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

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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 olis 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-Dimethlthiazol-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 (Kalemba & 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 (Kalemba & kunica 2003) [8]. Many of traditionally used plants have been scientifically evaluated with results yielding today's valuable drugs such as asprin, 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, 111]. 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) [1]. 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, kalemba & 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 (Kalemba & 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

Fig 2: Citronella oil

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

Fig 4: Rosemary oil

Rosemary oil

Rosmarinus officinalis belongs to labiatae family. Rosemary oil is mostly extracted from the leaves. The main chemical components of Rosmary oil are a-pinene, bronel, b-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 1: Some Human Cell Lines

Name of cell line	Meaning	Organism	Origin tissue	Target part	Characteristics
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 progesterone 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 kaempferols 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 membrance 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 2: Essential oil and their Phenolic compounds

Essential oil	Phenolic compounds
Citronella oil	Geraniol, Citronellol, Limonene
Neem oil	Triglycerides,Triterpynoids
Rosemary oil	Alpha- pinene, b-pinene, borneal, bornyl acetate
Clove oil	Eugenol.

Materials and Methods

Serum containers, 15ml centrifuge, 50ml Falcon, Glycerol, Minimal essential medium, Trypsin- 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) [4] 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

iii. Pour the medium in to a 15ml centrifugal vial
iv. Add 1-2ml of trypsin/EDTA and spread trypsin over cell layer.
v. Incubate at 37C or at RT for 5mins to cell detach.
vi. Add medium from centrifuge vial and pipette gently to break up the clumps.
vii. Once cells start to detach, tap flask gently and check the cells detachment using microscope.
viii. Preparing cells for cryopreservation, by pouring the cells medium culture flask in to a 15ml centrifuge tube.
ix. Centrifuge the cell suspension at 1500rpm for 5mins and discard supernatant.
x. 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.
v. Incubate at 37C or at RT for 5mins to cell detach.
vi. Add medium from centrifuge vial and pipette gently to break up the clumps.
vii. Once cells start to detach, tap flask gently and check the cells detachment using microscope.
viii. Preparing cells for cryopreservation, by pouring the cells medium from culture flask into a 15ml centrifuge tube.
ix. Centrifuge the cell suspension at 1500pm 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 liters 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 Na_2CO3 solution was added in to the mixture.
- Incubation is done for 20mins at room temperature.
- The absorbance was determined at 750nm against the parallely prepared blank (500micro litters of DMSO + 500micro liters of 10 % Folin - ciocalteu's phenol reagent + 350micro liters of 1M Na_2CO3 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 at a higher concentration of clove ot 20 % is showing highest Percentage of inhibition of 53.5 %.
Citronella oil is showing highest Percentage of inhibition of of 34.6%. Rosemary oil is showing pest percentage of inhibition i.e., 38.01 % and Neem oil is showing highest percentage of inhibition 62.3%. It is also noticed that at lowest concentration of 1.25 % Clove oil showing no inhibition of cells, Citronella oil showing 3.57%, Rosemary oil showing 8.5% and Neem oil showing 21.9 %.

Action of Essential oils on K-562 cell line

During this study while working with Essential oils (Citronella oil, Clove oil, Rosemary neem oil) from 20 % to 1.25 % against the canccr cells [K - 562cell line] it was examined that higher concentration of clove oil 20% is showing highest Percentage of inhibiuon 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 I00

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 3: Activity of clove essential oil on human cancer cell line

Conc. of Clove oil in %	MCF-7			HeLa			K562		
	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition%	IC 50
1.25	0	0%		0	0%		0.37	48.2%	
2.5	0.52	26.8%		0.62	12.16%		0.38	46.8%	
5	0.48	31.6%		0.60	14.9%		0.32	54.7%	
10	0.42	40.39%		0.45	36.1%		0.23	67.6%	
20	0.33	53.5%	46.5	0.47	32.8%	67.2	0.17	76.4%	23.6

Table 4: Activity of citronella essential oil on human cancer cell line

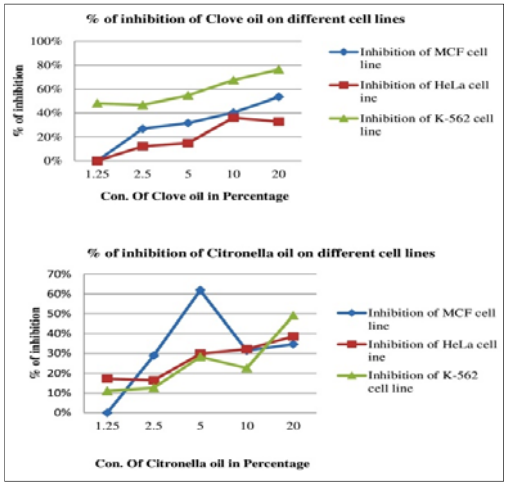
Conc. of Citronella oil in %	MCF-7			HeLa			K562		
	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition%	IC 50
1.25	0	0%		0.58	17.2%		0.62	11.1%	
2.5	0.50	28.9%		0.59	16.4%		0.61	12.6%	
5	0.26	61.9%		0.49	29.8%		0.50	28.3%	
10	0.48	31.4%		0.47	32.1%		0.54	22.6%	
20	0.46	34.6%	65.4	0.43	38.6%	61.4	0.35	49.1%	50.9

