
ISSN Print: 2231-1866
ISSN Online: 2231-1874

Journal of Biologically Active Products from Nature

A PEER REVIEWED INTERNATIONAL JOURNAL

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Journal of Biologically Active Products from Nature

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tbap20

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To cite this article: Priya Cheruvanachari, Subhaswaraj Pattnaik, Monika Mishra, Pratyush Pragyandipta & Pradeep K. Naik (2022) Terpinen-4-ol, An Active Constituent of Kewda Essential Oil, Mitigates Biofilm Forming Ability of Multidrug Resistant *Staphylococcus aureus* and *Klebsiella pneumoniae*, Journal of Biologically Active Products from Nature, 12:5, 406-420, DOI: 10.1080/22311866.2022.2154264

To link to this article: https://doi.org/10.1080/22311866.2022.2154264

Taylor & Francis
Taylor & Francis Group
ISSN: (Print) 2231-1866; ISSN: (Online) 2231-1874

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https://www.tandfonline.com/loi/tbap

Original Article

Terpinen-4-ol, An Active Constituent of Kewda Essential Oil, Mitigates Biofilm Forming Ability of Multidrug Resistant *Staphylococcus aureus* and *Klebsiella pneumoniae*

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Received 03 November 2022; Received in revised form 22 November 2022; Accepted 23 November 2022

Abstract: The increased incidence of microbial resistance to traditional antibiotics has urged the scientific community to look for alternative therapeutic regimens. In this context, mitigation of biofilm formation is considered as viable alternative. Since, plant derived essential oils are rich heritage of bioactive phytochemicals with widespread pharmacological values, in the present study Terpinen-4-ol, bioactive constituent of Kewda essential oil (KEO) extracted from *Pandanus odorifer* male flower was evaluated for its antimicrobial and antibiofilm activities against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC-740 and MTCC-109. The minimum inhibitory concentration (MIC) of Terpinen-4-ol against *S. aureus* and *K. pneumoniae* was 50 and 25 mM, respectively. At MIC level, Terpinen-4-ol exhibited antibacterial activities against both the reference strains i.e. MTCC-740 and MTCC-109 with a zone of 16 and 14 mm, respectively. On treatment with sub-MIC of Terpinen-4-ol, a significant reduction in exopolysaccharides (EPS) production was observed as evident from qualitative Congo red agar (CRA) assay. Further, the reduction in EPS production was quantified with a reduction of 67.51±1.29% against *S. aureus*. The light and fluorescence microscopic analysis also corroborated the antibiofilm potential of Terpinen-4-ol as a significant reduction in the thickness of biofilm formation was observed. *In silico* studies provided an insight into the action of Terpinen-4-ol in binding to target proteins associated with biofilm formation and drug resistance. Thus, Terpinen-4-ol could be considered as putative drug candidate in the fight against biofilm associated chronic infections and drug resistance.

Keywords: Biofilm, Congo Red agar, Drug resistance, Essential oil.

Introduction

As the deteriorating global health scenario is concerned, antimicrobial resistance is considered as one of the major threats and there is a need to overcome it through development of potential anti-infective therapeutics [1]. The antibacterial resistant bacteria have several ways to protect themselves from the antibiotic stress and formation of biofilm is one of the important characteristic strategy [2]. Biofilm is the extracellular polymeric substances (EPS) produced by a group of bacteria that helps in attachment to a surface [3]. About 40-80% of the bacteria are biofilm producers. Due to the formation of biofilm, the bacteria get protective clothing which helps to resist multidrug [4]. Specifically the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) group of pathogens

DOI: 10.1080/22311866.2022.2154264

are resistant to almost all the antibiotics by producing biofilms. Since drug-resistant microorganisms are constantly progressing, there is a need to develop alternative therapeutic or antibacterial drugs. To this end, medicinal plants are considered a rich source of bioactive compounds that can be utilised to develop novel antimicrobial drugs [5]. The medicinal plants also show significant ability against inhibition of biofilm production by different types of pathogenic bacteria. Therefore, natural products are a vital source for exploring new antibacterial and anti-biofilm therapies [6-7]. Certain plants are rich source of bioactive compounds that can be used against the infectious diseases [8]. These medicinal plants inhibit the growth and virulence factors of several microbes [9]. In the developing countries people still use the different parts of the plants for traditional medicines These plants produce secondary metabolites which exhibit many advantages like antibacterial, antioxidant, antiviral properties etc [10].

Among the different plant species with significant antimicrobial activity, the essential oil extracted from *Pandanus odorifer* (commonly known as Kewda (English) and Kevara (Hindi)) is used in several folkloric medicines. The flowers are used to treat disorder like Syphilis, tumor, skin diseases, leukoderma, leprosy, generating perspiration, earache, bronchitis, blood diseases, asthma, urinary tract illnesses, headache, rheumatism, constipation, and diabetes [11]. *P. odorifer* has several pharmacological activities like antidepressant, antidiabetic, anti-inflammatory, hepatoprotective, antioxidant and chemoprotective activities [12]. Monoterpenes and phenolic compounds are mostly responsible for its number of pharmacological properties. α-Terpienol has been reported with various biological activities. The activities include cardiovascular and antihypertensive effects, antioxidant & anticancer activities, antinociceptive potential, antiulcer & anticonvulsant activity, antibronchitis activity, skin penetration enhancing activity, and insecticidal activity [13]. Among the monoterpenes, Kewda essential oil contains 14.130% of Terpinen-4-ol, which is responsible for several pharmacological activities. It significantly reduced the production of tumor necrosis factor (TNF-α), interlukins (IL-1β), IL-10 and prostaglandin E2 by liposaccharide-activated monocytes [14]. Terpinen-4-ol extracted from *Melaleuca alternifolia* showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [15]. It has also shown antifungal properties against *Botrytis cinerea* and anti-viral properties [16-17]. In the fight against tumors and cancers, Terpinen-4-ol exhibited promising therapeutic avenues [18]. It has considerably inhibited the growth of pancreatic, colorectal, gastric and prostate cancer cells in dose-dependent manner [19]. . Owing to the widespread pharmacological importance, Terpinen-4-ol has been considered as promising alternative against several diseases and disorders. Since, Terpinen-4-ol also showed promising antimicrobial properties, it could be developed as an imperative remedy for the treatment of chronic microbial infections associated with biofilm dynamics. Hence, in the present study, the ability of Terpinen-4-ol to mitigate biofilm mechanics in ESKAPE pathogens, *S. aureus* and *K. pneumoniae* were determined with an insight into the mechanism of biofilm inhibition.

Materials and methods

Chemicals and reagents

The chemicals, Congo red agar, crystal violet and acridine orange were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. The dehydrated culture media Luria–Bertani (LB) broth and agar, Mueller Hinton (MH) broth and agar were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. The test sample, Terpinen-4-ol was purchased from Sigma Aldrich, USA.

Bacterial cultures and maintenance

The test bacteria, *S. aureus* and *K. pneumoniae* were collected from Veer Surendra Sai Institute of Medical Sciences and Research (VIMSAR), Burla, Sambalpur, Odisha. The respective reference strains of *S. aureus* (MTCC-740) and *K. pneumoniae* (MTCC-109) were procured from Microbial Type Culture Collection and Gene Bank (MTCC), IMTECH, Chandigarh, India. To maintain the 0.5 McFarland standard, the bacterial strains were cultured in Luria–Bertani (LB) broth at 37°C.

