Anticancer potential of Siddha formulations against oral cancer cell line in vitro

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Abstract

Treatment of cancer without any side-effects is still a challenge in the medical system. This leads to an increasing search for improved anticancer drugs. Plant products have been used as a traditional medicine for thousands of years as it has been drawing a great deal of attention to overcome cancer. The main objective of this study is to evaluate and compare the anticancer effect of MahaVallathy Leghiyam (MVL) and Neeradi Muthu Vallathy Leghiyam (NMVL) against human oral cancer (KB) cells. Different concentrations of aqueous extracts of MVL and NMVL were subjected to cytotoxic study. The antiproliferative effects were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and IC50 concentration was found at 3.25 mg/mL for MVL and 1.25 mg/mL for NMVL. The current investigation suggested that the phyto constituents of NMVL are responsible for anticancer activity.

Introduction

One of the most distressing diseases according to recent report is oral cancer and it ranks as third most common cancers in India, after cervical and breast cancer in female [1]. Hence, it is considered as a frivolous problem by the public officials, medical centres and private hospitals [2]. National Cancer Registry Programme (NCRP) reported Bhopal males precede Ahmedabad men by showing highest age-adjusted rate for mouth cancers [3]. Cigar smoking and alcohol consumption are the major risk factors in western countries, while chewing areca nut, betel-quids and smoking are major risk factors in South Asia, Southeast Asia and Taiwan [4]. The disease is highly allied to establish cultural risk factors that have been acquired traditionally by these populations to these specific risk factors [5]. Oral cancer is any malignancy neoplasm that appears as an abnormal growth within the oral cavity which includes buccal mucosa (cheek lining), gingiva, palate or the tongue, floor of the mouth and lip. Progression of OSCC is a multi-step process involving DNA fragmentation, membrane blebbing, chromatin condensation, genes related to the growth control, apoptosis, DNA damage response and other cellular regulators [6].

A quotidian treatment modality that has been used to control advanced stages of malignancies is Chemotherapy as it is known for its speedy prophylactic action against possible metastasis [7]. Several immune-modulating agents, chemotherapeutic drugs and cytotoxic drugs are available in Western medicine to treat various cancers. Apart from being enormously expensive, these drugs are associated with serious after effects. Ayurveda and polyherbal siddha preparations are emerging trends in curing many cancers which are being used by 80% of the World population, especially in developing countries. Siddha and Ayurveda are the traditional Indian system of medicines that has been successful since antediluvian times using natural herbal preparations, that can suppress and act against various tumors. Variety of them is being screened worldwide to validate their use as anti-cancerous drugs [8]. The phytochemical screening on quantitative analysis showed that the elements of MVL and NMVL are rich in popular phytochemical constituents such as flavonoids, terpenoids, carbohydrates, steroids and alkaloids. However, only limited scientific investigations has been carried out on anticancer activity of these two siddha formulations. The ability of these siddha formulations to efficiently inhibit oral cancer cell growth by promoting a competent rate of apoptosis is yet to be discovered and hence in this study, an attempt to congregate the anti-proliferative and anticancer potential of MVL and NMVL in their aqueus states have been closely monitored through an in-vitro analysis.

Materials and methods

Reagents

Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin methylthiozyl diphenyl- tetrazolium bromide (MTT)

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and Dimethyl sulfoxide (DMSO), Propidium iodide (PI), Acrifine
Orange (AO) and Ethidium bromide (EB) were procured from Hi-
Media Laboratories, other chemicals and reagents were obtained from
Sigma Aldrich Mumbai.

Preparation of MVL and NMVL

MVL and NMVL were procured from Indian Medical Practitioners
Co-operative Pharmacy and Stores Ltd., (IMPCOPS, Thiruvanmiyur,
Chennai, India), an authoritative source of Indian medicines (http://
www.impcops.org/), and its composition as per the ancient scripts [9].

Extract preparation

The MVL and NMVL were dissolved in different grading of solvent
system, to check the effective solubility. Based on the solubility test,
it was confirmed that formulations were soluble in water to employ
aqueous extract, and it was used for future studies.

Cell culture

Human oral cancer KB cell lines were obtained from National
Center for Cell Science (NCCS), Pune, India. The cells were nurtured in
T25 flasks at 37°C in Dulbecco’s Modified Eagle Media supplements
along with 10% FBS (Sigma-Aldrich, St. Louis, Mo, USA), 100 units/
ml penicillin G and 100 µg/mL streptomycin as antibiotic (Himedia,
mumbai, India). For all the experiments the cells were maintained in
100-mm culture dishes (Nunc) at humidified 5% CO₂/95% atmosphere
air (Heraeus, Hanau, Germany). Cells were sub cultured every 2-3 days
to improve the cell number and the cell population was assessed by
using haemocytometer, a standard procedure for cell counting.

