ELUCIDATION OF ANTIBACTERIAL ACTIVITY OF PANDANUS ODORIFER OIL (KEWDA OIL) AND ITS COMPONENT AGAINST MULTIDRUG RESISTANCE BACTERIA: PHYTOCHEMICAL PROFILING, MODE OF ACTION AND TOXICITY EVALUATION

A Dissertation Submitted to the Sambalpur University in Partial Fulfilment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

by

CHERUVANACHARI PRIYA

Regd. No. 001/2018/Bio.Tech.

DEPARTMENT OF BIOTECHNOLOGY & BIOINFORMATICS,
SAMBALPUR UNIVERSITY, JYOTIVIHAR, BURLA,
SAMBALPUR- 768019, ODISHA

August, 2022

ABSTRACT OF THE THESIS

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Under the Supervision of

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CERTIFICATE

*This is to certify that the research work entitled, "**Elucidation of antibacterial activity of pandanus odorifer oil (kewda oil) and its component against multidrug resistance bacteria: phytochemical profiling, mode of action and toxicity evaluation.**" submitted by **Mrs. Priya Cheruvanechari (Regd No: 001/2018/Bio.Tech.)** at **Sambalpur University, Orissa, India** is a bonafide record of her original work carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for any degree or diploma. I recommend this thesis in fulfillment of the award of the degree of **Doctor of Philosophy** in **Biotechnology.***

Prof. (Dr.) Pradeep Kumar Naik

Date:

DECLARATION

*I hereby declare that the matter embodied in the thesis entitled "**Elucidation of antibacterial activity of pandanus odorifer oil (kewda oil) and its component against multidrug resistance bacteria: phytochemical profiling, mode of action and toxicity evaluation**" the result of investigations carried out by me at Department of Biotechnology & Bioinformatics, Sambalpur University, Jyoti Vihar, Odisha under the supervision of Professor Pradeep Kumar Naik, Department of Biotechnology & Bioinformatics, Sambalpur University. The results of the investigation have not been submitted either in part or full for the award of any other degree or diploma in this institute or any other institute or university.*

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whatever the work described is based on the findings of other investigators. Any omissions there in may have been occurred by oversight or error in judgment is regretted.

Mrs. Cheruvanechari Priya

Date:

ACKNOWLEDGMENT

I would like to express my deep sense of gratitude and gratefulness to Prof. Pradeep Kumar Naik, Department of Biotechnology and Bioinformatics, Sambalpur University, for providing this lifetime opportunity to work under him. I express my heartiest thankfulness and indebtedness to him, for his guidance, support, inspiration and constant supervision as well as for providing all the necessary information, facilities and resources for the completion of my PhD research.

I'm grateful to extending my sincere thanks to all my reverenced teachers of Department of Biotechnology and Bioinformatics, Sambalpur University who have been kind enough to extend their generously and help at the sundry phases of my research whenever I approached them during my research tenure.

My gratitude is extended to all the staffs of Department of Biotechnology and Bioinformatics who always remain with me and have extended their assistance to me whenever I needed their help that cannot be forgotten.

I am highly grateful to Dr. Mahesh Chandra Sahu, Scientist-B Regional Medical Research Centre, ICMR Bhubaneswar (formerly at Medical Research Laboratory, IMS and Sum Hospital, Bhubaneswar), giving me an opportunity to carry my PhD work in his laboratory. I would also thank him for providing me extended facilities to complete my PhD research work.

I am grateful to Dr. Debasmita Dubey, Assistant Professor, IMS and SUM Hospital, Siksha 'O' Anusandhan University, Bhubaneswar for her expert guidance and necessary advice for Microbiological work and her constant help, continuous support and encouragement for the fulfillment of my thesis work.

I would like to place on record my sincere thanks to the Centre of Excellence in Natural Products and Therapeutics (COE-NPT), Department of Biotechnology and Bioinformatic for providing the laboratory facility to carry out my research work.

I am grateful to Dr. Goutam Ghosh, Assitant Professor, and Mr. Deepak Pradhan, Research Associate, Department of Pharmacognosy, School of Pharmaceutical Science, Siksha 'O' Anusandhan University, Bhubaneswar, for his expert guidance and necessary advice for pharmacognostical work.

I am sincerely grateful to Dr. Durga Madhaba Kara, Professor and Head, Department of Pharmacology, School of Pharmaceutical Science, Siksha 'O' Anusandhan University, Bhubaneswar, Dr. Subhaswaraj Pattnaik, DST-NPDF, COE-NPT, Mr. Pratyush Pragyandipta, Mr. Animesh Pattnaik and Ms. Monika Mishra Dept. of Biotechnology and Bioinformatics for their expert guidance and support for pharmacological, antimicrobial activity, transcriptome and bioinformatics study.

I extend my thanks to all my lab mates, COE-NPT laboratory, Dept. of Biotechnology and Bioinformatics for their inspirational words, co-operation and encouragement in carrying out my research work.

My sincere and deepest gratitude to my parents, parent in-laws and my sister and daughter for their unfaltering love, support and cooperation provided to me throughout my PhD work and life.

I also express my sincere gratitude to my beloved husband Mr. Vijeesh Viswanath for constant support and encouragement.

The financial assistance from OHEPEE, Dept. of Higher Education, Govt. of Odisha, World Bank initiative for pursuing my Ph.D work is greatly acknowledged.

Last but not least, I am thankful to the Almighty. You have made my life more bountiful. May your name be exalted, honored and glorified.

Cheruvanachari Priya

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ABSTRACT

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.

CHAPTER I

INTRODUCTION

1.1 Taxonomy and plant description

Pandanus fascicularis Lam. (Synonyms *P. tectorius, P. odorattissimus, P. odorifer* Forssk.), commonly known as Kewda, is an economically important essential oil bearing plant that belongs to the family Pandanaceae (Franck 2012; Kamble *et al*. ,2013; Jose *et al.*, 2016; Kumar *et al.*, 2017). The plant has different vernacular/ local names like Ketakee, Gandhapushpa, Sthiragandha, InduKalika, Jambala (in Sanskrit); Kewra, Keora, Kewda, Gagandhul (Hindi); Kedgi, Kevda, Keora (Marathi); Kewoda (Gujarati); Kiya, Ketakee (Odia); Keya, Kedki, Keori (Bengali); Thazhai, Thalay, Thazhampoo (Tamil); Mogali, Gajangi (Telugu); and Kaitha, Kaida, Thala (Malayalam). *Pandanus fascicularis* Lam., commonly known as Screw pine plant. The taxonomic classification of *Pandanus fascicularis* is as follows:

Kingdom	Plantae
Division	Angiospermae
Class	Monocotyledons
Order	Pandanales
Family	Pandanaceae
Genus	*Pandanus*
Species	*Pandanus fascicularis*

Figure 1.1. The Kewda plant

Pandanus fascicularis is a dioecious monocotyledonous aromatic plant with palm like appearance growing usually up to the height of 3-5 metres. It has a spinous trunk with prop or stilt roots (Panda *et al.*, 2009a). The leaves are glaucous, ensiform with coriaceous margins and spiny midribs, arranged spirally on the branches. Male flowers appear as clusters with a unique fragrance and are surrounded by bracts. The female flowers lack any fragrance and look like a pineapple. The fruits of this plant are ellipsoid, ovoid, globose or subglobose (Englberger *et al.*, 2009; Jose *et al.*, 2016). The plant is propagated vegetatively from root suckers or branch cuttings. It is adapted well to light and heavy well-drained soil. The plant is drought tolerant, salt tolerant and can withstand strong winds (Rashmi and Nadaf 2017). The flowering starts after 3-4 years of the plantation and the rainy season (July to October) sees the maximum flowers. The plant has a life span of 50-80 years which might last up to 100- 150 years also. But the fruiting stage is only for 20-25 years (Raina *et al.*, 2004; Raju *et al.*, 2011; Adkar and Bhaskar 2014). Senescence of the plant is mainly due to the infection of insect pests such as bagworm, beetles and thrips causing economic losses. Diseases like foot rot of

central shoot, leaf blight and fruit rot have also been reported in this plant (Jagadev *et al.,* 2001). Ecologically, the plant has been reported to bear immense potential in its complex root system and thereby controlling soil erosion, fixing sand dunes and protecting from damage caused by tsunami (Tanaka *et al.,* 2007; Tanaka *et al.,* 2009; Tanaka *et al.,* 20011; Thuy *et al.,* 2012; Thuy *et al.,* 2018). Arbuscular mycorrhizal fungi (AMF) association has also been reported in this plant which helps in strengthening the ecological efficacy in coastal regions (Kamble *et al.,* 2013).

Figure 1.2. The plant *Pandanus odorifer* (Forssk.), the various stages of flower, and fruit development

1.2 Origin and distribution

Pandanus is a paleotropic genus, belonging to an ancient family Pandanaceae, which represents dioecious monocotyledons having Gondwanan origin (Gallaher *et al.*, 2015). Among all the genera of Pandanaceae family, *Pandanus* is the largest genus and has the widest geographical distribution with immense economic and medicinal importance (Callmander 2000; Callmander *et al*. 2003; Beurki *et al.*, 2012). The diversity of habitats included by the genus occupies the tropical and sub-tropical zones, riversides, rocky or sandy coasts, swamp forests, mangrove forests, savannas, lowland dipterocarp forest and mountain forest (Susanti *et al.*, 2012).

In India, Pandanacae family represents about 30-40 species under three genera *Pandanus, Benstonea*, and *Freycinetia. Pandanus fascicularis* commonly known as Kewda or screwpine is an important member of the genus *Pandanus* with greater concentration in Andaman and Nicobar Islands and Northern and Southern India. It is native to South Asia including Indonesia and Philippines and some tropical parts of Australia. It is widely distributed in South America, Micronesia, Papua New Guinea, Melanesia, Polynesia, India and Pacific Islands (Zanan and Nadaf 2012; Adkar and Bhaskar 2014; Gurmeet and Amrita 2015). In India, *Pandanus fascicularis* is distributed in two biogeographic zones: the Western Ghats zone and the coastal zone. It is seen in the states of Odisha, Andhra Pradesh, Kerala, Tamil Nadu, West Bengal, some regions of Uttar Pradesh and Gujarat (Padhy *et al.,* 2016; Gurmeet and Amrita 2015). The coastal areas of Ganjam district of Odisha state are the luxuriant growth centres of the plant (Mohapatra and Sahoo 2007; Rout *et al.,* 2015). Other than India it occurs in the coastal regions of Iran, Malaysia, Mauritius, Myanmar, Java, China, Taiwan and south islands of Japan (Panda *et al.,* 2010). The distribution of the plant is in both west and east coast of India but abundance of the plant and also commercial utilization of Kewda flower is practiced only in the east coast region of Odisha state. Despite the distribution of the plant all along the coast of Odisha starting from Ganjam district in south-west to the district of Baleswar in North-east; the abundance of the plant is in the coastal belt of south-west region of Odisha specifically in Ganjam district. The stretch of about 4 kms along the coast and between the two rivers i.e., Bahuda from the west to Rushikulya in the east has observed luxuriant growth and having best of varieties.

1.3. Industrial importance of *Pandanus fascicularis* L. (Kewda)

Pandanus fascicularis (Kewda) is an economically important plant due to its high priced flower essential oil. Economically, it is an important natural bioresource for the perfumery industry due to the exquisite fragrance it possesses (Panda *et al.,* 2009a). The characteristic fragrance of the male flowers contributes to their utilisation for the production of various perfumery products through a process of distillation with water. *Pandanus fascicularis* is highly valued for the three products that include Kewda oil, Kewda attar and Kewda water. The Kewda oil has great demand in perfumery industry. The Kewda attar and Kewda water are mostly used for flavouring purposes in sweets, soft drinks and dishes (Panda *et al.,* 2012). Ganjam district of Odisha supplies about 50% of the world‘s Kewda essence and 85–90% of India‘s Kewda essence with estimated turnover of Rs. 50 crores (Padhy *et al.,* 2016). The cost of Kewda essential oil is approximately 2.5 to 4 lakhs per litre, Kewda attar is 0.2 lakh per litre and Kewda water is Rs 300 per litre (Padhy *et al.,* 2016). Essential oil of the kewda has various applications as food additive, aromatherapy, ayurvedic medicinal, hair oils, agarbattis (incense sticks), lotions, cosmetics, soaps and perfume (Panda *et al.,* 2012). As far as the essential oil quantity and quality is concerned the uniqueness of this region is significant because of the better yield and higher essence value of the flowers of the Kewda plants growing in this belt. Generally, the female plant does not bear floral bouquet; instead left to develop into fruit (Rout *et al.,* 2005). The small-scale perfumeries are thriving in the region due to abundance of kewda plantation and raw material for the perfumery i.e., kewda flowers along the coast of Ganjam, Odisha (Panda *et al.,* 2009). The demand for Kewda perfume has been rapidly increasing in the national and international market, especially in the Arab countries (Sahu and Misra 2007).

Besides the extensive use of Kewda male flowers for perfume production, other parts of the plant are also used in fibre, food, pharmaceuticals and handcraft industries. The leaves of Kewda are tough and have spines and thus employed as fences for crops to protect from cattle. They are also used for making mats, table lamps, ropes, purses, baskets, wall hangings, files etc (Akpabio and Akpakpan 2012; Abral *et al.,* 2012; Teli and Jadhav 2017). The leaf extracts are also used for food colouring purposes. In Sri Lanka, the leaves are used for cooking (Takeda *et al.,* 2008). The pulp and polyester composites of the leaves are used in the paper and fibre industry. The fibre obtained from *P. fascicularis* leaves also have a great potential for being used as textile and as

composite material. The prop roots are used as supports and fabrication of houses. The trunks of aged plants are used in thatched house construction. It is also used for preparing glue and making string. The branches are used to make compost and as wood fuel. In India and Sri Lanka, the flowers are used for decoration and are offered to God. Fruits are used as firewood and foodstuff (Zanan and Nadaf 2012; Baba *et al.,* 2016). Traditionally, the plants were used as fencing of the agricultural fields to protect the paddy fields from cattles (Panda *et al.,* 2009). The plants are also planted along the coastline as a wind breaker and soil binder (Panda *et al.,* 2010).

Hence, the cultivation practice along with the marketing of Kewda products has become an alternative source of income for the low-income coastal villagers resulting in their socio-economic growth. Thus, the plant has become an important bio-resource with a positive impact on local economy of Ganjam district, Odisha, India (Panda *et al.*, 2007a; Panda *et al*. 2009a; Panda *et al.,* 2010; Jose *et al.,* 2016).

1.4. Kewda in Indian system of medicine

Kewda (*Pandanus fascicularis* Lam.) is an important essential oil bearing plant. The plant also has a wide range of medicinal properties and is used as one of the ingredients in several Ayurvedic formulations (Andriani *et al.,* 2015). In Ayurveda, Kewda has been used for curing headache, rheumatism, anorexia, eye diseases, constipation, indigestion, leprosy etc (Madhavan *et al.,* 2008; Udupa *et al.,* 2011). The fruits and male flowers are used for treating skin and heart related infections (Adkar and Bhaskar 2014). Leaf extracts containing tablets are given for pain and inflammation in traditional medicine preparations (Panda *et al.,* 2009b). The leaves are used for treating leprosy, small-pox, syphilis, leucoderma and scabies (Padhy *et al.,* 2016). The extract of plant is known for its diuretic and anti-spasmodic properties (Rajeswari *et al.,* 2012; Adkar and Bhaskar 2014).

Studies have also demonstrated the role of Kewda oil in curing skin diseases, earache, headache, rheumatoid arthritis, smallpox, syphilis, sterility, cardiac troubles, colic infection laxative, spasms and leprosy (Adkar and Bhaskar 2014). Various pharmacological activities have been reported in this plant such as anti-viral, anti-allergy, anti-platelet, anti- tumour activity (Londonkar *et al.,* 2010; Adkar and Bhaskar 2014), anti-inflammatory and analgesic activities (Londonkar *et al.,* 2010; Udupa *et al*. 2011), antioxidant activity (Londonkar and Kamble 2009; Kaewklom and Vejaratpimol 2011; Kumar *et al.,* 2011), anticancer (Raj *et al.,* 2014), cardioprotective activity

(Sobhana *et al*. 2014; Kamala *et al*. 2016), CNS depressant activity (Raju *et al.,* 2011), adaptogenic (anti-stress) activity (Adkar *et al*. 2014), hepatoprotective activity (El-Shaibany *et al.,* 2016), protective effect on UV-B induced DNA damage (Kaewklom and Vejaratpimol 2011), wound healing activity (Panda *et al.,* 2009), antidiabetic activity (Kumari *et al.,* 2012; Rajeswari *et al.,* 2012), neuropharmacological activities (Kuber and Santhrani 2010), cytotoxic activity (Jitu *et al.,* 2017).

1.5. Characteristic features of essential oils

Essential oils are complex compounds, produced during the secondary metabolism of plants. In aromatic plants species, biosynthesis of essential oils occurs through two complex natural biochemical pathways involving different enzymatic reactions. Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the universal precursors of essential oil biosynthesis and are produced by the cytosolic enzymatic MVA (Mevalonic acid) pathway or by plastidic and enzymatic 1-deoxy-D-xylolose-5-phosphate (DXP) pathway, also called the 2-C-methylerythritol-4-phosphate (MEP) pathway (Betts, 2001). The majority of essential oils are composed of terpenes and terpenoids and other aromatic and aliphatic constituents, all characterized by low molecular weight. The terpene compounds are hydrocarbons of general formula $(C_5H_8)n$ formed from isoprene units and these compounds could be acyclic, monocyclic, bicyclic or tricyclic (Abed, 2007). On the basis of diversity in their chemical structure, they could be classified into several groups as monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}). The majority of the components of essential oils are monoterpenes represent approximately 90% of the essential oils. The chemical profile of essential oils varies in the number of molecules, stereochemical properties of molecules, and also depends on the type of extraction. Although the essential oil of Kewda consists of several phytochemicals its major constituent which is Phenyl ethyl methyl ether (PEME) ranges between 75-85 percent (Sahu and Misra, 2007; Misra *et al.,* 2000; Mahalingam *et al.,* 2012; Raina *et al.,* 2000)

1.6. Antimicrobial strength of essential oils

Essential oils have reported to have antimicrobial properties against the bacterial, fungal and viral pathogens (Duschatzky *et al.,* 2005). Presence of large number of alkaloids, phenols, terpenes and their derivatives make the essential oils more précised in their mode action against the ample variety of pathogenic microorganisms. Thus, the essential oils could be used as better supplements or alternatives against the pathogenic

microorganisms (Sakandamis *et al.,* 2002). The antimicrobial impact of essential oils and its various components extracted from medicinal plants has been well documented (Hammer *et al.,* 2002; Hood *et al.,* 2003; Duschatzky *et al.,* 2005). Thus, essential oils are key components in many applications, especially in food preservation, aromatherapy and medicine (Cantrell *et al.,* 1998).

Reports are in queue to execute the antibacterial efficiency of various essential oils. The essential oils of Cinnamon, Clove, Pimento, Thyme, Oregano, Peppermint, Lemongrass, Palmarosa, Acmella, Geranium, Tagetes, Jambolana had drawn attention of plethora of researchers who were actively involved to study antibacterial potency against the common bacterial infectious agents. The literature survey based upon the evaluation of antibacterial efficacy of essential oils and or its active components belonging to plants of Pandanaceae family declare about fact that the herbal members have active antibacterial metabolites (Elgayyar *et al.,* 2001).

1.7 Importance of present work in the context of current status

Kewda is an important Ayuvedic plant and has a unique aroma in the male flowers because of which it is having great commercial value. Kewda essential oil contributes largely for the economic value of the plant as it is used worldwide for perfume production. Kewda flower oil along with the roots and leaves extract have also been reported to possess various therapeutic properties (Sahu and Misra 2007; Padhy *et al.,* 2016). Even though the plant has great economic value, a comprehensive chemical profiling of the essential oil has not been done. Though reports are available to show that quality of Kewda essential oil from Ganjam district of Odisha is better than those available in other parts of the country, yet there is uncertainty about the quality of the flower oil. This necessitates the phytochemical analysis of Kewda essential oil. Further, it is necessary to carry out the detailed investigation on antibacterial activity against MDR bacteria. Therefore, the present work has been done to achieve the following objectives.

Objectives

1. Isolation, Identification, Biochemical and Molecular characterization of MDR (Multiple Drug Resistant) bacterial strains from the clinical samples.

2. Extraction of essential oils from *Pandanus odorifer* (Kewda) flower and photochemical characterization based on GC-MS, FTIR, NMR and HRMS as well as quantitative estimation of targeted secondary metabolite, PEME (Phenyl Ethyl Methyl Ether) and Terpinen-4-ol.

3. Evalution of antibacterial activity of Kewda essential oil and its active compounds, PEME and Terpinen-4-ol based on disc diffusion method and determine the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109.

4. Evaluation of antibioflim activity of Kewda essential oil and its active compounds, PEME and Terpinen-4-ol at sub-MIC concentration against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109.

5. Identification of targeted gene or protein of MDR bacteria for the mode of action of PEME based on comparative transcriptomics.

6. To determine the mode of action of PEME and Terpinen-4-ol against targeted protein based on computational techniques.

7. Toxicity evaluation of different doses of essential oil of Kewda using *in vivo* animal model based on histopathology and hematology study.

CHAPTER II

REVIEW OF LITERATURE

2.1. Antimicrobial activity of essential oils

The essential oils are the active principles produced from aromatic and medicinal plants, synthesized via shikimic-(mevalonate) acid pathway. The essential oils are secreted in specific tissue/gland or cells and the prime function is to protect the plants from gazing or hazardous elements. Researchers have taken keen interest to study the antimicrobial efficacy of various essential oils either from indigenously or cultivated varieties. Many essential oils were screened for their antibacterial activity against Gram-positive and Gram-negative bacteria along with antifungal properties. These essential oils are well studied for their antibacterial properties and beyond doubt they have shown some very promising results on salmonella, staphylococci and other oral pathogens. They can be very good alternatives for antibiotics if properly and thoroughly studied for these effects (Sienkiewicz *et al.,* (2015); Lee *et al.,* 2014).

Among the microbes, bacterial strains with virulence factors have immense importance in clinical consequences. The infective agents belonging to genera *Escherichia, Staphylococcus, Bacillus, Streptococcus, Enterococcus, Vibrio, Pseudomonas* and many more strains have drawn attention of researchers to be the test organisms in scientific studies. Chang *et al.,* 2001, had investigated antibacterial activity of oils from *Cinnamomum osmophloeum* against *Escherichia coli, Enterococcus faecalis, Staphylococcus aureas* (including the clinically methicillin resistant *S. aureus*), *Salmonella* sp. and *Vibrio parahemolyticus*. They reported that Cinnamom aldehyde was the main antibacterial component of the mixture. Like wise, it was reported that essential oil of oregano (*Origanum vulgare*), thyme (Thymus vulgaris), bay (*Pimenta racemosa*) and clove (*Eugenia caryophyllata* synonym: *Syzygium aromaticum*) are among the most active against strains of *E. coli* (Smith-Palmer *et al.,* 1998; Hammer *et al.,* 1999; Dorman and Deans, 2000) and peppermint oil (Imai *et al.,* 2001). Simon and Murray, (1990) had taken keen interest and also succeeded to test the antimicrobial activities of Basil essential oil.

2.2. Mechanism of action of essential oils against sensitive bacterial cells

Essential oils are lipophiles, thus they can easily enter cells, disrupt the membrane and/or permeable to membrane. The most important signs of membrane permeabilization are the loss of ions and the reduction of potential, the collapse of proton pump and the depletion of ATP pool (*Bakkali et al.,* 2008). Essential oils can cause the coagulation of cytoplasm and some damages to lipids and proteins (Burt, 2004). Intrinsic

and extrinsic conditions can be responsible of susceptibility and resistance of pathogens (Bajpai *et al.,* 2012). Some authors had categorized the test bacterial strains into essential oil sensitive and essential oil resistant. But many workers had preferred to work on sensitive cells (Pattnaik *et al.,* 1996, 1997; Dorman and Dean, 2000). Speranza and Corbo, (2010) suggested some speculations on essential oil resistant bacterial strains: (a) Gram-negative bacteria appear more resistant. This higher resistance could be attributed to the outer membrane. (b) Lactic acid bacteria (LAB) are the most resistant Gram-positive bacteria. This resistance was attributed to ATP generation by substrate level phosphorylation. (c) Among the Gram-negative bacteria, Pseudomonas show high resistance to these antimicrobials and (d) Essential oils were generally more active toward yeasts. Park S N *et al.,* (2012) had claimed that there were promising antibacterial potentialities of essential oils from *Achillea ligustica, Baccharis dracunculifolia, Croton cajucara, Cryptomeria japonica, Coriandrum sativum, Eugenia caryophyllata, Lippia sidoides, Ocimum americanum, and Rosmarinus officinalis* against cariogenic bacteria.

2.3. Phytoconstituent analysis and antimicrobial studies of essential oils

The pharmacognostical study of essential oils is an important facet in essential oil research. The essential oil is comprised of chiefly mono terpenes and sesqui terpenes. There is need for preliminary screening of essential oils by using standard pharmacognostical tests. As this study was initiated with extraction of essential oil and its phyto chemical analysis, therefore a short review based upon phytochemical analytical works was made. Previous studies reported that the chemical composition of essential oils isolated from the leaves of *Cosmos bipinnatus* was analyzed by GC-MS. The essential oil extracted from this plant was predominantly composed of monoterpenes (69.62%) and sesquiterpenes (22.73%). The antibacterial assay showed that the oil had significant inhibitory effects against both Gram-negative and Gram-positive bacteria isolates. The MIC of Gram-positive strains ranged between 0.16 and 0.31 mg/mL while those of Gram-negative bacteria ranged between 0.31 and 0.63 mg/mL. The Gram-positive bacteria were more susceptible to the essential oil than the Gram-negative bacteria. Most of the major components of this oil in other plants have been reported for antimicrobial activities (Olajuyigbe O and Ashafa, 2014). Malwal *et al.,* (2010) designed a study to extract and examine chemical composition, antimicrobial and antioxidant activity of the hydro-distillated essential oil of *Murraya koenigii* leaves from the South region of Tamilnadu, India. Major compounds detected in the oil were Linalool (32.83%), Elemol (7.44%), Geranyl acetate (6.18%), Myrcene (6.12%), Allo-Ocimene

(5.02), α-Terpinene (4.9%), and (E)-β-Ocimene (3.68%) and Neryl acetate (3.45%). From the identified compounds, they were classified into four groups that are oxygenated monoterpenes (72.15%), monoterpene hydrocarbons (11.81%), oxygenated sesquiterpenes (10.48%) and sesquiterpenes hydrocarbons (03.12%). The antibacterial activity of essential oil was pronounced by Disc Diffusion Method against various pathogenic microbes. The oil had a maximum zone of inhibition ability against *Corynebacterium tuberculosis, Pseudomonas aeruginosa, Streptococcus pyogenes, Klebsiella pneumonia* and *Enterobacter aerogenes.* The antioxidant profile of the sample was determined by different test systems. In all the systems, essential oil showed a strongest activity profile within the concentration range. Magina *et al.,* (2009) had taken leaf essential oil Eugenia brasilensis, *Eugenia beaurepaireana*, and *Eugenia umbelliflora* and anlalyzed by GC–MS. The major compounds found in the oil of *E. brasiliensis* were spathulenol (12.6%) and τ-cadinol (8.7%), of *E. beaurepaireana* were β-caryophyllene (8.0%) and bicyclogermacrene (7.2%), and of *E. umbelliflora* were viridiflorol (17.7%) and β-pinene (13.2%). These oils were assayed to determine their antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*. All of the oils analyzed showed antibacterial activity, ranging from moderate to strong, which was most accentuated for the *E. umbelliflora* and *E. brasiliensis* oils, which strongly inhibited the growth of *S. aureus* giving values of MIC = 119.2 and 156.2 μg/mL, respectively.

2.4. Phytochemical profiling of essential oil by GC-MS

The essential oil is comprised of mostly mono terpenes and sesqui terpenes. Specifically the essential oils of *Pandanus odorifer* consist of phytol (42.15%), squalene (16.81%), pentadecanal (6.17%), pentadecanoic acid (4.49%), 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol (3.83%), phytone (2.05%), etc. (Chen *et al.,* 2014). The GC-MS (gas chromatography and mass spectrometry) analysis of Kewda essential oil from the staminate infloresence had shown to consist of ether (37.7%), terpene-4-ol (18.6%), α-terpineol (8.3%), 2-phenylethyl alcohol (7.5%), benzyl benzoate (11%), viridine (8.8%), and germacrene-B (8.3%) along with a small amount of benzyl salicylate, benzyl acetate, benzyl alcohol (Raina *et al.,* 2004). Kusuma *et al.,* (2012) studied the major phytochemical constituents present in *Pandanus odoratissimus*, L. which consists of alkaloids (pandamarilactone-A, B), terpenoids, steroids, phenolic compounds, and alanine, and betasitisterol, citral ester of phthalic acid, fatty acids, aminoacids & proteins. Similarly, Busque *et al.,* (2002) extracted a number of active compounds from different

parts of *Pandanus odoratissimus*, by GC-MS analysis especially pandamarine pandamarilactone-1, pandamarilactone-31 and pandamarilactone-32, which were the crystalline piperidine type of alkaloids. However, Kumar *et al.,* (2010) had analysed the aqueous extracts of the leaves of *P. odoratissimus* and detected presence of alkaloids, carbohydrates, proteins, steroids, sterols, phenols, tannins, terpenes, flavonoids, gums and mucilage, saponins, and glycosides. The total phenolic content in the aqueous extract was ranged from 3.5 to 10.8% w/w. In addition, physcion, cirsilineol, n-triacontanol, β-sitosterol, camphosterol, daucosterol, palmitic acid and steric acid in rhizomes of *P. odoratissimus* were reported by Venkatesh *et al.,* (2012). Some workers (Jong and Chau, 1998) had made column chromatography study of methanol extract of *P. odoratissimus* and isolated a total of 15 compounds. Steroids, including phytosteroid mixtures; a-spin sterol and stigmast-7-en-3b-ol mixture; a-spinasterol caproate; stigmast-4-en-6b-ol-3-one and three phenolic compounds; vanillin; 2 (E)-3-(3′-methoxy-4-hydroxyphenyl)-prop-2-enal; 4-hydroxy-3-(2′,3′-dihydroxy-3′-methyl-butyl)-benzoic acid methyl ester and a new benzofuran derivative, 3-hydroxy-2-isopropenyl-dihydrobenzofuran-5-carboxylic acid methyl ester; plus six lignans; eudesmin; kobusin; pinoresinol; epipinoresinol; de-4′-O-methyleudesmin; and 3,4-bis(4-hydroxy-3-methoxy-benzyl)-tetrahydrofuran. The compounds were identified by mass, UV, IR, and 1H and 13C NMR spectra. In addition to this report, other group (Adkar *et al.,* 2014) found a number of active compounds extracted from different parts of *P. odoratissimus*, especially pandamarine pandamarilactone-1, pandamarilactone-31 and pandamarilactone-32. Mahalingam *et al.,* (2012) made GC-MS analysis of the methanol extract the whole plant material of *Pandanus odoratissimum* collected from Tamilnadu, India and reported about presence of -3-(4-(dimethylamino) cinnamoyyl)-4-hydroxycomarin. (69.82),3,3’-methylenebis(4-hydroxycomarin), erythro-9, 10-dihydroxyoctadecanoic acid, octadecanedioic acid and dihydroagathic acid.

