CHAPTER VI

PART - A : ANTIMICROBIAL SCREENING OF SOME INDIAN ESSENTIAL OILS

PART - B : EFFICACY OF ESSENTIAL OILS IN COMBINATIONS AGAINST SALMONELLA TYPHI.
Man has been depending on plants for his food, clothing, shelter and drugs from time to time. The last decade has witnessed a lot of research activity in the evolution of new antimicrobials. A large number of areas have been explored besides continuing investigations in known areas like sulphonamides, nitrofuran derivatives etc. About one third of all pharmaceuticals are derived from plants and over 60% of the pharmaceutical preparations are plant based. Side effects of drugs derived from plants are less than those of synthetic ones. Vane has pointed out that out of every 3000 chemicals synthesised only one is likely to be successful in its clinical trial.

India is known to possess about 2000 types of medicinal plants and those containing essential oils. Essential oils and their constituents find an amazingly wide and varied application in many industries, for flavouring of food, scenting beverages, drugs, paints, pharmaceuticals etc. The germicidal property of essential oils has been well known to mankind for a long time and these have been used as preserving agents. Scientific explanation of germ theory by Louis Pasteur further enhanced the interest to the understanding of infections, diseases and finding out their cures. Egyptians have made use of the embalming properties of essential oils since very early times.
Since the essential oils contain a variety of compounds, it is not possible to generalise their activity on any class of microorganisms. Essential oils can either 'static' if they inhibit the growth of the microorganisms or cidal if they kill them. Chamberland found that Anthrax bacilli contained in the blood were killed by the oil of vespetro in 18 hours, by the oil of angelica in 40 hours and by the oil of Ceylon cinnamon in 65 hours at 37°C. Later Cadea candoenier studied the bactericidal property of Ceylon cinnamon, wild thymol, geranium and clove oils against Salmonella typhi.

Starting from the close of the nineteenth century up to the beginning of the twentieth century, the antimicrobial activity of a number of essential oils has been investigated by Smith, Galti and Cayole, Dyche-Teague, Bryant, Kenkichi Saito, Khorana et al., Okazaki and Oshima, Lord and Husa, Maruzzella and Henry, Kliwe and Hathmacher and Tuckov.

Many constituents of the essential oils have also been studied thoroughly against many types of microorganisms. Gattabosse showed the useful utilisation of safrol for the diseases of foot and mouth. Smith and Khatoon studied many individual components of essential oils and found them useful as cough stimulants. Zetis experiments on menthol revealed its bacteriostatic effect on the species of Brucella. Thymol inhibits the wound bacterial growth even at the dilution 1:1000.
The curative value of essential oils in respiratory disorders of bacterial origin, anti-inflammation\textsuperscript{22,23}, to increase the volume output of the respiratory tract\textsuperscript{24,25}, for the treatment of Catarrhal disorders and antiseptic\textsuperscript{26} and relieving the tension of the stomach\textsuperscript{27} has been reviewed by many authors from time to time. Investigations\textsuperscript{28-31} of many essential oils of Indian origin revealed their possible utility as antimicrobial agents to combat the diseases caused by bacteria and fungi. Penfold and Grant\textsuperscript{32} and Rideal et al.\textsuperscript{33} studied the chemical and antimicrobial nature of many essential oils of Australian horizon. Similarly it has been found that vetiver, citronella, geranium, rose, sandalwood, neroli, lemon, bay, jasmine, anise, patchouli, ylang-ylang, oscimum, and many other oils exhibited antifungal activity. Several plant extracts\textsuperscript{34-37} have been shown possessing substances which may be fungicidal or fungistatic in nature.

Nigam and his co-workers\textsuperscript{38-47} have studied a large number of essential oils of Indian horizon for their antimicrobial activity against gram positive, gram negative and gram-variable bacteria and various classes of fungi including thermophilic fungi\textsuperscript{48} and dermatophytes with encouraging results.

Nigam and Banerjee\textsuperscript{49} have studied the anthelmintic properties of essential oils against tapeworms and have got encouraging results. The minimum doses of essential oils required to inhibit the growth of some organisms have also
been investigated. Monoterpenoids like geraniol, allocimene, citral etc. are well known for their antihistaminic activity.

