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Antimicrobial Activity of plant extracts and essential oils.

D.RamaBramhaReddy[1] ,K.Malleswari[2], T.Satish[3], V.Srinivas[4], V.Vinod Kumar[5].

Principal And Professor[1],Associate Professor[2],Student[3], Student[4], Student[5].

Department Of Pharmaceutical Chemistry[1],

Nalanda Institute Of Pharmaceutical Sciences[1],Guntur,India.

Abstract :

Antimicrobial properties of some essential oils against some pathogenic microorganisms. Investigations were carried out to assess the efficiency of five plant essential oils: thyme, myrtle, laurel, sage, and orange oils as natural food preservatives. The effect of the plant essential oils against Escherichia coli, Listeria monocytogenes, Staphylococcus aureus and Candida albicans at concentrations of 5–20 µl/disk (diameter 6 mm) and 0.5–3% (v/v) was studied in agar diffusion test medium and milk medium. The essential oils of these extracts exhibited markedly antibacterial and bacteriostatic activity, with thyme showing the highest inhibition and orange the lowest. However, with thyme extract, high inhibitory activity was observed for all tested concentrations, L. monocytogenes showed less sensitivity towards essential oil extracts.

Keywords: Essential oils; Antimicrobial activity; Pathogens; Milk.

Introduction :

Plant essential oils (EOs) and extracts have been long known to have beneficial properties but have recently been investigated for their use as antimicrobials. Essential oils and plant extracts are derived from various types of plant material such as the flowers, seeds, bark, wood, buds, fruits, and roots. These oils and extracts are obtained through various methods such as fermentation, extraction, expression, or enfleurage. Their active components can also be produced synthetically for commercial use. They can be composed of over 60 different components with particular compounds comprising up to 85% of the oil or extract, whereas others are only present in very small amounts. Compounds found in EOs and extracts include phenols, polyphenols, terpenoids, flavonoids, flavones, flavonols, tannins, quinones, coumarins, alkaloids, lectins, and polypeptides . According to these compounds can be analyzed using techniques such as chromatography and mass spectrometry. For centuries, EOs and extracts have been used for their aromatic nature with applications in perfumes and as flavor enhancers. In addition, a number of EOs are currently in use for their preservative properties. For instance, "Protecta One" and "Protecta Two" are made in the US and are composed of an herbal blend of extracts suspended in solutions of sodium citrate and sodium chloride, respectively .

Antimicrobial Activity of Essential Oils :

In recent years there has been a growing interest in researching and developing new antimicrobial agents from various sources to combat microbial resistance. Therefore, greater attention has been paid to the screening of antimicrobial activity and its evaluation methods. Several bioassays such as well diffusion, disk-diffusion, and broth or agar dilution are well known and commonly used methods. The lowest concentration of antimicrobial agent that completely inhibits growth of the organism in micro-dilution wells or tubes as detected by the unaided eye is called minimum inhibitory concentration (MIC). The most appropriate bioassays for the determination of MIC value are these dilution methods, as these bioassays offer the possibility of estimating the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macro dilution or micro-dilution). The most common estimation of bactericidal activity is the determination of minimum bactericidal concentration (MBC) which is defined as the concentration killing 99.9% or more of the initial inoculums (1).

Essential Oils :

• **Plant from which essential Oil is Derived**	• **Micro-Organism Targeted**	• **Mechanism of Action**
• Allium sativum	• Escherichia coli	• Induced Leakage
• Litsea cubeba	• Escherichia coli	• Destruction of outer and innner membrane.
• Foeniculum vulgare	• Shigella dysenteriae	• Loss of membrane integrity
• Forsythia koreana	• Food borne and other pathogenic bacteria	• Loss of membrane integrity and increased permeameability
• Piper nigrum	• Escherichia coli	• Cell becomes pitted,shriveled and leakage of inter cellular material
• Cuminum cyminum • Dipterocarpus gracilis	• Bacillus cereus • Bacillus subtilis • Bacillus cereus • Proteus mirabilis • Escherichia coli	• Changes in cytoplasm • Changes in cytoplasm
• Ocimum gratissimum • Origanum vulgare	• Pseudomonas aeruginosa • Staphylococcus aereus • Staphylococcus aureus	• Disruption of cell membrane • Permeabilized Membrane

Essential Oils :

- Essential oils are compounds extracted from plants.The oils capture the plant's scent and flavor,or "essence."Unique aromatic compounds give each essential oil its characteristic essence.Essential oils are obtained through distillation (via steam and/or water) or mechanical methods, such as cold pressing .

