In vitro and In silico study of Essential oil Components from *Eucalyptus tereticornis* as Antibacterial agents

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In

Department for Pharmaceutical Sciences and Natural Products

By

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June 2018

DECLARATION

I declare that the Project work entitled "**In vitro and In silico study of Essential Oil Components from *Eucalyptus tereticornis* Essential Oil as Antibacterial agents**" has been prepared by me under the guidance of Dr. Vikas Jaitak, Assistant Professor, Department of Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab. No part of this Project work has formed the basis for the award of any degree or fellowship previously.

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DECLARATION

I certify that

a) The work contained in this thesis is original and has been done by me under the guidance of my supervisor Dr. Vikas Jaitak.
b) The work has not been submitted to any other Institute for any degree or diploma;
c) I have followed the guidelines provided by the Central University of Punjab, Bathinda, in preparing the project report;
d) I have conformed to ethical norms and guidelines while writing the project report and;
e) Whenever I have used materials (data, models, figures, and text) from other sources, I have given due credit to them by citing them in the text of the project work, giving their details in the references, and taking permission from the copyright owners of the sources, whenever necessary.

Anupama Sharma

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CERTIFICATE

We certify that Anupama Sharma has prepared her project work entitled "**In vitro and In silico study of Essential Oil Components from *Eucalyptus tereticornis* Essential Oil as Antibacterial agents**", for the award of M.Sc. (Medicinal Chemistry) degree of the Central University of Punjab, under our guidance. She has carried out this work at the Department for Pharmaceutical Sciences and Natural Products, School of Basic and Applied Sciences, Central University of Punjab.

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ABSTRACT

"In vitro and In silico study of Essential Oil Components from *Eucalyptus tereticornis* Essential Oil as Antibacterial agents"

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ABSTRACT

Many essential oils are known for their antimicrobial activity and the main target is bacterial cell membrane which is important for bacterial viability and hence the study was done to find the target and effective binding of the components of the *Eucalyptus tereticornis* essential oil and comparison with the standard drug used for in vitro and in silico studies, A/S combination. In vitro and In silico studies has shown the potential of *Eucalyptus tereticornis* essential oil in modulating antibacterial resistance and its potential as an antibacterial agent at a conc. of 10µl that was comparable with the standard and also at 50 µl, the zone of inhibition was found to be equivalent to that of standard combinational drug of A/S (10:10) mcg. The In silico studies further confirms its potential to combat antibacterial resistance as the docking results has shown the effective binding of the components of essential oil than the standard in the order α-Terpinyl acetate (-2.754)> 8-epi-gama-eudermol> beta-eudesmol> L-alpha-Terpineol against PBP3 in comparison with the standard (-0.766). Docking simulation also suggests the effective binding of essential oil components with the beta lactamases as (-2.348) for Salbactam in comparison with (-3.671) Cis-p-mentha-1(7),8-dien-2-ol and also ADME/T studies has shown their ability to partition the bacterial cell membrane with logPo/w for the components for Aromadendrene (5.176), beta-myrcene (4.592). Along with 100% oral

absorption and the absorption through gut blood barrier QPPCaco found to be more than 500 for every component of the essential oil, and brain blood barrier that was found to be in range of 0.095 for alpha-terpinylacetate and 1.047 for Aromadendrene.

Anupama Sharma Dr. Vikas Jaitak

Grandpa, Papa, Mom, Dee and my Teachers since my childhood you supported me and I reached till here just because of you. Thank you to all for everything that you have done.

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	Antimicrobial Resistance Bacteria	ARB
2.	microlitre	µL
3.	WORLD HEALTH ASSEMBLY	WHA
4.	WORLD HEALTH ORGANISTAION	WHO
5.	Tuberculosis	TB
6.	*Escherichia coli*	*E.coli*
7.	Antimicrobial resistance bacteria	ARB
8.	Methicilin resistant staphylococcus aureus	MRSA
9.	Gas chromatography- mass spectrometry	GC-MS
10.	Essential oil	EO
11.	N-acetyl glucosamine	NAG
12.	N-acetyl muramic acid	NAM
13.	Mevalonic acid pathway	MVA
14.	Horizontal gene transfer	HGT
15.	Food and drug administration	FDA
16.	Quorum sensing Escherichia coli regulators B and C	QseBC
17.	Outer membrane protein	OmpA
18.	Shiga toxin	Stx
19.	Filamentation Temperature sensitive protein	FtsZ
20.	*t*-butyl hydroperoxide	*t*-BOOH
21.	Glucosyltransferase	GTFase
22.	Glucan binding protein	Gbp
23.	Toxic shock syndrome toxin	TSST1
24.	Heat shock protein	HSP

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

The emergence of Antibacterial resistance is one of the major causes of deaths around the world {Hart, 1998 #234}. The main factor behind antibacterial resistance is the overexploitation of antibacterials and bacteria itself, since they are ancient living creatures having tendency to adapt to various condition and hence unnecessary consumption of antibacterials is leading to resistance. The fact is already understood by the discoverer of PENICILIN (First generation of beta-lactam antibiotics), “SIR ALEXANDER FLEMINGS”(Tan & Tatsumura, 2015) as he mentioned in his speech after receiving the Nobel prize.

“Irrational use of penicillin treatment can make pathogenic bacteria defensive and we itself is morally responsible for deaths due to infection” (S. B. Levy, 2013).

Although knowing the fact that bacteria is having immense proclivity to mutate under stress for the survival, unrestricted consumption of drugs for various purposes making us to lose upon benefits of these wonder drugs (Gogarten, Doolittle, & Lawrence, 2002; Michael, Dominey-Howes, & Labbate, 2014). Due to overexploitation of antibacterials in animal farming and various agricultural practices, bacteria is gaining resistance and transferring it, which is leading to high mortality and morbidity rate which causes burden on economic system ranging as $20 billion with burden on health care system and facilities (Aarestrup, 2012; Ballal, 2016; Harrison & Svec, 1998). In studies it was shown that India was top antibiotic consumer in the world in 2010 next to china and the mortality rate reaching as high as 416.75 per 100,000 persons which is more than the burden in UK and almost double in comparison with US (Laxminarayan & Chaudhury, 2016). It was identified that around 88,000 type of infections resulted in 38,000 deaths annually (Sumpradit et al., 2017), Sepsis alone kills around one million infants worldwide along with TB that pose major burden on economic system especially in low income countries (Munro et al., 2007; Panigrahi et al., 2017). An estimated 18,650 deaths in the USA due to MRSA in 2005 was found which was more than the deaths due HIV/AIDS (D'Agata, Dupont-Rouzeyrol, Magal, Olivier, & Ruan, 2008). Around 27 Antibacterial-resistant infections cause over 50,000 deaths annually in Europe (Kapil, 2005). About 119 million people in the world that are estimated to be infected with lymphatic filariasis caused by Wuchereria bancrofti

are mainly from India (around 48 million especially from Tamil Nadu) alone, along with that the reported data suggest infectious diseases alone causes deaths that stands second in the world according to WHO reports (WHO 1994). Males are the most affected than females(Ramaiah, Ramu, Guyatt, Vijar Kumar, & Pani, 1998).

Generation time of bacteria is only 20-30 min and the rate with which new drugs are coming to health care system is very much negligible (Luepke et al., 2017). Apart from developing and feeding the drug pipeline with antibiotics various measures should be taken in order to insure proper antibacterials use, providing less competitive environment to bacteria, so that less ARB will now come into picture (S. Levy, 2014). Essential oils can be considered as a new realm in the discovery of antibacterials since they target the bacterial cell from multiple dimensions in comparison with available antibiotics which are specific in their target and prone to modification by bacteria and other microbes, many researches are going on in the field to prove the matter. These aromatic oils provides an alternative way to fight against antibacterial resistance and also the presence of antioxidant property shows its ability to use as food additives, preservatives and flavoring agents because they display little or no toxic side effects (S.-S. Han, Lo, Choi, Kim, & Baek, 2004), it maintains the natural flora within the body (as our body requires good bacteria to function properly and remove toxins), relatively less expensive and better biodegradability. Douglas fir tree were known to vary the composition and production of terpenes each year thus decreasing the ability of the budworm to develop widespread immunity to specific compound, which shows trees are naturally adopted and developed a system to combat the infection (Chen, Kolb, & Clancy, 2002). Plants were known for their ability to overcome the microbial intervention and contagion by cell wall strengthening and production of various antimicrobial compounds which are secondary metabolites (Delaney, Uknes, Vernooij, & Friedrich, 1994). Plant produces secondary metabolites which include alkaloids, tannins, terpenes and its derivatives, flavonoids, phenolic compounds etc. which shows pharmacological activity and are involved in defense mechanism, cell signaling, to enhance interaction with other microorganism and to avoid auto-toxicity (Bidlack, Omaye, Meskin, & Topham, 2000). *Eucalyptus* is an Australian native belongs to family *Myrtaceae* comprises around 700 to 800 species and is spread worldwide due to adaptability to various climates, soil and

growth condition {Fawad, 2012 #233}. This is the major source of essential oil for industrial and medicinal purposes, it is known for its various pharmacological properties. It has long been used for the treatment of colds, rhinitis, sinusitis and other respiratory infections. The literature study reveals that the botanical treatments have fewer side effects and are effective against a large number of ailments, the majority of research is focused on larvicidal activity of *Eucalyptus tereticornis* {Nathan, 2007 #229} but only few literature is available that concerns for the antibacterial activity of *Eucalyptus tereticornis* and hence the purpose of this project work is to characterize the chemical constituents and find out the potential antibacterial activity of *Eucalyptus Tereticornis* in-silico and in-vitro. Since many essential oils in various studies are found to target primarily the cell membrane and is known to be less effective against gram negative bacteria. Selection and comparison of beta-lactam antibiotic Ampicilin/Salbactam that mainly targets cell wall synthesis and is Beta-Lactamase inhibitor, along with that finding the drug equivalency and synergism is an approach that we had applied to predict *Eucalyptus tereticornis* essential oils as an alternative and which conc. of essential oil is required in comparison with the synthetic drug as these drugs pose various side effects sometimes that can be serious too while essential oils are natural, gets easily degraded and are less toxic to cell if used properly. The work program for this project work entitled as “In vitro and In silico study of Essential Oil Components from Eucalyptus tereticornis Essential Oil as Antibacterial agents” were.

1. Identification of components of essential oil of *Eucalyptus tereticornis* obtained from leaves by GC-MS technique.
2. In Vitro approach to find out the antibacterial activity of *Eucalyptus tereticornis* by using disc-diffusion and well-diffusion methods.
3. In Silico study of *Eucalyptus tereticornis* EO components against PBP3 and beta-lactamases bacterial enzyme.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITREATURE

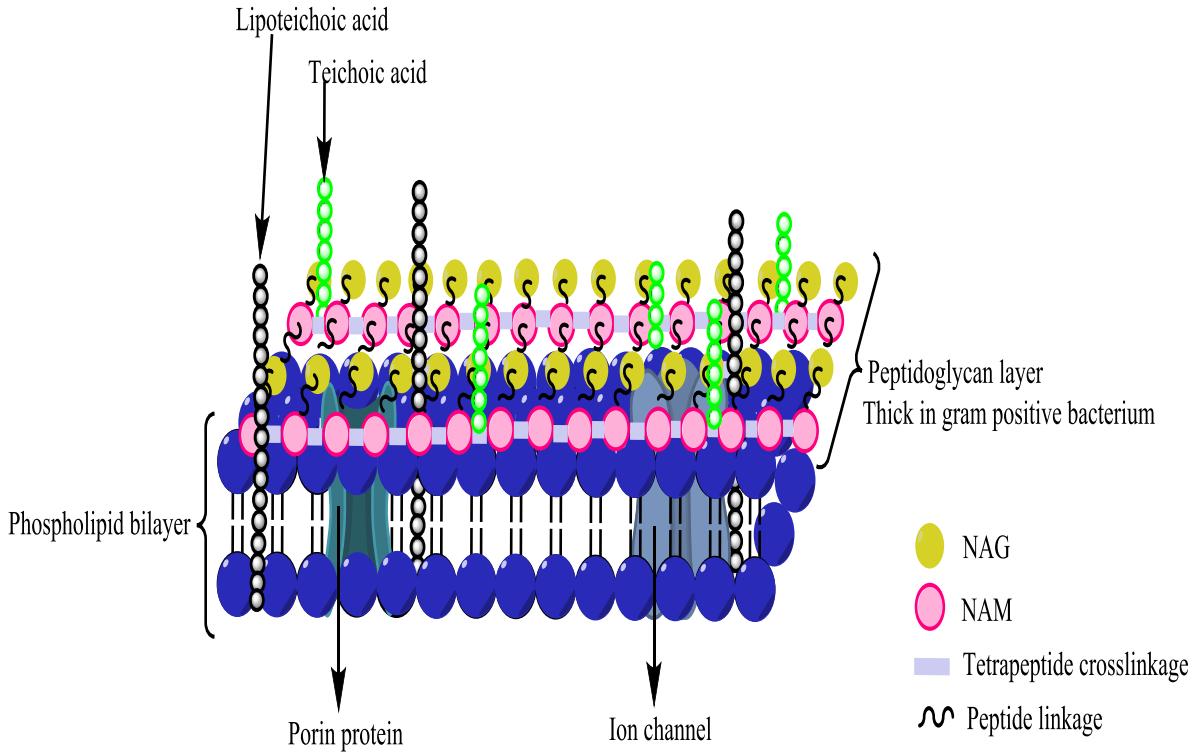
2.1 Antibiotic and Resistance

“SURVIVAL OF THE FITTEST”, (a process in which one organism may destroy another in order to preserve itself) the theory of evolution given by Charles Darwin has everything to do with bacterial evolution and antibacterial resistance as bacteria evolves, which is an old phenomenon, and the primary reason behind antibacterial resistance are the pressure that we put by excessive exploitation of antibiotics and microorganism themselves, fighting and surviving within the competitive environment has naturally led them to harvest many compounds to thrive that we use for the preparation of antibacterials (Burgess, Jordan, Bregu, Mearns-Spragg, & Boyd, 1999). Compounds that are obtained as a result of this competition from microorganism are termed as antibiotics that we use for treating infections. The term derived from the word ‘antibiosis’ which means survival of the fittest and it was first introduced by Vuillemin in 1889 (Rajbir Singh, 2002).

