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Effect of Electromagnetic Field on Antimicrobial Activity and Novel Antimicrobial Compounds

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Abstract

The objective of this study is to determine the antimicrobial activity of the essential oils and effect of electromagnetic field on the activity. To find out antimicrobial activity of essential oils (Thyme, Clove, Lavender), against test organisms disc diffusion assay was performed. MIC of all essential oils were determined under electromagnetic field and non-electromagnetic field. Both studies confirmed the antimicrobial activity of oils and effect of electromagnetic field. However, time killing experiment was conducted to calculate rate of inhibition for essential oils, lavender oil and thyme oil showed higher rate as compared to that of clove oil.

This study provides evidence to antimicrobial activity of essential oils, and also confirmed the effect of electromagnetic field on the activity. The proposed mechanism for antimicrobial activity is inhibition of metabolic enzymes which has supported with Analytical Profile Identification (API 20NE).

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1. Introduction

1.1 The Need for the new antimicrobial agents

Use of antimicrobial agents to avoid the growth of unwanted organisms has increased tremendously in various fields last few decades. Most of the commercially used antimicrobial agents are chemicals like alcohol. Repeated use of antimicrobial agents led to the resistance of organisms towards them. Antimicrobial agents can be in the form of antibiotics or disinfectants or preservatives. These agents mainly hamper the growth of organisms through various mechanisms. Since last 25 years resistance against most of the antimicrobials has become a huge problem. Various infectious organisms are showing resistance towards known antimicrobial agents as a result of which incidents of infections by these pathogens are increasing remarkably.e.g.Methicillin resistant *Staphylococci,* penicillin and erythromycin resistant *pneumococci,* vancomycin resistant *enterococci*.It has become compulsory to develop new antimicrobials, there is continuous effort in the pharmaceutical sector for new antimicrobial agents [7].

Though there are some alternative preservation techniques such as modified atmospheric packaging, high pressure, and use of wide range of food grade chemicals, these are not preferable as these are aggressive techniques and presence of chemicals in the food is not desirable by consumers. It can alter the quality of the food products. This situation generated the need of natural antimicrobial agent in the field of food industry and medicine. The search for novel antimicrobial compounds has been focused on the essential oils. The first experimental measurement of the bactericidal properties of the vapours of EO is said to have been carried out by De la Croix in 1881 [2].Essential oils have been used in medicine from the 18th century.

1.2 Essential oils and their uses

Essential oils (volatile oils) are the aromatic oily liquids obtained from plant materials like flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots. They are commercially derived by the steam distillation process. It is postulated that the importance of the essential oils to plants is to act as phytoprotective agents defending the tree from herbivores and pathogenic attack [10].

Essential oils and their components are widely used in medicine as constituents of different medical products, in the food industry as flavouring additives and also in cosmetics as fragrances [39]. On an average 3000 different types of essential oils are known so far, out of which 300 are commercially important. Oils of cinnamon,clove,eucalyptus,camphor,lemon,lime,nutmeg,orange,thyme,rosemary, basil have been used traditionally for various purpose by people from the different parts of the world [40]. Number of studies have been done on the antimicrobial activity of different essential oils and reported the antifungal, antimicrobial and insecticidal properties of some essential oils [36]. Some of the essential oils that had shown significant activity include, cinnamon oil, lemon oil, papermint oil, lime oil, lavender oil etc. Cinnamon oil has been used in the treatment of diabetes, lavender oil proved to have antifungal activity, it has been used in case of burns and insect bites as well [4]. Peppermint and orange oil have shown to posses anticancer activity [22]. Basil oil has proved to have anti-inflammatory activity [42].

Clove oil has reported to have antimicrobial, antioxidant, antifungal and antiviral activity. In addition to all these properties clove oil has anti-inflammatory, cytotoxic, insect repellent and anaesthetic properties [21].Thyme oil also proved to have antimicrobial and antifungal activity [32]. Essential oils are the mixtures of different single components that ultimately contribute to their antimicrobial activity and its impossible for the organism to mutate or to adapt to all the constituents as they do to the modern antimicrobial agents. This study mainly aims at the antimicrobial properties of clove oil (extracted from *Syzygium aromaticum*), lavender oil (extracted from *Lavandula officinalis,* Family; *Labiatae)*; and thyme oil (extracted from *Thymus vulgaris,*Family ; *Labiatae*). Clove oil is composed of 75-85% euganol, 8-15% eugenyl acetate [3], 10-64% thymol, 2-11% carvacrol, 2-31% Ɣ-terpinene, 10-56% p-cymene are the main constituents of the thyme oil(Burt, 2004).Lavender oil is composed of 43.73% of 1,5-Dimethyl-1-vinyl-4-hexenyl,25.10% of 1,3,7 octatriene,3,7-dimethyl-,7.32% eucalyptol and 3.79% camphor [24].

Respective constituents of oils make them to possess antimicrobial activity. The exact mechanism of killing of organisms by essential oils has not documented yet. The possible proposed mechanisms involve cytoplasmic membrane disturbance, disruption of proton motive force, electron flow, active transport and coagulation of cell contents [38].Chemical structure of the individual component of the essential oil affects their mode of action [15].The hydrophobicity of the essential oils enables them to partition in the lipids of cell membrane and mitochondria, making it permeable and leading to the leakage of cell contents [38].

1.3 Effect of Electromagnetic Field

Electromagnetic field has also considered being one of the promising methods of non-thermal food preservation. Published results agreed that various species of organisms are susceptible to electromagnetic field. It is applied as short time pulses to kill the living cells in aqueous solution [1]. The growth of *E.coli* decreased with increase in low frequency magnetic field and increase in exposure time. It proved the negative effect of electromagnetic field on the growth of *E.coli*. The mechanism of electromagnetic field in killing of organisms has not understood clearely. The proposed mechanisms involved the possible effect of electromagnetic field on the permeability of the ionic channels in the membrane. In the membrane it can affect ion transport in the cells. Other possible effect is the formation of free radical due to the magnetic field exposure. To attain the maximum antimicrobial activity within short period of time many scientist have used combination of methods. For example[19] has shown the use of high intensity pulsed electric fields method in combination with organic acids or with essential oils to inactive pathogenic organisms and extend the shelf life of fruit juices. [28] reported that the combined effect of electric field and chloramphenicol was additive.

1.4 Specific objective of the study

The aim of this study is to make use of these two insights of antimicrobial principles to attain the maximum activity. Here, antimicrobial activity of thyme oil, lavender oil and clove oil is investigated on two gram negative, two gram positive bacteria and yeast under different temperature conditions. Effect of electromagnetic field on their activity was also investigated. The study further involved time kill experiment. The effect of essential oils on the enzyme activity of the organisms was tested using Analytical Profile Identification 20NE.

2. Materials and Methods

The present study included the screening of test organisms for antimicrobial activity of essential oils by using Disc diffusion assay and by determining minimum inhibitory concentrations of oils under electromagnetic field, non electromagnetic field and at 37^0 C. Electromagnetic field was generated using the device with power requirements of 220 VAC,60 HZ and 70 WATTS. Time kill experiment and API 20 NE method was carried out in this experiment.

2.1. Essential oils

In this study, essential oils like clove oil, lavender oil and thyme oil were used as novel antimicrobial compounds. These oils were supplied by the Munero Pharmacy, Green Street, Upton Park, London. These oils were selected based on their traditional use in medicine. Numbers of studies revealed that all of these oils have strong antimicrobial activity. Lopez *et al* concluded that clove oil has maximum antimicrobial activity, whereas work of [13] showed that all of them have antimicrobial activity.

2.2. Culture

In this present study,two species of Gram negative bacteria, *Escherischia.coli* (11190), *Pseudomonas fluorescence* (ATCC13525/4607216) and two strains of Gram positive bacteria, *Staphylococcus epidermidis (*ATCC12228)*, Bacillus subtilis*(B6A16056) and yeast, *Saccharomyces cerevisae* (ATCC 60530) were tested These species were obtained from the Microbiology Department of the Science Laboratory of the London Metropolitan University, London.All strains were maintained on appropriate agar slants at 4°C throughout the study and used as stock cultures.

2.3. Growth and maintenance of microorganisms

Target microorganisms, *Escherichia coli, Staphylococcus epidermidis, Bacillus subtilis, Pseudomonas fluorescence, Saccharomyces cerevisae* were routinely maintained by sub culturing on nutrient agar (*E. coli, S. epidermidis, B.subtilis and P. fluorescens)* and malt extract agar (*S.cerevisiae).* Following subculture, they were then incubated at 30°C for 24 – 48 hours before storage at 4°C.

2.4. Media preparation

Preparation of media was the first step towards the experiment. Stock cultures of each organism were collected. For *E.coli,Staphylococcus epidermidis,Bacillus subtilis,Pseudomonas fluorescence* same medium i.e. Nutrient agar was used, and for *Saccharomyces cerevisae* Malt extract agar was used.