- Once the aromatic chemicals have been extracted, they are combined with a carrier oils to create a product that's ready for use.The way the oils are made is important, as essential oils obtained through chemical processes are not considered true essential oils.

- Biological activity of essential oils from five Lamiaceae species, namely, Mentha piperita, Lavandula angustifolia, Mentha pulegium, Salvia lavandulifolia and Satureja montana was determined by Nikolíc et al. for their antimicrobial, cytotoxic properties and chemical composition.

- Pseudomonas aeruginosa, Streptococcus pyogenes, Streptococcus mutans, Streptococcus sanguis, Streptococcus salivarius, Enterecoccus feacalis and Lactobacillus acidophilus were the seven bacterial species, representing clinical specimens, along with fifty-eight clinical oral Candida spp. isolates with three reference strains used in the study. Satureja montana essential oil proved to be the most potent, and also significant antimicrobial activity was exhibited by all essential oils against all tested microorganisms (2) .

- In another study, the antibacterial activity of the essential oil from dried leaves of oregano (Origanum vulgare) that were fully formed and leaves and flowers of lavender (Lavandula officinalis) was reported. The lowest values of minimum inhibitory concentration were yielded by oregano essential oil against E. coli with an MIC value of 1600–1800 ppm, whereas the MIC value of lavender essential oil was 2000 ppm.

- On the other hand, MIC value of oregano essential oil was 800–900 ppm and the MIC value of lavender essential oil was 1000–1200 ppm against S. aureus. The higher content of phenolic compounds was reported to be the cause for this inhibition(3) .

- Aumeeruddy-Elalfi et al. evaluated the antimicrobial properties of essential oils against eighteen microorganisms (bacterial and fungal isolates) that were isolated from seven exotic and two endemic medicinal plants of Mauritius.

- Using the micro broth dilution assay, significant antibacterial activities were recorded with low minimal inhibitory concentration for eight essential oils except for Salvia officinalis, where the recorded activity was comparable with the activity of antibiotics (4).

Materials & methods

Bacterial cultures

The following Gram - and Gram þ non-pathogenic cultures were used for testing the antibacterial activity of the essential oils and their major components (5).

1. Gram negative bacteria (non-pathogenic)

E. coli NCIM – 2089; E. coli HB 101/pBR 322 – ampr, tetr; Salmonella typhimurium NCIM – 2501; Proteus vulgaris NCIM – 2027; Pseudomonas aeruginosa NCIM – 2036.

2. Gram positive bacteria (non-pathogenic)

- Staphylococcus aureus NCIM – 2079; Streptococcus faecalis NCIM – 2080; Bacillus subtilis NCIM – 2063; Pure cultures of the above bacteria were obtained from National Chemical Laboratory (NCL), Pune, except E. coli HB 101 from University of Hyderabad.
- Plant Pathogenic Bacteria: Xanthomonas oryzae and X. malvacearum were also used to study the antibacterial activity of essential oils and their major components. X. Malvacearum was obtained from NCL, Pune, while X. oryzae was isolated from blight infected leaves collected from Directorate of Rice Research (DRR), Hyderabad. All the bacterial cultures, except plant pathogenic bacteria X. oryzae and X. alvacearum, were maintained in nutrient broth at 37 C. Xanthomonas oryzae and X. malvacearum were maintained on Sucrose peptone medium at 28 C.

Fungal cultures

- The following fungal cultures were used to assess the antifungal activity of the essential oils and their major components.
- Aspergillus niger, Fusarium oxysporum, Fusarium udum, Magnaporthe grisea
- Magnoporthe grisea was isolated from infected blast leaves collected from DRR, Hyderabad. Other three fungal cultures were obtained from the Dept. of Botany, Osmania University, Hyderabad.
- The fungal cultures were maintained on PDA slants. In case of M. grisea the incubation was for 7 days at 28 C. The cultures were sub cultured three times before using for antifungal assays (6).

