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ORIGINAL ARTICLE

Determination of antibacterial and anti-biofilm potential of Kewda essential oil against *Staphylococcus aureus* and *Klebsiella pneumoniae*

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Abstract

The Kewda essential oil (KEO) extracted from *Pandanus odorifer* male flower was evaluated for its phytochemical profiles, antimicrobial and anti-biofilm activities against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC 740 and MTCC 109 respectively. As evident from GC-MS analysis, the phytochemical profiles revealed the presence of 2-phenyl ethyl methyl ether (PEME) and Terpinen-4-ol as prominent secondary metabolites. The MIC of KEO was 5% (v/v) against all the test bacteria. The KEO exhibited strong antibacterial and anti-biofilm activities against test bacteria. A significant reduction in the biofilm production was observed with an inhibition of 67.51 ± 1.29% and 61.17 ± 3.75% against *K. pneumoniae* and 71.76 ± 4.56% and 59.3 ± 6.24% against *S. aureus.* The light and fluorescence microscopic analysis confirmed a significant decrease in the density and thickness of the biofilm matrix against both the clinical and reference strains when treated with sub-MIC of KEO.

Keywords Essential oil · Phytochemicals · MIC · Antimicrobial · Biofilm

Abbreviations

CLSI	Clinical and Laboratory Standards Institute
CRA	Congo Red Agar
EPS	Extracellular Polymeric Substances
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.
FT-IR	Fourier Transform Infrared
GC-MS	Gas Chromatography-Mass Spectroscopy
IMTECH	Institute of Microbial Technology
KEO	Kewda Essential Oil
KBr	Potassium bromide
MHB	Mueller Hinton Broth
BHI	Brain Heart Infusion
MIC	Minimum Inhibitory Concentration
PBS	Phosphate Buffer Solution
MTCC	Microbial Type Culture Collection
NIST	National Institute of Standards and Technology
PEME	2-phenyl ethyl methyl ether
TTC	2, 3, 5-triphenyltetrazolium chloride
VIMSAR	Veer Surendra Sai Institute of Medical Sciences and Research

Introduction

The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) are responsible for chronic microbial infections and are resistant to most available antibiotics by forming recalcitrant biofilms (Das et al. 2019). Biofilms are self-assembled structures that harbour microbial communities and facilitate the attachment of the microorganisms to the surface. The biofilms are mainly composed of extracellular polymeric substances (EPS), which contain extracellular proteins, carbohydrates and nucleic acids (Song et al. 2018). Biofilm forming bacteria exhibit more resistance towards antibiotics than their planktonic counterparts by modulation of

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metabolic state which ultimately results in the decreased antibiotic penetration into the matrix (Bazargani and Rohloff 2016). Owing to the importance of biofilms in drug resistance, it is imperative to quest for alternative therapies using natural products.

Plants produce highly diverse bioactive secondary metabolites with widespread pharmacological importance. The essential oil (EO) derived from different plants, owing to the presence of high phenolic and terpenoid constituents, reported for extensive pharmacological attributes including antimicrobial and anti-biofilm properties (Kim et al. 2016). Hence, EOs could be the potential therapeutic agents against biofilm mediated drug resistance (Rubini et al. 2018).

In the present study, the EO extracted from *Pandanus odorifer* (commonly known as Kewda) flowers was evaluated for its antimicrobial and anti-biofilm potential against ESKAPE pathogens, *Staphylococcus aureus* and *Klebsiella pneumoniae.* According to Adkar and Bhaskar (2014), Kewda essential oil (KEO) is extensively used for treatment of rheumatism, antispasmodic properties, antiseptic, antibacterial and degenerative diseases (Adkar and Bhaskar 2014). The soothing and calming properties of KEO are extensively explored in various aromatherapy regimens. Therefore, we have evaluated the presence of bioactive components in KEO, which might considerably influence its antimicrobial and anti-biofilm activities against test pathogens and could be instrumental in mitigation of chronic microbial infections.

