



Comparative *in-silico* docking, pharmacokinetic characterization, and antimicrobial evaluation of 2,4-DTBP and chlorhexidine against gingivitis-associated oral pathogens

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ABSTRACT

Gingivitis, the most common form of periodontal disease, affects nearly every individual at some stage of life. Chlorhexidine is widely used as the gold standard for its treatment; however, its potential cytotoxicity and irritant effects at higher concentrations necessitate the development of safer alternatives. This study investigates the *in-silico* docking potential of 2,4-di-tert-butylphenol (2,4-DTBP), a phenolic compound, against four key oral pathogens associated with gingivitis—*Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. Two proteins involved in virulence and biofilm formation were selected from each species. Docking analysis with Autodock was done, which revealed, 2,4-DTBP displays binding affinities comparable to chlorhexidine. Furthermore, its absorption, distribution, metabolism, excretion and toxicity profile and drug-likeness were evaluated using Swiss Absorption, Distribution, Metabolism, Excretion, Protox, Predicting Small-Molecule Pharmacokinetic and Toxicity Properties Using Graph-Based Signatures and Molsoft tools, indicating acceptable pharmacokinetic properties with mild irritability. In addition, minimum inhibitory concentration evaluation demonstrated that both 2,4-DTBP and chlorhexidine exhibited measurable antibacterial activity, with 2,4-DTBP showing comparatively stronger inhibition, further supporting the *in-silico* findings. To improve therapeutic efficacy and minimize toxicity, the compound holds strong potential for delivery through advanced systems such as nanoparticles, nanoemulsions, liposomes, phytosomes and solid lipid nanoparticles, especially when encapsulated with biocompatible polymers. These platforms can enhance stability, targeted delivery and sustained release. Therefore, 2,4-DTBP can be considered a preliminary candidate for further development in gingivitis management, particularly within biocompatible, controlled-release delivery systems.

1. INTRODUCTION

Periodontal diseases are among the most prevalent inflammatory conditions in the oral cavity. They affect the

periodontium, which includes the supporting structures of the teeth. These diseases are mainly caused by bacterial biofilms and typically impact people at least once in their lives. The most common type is gingivitis, which can become chronic and is troubling because it may advance to periodontitis. This more severe form involves not only the gums but also the teeth and the surrounding bones and ligaments, which could result in destruction of periodontium [1]. The mouth is very prone to biofilm formation due to a variety of microbes living in

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balance; over 700 species are found in a healthy person. Their ongoing metabolic activities lead them to cluster into biofilms, which help them get the nutrients they need, providing survival advantages [2]. This biofilm formed is known as dental plaque; it's a colourless, sticky layer that sticks to tooth enamel or gum surfaces. In its early stages, plaque can be easily removed with regular brushing and flossing. It mainly consists of beneficial oral microbes and is generally harmless [3]. However, if not cleaned, microbial communities grow and increase the number of harmful bacteria. This change in microbial balance, known as dysbiosis, can lead to inflammatory issues like gingivitis and periodontitis. Over time, plaque hardens into tartar or calculus, which holds more harmful species, worsens inflammation and needs professional removal [4]. These harmful infections can extend beyond the mouth, affect gut microbiota and cause systemic inflammatory responses in other parts of the body. These interconnected inflammatory pathways are increasingly linked to the development or worsening of conditions related to the heart, brain, lungs and digestive system, raising significant concerns [5].

Over the past decades, researchers around the world have mainly concentrated on managing and treating periodontitis because of its effect on systemic circulation. As a result, gingivitis has received less attention. Early detection and treatment of gingivitis could help stop its development into the chronic and more harmful form, periodontitis. This preventive strategy could decrease the need for regenerative and surgical procedures. It would also lessen the complexity of treatment and the financial strain on patients [6].

Many bioactive compounds in nature can help reduce gingivitis. Many of these come from traditional home remedies used by different cultures. Recent advances have made it possible to isolate and identify specific bioactive molecules from raw plant, algal, fungal and lichen extracts. This allows for more focused treatment effects than what the full mix of chemicals provides [7]. However, issues like poor absorption, instability and possible toxicity have limited the practical use of some of these powerful compounds. This can now be addressed through new drug delivery methods such as nanoparticle formulation, nanoemulsions, liposomes, phytosomes, solid lipid nanoparticles and the use of biocompatible polymers for encapsulation. These methods improve stability, absorption, offer better targeted delivery, controlled release and overall safety [8]. This makes them promising options for effectively treating gingivitis. One such compound, 2,4-di-tert-butylphenol (2,4-DTBP), is a lipophilic phenolic molecule that shows a wide range of bioactivities *in vitro*. These include antimicrobial, antifungal, antioxidant, anti-inflammatory, anticancer and antibiofilm effects [9–11]. However, the use of 2,4-DTBP in medicine is limited because of its potential to be mildly toxic. It has been reported to cause autotoxicity in producing organisms and harmful effects in various human cell lines [12]. Interestingly, 2,4-DTBP is produced by certain plants, fungi or lichens, especially when they are under stress. It provides benefits such as better stress tolerance and protection against pathogens [11].