Plant essential oils

Seven essential oils were selected for the present study. These oils were extracted by steam distillation from Cymbopogon flexuosus (lemon grass-LG), C. martini (palm rosa-PR), Eucalyptus citridora (EC), Tagetus minuta (TM), Pelargonium sp (geranium- GE), C. winterianus (citronella-CI) and Mentha arvensis (MA). These essential oils contain terpenoids and their derivatives. All the essential oils used in the present study were kindly supplied by Central Institute for Medicinal and Aromatic Plants, Boduppal, Hyderabad.

Major components of essential oils

Citral, citronellal and geraniol were obtained from M/s Sigma Chemicals.

The concentration of essential oil/the components provided by manufacturers was determined by Semenova et al., and then the final concentration was brought to 10 mg/ml and then used for the experiments.

4. Components of Essential Oils with Antimicrobial Activity :

The major constituents of EOs can constitute up to 85%, whereas other components are present in trace amounts. α-phellandrene (36%) and limonene (31%) in Anethum graveolens leaf oil, d-limonene (over 80%) in citrus peel oils, α-phellandrene (36%) and limonene (31%) in Anethum graveolens leaf oil, carvacrol (30%) and thymol (27%) in Origanum compactum oil, α/β-thujone (57%) and camphor (24%) in Artemisia herba-alba oil, carvone (58%) and d-limonene (37%) in Anethum graveolens seed oil, and menthol (59%) and menthone (19%) in Mentha piperita oil are among the constituents present at relatively higher concentrations in essential oils. Generally, the biological properties Medicines of the essential oils are determined by their major components including two groups of distinct bio-synthetical origin. Terpenes and terpenoids comprise the main groups whereas aromatic and aliphatic constituents comprise the other group, all characterized by low molecular weight (7).

4.1. Terpenes and Terpenoids :

Several isoprene units (C5H8) upon combination result in the production of hydrocarbons called terpenes. Occurring in the cytoplasm of plant cells, biosynthesis of terpenes proceeds via the mevalonic acid pathway starting from acetyl-CoA. Having a backbone of hydrocarbons, cyclases can rearrange terpenes into cyclic structures, thus forming monocyclic or bicyclic structures. Terpene biosynthesis consists of synthesis of the isopentenyl diphosphate (IPP) precursor, IPPs being added repetitively to form the prenyldiphosphate precursor of the various classes of terpenes, terpene-specific synthetase modification of the allylic prenyldiphosphate to form the terpene skeleton, and finally, secondary enzymatic modification (redox reaction) of the skeleton to attribute functional properties to the different terpenes. Monoterpenes (C10H16) and sesquiterpene (C15H24) are the main terpenes, but longer chains such as diterpenes (C20H32), triterpenes (C30H40), etc., also exist. p-

cymene, limonene, menthol, eugenol, anethole, estragole, geraniol, thymol, γ-terpinene, and cinnamyl alcohol are among the examples of some constituents of essential oils with antimicrobial activity (Figure 1). Angelica, bergamot, lemongrass, mandarin, mint, caraway, celery, citronella, coriander, eucalyptus, geranium, petitgrain, pine, juniper, lavandin, lavander, lemon, orange, peppermint, rosemary, sage, and thyme are among the representatives of plants with some of these compounds. Oxygenated monoterpene (β-fenchol) and oxygenated sesquiterpene (α-eudesmol) were identified as the two main bioactive constituents in the essential oil obtained from fresh leaves of Eucalyptus teretecornis with a minimum inhibitory amount (MIA) of 28 µg and 10 µg against Alternaria alternata. Similarly, another study reported β-fenchol and linalool as the two antimicrobial components in essential oil obtained from the fresh leaves of Zanthoxylum alatum (8-9).

