

RESEARCH PAPER

Comprehensive Phytochemical Characterization Of *Aloe Barbadensis* Leaf Gel Extract And Its Correlation With Antibiofilm Efficacy Against Cross Kingdom *Streptococcus Mutans* And *Candida Albicans* Polybiofilm

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ABSTRACT

Objective: Polymicrobial biofilms formed by *Streptococcus mutans* and *Candida albicans* contribute significantly to oral disease pathogenesis, wherein interkingdom interactions enhance extracellular matrix production, virulence factor expression and antimicrobial tolerance, limiting the use of conventional chemotherapeutics. This study investigates the phytotherapeutic role of *Aloe barbadensis* Miller (or *Aleo vera*) leaf gel, which exhibits dual antibacterial and antifungal potential.

Methods: Phytochemical characterization of the extract using FTIR, GC-MS and LC-MS are reported. Antimicrobial and antibiofilm activity of *S. mutans* and *C. albicans* are reported using zone of inhibition, MTT viability and biofilm inhibition using crystal violet assay. *In silico* molecular docking and toxicity are performed for aloe-emodin.

Results: Phytochemical screening confirmed the presence of flavonoids, saponins in the ethanolic extract of *Aloe barbadensis* Miller. FTIR spectroscopy revealed the presence of acemannan and anthraquinone (eg. emodin, aloin, aloesin, chrysophanol). GC-MS profiling of *A. vera* gel ethanolic extract revealed lipid rich phytochemicals with phenolic, oxygenated hydrocarbons and heterocyclic compounds. Various non-volatile metabolites such as phenolic, chromone, anthraquinone, and flavonoid derivatives were identified through LC-MS analysis. Antimicrobial susceptibility assays exhibited a dose-dependent inhibition of mono and dual cultures. Biofilm inhibition and eradication assays demonstrated >90% inhibition of *S. mutans*, ~80% in *C. albicans* and ~85% in polymicrobial biofilm using 12.5 mg/ml similar to 50 µg/ml of chlorhexidine. Biofilm eradication of less than 60% was recorded for mono and dual species even as % inhibition was observed using 12.5 mg/ml of *A. vera* gel as 50 µg/ml of chlorhexidine. *In silico* blind molecular docking revealed high binding energies of aloe emodin, the dominant anthraquinone with key virulence and adhesin proteins glucosyl transferase (GtfB) and sortase A (Sor A) of *S. mutans* and *C. albicans* agglutinin like sequence 3 (Als3) and secreted aspartyl proteinase 5 (Sap5). These interactions suggest inhibition of glucan, cell-surface anchoring, fungal adhesion, and proteolytic activity as underlying mechanisms. *In silico* toxicity and ADME profiling using SwissADME and StopTox predicted a favourable safety and drug-likeness profile for the dominant Aloe emodin compound.

Conclusion: These findings support *Aloe barbadensis* Miller as a mechanistically validated phytotherapeutic candidate for targeting polymicrobial biofilms.

Keywords: *Aloe barbadensis* Miller, *Streptococcus mutans*, *Candida albicans*, Dual-species biofilm, antimicrobial

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1. INTRODUCTION

Aloe barbadensis Miller (common name Aloe vera) belonging to Family Aloaceae, subdivision Liliaceae or Asphodelaceae, is a succulent plant widely used in traditional and modern medicine due to the presence of a rich array of diverse phytochemicals that impart potent

anti-oxidant, anti-inflammatory and tissue regeneration properties¹⁻³. Its leaf gel and rind are rich in bioactive molecules such as anthraquinones (eg. aloin, aloe-emodin), flavonoids, saponins and polysaccharides giving it broad therapeutic potential with excellent biocompatibility⁴. Given the increase in antibiotic resistance for managing

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infectious diseases, *A. vera* is emerging as a promising natural candidate for its applications in oral healthcare products such as gels and mouthwashes⁵.

The oral cavity comprises of a complex and dynamics microbiome consisting of viruses, bacteria, archaea and fungi that collectively contribute to oral homeostasis⁶. However, dietary sugars, imbalanced nutrition, poor oral hygiene, systemic immunosuppression, or frequent antibiotic exposure may result in dysbiosis⁷. Under such conditions, opportunistic pathogens such as *Candida albicans* and virulent strains of *Streptococcus mutans* exploit the ecological imbalance to establish resilient biofilms and recurrent infections^{6,8,9}. Dental caries initiation occurs through glycan-mediated adhesion and acidogenic metabolism of *S. mutans*, while *C. albicans* enhances biofilm thickness and invasiveness by interacting with bacterial glycans and adopting hyphal forms⁹⁻¹¹. Cooperative interactions within multi-kingdoms biofilms increases resistance to mechanical removal (teeth brushing) and conventional antimicrobials compared to mono species growth forms, playing a major role in progression of dental caries and oral mucosal infections^{8,9,12}.

Several studies have shown the antibacterial and antifungal activities of *A. vera* extracts against oral pathogens, largely focused on single species effects^{13,14}. *A. vera* also has documented efficacy against oral infections with reported plaque reduction and healing in clinical settings¹⁴⁻¹⁸. There are however very limited direct studies specifically on *A. vera* against *S. mutans* and *C. albicans* cross kingdom biofilms. In this context, the present study investigates the antimicrobial, antibiofilm and antioxidant activity of *A. vera* leaf extract against *S. mutans* and *C. albicans* cross kingdom polymicrobial biofilm models. Phytochemical screening, DPPH radical scavenging activity capacity, spectrophotometric and chromatographic characterization are integrated to identify the key bioactive compounds. Docking experiments with dominant phytochemicals with biofilm inducing genes further corroborates the activity of the *A. vera* extract. By correlating phytochemical composition with functional outcomes, the study aims to understand development of *A. vera* based natural therapeutics to control polymicrobial oral biofilms and reduce cariogenesis using traditional products.

2. MATERIALS AND METHODS

2.1. Test Organisms: *Streptococcus mutans* MTCC 497 and *Candida albicans* MTCC 227 were procured from IMTECH, Chandigarh, India. *S. mutans* was maintained on Mitis Salvarius agar media were incubated at 37 °C for 24 h. *C. albicans* was grown on Sabourauds Dextrose agar and incubated at 37 °C for 48 h. Cultures were stored in the refrigerator at 4 °C till further use. All media and chemicals were procured from HiMedia, India and Merck, India respectively.