Genomic DNA extraction

The overnight clinical bacterial strains, *S. aureus* and *K. pneumoniae* were centrifuged at 30,000 rpm for 30 sec. The supernatant was discarded and the pellet was resuspended with 700 µl extraction buffer. The tube was incubated at 60°C for 30 mins. 650 µl of chloroform isoamyl alcohol was added and centrifuged. The upper aqueous phase was added with 200 µl extraction buffer without proteinase with addition of 650 µl chloroform isoamyl alcohol. The tube was vortexed and the upper aqueous phase was transferred in a fresh tube. Chloroform isoamyl alcohol extraction were performed twice using 650 µl of the chemicals. The DNA was precipitated by adding equal amount of isopropanol and centrifuged. The isopropanol was removed and the pellet was washed with 70% ethanol. The DNA pellet was air dried and resuspended with TE buffer, EDTA and RNAase and incubated at 37°C for 30 min [20].

Molecular characterization of clinical bacterial isolates

The genomic DNA of isolated clinical bacterial strains was isolated and the 16S rDNA gene was amplified using the universal primers **27F** (5′AGAGTTTGATCCTGGCTCAG3′) and **1492R** (5′TACGGTTACCTTGTTACGACTT3′). Amplified PCR products (Amplification parameter set: pre-denaturation of template at 95°C for 5 min followed by 39 cycles of denaturation 30 sec. at 95°C, 45 sec. annealing and 1 min. elongation at 72°C, with a final extension of 7 min. at 72°C) were purified and sequenced. The obtained sequences were BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with NCBI (National Centre for Biotechnology Information, U.S. National Library of Medicine) database to find homologous sequences. Phylogenetic tree was constructed with 16S rDNA sequences of the two bacterial isolates and their closely related sequences using MEGA 5.0 software [21-22].

Determination of minimum inhibitory concentration (MIC)

The lowest concentration of Terpinen-4-ol, which inhibited the growth of *S. aureus* and *K. pneumoniae* and their reference strains, MTCC-740 and MTCC-109 was considered as MIC. The MIC of Terpinen-4-ol against test bacteria was determined as per the Clinical and Laboratory Standards Institute (CLSI) guidelines using the two-fold broth microdilution method. Several dilutions of Terpinen-4-ol (i.e. 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 mM) were prepared in MHB media from stock concentration and an aliquot of bacterial suspension was added and incubated at 37°C overnight. MHB media without sample and bacterial suspension is considered as blank whereas MHB media with only bacterial suspension served as negative control. After incubation, an aliquot of 5 µL of 0.125% TTC (2, 3, 5-triphenyltetrazolium chloride) was added to each well and the micro-titter plate was incubated at 37°C for 15 min. The wells were examined for the development of the pink colour, which inferred bacterial growth, and the absence of the pink colouration was considered an inhibition of bacterial growth. The minimum concentration of the sample where no colour change was considered as the MIC value. From the MIC values, sub-MICs (i.e. ½ MIC) were calculated and considered for antibacterial and biofilm inhibition assays [23-24].

Antibacterial activity of Terpinen-4-ol

Agar well diffusion assay was performed to determine the antibacterial activity of Terpinen-4-ol. Antibacterial activities were evaluated by measuring the diameter of halo zones formed around the well [25].

Inhibition of biofilm formation by Terpinen-4-ol

Qualitative biofilm formation assay

The inhibition of biofilm production by Terpinen-4-ol was evaluated by both quantitative and qualitative methods. For qualitative analysis of biofilm formation, Congo red agar (CRA) method was followed, which characteristically provides a platform for the presence or absence of EPS production on the molten agar plates [26]. Bacterial culture without treatment was considered as a control. In addition, the tube method was used for early detection of biofilm matrix by staining with crystal violet.

Quantitative biofilm formation assay

Polystyrene-based 24-Microtiter plate (MTP) was employed to quantify the inhibition of biofilm formation with the treatment of sub-MIC level of Terpinen-4-ol compared to untreated control using the crystal violet staining method [27].

Microscopic analysis of biofilm formation

For light and fluorescence microscopic studies, the test bacteria (0.5 McFarland standard) were treated with sub-MIC of Terpinen-4-ol and the setup was incubated overnight at 37°C to allow biofilm formation. After incubation, the media were discarded and biofilms attached to the surface were rinsed with PBS followed by staining with crystal violet and acridine orange. After staining the biofilms for 10-15 min, the adhered biofilms were visualized under light microscope (QUASMO, PZRM-26) and fluorescence microscope (Nikon, Eclipse TS2) [28-29].

In silico study of molecular interaction of Terpinen-4-ol with targeted proteins

Preparation of target proteins

The proteins that are involved in pathogenesis, biofilm production and drug resistance of *S. aureus* such as SarA (Global regulatory protein; PDB ID: 2FNP), Sortase A (surface associated protein; PDB ID: 1T2P), AgrA (transcriptional regulator; PDB ID: 4G4K), MepR (transcriptional regulator of multidrug efflux pump, MepA; PDB ID: 3ECO) and Rot (global regulator of virulence genes; PDB ID: 4Q77) were considered as target proteins. Protein preparation wizard (Schrödinger, Inc., NY) tool was used for pre-processing of the selected target protein structures followed by addition of missing hydrogen atoms [30]. Prime side-chain prediction tool was employed to detect the missing side chain atoms of amino acids and further repaired using Prime tool (Schrödinger, Inc., NY). Further energy minimization steps were performed using Macromodel (Schrodinger) and OPLS 2005 force field by following Polak-Ribiere Conjugate Gradient (PRCG) algorithm with an energy gradient of 0.01 kcal/mol [30].

Preparation and optimization of molecular structure of Terpinen-4-ol

The molecular structure of ligand, Terpinen-4-ol was constructed using ChemDraw software and imported to Maestro (Schrödinger package) [30]. Similar to target proteins, for ligand mole-cule, PRCG algorithm was used for energy minimization using Macromodel (Schrödinger package) and OPLS 2005 force field [30]. Further, geometric optimization of ligand molecule was performed using Jaguar (Schrödinger, package). After energy and geometric optimization of ligand, Ligprep (Schrödinger package) was used to generate various conformations of Terpinen-4-ol [30, 31].

Molecular docking of Terpinen-4-ol

Since the co-crystal structures of Terpinen-4-ol with target proteins are not available, blind docking approach was considered for determining the suitable interactions between the ligand and proteins. To achieve this, SiteMap (Schrodinger package) was employed to predict all the suitable binding sites of proteins of interest [30]. Once the binding sites were predicted, the receptor grid boxes (dimension: 12Å × 12Å × 12Å) were generated using Glide Grid-receptor generation program [30]. The various conformations of Terpinen-4-ol generated earlier were docked onto each predicted binding site using Glide XP (extra precision) algorithm (Schrodinger, Inc., NY) and evaluated their binding poses using Glide XP_{Score} function [32-33]. The single best conformation of Terpinen-4-ol with the lowest minimum docking score with target protein was used for further analysis.

Results

Molecular characterization of clinical bacterial isolates

From the confirmative 16S rDNA gene sequencing analysis, bacterial isolate (**6**) was identified as *K. pneumoniae* strain based on its high similarity in nucleotide homology and molecular phylogenetic analysis (Figure. 1a). Similarly, the other bacterial isolate (**10**) was identified as *Staphylococcus* sp. strain based on the sequence homology and molecular phylogenetic analysis (Figure. 1b).

MIC determination

The MIC of Terpinen-4-ol against clinical as well as reference strain (MTCC-740) of *S. aureus* was found to be 50 mM, whereas the MIC against clinical as well as reference strain (MTCC-109) of *K. pneumoniae* was found to be 25 mM (Figure 2).

Antibacterial activity of Terpinen-4-ol

Terpinen-4-ol (at MIC level) showed promising antibacterial properties against both the reference strains, MTCC 740 and MTCC 109, with a zone of inhibition of 16 and 14 mm respectively (Figure 3).

