



Original article

# An *in vitro* study of *Ocimum sanctum* as a chemotherapeutic agent on oral cancer cell-line

A.M. Luke <sup>\*,a</sup>, R. Patnaik <sup>a</sup>, S.T. Kuriadom <sup>a</sup>, M. Jaber <sup>a</sup>, S. Mathew <sup>b</sup><sup>a</sup> Department of Basic Medical and Dental Sciences, College of Dentistry, Ajman University, Al Jurf, Ajman, UAE<sup>b</sup> Department of Clinical Sciences, College of Dentistry, Ajman University, Al Jurf, Ajman, United Arab Emirates

## ARTICLE INFO

## Article history:

Received 7 September 2020

Revised 2 November 2020

Accepted 3 November 2020

Available online 11 November 2020

## Keywords:

MTT assay

NRU assay

Ca9-22 cell line

*Ocimum sanctum*

Lethal concentration

## ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common cancer in the world. If remain untreated for several years, it may be fatal. Hence, it is important to prevent and treat OSCC at an early stage. In this study the effect of aqueous and dry leaves extract of *Ocimum sanctum* was observed on Ca9-22 cell line, which is an OSCC cell line. For this, Ca9-22 cell line was cultured and maintained. After 24 h, the cells were treated with aqueous and dry leaves extract of *Ocimum sanctum* plant. Viability of the cancerous cells were studied by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay and neutral red uptake (NRU) assay. Minimum inhibitory concentrations (MIC), lethal concentration<sub>25</sub> (LC<sub>25</sub>), lethal concentration<sub>50</sub> (LC<sub>50</sub>) and highest permissive concentration (HPC) was calculated by probit computational method. Experimentally, the MIC value was 5 mg/L, whereas the HPC was 30 mg/L of the plant extract in aqueous state. For the dry extract the MIC was 5 mg/L whereas the HPC was 35 mg/L for both MTT and NRU assays. For MTT assay LC values: 7.41 (LC<sub>25</sub>), 14.79 (LC<sub>50</sub>) and 26.91 mg/L (LC<sub>75</sub>) for aqueous extract and 12.58 (LC<sub>25</sub>), 20.89 (LC<sub>50</sub>), 29.51 mg/L (LC<sub>75</sub>) for dry extract. For NRU assay LC values were 10.23 (LC<sub>25</sub>), 14.79 (LC<sub>50</sub>) and 20.89 mg/L (LC<sub>75</sub>) aqueous extract, and 16.59 (LC<sub>25</sub>), 23.44 (LC<sub>50</sub>), 30.19 mg/L (LC<sub>75</sub>) dry extract of the plant. From the above study it was concluded that, *Ocimum sanctum* have anti-cancerous activity. It can further be used for therapeutic purposes.

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## 1. Introduction

Oral cancer is the most common and prevalent cancer worldwide which is a leading cause of mortality in several regions (Gupta et al., 2016). Basing upon a report of the World Health Organization, in some countries of Asian-Pacific, the cancer of lip and oral cavity lies within the top three of all cancers (WHO, 2019). Keeping an eye on this evidences, researchers showed attention towards the prevention and cure of human squamous cell carcinoma (Suparp et al., 2001).

*Ocimum Sanctum* Linn (Tulsi or Holy Basil), belongs to Labiatae family, is most abundantly cultivated throughout the world. *O. Sanctum* has many medicinal properties such as, anti-fungal

(Balakumar et al., 2014; Gopalkrishna et al., 2016; Zaidi et al., 2018), anti-bacterial (Kaypetch et al., 2015), anti-caries (Agarwal and Nagesh, 2010), anti-viral (Ghoke et al., 2018), and anti-cancer effect (Bhattacharyya and Bishayee, 2013). The plant has reported to contain several eugenol, urosolic acid, alkaloids, flavonoids, tannins, and carbohydrates (Sandip et al., 2014). A significant reduction in Sarcoma-180 tumor cell size and an increase in lifespan of mice with this tumor was reported in a study by the use of ethanolic extract of *O. sanctum* (Nakamura et al., 2004). The anti-tumorigenic effects on several cancer types including gastric cancer (Manikandan et al., 2007), pancreatic cancer (Shimizu et al., 2013), non-small cell lung cancer (Kwak et al., 2014), and lung cancers (Magesh et al., 2009; Kim et al., 2010) have been studied by the ethanolic extract of *O. sanctum* leaves. In a study, on rat gastric carcinoma induced by expression of proteins involved in proliferation, invasion, and angiogenesis of carcinogen decreased by ethanolic extract of *O. sanctum* (Manikandan et al., 2007). The vascular endothelial growth factor production and MMP-9 activity in metastatic-induced NCI-H460 non-small cell lung cancer cells was reduced by the activity of ethanolic extract of *O. sanctum* by inhibiting the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (Kwak et al., 2014). However, there is lack of information

