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Original Article

Evaluation of anticancer activity of *Melaleuka Alternifolia* on Cervical cancer cell line (Hela) - an in-vitro study

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ABSTRACT

Introduction and aims Cervical cancer has become the third most common cancer among women and second most common cause of death. The current treatment modality has issues of drug resistance and side effects. Hence, investigation of plant species as a source of experimental therapeutic agents, in treating cancer is currently gaining a lot of importance. One such naturally available plant extract is *Melaleuka alternifolia* (TTO) which belongs to the family of essential oils is a very good antibacterial, antifungal, antiviral, antiprotozoal and anti-inflammatory agent. But currently there is a lot of importance is given for its anticancer effect. Hence our aim is to evaluate anticancer activity of *Melaleuka alternifolia* on cervical cancer cell line (HeLa) by MTT assay an in vitro method. **Methodology**: Before the start of the study ethical clearance was obtained from Institutional Review Board. The cytotoxicity checked for cervical cancer (Hela) cell line and Vero Monkey kidney cell line which was used as a control in our current study. These cell lines were procured from NCCS Pune, India. 1. MTT solution preparation (stock solution): 5 mg in 1 ml of PBS. 2. Cell culture : The cell lines were maintained in 96 wells micro titer plate containing MEM media supplemented with 10% heat inactivated fetal calf serum (FCS), containing 5% of mixture of Gentamicin (10ug), Penicillin (100 Units/ ml) and Streptomycin (100µg/ml) in presence of 5% CO2 at 37°C for 48-72 hours. 3. Cytotoxicity Assay: In vitro growth inhibition effect of test compound was assessed by calorimetric or spectrophotometric determination of conversion of MTT into Formazan blue by living cells. **Results** The results represent the mean of five readings. The IC50 value of tea tree oil for cervical cancer cell line after 48 hrs was **3.125µg/ml**. Spearmans rho's Correlation showed P value <0.05 indicating there was statistical significant results obtained when TTO was treated with HeLa cervical cancer cell line for 48 hrs incubation period. **Conclusion:** TTO has a promising anticancer property against cervical cancer cell line (HeLa) with its IC50 value **3.125µg/ml**. Hence this TTO with its greater efficacy related to its anticancer activity can be brought to the level of clinical trials in the coming future.

Keywords: Anticancer,Cytotoxicity,MTT assay,Tea tree oil.

1. INTRODUCTION

Breast and cervical cancers are the most common cancers in women worldwide. [1] In India, cervical cancer is a leading cancer among women with annual incidence of about

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1,30,000 cases and 70-75,000 deaths[1]. India shares about one fourth of the global cervical cancer burden.[2]

Conventional medicine, surgery, chemotherapy and radiotherapy are primary approaches for cancer treatment, but they are not always effective. But, for many years, traditional medicines have been applied for the treatment of cancers in the world. Herbal medicines are generally low in cost, plentiful, and show very little toxicity or side effects in clinical practice. [1]

One such naturally available plant extract derived from Australian native plant *Melaleuca alternifolia* i.e tea tree oil (TTO) , invented by Penfold[3] in the year 1920 has many medicinal properties. Hence the aim of the current study was to evaluate the anticancer activity of one such naturally available plant extract *Melaleuca alternifolia*,i.e. Tea tree oil (TTO) against Cervical (HeLa) cancer cell line and Vero cell line by in vitro method MTT Assay.

2. MATERIAL AND METHODS

Source of data:

Before the start of the study ethical clearance was obtained by Institutional Review Board. We evaluated the anticancer activity of M.Alternifolia (i,.e.TTO) on cervical cancer (Hela) and (Vero) Monkey kidney cell line which was used as contol. The cell lines were procured from NCCS National Centre For Cell Science, Pune, India. Then subjected for MTT Assay to assess the cell viability and cell cytotoxicity (Cell Lysis). We received commercially available Tea tree oil from Crystal aromatics New Delhi, imported from Australia with Refractive index 1.475, at 25^0 weight/ml was 0.8850gm/ml.

MTT Assay

1. MTT solution preparation (stock solution): 5 mg in 1 ml of PBS.

MTT (yellow dye) reduced by succinic dehydrogenase in the mitochondria of viable cells to purple formazan crystals.Formazan crystal production is directly proportional to the viable cells and inversely to the degree of cytotoxicity.When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells.