GC-MS analysis of *P. odoratissimum* whole plant methanol extract revealed the presence of the either compound -3-(4-(dimethylamino) cinnamoyyl)-4-hydroxycomarin, 3,3’-methylenebis (4-hydroxycomarin), erythro-9, 10-dihydroxyoctadecanoic acid, octadecanedioic acid and dihydroagathic acid (Mahalingam *et al.,* 2012). MacLeod and Pieris (1982) analysed the essential oil of *Pandanus latifolius* leaves and reported the presence of mainly sesquiterpene hydrocarbons and the only monoterpene that was reported was linalool. The chemical profile of the red fruit oil from *Pandanus conoideus* was reported by Rohman *et al.,* (2012). The main fatty acid composition of *P. conoideus*

oil is oleic acid (68.80%) followed by linoleic acid (8.49%). The main volatile compounds of *P. conoideus* oil as determined using GC-MS and headspace analyser are 1,3-dimethylbenzene (27.46%), N-glycylL-alanine (17.36%), trichloromethane (15.22%), and ethane (11.43%). *Pandanus amaryllifolius* leaf oil chemical composition was reported by Jiang (1999). About twenty-two compounds including 9 alcohols, 4 carboxylic acids, 3 ketones, 2 esters, 3 hydrocarbons and 1 furanone were identified and the major components were 3-methyl-2-(5H)-furanone, 3- hexanol, 4-methylpentanol, 3-exanone and 2-hexanone. Isopentenyl and dimethylallyl acetates and cinnamates were reported as the dominant phytoconstituents in the essential oil of ripe fruit of *Pandanus tectorius* (Vahirua-Lechat *et al.,* 1996). The leaves of *Pandanus odorus* was extracted by supercritical carbon dioxide as solvent in the freeze-dried extraction process and the extracts were analysed with GC-MS. Analysis of the extract with GC-MS showed the presence of *α*-tocopherol, *β*-sitosterol, hexadecanoic acid, campesterol, squalene, stigmasterol and 9,12,15-octadecatrien-1-ol as major components (Ab Rahman *et al.,* 1999). Raina *et al.,* (2004) compared the chemical composition of *Pandanus odoratissimus* L. flower oil obtained by hydrodistillation with the local market Kewda oil. The major compounds of the hydrodistilled Kewda flower oil were identified as 2-phenyl ethyl methyl ether (37.7%), terpinen-4-ol (18.6%), *α*-terpineol (8.3%) and 2-phenyl ethyl alcohol (7.5%), and the major components of market Kewda oil were 2-phenyl ethyl alcohol (33.2%), 2-phenyl ethyl methyl ether (16.1%), benzyl benzoate (11.0%), viridine (8.8%) and germacrene B (8.3%). Rout *et al*. (2015) reported the chemical composition of the extract obtained by liquid CO_2 extraction of the *P. fascicularis* flowers. It was enriched with phenylethyl methyl ether (65–77%), terpinen-4-ol (13–18%) and *α*-terpineol (1–3%), which were definitely soluble in distillation condensate. The main chemical components of the essential oil were phytol (42.15%), squalene (16.81%), pentadecanal (6.17%), pentadecanoic acid (4.49%), 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol (3.83%), phytone (2.05%) and the other 74 chemical compositions by Chen *et al.*, (2014).

2.5. Assessment of antimicrobial efficacy of the Kewda essential oil

Kewda (*Pandanus odorifer* Lam.) is an important essential oil bearing plant. The plant also has a wide range of medicinal properties and is used as one of the ingredients in several Ayurvedic formulations (Andriani *et al.,* 2019). In Ayurveda, Kewda has been used for curing headache, rheumatism, anorexia, eye diseases, constipation, indigestion, leprosy etc (Madhavan *et al.,* 2008; Udupa *et al.,* 2011). Studies have also demonstrated

the role of Kewda oil in curing skin diseases, earache, headache, rheumatoid arthritis, smallpox, syphilis, sterility, cardiac troubles, colic infection laxative, spasms and leprosy (Adkar and Bhaskar 2014). Various pharmacological activities have been reported in this plant such as anti-viral, anti-allergy, anti-platelet, anti-tumour activity (Londonkar *et al.*, 2010; Adkar and Bhaskar 2014).

Antifungal activity of *Pandanus odoratissimus* oil extracted from the flower was reported against seven species of dermatophytic fungi, and it was compared against the two commonly used antifungal agent's fluconazole and griseofulvin. The fungal strains were *Microsporum gypseum*, *Epidermophyton floccosum*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Trichophyton rubrum*, *Trichophyton verrucosum* and *Trichophyton violaceum.* Significantly, the antifungal activity of *P. odoratissimus* was found to be similar to the control of antifungal drugs griseofulvin and fluconazole against most of the dermatophytic test fungi. Screening by using agar-well diffusion method revealed that *P. odoratissimus* showed excellent antidermatophytic activity against E. *floccosum* and *T. Violaceum* (Babu *et al.*, 2018).

Kaiser *et al.,* (2011) had reported about the antibacterial activity showed potent growth inhibitory activity against *Candida albicans* and *Saccharomyces cerevisiae*. They had suggested that the observed antimicrobial activity of *P. odorus* might be due to the presence of volatile terpenes alcohols, borneol and monoterpene hydrocarbons. In this context, Kim and Marshall (1995) claimed that p-cymene and pinene constituents of Kewda essential oil had synergic antimicrobial effects. Kumar *et al.,* (2010) had analysed the antimicrobial effects of petroleum ether, chloroform, and hydroalcoholic extracts of *P. odoratissimus* leaves against *Bacillus subtilis*, *Escherichia coli*, *Staphlococcus aureus*, and *Candida albicans*. In terms of antimicrobial effects, all the three extracts exhibited effective inhibition zones against gram-positive bacteria, that is, *S. aureus, B. subtilis*. However, they were ineffective against gram-negative bacteria (*E. coli* and *P. aeruginosa*) and fungi (*C. albicans*). Out of three extracts, hydroalcoholic extract showed good antimicrobial activity. Kumar *et al.,* (2010) and found the presence of alkaloids and flavonoids in hydroalcoholic extract of Pandanus sp. But Jong *et al.,* (2013) suggested that the peduncle extracts, the hydroalcoholic, chloroform and petroleum ether extracts of the leaves had exhibited effective inhibition zones against gram-positive bacterial strains and being ineffective against gram-negative bacteria and yeast. These authors reported that there was indication of good antimicrobial activity of *Pandanus Odoratissimus L.* (whole plant)

against both gram-positive and gram-negative bacteria, such as *Escherichia coli, Staphylococcus aureus, Proteus ulgaris, Pseudomonas aeruginosa, Klebsiella pneumoniae.*

The antimicrobial effects of petroleum ether, chloroform, and hydroalcoholic extracts of *P. odoratissimus* leaf was carried out against *Bacillus subtilis, Escherichia coli, Staphlococcus aureus*, and *Candida albicans*. In terms of antimicrobial effects, all the three extracts exhibited effective inhibition zones against gram-positive bacteria, that is, *S. aureus, B. subtilis*. However, they were ineffective against gram-negative bacteria (*E. coli* and *P. aeruginosa*) and fungi (*C. albicans*). Out of three extracts, hydroalcoholic extract showed good antimicrobial activity. The phytochemical study showed the presence of alkaloids and flavonoids in hydroalcoholic extracts (Kumar *et al.,* 2010)

The disc diffusion method was used by (Kaiser *et al.*, 2011; Kumar *et al.*, 2010) to test antimicrobial activity of Leaf extract and Root extract of *Pandanus odorus* against gram-positive, gram-negative bacteria, fungi and yeast. It was reported that most of the fractions of *Pandanus odorus* showed moderate antibacterial activity against the tested microorganisms. They had claimed that the observed antimicrobial activity of *P.odorus* may be due to the presence of volatile terpenes alcohols, such as borneol and monoterpene hydrocarbons, such as p-cymene and pinene symergistic effect.

From this concise note regarding the phytochemical analysis and the antimicrobial efficacy study of *Pandanus odoratissimus* essential oil in neat form or its active constituents, it was observed that there were several lacunae behind the studies. Researchers had added only the cataloguing values. Most of the reported studies were observed to be incomplete. The vertical experiments related to phytochemical analysis of KEO (a) extraction of Kewda essential oil, (b) phytochemical analysis GC-MS, FTIR or NMR analysis to identify the components were wanting. Moreover, it was found that authors had not taken interest for contributing a paper to determine the bactericidal or bacriostatic effect of the said oil against the test organisms. The bacterial strains were categorized into either resistant or sensitive towards the Kewda oil. But a depth study like Scanning Electron microscopy, Transmission electron Microscopy of Atomic Force microscopic studies are totally lacking in the literature. Very few workers had taken the Kewda oil in hydro distilled form and most of the researchers had taken the solvent extracts of the plant. In the last part, it may be mentioned here that the streamlined vertical scientific study for evaluating antibacterial activity of indigenous Kewda essential oil against bacterial strains is missing.

CHAPTER III

MATERIALS AND METHODS

3.1. Collection of plant materials of Kewda

Kewda (*Pandanus odorifer*) plants are luxuriantly grown in the Ganjam district of Odisha, specifically in the area of Rushikulya River Bank (RRB), Kalipalli, Keluapalli, Markandi, Indrakhi, Mantridi, Kaliabali, Basanaputty, Chamakhandi, Podapadar, Chilika and Tampara (Figure 3.1). The male flowers of Kewda plant were harvested early in the morning (7-9 a.m) in the month of July and August which is the blooming season for Kewda. The sampling was done in the morning because the fragrance of the flowers was lost quickly after opening of calyx. Freshly plucked flowers collected from aforesaid locations were chopped to smaller pieces of about 1-2 inches length and added (about 800 grams) to the round bottom flask of 5 litres capacity with water in 1:3 ratios and were subjected to hydro distillation for a period of 4 h using a Clevenger type apparatus. After completion of hydro-distillation the extracted essential oil of Kewda which consists of aroma was dried over anhydrous sodium sulphate (Sigma Corporation, USA) to remove moisture traces and transfered to glass vials and kept at 4 ^{o}C until further analysis. PEME and Terpinen-4-ol compounds are purchased from Sigma Aldrich

The average oil yield of the flower essential oil was calculated by using formula:

$$Oil\ yield\ (\%\ v.w\ of\ fesh\ weight) = \frac{volume\ of\ essential\ oils\ (ml)}{weight\ of\ raw\ flowers\ taken}\ X\ 100\%$$

3.2. GC-MS analysis of Kewda essential oil

Kewda essential oil was analysed by GC–MS coupled with SQ8 Mass Detector; ionization voltage 70 eV. The GC analysis was carried out on Perkin Elmer Clarus 580 (Perkin Elmer, USA) gas chromatograph fitted with a Flame ionization detector (FID) fitted with 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). Injector temperature was set at 250 °C with 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.

Figure 3.1. Collection site of Kewda flowers and Kewda leaves

3.3. High Resolution Mass Spectrometer (HRMS) analysis of Kewda essential oil

The qualitative and quantitative analysis of the phytochemical composition of the extracted essential oil was done in Exactive^{TM} Plus Orbitrap high-resolution mass spectrometer connected in tandem with Ultimate 3000 high-performance liquid chromatography (Thermo Scientific, USA). LC-MS grade methanol, water, formic acid, acetonitrile, and acetone were procured from fisher scientific (USA). In a 2.0 ml centrifuge tube, 0.5 ml of the extracted essential oil and 1.5 ml of 1:1 methanol:water were taken. The solution was thoroughly mixed and centrifuged for 10 minutes at 4 ^{o}C at a speed of 10000 rpm to remove any solid particles. The solution was then filtered in a 0.22 µM syringe filter (to get rid of any additional particles). Around 0.5 mL of the filtrate was aspirated and put in the DP ID vial (Cat#C4000-1W, Thermo Scientific, USA) for mass spectrometry. A Hypersil BDS C18 (250 mm × 2.1 mm, 5 µm; Thermo

Scientific, USA) column was used to separate the compounds in the solution. The mobile phage consists of a 1:1 solution of methanol: water with 0.1% formic acid. The flow rate of the sample was set at 3 µL/Min, column temperature maintained at 30 ^{o}C, and pressure maintained at 700 bar. The molecules were ionized through an electrospray ionization mechanism at the potential of 3eV and were sourced through nitrogen gas into the orbitrap chamber. The sample was run for 15 minutes and the ions were detected in both positive and negative polarity with the scan range of 50-750 m/z. The mass peak intensities were compared with the standard curve which was made by normalizing the peak intensities of the 5 standard compounds in the mass range of 50-500 m/z to convert the peak intensities into concentration. The mass peaks were then annotated through a python in-house program by parsing it with the PubChem library with the error range of ±0.01 m/z.

3.4. FTIR analysis of Kewda essential oil

The Kewda essential oil was analysed by Fourier transform infrared spectroscopy (FTIR) by 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.

3.5. NMR analysis of Kewda essential oil

The Kewda essential oil was analyzed by nuclear magnetic resonance (NMR) spectroscopy available in the sophisticated analytical instrumentation facility, Cochin, using Bruker-400MHz NMR. The NMR instrument was equipped with the system of inverse broadband probe fitted with a Z-axis gradient and with automatic tuning and matching. The inner coil is optimized for 1H and the outer coil can be tuned from 31P to 97Mo (and others in between). So X-frequencies are ranging from 162 to 27 MHz. The whole system is controlled by Bruker's Topspin software. The solvent used was PBCL (Chloroform with D_2O).

3.6. Isolation, Biochemical Identification, Antibiotic Susceptibility of Bacterial Isolates

3.6.1. Isolation of bacteria from clinical samples

Clinical samples (urine, stool, pus, blood, swabs and body fluids) were collected from both hospitalized patients and patients attending the OPD (IMS and Sum Hospital, Bhubaneswar, Odisha) aseptically (Figure 1). Routine microscopical examination of the samples was performed to check the presence of bacteria and pus cells. The clinical

samples were processed and cultured on suitable medium to check for the growth of bacteria followed by Gram-staining (Figure 2 A, B). This study was approved by the institutional ethical committee of IMS and Sum Hospital (IEC Code No. IMS SH/IEC/2018/37).

Figure 3.2. A. Sterilized Swab stick; B. Urine sample of patient

3.6.2. Identification of bacteria

All the collected clinical samples except urine sample were inoculated onto freshly prepared sterile MacConkey and blood agar plates. Meanwhile, the urine samples were inoculated on sterile cystine lysine electrolyte deficient (CLED) agar plates. The inoculated plates were incubated aerobically at 37 °C for 24 h. On the other hand, collected blood samples were incubated at 37 °C overnight to check the growth of microorganisms. After incubation, those blood samples were inoculated on blood, chocolate and MacConkey agar plates and plates were kept for incubation at 37 °C for 24 h. These blood samples were inoculated at 2^{nd}, 4^{th}, and 7^{th} day of sample collection. Bacteria were identified based upon the colony characters, VITEK2 (Biomereux) and standard biochemical procedures followed by Clinical and Laboratory Standards Institute (CLSI) guidelines. Standard microbial type culture collection (MTCC) strains of bacteria were used as reference controls. 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. Two reference bacterial strains i.e. *Staphylococcus aureus* (MTCC 740) and *Klebsiella pneumoniae* subsp. *pneumoniae* (MTCC 109) were also procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.6.3. Biochemical characterization of isolated bacterial strains

For pure-culture samples of Gram-positive bacteria, confirmative catalase and coagulase tests were performed.

I. **Catalase test:** On a clean grease free slide, a drop of 3% H_2O_2 was mixed with a loopful of test bacterial culture. Occurrence of effervescence indicate the presence of catalase enzyme (Figure 2C).

II. **Coagulase test:** A lump of test organism was emulsified with a drop of normal saline water (0. 89%). A drop of human blood serum was added to the suspension; clumping of cells, if observed within 10s, confirmed the presence of bound coagulase enzyme.

When a sample of Gram-positive cocci responded positively to both catalase and coagulase tests, the strain was confirmed to be identified as *S. aureus* (Figure 2D). But catalase negative colonies were cultured on blood agar to check for their haemolytic patterns, and bacitracin test was conducted. Catalase negative Gram-positive colonies having beta-haemolysis (complete haemolysis of erythrocytes) on blood agar and simultaneously sensitive to the bacitracin are identified as Group A *Streptococci* or *S. pyogenes*. Meanwhile, Catalase negative, alpha-haemolytic (partial or green haemolysis of erythrocytes) colonies were subjected to bile-esculin test (Figure 2E). The bile-esculin medium contains esculin and peptone for nutrition and bile to inhibit growth of Gram-positive bacteria, other than Group D *Streptococci* or *Enterococci*. Ferric citrate was used as an indicator. Organisms, which split esculin molecules and use the liberated glucose to supply energy, release esculin into the medium. The free esculin reacts with ferric citrate in the medium to form a phenolic iron complex, which turns the agar-slant from dark brown to black. An agar-slant that was more than half darkened within 48 h of incubation was bile-esculin positive, for the confirmation of *E. faecalis*; but the alternative non-darkening of the agar was taken as the negative result (Forbes *et al.*, 2007).

For pure-cultures of Gram negative bacilli, the following tests were performed in succession along with the catalase test.

III. Oxidase test: A bacterial colony was rubbed onto a filter paper, impregnated with tetramethyl-p-phenylenediamine dihydrochloride and the dye indophenols; the zone of the filter paper turns blue/purple (considered as positive result), while no change of colour (considered as negative result) (Figure 2F).

IV. **Indole test**: To an aliquot of 5mL of 48 h old grown culture (test culture), an aliquot of 0.5mL of Kovac's reagent (p-dimethylaminobenzaldehyde, isoamyl alcohol and HCl) was added. A formation of a cherry-red or purple-red ring at the interface of the broth culture and the reagent indicated the indole production (Figure 2G).

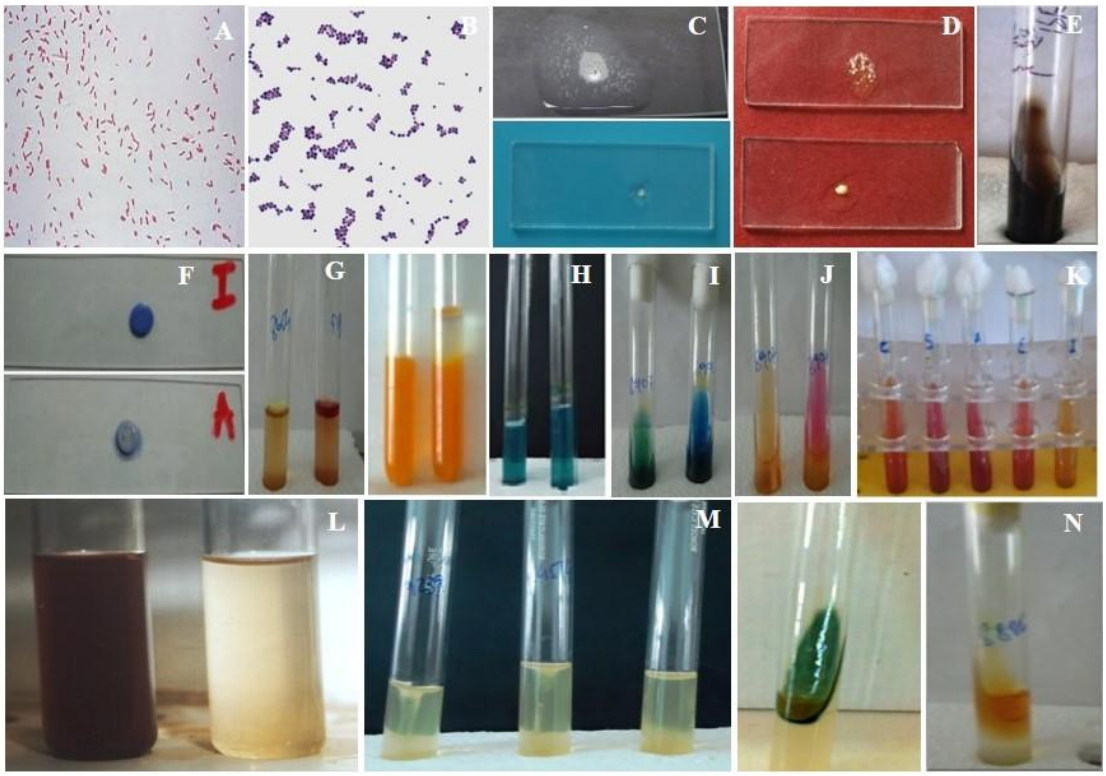


Figure 3.3. A. Gram negative bacilli; **B**. Gram positive cocci; **C**. Catalase +ve and –ve; **D.** Coagulase +ve and –ve; **E**. Bile-esculin +ve and –ve; **F.** Oxidase +ve and –ve; **G.** Indole +ve and –ve; **H.** Oxidation fermentation +ve and –ve; **I.** Citrate +ve and –ve; **J.** Urease Test +ve and –ve; **K.** Triple-Sugar iron test; **L.** Nitrate test +ve and –ve; **M.** Motility +ve and –ve; **N.** Phenylalanine deaminase test+ve and –ve.

V. **Methyl red test** (MR test): To an aliquot of 5 mL of sterile MRVP broth (peptone 7 g, glucose 5 g, potassium phosphate 5 g, pH 6.9), the test culture was inoculated and incubated at 37 °C for 48 h. To this culture, 5 drops of methyl red solution were added as an indicator. If the total solution turned red, the test was taken as positive for the formation of organic acids as products.

VI. **Voges-Proskauer test** (VP test): To an aliquot of 5 mL sterile MRVP broth, a loopful of the test culture was inoculated and the mixture was incubated at 37 °C for 48 h. To this culture tube, 10 drops of VP I reagent (5% α-napthol, in absolute alcohol) and 2-3 drops of VP II reagent (40% KOH solution) were added and the mixture was allowed to stand for 15-20 min for the reaction to complete. The positive result was the

appearance of red colour of the mixture, i.e., production of a neutral product, acetoin from the fermentation of glucose by the organism, and alternately yellow colour production indicated the negative result.

VII. Oxidation fermentation test (O/F Test): Hugh-Leifson glucose broth (HLGB) with glucose, bromocresol purple, as main component (pH 7.4) was prepared. A small amount of agar was used to get semisolid media to facilitate stab. After sterilization, inoculation was done by stabbing of loop. Over one set of tubes paraffin was poured to give anaerobic condition and then incubated for 24 h and the other set incubated. Colour changed from purple to yellow in both tubes: fermentative. Colour changed only in tubes without paraffin: oxidative. Colour change in any tube microorganism is inert to the media (Figure 2H).

VIII. **Citrate test**: The test culture was inoculated onto a slant of Simon citrate agar that was incubated at 37 °C for 48 h. The change of colour of agar from green to blue indicated that organism used citrate as the sole source of carbon (Figure 2I).

IX. Urease test: The test organism was inoculated onto a slant of Christensen's urea agar (peptone, glucose, sodium chloride, mono-potassium phosphate, urea, phenol red, distilled water, and at pH 6.8). The hydrolysis of urea yielding ammonia gas increases the pH that changes the colour of the medium from off-white to pink/orange, the positive result **(Figure 2J**).

X. **Triple-sugar-iron test (TSI test)**: Two or 3 drops of test broth culture were inoculated on TSI-agar slant and subsequently, a stab was made up to the butt of the slant. The tube was incubated at 37 °C for 48 h; the black colour appearance indicated the H_2S production (Figure 2K).

XI. Nitrate test: An aliquot of 5 mL of nitrite broth (peptone 5 g, beef-extract 3 g, KNO_3 1 g and distilled water 1,000 mL) was inoculated with 1 drop of 24 h old broth test culture and was incubated for 48 h at 37 °C. From the development of red colour within 30 sec of adding a few drops of the reagent A (α-napthol 5 g in 1,000 mL of 30% acetic acid) and reagent B (sulphanilic acid 5 g in 1,000 mL acetic acid) the positive result was inferred. No colour change suggested the negative result. MTCC strain of each Gram positive or Gram-negative bacterium was used as the reference control in each biochemical test (Figure 2L) (Forbes *et al.,* 2007).

XII. Carbohydrate fermentation tests: Phenol red broth is a general-purpose differential test medium typically used to differentiate Gram negative enteric bacteria. It contains peptone, phenol red (a pH indicator), a Durham tube, and one carbohydrate (or

sugar). Five different kinds of phenol red broths were prepared each containing one kind of sugar, i.e., glucose, lactose, maltose, mannitol and sucrose. Phenol red is a pH indicator, which turns yellow below a pH 6.8 and fuchsia/pink colour above pH 7.4. The test bacteria were inoculated to the each broth/tube and were incubated at 37 °C for 48 h. If the bacteria was able to utilize the carbohydrate, an acid by-product is formed, which turns the media yellow. If the bacterium was unable to utilize the carbohydrate but does use the peptone, the by-product formed is said to be ammonia, which raises the pH of the medium and turns it fuchsia/pink colour. When the bacterium was able to use the carbohydrate, a gas by-product may be produced; an air bubble will be trapped inside a Durham tube. If the bacteria unable to utilize the carbohydrate, gas will not be produced, and no air bubble will be formed (Forbes *et al.*, 2007).

XIII. Motility test by hanging drop method: Motility test agar is a semi-solid medium used for the detection of bacterial motility, which can be observed directly from examination of the tubes following incubation. Growing colony spreads out from the line of inoculation, if the bacteria are motile. Highly motile bacteria have growth throughout the tube. Growth of non-motile organisms only occurs along the stab line. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (considered as motile) (**Figure 2M**).

XIV. Phenylalanine deaminase test: To determine ability of bacteria to deaminate phenylalanine to phenyl pyruvic acid (PPA). Inoculate a medium containing phenylalanine is with a bacterial culture and incubate at 37 °C for 18 to 24 h. Add a few drops of $FeCl_2$ solution. Green colour is produced in the formation of PPA. Green colour of the media was positive and colour remain unchanged was negative (Figure 2N).

3.7. Identification with VITEK 2

The test panels (ID-GPC) contained 46 fluorimetric tests that included pH change tests and derivatives to detect aminopeptidases and -osidases. Substrates used for detection of aminopeptidases are coupled with 7-amino-methylcoumarin (7AMC); substrates for the detection of -osidases are usually coupled with 4-methylumbelliferone (4MU). The 21 test substrates are as follows: 4MU-α-L-arabinofuranoside, 4MU-α-D-galactoside, 4MU-α-D-glucoside, 4MU-α-D-*N*-acetylneuraminic acid, 4MU-β-D-galactoside, 4MU-β-D-glucoside, 4MU-β-D-glucuronide, 4MU-β-D-mannoside, 4MU-*N*-acetyl-β-D-glucosaminide, 4MU-phosphate, alanine-7AMC, arginine-7AMC, aurease (butiloxicarbonyl-Val-Pro-Arg-AMC), histidine-7AMC, α-glutamic acid-7AMC,

threonine-7AMC, lysine-7AMC, phenylalanine-7AMC, proline-7AMC, pyroglutamic acid-7AMC, and tyrosine-7AMC. Furthermore, the ID-GPC card includes 16 fermentation tests (for D-raffinose, D-amygdaline, arbutine, D-galactose, glycerol, D-glucose, L-arabinose, lactose, D-maltose, D-mannitol, *N*-acetylglucosamine, salicin, D-sorbitol, D-Trehalose, D-melibiose, and D-xylose), two decarboxylase tests (for ornithine and arginine), and six miscellaneous tests (for urease, pyruvate, optochin, novobiocin, polymyxin B sulfate, and 6% NaCl). The GN test card is used in the identification of fermenting and non-fermenting Gram-negative bacilli. The VITEK 2 GN card is based on 47 biochemical tests measuring carbon source utilization, inhibition and resistance, and enzymatic activities.

The card was automatically filled by a vacuum device, sealed and inserted into the VITEK 2 reader-incubator module (incubation temperature, 35.5 °C), and subjected to a kinetic fluorescence measurement every 15 min. The results were interpreted by the ID-GPC and ID –GNB database and final results were obtained automatically.