Nutrient agar (NA) was prepared by suspending 14g nutrient agar powder in 500ml distilled water. Malt extract agar (MEA) was prepared as in NA but 25g MEA powder was used. Nutrient broth was prepared by suspending 6.5g nutrient broth powder in 500ml distilled water whereas for malt extract broth, 10g powder was used for 500 ml distilled water.

All media, unless stated otherwise was sterilised by autoclaving at 121°C for 15 min. The media was then allowed to cool to about 60°C before being poured into Petri dishes. Agar was poured into the 25 autoclaved petridishes in aseptic conditions, allowed it to solidify. After solidification of the medium petridishes were kept in the incubator at 30ºc for 24 hours. Petri dishes were kept in the upside down manner to avoid dropping of the condensed water on sterile medium. After incubation, these plates were checked for any contamination, plates showing any contamination were thrown straight away. This procedure is going to be repeated for every time whenever the agar plates are required.

2.5. Screening for antimicrobial activity

2.5.1. Disc diffusion methods

The most used screening methods for antimicrobial assay are disc diffusion, well diffusion, agar dilution and broth dilution. In this experiment, antimicrobial activity of oils was assayed by using disc diffusion method. This is the routine, economical and easy method for detection of resistance of microorganisms. Permeability of agar allows small molecules movement through it, under controlled conditions this movement of the molecules referred to the concentration of the molecule. This method is recommended by the National Committee of Clinical Laboratory Standards (NCCLS) for the determination of susceptibility of microorganisms towards antimicrobial product. This is the preliminary check for antimicrobial activity prior to more detailed studies. Paper discs used for this method were made by punching Whattman paper no.1(5mm); this paper allows diffusion of oil into agar. Paper discs were autoclaved at 121ºc for 15 minutes. For this experiment, 3 plates of respective medium were used for each microorganism. As this assay was performed for clove oil, lavender oil, thyme oil, total 9 plates were required for each microorganism. Suspension of each microorganism was prepared by transferring loopful of microorganisms into sterile broth (nutrient and malt extract), these suspensions were incubated at 37ºc for 24 hours.0.1 ml of this suspension was transferred on to the medium and spread using sterile glass spreader. Spreader was sterilised by dipping into the 70% alcohol and then flamed into burner to remove excess alcohol. Then using a pair of sterile forceps one disc was picked, dipped into the 100% oil, excess oil was removed by touching to the edge of bottle. This oil soaked disc was then placed on the plate carefully, pressed it slightly on the medium surface with the help of forcep. Four discs were placed on the same plate in such a manner that inhibition zones will be observed clearly. Same procedure was repeated for each microorganism and all oils. All aseptic conditions were maintained throughout the experiment.

Controls were made by dipping sterile paper discs into distilled water and alcohol and placing onto the sterile media plates. All plates were incubated at 37ºc for 24 hours for bacteria and 48 hours for yeast. After incubation, plates were observed for the inhibition zones and results were noted down for the record. This study was repeated twice to confirm the results.

2.5.2. Determination of minimum inhibitory concentration and microbiocidal concentration

MIC is the minimum inhibitory concentration of oil for respective microorganism. It was determined by liquid dilution or broth dilution method.

Here, two fold dilution methods were used; three sets of universal bottles were taken for clove oil, lavender oil and thyme oil. Four dilutions were prepared for each organism in one set. In total 20 tubes were there in each set. Tubes were labelled as 50%, 25%, 12.5% and 6.25% for each organism. In the first tube that is to the 50%, 2 ml of nutrient broth and 2 ml of oil was added. As oil is immiscible in aqueous solution it formed the separate layer on the top of the broth, to dissolve this layer, 0.02% of surfactant, Tween 80 was added, mixed it thoroughly. Tween 80 is approved surfactant for antimicrobial studies of oils. It's been used in previous studies of investigating antimicrobial activity of essential oils [40].2 ml from this mixture was transferred to 25% bottle containing 2 ml of nutrient broth, mixed it thoroughly and again 2 ml from this, transferred to the next bottle with 2 ml nutrient broth to make the concentration of oil 12.5%.Similarlly, 6.25% dilution was made. Malt extract broth was used in the place of nutrient broth for *Saccharomyces cerevisiae*. These tubes were observed carefully for the dissolution of oil into the broth. All dilutions were prepared in the same way for all sets. Caps of the bottles were quarter loosed to avoid the bumping of the solution after autoclaving.

These dilution series was then autoclaved at 121ºc for 15 minutes. After autoclaving, cooled down the temperature to the normal and inoculated all the four dilutions using suspension of respective organism, volume used for the inoculation was 0.1 ml. Inoculation was done in aseptic conditions. All dilution sets were incubated at 37ºc for 24-48 hrs. After incubation, tubes were observed for growth, MIC and MBC were determined by spreading 0.1ml of dilution mix onto the agar plate. Spreading was done for all dilutions and plates were incubated at 37ºc for 24-48 hrs. Results were observed and recorded.

Similar kind of sets of dilution series were prepared as mentioned above for all oils and kept one in the electromagnetic field and another one on the bench at room temperature named as non electromagnetic field for 24 hrs.MIC and MBC of these sets also determined using spread plate technique. Results were noted down.

2.6. Time kill studies of lavender ,clove and thyme oil

The aim of this particular study is to determine the viability of the organisms after contact with the oil for a specified time period [20].Out of three oils; lavender and clove oil were used to perform time kill plots against all test organisms. Time kill plot has been performed by [45] using *Staphylococcus aureus, Escherichia coli* and *Candida albicans* as test organisms to investigate time kill plots of clove oil and rosemary oil respectively. May J. also performed time kill experiments of tea tree oils on clinical samples like vancomycin resistant *Enterococci,* gentamycin resistant *Klebsiella.*

Here, the organisms under study were same that used for determination of MIC. Microorganisms were grown overnight at 37°c to obtain culture in exponential phase. These cultures were then used for preparing inoculums for the experiment. Unlike the above mentioned studies, only one concentration of oil was used in this study.50% v/v concentration was used for both the oils, autoclaved and then inoculated with the 0.1 ml of organisms. Right after the inoculation, 0.1ml liquid was taken out and spread on the agar plate, Liquid was then incubated at 37°c, after every 20 minutes; sample was taken out and spread on the agar plate. Experiment was continued till 160 minutes. All the plates were incubated at 37°c for 24 hrs (bacteria), 48 hrs (yeast).After incubation, plates were observed for inhibition and respective time was recorded.

Viable colonies on the plates were counted and cfu/ml was calculated using the formula. Graph was drawn for each organism with logarithm of cfu/ml against time to determine the rate of inhibition.

2.7. Determination of enzyme inhibition by API 20 NE method

Basically, API 20 NE is the standardised identification system for non fastidious, non enteric gram negative rods. It is the set of biochemical tests that includes 8 enzymatic tests and 12 carbohydrate assimilation tests. It gives 99-100% reliable results except for indole production(96.5%).Test system is in the form of strip having 20 cupules containing dehydrated substrates. The principle behind the test is that the suspension of test organism is filled in the cupules having substrates inside, during incubation, metabolism produces change in colour which is spontaneous or need an additional reagent. This result has to match with the standard reading table results. In case of assimilation tests, organism need to grow in minimal medium provided, i.e. API AUX medium.

Here, the purpose behind using this method was different than identification .We got the pure culture of organisms with us. The another aspect of the test was consider in this study i.e. enzyme activity. To figure out the exact effect of essential oils on the enzyme activity of organisms API 20 NE was performed. Procedure was same as that for an identification.24 hours incubated fresh cultures of organisms were prepared, 1/2 MIC, MIC and control (pure suspension) were used for each organism and each oil. MIC and ½ MIC concentrations of oils were made as per previous methods.

These dilutions were autoclaved, along with same amount of medium for control. All dilutions and controls were inoculated with 0.1ml of respective organism, incubated for 24 hours at 37°c.Actual test i.e. inoculation of test strips was done on 2nd day, an incubation kit (box, lid, tray) was prepared, 5ml distilled water was distributed in the tray to create a humid atmosphere, strip was taken out and placed on the tray.24 hrs incubated MIC was taken with the help of PSIpette and filled the cupules with precautions to avoid bubble formation. Tests NO3 to PNPG were inoculated in the same manner, For remaining tests, 200µl of MIC dilution was mixed in the provided AUX medium, mixed thoroughly and this mixture was filled in the rest of the tubes and cupules of the tests. Mineral oil was added to the cupules of three underlined tests i.e. GLU,ADH,URE to maintain an anaerobic conditions. Incubation box was closed and incubated at 37°c for 24 hours. Same procedure was repeated for each organism and both oils. Results were observed on the 3rd day of experiment and recorded.