In the present investigations five essential oils have been studied for their activity against human and plant pathogens at different concentrations. The details regarding selecting the micro-organisms, different media required for their growth, the type of techniques followed etc. are explained in the experimental part.

EXPERIMENTAL

Identification:

The authenticity of the plant materials and the micro-organisms\(^{50-52}\) has been established from the Botany Department of the University of Sagar, Sagar.

Isolation of Essential Oils:

The essential oils isolated from the leaves of *Skimmia laurisola*, *Cymbopogon flexuosus*, *Cinnamomum zeylanicum*, *Geranium* and *Eucalyptus citriodora* by water-steam distillation method were dried over anhydrous sodium sulphate and stored in amber coloured bottles at 5\(^{\circ}\)C. The dilutions were made in ethylene glycol since it did not show any activity as such.

Pathogenesis and toxicity of bacteria\(^{53}\) and fungi selected for antimicrobial screening of essential oils are as follows:
**Bacillus anthracis**: An infectious disease of wild and domesticated animals especially cattle and sheep that can be transmitted to man very easily causing Anthrax disease. It is characterized by black pustules.

**Salmonella paratyphi**: It is a natural pathogen of man causing enteric fever.

**Vibrio cholerae**: It is a well known human pathogen causing dreadful cholera.

**Xanthomonas malvacearum**: It causes rot disease on all parts of the plants of Malvaceae family.

**Keratinophytton terreum**\(^54\) It is a high level keratinophilic fungus destroying keratin of animals and human beings.

**Malbranchea pulchella var. Sulphurea**\(^55\): It is a highly cellulolytic form.

**Trychophytton mentagrophytes** syn. **T. interdigitale**\(^56,57\): It is a dermatophyte causing the well known ring worm disease.

For the perfect evaluation of antimicrobial activity, the following conditions\(^58\) must be fulfilled:

1. The substance to be evaluated must be brought in an intimate contact with the test organism against which the activity is to be studied.

For this purpose several methods\(^59-61\) as shown below have
been proposed from time to time.

I. Agar-streak dilution method.

II. Serial dilution method.

III. Agar-diffusion method.
   (a) Cup-plate method.
   (b) Strip diffusion method.
   (c) Replica plate method.
   (d) Paper disc method.
   (e) Cup-cylinder method.

IV. Special methods for measuring the activity of certain substances.

Out of these, the agar diffusion technique\textsuperscript{62} has been followed by using special microbial filter paper discs\textsuperscript{63}.

2. Favourable conditions must be provided for the optimum growth of the micro-organism in the absence of any antimicrobial agents other than those being studied.

Some of the important factors\textsuperscript{64} that affect the antimicrobial activity are as follows:

I. Composition of the medium and its suitability.

II. pH of the medium.

III. Authenticity of the culture.

IV. Density of the essential oil.

V. Stability of the oil at incubation temperature.

VI. Time of incubation required.

VII. Size of the inoculum.
3. There should be a suitable device to measure the antimicrobial activity of the substance.

In the present investigations the antimicrobial activity has been measured in terms of inhibitory zones appearing around the filter paper disc.

The following media\textsuperscript{65} have been used for the growth of bacteria and fungi respectively.

1. Peptone - beef extract nutrient agar medium with the following composition has been used in case of bacteria for preparing the slants and the agar plates.

\begin{align*}
\text{Peptone} & \quad 5.0 \text{ g.} \\
\text{Beef extract} & \quad 5.0 \text{ g.} \\
\text{Sodium chloride} & \quad 5.0 \text{ g.} \\
\text{Dextrose} & \quad 10.0 \text{ g.} \\
\text{Agar-agar} & \quad 20.0 \text{ g.} \\
\text{Distilled water} & \quad 1000 \text{ ml.}
\end{align*}

2. Sabouraud's dextrose agar medium with the following composition was utilized in case of fungi.

\begin{align*}
\text{Peptone} & \quad 10.0 \text{ g.} \\
\text{Dextrose} & \quad 40.0 \text{ g.} \\
\text{Agar-agar} & \quad 20.0 \text{ g.} \\
\text{Distilled water} & \quad 1000 \text{ ml.}
\end{align*}
Weighed quantities of the ingredients of the above media were mixed and sterilized by autoclaving at 15 lbs pressure for about 30 minutes and cooled to 40°C. Thereafter homogeneous suspension was prepared by transferring aseptically a few loopfuls of spores from the fresh subculture into the corresponding medium followed by a thorough shaking. 20-25 ml of this medium was poured into each petridish (10 cm diameter) which had already been sterilized at 140°C for about 30 hours and allowed to gel. After gelling, paper discs (6 mm) prepared from Whatman No. 1 filter paper and soaked well in different dilutions of the essential oils were placed and were incubated.

