Anti-cancer and Anti-oxidant activity of essential oils of *Rosmarinus officinalis*, *Azadirachta indica*, *Syzygium aromaticum* and *Cymbopogon nardus*

Dr. K Sujatha and K Bala Sirisha

Abstract
Natural extracts from such as essential oils can help to prevent some side effects. Essential oils and their compounds have potential to be valuable resources in the production of new drugs useful against human diseases. The main purpose of using essential oils for this treatment is to prevent the side effects caused by other traditional therapies.

Our study was conducted to investigate the anticancer activity and total phenolic content of essential oils extracted from *Rosmarinus officinalis*, *Azadirachta indica*, *Syzygium aromaticum* and *Cymbopogon nardus*. The activity of these oils was tested on breast cancer cell line (MCF-7), leukemia cell line (K-562) and cervical cancer cell line (HeLa). The anti-cancer activity was determined using MTT-[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide] assay. The total phenolic content was determined by using FC (Folin-ciocalteu) assay.

Among the four essential oils investigated in the present study viz., Clove, Citronella, Rosemary and Neem essential oils, each exhibited the cytotoxic activities towards MCF-7, K-562 and HeLa human tumor cell lines. On the basis of our results, Clove oil showed greater degree of cytotoxic activity on the K-562 cell line, Citronella oil showed more cytotoxic activity on MCF-7 cell line and Neem oil showed higher cytotoxic activity on HeLa cell line. On the basis of result of F-C assay, Clove oil showed greater percentage of phenolic viz., 45%.

Keywords: Anticancer activity, *Azadirachta indica*, *Cymbopogon nardus* *Rosmarinus officinalis*

Introduction
Essential oils are known to be complex mixtures of monoterpenes, sesquiterpenes and volatile phenolics (Carson & Riley 1995) [3], as well as alcohols, aldehydes, ethers, hydrocarbons and ketones (Kalmba & Kunica 2003) [8]. Phenols have been credited as being the most active components with the broadest spectrum of antimicrobial activity followed by aldehydes, ketones and alcohols (Kalmba & Kunica 2003) [8]. Many of traditionally used plants have been scientifically evaluated with results yielding today’s valuable drugs such as aspirin, digitoxin, morphine and quinine (Butler, 2004) [2]. Essential oils are potential sources of novel antimicrobial compounds (Simic et al., 2004) [10] especially against bacterial pathogens. Essential oils and extracts have been used for many thousands of years in food preservation, pharmaceuticals, alternative medicine and natural therapies (Cimanga et al., 2002; Sylvestre et al., 2006) [5, 11]. Successful plant remedies and their preparations as medicinal treatments has been used for thousands of years in indigenous cultures around the world (Balunas & Kinghorn, 2005) [11]. Essential oils and their volatile constituents are used widely to prevent and treat human disease (Edris AE, 2007) [6]. Essential oils are common in plants that are used traditionally as medicinal treatments and currently are more systematically studied (Edris, 2007, Kalmba & Kunica, 2003; Lahlou, 2004) [6, 8, 9]. The level of activity is dependent on the combination and ratio of different components as opposed to quantity of the primary constituent (Kalmba & Kunica 2003; Houghton et al., 2007) [8, 7].

Essential oils used

**Clove oil**
Clove essential oil (*Syzygium aromaticum*) is rich in minerals such as iron, calcium, potassium, sodium and phosphorus and hydrochloric acid. Majority of people believe that it is useful in preventing and treating cancer. In homeopathic medicine credits clove oil with the ability to help treat cancer when combined with the right parts of Wormwood and Black Walnut Hulls (Fig:1).
Citronella oil
It is one of the essential oils obtained from the leaves & stems of different species of *Cymbopogon*. The united states environmental protection agency considers oil of citronella as a bio pesticide with a non-toxic mode of action. Research also shows that citronella oil has strong antifungal, anticancer properties and effective in calming barking dogs (Fig:2).

![Fig 1: Clove oil](image1)
![Fig 2: Citronella oil](image2)

Neem oil
Neem (*Azadirachta indica*) helps to improve the antioxidant levels which act against carcinogen. Neem produces an antioxidant compound glutathione which is a carcinogen detoxifying enzyme. Neem seed oil has oxygen radical absorbance capacity ranging from 350-500. By improving the immune capacity, the human cells get defended against cancer cells. Neem oil comprises of compounds polysaccharides and liminoids which can also treat tumour. It is found successful against lymphocytic leukemia (Fig:3).

