Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating: 3.53
JMPS 2018; 6(5): 01-06

Received: 01-07-2018
Accepted: 02-08-2018

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Evaluation of anticancer activity of *Melaleuca alternifolia*. (i. e. tea tree oil) on Leukemia cancer cell line (K562): An *in vitro* study

Sujata Byahatti, Chetana Bogar, Kishore Bhat and Girish Dandagi

Abstract
Introduction and aims: Among various types of cancer, leukaemia is one of the most common types of cancer worldwide. Although the current treatment modality has various issues of drug resistance and side effects. Many anticancer drugs currently used clinically have been isolated from plant species. Studies suggested that herbal medicines have a great potential in combating leukaemia. However, the use of therapeutic herbs in developing countries as a curative agent against leukaemia is predominantly associated. 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 *Melaleuca 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 has been given for its anticancer effect. Hence our aim is to evaluate anticancer activity of *Melaleuca alternifolia* on Leukemia cancer cell line (K562) 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 Leukemia cancer (K562) cell line. The 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 ºC 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 IC_{50} value of tea tree oil for Leukemia cancer (K562) 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 Leukemia cancer (K562) cell line for 48 hrs incubation period.
Conclusion: TTO has a promising anticancer property against Leukemia cancer (K562) cell line with its IC_{50} 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: Cytotoxicity, MTT assay, Tea tree oil, K562

Introduction
More than 95% of leukemia's are in acute form which constitute the most common diagnostic group of childhood cancers in India and worldwide [1, 2] GLOBOCAN has reported that there are nearly 25,000 children who are diagnosed with cancer in India every year and around 9000 of these have leukemia. With this we can even estimate, that there would be 90,000 children who can develop with leukemia in a decade in India [3]. In the world the highest incidence of leukemia (29% incidence per 100,000 individuals) noted in Denmark [4] The diagnosis of leukemia is 10 times higher in adults as compared to children and often noted in males as compared to females [5] According to the literature, nearly 2, 6,000 children and adults around the world developed some form of leukemia in the year 2000 and 2,09,000 had lost their lives due to this condition [6] The treatment includes a combination of chemotherapy, antibiotic, blood transfusion, radiation therapy, and bone marrow transplantation. Although some of these treatments are found difficulty in handling, but have definitely increased the survival rate of these patients [7]
Hence currently a great source of scientific research focusing mainly on naturally available plant derivatives. India being traditionally rich in many sources of plant extracts, the use of

therapeutic herbs in developing countries as cures against leukemia is quite predominant [8].

When compared to synthetic treatment modality, medicinal herbs have symbolized safety in many ways [9] Hence, they play an important role in the treatment of cancer [10] It has been found that many limitations were noted in number of *in vitro* studies wherein direct cytotoxicity was performed. Various plant species have been studied for their anticancer properties, and it is known fact that approximately 40%–50% of the drugs which are currently available in the market today are either derivative from natural resources or they are natural products themselves. Hence, these naturally available plant extracts provide a greater source for therapy and solid base for synthetic drugs [11]

Hence, keeping theses hypothesis in mind we aim to evaluate the anticancer activity of one such naturally available plant extract *Melaleuca alternifolia*, i.e. Tea tree oil (TTO) against leukemia cancer (K562) cell line by *in vitro* method using MTT Assay.

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 Leukemia cancer cell line (K562). We received this 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. The cell lines which 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).

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 line used for the study were K562 (human procured from NCCS, Pune).The cell line then 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 37 ºC 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. Remove the supernatant from the plate and add fresh MEM solution and treat with different concentrations of extract or compound appropriately diluted with 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 37 ºC 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:

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

Principle of assay

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-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 [12]