3.8. Identification of bacteria by 16SrRNA sequencing

The genomic DNA of the bacterial isolates were isolated based upon the protocols prescribed by Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research; Catalogue No. D6005). The concentration of extracted DNA sample was measured by Nanodrop (Biotech instruments, USA). DNA was stored at −80 °C for further use. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 to 2.0 is generally accepted as "pure" for isolated DNA samples. The 16S rRNA gene was amplified by prescribed polymerase chain reaction (PCR) (reaction mixture contains: 10 pmol each of forward and reverse primers, 2.5 mM of $MgCl_2$, 200 µM each of the four deoxyribonucleotide triphosphates (dNTPs), 0.5 U of Taq DNA polymerase, 1x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50 to 100 ng of isolated bacterial genomic DNA) using the specific primers **27F** (5′AGAGTTTGATCCTGGCTCAG3′) and **1492R** (5′TACGGTTACCTTGTTACGACTT3′).

The PCR reaction steps include the denaturation of the template by heating at pre-denaturation of 95 °C for 5 min. This step was followed by 39 cycles of denaturation for 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. The amplicons (the amplified DNA products) were subjected for electrophoresis in 0.8% agarose gel run in 1× TAE buffer at 50V for 30 to

45 minute till DNA fragments are migrated well. The amplicons in the form of bands in the agarose gel was analyzed in Gel documentation system. The PCR products were further purified and sequenced (ABI 3730XL, Thermo Fisher Scientific). The sequences were compared for homology using the NCBI BLAST database (Martinez-Absalon *et al.,* 2014). The 16S rRNA gene sequences were subjected for multiple sequence alignment and subsequent phylogenetic analyses using MEGA 5.0 software (Tamura *et al.,* 2011).

3.9. Antibiotic susceptibility test by Kirby-Bauer's method

All bacterial strains including MTCC standard strains were subjected to antibiotic sensitivity tests by the Kirby-Bauer's/disc-diffusion method, using a 4 mm thick Mueller–Hinton agar (MHA) (HiMedia, Mumbai) medium, in duplicates. An aliquot of 0.01 mL of 0.5 McFarland equivalents, approximately from an exponentially growing overnight culture was spread on agar for the development of lawn of a bacterium at 37 °C in a BOD incubator (Remi CIM-12S). Further, on the lawn-agar of each plate, 8 high potency antibiotic discs (HiMedia) of 16 prescribed antibiotics were placed, separately at equal distances from one another. Plates were incubated for 18 h at 37 °C and were examined for size of zones of inhibition around each disc, following the standard antibiotic susceptibility test chart of Clinical Laboratory Standard Institute (CLSI) guidelines (CLSI, 2011). Experiments were done three times and data of the third set of experiments were presented (Forbes *et al.,* 2007).

For Gram negative bacteria; ampicillin (AMP) (10 µg/disc), gentamicin (GEN) (30 µg/disc), amikacin (AK) (30 µg/disc), levofloxacin (LV) (10 µg/disc), ciprofloxacin (CIP) (5 µg/disc), cefepime (CPM) (30 µg/disc) were used. Similarly, for Gram positive bacteria, ampicillin (AMP) (10 µg/disc), gentamicin (GEN) (10 µg/disc), amikacin (AK) (30 µg/disc), ciprofloxacin (CIP) (5 µg/disc), Amoxyclav (AMC) (30 µg/disc); Collistin (CL) (10 µg/disc), Ofloxacin (OF) (5 µg/disc), Tigecycline (TGC) (10 µg/disc), Nallidixic acid (NA) (10 µg/disc) were used. The plates were then incubated at 37 °C for 24-48 h. Diameters of the zone of inhibition around the disc were measured using a zone measurement scale (caliper) and the isolates were classified as sensitive, intermediate, and resistant according to the standardized table supplied by the CLSI guidelines.

3.10. Antibacterial activities of Kewda essential oil and its constituents, 2-Phenyl ethyl methyl ether (PEME) and Terpinen-4-ol against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109

3.10.1. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of crude Kewda essential oil and its pure compounds (PEME and Terpinen-4-ol) against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109 was determined using microdilution method as prescribed by CLSI guidelines, 2012. An exponential culture of each strain in Muller-Hinton (MH) broth (HiMedia) were suitably diluted with the normal saline solution to obtain the level of equivalent to the 0.5 McFarland standard. Suitable dilutions from original stock solutions of each sample (for kewda oil concentration range used: from 5% to 0.039% in 2-fold serial dilution; for PEME and Terpinen-4-ol, concentrations range used: 50mM to 0.39 mM in 2-fold serial dilution) were prepared in MH broth followed by inoculation of 0.5 McFarland bacterial culture. The experimental setupwas incubated at 37 °C for 16-18 h. Bacterial culture inoculated into MH broth without sample treatment was considered as negative control. After incubation, an aliquot of 5 µL 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 minutes. 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 (Veiga *et al.,* 2019).

3.10.2. Antibacterial activity using agar well diffusion assay

Antibacterial activities of crude Kewda essential oil and its pure compounds (PEME and Terpinen-4-ol) were determined by the agar-well diffusion method against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109, which exhibited resistance to maximum numbers of antibiotics screened. Bacterial lawns were prepared in sterile MHA plates using sterile cotton swabs followed by preparation of wells of 8 mm diameter using a sterile borer. Further, wells were filled with 100 µL aliquots of 30 mg/mL oil, diluted from the original stock of crude oil and pure compound of individual organic solvents with the aqueous extract, by 10% DMSO solution. Plates were incubated at 37 °C for 18-24 h. Antibacterial activities

were evaluated by measuring the diameter values of zones of inhibition. Experiment of each solvent-extract was conducted thrice and data of the third repeated experiment are presented (Negreiros *et al.,* 2016).

3.11. Anti-biofilm activities of Kewda essential oil and its constituents, 2-Phenyl ethyl methyl ether (PEME) and Terpinen-4-ol against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109

3.11.1. Qualitative biofilm formation assay (Congo red agar plate method)

Congo red agar (CRA) method is a qualitative assay for detection of biofilm producer microorganism, as a result of colour change of colonies inoculated on CRA medium, is described by Chhibber *et al.,* (2017). Briefly, overnight culture of test bacteria (treated with sub-MIC level of kewda oil, PEME and Terpinen-4-ol) streaked onto the congo red agar (CRA) plates containing combination of 0.8 mg/ml congo red dye (w/v)) and sterile medium of BHI agar (37 mg/ml), sucrose (50 mg/ml) and agar agar (10 mg/ml). The streaked CRA plates were incubated at 37 °C for 48 h and the colour of the colonies were observed. Bacterial culture without treatment was considered as control (Chhibber *et al.,* 2017).

3.11.2. Qualitative biofilm formation assay (Tube Method)

Tube method (TM) that is a qualitative assay for detection of biofilm producer microorganism, as a result of the occurrence of visible film, is described by Christensen *et al.,* (1985). Bacterial isolates (untreated and treated with sub-MIC level of kewda oil, PEME and Terpinen-4-ol) are inoculated in polystyrene test tube which contained TSB and incubated at 24 h at 37 °C. The sessile isolates of which biofilms formed on the walls of polystyrene test tube are stained with crystal violet for 10-15 min, after planktonic cells are discharged by rinsing twice with phosphate-buffered saline (PBS). Then, crystal violet-stained polystyrene test tube is rinsed twice with PBS to discharge excess stain. After air drying of test tube process, the occurrence of visible biofilm lined the walls, and the bottom of the tube indicates biofilm production.

3.11.3. Quantitative biofilm formation assay (Crystal violet staining method)

For this experiment, polystyrene based 24-MTP was used to allow pathogenic microorganisms to form biofilm matrix onto the polystyrene surface. Briefly, overnight culture of test bacteria was inoculated into MH broth supplemented with or without sub-MIC level of kewda oil, PEME and Terpinen-4-ol and incubated for 24 h at 37 °C. After incubation, the culture medium was discarded and the attached biofilm matrix was

washed 2-3 times for complete separation of cell debris. In the next step, the attached biofilm matrix was stained with 0.1 % CV (w/v) for 20 min followed by removal of excess stain. The stained biofilm matrix were then washed with PBS for 2-3 times followed by addition of 95 % ethanol and optical density was quantified at 540 nm (Luciardi *et al.,* 2016).

3.11.4. Biofilm formation assay (microscopic observation)

Briefly, microbial culture of 0.5 McFarland standard was added to the 24-MTP containing sterile coverslip immersed in the freshly prepared sterile LB growth medium supplemented with or without sub-MIC level of Kewda oil, PEME and Terpinen-4-ol. The experimental setup was incubated at 37 °C for 16–18 h and allowed formation of biofilm on the surface of coverslips. At the end of the incubation period, the LB medium was discarded and the planktonic debris was removed from the coverslips by 2-3 washing steps in sterile PBS. The biofilm matrix grown over the coverslips were then subjected for microscopic analysis. For light microscopic analysis, the adhered biofilms on the coverslips were stained with 0.4 % CV (w/v) for 10 min followed by visualization under light microscope (Packiavathy *et al.,* 2014). For observation under fluorescence microscope, the biofilms adhered over the coverslips were stained with 0.01% acridine orange (w/v) and incubated for 5–10 min under dark conditions. At the end of incubation, excess stain was removed and the biofilms were observed under fluorescence microscope (Wu *et al.,* 2016).

3.12. Whole transcriptome analysis by RNA-seq using illumine HiSeq 2000 platform 15

Whole transcriptome sequencing has been performed between treated and untreated MDR strain of *Staphylococcus aureus* with PEME (the principal compound of essential oil of Kewda) to understand the mode of action. Total RNA were isolated from the bacterial samples by Trizol method (Invitrogen) as per the manufacturer's protocol followed by DNAse treatment to ensure that the samples were not contaminated with genomic DNA. RNA purity was assessed on Nanodrop and 1% agarose gel electrophoresis. RNA samples having an A260:A280 ratio above 1.8 and A260:A230 ratio above 2.2 has been considered good. The integrity of the RNA were evaluated on Agilent Bioanalyzer, and samples with RNA integrity numbers greater than and equal to 6.5 will prepared for sequencing. The total RNA has been amplified and converted to double stranded cDNA and were then used for Illumina paired end sequencing library

preparation followed by high throughput sequencing of transcriptome and comparative analysis. Briefly, the purified cDNA library products were evaluated using the Agilent bioanalyzer followed by cluster generation on the HiSeq paired-end flow cell and massively-parallel sequencing (2×100 bp) on Illumina HiSeq 2000. The paired end data generated by Hiseq 2000 has been submitted to the NCBI Short Read Archive as well. The sequence reads were pre-processed using tools like Trimmomatic and Printseq to filter out low quality reads. The sequences were then aligned to the available reference genome sequence using TopHat, which was integrated with Bowtie software. TopHat removes reads based on quality scoring accompanying each read in FASTQ files and then maps the reads to the reference genome. The aligned reads resulted from Tophat-Bowtie pipeline were analyzed further by Cufflinks which will report the expression of the transcripts in Fragments/kb of exon per Million fragments mapped (FPKM). FPKM was an expression of the relative abundance of transcripts. For determination of differential expression of genes between the treated and untreated *Staphylococcus aureus*. Cuffdiff and Cuffcompare were used using the reference genome comparing the experimental groups. Alternatively, differential expression has also been performed using DESeq, edgeR etc. from R Bioconductor package which also adjusts biases arising from data normalization, small number of highly expressed genes etc. and uses suitable statistical and FDR tests. To identify novel target, Cufflinks has be further used without a reference genome. It allows the construction of the minimum number of transcripts that described the data without a bias towards already discovered transcripts. The differentially expressed gene lists with fold change greater than 2, obtained from above further analyzed using tools and databases like Cytoscape plugins, DAVID, StringDB, Ingenuity Pathway Analysis (IPA) etc to obtain further downstream results like, Canonical pathways, 16 GO Ontologies, Gene and Protein Networks, Gene Set analysis, Gene clustering, Target protein identification etc.

3.13. *In silico* study of molecular interaction of Terpinen-4-ol and PEME with targeted proteins

3.13.1 Protein preparation

The proteins that are involve in pathogenesis, bioflim 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, 3BS1), MepR (transcriptional regulator of multidrug efflux pump, MepA; PDB ID: 3ECO) and Rot (global regulator of virulence genes; PDB ID: 4Q77) were used in

the study. The PDB structures of these proteins were preprocessed using a multistep procedure of protein preparation wizard (Schrödinger, Inc., NY). The missing hydrogen atoms were added using protein preparation wizard. The missing side chain atoms of the amino acids were identified using Prime side-chain prediction tool and repaired using Prime (Schrödinger, Inc., NY). Further, the structures were refined by energy minimization using Macromodel (Schrodinger) and OPLS 2005 force field. Polak-Ribiere Conjugate Gradient (PRCG) algorithm with an energy gradient of 0.01 kcal/mol was used for the energy minimization.

3.13.2 Preparation of molecular structure of Terpinen-4-ol and PEME

The molecular structure of Terpinen-4-ol and PEME (Figure 3.4) was built using ChemDraw and imported into Maestro (Schrödinger package). The molecular structure was energy minimized using Macromodel (Schrödinger package) and OPLS 2005 force field with PRCG algorithm (energy gradient of 0.001). We used DFT (hybrid density functional theory) with Becke's three-parameter exchange potential and the Lee-Yang-Parr correlation functional (B3LYP) with basis set 6-31G** using Jaguar (Schrödinger, package) (Santoshi & Naik, 2014) for the geometric optimization of the structure. Further, the various conformations of Terpinen-4-ol were generated using Ligprep (Schrödinger package).

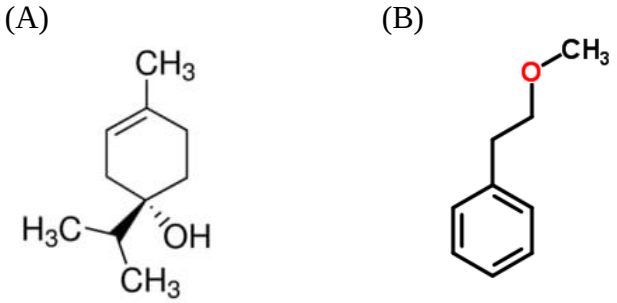


Figure 3.4. Molecular structure of (A) Terpinen-4-ol and (B) Phenyl Ethyl Methyl Ether (PEME), the bioactive molecules from the essential oil of Kewda.

3.13.3 Molecular docking of Terpinen-4-ol and PEME

We have followed blind docking approach to investigate the molecular interaction of Terpinen-4-ol and PEME with different proteins, in the absence of co-crystal structures. In the process of blind docking, all the binding sites of protein were predicted using SiteMap (Schrodinger package) and the receptor grid boxes were generated for each predicted binding site. With the help of a Glide grid-receptor generation program, an inner grid box of size 12Å x 12Å x 12Å was defined at the centroid of the binding site. Within this search space, the diameter midpoint of each

docked ligand was required to be present. Further, an outer grid box was also defined with an edge length of 20Å. Herein, all ligand atoms of a valid pose must be located. The various conformations of Terpinen-4-ol generated above were docked onto each predicted binding site using Glide XP (extra precision) algorithm (Schrodinger software package) and evaluated their binding poses using Glide XP_{Score} function (Friesner *et al.*, 2004; Halgren *et al.*, 2004). The single best conformation of Terpinen-4-ol with the lowest minimum docking score with protein was used for further analysis.

3.14. Evaluation of toxicity of Kewda essential oil in *in vivo* mice model

3.14.1. Toxicity assay

Both the male and female rats (110-150g) were obtained at 5-8 weeks from Neelachal tirati, Kolkata, Saha enterprises (1828/po/Bt/S/15/CPCSEA). The Animal Ethics committee of the SOA University of Bhubaneswar approved all the experimental procedures (Protocol IAEC/SPS/SOA/18/2019). Experiments were carried out at a lab with registration number 1171/Po/Re/S/08/CPCSEA. The animals were maintained individually in 10×16 iron cages at room temperature with a 12-h day and light period at the animal house of SOA University. Before beginning the trial, the animals were given a 7-day acclimatization period.

3.14.2. LD_{50} assay

The LD_{50} (Lethal Dose) is the amount of a drug or oil given all at one time that cause 50% of targeted animals to die. This is one method of determining the drugs or extracts short-term toxicity. A single dosage of oil of Kewda was given orally to selected rats at different doses of 200, 400 and 800 for LD_{50} testing. The test animals were then monitored for 72 h.

3.14.3. Sub-acute toxicity study

For both the acute and sub-acute experimental purposes, both the male and female rats were divided into five different experimental groups with each group having six animals. 1^{st} group of animals was fed with a regular diet with distilled water using oral gavage and considered it a control group for acute toxicity analysis. 2^{nd} to 5^{th} were feed with a regular diet including distilled water containing Kewda oil of the plant, as doses range from 200 to 800 mg/Kg body weight for acute and sub-acute toxicity analysis. General behaviour body weight of the animals were recorded, and clinical toxicity symptoms were evaluated for 14 days. Animals were sacrificed at the end of this study animals were anesthetised by giving ketamine at 20 mg/kg body weight. The heart

puncture was conducted after the anaesthesia had achieved depth to collect blood for biochemical and haematological assessments.

3.1.4.4. Haematological analysis

All surviving animals were haematologically examined at the end of the experiment. A comprehensive blood count was performed using an automated haematology analyser. Among the haematological tests performed were haemoglobin concentration (HGB), red blood cell count (RBC), platelet count (PLT), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and white blood cell count (WBC) (WBC).

3.14.5. Blood serum biochemistry analysis

After the experiment, biochemical testing was performed on all surviving animals. Each blood sample serum was extracted and kept in cryogenic tubes at a temperature of -80 °C. The blood was taken and transferred to tubes without anticoagulant, which were left at room temperature for 60 min before being centrifuged for 10 min at 4000 rpm. The following tests were performed: glucose (G), total cholesterol (TC), triglycerides (TG), aspartate amino transferase (AST), alanine aminotransferase (ALT), urea (Ur), creatinine (Cr), and total protein.

3.14.6. Histopathology

Organs were removed from all surviving animals, cleaned in a 0.9 % (w/v) saline solution, weighed, and preserved in a 40 percent formaldehyde solution. Following that, the organs were prepared for paraffin embedding. Haematoxylin and eosin staining were used to create 5 mm thick slices (H&E). Using haematoxylin and eosin staining, the tissues were examined under an optical microscope for the overall structure, indications of inflammation, tubular swelling and oedema, degenerative alterations, and necrosis evidence. The photographs were taken using a microscope (Nikon Eclipse Ts2R-FL).

3.14.7. Statistical analysis

The mean SEM of the comparative haematological and biochemical data was provided (SEM). The data were subjected to one-way analysis of variance (one-way ANOVA) .The information was given in a mean, standard error of the mean (SEM). P (0.05) was used to determine statistical significance. Samples were analyzed with Student's *t*-test and it was found that $p > 0.05$, which was not statistically significant.

CHAPTER IV

RESULTS AND DISCUSSION

P. odorifer is a high value medicinal plant. It has several therapeutic properties like analgesic, anticancer, antidiabetic, antimicrobial, anticonvulsant, antioxidant, antidepressant and antineurotic activity. In Ayurveda, the Kewda oil is used to treat rheumatism and general body pain. It is mainly composed of secondary metabolites such as terpenoids lipids, coumarins, alkaloids, flavonoids, phenols, lignans and steroids. [13, 14] We investigated the antibacterial activity of the essential oil and two of its major chemical compounds, PEME and Terpinol against both Gram positive and Gram negative MDR bacteria mainly because of the exponential rise in MDR bacteria strains with the currently used antibiotics.[15, 16] The significant antibacterial activity noted with the essential oil and two of its major chemical compounds can assume a pivotal part in controlling MDR bacterial strains where the majority of the synthetic based antimicrobials drugs may have serious side effects.

4.1. Extraction of essential oils from *P. odorifer* (Kewda) flower and its phytochemical characterization

As per the standard operating procedures of double hydro-distillation, essential oil of Kewda flower was extracted and analyzed for its phytochemical using various analytical equipments such as GC-MS, HRMS, FTIR and NMR. The oil yield and quality mainly affects the price of Kewda oil in the national as well as in the international market. It has also been reported that the oil available in Ganjam district is of superior quality as compared to other states. The distilled essential oils were slightly yellowish in colour. The average essential oil content was 0.24 µl/1000g.

4.1.1. GC-MS based quality evaluation of essential oil

The GC-MS analysis of Kewda essential oil revealed the presence of several phytochemicals of interest as referred by NIST library (Table 4.2). As evident from GC-MS chromatogram, low molecular weight ester i.e. 2-Phenyl ethyl methyl ether (PEME) exhibited highest peak area percentage with occurrence of 80.435 %. The presence of PEME in essential oil of kewda flower suggested its widespread potential in cosmetic industries as fragrances (Sparkman *et al.,* 2011). The next phytochemical in the panel is Terpinen-4-ol (14.13 %), which was highly reported to be a major component of several essential oils of different aromatic plants. Terpinen-4-ol, an important member of terpene family also exhibits promising therapeutic values (Shapira *et al.,* 2016). The other important constituents identified were α terpeneol (tertiary monoterpenoid; 1.829%) and γ-terpenene (monoterpene; 1.79%). All these components belonged to

terpene family and were reported as major components of plant derived essential oils (Bourgou *et al.*, 2012; Khaleel *et al.*, 2018).

Table 4.1. List of identified phytochemicals from the essential oil of *P. odorifer* flower by GC-MS analysis.

S. NO	*PHYTOCHEMICALS*	*RETENTION TIME (MIN)*	*PEAK HEIGHTS*	*CORRELATION AREA*	*PERCENTAGE (%)*
1.	p cineole	3.1	446068	9620777	0.405%
2.	γ terpinene	3.4	1313773	42386649	1.790%
3.	2-Phenyl ethyl methyl ether (PEME)	3.9	23401847	1905199478	80.435%
4.	p meth2en1ol	4.5	523708	13876298	0.586%
5.	cis p meth-2-en-1-ol	4.8	361742	9242053	0.390%
6.	Terpinen-4-ol	5.6	8529820	334683018	14.130%
7.	α terpineol	5.8	1797801	43327170	1.829%
8.	piperitone	7.2	236538	8220680	0.347%
9.	nerolidol	14.6	62365	2065528	0.087%

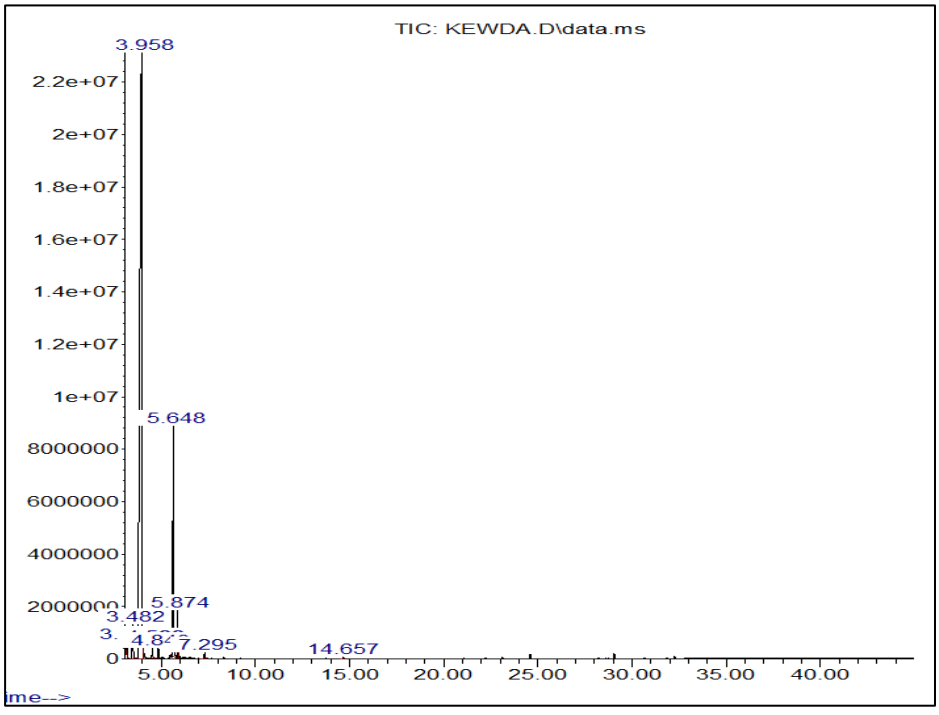


Figure 4.1. GC-MS chromatogram of Kewda essential oil depicting different peaks corresponding to the presence of phytochemicals.

4.1.2 HRMS Analysis of crude essential oil of *P. odorifer*

The phytochemical screening of essential oil of *P. odorifer* revealed the presence of various secondary metabolites (Table 4.2 and Figure 4.2). Different phytochemicals in the essential oil were identified on the analysis of fragmentation pattern of mass spectra and direct comparison of their spectral data with the chemical profiles, using the NIST library, and comparisons of published mass spectra (Figure 4.2). The chemical profiles of the identified compounds with their retention time, percentage peak area, molecular formula, molecular weight, structure, nature of the compound, and reported activity are collated in Table 4.2. The studies on biologically active compounds in *P. odorifer* by 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, where it was found to be effective against both Gram-positive and Gram-negative bacteria. Further, it was found from the HRMS study that the major compound present in crude oil was PEME, which is responsible for its antimicrobial activity.

Table 4.2. Phytochemical constituents identified in the essential oil of *P. odorifer* flower by HRMS analysis.

Sl. No.	Compound name	Molecular weight	Concentration (picomole)
A.	p-benzoquinone	108.021	31.641
B.	2-phenethyl alcohol	122.073	14.569
C.	p-cymene	134.109	48.500
D.	2-phenethyl methyl ether	136.089	112.22
E.	alpha-Terpineol	154.136	221.24
F.	Psoralen	186.032	20.377
G.	Isoplumbagin	188.047	2.3489
H.	Genipin	226.084	1.2797
I.	Artemidiol	234.089	5.6275
J.	Pinocembrin	256.074	0.5880
K.	(-)-Glycinol	272.068	1.75057
L.	Iprobenfos	288.095	0.4641

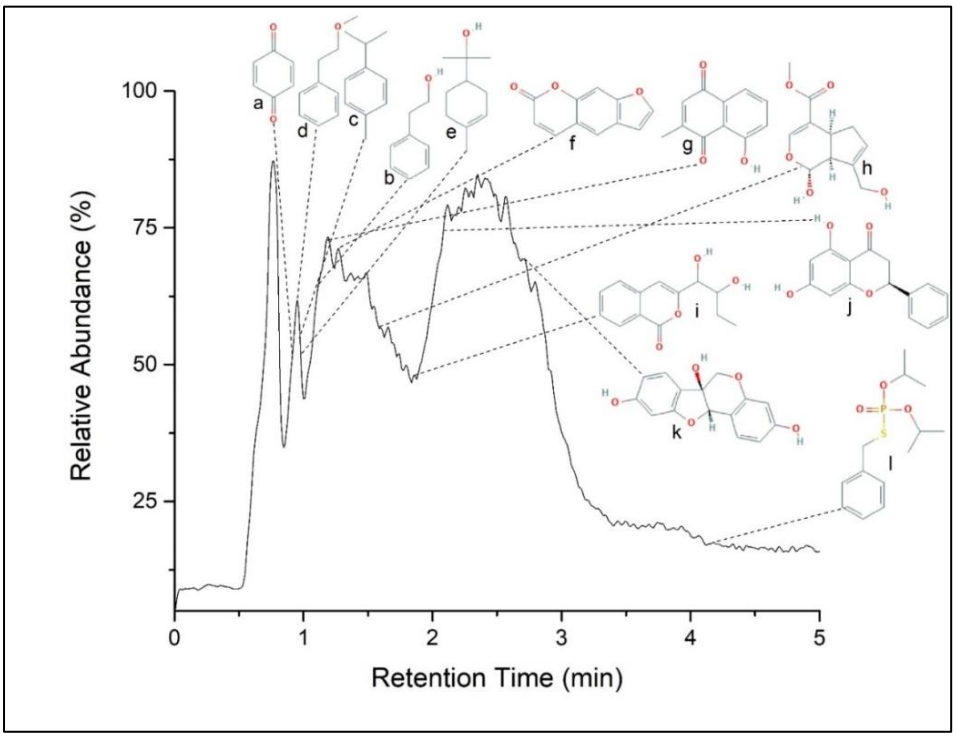


Figure 4.2. Mass spectra of the essential oil of *P. odorifer* indicating the presence of several major phytochemicals.

4.1.3 Fourier transform infrared spectroscopy (FTIR) analysis of Kewda essential oil

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} (Figure 4.3; Table 4.3). The peak at 3493.30 and 3485.90 cm^{-1} correspond to the presence of Alcohol C-OH group within the stretching vibration absorption. Meanwhile, the absorption peaks at 3027.52, 2925.31 and 2870.97 cm^{-1} attributed to C-H Alkanes. The absorption peaks at 2736.02 and 1728.25 cm^{-1} corresponds to Aldehyde group which was in accordance with earlier report (Ogwuche and Edema, 2020). Other important absorption peaks at 1487.84 and 1453.65 cm^{-1} (corresponds to C=C Aromatic ring with bending vibrations) and 1382.02 cm^{-1} (corresponds to Phenolic group with bending vibrations). The FTIR analysis of Kewda essential oil revealed the presence of characteristic functional groups of interest which were in accordance with earlier report of composition of functional groups in several essential oils (Ogwuche and Edema, 2020).