Materials and methods

Collection of male flowers of Kewda and extraction of essential oil

The wild male flowers of *P. odorifer* were collected from the coastline of Ganjam, Odisha, early in the morning (7–9 AM) in the month of July and August, which is the blooming season for Kewda. The sampling was carried out in the morning because the fragrance of the flowers was lost quickly after opening of calyx. Hydrodistillation method was employed for the extraction of essential oil using a Clevenger type apparatus (Pontes et al. 2019). The extracted essential oil of Kewda consisted of aroma, was dried over anhydrous sodium sulphate (Sigma Corporation, USA) to remove moisture and transferred to glass vials and kept at 4 °C until further analysis.

GC-MS analysis of KEO

KEO was analysed by Gas chromatography coupled with SQ8 mass detector; ionization voltage 70 eV. The G.C. analysis was carried out on Perkin Elmer Clarus 580 (Perkin Elmer, USA) gas chromatograph fitted with a flame ionization detector (FID) and an Elite-5 MS capillary column (5% phenyl, 95% dimethyl polysiloxane) having 30 m length x 0.25 mm I.D. x 0.25 µm film thickness. Helium was the carrier gas (1 mL/min). The injector temperature was set at 250 °C with the source temperature 180 °C. The oven temperature was initially kept at 60 °C and then gradually increased to 220 °C at 3 °C/min with 7 min hold at 220 °C. The total run time was 60.33 min. Mass units were monitored from *m*/*z* 40 to 500. Neat oil of 0.1 µL was injected into the system for performing analysis. Column and oven temperature of GC was kept same as that of GC-MS. Injector and detector (FID) temperature was set as 250 °C. The area percentage of the detected compounds was determined from the GC-FID peak areas. Identification of different compounds was carried out by comparing the mass spectra of each detected compound with the NIST (National Institute of Standards and Technology) library. By peak-area normalization, the relative percentage of the detected peaks was obtained.

FT-IR analysis of KEO

The KEO was analysed by fourier transform infrared spectroscopy (FT-IR) using the Bruker, Alpha II model. The spectral range was 4000 – 400 cm^{-1}. Resolution was 4 cm^{-1} with KBr beam splitter and DTGS detector. There was HGTR assembly for measurement (Cebi et al. 2021).

Antibacterial activity of KEO

The clinical bacterial isolates were collected from Veer Surendra Sai Institute of Medical Sciences and Research (VIMSAR), Burla, Sambalpur, Odisha. From the urine sample of the patients, the bacterial strains were isolated. Further, the isolated bacterial strains were subjected for biochemical characterization followed by molecular characterization (16S rRNA sequencing) and VITEK-II analysis for identification of the bacterial strains. The standard MTCC cultures i.e. MTCC 740 (*Staphylococcus aureus*) and MTCC 109 (*Klebsiella pneumoniae*) were procured from IMTECH, Chandigarh, India. Mueller-Hinton broth (MHB) and Mueller Hinton Agar (MHA) medium were used for harvesting bacterial cells and maintaining the bacterial cultures. Prior to experimental setup, the overnight grown bacterial cultures were maintained at 0.5 McFarland standard. The minimum inhibitory concentration (MIC) of KEO against test microorganisms was determined according to CLSI guidelines, 2012 (M26-A) with slight modifications. Two-fold serial microdilution method was employed for the determination of the MIC. The highest concentration