2.2. Sampling and Phytoextraction: *Aloe barbedensis* Miller (AV) leaf samples were obtained from medicinal garden, CSJM University Kanpur, and authenticated by the Ministry of Environment, Forest and Climate Change,

Botanical Survey of India, Prayagraj, India. Mature and healthy leaves (30 cm length) were rinsed thoroughly to remove dirt. The bottom of the leaf was cut and left standing upright in a pot for 10 to 15 min until the latex completely drained out. The edges of the leaf were then cut lengthwise and the transparent gel removed without taking the green part with the help of a spoon. The gel was blended to make it smooth homogenous solution prior to spreading in a thin layer on parchment paper. The gel was set in an oven at 60 °C for 3 h to dry. Upon cooling the gel powder was stored in sterile containers at 4 °C. For further experiments, *A. vera* gel powder was mixed in ethanol (90%) (1:10 ratio) at room temperature for 48 h. Extract was filtered using sterile Whatman filter paper No. 1 and the concentrated extract obtained post evaporation was solubilized in sterile Milli Q water or stored at 4 °C until further use.

2.3. Phytochemical Screening: Preliminary phytochemical screening was performed for the detection of phytoconstituents (alkaloids, flavonoids, phenols, glycosides, proteins and anthraquinone) as described in previous protocols¹⁹. Total phenolic content estimation and the flavonoid content estimation was based upon the formation of a flavonoid-aluminum complex as described previously²⁰. Antioxidant activity was determined by the ability of the extract to inhibit or scavenge the DPPH free radical activity²¹.

2.4. FTIR Spectroscopy: Fourier Transform Infra-Red spectrometer, in the range of 400–4000 cm⁻¹ (PerkinElmer) of the extract was done in Indian Institute of Technology, Kanpur. Interpretation of IR spectra obtained from extract was achieved by comparing the spectral data with references for identification of functional groups existing in the leaf sample²².

2.5. Gas Chromatography- Mass spectrometry (GC-MS/MS): GS-MS/MS analysis was performed using a triple quadrupole gas chromatography-mass spectrometer equipped with AOC-20i autosampler (Model: GCMS-TQ8040). Separation was achieved on a SH-Rxi-5Sil capillary column (5% biphenyl, 95% dimethylpolysiloxane, 30m x0.25m i.d., 0.25 µm film thickness) suitable for the analysis of semi-volatile phytochemicals. The oven temperature was programmed from 50 °C (2 min hold) to 310 °C at a rate of 10 °C / min, followed by a 25 min final hold. The total analysis time was 53 min. Samples were injected in splitless model at an injector temperature of 250 °C. The ion temperature was set at 230 °C, and the interface temperature was maintained at 250 °C. Mass spectra were acquired in Q3 scan mode. Compound identification was carried out by comparison of acquired mass spectra with the NIST mass spectral library. Where available, authentic analytical standards were analysed under identical GC-MS conditions, and compound identities were confirmed by matching retention times and mass fragmentation patterns²³.

2.6. Liquid Chromatography/ Mass Spectrometry LC-MS/MS analysis was performed using a system coupled to triple quadrupole mass spectrometer, equipped with an electrospray ionization (ESI) source operating in positive ion mode (ES) (Waters, Acquity-H Class UPLC with Xevo TQD-MS, Mass range 50-2000 KD, Patanjali Food and Herbal Park Pvt Ltd, Haridwar). The sample (20 µL) was injected in infusion mode without a column or solvent. The ESI parameters were as follows: capillary voltage 3.5 kV, cone voltage 35V, source temperature 150 °C. Data were acquired in MS2 mode over an m/z range of 50-2048 at 3 eV collision energy. Mass-lynx V4.1 software was used for data acquisition and processing. Base peak intensity (BPI) chromatograms and peak lists were generated from MassLynx plots²⁴.

2.7. Antimicrobial activity: Antimicrobial activity of the plants extracts was carried out as described previously²⁵. 0.1 ml of log phage bacterial suspension (10^8 cfu ml⁻¹) was used in spread plate method on Mueller Hinton Agar (MHA). Chlorhexidine (CHX, 100 µl per well at 50 µg ml⁻¹ concentration, CHX) was used as positive control. 0.1 ml (25, 50, 100, 200 mg ml⁻¹ in milliQ water of *A. vera* extract was added to wells. Plates were incubated at 37 °C overnight and zone of inhibition measured. The values of the antimicrobial activity were expressed as the mean of inhibition zones (mm) with three replicates for each treatment.

Minimum inhibitory concentration was evaluated using the broth microdilution method, in a 96-well microtiter plate and percent viability determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay²⁶. 0.1 ml of *S. mutans*, *C. albicans* and mixed cultures (1:1) (0.5 McFarland standard) and 0.1 ml of 2-fold diluted (200 to 1.5 mg ml⁻¹ concentrations were added to the microtiter plate and plates incubated at 37 °C for 24 h. Sterile MilliQ was added as a solvent control. Post incubation, 30 µL of MTT solution was added, with 20 min. No colour change was observed in the control well, while microbial presence-based reduction resulted in purple formazan color which was measured at 540 nm using spectrophotometer. Percentage viability was calculated as:

$$\text{Percent Viability} = \frac{\text{Test Absorbance} - \text{Blank absorbance}}{\text{Control Absorbance} - \text{Blank Absorbance}} \times 100$$

2.8. Biofilm Formation and Eradication Assay using Crystal Violet Assay: Biofilm formation and eradication of 24h preformed biofilm was evaluated using crystal violet assay²⁷. Log phase *S. mutans*, *C. albicans* and mixed cultures (1:1) with two-fold dilutions of *A. vera* ethanol leaf extracts (3.12-200 mg/ml) were then added to each well and the plates were incubated at 37 °C for 24 h. Positive control included culture and medium without an antimicrobial agent. Planktonic cells were aspirated and transferred to fresh microtiter plate and growth measured at 540 nm using microtiter plate reader (BR-580, BR

Biochem, Lifesciences Pvt. Ltd.). Log phase *S. mutans*, *C. albicans* and mixed cultures were allowed to grow and form biofilm on the bottom surface of the wells at 37 °C for 24h to generate 24h preformed biofilm. Media was replaced with different concentrations of *A. vera* ethanol leaf extracts (3.12-200 mg/ml) and incubated at 37 °C for 24 h. For crystal violet biofilm assay, the biofilm was washed with twice with phosphate buffer saline (1X PBS). Staining of biofilm was performed with 0.1 ml of 0.1 % (w/v) crystal violet for 30 min at 37 °C. Excess crystal violet was aspirated and that staining the biofilm was solubilized in 0.1 ml of dimethyl sulfoxide, and the absorbance was measured at 540 nm. The percentage inhibition of the planktonic and biofilm formed or eradicated was calculated as:

$$\text{Percent Inhibition} = \frac{\text{Test Absorbance} - \text{Control Absorbance}}{\text{Control Absorbance}} \times 100$$

2.9. In silico molecular docking analysis: Molecular docking of major bioactive compound derived from phytochemical analysis, Aloemodin with target protein identified through literature survey was carried out using the CB-Dock2 web server, which enables automated blind docking through cavity detection and scoring²⁸. The 3D structure of Aloe emodin ligand was obtained from PubChem database and prepared by ensuring correct geometry optimization, protonation, and geometry optimization in compatible format. Protein structures were downloaded from Protein Data Bank (PDB) and pre-processed by removing crystallographic water molecules and non-essential heteroatoms, followed by structural validation. Each prepared protein and ligand were uploaded to the CB-Cock2 server. For each protein multiple cavities were detected and ranked based on size and suitability for ligand binding using CurPocket algorithm. Cavity with least binding energy was used for docking with Autodock Vina, and best docking pose for each protein-ligand complex was selected based on Vina binding energy scores (kcal/mol) and key interactions between interacting amino acids within the binding site were analysed to support binding stability^{29,30}.