\* Corresponding author.

E-mail address: [a.luke@ajman.ac.ae](mailto:a.luke@ajman.ac.ae) (A.M. Luke).

Peer review under responsibility of King Saud University.



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regarding the effect of *O. sanctum* on human squamous cell carcinoma. Therefore, the aim of this study was to investigate effect of *O. sanctum* on oral squamous cell carcinoma.

## 2. Materials and methods

### 2.1. Plant extract preparation

Fresh leaves of *O. sanctum* were used for this study. Leaves were grinded using a mortar and pestle to the paste form. The paste was stored overnight at room temperature in a flask after adding 300 mL of distilled water into it. The mixture was filtered into a beaker using Whatman Filter Paper (GF/A, 110 mm) while the residue remained left in the borosilicate glass bottle. Another 300 mL of distilled water was added into the borosilicate glass bottle to soak the remaining residue, which was kept overnight at room temperature. The same step was repeated for next two consecutive days. A rotary evaporator (RV 10 Basic IKA Rotary Evaporator) was used to evaporate the aqueous extract of Tulsi leaves at 60 °C. With the help of a 0.22 µm filter, the suspensions after filtration was stored at –20 °C until further use. Several concentrations (0, 5, 10, 15, 20, 25 and 30 mg/ml) of extracts was prepared for this study. The dry extract of Tulsi leaves were prepared too after shading leaves to dry for two days. After drying the leaves were grinded using mortar and pestle to fine powdered form for this study. In case of dry extract several concentrations too prepared (0, 5, 10, 15, 20, 25, 30 and 35 mg/L)

### 2.2. Cell culture and viability study

For this study, Ca9-22 cell line, an oral squamous cell carcinoma cell line (OSCC) was used. The cell line was purchased. After getting the cell line, cells were washed thoroughly with PBS and the cell number was counted. The cells were cultured in minimum essential media (MEM) containing 10% FBS (Fetal Bovine Serum) and 1% penicillin-streptomycin solution in six well culture plate. The cells were kept in an incubator at 5% CO<sub>2</sub> and at 37 °C temperature. The cells were maintained by changing the media in two days interval. After getting confluent cells were transferred to 96-well culture plates at concentration of 1X10<sup>4</sup> cells/well for study of effect of *O. sanctum*. The cells were treated with several concentrations of the leave extract (0, 5, 10, 15, 20, 25 and 30 mg/L) and were incubated for 24 and 48 h. After the incubation period, viability of OSSCs were studied by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay and neutral red uptake (NRU) assay.

### 2.3. MTT assay

The MTT solution was prepared at the concentration of 5 mg/mL in PBS. After 24 and 48 h of leave extract treatment in 96-well culture plate, 80 µL of MTT solution was added to each well to study the effect. The plate was kept in an incubator (37 °C, 5% CO<sub>2</sub>) for 4 hr. The mixture was gently centrifuged at 1,000 rpm for 10 min at 22 °C. The supernatant was removed and the pellet was dissolved by an 1.0 mL aliquot of 100% dimethyl sulfoxide (DMSO) and the suspension was kept in an incubator for 1 h. Optical density<sub>570</sub> of the suspension was measured with a spectrophotometer.

Percentage of cell density =  $100 \times (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / \text{OD}_{\text{control}}$ . MTT in DMSO solution was taken as the blank. Probits of observed lethality percent values were used for the analysis of toxicity.

Yellow MTT is reduced to purple formazan crystals in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a wavelength between 540 and

630 nm by a spectrophotometer. The absorption maximum is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable/living cells.