of KEO used for the determination of MIC was 5%. From this concentration, two-fold serial dilution was employed with the lowest concentration of 0.039%. The above mentioned serial dilutions were prepared in MH Broth medium followed by inoculation of test pathogens. The MIC was determined in 96 Microtiter plate setup (microdilution), where in each well, 100 µL of sterile Muller Hinton Broth was used. 0.5 McFarland of test bacterial cultures were considered for the inoculation into the experimental setup in 96 Microtiter plate. The experimental setup was incubated at 37 °C for 16–18 h. Bacterial culture inoculated into MHB without sample treatment considered 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-titer plate was incubated at 37 °C for 15 min. The wells were examined for the development of the pink colour which inferred the bacterial growth, and the absence of the pink colouration was considered as inhibition of bacterial growth. The minimum concentration of sample where no change in the colour was observed was considered as the MIC value. The results were taken in triplicate. In the experimental setup, MH broth in the wells were treated as blank whereas MH broth with 0.5 McFarland test bacterial solutions were considered as negative control (Cheruvanachari et al. 2022). Agar well diffusion assay was performed to determine the antibacterial actions of KEO. 50 µL of KEO (MIC and sub-MIC concentrations prepared in n-Hexane) were incorporated in the prepared wells in the solidified MH Agar medium. Antibacterial activities were evaluated by measuring the diameter of halo zones formed around the well. For the experiment, n-Hexane was considered as control (Negreiros et al. 2016).

Table 1 List of identified phytochemicals from the essential oil of *Pandanus odorifer* flower by GC-MS analysis

Sl. No.	Phytochemicals	Retention Time (min)	Peak heights	Correlation area	Peak area Percentage (%)
1.	p-Cineole	3.1	446,068	9,620,777	0.405%
2.	γ-Terpinene	3.4	1,313,773	42,386,649	1.790%
3.	2-Phenyl ethyl methyl ether (PEME)	3.9	23,401,847	1,905,199,478	80.435%
4.	p-Meth2en1ol	4.5	523,708	13,876,298	0.586%
5.	cis p meth-2-en-1-ol	4.8	361,742	9,242,053	0.390%
6.	Terpinen-4-ol	5.6	8,529,820	334,683,018	14.130%
7.	α-terpineol	5.8	1,797,801	43,327,170	1.829%
8.	Piperitone	7.2	236,538	8,220,680	0.347%
9.	Nerolidol	14.6	62,365	2,065,528	0.087%

Inhibition of biofilm production by KEO

The inhibition of biofilm production by KEO 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 Congo red agar (composed of Brain Heart Infusion broth: 37 mg/mL; Sucrose: 50 mg/mL; Agar Agar: 10 mg/mL; Congo Red dye: 0.8 mg/mL) plates (Chhibber et al. 2017). Bacterial culture without treatment was considered as a control.

Polystyrene-based 24-Microtiter plate (MTP) was employed to quantify the extent of biofilm formation in the treatment setup with sub-MIC level of KEO compared to untreated control using crystal violet staining method (Cannas et al. 2014). After the addition of the sub-MIC concentration of KEO and bacterial culture (0.5 McFarland), the setup was incubated overnight at 37 °C. After the formation of biofilm matrix, the wells were washed with sterile PBS. The biofilms were stained with 0.1% crystal violet followed by removal of excess stain. To the wells, 95% ethanol was added in each of the wells. The absorbance was taken at 540 nm using ELISA plate reader (Kang et al. 2019). The percentage (%) of inhibition was calculated using:

$$\%of\,Inhibition = (OD_{Control} - OD_{Treatment})/OD_{Control} \times 100\,(OD : Opticaldensity)$$

For light and fluorescence microscopic analysis, test bacteria (0.5 McFarland standard) were treated with sub-MIC of KEO and the setup was incubated overnight to allow biofilm formation in Luria Bertani (LB) broth. After incubation, media were discarded and biofilms were rinsed with sterile 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) (Packiavathy et al. 2014; Wu et al. 2016). The fluorescent images were subjected for analysis using COMSTAT2 software (Heydorn et al. 2000).