3. Results

3.1. Sensitivity of essential oils

The sensitivity of clove, lavender and thyme oil against test organisms studied by disc diffusion assay. Susceptibility of all organisms for essential oils was measured in terms of the inhibition zone surrounded by the disc and represented as a means±standard deviation, calculated by one way analysis of variance. As p value was less than 0.05, multiple comparisons was done to ascertain where the difference lie. LSD (Fisher's test), Bonferroni tests were used. SPSS 17.0 was used to perform all the statistical analysis. Results for ANOVA and multiple comparisons are summarised in table 1.

- implies growth but no zones of inhibition; For each row, if letters following mean±S.D are the same, then there are no significant differences between the means using LSD test at $p = 0.05$.

Table 1: Summary of zones of inhibition (in mm) of essential oils (clove,lavender and thyme oil)against target organisms(*E.coli,P.fluorescence,B.subtilis,S.epidermidis* and *S.cerevisiae*).

MICROORGANISMS	Means±S.D of zones of inhibition by essential oils in mm		
	CLOVE OIL	LAVENDER OIL	THYME OIL
Escherischia.coli (11190)	15 ± 4.34^{a}	12.62 ± 1.68^{a}	31.12 ± 1.12^{b}
Staphylococcus epidermidis (ATCC12228)	14.3 ± 3.77^{x}	11.87 ± 3.27^{x}	27.87 ± 1.80^{y}
Pseudomonas fluorescence (ATCC 13525/4607216)	18.25 ± 4.46^{p}	13.62 ± 3.15^{q}	24.87 ± 3.13^{z}
Bacillus subtilis (B6A16056)	22.12 ± 1.55^{c}	20.87 ± 1.55^{c}	31.37 ± 1.68^{d}
Saccharomyces cerevisiae (ATCC60530)	18.12 ± 2.58^{s}	–	20.25 ± 2.1^{s}

Each bacterial strain demonstrated a significant degree of sensitivity to all essential oils (clove, lavender and thyme oil) with >10 mm zones of inhibition. Amongst all, thyme oil exhibited highest antimicrobial activity against all organisms with >20mm of zones of inhibition.

According the LSD test, there was no significant difference between the means of zones of inhibition of lavender oil and clove oil against all organisms except *P.fluoroscence,* There were significant differences between the means of zones of inhibition of all essential oils in case of *P.fluorescence* which are illustrated in fig1.Whereas there was significant difference between means of zones of inhibition of thyme oil in case of all organisms. Clove oil has been proved to be executing antifungal activity in the other studies [8,21], it exhibited sensitivity towards yeast, *Saccharomyces cerevisae* in this study. Lavender oil didn't show inhibition against *S. Cerevisae* but all other organisms were sensitive to it.

Bacillus subtilis demonstrated maximum sensitivity to the all essential oils. It was the most susceptible bacteria as compared to the rest of others.

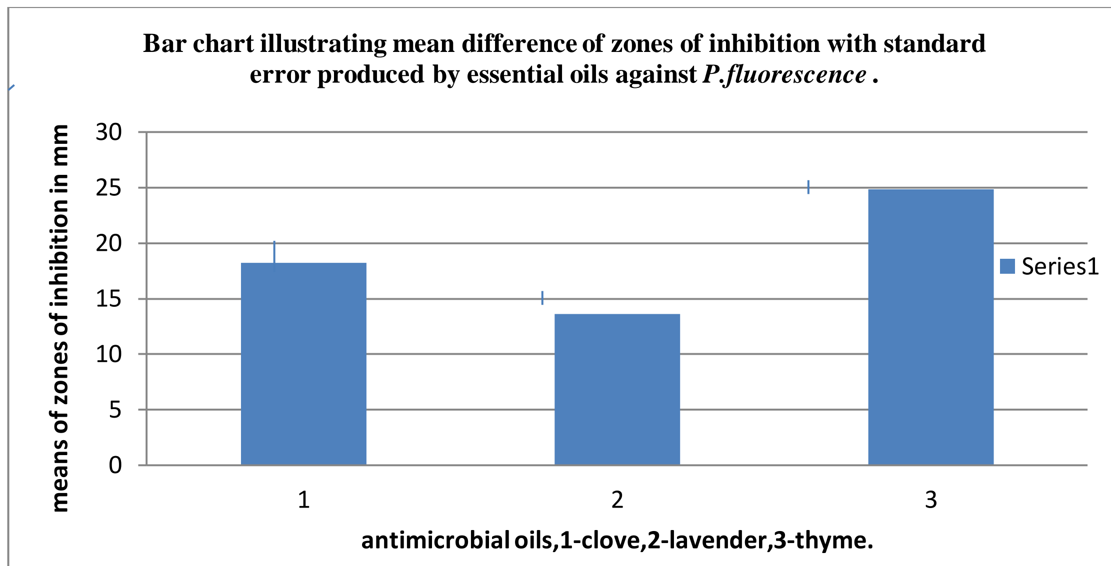

Figure 1: Bar chart illustrating mean difference of zones of inhibition with standard error produced by essential oils against *P.fluorescence*.

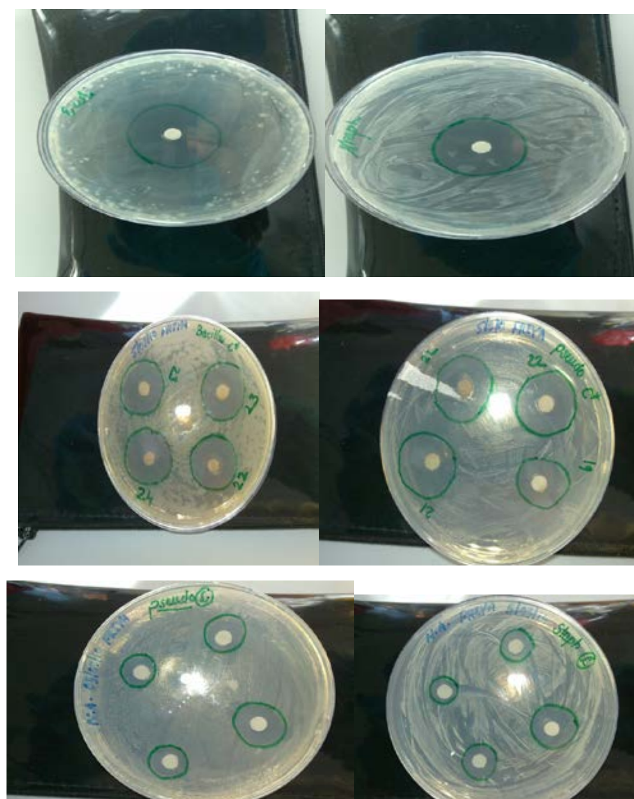

Figure 2: Illustrates the zones of inhibition produced by the thyme oil against *E.coli* and *S.epidermidis*(first row),zones of inhibition produced by clove oil against *B.subtilis* and *P.fluorescednce*(second row),zones of inhibition produced by lavender oil against *P.fluorescence,S.epidermidis(*third row)

3.2. Determination of MIC of essential oils under electromagnetic field and non electromagnetic field

As it can be seen in Table no.2, all oils exhibited inhibitory activity against test organisms supporting the results of disc diffusion assay. MICs of all essential oils under different incubation conditions range from 3.125%-50%.Thyme oil was having maximum antimicrobial activity under all incubation conditions, MIC of thyme oil range from 3.125% - 25%. The activity of thyme oil increased when incubated under electromagnetic field with *E.coli,S.epidermidis,P.fluoroscence*.Clove oil didn't exhibit sensitivity with *E.coli* when incubated at R.T., On the contrary it inhibited *E.coli* under electromagnetic field and at 37°C.With all essential oils and under any incubating condition *S.epidermidis* executed maximum susceptibility. In case of *E.coli, S.epidermidis, P.fluoroscence,*the inhibitory concentration for each oil decreased, when placed under electromagnetic field. The MIC values range from 6.25%-50% for both clove and lavender oil. Lavender oil was inactive against *S.cerevisae* at Room temperature (non-electromagnetic field) and even when incubated under electromagnetic field but showed little activity at 37°C with MIC value of 50%.MBC values of the all essential oils were similar to that of their MIC values in most of the cases. From the MIC values of various essential oils under different incubation conditions, a clustered bar chart was drawn to compare the antimicrobial activity of oils under electromagnetic field and non electromagnetic field at room temperature. The bar chart clearly illustrated the decrease in inhibitory concentration of oils when incubated at electromagnetic field. In other words, electromagnetic field increased the antimicrobial activity of all essential oils. Number of colonies on the plates showing growth were recorded and determination of the number of viable organisms after the addition of oil was done using the formula, Total viable count (TVC) = No. of colonies x1/dilution x1/inoculum vol.The cfu/ml calculated for each organism was used in the further statistical analysis of antimicrobial activity of essential oils under different incubation conditions.

Table 2: MICs of essential oils under electromagnetic field and non-electromagnetic field at room temperature.

