March - April 2020
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Antibacterial Efficacy of Selected Essential Oils against Bacillus Megaterium and their Application in Food Preservation

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Abstract
Antibacterial effect of ten different essential oils like Clove oil (*Syzygium aromaticum*), Lemongrass oil (*Cymbopogon*), Lemon oil (*Citrus limonum*), Peppermint oil (*Mentha piperita*), Tea tree oil (*Melaleuca alternifolia*), Eucalyptus oil (*Eucalyptus globulus*), Orange oil (*Citrus sinensis*), Basil oil (*Ocimum basilicum*), Rosemary oil (*Rosmarinus officinalis*) and Cinnamon oil (*Cinnamomum verum*) were studied against the food spoilage bacteria *Bacillus megaterium* for its application as food preservatives. The oils were tested at the concentrations of 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/mL. Essential oil with potent antibacterial efficacy was determined individually using agar well diffusion method. Results revealed that among the tested essential oils clove and cinnamon oil exhibit high antibacterial effect. Synergy test was further studied in order to determine the combined efficacy of clove and cinnamon oil. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also observed. MIC was found to be as 15.625 and 62.5 mg/mL for clove and cinnamon oil. Similarly MBC were recorded as 31.25 and 62.5 mg/mL.

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Introduction:

In spite of modernization and changing lifestyle, our society requires safer, high quality and preservative-free food products with extended shelf life (Gould et al., 1996). But food- borne illness resulting from the pathogenic bacteria and their toxins is a major concern for both the consumers and food industry despite the use of various preservatives. According to the estimation done by World Health Organization, 30 % of people in industrialized countries suffer from a food borne disease every year. In the year of 2000, at least two million people died from diarrheal disease worldwide. The experts in food processing and regulatory agencies are continuously facing the concerns with the high and growing number of illness outbreaks caused by some pathogenic and

March - April 2020
ISSN: 0193-4120 Page No. 1909 - 1916

spoilage microorganisms in food (Shan et al., 2011).

Conventional methods of food preservation involves the usage of synthetic chemical preservatives, which leads to many health issues such as nausea, vomiting, diarrhea, respiratory problems like wheezing, running nose, allergic reactions like itching, rashes, flushing and they also cause cancer. In general, artificial preservative can be classified in to 3 major group's antimicrobial agent, antioxidants and anti-enzymatic preservatives (Sati et al., 2013). Anti microbial agents are normally used to destroy or inhibit the growth of microbes on foods. Some of the components used as antimicrobial agents are benzoates, sorbates, propionates and nitrites. Addition of these chemicals as food preservatives causes DNA damage and is more toxic to health. Antioxidants play a crucial role in slowing down or stopping the breakdown of fats and oils in the food that occurs in the presence of oxygen leading to rancidity.

Sulfite is the commonly used antioxidant in food preservation process. Food substances having Sodium sulfite, sodium bisulfite, sodium metabisulfite, potassium bisulfite and potassium metabisulfite results in allergic reactions and exacerbation of asthma. Anti-enzymatic preservatives control the enzymatic processes and prevent the harmful effects of chemical enzymes in food products. Some of the components used as this kind of preservatives are erythorbic acid and citric acid. They usually stop the action of the enzyme phenolase that leads to browning reactions in cut fruits or potato. The short-term side effects associated with the usage of erythorbic acid includes headache, dizziness, fatigue, body flushing and hemolysis. The long-term side effects are kidney stones and triggering gout symptoms. The usage of such synthetic preservatives is also not efficient in the removal of food organisms. So, the natural substances with antimicrobial properties, that do not cause any side effects, can be considered as alternatives in food industries.

Essential oils derived from the aromatic plants are acting as the best alternatives for synthetic food preservatives (Zaika et al., 1988, Beuchat et al., 1989). Essential oils, otherwise known as volatile or ethereal oils are the complex mixtures of volatile, hydrophobic components from plants which are containing volatile aromatic compounds derived from aromatic plants. Essential oils are made up of smaller organic molecules known as terpenes (Faleiro, 2011). Terpenes are the polymers of a volatile, five carbon compound called as isoprene. The active compounds, which are responsible for flavor and aroma of essential oils, exist at various concentration levels in their sources and are responsible for multiple effects. These oils are extracted from the plants by several methods like steam distillation, organic solvent extraction, cold pressing, supercritical fluid extraction.

