits, inhibits the inter-kingdom biofilm formation and thereby supports overall oral hygiene. A 1.0% (v/v) concentration of the essential oil mix was tested on biofilms, which is nearly half the concentration of essential oils present in the oil-pulling formula of DKG. Scanning electron microscopy (SEM) revealed a dense mesh of hyphae and yeast cells of *C. albicans* with coccus shaped *S. mutans* building a complex architecture and extensive extracellular polymeric substance (EPS) matrix. At lower magnification, SEM imaging of the entire bracket and elastomeric ligatures revealed a dense, thick layer of dual-culture biofilm. In contrast, brackets and elastomeric ligatures exposed with DKG exhibited a notably reduced microbial adherence, accompanied by significant structural alterations, cell lysis and scattered cellular debris. Additionally, crystal violet staining, followed by biofilm biomass quantification and metabolic activity, served as an effective and reliable method to study biofilms on elastomeric ligatures. The current study was limited to in vitro antimicrobial assessment of DKG in oral pathogens, with one clinical isolate (*S. mutans*). Further validation of these findings in the drug-resistant clinical isolates from different geographies would add robust body-of-evidence of DKG functionality. In addition, deciphering host-pathogen interactions and preclinical efficacy in animal models of oral infection would further substantiate the therapeutic relevance of the present findings. Long-term non-clinical and clinical safety assessment of DKG would also be useful in ascertaining its clinical relevance in real-world oral healthcare. Nonetheless, the present study highlighted the potential of DKG in mitigating microbial adherence and biofilm formation on orthodontic surfaces, and in promoting oral hygiene and overall health (as shown in the study summary, Fig. 9).

Conclusion

Dant-Kanti-Gandush (DKG) serves as a valuable natural intervention for maintaining oral hygiene and preventing biofilm-associated complications, even in the presence of orthodontic fixtures, where microbial adhesion and plaque accumulation are heightened. DKG could inhibit multiple oral pathogens, disrupt dual-species biofilms, reduce their adherence on orthodontic fixtures, highlighting DKG as an effective adjunct to conventional oral care, particularly for individuals undergoing orthodontic treatment. Further *in-vivo* studies and randomized clinical trials are needed to validate its therapeutic potential and clinical efficacy in real-world oral healthcare applications.