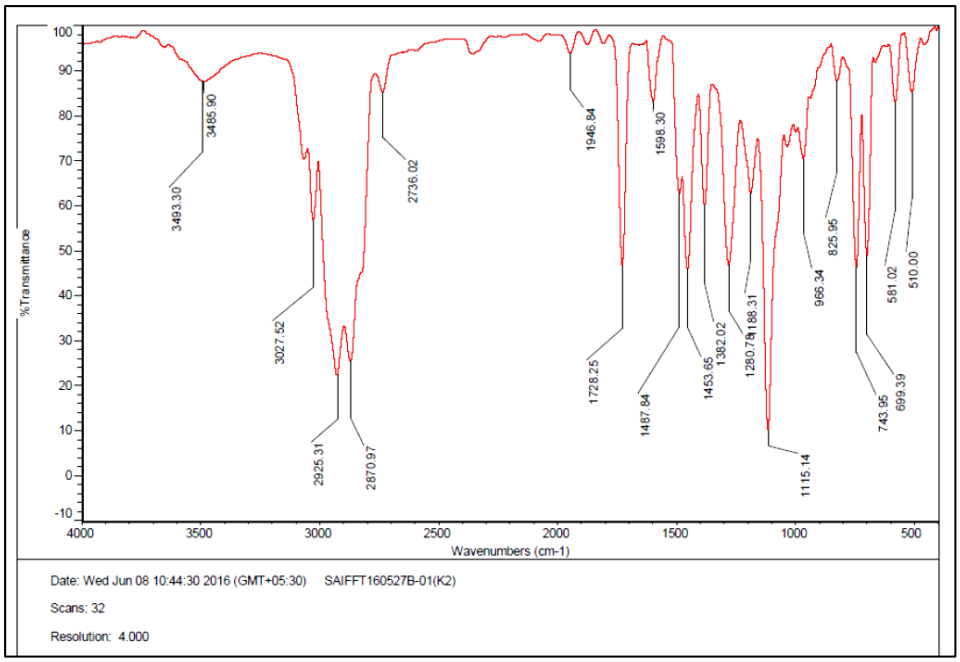


Figure 4.3. The FTIR spectral analysis of Kewda essential oil.

Table 4.3. List of identified molecules from FTIR analysis of kewda essential oil

Sl. No.	*Frequency (cm^{-1})*	*Types of vibration*	*Compounds*	*Groups*
1.	3493.30	Stretching	Alcohol	O-H
2.	3485.90	Stretching	Alcohol	O-H
3.	3027.52	Stretching	Alkane	C-H
4.	2925.31	Stretching	Alkanes	C-H
5.	2870.97	Stretching	Alkanes	C-H
6.	2736.02	Stretching	Aldehyde	C-H
7.	1946.84	Stretching	Alkene	C=C=C
8.	1728.25	Stretching	Aldehyde	C=O
9.	1487.84	Bending	Aromatic Ring	C=C
10.	1453.65	Bending	Aromatic Ring	C=C
11.	1382.02	Bending	Phenol	O-H
12.	1280.78	Stretching	Sulfonate	S=O
13.	1188.31	Stretching	Sulfonate	S=O
14.	1115.14	Stretching	Secondary Alcohol	C=O
15.	966.34	Stretching	Alkenes	C=H
16.	825.95	Bending	Alkene	C=C
17.	743.95	Stretching	Ikyl Sulfides	C=S
18.	699.39	Bending	Alkene	C=H
19.	581.02	Stretching	Alkyl Halides	C=Cl
20.	510.00	Stretching	Holocompound	C-I

4.1.4 Nuclear Magnetic Resonance (NMR) analysis of Kewda essential oil

The NMR spectra of Kewda essential oil revealed the presence of ketones, Esters, alcohols, aromatic phenols, Vinylics which were compiled from e-library source (Figure 4.4; Table 4.4).

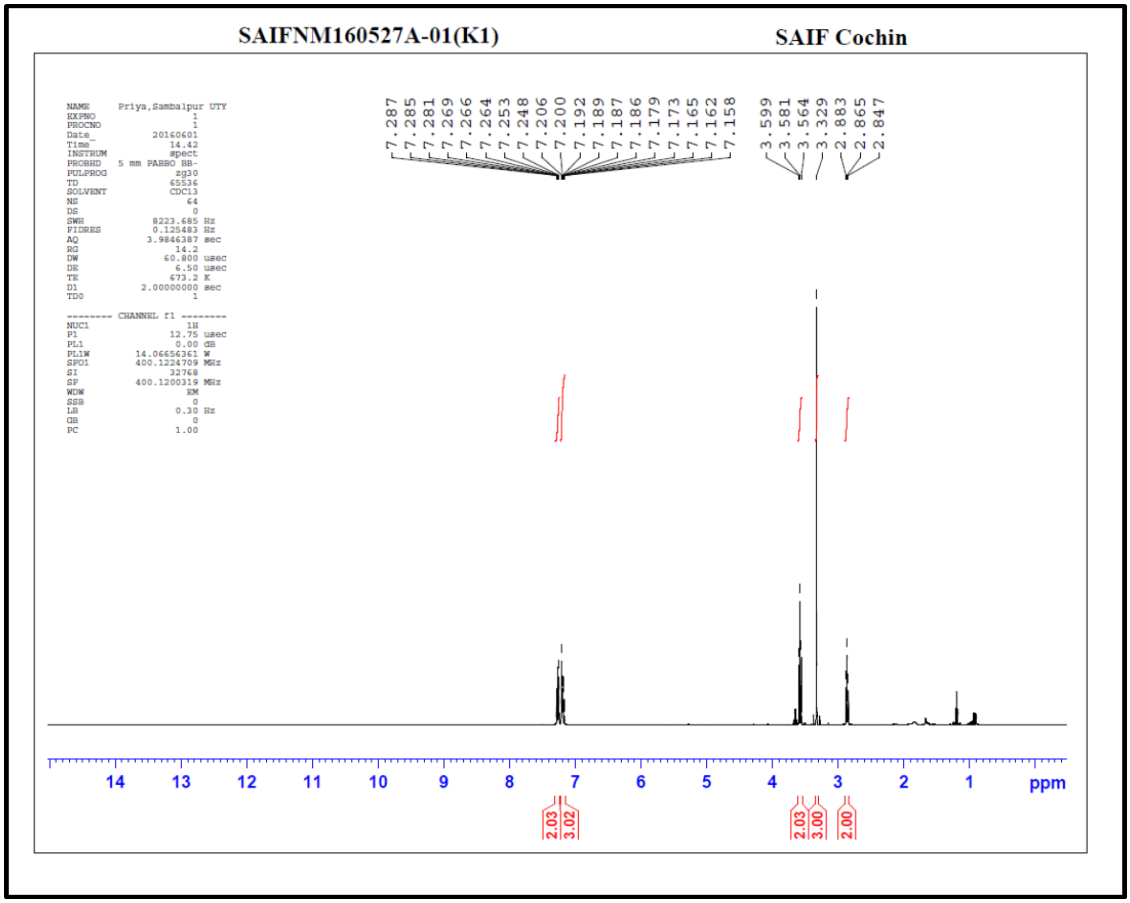
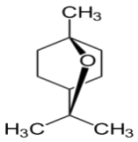
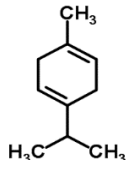
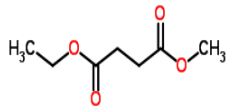
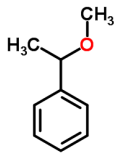
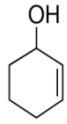
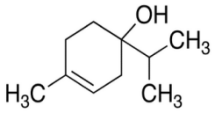
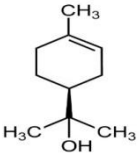
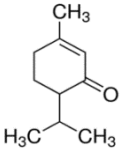
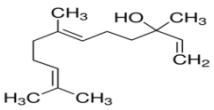


Figure 4.4. The absorption NMR spectra of Kewda essential oil.

Table 4.4. List of compounds identified from NMR analysis of Kewda essential oil.

Components	***IUPAC name***	***Structures***
p Cineole	1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane	
γ-Terpinene	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)	

Ethylmethyl ester	Methoxyethane	
p Meth2en1ol	2-Methoxyethyl benzene	
cis p Meth2en1ol	1-methyl-4-propan-2-ylcyclohex-2-en-1-ol	
Terpinen 4 ol	4-methyl-1-propan-2-ylcyclohex-3-en-1-ol	
α Terpineol	2-(4-methylcyclohex-3-en-1-yl)propan-2-ol	
Piperitone	3-methyl-6-propan-2-ylcyclohex-2-en-1-one	
Nerolidol	3,7,11-trimethyldodeca-1,6,10-trien-3-ol	

4.2 Isolation, identification and molecular characterisation of isolated bacteria

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. Further for the present study, two reference bacterial strains i.e. *Staphylococcus aureus* (MTCC 740) and *Klebsiella pneumoniae* subsp. *pneumoniae* (MTCC 109) were also procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. All the clinical bacterial strains were sub-cultured and subjected for identification by standard biochemical procedures followed by Clinical and Laboratory Standards Institute (CLSI) guidelines.

Gram positive bacteria as medium to large, smooth, entire, slightly raised, creamy yellow, with green/β-haemolytic colonies on blood agar, found positive to catalase and

coagulase tests were confirmed as *S. aureus* (Table 4.5); blue-pigmented, round and opaque colonies on chromogenic agar were considered as MRSA strains. Further confirmation of colonies as MRSA was done with observing growth in presence of oxacillin 1 µg/disc on MH agar plate. Catalase negative GP colonies giving beta-haemolysis (complete haemolysis of erythrocytes) on blood agar and simultaneously sensitive to the bacitracin are identified as Group A streptococci or *S. pyogenes*. Further, the GN bacterium, *K. pneumonia* was identified basing on colony characteristic on MacConkey and cysteine lactose electrolyte deficient (CLED) agar, and results obtained from standard biochemical procedures.

Table 4.5. Biochemical test results for identification of clinically isolated bacterial strains.

SL. NO.	*CLINICAL BACTERIAL STRAINS*	*UREASE TEST*	*METHYL RED (MR) TEST*	*VP TEST*	*INDOLE TEST*
1.	*Escherichia coli*	-	+	-	-
2.	*Salmonella typhi*	-	+	-	-
3.	*Salmonella paratyphi A*	-	+	-	-
4.	*Salmonella paratyphi B*	-	+	-	-
5.	*Proteus* sp.	+	+	-	-
6.	*Klebsiella pneumoniae*	+	-	+	+
7.	*Shigella* sp.	-	+	-	-
8.	*Pseudomonas aeruginosa*	Catalase (+)	-	-	-
9.	*Enterococcus* sp.	Catalase (-)		+	+
10.	*Staphylococcus aureus*	Catalase (+)	Coagulase (+)		

4.3. Antibiotic susceptibility of clinically isolated bacteria

All the clinically isolated bacteria (Two Gram positive and eight Gram negative 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 microrganisms were either resistant, sensitive or intermediate towards different antibiotics based on the zone of inhibition (Table 4.6).

Table 4.6. Antibiotic susceptibility of clinically isolated Gram negative bacteria.

Bacterial isolates	*List of Antibiotics*											
	Zone of inhibition (mm)											
	LE	**CPM**	**AK**	**CIP**	**MET**	**GEN**	**AMP**	**AMC**	**CL**	**OF**	**TGC**	**NA**
Escherichia coli	31±0	21±5.5	**14.6±0.5**	34.3±3.5	**0±0**	23.3±2	16.6±3	17.5±1.5	**0±0**	28±3	**11.6±2**	18.3±0.5
Salmonella typhi	35.5±4.5	26.5±2.5	20.6±1.5	37±4.3	**0±0**	26.3±1.5	19.6±3.2	19.6±0.5	**1±2**	35.3±3.2	**1.6±3**	22.3±2.8
Salmonella paratyphi A	35.5±0.7	25±4	21±3.6	40.3±5.1	**0±0**	26±2	24.6±4	22.3±2	**13.6±1.5**	31.6±3	16±2	19±1.7
Salmonella paratyphi B	27.3±4.1	31±4.2	21±1	39.3±2.8	**0±0**	25±4.5	20±3.4	18±5.6	**13±1.7**	21.3±2.5	**10.5±3.6**	18.3±0.5
Proteus sp.	29±3.6	24.6±3	16±2	36.5±3.5	**0±0**	17.3±4.7	28±4.2	20±3.6	**0±0**	25.6±4.5	**7±1.8**	16±2.8
Klebsiella pneumoniae	15.3±4.9	**11±4**	**13.6±0.5**	21.5±0.5	**0±0**	20.3±4	**0±0**	**0±0**	**12.3±3.5**	**13±3**	**13±3**	**12.6±2.8**
Shigella sp.	32.3±1.5	22.6±1.1	**12.6±2**	34±3	**0±0**	21.3±2.5	17±1.7	16±2	**0±0**	19.3±5.1	**4.3±2**	19±0
Pseudomonas aeruginosa	25.6±2	22±3.6	24.5±2.1	38±0	**0±0**	26.3±5.5	**21.4±0**	**0±0**	15.3±4	20.6±2.8	**19.2±0**	**8±1.4**

R: Resistant; **S: Sensitive** and **I: Intermediate**

Note: Antibiotics (µg/disc): LE: Levofloxacin (10 µg/disc), CPM: Cefepime (30 µg/disc), AK: Amikacin (30 µg/disc); CIP: Ciprofloxacin (5 µg/disc), Met: Methicillin (10 µg/disc), GEN: Gentamicin (30 µg/disc), AMP: Ampicillin (10 µg/disc), AMC: Amoxyclav (30 µg/disc); CL: Collistin (10 µg/disc), OF: Ofloxacin (5 µg/disc), TGC: Tigecycline (10 µg/disc), NA: Nallidixic acid (10 µg/disc).

As depicted in Table 4.6, *E. coli* was highly sensitive towards Ciprofloxacin (CIP) and Levofloxacin (LE) with a zone of inhibition of 34.3 and 31 mm, respectively. Meanwhile, *E. coli* exhibited resistance against both Methicillin (MET) and Collistin (CL). Among the Gram negative bacteria, *Klebsiella pneumoniae* as well as *Pseudomonas aeruginosa* showed resistance against the most number of antibiotics used in this study with resistant against Methicillin (MET), Ampicillin (AMP), Amoxyclav (AMC) and Tigecycline (TGC) (Table 4.6). Among the antibiotics tested, Tigecycline (TGC) was observed to be neither sensitive nor resistant i.e. intermediate for all the clinically isolated Gram negative bacteria with moderate zone of inhibition. Interestingly, all the bacterial isolates were found to be resistant against Methicillin (MET). The antibiotic susceptibility of the clinically isolated Gram negative pathogens were also presented in Figure 4.5 to Figure 4.12.

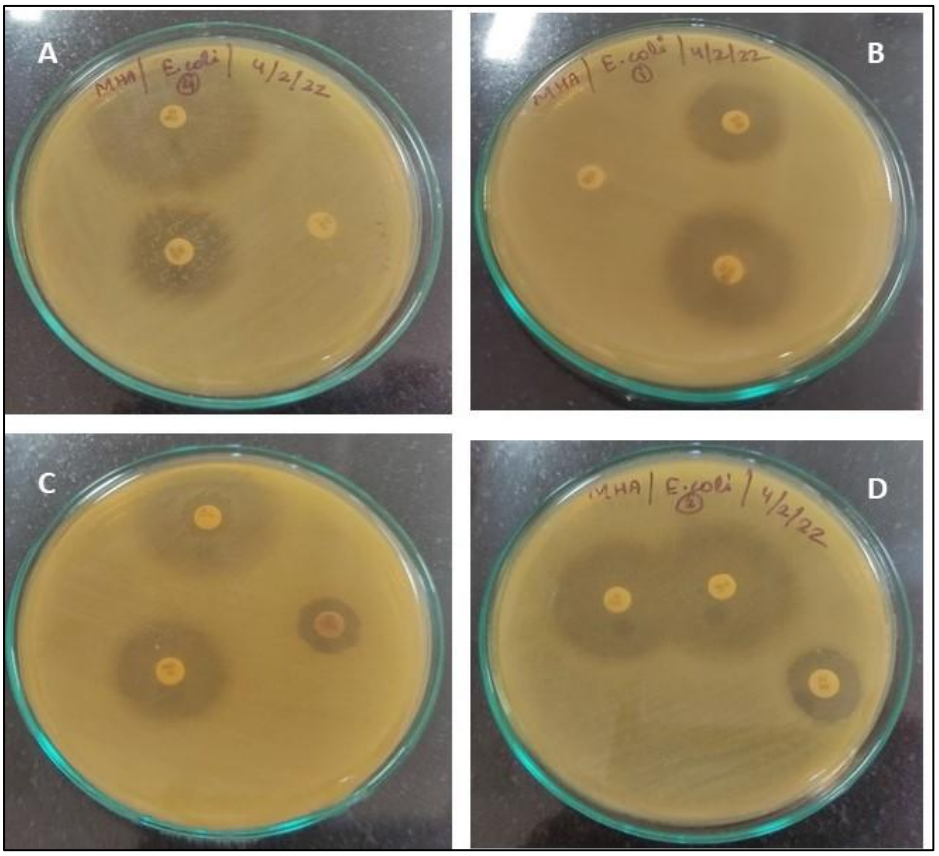


Figure 4.5. Antibiotics susceptibility test of Escherichia coli against **A.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **B.** Ampicillin (AMP), Collistin (CL) and Nallidixic acid (NA); **C.** Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **D.** Amikacin (AK), Levofloxacin (LE) and Cefepime (CPM).

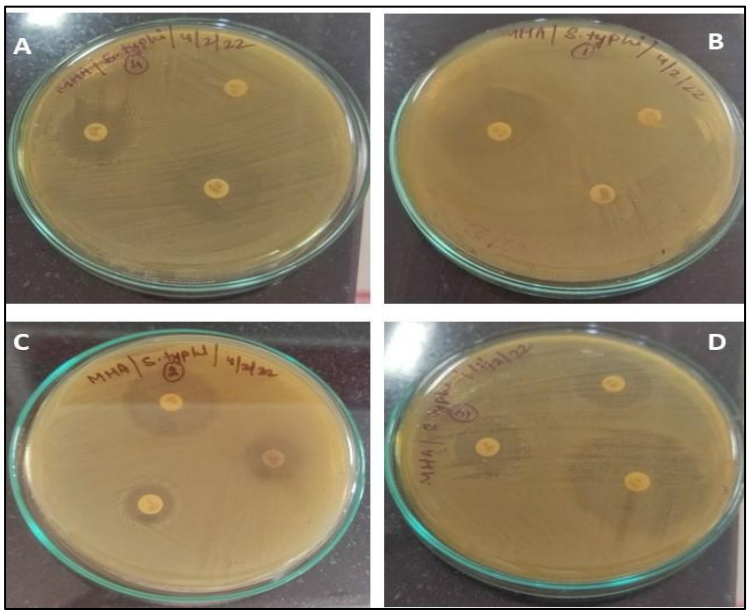


Figure 4.6. Antibiotics susceptibility test of *Salmonella typhi* against **A.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **B.** Ampicillin (AMP), Collistin (CL) and Nallidixic acid (NA); **C.** Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **D.** Amikacin (AK), Levofloxacin (LE) and Cefepime (CPM).

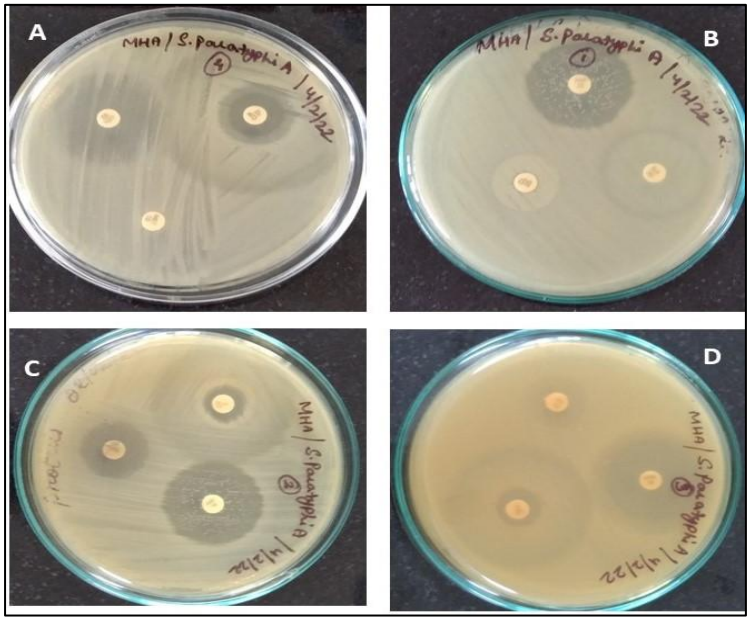


Figure 4.7. Antibiotics susceptibility test of *Salmonella paratyphi* A against **A.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **B.** Ampicillin (AMP), Collistin (CL) and Nallidixic acid (NA); **C.** Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **D.** Amikacin (AK), Levofloxacin (LE) and Cefepime (CPM).

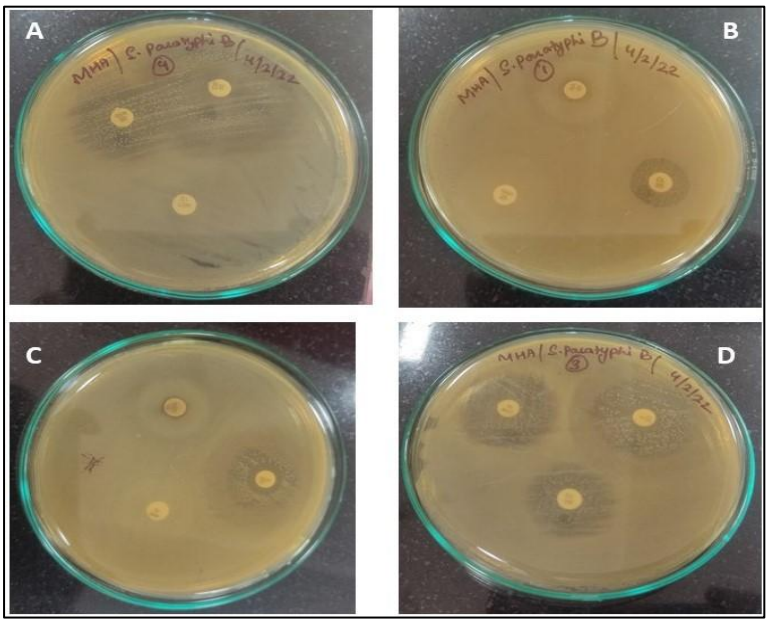


Figure 4.8. Antibiotics susceptibility test of *Salmonella paratyphi* B against **A.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **B.** Ampicillin (AMP), Collistin (CL) and Nallidixic acid (NA); **C.** Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **D.** Amikacin (AK), Levofloxacin (LE) and Cefepime (CPM).

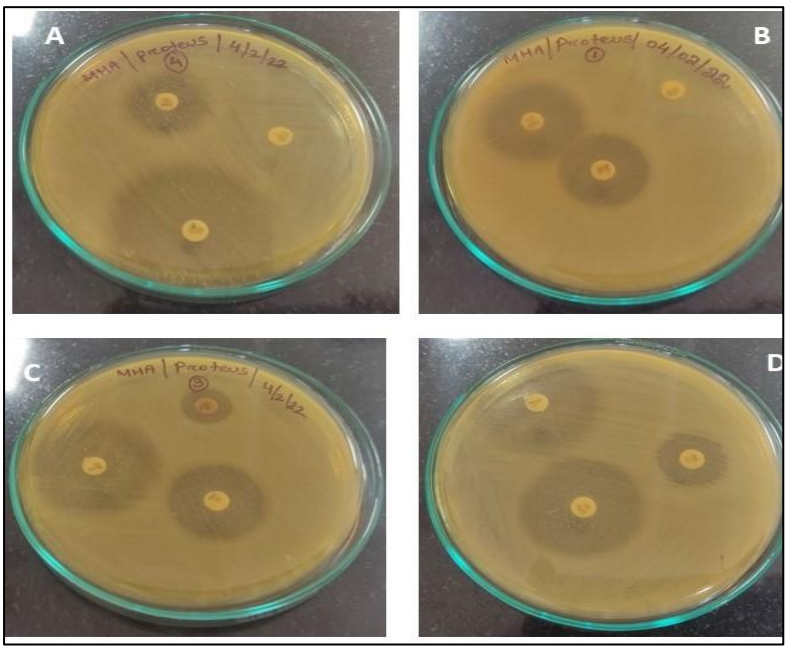


Figure 4.9. Antibiotics susceptibility test of *Proteus* sp. against **A.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **B.** Ampicillin (AMP), Collistin (CL) and Nallidixic acid (NA); **C.** Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **D.** Amikacin (AK), Levofloxacin (LE) and Cefepime (CPM).

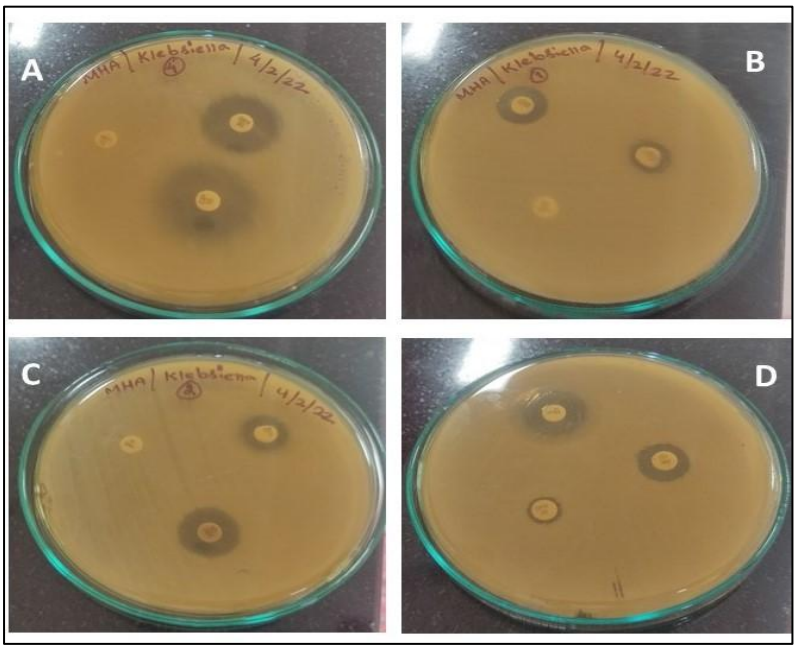


Figure 4.10. Antibiotics susceptibility test of *Klebsiella pneumoniae* against **A.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **B.** Ampicillin (AMP), Collistin (CL) and Nallidixic acid (NA); **C.** Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **D.** Amikacin (AK), Levofloxacin (LE) and Cefepime (CPM).

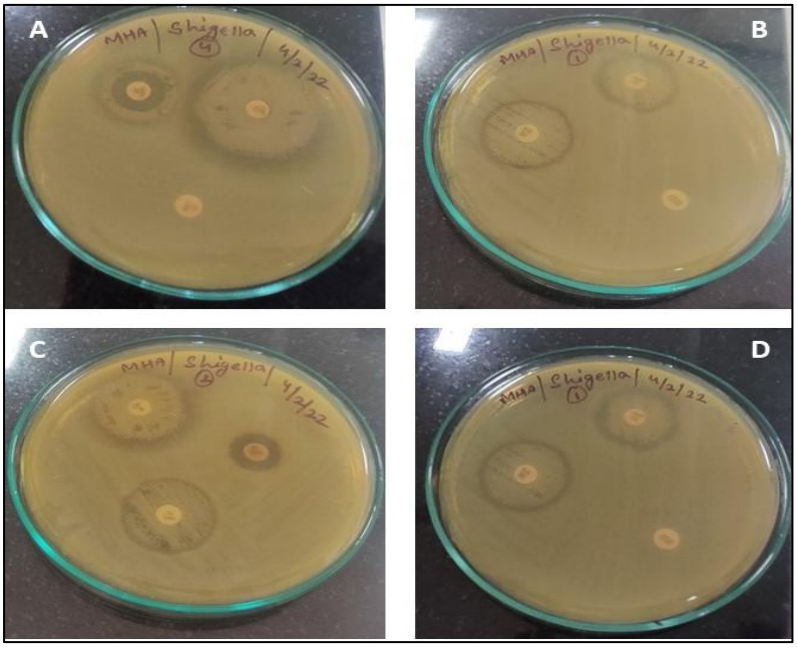


Figure 4.11. Antibiotics susceptibility test of *Shigella* sp. against **A.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **B.** Ampicillin (AMP), Collistin (CL) and Nallidixic acid (NA); **C.** Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **D.** Amikacin (AK), Levofloxacin (LE) and Cefepime (CPM).

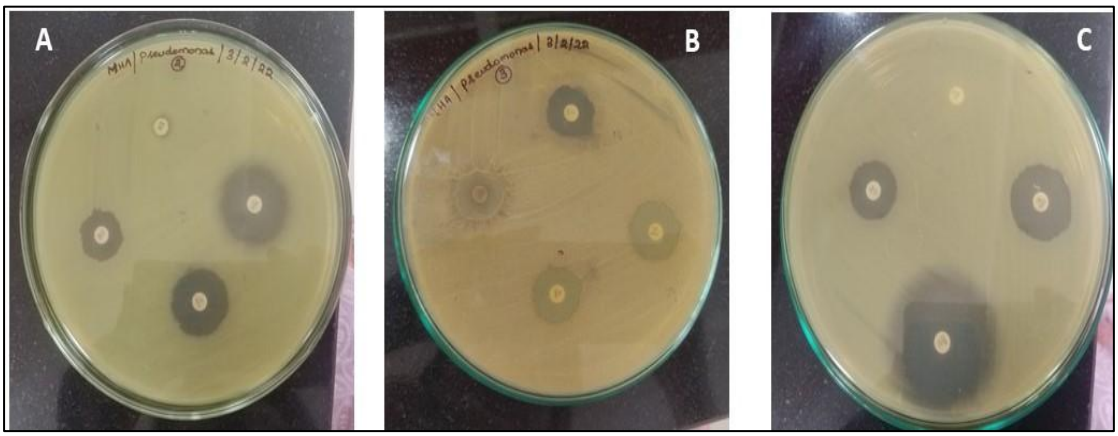


Figure 4.12. Antibiotics susceptibility test of *Pseudomonas aeruginosa* against **A.** Levofloxacin (LE), Cefepime (CPM), Collistin (CL) and Nallidixic acid (NA); **B.** Ampicillin (AMP), Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF); **C.** Amikacin (AK), Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET)Amoxyclav (AMC), Tigecycline (TGC) and Ofloxacin (OF).