Chlorhexidine is currently regarded as the gold-standard antiseptic in the management of plaque-induced gingivitis and is widely incorporated into mouthrinses and other oral-care formulations. However, its clinical utility is constrained by several local adverse effects, including tooth and tongue staining, taste disturbance, mucosal irritation and in some cases, desquamation of the oral epithelium. *In vitro* studies have also demonstrated pronounced cytotoxicity of chlorhexidine towards a variety of mammalian cell types, particularly oral fibroblasts and osteoblast-like cells, raising concerns about damage to host tissues during prolonged use [13]. Chlorhexidine exerts its antibacterial activity primarily through cationic binding to bacterial cell walls, followed by disruption of cytoplasmic membranes and precipitation of intracellular components. In contrast, phenolic alkyl compounds such as 2,4-DTBP are proposed to intercalate into lipid bilayers, perturb membrane integrity and generate oxidative stress through redox-active phenolic groups. This mechanistic distinction is relevant when considering differential toxicity towards host cells and the potential for resistance development [14].

In this study, an *in-silico* approach was employed to evaluate the efficacy of 2,4-DTBP against biofilm-forming oral bacteria associated with gingivitis. The selected bacteria include two facultative anaerobes, *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans*, and two obligate anaerobes, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. All four bacterial species are recognised to contribute to oral dysbiosis and worsen the progression of gingival and periodontal diseases. *Streptococcus mutans* is a primary colonizer that starts dental caries; increasingly found in patients with chronic gingivitis and periodontitis, likely due to shared inflammatory pathways between these conditions [15,16]. *Fusobacterium nucleatum* acts as a bridge organism, helping early and late colonizers to co-aggregate in the oral biofilm. As an opportunistic pathogen, it plays a crucial role in the shift from healthy to dysbiotic microbial community [17]. *Aggregatibacter actinomycetemcomitans* is a capnophilic, facultative anaerobe strongly linked to aggressive periodontitis, especially in juvenile periodontitis cases. Its ability to produce leukotoxins and other immune-modulating factors is related to its virulence [18]. *Porphyromonas gingivalis*, an obligate anaerobe, is seen as a key pathogen in periodontal disease. It can manipulate the host immune response and disrupt microbial balance, which promotes a dysbiotic environment that leads to alveolar bone loss [19].

Following an extensive literature review, two key antibiofilm protein targets were selected from each of these oral pathogens and subjected to molecular docking with 2,4-DTBP using AutoDock to assess binding affinities [20]. The binding efficacy of 2,4-DTBP was compared with chlorhexidine, the gold standard in the treatment of periodontal diseases. To further assess the drug candidacy of 2,4-DTBP, an in-depth pharmacodynamic evaluation was essential and thus, subsequently, its absorption, distribution, metabolism, excretion and toxicity (ADMET) characteristics were predicted using Swiss Absorption, Distribution, Metabolism, Excretion

(SwissADME), Predicting Small-Molecule Pharmacokinetic and Toxicity Properties Using Graph-Based Signatures (pkCSM) and ProTox II, along with molecular descriptors and drug-likeness potential analysed using the Molecular Software (MolSoft) platform [21,22]. This comprehensive computational analysis aims to provide preliminary insights into the therapeutic potential and pharmacokinetic behaviour of 2,4-DTBP as a prospective antibiofilm agent against gingivitis-associated oral pathogens.

2. METHODOLOGY

2.1. Protein target selection and preparation for docking

Four gingival pathogens- *S. mutans*, *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*, were chosen for this study because of their known roles in damaging affecting gum health and worsening inflammation. Two critical target proteins were selected based on their known roles in bacterial adhesion, colonization, immune evasion and biofilm formation, all critical factors that lead to the onset and persistence of oral infections. Three-dimensional structures of these proteins were retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB) in PDB format using

their accession IDs (Table 1). To ensure accurate and reliable docking simulations, the proteins were prepared prior to molecular docking by removing nonstandard residues and water molecules using “UCSF ChimeraX” (University of California, San Francisco ChimeraX), as the ligand may interact with these entities, affecting the docking score [24]. Table 1 details the specific protein targets from each pathogen and their roles in disease progression, with their respective PDB Identifiers.

2.2. Optimization of the ligand molecule

The 3D structure of 2,4-DTBP was retrieved from PubChem (CID: 7311) in structure data file format. Geometry optimisation was carried out in Avogadro using the MMFF94 force field with the steepest-descent algorithm, a convergence criterion of 10^{-7} kcal·mol⁻¹ and a maximum of 5,000 optimisation steps to ensure local energy minimisation of the ligand conformers before docking [30–33] and the energy-minimized 3D conformer was saved in Molecule format, which was then converted to PDB format using Open Babel [34] for docking in AutoDock. The same procedure was followed for chlorhexidine (CID: 9552079), which was used as a reference ligand to compare its binding efficacy with that of 2,4-DTBP.

Table 1. Selected bacteria and protein targets with functional roles in biofilm formation and virulence; along with PDB identifiers.

Organism	Role in gingivitis	Protein target	Function	Classification	PDB ID	Reference
<i>S. mutans</i>	Early colonizer	Antigen I/II (V-region)	Biofilm attachment	Membrane protein	1 JMM	[23]
		ComA (ATP-binding protein)	Quorum sensing	Transport protein	3 VX4	
<i>F. nucleatum</i>	Links early and late colonizers in gingivitis	FadA (L14A mutant)	Biofilm attachment	Cell adhesion protein	3 ETX	[24]
		CbpF (Autotransporter adhesin)	Biofilm attachment	Cell adhesion protein	9 GH4	[25]
<i>A. actinomycetemcomitans</i>	Increases inflammation leading to aggressive periodontitis	DspB (Dispersin B)	Biofilm dispersion	Hydrolase	1 YHT	[26]
		CDT (Cytotoxic distending toxin)	Inflammation	Toxin	2 F2F	[27]
<i>P. gingivalis</i>	Key periodontal pathogen	Kgp (Gingipain K)	Disrupts host proteins	Hydrolase	4 RBM	[28]
		FimA (pilus subunit)	Biofilm attachment	Cell adhesion protein	6 KMF	[29]

Table 2. Predicted active binding sites of target proteins with average conservation scores and centroid coordinates (via PrankWeb).