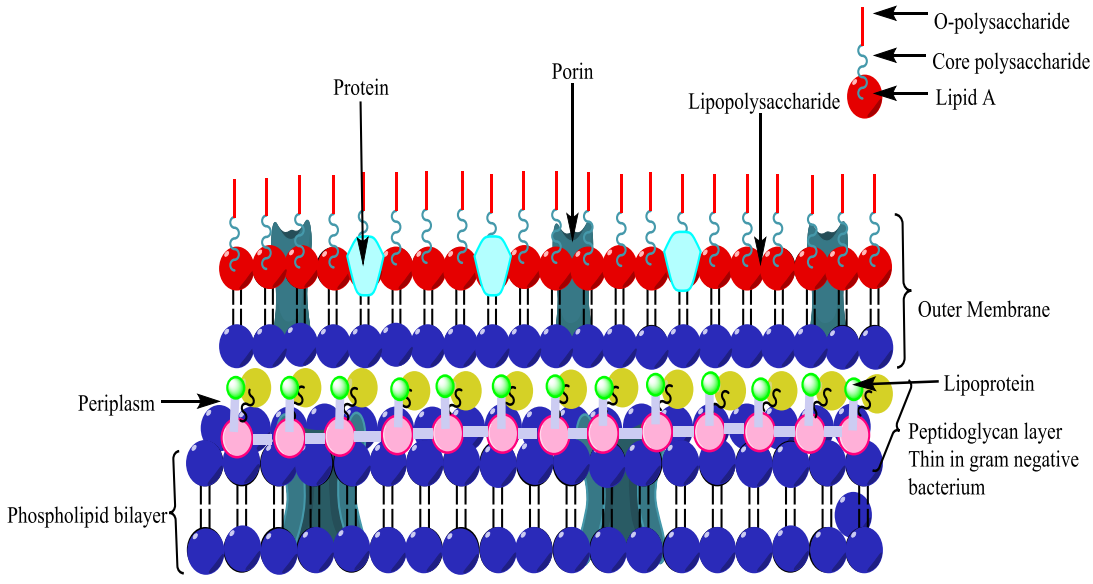
Ampicillin is a first broad spectrum, third generation of β-lactam antibiotics used for the treatment of various infection such as respiratory tract infections, meningitis, urinary tract infections, salmonellosis, and endocarditis etc., they are resistant to acid hydrolysis and are sensitive to beta- lactamases {Gilbert, 1997 #235}. The adverse effects associated with the use of antibacterials are also observed with Ampicillin that includes Diarrhea, pseudomembranous colitis, vomiting, and skin rashes {Cunha, 2001 #237}{Lusk, 1977 #236}. To overcome the effect of hydrolysis of beta-lactam ring by the enzyme, Salbactam (semi synthetic beta lactamases inhibitor) was used in combination, that was an easy target for the enzyme but the combination has more adverse effects on human health than benefits, as found in the study Nausea, vomiting, diarrhea, and rashes are the most common side effects found along with that the combination was ineffective against pseudomonal infection {Noguchi, 1988 #241; Walker, 1993 #240}. In clinical studies 43% of 768 bacterial strains that are harmful pathogens for humans are found to be resistant to Ampicillin {Lees, 1986 #242}

The evolution in the bacterial biology is an important aspect to understand to revolutionize the drugs used to cure bacterial infection and diseases as gram negative bacterium was found to develop fast mutation in many studies specially *E.coli* having symbiotic relation with the eukaryotic cells, is a potential pathogen to humans and livestock, whose certain strain can cause infection related to gastrointestinal tract, central nervous system, urinary tract, or bloodstream (Merkx-Jacques et al., 2013). *Escherichia coli* BL21 DE3 MTCC 1679 are facultative anaerobe where DE3 stands for λ prophage carrying the T7 RNA polymerase gene and lacI. The strain is famous for the production of proteins via T7 system having low error rate, T7 RNA polymerase system is a very active enzyme having higher turnover number than the normal *E. coli* RNA polymerase and is resistant to certain antibiotics such as rifampicin (Tabor, 1990).

The structural difference that bacteria possess is one of the factors behind antibacterial resistance i.e., gram positive bacteria is more susceptible towards antibiotics than do the gram negative bacteria (spectrum of antibiotics). Many antibiotics inhibit the growth of bacteria via action on enzymes involved in peptidoglycan synthesis such as beta lactam antibiotics, that are now becoming ineffective due to its overexploitation and resistance mechanism adopted and developed by bacteria (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013).



Cell wall of gram positive bacteria



Cell wall of gram negative bacteria

Figure.1. Difference of bacterial cell

2.2 Why Essential oils?

To counter the antibiotic resistance, ‘essential oil’ is the key, the term which is the diminution of the originals ‘quintessential oil’ which means spirit or life force (related to plants) (Rhind, 2012). These secondary metabolites are mainly obtained using distillation and evaporation in the form of complex mixture of volatile compounds having aroma in the order of their maximum concentration monoterpenoids > sesquiterpenoids > hemiterpenoids, which also depends upon the growth condition, preservation and extraction technique, along with it they may contain phenylpropanoids, fatty acids and some sulfur derivatives (Attokaran; Baser & Buchbauer, 2015). Practically essential oil can be obtained from any part of the plants that has long been exploited for their medicinal purposes that dates back to history, which was tested for their anticancer, anti-diabetic, antifungal, antibacterial, anti-malarial, antiviral activities etc. in modern era (Abdollahi et al., 2003; Edris, 2007; Kalemba & Kunicka, 2003; Sylvestre, Legault, Dufour, & Pichette, 2005). Low pH, low temperature and low oxygen levels are some of the condition that enhances the antibacterial activity of essential oils (S. Burt, 2004). Essential oil possess anti-inflammatory and immuno modulatory activity and Cinnamaldehyde, a major constituent of cinnamon essential oil, found to possess anti-mutagenic and anti-tumor properties in studies along with it many essential oils such as clove, rosemary, lavender are found to enhance the memory power in children’s aged 13–15 years, anticancer activity of essential oils that proves their therapeutic value in comparison with the antibiotics that have various adverse effects on human body (Anastasiou & Buchbauer; X. Han, Parker, & Dorsett, 2017). Essential oil gets absorbed easily from skin due to its lipophillic nature and in studies it was found to cause a better local blood-circulation, providing feeling of warmth and mood relaxant and pain relief to humans (Schilcher, 1985). Essential oils obtained from plant parts are found to have no or mild side effects as claimed in some of the studies along with it mild antibiotic effect in gut flora and have disinfecting effect on respiratory tract while in another study it was found that essential oils inhibit the growth of potential pathogen while not causing any harm to beneficial bacteria in the gut thus allowing them to boost the immune system by inhibiting the colonization of pathogens in the gut {Ouwehand, 2010 #239}{Groot, 2011 #238}. Linalool and eucalyptol reduced the DNA damage by 30% to 40%, by

suppression of *t*-BOOH induced mutagenesis in human cells, while myrcene was ineffective and eucalyptol have only moderate effect (Mitić-Ćulafić et al., 2009). In India the use of medicinal plants as folk medicine has been mentioned in rigveda that was believed to be as old as 4500 to 1600 B.C. (Chopra & Chopra, 1933). Egyptian used these botanical treatment for embalming in order to prevent the decaying of body and growth of the bacteria and other microbes (Edris, 2007). These medicinal plants are major sources of ailment treatment in developing countries providing medicine at low cost and fewer side effects and belief of older remedies used by ancestors in developing countries like India, Indonesia, china etc. (Gurib-Fakim, 2006). Over 100,000 known secondary metabolites produced via MVA Or MEP are involved mainly in plants defense, signaling mechanism and an estimated 3,000 essential oils are known out of which 300 are commercially exploited for industrial and other purposes (Bassolé & Juliani, 2012). Many essential oils are approved by FDA as safe for use in food and medicinal purposes (Smith & Navilliat, 1995).

2.3 BACTERIAL GENE AS TARGET OF ESSENTIAL OIL COMPONENTS

Genes that are associated with antibiotic resistance affects the expression levels of bacterial cellular components and are mainly acquired by HGT also known as lateral gene transfer that includes transformation, transduction, and conjugation, which is the introduction of genetic material from one species to another by mechanisms other than the vertical transmission from parent to offspring leading to bacterial evolution (Thomas & Nielsen, 2005). Molecular elements carrying these genes can be bacterial chromosome, plasmids, transposons, integrons, and bacteriophage (de la Cruz & Davies, 2000;Solheim, Sekse, Urdahl, Wasteson, & Nesse, 2013; Zhaxybayeva & Doolittle, 2011). Other mechanism using which bacteria can acquire resistance and protection against external factors are Bacterial biofilm formation and SOS response generated during bacterial DNA damage and bacterial biofilm formation which is adopted by bacteria to form bacterial community in order to survive and thrive in various harsh conditions (Miller et al., 2004).

Table. 1. Targets of essential oil components in bacterial cell.

ESSENTIAL OIL	ESSENTIAL OIL COMPONENT	PROTEINS	GENES	REFRENCES
Clove Nutmeg Cinnamon Basil Bay leaves	EUGENOL	Fimbrial proteins curli fimbriae (Csg), type I fimbriae (Fim), E.coli common pilus (Ecp), F9 fimbriae (Z2200)	Ler Ler-controlled genes(espD, escJ, escR, and tir) Curli producing genes (*csgABDFG*)	(Y.-G. Kim et al., 2016)
Hamamelis virginiana L	HAMAMELITANNIN	TraP receptor AgrC	Trap	(Brackman et al., 2016)
Cinnamon	CINNAMALDEHYDE	LuxR		(Wong, Ahmad-Mudzaqqir, & Wan-Nurdiyana, 2014) (Brackman et al., 2011) (Sheng, Rasco, & Zhu, 2016) (Domadia, Swarup, Bhunia, Sivaraman, & Dasgupta, 2007)
		Stx2 (SHIGA TOXIN)	stx2 gene	
		FtsZ		
			QseBC, luxS	
Moroccan thyme	CARVACROL AND THYMOL	AcrAB pump		(Aelenei et al., 2016) (Fadli, Chevalier, Hassani, &

				Mezrioui, 2014)
Pine , Rosemary, Satureja myrtifolia	(+)-α-pinene	Phospholipase A		(Silva et al., 2012)
C. Boreale		GTFase		(B.-S. Kim et al., 2015)
	THYMOL	Upregulates OmpX and OmpA		(Rai & Kon, 2013)
Garlic	ALLICIN		PelF	(Ninyio et al., 2017)
Ferula and dorema		LasI , LasR system Elastase		(Sepahi, Tarighi, Ahmadi, & Bagheri, 2015)
Bay, clove and pimento berry	EUGENOL	Curli	CsgABDFG	(Y.-G. Kim et al., 2016)
		Fimbriae	fimCDH	
		Toxins	espD escJ, escR tir	
Perilla oil	PERILLALDEHYDE, β-CARYOPHYLLENE AND LIMONENE ELIMICIN, MYRISTICIN	α-toxins		(Qiu et al., 2011) (Bumblauskiene , Jakstas, Janulis, Mazdzieriene, & Ragazinskiene, 2009) (Solórzano-Santos & Miranda-Novales, 2012)
		SEA	sea	
		SEB	seb	
		TSST-1	tst agrA	
Oregano	CARVACROL	Shiga toxin	luxS ler stx2B fliC	(Mith, Clinquart, Zhiri, Daube, & Delcenserie, 2015)
Arabidopsis thaliana Zingiber nimmonii	(E)-β-CARYOPHYLLENE			(Huang et al., 2012)

	CARVACROL	Induction of heat shock protein 60 Inhibition of flagellin ATPase		(Barros-Velazquez, 2015) (Burt et al., 2007)
	FARNESOL	Inhibit recycling of the C55 lipid carrier of the murein monomer precursor β-lactamases		(Kurek, Nadkowska, Pliszka, & Wolska, 2012)
Myrtle		Interacts with Lipoproetic bilayer of cytoplasmic membrane		(Galizzi, Cacco, Siccardi, & Mazza, 1975) (Aelenei et al., 2016)
Garlic	ALLICIN	Thiol protease papain NADP+ dependent Alcohol dehydrogenase		(Bizerra, Da Silva Junior, & Hayashi, 2012)
Perilla	α-LINOLENIC ACID	α-toxin , enterotoxin A and B		(Rai & Kon, 2013)
Immortelle	GERANIOL	AcrEF efflux pump		(Lorenzi et al., 2009)

An important class of proteins is a transcription factor which regulates differential gene expression in an organism that can be modulated by phenolic or other components of essential oil having tendency to form covalent bond, hydrogen bonds or interacts with other ionic or non ionic interactions, that directly or indirectly affects the gene regulation. In many studies done on effects of essential oil components on gene expression, many genes were found to be either up or down regulated (Muthaiyan et al., 2012).

2.4 MECHANISM OF ACTION OF ESSENTIAL OIL AND THEIR COMPONENTS

Various mechanisms of action were proposed in numerous literatures and varied data on activity was available which depends on the bacterial species taken and

the condition of storage, growth and method of extraction of essential oils. Essential oils shows broad spectrum of activity against large number of bacteria's though it is mentioned in texts that gram negative bacterium is less susceptible than gram positive bacterium due to the differences in the cell wall structure of bacterium that not only protects the bacterium but are also involved in pathogenesis. Essential oils are complex mixture of various compounds in variable amounts that are mainly composed of terpenes, terpenoids which are hydrophobic in nature that primarily targets the bacterial cell Cytoplasmic membrane followed by secondary action that result in potassium ion efflux, pH decrease, ATP loss etc. (Gayán, Torres, & Paredes-Sabja, 2012).

2.4.1 Effect on Cytoplasmic Membrane and Outer Membrane

Primary site of action of essential oil components is Cytoplasmic membrane which plays a vital role in proper functioning of bacterial cell (Weiner & Rothery, 2007). Mainly composed of phospholipids bilayer and proteins, it is protein embedded membrane that performs variety of function in order to maintain cellular functions such as energy production, ATP synthesis, osmotic pressure and along with it providing a protection barrier and selectivity for solutes and compounds to enter inside of the cell (Barák & Muchová, 2013). Components of various essential oil are known to accumulate with in lipid bilayer of Cytoplasmic membrane where the hydrophobic (less polar) components of essential oils mainly interacts with the hydrophobic part of the lipid bilayer and dissolve in it leading to expansion and hence permeability of the membrane increases though the outer membrane remains intact might be due to the Mg^{+} ion that protects the membrane against such deleterious effects of hydrocarbons (Sikkema, de Bont, & Poolman, 1995; Trombetta et al., 2005). This accumulation tends to reduce lipid-protein and lipid-lipid interaction and destruction of cell morphology. The antibacterial activity of the essential oil varies according to the concentration and the type of bacterium, which was mainly attributed to a variation in the role of penetration of active compounds of the essential oil through the cell membrane (S. M. Silva et al., 2011). Predicted mechanism is that essential oil might bind to the cell surface and then penetrates to the target sites, possibly the plasma membrane and there it interacts with membrane-bound enzymes that result in the disruption of cell wall structure that renders the synthesis of some macromolecules, such as DNA, RNA, protein, or

polysaccharides which eventually leads to cell death (Rhayour, Bouchikhi, Tantaoui-Elaraki, Sendide, & Remmal, 2003). Carvacrol affect the protein folding or interaction of outer membrane which is evident with the fact that cell produces GroEL chaperonins (indicative of protein misfolding) and Thymol are found to interact with outer membrane of gram-negative bacteria making it perturbed, thus releasing lipopolysaccharides (LPS) and the permeability increases along with that many Heat shock proteins were also up-regulated (S. Burt, 2004). Isoeugenol and Thymol mainly interacts with the polar head group region of the lipid bilayer where they are found to form H-bond between the polar side chains and phosphate or carbonyl group of phospholipid headgroup and also interacts with propenyl chain intercalating into the acyl chains to destabilize membrane. There they enhances the interfacial lateral pressure of the outer leaflet of the membrane that ultimately affects the lipid packing and membrane becomes leaky and distorted shape is observed in many cases (Hyldgaard et al., 2015; Preedy, 2015). dltABC operon that are mainly involved in the alanylation of wall teichoic acid which controls the autolysin activity in S. aureus, repression of the gene in the presence of cold pressed orange essential oil leads to lysis, along with that SOS system was inhibited that bacteria uses mainly in adverse condition for survival and hence ability to overcome the action of essential oil seems difficult for bacteria and eventually death occurs (Muthaiyan et al., 2012). Inhibition of toxin production is a consequence of sequential action of essential oil on bacterial cell that includes the attachment of essential oil which might disturb the phospholipid bilayer with consequences to the trans-membrane transport process by action on membrane proteins that limits the way the release of toxins to the contiguous environment (Faleiro, 2011)