2.10. In silico ADMET (absorption, distribution, metabolism, excretion and toxicity) and drug likeness assessment: The canonical Simplified Molecular Input Line Entry System (SMILES) string for Aloe Emodin is O=C1C=2C=CC=C(O)C2C(=O)C3=C(O)C=C(C=C13)CO was downloaded from PubChem. The pharmacokinetic and ADMET properties were assessed using the Swiss AME server³¹. Molecular fingerprinting approach was applied to evaluate drug-likeness, based in Lipinski's rule of five (LRF). Toxicological assessments were conducted using StopTox servers³².

2.10. Statistical Analysis: Data were expressed as means ± standard deviation. The data were subjected to student t test and one-way ANOVA with post hoc test wherever

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applicable at $p < 0.05$ significant levels. Graphs were prepared using the GraphPad Prism 10.4.2 program.

3. RESULTS AND DISCUSSION

3.1. Phytochemical extraction, yield and phytochemical analysis: Qualitative phytochemical screening revealed the presence of different phytochemical components such as steroids, terpenoids, flavonoids, tannins, phenols,

glycosides, and alkaloids in the *A. vera* ethanolic extracts (ALE). The total phenol content of 13.3 ± 1.9 mg GAE/g and total flavonoid content of 3.4 ± 1.7 mg QE/g was record (Table 1). DPPH assay was used to determine the percentage radical scavenging activity (% RSA) of the various fractions of *A. vera* across a concentration range of 10-100 $\mu\text{g/ml}$, with quercetin (QUR) serving as a standard (Figure 1). Comparable quenching activity was obtained at 20 mg/ml of *A. vera* with quercetin standard.

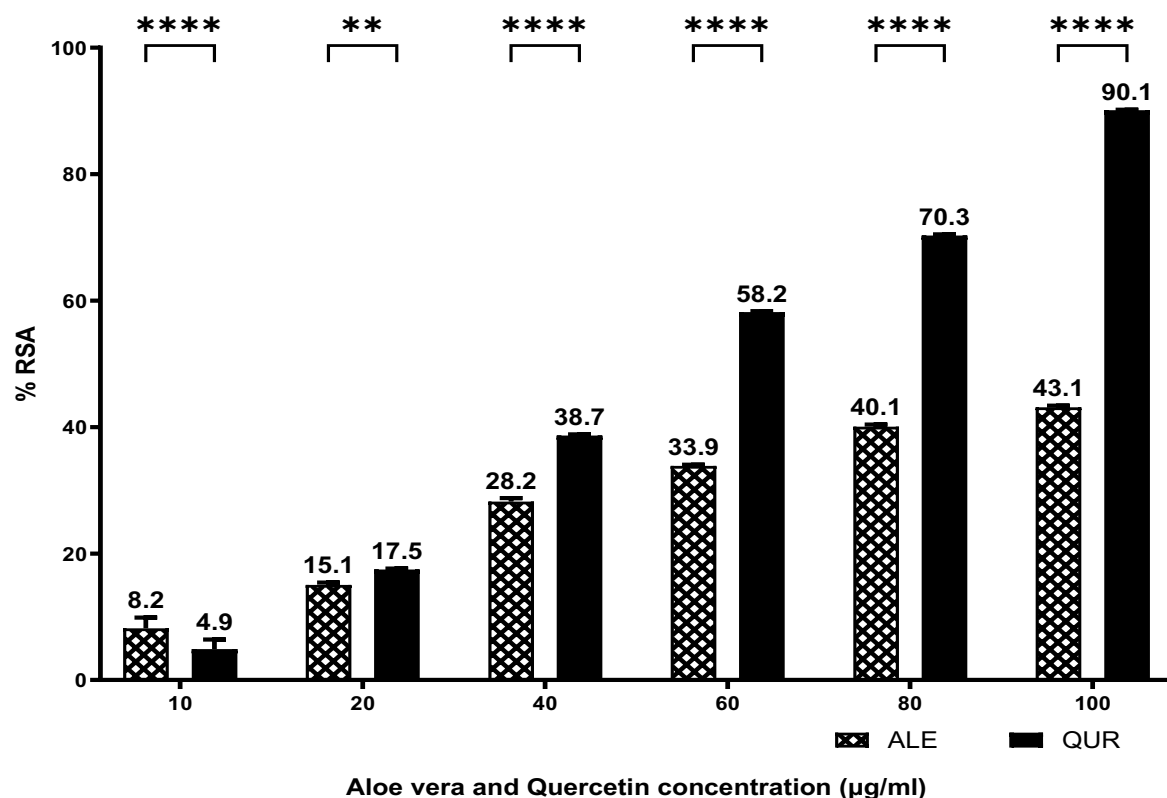


Figure 1. Antioxidant activity of *A. vera* ethanolic extract

The FTIR spectrum of *A. vera* was similar to that reported in similar reports dominated by acemannan and mucilaginous polysaccharides (1082 , 1041 , 3321 , 2980 cm^{-1}) with an admixture of phenolic/anthraquinone signals (Table 1, Figure 2). Broad O-H stretching band at 3321 cm^{-1} was with strong carbohydrate bands at 1082 and 1041 cm^{-1} confirms hydrated mucilage and acemannan-rich extract document in previous studies²². Weak -CH₂ stretching peak at 2980 cm^{-1} align with

aliphatic carbohydrate in Aloe polysaccharides. The fingerprint region between 1041 and 878 cm^{-1} reflects C-O stretching and aromatic ring vibrations, indicative of flavonoids, tannins and phenolic compounds. The 1632 cm^{-1} band together with absorption at 1320 , 878 and 622 cm^{-1} is consistent with conjugated carbonyl and aromatic vibrations of anthraquinone (eg. emodin, aloin, aloesin, chrysophanol) found in gel-rind mixtures.