Inhibition of biofilm formation by Terpinen-4-ol

Qualitative biofilm formation assay

When both clinical and reference strains of *S. aureus* and *K. pneumoniae* were grown in the presence of sub-MICs of Terpinen-4-ol; a significant decrease in the exopolysaccharide matrix production as evident from comparatively less blackened crystalline colonies unlike to that in the untreated control (Figure 4a). On treatment with sub-MICs, the profuse production of exopolysaccharide matrix on the wall of the test tube also severely affected depicting the reduced adherence capabilities of test pathogens to the surface (Figure 4b).

Quantitative biofilm formation assay

Terpinen-4-ol exhibited highest inhibitory effect on biofilm formation on *S. aureus* and its reference strain, MTCC-740 with an inhibition of 67.51±1.29 and 67.77±2.95%, respectively (Figure 5). Meanwhile, 59.07±4.38 and 56.12± 2.78% of biofilm inhibition was observed when *K. pneumonia* and MTCC-109 were grown in the presence of Terpinen-4-ol at sub-MIC level (Figure 5).

Microscopic analysis of biofilm formation

In presence of sub-MICs of Terpinen-4-ol, a significant decrease in the biofilm matrix formation on sterile glass coverslips was observed as evident from light microscopic analysis of bacterial biofilms (Figure 6). Unlike

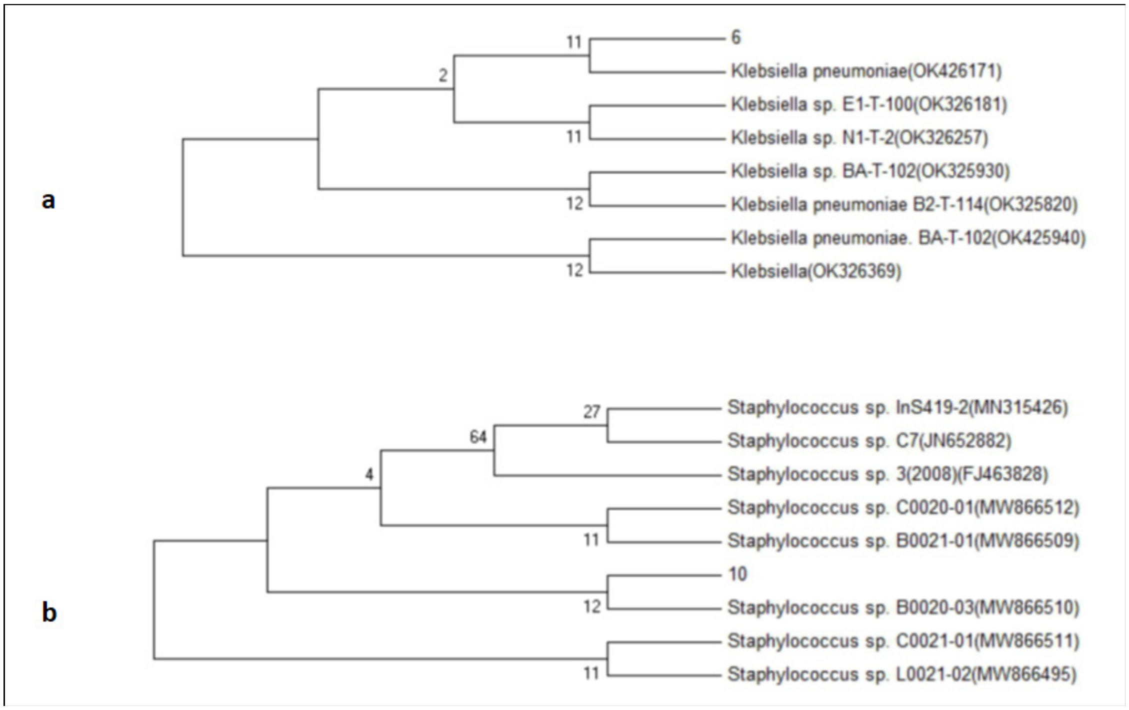


Figure 1. Molecular phylogenetic analysis of clinical bacterial isolates (06 and 10) identified as (a) *Klebsiella pneumoniae* and (b) *Staphylococcus* sp. using the Maximum Likelihood Method based on the Tamura-Nei Model

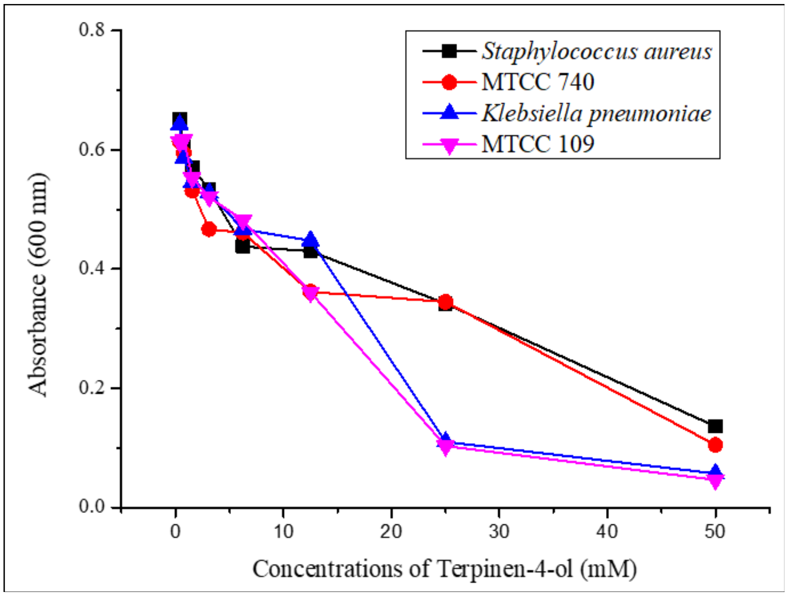


Figure 2. Minimum inhibitory concentration of Terpinen-4-ol against clinical isolates of *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC-740 and MTCC-109

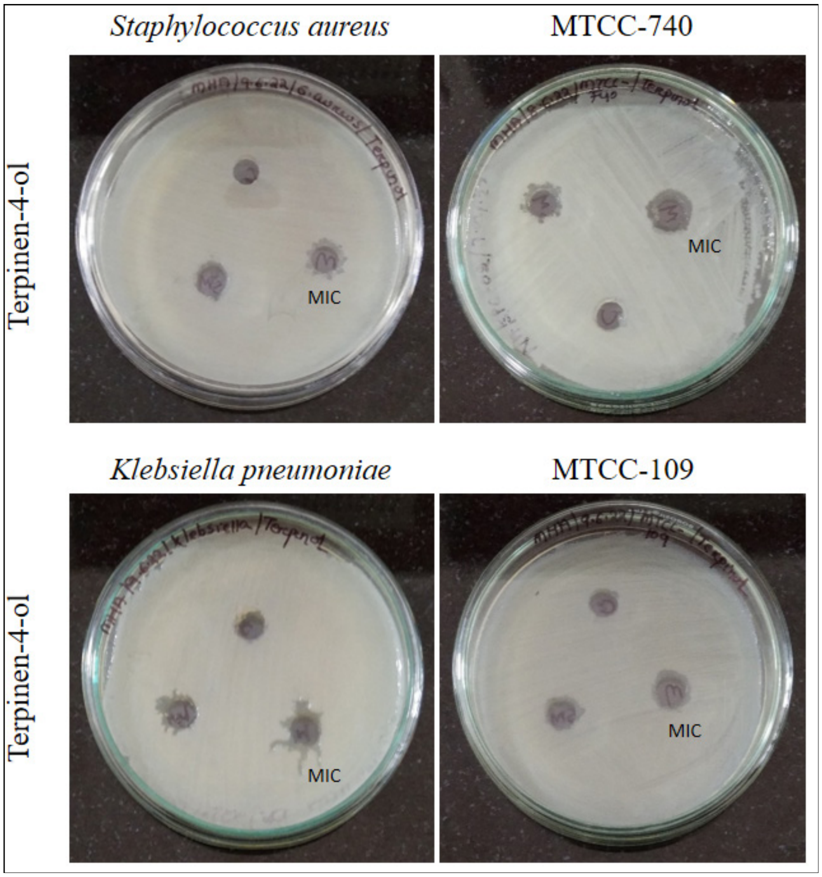


Figure 3. Effect of Terpinen-4-ol (at MIC level) on the growth of clinical bacterial isolates, *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC-740 and MTCC-109