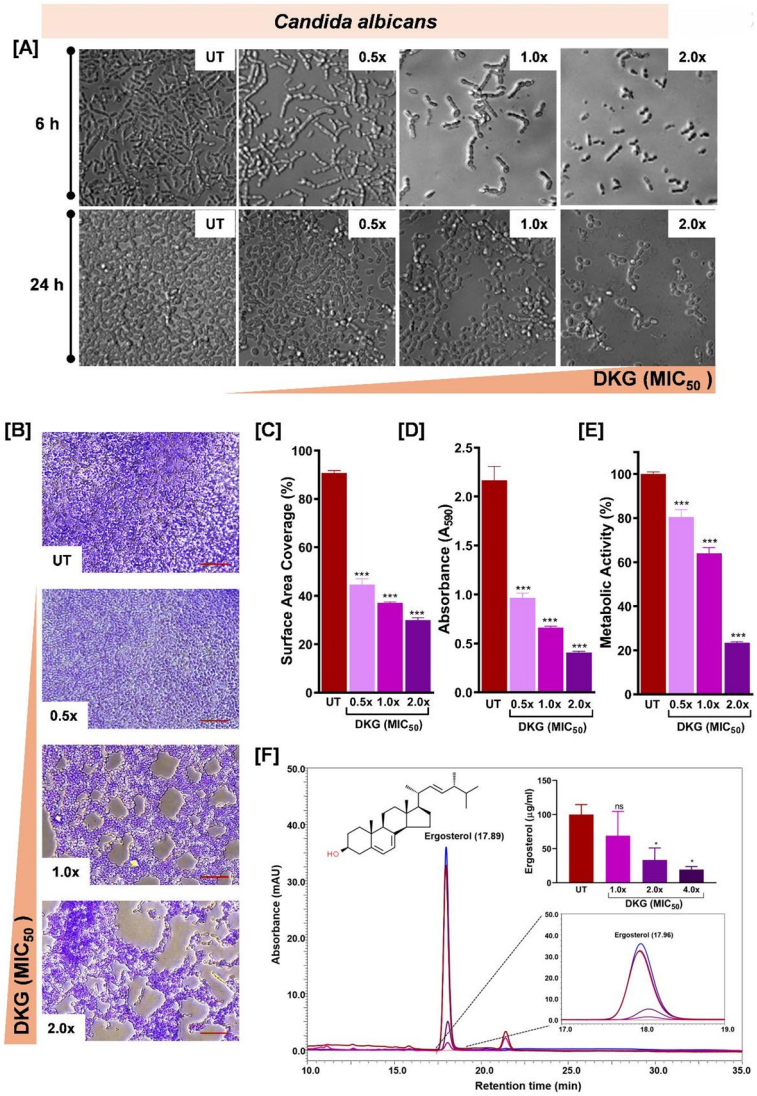


Fig. 5. DKG inhibited yeast-to-hypha conversion and biofilm formation in *C. albicans*. (**A**) Yeast cells treated with DKG at increasing concentrations (0.5×, 1.0×, and 2.0× MIC_{50}). Representative phase-contrast microscopy images show untreated (UT) and DKG-treated yeast cells. Scale bars represent 2 µm. (**B**) Microscopic observation of *C. albicans* biofilm strained with crystal violet. The untreated (UT) *C. albicans* biofilm shows dense coverage, while DKG treatment (0.5×, 1.0×, and 2.0× MIC_{50}) progressively induced disruption of biofilm. Scale bars indicate 50 µm. (**C**) Subsequent quantification of the surface area covered by biofilm in (**B**) using ImageJ. (**D**) Bar graph showing absorbance units of ethanol-solubilized crystal violet stain extracted from biofilms formed by *C. albicans* (**E**) Metabolic activity (%) determined by alamar blue assay in biofilms formed by *C. albicans*. (**F**) Representative chromatogram for ergosterol detection using UHPLC-DAD. The chromatograms displaying peaks corresponding to ergosterol detected in *C. albicans* treated with different concentrations of DKG. Quantification performed with reference to the ergosterol standard (blue). Inset provides a magnified view of the main ergosterol peak, highlighting differences in peak shape and intensity across DKG treatments. Bar graph in the right panel shows quantification of ergosterol content. Error bars represent mean ± SEM from three independent experiments; significance of data has been represented as, $^{***}p < 0.001$.