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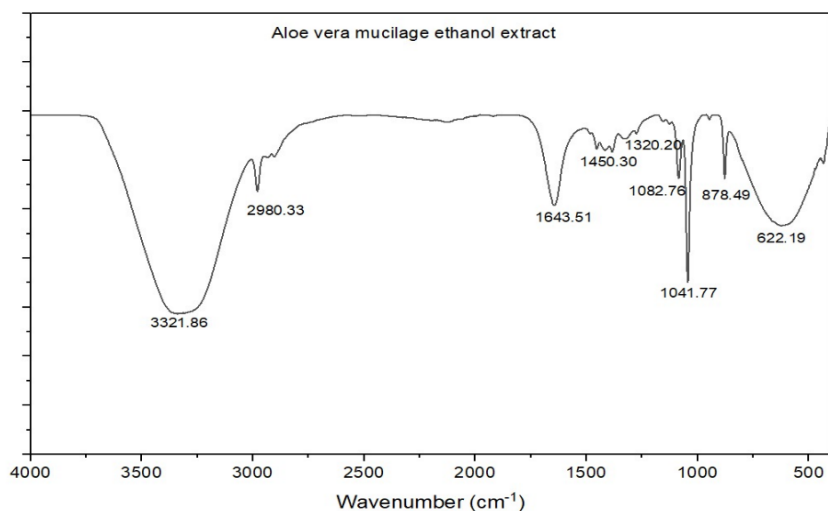


Figure 2. FTIR spectrum of *A. vera* get showing characteristic functional groups corresponding to polysaccharides, phenolic compounds and flavonoids.

Table 2. FTIR profiling of *A. vera* ethanolic extract

Peaks (cm ⁻¹)	Description	Functional Groups	Justification
622	Strong Broad Peak	C-H plane bending in aromatic rings	Flavonoids and phenolic compounds
878	Fingerprint region	Ortho or mono substituted aromatic ring	High concentration of flavonoids, phenols and tannins
1041-1320	Fingerprint region	C-O or C-N stretching vibration of aromatic rings	Flavonoids and Glycosidic linkages
1450	Fingerprint region	C-H bending, O-H bending, COOH stretching	Phenolic constituents
1643	Strong narrow peak	C=C aromatic stretching, C-H bending	Ethanol extraction/ Phenolics
2980	Fingerprint region	Asymmetric and symmetric stretching vibrations of C-H in -CH ₂ -	Aldehyde
3321	Strong Broad Peak	O-H stretching vibration	Mucilage polysaccharides

3.2. GC-MS profiling: GC-MS profiling of *A. vera* show a complex mixture of sixteen volatile and semi-volatile phytoconstituents eluting between 5.2 and 23.2 min (Figure 3, Table 3). The chromatographic profile was dominated by oxygenated hydrocarbons, fatty acids, phenolics and heterocyclic compounds. Along the detected constituents, cyclopropyl carbinol (RT 7.99 min) with an area percentage of 39.33% represents the most abundant compound in the volatile fraction. Significant proportions of n-hexadecanoic acid (palmitic acid; 7.61%), cis-vaccenic acid (2.62%), and octadecanoic acid (Stearic acid; 3.13%) represent a lipid rich phytochemicals profile. These fatty acids are well recognized for their membrane disruption, enzyme system inhibition and suppression of quorum sensing and biofilm formation. Short chain oxygenated compounds such as 3-hydroxybutanal and 6-oxa-bicyclic derivatives are reported to disrupt microbial membranes and interfere with metabolic pathways.

Presence of anti-oxidant and redox active 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one also may contribute to inhibition of microbial growth^{33,34}.

3.3.LC-MS Analysis: LC-MS analysis of the *A. vera* extract revealed a chemically diverse non-volatile metabolite profile dominated by phenolic, chromone, anthraquinone, and flavonoid derivatives (Figure 4, Table 4). Major ions were detected in the m/z range of 100-750, indicating the presence of both low molecular weight phenolics and high molecular weight glycosylated compounds. Prominent signals were observed at m/z 325, corresponding to aloe-emodin, along with multiple chromone and anthraquinone related fragments, including oxidized and glycosylated derivatives. The detection of aloin (m/z 457) and glycosylated anthraquinones (m/z 527 and 707) confirms the presence of bioactive secondary metabolites previously associated with antimicrobial and antibiofilm properties. Synergistic activity of

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multiple phytochemicals rather than a single compound is responsible for inhibiting the polymicrobial biofilm, where membrane active compounds reduce microbial

adhesion and extracellular matrix stability while other affect metabolic regulation.

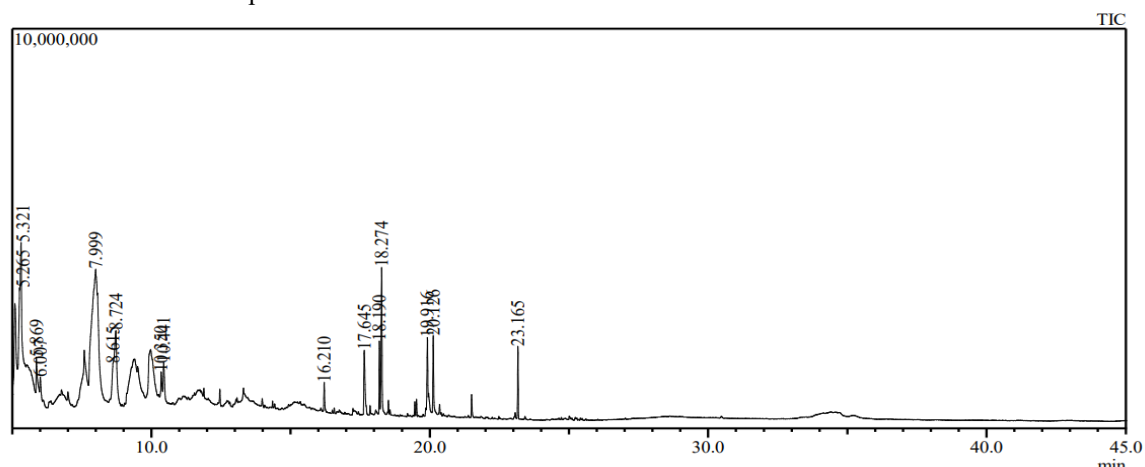


Figure 3. Gas Chromatography Mass Spectrum of *A. vera* gel extract. Compound identification is based on control compounds.

