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ORIGINAL RESEARCH

Effect of *Ocimum sanctum* on Oral Cancer Cell Line: An *in vitro* Study

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ABSTRACT

Background: Cancer till today remains the leading cause of death in both developed and developing countries. Plants have been beacon of therapeutic sources for curing diseases from times immemorial. Hence, the present study aimed at evaluating the antiproliferative activity of extract of *Ocimum sanctum* leaves on oral cancer cell line.

Objectives:

- To evaluate the antiproliferative effect and to analyze dose dependent cytotoxic activity of aqueous extract of *O. sanctum* leaves on KB mouth cell line.
- To compare the effectiveness among different variety of *O. sanctum*.

Materials and methods: KB cells (Mouth Epidermal Carcinoma Cells) were used for the present study. Aqueous and dry extract of *O. sanctum* with both dark (Krishna Tulsi) and light (Rama Tulsi) leaves were prepared in the institution. The antiproliferative and cytotoxic activity on KB cell line was evaluated by MTT assay. Statistical analysis with Mann-Whitney U-test and Wilcoxon matched pairs test was carried out.

Results: The aqueous extract of *O. sanctum* of both the leaves exhibited significant cytotoxic effect against oral cancer cell line.

Conclusion: Aqueous extract of *O. sanctum* leaves was effective as an antiproliferative agent which caused apoptosis in oral cancer cell line.

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Clinical significance: *Ocimum sanctum* herb which is abundantly grown in India can be used for its anticancer properties for treating oral cancer. This will not only be cost-effective but will also have less or no side effects.

Keywords: *Ocimum sanctum* leaves, Oral cancer cell line, Oral squamous cell carcinoma.

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INTRODUCTION

Cancer which can affect any part of the body is a group of disease caused by loss of cell cycle control. It is associated with abnormal and uncontrolled cell growth.[1] Oral cancer is the sixth most common cancer affecting mankind, which also presents with low rate of survival. More than 90% of oral cancers are histopathogically squamous cell carcinomas (SCC).[2] Oral SCCs typically affect males over 40 years of age with a history of regular exposure to etiological risk factors, like tobacco products, alcohol, betel quid or micronutrient deficiency. However, today even younger patients with lower cumulative tobacco or alcohol exposure are increasingly presenting with OSCC.[3]

These early onset of oral squamous cell carcinoma (OSCC) are often located at the base of the tongue, tonsils and oropharynx and are associated with human papilloma virus.[3] The current standard approach of western medicine for treatment of oral cancer consists of attempts to eradicate established tumor with combined treatment, like surgery, chemotherapy and radiation. However, these therapies have failed in many aspects making human life miserable and reducing the life span of patients. Also after surgical treatment there will be distortion of face, trouble in breathing, swallowing and speaking. Patients remain sick, due to toxic effect of

radiation and chemotherapies as these cells not kill only cancer cells; but also kill normal cells. This makes patients prone to various diseases along with opportunistic infections.[4] Therefore, the need of the hour is to develop treatment modalities by using plants derivatives which obscure potent side effects and act as effective therapeutic agents.

Herbs and various plant products are considered as one of the important source of medicine.[5] These herbs are mentioned in ayurveda for treatment of various tumors including cancer therapy resulting in complete healing and reducing the side effects associated with cancer.[6,7] In ayurveda (Rigveda) *Ocimum sanctum* has been well documented for its therapeutic potential.

Ocimum sanctum (Sanskrit–Tulsi, English-Holy Basil) is one of the holiest and sacred herbs grown widely in India and is known to possess anti-fungal, anti-microbial and anti-inflammatory actions.[8-10] In few studies, it has been demonstrated that *O. sanctum* has significant anticancer properties.[11-13] Hence in the present study, we would like to evaluate the effectiveness of anticancer properties of *O. sanctum* (Tulsi) on a commercially available oral cancer cell line.

OBJECTIVES

- To evaluate the antiproliferative activity of extracts of *O. sanctum* leaves on OSCC line (KB mouth cell line).
- To analyze dose dependent cytotoxic effect of extracts of *O. sanctum* leaves on OSCC line (KB mouth cell line).
- To compare the effectiveness among different variety of *O. sanctum*, such as Rama Tulsi and Krishna Tulsi.

MATERIALS AND METHODS

Fresh leaves of *O. sanctum* were obtained from Regional Medical Research Centre (RMRC), Belgaum. The identity of the plant was confirmed by scientist of RMRC. The collection of *O. sanctum* leaves and preparation was carried out according to the following protocol. The study protocol was submitted to the Institutional Review Board of our institution and it was carried out after the IRB approval.