Results and discussion

The EO obtained from *P. odorifer* male flower by the hydro-distillation process was transparent in colour with a characteristic aroma. From the gas chromatography-mass spectrometric (GC-MS) analysis, nine phytochemicals were identified based on their retention time, and peak area percentage (Table 1). Table 1 inferred the peak area percentage of 2-Phenyl Ethyl Methyl Ester (PEME) was highest at 80.435% followed by Terpinen-4-ol with a 14.13% of

peak area percentage. The results were in accordance with the earlier report, depicting the highest peak area percentage of PEME was 37.7% followed by Terpinen-4-ol (18.6%) (Raina et al. 2004). The analysis was also informative about the presence of bioactive components like α-terpeneol (1.829%) and α-terpenene (1.79%). All these identified components were reported as significant components of plant-derived essential oils (Khaleel et al. 2018; Ghazal et al. 2022) also reported the presence of Terpinen-4-ol (4.43%) in *Origanum majorana* (Ghazal et al. 2022). In an earlier study, the essential oil extracted from *P. odorifer* (Forssk.) Kuntze also reported to constitute PEME followed by Terpinen-4-ol as the most abundant bioactive secondary metabolites which corresponds to the results obtained in the present study (Nasim et al. 2018).

The FTIR analysis inferred the presence of several chemotypes including alcohols, alkanes, aldehydes, phenols, sulfonates, alkyl halides, alkyl sulfides, and halo-compounds. Some of the components were with aromatic rings. Most of the identified monoterpenes were observed at 3493.30 cm^{-1} to 510.00 cm^{-1} as observed from the FTIR spectra with a distinctive molecular fingerprint profile (Fig. 1). The presence of distinct functional groups of interest as evident from FTIR spectra were in accordance with the earlier report depicting functional groups in several plant-derived essential oils (Agatonovic-Kustrin et al. 2020).

The minimum inhibitory concentration (MIC) of KEO was found to be 5% (v/v) against both the clinical strains of *S. aureus* and *K. pneumoniae* and their reference strains, MTCC 740 and MTCC 109 (Fig. 2a). At the MIC level, KEO exhibited promising antibacterial activity. All the test