Protein targets	Predicted pocket priority	Average evolutionary conservation of pocket residues	Centroid coordinates		
			X	Y	Z
1 JMM	1	1.192	36.8	25.5	-5.2
3 VX4	1	2.161	42.5	21.4	11.4
3 ETX	1	2.549	19.0	-23.0	20.0
9 GH4	3	0.099	159.8	159.3	199.7
1 YHT	1	1.703	15.6	2.0	27.3
2 F2F	2	1.943	20.9	72.8	31.4
4 RBM	1	2.127	26.3	27.5	25.9
6 KMF	2	1.452	197.4	218.6	229.7

2.3. Prediction of ligand-binding regions in proteins

The 3D structures of the protein targets were examined with the active site prediction tool “PrankWeb” [35], which was then cross-verified with another tool, Computed Atlas of surface topography of proteins (CASTp) before further processing [36], to find the most probable ligand-binding sites. The predicted active sites were ranked according to spatial-physicochemical factors and average evolutionary conservation of amino acid residues present in them. Each of these sites was then tested through molecular docking with ligands 2,4-DTBP and chlorhexidine. The binding pocket that showed the least binding energy on interaction was chosen as the best. The structural coordinates and key features of the selected ligand binding pockets are shown in Table 2.

2.4. Ligand protein binding using AutoDock

The pre-optimized 3D structures of the protein and ligands, generated using UCSF Chimera and Avogadro, were imported into AutoDock in PDB format. For the proteins, polar hydrogens were added, Kollman charges assigned and AD4 atom types specified, after which the structure was saved in PDBQT (PDB, Partial Charge and Torsions) format. The ligand structures were also similarly pre-processed by adding Gasteiger charges, defining rotatable bonds and finally, converted into PDBQT format. Grid parameters were set up using the centroid coordinates in Table 2, predicted using the PrankWeb with a grid spacing of 0.500 Å. AutoGrid was used to compute the grid maps, followed by molecular docking with AutoDock. Both 2,4-DTBP and chlorhexidine followed the same docking protocol, with 100 genetic algorithm (GA) runs generated for each ligand. For each protein–ligand pair, 100 independent Lamarckian GA runs were performed. Binding energies reported correspond to the mean \pm standard deviation of the lowest-energy poses within the most populated cluster, with cluster Root mean square deviation cut-offs set to 2.0 Å [34]. The docking pose with the lowest binding energy was chosen and visualized using the ProteinPlus server. Two-dimensional interaction diagrams were created using PoseView, which is also part of ProteinPlus [37]. Finally, the binding affinities of 2,4-DTBP were compared with those of chlorhexidine to evaluate its potential bioactivity and effectiveness.

2.5. Molecular properties and drug-likeness

The molecular properties and drug-likeness of 2,4-DTBP were evaluated using the MolSoft online platform. This tool predicts how likely a compound is to have drug-like features by comparing its properties against a dataset of over 5,000 approved drugs and 10,000 nondrug molecules. The drug-likeness score gives insight into how well the compound fits with pharmacologically active molecules [38].

2.6. Pharmacokinetic and toxicological profiling

Pharmacokinetic properties, including ADMET of 2,4-DTBP were assessed using two online prediction tools: SwissADME [39] and pkCSM [40]. These tools use structure-based models to estimate important parameters such as gastrointestinal absorption, blood–brain barrier (BBB) permeability, cytochrome

P450 enzyme inhibition, renal clearance and potential toxic effects. In addition, a comparison of the toxicological profile of 2,4-DTBP and chlorhexidine was done using ProTox II [41,42]. Using these platforms improves the reliability of the predictions and provides a thorough *in silico* evaluation of the compound’s drug-like behaviour and safety profile.

2.7. *In vitro* Minimum Inhibitory Concentration (MIC) determination of 2,4-DTBP and chlorhexidine against gingivitis-associated oral pathogens

The MIC of 2,4-DTBP and chlorhexidine was determined by the broth dilution method using test tubes. *Streptococcus mutans* cultures were incubated for 24 hours and *A. actinomycetemcomitans* cultures were incubated for 48 hours with varying concentrations of both antibacterial agents (2, 4, 8, 16, 32, 64, 128, 256, 512 and 1,024 $\mu\text{g/ml}$) at 37°C in 5% CO₂. The experiment was performed in triplicate and the tubes were visually examined for bacterial growth after incubation. The lowest concentration of the compound showing complete absence of visible growth (no turbidity) was recorded as the MIC, while tubes showing visible turbidity were indicative of bacterial growth [43].

3. RESULTS

3.1. Molecular docking

The ligand 2,4-DTBP was docked against 8 selected protein targets from the four test pathogens. The binding affinities of 2,4-DTBP ranged between -5.0 and -7.0 kcal/mol. These values are very similar to those of the docking energies of reference compound chlorhexidine, which showed comparable binding energies across the same targets. This similarity in docking scores indicates that 2,4-DTBP may have binding effectiveness like chlorhexidine against these bacterial proteins. Analysis of docking results was conducted using AutoDock and ProteinPlus server. The binding affinities of ligands 2,4-DTBP and chlorhexidine for all protein targets are summarized in Table 3. Across all targets, the standard deviation of binding energies within the best-scoring cluster

Table 3. Comparative binding energies (kcal/mol) of 2,4-DTBP and chlorhexidine docked against protein targets from selected oral pathogens.