Preparation of Plant Extract

The extract (aqueous and dry) was prepared in the institution with the help of a pharmacist. The aqueous extract was prepared as 30 gm of leaves were ground using a mortar and pestle to obtain the paste form, and the paste was then kept in a flask with 300 ml of distilled water in it. The ingredients were mixed and kept overnight at room temperature. Next day, the mixture was filtered into a beaker using Whatman Filter Paper (GF/A, 110 mm) while the residue was left in the borosilicate glass bottle. Another 300 ml of distilled water was then poured into the borosilicate glass bottle to soak the remaining residue, which was then kept overnight at room temperature. These steps were then repeated for the next two consecutive days. Aqueous extract of Tulsi leaves was evaporated using a rotary evaporator (RV 10 Basic IKA Rotary Evaporator) at 60°C.[14,15] The suspensions were then filtered with a 0.22 µm filter and stored at –20°C until further use. Five concentrations (10, 20, 25, 30 and 50 µg/ml) of extracts were prepared. The same process was carried out for dry extract with the only difference of that the fresh leaves were shade dried for 2 days and ground using a mortar and pestle to obtain the fine powdered form.

Cell Line and Cell Culture

Oral squamous cell carcinoma cell line (KB mouth cell line) procured from National Centre for Cell Science, Pune, India, were used for the present study. Oral squamous cell carcinoma cell line (KB mouth cell line) were maintained in 96 wells micro titer plate containing MEM (modified Eagle's medium) media supplemented with 10% heat inactivated fetal calf serum (FCS), and 5% of mixture of Gentamycin, 100 units/ml penicillin-streptomycin (Invitrogen). The cells were grown in 5% CO_2 in humid condition at 37°C for 48 to 74 hours.[16]

Treating Oral Cancer Cell Line with Various Concentrations of *O. sanctum*

Oral squamous cell carcinoma cell lines (KB mouth cell line) were seeded into 96 well plates at a density of 3×10^3 cells/well. After 24 hours, the cells were treated with *O. sactum* extracts at five concentrations (10, 20, 25, 30 and 50 µg/ml) diluted with DMSO (dimethyl sulphoxide) and incubated for 24 and 48 hours and untreated cells were also incubated for same period.

Cell Viability Assay

Cell viability assay was done to determine the sub-lethal concentrations (IC50) of extracts and proliferative activity of the cells in presence of extracts. After completion of incubation period, working solution (20 µl, 5 mg per ml in sterile PBS) of MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added to the wells to detect the cell viability. The plate was further incubated for 4 hours. After incubation, the media was removed carefully without disturbing formazan precipitate and dissolved in 100 µl of 100% dimethyl sulfoxide (DMSO) and optical density was measured at wavelength of

492 nm by using spectrophotometer (LISA plus). The quantification of inhibition of proliferation was done by manual counting of the cells under the microscope by using Neubaur Counting Chamber by a Microbiologist.[16] The following were the groups made to analyze the dose at which *O. sanctum* will be effective:

Group I: Dry extract with Rama Tulsi or light leaves.

Group II: Dry extract with Krishna Tulsi or dark leaves

Group III: Aqueous extract with Rama Tulsi or light leaves

Group IV: Aqueous extract with Krishna Tulsi or dark leaves

Group V: Control normal group.

STATISTICAL ANALYSIS

All *in vitro* assay data signify the mean ± standard deviation of triplicates and IC_{50} was calculated by using one way analysis of variances (ANOVA) followed by Mann-Whitney U-test which was used for comparisons between independent groups. The differences were considered significant when the probability was $p < 0.05$.

RESULT AND OBSERVATIONS

The dry extract (powdered extract) of *O. sanctum* leaves both light leaves (group I) and dark leaves (group II) did not show any significant cytotoxic activity on oral cancer cell line. The aqueous extract of *O. sanctum* both light leaves (group III) and dark leaves (group IV) exhibited significant cytotoxic effect against oral cancer cell line. All the four groups were compared with control group (group V) which was untreated oral cancer cell line.

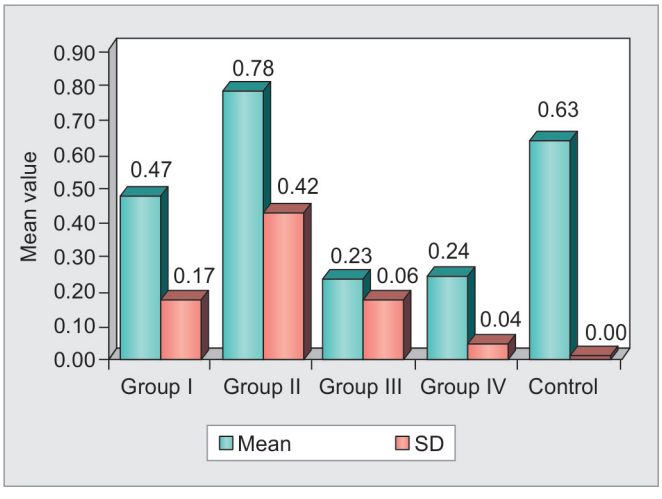
Graph 1: Comparison of five groups (I, II, III, IV and V) with optical density (nm) by Kruskal Wallis ANOVA. Comparison of five groups with mean optical density which showed that group V (control) was 0.63 and group I mean was 0.47 were as group II mean was 0.78, this suggests that groups I and II are same as group V. Standard deviation showed in group V control 0.00 were as almost similar with group III 0.06 and group IV 0.04. This signifies that groups III and IV were more effective. The result is graphically represented in Graph 1.