Essential oils possess antimicrobial (Kim et al., 1995, Chutia et al., 2009), anti-oxidant, anti-mutagenic, anti-carcinogenic and insecticidal properties (Moretti et al., 2002). They are being highly investigated for their application in food preservation. The antioxidants present in the essential oils and extracts obtained from the plants are used to increase the shelf-life of food products and to treat human diseases such as atherosclerosis and cancer (Ebrahimi et al., 2013). It is generally recognized that the antimicrobial action of essential oils depends on their hydrophilic or lipophilic character. Being the hydrophobic compounds, essential oils dissolve in the hydrophobic domain of the cytoplasmic membrane of bacterial cells, between the lipid acyl chains and alter membrane fluidity. This results in swelling of membrane and increase the

March - April 2020
ISSN: 0193-4120 Page No. 1909 - 1916

permeability of the cytoplasmic membrane as observed in absorbance measurements of UV absorbing materials of intracellular components and impairment of enzymes may occur.

The irreversible gross damage to membrane results dissipation of pH gradients, leakage of divalent cations, ATP and the excessive loss lead to death of cells. The mode of action of antimicrobial agents also depends on the type of microorganisms and is mainly related to their cell wall structure and the outer membrane arrangement. Nowadays essential oils are being used in food industries with wide applications.

Bacillus megaterium a gram positive, endospore-forming, rod shaped bacteria can grow at the temperature between 3°C to 45°C. It was reported that *Bacillus megaterium* causes spoilage in food products which are of importance. It results bitter flavored milk due to proteolysis which leads to food poisoning (Sutherland et al., 1994). It also causes food spoilage in cocoa products, fish products and in spices which are not dried properly (Fritze et al., 2003). In bakery products like bread, it gives ropiness. Transfer of *B. megaterium* spores from the soil to the wheat grains from which the bread is prepared is the key factor in spoilage (Thompson et al., 2003).

The aim of this present study is to investigate the antibacterial effect of selected essential oils like Clove, Cinnamon, Tea Tree, Lemon grass, Eucalyptus, Lemon, Rosemary, Peppermint, Basil, Orange oil on *B. megaterium* in order to utilize essential oils as food preservatives. Moreover the ability of some essential oils to be used in combination make them a potent candidate in food preservation (Tejeswani et al., 2014). Further the effect of essential oils on cell morphology was studied by SEM analysis.

Materials and Methods:

Chemicals and strain:

The selected EOs were procured from RV Essentials Pvt. Ltd, Delhi, India and stored at 4°C till further used. The strain used for the present study was *B. megaterium* was procured from Indian Type Culture Collection (ITCC). The culture was maintained by sub culturing on Muller Hinton Agar (MHA) incubated at 28°C for overnight and stored at 4°C for long term use. Chemicals such as Muller Hinton agar, Muller Hinton broth were purchased from Himedia India Pvt. Ltd Mumbai, India. Dimethyl Sulfoxide (DMSO), Ethanol, Tween-20, Agar-Agar, Glutaraldehyde, Disodium hydrogen phosphate and Sodium dihydrogen phosphate were supplied from Spectrum Chemicals, Mumbai.

Screening for Antibacterial activity:

In vitro antibacterial activity of essential oils was studied by agar well diffusion method (Balouiri et al., 2016). The inoculum size of the culture is considered as 1×10^8 CFU/ml as per the guidelines provided by Clinical Laboratory Standards Institute (CLSI). A single colony from an overnight bacterial culture plate was seeded into 5 mL of an appropriate pre-warmed growth medium broth (MHB). Culture tubes were shaken at 180 rpm and 28°C for overnight. The optical density of the culture was adjusted within the range of 0.08-0.13 at 600 nm with reference to 0.5 McFarland standards (which has OD between 0.08-0.13 at 600 nm) for the sensitivity assay. Stock concentration of 500 mg/mL was prepared by dissolving the oil in the mixture of 10 % aqueous dimethylsulfoxide (DMSO) and 0.5 % (v/v) Tween-20. Ethanol was chosen as a suitable solvent for basil since the diffusivity of the oil is less in the mixture of DMSO and Tween-20. Concentrations ranging from 50 mg/mL to 450 mg/mL were prepared by dissolving the stock oil solution in sterile distilled water.