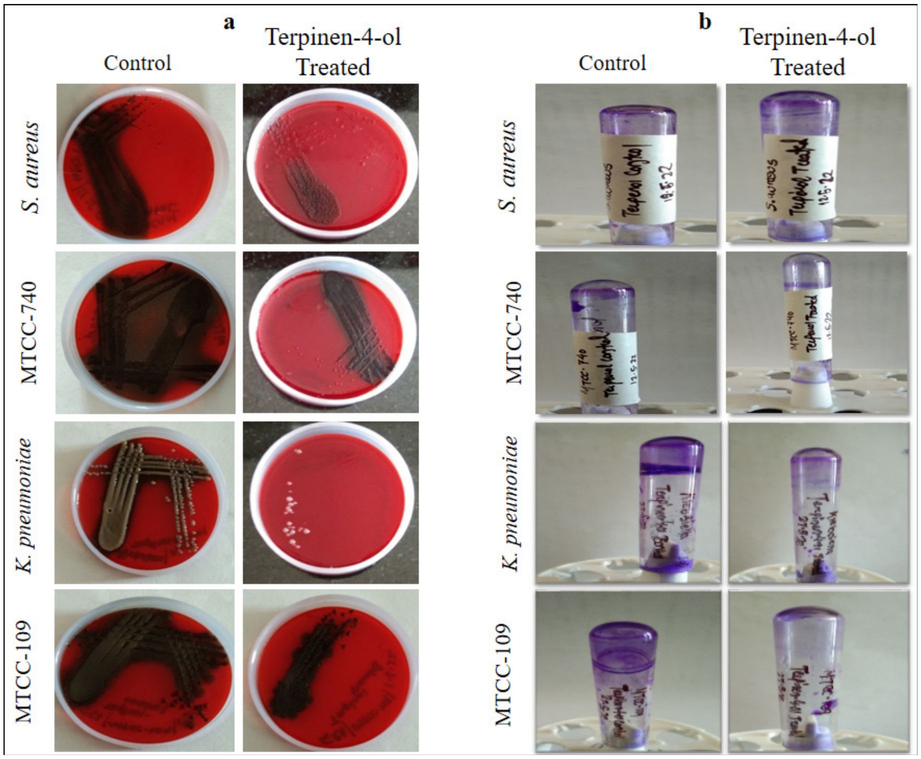


Figure 4. Effect of sub-MICs of Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC-740 and MTCC-109 using (a) Congo red agar plate method and (b) Tube method using crystal violet staining

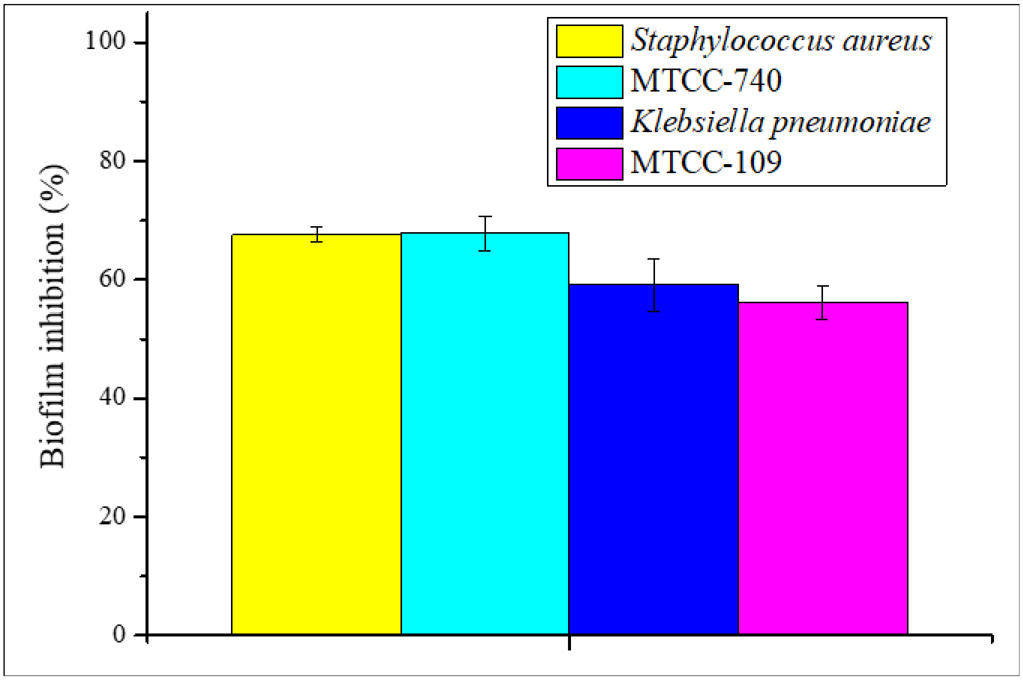


Figure 5. Effect of sub-MICs Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC-740 and MTCC-109 using quantitative crystal violet staining method

that of aggregated cells in the case of untreated control, the bacterial cells were comparatively less aggregated when treated with Terpinen-4-ol. The results were further corroborated by fluorescence microscopic analysis (Figure 7).

Molecular docking analysis

From the predicted binding sites (blind docking) against which the docking score is lowest, was considered to be the putative binding site for Terpinen-4-ol (Table 1). Terpinen-4-ol revealed the lowest minimum docking score of -3.971 Kcal/mol and docking energy of -18.471 Kcal/mol with AgrA (a transcriptional regulator protein). In contrast, the docking score and docking energy of Terpinen-4-ol with other proteins such as MepR (transcriptional regulator of multidrug efflux pump), AgrA (transcriptional regulator protein), SarA (Global regulatory protein) and Rot (global regulator of virulence genes) were found to be almost similar (Table 1). Terpinen-4-ol was found to accommodate well inside the binding cavity (Figure 8, 9). The binding of Terpinen-4-ol involved both

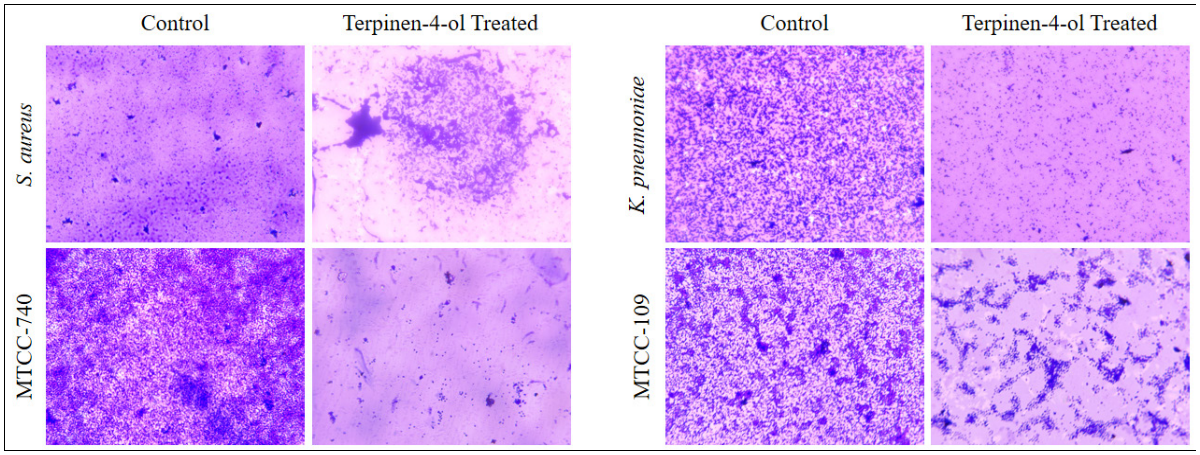


Figure 6. Light microscopic observations on the effect of sub-MICs of Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC-740 and MTCC-109

