



Evaluation of anticancer activity of *Melaleuca alternifolia* (i.e., tea tree oil) on colon cancer cell line (HT29) - An *in vitro* study

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Abstract

Introduction and Aim: Exploring naturally available plants as a source of new therapeutic agents, in treating the different types of cancer is currently gaining a lot of importance. One such naturally available plant extract derived from Australian native plant *Melaleuca alternifolia* (i.e., tea tree oil [TTO]) which belongs to the family of essential oils is a very good antibacterial, antifungal, antiviral, antiprotozoal, and anti-inflammatory agent. However, currently, a lot of importance is given for its anticancer effect. Hence, our aim is to evaluate anticancer activity of *M. alternifolia* on colon cancer cell line (HT29).

Materials and Methods: Before, we started our study ethical clearance was obtained from the institutional review board. The cytotoxicity checked for colon cancer (HT29) cell line with positive control being Cisplatin by an MTT assay. MTT is an *in vitro* method of analysis of cytotoxicity of TTO on colon cancer cell line. TTO was obtained from crystal aromatics, New Delhi, imported from Australia with refractive index of 1.475. These cell lines which were used in our study were procured from NCCS Pune, India. (1) MTT solution preparation (stock solution): 5 mg in 1 mL of PBS. (2) Cell culture: During culture, the cell lines were maintained in 96 wells microtiter plate which contains MEM media supplemented with 10% heat-inactivated fetal calf serum (FCS), containing 5% of mixture of gentamicin (10 µg), penicillin (100 units/mL), and streptomycin (100 µg/mL) in the presence of 5% CO₂ at 37°C for 48–72 h. (3) Cytotoxicity assay: Wherein, there is an *in vitro* growth inhibition effect of test compound which was assessed by calorimetric or spectrophotometric method where the yellow-colored MTT converted into purple-colored Formazan crystals by mitochondria in the living cells.

Results: Hence, in the results of the current study, mean of five readings was represented. The IC₅₀ value of TTO for colon cancer cell line after 24 h was 12.5 µg/mL. Spearman's rho's correlation showed $P < 0.060$ indicating that there were no significant results obtained when TTO was treated with HT29 colon cancer cell line.

Conclusion: TTO has a promising anticancer property against colon cancer cell line (HT29) with its IC₅₀ value of 12.5 µg/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.

Introduction

Naturally available essential oils are having an important role in the prevention and treatment of cancer.^[1] These essential oils with therapeutic potential can act by two ways - chemoprevention and cancer suppression.^[2] These naturally available essential oils which were also called as volatile

or ethereal oils are aromatic, highly volatile, and hydrophobic liquids which are produced by as secondary metabolites by aromatic plants. There are nearly about 3000 essential oils, of which about 300 are relevant for pharmaceutical, agronomic, food, cosmetic, and perfume industries. These aromatic plants which are sources of essential oils they mainly grow in Mediterranean and tropical countries.

^[3]The complex mixture of essential oils has a wide range of biological activities, which includes antiseptic, anti-inflammatory, spasmolytic, sedative, analgesic, and anesthetic properties. Currently, there is a lot of scientific reports focuses on the anticancer potential of essential oils which further helps to overcome the development of multidrug resistance and important side effects which are associated with the currently used anticancer drugs.^[3]

Many naturally available plant extracts are forming a greater platform for more than 60% of anticancer agents.^[4] One such naturally available plant derivative known from Australian native plant *M. alternifolia* (i.e., tea tree oil [TTO]) which is processed through steam distillation process belongs to family originated from Myrtaceae. It has more than 100 components in it. Prominent constituents of TTO are γ -terpinene, α -terpinene, terpinen-4-ol, 1,8-cineole, and ρ -cymene. Terpinen-4-ol, which is the most abundant and active component in the oil, which is responsible for the many *in vitro* and *in vivo* actions reported for TTO.^[3]

Various studies have evaluated cytotoxicity of TTO on cultured cells which were initially performed to determine its potential toxic effects. The cytotoxicity was performed on a wide panel of cancer cell line and human normal cell lines.^[5-9]

The cytotoxicity effect of many naturally available plant derivatives have effectively measured by an *in vitro* method in number of previous literature on colon cancer cell line.^[10-12]

However, there is no much information related to anticancer activity of TTO on colon cancer cell line. Hence, we are aiming at one such rare *in vitro* study to evaluate whether TTO has any anticancer activity on colon cancer cell (HT29) lines.