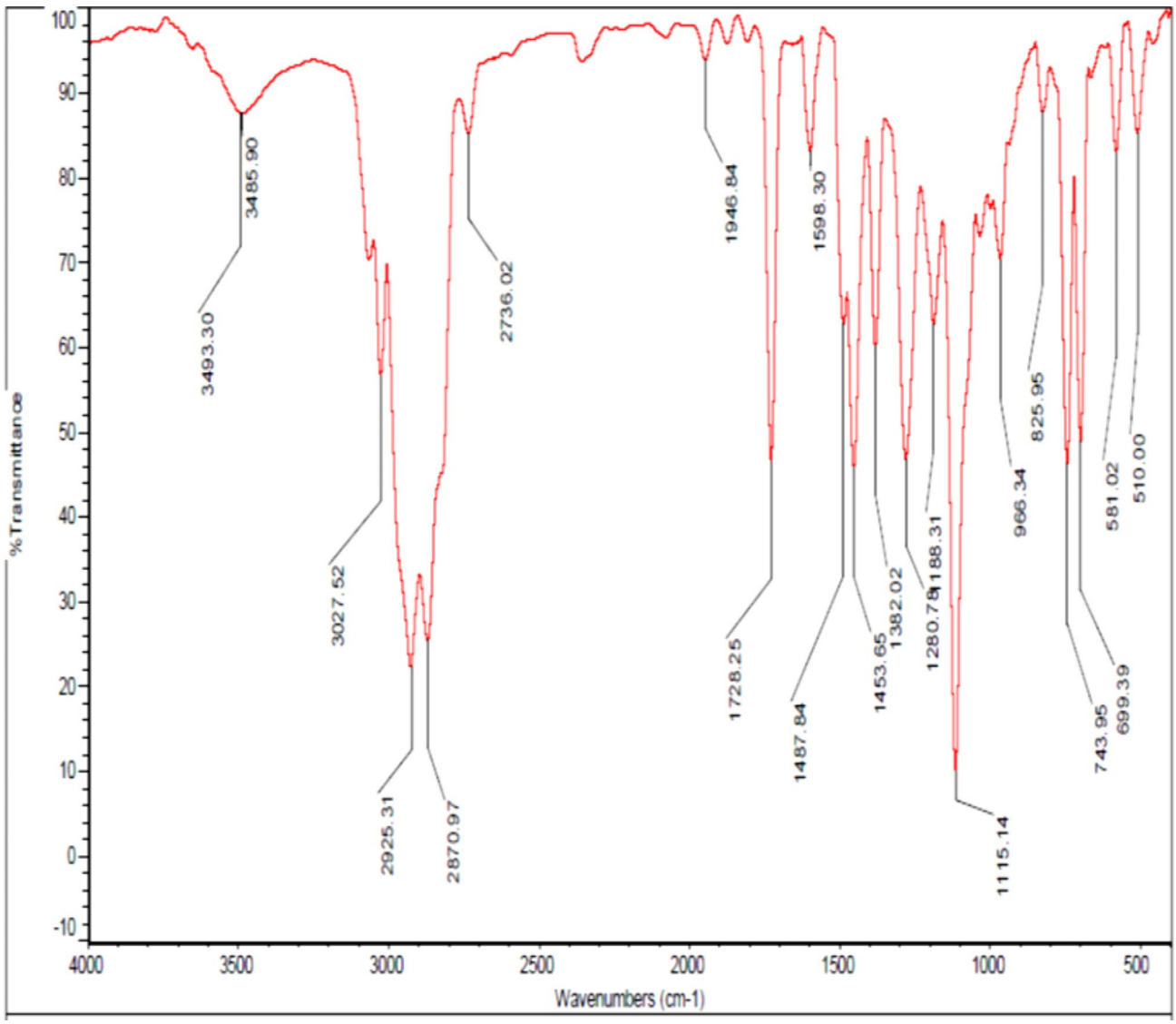

Fig. 1 The FTIR spectral analysis of *Pandanus odorifer* (Kewda) essential oil

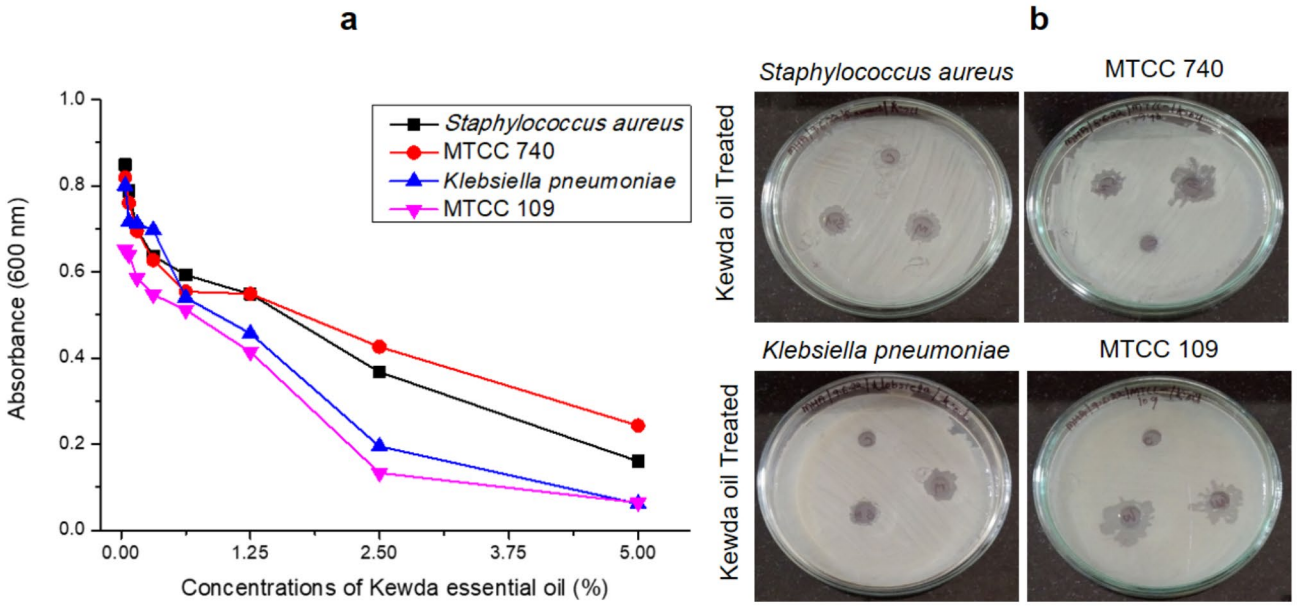

Fig. 2 (a) Minimum inhibitory concentration (MIC) of Kewda essential oil (KEO) against clinical isolates of *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109; (b) Effect of KEO (at MIC level) on the growth of test bacteria, *S. aureus* and *K. pneumoniae* and their reference strains, MTCC 740 and MTCC 109

pathogens were observed to be highly sensitive towards the treatment of KEO at MIC with highest zone of inhibition against standard strains MTCC 109 followed by MTCC 740 with a zone of inhibition of 22 and 19 mm, respectively (Fig. 2b). The results were in accordance with earlier studies depicting the promising antibacterial activities of plant derived essential oils (Man et al. 2019; Purkait et al. 2020). Abd Wahab et al. (2022) also described that the essential oil extracted from *Melaleuca cajuputi* shows antibacterial activity against the clinical isolates which can be used to treat several bacterial diseases (Abd Wahab et al. 2022).

On treatment with sub-MIC concentration of KEO, a significant decrease in the biofilm production in test microorganisms was observed with a concomitant reduction in the production of exopolysaccharides (EPS). Compared to the crystalline black colonies in the untreated control (indicating the profuse production of EPS), the treated bacteria with KEO produced less EPS (Fig. 3a). The quantitative biofilm formation assay using crystal violet staining method further revealed that treatment with sub-MIC concentration of KEO significantly inhibited biofilm formation against MTCC-740 and MTCC-109 with a reduction