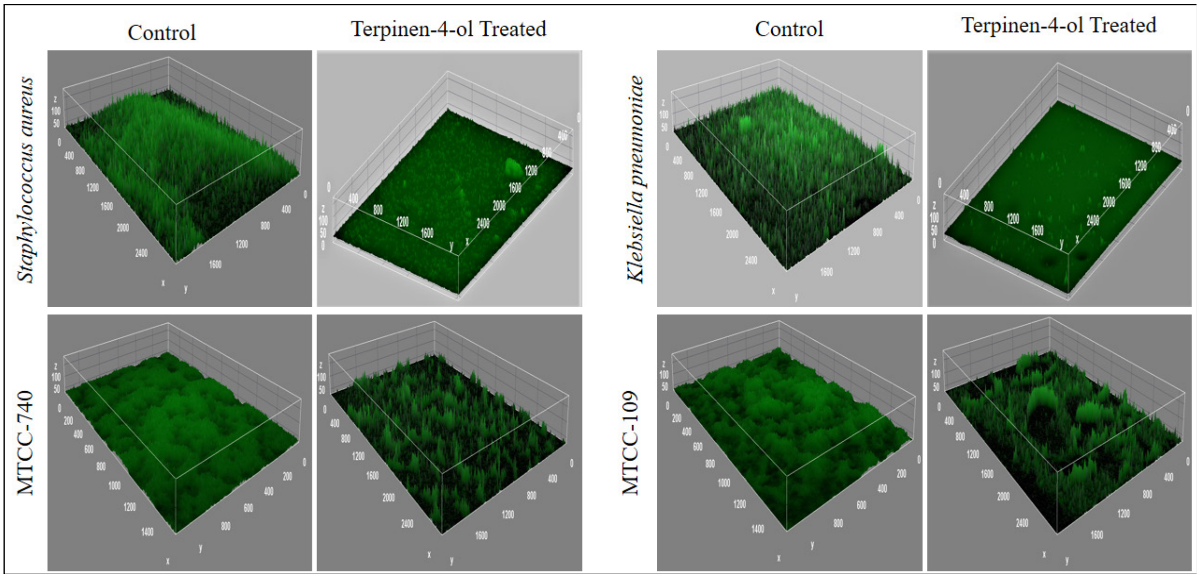


Figure 7. Fluorescence microscopic observations on the effect of sub-MICs of Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC-740 and MTCC-109

Table 1. Docking results of Terpinen-4-ol with respect to different binding sites onto the proteins involve in pathogenesis, biofilm production and drug resistance of *S. aureus*

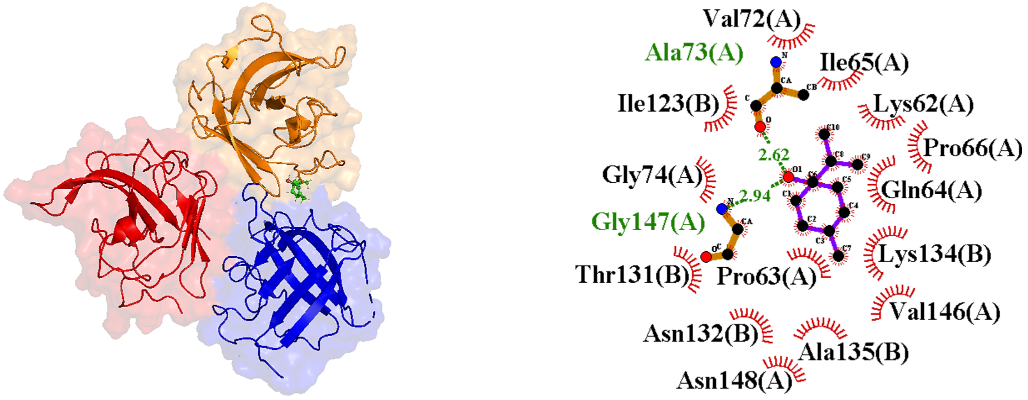
Site ID	Site score	Volume (Å)^3	Glide XP score (Kcal/mol)	Glide docking energy (Kcal/mol)
(a) PDB ID: 1T2P (Sortase A, a surface associated protein)				
1	1.023	482.2	-4.032	-22.44
2	0.959	415.4	-1.009	-15.92
3	1.049	342.6	-3.257	-18.61
4	0.875	220.5	-3.053	-17.07
5	0.786	116.3	-2.139	-17.99
6	0.790	110.8	-3.153	-17.85
7	0.651	105.6	**-4.405**	**-21.86**
8	0.688	92.27	-2.466	-19.50
(b) PDB ID: 2FNP (SarA, a global regulatory protein)				
1	0.941	142.7	-1.024	-18.731
2	0.853	148.2	-3.557	-18.168
3	0.780	128.3	**-3.862**	**-18.248**
4	0.648	75.12	-3.470	-20.180
5	0.727	162.6	-1.029	-15.181
6	0.599	61.05	-2.742	-19.966
7	0.755	76.15	-2.999	-10.144
8	0.626	58.99	-3.163	-17.158
(c) PDB ID: 3ECO (MepR, a transcriptional regulator of multidrug efflux pump)				
1	0.944	262.7	**-3.904**	**-17.752**
2	0.977	248.3	-3.847	-15.162
(d) PDB ID: 4G4K (AgrA, a transcriptional regulator)				
1	1.027	531.6	**-3.971**	**-18.471**
2	0.609	78.55	-3.404	-17.159
(e) PDB ID: 4Q77 (Rot, a global regulator of virulence genes)				
1	0.663	130.7	-2.396	-17.789
2	0.530	45.62	-2.385	-16.045
3	0.574	101.5	**-3.313**	**-16.957**

hydrogen bonding and hydrophobic interactions as shown in the ligplot (Figure 8, 9). The binding of Terpinen-4-ol involved two hydrogen bonds (dashed line) with the binding site amino acid (Ala 73A and Gly 147A) of 1T2P (Figure 8d), two hydrogen bonds with Thr 117A and Leu 160B of 2FNP (Fig. 8e), one hydrogen bond with binding site amino acid (His 35A) of 3ECO (Figure 8f), one hydrogen bond with Gln 155B of 4G4K (Figure 9c) and one hydrogen bond with Gln 14B of 4Q77 (Figure 9d).

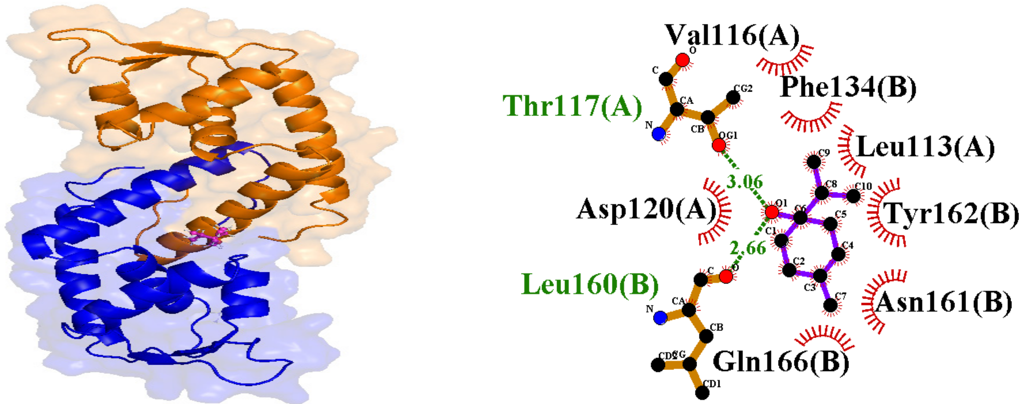
Discussion

Based on the traditional uses of *P. odorifer*, the plant was selected to examine the antibacterial and anti-biofilm activities in a series of *in vitro* assays. The infectious pathogens were selected based on their multi-drug resistant mechanisms. Kewda essential oil (KEO) derived from the plant contains a number of bioactive secondary metabolites. Among these secondary metabolites, Terpinen-4-ol is one of the leading component present in the KEO. Based upon the preliminary results of KEO, Terpinen-4-ol was selected for the present study. No doubt several environmental factors (i.e. location, condition and soil factors) modulate the composition of plant-derived essential oil, Terpinen-4-ol found