2. Cell culture :

The cell lines were maintained in 96 wells micro titer plate containing MEM media supplemented with 10% heat inactivated fetal calf serum (FCS), containing 5% of mixture of Gentamicin (10ug), Penicillin (100 Units/ ml) and Streptomycin (100µg/ml) in presence of 5% CO_2 at 37ºC for 48-72 hours.

3. Cytotoxicity Assay :

Invitro growth inhibition effect of test compound was assessed by calorimetric or spectrophotometric determination of conversion of MTT into " Formazan blue" by living cells. Remove the supernatant from the plate and add fresh MEM solution and treat with different concentrations of extract or compound appropriately diluted with DMSO. Control group contains only DMSO. In the current study, 10, 20, 25, 30 and 50ul of the stock solution (10mg / ml prepared in DMSO) were added to respective wells containing 100ul of the medium. So, the final concentrations were 10, 20, 25, 30 and 50ug / ml. That means to say the various concentration of TTO used to evaluate its anticancer activity were 100%, 50%, 25%, 12.5%,6.25%,3.125%, 1.562%, 0.781%, 0.390%, 0.195% respectively.

4. After 24hrs incubation at 37ºC in a humidified atmosphere of 5% Co2, stock solution of MTT was added to each well (20µl, 5mg per ml in sterile PBS) for further 4 hr incubation.
5. The supernatant carefully aspirated, the precipitated crystals of "Formazan blue' were solubilised by adding DMSO (100µl) and optical density was measured at wavelength of 570nm by using LISA plus.
6. The results represent the mean of five readings. The concentration at which the OD of treated cells was reduced by 50% with respect to the untreated control.

Formula :

Surviving cells (%) =

Mean OD of test compound/Mean OD at control ×100

Principle of assay:

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. DMSO, Isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.[4]

3. RESULTS

Table 1 shows the various concentrations of TTO, Mean OD noted for cervical cancer cell line with Cell viability and Cell Inhibition after incubation period of 48 hrs.

Table 1: The various concentrations of TTO, Mean OD noted for cervical (HeLa) cancer cell line with Cell viability and Cell Inhibition.

S.No.	Cell line	Concentration	Absorbance (nm)	% cell lysis	% of Cell Viablity
1	Hela	100			
	48hrs				
2		50			
3		25	0.321	36.43%	63.57%
4		12.5	0.340	38.59%	61.41%
5		6.25	0.381	43.24%	56.76%
6		3.125	0.450	51.07%	48.93%
7		1.562	0.491	55.73%	44.27%
8		0.781	0.526	59.70%	41%
9		0.390	0.591	67.08	32.92%

10		0.195	0.681	77.29	22.71%
control		00	0.881		

Table 2 shows the various concentrations of TTO, Mean OD noted for Vero Monkey kidney cell line with Cell viability and Cell Inhibition after incubation period of 24 hrs.

Table 2: The various concentrations of TTO, Mean OD noted for (Vero) Monkey Kidney cell line with Cell viability and Cell Inhibition.

S.No.	Cell line	Concentration	Absorbance (nm)	% Cell Lysis	% Cell viability
1	Vero	100	0.473	3.3	96.63%
	(Control)		0.445		
2		50	0.475	97.78	2.21%
			0.454		
3		25	0.476	102.42	The proliferation of cells noted from this concentration
			0.497		
4		12.5	0.506	107.47	
			0.515		
5		6.25	0.523	109.57	
			0.518		
6		3.125	0.553	113.47	
			0.525		
7		1.562	0.580	118.42	
			0.545		
8		0.781	0.618	122.94	
			0.550		
9		0.390	0.686	130.94	
			0.559		
10		0.195	1.149	184.10	
			0.600		
control		00	0.482	0.475	
		00	0.469		

Table 3 shows Spearman's rhos Correlation with P value <0.050 indicating statistical significant results when TTO was treated with Cervical (HeLa) cancer cell line and Vero cell line.

Table 3: Spearmans rho Correlation Coefficient Correlation with TTO.

Hela Cell Line	SPEARMANS CORRELATION COEFFICIENT	P VALUE	SIGNIFICANT
Concentration Vs Cell Viability	-1.000	<0.050	Significant
Concentration Vs Cell Inhibition	+1.000	<0.001	Significant

Fig 1 shows TTO treated with cervical (Hela) cancer Cell line after 48h showing cytotoxic effect with 50% cell lysis.

Fig 1: The Photograph showing TTO treated with cervical (Hela) cancer Cell line after 48h showing cytotoxic effect with 50% cell lysis.