of 72.72 and 62.86% respectively (Fig. 3b). The EO extracted from *O. majorana* showed biofilm inhibition by 36–86% (Ghazal et al. 2022). The inhibition to biofilm production could be attributed to the presence of Terpinen-4-ol, which was earlier reported to exhibit anti-biofilm properties (Cordeiro et al. 2020). Terpinen-4-ol, the bioactive secondary metabolite from KEO showed promising antibacterial and anti-biofilm activities against drug resistant pathogens, *S. aureus, K. peumoniae*, and the reference MTCC 740 and MTCC 109 (Cheruvanachari et al. 2022).

The significant reduction in EPS matrix production on treatment with sub-MIC concentrations of KEO was further confirmed by microscopic evaluations. A significant decrease in the density and thickness of the biofilm matrix formed onto glass coverslips was observed in both the clinical isolates and reference strains of the bacteria when treated with sub-MIC of KEO as evident from both light and fluorescence microscopic analysis (Fig. 4). Apart from the thickness, the distribution of biofilm matrix formed over the glass surface were also considered for the assessment of the ability of KEO in mitigating biofilm formation as compared to untreated control. The microscopic analysis provided an advantage over the quantitative method by allowing to visualize the formed biofilm matrix over the abiotic surface. The results were in accordance with the report of Kim et al. (2015), in which Cinnamon bark oil and its bioactive constituents significantly altered the biofilm formation in multidrug-resistant bacteria. The antibacterial and anti-biofilm activities of KEO could be due to the presence of bioactive secondary metabolites, PEME and Terpinen-4-ol. As the