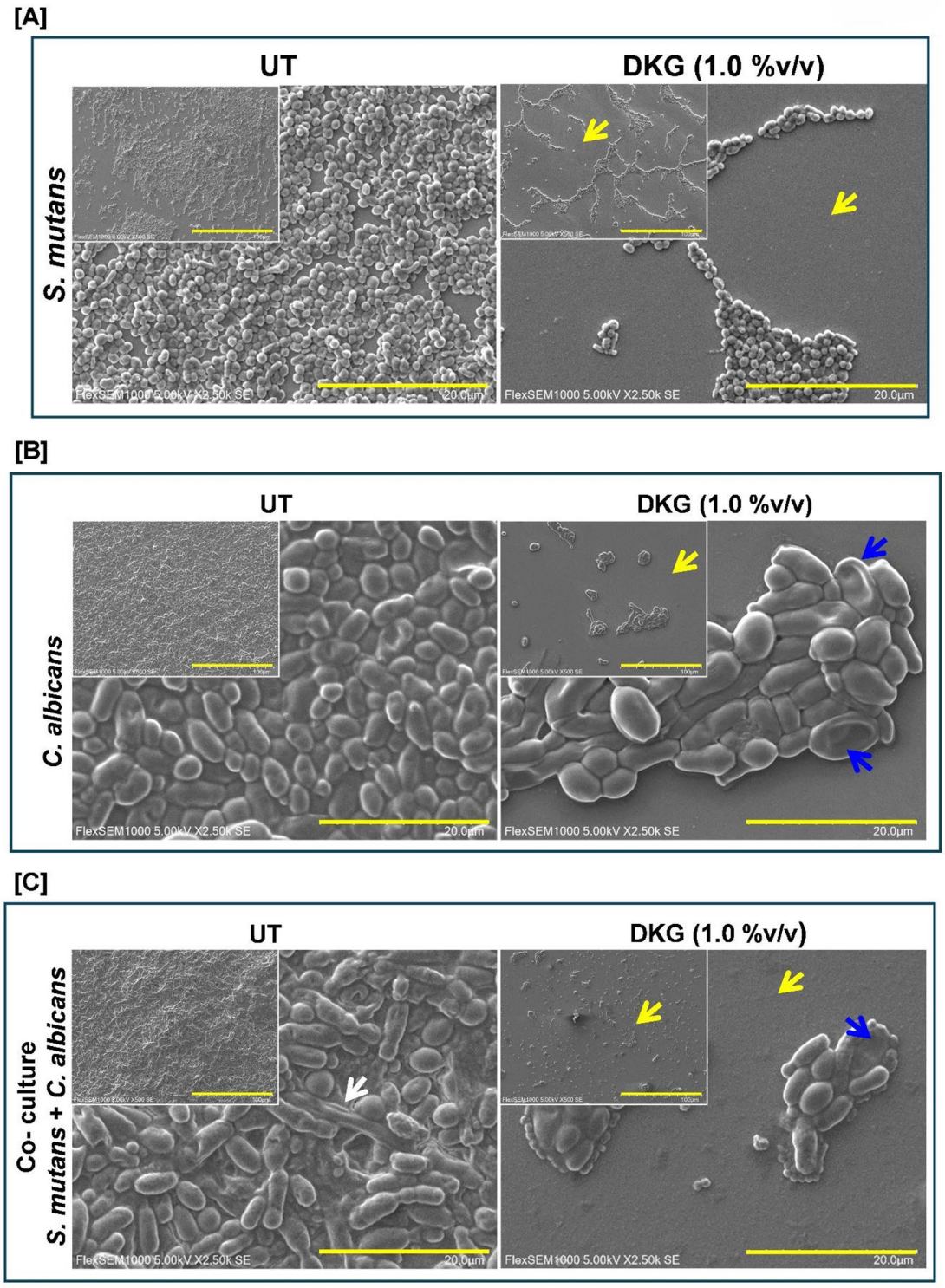


Fig. 6. DKG inhibited biofilm formation of *S. mutans* and *C. albicans* in mono- and co-culture conditions. (**A**-**C**) Scanning Electron Microscopy (SEM) images showing biofilm formation of *S. mutans* (**A**), *C. albicans* (**B**) and co-cultured *S. mutans* and *C. albicans* in the presence 1.0% (v/v) and absence of DKG. Left panel depicts a dense biofilm matrix of clustered microbial cells (control), while the right panel shows a disrupted biofilm structure with scattered cells, indicating reduced biofilm formation (treated with 1.0% (v/v) of DKG). Inset in all images provide a visual overview of the biofilms at lower magnification. Scale bars indicate 20.0 µm and 100.0 µm, respectively. Blue arrowheads: deformed yeast cells; white arrowheads: healthy hyphal structures in untreated but missing in DKG treatment; yellow arrowheads: reduced microbial density.