Microorganisms	Minimum Inhibitory Concentration (%v/v) of different essential oils.								
	Control			Electromagnetic Field			Incubated at 37°C		
	Clove	Lavender	Thyme	Clove	Lavender	Thyme	Clove	Lavender	Thyme
E.coli(11190)	_*	25	12.5	50	12.5	6.25	25	25	12.5
S.epidermidis (ATCC12228)	6.25	50	6.25	6.25	12.5	3.125	25	25	6.25
P.fluoroscence(ATCC 13525/4607216)	25	12.5	12.5	12.5	12.5	6.25	25	25	25
B.subtilis(B6A16056)	_*	50	25	50	50	25	50	_*	25
S.cerevisae (ATCC60530)	25	_	6.25	25	_	6.25	25	50	6.25

_implies microbes not susceptible to oil, _* implies all plates showing growth.

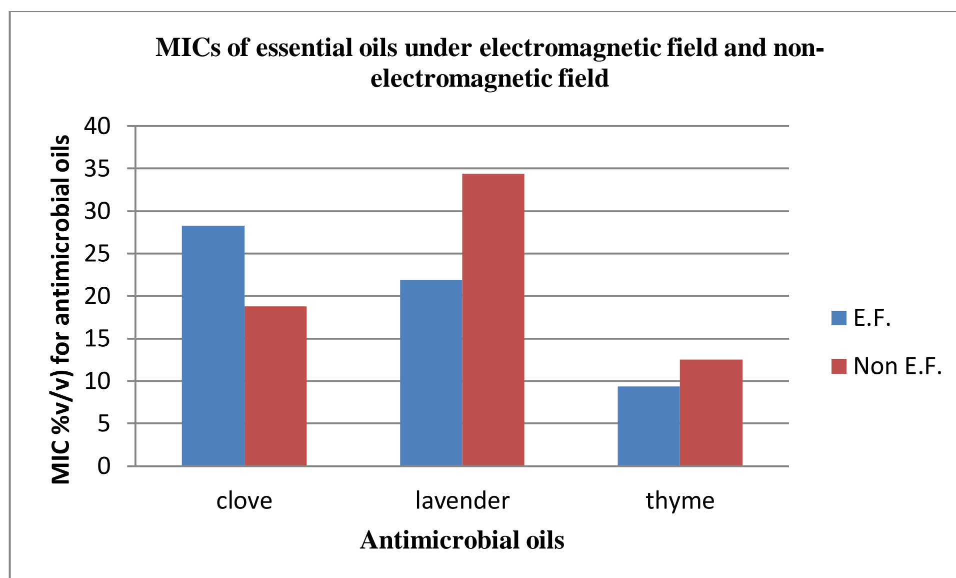

Figure 3: Clustered bar chart illustrating the MICs of essential oils under electromagnetic field and non-electromagnetic field.

The MIC values for all essential oils, suggested that there was an effect of electromagnetic field on the antimicrobial activity of essential oils. To make this point clear, One way analysis of variance (ANOVA) was performed to find out the significant difference in the activity of different essential oils under electromagnetic field and non electromagnetic field at room temperature. By using the SPSS 17.0 software, analysis of antimicrobial activity of essential oil was done based on cfu/ml for each organism.

The output file for *P.fluoroscence* showed that the level of significance was 0.011 which was less than 0.05 so it can be concluded that there was significant difference on average between cfu/ml of essential oils under electromagnetic field and non electromagnetic field. That means there was effect of electromagnetic field on antimicrobial activity of oils against *P.fluoroscence.* Following bar chart illustrated the fact more precisely.

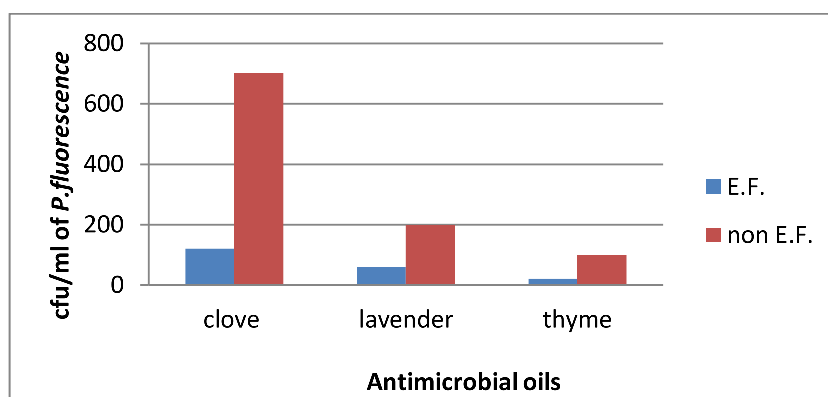

Figure 4: Clustered bar chart representing significant difference in the cfu/ml of *P.fluorescence* under electromagnetic field and non-electromagnetic field.

In case of *E.coli*, significant difference was observed on average between the cfu/ml of essential oils when

incubated under electromagnetic field and non electromagnetic field as p value was 0.016 i.e. less than 0.05. This can be best explained by clustered bar chart below:

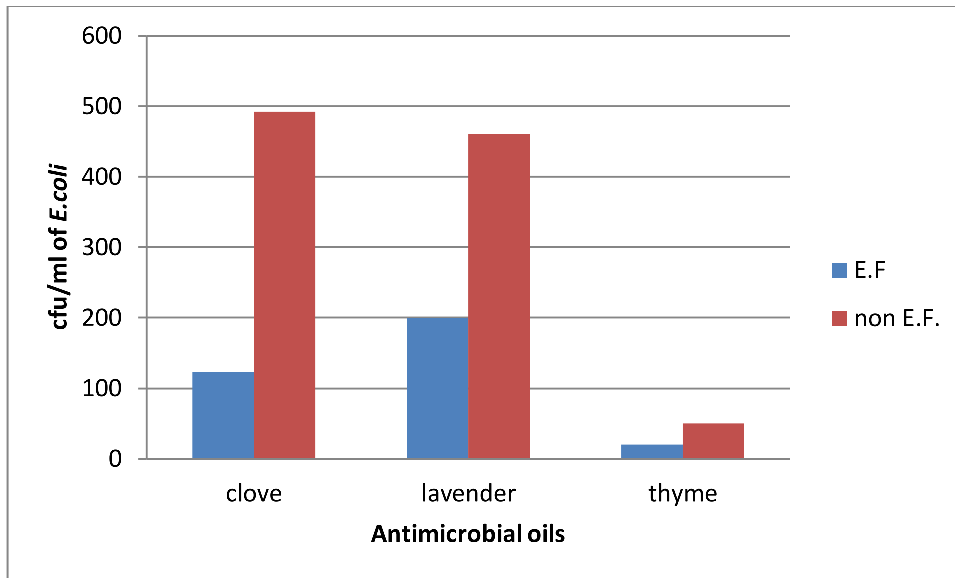

Figure 5: Clustered bar chart representing significant difference in the cfu/ml of *E.coli* under electromagnetic field and non electromagnetic field.

From the output file, significance level value of one way analysis of variance observed to be 0.073 which was greater than 0.05.That means there was no significant difference between the cfu/ml of *B.subtilis* under electromagnetic field and non electromagnetic field. So it can be said that electromagnetic field didn't show any effect on the activity of the essential oils. In case of *S.epidermidis, p* value was 0.010 which was less than 0.05,it gave an evidence that test was significant and the cfu/ml of *S.epidermidis* were different when incubated at electromagnetic field and non electromagnetic field. The activity of essential oils towards *S.epidermidis* changed when incubated under electromagnetic field. The following bar chart represents the positive effect of electromagnetic field on antimicrobial activity of essential oils.

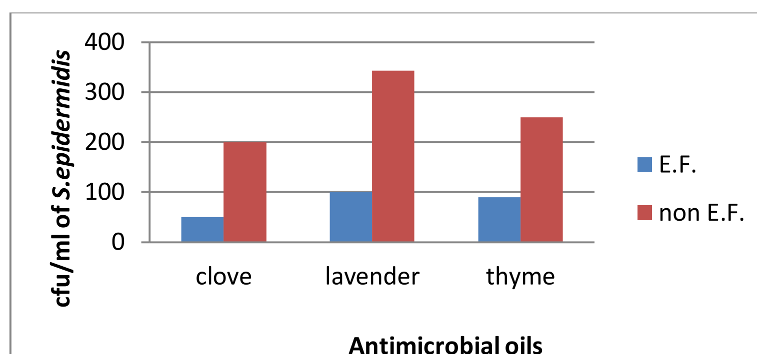

Figure 6: Clustered bar chart representing the significant difference in the cfu/ml of *S.epidermidis* under electromagnetic field and non-electromagnetic field.