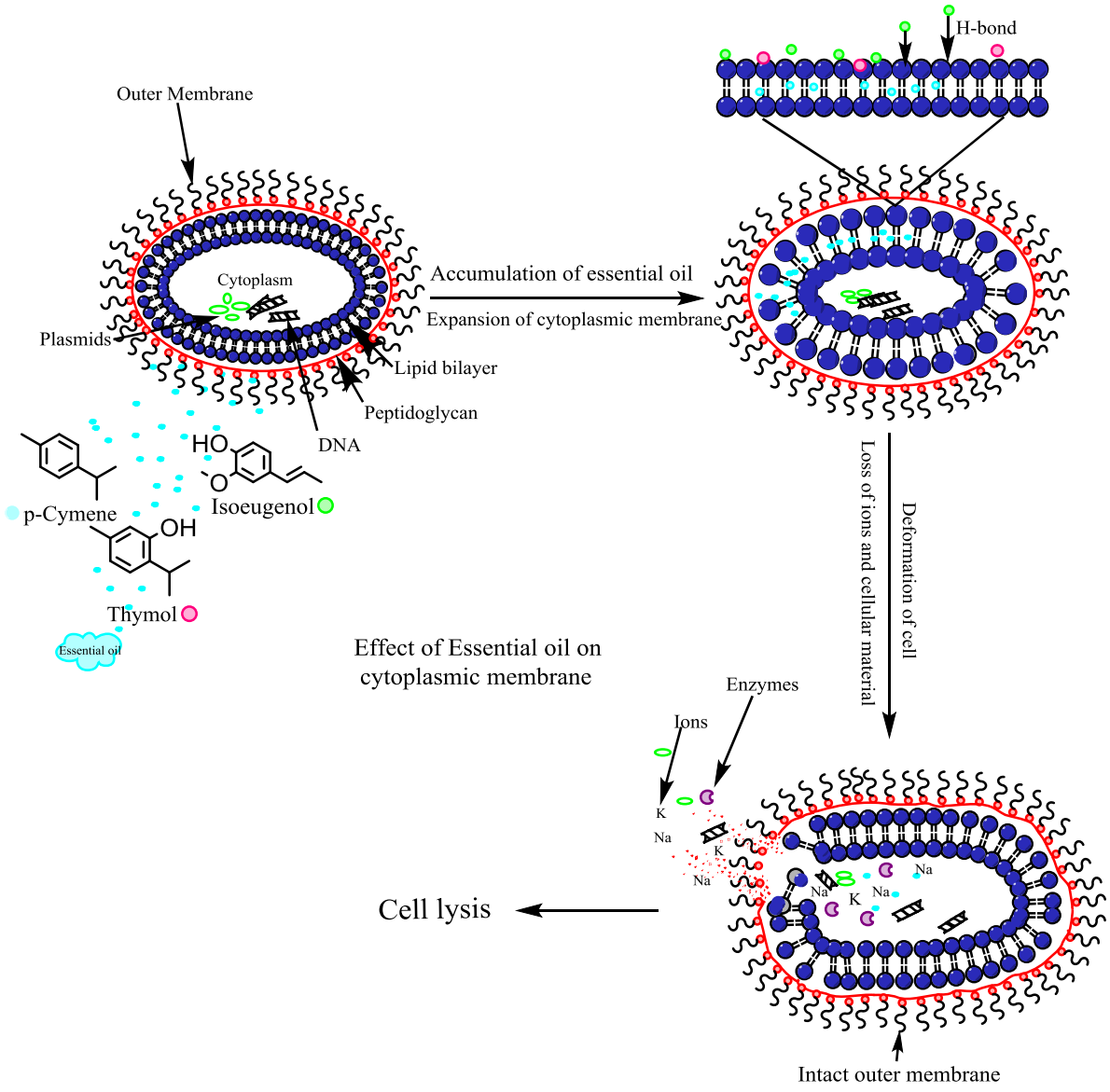
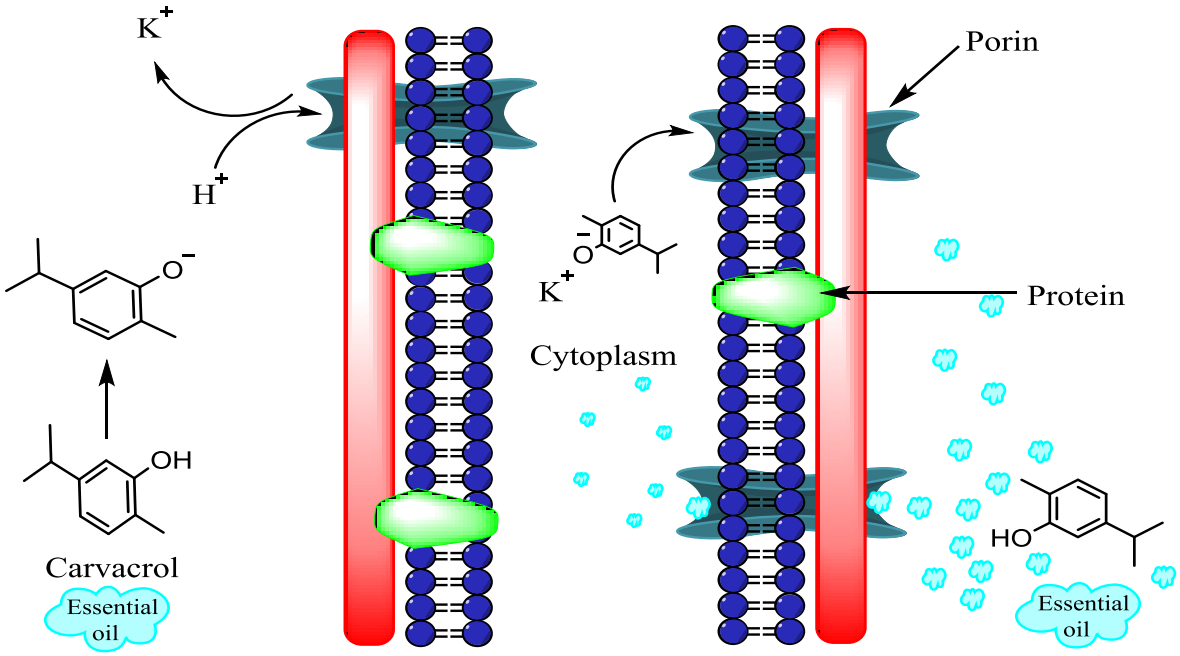


Figure.2. Mechanism of accumulation and expansion of phospholipid bilayer

2.4.2 Disruption of potassium gradient

Potassium ion, an important cation that is required for the activity of intracellular enzymes, involved in the maintenance of a constant internal pH and membrane potential along with that it act as second messenger in bacterial cell. The gradient of K^+ ion was maintained across the Cytoplasmic membrane by membrane embedded energy-dependent pumps such as Kdp, Trk, Kup (Gründling, 2013). Bacteria controls membrane potential ΔΨ and proton gradient ΔpH in order to maintain a constant value of proton motive force, dissipation in either of the component is compensated by a counteracting increase in the other, Carvacrol

phenolic monoterpene acts as a trans membrane carrier by exchanging its hydroxyl H^+ for another ion such as K^+ (Farha, Verschoor, Bowdish, & Brown, 2013; Fitzgerald et al., 2004; Ultee, Bennik, & Moezelaar, 2002). Many phenolic compound such as Carvacrol, Thymol found in essential oils are weak organic acid that partially dissociates and involved in ion exchange along with it they also possess lipophilicity that allows them to penetrate the cell membrane and causes damage to membrane and the observed tendency for phenolic compounds to cause K^+ ion efflux (p-coumaric acid>ferulic acid>caffeic acid>gallic acid >protocatechuic and vanillic acid) along with it phosphate efflux was also observed (Campos et al., 2009).



Action of Essential oil on Ion Gradient

Figure.3. Mechanism of phenolic component of Essential Oil acting as transmembrane proton carrier

2.4.3 Dissipation of ATP synthesis

ATPase is a synthase enzyme consisting of a stalk, the F_0 protein and spherical bulb shaped F_1 part embedded in cell cytoplasm, mainly involved in the synthesis of ATP from ADP. The enzyme is powered by inflowing protons that flows through a channel in the F_0 stalk into the F_1 portion which contains the enzymes active site, that starts rotating in a same way like flagella and the rotational energy gets

converted into chemical energy (Deckers-Hebestreit & Altendorf, 1996; Toei et al., 2007). Perturbation in membrane protein, loss of ion gradient ultimately leads to loss of pH homeostasis and dissipation of ATP synthesis within the cell cytoplasm along with that the increased permeability of plasma membrane causes increase of extracellular ATP that shows leakage in the cell membrane (Adiguzel, Ozer, KiliC, & CetiN, 2007). The proton motive force is essential for flagellar motility, nutrient import and the production of ATP by F_0F_1-ATPase, such that dissipation of electrochemical gradient halts the ATP production, flagellar motility and import of nutrients (Farha et al., 2013). In some studies no proportional extracellular ATP increase was found instead intracellular decrease which suggests the rate of ATP synthesis was reduced or that the rate of ATP hydrolysis was increased (Preedy, 2015). Carvacrol was found to dissipate the pH gradient across the cell membrane and efflux rate of phosphate ion along with loss of phosphate ions required for ATP production (S. Burt, 2004; Campos et al., 2009). Thymol affects the citrate acid cycle and many other enzymes directly or indirectly involved in ATP synthesis along with it Carvacrol disrupts the membrane potential and hence the proton motive force needed to drive flagellar movement which ultimately leads to non motile cells (Hyldgaard, Mygind, & Meyer, 2012)

2.4.4 Action on membrane proteins

Thymol mainly obtained from thymus vulgaris is found to target membrane bound proteins and causes misfolding and upregulation of several genes involved in outer membrane formation. Along with it also affects the citrate acid cycle which directly or indirectly affects the ATP synthesis which leads to loss in energy and various cellular activities and eventually death (Chouhan, Sharma, & Guleria, 2017). Eucalyptus globulus obtained from fruit was rich in Aromadendrene that has a reactive exocyclic methylene group and a cyclopropane ring that can alkylate the protein making it inactive (Mulyaningsih, Sporer, Reichling, & Wink, 2011). In the presence of Thymol cell produces various heat shock proteins such as HSP 60 (GroEL) and HSP70 (DnaK) that indicates protein misfolding (Hyldgaard et al., 2012).

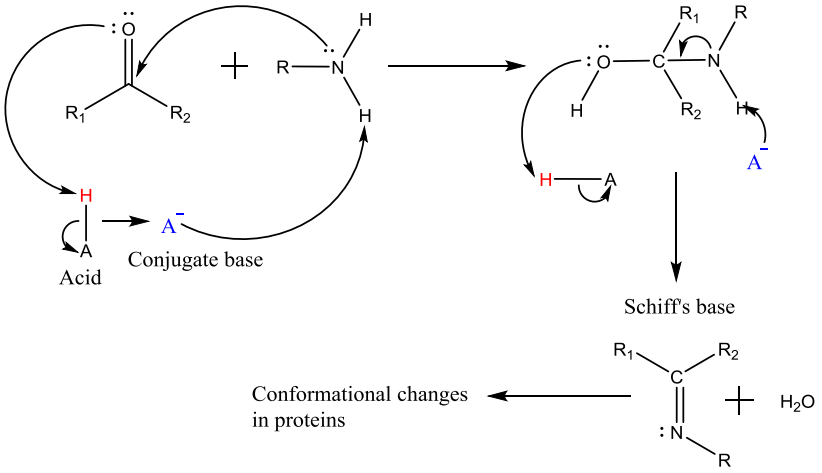


Figure. 4. Effect of essential oil components containing aldehyde group on proteins

Components of essential oils with an aldehyde group can form Schiff's base with the amino group of proteins, amino acid residues or DNA bases; such reaction induces conformational changes in the proteins

Allyl isothiocyanates found in mustard, garlic essential oils, contains isothiocyanate group which is highly electrophilic that induces oxidative stress on proteins and reacts readily under normal physiological conditions with nucleophiles containing oxygen, sulphur or nitrogen at the centre and hence can cleave the cystine disulfide bond in protein as an oxidative process that results in inhibition and or inactivity of proteins (Hayashi, Bizerra, & Da Silva Junior, 2013; Kawakishi, Goto, & Namiki, 1983).

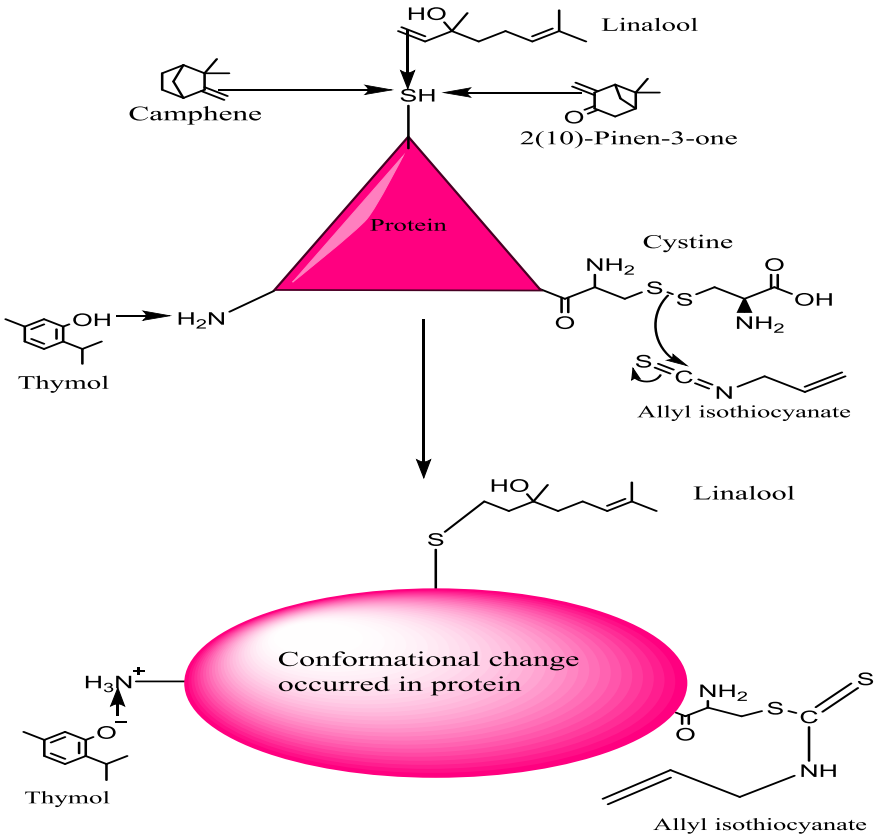


Figure. 5. Conformational changes occurred on action of essential oil components

2.4.5 Effect on fatty acid synthesis

Fatty acid synthesis is important for maintaining the cell homeostasis, growth, energy production and the production of a number of lipid-containing components, including the cell membranes, lipid A part of outer membrane of gram negative bacterium, which is synthesized by the bacteria using the FAS II pathway unlike mammals who uses FAS I pathway providing selective inhibition target for antibacterials (Kaneda, 1991; Muraleedharan & Avery, 2007). The fatty acids synthesized by the bacterium are shorter, lacks polyunsaturation and monoenoic C18 acids, FabI the enoyl-ACP reductase enzyme, involved in the final and rate limiting step of the chain elongation process of type II FAS, was the most explored target and inhibited using drugs like triclosan and isoniazid (Zhang, White, & Rock, 2006). Linoleic acid inhibits the incorporation of acetate into phospholipids thereby inhibiting the FabI, it was shown that linoleic acid can bind both free enzyme (to prevent the binding of the nucleotide cofactor NADH or NADPH) and also binds to the FabI–NADPH complex (to prevent the binding of the substrate) (Zheng et al.,

2005). Essential oils may inhibit the desaturase enzyme involved in saturated fatty acid synthesis and also affects the pool of fatty acid synthesizing enzymes that lead to an increase in the cis isomers, reduction of the chain length and a general decrease of UFAs and increase of SFAs in the membrane lipid bilayer that results in a loss of membrane fluidity and a consequent increase in membrane rigidity that leads to loss of metabolites and eventually cell death (Nazzaro et al., 2013).