Organism	Protein target	Binding energy (Kcal/ mol)	
		2,4- DTBP	Chlorhexidine
<i>S. mutans</i>	1 JMM	-5.77 ± 0.4	-6.73 ± 0.5
	3 VX4	-6.03 ± 0.1	-6.49 ± 0.05
<i>F. nucleatum</i>	3 ETX	-5.15 ± 0.5	-6.11 ± 0.4
	9 GH4	-4.04 ± 0.09	$+2.68 \pm 0.5$
<i>A. actinomycetemcomitans</i>	1 YHT	-5.62 ± 0.1	-6.00 ± 0.2
	2 F2F	-5.02 ± 0.4	-5.11 ± 0.05
<i>P. gingivalis</i>	4 RBM	-5.86	-5.72 ± 0.4
	6 KMF	-5.70 ± 0.1	-6.11 ± 0.2

was ≤ 0.5 kcal/mol, indicating good convergence of the docking protocol. Given the intrinsic approximations of the scoring function and the limited sampling of protein flexibility (FLEX), the reported binding energies should be interpreted as relative indicators of binding preference rather than precise thermodynamic quantities. The docked conformations of 2,4-DTBP with the lowest binding energies were visualized using the ProteinPlus server and detailed 2D interaction maps were created with the PoseView tool. Representative 3D docking poses and corresponding 2D interaction plots are shown in Figures 1–8.

3.2. Physicochemical and drug-likeness evaluation of 2,4-DTBP

The MolSoft computational tool was used to assess the spatial arrangement and molecular interaction potential of 2,4-DTBP ($C^{14}H^{22}O$; Molecular weight 206.17 g/mol). The results revealed the presence of one hydrogen bond donor and hydrogen bond acceptor atom, attributed to phenolic –OH. The calculated (Molecular Logarithm of the Partition Coefficient (p)) value is 5.22, showing significant lipophilicity (LIPO). The predicted Molecular Logarithm of Solubility value was -4.76 , which indicates poor aqueous solubility around 3.55 mg/l. The topological polar surface area measures 16.79 \AA^2 , suggesting limited polarity (POLAR). The estimated molecular volume of 257.37 \AA^3 , points to a compact structure. The pKa (negative logarithm of the acid dissociation constant) values for the most basic and acidic functional groups are reported as less than 0 and 9.74, respectively, thus confirming the presence of both strongly acidic and weakly basic moieties.

The BBB permeability score of 4.86 implies that the molecule may enter the central nervous system (CNS). Importantly, the structure has no stereocenters, which simplifies synthesis and reduces issues with stereoisomerism. The predicted drug-likeness score of 1.24 (Fig. 9) supports its potential as a lead compound in drug discovery. However, its LIPO and low solubility may require structural changes to improve pharmacokinetics.

3.3. Pharmacokinetics- ADME and toxicity

Online tools SwissADME, pkCSM and ProTox II” were used to assess the pharmacokinetic and drug-like

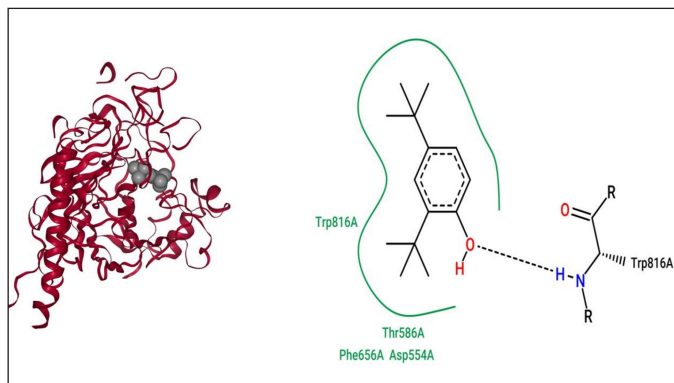


Figure 1. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target IJMM-antigen I/II (V-region) of *S. mutans*.

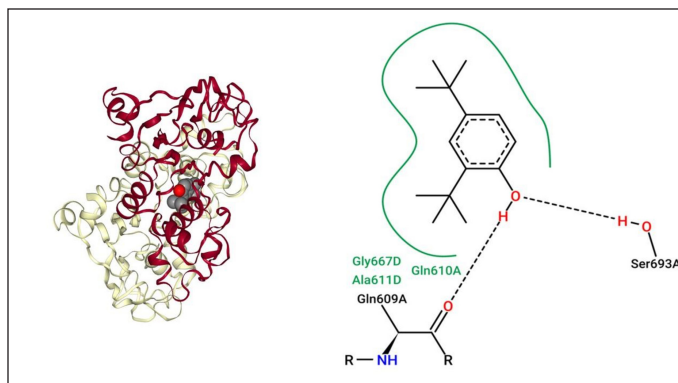


Figure 2. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target 3 VX4-ComA (ATP-binding protein) of *S. mutans*.

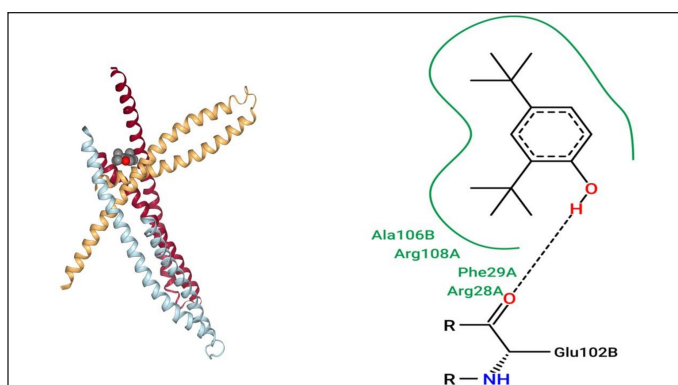


Figure 3. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target 3 ETX-Fad A (L14A mutant) of *F. nucleatum*.