Cell viability of KB mouth cell line treated with both the extracts (dry and aqueous) with 5 concentrations of Tulsi leaves was determined by MTT Assay. The IC_{50} values for both the extracts were calculated. The optimized IC_{50} values of extract are 20 µg/ml in group III (aqueous extract with light leaves) and 10 µg/ml in group IV (aqueous extract with dark leaves) for 48 hours in KB oral cancer cells. Among four extracts tested, two extracts exhibited promising activity with IC_{50} values of less than 50 µg/ml. The aqueous extract presented the best cytotoxic effect with an IC_{50} value of 10 and 20 µg/ml which showed lysis with groups III and IV. The result graphically represented in Graph 2.

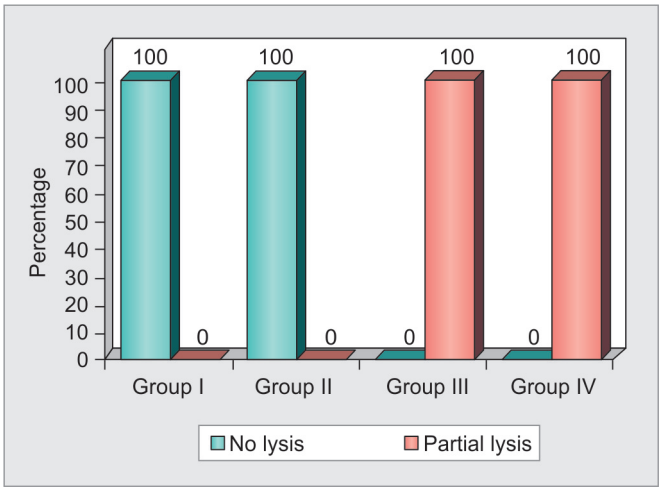
The comparison between groups III and IV that is aqueous solution with light and dark leaves did not show any difference related to cytotoxic activity.

Morphological Observation

The light microscopic observation of aqueous extract treated with KB oral cancer cell line after 48 hours of exposure showed typical morphological features of apoptosis as aqueous concentrations increased (10, 20, 25, 30 and 50 µg/ml). The cell death inducing ability of aqueous extract is determined by visual observation under inverted microscope. This is indicated by formation of fragmented apoptotic bodies which are highly condensed. Here, we found that the number of condensed nuclei in aqueous extract (groups III and IV) of Tulsi leaves were comparatively more than that of dry extract (groups I and II) in KB oral cancer cells. The untreated control cell lines showed no condensed or fragmented nuclei (Fig. 1).



Graph 1: Comparison of five groups with mean optical density (nm)