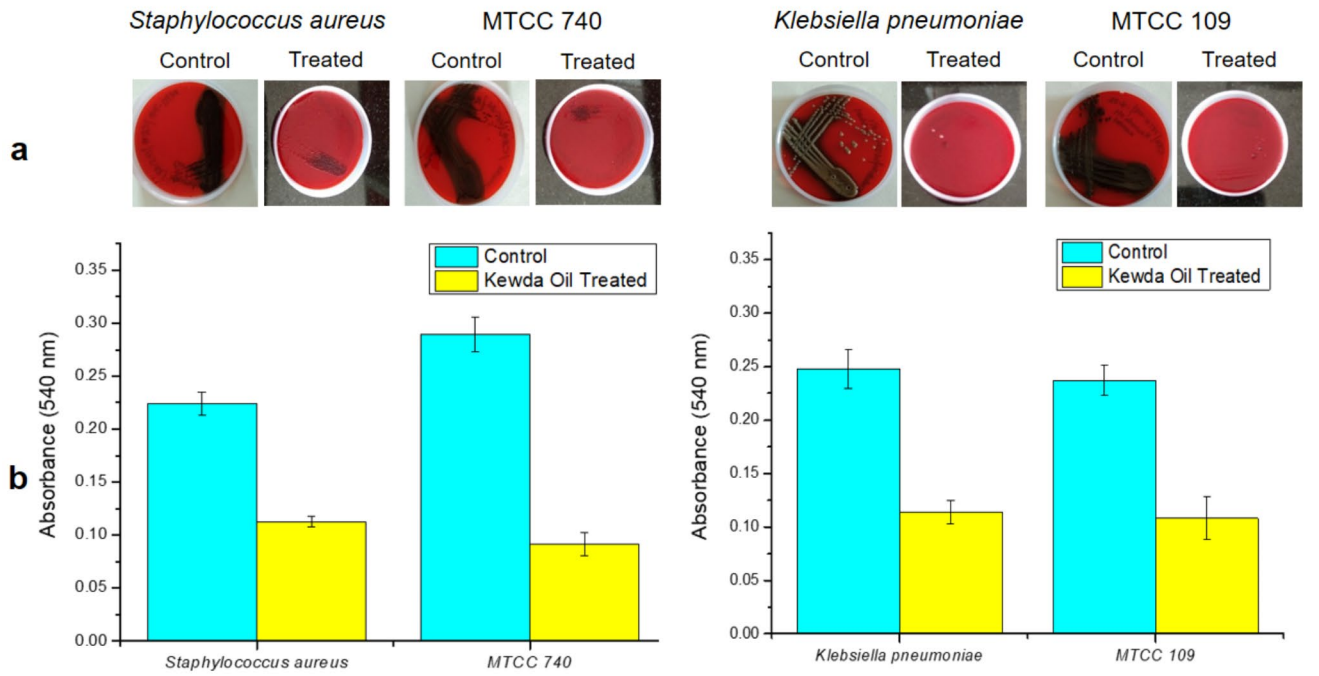

Fig. 3 (a) Effect of sub-MIC of Kewda essential oil (KEO) on biofilm formation in clinical *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109 using Congo red agar plate method; (b) Effect of sub-MIC of KEO on biofilm formation in clinical *S. aureus* and *K. pneumoniae* and their reference strains MTCC 740 and MTCC 109 using quantitative crystal violet staining method