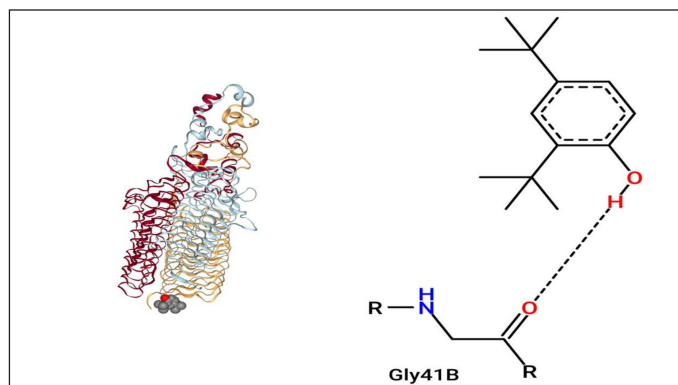


Figure 4. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target 9 GH4-CbpF (Autotransporter adhesin) of *F. nucleatum*.

profiles of 2,4-DTBP. A detailed ADMET analysis was done to evaluate the compound's potential as a drug candidate, as shown in Table 4. Figure 10 displays the chemical structure of 2,4-DTBP, along with a radar plot showing key physicochemical descriptors such as LIPO, molecular size, POLAR, water insolubility (INSOLU), molecular FLEX and degree of unsaturation (INSATU). The SwissADME bioavailability radar indicated that the LIPO and INSOLU parameters of 2,4-DTBP fall outside the optimal range for

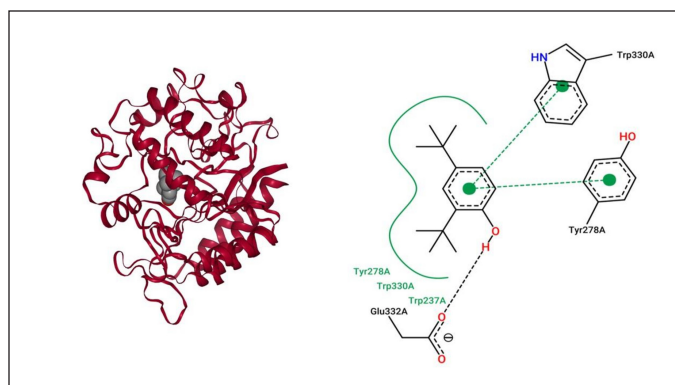


Figure 5. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target IYHT DspB (Dispersin B) of *A. actinomycetemcomitans*.

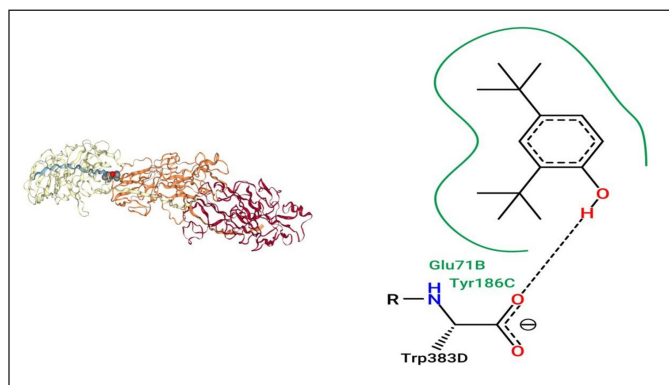


Figure 8. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target 6 KMF-FimA (Pilus) subunit of *P. gingivalis*.

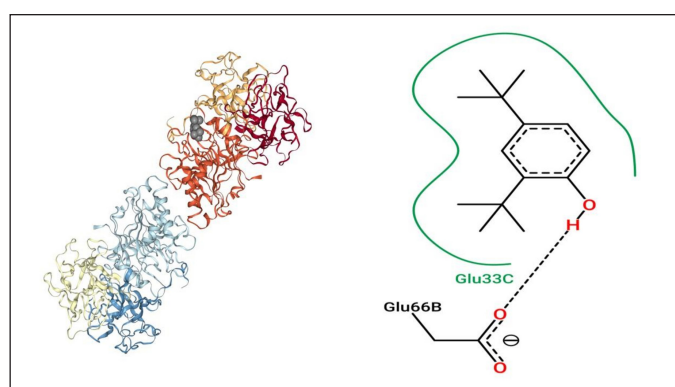


Figure 6. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target 2 F2F- CDT (Cytotolethal distending toxin) of *A. actinomycetemcomitans*.

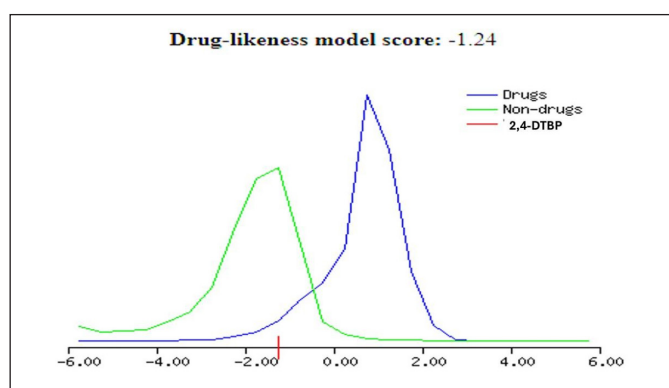


Figure 9. Drug-likeness model score distribution for 2,4-DTBP compared to drugs and nondrugs.