2.4.6 Effect on biofilm formation and quorum sensing activity

Quorum Sensing is a mechanism using which bacteria, either gram positive or negative keep check on density population via. Gene expression with the help of chemicals called as autoinducers or bacterial pheromone. It modulates both intra- and inter-species cell to cell communication which helps in building complex community structure and in the regulation of activities, such as virulence factor expression, bioluminescence, sporulation, biofilm formation and mating (Szabó et al., 2010). Biofilms associated infections was estimated around 65% and that treatment of these biofilm-based infections costs >$1 billion annually (Mah & O'Toole, 2001). *C. boreale* EO was found to affect the mRNA expression level of several virulence factor genes mainly involved in biofilm formation such as gtfB, gtfC, gtfD that encodes for GTFase enzyme important for glucan synthesis from sucrose that helps in decreasing free binding energy to the surface in contact for bacterial adhesion along with the decrease in vicR level that regulates the expression level of genes mentioned above, It also decreases the expression level of gbpB that encodes for surface associated glucan binding protein which mediates the interaction between cell surface and glucan (B.-S. Kim et al., 2015). Cinnamaldehyde was found to posses inhibitory action against the transcription of LuxR led by promoter P_{LUXI} by 70% (Faleiro, 2011).

2.4.7 Inhibition of bacterial cell division and growth

FtsZ is a GTP-dependent protein that polymerizes and creates Z-ring at the centre of the cell division of Cytoplasmic membrane, it is a homolog a tubulin found in eukaryotes important for cell division and proliferation (Randich & Brun, 2015). Many compounds present in essential oils are known to inhibit the growth and cell division of bacterium by targeting the FtsZ protein (Filamentation temperature sensitive protein Z) such as Cinnamaldehyde, Curcumin etc., H2 and H3 of

Cinnamaldehyde interacts with G295 and V208 of FtsZ (Hyldgaard et al., 2012). Cinnamaldehyde inhibits the cell separation in bacillus cereus as it perturbs the Z-ring morphology and also reduces the Z- ring per unit length in E.coli, binding of which inhibits the GTP hydrolysis and polymerization by FtsZ protein and inhibits the cytokinesis and hence cell could not divide though septa were present after division (Domadia et al., 2007; Hyldgaard et al., 2012).

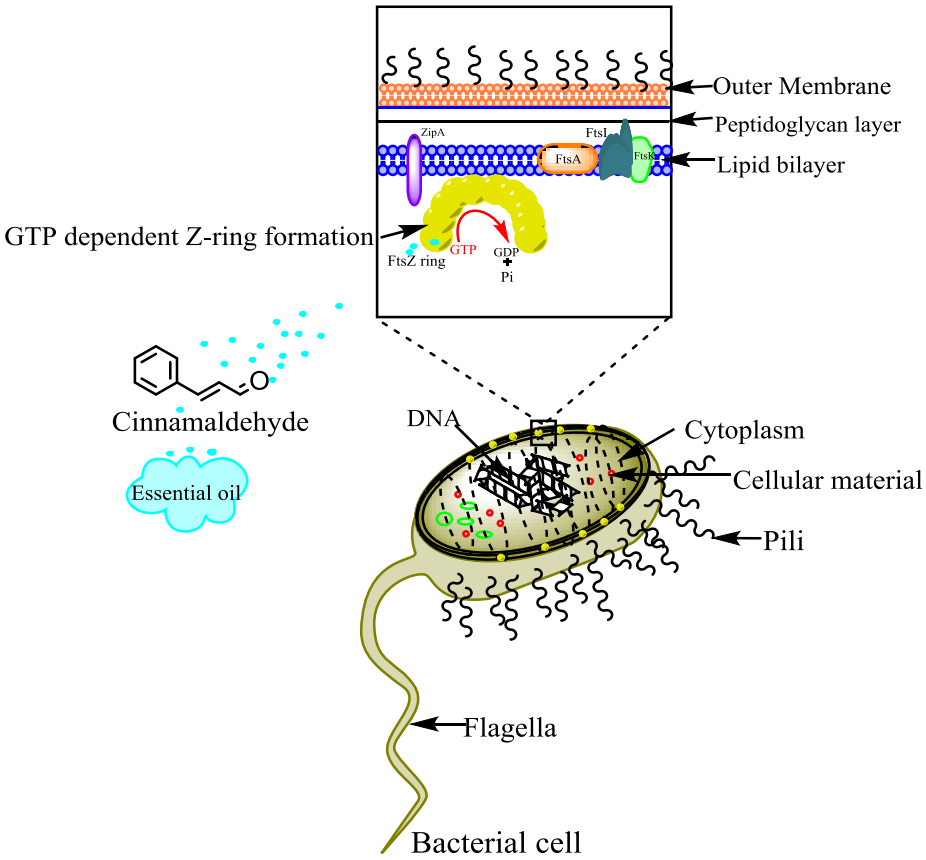


Figure.6. Inhibition of Z-ring formation by components of essential oil

Curcumin present in turmeric essential oil is found to bind on the same site as that of GTP and hence inhibition of gunanine nucleotide hydrolysis, it also perturbs the Z-ring formation by inhibiting FtsZ assembly (Kaur, Modi, Panda, & Roy, 2010). Thioredoxin-1 (Trx-1) important for cell division is E12 kDa redox protein and is essential for regeneration of methionine sulfoxide reductase was not expressed in the presence of Thymol (Nazzaro et al., 2013).

Allicin inhibited the degradation of mRNA as well as RNA synthesis, thus inhibiting the growth of the cell (Feldberg et al., 1988). Zeylasteral and demethylzeylasteral a triterpenoid obtained from *Maytenus blepharodes* block the bacterial cell division

by inhibiting DNA synthesis, macromolecules synthesis and inhibition of incorporation of N-acetylglucosamine in the cell wall is the main target in Bacillus subtilis (Moujir, 2005).

2.4.8 Inhibition of toxin production

SLO (streptolysin O) is a potent streptococcal cytolytic toxin that contributes to the pathogenesis of streptococcal infections is inhibited by the sulfur compound present in garlic essential oil and is found to bind the cysteine residue, essential for activity, using thiol-disulphide exchange reactions though the activity is low due to its tendency to bind free thiol group (Arzanlou & Bohlooli, 2010).

2.4.9 Modulation of drug resistance

The potential of these phytochemicals as antibacterial agents is known and tested to evaluate the result, apart from this they also possess tendency to enhance the susceptibility of bacterial strains that are known to be resistance to certain antibiotics available in the market. Many essential oil components such as geraniol, are found to modulate the drug resistance by targeting the efflux pumps over expression thus restoring the susceptibility of bacterium against the drug (Chouhan et al., 2017). *C. grewioides* essential oil mainly consists of α-Pinene, sabinene and limonene acts as putative efflux pump inhibitors in bacteria (de Medeiros et al., 2017). Pentacyclic triterpenoids oleanolic acid and ursolic acid can modulate resistance to β-lactam antibiotics, Ampicilin and oxacillin, along with that sesquiterpene farnesol possess ability to inhibit the recycling of the lipid carrier of murein monomer precursor and reduces the secretion and activity of β-lactamase thereby making bacterium susceptible towards β-lactam antibiotics, reduced biofilm formation was also observed (Kuroda, Nagasaki, & Ohta, 2007; Wang, Jhan, Tsai, & Chou, 2016)

2.4.10 Synergistic activity of essential oils

Since essential oil contains various compounds in variable amounts, additive, antagonist and synergistic effect may be observed between the components and antibiotics used in combinations (Fahimi, Hajimehdipoor, Shabanpoor, Bagheri, & Shekarchi, 2015). The additive effect occurs when the combined effect of the components is equal to the sum of the individual effects. Synergism is observed

when the activity of the combined substances is higher than the sum of the activity of individual activities. In contrast the antagonistic effect is observed when the activity of components in combination is inferior in comparison when they are applied separately (White, Burgess, Manduru, & Bosso, 1996). p-cymene alone is a weak antibacterial agent but it causes cell membrane to swell more than Carvacrol since p-Cymene is more hydrophobic than Carvacrol that enables it to be transported more easily into the cell and act intracellularly (Fahimi et al., 2015). HEPu (hydroethanolic extract of *Piper umbellatum*) was able to increase the permeability of the OM of *Shigella flexneri* thus allowing the entry of many antibiotics and hence shows synergism (da Silva Jr et al., 2014).

2.5 Resistance to essential oil components

As a whole essential oil is multiple target drug that targets the bacterial cell from multiple dimensions and resistance to essential oils as a whole is less probable and isn't reported but there are some reports that suggests use of individual compounds presents in essential oils is not beneficial and that bacteria are capable of metabolizing and making it flux out of the cell by expression of porin proteins that readily removes toxic material from the cell. Allicin a sulfur compound present in the essential oil of garlic is capable of inhibiting the RNA synthesis thus act as bacteriostatic, is found to get metabolized within the cell or they prevent its interaction with critical targets though cells are not able to recover fully after treatment with allicin was also observed (Feldberg et al., 1988).

2.6 Eucalyptus

Comprising around 700 species, eucalyptus an Australian native is fast growing evergreen tall trees known to grow in various climatic conditions whose first plantation was done in India by Tippu Sultan, the ruler of Mysore in his palace garden on Nandi hills in around 1790 and later on blue gum was introduced (E.Globulus) by Britishers in Nilgiri hills(Batish, Singh, Kohli, & Kaur, 2008; Naithani). Popular for agro-forestry, *eucalyptus* mainly planted in India to meet the need for timber, fuel-wood and wood production(Patil, Patil, Mutanal, & Shahapurmath, 2012). Mainly five eucalyptus species was planted in India namely

E. camaldulensis (River red gum or Murray red gum), *E. globules* (Blue gum or Tasmanian blue gum), *E. grandis*, *E. tereticornis* (Forest red gum), *Eucalyptus citriodor* (Lemon scented gum) out of which *Eucalyptus tereticornis* was mainly planted in Punjab, Haryana, Gujarat, Madhya Pradesh, U.P, Bihar etc. due to its ability to grow in dry tropical to moist tropical areas with annual rain fall of 400 to 4,000mm(Luna). Plantation of eucalyptus species mainly began in the mid-eighties in Punjab, but in recent years it is hold responsible for declining ground water table specially in south India(Calder, 1986; Puri, 2007). *Eucalyptus* is known for its wide spectrum of biological activity such as anti-microbial, fungicidal, insecticidal/insect repellent, herbicidal, acaricidal and nematicidal etc. (Badrunnisa, Ramanath Pai, & Shantaram, 2011; Batish et al., 2008) and is known for treating various respiratory disorders such as bronchitis and sinusitis (Elaissi et al., 2012). *Eucalyptus Camaldulensis* is shown to possess antioxidant and anti-diabetic activity (Basak & Candan, 2010). Indigenous to various countries *Eucalyptus* has been described in France, United States, China, British and European Pharmacopoeias having a wide range of pharmacological properties and must contain at least 70% of 1,8-cineole for its medicinal uses obtained via steam distillation (Dey & Mitra, 2013).

Taxonomical classification

TREE**:** *Eucalyptus Tereticornis*

Genus: *Eucalyptus*

Family**:** *Myrtaceae*

Kingdom: *Plantae*

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Myrtales

Figure.7. *Eucalyptus* Tree (Image taken nearby Central University of Punjab)

Mysoore gum or forest redgum is a hybrid of *eucalyptus* also known as *Eucalyptus Tereticornis* (Chaturvedi, 1989) has long been known for its larvicidal, mosquito repellent activity and tested for its radical scavenging activity and is considered to be safe for human use in food and eco-friendly (Nathan, 2007; H. P. Singh, Mittal, Kaur, Batish, & Kohli, 2009). Essential oil from *Eucalyptus tereticornis* essential oil is mainly obtained from leaves of the tree but very few reports have been found that mainly focuses on its antibacterial activity. Other than the essential oil, the leaf are a good source of important triterpenoids such as ursolic acid and betulinic acid, which has wide range of pharmacological activities and therapeutic use and found to contain various non-volatile phyto constituents such as acyl phloroglucinol derivatives, triterpenoids, tannins, lignins, long chain fatty acids, flavonoids and other phenolic compounds {Maurya, 2016 #230}. From historical era our ancestors relied upon the medicines obtained from plants sources and they were proved to be beneficial for the health and well being and hence belief of population to these

treatments are more than the synthetic one. The products that are available in the market containing eucalyptus are herbal syrup, tea, cough candies, sinus comfort tea used for aromatherapy in bronchial wellness, herbal oils used for the treatment of lice and itchy scalp in hairs, soaps, sanitary balms , mouthwash, toothpastes etc. {Farrar, 2013 #243}. *Eucalyptus tereticornis* was a major source for honey, nectar and was found to exhibit hypoglycemic effect mainly with aqueous extract that decreases the blood glucose level by affecting the metabolism {Dey, 2013 #244}.

CHAPTER 3

RATIONALE AND OBJECTIVES

3. Rationale and Objectives

3.1 Rationale

While going through the texts that are available on antibacterial activities of essential oils, we have observed that essential oils possess strong cytotoxic activity and active against the large sets of microbes as the compounds present in essential oils are mainly involved in the plant's defense system. Though many literature talks about the mechanism of action but the in-depth knowledge lacks still. We are trying explaining and find out how essential oils can act as an alternative or work in synergism with antibiotics, along with the mechanism of action using in-silico and in-vitro approach.

3.2 Objectives

The main objectives of this dissertation work are:

- Identification of components of essential oil of *Eucalyptus tereticornis* obtained from leaves by GC-MS technique.
- In Vitro approach to find out the antibacterial activity of *Eucalyptus tereticornis* by using disc-diffusion and well-diffusion methods.
- In Silico study of *Eucalyptus tereticornis* EO components against PBP3 and beta-Lactamase bacterial enzyme.