For *S.cerevisae, no* significant difference was observed in average cfu/ml values under electromagnetic field and

non electromagnetic field. The p value was 0.920 which was less than 0.05 hence we accepted the fact that cfu/ml values were similar in both electromagnetic field and non electromagnetic field. There was no effect of electromagnetic field on the antimicrobial activity of essential oils in case of For *S.cerevisae.*

To sum it all, electromagnetic field had effect on the antimicrobial activity of essential oils with all organisms except *B.subtilis* and *s.cerevisae*.Both Gram negative(*E.coli* and *P.fluoroscence*) and Gram positive (*S.epidermidis* and *B.subtilis*) exhibited susceptibility towards atleast 2 oils either in electromagnetic field or at 37°C.Thyme oil showed the highest antimicrobial activity at 37°C as well as in electromagnetic field against *S.epidermidis.*

3.3 Time kill studies of lavender,clove and thyme oil

It was conducted to measure the time of killing of test organisms with essential oils and activities of oils were compared accordingly. Lavender oil and thyme oil showed higher activity against all organisms than that of clove essential oil. Lavender oil killed all the organisms within 60 minutes except *B.subtilis,* it took 100 minutes. Though *S.cerevisae* showed typical pattern of time kill curve, it was not taken into consideration as lavender oil failed to inhibit *S.cerevisae* in previous studies. *E.coli* was completely killed by lavender oil within 20 minutes. The rate of killing of *P.fluoroscence* was also same as that of the *E.coli* in case of the lavender oil.Time kill rate of all organisms was less in case of thyme oil. It showed significant activity within 60 minutes for all organisms.More so,*Saccharomyces cerevisae* and *E.coli* were killed within 20 minutes indicating rapid antimicrobial effect of thyme oil. Lavender oil and thyme oil reported almost same time of inhibition for all organisms except *Bacillus subtilis* and *Saccharomyces cerevisae*. On the contrary, clove oil took time to kill the organisms, though there was gradual decline in the viable number of organisms.*S.cerevisae* and *P.fluoroscence* were killed within 100 minutes and that was the earliest time of killing recorded by the clove oil.The time kill plot for *E.coli* in case of all essential oils is shown below. *E.coli* was completely killed within 20 minutes by both lavender oil and thyme oil while clove oil took 160 minutes to kill it.

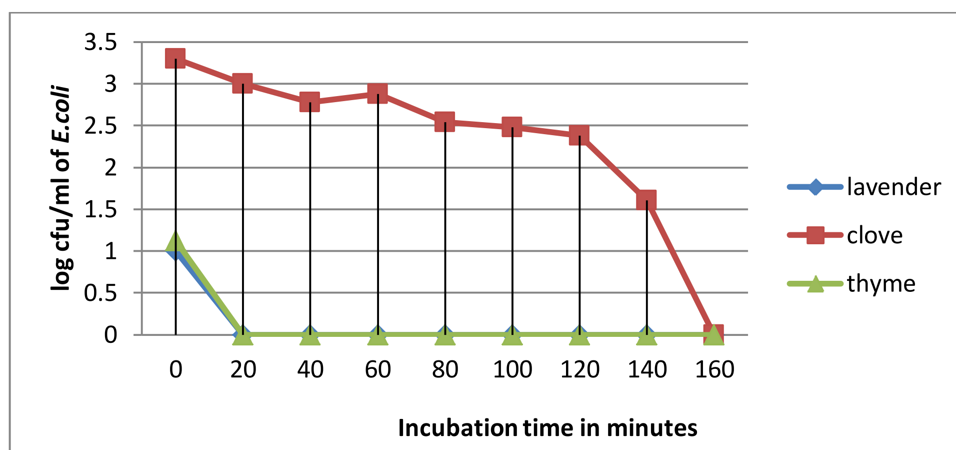

Figure 7: Time kill curve representing the rate of killing of *E.coli.*

S.epidermidis was killed within 60 minutes by both lavender oil and thyme oil.Whereas, clove oil took 160

minutes to kill *S.epideermidis.*The time kill plot for *S.epidermidis* with all essential oils is given below:

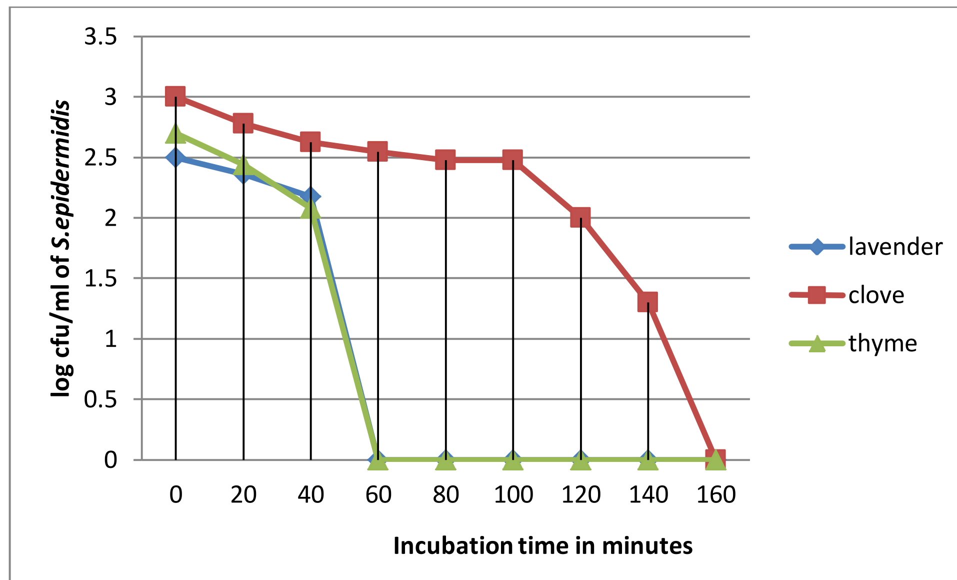

Figure 8: Time kill curve representing the rate of killing of *S.epidermidis.*

P.fluoroscence was killed within 100 minutes in case of clove oil whereas 40 minutes were sufficient for the lavender oil as well as thyme oil. The plot illustrating this rate of killing with all the oils is drawn below.

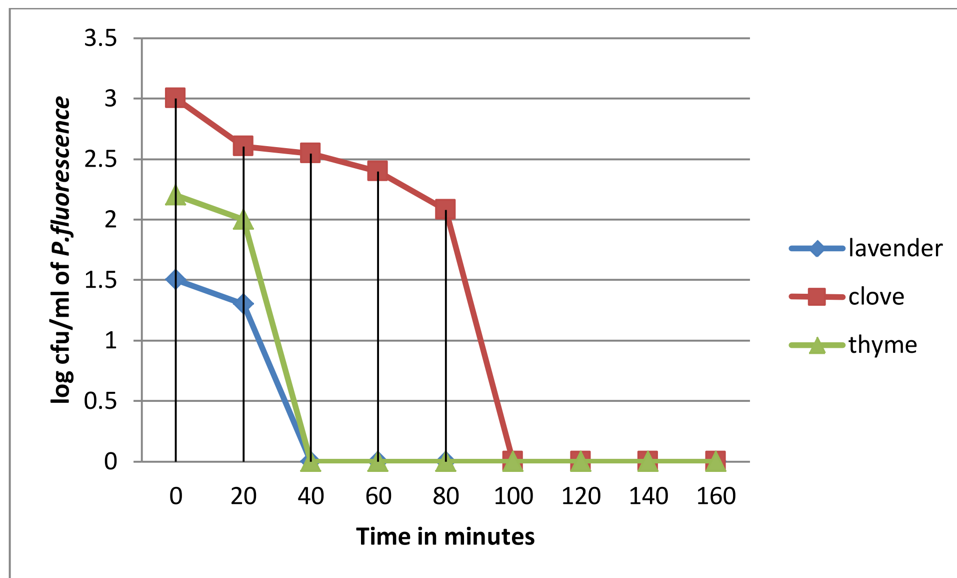

Figure 9: Time kill curve representing the rate of killing of *P.fluorescence.*

Fig no.10. Represents the rate of killing for *B.subtilis* with clove, lavender and thyme essential oils. Time of killing was 140 minutes for both lavender oil and clove oil, and for thyme oil it was 60 minutes. *S.cerevisae* was killed within 80 and 100 minutes by lavender and clove oil respectively. Rapid inhibition of *Saccharomyces cerevisae* was observed with thyme oil (within 20 minutes).

The time kill curve for *S.cerevisae* is presented in the fig.no.11.