Cell proliferation assay

MTT assay was carried out following as per the method of Safadi
et al. [10]. Cells (1×10⁴/well) were plated in 24-well plates and incubated
in 37°C with 5% CO₂ condition. After the cell reached the confluence,
various concentrations (25, 12.5, 6.5, 3.25, 1.625, 0.81, 0.4, 0.1 mg/mL)
of MVL and (25,12.5, 6.5, 3.25, 2.5, 1.25, 0.75, 0.35, 0.18 and 0.9 mg/
ml) of NMVL were treated and incubated for 24 hrs and 48 hrs. After
incubation, the sample was removed from the well and washed with
phosphate-buffered saline (PBS) (pH 7.4). 100 µL/well (5 mg/mL) of
0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide
(MTT) was added and incubated for 4 hours. After incubation, 1 mL
of DMSO was added in all the wells. The absorbance at 570 nm was
measured with UV- Spectrophotometer using DMSO as the blank.
Measurements were performed and the concentration required for a
50% inhibition (IC50) was determined graphically. The % cell viability
was calculated using the following formula:

\[
\% \text{ inhibition} = \frac{\text{Mean OD of untreated cells} - \text{Mean OD of treated cells}}{\text{Mean OD of untreated cells}} \times 100
\]

Graphs were plotted using % of cell viability at Y-axis and
centration of the sample in X-axis. Cell control and sample control
were included in each assay to compare the full cell viability assessments.
Based on MTT assay, IC₅₀ values obtained were 3.25 mg/mL for MVL
and 1.25 mg/mL for NMVL respectively, which were further employed to
assess their extent of effects on nucleus, morphology and apoptotic
effects of DNA.

PI staining

Propidium iodide (PI) staining was carried out by the method of
Siripur Natesh et al. [11]. KB cells were plated at a density of 5×10⁴ in
6-well plates. They were allowed to grow at 37°C in a humidified CO₂
incubator until they are 70-80% confluent. Then cells were treated
with 3.25 mg/mL for MVL and 1.25 mg/mL for NMVL for 24 hrs and
48 hrs. Culture medium was aspirated from each well and cells were
gently rinsed twice with PBS at room temperature, before fixing in
methanol: acetic acid (3:1 v/v) for 10 min, and stained with 50 µg/mL
PI for 20 min. Nuclear morphology of apoptotic cells with condensed/
fragmented nuclei was examined by fluorescence microscopy and at
least 1x10³ cells were counted for assessing apoptotic cell death.

AO/EB dual staining

Acrifine orange/Ethidium bromide staining was carried out by the
method of Siripur Natesh et al. [12]. KB cells were plated at a density of
1x10⁵ in 6-well plates. They were allowed to grow at 37°C in a humidified
CO₂ incubator until establishing 70-80% confluency. Then cells were
treated with 3.25 mg/mL for MVL and 1.25 mg/mL for NMVL (selected
based on the IC₅₀ concentration) of the test drugs for 24 hrs and 48 hrs.
The culture medium was aspirated from each well and cells were gently
rinsed twice with PBS at room temperature. Then equal volumes of cells
from control and drugs treated were mixed with 100 µL of dye mixture
(1:1) of ethidium bromide and acridine orange and viewed immediately
inverted fluorescence microscope at 20X magnification. A minimum of
300 cells were counted in each sample at two different fields. Three
century cells per sample were counted at each time points such as 24
hrs and 48 hrs. The cells were scored as viable or dead, and if dead,
whether by apoptosis or necrosis as judged from nuclear morphology
and cytoplasmic organization. The percentage of apoptotic and necrotic
cells were then calculated. Morphological features of interest were
photographed and calculated,

% of apoptotic cells = \frac{\text{total number of apoptotic cells}}{\text{total number of cells counted}} \times 100

Viable cells with organized structure were identified by green
fluorescence. Early apoptosis were presented with highly condensed
or fragmented yellow chromatins. The same, condensation and
fragmentation with orange chromatins were marked as late apoptosis.
Bright orange chromatin in round nuclei was appreciated in necrotic
cells. Membrane blebbing also indicates apoptosis.

Statistics

Numerical data are expressed as mean ± standard deviation (SD).
Statistical differences were evaluated by a one-way analysis of variance
(ANOVA) using statistical package for social sciences (SPSS) software
for window 9 Version 11.5 (SPSS Inc., Chicago, Ill, USA). Post Hoc test
was performed for comparisons using the least significant difference
(LSD) test, were considered statistically significant when P<0.05.

Results

Cell viability Assay

KB cells were incubated and tested with several tetrazolium salts.
Finally only the pale yellow MTT reagent generated the most expected
results by producing dark blue formazan. MTT reaction product was
only partially soluble in the medium, therefore an alcohol (isopropanol
– acid-alcohol mixture) was employed to dissolve the formazan to
produce a homogenous solution suitable for measuring the optical
density. Due to pH changes, a variable colour of phenol red was produced
by normal tissue culture medium which interfered with the wavelength
of blue MTT. At the end of the assay this was bypassed by converting
the phenol red to absolute yellow acidic form. After the final procedure,
MTT cleavage was achieved after 4 h (37°C). The OD was measured at


Volume 19: 2-6
Ganapathy P (2019) Anticancer potential of Siddha formulations against oral cancer cell line in vitro

The percentage of apoptotic nuclei after treating with 1.25 mg/mL NMVL (Figure 4) was more than in 3.25 mg/mL MVL treated group as compared to MVL.