Materials and Methods

Source of data

Before the start of the study, ethical clearance was obtained by the institutional review board. We evaluated the anticancer activity of *M. alternifolia* (i.e., TTO) on colon cancer cell line (HT29). We received this commercially available TTO from crystal aromatics New Delhi, imported from Australia with refractive index 1.475, at 25° weight/mL was 0.8850 g/mL. The cell lines which were procured from NCCS National Centre for Cell Science, Pune, India. Cell viability and cell cytotoxicity (cell lysis) was assessed by subjecting the cell lines for MTT assay.

MTT assay

MTT solution preparation (stock solution): 5 mg in 1 mL of PBS MTT (yellow dye) which is reduced by succinic dehydrogenase present in the mitochondria of viable cells into purple-colored formazan crystals. More the purple-colored formazan crystal more the cells are viable and inversely proportional to the degree of cytotoxicity. Dead cells or cells which have undergone lysis do not have the capacity to change MTT into formazan crystals. So they appear lighter. Hence, this change in color shall serve as a great convenient marker of only the viable cells.

Cell culture

The cell line used for the study was HT29 colon cancer cell line (human procured from NCCS, Pune). The cell line then maintained in separate 96 wells microtiter plate which contains MEM media supplemented with 10% heat-inactivated FCS, containing 5% of mixture of gentamicin (10 μ g), penicillin (100 units/mL), and streptomycin (100 μ g/mL) in the presence of 5% CO₂ at 37°C for 48–72 h.

Cytotoxicity assay

The cytotoxicity of TTO determined by *in vitro* method, wherein there is conversion of yellow-colored MTT into purple-colored formazan crystals which was assessed by calorimetry or spectrophotometry.

Later, the supernatant was removed from the plate and add fresh MEM solution and treated with different concentrations of extract or compound appropriately diluted with DMSO. In the current study, 10, 20, 25, 30, and 50 μ L of the stock solution (10 mg/mL prepared in DMSO) were added to respective wells containing 100 μ L of the medium. Hence, the final concentrations were 10, 20, 25, 30, and 50 μ g/mL. That means to say the various concentrations of TTO used to evaluate its anticancer activity were 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562%, 0.781%, 0.390%, and 0.195%, respectively.

- A. After incubating at 37°C for 24 h in a humidified atmosphere of 5% CO₂, stock solution of MTT was added to each well (20 μ L, 5 mg/mL in sterile PBS) for further 4 h incubation.
- B. The supernatant carefully aspirated, the precipitated crystals of “formazan blue” were solubilized by adding DMSO (100 μ L), and optical density was measured at wavelength of 570 nm using LISA plus.
 1. 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:

$$\text{Surviving cells (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control}} \times 100$$

Principle of assay

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-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, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g., DMSO and isopropanol) and the released; solubilized formazan reagent is measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.^[13]

Results

Table 1 shows the various concentrations of TTO, mean OD noted for colon cancer cell line with cell viability and cell inhibition after incubation period of 24 h.

Table 2 shows Spearman's rho's correlation with $P < 0.001$ indicating statistically significant results when TTO was treated with colon cancer cell line (HT29) cell line.

Figure 1 shows TTO treated with colon cancer HT29 cell line after 48 h showing cytotoxic effect with 50% cell lysis.

Discussion

The cytotoxicity of TTO on colon cancer cell line was conducted by MTT assay. Table 1, Graph 1, and Figure 1 shows that cytotoxic effect of TTO on colon cancer cell line (HT29). The IC_{50} value obtained after treating TTO for 24hr incubation is 12.5 $\mu\text{g/mL}$. The Spearman's rho correlation coefficient correlation with TTO was not having statistically significant result with $P < 0.060$ [Table 2].