(a) Complex of Terpinen-4-ol and 1T2P **(d) Ligplot of Terpinen-4-ol and 1T2P**



(b) Complex of Terpinen-4-ol and 2FNP **(e) Ligplot of Terpinen-4-ol with 2FNP**



(c) Complex of Terpinen-4-ol and 3ECO **(f) Ligplot of Terpinen-4-ol with 3ECO**

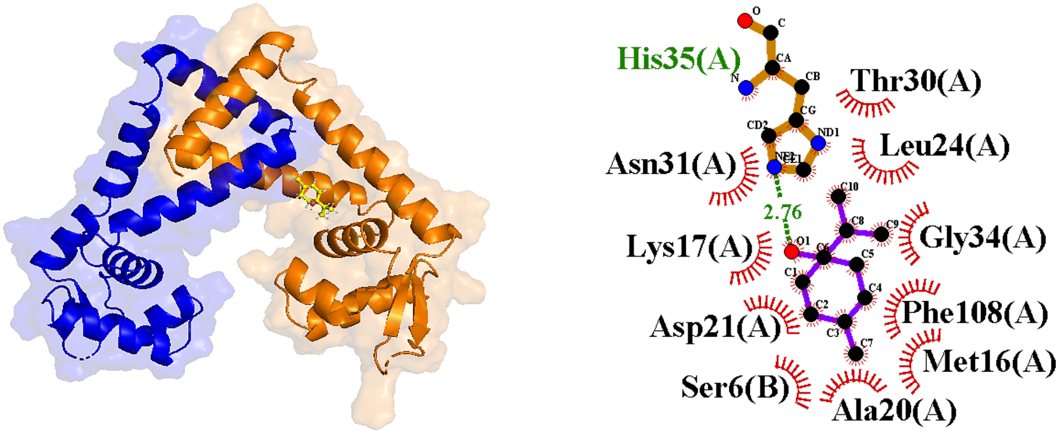
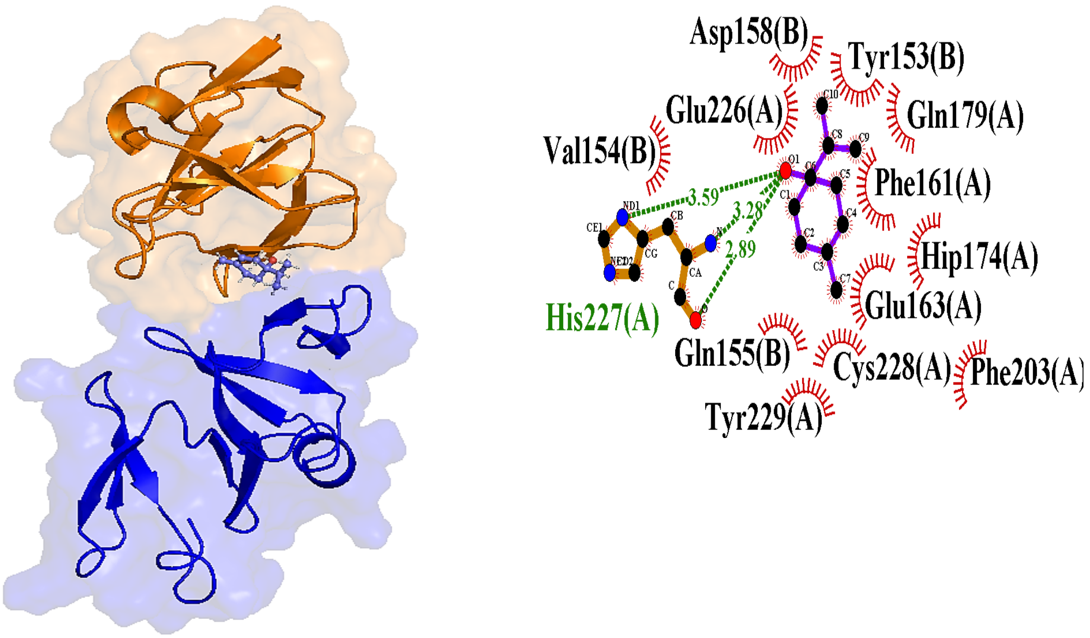


Figure 8. Molecular docking of Terpinen-4-ol onto the proteins involve in pathogenesis, biofilm production and drug resistance of *S. aureus*. Terpinen-4-ol is well accommodated inside the binding site of (a) 1T2P, (b) 2FNP and (c) 3ECO. The ligplot analysis of Terpinen-4-ol showing the interactions with binding site amino acids of (d) 1T2P, (e) 2FNP and (f) 3ECO. Its binding involved both hydrogen bonds represented as dotted (green) lines and hydrophobic interactions denoted with curved (red) lines

(a) Complex of Terpinen-4-ol and 4G4K **(c) Ligplot of Terpinen-4-ol and 4G4K**



(b) Complex of Terpinen-4-ol and 4Q77 **(d) Ligplot of Terpinen-4-ol with 4Q77**

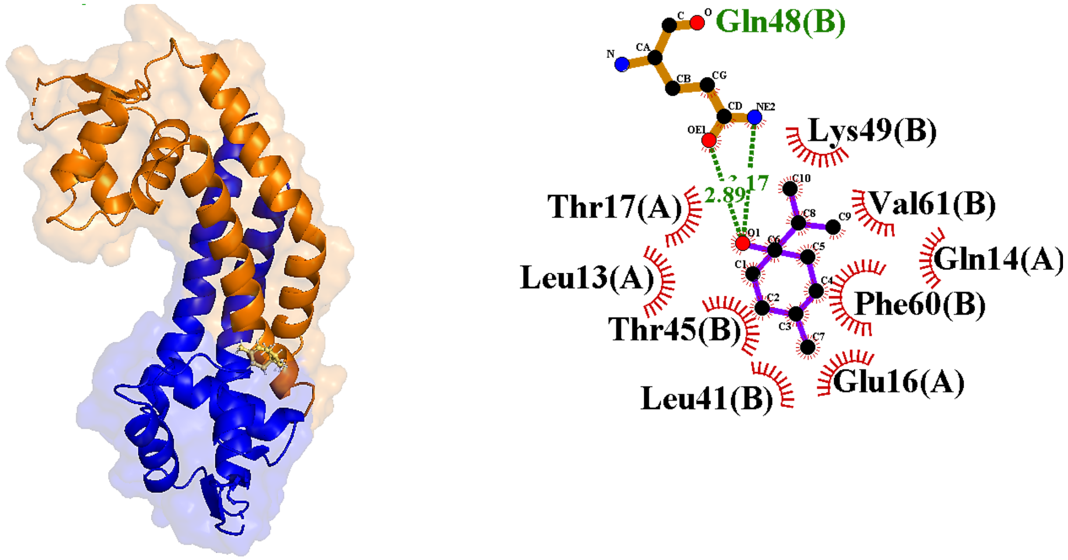


Figure 9. Molecular docking of Terpinen-4-ol onto the proteins involve in pathogenesis, biofilm production and drug resistance of *S. aureus*. Terpinen-4-ol is well accommodated inside the binding site of (a) 4G4K and (b) 4Q77. The ligplot analysis of Terpinen-4-ol showing the interactions with binding site amino acids of (c) 4G4K and (d) 4Q77. Its binding involved both hydrogen bonds represented as dotted (green) lines and hydrophobic interactions denoted with curved (red) lines.

to be an important component of KEO [34]. Thus, the microbial drug resistant mechanisms could be controlled by the bioactive compounds present in the essential oil.