The antibiotic susceptibility of clinically isolated Gram positive pathogenic bacteria *Staphylococcus aureus* and *Enterococcus faecalis* was depicted in **Figure 4.13** and **Figure 4.14** respectively (**Table 4.7**).

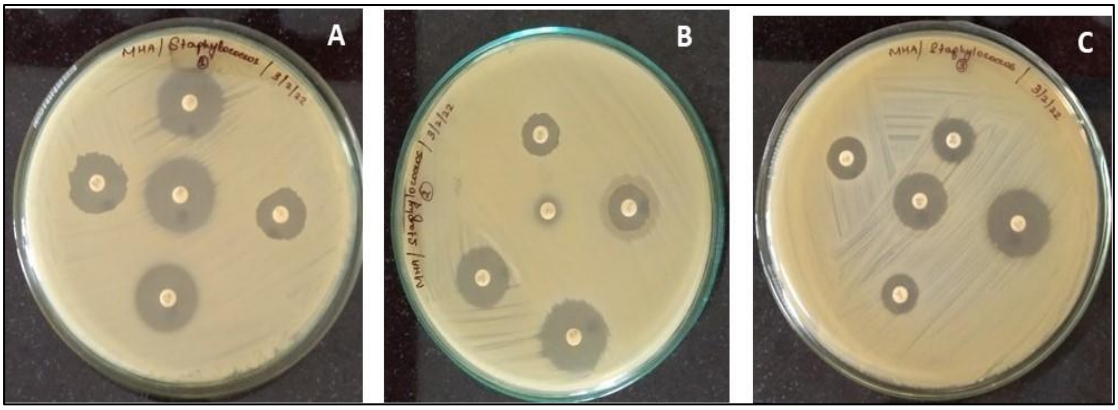


Figure 4.13. Antibiotics susceptibility test of *Staphylococcus aureus* against **A.** Cefotaxime (CTX), Penicillin (P), Linezolid (LZ), Ampicillin (AMP) and Clindamycin (CD); **B.** Erythromycin (EM), Tetracycline (TE), Methicillin (MET), Azithromycin (AZM) and Vancomycin (VA); **C.** Amikacin (AK), Ciprofloxacin (CIP), Gentamicin (GEN), Cefepime (CPM) and Levofloxacin (LE).

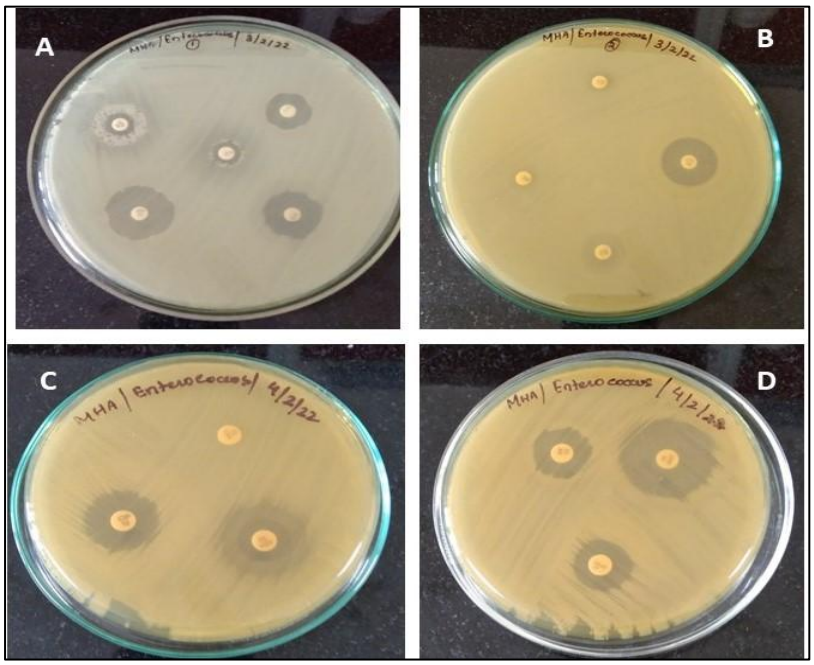


Figure 4.14. Antibiotics susceptibility test of *Enterococcus faecalis* against **A.** Erythromycin (E), Tetracycline (TE), Ampicillin (AMP), Azithromycin (AZM) and Penicillin (P); **B.** Cefotaxime (CTX), Linezolid (LZ), Vancomycin (VA) and Clindamycin (CD); **C.** Ciprofloxacin (CIP), Gentamicin (GEN) and Methicillin (MET); **D.** Amikacin (AK), Cefepime (CPM) and Levofloxacin (LE).

Table 4.7. Antibiotic susceptibility of clinically isolated Gram positive bacteria.

List of Antibiotics	Staphylococcus aureus	Enterococcus faecalis
	Zone of Inhibition (mm)	
LE	**14.5±2.1**	16±0
CPM	23.6±1.5	23.3±2.0
AK	**14.3±3.5**	16±2
CIP	**13.3±3.7**	18.5±0.7
MET	**0±0**	**0±0**
GEN	24.3±3.7	28.0±1.0
AMP	18.3±4.0	23.0±1.7
AZM	22.6±2.3	29.3±3.5
VA	**13.6±1.5**	**6.2±2.0**
E	27.0±2.6	22.6±5.0
CTX	16.6±3.2	**4.5±2.8**
TE	23.0±2.6	19.6±4.0
P	17.3±1.1	18.3±0.5
LZ	27.3±2.0	24.5±3.5
CD	30.0±3.6	33.5±3.5

R: Resistant; **S: Sensitive**; **I: Intermediate**

As depicted in Table 4.9, both *S. aureus* and *E. faecalis* were highly resistant against Methicillin (MET). Meanwhile, *S. aureus* was observed to be neither highly resistant nor highly sensitive towards Levofloxacin (LE), Amikacin (AK), Ciprofloxacin (CIP) and Vancomycin (VA) (Table 4.7).

4.4. Molecular characterization of selected clinical bacterial isolates

The molecular identification of isolated bacteria was done based on 16S rRNA sequencing analysis. The bacterial isolate 06 was identified as *Klebsiella pneumoniae* strain based upon its high sequence similarity and molecular phylogenetic analysis (Figure 4.5A). Similarly, bacterial isolate 10 was found to be *Staphylococcus* sp. strain based upon the sequence similarity and molecular phylogenetic analysis (Figure 4.5B). These two bacterial isolates were used for their susceptibility of Kewda oil and two of its active compounds.

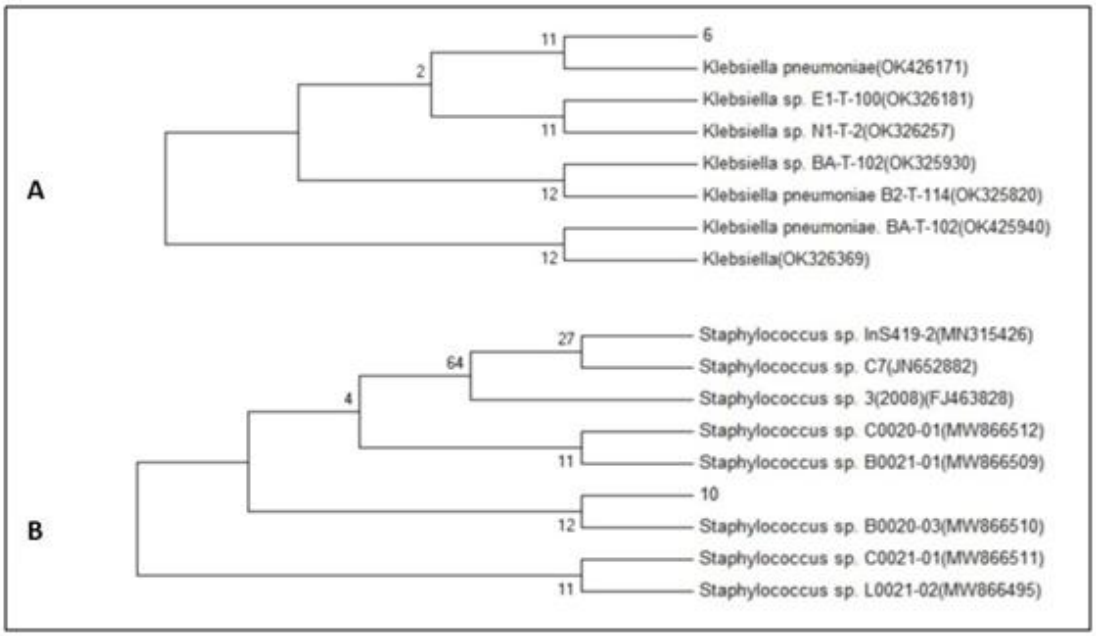


Figure 4.15. Molecular phylogenetic analysis of clinical bacterial isolates (06 and 10) identified as **A.** *Klebsiella pneumoniae* and **B.** *Staphylococcus* sp. using the Maximum Likelihood Method based on the Tamura-Nei Model.

4.5. Molecular identification and antibiotic sensitivity of two clinically isolated bacteria using Vitek-II genotyping

Vitek-II genotyping of selected bacterial isolates revealed the rapid identification of clinical isolates 06 and 10 as *Klebsiella pneumoniae* and *Staphylococcus aureus*,

respectively. Further, Vitek-II genotyping also provided a reliable data on the extent of the identified bacteria towards a range of conventional antibiotics. As evident from Vitek-II analysis, *Klebsiella pneumoniae* (Lab ID: 22598712) exhibited resistance against potent antibiotics such as Ceftazidime, Aztreonam, Imipenem, Cefepime, Amikacin, Ciprofloxacin, Levofloxacin and Tigecycline (Table 4.8). Meanwhile, *Staphylococcus aureus* (Lab ID: 22599797) showed resistance towards Oxacillin, Gentamicin, Ciprofloxacin and Levofloxacin (Table 4.9).

Table 4.8. Antibiogram of clinically isolated *Klebsiella pneumoniae* using Vitek-II system.

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin/Clavulanic Acid	>=128	R	Amikacin	>=64	R
Piperacillin/Tazobactam	>=128	R	Gentamicin	>=16	R
Ceftazidime	>=64	R	Ciprofloxacin	>=4	R
Cefoperazone/Sulbactam	>=64	R	Levofloxacin	>=8	R
Cefepime	>=64	R	Minocycline	>=16	R
Aztreonam	>=64	R	Tigecycline	>=8	R
Doripenem	>=8	R	Colistin	<=0.5	I
Imipenem	>=16	R	Trimethoprim/Sulfamethoxzole	>=320	R
Meropenem	>=16	R			

R: Resistant; S: Sensitive; I: Intermediate

Table 4.9. Antibiogram of clinically isolated *Staphylococcus aureus* using Vitek-II system.

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Cefoxitin Screen	POS	+	Linezolid	2	S
Benzylpenicillin	>=0.5	R	Daptomycin	0.5	S
Oxacillin	>=4	R	Teicoplanin	<=0.5	S
Gentamicin High Level (synergy)			Vancomycin	<=0.5	S
Gentamicin	>=16	R	Tetracycline	<=1	S
Ciprofloxacin	4	R	Tigecycline	<=0.12	S
Levofloxacin	4	R	Nitrofurantoin	32	S
Inducible Clindamycin Resistance	NEG	-	Rifampicin	<=0.03	S
Erythromycin	1	I	Trimethoprim/sulfamethoxazole	160	R
Clindamycin	0.25	S			

R: Resistant; S: Sensitive; I: Intermediate

4.6. Determination of antibacterial activity of Kewda essential oil and its primary constituents, PEME and Terpinen-4-ol

4.6.1. Determination of Minimum Inhibitory concentration (MIC)

As per CLSI guidelines, MIC of Kewda essential oil and its constituent phytochemicals, PEME and Terpinen-4-ol were determined. From the results, the MIC of Kewda oil was observed to be 5% against both clinical as well as reference strains of *S. aureus* and *K. pneumonia* (Figure 4.16). Meanwhile, the MICs of PEME were found to be 50 mM against both clinical as well as reference strain of *S. aureus* and *K. pneumoniae* (Figure 4.17). 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 25 mM (Figure 4.18).

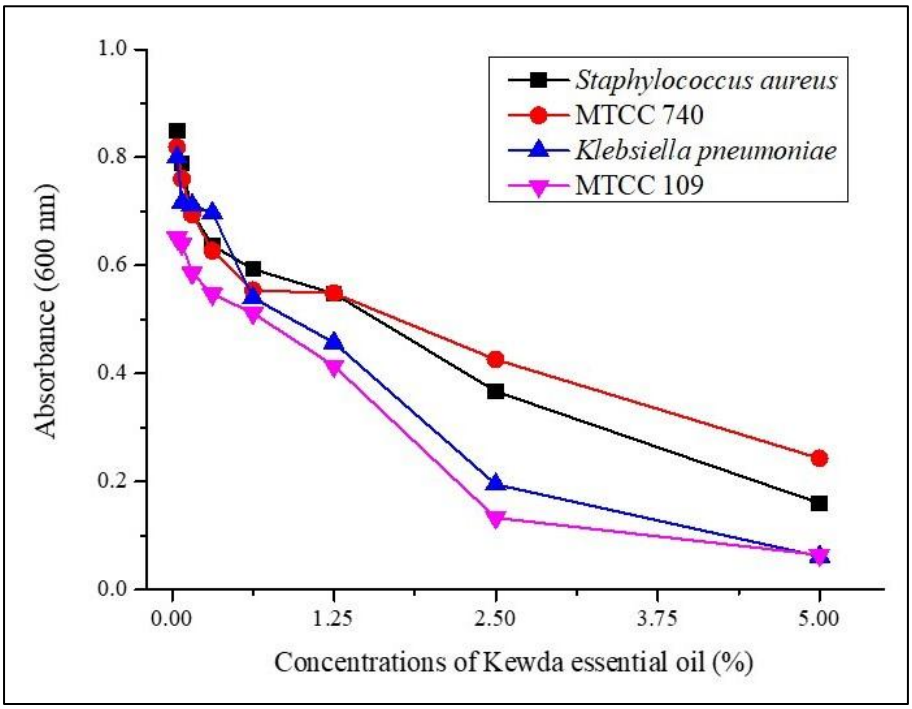


Figure 4.16. Minimum inhibitory concentration of Kewda essential oil against clinical isolates of *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109.

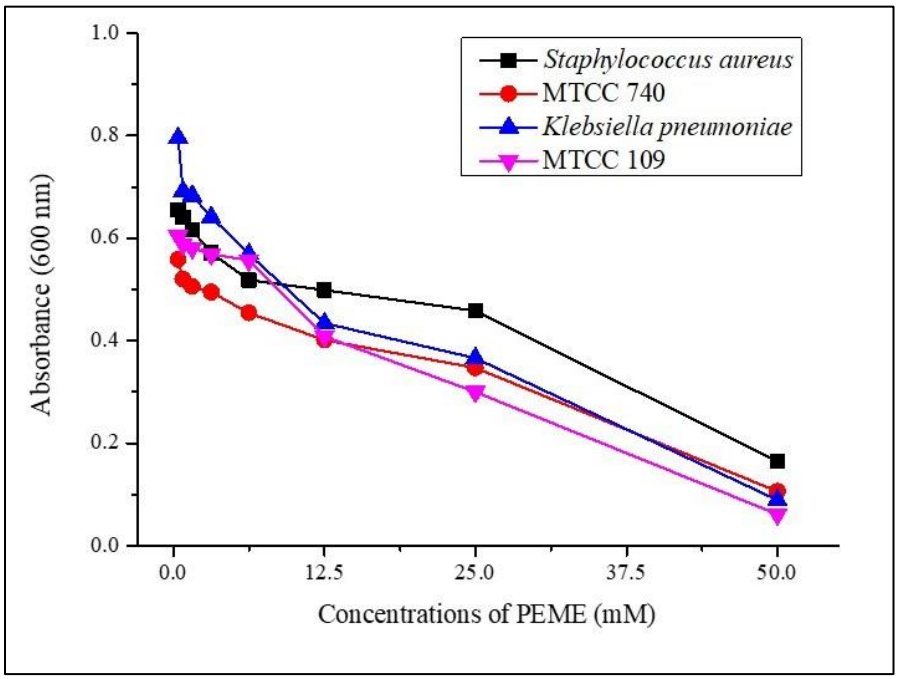


Figure 4.17. Minimum inhibitory concentration of PEME against clinical isolates of *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109.

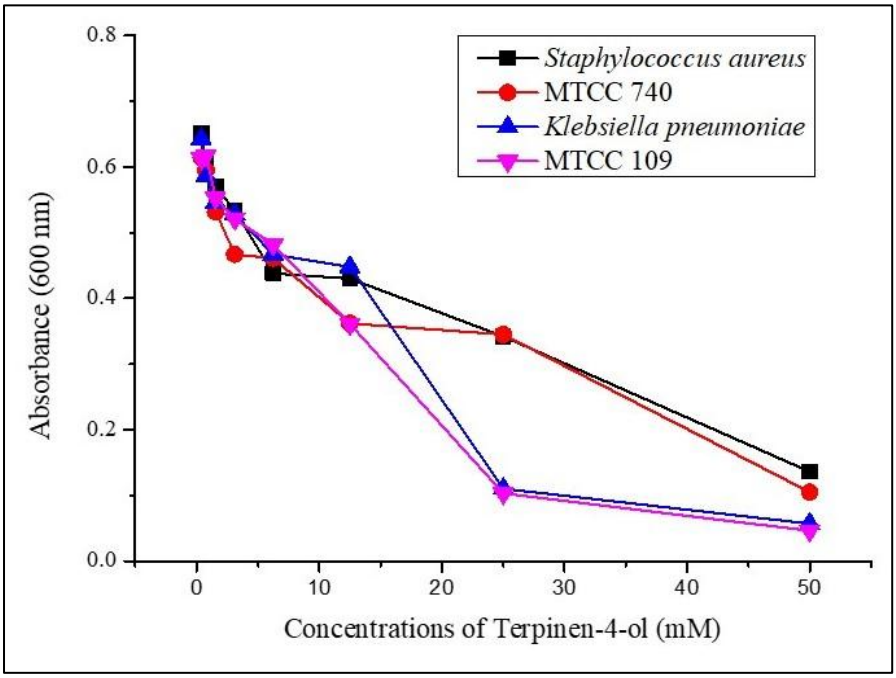


Figure 4.18. Minimum inhibitory concentration of Terpinen-4-ol against clinical isolates of *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains MTCC 740 and MTCC 109.

4.6.2. Antibacterial activity using Agar well diffusion assay

The results demonstrated that Kewda essential oil exhibited strong antibacterial activities against both *K. pneumoniae* and its reference strain MTCC 109 with zone of inhibition of 17 and 22 mm, respectively at MIC level. Meanwhile, Terpinen-4-ol (at MIC level) showed promising antibacterial properties against both the reference strains i.e. MTCC 740 and MTCC 109 with a zone of inhibition 16 and 14 mm, respectively (Figure 4.19). Compared to Kewda essential oil and Terpinen-4-ol, PEME exhibited comparatively reduced antibacterial effect on the tested bacteria (Figure 4.19). The widespread antibacterial activities of Terpinen-4-ol as evident from agar well diffusion assay was in accordance with earlier results depicting its antibacterial properties against different isolates of *S. aureus* (Cordeiro *et al.*, 2020).

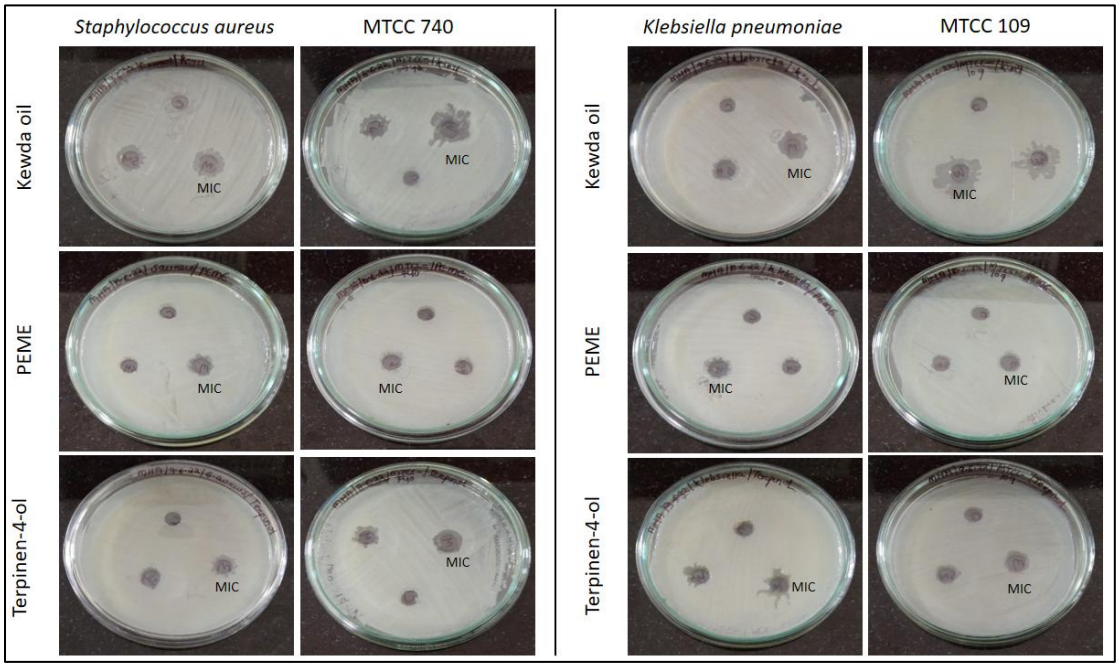


Figure 4.19. Effect of Kewda essential oil, PEME, and Terpinen-4-ol (at MIC level) on the growth of clinical bacterial isolates, *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC 740 and MTCC 109.

4.7. Determination of anti-biofilm activities of Kewda essential oil, PEME and Terpinen-4-ol

4.7.1. Qualitative biofilm formation assay (Congo red agar method)

From the congo red agar plate assay, it was observed that in the untreated control of both clinical *S. aureus* and MTCC 740, crystalline black colonies were formed depicting the profuse production of exopolysaccharide matrix which corresponds to the

formation of biofilm (Figure 4.20). When both clinical *S. aureus* and MTCC 740 were grown in presence of sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol; a significant decrease in the exopolysaccharide matrix production was noticed, indicating inhibition to the biofilm forming ability of tested bacteria. The results were in accordance with the earlier report depicting the gradual reduction in exopolysaccharides production when Methicillin resistant *S. aureus* were treated with Thymol, an active constituent of *Thymus vulgaris* essential oil (Valliammai *et al.*, 2020).

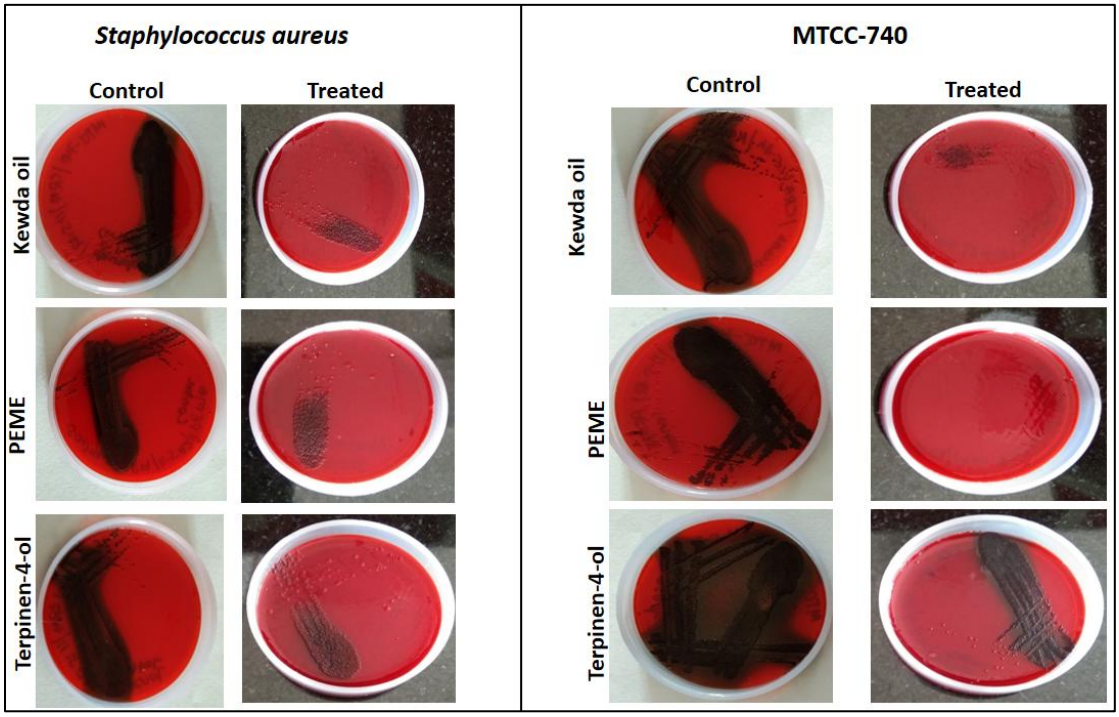


Figure 4.20. Effect of sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and its reference strain MTCC 740 using Congo red agar plate method.

From the congo red agar plate assay, it was observed that in the untreated control of both clinical *K. pneumoniae* and MTCC 109, crystalline black colonies were formed depicting the profuse production of exopolysaccharide matrix which corresponds to the formation of biofilm (Figure 4.21). When both clinical *K. pneumoniae* and MTCC 109 were grown in presence of sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol; a significant decrease in the exopolysaccharide matrix production was found indicating inhibition to biofilm forming ability of tested bacteria.

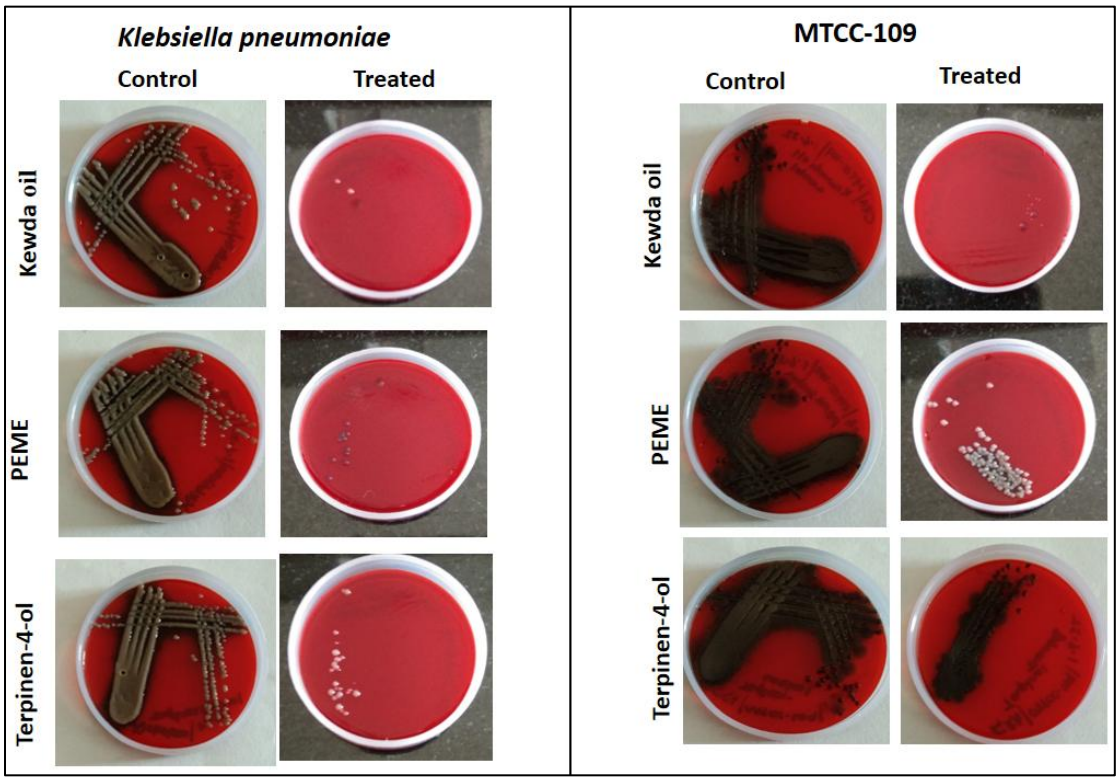


Figure 4.21. Effect of sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol on biofilm formation in clinical *Klebsiella pneumoniae* and its reference strain MTCC 109 using Congo red agar plate method.

4.7.2. Biofilm formation assay (Tube method)

From the tube method, it was observed that in the untreated control of both clinical *S. aureus* and MTCC 740, biofilm matrix were formed depicting the profuse production of exopolysaccharide matrix on the wall of the test tube (Figure 4.22). When both clinical *S. aureus* and MTCC 740 were grown in presence of sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol; a significant decrease in the biofilm formation was observed due to reduced adherence capabilities of test pathogens owing to decrease in the exopolysaccharide matrix production. Similar trends were also observed when clinical *K. pneumoniae* and MTCC 109 treated with sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol. A significant reduction in the attachment of crystal violet stained sessile bacterial cells (both) on the wall of test tube was observed as compared to untreated control (Figure 4.23).