### 2.4. NRU assay

A 100 µg/mL neutral red solution was prepared in serum free DME medium. After 24 h growth of cells in the presence of a toxic leaf extract in 96-well culture plate, an aliquot of 200 µL of neutral red dye solution was added to each well. The plate was kept in an incubator (37 °C, 5% CO<sub>2</sub>) for 3 hr. Indeed, lysosomes of viable cells absorb the dye and the dead cells remain unstained. Lysosomal activity of live cells is more. As a result live cells will absorb and remove more stain as compare to dead cells. Thereafter, an 1 mL aliquot of separately prepared 'neutral red desorption solution' (1% acetic acid, 50% ethanol and 49% distilled water) was added to each well for removal of the dye from lysosomes of live cells. Optical density<sub>540</sub> of the washout was measured with a spectrophotometer to assess live cell density whose percent values were calculated.

## 3. Results

### 3.1. MTT assay

The cell density of Ca9-22 mouth cell line at OD<sub>570</sub> gradually decreased from the level of 5 mg/L to 30 mg/L of both aqueous and dry extract of the plant. Experimentally, the MIC value was 5 mg/L, whereas the HPC was 30 mg/L of the plant extract in aqueous state. For the dry extract the MIC was 5 mg/L whereas the HPC was 35 mg/L. Extrapolated log<sub>10</sub> values of aqueous extract from the plot were 0.87, 1.17 and 1.43, respectively as LC<sub>25</sub>, LC<sub>50</sub>, and LC<sub>75</sub> log<sub>10</sub> values; whereas similar values for dry extract were 1.10, 1.32, and 1.47, respectively. Thus, these log<sub>10</sub> concentration values generated LC values: 7.41 (LC<sub>25</sub>), 14.79 (LC<sub>50</sub>) and 26.91 mg/L (LC<sub>75</sub>) for aqueous extract and 12.58 (LC<sub>25</sub>), 20.89 (LC<sub>50</sub>), 29.51 mg/L (LC<sub>75</sub>) for dry extract (Table 1, Fig. 1).

### 3.2. NRU assay

Cell density, monitored at OD<sub>540</sub> gradually decreased from the level of 5 mg/L to 30 mg/L of both aqueous and dry extract. Experimentally, the MIC value was 5 mg/L; whereas the HPC was 30 mg/L for aqueous extract of the plant. For dry plant extract the MIC was 5 mg/L and the HPC was 35 mg/L. Log<sub>10</sub> values of aqueous extract concentrations extrapolated from the probit plot yielded log<sub>10</sub> values 1.01, 1.17 and 1.32, for values of LC<sub>25</sub>, LC<sub>50</sub>, and LC<sub>75</sub> respectively; and for dry extract these values were 1.22, 1.37 and 1.48, respectively. Further, these log<sub>10</sub> concentration values generated LC values: 10.23 (LC<sub>25</sub>), 14.79 (LC<sub>50</sub>) and 20.89 mg/L (LC<sub>75</sub>) aqueous extract, and 16.59 (LC<sub>25</sub>), 23.44 (LC<sub>50</sub>), 30.19 mg/L (LC<sub>75</sub>) dry extract (Table 1, Fig. 1).