![Fig 3: Neem oil](image3)
![Fig 4: Rosemary oil](image4)

Rosemary oil
*Rosmarinus officinalis* belongs to labiatae family. Rosemary oil is mostly extracted from the leaves. The main chemical components of Rosmary oil are α-pinene, bronel, β-pinene, camphor, bornyl acetate, camphene, 1,8-cineole and limonene. Rosemary oil has strong antioxidant which means it protects fats from being attacked by oxygen (Fig:4).

Cell Line
A cell line is a homogeneous population of cell, stable after successive mitoses, and in theory have an unlimited capacity for division. They are of daily use in research laboratories of biology. Their uses are easier than that of cells in primary culture provide a tool of choice for certain applications. They can be used to produce drugs with high added value, such as the interferon beta-1a. A line after a tumour is not necessarily tumorigenic (Table:1).

<table>
<thead>
<tr>
<th>Name of cell line</th>
<th>Meaning</th>
<th>Organism</th>
<th>Origin tissue</th>
<th>Target part</th>
<th>Characteristics</th>
</tr>
</thead>
</table>
| MCF-7             | Michigan Cancer Foundation-7 | Human | Mammary gland | Invasive breast ductal carcinoma | Primary tumor- Invasion breast ductal carcinoma.  
|                   |         |          |               |             | Origin of cells- pleural effusion  
|                   |         |          |               |             | Proliferative response of estrogens  
|                   |         |          |               |             | Presence of progestosterone receptors |
| HeLa              | Human epithelial carcinoma cell line | Human | Shortening of telomerase | Cervical cancer cells | It is a human epithelial cell line derived from cervical carcinoma  
|                   |         |          |               |             | It have been transformed by human papillomavirus 18 (HPV18)  
|                   |         |          |               |             | These cells are adherent cells meaning that they will stick to the cell culture flask  
|                   |         |          |               |             | The replication time is 23 hours. HeLa cells can easily contaminate other cell lines as its often difficult to control |
| K-562             | Kadsura kaemperols kainic acid | Human | Bone marrow | Lympho blast | They exhibit much less clumping that many other suspension lines in culture  
|                   |         |          |               |             | The K-562 cell line over expresses a 4.5-kilobase mRNA, which is thought to code for the Mr 170,000 membrane glycoprotein associated with multidrug resistance |

Essential oils contain non-structural phenolic compounds which act as antioxidants. Antioxidants are, in effect, sponges that soak up the free radicals in our system. By adding young living essential oils, we are better able to maintain our good health and reduce the risk of developing some cancer and even heart diseases and stroke (Table:2).

<table>
<thead>
<tr>
<th>Essential oil</th>
<th>Phenolic compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citronella oil</td>
<td>Geraniol, Citronellol, Limonene</td>
</tr>
<tr>
<td>Neem oil</td>
<td>Triglycerides, Triterpynoids</td>
</tr>
<tr>
<td>Rosemary oil</td>
<td>Alpha- pinene, β-pinene, borneal, bornyl acetate</td>
</tr>
<tr>
<td>Clove oil</td>
<td>Eugenol</td>
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</tbody>
</table>

Materials and Methods
Serum containers, 15ml centrifuge, 50ml Falcon, Glycerol, Minimal essential medium, Tryptsin- EDTA, Conical flasks, Culture plates, MTT- [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide] dye, DMSO- Dimethyl sulfoxide, Folin- Ciocalteu’s phenol reagent, Sodium bicarbonate. MITT Assay protocol by (Chen; 2011) and Folic- Ciocalteau Assay methodology.

Sub culturing procedure for cell lines
For attached cell lines
i. Check the cells for microbial contamination by microscopic examination  
ii. Check cells for attachment, then decant medium
i. Pour the medium in to a 15ml centrifugal vial
ii. Add 1-2ml of trypsin/EDTA and spread trypsin over cell layer.
iii. Incubate at 37C or at RT for 5mins to cell detach.
iv. Add medium from centrifuge vial and pipette gently to break up the clumps.
v. Once cells start to detach, tap flask gently and check the cells detachment using microscope.
vi. Preparing cells for cryopreservation, by pouring the cells medium culture flask in to a 15ml centrifuge tube.

- Centrifuge the cell suspension at 1500rpm for 5mins and discard supernatant.
- Resuspend the cells or add 3ml of fresh medium to the cell pellet and mix gently.

**For suspension cell lines**

i. Check the cells for microbial contamination by microscopic examination.
ii. Leaving approximately 5ml medium at the bottom of the flask, decant slowly the remaining medium into a waste container.
iii. Pour the medium into a 15ml centrifuge vial.
iv. Add 1-2ml of tyrpsin or EDTA and spread trypsin over cell layer.

- Incubate at 37C or at RT for 5mins to cell detach.
- Add medium from centrifuge vial and pipette gently to break up the clumps.