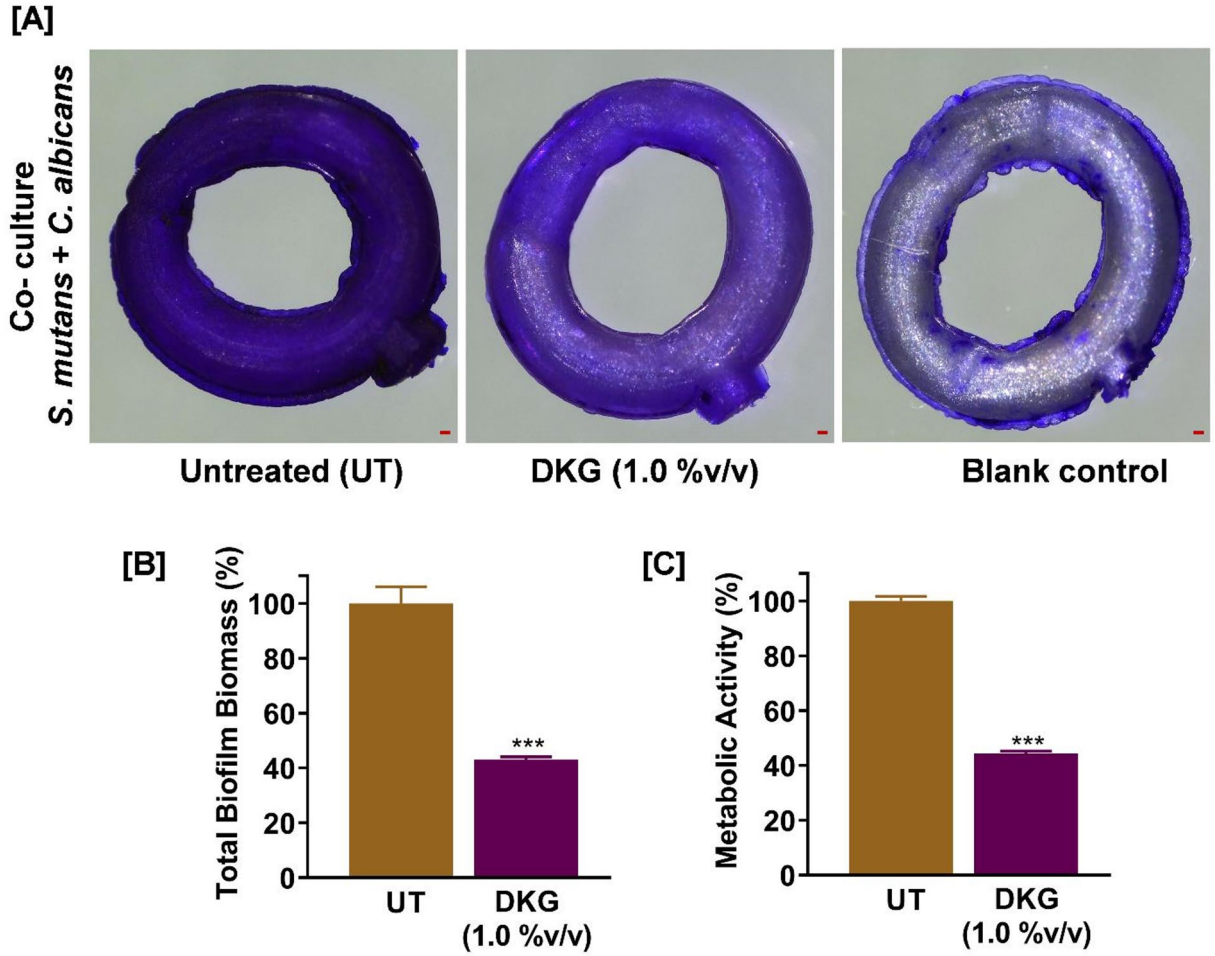


Fig. 7. DKG inhibited cross kingdom biofilm formation on elastomeric ligatures. (**A**). Microscopic images of crystal violet (CV) stained orthodontic ligatures. Scale bar: 100 µm. (**B**). Bar graph representing total biofilm biomass in percentage ($n = 3$). (**C**). Bar graph showing metabolic activity determined from alamar blue assay ($n = 3$). Error bars represent mean ± SEM from three independent experiments; significance of data has been represented as, $^{***}p < 0.001$.