CHAPTER 4

MATERIAL AND METHODS

4. MATERIAL AND METHODS

4.1 Essential oil

Essential oil of *Eucalyptus tereticornis* was humbly provided by Dr. Vikas Jaitak (Assistant Professor; Department of Pharmaceutical Sciences and Natural Products) previously extracted by Abhilash Rana the student of MSc. Medicinal Chemistry (2015-17), kept in airtight vile to prevent subsequent leakage due to the volatility of essential oils.

4.2 Bacterial Strain and Growth Conditions

The in-vitro activity of the *Eucalyptus tereticornis* essential oil was assayed against the bacterial strain *E. coli* BL21 (D3) MTCC 1679 was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, stored in MHA agar plates at -20^{o}C temperature was obtained from Biochemistry Lab, Central University of Punjab with the permission of Dr. Ramakrishna Wusirika (Professor; Department of Biochemistry and Microbial Sciences). The strain is famous for the production of proteins via T7 system, T7 RNA polymerase system is a very active enzyme having higher turnover number than the normal *E. coli* RNA polymerase and is resistant to certain antibiotics such as rifampicin (Tabor, 1990).

4.3 Antibiotic Disc

A combination of Ampicilin/Salbactam (10:10 mcg) used for this project work was provided by the Biochemistry lab. A combination drug used to inhibit the beta-lactamases and Transpeptidases enzyme along with that they are having various serious side effects as well. The images of the antibiotics used are shown in figure

4.4 Reagents and Chemicals

HPLC grade Dichloromethane, $BaCl_2.H_2O$, H_2SO_4, Nutrient broth, MHA from Hi Media, Sodium chloride, Distilled water, DMSO, Ethanol.

4.5 Apparatus Used

Disposable Petri Plates, Inoculation loop, conical flasks (500 ml), L-Spreader, Test tubes, Autoclave, vile, Eppendorf, Whatman Filter paper.

4.6 Instrument used and Software Used

- 96 Well microplate reader UV spectrophotometer (Synergy H1) from Biotek Company is used for obtaining OD in order to standardize the concentration of cell per mL of the solution, equipped with Agiliant biosystem (Software to calculate CFU/mL from OD).
- Laminar Air Flow workstation from Klenz Flow is an enclosed stainless steel bench, which is thoroughly designed to maintain aseptic condition. It is equipped with High Efficiency bacterial-retentive filter that filters and kills nearly all bacteria from the air.
- Molecular docking analyses were carried out on windows 7 professional platform running on an HP-Work Station K800 series with Intel Xeon processor and 64 GB RAM, 32 core processor, E5-2620 version4@2.10 GHz. The latest version of Schrodinger 2018-1-LLC (NY-USA0 and ChembioDraw Ultra-14 developed by Cambridge Pvt. Ltd. was used.

4.7 GC-MS Analysis

The compounds presents in the *Eucalyptus tereticornis* EO was analyzed using Gas Chromatograph coupled with Mass Spectrophotometer (GC-MS) developed by Shimadzu on Shimadzu QP 2010 Mass Spectrophotometer. Separation in the GC-MS was carried out using Rxi®-5Sil MS Column situated in Oven and a carrier gas used was He. It is Low Bleed Column, inert for acidic, basic and polar compounds. Column was programmed from 40°C to 240°C at 4°C/min and then kept constant at 250°C for 10 min for both the analyses. The gas carrier was Helium maintained at a flow rate of 1.00mL/min at a pressure of 49.5 kPa. The syringe was washed with 8 µL of chloroform and 2 µL *Eucalyptus* essential oil solutions in chloroform was injected through autosampler and analyzed with Rxi®-5Sil MS column. Peak areas were measured by electronic integration and the relative amounts of the individual components are based on the peak areas.

4.8 In vitro studies

Antibacterial susceptibility testing of the *E.coli* isolates was performed by the Kirby-Bauer disc diffusion method and well diffusion method using Mueller-Hinton agar (Hi-Media) according to the NCCLS Standards. Antibacterial susceptibility

test is a well defined and established method used to determine the susceptibility of a particular microorganism to a drug. It is a qualitative assay in which discs of filter paper are impregnated with a single concentration of different antibiotics or any chemicals that will diffuse from the disk into the agar. The discs are then placed on the surface of an agar plate which has already been inoculated with test bacteria. During the incubation period, the compound taken diffuses into the agar that creates a concentration gradient in the agar which depends on the solubility and molecular size of the compound. The absence of growth of the organism around the antibiotic discs is indicative of growth inhibition of respected organism. This area around the disc is known as a zone of inhibition, which is uniformly circular with a confluent lawn of growth in the media. This test was performed with protocols based on guidelines of CLSI, formerly known as NCCLS (CLSI, 2006) with slight modification mentioned in the paper (Rajinder Singh, Shushni, & Belkheir, 2015) for final results.

4.8.1 Revival of bacteria

50ml of nutrient broth was prepared using 0.65gm of powder in a conical flask and sterilized for about an hour. Bacterial strain of *E.coli* stored in -20^{o}C was taken using inoculation loop and transferred to nutrient broth already prepared. The flask was then kept in incubator for almost 40 hrs 42 min (dated 12/3/2018 at 6:00 p.m. to 14/3/2018 at10:42 a.m.) at 37^{o}C and RPM of 140 s. The cell count was measured using UV spectrophotometer by comparing various dilutions of water/saline (prepared using 0.08% of NaCl) containing bacteria with standard Mc Farland solution prepared using 0.05ml of 1.175% $BaCl_2.H_2O$, 9.95ml of 1% H_2SO_4.

4.8.2 Preparation of plates

Muller Hinton Agar from Hi media (contents: peptic digest of animal tissue, sodium acetate, beef extract 5-10% blood and other biological fluids, yeast extract, agar) was prepared (best suited for strains of *E.Coli*) as recommended by the manufacturer 3.8gm in 100mL of Distilled water. Sterilized agar was allowed to cool to room temperature. Approximately 15mL of molten agar was poured into prelabeled sterile Petri dishes on a level surface and plates are allowed to set at

room temperature to dry, so that no drops of moisture remain on the surface of the agar.

4.8.3 Evaluation of Antibacterial Activity

The disk diffusion microbial susceptibility method along with well diffusion methods was used for the evaluation of antibacterial test of *Eucalyptus tereticornis* essential oil. The test was performed by applying bacterial inoculums using sterilized L-spreader (standardized by matching its turbidity with McFarland No 1 standard) of approximately 1×10^6CFU/mL on the surface of pre-sterilized plastic petri dish containing solidified Mueller-Hinton agar. A/S combination of commercially-prepared, fixed concentration 10:10 ratio, paper antibiotic discs are placed on the inoculated agar surface, along with paper discs prepared of 6mm using Whatman filter paper.

The zone of growth inhibition around each of the antibiotic discs is measured to the nearest millimeter that is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium.

4.9 In silico studies

Molecular docking simulation is one of the key tools in medicinal chemistry and computer-assisted drug design that removes the less effective drug from the population, thereby reducing the cost and extra task for synthesis of unwanted molecules and lead drug are easily identified using this method. Schrödinger suite provides the molecular docking based on the Induced Fit model that was first introduced by Daniel Koshland in 1958, the protocol predicts the effect of Ligand docking on protein structure. In silico studies were performed using X- ray protein structures of PBP's and beta lactamases having a resolution of 2.5Å and 0.79 Å respectively retrieved from PDB (online data base for protein structure and their information) that was prepared using proteinprep wizard in Schrödinger suite software and docked with the molecules of essential oil components and the dock score was obtained for analysis.

4.9.1 Target proteins and preparation

From the RCSB Protein Data Bank the three-dimensional X-Ray structure of the proteins 4BJP and 4UA6 were obtained for the present study. The structure taken from PDB has no information of bond order and formal atomic charges in general and hence they are prepared in suitable manner before use using protein preparation wizard in Maestro 11.1. In the preparation missing hydrogen's were added and optimized using "protassign", at pH 7 was achieved and water molecules were deleted having distance more than 5Å, along with that missing disulfide bonds were added.

4.9.2 Ligand selection and Preparation

The compounds present in the *Eucalyptus tereticornis* essential oil obtained from leaves and antibiotics taken as standard shown in figure was selected for docking studies. The structure of the compounds was drawn using ChembioDraw Ultra-14 and converted to MOL SDF format and to generate atomic coordinates Ligand preparation tool LigPrep was used in Schrödinger suite software.

4.9.3 Grid Generation

The grid box was generated around the co-crystallized Ligand in PBP's (4BJP) of the prepared protein. Grid generation helps to specify the active binding pocket of the receptor where docking is to be performed. Grids were generated using the default parameter within the centroid of the co-crystallized Ligand by selecting the residue, after protein preparation task was done. For beta- lactamase sitemap was used in order to find the probable binding site for the components. It is a tool used to identify the potential active site of a protein if co-crystallized ligand was not present in case, after which grid generation was done that makes the grid around the target site in which ligand can specifically bind.

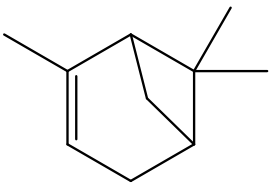
4.9.4 Protein-Ligand Docking

The docking was done using the Induced Fit Docking (IFD) protocol (Schrödinger Suite 2011 Induced Fit Docking protocol; Glide version 5.7, Schrödinger, LLC, New York, NY, 2011; Prime version 3.0, Schrödinger, LLC, New York, NY, 2011) as implemented in Schrödinger Suite 2011. All default settings were used, except the XP (Extra Precision) scoring function. Co-crystallized ligand is removed from the respective protein by splitting it into water, ligand and protein before docking

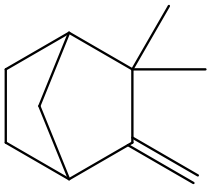
and G-score was used. During the Glide docking process, the van der Waals radii of protein and ligands were both scaled by a factor of 0.5. In addition, only the residues within 5 Å of the ligands taken as flexible were refined. Maximum 10 posses were allowed per docking out of which only one pose per ligand was allowed to be written. Essential oil components and the Ampicilin docking against PBP's and Salbactam docking against beta-lactamases were done using XP so as to get accurate and more precise results. RMSD was computed for the input ligands geometry and no constraints were used. G-score was used to indicate the binding potential of ligands as it has several components such as hydrophobic interaction (lipo), columbic interaction (coul), polar interactions and hydrogen bonds (H-bond) etc. Most negative G-score was selected that predicts the most favorable conformation of each ligand integrated at the active of the target protein.

4.10 ADME/T Studies

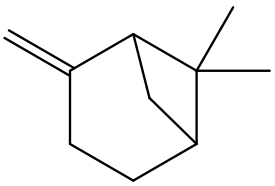
ADME stands for Absorption, Distribution, Metabolism, and Excretion, the study of which defines the properties of the compounds taken for study. This study was performed using Qikprop module in the Schrödinger suite that predicts the properties like molecular wt., permeability through MDCK cells ($QPlog_{HERG}$), gut-blood barrier (QPP_{Caco}). Module convenient for ADME studies that provides information regarding the pharmacokinetics and dynamics of the molecule and helps in the elimination or modification of compounds with poor ADME in order to save time for research and development.



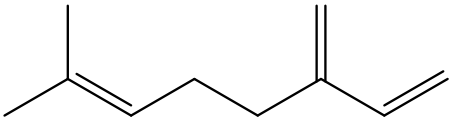
1.alpha-Pinene



2.Camphene



3.beta-Pinene



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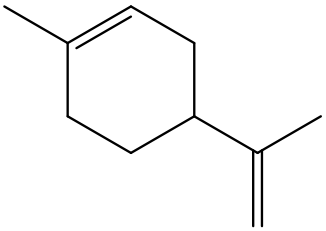
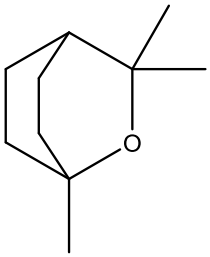


5.D-limonene



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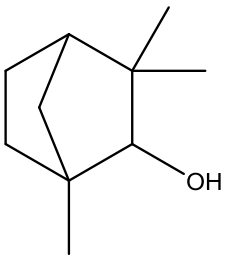
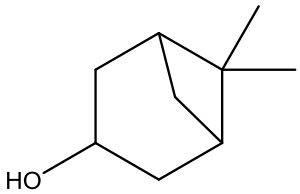
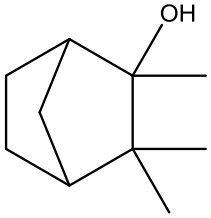


7.Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl



8.Bicyclo[3.1.1]heptan-3-ol,6,6-dimethyl



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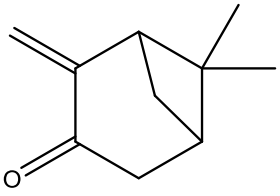

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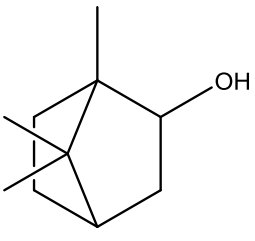

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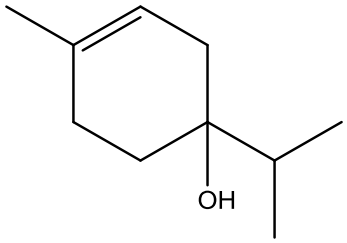

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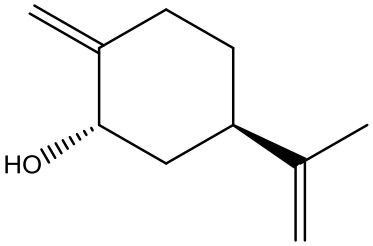
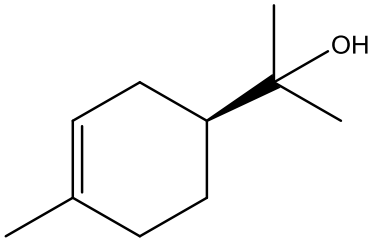
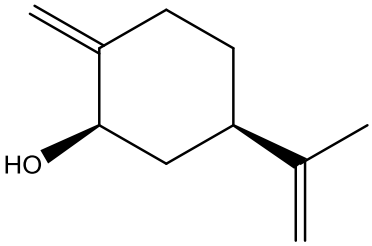