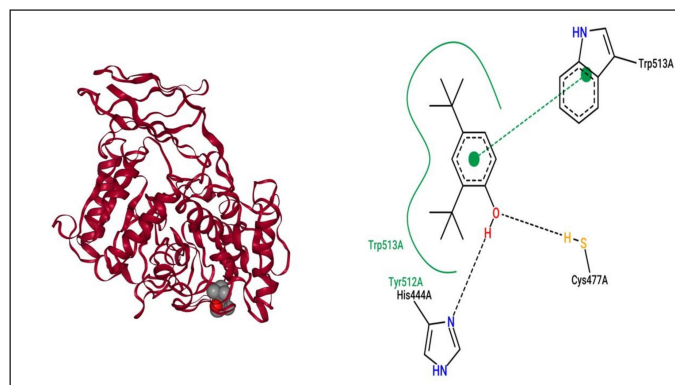


Figure 7. The 3D docking pose and 2D interaction diagram of 2,4-DTBP with protein target 4 RBM-Kgp (Gingipain K) of *P. gingivalis*.

oral drug-like molecules, reflecting its high LIPO and poor aqueous solubility. 2,4-DTBP is also predicted as a substrate of CYP3A4 and an inhibitor of CYP1A2, which suggests it may interfere with the efflux of co-administered CYP1A2 substrates.

The Boiled-Egg model, a popular graphical tool in early drug discovery, is also included in the figure, which predicts the molecule's chances of passive gastrointestinal absorption (HIA) and its ability to cross the BBB. In toxicity

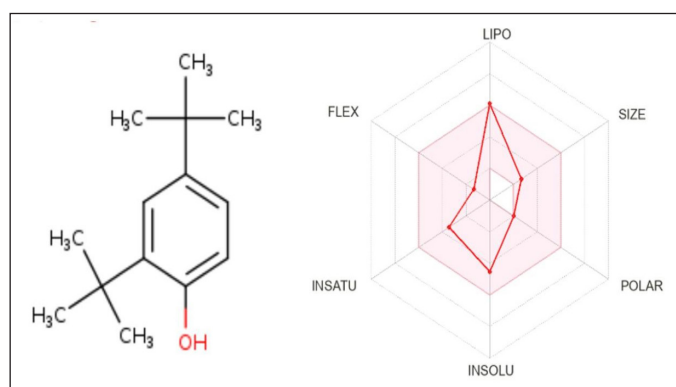
prediction using ProTox-II, both chlorhexidine and 2,4-DTBP were classified under the same Toxicity Class 4, indicating a comparable level of acute oral toxicity. Chlorhexidine showed no major predicted organ-specific toxicities, while 2,4-DTBP displayed certain *in-silico* alerts that require further evaluation in cell lines. Overall, both compounds demonstrate similar acute toxicity profiles, with 2,4-DTBP needing additional experimental validation to clarify its safety characteristics.

3.4. Comparative MIC determination of 2,4-DTBP and chlorhexidine against gingivitis-associated oral pathogens

The MIC values of 2,4-DTBP and chlorhexidine were determined against *S. mutans* and *A. actinomycetemcomitans* using the broth dilution method. *Streptococcus mutans* showed complete inhibition of visible growth at a concentration of 4 µg/ml for 2,4-DTBP, while *A. actinomycetemcomitans* growth was inhibited only at 8 µg/ml concentration, whereas chlorhexidine exhibited growth inhibition of *S. mutans* at 8 µg/ml and *A. actinomycetemcomitans* at 16 µg/ml concentrations. The results indicate that 2,4-DTBP demonstrated comparatively higher antibacterial efficacy than chlorhexidine against both test organisms.

Table 4. Detailed analysis of ADMET properties of 2,4-DTBP predicted from SwissADME and PKCSM.

Category	Property	Predicted value	Unit	Interpretation
Absorption	Water solubility	-3.924	log mol/l	Low solubility (~12.0 mg/l)
Absorption	Caco-2 permeability	1.666	log Papp (10 ⁻⁶ cm/s)	High permeability
Absorption	Intestinal absorption (Human)	92.034	% Absorbed	Good absorption potential
Absorption	Skin permeability	-2.301	log Kp	Moderate skin permeability
Absorption	P-glycoprotein substrate	No	Categorical	Not a substrate
Absorption	P-glycoprotein I/II inhibitor	No / No	Categorical	No inhibition
Distribution	Volume of distribution (VDss)	0.611	log L/kg	Moderate tissue distribution
Distribution	Fraction unbound (Human)	0.044	Fu (unitless)	High plasma protein binding
Distribution	BBB permeability	0.478	log BB	Moderate permeability to CNS
Distribution	CNS permeability	-0.848	log PS	Low passive CNS diffusion
Metabolism	CYP3A4 substrate	Yes	Categorical	Potential metabolic liability
Metabolism	CYP1A2 inhibitor	Yes	Categorical	Possible enzyme inhibition
Metabolism	CYP2D6/2C9/2C19/3A4 inhibitor	No	Categorical	Low risk of metabolic inhibition
Metabolism	CYP2D6 substrate	No	Categorical	Not a substrate
Excretion	Total clearance	0.759	log ml/min/kg	Moderate clearance rate
Excretion	Renal OCT2 substrate	No	Categorical	Not eliminated via OCT2 pathway
Toxicity	AMES toxicity	No	Categorical	Nonmutagenic
Toxicity	Max. tolerated dose (Human)	0.42	log mg/kg/day	Moderate tolerance
Toxicity	hERG I/II inhibition	No / No	Categorical	No cardiac toxicity risk
Toxicity	Oral rat acute toxicity (LD ₅₀)	2.351	mol/kg	Moderate acute toxicity
Toxicity	Oral rat chronic toxicity (LOAEL)	1.696	log mg/kg-bw/day	Moderate chronic toxicity
Toxicity	Hepatotoxicity	No	Categorical	No liver toxicity predicted
Toxicity	Skin sensitization	Yes	Categorical	Potential allergen
Toxicity	<i>T. pyriformis</i> toxicity	1.572	log µg/l	Moderate aquatic toxicity
Toxicity	Minnnow toxicity	0.006	log mM	Low fish toxicity

