SYNOPSIS OF THE THESIS SUBMITED TO SAMBALPUR UNIVERSITY

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ABSTRACT OF THE DISSERTATION

The indiscriminate and irrational use of antibiotics to treat chronic microbial infections non-specifically results in the emergence of drug resistant microorganisms. Multidrug resistant microorganisms are constantly progressing, posing a serious threat to current healthcare settings. Particularly, the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) group of pathogens are considered as the most critically acclaimed drug resistant bacterial community owing to their widespread resistance patterns to several groups of antibiotics. World Health Organization (WHO) also categorizes these pathogens as priority, which corresponds to the urgent need to develop new therapeutic modules to counteract bacterial pathogenesis. Biofilms are adaptive mechanisms used by pathogenic bacterial populations to withstand environmental stress, including antibiotics. The biofilm forming pathogens can be encased within the self-assembled polymeric matrix (composed of carbohydrates, proteins, nucleic acids and lipids), providing a protective environment against antibiotic treatment. Since biofilms play a pivotal role in drug resistance mechanisms, targeting biofilm mechanics could be a viable therapeutic module. In this regard, it is imperative to develop alternative therapeutic or antibacterial drugs targeting the biofilm matrix. In this context, it is important to consider the putative antibacterial drug candidates that should not only possess an inhibitory effect on the growth of drug resistant bacteria but also facilitate the decrease in selective pressure associated with antibacterial drugs towards the bacterial community. To this end, pharmacologically important medicinal plants are considered as a rich source of bioactive compounds that can be utilized to develop novel antimicrobial drugs (Chandra et al., 2017). The medicinal plants also show significant ability against inhibition of biofilm production by different types of bacteria, including ESKAPE pathogens.

Therefore natural products are a vital source for exploring new antibacterial and anti-biofilm therapies. So keeping this on mind, natural products, their derivatives and putative drug candidates have emerged and posed a potential challenge to these pathogens. These natural products are widely accepted because they had been traditionally used in folkloric medicine to cure diseases and are safe to use, having no side effects. Among the different plant species with significant antimicrobial activity, the essential oil extracted from *Pandanus odorifer* (known as kewda oil) is used in traditional medicines mainly because it consists of a number of bioactive monoterpenes and phenolic compounds. Among the bioactive constituents, Kewda essential oil contains 2-Phenyl ethyl methyl

ether (PEME) and Terpinen-4-ol as the most prominent phytoconstituent, which are responsible for several pharmacological activities, including antimicrobial properties.

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 a viable alternative. Since plant-derived essential oils are the rich heritage of bioactive phytochemicals with widespread pharmacological values, in the present study Kewda essential oil (KEO) and its two bioactive compounds such as Terpinen-4-ol and 2-Phenyl Ethyl Methyl Ether (PEME) were evaluated for their antimicrobial and antibiofilm activities against ESKAPE pathogens, *Staphylococcus aureus* and *Klebsiella pneumoniae* and their standard reference strains, MTCC-740 and MTCC-109, respectively. A total of ten clinical bacterial isolates (*Escherichia coli*, *Salmonella typhi*, *Salmonella paratyphi* A, *Salmonella paratyphi* B, *Proteus* sp., *Klebsiella pneumoniae*, *Shigella* sp., *Pseudomonas aeruginosa*, *Enterococcus* sp. and *Staphylococcus aureus*) were isolated from the clinical samples. The bacterial strains were sub-cultured and subjected for identification by standard biochemical procedures followed by Clinical and Laboratory Standards Institute (CLSI) guidelines. These isolated bacteria were subjected for antibiotic susceptibility test with 12 antibiotics of 5 classes (4 aminoglycosides, 3 β-lactams, 5 cephalosporins, 1 carbapenem and 4 fluoroquinolones). The microorganisms were either resistant, sensitive or intermediate towards different antibiotics based on the zone of inhibition. In particular, the Gram negative bacteria, *Klebsiella pneumonia* and Gram positive bacteria, *Staphylococcus aureus* showed resistance to most of the antibiotics were sensitive to KEO and its two bioactive compounds.

The Kewda essential oil (KEO) extracted from *Pandanus odorifer* male flower was evaluated for its phytochemical profiles using various analytical equipments such as Gas chromatography-mass spectrometer (GC-MS), High resolution mass spectrometer (HRMS), Fourier transform infrared (FTIR) spectroscopy and Nuclear magnetic resonance (NMR) spectroscopy. The GC-MS analysis of Kewda essential oil revealed the presence of several phytochemicals, out of which 2-Phenyl ethyl methyl ether (PEME) exhibited the highest peak area percentage with an occurrence of 80.435 %. The presence of PEME in the essential oil of the Kewda flower suggested its widespread potential in cosmetic industries as fragrances. The next phytochemical in the panel is Terpinen-4-ol (14.13 %) which has promising therapeutic values. The other important constituents identified were α terpeneol (tertiary monoterpenoid; 1.829 %) and γ-terpenene (monoterpene; 1.79 %).