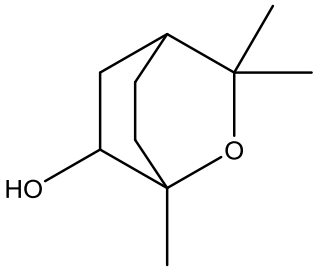
13.Trans-p-mentha-1(7),8-dien-2-ol



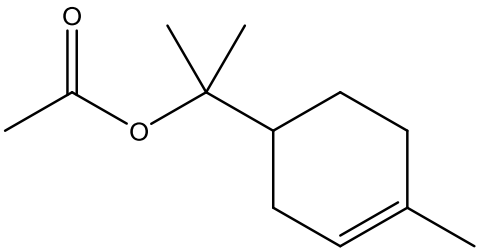
14.L-alpha-terpineol



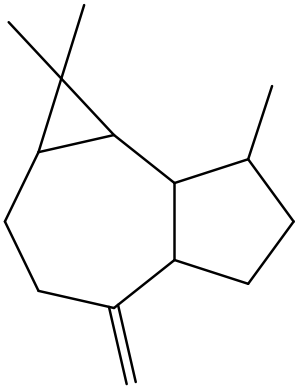
15.Cis-p-mentha-1(7),8-dien-2-ol



16. 2-Oxabicyclo[2.2.2]octan-6-ol,1,3,3-trimethyl



17. alpha-Terpinyl acetate



1H-Cycloprop[e]azulene,decahydro-1,1,7-trimethyl-4-methylene

18.Aromadendrene

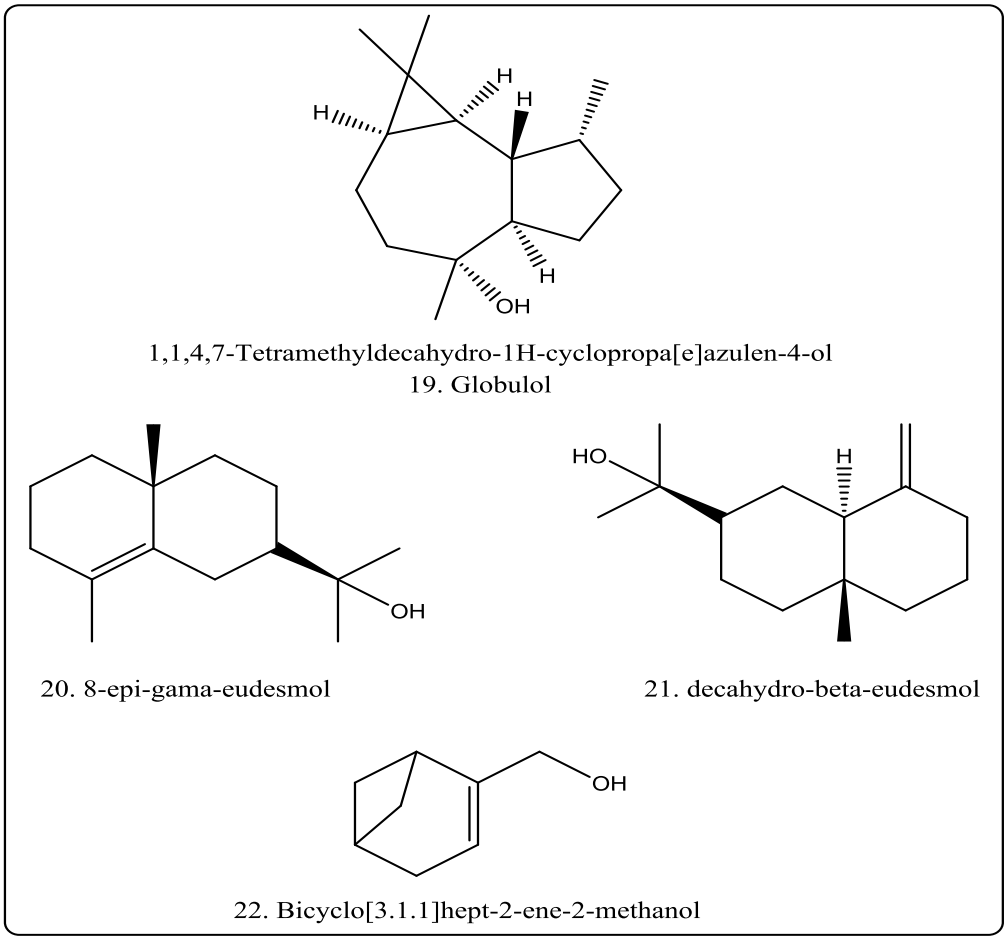


Figure. 8. Structures of components of *Eucalyptus tereticornis* essential oil

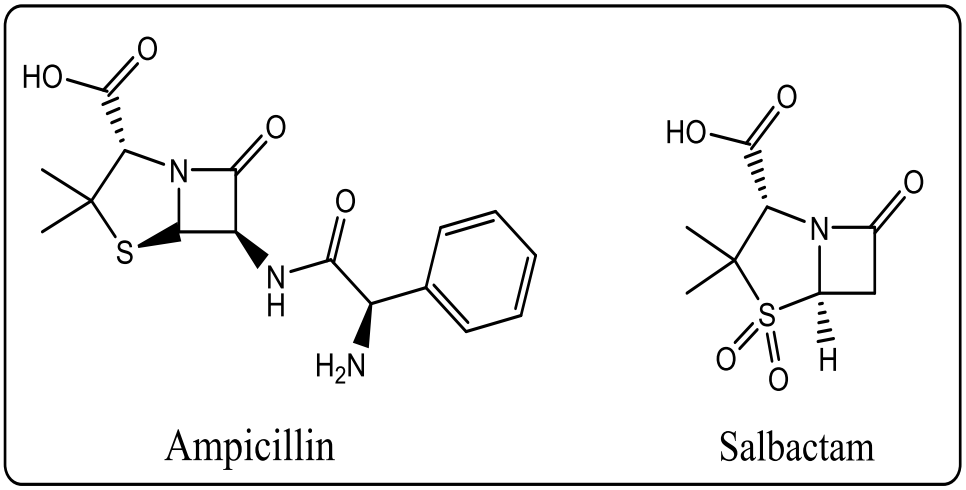


Figure. 9. Structure of Antibiotics used

CHAPTER 5

RESULTS AND DISCUSSION

5. Results and Discussion

5.1 GC-MS Analysis of *Eucalyptus Tereticornis* Essential Oil

Percentage composition of *Eucalyptus Tereticornis* Essential Oil was studied by GC-MS using non polar, low bleed column Rxi®-5Sil MS which was coated by 5% diphenyl/95% dimethyl polysiloxane stationary phase. From the sample of essential oil 22 compounds were identified out of which 1,8-Cineole (Eucalyptol) (56.38%) was the main compound along with α-Pinene (8.41%), β-Pinene (14.63%), α-Terpinyl acetate (6.75%), L-α-Terpineol (2.48%). The lists of constituents in the *Eucalyptus Tereticornis* Essential oil are listed in the Table.

5.2 IN VITRO Activity of ET Essential oil

Many antibiotics are found to be ineffective against various strains due to their overexploitation and increased demand for compounds of natural origin with least side effects and more efficacious, has lead researchers to find alternative of the synthetic drugs. Antibacterial activity of *Eucalyptus tereticornis*, a tree from Australian native, widely distributed due its ability to survive in various condition has been chosen to test the antibacterial efficacy against the pathogenic strain of *E.coli*, since many gram negative bacteria has shown little or no susceptibility towards the components of essential oil due to their hydrophobic nature.

The *Eucalyptus tereticornis* essential oil has shown activity even at low conc. not comparative with antibiotic but activity was observed even at 1-2µl and also with dilution, against bacterial strain of *E.coli* MTCC 1679 and hence the test was performed to check the activity at some more higher concentration and also the synergism and the results obtained for synergistic effect of essential oil with antibiotic and alone by antibacterial analysis was presented in Table 2. The result obtained at 10 µL concentration of *E. tereticornis* essential oil was comparable though the zone of inhibition obtained 15mm (avg.) for EO only in comparison the antibiotic disc 27mm (avg.) at a conc. of 10:10 mcg. Also the combination of EO with Antibiotic disc has shown larger zone of inhibition 29.5mm (avg.) at a concentration of (10:10mcg; 10 µL) and hence there is synergistic effect between essential and antibiotic can be predicted. The experimental results were shown in figure10.

Table. 2. The zone of inhibition of A/S antibiotic and *Eucalyptus tereticornis* essential oil alone and in combination with the antibiotics taken as standard.

Compound	A/S	*Eucalyptus tereticornis* essential oil	A/S + *Eucalyptus tereticornis* essential oil
Zone of inhibition (Average diameter in mm)	27mm	15mm	29.5mm
	28mm+ 26mm	14mm+16mm	29mm+30mm
Concentration used In µL	10:10	10 µL	10:10 + 10 µL

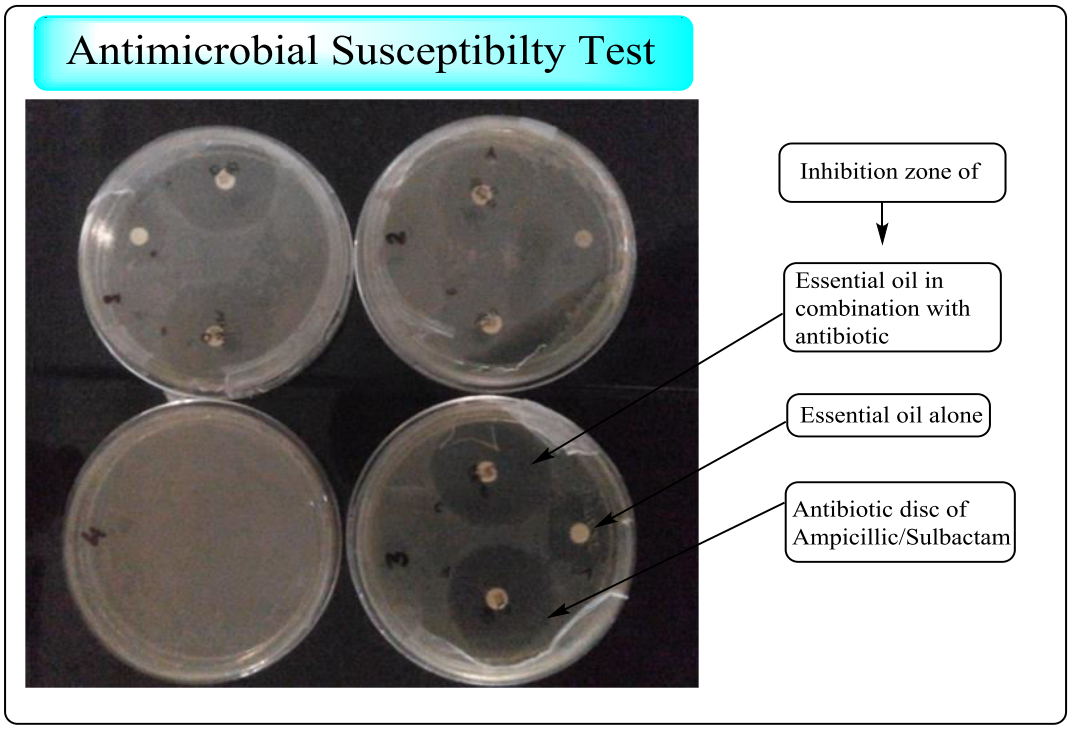


Figure. 10. In vitro results showing Synergistic effect of essential oil and antibiotic

Since essential oil can be used in combination to enhance the efficacy of antibiotics currently in use and synergistic activity was checked using antibacterial susceptibility test. The components of essential oils helps in retarding the microbial resistance towards the drugs available in market along with minimizing the dosage

required for action and are safe for use. In order to find the alternative of antibiotics in use we have performed experiment for the determination of equivalency of essential oil with antibiotic and the results obtained has shown drug equivalency in terms of concentration at 50 µL, in comparison with the standard drug that has shown same zone of inhibition of 26mm (avg.) at 10:10 mcg and the results were shown in table 3.

Table. 3. Zone of Inhibition of antibiotic and *Eucalyptus tereticornis* essential oil showing equivalency at 50µL concentration of *Eucalyptus tereticornis* essential oil.

Compound	A/S	*Eucalyptus tereticornis* essential oil
Zone of inhibition	26mm	26mm
Concentration used (In µL)	10:10	50 µL

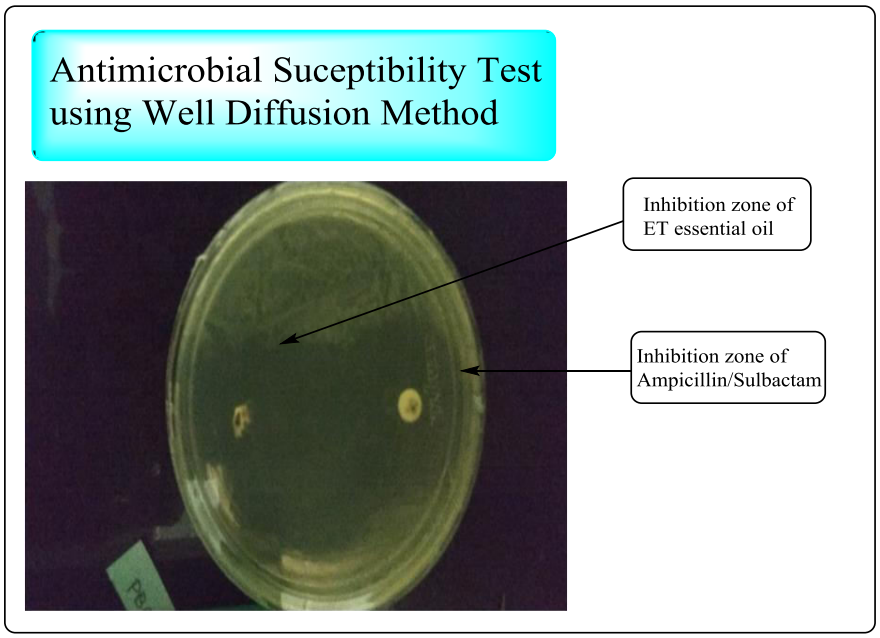


Figure. 11. Experiment to find out the drug equivalency of essential oil

Along with synergism and drug equivalency, we have observed the problems while handling essential oil during the susceptibility test. Due to its volatile nature it gets evaporated easily at the incubation temperature of 37^0C. Because of lower vapour pressure and subsequent leakage was observed from the disc into the entire petri

plate that leads to error in the results. In many papers modified method have been used i.e., well diffusion methods that allows the oil to evenly distribute within the agar thus proper zone of inhibition was observed. Along with that when dilution were made in DMSO, again activity was shown to decline due to the presence of essential components present per ml of vol. decreases.

5.3 In Silico activity of *Eucalyptus tereticornis* essential oil

After getting the results from antibacterial susceptibility test and finding Eucalyptus tereticornis essential oil's to be effective against the strain of E.coli, it is reasonable to find the target binding site of the components and their effectiveness at binding to the target protein. Since we had taken Ampicilin/Salbactam combination for the comparison as standard and hence PBP's and β-lactamases was the choice of protein to confirm the obtained result and efficacy of the components of essential oil that are actually binding and inhibiting the target protein taken from PDB (4BJP).

5.3.1 The docking result of *Eucalyptus tereticornis* essential oil against PBP's

The essential oil constituents of *Eucalyptus tereticornis* are docked to the active sites of protein using XP precision in order to validate the docking approach. Penicillin binding proteins 3 (PBP's 3) also known as FtsI are biosynthetic enzymes of bacterial cell wall assembly that are found anchored in the cell membrane and are involved in the cross linking of bacterial cell wall in the final step during cell division obtained from PDB (4BJP) (Sauvage et al., 2014). The enzyme is one of the famous targets for penicillin, one of first discovered antibiotics that revolutionized the bacterial infection treatment, but to its excessive use in various fields its efficacy was reduced or strains become resistance and hence need of finding the alternative is suggestive. Out of 22 compounds present in *Eucalyptus tereticornis* essential oil 14 has shown binding affinity towards the target protein and only seven compounds has shown good binding potential in comparison with the standard drug Ampicilin in the order α-terpinyl acetate (-2.754)> 8-epi-gama-eudermol (-1.787)> beta-eudesmol (-1.603)> L-alpha-terpineol>Globulol>Bicyclo[3.1.1]hept-2-ene-2-methanol>2Oxabicyclo[2.2.2]octan-6-ol,1,3,3-trimethyl > Ampicilin.

