Original Article

Effect of *Ocimum sanctum* extract on leukemic cell lines: A preliminary *in-vitro* study

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Abstract

Background: Surge of cancer incidence, effects of chemotherapeutic agents and their cost and reduced survival and responsiveness to treatment have led to shift of attention of researchers toward herbal remedies to look for newer dimension in cancer therapy. *Ocimum sanctum*, Holy Basil or Tulsi, holiest herb well used in the Indian household, has drawn much attention toward its various health benefits, especially anti-cancer property. The present study was carried out to evaluate the cytotoxic effect of *O. sanctum* on leukemic cell lines K562.

Materials and Methods: Dry and aqueous extracts of two types of Tulsi leaves (Rama Tulsi and Krishna Tulsi) were evaluated for a dose-dependent cytotoxicity and anti-proliferative against K562 cell lines, leukoerythroid progenitor leukemic cell lines by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Half-maximal inhibitory concentration was evaluated for each of the extracts.

Results: Both dry and aqueous extracts of both types of Tulsi leaves demonstrated a significant amount of cytotoxicity against the studied cell lines.

Conclusion: This being preliminary study, we propose the initial finding of cytotoxic abilities of the herb against the leukemic cell lines and recommend a more detailed evaluation of the herb and its components.

Keywords: 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, apoptosis, cytotoxicity, K562 cell lines, Krishna Tulsi, *Ocimum sanctum*, Rama Tulsi, Tulsi

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INTRODUCTION

The magnitude of the impact of cancers on humanity is devastating. The International Agency for Research on Cancer quotes the statistics to be 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of the diagnosis) in 2012 worldwide.[1] By the year 2030, it is projected that there will be ~26 million new cancer cases and 17 million cancer deaths per year. The global distribution of cancer and types of cancer that predominate continues to change, especially in economically developing countries. Low- and middle-income countries accounted for about half (51%) of all cancers worldwide in 1975; this proportion increased to 55% in 2007 and is projected to reach 61% by the year 2050.[2]

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The existing cancer therapeutics and advancements in research have failed to promise the expectations toward curability of cancer. Understanding of complex cancer genetics and genesis remains still an unsolved mystery to humans, at least the growing cancer deaths and predictions suggest so. The magnitude of this problem necessitates the exploration of newer avenues.

Natural products have been a rich source of valuable agents to medicine. Natural compounds or compounds related to them contribute to more than 50% of currently available drugs. Above 60% is the contribution of natural products to cancer therapeutics. Currently, more than 30 compounds of natural origin are in different phases of the clinical study for the treatment of different types of cancer.[3]

Ocimum sanctum

Scientific classification

Kingdom: Plantae
(Unranked) Angiosperms
(Unranked) *Eudicots*
(Unranked) *Asterids*
Order: Lamiales
Family: *Lamiaceae*
Genus: *Ocimum*
Species: *Ocimum tenuiflorum*
Binomial name: *O. tenuiflorum* or *Ocimum sanctum* L.

O. sanctum (Sanskrit – Tulsi and English – holy basil) is considered to be one of the holiest and sacred herbs documented in Ayurveda. Tulsi is an aromatic shrub and is known in Ayurveda as "The Incomparable One," "Mother Medicine of Nature" and "The Queen of Herbs" and is revered as an "elixir of life" that is without equal for both its medicinal and spiritual properties. Tulsi belongs to the basil family *Lamiaceae* (tribe ocimeae) that is thought to have originated in North Central India.[4,5]

Tulsi assists with the body's internal physiologic balance and protection of the body from toxin-induced damage. These functions are often attributed to Tulsi's high content of phenolic compounds and anti-oxidant properties, with Krishna Tulsi (black/purple variety) having a higher phenolic content and anti-oxidant capacity than white Vana (wild) Tulsi.[5] Various documented beneficial effects of *O. sanctum* include anti-inflammatory, analgesic, antipyretic, antidiabetic, hepatoprotective, hypolipidemic, immune modulatory and anti-stress activity. Evidence on *O. sanctum* leaves having anti-cancer properties has been well documented.[4-7] With this background, the present study was conducted to evaluate the cytotoxic effects of *O. sanctum* on leukemic cell lines to explore the anti-cancer potential against leukemic cell lines K562.