Table 3: Biological relevance of volatile and semi-volatile compounds identified in *A. vera* extract by GC-MS

Peak#	Retention Time	Area %	Height%	Compound Name	Class	Reported biological relevance
1.	5.265	6.34	8.29	Butanal, 3- hydroxy-	Hydroxy aldehyde	Antimicrobial with reactive aldehyde activity
2.	5.321	10.04	11.98	6-Oxa-bi	Oxygenated heterocycle	Antimicrobial, anti-oxidant
3.	5.869	2.41	3.33	Hexane,2-Bromo	Halogenated alkane	Antibacterial
4.	6.007	1.59	1.95	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	Furanone	Anti- quorum sensing
5.	7.999	39.99	11.10	Cyclopropyl carbinol	Alcohol	Membrane disruptive effects
6.	8.615	5.29	3.2	2-Propanamine, N-methyl-N-nitroso	Nitrosamine	Antimicrobial stress inducing activity
7.	8.724	6.77	6.43	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	Pyranone	Anti-oxidant
8.	10.35	1.48	2.29	Ethanone,1-(2,5-dihydroxyphenyl)-	Phenolic ketone	Anti-oxidant and Antimicrobial
9.	10.441	2.48	3.31	Cyclotetrasiloxane,octamethyl-	Siloxane	Mild antimicrobial
10.	16.21	1.17	2.64	Tetradecanoic acid	Saturated fatty acid	Microbial membrane disruption
11.	17.645	4.19	5.88	Lidocaine	Alkaloid like amide	Antibiofilm in oral pathogens
12.	18.19	2.36	6.65	Dibutyl phthalate	Phthalate ester	Antimicrobial

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13.	18.274	7.61	13.31	n-Hexadecanoic acid	Saturated fatty acid	Antibacterial, antifungal, antibiofilm
14.	19.916	2.62	5.97	Cis-Vaccenic Acid	Unsaturated fatty acid	Antimicrobial
15.	20.126	3.13	7.01	Octadecanoic acid (stearic acid)	Saturated fatty acid	Antibacterial. Antifungal
16.	23.165	2.53	6.66	Bis (2-ethylhexyl) phthalate S	Phthalate ester	Antimicrobial, Anti-biofilm

Biological relevance is inferred from previously reported antimicrobial, antibiofilm, and antioxidant studies of structurally similar compounds detected by GC-MS²³.

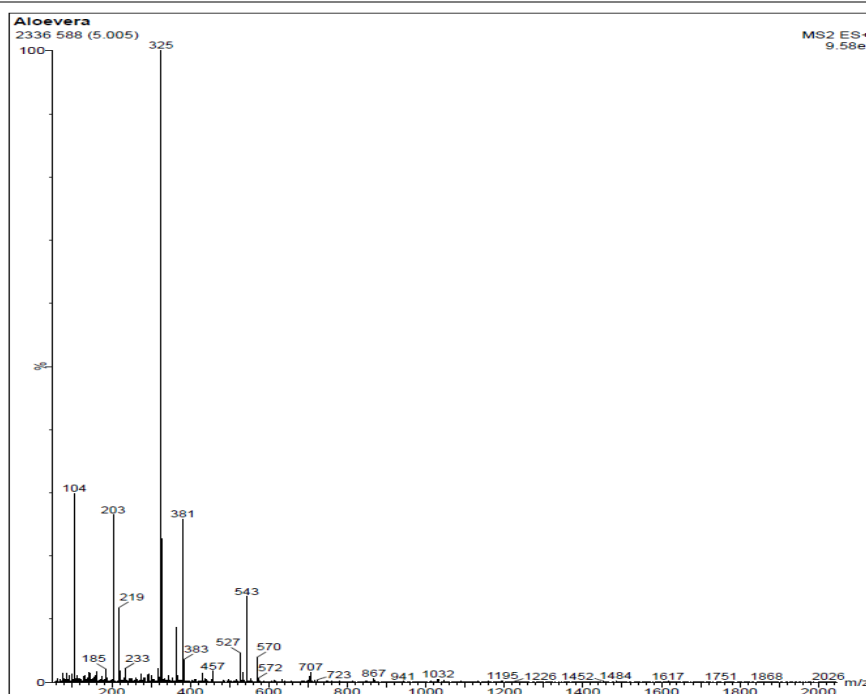


Figure 4. LC-MS/MS spectrum of *A. indica* ethanolic leaf extract. Positive-mode ESI tandem MS (MS²) acquired on the [M+H]⁺ precursor. Spectra are plotted on a relative intensity scale (0-100%) across m/z 0-2000.

Table 4. Putative phytochemical constituents of *A. vera* ethanolic leaf gel extract identified by Liquid Chromatography- Mass spectroscopy in ESI+ model

No	Observed m/z	Putative nature of fragment	Chemical	Molecular Formula (putative)	Reported biological relevance
1	104	Phenolic		C ₆ H ₄ O ₂ ⁺	Anti-oxidant activity with interference with initial microbial adhesion
2	185	Chromone		C ₁₀ H ₈ O ₄ ⁺	Antimicrobial and anti-inflammatory activity
3	203	Carbohydrate		C ₆ H ₁₂ O ₆ ⁺	Polysaccharide-rich components involved in biofilm matrix interactions
4	219	Chromone		C ₁₁ H ₈ O ₅ ⁺	Fragmentation pattern consistent with chromone backbone
5	233	Anthraquinone		C ₁₄ H ₈ O ₄ ⁺	Disrupt microbial membranes and EPS synthesis
6	325	Aloemodin		C ₁₅ H ₁₀ O ₅ ⁺	Anthraquinone with antibacterial, antifungal and antibiofilm with anti-quorum sensing properties
7	381	Oxidized chromone glycoside		C ₁₅ H ₁₀ O ₅ ⁺	Enhance solubility and biological availability
8	457	Aloin (barbaloin)		C ₂₁ H ₂₂ O ₉ ⁺	Major Aloe bioactive inhibiting microbial adhesion and biofilm formation

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9	527	Glycosylated anthraquinone	C ₂₄ H ₂₄ O ₁₃ ⁺	Sugar conjugation may modulate stability and activity
10	543	Aloin derivative	C ₂₆ H ₂₈ O ₁₃ ⁺	Structural variant
11	570	Flavonoid derivative	C ₂₇ H ₂₂ O ₁₃ ⁺	Antioxidant, antibiofilm and quorum quenching effects
12	707	Glycosylated anthraquinone	C ₃₂ H ₃₄ O ₁₈ ⁺	High molecular weight glycoside with antimicrobial effects

Molecular formulas and compound identities are putative, inferred from m/z values, ionization mode (ESI⁺), and comparison with previously reported LC-MS data of *Aloe vera*²⁴. Reported biological relevance was derived from published studies. Confirmation using authentic standards or NMR was not performed

3.4. Antimicrobial Activity and Viability testing of *A. vera* extract:

The antimicrobial efficacy of *A. vera* was evaluated against *S. mutans*, *C. albicans* and mixed polymicrobial culture using the agar well diffusion assay (Figure 5). A clear concentration dependant increase in zone of inhibition was observed for all test groups. For both *S. mutans* and *C. albicans* increasing concentration resulted in a progressive increase in antibacterial activity with maximum inhibition at 200 mg/ml (Figure 5A). Statistically significant difference (****, p ≤ 0.0001) were noted between lower concentrations (25-50 mg/ml) and higher concentrations (100-200 mg/ml) indicating a dose responsive antibacterial and antifungal effect. Importantly, polymicrobial mixed culture showed substantial inhibition with zones comparable or slightly lower than those observed in mono-species cultures. The highest concentration (200 mg/ml) produced largest

inhibition zone comparable to chlorhexadiene (50 µg/ml). Representative agar plants (Figure 5C, lower panel) show well defined zone of inhibition around wells containing *A. vera*.