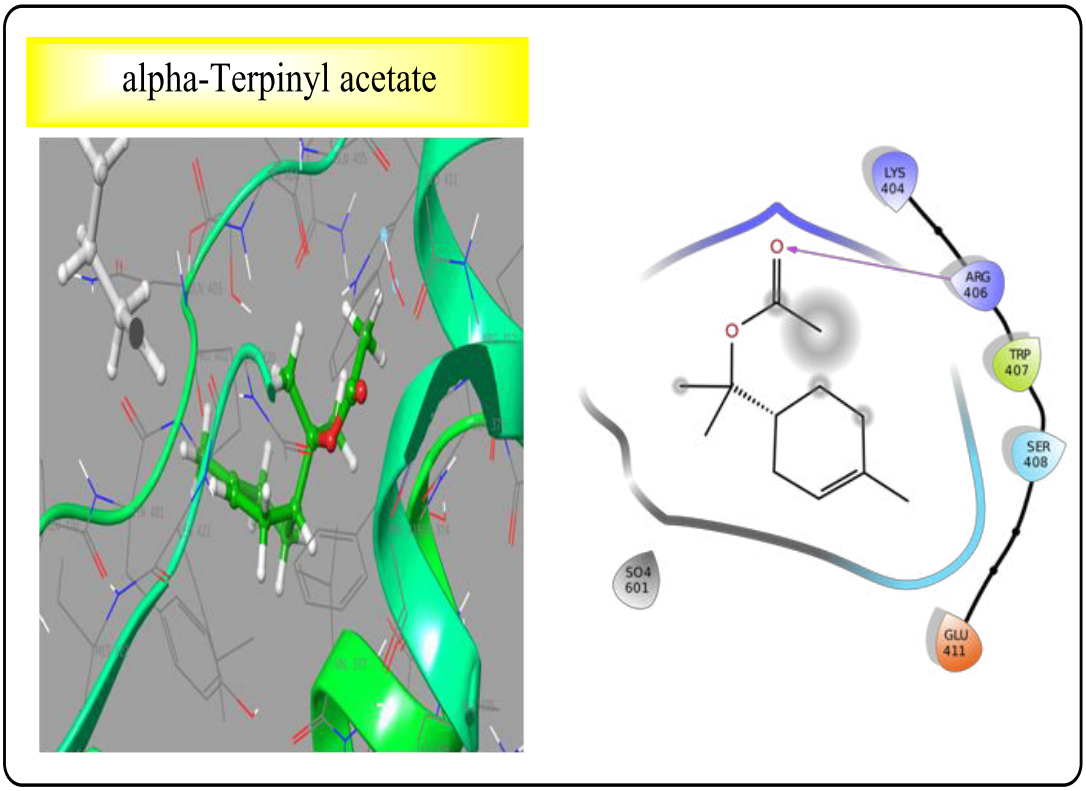


Figure. 12. Interaction of alpha-Terpinyl acetate at the binding pocket of PBP3

With the docking result we have observed that alpha-terpinyl acetate (-2.754) has the best binding affinity towards the PBP3, showing hydrogen bonding interaction with Arg406 at the active site along with it other components has also shown strong binding affinity than Ampicillin such as 8-epi-gama-eudermol (-1.787), alpha-terpineol (Gln403) via hydrogen bonding and Globulol with the Arg406 residue. There is no such literature is available that specifies the activity of terpinyl acetate though bornyl acetate activity has been evaluated but very few work has been done on the activity of Terpinyl acetate. The results shows good binding affinity of Terpinyl acetate with PBP3 and can be tested for its efficacy in vitro as well, as docking results provides the potential targets and compounds for the development of new drugs. Ampicilin belongs to 3rd generation of β-lactam antibiotics aminopenicillins that has broad spectrum of activity but is sensitive to hydrolysis by β-lactamase that results in the ring opening of strained lactam ring and subsequent inhibition. It has shown minor activity against the target protein PBP3 in comparison with the components of essential oil that predicts essential oil is a better choice of drug with more efficacies and less side effects that is known since historical era.

The docking result of *Eucalyptus tereticornis* essential oil against PBP3

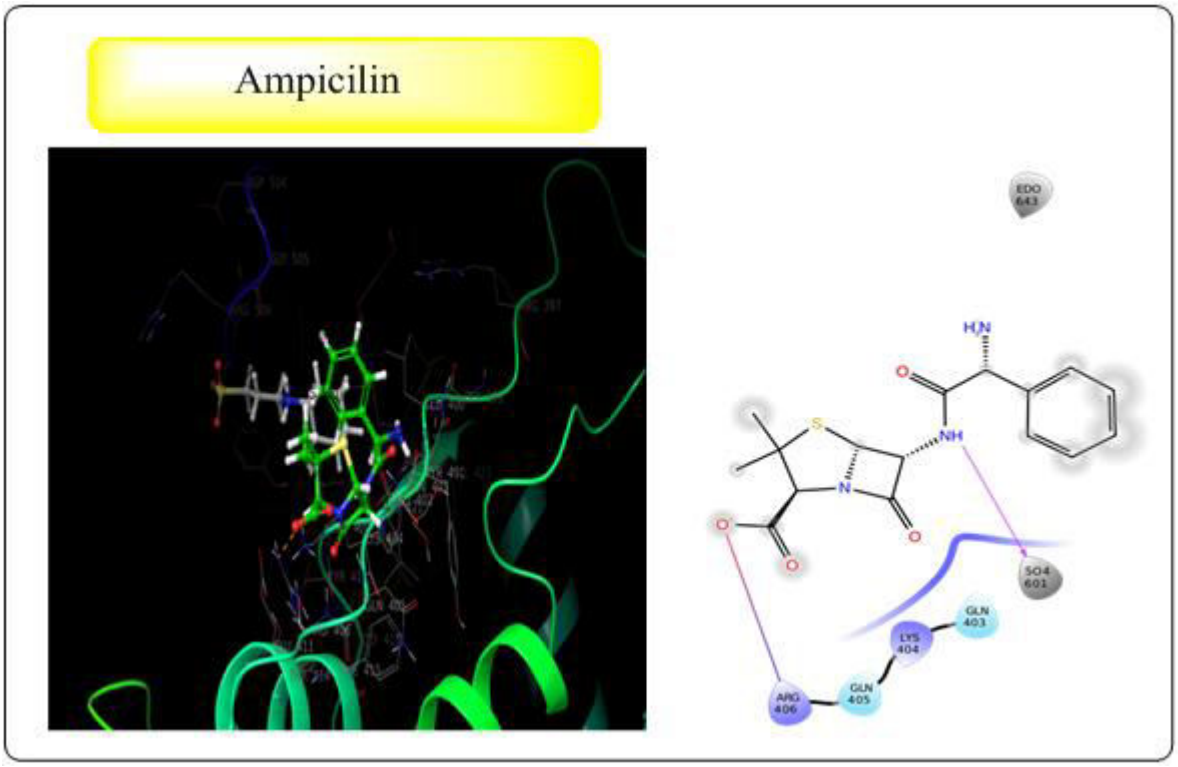


Figure. 13. Interaction of Ampicillin with the binding site in PBP3

5.3.2 The docking result of *Eucalyptus tereticornis* essential oil against beta-Lactamase

Pencillinase enzyme known to hydrolysis the penicillin ring system due do its highly strained ring system thereby making it inactive for the action i.e., inhibition of peptidoglycan protein required for the cross linking of the peptidoglycan layer. Many drugs are used to inhibit the β-lactamases such as Salbactam, Clavulanic acid etc. in addition with antibiotics that inhibits the PBP and hence combination seems to be one of better options but they also possess adverse effects such as headache with a severe blistering, peeling, and red skin rash, diarrhea that is watery or bloody. In comparison if we check the therapeutic value of the essential oils, it seems much better than these synthetic drugs, because of their natural origin they possess less or no side effects in comparison and also have mood relaxant, antioxidant property that helps body to get rid of toxic free radicals.

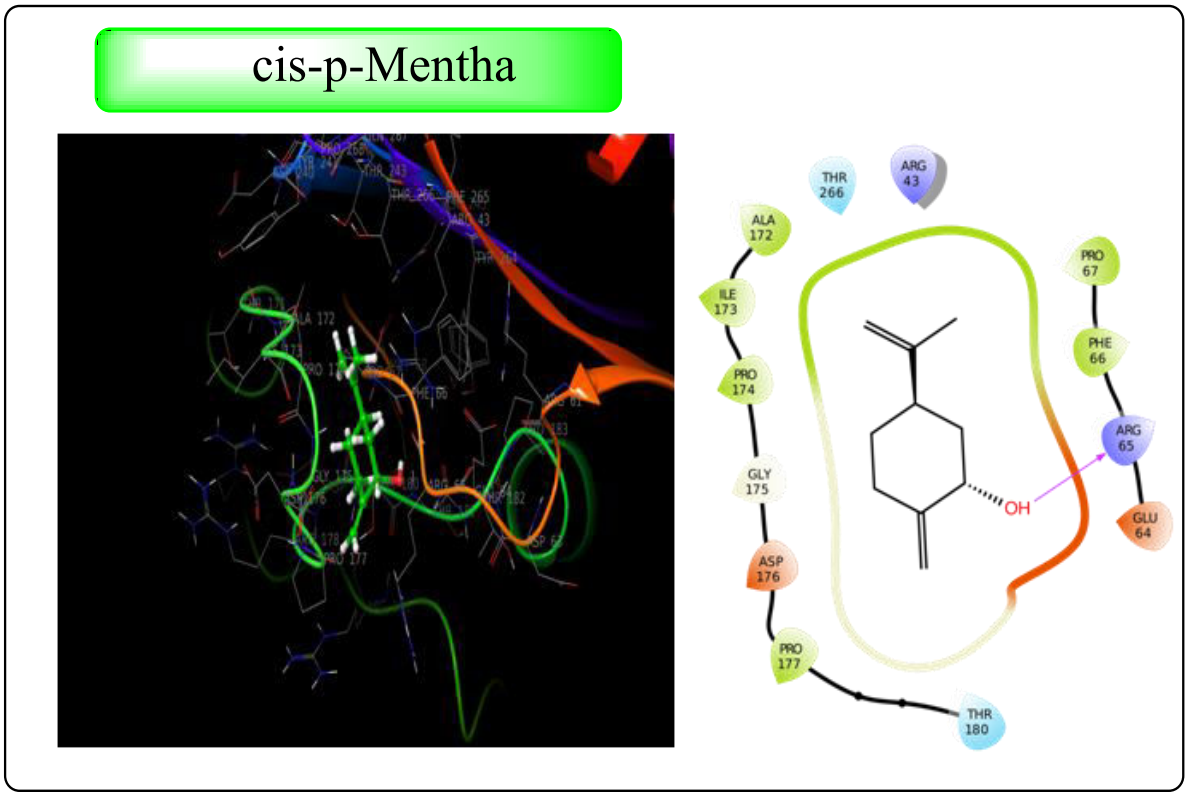


Figure. 14. Interaction of cis-p-Mentha at the active site of beta- lactamases

Cis-p-mentha (-3.671) has shown best binding affinity towards the beta-lactamases, might be due to its small size that can fit into the cavity easily and interact with the active site as evident with ligand protein interaction dig in the figure.11 shows H-bond interaction with the ARG65 residue, because of OH group that can interact with either NH_2 or the COOH group of the residual protein. Salbactam (-2.348) has also shown the binding with the same residue ARG65 but the dock score suggests better binding affinity of the essential oil component present in *Eucalyptus tereticornis* essential oil such as terpinen-4-ol (-3.452) followed with Camphene (-2.193) and alpha-pinene (-2.181) and hence we can assume that essential oil is better at inhibiting the beta-lactamases and posses the resistance modulatory effect as mentioned in some of the literatures (Kuroda, Nagasaki, & Ohta, 2007; Wang, Jhan, Tsai, & Chou, 2016) about antibiotic resistance modulation property of the essential oil components. The above result suggests that *Eucalyptus tereticornis* essential oil might be inhibiting the beta-lactamases production in the bacterium that produces beta-lactamase, thus making them susceptible towards antibiotic which is one synergism that they possess.

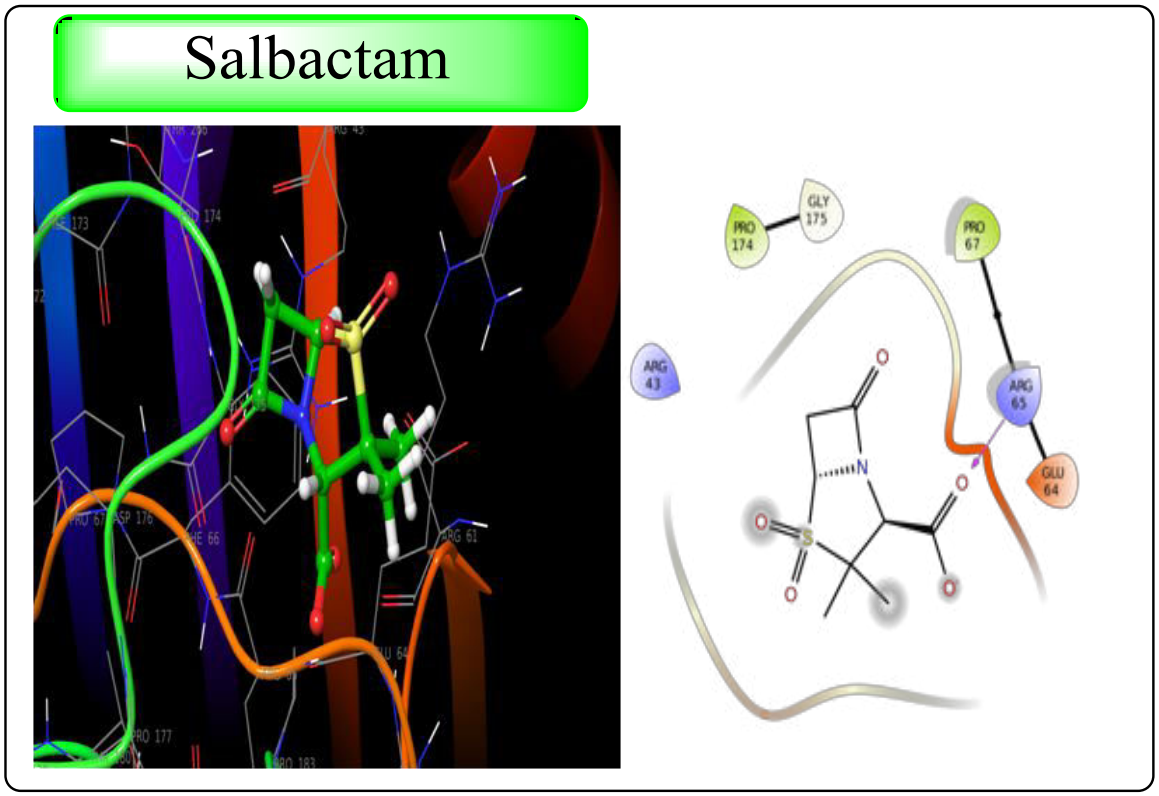


Figure. 15. Interaction of Salbactam with beta-lactamases active site

5.4 ADME /T Study

ADME/T study helps to predict the adsorption, distribution, metabolism, excretion, and toxicity that were studied using QikProp module of Schrödinger suite. It is an easy to use and accurate method that helps in defining the pharmacokinetics of the compounds.