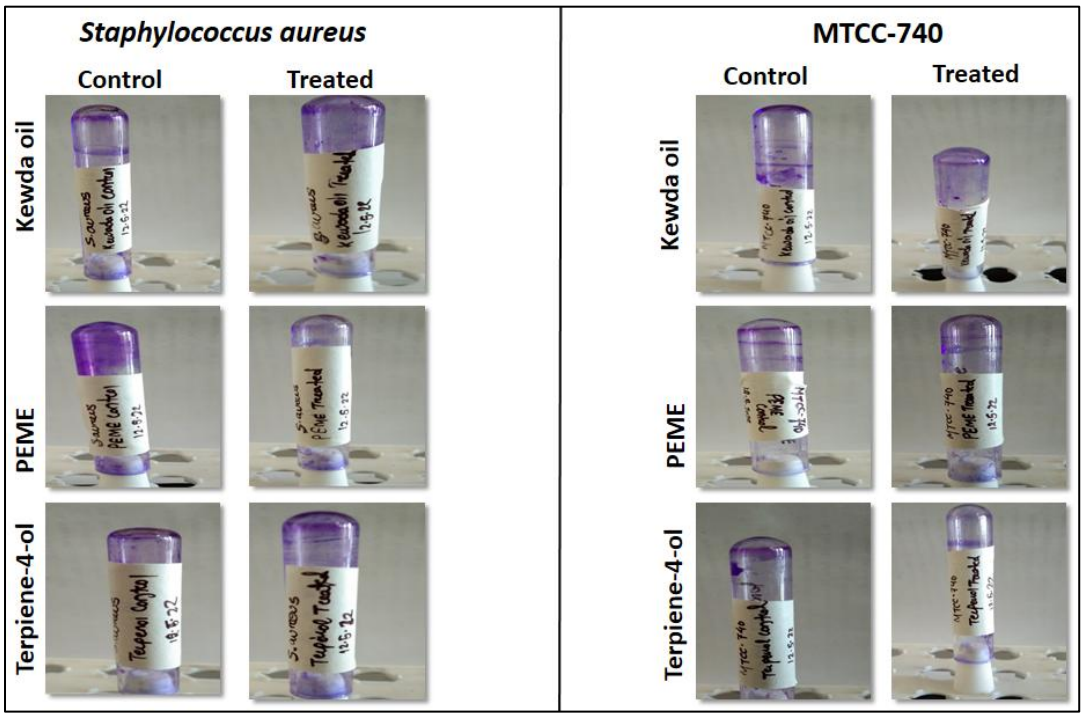


Figure 4.22. Effect of sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and its reference strain MTCC 740 using test tube method.

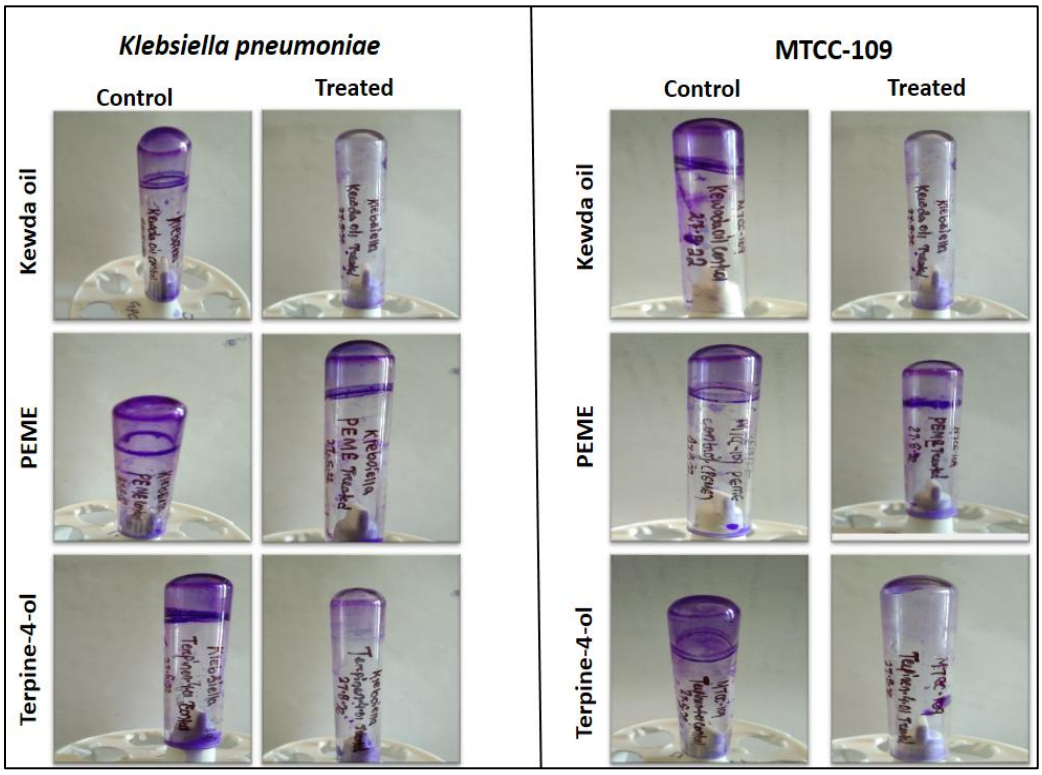


Figure 4.23. Effect of sub-MICs of Kewda essential oil, PEME and Terpinen-4-ol on biofilm formation in clinical *Klebsiella pneumoniae* and its reference strain MTCC 109 using test tube method.

4.7.3. Quantitative biofilm formation assay (Crystal violet method)

As evident from quantitative biofilm formation using crystal violet staining method, all the test samples significantly inhibited the biofilm formation in clinical bacterial isolates (*S. aureus* and *K. pneumoniae*) and their reference strains MTCC 740 and MTCC 109 (Figure 4.24, 4.25). Among the test samples, Terpinen-4-ol exhibited highest inhibitory effect on biofilm formation on *S. aureus*, whereas PEME showed highest inhibitory effect on *K. pneumoniae* with an inhibition of 67.51±1.29 and 61.17± 3.75 %, respectively. Meanwhile, PEME showed highest inhibitory effect on biofilm formation in both the reference strains (i.e. MTCC 740 and MTCC 109) with a reduction of 71.76±4.56 and 59.3±6.24%, respectively. The results of Terpinen-4-ol in biofilm inhibition was in accordance with the report depicting the anti-biofilm activities of Terpinen-4-ol against several clinical isolates of *S. aureus* (Cordeiro *et al.*, 2020).

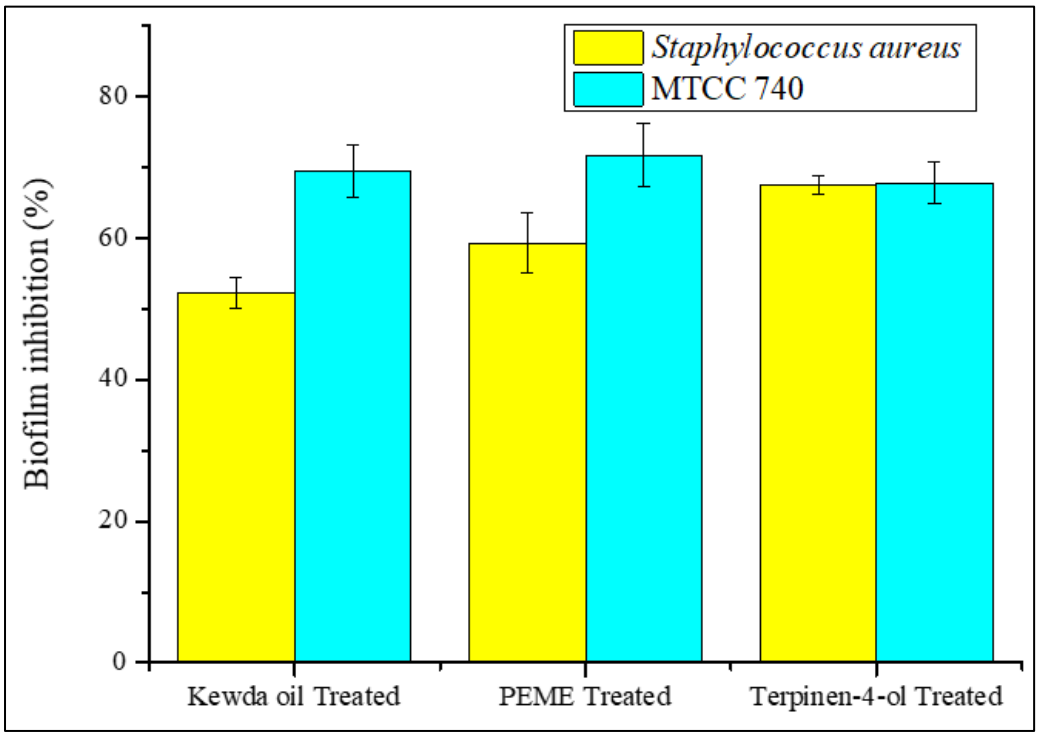


Figure 4.24. Effect of sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and its reference strain MTCC 740 using quantitative crystal violet staining method.

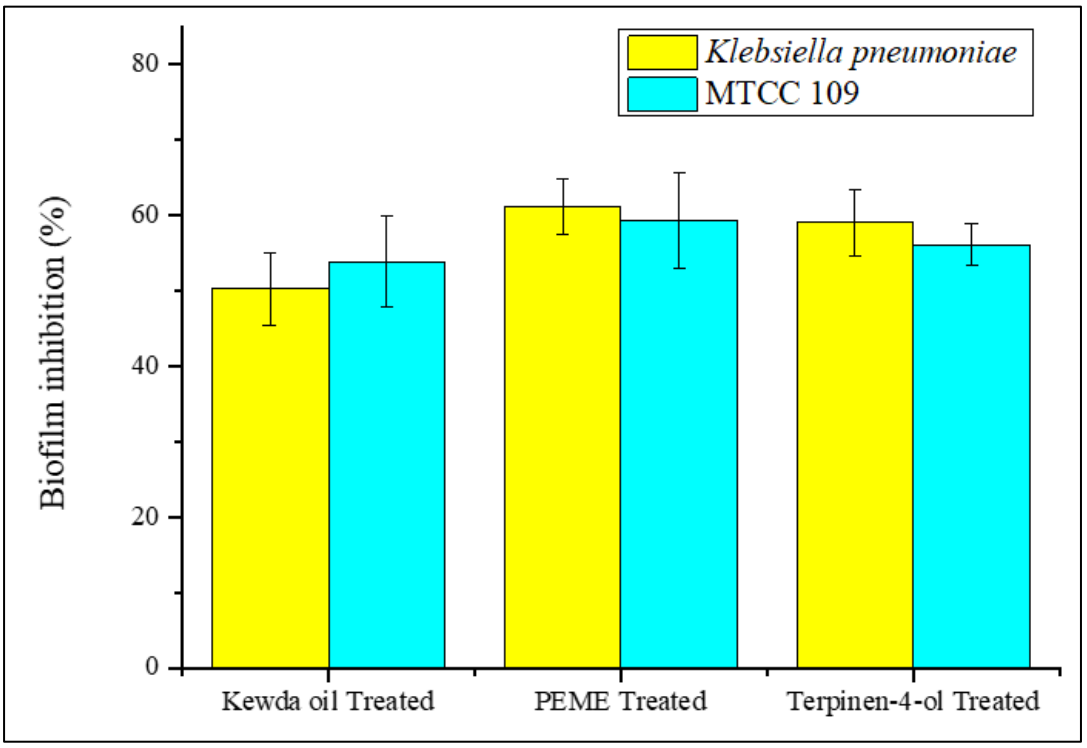


Figure 4.25. Effect of sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol on biofilm formation in clinical *Klebsiella pneumoniae* and its reference strain MTCC 109 using quantitative crystal violet staining method.

4.7.4. Anti-biofilm activities using microscopic analysis

From the light microscopic analysis, it was observed that a significant decrease in the density and thickness of the biofilm matrix formed onto glass coverslips in clinical isolates of *S. aureus* as well as its reference strain MTCC 740 when grown in presence of Kewda essential oil, PEME, and Terpinen-4-ol at their sub-MIC levels. The sessile bacterial cells were found to be comparatively scattered compared to thick assemblage of cells in untreated control (Figure 4.26). Similar trend was also observed on treatment with sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol against *K. pneumoniae* and MTCC 109 (Figure 4.27). The results were in accordance with the report where Cinnamon bark oil and its bioactive constituents significantly altered the biofilm formation in multidrug resistant *P. aeruginosa* (Kim *et al.*, 2015).

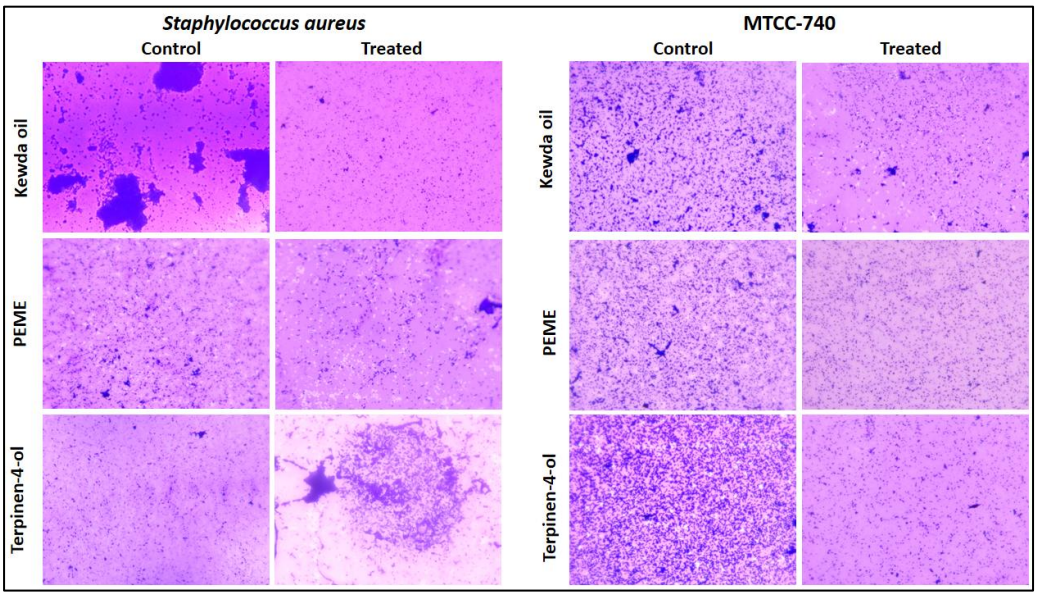


Figure 4.26. Light microscopic observations on the effect of sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and its reference strain MTCC 740.

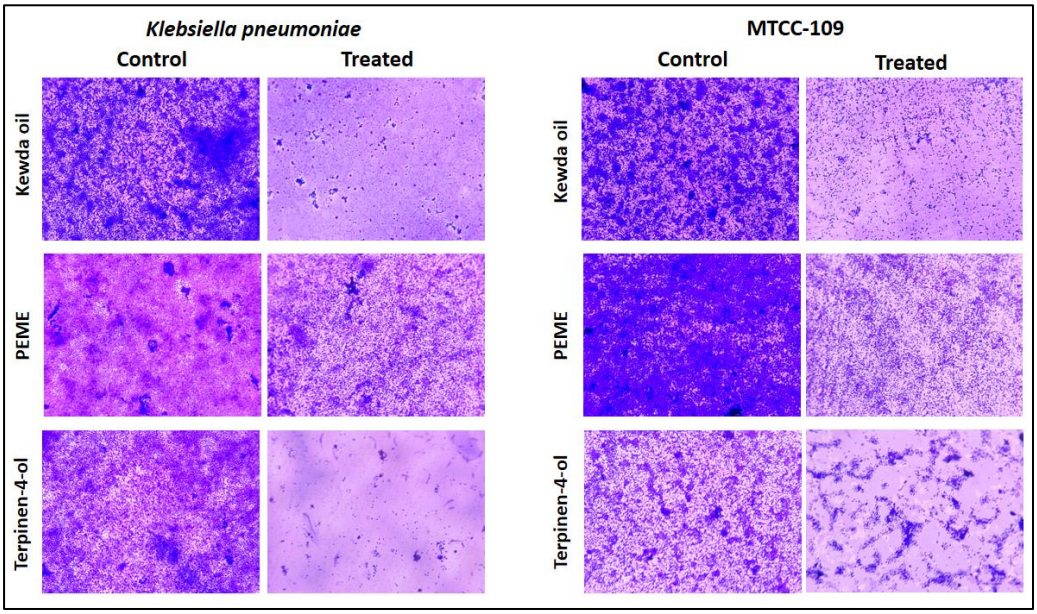


Figure 4.27. Light microscopic observations on the effect of sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol on biofilm formation in clinical *Klebsiella pneumoniae* and its reference strain MTCC 109.

Fluorescence microscopic studies on biofilms formed by clinical isolates of *S. aureus* and its reference strain MTCC 740 when supplemented with sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol revealed the reduction in aggregation of biofilm forming cells with disperse localization compared to aggregate biofilm mass as observed in untreated control (Figure 4.28). Similarly, when clinical isolate of *K. pneumoniae* and

its reference strain, MTCC 109 grown in the media supplemented with sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol; concomitant decrease in the aggregation of biofilm cells were observed. Meanwhile, in the untreated control, thickened and more aggregated biofilms were observed suggesting the anti-biofilm activities of test samples (Figure 4.29).

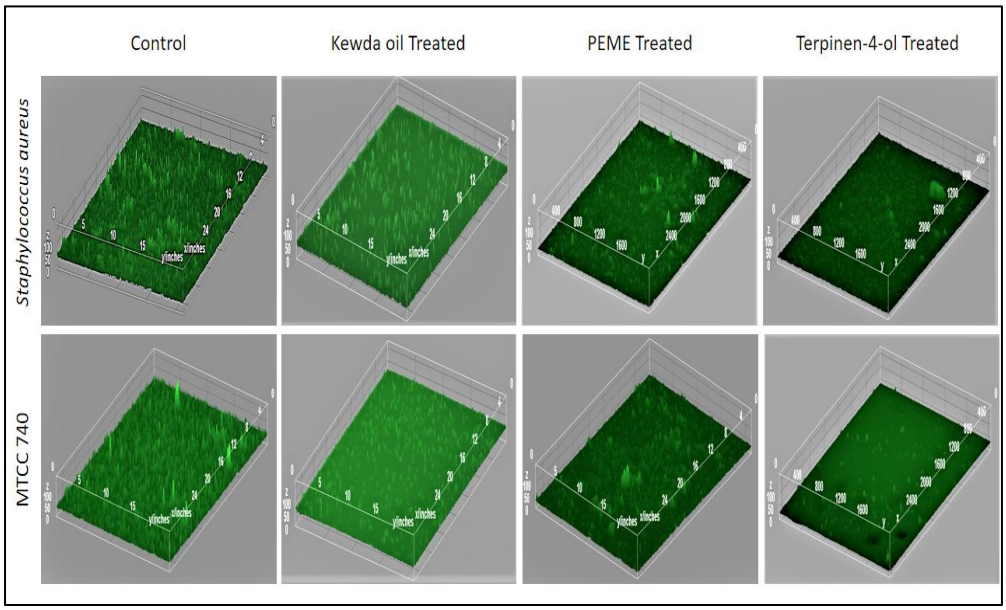


Figure 4.28. Fluorescence microscopic observations on the effect of sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol on biofilm formation in clinical *Staphylococcus aureus* and its reference strain MTCC 740.

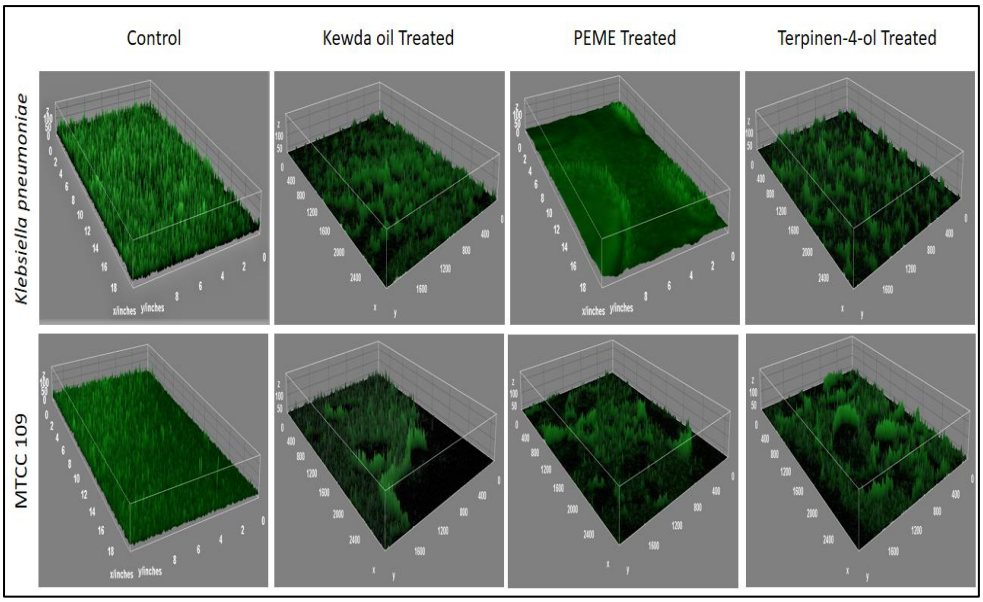


Figure 4.29. Fluorescence microscopic observations on the effect of sub-MICs of Kewda essential oil, PEME, and Terpinen-4-ol on biofilm formation in clinical *Klebsiella pneumoniae* and its reference strain MTCC 109.

4.8 Transcriptome analysis

Total 18.5 GB raw data of *Staphylococcus aureus* (G-Control_1 and G-Control_2) and *Staphylococcus aureus* treated with PEME (G-PEM_1 and G-PEM_2) were used to quality check and trimming for accuracy. The basic statistical percentage was passed the quality check. Table 1 shows the statistical summary of transcriptome sequencing.

Table 4.10. Statistical summary of Quality control of transcript sequencing

Filename	G-Control_1.fastq	G-Control_2.fastq	G-PEM_1.fastq	G-PEM_2.fastq
Total Sequences	10379840	10390243	15124576	15141921
Encoding	Sanger/ Illumina 1.9	Sanger/ Illumina 1.9	Sanger/ Illumina 1.9	Sanger/ Illumina 1.9
Sequence length	18-151	151	18-151	151
%GC	57	56	58	57
Basic Statistics	pass	pass	pass	pass

The *Staphylococcus aureus* genome was used as reference to align the reads by using the HISAT2 software. About 99.98% and 100.00% reads obtained from the G-Control_1 and G-Control_2 were mapped to the reference genome, whereas 100.00% reads obtained from the G-PEME_1 and G-PEME_2 were mapped to the reference genome. The statistical summary of the mapping results is shown in Table 2.

Table 4.11. Statistical summary of the mapping results.

	Staphylococcus aureus		***Staphylococcus aureus*/PEME**	
	G-Control_1	G-Control_2	G-PEME_1	G-PEME_2
Total reads	10379840 (100.00%)	10390243 (100.00%)	15124576 (100.00%)	15141921 (100.00%)
Aligned 0 times	10378001 (99.98%)	10390136 (100.00%)	15123875 (100.00%)	15141627 (100.00%)
Aligned exactly 1 time	88 (0.00%)	72 (0.00%)	232 (0.00%)	224 (0.00%)
Aligned >1 times	1751 (0.02%)	35 (0.00%)	469 (0.00%)	70 (0.00%)

Table 4.12. Statistical summary of the prediction of essential genes by comparison with data base of essential genes (DEGs).

	P VALUE	FOLD CHNAGE (log2)	DEGS
Total significant genes	Pvalue < 0.5		1024
Total up regulated genes	Pvalue < 0.5	>=0.8	9
Total down regulated genes	Pvalue < 0.5	<=-0.8	16

The cufflinks package was used to calculate the transcript abundances. The FPKM were estimated and the Cuffdiff package was used to find the different DEGs. The p value was adjusted using the q value. A q value < 0.5 and log2 (fold change) > 0.8 (UP regulated gene) < -0.8 (Down regulated gene) was set as the threshold for significantly differential expression. Table 3 shows the calculation of number of DEGs find out.

4.8.1 PPI network analysis

Protein-protein interaction network analysis was performed taking into consideration of up regulated essential genes (n=9) and constructed the PPI network. From the PPI network analysis it was found that six genes were involved in different signal pathway and were integrated using STRING website to explore the association between these DEGs. The gene glms and SAXN108_1366 showing the interaction with each other (Figure 4.30).

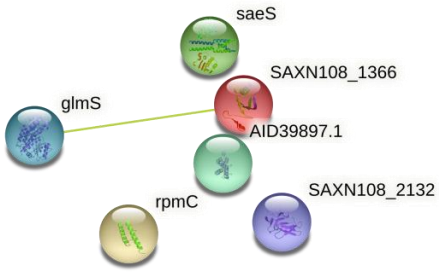


Figure 4.30. Protein-protein interaction network analysis of up regulated proteins from *Staphylococcus aureus* treated with PEME showing the interaction between two proteins, glmS and SAXN108_1366.

Similarly, the protein-protein interaction network analysis for the down regulated genes (n=16), were used to construct the PPI network and the genes involved in different signal pathway were integrated using STRING website to explore the association between these DEGs. In addition the interactions between the proteins involved in biofilm produce were also deciphered and included in Figure 4.31.

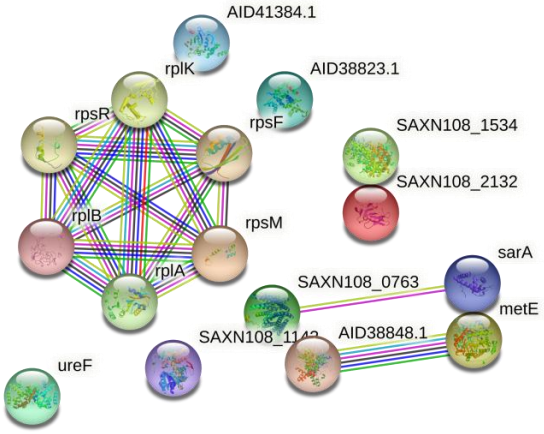


Figure 4.31. Protein-protein interaction network analysis of down regulated proteins from *Staphylococcus aureus* treated with PEME showing the interaction between different proteins involved in bioflim production.

4.8.2 Functional annotations of up regulated and down regulated genes

Among the nine up-regulated genes, functional annotations of only five genes were available and were collated in Table 1. Similarly, among the sixteen down-regulated genes, only four genes such as *agrA*, *sarA*, *norA* and *mopR* are directly associated with the pathogenesis of *S. aureus* through quorum sensing interactions and biofilm formation. Hence, treatment with PEME, the down regulation of above-mentioned genes suggested the influence of PEME on regulation of quorum sensing associated virulence and mitigation of biofilm formation (Table 2).

Table 4.13. Functional annotation of up-regulated gens of *Staphylococcus aureus* identified with the treatment of 2-Phenylethyl methyl ether (PEME).

Sl. No.	Gene	Biological process	GO Term ID	Molecular function	GO Term ID	Cellular component	GO Term ID
1.	AID39897.1	Pathogenesis	GO:0009405	Nucleic acid binding	GO:0003676	Integral component of membrane	GO:0016021
				Catalytic activity	GO:0003824		
		Interspecies interaction between organisms	GO:0044419	Endonuclease activity	GO:0004519	Cell periphery	GO:0071944
				Hydrolase activity	GO:0016787	Cellular anatomical entity	GO:0110165
2.	SAXN108_1366	Metabolic process	GO:0006082	Nucleic acid binding	GO:0003676	Intracellular	GO:0005622
		Fatty acid metabolism,	GO:0006631	Catalytic activity	GO:0003824	Cytoplasm	GO:0005737
		Carboxylic acid metabolic process	GO:0019752	Lyase activity	GO:0016829	Cytosol	GO:0005829
		Cellular respiration	GO:0045333	Hydrolyase activity	GO:0016836	Cellular anatomical entity	GO:0110165
3.	*glmS*	Metabolic process	GO:0008152	Catalytic activity	GO:0003824	Intracellular	GO:0005622
		Biosynthetic process	GO:0009058	Transferase activity	GO:0016740	cytoplasm	GO:0005737
		Nitrogen compound metabolic process	GO:0006807				
		Carboxylic acid metabolic process	GO:0019752			Cellular anatomical entity	GO:0110165
		Cellular process	GO:0009987				
4.	*rpmC*	Metabolic process	GO:0008152	Structural constituent of ribosome	GO:0003735	Intracellular	GO:0005622
		Biosynthetic process	GO:0009058			Cytoplasm	GO:0005737
		Gene expression	GO:0010467	Structural molecule activity	GO:0005198	Cellular anatomical entity	GO:0110165
		Translation	GO:0006412			Large ribosomal subunit	GO:0015934

5.	*saeS*	Phosphorelay signal transduction system	GO:0000160	Protein kinase activity	GO:0004672	Integral component of membrane	GO:0016021
		Protein phosphorylation	GO:0006468	Transferase activity	GO:0016740	Cell periphery	GO:0071944
		Signal transduction	GO:0007165				
		Pathogenesis	GO:0009405	Phosphorelay sensor kinase activity	GO:0000155	Cellular anatomical entity	GO:0110165
		Intracellular signal transduction	GO:0035556				

Table 4.14. Functional annotation of down-regulated genes of *Staphylococcus aureus* identified with the treatment of 2-Phenylethyl methyl ether (PEME).