K562 cells

K562 cells were the first human immortalized leukoerythroid progenitor leukemic cell lines of myeloid cell lineage. These cells were derived from a 53-year-old female with chronic myelogenous leukemia in blast crisis and are positive for BCR-ABL fusion gene (Philadelphia chromosome).[8,9] K562 leukemic cell lines are a part of NCI-60 cancer cell line panel that have been used extensively in bench and translational research by the National Cancer Institute.[10] As with many cancer cell lines, there seems to be a plethora of aurora kinase that results in uncontrolled cell division and cancer growth through excess formation of spindles, chromosomal separation and cytokinesis. Inhibition of these kinases plays a significant role in limiting the mitotic potential of these cells.[11]

Apoptosis plays a vital role in regulating K562 cells and can be induced by changes in these cell lines.[12] BCR-ABL, BCL-2 and various other cellular components play a role in apoptosis, thereby regulating the K562 cell-cycle.[13] Similarly, the p53 gene, tumor suppressor gene, plays a key role in apoptosis. These p53 genes target various cyclin-dependent kinase inhibitor that causes cell differentiation and arrests in the G1 phase of the cell cycle and subsequently triggering apoptotic pathway.[14] K562 leukemic cell lines are known to have variations in the levels of components induced by BCR-ABL gene and other cellular components that results in reduced apoptosis and cancer cell growth.[13] The above given properties and the ability to generate these changes in cell cycle regulation provide a potential target for anti-cancer drugs. One of the landmark drugs that utilized these cell lines and led to breakthrough discovery was tyrosine-kinase inhibitor, which is capable of inducing molecular remission in patients with chronic myelogenous leukemia. K562 leukemic cell lines are a part of panel of human cancer cell lines that are currently being used to screen agents that have the potential for anticancer activity.[15]

The objective of the present study was to evaluate and compare the dose-dependent cytotoxic effect of dry and aqueous extract of two different subspecies of *O. sanctum* on leukemic cell line K562.

MATERIALS AND METHODS

After confirming the authenticity of the plant, (NISCAIR, New Delhi) fresh aqueous and dry extracts

of *O. sanctum* leaves were prepared at Bapuji Institute of Pharmacy, Davangere, Karnataka, India. The two different types of Tulsi leaves were collected which were freshly plucked, namely light-colored leaves termed as Rama Tulsi (R) and dark-colored leaves termed as Krishna Tulsi (K). Confirmation for both the plants was obtained by NISCAIR, New Delhi.

The extracts tested in the present study were categorized into the following four groups:

- R1 = Dry extract from Rama Tulsi
- R2 = Aqueous extract of Rama Tulsi
- K1 = Dry extract from Krishna Tulsi
- K2 = Aqueous extract of Krishna Tulsi.

Ocimum sanctum extract preparation

Thirty gram of *O. sanctum* leaves were grounded using mortar and pestle and paste form was then kept in the flask with 300 ml of distilled water. The ingredients were mixed and kept overnight. The mixture was filtered while the residue was left in the flask. Another 300 ml of distilled water was poured to bottle to soak the remaining residue. These steps repeated for 2 days. Aqueous extract of Tulsi was evaporated at 60°C. The suspension was then filtered with a 0.22 μm filter paper.

For dry extracts, fresh leaves were shade dried for 5 days and grounded with mortar and pestle to obtain a fine powder.