Results

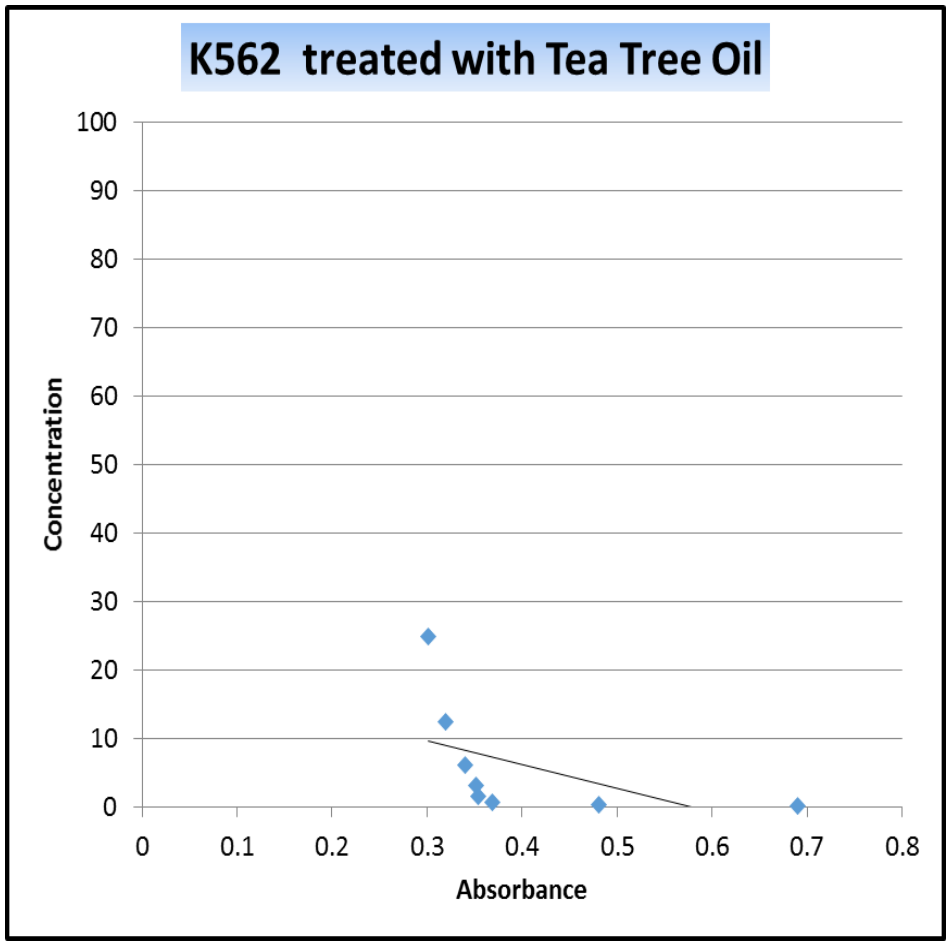
Table 1 explains about cytotoxic effect TTO at concentrations (100,50,25,12.5,6.25,3.125,1.562,0.781,0.390,0.195) in serial dilution. 50% of cell inhibition noted at the concentration of 3.125µg/ml. (Table 1 Graph 1 and Fig 1). Table 2 shows the Spearmans Rhos correlation showed the statistical significant results with p-value <0.05. Graph 1. Scatter plot shows TTO concentration, Cell death and viability for K562 cell line with IC_{50} value of 3.125µg/ml. Fig 1 shows TTO treated with Leukemia cancer K562 cell line Cell line after 48h showing cytotoxic effect with 50% cell lysis. Table 3. depicts the results of positive control (Cisplatin) used in the current study.

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	K562 Concentration	Absorbance (nm)	% cell lysis
1	k562	100		
2		50		
3		25	0.301	43.62
4		12.5	0.320	46.37
5		6.25	0.340	49.27
6		3.125	0.351	50.86
7		1.562	0.354	51.30
8		0.781	0.369	53.47
9		0.390	0.481	67.71
10		0.195	0.690	
control		00		

Table 2: Spearman's rhos Correlation.

K562 cell line	-1.000	p-value <0.05;Significant



Graph 1: Scatter plot showing TTO concentration, Cell death and viability for K562 cell line.

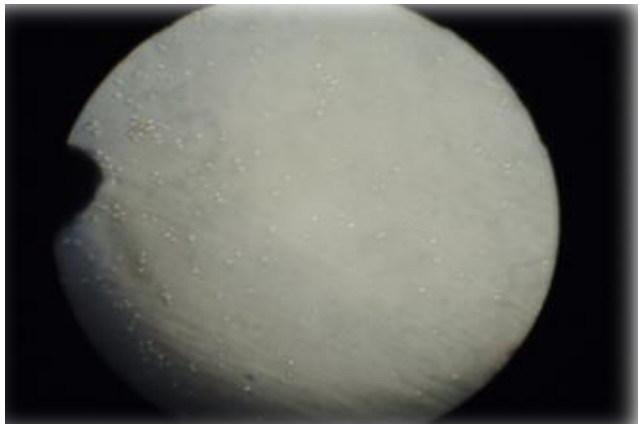


Fig 1: Shows TTO treated with Leukemia cancer K562 cell line Cell line after 48h showing cytotoxic effect with 50% cell lysis.