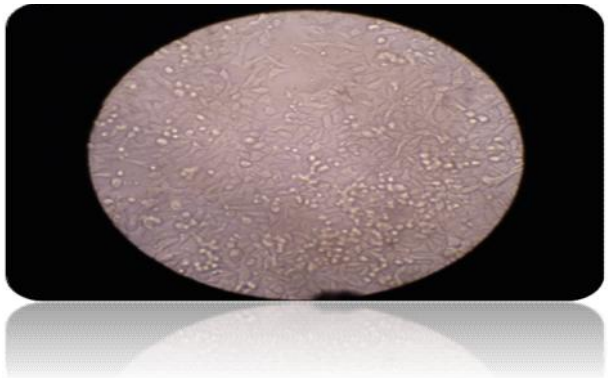
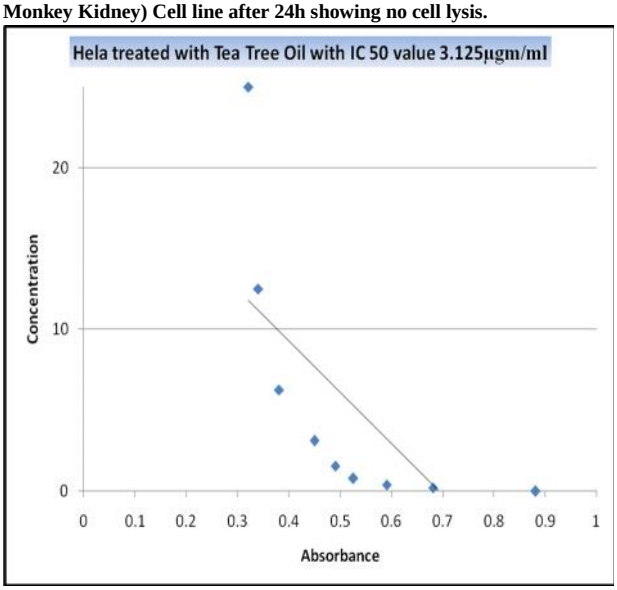
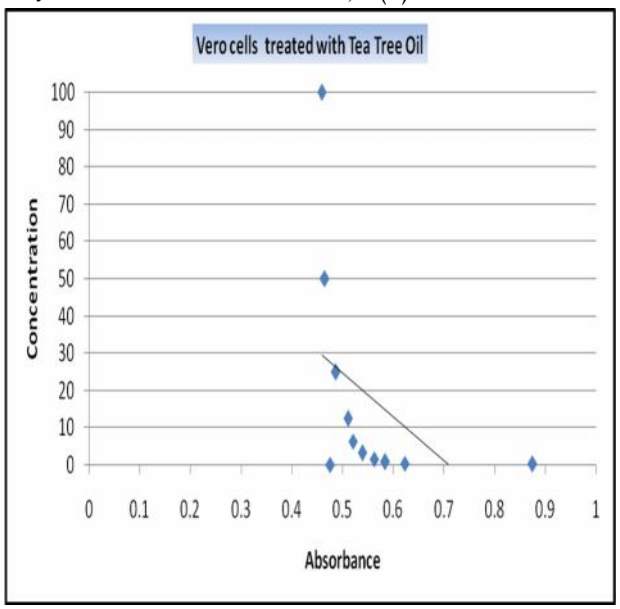


Fig 2.The Photograph showing TTO treated with VERO (Normal Monkey Kidney) Cell line after 24h showing no cell lysis.



Graph 1: Scatter plot showing TTO concentration, Cell death and viability for cervical (Hela) cancer cell line.

*IC_{50} Value of TTO for Cervical (Hela) Ca cell line after 48hrs is 3.125µg/ml.



Graph 2: Scatter plot showing TTO concentration, Cell death and viability for Monkey kidney (vero) cell line.

*At higher concentration of Tea tree oil 2-4 cells death were noted. As the concentration reduced to 50% in Serial dilution the excessive proliferation of cells noted. This indicates that higher concentration of TTO had minimal cytotoxic effect, so lower concentration can be used for anticancer activity.