- Once cells start to detach, tap flask gently and check the cells detachment using microscope.
- Preparing cells for cryopreservation, by pouring the cells medium from culture flask into a 15ml centrifuge tube.
- Centrifuge the cell suspension at 1500rpm for 5mins and discard supernatant. Resuspend the cells or add 3ml of fresh medium to the cell pellet and mix gently.

**Glycerol stock preparation**

- **For 7.5 % stock** - Medium 4625.00 micro litres, Glycerol-375.00 micro litres, And add 1ml to 500 micro litres of cell suspension (Centrifuged above).

- **For 10 % stock** - Medium- 4500.00 micro litres, Glycerol-500.00 micro litres, And add 1ml to 500 micro litres of cell suspension (Centrifuged above). Add 1ml to 500 micro litres of cell suspension to the sub culturing culture flasks. (Medium and trypsin EDTA which is using for cell lines should be pre warmed at 37C).

**Activity of essential oils on human cancer cell lines**

- Take sub cultured human cancer cells viz., MCF7, HeLa and K562.
- Plate 500-10,000 human cancer cells viz. MCF7, Hela, K562, in 200µl media per well in a 96 well plate. Leave 8 wells empty for blank controls. Further steps are followed as per the protocol of (Chen; 2011) [4].
- Read optical density at 560nm and subtract background at 670nm. Optical density should be directly correlated with cell quantity.
- Draw a graph by taking concentration of clove oil in % on X- axis and % of inhibition on Y-axis.

**Total phenolic assay**

- Test tubes containing 500 micro litres of gallic acid [dilated 400-fold with DMSO] (5, 10, 15, 20, 25, 30 and 35 mcG/ml) were prepared.

- Test tubes containing 500 micro litres of four essential oils viz, Clove oil, Citronella oil, Rosemary oil and Neem oil, were prepared.
- 500micro litres of 10 % Folin - ciocalteu's phenol reagent (in DMSO) was added in to each test tube and mixed.
- After 20mins, a 350micro litres of 1M Na2CO3 solution was added in to the mixture.
- Incubation is done for 20mins at room temperature.
- The absorbance was determined at 750nm against the parallelly prepared blank (500micro litters of DMSO + 500micro litres of 10 % Folin - ciocalteu's phenol reagent + 350micro litres of 1M Na2CO3 solution).
- All samples were analyzed in triplicate.
- Standard graph of gallic acid was plotted by taking concentration on X-axis and O.D. values on Y-axis.
- The concentrations in standard graph, corresponding to O.D values of essential oils were determined.
- From the above determined concentrations, total phenolic content of oils were determined.

**Result & Discussion**

Essential oils are complex compounds, very compatible with human physiology, with a host of research-supported health benefits. Included in this study are essential oils derived from different plant sources and have played significant roles in traditional medicine sytems.

**Action of essential oils On HeLa cell line**

During this study work up with Essential oils (Citronella oil, Clove oil, Rosemary oil, neem oil) from 20 % to 1.25 % against the cancer cells [HeLa cell line] was undertaken. It was examined that higher concentration of clove oil 20 % is showing highest percentage of inhibition i.e., 32.8 %, Citronella Oil is showing highest Percentage of inhibition i.e., 386 %, Rosemary oil is showing highest percentage of inhibition i.e., 34.9 % and Neem oil is showing highest percent of inhibition i.e., 64.5 %. It is also observed that at lowest concentration of 1.25 % Clove oil is showing no inhibition of cells, Citronella Oil showing 3.57%, Rosemary oil showing 10.4 % and Neem oil showing 23.4%.

**Action of essential oils on breast cancer cells**

During this study while working with Essential oils (Citronella oil, Clove oil, Rosemary oil, Neem oil) from 20 % to 1.25 % against the cancer cells (MCF - 7 cell line), it was examined that a higher concentration of clove oil 20 % is showing highest Percentage of inhibition i.e., 38.01 % against the cancer cells [K - 562 cell line] it was examined that higher concentration of clove oil 20% is showing highest Percentage of inhibition of 764 %, Citronella oil is showing highest percentage of inhibition at 49.1%, Rosemary oil is showing highest percent of inhibition i.e., 38.01% and Neem oils is showing highest percentage of
inhibition i.e., 62.3%. It is also noticed that at lowest concentration of 1.25 % Clove oil showing lowest Percentage of inhibition of 48.2% on cells, Citronella oil showing 11.1%, Rosemary oil showing 13.44 % and Neem oil showing 21.3 %.

Calculation
% cell inhibition = 100-{(At-Ab)/(Ac-Ab)}x 100

IC50 = Ac-At x 100

Where as At = Absorbance value of test compound; Ab = Absorbance value of blank; Ac = Absorbance value of control.