Most of the essential oils are extracted through steam distillation. Whereas, TTO, i.e., *M. alternifolia*^[14] is the type of an essential oil which belongs to Myrtaceae family, which is a plant native from Australia. Anciently, this oil was used by aboriginal Australian soldiers for insect bites and many other skin infections and later its topical antiseptic effects were rediscovered in the 1920s. The safety/toxicity associated with topical uses of the essential oil for skin and wound care has been

Table 1: The various concentrations of TTO, mean OD noted for colon cancer cell line with cell viability and cell inhibition

S. No.	Cell line	Concentration	Absorbance (nm)	% cell lysis	% cell viability
1	HT29 for 48 h	100	0.485	100	0
2		50	0.323	67	33
3		25	0.217	45	55
4	* IC_{50}	12.5	0.266	55	45
5		6.25	0.215	44.69	55.31
6		3.125	0.212	44.07	55.93
7		1.562	0.185	38.46	61.54
8		0.781	0.176	36.59	63.41
9		0.390	0.111	23.00	77
10		0.195	0.103	21.41	78.59
Control		00	0.481		

* IC_{50} value for TTO for colon cancer cell line (HT 29) after 24 hrs is 12.5 $\mu\text{g/mL}$.

Table 2: Spearman's rho correlation coefficient correlation with TTO

HEP2 cell line	Spearman's correlation coefficient	P value	Significant
Concentration versus cell viability	-0.582	<0.060	Not significant
Concentration versus cell inhibition	+0.582	<0.001	Significant

TTO: Tea tree oil

rigorously examined.^[15] The large amount of monoterpenes which are present in TTO in which half are oxygenated and the rest are hydrocarbons.^[16]

International Organization for Standardization, ISO 4730: 2000 has done the standardization of 14 components of the oil.^[15] The results obtained from many in vitro and in vivo method studies are basically because of terpinen-4-ol, which is the main ingredient present in TTO.^[17] TTO has wide range of mechanism of actions. It is known for its antibacterial,^[18,19] antifungal,^[5] antiviral,^[6] and anti-inflammatory properties,^[7] but recently, its anticancer activities are given more importance.^[8,20,21]

To study the anticancer effect of TTO, the cytotoxicity was checked initially on cultured cells to determine its potential toxic effects. The cytotoxicity of TTO was tested on a wide range 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 leukemia (CTVR-1), and for normal fibroblast, and epithelial cells.

Hence, all these studies conducted on TTO have showed an IC_{50} value which ranged as 20–2700 $\mu\text{L/mL}$.^[6,9,20,22] This IC_{50} value was higher than our current study which could be due to variation in type of TTO, cell line and the method followed. Apart from this, the potential anticancer activity of TTO which was reported in a study conducted by Calcabrini *et al.* in human melanoma M14 wild-type cells and their drug-resistant counterparts, M14 adriamycin-resistant cells. The higher concentrations of TTO (0.02% and 0.03%), as well as terpinen-4-ol, which inhibit the growth and further induce caspase-dependent apoptotic cell death in both wild-type and drug-resistant melanoma cells.

Drug-resistant melanoma cells were more susceptible to the cytotoxic effect^[9] due to terpinen-4-ol present in TTO than other cells. The different lipid composition of the plasma membrane could be attributed to the greater sensitivity of drug-resistant cells to the TTO treatment.^[23,24]

The cytotoxicity of TTO can be decided on the basis of lipophilicity with cell membrane leading to alteration in cell growth and activity. It was also noted that there was no cytotoxic effect of topical TTO agents on "normal" epithelial cells, fibroblast cells, and basal keratinocytes.^[21,25] Hence, it confirms that TTO having its effect on rapidly proliferating, highly sensitive tumor cells when compared to normal cells.