Cell lines and cell culture

The cell lines used for the study were K562 cell lines (human, procured from NCCS, Pune). The cell lines were maintained in 96-well microtiter plate containing MEM media supplemented with 10% heat-inactivated fetal calf serum containing 5% of mixture of gentamicin (10 μg), penicillin (100 units/ml) and streptomycin (100 μg/ml) in the presence of 5% CO_2 at 37°C for 48–72 h.[16]

Cytotoxicity assay (3-(4,5-dimethythiazol-2-yl) -2,5-diphenyl tetrazolium bromide assay)

MMT cytotoxic assay was carried out to evaluate the cytotoxic effect of four groups of *O. sanctum* extracts on K562 cell lines followed by evaluating the dose-dependent cytotoxic effect of the extracts and to estimate the half-maximal inhibitory concentration (IC_{50}), the concentration of drug which induces 50% cytotoxicity.

Principle of the 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, colored (dark purple) Formazan product. The cells are then solubilized with an organic solvent (e.g., dimethyl sulfoxide [DMSO], 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.[16]

Procedure of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

In-vitro growth inhibition effect of the 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 contained only DMSO. In this study, 10, 20, 25, 30 and 50 μl of the stock solution (10 mg/ml prepared in DMSO) were added to respective wells containing 100 μl of the medium. Hence, the final concentrations were 10, 20, 25, 30 and 50 μg/ml. After 48 h incubation at 37°C in a humidified atmosphere of 5% CO_2, stock solution of MTT was added to each well (20 μl, 5 mg/ml in sterile phosphate-buffered saline) and subjected for further 4 h of incubation. The supernatant was carefully aspirated, the precipitated crystals of "Formazan blue" were solubilized by adding DMSO (100 μl) and optical density (OD) was measured at the wavelength of 570 nm by using LISA plus. The results represent the mean of five readings. The concentration at which the OD of treated cells was reduced by approximately 50% with respect to the untreated control was determined to be IC_{50}.

Surviving cells were calculated in percentage with the following formula mentioned below and tabulated.

Formula: Surviving cells (%) = Mean OD of test compound × 100.

RESULTS

All the groups tested for cytotoxicity showed the cytotoxic effect on K562 cell lines. The surviving cells percentage was calculated using the above-mentioned formula.

Cytotoxic activity of *Ocimum sanctum*

The ANOVA test to test the mean surviving cells when treated with various groups of extracts and control suggest a significant difference between the groups and control. Among the various groups, there was no significant difference [Table 1].

The increasing concentration gradient and surviving cell percentage were evaluated by Karl Pearson's correlation analysis which suggested high negative correlation (−0.78) as depicted in Graph 1.

Half-maximal inhibitory concentration values

Different concentrations among each group of the extracts were checked for half-maximum concentration, i.e., the centration at which the cell count diminished by 50%. The concentrations inducing cytotoxic effect of IC_{50} are shown in Table 2. R1 had least IC_{50} at 30 μg/ml indicating higher cytotoxicity at lower concentration.

DISCUSSION

Tulsi, *O. sanctum,* is part of Indian traditional history and regarded as mother medicine among the herbs with medicinal values. Holy basil is indeed a holy plant worshipped in the Indian household. The Ayurvedic texts elaborate the medicinal value of the plant and its parts.

The Charaka Samhita documents Tulsi to be of immense use in the treatment of headaches, rhinitis, stomach disorders, inflammation, heart diseases, various forms of poisoning and malaria. Each part of the plant has proven to offer protection against various diseases; the aqueous and alcoholic extract from the leaves has anti-inflammatory, antipyretic, analgesic, antiasthmatic, antiemetic, antidiabetic, hepatoprotective, hypotensive, hypolipidemic and antistress agents. Oil derived from the leaves of the plant possess antibacterial, antioxidant and anti-inflammatory properties.[4,6,17]

Table 1: Surviving cells in percent after MTT assay with different groups of *Ocimum sanctum* extract