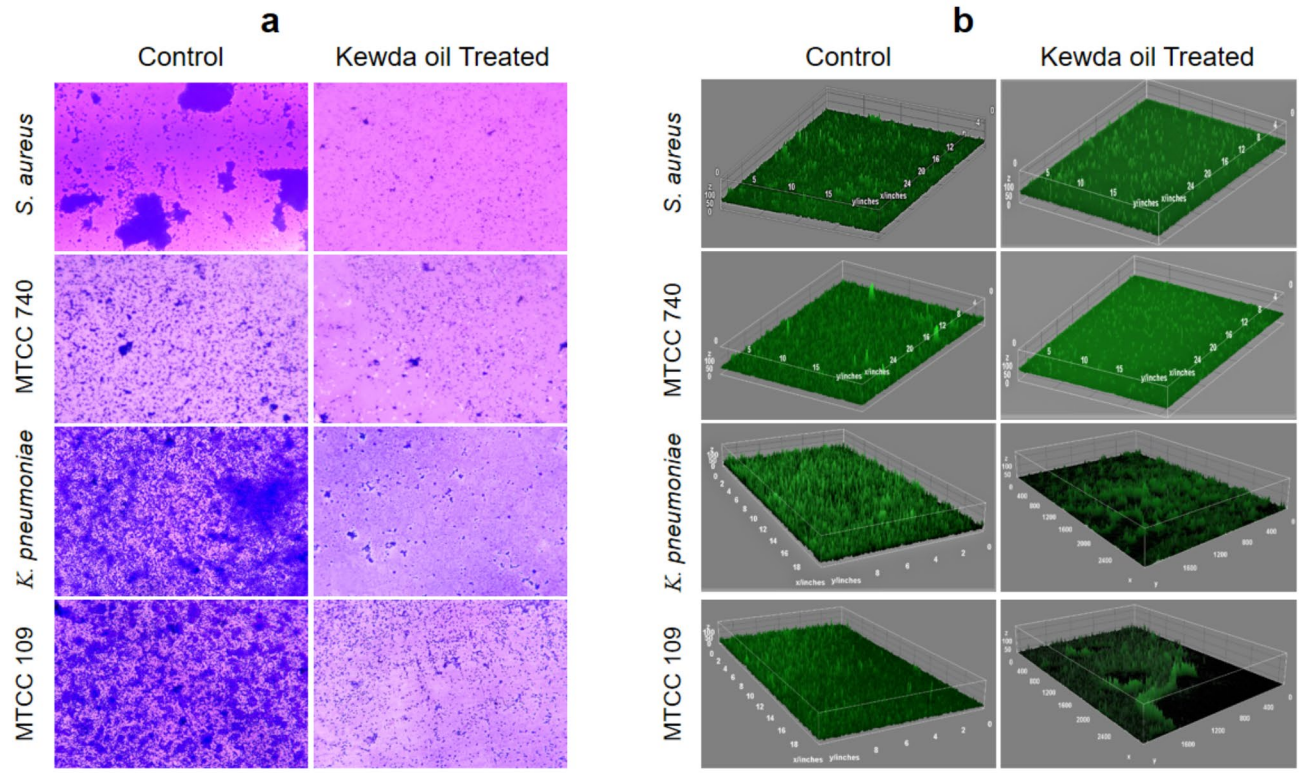

Fig. 4 (a) Light microscopic and (b) Fluorescence microscopic observations on the effect of sub-MIC of Kewda essential oil (KEO) on biofilm formation in *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC 740 and MTCC 109

study revealed that KEO exhibited promising antibacterial and also showed biofilm inhibitory properties, further in depth analysis could be employed to determine its role in mitigation of chronic microbial infections. Moreover, KEO could also be used to study its role in efflux pump mechanisms. The major drawback in the treatment of biofilm related diseases is due to the protective layers formed by the biofilm causing organisms which prohibits the penetration of antibiotics. The biofilms are resistant to antibiotics due to outer membrane structure, less penetration of antibiotics, enzyme mediated resistance, efflux pump, genetic adaptation (Singh et al. 2017). Hence, Kewda essential oil (KEO) could be used in combination with other effective bioactive agents such as antibiotics to tackle the drug resistance phenomena in the near future.

Conclusion

The encouraging antimicrobial and anti-biofilm activities of KEO against drug-resistant pathogens, could be attributed to the presence of bioactive compounds of pharmacological importance, particularly PEME and Terpinen-4-ol. In addition, the anti-biofilm potential of KEO could be instrumental in mitigating biofilm-associated infections in the near future.

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Author's contribution statement Priya Cheruvanachari: Methodology, Data analyses, Original draft preparation. Monika Mishra: Design of the study, Methodology, Data analysis, Original draft preparation, Writing - Review and editing. Subhaswaraj Pattnaik: Conceptualization, Design of the study, Methodology, Data analysis, Original draft preparation, Writing - Review and editing, Funding acquisition. Pradeep Kumar Naik: Conceptualization, Design of the study, Writing - Review and Editing, Funding acquisition.

Declarations

Competing Interest The authors declare that no potential conflict of interest.

Ethical approval statement No ethical approval is required for the present study.

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