(Activity of Essential oils on Human Cancer cell lines Tables & Graphs are below)

<table>
<thead>
<tr>
<th>Table 3: Activity of clove essential oil on human cancer cell line</th>
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<tbody>
<tr>
<td>Conc. of Clove oil in %</td>
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</tr>
<tr>
<td>1.25</td>
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<td>2.5</td>
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<tr>
<td>5</td>
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<thead>
<tr>
<th>Table 4: Activity of citronella essential oil on human cancer cell line</th>
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<tbody>
<tr>
<td>Conc. of Citronella oil in %</td>
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<td>1.25</td>
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<td>2.5</td>
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<th>Table 5: Activity of rosemary essential oil on human cancer cell line</th>
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<tr>
<td>Conc. of Rosemary oil in %</td>
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<td>2.5</td>
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<th>Table 6: Activity of neem essential oil on human cancer cell line</th>
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<tbody>
<tr>
<td>Conc. of Neem oil in %</td>
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<td>5</td>
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<td>10</td>
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<tr>
<td>20</td>
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</tbody>
</table>

% of inhibition of Clove oil on different cell lines

% of inhibition of Citronella oil on different cell lines

% of inhibition of Rosemary oil on different cell lines

% of inhibition of Neem oil on different cell lines
Anti oxidant activity and percentage of phenolic

**Calculation**

\[ T = \frac{CV \times 100}{M} \]

Where as, \( T \) = Total phenolic content of oil; \( C \) = Concentration correlation with standard and \( M \) = initial weight of oil taken. Here, 2ml of oil by volume represents 0.1mg by weight. So, 0.5 ml represents 0.025mg. Therefore, \( M = 0.025mg \). (Table: 3)

**Citronella oil**

From the above graph, \( C = 5mcg / ml \).

Total phenolic content, \( T = \frac{(5mcg/ml \times 500mcL) \times 100}{25mcg} = 10\% \)

**Rosemary oil**

From the below graph, \( C = 7.5 mcg / ml \).

Total phenolic content, \( T = \frac{(7.5 mcg / ml \times 500mcL) \times 100}{25mcg} = 15\% \)

**Neem oil**

From the above graph, \( C = 5 mcg / ml \).

Total phenolic content, \( T = \frac{(5 mcg / ml \times 500mcL) \times 100}{25mcg} = 10\% \)

**Table 3: Anti oxidant activity & % of phenolic**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Essential oil</th>
<th>O.D. at 750nm</th>
<th>Phenolic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Citronella</td>
<td>0.0845</td>
<td>10%</td>
</tr>
<tr>
<td>2</td>
<td>Neem</td>
<td>0.081</td>
<td>10%</td>
</tr>
<tr>
<td>3</td>
<td>Rosemary</td>
<td>0.103</td>
<td>15%</td>
</tr>
<tr>
<td>4</td>
<td>Clove</td>
<td>0.286</td>
<td>45%</td>
</tr>
</tbody>
</table>

Among the four essential oils investigated in the present study viz, Clove, Citronella, Rosemary and Neem essential oils, each exhibited the cytotoxic activities towards MCF-7, HeLa and K-562 human tumor cell lines. Essential oils were serially diluted in TWEEN 80 because of their highly volatile nature. On the basis of our results, Clove oil showed greater degree of cytotoxic activity on the K-562 cell line, Citronella oil showed more cytotoxic activity on MCF 7 cell line and Neem oil showed more cytotoxic activity on HeLa cell line.

Free radicals/reactive oxygen species are associated with many biological phenomena such as inflammation, ageing, and carcinogenesis. The antioxidant activity of polar extracts of essential oils are related to the content of phenolic compounds Constituents in essential oils have shown a variety of pharmacological activities for cancer chemoprevention and therapy in *in-vitro*, and *in-vivo* models.

Among the four essential oils investigated in the present study viz, Clove, Citronella, rosemary and neem essential oils, each oil was examined for their total phenolic content Folin ciocalteu assay was used to test the anti-oxidant activity. On the basis of our results, Clove oil showed greater percentage of phenolics viz., 45%.
Conclusion
Current study vindicated the bioactivity of certain essential oils viz, clove, citronells, rosemary and neem oils, human tumor cell lines to different extent. Further research can be done by studying the induction of apoptotic behaviour of these oils on the studied cancer cell lines by analysing the protein expression and cell viability. Westen blot, Flow cytometry and Quantitative analysis using Real time PCR for expression of tumour inducing proteins and their inhibition rate using these essentials oils can be highly advantageous. Thus, it is possible to observe that the essential oil analyzed in this study may be used as an alternative for food, cosmetics and medicine. In addition to their use for food and cosmetics, the potential of essential oils for the treatment of acne and cancer merits further exploration in the future.

References