**Figure 10.** Chemical structure along with a radar plot of physicochemical properties of 2,4-DTBP.

4. DISCUSSION

The global rise in emerging diseases has significantly increased the demand for new therapeutic agents. This has intensified the search for natural bioactive compounds, many of which are still unidentified. The 2,4-DTBP is one such

compound of great interest. This phenolic compound is known for its antibacterial, antifungal and antioxidant properties. These biological activities make it a strong candidate for therapeutic development. However, its lipophilic nature and relatively high molecular weight may create challenges with bioavailability and toxicity. Molecular docking studies revealed that 2,4-DTBP has binding energies between 5 and 7 kcal/mol against protein targets from selected oral pathogens (*S. mutans*, *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*). These values are acceptable for drug-like compounds and are similar to those of the reference drug chlorhexidine. For some proteins, 2,4-DTBP even showed a better binding affinity than chlorhexidine. This suggests it may also have similar or enhanced biological activity *in vitro* settings against these bacteria.

Despite these promising findings, toxicity remains a significant concern. Chlorhexidine has been extensively tested in various noncancerous human cell lines, including gingival epithelial cells, fibroblasts, myoblasts, osteoblasts, keratinocytes and macrophages. Studies report substantial cytotoxic and irritant effects and apoptotic-like nuclear morphology in human gingival epithelial cells even at lower values (0.02%–0.2%) after 1–3 of exposure [44,45]. Concentrations of $\geq 0.02\%$

have been shown to reduce cell viability and high toxicity in odontoblast-like cells MDPC-23 [46,47]. Chlorhexidine also causes strong, dose- and time-dependent cytotoxicity in RAW 264.7 macrophages and leads to significant cell death in HaCaT keratinocytes and fibroblasts after prolonged exposure [48]. In osteoblast-like U2OS cells, the IC₅₀ was approximately 0.005%, highlighting the high sensitivity of bone-related cells to the compound [50].

In contrast, 2,4-DTBP has primarily been tested in cancer cell lines such as HeLa, MCF-7, A431 and AGS. These studies have shown dose-dependent cytotoxicity and pro-apoptotic activity [48; 49]. However, there is currently a lack of data regarding its effects on normal, noncancerous human cells. This limitation restricts the ability to directly compare the safety profile of 2,4-DTBP to that of chlorhexidine in clinical or dental applications.

The ADMET profile of 2,4-DTBP shows several favourable pharmacokinetic properties that support its potential as a therapeutic candidate. It demonstrates high intestinal absorption (92.03%), moderate skin permeability (log K_p = -2.301) and is neither a substrate nor inhibitor of P-glycoprotein, which suggests minimal interference with common drug transporters. Its moderate volume of distribution (log VD_{ss} = 0.611) and strong plasma protein binding (fraction unbound = 0.044) imply sustained circulation with limited free drug availability. The deviation of 2,4-DTBP from the optimal LIPO and INSOLU windows on the SwissADME radar plot suggests an increased risk of formulation-dependent absorption and nonspecific tissue accumulation. These issues will likely necessitate solubility-enhancing strategies such as nanoemulsions or polymeric nanoparticles and careful dose optimisation to avoid off-target effects associated with excessive LIPO. The 2,4-DTBP also shows moderate CNS permeability (log BB = 0.478), although passive diffusion into the brain is low (log PS = -0.848), which may reduce off-target neurological effects. *In silico* metabolism predictions indicated that 2,4-DTBP is a putative substrate of CYP3A4 and an inhibitor of CYP1A2. Although specific metabolic pathways and metabolites were not characterised experimentally in this study, these findings suggest that 2,4-DTBP could undergo extensive phase I oxidative metabolism and may interfere with the clearance of co-administered CYP1A2 substrates. Future work should therefore include *in vitro* microsomal stability assays, metabolite identification and CYP inhibition/induction studies to delineate potential drug–drug interaction risks. Importantly, toxicity evaluations suggest that 2,4-DTBP is nonmutagenic (negative AMES test), nonhepatotoxic and noncardiotoxic (no hERG I/II inhibition), with a moderate oral LD₅₀ of 2.351 mol/kg and no predicted liver toxicity. There was observed potential for skin sensitization, which could be a concern in topical applications. The toxicity and ADMET data presented here are entirely model-based and should be interpreted with caution. SwissADME, pkCSM and ProTox-II are trained on heterogeneous datasets and may not fully capture idiosyncratic or long-term toxicities of phenolic compounds. Moreover, prediction accuracies decrease outside the chemical space of the training sets and the tools do not address local tissue irritation, immunogenicity or complex mixture effects relevant to oral formulations. Therefore, the comparable toxicity classes

predicted for 2,4-DTBP and chlorhexidine cannot substitute for systematic *in vitro* cytotoxicity, genotoxicity and *in vivo* safety studies. Beyond acute toxicity, phenolic alkyl compounds structurally related to 2,4-DTBP have been implicated in endocrine-modulating and pro-oxidant effects in mammalian systems, including interference with steroid hormone receptors and induction of oxidative stress pathways. Although such endpoints were not specifically modelled in the present ADMET workflow, they represent plausible off-target mechanisms of toxicity. Detailed endocrine and redox-stress profiling in relevant human cell models, together with *in vivo* sub-chronic exposure studies, will be required before considering long-term oral use [50].