Terpinen-4-ol is the major constituent of several plant derived essential oils and has been reported for widespread pharmacological potential. Terpinen-4-ol, derived from essential oil of *Melaleuca alternifolia*, also known as tea tree oil (TTO) and *Thymus vulgaris* exhibited promising antibacterial properties against several pathogenic bacteria including *Escherichia coli*, *P. aeruginosa*, *S. aureus* and *Lactobacillus acidophilus* [35-36]. In the present study, at MIC levels, Terpinen-4-ol (identified from Kewda essential oil) exhibited promising antibacterial properties against multidrug resistant *S. aureus* and *K. pneumoniae* and their standard reference strains, MTCC-740 and MTCC-109. These results were in accordance with earlier studies depicting the antimicrobial actions of Terpinen-4-ol [15].

In earlier studies, Terpinen-4-ol reported for biofilm inhibition against oral pathogens, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, suggesting its role in biofilm mitigation. In the present study, as evident from both qualitative and quantitative biofilm formation assays, a significant reduction in biofilm formation in MDR *S. aureus* and *K. pneumoniae* and their standard reference strains, MTCC-740 and MTCC-109 was observed when treated with sub-MIC of Terpinen-4-ol. The promising anti-biofilm activities suggested the avenues of Terpinen-4-ol as a potential candidate for developing anti-biofilm agents against drug resistant pathogens in the near future [15, 37].

From the molecular docking analysis, Terpinen-4-ol showed highest binding affinity towards Sortase A (PDB ID: 1T2P) with a Glide XP score of -4.405 Kcal/mol. The promising binding affinity suggested the influence of Terpinen-4-ol in mitigation of biofilm formation in *S. aureus* as Sortase A (SrtA) is considered as a key regulator for bacterial virulence mechanisms, including biofilm formation and development [38]. Similarly, the effective binding affinity of Terpinen-4-ol with the active side residues of SarA (Global regulatory protein, PDB ID: 2FNP) and AgrA (Transcriptional regulator, PDB ID: 4G4K) with respective Glide XP score of -3.862 and -3.971 Kcal/mol suggested its role in the management of bacterial pathogenesis and biofilm dynamics in drug resistant pathogens [39]. The binding affinity of Terpinen-4-ol towards the transcriptional regulatory proteins responsible for bacterial pathogenesis and biofilm formation suggested its candidature as potential anti-biofilm agent and hence could be explored for its role in the management of biofilm associated chronic infections. As evident from *in vitro* biofilm formation assays followed by molecular docking studies, Terpinen-4-ol exhibited promising biofilm inhibitory potential by targeting the specific proteins such as Sortase A, SarA, and transcriptional regulatory protein, AgrA which are involved in inducing bacterial virulence by promoting biofilm formation and drug resistance. Hence, Terpinen-4-ol could be considered as potential drug candidate in our fight against bacterial drug resistance phenomena.

Conclusion

The ESKAPE pathogens have the ability to resist several antibiotics in clinical practices, for which development of new and potent antibacterial drugs is required. Since, the drug discovery pipelines take extended duration for approval of new drugs, effective alternative therapeutics could be considered as promising tools. In this regard, biofilm mitigation is instrumental in attenuating the bacterial chronic infections. In this context, we have observed the influential role of Terpinen-4-ol from KEO in mitigating the biofilm mechanics in ESKAPE pathogens, *S. aureus* and *K. pneumoniae*. The promising anti-biofilm properties of Terpinen-4-ol against ESKAPE pathogens suggested its role as potential anti-biofilm agents against drug resistant bacterial pathogens in the post antibiotic era. Apparently, Terpinen-4-ol could also be used in combination with other therapeutic drugs for improving the therapeutic efficacy for disruption of recalcitrant biofilm matrix. Since there are only few scientific evidences available on *P. odorifer* and its bioactive phytochemicals in the fight against biofilm associated chronic infections and drug resistance, an insight phytochemical, pharmacological and microbiological investigation could be explored for widespread therapeutic avenues in the near future.

Acknowledgement

The authors like to acknowledge OHEPEE, Govt. of Odisha, through the World Bank initiative for providing financial support. The author, Subhaswaraj Pattnaik also acknowledges the Science and Engineering Board (SERB), Department of Science and Technology (DST), Govt. of India for the award of National Post-Doctoral Fellowship (NPDF) programme (Ref. Number: PDF/2021/001260).

Conflict of interest

The authors declare no conflict of interests.

References

1. **Thabit, A. K., Crandon, J. L., Nicolau, D. P. (2015).** Antimicrobial resistance: impact on clinical and economic outcomes and the need for new antimicrobials. Expert Opin. Pharmacother. 16(2): 159-177.
2. **Wright, G.D. (2017).** Opportunities for natural products in 21st century antibiotic discovery. Nat. Prod. Rep. 34(7): 694-701.
3. **Yin, W., Xu, S., Wang, Y., Zhang, Y., Chou, S.H., Galperin, M.Y., He, J. (2021).** Ways to control harmful biofilms: prevention, inhibition, and eradication, Crit. Rev. Microbiol. 47: 57-78.
4. **Srinivasan, R., Santhakumari, S., Poonguzhali, P., Geetha, M., Dyavaiah, M., Xiangmin, L. (2017).** Bacterial biofilm inhibition: A focused review on recent therapeutic strategies for combating the biofilm mediated infections. Front. Microbiol. 2: 676458.
5. **Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A.P., Nautiyal, A.R. (2017).** Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials-A review. Plants. 6: 16.
6. **Simoes, M., Bennett, R.N., Rosa, E.A. (2017).** Understanding antimicrobial activities of phytochemicals against multidrug resistant bacteria and biofilms. Nat. Prod. Rep. 26: 746-757.
7. **Unlu, A., Sar, T., Seker, G., Erman, A.G., Kalpar, E., Akbas, M.Y. (2018).** Biofilm formation by *Staphylococcus aureus* strains and their control by selected phytochemicals. Int. J. Dairy Technol. 71: 637-646.
8. **Rossiter, S.E., Fletcher, M.H., Wuest, W.M. (2017).** Natural products as platforms to overcome antibiotic resistance. Chem. Rev. 117(19): 12415-12474.
9. **Cioch, M., Satora, P., Skotniczy, M., Semik-Szczurak, D., Tarko, T. (2017).** Characterisation of antimicrobial properties of extracts of selected medicinal plants. Polish J. Microbiol. 66(4): 463.
10. **Maroyi, A. (2013).** Traditional use of medicinal plants in south-central Zimbabwe: review and perspectives. J. Ethnobiol. Ethnomed. 9(1): 1-18.
11. **Raina, V.K., Kumar, A., Srivastava, S.K., Syamsundar, K.V., Kahol, A. P. (2004).** Essential oil composition of 'kewda' (*Pandanus odoratissimus*) from India. Flavour and Fragrance J. 19(5): 434-436.
12. **Vivekanandarajah, S., Rajamanoharan, P.R., Heinrich, M., Munday, M. (2015).** Preparations and plants used to treat diabetes in Sri Lankan Siddha Medicine. In 3rd International Conference on Ayurveda, Unani, Siddha and Traditional Medicine, Institute of Indigenous Medicine, University of Colombo, Colombo (Vol. 67).
13. **Khaleel, C., Tabanca, N., Buchbauer, G. (2018).** α-Terpineol, a natural monoterpene: A review of its biological properties. Open Chem. 16(1): 349-361.
14. **Nogueira, M.N.M., Aquino, S.G., Rossa Junior, C., Spolidorio, D.M.P. (2014).** Terpinen-4-ol and alpha-terpineol (tea tree oil components) inhibit the production of IL-1β, IL-6, and IL-10 on human macrophages. Inflamm. Res. 63(9): 769-778.
15. **Cordeiro, L., Figueiredo, P., Souza, H., Sousa, A., Andrade-Júnior, F., Medeiros, D., Nóbrega, J., Silva, D., Martins, E., Barbosa-Filho, J. and Lima, E. (2020).** Terpinen-4-ol as an Antibacterial and Antibiofilm Agent against *Staphylococcus aureus*. Int. J. Mol. Sci. 21: 4531.
16. **Yu, D., Wang, J., Shao, X., Xu, F., Wang, H. (2015).** Antifungal modes of action of tea tree oil and its two characteristic components against *Botrytis cinerea*. J. Appl. Microbiol. 119: 1253-1262.