The seeded agar plates were incubated at 37°C for 24 hours in case of bacteria and 27-28°C for 4 days in case of fungi. As the experiments were conducted in duplicate, the average inhibition zones observed are recorded in the Table I to VII followed by graphical representations to assess the variations visually.
Table - I

Inhibitory Zones Obtained from Essential Oils (different % compositions) against *Vibrio cholerae* (mm*)

<table>
<thead>
<tr>
<th>Essential Oil</th>
<th>% composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Skimmia laureola</td>
<td>31</td>
</tr>
<tr>
<td>Cymbopogon flexuosus</td>
<td>29</td>
</tr>
<tr>
<td>Cinnamomum zeylanicus</td>
<td>21</td>
</tr>
<tr>
<td>Geranium</td>
<td>22</td>
</tr>
<tr>
<td>Eucalyptus citriodora</td>
<td>34</td>
</tr>
</tbody>
</table>

* includes the diameter of the paper disc (6 mm).
Table - II
Inhibitory Zones Obtained from Essential Oils (different % compositions)
against *Salmonella paratyphi* (**mm**)

<table>
<thead>
<tr>
<th>Essential Oil</th>
<th>% composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>Skimmia laureola</em></td>
<td>22</td>
</tr>
<tr>
<td><em>Cymbopogon flexuosus</em></td>
<td>32</td>
</tr>
<tr>
<td><em>Cinnamomum zeylanicum</em></td>
<td>26</td>
</tr>
<tr>
<td><em>Geranium</em></td>
<td>20</td>
</tr>
<tr>
<td><em>Eucalyptus citriodora</em></td>
<td>26</td>
</tr>
</tbody>
</table>

* includes the diameter of the paper disc (6 mm).
Table - III

Inhibitory Zones Obtained from Essential Oils (different % compositions) against *Bacillus anthracis* (mm²).

<table>
<thead>
<tr>
<th>Essential Oil</th>
<th>100</th>
<th>80</th>
<th>60</th>
<th>40</th>
<th>20</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Skimmia laureola</em></td>
<td>14</td>
<td>12</td>
<td>9</td>
<td>8</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><em>Cymbopogon flexuosus</em></td>
<td>20</td>
<td>18</td>
<td>14</td>
<td>12</td>
<td>9</td>
<td>R</td>
</tr>
<tr>
<td><em>Cinnamomum zeylanicum</em></td>
<td>21</td>
<td>19</td>
<td>16</td>
<td>15</td>
<td>13</td>
<td>R</td>
</tr>
<tr>
<td><em>Geranium</em></td>
<td>20</td>
<td>15</td>
<td>9</td>
<td>8</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><em>Eucalyptus citriodora</em></td>
<td>18</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>9</td>
<td>R</td>
</tr>
</tbody>
</table>

* includes the diameter of the paper disc (6 mm.).
Table - IV
Inhibitory Zones Obtained from Essential Oils (different % compositions) against *Xanthomonas malvacearum* (mm*)

<table>
<thead>
<tr>
<th>Essential Oil</th>
<th>% composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>Skimmia laureola</em></td>
<td>31</td>
</tr>
<tr>
<td><em>Cymbopogon flexuosus</em></td>
<td>29</td>
</tr>
<tr>
<td><em>Cinnamomum zeylanicum</em></td>
<td>21</td>
</tr>
<tr>
<td><em>Geranium</em></td>
<td>22</td>
</tr>
<tr>
<td><em>Eucalyptus citriodora</em></td>
<td>34</td>
</tr>
</tbody>
</table>

*includes the diameter of the paper disc (6 mm.).
Table - V

Inhibitory Zones obtained from Essential Oils (Different % Compositions) against *Trichophyton mentagrophytes* (mm*).