Sl. No.	Gene	Biological process	GO Term ID	Molecular function	GO Term ID	Cellular component	GO Term ID
1.	*agrA* (SAXN108_2132)	Phosphorelay signal transduction system	GO:0000160	Nucleic acid binding	GO:0003676	Intracellular	GO:0005622
		Cell communication	GO:0007154				
		Signal transduction	GO:0007165	Binding	GO:0005488	Cytoplasm	GO:0005737
		Cellular process	GO:0009987				
		Signaling	GO:0023052	Organic cyclic compound binding	GO:0097159	Cellular anatomical entity	GO:0110165
		Intracellular signal transduction	GO:0035556				
2.	*mepR* (AID38823.1)	Regulation of transcription, DNA-templated	GO:0006355	Nucleic acid binding	GO:0003676	Intracellular	GO:0005622
		Regulation of gene expression	GO:0010468				
		Regulation of metabolic process	GO:0019222	Organic cyclic compound binding	GO:0097159	Cytoplasm	GO:0005737
		Regulation of biological process	GO:0050789				
		Regulation of cellular	GO:0050794	Transcription	GO:0140110	Cellular	GO:0110165

		process		regulator activity		anatomical entity	
		Biological regulation	GO:0065007				
3.	SAXN108_0407	Metabolic process	GO:0008152	Binding	GO:0005488	Intracellular	GO:0005622
		Cellular process	GO:0009987	Transferase activity	GO:0016740	Cytoplasm	GO:0005737
		Cellular metabolic process	GO:0044237	Catalytic activity	GO:0003824	Cellular anatomical entity	GO:0110165
4.	*norA* (SAXN108_0763)	Transport	GO:0006810	Transporter activity	GO:0005215	Plasma membrane	GO:0005886
		Cellular process	GO:0009987			Integral component of membrane	GO:0016021
		Response to antibiotic	GO:0046677	Transmembrane transporter activity	GO:0022857	Cell periphery	GO:0071944
		Transmembrane transport	Transmembrane transport			Cellular anatomical entity	GO:0110165
5.	SAXN108_1143	Metabolic process	GO:0008152	Catalytic activity	GO:0003824	Plasma membrane	GO:0005886
		Cellular process	GO:0009987	Binding	GO:0005488	Membrane	GO:0016020
		Cellular metabolic process	GO:0044237	Electron transfer activity	GO:0009055	Cell periphery	GO:0071944
		Cellular respiration	GO:0045333	Organic cyclic compound binding	GO:0097159	Cellular anatomical entity	GO:0110165
6.	SAXN108_1534	Metabolic process	GO:0008152	Catalytic activity	GO:0003824	Intracellular	GO:0005622
		Cellular process	GO:0009987	Binding	GO:0005488	Cytoplasm	GO:0005737
		Cellular metabolic process	GO:0044237	Organic cyclic compound binding	GO:0097159	Cytosol	GO:0005829
		Primary metabolic process	GO:0044238	Oxidoreductase activity	GO:0016491	Cellular anatomical entity	GO:0110165
7.	*metE* (SAXN108_0406)	Metabolic process	GO:0008152	Catalytic activity	GO:0003824	Intracellular	GO:0005622
		Cellular process	GO:0009987	Binding	GO:0005488	Cytoplasm	GO:0005737
		Cellular metabolic process	GO:0044237	Transferase activity	GO:0016740	Cytosol	GO:0005829
		Small molecule biosynthetic process	GO:0044283	Metal ion binding	GO:0046872	Cellular anatomical entity	GO:0110165
8.	*rplA* (SAXN108_0592)	Metabolic process	GO:0008152	Nucleic acid binding	GO:0003676	Intracellular	GO:0005622

		Cellular process	GO:0009987	Structural constituent of ribosome	GO:0003735	Cytoplasm	GO:0005737
		Cellular metabolic process	GO:0044237	Organic cyclic compound binding	GO:0097159	Cytosol	GO:0005829
		Biological regulation	GO:0065007	Heterocyclic compound binding	GO:1901363	Cellular anatomical entity	GO:0110165
9.	*rplB* (SAXN108_2496)	Metabolic process	GO:0008152	Nucleic acid binding	GO:0003676	Intracellular	GO:0005622
		Cellular process	GO:0009987	Catalytic activity	GO:0003824	Cytoplasm	GO:0005737
		Cellular metabolic process	GO:0044237	Transferase activity	GO:0016740	Cytosol	GO:0005829
		Gene expression	GO:0010467	Heterocyclic compound binding	GO:1901363	Cellular anatomical entity	GO:0110165
10.	*rplK* (SAXN108_0591)	Metabolic process	GO:0008152	Nucleic acid binding	GO:0003676	Intracellular	GO:0005622
		Cellular process	GO:0009987	Binding	GO:0005488	Cytoplasm	GO:0005737
		Cellular metabolic process	GO:0044237	Heterocyclic compound binding	GO:1901363	Cytosol	GO:0005829
		Cellular component biogenesis	GO:0044085	Organic cyclic compound binding	GO:0097159	Cellular anatomical entity	GO:0110165
11.	*sarA* (SAXN108_0683)	Pathogenesis	GO:0009405	Nucleic acid binding	GO:0003676	Intracellular	GO:0005622
		Regulation of biosynthetic process	GO:0009889	Binding	GO:0005488	Cytoplasm	GO:0005737
		Regulation of gene expression	GO:0010468	Heterocyclic compound binding	GO:1901363		
		Interspecies interaction between organisms	GO:0044419	Organic cyclic compound binding	GO:0097159	Cellular anatomical entity	GO:0110165

Table 4.15. KEGG pathways analysis of up and down-regulated genes of *Staphylococcus aureus* with treatment of 2-Phenylethyl methyl ether (PEME).

UP-REGULATED GENES			**DOWN_REGULATED GENES**		
Gene name	**KEGG_ID**	**Pathways**	**Gene name**	**KEGG_ID**	**Pathways**
glmS	sae01250	Biosynthesis of nucleotide sugars	*metE*	sae01100	Metabolic pathways
	sae00250	Alanine, aspartate and glutamate metabolism		sae01230	Biosynthesis of amino acids
	sae00520	Amino sugar and nucleotide sugar metabolism		sae00270	Cysteine and methionine metabolism
	sae01100	Metabolic pathways		sae00450	Selenocompound metabolism
rpmC	sae03010	NWMN_2150 (rplW) NWMN_2141 (rplX) NWMN_2144 (rpmC) NWMN_2134 (rpmD) NWMN_2151 (rplD) NWMN_0014		sae01110	Biosynthesis of secondary metabolites
saeS	sae02020	Two-component system	*rplB*/*rpsM*	sae03010	Ribosome
			agrA	sae00261	Monobactam biosynthesis
				sae01210	2-Oxocarboxylic acid metabolism
				sae01220	Degradation of aromatic compounds
				sae02024	Quorum sensing
				sae02020	Two-component system
			norA	sae01110	Biosynthesis of secondary metabolites
				sae02020	Two-component system
				sae00350	Tyrosine metabolism
				sae02024	Quorum sensing
				sae01100	Metabolic pathways

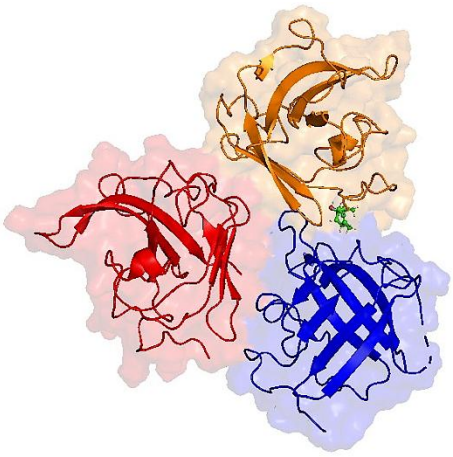
4.9 Molecular docking of Terpinen-4-ol and PEME with selected proteins

The binding site of Terpinen-4-ol and PEME onto the proteins involve in pathogenesis, bioflim 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 protein; PDB ID: 4G4K), MepR (transcriptional regulator of multidrug efflux pump, PDB ID: 3ECO) and Rot (global regulator of virulence genes; PDB ID: 4Q77) are not known due to not availability of its co-crystal structure with these proteins. Hence, we followed the blind docking approach to dock Terpinen-4-ol and PEME to its suitable binding site on tubulin. In this approach, we have considered all the predicted binding sites of proteins for the docking of Terpinen-4-ol and PEME, followed by evaluation of their docking score against all the binding sites. The binding site against which the docking score is lowest minimum was considered to be the putative binding site for Terpinen-4-ol and PEME (Table 4.16 and 4.17). Terpinen-4-ol revealed the lowest minimum docking score of -4.405 Kcal/mol and docking energy of -21.86 Kcal/mol with Sortease A (a surface associated protein), followed with a docking score of -4.025 Kcal/mol and docking energy of -21.24 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 4.16). Terpinen-4-ol was found to accommodate well inside the binding cavity (Figure 4.32). 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 4.32D), two hydrogen bonds with the amino acids Thr 117A and Leu 160B of 2FNP (Figure 3.2E), one hydrogen bond with His 35A of 3ECO (Figure 4.32F), two hydrogen bonds with amino acid Gln 48B of 4G4K (Figure 4.32I) and two hydrogen bonds with amino acids, Thr 117A and Leu 160B of 4Q77 (Figure 4.32J). Besides hydrogen bonding the binding of Terpinen-4-ol involved many hydrophobic interactions with the binding site amino acids.

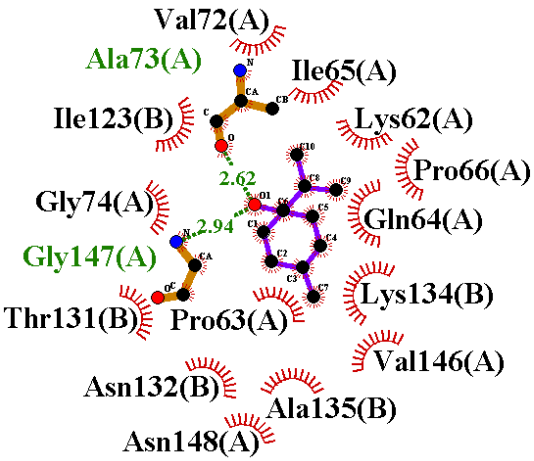
Table 4.16. Docking results of Terpinen-4-ol with respect to different binding sites onto the proteins involve in pathogenesis, bioflim production and drug resistance of *S. aureus*.

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
(d) 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
(e) 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
(f) 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**

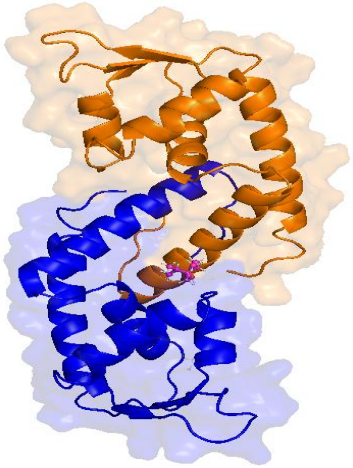
(A) Complex of Terpinen-4-ol and 1T2P



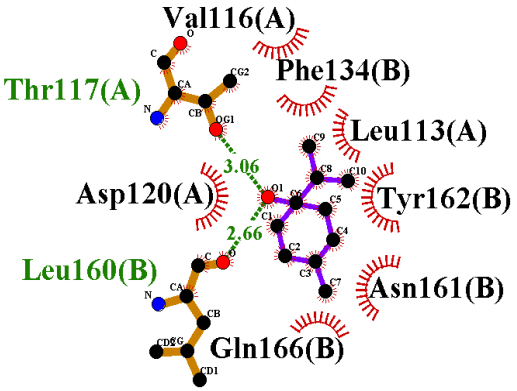
(D) Ligplot of Terpinen-4-ol and 1T2P



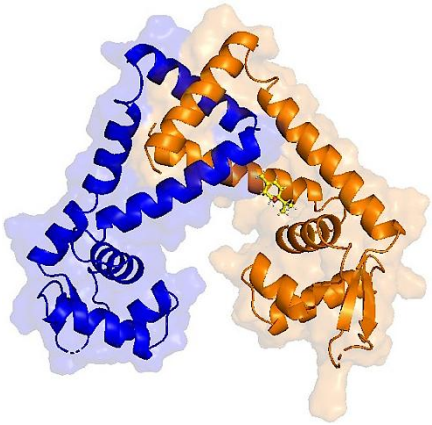
(B) Complex of Terpinen-4-ol and 2FNP



(E) Ligplot of Terpinen-4-ol with 2FNP



(C) Complex of Terpinen-4-ol and 3ECO



(F) Ligplot of Terpinen-4-ol with 3ECO

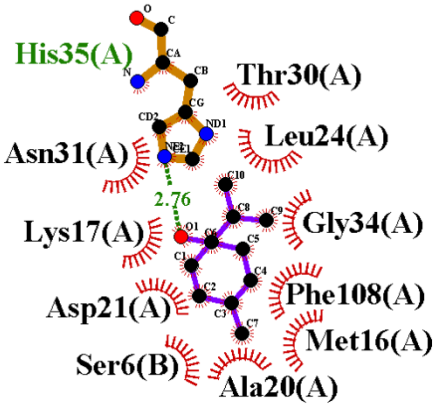
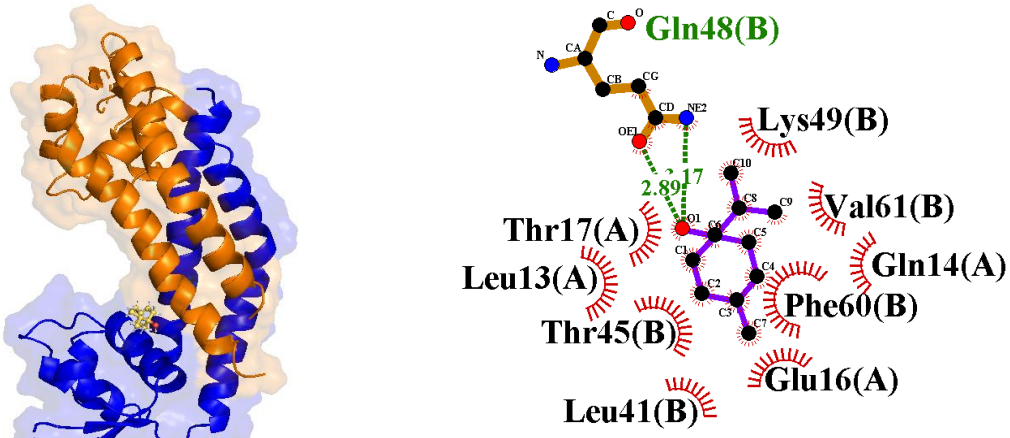


Figure 4.32. Molecular docking of Terpinen-4-ol onto the proteins involve in pathogenesis, bioflim production and drug resistance of *S. aureus*. Terpinen-4-ol is well accommodated inside the binding site of (A) 1T2P, (B) 2FNP and (C) 3ECO. The ligplot analysis of Terpinen-4-ol showing the interactions 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.

(G) Complex of Terpinen-4-ol and 4G4K **(I) Ligplot of Terpinen-4-ol and 4G4K**



(H) Complex of Terpinen-4-ol and 4Q77 **(J) Ligplot of Terpinen-4-ol with 4Q77**

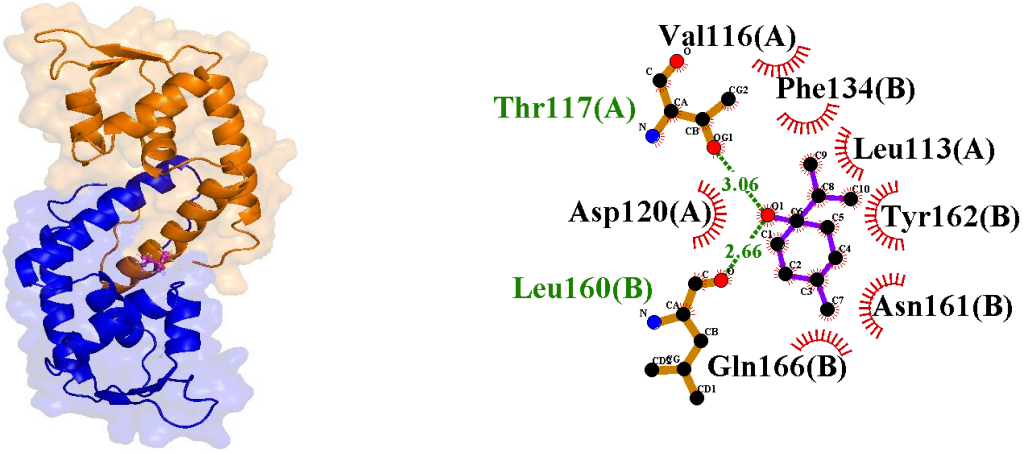


Figure 4.33. Molecular docking of Terpinen-4-ol onto the proteins involve in pathogenesis, bioflim production and drug resistance of *S. aureus*. Terpinen-4-ol is well accommodated inside the binding site of (G) 4G4K and (H) 4Q77. The ligplot analysis of Terpinen-4-ol showing the interactions with binding site amino acids of (I) 4G4K and (J) 4Q77. Its binding involved both hydrogen bonds represented as dotted (green) lines and hydrophobic interactions denoted with curved (red) lines.

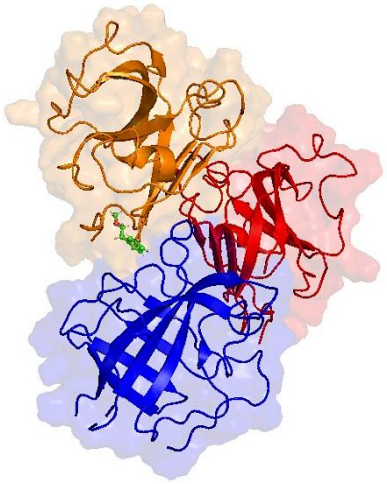
Similarly, PEME revealed the lowest minimum docking score of -3.757 Kcal/mol and docking energy of -18.129 Kcal/mol with AgrA, a transcriptional regulator protein, followed with a docking score of -3.559 Kcal/mol and docking energy of -17.803 Kcal/mol with MepR, a transcriptional regulator of multidrug efflux pump; docking score of -3.342 Kcal/mol and docking energy of -22.499 Kcal/mol with Sortase A, a surface associated protein. In contrast, the docking score and docking energy of PEME with other proteins such as SarA, a global regulatory protein docking score -2.684 Kcal/mol and docking energy -19.25 Kcal/mol) and Rot, a global regulator of virulence genes (docking score -2.526 Kcal/mol and docking energy -17.633 Kcal/mol) were found to be minimum (Table 4.17).

Table 4.17. Docking results of PEME with respect to different binding sites onto the proteins involve in pathogenesis, bioflim production and drug resistance of *S. aureus*.

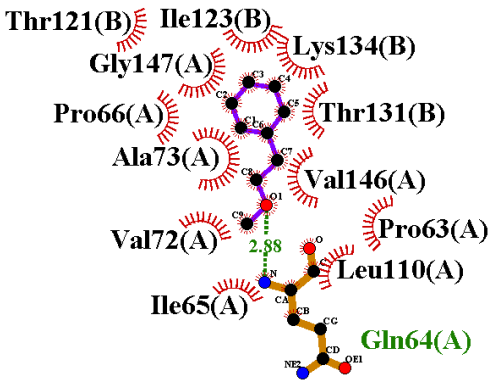
Site ID	Site score	Volume (Å)^3	Glide XP score (Kcal/mol)	Glide docking energy (Kcal/mol)
(a) PDB ID: 1T2P (Sortase A, a surface associated protein)				
1	1.023	482.2	-2.889	-18.857
2	0.959	415.4	-3.309	-19.783
3	1.049	342.6	-2.244	-21.089
4	0.875	220.5	-2.041	-15.240
5	0.786	116.3	-2.616	-16.980
6	0.790	110.8	-1.888	-15.487
7	0.651	105.6	**-3.342**	**-22.499**
8	0.688	92.27	-2.609	-20.189
(b) PDB ID: 2FNP (SarA, a global regulatory protein)				
1	0.941	142.7	-2.072	-18.339
2	0.853	148.2	-2.576	-18.839
3	0.780	128.3	**-2.684**	**-19.25**
4	0.648	75.12	-3.172	-15.202
5	0.727	162.6	-1.219	-16.987
6	0.599	61.05	-2.290	-16.743
7	0.755	76.15	-4.023	-12.138
8	0.626	58.99	-2.175	-15.914
(d) PDB ID: 3ECO (MepR, a transcriptional regulator of multidrug efflux pump)				
1	0.944	262.7	**-3.559**	**-17.803**
2	0.977	248.3	-3.441	-17.008
(e) PDB ID: 4G4K (AgrA, a transcriptional regulator)				
1	1.027	531.6	**-3.757**	**-18.129**
2	0.609	78.55	-2.254	-16.783
(f) PDB ID: 4Q77 (Rot, a global regulator of virulence genes)				
1	0.663	130.7	-2.526	-17.633
2	0.530	45.62	-1.940	-18.192
3	0.574	101.5	**-1.149**	**-21.606**

PEME was found to accommodate well inside the binding cavity (Figure 4.33). The binding of PEME involved one hydrogen bond (dashed line) with the binding site amino acid (Gln 64A) of 1T2P (Figure 4.33C), one hydrogen bond with the amino acid Lys 154A of 2FNP (Figure 4.33D), two hydrogen bonds with amino acids, His 35A and Asn 31A of 3ECO (Figure 4.33H), one hydrogen bond with amino acid Gln 155B of 4G4K (Figure 4.33I) and one hydrogen bond with amino acid, Gln 14B of 4Q77 (Figure 4.33J). Besides hydrogen bonding the binding of PEME involved many hydrophobic interactions with the binding site amino acids.

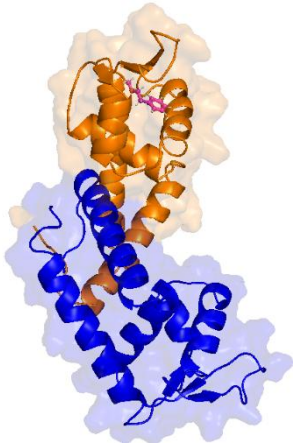
(A) Complex of PEME and 1T2P



(C) Ligplot of PEME and 1T2P



(B) Complex of PEME and 2FNP



(D) Ligplot of PEME with 2FNP

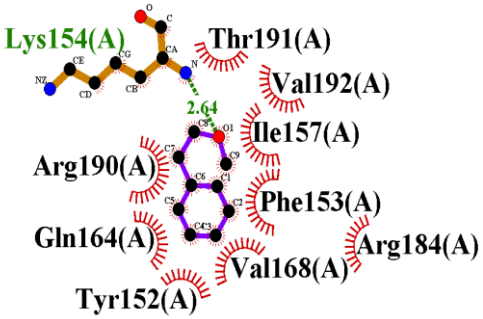
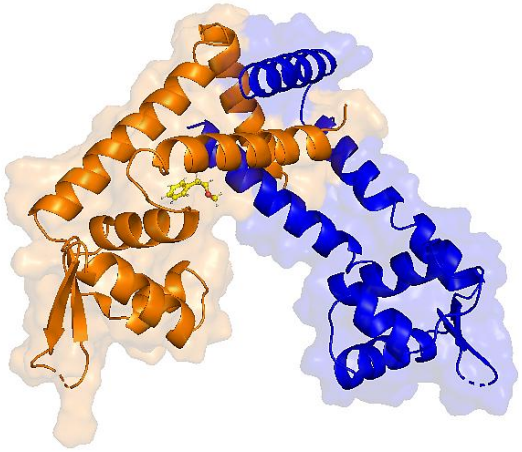
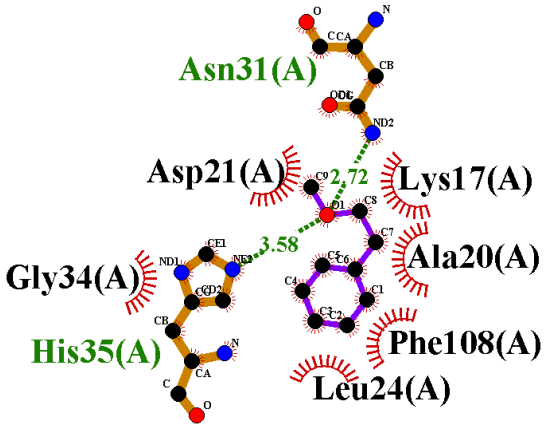


Figure 4.34. Molecular docking of PEME onto the proteins involve in pathogenesis, bioflim production and drug resistance of *S. aureus*. Terpinen-4-ol is well accommodated inside the binding site of (A) 1T2P and (B) 2FNP. The ligplot analysis of PEME showing the interactions with binding site amino acids of (C) 1T2P and (D) 4Q77. Its binding involved both hydrogen bonds represented as dotted (green) lines and hydrophobic interactions denoted with curved (red) lines.

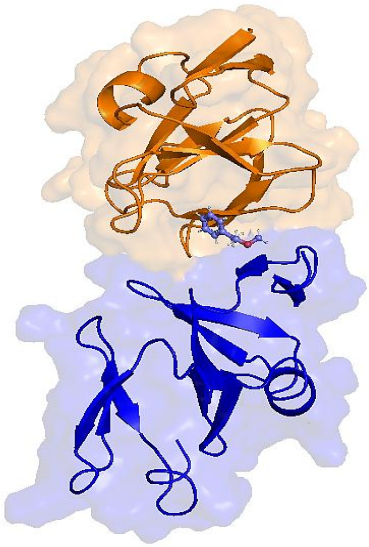
(E) Complex of PEME and 3ECO



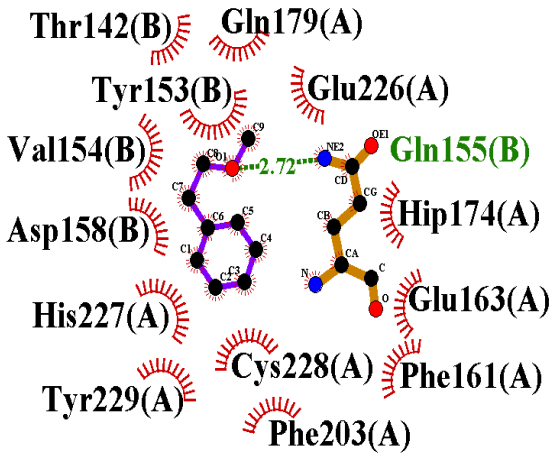
(H) Ligplot of PEME with 3ECO



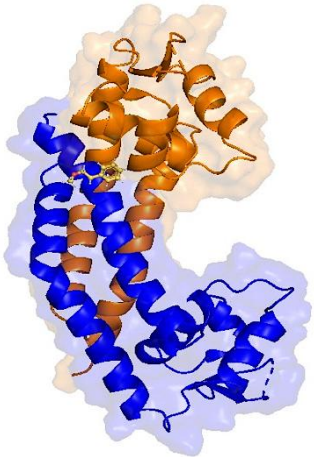
(F) Complex of PEME and 4G4K



(I) Ligplot of PEME and 4G4K



(G) Complex of PEME and 4Q77



(J) Ligplot of PEME and 4Q77

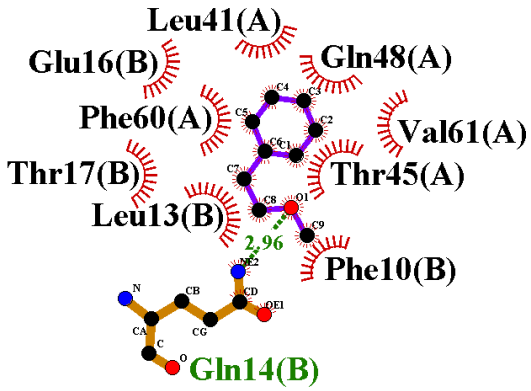


Figure 4.35. Molecular docking of PEME onto the proteins involve in pathogenesis, bioflim production and drug resistance of *S. aureus*. Terpinen-4-ol is well accommodated

inside the binding site of (E) 3ECO, (F) 4G4K and (G) 4Q77. The ligplot analysis of PEME showing the interactions with binding site amino acids of (H) 3ECO, (I) 4G4K and (J) 4Q77. Its binding involved both hydrogen bonds represented as dotted (green) lines and hydrophobic interactions denoted with curved (red) lines.

4.10. Toxicity evaluation of essential oil

The treated animals with essential oil of *P. odorifer* showed neither any toxic effect nor any lethal effect. Even if with the administration of highest dose up to 800 mg/kg did not reveal any signs of toxicity or mortality in rats during the entire period of study. Therefore, LD_{50} of the essential oil was considered to be greater than 800 mg/kg. No significant difference in blood biochemical parameters and organ functions between the treated and untreated groups was noticed (Table 4.18). Also during the doses regimen no abnormal behaviour regarding food and water intake and body weights was observed. To further determine whether treatment with essential oil results in toxicities to normal tissues, we examined the liver and kidney of rats (Figure 4.34).

Table 4.18. Observations on the normal symptoms of the animals with the treatment of different doses of essential oil of *P. odorifer***.**

Behaviour Type	Control and treated groups of animals			
	Control	200 mg/kg	400 mg/kg	800 mg/kg
Spontaneous type	Normal	Normal	Normal	Normal
Alertness	Normal	Normal	Normal	Normal
Awareness	Normal	Normal	Normal	Normal
Sound response	Normal	Normal	Normal	Normal
Touch response	Normal	Normal	Normal	Normal
Pain response	Normal	Normal	Normal	Normal
Righting reflex	Normal	Normal	Normal	Normal
Pinna reflex	Normal	Normal	Normal	Normal
Grip strength	Normal	Normal	Normal	Normal
Food intake	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal
Mortality	Absent	Absent	Absent	Absent

4.10.1 Sub acute toxicity study

Different parameters such as hematological parameters, biochemical parameters and histopathological study of kidney and liver of the control and experimental animals were noted at the end of the experimental period to reveal any toxiciy. Treatment with Kewda essential oil with doses of 200, 400 and 800 mg/kg body weight have not

produced any remarkable changes on animal hematological parameters (Table 4.19). Similarly treatment with Kewda oil at the doses of 200, 400 and 800 mg/kg body weight have no significant effects on blood biochemical parameters compared to untreated group of animals (Table 4.20) indicating no side effects of Kewda oils with organ function. As an example a non significant difference ($p > 0.05$) in ALT, AST indicates no toxicity effect to liver. Urea level remained unchanged, while creatinine levels in blood were slightly reduced after administration of oil in all the treatment groups, indicating no effect to kidney**.** Treatment of essential oil at a doses of 200, 400 and 800 mg/kg body weight fails to reveal any detectable pathological abnormalities in both liver and kidney based on histopathology. Panels (Figure 4.34) represent H&E staining of paraffin-embedded 5.0 micron-thick sections of the liver and kidney at magnification of 200x. The liver showed normal hepatic lobular architecture. The kidneys revealed normal glomeruli, proximal and distal tubules, interstitium, and blood vessels.