17. **Garozzo, A., Timpanaro, R., Bisignano, B., Furneri, P.M., Bisignano, G., Castro, A. (2009).** *In vitro* antiviral activity of *Melaleuca alternifolia* essential oil. Lett. Appl. Microbiol. 49: 806-808.
18. **Shapira, S., Pleban, S., Kazanov, D., Tirosh, P., Arber, N. (2016).** Terpinen-4-ol: A novel and promising therapeutic agent for human gastrointestinal cancers. PLoS ONE. 11: e0156540.
19. **Yadav, E., and Rao, R. (2016).** A promising bioactive component terpinen-4-ol: a review. Int. J. Pharmacog. 1: 336-345.
20. **Chapaval, L., Moon, D.H., Gomes, J.E., Duarte, F. R., Tsai, S.M. (2008).** An alternative method for *Staphylococcus aureus* DNA isolation. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 60: 299-306.
21. **Martínez-Absalón, S., Rojas-Solís, D., Hernández-León, R., Prieto-Barajas, C., Orozco-Mosqueda, M. D. C., Peña-Cabriales, J.J., Sakuda, S., Valencia-Cantero, E., Santoyo, G. (2014).** Potential use and mode of action of the new strain *Bacillus thuringiensis* UM96 for the biological control of the grey mould phytopathogen *Botrytis cinerea*. Biocont. Sci. Technol. 24(12): 1349-1362.
22. **Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011).** MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28(10): 2731-2739.
23. **Das, A., Dey, S., Sahoo, R.K., Sahoo, S., Subudhi, E. (2019).** Antibiofilm and antibacterial activity of essential oil bearing *Zingiber officinale* Rosc.(Ginger) Rhizome against multi-drug resistant isolates. J. Essential Oil Bearing Plants. 22: 1163-1171.
24. **Al Saqr, A., Aldawsari, M.F., Khafagy, E.S., Shaldam, M.A., Hegazy, W.A.H., Abbas, H.A. (2021).** A novel use of allopurinol as a quorum-sensing inhibitor in *Pseudomonas aeruginosa*. Antibiotics. 10: 1385.
25. **Negreiros, M.D.O., Pawlowski, Â., Zini, CA., Soares, GLG., Motta, ADS., Frazzon, APG. (2016).** Antimicrobial and antibiofilm activity of *Baccharis psiadioides* essential oil against antibiotic-resistant *Enterococcus faecalis* strains, Pharm. Biol. 54: 3272-3279.
26. **Chhibber, S., Gondil, V.S., Sharma, S., Kumar, M., Wangoo, N., Sharma, R.K. (2017).** A novel approach for combating *Klebsiella pneumoniae* biofilm using histidine functionalized silver nanoparticles. Front. Microbiol. 8: 1104.
27. **Luciardi, M.C., Blázquez, M.A., Cartagena, E., Bardón, A., Arena, M.E. (2016).** Mandarin essential oils inhibit quorum sensing and virulence factors of *Pseudomonas aeruginosa*. LWT - Food Sci. Technol., 68: 373-380.
28. **Packiavathy, I.A.S.V., Priya, S., Pandian, S.K., Ravi, A.V. (2014).** Inhibition of biofilm development of uropathogens by curcumin-An anti-quorum sensing agent from *Curcuma longa*. Food Chem. 148: 453-460.
29. **Wu, S., Liu, G., Jin, W., Xiu, P., Sun, C. (2016).** Antibiofilm and anti-infective of a marine bacterial polysachharide against *Pseudomonas aeruginosa*. Front. Microbiol. 7: 102.
30. **Radha, G., Naik, P.K., Lopus, M.** (2022). *In vitro* characterization and molecular dynamic simulation of shikonin as a tubulin-targeted anticancer agent. Comput. Biol. Med. 147: 105789.
31. **Santoshi, S., Naik, P.K. (2014).** Molecular insight of isotypes specific β-tubulin interaction of tubulin heterodimer with noscapinoids. J. Comput. Aided. Mol. Des. 28: 751-763.
32. **Friesner, R.A., Banks, J.L., Murphy, R.B., Halgren, T.A., Klicic, J.J., Mainz, D.T., Repasky, M.P., Knoll, E.H., Shelley, M., Perry, J.K., Shaw, D.E., Francis, P., Shenkin, P.S. (2004).** Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J. Med. Chem. 47: 1739-1749.
33. **Halgren, T.A., Murphy, R.B., Friesner, R.A., Beard, H.S., Frye, L.L., Pollard,**

W.T., Banks, J.L. (2004). Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. J. Med. Chem. 47: 1750-1759.

34. **Correa, M.S., Schwambach, J., Mann, M.B., Frazzon, J., Frazzon, A.P.G. (2019).** Antimicrobial and antibiofilm activity of the essential oil from dried leaves of *Eucalyptus staigeriana*. Arq. Inst. Biol. 86: 1-8.
35. **Schmidt, E., Wanner, J., Hoferl, M., Jirovetz, L., Buchbauer, G., Gochev, V., Girova, T., Stoyanova, A., Geissler, M. (2012).** Chemical composition, olfactory analysis and antibacterial activity of *Thymus vulgaris* chemotypes geraniol, 4-thujanol/ terpinen-4-ol, thymol and linalool cultivated in Southern France. Nat. Prod. Commun. 7: 1095-1098.
36. **Bordini, E.A.F., Tonon, C.C., Francisconi, R.S., Magalhaes, F.A.C., Maquera-Huacho, P.M., Bedran, T.L., Pratavieira, S., Spolidorio, L.C., Spolidorio, D.P. (2018).** Antimicrobial effects of terpinen-4-ol against oral pathogens and its capacity for the modulation of gene expression. Biofouling. 34: 815-825.
37. **Maquera-Huacho, P.M., Tonon, C.C., Correira, M.F., Francisconi, R.S., Bordini, E.A.F., Marcantonio, E., et al.(2018).** *In vitro* antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces. Biofouling., 34:699-709.
38. **Thappeta, K.R.V., Zhao, L.N., Nge, C.E., Crasta, S., Leong, C.Y., Ng, V., Kanagasundaram, Y., Fan, H., and Ng, S.B.(2020).** In-Silico identified new natural Sortase A inhibitors disrupt *S. aureus* biofilm formation. Int. J. Mol. Sci., 21: 8601.
39. **Ganesh, P.S., Veena, K., Senthil, R., Iswamy, K., Ponmalar, E.M., Mariappan, V., Girija, A.S.S., Vadivelu, J., Nagarajan, S., Challabathula, D., and Shankar, E.M. (2022).** Biofilm-Associated Agr and Sar Quorum Sensing Systems of *Staphylococcus aureus* Are Inhibited by 3-Hydroxybenzoic Acid Derived from *Illicium verum*, ACS Omega, 7: 14653-14665.