The minimum inhibitory concentration (MIC) of *A. vera* was determined using the MTT assay that measures cellular viability of *S. mutans*, *C. albicans* and mixed cultures. As shown in Figure 5B, *A. vera* treatment resulted in a dose-dependent reduction in microbial viability across all tested groups with ≥ 90-99 % reduction at 100 mg/ml concentrations. CHX (50 µg/ml) was used as a positive control to benchmark the antimicrobial activity of *A. vera* extract. Concentrations of ~6.25 mg/ml of extract was found to give similar antimicrobial activity against monospecies and polymicrobial species.

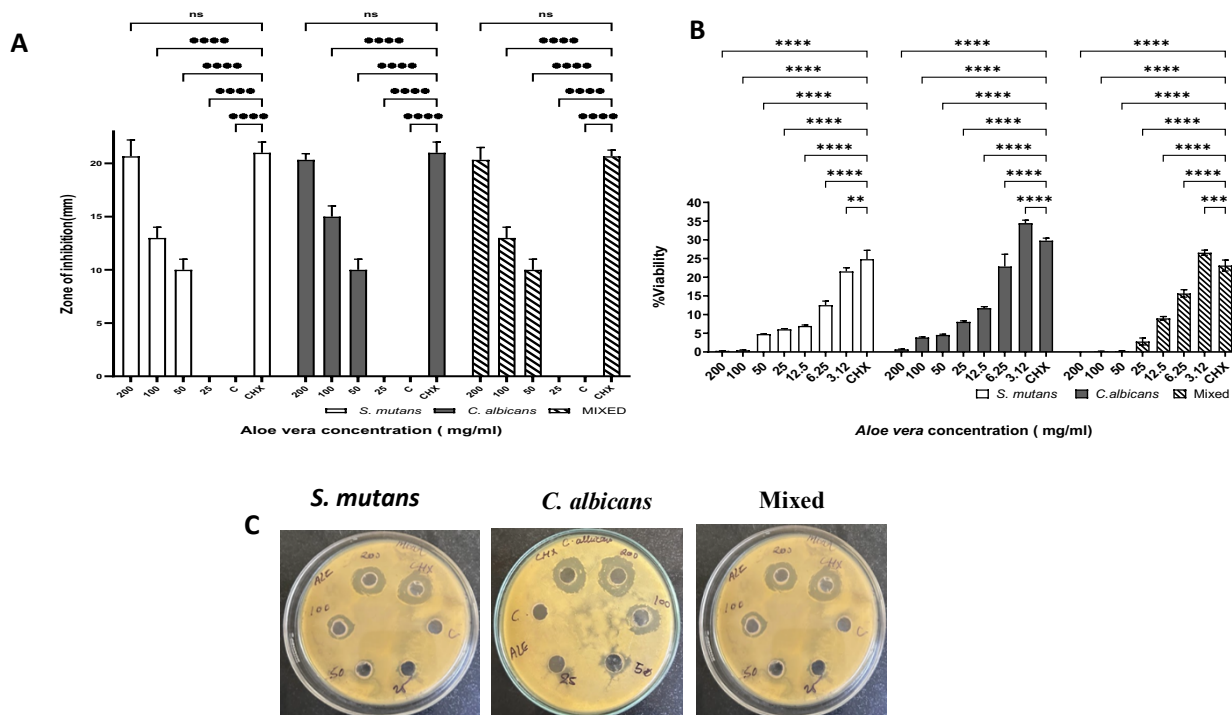


Figure 5. (A) Antimicrobial activity of *S. mutans*, *C. albicans* and Mixed Culture treated with different concentrations of *A. vera* ethanolic extract using well diffusion assay zone of inhibition. Experiments were performed in triplicates and data are expressed as mean

± SD. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. Statistically significant effects are indicated with asterisks p ≤ 0.05 (*). **(B)** Percentage viability of *S. mutans*, *C. albicans* and mixed culture treated with

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different concentrations of *A. vera* ethanolic extract as assessed by MTT assay. Chlorhexidine (CHX, 50 µg/ml) was used as a positive control. Data are expressed as mean ± SD. Statistical significance was determined by two-way ANOVA followed by Dunnett's multiple comparison post hoc test. Asterisks above each bar denote no significance compared to CHX control for that organism or mixed culture $p > 0.05$ (**)

3.5. Effect on Biofilm Formation and Eradication:

Polymicrobial oral biofilms are highly resistant to antimicrobial chemotherapeutic agents due to their ability to form biofilms. Polymicrobial biofilms between *S. mutans* and *C. albicans* are reported to be resistant compared to monospecies. Biofilm formation and eradication assays were set up in microtiter plates which

were treated with increasing concentrations of *A. vera* extract (3.12-200 mg/ml). Figure 6A indicates a dose-dependent inhibition of biofilm formation and preformed biofilm eradication was found in all tested groups. *S. mutans* biofilm formation reached ~90-95%, *C. albicans* was inhibited at 80% at 200 mg/ml concentrations (****, $p < 0.0001$), while polymicrobial biofilm achieved ~85% inhibition at the same concentrations. Biofilm eradication percentages were lower at all *A. vera* concentrations, reflecting the increased resilience of mature biofilms (Figure 6B). At 200 mg/ml, *A. vera* achieved ~55% eradication of *S. mutans* biofilm ~inhibitory effect of *A. vera* exceeded biofilm inhibition achieved by CHX across all biofilm models, particularly for mixed biofilm group. The role of *A. vera* in biofilm inhibition has been reported for *Enterococcus* and *C. albicans* previously but not for mixed biofilms³⁵⁻³⁷.