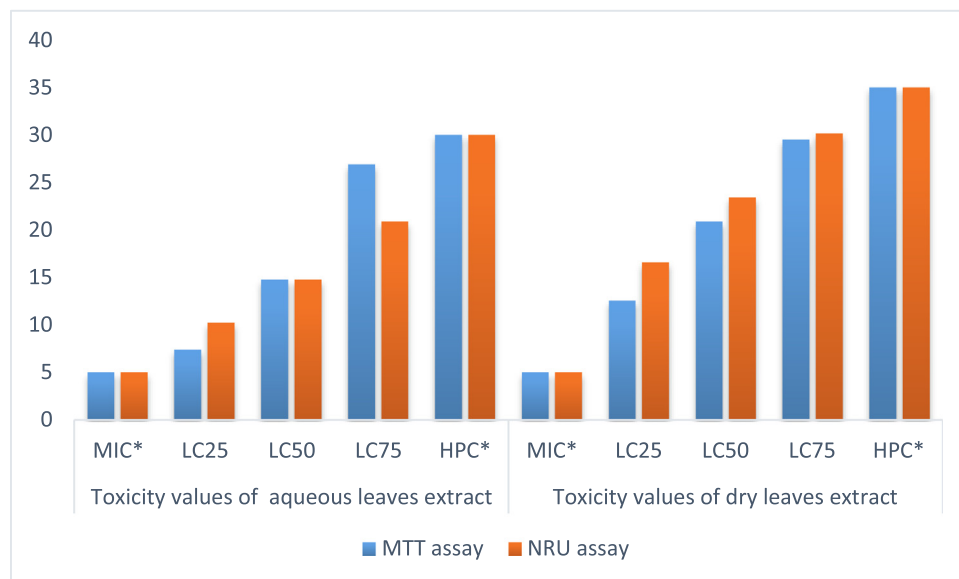
## 4. Discussion

The present study described the effect of both aqueous and dry extract of *ocimum sanctum* leaves in oral squamous cell carcinoma cell line. In a previous study it was found that in A549, a lung cancer cell line, effect of the same plant extract was found at 0.2 mg/ml (Magesh et al., 2009). However, the same concentration was found non-toxic to NCI-H460 (a lung cancer cell line) cell line (Kwak et al., 2014). Our results indicated that 5, mg/L is the MIC for Ca9-22 cell line in both aqueous and dry plant extract. Since,

**Table 1**Toxicity values of aqueous and dry leaves extract of *Ocimum sanctum* plant to Ca9-22 cells obtained by experimentation and the probit computation (mg/L).

Assay methods	Toxicity values of aqueous leaves extract					Toxicity values of dry leaves extract				
	MIC*	LC <sub>25</sub>	LC <sub>50</sub>	LC <sub>75</sub>	HPC*	MIC*	LC <sub>25</sub>	LC <sub>50</sub>	LC <sub>75</sub>	HPC*
MTT assay	5	7.41	14.79	26.91	30	5	12.58	20.89	29.51	35
NRU assay	5	10.23	14.79	20.89	30	5	16.59	23.44	30.19	35

Note: \*Mean from number of experiments.

**Fig. 1.** Histogram of MIC, LC<sub>25</sub>, LC<sub>50</sub>, LC<sub>75</sub> and HPC values in mg/L, corresponding to MTT and NRU assay methods (see text for abbreviations) after the Ca9-22 cells treated with both aqueous and dry leaves extract of *Ocimum sanctum* plant.

oral cancer is a dangerous disease in the world which can be prevented. Lowest survival rates of <50% within a 5-year period is found in oral cancer patients. Still less cases are diagnosed and treated in relation to oral cavity instead of advances in diagnosis and treatment procedure due to lack of public awareness (Vimalakshan and Kumar, 2017). As use of natural plant products as therapeutic agents is practiced worldwide from immortal time. *Ocimum sanctum* famously known as 'Tulsi' is found everywhere, since the plant is known to contain many constituents. In a study antioxidant properties were reported in ursolic acid which is a product of *O. sanctum* (Balanehru & Nagarajan, 1992). In the neoplastic development, to scavenge free radical *O. sanctum* may have the ability. It was reported that in mice glutathione and glutathione S-transferase activity was elevated to 78% due to *o.sanctum* administration by preventing tumors and hepatomas (Aruna and Sivaramakrishnan 1990, 1992).

In this study it was found that *O. sanctum* is a cytotoxic agent against oral squamous cancer cell line (Ca9-22). Due to presence of phytochemicals in the leaves of this plant, it has the ability to fight against oral cancer. Both aqueous and dry extract of the plant is effective where as in a comparative study aqueous extract has more promising result than dry extract. The most effective dose, HPC was 30 mg/L for aqueous extract of leaves and 35 mg/L for dry extract of leaves.

## 5. Conclusion

It was concluded that *O. sanctum* can be used as a potential anticarcinogenic and chemotherapeutic agents. As the extracts of *O. sanctum* have anticancer activity due to presence of active principle components.

## Declaration of Competing Interest

Authors declare no conflict of interest.

## Acknowledgement

We are thankful to Dean of graduate studies and research (DGSR) for giving us the opportunity. This research was done from the internal grant of Ajman University (Grant Id: 2019-IRG-DEN-1). We are grateful to Dean, College of Dentistry, Ajman University for providing support for this research work.

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