March - April 2020
ISSN: 0193-4120 Page No. 1909 - 1916

Muller Hinton agar (20 ml) was poured into sterile Petri plates (90 mm diameter) and 100 µl of the culture was spread evenly onto pre-warmed 37°C agar plates using a sterile swab. Wells were punched in the agar plate and the diluted oil samples were loaded into the wells and sealed with parafilm (Anis Ahmed et al., 2013). Plates were then inverted and incubated for approximately 24 h at 28°C and the diameter of the inhibition zones was measured in mm. The control sets were prepared using equal amounts of 10% DMSO in place of oil. The sensitivity was classified according to Balouiri et al., 2016 as follows: not sensitive for a diameter less than 8 mm, sensitive for a diameter of 9–14 mm, very sensitive for a diameter of 15–19 mm, and extremely sensitive for a diameter larger than 20 mm. Each test was performed in three replicates (Goni et al., 2009).

Evaluation of MIC and MBC of essential oils:

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of EOs were determined using a broth microdilution method in 96-well strip tubes with transparent strip-caps. Bacterial suspensions were adjusted to a final concentration of 1×10^8 CFU/ml as per the guidelines provided by Clinical Laboratory Standards Institute (CLSI) in MHB. 100 µL of 2 fold serially diluted EOs using MHB were added into the wells of microtitre plate. 100 µL of bacterial suspension was finally added to each well except sterility control (which consists of MHB only). The growth control wells contained 100 µL of the corresponding inoculum suspension and 100 µL of the sterile oil-free medium and DMSO-Tween-20 mixture. The plate was sealed with parafilm and incubated at 28°C for 24 h. The lowest concentration at which growth did not occur is the MIC value. All tests were performed in triplicates. The plate was visually observed for turbidity in the wells to determine the MIC (Wiegand et al., 2008, Seenivasan et al., 2006).

Now an aliquot of 50 µl from the wells remaining without visible growth were plated onto MHA and incubated for 24 h at 28°C. The least concentration of the essential oil with no visible bacterial growth after incubation was taken as minimum bactericidal concentration (MBC). (Andrews 2001, Lambert et al., 2001).

SYNERGY TEST:

Synergy test was also performed to determine the antibacterial activity of combination of essential oils against *B. megaterium*. From the agar well diffusion method, it was observed that the clove and cinnamon oils were found to be extremely sensitive among the tested oils against *B. megaterium.* EOs were combined in the ratio of 1:1 and the experiments were repeated as mentioned with the concentrations ranging from 50 mg/mL to 500 mg/mL.

Statistical Analysis:

All experiments were repeated at least twice. Data were analyzed using statistical software. Effect of treatments on *B. megaterium* were analysed using one way ANOVA. Significance of differences among treatments was observed by considering $p < 0.5$.

Results and Discussion:

Antibacterial activity assay:

All essential oils inhibited the growth of *B. megaterium* in a dose dependent manner. A concentration of 50 mg/ml of clove and cinnamon oil is extremely sensitive towards the bacteria whereas Lemon grass and Tea Tree oil is very sensitive at 250 mg/ml. Eucalyptus oil exhibit only mild inhibition at 350 mg/ml. Lemon oil, Basil, Orange oil, Peppermint oil and Rosemary

March - April 2020
ISSN: 0193-4120 Page No. 1909 - 1916

oil does not have any effect on the bacteria. The efficacy of essential oil was in the order of clove > cinnamon > lemon grass, Tea tree > Eucalyptus. The antibacterial effect exhibited by these oils could be attributed to the presence of bioactive terpenes, phenolic acids, alcohols, hydrocarbons and aldehydes. (Singh et al., 2002, Burt, 2004).