Group	Concentration (μg/ml)	Surviving cells percentage (calculated)
R1	10	90.1
	20	89.3
	25	77.7
	30	54.5
	50	33.4
R2	10	84.9
	20	79.3
	25	75.5
	30	71.8
	50	55.8
K1	10	110.1
	20	99.9
	25	85.4
	30	73.3
	50	54.3
K2	10	81.9
	20	58.3
	25	57.2
	30	55.9
	50	54.8
Control	0	109.7

MTT: 3(4,5dimethythiazol2yl) 2,5diphenyl tetrazolium bromide assay

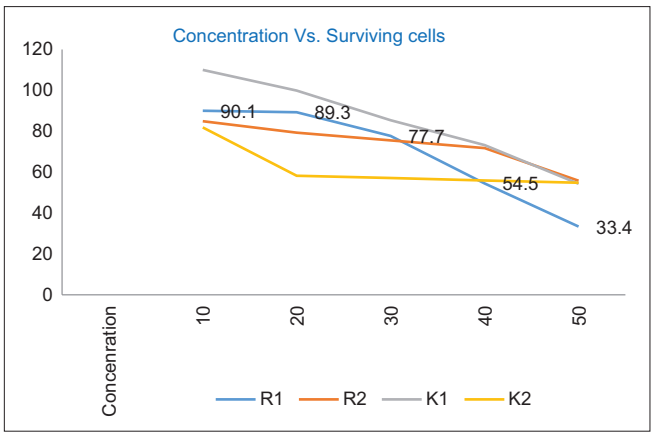
Table 2: IC_{50} value for each group of *Ocimum sanctum* extract

Group	IC_{50} (μg)
R1	30
R2	50
K1	50
K2	50

Conquest against cancer has persuaded the science to evaluate the claims of anticancer property of this herbal extract. With the intent, the present study evaluated the cytotoxic ability of *O. sanctum* extract in four different forms against the K562 cell lines, which are said to be the first human immortalized myelogenous leukemic line to be established. They are erythroleukemia type and cells are nonadherent and rounded, bear some proteomic resemblance to both undifferentiated granulocytes and erythrocytes.

The cytotoxic efficacy of five different concentrations of two forms of *O. sanctum* was tested and evaluated by the MTT assay. The results suggested the surviving cells count were significantly lower when treated with various forms of the extracts as compared to the controls [Table 1]. Whereas among various groups, the difference in the viable cell count was not significant. These suggest the stark cytotoxic activity exhibited by various groups of extracts. Results also imply all the forms and types evaluated express cytotoxic effects on the tested cell lines.

When the dose-dependent effect was evaluated, all the groups showed a steady decrease in the surviving cell count percentage. This implies as the concentration of the *O. sanctum* increased, cytotoxic activity of the extract increased steadily with a significant correlation as per the Karl Pearson's correlation assay. This steady increase in the cytotoxic ability is attributable to the various components of the extracts which induce cytotoxicity.



Graph 1: The reduction in surviving cells with an increase in concentration of extract in all groups

The IC_{50}, half-maximal inhibitory concentration, of all the types of extracts was assessed, of which the dry extracts of light-colored leaves of *O. sanctum* showed 50% reduction in surviving cells of K562 at 30 μg/ml concentration. All other groups showed such potential at 50 μg/ml concentration. Suggesting R1 extract to be more potent than other groups, as per the present findings.

O. sanctum is an herb with multiple health benefits. Studies, both *in-vitro* cell culture studies and animal studies, have revealed the anticancer effect of the herbs extract. Studies on mouse Lewis lung carcinoma (LLC) cells, A549 (adenocarcinomic human alveolar basal epithelial cells) have showed cytotoxic effects on the tumor cells. The results of studies demonstrated that ethanolic extract of Tulsi induces apoptosis in A549 cells through a mitochondria caspase-dependent pathway and also inhibits the *in vivo* growth of LLC in a dose-dependent manner.[18]