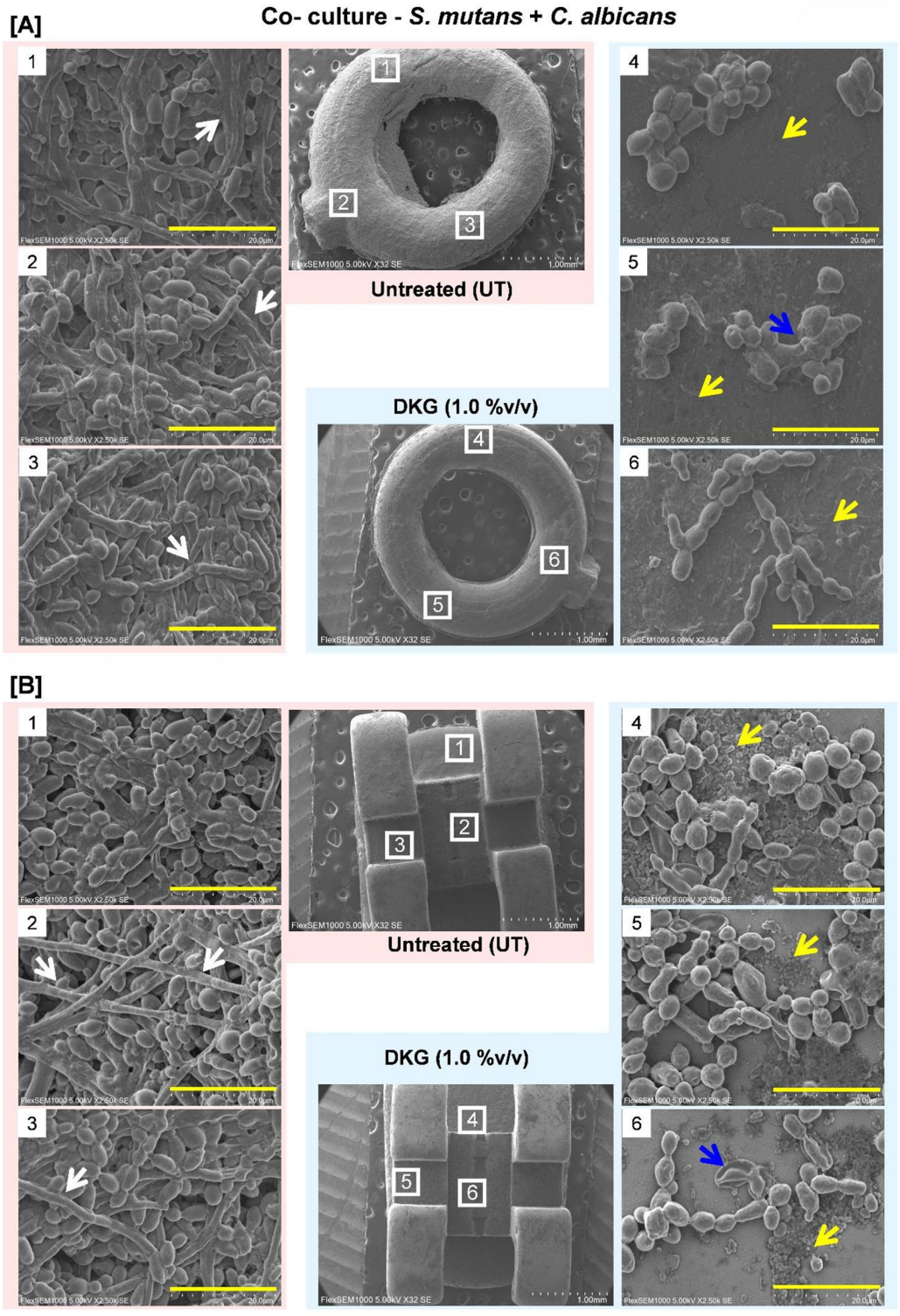


Fig. 8. DKG prevented cross-kingdom biofilm formation by *S. mutans* and *C. albicans* on the Orthodontic fixtures. Scanning electron microscopy (SEM) images of (**A**) Elastomeric ligature and (**B**) Orthodontic brackets showing biofilms formed by co-cultured *S. mutans* and *C. albicans*. The untreated (UT) samples (pink background) exhibit dense and well-established biofilm structures (panel 1, 2 and 3) characterized by extensive microbial colonization and extracellular matrix production. In contrast, DKG (1.0%v/v treated samples (blue background) show significant biofilm disruption and reduced microbial adhesion (panel 4, 5 and 6), with altered microbial morphology and reduced biofilm density on the surface of the elastomeric ligature and orthodontic bracket. Blue arrowheads: deformed yeast cells; white arrowheads: healthy hyphal structures in untreated but missing in DKG treatment; yellow arrowheads: reduced microbial density.