<table>
<thead>
<tr>
<th>Essential Oil</th>
<th>% Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>Skimmia laureola</em></td>
<td>32</td>
</tr>
<tr>
<td><em>Cymbopogon flexuosus</em></td>
<td>23</td>
</tr>
<tr>
<td><em>Cinnamomum zeylanicum</em></td>
<td>38</td>
</tr>
<tr>
<td><em>Geranium</em></td>
<td>34</td>
</tr>
<tr>
<td><em>Eucalyptus citriodora</em></td>
<td>29</td>
</tr>
</tbody>
</table>

* includes the diameter of the paper disc (6 mm).
Table VI

Inhibitory Zones Obtained from Essential Oils (different % composition) against Malbrenchea pulchella (mm*).

<table>
<thead>
<tr>
<th>Essential Oil</th>
<th>% composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Skimmia laureola</td>
<td>19</td>
</tr>
<tr>
<td>Cymbopogon flexuosus</td>
<td>22</td>
</tr>
<tr>
<td>Cinnamomum zeylanicum</td>
<td>24</td>
</tr>
<tr>
<td>Geranium</td>
<td>32</td>
</tr>
<tr>
<td>Eucalyptus citriodora</td>
<td>41</td>
</tr>
</tbody>
</table>

* includes the diameter of the paper disc (6 mm.).
Table - VII

Inhibitory Zones Obtained from Essential Oils (different % composition) against *Keratinophyton terreum* (mm*).

<table>
<thead>
<tr>
<th>Essential Oil</th>
<th>% composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>Skimmia laureola</em></td>
<td>20</td>
</tr>
<tr>
<td><em>Cymbopogon flexuosus</em></td>
<td>30</td>
</tr>
<tr>
<td><em>Cinnamomum zeylanicum</em></td>
<td>31</td>
</tr>
<tr>
<td><em>Geranium</em></td>
<td>40</td>
</tr>
<tr>
<td><em>Eucalyptus citriodora</em></td>
<td>33</td>
</tr>
</tbody>
</table>

* includes the diameter of the paper disc (6 mm).
Activity of essential oils (different concentrations) against Vibrio cholerae.

- Skimmia laureola
- Cymbopogon flexuosus
- Cinnamomum zeylanicum
- Geranium
- Eucalyptus citriodora
Activity of essential oils (different concentrations) against *Salmonella paratyphi*

1 - *Skimmia laureola*
2 - *Cymbopogon flexuosus*
3 - *Cinnamomum zeylanicum*
4 - *Geranium*
5 - *Eucalyptus citriodora*
Activity of essential oils against Bacillus anthracis
Activity of essential oils against *Xanthomonas malvacearum*.

**Different concentrations (%)**

<table>
<thead>
<tr>
<th>Inhibition zone</th>
<th>Skimmia laureola</th>
<th>Cymbopogon flexuosus</th>
<th>Cinnamomum zeylanicum</th>
<th>Geranium</th>
<th>Eucalyptus citriodora</th>
</tr>
</thead>
</table>

Graph showing the inhibition zone for different essential oils at varying concentrations.
Activity of essential oils against *Trichophyton mentagrophytes*.
Activity of essential oils (different concentrations) against

1 - Skimmia laureola
2 - Cymbopogon flexuosus
3 - Cinnamomum zeylanicum
4 - Geranium
5 - Eucalyptus citriodora

Keratinophyton terreum

Malbranchea pulchella

Inhibition zone
DISCUSSION

The zone of inhibition which is the indirect measure of the antimicrobial activity depends naturally on the ability of the oil to diffuse in the medium. The results show that almost all essential oils possess antibacterial property. It is interesting to note that the essential oil isolated from the leaves of *Eucalyptus citriodora* has shown good activity against *Xanthomonas malvacearum*, thereby suggesting the possibility of its application for combating the rot diseases caused by the organism to the plants belonging to the natural order Malvales.

The essential oils of *Cymbopogon flexuosus* and *Cinnamomum zeylanicum* have shown the possibility of checking the growth of the two human pathogens i.e., *Vibrio cholerae* and *Salmonella paratyphi*. All the essential oils tested have been found to be ineffective against *Bacillus anthracis*. The only resistant bacteria amongst those tested.

The antifungal activity of the essential oils has also been marked but all were not capable of inhibiting cent per cent growth of all the test organisms.