All the 22 components identified using GC-MS were found to have 100% absorption. It is result suggestive since essential oil because of its hydrophobicity gets absorbed through skin easily. ADME/T studies has shown greater QPlogPo/w values for alpha-Terpinyl acetate (3.211), Cis-p-mentha-1(7)8-dien-2-ol (2.379), ligands that has shown greater binding affinity than the standard drug In-Silico. The result is suggestive of why the components of essential oil easily penetrate the cell membrane made of lipid bilayer and causes expansion and swelling leading to loss of ion gradient and cell death. The QPlogKhase suggestive of binding affinity to human albumin serum, the values obtained for the ligand shows they are more effective than the standard drug as values for alpha-Terpinyl

acetate (0.28), Cis-p-mentha-1(7)8-dien-2-ol (-0.087). The components remain unbound to human albumin serum and hence free to exert their effect. The QPPCaco shows the tendency of the components to penetrate the gut blood barrier and all the 22 components of *Eucalyptus tereticornis* essential oil has shown greater affinity to cross the gut blood barrier since they are hydrophobic. Essential oil mainly contained terpenes and oxygenated terpenes containing hydrophilic group a perfect balance of complex mixture that helps in penetration and prolong action and effective than the routine drug available that has many adverse effects on human body.

CONCLUSION

CONCLUSION

This study has shown that essential oil of *Eucalyptus tereticornis* obtained from leaves possesses rather a significant activity against *E.coli*. These results confirm the potential use of *E. tereticornis* essential oil as an alternative antibacterial agent in natural medicine for the treatment of numerous infectious diseases even with gram negative bacterium. Study suggests the antibiotic resistance modulatory activity of the components of *Eucalyptus tereticornis* essential oil and it also targets the synthesis of peptidoglycan by inhibiting the PBP enzyme. Multitarget activity of essential oil was reported in many studies and our study also suggests the same with *Eucalyptus tereticornis* essential oil. In silico studies has shown the binding affinity towards the enzymes with better affinity than the standard drugs and also ADME/T studies are suggestive why they are capable of penetrating the cell and exerting its effects and also 100% absorption due to their hydrophobic nature along with balanced hydrophilicity since these compound contain certain hydrophilic group and hence a balance is obtained between hydrophobic/ hydrophilic character.

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APPENDIX

APPENDIX A

Table. 4. Components of *Eucalyptus tereticornis* essential oil indentified by GC-MS

Ligand	Name	R.Time	Area %
1	α-pinene	10.958	8.41
2	Camphene	11.539	0.30
3	β-pinene	12.777	14.63
4	β-myrcene	13.243	0.60
5	D-limonene	14.817	0.45
6	Eucalyptol	15.107	56.38
7	exo-norborneol Bicyclo[2.2.1]heptan-2ol, 1,3,3-trimethyl	18.502	0.90
8	Pinocarveol Bicyclo[3.1.1]heptan-3-ol,6,6-dimethyl	19.393	1.94
9	Bicyclo[2.2.1]heptan-2-ol,2,3,3-trimethyl	19.889	0.15
10	Pinocarvone	20.151	0.37
11	endo-borneol	20.594	0.78
12	terpinen-4-ol 3-Cyclohexyl-1-ol,4-methyl-1-(1-methylethyl)	20.920	0.71
13	Trans-p-mentha-1(7),8-dien-2-ol	21.246	0.45
14	L-α-terpineol	21.557	0.81
15	Cis-p-mentha-1(7),8-dien-2-ol	22.798	0.19
16	2-Oxabicyclo[2.2.2]octan-6-ol,1,3,3-trimethyl	26.727	0.28
17	α-terpinyl acetate 2-(4-methylcyclohex-3-en-1-yl)propan-2-yl acetate	27.098	6.75
18	Aromadendrene 1H-Cycloprop[e]azulene,decahydro-1,1,7-trimethyl-4-methylene	30.203	0.13
19	Globulol 1,1,4,7-Tetramethyldecahydro-1H-	34.991	0.71

	cycloprop[e]azulene-4-ol		
20	8-epi-γ-eudesmol	36.357	0.50
21	2-Naphthalenemethanol,decahydro-β-eudesmol	37.113	2.07
22	Bicyclo[3.1.1]hept-2-ene-2-methanol	21.617	0.81
	TOTAL		99.13%

APPENDIX B

Table. 5. Dock score of *Eucalyptus tereticornis* essential oil components with bacterial enzyme Transpeptidases (PDB ID: 4BJP)

Ligand	Docking Score	XP HBond	XP PhobEN	XP LipophilicEvdW	XP Electro
Ampicilin	-0.766	-1.632	0	-0.509	-0.366
Pinocarveol	-0.704	-0.35	0	-0.238	-0.307
exo-norborneol	-0.557	0	0	-0.124	-0.562
α-pinene	-0.091	0	0	-0.247	-0.039
D-limonene	0.496	0	0	-0.577	-0.032
β-myrcene	1.134	0	0	-0.327	-0.122
L-alpha-terpineol	-1.502	-0.693	0	-0.424	-0.289
Bicyclo[3.1.1]hept-2-ene-2-methanol	-1.008	-0.852	0	-0.296	-0.305
2-Oxabicyclo[2.2.2]octan-6-ol,1,3,3-trimethyl	-0.857	-0.48	0	-0.272	-0.292
trans-p-mentha-1(7),8-dien-2-ol	-0.558	-0.35	0	-0.174	-0.398
cis-p-mentha-1(7),8-dien-2-ol	-0.425	-0.35	0	-o.321	-0.435
8-epi-gama-eudermol	-1.787	0	0	-1.556	-0.043
beta-eudesmol	-1.603	0	0	-1.424	-0.029
Globulol	-1.169	-0.421	0	-0.178	-0.661
α-terpinyl acetate	-2.754	-0.7	0	-1.231	-0.11

APPENDIX C

Table. 6. Dock score of *Eucalyptus tereticornis* essential oil components with bacterial enzyme beta-lactamases (PDB ID: 4UA6)

Ligand	Docking Score	XP HBond	XP PhobEN	XP LipophilicEvdW	XP Electro
Salbactam	-2.348	-0.653	0	-1.045	-0.151
α-pinene	-2.181	0	0	-1.814	-0.049
Camphene	-2.193	0	0	-2.063	0.012
β-pinene	-2.262	0	0	-1.196	0.015
β-myrcene	-1.36	0	0	-1.975	-0.189
D-limonene	-2.502	0	0	-2.432	-0.123
Eucalyptol	-1.157	0	0	-1.559	-0.008
exo-norborneol	-2.249	-0.347	0	-1.444	-0.258
Pinocarveol	-2.671	-0.346	0	-1.823	-0.184
Aromadendrene	-2.484	0	0	-2.266	-0.018
Pinocarvone	-2.388	0	0	-2.079	-0.038
endo-borneol	-2.127	0	0	-1.858	-0.004
Terpinen-4-ol	-3.452	-0.777	0	-2.092	-0.321
L-α-terpineol	-2.892	-0.424	0	-2.308	-0.256
Cis-p-mentha-1(7),8-dien-2-ol	-3.671	-0.7	0	-2.393	-0.351
2-Oxabicyclo[2.2.2]octan-6-ol,1,3,3-trimethyl	-2.682	-0.633	0	-2.006	-0.12
Globulol	-3.136	0	0	-2.66	-0.231
8-epi-γ-eudesmol	-3.114	0	0	-3.103	-0.049
Bicyclo[3.1.1]hept-2-ene-2-methanol	-2.751	-0.7	0	-1.708	-0.238

APPENDIX D

Table. 7. ADME/T study of *Eucalyptus tereticornis* essential oil components

Ligand	MW (130-725)	Donor HB (0-6)	Acpt HB (2-20)	QPlog Po/w (2-6.5)	QPlog HERG (<-5)	QPPCaco (<25poor >500high)	QPlog BB (-3-1.2)	QPlog Khsa (-1.5-1.5)	% Human oral Absorption >80%high <25%poor
α-pinene	136.236	0	0	3.64	-2.772	9906.038	0.872	0.349	100
Camphene	136.24	0	0	3.3461	-2.591	9906.038	0.859	0.321	100
β-pinene	136.23	0	0	3.602	-2.719	9906.038	0.865	0.344	100
β-myrcene	136.22	0	0	4.592	-3.832	9906.038	0.86	0.401	100
D-limonene	136.238	0	0	3.987	-3.262	9960.038	0.833	0.384	100
Eucalyptol	154.252	0	0.75	2.461	-2.593	9906.038	0.605	0.224	100
exo-norborneol	154.252	1	1.7	2.204	-2.409	4805.527	0.256	-0.074	100
Pinocarveol	154.236	1	1.7	2.11	-2.753	4056	0.19	-0.089	100
Bicyclo[2.2.1]heptan-2-ol,2,3,3-trimethyl	154.252	1	0.75	2.706	-2.14	5376	0.302	0.052	100
Pinocarvone	150.22	0	2	1.979	-2.747	3775.52	0.166	-0.212	100
endo-borneol	154.252	1	1.7	2.056	-2.348	4110.09	0.205	-0.101	100
Terpinen-4-ol	154	1	0.75	2.978	-2.919	5684.437	0.244	0.118	100

Trans-p-mentha-1(7),8-dien-2-ol	152.236	1	1.7	2.472	-3.232	3903.336	0.102	-0.053	100
L-α-terpineol	154.252	1	0.75	2.964	-3.101	4922.658	0.187	0.125	100
Cis-p-mentha-1(7),8-dien-2-ol	152.236	1	1.7	2.379	-3.069	3952.262	0.114	-0.087	100
2-Oxabicyclo[2.2.2]octan-6-ol,1,3,3-trimethyl	170.251	1	2.45	1.995	-2.417	4592.804	0.241	-0.131	100
α-terpinyl acetate	196.289	0	2	3.211	-3.675	4113.665	0.095	0.28	100
Aromadendrene	204.355	0	0	5.176	-3.017	9966.038	1.047	0.969	100
Globulol	222.37	1	0.75	3.909	-2.987	5338.602	0.275	0.691	100
8-epi-γ-eudesmol	222.37	1	0.75	4.075	-3.048	5043.8334	0.192	0.701	100
2-Napthalenemethanol,decahydro-β-eudesmol	222.37	1	0.75	4.024	-3.123	5034.538	0.193	0.666	100
Bicyclo[3.1.1]hept-2-ene-2-methanol	152.236	1	1.7	2.106	-2.788	3776.962	0.103	-0.126	100

QPlogPo/w: Predicts octanol/water partition coefficient. The more positive o/w value means more lipophilic the compound. (-2-6)

QPlogKhsa: Predicts the binding of compound to human serum albumin. (-1.5-1.5)

QPlogBB: Ability to cross blood brain barrier. (-3-1.2)

QPPCaco: Predicts the apparent Caco-2 cell permeability in mm/sec. Caco cells are model for the gut blood brain barrier for the non-active transport. (range if <25 then poor, >500 then great)

APPENDIX E

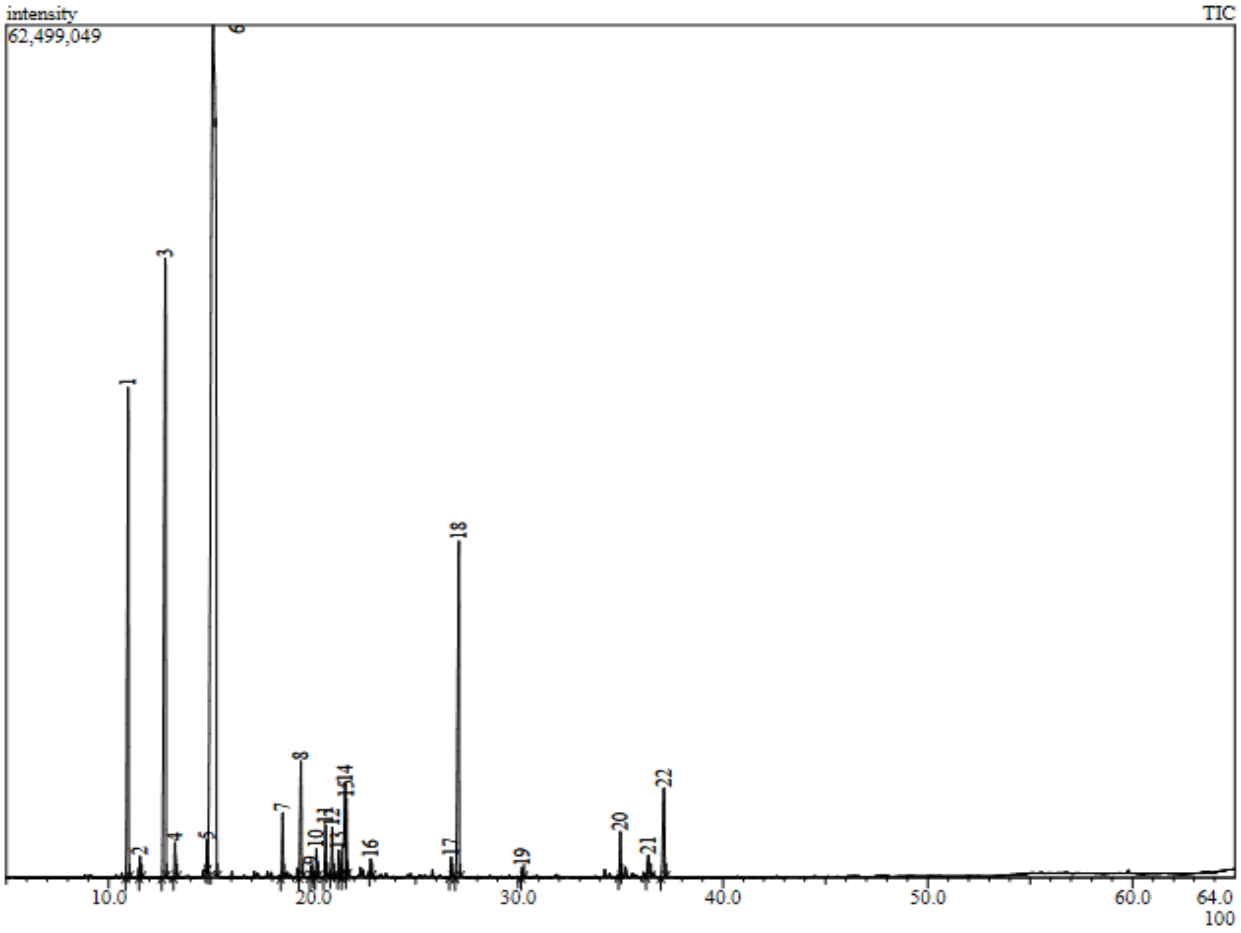

Figure. 16. GC-MS spectra of *Eucalyptus tereticornis* essential oil