4.2. Phenylpropenes :

In plants, synthesis of phenylpropenes occurs from the amino acid precursor phenylalanine, constituting a subfamily among the various groups of organic compounds called phenylpropanoids. A relatively small proportion of essential oils is composed of phenylpropenes, and the phenylpropenes that have been most thoroughly studied are safrole, eugenol, isoeugenol, vanillin, and cinnamaldehyde. Eugenol, which is a clear to pale yellow oily liquid is extracted from clove oil, nutmeg, cinnamon, basil, and bay leaves. A study reported eugenol as the antifungal bioactive molecule from Cinnamomum tamala, with a minimum inhibitory amount of 9.5 and 8.2 µg against Alternaria alternata and Curvularia lunata, respectively. Eugenol has also been shown to cause deterioration of the cell wall, lysis of cells, and prevention of enzyme action in Enterobacter aerogenes. The antimicrobial activity of phenylpropenes is dependent on the selected microbial strains, the kind and number of substituents on the aromatic ring, and experimental parameters such as temperature and medium chosen for growth, etc. Cinnamaldehyde is a flavor- and odor-giving organic compound. Being a pale yellow viscous liquid, it occurs naturally in the bark of cinnamon trees and other species of the genus Cinnamomum. It is found as growth inhibitor of Escherichia coli and Salmonella typhimurium but does not disintegrate the outer membrane or deplete the intracellular ATP pool (10).

MATERIALS AND METHODS:

Agar dilution method :

The agar dilution method followed that approved by the NCCLS with the following modification: a final con-centration of 0·5% (v/v) Tween-20 (Sigma) was incorporated into the agar after autoclaving to enhance oil solubility. Brief- ly, a series of twofold dilutions of each oil, ranging from 2% (v/v) to 0·03% (v/v), was prepared in Mueller Hinton agar with 0·5% (v/v) Tween-20. Plates were dried at 35°C for 30min prior to inoculation with 1–2 ml spots containing approximately 104 cfu of each organism, using a multipoint replicator (Mast Laboratories Ltd, Liverpool, UK). Mueller Hinton agar, with 0·5% (v/v) Tween-20 but no oil, was used as a positive growth control. Inoculated plates were incubated at 35°C for 48h. Minimum inhibitory concentrations (MICs) were determined after 24h for the bacteria and after 48h for C. albicans. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate. The presence of one or two colonies was disregarded (11).

Broth microdilution method :

The broth microdilution assay was performed as described previously (Hammer etal. 1996) with the following modifications: MHB was used instead of heart infusion broth and,in tests with C. albicans, sub-cultures were performed after 48h incubation. For most oils, the highest concentration tested was 4·0% (v/v), although for some this was 8·0% (v/v). The lowest concentration tested was 0·008% (v/v).

organisms, inhibited at ¾2·0% (v/v) by 41 and 40 extracts, respectively. Table2 shows MICs and minimum cidal concentrations (MCCs) of 20 plant oils and extracts obtained by the broth microdilution method. Thyme had the lowest MIC of 0·03% (v/v) against C. albicans and E. coli, and vetiver had the lowest MIC of 0·008% (v/v) against Staph. aureus. Comparison of MICs obtained by agar and broth methods showed that differences exceeding two serial dilutions were seen withpeppermint, patchouli, sandalwood, and vetiver. The greatest

difference was for C. albicans and sandalwood, where the MIC obtained by agar dilution was 0·06% (v/v) compared with the MIC by broth microdilution of ×8·0% (v/v) (13).

All oils were diluted v/v in both agar and broth dilution methods.