Table 3: Positive control used in the current study.

S. No.	Sample	Concentration	Absorbance (nm)	Results as observed	IC_{50} (µg)
1.	Cisplatin	2.5	1.413	< 50% lysis	10ug
2.		5	1.280	< 50% lysis	
3.		7.5	1.235	< 50% lysis	
		10	0.767	50% lysis	
4	control	00	1.529	No lysis	

Discussion

The cytotoxic effect TTO evaluated at concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390 and 0.195) which was done in serial dilution. Wherein, 50% of cell inhibition noted at the concentration level 3.125µg/ml of TTO**.** (Table 1 Graph 1 and Fig 1). The Spearmans Rhos correlation showed the statistical significant results with p-value <0.05. (Table 2). The results of positive control (Cisplatin) which was used in the current study shows that at 10, 7.5, 5, 2.5 µg/ml concentrations exhibited cytotoxic effects noted at all concentration levels. (Table 3)

Melaleuca alternifolia i.e tea tree oil (TTO) is the essential oil obtained through steam distillation process belongs to Myrtaceae family, a plant native from Australia. Traditionally, the oil was used by aboriginal Australian soldiers for insect bites and skin infections and reinvented in 1920s for its topical antiseptic effects [13] TTO basically has high number of monoterpenes; in which about half are

oxygenated and the rest are hydrocarbons [14] TTO made up of more than 100 constituents, predominantly has terpinen-4-ol, γ-terpinene, α-terpinene, 1,8-cineole, and ρ- cymene. The international standard for "Oil of Melaleuca terpinen-4-ol type" which sets the upper and lower limits for 14 components of the oil (International Organization for Standardization, ISO 4730: 2000) [13]. Terpinen-4-ol, is rich component of the oil, which is thought to be the main energetic content which is responsible for the several *in vitro* and *in vivo* activities reported for TTO[15]. TTO has wide range of actions. It is a very good antibacterial agent [16, 17] antifungal agent [18], antiviral agent [19], and anti-inflammatory agent [20], but currently the phytocomplex and some of its components have been screened for anticancer activities [21-23]. Calcabrini and colleagues (2004) have reported the potential anticancer activity of TTO in human melanoma M14 wild type cells and their drug-resistant counterparts, M14 adriamycin-resistant (ADR) cells. The higher concentrations (0.02 and 0.03%) of TTO, as well as terpinen-4-ol, was inhibiting the growth and inducing caspase-dependent apoptotic cell death in both wild type and drug-resistant melanoma cells with the latter being more susceptible to the cytotoxic effect [23]. Drug-resistant cells had greater sensitivity to the TTO treatment that could be attributed to the different lipid composition of the plasma membrane since there is evidence indicating that multidrug resistance phenotype is also associated with changes in membrane lipid composition [3, 24, 25]. Hence, this suggests that, the cytotoxic effect of TTO might be due to the interaction of the lipophilic components of the oil with the phospholipid bilayer of cell membranes with consequent alteration of cell growth and activity. But there was no cytotoxic effect of TTO on "normal" epithelial cells, fibroblast cells and basal keratinocytes [26], when compared to melanoma cells [27]. This confirms a point that a rapidly dividing tumor cells were highly sensitive to TTO when compared to normal cells. However, earlier reports have mentioned about cytotoxic effect of TTO in murine mesothelioma (AE17) and melanoma (B16) cell lines with different IC_{50} values, which could be due to the different types of cell lines used[28]. Recently TTO and its major element, terpinen-4-ol, has been also reported to interfere with the migration and invasion processes of drug-sensitive and drug-resistant melanoma cells [21].

The efficacy of topical TTO was evaluated on aggressive, subcutaneous, chemoresistant tumors in fully immune-competent mice [29, 30]. Results of the study have shown that topical treatment with 10% TTO, given once a day for 4 consecutive days induced a significant, though temporary, regression of subcutaneously established AE17 tumors and further slowed the growth of B16-F10 tumors. DMSO which was used as a penetration enhancer, at concentrations which was necessary to induce the antitumor effect. Similar kind of effects were also noted on tumor aggression when a combination of the five major constituents of TTO (terpinen-4-ol, γ-terpinene, α- terpinene, 1, 8-cineole, and ρ-cymene) were used at equivalent doses to those found in 10% of TTO [28]. Another follow-up study evaluated the mechanism of action underlying the antitumor activity of topical TTO reporting that topically applied 10% TTO induced a direct cytotoxic effect on subcutaneous AE17 tumor cells, which was associated with non-tumor specific activation of local immune response (i.e., neutrophils, dendritic, and T cells) [30]. Tumor cells undergoing primary necrosis by *in vivo* tissue phenomena using TTO treatment were similar to previously suggested *in vitro* method [23, 29]. Interestingly, it was noted that the topical treatment with TTO had no influence on the fibroblasts which were neighboring tumor cells, nor on lymphocytes and skeletal muscle fibers adjacent to tumor suggesting that normal cells had higher tolerance to the cytotoxic effect when compared to rapidly progressing tumor cells.