Essential oil isolated from the leaves of *Cinnamomum zeylanicum* has shown good activity against *Trichophyton mentagrophyte* even at different concentrations. None of the essential oils was equally effective on *Malbranchea pulchella* as the oil of *Eucalyptus citriodora* (leaves).
Similarly the oil of Geranium has been found highly active against *Keratinophyton terreum*.

**PART - B : STUDIES OF ESSENTIAL OILS IN COMBINATION AGAINST **

**SALMONELLA TYPHI.**

Marussella et al. 66 have reported the antimicrobial activity of some essential oils individually and in combination also.

In the present study sixteen essential oils from the leaves of *Cinnamomum zeylanicum*, *Citronella*, *Cymbopogon flexuosus*, Geranium, Lemon grass, *Palmarosa*, *Eucalyptus citriodora*, *Mentha arvensis*, *Mentha spicata*, *Mentha piperita*, *Skimmia laureola*, from the seeds of *Anethum sowa* and from the fruits of *Carum carvi*, *Cuminum cyminum*, *Trachyspermum ammi* syn. *Carum capticum*, *Trychospermum roxburghianum* syn. *Carum roxburghianum* have been obtained by steam and water distillation method and tested in different combinations for their activity against *Salmonella typhi* and compared with chloramphenicol as the reference antibiotic in nutrient agar medium by the agar filter paper disc diffusion technique as explained earlier.

*Salmonella typhi*: It causes typhoid fever in human beings. It contaminates the water and food stuffs to a large extent.
It attacks the chicks and sometimes leads to their death.

As the above isolated essential oils were reported active against *Salmonella typhi* by different research workers, the present author tried their combinations also.

**RESULTS**

All the mixtures contain equal volumes of all component essential oils. The weights of the essential oils of the two mixtures nos. 1 and 8 with zones of inhibition as 42 and 52 mm respectively (including the diameter of the paper disc 6 mm) compared favourably with the reference antibiotic chloramphenicol, with zone of inhibition 58 mm (including the diameter of the paper disc 6 mm), and could be used in its place.

**Mixture - 1:**

* *Mentha piperita* (0.1860g), *Eucalyptus citriodora* (0.1880g), *Cinnamomum zeylanicum* (0.2394g), *Trachyspermum ammi* (0.2228g), *Carum cervi* (0.2444g) and *Cuminum cyminum* (0.2214g) : 42 mm*.

**Mixture - 2:**

* *Mentha spicata*, *Cuminum cyminum*, *Anethum sowa* and *Trachyspermum roxburghianum* : 15 mm*.

* includes the diameter of the paper disc ( 6 mm ).
Mixture - 3:

Citronella, Palmarosa, Lemon grass and Geranium : 11 mm*.

Mixture - 4:

Trachyspermum ammi, Mentha piperita, Eucalyptus citriodora, Cinnamomum zeylanicum and Cuminum cyminum : 23 mm*.

Mixture - 5:

Mentha arvensis, Anethum sowa, Cinnamomum zeylanicum and Cymbopogon flexuosus : 9 mm*

Mixture - 6:

Eucalyptus citriodora, Cinnamomum zeylanicum, Mentha piperita, Carum cepaicum and Cuminum cyminum : 29 mm*.

Mixture - 7:

Skimmia laureola, Cinnamomum zeylanicum, Mentha piperita, Carum cepaicum and Cuminum cyminum : 17 mm*.

Mixture - 8:

Eucalyptus citriodora (0.1884g), Cinnamomum zeylanicum (0.2402g), Cuminum cyminum (0.2208g), Mentha arvensis (0.1928g), Mentha piperita (0.1860g) and Mentha spicata (0.1906g) : 52 mm*.

Mixture - 9:

Lemon grass, Geranium, Palmarosa, Citronella, Cinnamomum
zyylanicum, Eucalyptus citriodora, Mentha piperita, Carum cepatrum, Carum carvi and Cuminum cyminum: 16 mm*.

For reference antibiotic chloramphenicol: 58 mm*.

SUMMARY

Five essential oils have been screened for their antimicrobial activity against human and plant pathogens at different concentrations. The studies reveal the possible utility of these essential oils as antimicrobial agents to combat the diseases caused by the microbes tested.

The results obtained during the investigation of the efficacy of sixteen Indian essential oils in combinations against Salmonella typhi could be of use medicinally utilising some Indian medicinal and aromatic plants.

* includes the diameter of the paper disc (6 mm).
REFERENCES