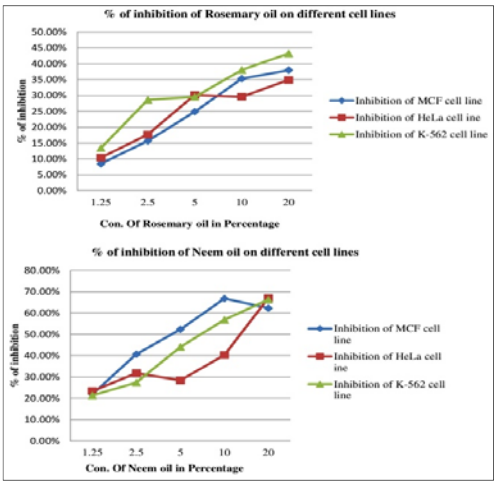
Table 5: Activity of rosemary essential oil on human cancer cell line

Conc. of Rosemary oil in %	MCF-7			HeLa			K562		
	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition%	IC 50
1.25	0.65	8.5%		0.63	10.4%		0.61	13.44%	
2.5	0.59	15.7%		0.58	17.7%		0.50	28.63%	
5	0.53	24.9%		0.49	30.2%		0.49	29.56%	
10	0.46	35.3%		0.50	29.6%		0.43	38.05%	
20	0.44	38.01%	33.7	0.45	34.9%	24.5	0.39	43.27%	43.4

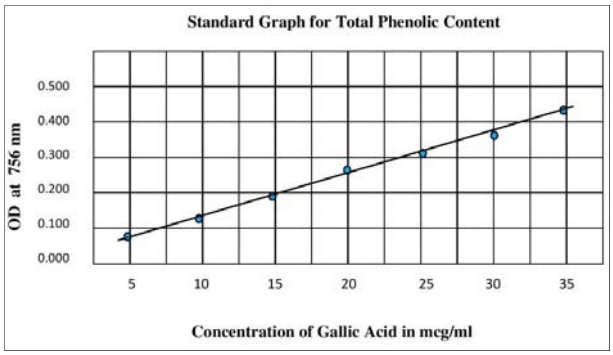
Table 6: Activity of neem essential oil on human cancer cell line

Conc. of Neem oil in %	MCF-7			HeLa			K562		
	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition%	IC 50	OD at 492nm	Growth Inhibition %	IC 50
1.25	0.55	21.9%		0.54	23.4%		0.55	21.3%	
2.5	0.42	40.7%		0.48	31.8%		0.51	27.4%	
5	0.34	52.3%		0.50	28.4%		0.39	44.1%	
10	0.23	66.8%		0.42	40.3%		0.30	56.8%	
20	0.27	62.3%	49.2	0.25	67.1%	37.6	0.23	66.3%	36.8





Anti oxidant activity and percentage of phenolic

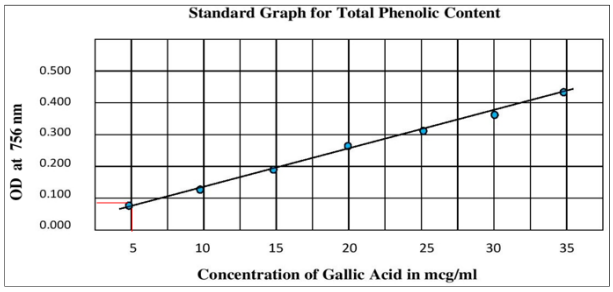


Calculation

$$T = \frac{CV \times 100}{M}$$

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

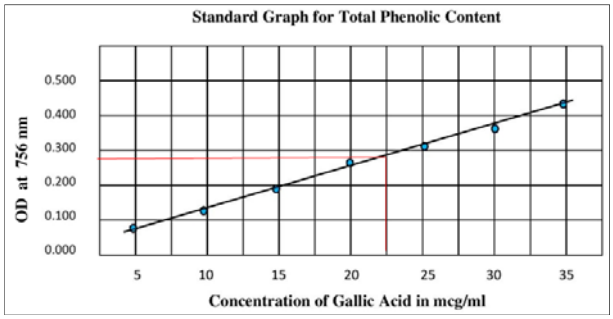


From the above graph, C = 5mcg / ml.

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

= 10%

Clove oil



From the above graph, C = 22.5 mcg / ml.

Total phenolic content, T = $\frac{(22.5 \text{ mcg / ml} \times 500mcL) \times 100}{25mcg}$

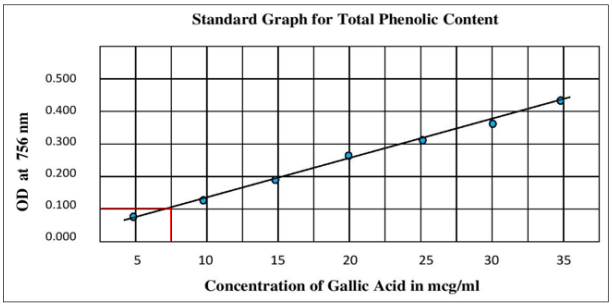
= 45%

Rosemary oil

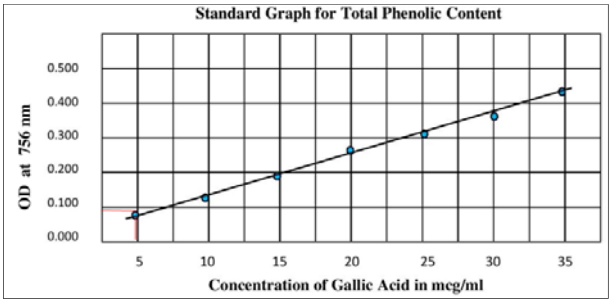
From the below graph, C = 7.5 mcg / ml.

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

= 15%



Neem oil



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

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

= 10%

Table 3: Anti oxidant activity & % of phenolic

S. No	Essential oil	O.D. at 750nm	Phenolic%
1	Citronella oil	0.0845	10%
2	Neem oil	0.081	10%
3	Rosemary oil	0.103	15%
4	Clove oil	0.286	45%

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, HeL 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 HcLa 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 vit. Clove, Citonella, rosemary and neem essential oils, cach 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.

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