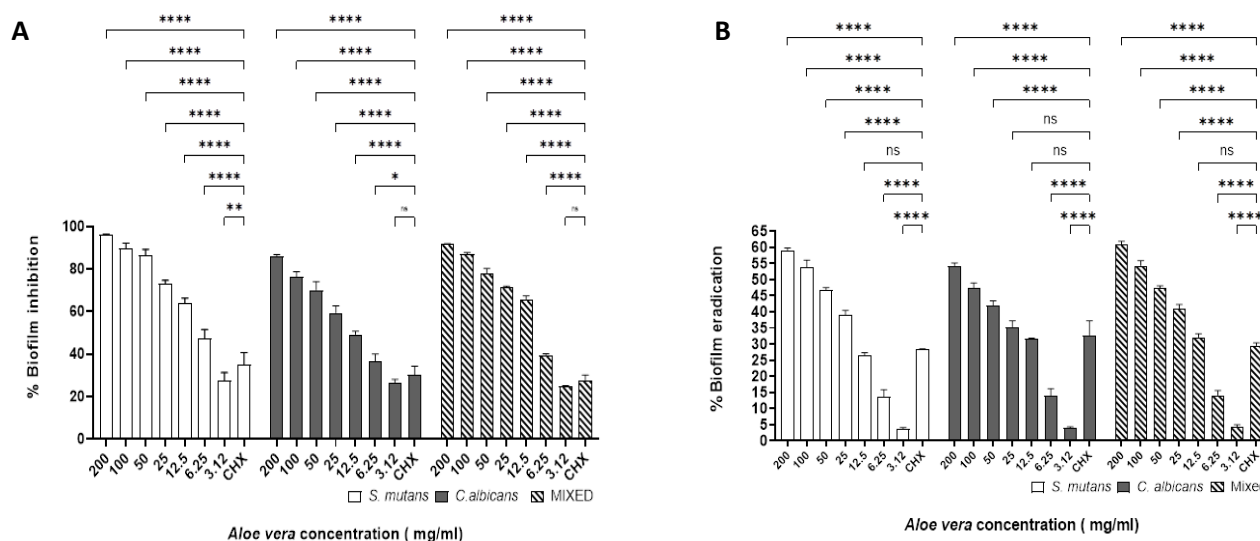


Figure 6. Inhibition of biofilm formation and eradication of formed biofilm by *A. vera* ethanolic extract.

Percentage inhibition of *S. mutans*, *C. albicans* and mixed biofilm using crystal violet assay at different concentrations (3.12 to 200 mg/ml) of *A. indica* ethanolic leaf extract on (A) Biofilm formation (B) Biofilm eradication assay. Chlorhexidine (50 µg/ml CHX) used as positive control for the assay. Data are expressed as mean ± SD. Statistical significance was determined by two-way ANOVA followed by Dunnett's multiple comparison post hoc test. Statistically significant effects are indicated with asterisks above each bar for that organism or mixed culture. ** $p < 0.05$; *** $p < 0.001$.

3.6. Molecular docking of Aloe emodin with polybiofilm forming proteins:

Molecular docking analysis was performed to elucidate the anti-biofilm mechanism of *A. vera* gel extract by evaluating the

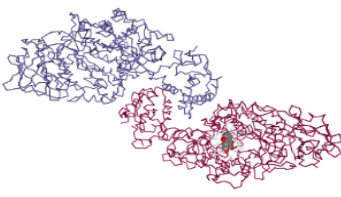
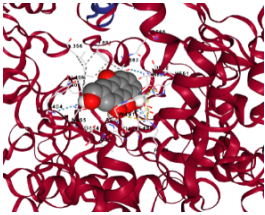
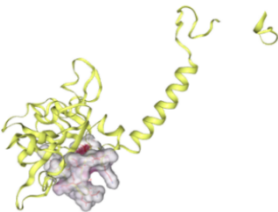
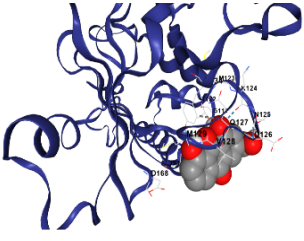
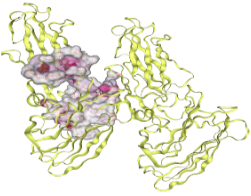
interaction of its major bioactive compounds, aloe emodin, with key virulence and biofilm associate proteins of *S. mutans* and *C. albicans*. The docking results demonstrate favourable binding affinities of Aloe emodin towards glucosyl transferase B (Gtf B; PDB:8FT9) and Sortase A (Srt A; PDB:4TQX) and of *S. mutans* and Als3, PDB: 4LE5) and Sap5; PDB:2QZC) of *C. albicans*. Blind docking of Aloe emodin with four protein targets was performed using CB-Dock2. For each protein, the top five cavities were detected and ligand docked. The most favourable binding energies (kcal/ml) and corresponding binding poses are presented in Figure 7. Aloe emodin was found to interact with each of the protein implicated in polymicrobial biofilm formation further validating its role in control biofilm formation (Table 5). Among the bacterial targets. Aloe

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emodin showed stronger binding affinity towards Gtf B (-8.2 kcal/mol) that SrtA (-6.4 kcal/mol), indicating a predominant effect on EPS synthesis. In parallel, inhibition of SrtA compromises anchoring of surface adhesins weakening initial attachment and clustering. For *C. albicans*, strongest binding affinity for Sap 5 (-8.5 kcal/mol), essential for hyphal development followed by Als3 (-7.8 kcal/mol), which plays a central role in cross kingdom adhesion. Synergy arising from concurrent targeting of bacterial EPS synthesis, adhesin anchoring,

hyphal adhesion and protease mediated biofilm maturation. Previous *in silico* studies demonstrate that bioactive compounds of *A. vera* such as aloesin, aloemodin and chrysophanic acid exhibited significant docking affinities with bacterial and virulence associated protein in *Helicobacter pylori*³⁸. Emodin derivative have been shown to bind kinases relevant to *C. albicans* biofilm formation further validating computational approaches for exploring phytotherapeutic targets³⁹.

Figure 7. Favourable binding energies (kcal/ml) and corresponding binding for *S. mutans* GtfB, Sortase A and *C. albicans* Als3 and Sap5 with Aloe-emodin

Cavity	Protein-Ligand	Interacting residues
<i>S. mutans</i> GtfB		
<i>S. mutans</i> Sortase A		
<i>C. albicans</i> Als3		

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Table 5. Structural and docking-based characterization of biofilm-associated targets, GtfB and SrtA in *S. mutans*, and Als 3 and Sap5 in *C. albicans* including binding energies, interacting residues, and cavity metrics

Organism	PDB	Gene	Biological Role	Vina score Binding Energy kcal/mol	Contact residue	Cavity volume A ³	Cavity Size
<i>S. mutans</i>	8FJ9	GtfB	Glucosyltransferase involved in extracellular polysaccharide synthesis and biofilm matrix formation	-8.2	Chain A: LEU356 TYR404 LEU407 LEU408 ARG449 ASP451 ALA452 VAL453 ASP454 ASN455 GLU489 TRP491 HIS561 ASP562 GLN566 PHE881 ASP883 ASN888 TYR890	727	17,12,9
<i>S. mutans</i>	4TQX	SrtA	Sortase A responsible for anchoring surface adhesins and biofilm establishment	-6.4	Chain A: ILE93 LYS109 THR122 LYS124 ASN125 ASP126 GLN127 MET129 TYR134 ASP168	632	13,13,7
<i>C. albicans</i>	4LE5	Als3	Agglutinin-like sequence protein 3 (Als 3) -a major adhesin involved in cross kingdom interactions	-7.8	Chain A: ALA19 THR20 TYR21 ASN22 TYR23 THR28 SER159 VAL161 LEU167 THR168 ASP169 TRP224 ASN225 TYR226 ARG299	2449	22,26,25
<i>C. albicans</i>	2QZX	Sap 5	Secreted aspartic proteinase involved in tissue invasion and biofilm maturation	-8.5	Chain A: ALA11 ALA162 CYS256 SER277 GLU278 LEU280 PHE281 GLN282 CYS294 ARG312 Chain B: CYS256 LYS257 THR258 SER259 SER277 LEU280	2100	17,17,30