4. DISCUSSION

Currently, a great deal of research is focusing on the investigation of certain plant species as a source of experimental therapeutic agents. Although it has been know from the literature that more than 60% of anticancer agents which are currently in use are derived from natural resources[5], but the toxic side effects generated and resistance to these and other synthetic drugs makes it necessary for the search of new novel agents. [5]

In our study we evaluated the anticancer activity of Tea tree oil by using in vitro method MTT assay. The cytotoxic effect was checked for HeLa and Vero cell lines after 48hrs and 24hrs respectively. IC_{50} Value of TTO for Cervical (Hela) Ca cell line after 48hrs obtained was 3.125µg/ml (Table 1, Graph 1and Fig 1). This indicates that higher concentration of TTO had great cytotoxic effect, so lower concentration can be sufficient to be used for anticancer activity. TTO had minimal cytotoxic effect on Vero cell line which was used as a control in our study than HeLa cell line. For Vero cell line, TTO at higher concentration showed 2-4 cells death. As the concentration reduced to 50% in Serial dilution there was excessive proliferation of cells noted (Table 2, Fig 2 and Graph 2). Spearman's rhos Correlation has shown statistical significant results when TTO was treated with Cervical (HeLa) cancer cell line and Vero cell line with P value <0.050 (Table 3).

Tea tree oil (TTO) which is an Australian native plant derivative belongs to the family of Myrtacea is an essential oil which is obtained through steam distillation process from *Melaleuca alternifolia,* [6] TTO has more than 100 components present in it, with terpinen-4-ol, γ-terpinene, α-terpinene, 1,8-cineole, and ρ-cymene are predominant. Terpinen-4-ol, being the most abundant and active component of the oil, which is responsible for the several *in vitro* and *in vivo* results reported for TTO [7, 8]. TTO has a wide range of mechanism of actions and it's a very good antibacterial[9,10], antifungal[11] , antiviral[12], and anti-inflammatory agent[13], currently lot of importance has been given for its anticancer activity[14-16]. The cytotoxicity of the TTO was tested on a wide panel of human cell cultures which includes cervical cancer (HeLa), acute lymphoblastic leukemia (MOLT-4), erythromyeloblastoid leukemia (K562), B cell derived from bone marrow of a patient with acute myeloid leukaemia (CTVR-1), fibroblast, and epithelial cells. The results of these studies showed an IC50 value of TTO on cell growth ranging from 20 to 2700 μg/mL[12,15,17,18].

The most potential anticancer activity of TTO was initially reported in a study conducted by Calcabrini and colleagues (2004) [19] in human melanoma M14 wild type cells and their drug-resistant counterparts, M14 adriamycin-resistant (ADR) cells.

TTO, at the higher concentrations (0.02 and 0.03%), as well as terpinen-4-ol, was able to inhibit the growth and induce caspase-dependent apoptotic cell death in both wild type and drug-resistant melanoma cells with the latter being more susceptible to the cytotoxic effect[16].

The earlier studies also confirm the cytotoxic effect of TTO on murine mesothelioma (AE17) and melanoma (B16) which was higher than "normal" epithelial and fibroblast cells, [20] . IC50 values obtained were slightly different, which is probably due to the different cell types [21] .

TTO and terpinen-4-ol might elicit its effect by inhibiting rapidly dividing cells more readily than that of slower growing noncancerous cells[21].More recently, the ability of TTO and its major component, terpinen-4-ol, has been also reported to interfere with the migration and invasion processes of drug-sensitive and drug-resistant melanoma cells[14]. Two recent studies investigated the efficacy of topical TTO on aggressive, subcutaneous, chemoresistant tumors in fully immune-competent mice[22,23]. A follow-up study investigated the mechanism of action of TTO reporting that topically applied 10% TTO induced a direct cytotoxicity on subcutaneous AE17 tumor cells[23]. However, in vivo findings were similar to previously suggested *in vitro* results[16,22].

Hence, terpinen-4-ol that is the likely to be the great mediator of an in vitro and in vivo efficacy of TTO [24]. Only one study has demonstrated in vitro anticancer efficacy of 1,8-cineole against two human leukaemia cells lines through apoptosis[25].

IC50 values of TTO reported for human cancer cell lines, HepG2 and HeLa were 0.002%-0.27% respectively[26] which was highly variable, depending on the type of cell examined. The observation that concentrations of 0.02% and 0.03% of

TTO which significantly reduce viability of M14 human melanoma cells after 48h [19] supports our present data.

Hence, anticancer activity tea tree against various cell lines has shown better results in each experimental study which provokes the thought that the cytotoxic effect was predominant on the excessive proliferating cells than normal cells. So further work need to be performed to the level of animal studies then human trials in the coming future.

5. CONCLUSION

Efficacies of TTO as an anticancer agent with its IC50 Value obtained from other studies were different and varying. This depends on the type of plant extract used, its concentration and method used. Hence, this IC_{50} value of TTO with its greater efficacy related to its anticancer activity can be brought to the level of clinical trials in the coming future.

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