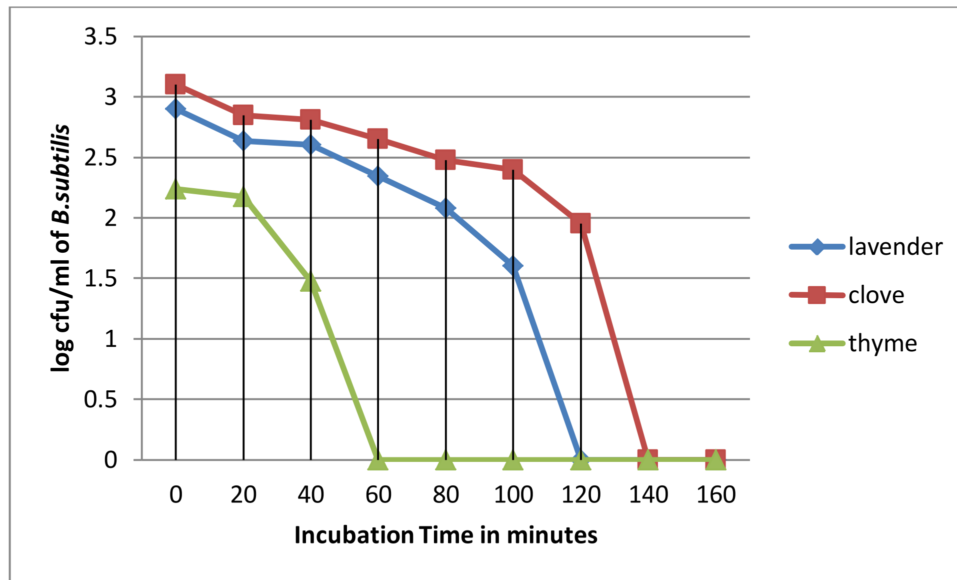

Figure 10: Time kill curve representing the rate of killing for *B.subtilis.*

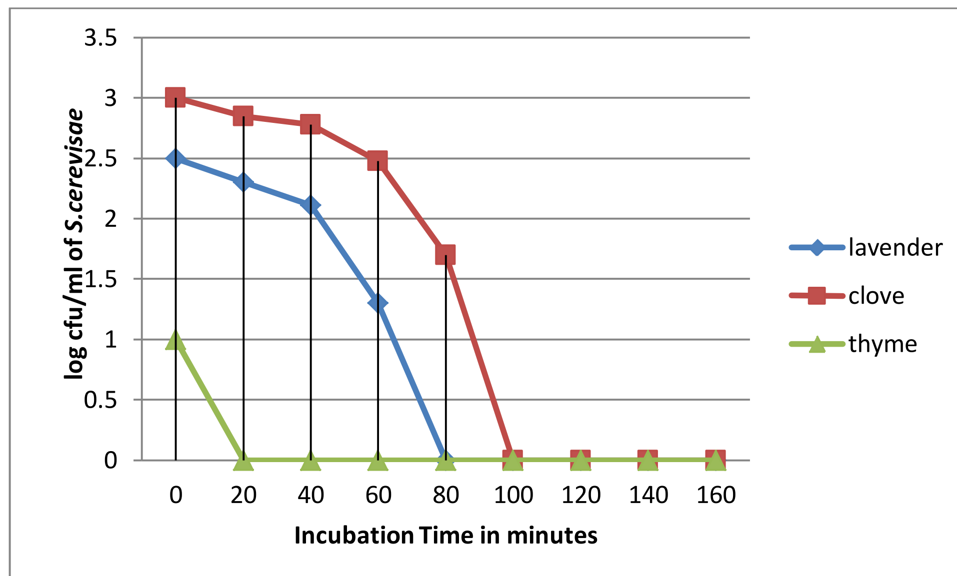

Figure 11: Time kill curve representing the rate of killing for *S.cerevisiae.*

3.4 Determination of enzyme inhibition by API 20 NE method

After incubation of 24 hrs, all the API 20NE strips were observed for colour change, this change is spontaneous for some tests while some tests need addition of reagents.NIT1 and NIT2 reagents were added to NO_3 test if negative reaction occur then Zn reagent powder was added to investigate further reduction test. For TRP test i.e. indole production James reagent was added, and results were noted down according to the colour change. The results for control, MIC and 0.5 MIC of each organism were recorded in case of both the oils and represented in the following tables. In case of clove oil, results obtained for MIC and 0.5MIC were same.

The purpose of this study was to compare the enzyme activity of organisms after addition of oil. Therefore the results obtained for control were compared with that of the MIC and 0.5MIC of oil. Different kinds of reactions were tested in this particular method, i.e.enzyme tests and assimilation tests. For best of my knowledge, no study has used API 20NE to investigate enzyme inhibition in case of essential oils.

In case of *E.coli,* reduction of nitrates to nitrites and fermentation of glucose was inhibited by both 0.5MIC and MIC of both the oils.β-galactosidase enzyme also inhibited by both 0.5MIC and MIC of both the oils. Glucose assimilation was hampered in MIC of the both oils but 0.5MIC of oil was showing glucose assimilation meaning that the glucose assimilation was inhibited when oil concentration increases from 0.5MIC to MIC. Same kind of results were observed in case of maltose assimilation, inhibition occurs at MIC but not at 0.5MIC.Assimilation of mannose was also found to be inhibited by oil. Overall, both clove oil and lavender oil inhibited the same enzymes and reactions in *E.coli.* In case of *S.epidermidis,* arginine dihydrolase and urease were inhibited at MIC of both the oils while same enzymes were active at 0.5MIC indicating that concentration more than 0.5MIC is necessary to inhibit the activity of these enzymes.

Table 3: Results of API 20 NE for *Escherischia coli* and *Staphylococcus epidermidis.*

	E.coli				*S.epidermidis*			
Tests	Control	Lavender		Clove	Control	Lavender		Clove
NO_3		MIC	0.5MIC	MIC		MIC	0.5MIC	MIC
$-NO_2$	+	-	-	-	-	-	-	-
$-N_2$	-	-	-	-	+	+	+	+
TRP	-	-	-	-	-	-	-	-
GLU	+	-	-	-	+	+	+	+
ADH	-	-	-	-	+	-	+	-
URE	-	-	-	-	+	-	+	-
ESC	+	+	+	-	+	+	+	+
GEL	-	-	-	-	-	-	-	-
PNPG	+	-	-	-	-	-	-	-
GLU	+	-	+	-	+	+	+	+
ARA	+	+	+	+	-	-	-	-
MNE	+	-	-	-	-	-	-	-
MAN	+	+	+	+	-	-	-	-
NAG	+	+	+	+	+	+	+	+
MAL	+	-	+	-	+	+	+	+
GNT	+	+	+	+	-	-	-	-
CAP	-	-	-	-	-	-	-	-
ADI	-	-	-	-	-	-	-	-
MLT	+	+	+	+	+	+	+	+
CIT	-	-	-	-	-	-	-	-
PAC	+	+	+	+	-	-	-	-

In case of *P.fluoroscence,* lavender oil inhibited reduction of nitrates to nitrogen, mannitol assimilation and trisodium citrate assimilation at both MIC and 0.5MIC.Gelatin hydrolysis was inhibited at MIC of both the oils but not at 0.5MIC.Maltose assimilation was also inhibited by both the oils.

In case of *B.subtilis,* both the oils inhibited the reduction of nitrates to nitrites, but reduction of nitrates to nitrogen was observed. Both the oils had inhibitory action on the same enzymes activity.Arginine dihydrolase, hydrolysis of gelatin, assimilation of mannitol and assimilation of potassium gluconate were affected by the oils.

To sum it all, API 20NE studies were very useful to understand the effect of oils on the different enzyme systems of the organisms. Different studies have been done on the antimicrobial activity of essential oils, but very few of them had insight on the exact mechanism behind the inhibition. This study showed that essential oils have altered the nitrogen metabolism, in most of the organisms, nitrogen reduction has hampered due to the oils. Subtle changes in the nitrogen metabolism of rumen microorganisms, on addition of blend of essential oils were observed by [5].This study also emphasised on the protease inhibition by the oil.

Table 4: Results of API 20 NE for *Pseudomonas fluorescence* and *Bacillus subtilis.*

	P.fluoroscence				*B.subtilis*			
Tests	Control	Lavender		Clove	Control	Lavender		Clove
NO_3		MIC	0.5MIC	MIC		MIC	0.5MIC	MIC
$-NO_2$	-	-	-	-	+	-	-	-
$-N_2$	+	-	-	+	-	+	+	+
TRP	-	-	-	-	-	-	-	-
GLU	+	+	+	-	-	-	-	-
ADH	-	-	-	-	+	-	-	-
URE	-	-	-	-	-	-	-	-
ESC	+	+	+	+	+	+	+	+
GEL	+	-	+	-	+	-	-	-
PNPG	-	-	-	-	-	-	-	-
GLU	+	+	+	+	+	+	+	+
ARA	-	-	-	-	+	+	+	+
MNE	-	-	-	-	-	-	-	-
MAN	+	-	-	+	+	-	-	-
NAG	+	+	+	+	+	+	+	+
MAL	+	-	-	-	+	+	+	+
GNT	-	-	-	-	+	-	-	-
CAP	-	-	-	-	-	-	-	-
ADI	-	-	-	-	-	-	-	-
MLT	+	+	+	+	+	-	-	-
CIT	+	-	-	+	+	-	-	-
PAC	-	-	-	-	-	-	-	-

Figure 12: demonstrates the results of API 20NE method for *P.fluorescence* with lavender oil,control (uppermost),MIC (middle),1/2 MIC (lower).

Figure 13: represents the results of API 20NE method for *B.subtilis* with lavender oil,control (uppermost),MIC (middle),1/2 MIC (lower).