3.7 Integrated *In silico* ADMET and SMILES based acute oral toxicity of Aloe-emodin:

The ADMET properties for Aloe-emodin were evaluated using SwissADME followed by toxicity prediction using ProTox-3.0 (Prediction of Toxicity of

Chemicals) and StopTox, that evaluated various toxicity endpoints using machine learning and QSAR models^{31,32}. Aloe-emodin exhibited favourable drug-likeness, satisfying Lipinski, Ghose, Veber, Egan and Muegge criteria with no rule's violation and a predicted

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availability of 0.55. The compound showed moderate lipophilicity (consensus log P=1.5) and a topological polar surface area (TPSA) of 94.83Å², consistent with its high predicted gastrointestinal absorption and lack of blood-brain barrier permeability as also determined by boiled egg plot (Supplementary Figure). Swiss ADME solubility models (ESOL, Ali, SILICO-IT) uniformly classified is a water soluble (Predicted solubility 3.22×10^{-2} - 2.46×10^{-1} mg/mL)

Prior to toxicity interpretation, the applicability domain (AD) of the model was assessed using Dice similarity analysis, which was found to lie within the AD of the training set, thereby supporting the reliability of the prediction. The Stop Tox model predicted an acute oral toxicity probability of 64% for Aloe-emodin (Figure 7B). The SwissADME bioavailability radar analysis revealed that the Aloe emodin resides within the optimal

physicochemical space for oral drug-likeness, with balanced lipophilicity, polarity, solubility and structural flexibility (Figure 7B). Fragment contribution analysis revealed that the anthraquinone scaffold and multiple hydroxyl and certain carbonyl associated sites contributed to predicted toxicity signals (Figure 7C). These findings suggest that the overall predicted oral toxicity arises from the combined influence of quinone and bioactive phenolic structural features. The predicted high gastrointestinal absorption and moderate oral toxicity of Aloe-emodin suggest that its oral exposure requires careful dose optimization, when considering interventions targeting oral communities such as *S. mutans* and *C. albicans*. These are computational predictions and require experimental validation to confirm their biological relevance.

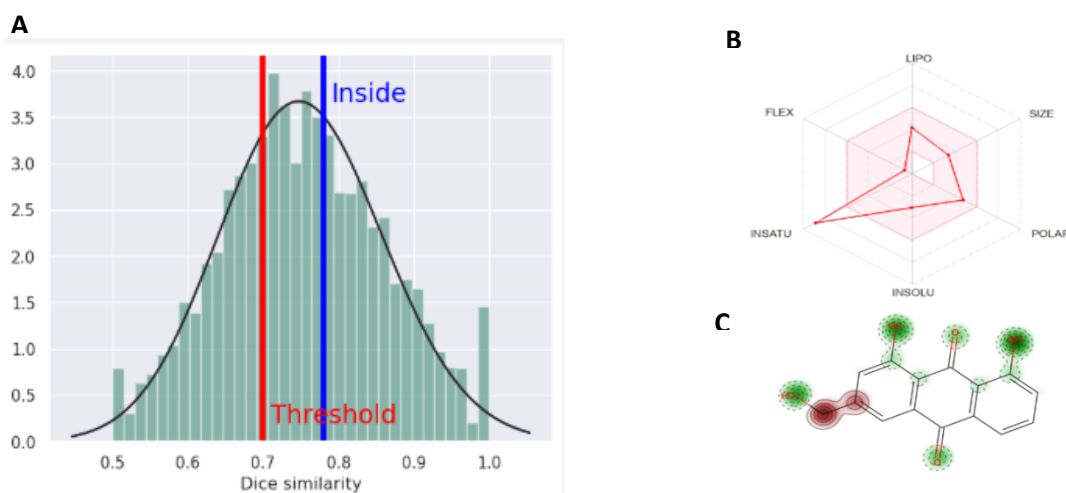


Figure 8. *In silico* toxicity prediction and ADME profiling of Aloe emodin (A) Dice similarity distribution using Stop Tox platform, where red represent predefined toxicity alert threshold and blue link indicates compounds in acceptable similarity range (B) Swiss ADME bioavailability radar summarizing lipophilicity, size, polarity, solubility, saturation and flexibility (C) 2D molecular representation wherein atomic or sub structural elements are indicated as favourable (green), unfavourable (red) influence on ADME-related properties for qualitative guidance for structure-property optimization.

4. Conclusion

Mixed biofilms are typically more resistant to antimicrobial agents due to synergistic interactions, enhanced extracellular matrix production. Some investigations demonstrate that *A. vera* are often required higher doses, based on formulations to achieve similar anti-biofilm effects, consistent with the findings of this study. In mixed biofilms, *S. mutans* enhances *C. albicans* adhesion through glucosyl transferase-

mediated extracellular polysaccharide production while *C. albicans* provides surface for bacterial colonization. *A. vera* phytochemicals are reported to inhibit GTF activity and reduce EPS synthesis thereby weakening the matrix that stabilizes the biofilms. Phenolics and anthraquinone may also disrupt membrane integrity, increasing permeability leading to reduced metabolic activity, as observed in the MTT assay outcomes. Surfactant-like properties may also reduce surface hydrophobicity limiting adhesion and biofilm formation. In the present study, higher concentrations of *A. vera* demonstrated comparable metabolic inhibition as compared to CHX. Other studies have investigated oral antimicrobial properties in low to mid microgram range, with CHX exhibiting superior antimicrobial potency than *A. vera* alone. The ability of *A. vera* to achieve CHX comparable inhibition supports its scientific rationale behind using a biocompatible, plant derived natural adjunct in oral care formulations aimed at controlling polymicrobial infections.

Abbreviations

ADMET: Absorption, CV: Crystal Violet; DMSO: Dimethyl Sulfoxide; MBC: Minimum Bactericidal

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Concentrations; MIC: Minimum Inhibitory Concentration; MHA: Muller Hinton Agar; MHB: Muller Hinton Broth; TSB: Tryptone Soy Broth; QS: Quorum Sensing; TFC: Total Flavonoid Content; TPC: Total Phenolic Content. LC-MS/MS: Liquid chromatography- Tandem Mass Spectrometry

Author Contribution Statement

Conceptualization, experimentation, analysis and writing—original draft preparation, SR, SDK and NS; writing—review and editing, SDK, SS, AK, NS and EK. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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