HRMS analysis clearly showed the presence of 12 antimicrobial compounds namely p-benzoquinone, 2-phenethyl alcohol, p-cymene, 2-phenethyl methyl etheralpha-Terpineol, Psoralen, Isoplumbagin, Genipin, Artemidiol, Pinocembrin, (-)-Glycinol, Iprobenfos. The presence of such secondary metabolites can be attributed to its antimicrobial activity, which were effective against Gram-positive and Gram-negative bacteria. Further, it was found from the HRMS study that the major compound present in crude oil was PEME. Fourier Transform Infrared Spectroscopic (FTIR) analysis of Kewda essential oil identified the presence of several chemical moieties such as Alcohols, Alkanes, Aldehydes, Phenols, Sulfonates, Alkyl halides, Ikyl Sulfides, Alkyl Halides and Holo-compounds with characteristic IR fingerprints ranging from 3493.30 cm^{-1} to 510 cm^{-1}. The NMR spectra of Kewda essential oil revealed the presence of ketones, esters, alcohols, and aromatic phenols.

The minimum inhibitory concentration (MIC) of KEO was 5% (v/v) against the Gram negative bacteria, *Klebsiella pneumonia* and Gram positive bacteria, *Staphylococcus aureus* and their reference strains, MTCC-740 and MTCC-109, respectively. Further, KEO exhibited a strong antibiofilm activities against these bacteria. A significant reduction in the exopolysaccharides (EPS) production was observed with an inhibition of 67.51±1.29 and 61.17±3.75 % against *K. Pneumoniae* and its reference strain, MCC-109 and 71.76±4.56 and 59.3±6.24 % against *S. aureus* and its reference strain, MTCC-740. 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.

The 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 also observed as evident from qualitative congo red agar (CRA) and tube method. 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 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) associated with biofilm formation and drug resistance. Terpinen-4-ol exhibited the highest binding affinity towards Sortase A (PDB ID: 1T2P) with a Glide docking score of -4.405 Kcal/mol, which suggested the ability of Terpinen-4-ol in quenching biofilm formation in *S. aureus* by targeting the surface associated protein, sortase A. Thus, Terpinen-4-ol could be considered as a putative drug candidate in the fight against biofilm associated chronic infections and drug resistance.

Another Kewda essential oil bioactive compound, 2-Phenyl Ethyl Methyl Ether (PEME), 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 PEME against *S. aureus* and *K. pneumoniae* was 50 mM, respectively. At MIC level, PEME exhibited antibacterial activities against test bacteria. On treatment with sub-MIC of PEME, a significant reduction in exopolysaccharides (EPS) production was observed, as evident from the qualitative Congo red agar (CRA) assay. Further, the reduction in EPS production was quantified using crystal violet staining method, which showed the highest inhibition was observed against MTCC-740 with a reduction of 71.76±4.56%. The light and fluorescence microscopic analysis also corroborated the anti-biofilm potential of PEME as a significant reduction in the thickness of biofilm formation was observed. *In silico* studies of PEME in binding to target proteins 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) associated with biofilm formation and drug resistance was studied. Among the target proteins used in this study, PEME exhibited the highest binding affinity towards SarA (Global regulatory protein; PDB ID:2FNP), which is directly associated with bacterial virulence and biofilm mechanics. Thus, targeting SarA, PEME could interfere with the biofilm dynamics of *S. aureus*. Further, transcriptomic data analysis suggested the role of PEME in down regulation of specific genes, *agrA, sarA, norA* and *mepR,* which are linked with bacterial virulence, biofilm dynamics and drug resistance patterns. Thus, PEME could be considered as a putative drug candidate in the fight against biofilm associated chronic infections and drug resistance. Thus, the present study provided encouraging results on the use of plant-derived Kewda essential oil (KEO) and its derivative PEME and Terpinen-4-ol as potential therapeutic agents against bacterial virulence and biofilm

mechanics in drug resistant ESKAPE pathogens, *S. aureus* and *K. pneumoniae*. The promising antibiofilm potential of crude essential oil and its bioactive derivatives could also be used to target the biofilm associated infections in other multidrug resistant pathogenic microorganisms in the near future. The present study also provides a platform to decipher the combinatorial effect of the two bioactive compounds, PEME and Terpinen-4-ol with the available antibiotics for improved biological activities.