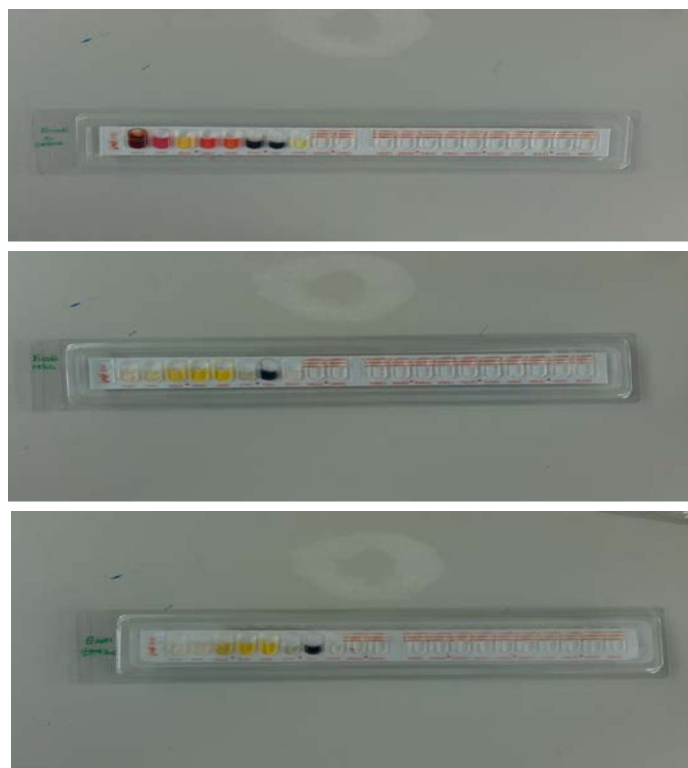

Figure14: demonstrates the results of API 20NE method for *E.coli*,control (uppermost),MIC (middle),1/2 MIC(lower).

4. Discussion

More than 500 studies have reported antimicrobial and antifungal activity of the essential oils. It is important to investigate scientifically those oils which have been used in traditional medicines as a novel antimicrobial compounds [13].Various studies have documented the significant antimicrobial activity of clove, lavender and thyme oil [13,40].These oils found to be significant against common pathogens under some in vitro studies, but most of these studies provide general information whether or not an oil possesses activity against Gram positive, Gram negative and fungi. Some studies provide the relative activity of essential oils by comparing the results from the different oils against same organism [13].However, comparison of results obtained with previous studies is not feasible as different essential oils vary in their action with different organisms and under different environmental conditions.

In the present study, three essential oils (clove, lavender and thyme) were analysed for their activity against common pathogens. From the results of disc diffusion assay, it can be concluded that thyme oil had the highest activity amongst three of them. Lavender oil and clove oil seemed to have almost same activity against all organisms except *P.fluoroscence*. The results obtained to [40] regarding disc diffusion assay were similar to some extent as that of the results obtained in this study. Considering the results of minimum inhibitory concentration assay, lowest MIC was reported by thyme oil supporting the results of disc diffusion assay.

Although; many studies have reported about the MICs of clove, lavender and thyme oil, it is problematic to compare them with the results of this study as composition of oils can differ with the local climatic conditions of plants. Apart from this, there are various factors that may influence the results of the study such as culture conditions, solvent, age of inoculums etc. [6]. The finding that thyme oil has maximum antimicrobial activity amongst all 3 oils against all test organisms in this investigation is in accord with the previous studies [41].Thyme oil showed promising activity even at low concentrations. [13] reported the MICs of clove, lavender and thyme oil against *E.coli, as* 0.5 v/v, 0.12 v/v and 0.25 accordingly. This study differs to the previous results as MICs of clove, lavender and thyme oil against *E.coli* were 0.5 v/v, 0.25 v/v and 0.5 v/v respectively. However, under different incubation conditions, all the three oils behaved differently. In case of thyme oil, MIC values at room temperature and at 37°C were same for all organisms, but these values decreased under electromagnetic field stating the positive effect of electromagnetic field on antimicrobial activity of thyme oil.*Saccharomyces cerevisae* was exception to this finding as it was having same MIC values almost in every incubation condition. MIC values of clove oil significantly decreased under electromagnetic field only against *Pseudomonas fluorescence* ,all other organisms had almost same activity under all conditions. The results obtained in case of lavender oil under different incubation conditions (i.e.under electromagnetic field and non-electromagnetic field) were inconsistent, *Staphylococcus epidermidis* showed significant decrease in MIC under electromagnetic field and even at 37°C.On the contrary, MIC value elevated from 12.5% to 25% at 37°C,in case of *Pseudomonas fluorescence.* According to the statistical analysis, it can be concluded that under electromagnetic field, all essential oils showed significant increase in the antimicrobial activity against *E.coli,P.fluoroscence,S.*epidermidis in terms of cfu/ml. When antimicrobial activity of essential oils under electromagnetic field and non electromagnetic field was compared, it was observed that both Gram positive and Gram negative organisms were killed at low concentration of essential oils under electromagnetic field but yeast had no effect on its susceptibility towards essential oils under electromagnetic field. Strasak has studied the effect of low frequency magnetic field on *E.coli* and reported that decrease in the viable organisms was due to the exposure of electromagnetic field. But it's not yet solved how bacteria get killed by the electromagnetic field. Studies have supported possible mechanisms like alteration in the permeability of ionic channels in the membrane that affect the ion transport in the cell finally resulting into changes in the biological function of the organisms. Other possible effect is the formation of free radicals due to electromagnetic field.

Although, maximum studies on antimicrobial activity of essential oils dealt with the MICs and relative activity of oils, it's important to find out how rapidly it kills microorganisms. This study focuses on this particular point, time kill studies were done on the all oil against 5 test organisms. Time kill studies of tea tree oil have done on clinical samples by May *etal* in 2000.Time kill plot of clove and rosemary oil was determined against *Staphylococcus aureus, Escherischia coli* and *Candida albicans* by Fu *etal.* These previous studies have provided base for the determination of time kill plot, results of time kill studies have helped in determining time of bacterial eradication. Viabillity of the organisms after contact with oils for specified period of time was checked in this study. Based on the results obtained in this study, thyme oil proved to have rapid activity as compared to other oils. *Escherischia coli* and *Saccharomyces cerevisae* were killed within 20 minutes with thyme oil. The rate of killing was so fast for thyme oil that all organisms showed complete inhibition within 60 minutes.

After thyme oil, lavender oil had considerable effect on the test organisms, it killed *Pseudomonas florescence, Staphylococcus epidermidis* within 40 and 60 minutes respectively, whereas *E.coli* was killed within first 20 minutes of exposure. Lavender oil showed slow killing to *Bacillus subtilis* and *Saccharomyces cerevisae*. Clove oil seemed to have slowest activity in this study as the time taken by the clove oil to kill the test organisms was more. The slowest activity showed by clove oil was 160 minutes towards *E.coli* and *Staphylococcus epidermidis.* With clove essential oil *E.coli* appeared to show an increase in the total viable count after first 40 minutes following the decline phase. This proved the weakness of clove oil as that compared to other two. The lowest rate of killing reported with clove oil was 100 minutes in case of *Staphylococcus epidermidis* and *Saccharomyces cerevisae.* There were discrepancies in the activity of various oils with different group of organisms used in this study. With the Gram positive bacteria, lavender oil showed complete killing of *Staphylococcus epidermidis* after 60 minutes and complete killing of *Bacillus subtilis* was observed after 120 minutes. With that of clove oil,*Satphylococcus epidermidis* and *Bacillus subtilis* were completely killed after 160 and 140 minutes. While thyme oil inhibited both of them after 60 minutes. Rapid killing was observed in case of Gram negative bacteria (*E.coli* and *Pseudomonas florescence)* by lavender oil and thyme oil. In that also *E.coli* had more sensitivity towards both the oils as it was completely killed within 20 minutes.

Authors in reference [13] found that clove, thyme and lavender oil inhibited *Eschereschia coli, Pseudomonas aerogenosa, Staphylococcus aureus, Salmonella enteric.* Out of which *Staphylococcus aureus* was Gram positive and *Escherischia coli,Pseudomonas fluorescence,Salmonella enteric* were Gram negative. In this study, clove oil was effective against *Escherischia coli, Pseudomonas fluorescence* which are Gram negative bacteria and *Staphylococcu epidermidis* and *Bacillus subtilis* which are Gram positive bacteria. Clove oil was also effective against yeast, *Saccharomyces cerevisae.*Similarally,lavender oil and thyme oil showed antimicrobial activity against Gram positive bacteria (*Bacillus subtilis,Staphylococcu epidermidis)* and also against Gram negative bacteria (*Escherischia coli* and *Pseudomonas fluorescence).*Lavender oil appeared to be inactive against yeast,*Saccharomyces cerevisae* but thyme oil had significant activity against yeast. The mode of action of essential oil was not discussed by these workers but most of the known modes of action have been based on those of antibiotics and disinfectantas. As an example,penicillin inhibits the cell wall synthesis[34].