Table 4.19. Different blood parameters of the treated animals with different doses (200, 400 and 800 mg/kg body weight) of Kewda essential oil for 28 days.

Parameters	**Group-I**	**Group-II**	**Group-III**	**Group-IV**
HGB (g/dL)	12.02 ± 0.44	10.4 ± 0.26	13.0 ± 0.46	11.9 ± 0.38
RBC (106 /µL)	8.24 ± 0.67	5.30 ± 0.30	7.40 ± 0.17	8.33 ± 0.24
PLT(103 /µL)	793.9 ± 24.1	902.3 ± 17.2	743.3 ± 36.8	935.4 ± 41.0
HCT (%)	44.86 ± 2.02	40.2 ± 1.09	35.5 ± 1.07	43.7 ± 1.06
MCV (pg)	57.0 ± 0.96	57.0 ± 0.36	54.4 ± 1.56	55.0 ± 0.96
MCH (pg)	15.4 ± 0.50	17.2 ± 0.72	21.4 ± 1.06	16.06 ± 0.76
MCHC (g/dL))	32.3 ± 1.01	34.3 ± 1.65	35.7 ± 1.61	33.36 ± 0.63
WBC (10^3 /µL)	6.86 ± 0.20	7.67 ± 0.53	4.69 ± 0.42	6.44 ± 0.63

Group I: Control, Group-II: 200 mg/kg body weight, Group-II: 400 mg/kg body weight, Group-IV: 800 mg/kg body weight

Table 4.20. Blood biochemical parameters and organ functions between the control and the treated groups with different doses of essential oil of *P. odorifer*.

Parameters	**Group-I**	**Group-II**	**Group-III**	**Group-IV**	**Normal value**
Glucose(mg/dl)	82.05±2.1	82.89±1.98	83.02±1.4	82.93±1.9	70-110
Urea(mg/dl)	30.02±3.1	30.04±2.98	30.86±1.78	30.29±2.14	15-45
Creatinine(mg/dl)	0.78±1.05	0.72±1.34	0.72±1.25	0.73±1.47	0.5-1.5
Total protein(mg/dl)	5.98±2.56	6.18±1.45	6.26±1.76	6.14±1.5	6.0-8.0
Total cholesterol(mg/dl)	126.02±1.78	126.32±2.22	126.89±2.3	126.87±2.5	140-250
Tri glycerides(mg/dl)	89.02±3.1	89.01±2.9	90.01±2.8	90.56±2.6	25-160
Asparateamino transferage(AST)(IU/L)	30.02±2.5	29.99±2.6	29.32±1.9	29.12±3.2	Up to 46
Alanineaminotransferase (ALT)(IU/L)	25.89±2.1	25.27±1.6	25.12±1.9	25.39±2.2	Up to 40

GroupI: Control, Group-II: 200 mg/kg body weight, Group-II: 400 mg/kg body weight, Group-IV: 800 mg/kg body weight.

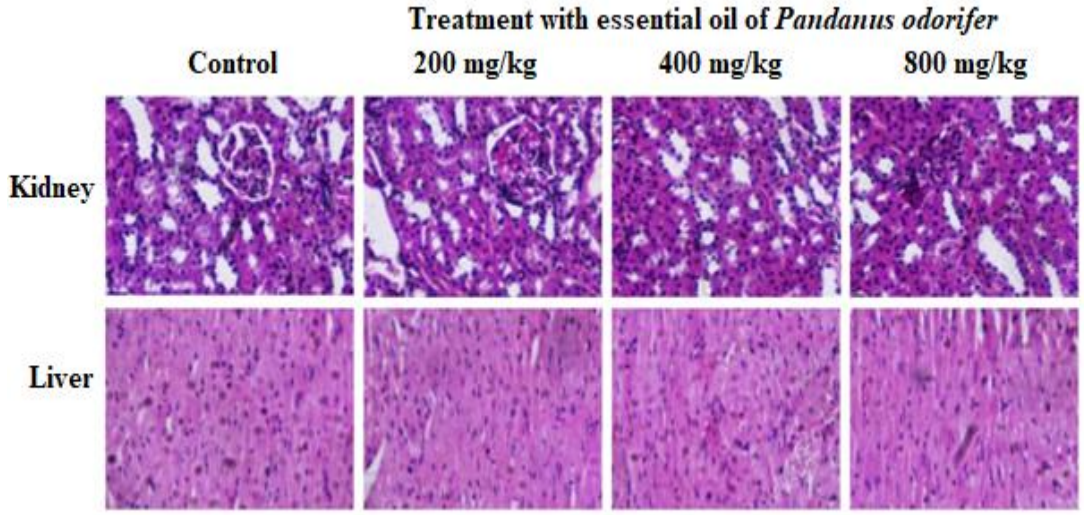


Figure 4.36. Panels represent H&E staining of paraffin-embedded 5.0 micron-thick sections of the liver and kidney at magnification of 200x. There is no evidence of any pathological abnormalities between the control and treated groups. The liver showed normal hepatic lobular architecture. The kidneys revealed normal glomeruli, proximal and distal tubules, interstitium, and blood vessels.

Further, no any detectable pathological abnormalities was noticed even if with the treatment of highest dose of 800 mg/kg body weight. To know about liver injury ALT and AST level were tested. Result showed a nonsignificant reduction of ALT and AST. This indicates that kewda oil do not have toxic effects in liver. Urea and creatinine level are considered as markers with respect to kidney funcation. Urea level remain same whereas creatinine levels decreased in treated groups. According to (Nisha *et al.*, 2017) renal toxicity should be considered only when creatinine and urea level increased parallel to each other**.** To identify hyperlipidemia, total cholesterol and triglycerides were evaluated, which are risk factor for heart diseases. Both cholesterol and triglycerides were lies in between normal range. We found that the LD_{50} value for Kewda oil was more than 800 mg/kg body weight.

4.11 Disscussion

As antibiotics are spine to hospital/clinical management, the emergence of strains resistant to several antibiotics of different classes in pathogenic bacteria creates utmost annoyance in microbial stewardship programme. Especially in empiric therapy, intended for acute diseases and at the end of a surgical protocol, the treating physician circumspect about the choice on antibiotics. Prescribing a moribund antibiotic would

lead to the failure of the treatment, as drug resistant bacterial strains are more virulent than their sensitive strains. As it is, because of natural evolutionary capabilities of having both intrinsic and extrinsic modes of arrival of resistant strains (Perez *et al.,* 1990), colossal emergence of MDR bacteria becomes the obvious aftermath at a blistering pace, after the introduction of some newer antibiotic along with resistance to others introduced earlier. Pathogenic bacteria evolve new strains gaining resistance to recently used antibiotics and drugs, an event which repeats by itself; and in the last few decades there have been an increase in the prevalence of MDR bacteria, worldwide. Since, bacteria have cryptic inter-continental migration, their drug resistant strains slowly escalate to other areas. Concomitant to the search for newer generations of antibiotics for the control of increased torrent of MDR pathogenic bacteria, continual efforts for search of control agents from plants, non-conventional sources and structural modifications of moribund control agents have been undertaken (Gericke *et al.*, 2002; Burt *et al.*, 2004), as apothecary follows eclectic principles in drug discovery.

MDR strains of both Gram-negative (GN) and Gram-positive (GP) bacteria have emerged increasingly as public health perils. MDR GP bacteria are less prevalent than MDR GNs, in any hospital/community setting (Falagas and Bliziotis, 2007), but species of *Staphylococcus* and *Enterococcus* spearhead as belligerent MDR GP cocci (Subedi and Bramahadathan, 2005; Sood *et al.,* 2008), which are considered as the important determinants of public health problems, worldwide. The originally known commensal, *S. aureus* causes mild to severe or potentially fatal illness in the MRSA form, when its strains gain multiple-drug resistance; eventually, MRSA has become ill-famed as the superbug of the health domain, to put in sotto voce. As it is known, long term hospitalization causes increase of susceptibility of a patient to the MRSA infection (Chambers, 2001), particularly causing suppurations at surgical sites and invading urinary tracts. It was also reported that 51.5% patients had already been infected with MRSA at their time of the admission to hospitals, which could cause an introduction of newer/differently resistant MRSA strains to hospitals from community (Slonczewski and Foster, 2009).

Enterococci are primarily opportunistic pathogens. Intensive use of broad spectrum antibiotics in the hospitals has been responsible for emergence of these organisms as important nosocomial pathogens (Gold, 2001). The first report of VRE was reported in 1988 (Uttley *et al.,* 1988). Thereafter, VRE have spread rapidly all over the world. For example, from the year 1989 to 1993 the percentage of nosocomial infections

due to VRE reported to the Centers for Disease Control and prevention, USA increased from 0.3 to 7.9 per cent. Though the major problem in treatment of VRE infection arises in endocarditis, the urinary tract is the commonest site from where bacteremia can occur. There are very few reports on isolation of VRE from India (Mathur *et al.,* 2003), though epidemiology of nosocomial VRE bacteremia has been quite extensively studied. Studies on problems posed by the VRE as pathogens in UTI are very few. *Enterococci* in mixed culture are very commonly isolated from urine samples. It is not always easy to assess the clinical significance of VRE in routine cultures or to differentiate colonization from infection (Wong *et al.,* 2000). The present study was undertaken to look for vancomycin resistance in *Enterococci* obtained in significant numbers from various nosocomial and community acquired samples, and to study the infection dynamics of this MDR pathogen.

Nosocomial acquisition and its subsequent colonization of VRE is an emerging international threat to public health, and it has been emphasized in the US; colonization of VRE among non-hospitalized persons has been also reported. In contrast, in European countries, colonization is frequently reported in persons outside the health-care setting (McDonald *et al.,* 1997). An important factor associated with VRE in the community in Europe has been the avoparcin, a glycopeptide antimicrobial drug used for years in many European nations at sub-therapeutic doses as a growth promoter in food-producing animals. In Europe, evidence suggests that food borne VRE may cause colonization in man (Aarestrup *et al.,* 1996; Kruse and Rorvik, 1996).

The methylase gene, *erm(A),* formerly termed as *erm(TR)* was identified in *S. pyogenes* conferring erythromycin resistance (Seppala *et al.,* 1998). A work from Spain recorded different species of *Enterococcus* with erythromycin resistance, with genes, *erm(A), erm(B), erm(C) erm(TR), mef(A/E)* and *msr(A)*. Each group has 2 or more variants; for example, *erm(A)* has two variants *erm(A)* and *erm(TR)*. Likewise for *erm(T),* 17 variants of genes were recognized encoded by different transposons and plasmids (Roberts *et al.,* 1999). Indeed, MLSB resistance in Cd-s strains of *Streptococci* carrying any version of *erm* gene is inducible to express clindamycin resistance, by the Er-r character. Besides *S. pyogenes,* other *Streptococcus* sp. such as, group C and group G species (*S. agalactia* and *S. pneumonniae*) also have *erm(A).* Further, in the other species originally isolated from fermentation units, *Peptostreptococcus magnus* also was reported to have *erm(A)* gene (Reig *et al.,* 2001), conferring it virulence along with drug resistance, as both characters are linked (Martinez *et al.,* 2002). Conjugative transfer of

the *erm(A)* gene from *erm(A)* positive isolates of *S. pyogenes* to Er-s strains of *S. pyogenes*, *E. faecalis* and *Listeria innocua* was recorded (Giovanetti *et al.*, 2002). The other methylase gene, *erm (AM)* now called as *erm(B),* coding resistance to MLSB antibiotics was associated with the constitutive, as well as the inducible MLSB phenotypes (Giovanetti *et al.*, 2002). This bacterial pathogen was reported to be resistance to streptogramin A too (Emborg *et al.*, 2004). Over the last 3 decades, N6 methyl transferase had been isolated from different bacterial species, that causes DNA adenine methylation; similar methylations were recorded causing the methylation of the 23s rRNA in *Enterococci* and *Staphylococci*, which have been involved in MLSB resistance with a lot of variances, named alphabetically from *erm(A)* up to *erm(Y)* (Graham *et al.*, 2009).

In the US, *Staphylococcus pyogenes* (Group A *Streptococcus*) had records of nosocomial transmission, from patients to health care workers in causing fulminant invasive diseases, sore throat with hip and joint pains (Lacy and Horn, 2009). Levofloxacin resistant *S. pyogenes* with MIC of 16 µg/mL were prevalent in the US (Richter, 2003). The multidrug resistance of *S. pyogenes* was linked to major virulence factor, the M-protein, which is coded by the *emm* gene (Wahl *et al.*, 2007). It had been seen that the most GP bacteria such as, *Enterococcus* sp. (including VRE), *S. aureus* (including MRSA) and *S. pyogenes* survive for months on dry surfaces. Many GN species such as, *A. baumannii*, *Citrobacter* sp., *P. aeruginosa* and *Proteus* sp. also survived equally for months on dry surfaces. Bacteria such as, *Bordetella pertussis*, *Haemophilus influenzae*, *P. mirabilis* and *V. cholerae* persisted only for a few days on dry surfaces. These reports clearly suggested potency of nosocomial spreads of the above-mentioned bacteria (Kramer *et al.*, 2006).

The β-lactam group of antibiotics comprising penicillin and its derivatives including broad spectrum cephalosporins and monobactam are hydrolyzed by ESBL producing bacterial strains. Sometimes precipitated health episodes in hospitals and communities due to GN bacteria, *Citrobacter baumannii*, *Proteus* sp., *A. baumannii* are the uropathogens, while *S. aureus* and *P. aeruginosa* cause suppurations (Mittal *et al.*, 2009). Slowly, strains of, *P. aeruginosa* and *Citrobacter* sp. are becoming prominent to cause invasive infections in under-5 children, alike in both developed and developing worlds because of multidrug resistance. The third generation cephalosporins (3GCs) namely, cefotaxime, ceftazidime and ceftriaxone were developed because of the production of ampicillin-hydrolyzing β-lactamases carried by plasmids, TEM1

(temoneira), TEM2 and SHV1 (sulphydryl variable). Moreover, about 150 ESBL bacterial strains had been described to have a worldwide distribution, a decade ago (Bradford, 2001) that clearly demonstrated that β-lactam antibiotic resistance emerged in geographic zones, where a particular antibiotic was used first (Jarlier *et al.,* 1988). Further, ESBL producing bacteria often show cross resistance to other groups of antibiotics like, fluoroquinolones. Further, the matter of utter clinical annoyance incidentally was the close relationship between ciprofloxacin resistance and ESBL production in antimicrobial stewardship programme (Paterson *et al.,* 2000). Indeed, surveillance studies on a pathogen or a group provide a mirror of infection dynamics of a hospital/community. It has been known that ESBL harbouring patients require longer hospital stay in wards/ICUs, longer use of devices ventilation, catheterization and a few more that are inductive often for longer hospitalization — all stemming from as well as leading to severity of an illness arising from an infection episode and exposure to more and more nosocomial infections, followed by the use of higher generation of antibiotics (WHO, 2011; Mangeney, 2000), sometimes impassably compelling a patient's transfer to hospice.

Spread of ESBL strains is multifactorial: they are from improperly washed hands of indurate health care providers and inanimate objects of hospitals and nursing homes, as well as the over-crowed hospital corridors and communal living settings, to cite a few. Eventually patients, who carry antibiotic resistant organisms when in contact with immunocompromised/ aged patients as well as, healthcare workers cause the spread, the later often serve as reservoirs or exchangers. In indoor hospital units, viz., wards, cabins and ICUs, one infected patient is sufficient to cause infections to many, by devices, fomites and health care workers.

Indeed, the indiscriminate use of antibiotics could be regarded as the cause of emergence of MDR bacteria, as in regions where the availability of antibiotics is limited, the prevalence of MDR *A. baumannii* and *P. aeruginosa* were low (Tiwari *et al.,* 2009). Thus, the problem of saturnine emergence of the cohort of MDR pathogens did not happen obliviously, but those are welcomed by indurate attitudes in the antibiotic use, despite availability of information on mechanism of bacterial mutations and their rates.

Further, nosocomial infections are reported from ICUs, because of the severity of infection by one or other pathogen in patients on wounds. This situation causes spreads of several infectious bacteria at a time by cross-infections. Frequently, device associated nosocomial infections have been reported from many hospitals due to human errors,

despite better cleanliness of general hospital environments (Eiff *et al.,* 2005), at least in poverty stricken developing countries. Indeed, nosocomial/community spreads of infections could be attributed to the lack of general awareness among public, an indurate attitude or the lack of specific awareness among paramedical staff, and at least to the plethora of physiological and genetic survival mechanisms of MDR bacterial strains.

This thesis recorded prevalence of GP and GN bacteria to identify the present status of the philanthropic hospital attended by patients from rural areas, urban slums as well as from well heeled mass, in resource limited settings. This Indian epitome should strengthen the epidemiological database and would help fixing by facilitation of quality improvement in hospital management and for the reduction in cost of hospitalization, as well as in the reduction of morbidity and mortality due to this GP and GN MDR pathogens. The pharmacy world too is anticipated to be benefitted by this and similar studies on subtle MDR pathogens all over, for finesse in dovetailing suitable drugs of non-microbial origin even, as antimicrobials (Mesquita *et al.,* 2007). Thus, complementary medicines are thought up in principles of 'comparative effectiveness research', and isolated phyto-compounds could be promoted.

Phytochemicals are a large group of chemical compounds naturally occurring in plants, conferring color, flavor, aroma and texture. These compounds have been developed over thousands of years of evolution to defend organisms from the effects of free radicals, viruses, bacteria and fungi. They are widely distributed in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs and spices and in plant-based beverages such as wine and tea (Ramona Barbieri *et al.*, 2017). In recent years, many studies have shown that phytochemicals exert their antibacterial activity through different mechanisms of action, such as damage to the bacterial membrane and suppression of virulence factors, including inhibition of the activity of enzymes and toxins, and bacterial biofilm formation. Since earliest times, many plants have been known to exert healing properties against human infections due to their content of secondary metabolites. Which in more recent times have been found to act as antimicrobial agents against human pathogens (Borges *et al.,* 2015). Higher and aromatics plants have traditionally been used in folk medicine as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeasts Most of their properties are due to essential oils produced by their secondary metabolism (Adilson Sartoratto *et al.,* 2004). Plants produce a large and diverse array of organic compounds that appear to have no direct function in growth and development, and these are named

as secondary metabolites (Mangalagiri *et al.,* 2021). Plant oils and extracts have been used for a wide variety of purposes for many thousands of years. Essential oils are a mixture of volatile constituents produced by the secondary metabolism of aromatic and other variety of plants **(**Herman *et al.,* 2020**).** The oil droplets being stored in the oil glands or sacs can be removed by either accelerate diffusion through the cell wall or crush the cell wall. The adopted techniques depend on the part of the plants where the oil is to be extracted, the stability of the oil to heat and susceptibility of the oil constituents to chemical reactions. Common techniques used for the extraction of essential oils are. Hydrodistillation, Effleurage, Cold pressing, Steam distillation, Solvent extraction, Microwave Assisted Process (MAP), Carbondioxide extraction (Hamid, A.A., *et al.*, 2011).

P. odorifer is a highly potential medicinal plant. It has several therapeutic properties like analgesic anticancer, antidiabetic, antimicrobial anticonvulsant, anti-oxidant, antidepressant and antineurotic activity. Ayurveda also In Ayurveda, the Kewda oil has been used to treat rheumatism and general body pain. It is mainly composed of secondary metabolites such as terpenoids lipids, coumarins, alkaloids, flavonoids, phenols, lignans and steroids. (Rajeswari J *et al.,* 2012). The exponential rise in multidrug-resistant strains has led to the search for newer and effective, drugs which can be easily available and less expensive. This research was conducted to rationalize the antimicrobial activity of *P. odorifer's* essential oil and its use as an alternative to currently used antibiotics.

In the present study, the essential oil extracted from fresh flowers of *Pandanus odorifer* (commonly known as Kewda) was evaluated for its antimicrobial and antibiofilm potential against ESKAPE pathogens, *Staphylococcus aureus* and *Klebsiella pneumoniae.* According to Adkar and Bhaskar (2014), Kewda essential oil (KEO) is recommended for treating headaches, rheumatism, spasm, boils, etc. Previous studies also revealed that green synthesized zinc oxide nanoparticles (ZnO N.P.s) using *P. odorifer* leaf extract exhibited promising antimicrobial activities (Hussain *et al.,* 2019b). Hussain *et al.,* (2019a) also reported the antibiofilm potential of biosynthesized silver nanoparticles (AgNPs) from Kewda leaf extract. Therefore, we have evaluated the presence of bioactive components in KEO, which might considerably influence its antimicrobial and antibiofilm activities against test microorganisms. The gas chromatography-mass spectrometric (GC-MS) analysis of KEO followed by NIST library database identification; nine phytochemicals were identified based on their

retention time, peak height, peak area percentage. It was inferred that 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 belonged to the terpene family and were reported as significant components of plant-derived essential oils (Bourgou *et al.*, 2012; Khaleel *et al*. 2018). Further, GCMS analysis of the extracted crude oil revealed the presence of 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, where it was found to be effective against both Gram-positive and Gram-negative bacteria. It was found from the HRMS study that the major compound present in crude oil was PEME, which responsible for its antimicrobial activity.A more comprehensive investigation should be carried out because of the isolation and characterization of specific chemical compounds responsible for the previously said medicinal values.

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. At the MIC level, KEO exhibited promising antibacterial activity. Among the test bacteria, both *K. pneumoniae* and its reference strain MTCC-109 were highly sensitive to the treatment of KEO at MIC with a zone of inhibition of 17 and 22 mm, respectively. In contrast, the clinical strain of *S. aureus* showed 16 mm of a zone of inhibition with MIC of KEO, as evident from the agar well diffusion assay. The results were in accordance with earlier studies depicting the promising antibacterial activities of plant derived essential oils (Man *et al.,* 2019). 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). The inhibition to bioflim production with the treatment of KEO was mainly because of its bioactive compound, Terpinen-4-ol. Previously it was demonstrated that Terpinen-4-ol inhibits the biofilm production by bacteria (Cordeiro *et al.,* 2020).

The present study shows administration of dose up to 800mg/kg did not reveal any signs of toxicity or mortality in rats during the entire observation period. Therefore, LD_{50} of extract may be considered to be greater than 800mg/kg. Lulekal *et al.,* 2019 observed no mortality and sign of toxicity in female mice treated with 2000 mg/kg of the of *C. citratus* essential oil and considered the lethal dose (LD_{50}) of *C. citratus* essential oil oral administration was greater than 2000 mg/kg.Treatment with kewda oil of all doses had not produced any remarkable changes on animal hematological parameters. The liver, kidneys, and lungs histopathology studies with varied dosages (300, 600, 1200, and 2000 mg/kg body weight) revealed no significant differences when compared to the control group. Lulekal *et al.,* 2019 determined normal hepatocellular morphology, normal periportal area with no evidence of necrosis and inflammation. For *C. citratus* essential oil. Liaqat *et al.,* 2018 reported non toxic evidence of essential oils Aegle *marmelos, Murraya koenigii, Citrus reticulata* Blanco, *Zanthoxylum armatum, Skimmia laureola, Murraya paniculata*, and *Boenninghausenia albiflora*.

4.12. Conclusions and Future Scope

In the present study, the essential oil extracted from fresh flowers of *Pandanus odorifer* (commonly known as Kewda) was evaluated for its chemical profiling as well as antimicrobial and antibiofilm potential against ESKAPE pathogens, *Staphylococcus aureus* and *Klebsiella pneumoniae.* The gas chromatography-mass spectrometric (GC-MS) analysis followed by NIST library database identification and HRMS analysis of Kewda oil revealed presencr of several phytochemicals, however, the two compounds 2-Phenyl Ethyl Methyl Ester (PEME) at 80.435% followed by Terpinen-4-ol with a 14.13% of peak area percentage were found in highest percentage. The FTIR analysis of Kewda oil revealed the presence of distinct functional groups of interest, which were in accordance with earlier report of composition of functional groups in several plant-derived essential oils. NMR spectral analysis revealed the major essential oil constituents as ketones, esters, alcohols, aromatic phenols, vinylics, etc.

The minimum inhibitory concentration (MIC) of KEO was found to be 5% (v/v) against both the isolated multi drug resistant clinical strains of *S. aureus* and *K. pneumoniae* and their reference strains, MTCC-740 and MTCC-109. Among the test bacteria, both *K. pneumoniae* and its reference strain MTCC-109 were highly sensitive to the treatment of Kewda oil compared to the clinical strain of *S. aureus*. Further, treatment with sub-MIC concentration of Kewda oil, a significant decrease in the biofilm production in both the clinical strains of *S. aureus* and *K. pneumoniae* and their reference

strains, MTCC-740 and MTCC-109 was observed with a concomitant reduction in the production of exopolysaccharides (EPS). The encouraging antimicrobial and antibiofilm activities of Kewda oil, as evident from experimental studies, could be attributed due 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 our efforts to mitigate biofilm-associated infections and drug resistance profiles of pathogenic microorganisms in the near future.

Terpinen-4-ol, bioactive constituent of Kewda essential oil was evaluated for its antimicrobial and antibiofilm activities against *Staphylococcus aureus* and *Klebsiella pneumoniae* and their reference strains, MTCC-740 and MTCC-109. At minimum inhibitory concentration it exhibited promising antibacterial and antibioflim activity against multidrug resistant *S. aureus* and *K. pneumoniae* and their standard reference strains. *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. Terpinen-4-ol could also be used in combination with other therapeutic drugs for improving the therapeutic efficacy by disrupting the biofilm matrix.

2-Phenyl Ethyl Methyl Ether (PEME), bioactive constituent of Kewda essential oil also exhibited promising antibacterial and antibiofilm activities against test bacterial pathogens, *S. aureus* and *K. pneumoniae* and their reference strains, MTCC-740 and MTCC-109. As evident from qualitative and quantitative biofilm formation assays, PEME strongly inhibited biofilm formation in test bacteria. Transcriptomic analysis of mRNA isolated from *S. aureus* treated with PEME suggested the down-regulation of several genes (e.g. *agrA*, *norA*, *mepR*, *sarA*), which are directly or indirectly associated with bacterial virulence, biofilm formation and drug resistance patterns. From the molecular docking analysis, it was evident that PEME showed highest binding affinity towards SarA and AgrA, which plays crucial role in attenuation of bacterial pathogenesis and biofilm dynamics in drug resistant bacteria. Thus, PEME could be explored for its possible role in the management of biofilm associated infections in the near future.

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LIST OF PUBLICATIONS

1. **Cheruvanachari Priya** and Pradeep K. Naik (2022). Isolation, Characterization and Antibiotic Susceptibility of Gram Positive and Gram Negative Bacteria from the Clinical Samples. Zeichen Journal, Volume: 08, Issue: 08, 155-169. **DOI:15.10089.ZJ.2020.V08I08.285311.2895**
2. **Cheruvanachari Priya**, Monika Mishra, Subhaswaraj Pattnaik, Pradeep K. Naik (2022). Phytochemical characterization, antibacterial and antibioflim activity of Kewda essential oil against *Staphylococcus aureus* and *Klebsiella pneumonia.* Biologia (Under review)
3. **Cheruvanachari Priya**, Subhaswaraj Pattnaik, Monika Mishra, 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 pneumonia.* Natural Product Research, 12(5): 406-420, DOI: 10.1080/22311866.2022.2154264
4. **Cheruvanachari Priya**, Subhaswaraj Pattnaik, Monika Mishra, Pratyush Pragyandipta, Animesh Pattnaik, Pradeep K. Naik (2022). Deciphering the anti-biofilm potential of 2-Phenylethyl methyl ether (PEME), a bioactive compound of Kewda essential oil against multidrug resistant *Staphylococcus aureus* and *Klebsiella pneumonia.* Microbiological Research (Under review)
5. **Cheruvanacharia Priya**, Debasmita Dubeya, Manish Paulb Biswaranjan Pradhanc, Bikash Chandra Beherad, Pradeep K. Naik (2022). Antimicrobial, Phytochemical and toxicological profile of essential oil extracted from *Pandanus odorifer* with an available simulation study. Journal of Traditional and Complementary Medicine (Under review)

LIST OF CONFERENCES ATTENDED

1. **Cheruvanacharia Priya** and Pradeep Kumar naik. "Evaluation of antibacterial activity of Terpenin-4-ol." Oral Presentation in National Conference on Current Research Trends in Biotechnology, Bioinformatics and Intellectual Property Management from 3rd to 4th March, 2020 organized by Department of Biotechnology and Bioinformatics, Sambalpur University.
2. **Cheruvanacharia Priya** and Pradeep Kumar Naik. "2-Phenylethyl methyl ether (PEME) a potential lead molecule against MDR bacteria." Oral Presentation in National Seminar on Harnessing Science & Technology for a better future from 23rd to 24th November, 2019 jointly organized by Odisha Environmental Society, Bhubaneswar and Siksha 'O' Anusandhan (Deemed to be University).