Graph 2: Comparison of five groups with status of lysis

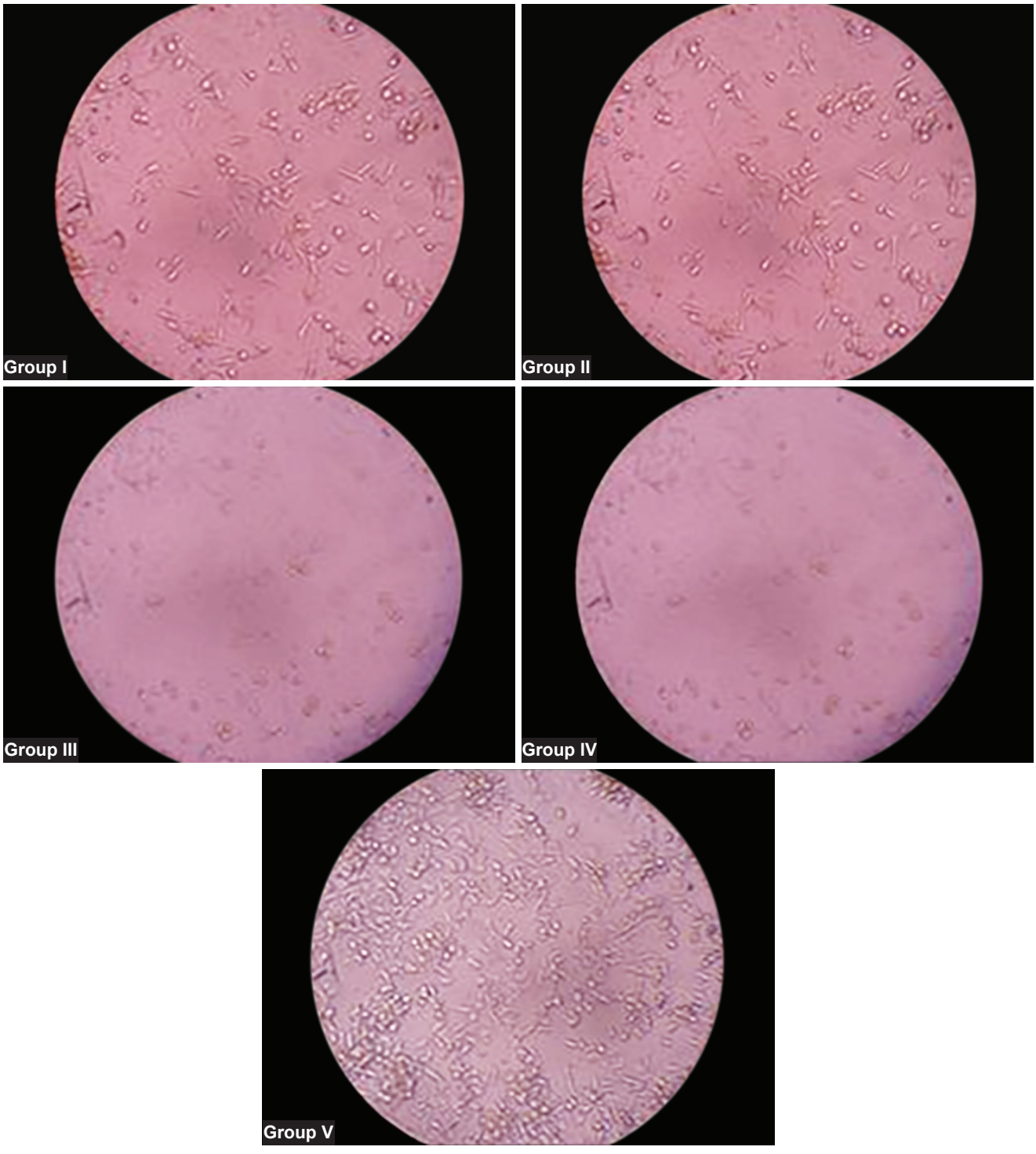


Fig. 1: Morphological slides of all five groups representing condensed chromatin in groups III and IV treated with KB oral cancer cell line

DISCUSSION

The use of herbs and other plant products as medicine is practiced worldwide from time immortal. In India, the major way of treating diseases in ancient time was Ayurveda which mainly concentrates on using natural plant products as therapeutic agents. *Ocimum sanctum* famously known as 'Tulsi' is found everywhere in India. It is not only worshipped and thought to be scared is also used as home remedy for many respiratory ailments.

This was a preliminary *in vitro* study wherein we evaluated the effectiveness of Tulsi as a cytotoxic agent against oral cancer cells. There was an overwhelmingly positive response of aqueous Tulsi extract on oral cancer cells. The cytotoxic effect was mainly because of the apoptosis caused by the aqueous solution. This effect can be attributed to the presence of phytochemical compounds which are abundant in Tulsi leaves. The phytochemical compounds present in Tulsi are dimethyl

benzene oleic acid, ethyl benzene camphene eugenol, linolenic acid, vicenin-2, citronellal, ocimarin, isorientin, circineol, myrecene, orientin, chlorogenicacid, esculectin isovitexin, gallic acid, limocene, galuteolin, rosmarinic acid, vitamin C sabinene, calcium, phosphorous and various other micronutrient which may have the ability to prevent the early changes of carcinogenesis.[6,17-19] Also be the reason for its effectiveness.

In our study, we found that aqueous solution of Tulsi (both dark and light leaves) is quite effective as compared to the dry extract, which was also the conclusion drawn by Aggarwal et al.[20] The most effective dose was 20 µg/ml of aqueous extract of light leaves and 10 µg/ml of aqueous extract of dark leaves.

The morphological evaluation of treated oral Cancer cells line in our study revealed nuclear condensation, cell shrinkage, membrane blebbing and apoptotic body formation which is the characteristic features of apoptosis.[21-24] This suggests a positive cytotoxic ability of Tulsi extract on oral cancer cell lines. There was no major difference between light and dark leaves of Tulsi in aqueous solution. Our results showed morphological changes were typical of apoptosis indicating the anti cancer activities of *O. sanctum* leaves with aqueous extract.

CONCLUSION

We recommend more studies especially randomized control trials among patients with oral premalignant and malignant lesions to confirm the effect of *O. sanctum* herb. The time has come where we all as clinicians and researchers should explore more into nature and find solutions to the problems faced by mankind.

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