The morphological evaluation of *O. sanctum* treated oral cancer cells line revealed a positive cytotoxic ability of Tulsi extract on oral cancer cell lines. Morphological changes were typical of apoptosis indicating the anticancer activities of *O. sanctum* leaves with aqueous extract. The same study also evaluated the variations in dark- and light-colored Tulsi extracts, which revealed concurrent results as our study with no significant variation in cytotoxic efficacy.[19] Furthermore, the study on a mouse model revealed the *O. sanctums*, ability to prevent the early events of carcinogen-induced hamster buccal pouch carcinogenesis.[20]

The treatment of SiHa cell line, a cervical cancer cell line, with the ethanolic extracts of leaves of *O. sanctum* and *Azadirachta indica* and roots of *Withania somnifera* at IC_{50} values for 48 h resulted the formation of apoptotic bodies. Other than these, *O. sanctum* has been evaluated for and demonstrated its anticancer properties against prostate cancer, breast cancer and gastric cancer.[21]

The *O. sanctum* has numerous ingredients which induce beneficial effects. *O. sanctum* has a rich source of phytochemical compounds such as oleic acid, rosmarinic acid, eugenol, linoleic acid, vicenin-2, ocimarin, isorientin, esculetin, orientin, chlorogenic acid, isovitexin, aesculin, gallic acid, citronellal, galuteolin, circineol, dimethyl benzene, camphene, myrcene, ethyl benzene, limocene, sabinene, Vitamin C, calcium, phosphorous and various other micronutrients.[21]

Summary of some of the components of *O. sanctum* extracts which have been studied and established to have anticancer activity is listed in Table 3.[21]

Any or multiple ingredients included in Table 3 can be responsible for the anticancer demonstrated in this study. As initially mentioned, the drugs which target the cell cycle regulation factors possess the anticancer ability.[15] *O. sanctum* extracts have demonstrated apoptosis-induced cytotoxicity in the present study. Previous studies have confirmed the cytotoxic action by inducing apoptosis through Bcl-2 regulated mitochondria caspase-dependent pathways in cancerous cell lines. Along with this, proven free-radical scavenging properties, the *O. sanctum* extract can be explored for its anticancer potentials.

CONCLUSION

This study evaluated the cytotoxic effect of *O. sanctum* leaf extract on the leukemic cell line and established it on K562 cell lines. We could also demonstrate the cytotoxic effect was dose-dependent and established that activity was similar in all the groups evaluated. The medicinal herbs have always provided insight and lead to the pharmacological industry since time immemorial. The present study also provides some insight into the novel anti-cancer effects of the herb on leukemic cell lines. The exact mechanism of the action was not evaluated in this study; however, various mechanisms of the components of the herb with anticancer activity discussed could be the mode of activity exhibited.

Future prospects

This being preliminary study, we propose the initial finding of cytotoxic abilities of the herb against the leukemic cell lines and recommend a more detailed evaluation of the herb and its components. Since various animal and *in-vitro* studies have established anti-cancer response and also the herb has been used regularly both in Ayurvedic

Table 3: Various phytochemical components present in the extracts of *Ocimum sanctum*, their anticancer activity and their mode of action

Phytochemical component of *Ocimum sanctum*	Mechanism of action	Anticancer effect
Orientin, a flavonoid	Free radical-inhibiting activity and free-radical scavenging	Radiation protection
Vicenin-2	Antiproliferative, antiangiogenic and proapoptotic	Cytotoxicity
Eugenol, allylbenzene class of chemical compounds	Apoptosis via the mitochondrial pathway	Prevents tumor progression
Linolenic acid, an omega-3-fatty acid	Inactivation of Bcl-2	Proapoptotic, chemopreventive agent
Ursolic acid (3-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpenoid	Inhibit tumorigenesis, tumor promotion and suppress angiogenesis	Cytotoxic

medicine and household, without any adverse reactions, the medicinal use of this herbal extract needs to evaluated.

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Conflicts of interest

There are no conflicts of interest.

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