Chloramphinicol inhibits the protein synthesis [17], Norfloxacin inhibits the nucleic acid replication[37]. The present studies investigated likely modes of action by investigating the effects of microbial metabolic enzymes. Clove oil was found to inhibit enzymes involved in nitrate reduction, glucose fermentation, mannose assimilation, it also inhibited the β-galactocidase in *E.coli(*Table no 3),whereas arginine dihydolase and urease were inhibited in *S.epidermidis* (Table no.3). *E.coli* is Gram negative and *S.epidermidis* is Gram positive. Antibiotics having antimicrobial activity against both Gram positive and Gram negative bacteria are classified as having broad spectrum activity. These studies suggested that clove oil has broad spectrum activity by inhibiting the enzymes involved in gelatine hydrolysis, malic acid assimilation, potassium gluconate assimilation and urease in Gram positive organisms and β galactocidase, enzymes involved in glucose fermentation, maltose assimilation in Gram negative organisms. Lavender oil inhibited the enzymes involved in the nitrate reduction to nitrogen, mannitol assimilation, trisodium citrate assimilation and proteases (involved in gelatine hydrolysis) in *Pseudomonas fluorescence*(Table no.4) ,whereas arginine dihydrolase, enzymes involved in hydrolysis of gelatine, assimilation of potassium gluconate were inhibited in *B.subtilis* (Table no.4).

Thus, it was concluded that lavender oil too posses broad spectrum activity. Its worth mentioning one thing about the lavender oil that in case of *Pseudomonas fluorescence*, gelatine hydrolysis inhibited at MIC but not at 0.5 MIC indicating that as concentration of oil increased from 0.5 MIC to MIC, enzymes involved in the gelatine hydrolysis inhibited. That means the critical concentration of lavender oil to inhibit the gelatine hydrolysis is in between 0.5MIC and MIC.In *Staphylococcus epidermidis*,arginine dihydrolase was inhibited at MIC of both the oils but not at 0.5 MIC indicating that critical concentration of both the oils to inhibit arginine dihydrolase was in between 0.5MIC and MIC.

Thus, both lavender and clove oil showed the inhibition of some enzymes in each organism. So, it can be concluded that antimicrobial activity of essential oils may be due to the inhibition of these enzymes. Though exact modes of action of essential oils are being debated, enzyme inhibition can be a possible mechanism. Enzymes are the very important components of microbial system, all cell functions are based on enzymes. Inhibition of enzymes certainly has impact on viability of cell. In this study, essential oils have inhibited more than two enzymes in each organism which can be a possible mode of action. Any direct evidence has not reported yet about the effect of essential oil on genetic material but present study has reported the effect of oils on enzyme system, enzymes are encoded by genes so it is possible that essential oils have effect on genetic material of organisms. Essential oils appeared to be effective against both Gram positive and Gram negative organisms unlike results observed in previous studies [43].This study supported the inhibition of metabolic enzymes as a mode of action of essential oils. More so study revealed about the positive effect of electromagnetic field on the antimicrobial activity of essential oils. There was significant increase in the activity of all essential oils under electromagnetic field.

5. Conclusion

Based on the results of different methods used in this study it can be concluded that clove, lavender and thyme oil exhibited significant antimicrobial activity against all test organisms. Both Gram positive and negative organisms were susceptible to the essential oils however *B.subtilis* showed highest zones of inhibition with all essential oils. LSD test was performed to investigate relative activity of essential oils and it was observed that thyme oil was having significantly different zones of inhibition than other two oils in case of all test organisms, whereas there was no significant difference in the zones of inhibition of lavender oil and clove oil for most of the organisms except for the *P.fluorescence*. Effect of electromagnetic field on the antimicrobial activity of essential oils was tested against all organisms in terms of their MICs and cfu/ml. Under electromagnetic field most of the test organisms showed lower (or same) MIC values as compared to non electromagnetic field. More so ANOVA test was performed using cfu/ml of test organisms with all essential oils and results of the test indicated the strong effect of electromagnetic field on the antimicrobial activity of essential oils in case of *E.coli, P.fluorescence* and *S.epidrmidis*. Thyme oil was found to be more effective under electromagnetic field. According to the time kill curves, slowest antimicrobial activity was reported by clove oil against each organism. Rapid inhibition of organisms was observed in case of both lavender oil and thyme oil, in that to thyme oil was very active even at low concentrations. Finally, the effort was made to investigate the mechanism behind the antimicrobial activity of essential oils, and the study ended up with the inhibition of metabolic enzymes as a possible mechanism as different types of enzymes were found to be inhibited by the oils.

6. Result

TVC for all dilutions incubated at room temperature (non electromagnetic field) were calculated and given in the table 3.

Table 3: TVC of all organisms when incubated with clove oil at room temperature.

Total viable count of microorganisms incubated at room temp.	Concentration of clove oil			
	50	25	12.5	6.25
E.coli (cfu/cm3)	4×10^2	6×10^2	86×10^1	9×10^2
S.epidermidis(cfu/cm3)	_	_	_	_
P.fluoroscence(cfu/cm3)	_	_	5×10^1	1×10^2
B.subtilis(cfu/cm3)	_	5×10^1	28×10^1	5×10^2
S.cerevisiae(cfu/cm3)	_	_	25×10^1	65×10^1

_ implies to no growth.

Total viable count for all dilutions incubated at electromagnetic field were calculated and mentioned in the table 4.

Table 4: TVC of microorganisms when incubated with clove oil at electromagnetic field.

Total viable count of microorganisms incubated at electromagnetic field.	Concentration of clove oil			
	50	25	12.5	6.25
E.coli (cfu/cm3)	_	5×10^2	7×10^2	10×10^2
S.epidermidis(cfu/cm3)	_	9×10^1	35×10^1	4×10^2
P.fluoroscence(cfu/cm3)	_	_	_	8×10^2
B.subtilis(cfu/cm3)	45×10^1	5×10^2	5×10^2	7×10^2
S.cerevisiae(cfu/cm3)	_	_	3×10^2	63×10^1

_ implies to no growth.

Total viable count of all dilutions incubated at 30ºc were calculated and noted down below in table 5.

Table 5: TVC of all organisms incubated with clove oil at 30ºc._ implies to no growth.

Total viable count of microorganisms incubated 30ºc.	Concentration of clove oil			
	50	25	12.5	6.25
E.coli (cfu/cm3)	_	4 x 10^1	10 x 10^1	17 x 10^1
S.epidermidis(cfu/cm3)	_	_	79 x 10^1	10 x 10^2
P.fluoroscence(cfu/cm3)	_	_	13 x 10^1	28 x 10^1
B.subtilis(cfu/cm3)	_	_	10 x 10^1	31 x 10^1
S.cerevisiae(cfu/cm3)	_	7 x 10^2	11 x 10^1	5 X 10^1

Tests of Between-Subjects Effects

Dependent Variable:cfu

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	490533.333[a]	4	122633.333	1.287	.435
Intercept	250934.489	1	250934.489	2.633	.203
conditions	67213.333	1	67213.333	.705	.463
oils	226433.333	2	113216.667	1.188	.417
conditions * oils	46413.333	1	46413.333	.487	.535
Error	285866.667	3	95288.889		
Total	1621400.000	8			
Corrected Total	776400.000	7			

a. R Squared = .632 (Adjusted R Squared = .141)

Tests of Between-Subjects Effects

Tests of Between-Subjects Effects

Dependent Variable:cfu

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	490533.333[a]	4	122633.333	1.287	.435
Intercept	250934.489	1	250934.489	2.633	.203
conditions	67213.333	1	67213.333	.705	.463
oils	226433.333	2	113216.667	1.188	.417
conditions * oils	46413.333	1	46413.333	.487	.535
Error	285866.667	3	95288.889		
Total	1621400.000	8			
Corrected Total	776400.000	7			

Dependent Variable:cfu

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	175233.333[a]	3	58411.111	1.857	.369
Intercept	197633.333	1	197633.333	6.284	.129
conditions	43200.000	1	43200.000	1.374	.362
oil	93633.333	1	93633.333	2.977	.227
conditions * oil	13333.333	1	13333.333	.424	.582
Error	62900.000	2	31450.000		
Total	574200.000	6			
Corrected Total	238133.333	5			

a. R Squared = .736 (Adjusted R Squared = .340)

For E.coli

cfu

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
EF	4	122.5000	68.49574	34.24787	13.5080	231.4920	40.00	200.00
non EF	4	592.5000	291.36175	145.68087	128.8784	1056.1216	350.00	1000.00
Total	8	357.5000	318.60186	112.64277	91.1422	623.8578	40.00	1000.00

ANOVA

cfu

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	441800.000	1	441800.000	9.863	.020
Within Groups	268750.000	6	44791.667		
Total	710550.000	7			

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