IC_{50} value of TTO in murine mesothelioma (AE17) and melanoma (B16) cell lines was slightly different which was probably due to the different cell types.^[26] Hence, it confirms that TTO might obtain its effect by inhibiting rapidly dividing cells more readily than slower growing non-cancerous cells.^[27] It is also noted that drug-sensitive and drug-resistant melanoma cells were mainly interfered with the migration and invasion processes by TTO and its major component, terpinen-4-ol.^[8]

Other studies which investigated the efficacy of topical TTO on aggressive, subcutaneous, and chemoresistant tumors in fully immune-competent mice.^[3,27] The other studies have shown that topical treatment with 10% TTO, which was given once a day for 4 consecutive days, induced a significant, regression of established

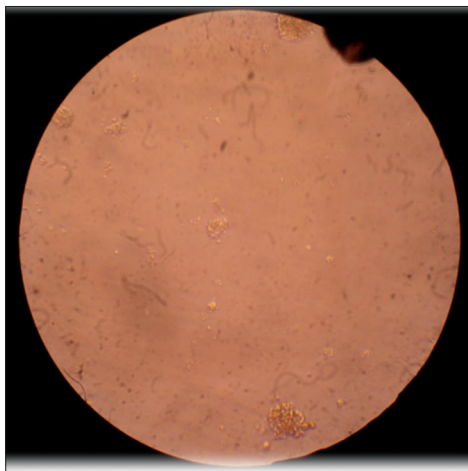
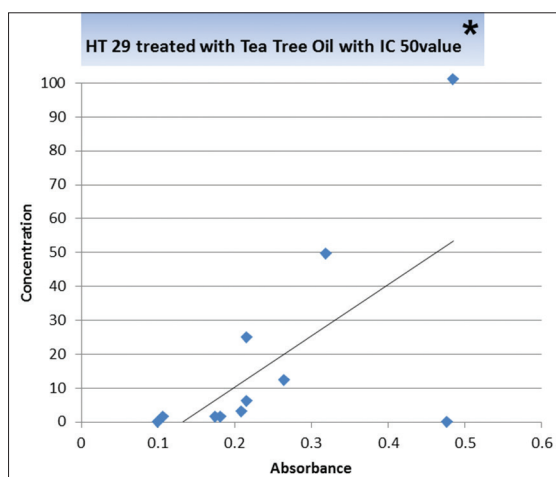


Figure 1: Tea tree oil treated with colon cancer HT29 cell line after 48 h showing cytotoxic effect with 50% cell lysis



Graph 1: Scatter plot showing tea tree oil (TTO) concentration, cell death, and viability for colon cancer cell line. *IC₅₀ value of TTO for colon (HT29) Ca cell line after 24 h is 12.5 µg/mL

subcutaneous AE17 tumors and slowed the growth of B16-F10 tumors. A penetration enhancer DMSO was necessary to induce the antitumor effect; no effects were evident when using TTO or solvents other than DMSO (i.e., isopropanol or acetone).

Similar effects on tumor growth were also obtained using a combination of five main ingredients of TTO (terpinen-4-ol, γ-terpinene, α-terpinene, 1,8-cineole, and β-cymene) in the doses to those found in 10% TTO.^[26]

Anticancer effect related to topical TTO was accompanied by skin irritation that which got resolved quickly and completely. A follow-up study investigated the mechanism of action of topically applied 10% TTO which reported that a direct cytotoxic effect was noted on subcutaneous AE17 tumor cells, which was associated with non-tumor-specific activation of local immune response (i.e., neutrophils, dendrite, and T

cells).^[3] The transmission electron microscopy analysis of AE17 tumor sections from mice treated topically with TTO revealed that following *in vivo* TTO treatment, tumor cells undergo primary necrosis as originally suggested *in vitro* studies.^[9,27]

Conclusion

AS far as potential anticancer activity of essential oils is concerned, still there is no much information available. Hence, lot of research needs to be progressed in this regard. Efficacy of TTO as an anticancer agent with its IC₅₀ value in our study has shown better results than the other naturally available plant extracts from the previous literature. This variation in the result could be due to differences in the method and concentrations used from the previous studies. Hence, this IC₅₀ 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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