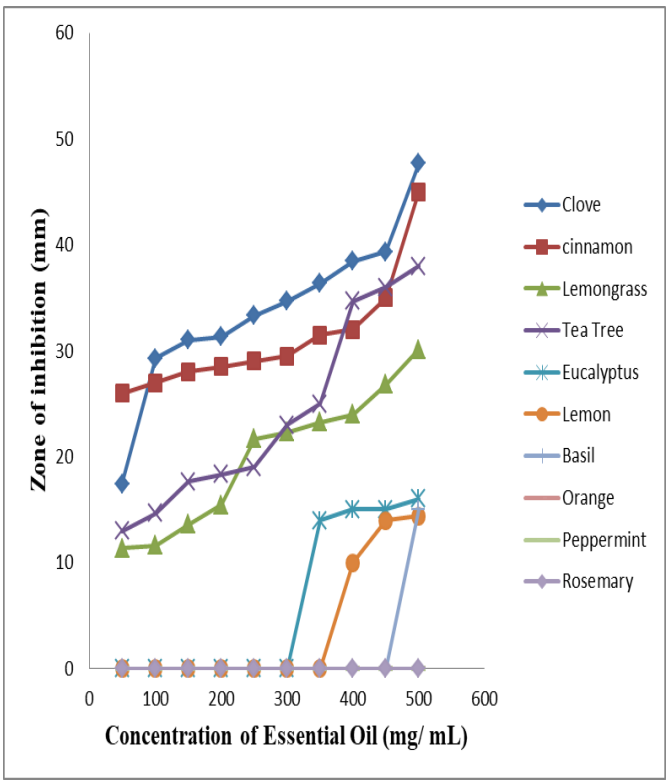

Fig 1: Inhibitory Effects of Essential oil on the growth of *Bacillus megaterium*

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of essential oils:

Since Clove, Cinnamon, Tea Tree and Lemon grass oil exhibited better antibacterial effects, these were further analyzed for determining the optimal concentration whereas Eucalyptus oil Lemon oil, Basil, Orange oil, Peppermint oil and Rosemary oil were not considered. Clove, Cinnamon, Tea Tree and Lemon grass essential oils MIC and MBC values against *B. megaterium* determined by visualizing the turbidity in the microtitre wells. Clove and Cinnamon oil showed potent antibacterial activity with MIC and MBC values of 15.625 mg/ml and 31.25 mg/ml respectively. Tea Tree and Lemon grass oil displayed the MIC and MBC values ranging between 31.25 mg/ml to 125 mg/ml. Essential oils selected inhibited the bacterial growth and showed bactericidal activity. Because of its lowest MIC and higher inhibition percentage clove and cinnamon oil was selected for further analysis. The Lower value of MIC compared to MBC indicated that clove and cinnamon oil is bacteriostatic at lower concentration and bactericidal at higher concentration (Sharma et al., 2017).

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of essential oils against *B. megaterium:*

S. No	Essential Oil	MIC (mg/ml)	MBC (mg/ml)
1	Clove	15.625	31.25
2	Cinnamon	15.625	31.25
3	Tea Tree	31.25	62.5
4	Lemon grass	62.5	125

Synergy Test:

Better antibacterial activity was observed by treating the *B. megaterium* with clove and cinnamon oil only when compared to Tea Tree and Lemon grass. Synergistic effect of the clove and cinnamon oils were studied and the antibacterial activity was tested in combination with the concentrations ranging from 50 mg/mL to 500 mg/mL. It was noted that the antibacterial effect in combination is also very sensitive at 50 mg/mL (Ghabraie et al., 2016).

March - April 2020
ISSN: 0193-4120 Page No. 1909 - 1916

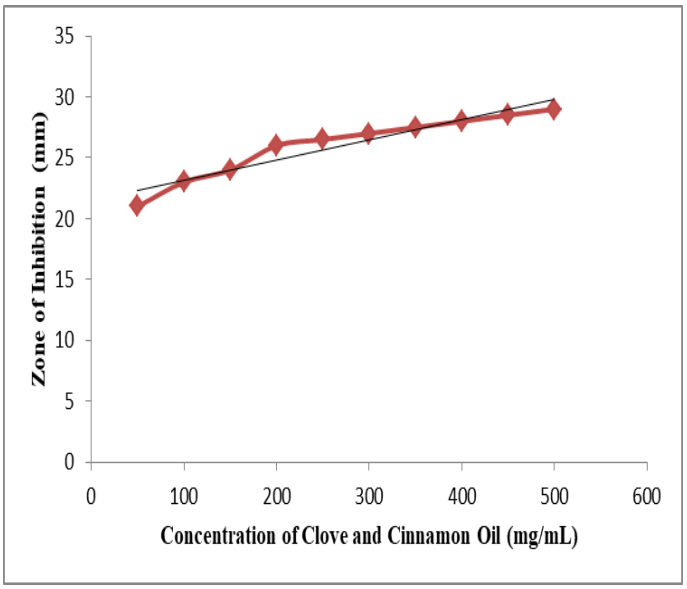

Fig 2: Synergistic effect of Clove and Cinnamon oil on *B. megaterium*

SEM Analysis of Bacteria:

For SEM analysis, *B. megaterium* grown on LB agar plates treated with and without essential oil at 28°C for 24 h. Sample was prepared for SEM visualization by primary fixation with a 2.5% glutaraldehyde solution for overnight at 4°C. Then, they were washed with 0.1 M sodium phosphate buffer solution (pH 7.2) three times for 20 min each and then the samples were dehydrated in an ethanol series (30%, 50%, 70%, and 95%) for 20 min in each alcohol dilution and finally with absolute ethanol for 45 min. Samples were then critical point dried in liquid carbon dioxide. The samples were mounted on silver stub and gold covered by cathodic spraying (Polaron gold). Morphology of bacteria was observed on a scanning electron microscope (Zeiss EVO 50) operating at 20.00 kv.