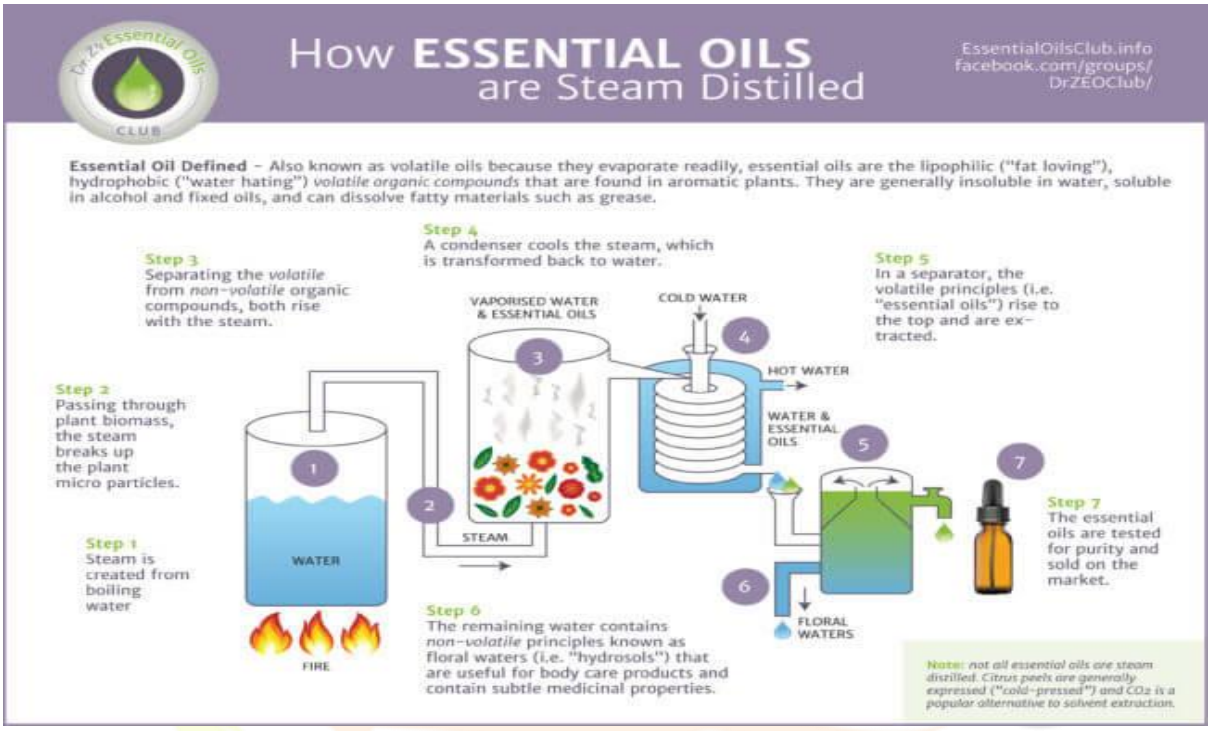

Figure 1:-Steam Distillation Of Essential Oils

Agar dilution method:

The agar dilution method followed that approved by the NCCLS with the following modification: a final concentration of 0·5% (v/v) Tween-20 (Sigma) was incorporated into the agar after autoclaving to enhance oil solubility. Briefly, a series of twofold dilutions of each oil, ranging from 2% (v/v) to 0·03% (v/v), was prepared in Mueller Hinton agar with 0·5% (v/v) Tween-20. Plates were dried at 35°C for 30min prior to inoculation with 1–2 ml spots containing approximately 104 cfu of each organism, using a multipoint replicator (Mast Laboratories Ltd, Liverpool, UK). Mueller Hinton agar, with 0·5% (v/v) Tween-20 but no oil, was used as a positive growth control. Inoculated plates were incubated at 35°C for 48h. Minimum inhibitory concentrations (MICs) were determined after 24h for the bacteria and after 48h for C. albicans. The MICs were determined as the lowest concentration of oil inhibiting the visible growth of each organism on the agar plate. The presence of one or two colonies was disregard (12).