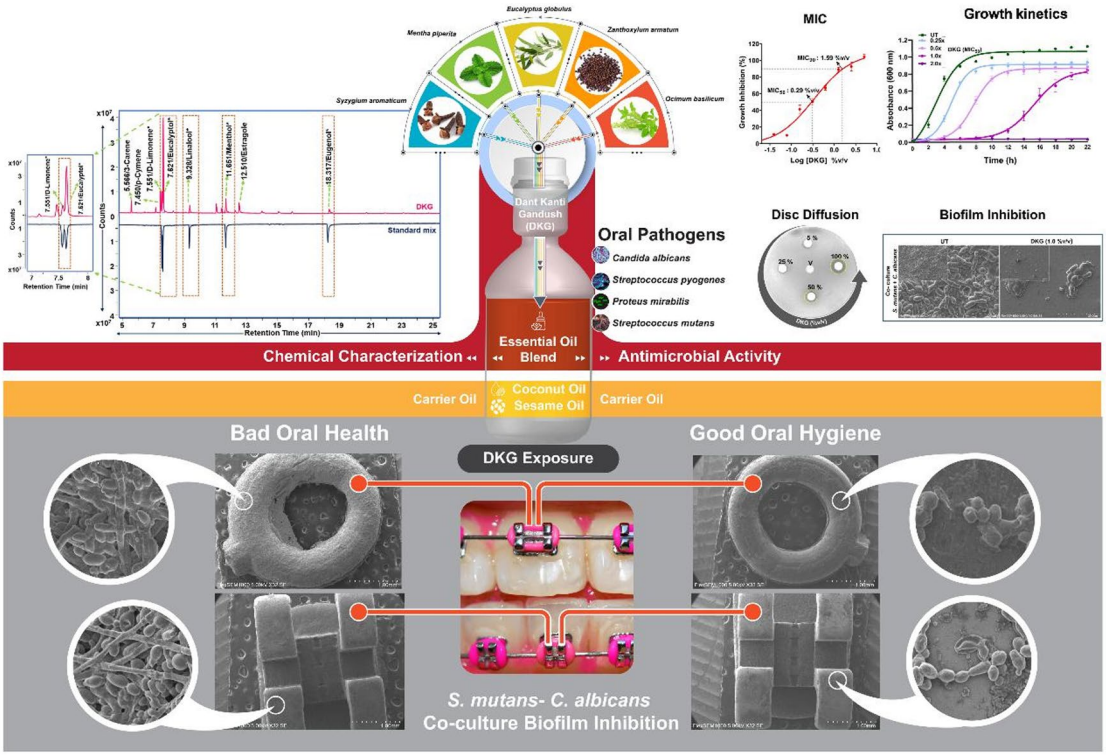


Fig. 9. Schematic representation summarizing present study outcomes.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

AB: Conceptualization, Supervision, Resources, Writing-review & editing; HJ: Methodology, Data curation, Formal analysis, Writing-original draft; NKN: Methodology, Investigation; YV: Methodology, Data curation, Formal analysis, Writing-original draft; MT: Methodology, Data curation, Formal analysis; MJ: Methodology, Investigation; KS: Methodology, Investigation; PN: Supervision; Writing-review & editing; SL: Visualization, Methodology, Data curation, Formal analysis, Writing-review & editing; AV: Project administration, Conceptualization, Visualization, Supervision; Writing-review & editing.

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Declarations

Competing interests

The test article, Dant-Kanti-Gandush was sourced from Divya Pharmacy, Haridwar, India. AB is an honorary trustee in Divya Yog Mandir Trust, which governs Divya Pharmacy, Haridwar. In addition, he holds an honorary managerial position in Patanjali Ayurved Ltd., Haridwar, India. Divya Pharmacy and Patanjali Ayurved Ltd commercially manufacture and sell several Ayurvedic products. Other than providing the test article, Divya Pharmacy was not involved in any part of this study. Other authors have no conflict of interest to disclose.

Additional information

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