The MIC values determined in this study show that 2,4-DTBP inhibits *S. mutans* at 4 µg/ml and *A. actinomycetemcomitans* at 8 µg/ml, while chlorhexidine requires 8 and 16 µg/ml, respectively. This indicates a comparatively higher antibacterial efficacy of 2,4-DTBP against both pathogens. Literature reports chlorhexidine MICs for *S. mutans* ranging from 2 to 18 µg/ml, placing the observed 8 µg/ml MIC well within this range, while chlorhexidine MIC data for *A. actinomycetemcomitans* are limited but generally considered effective at clinical concentrations used in periodontal therapy. The lower MICs for 2,4-DTBP align with its broad antimicrobial activity and support its potential as a promising alternative agent for oral infections [51–53]. Although its systemic toxicological profile may be similar to chlorhexidine, its incorporation into nanoparticle-based/pH-sensitive delivery systems could enable targeted and controlled release, potentially minimizing local cytotoxic effects while maintaining effective antimicrobial action. Despite the encouraging docking results, the *in-silico* approach used in this study has inherent limitations. The docking was performed in rigid receptor conformations in an implicit solvent environment and, therefore, might not capture protein FLEX, explicit solvent effects or dynamic conformational changes within the binding pocket. In addition, the empirical scoring functions used by AutoDock may generate false-positive poses that do not directly translate into absolute binding affinities. Consequently, the uncertainty associated with the docking scores is likely on the order of 1–2 kcal/mol, so small differences between ligands or targets fall within this error margin and the docking results should be regarded as qualitative indicators of relative binding propensity rather than definitive evidence of *in vivo* efficacy. The present work combines docking, ADMET prediction and MIC determination, but further wet-lab validation is essential. Future studies should include quantitative biofilm disruption and time-kill assays against mono- and multispecies oral biofilms, together with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or resazurin-based cytotoxicity assays on relevant oral cell lines (gingival keratinocytes, fibroblasts and osteoblast-like cells). These experiments are required to confirm whether the superior MIC of 2,4-DTBP translates into effective and safe antibiofilm activity under physiologically relevant conditions. Thus, 2,4-DTBP exhibits measurable antibacterial activity and *in-silico* pharmacokinetic properties that are broadly comparable to those predicted for chlorhexidine; however, its clinical utility remains hypothetical until validated by comprehensive *in vitro* and *in vivo* studies.

5. CONCLUSION

Given its strong antimicrobial and antibiofilm activity, along with favourable properties such as high intestinal absorption, nonmutagenicity and a lack of predicted liver or heart toxicity, 2,4-DTBP represents a preliminary lead for further preclinical evaluation. Its ability to disrupt biofilms and inhibit key oral pathogens shows its potential in products aimed at treating microbial infections, especially in oral and dental care. There is a lack of toxicity data for 2,4-DTBP in normal human cells; moreover, findings from cancer-cell experiments, together with *in-silico* toxicity predictions, are insufficient to draw definitive conclusions about its safety in comparison with chlorhexidine, which is well documented to exert marked cytotoxic effects. Therefore, 2,4-DTBP can be effectively used in the creation of biogenic nanoparticles, antimicrobial coatings or therapeutic gels. This enables researchers to take advantage of its healing properties while reducing safety concerns through careful formulation and further toxicity testing.

6. LIST OF ABBREVIATIONS

ADMET, Absorption, distribution, metabolism, excretion and toxicity; BBB, Blood–brain barrier; CASTp, Computed atlas of surface topography of proteins; CHX, Chlorhexidine; 2,4-DTBP, 2,4-Di-tert-butylphenol; GA, Genetic algorithm; HBA, Hydrogen bond acceptor; HBD, Hydrogen bond donor; HOMO, Highest occupied molecular orbital; Lipinski RO5, Lipinski's rule of five; LogP, Partition coefficient (octanol-water); LUMO, Lowest unoccupied molecular orbital; MIC, Minimum inhibitory concentration; MMFF94, Merck molecular force field 94; MW, Molecular weight; MWCO, Molecular weight cut-off; NP, Nanoparticle; pkCSM, Predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures; ProTox II Prediction of toxicity (Version II); QSAR, Quantitative structure–activity relationship; SwissADME, Swiss absorption, distribution, metabolism and excretion; TPSA, Topological polar surface area; UCSF, University of California, San Francisco; WHO, World Health Organization.

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8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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One of the authors, Dilipraj Sathyamurthy, is affiliated with Mediomix Diagnosis and Bioresearch Private Limited, Bengaluru, India. This industry affiliation had no role in the study design, data collection, analysis, interpretation of results, or manuscript preparation. The authors declare no financial or non-financial conflicts of interest related to this work. This study did not receive any specific funding from Mediomix Diagnosis and Bioresearch Private Limited or from any public, commercial, or not-for-profit funding agencies. The organization had no involvement in the conduct of the study or in the preparation of the manuscript.

11. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

12. DATA AVAILABILITY

All data generated and analyzed are included in this research article.

13. PUBLISHER'S NOTE

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14. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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