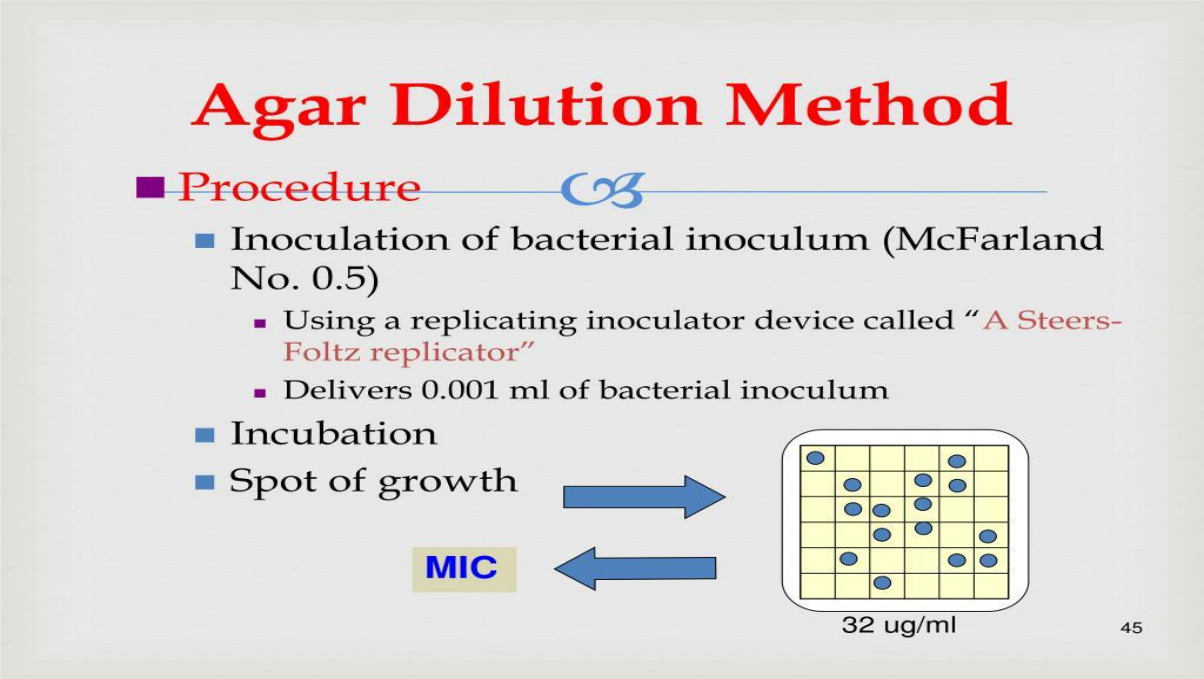

Figure 2:- Agar Dilution Method

Broth microdilution method:

The broth microdilution assay was performed as described previously (Hammer etal. 1996) with the following modifications: MHB was used instead of heart infusion broth and, in tests with C. albicans, sub-cultures were performed after 48h incubation. For most oils, the highest concentration tested was 4·0% (v/v), although for some this was 8·0% (v/v). The lowest concentration tested was 0·008% (v/v). The MICs of 52 plant oils and extracts obtained by the agar dilution method are shown in Table1. Lemongrass, oregano and bay inhibited all organisms at ¾2·0% (v/v). Rosewood, coriander, palmarosa, tea tree, niaouli, peppermint, spearmint, sage and marjoram inhibited all organisms except Psaeruginosa at ¾2·0% (12).

Several common plant extracts have demonstrated significant antimicrobial activity. Here are a few examples(13-17):

2.1.**Examples**

1. (Allium Sativum) Garlic:

➢ Garlic extract exhibits broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as some fungi and viruses. Allicin, a compound found in garlic, is responsible for its antimicrobial properties.

2. Tea tree oil (Melaleuca alternifolia) :

➢ Tea tree oil has strong antimicrobial properties, particularly against bacteria and fungi. It contains terpenes, such as terpinen-4-ol, which possess antimicrobial activity.

3. Cranberry (Vaccinium macrocarpon) :

➢ Cranberry extract is known for its ability to prevent urinary tract infections caused by bacteria. It contains proanthocyanidins, which inhibit the adhesion of bacteria to the urinary tract walls.

4. Eucalyptus (Eucalyptus spp.) :

➢ Eucalyptus extracts, particularly essential oils, have shown antimicrobial activity against various bacteria and fungi. Eucalyptol, a major component of eucalyptus oil, exhibits strong antimicrobial properties .

CONCLUSION:

Thyme, sage, myrtle, laurel, and orange essential oils have a potential to inhibit and inactivate four microorganisms in agar and milk medium at different concentrations. The inhibitor effects of essential oils increased with increasing concentration. It is suggested to investigate higher essential oils concentrations than were those used in research, and to study the effects over a longer time period in milk and other available milk products to access the potential of plant species essential oils as preservatives.

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