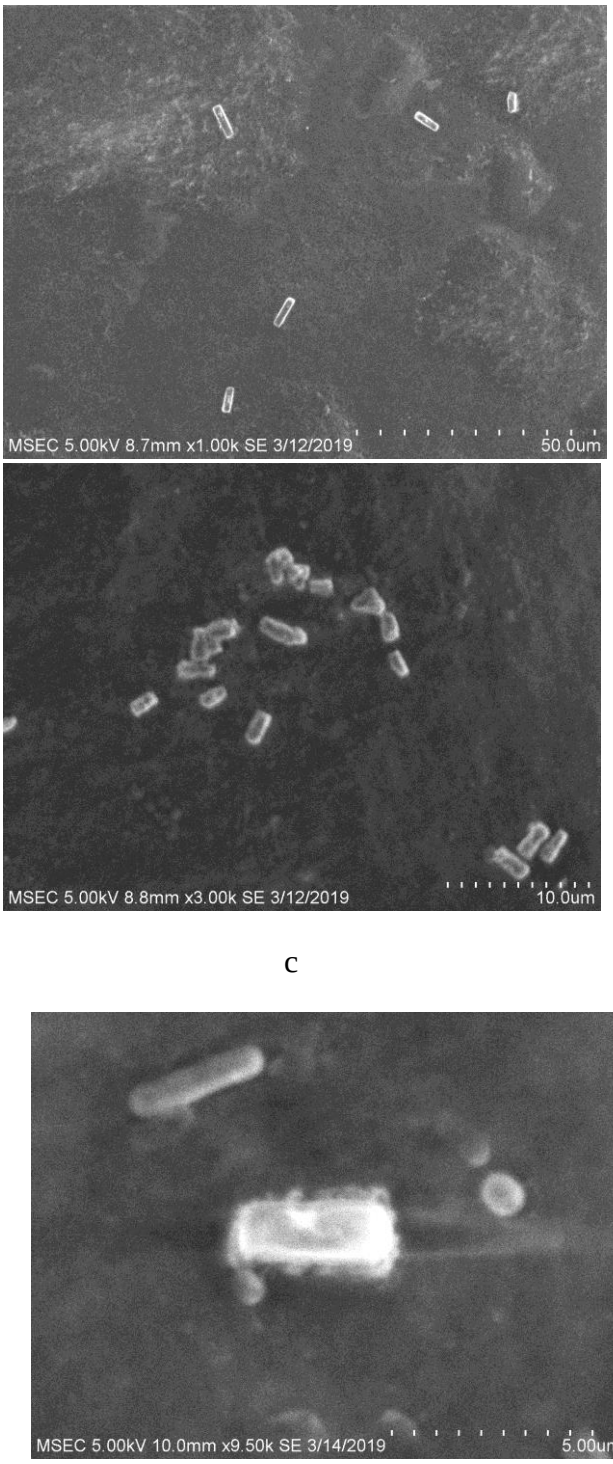

Fig 3 : SEM Images showing morphology of a) Untreated b) Clove oil treated c) Cinnamon oil treated. SEM image depicts the morphological changes is observed in the case of Clove and Cinnamon oil treated essential oils.

Conclusion:

March - April 2020
ISSN: 0193-4120 Page No. 1909 - 1916

It is believed that lipophilic nature of essential oils may facilitate in the penetration of lipid bilayer membrane and cause membrane disruption. Results obtained are in accordance with the previous studies wherein the clove oil and cinnamon oil or its major constituent, Eugenol presence or the cinnamon oil with its bioactive compound cinnamaldehyde, disintegrates the cell membrane causing a major alteration in cell permeability. It leads to the loss of cell constituents and subsequently results in cell death. Essential oils exhibit antibacterial effect in a dose dependent manner. In conclusion, experimental validation provides ample information in the use of essential oils as natural agents to control *B. megaterium* and its potential application as preservatives in food substances (Brul, 1999).

Conflict of Interest:

There is no conflict of interest.

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