The potential toxic effect of TTO was initially performed to determine the cytotoxicity on cultured cells. TTO toxicity 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. In these studies TTO showed an IC_{50} on cell growth ranging from 20 to 2700 μg/mL [19, 22, 27, 31].

The effect of Melatonin (MLT) on the cytotoxicity of different chemotherapeutic agents used in leukemia cells was evaluated by *in vitro* method. The experiments were carried out on human leukemia cell lines, Jurkat, MOLT-4, Daudi, HL-60, CMK, and K562, and two different patient samples. Leukemia cells were incubated with cytarabine, daunorubicin, and etoposide with or without 10–5 M and 10–3 M concentrations of MLT which did not interfere with the cytotoxic effect caused by those chemotherapeutic drugs. [32]

The inhibition of metabolic processes of leukemic cell lines to Spir extract demonstrated little effect on the growth or viability of either cancer cell lines or normal hematopoietic or stem cells at the doses tested. The significant effects observed that cyano- and carotenoid-algae strongly inhibit the AML cell lines HL-60 and MV-4-11 and primary leukemia blasts. [33]

The two most common edible cyanobacteria include Spirulina and AFA [34], both of which contain phycocyanin, a molecule which is shown to induce apoptosis in the chronic myeloid leukemia cell line, K562 [35] and other types of cancer [36-38]. Spirulina as a nutritional supplement used for many years and has no undesirable side effects [39]. It is known as nature's richest source of nutrition [40] Orally administered spirulina extract exerted a synergistic antitumor activity with BCG-cell wall skeleton when used as immunotherapy of melanoma [41].

The cytotoxic activity of M. officinalis essential oil investigated, [42] by MTT assay in 2004, by de Sousa and colleagues, on lung (A549), colon (Caco-2), breast (MCF-7), and leukemia (HL-60 and K562) human cancer cell lines and in a mouse melanoma cell line (B16F10). A. C. de Sousa

Natural food based compounds has potential for prevention and biotherapy of cancers including leukemia. The common dietary flavonoid apigenin has anticancer activity, but also may decrease chemotherapy sensitivity, depending on the cell type [43].

Anti-proliferative effect of the oil extracted from seeds of Argania spinosa was assessed through an *in vitro* test on three T-ALL cell lines: JURKAT, MOLT3 and DND41. The cytotoxic effects of A. spinosa oil extract were checked by MTT assay which demonstrates that which inhibited the growth at the dose of 100 µg/mL of JURKAT, MOLT3 and DND41 cells [44].

Hence, it has been noted that cytotoxic effect which was produced by many naturally available plant derivatives has significant better results with least number of side effects on adjacent normal cells. Further research on TTO can provide a better ailment in treating the various types of cancer which needs to be preceded with animal and human trials.

Conclusion

Formulation of tea tree oil which induces tumor degeneration

is well tolerated and is accountable for inhibiting growth required to be worked upon. Hence the formulations of TTO should be developed in such a way that it can be regulated over a more extended time frame with a specific goal to permit more prolonged tumor growth inhibition as well as tumor regression. Hence, the mechanism of action of these plant extract should be thoroughly investigated, explicitly looking at the possible direct and indirect effects of *Melaleuca alternifolia*. TTO if it is taken in combination with other phytocompounds, it shows synergistic effect and remains more effective for curing cancer. Alternately, a preparation having known amount of each component can be used in a standardized manner to initiate a complete and perpetual tumor growth regression would be consummate [45]. Efficacy of TTO as an anticancer agent with its IC_{50} 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 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.

Acknowledgements

I would like to thank Rajiv Gandhi University of health sciences for providing grant for conducting the above research. I would like to thank our beloved principal sir Dr. Ramakant Nayak, Head of the department of Oral Medicine and Radiology Dr. Renuka Ammanagi, Dr. Ravi Shirahatti, Head of the department of Public health Dentistry for support and encouragement.

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