Apoptosis enhanced in NMVL treated groups showing prominent cell decrement at 48 hours treatment marking compared to MVL. These observations were considered as morphological hallmarks of the terminal stage of apoptosis. KB cells response to NMVL was well marked compared to MVL. Apoptosis enhanced in NMVL treated groups showing prominent cell decrement at 48 hours treatment compared to MVL.

The percentage of apoptotic nuclei after treating with 1.25 mg/mL NMVL (Figure 4) was more than in 3.25 mg/mL MVL treated group (Figure 4), while appreciating the apoptotic changes on the DNA, the
fluorescence images with phase contrast images were merged to analyze the morphological changes over the cell membrane such as loss of integrity of the plasma membrane [16].

**MVL and NMVL impact on morphology through AO/EB staining after apoptosis**

Morphological features of apoptosis were further scrutinized by viewing through fluorescence microscopy after AO/EB dual staining. Figure 5 represents control, MVL and NMVL treated when the cell lines were administered with 3.25 mg/mL MVL and 1.25 mg/mL NMVL. Staining with AO and EB revealed condensed state of chromatin, fragmented nucleus, abnormal shape and size in KB cells when treated with 24 and 48 h while the control cells exhibited a normal cell morphology characterized by a diffused chromatin structure and whereas for light green (EB) staining shows pronounced discriminative results. The percentage of apoptotic cells after 24 hrs and 48 hrs treatment with 3.25 mg/mL of MVL and 1.25 mg/mL of NMVL increased (p≤0.05) drastically to 41% and 61% in case of MVL and 31% and 75% respectively.

**Discussion**

Oral and tongue cancer is an inordinate, complex multifocal process that mainly halted in three main anatomical sites of an oral cavity by causing several genetic alterations [17]. Chemotherapy, an expensive, most intended cancer treatment not only provoked serious side effects but also enormously worsens the financial stability of a family thereby affects the overall province of a country. Ever since olden days, herbal based medicines are most preferred for curing various challenging ailments [18,19]. Currently indigenous medicinal therapy is commonly in practice by CAM practitioners. MVL constitutes more than 34 different botanicals while NMVL bears more than 20 daily used herbs in it and was used against various chronic ailments. So far studies reporting anticancer property of these two drugs are lacking. Therefore, their crude and fractioned extracts were employed to evaluate their cytotoxicity and antiproliferative activity on KB cell lines.

The outcome of cytotoxicity assay using MTT in this study is clearly revealed through the respective IC₅₀ dosage values. Crude extracts of both test drugs were employed to know whether the formulation is toxic to even native cells. MTT results of MVL revealed 85% of viability at 1.25 mg/mL concentration and 50% viability at 3.25 mg/mL when compared to the standard anti-cancer drug Doxycrubcin. Previous reports supports our current outcome that the components of NMVL possess anticancer, antioxidant, anti-inflammatory and analgesic properties [20-23]. NMVL could exert 50% viability even at the minimum concentration, while MVL could exert only at an added dosage. Thus both the drugs were capable of interfering in cell proliferation and induce cell mortality at various concentrations. The cells that underwent programmed cell death appeared floating in the culture medium as they lost their adhering property and most of the cells got detached from the surface of the culture plates. Anoikis - early detachment from their basal membrane is one of the prime features observed in monolayer adherent cells during apoptosis [24]. The morphological changes on the apoptotic cell surfaces indicates that whatever apoptosis induction may be initiated, once if the apoptosis begins, the cells starts to follow similar morphological changes [25]. Cells that underwent apoptosis revealed typical features such as membrane blebbing, nuclear condensation, formation of pyknotic bodies and chromatin cleavage [14]. These distinctive hallmark morphological features are widely used for identification and quantification of apoptosis [15]. Therefore, determination of the morphological changes to define apoptosis was visualized using inverted phase contrast microscope. On delayed hours of exposure of KB cells with the test formulations, they underwent necrosis which was evaluated based on changes that affected the plasma membrane [26], the present study forwarded to analyze the morphological changes cytologically using Propidium iodide (PI) via fluorescence microscope.

The dye is permeable to cell nuclei and is useful to identify chromatin condensation and fragmentation in the apoptotic cell nuclei. The red fluorescence emitting propidium iodide is a cell impairment DNA-binding dye which means the dye entry is qualified only when there is decrease in sturdiness and increase in porosity of the plasma membrane [27]. As observed in control or untreated cells appeared to be intact in oval shape and the nuclei appeared less fluorescent or absence of red fluorescence. Necrotic cells were swollen and possess irregular membranes and fluorescent bright pink stained chromatin. Due to weak membrane integrity, PI seeped into intact, even shrunken cells...
and apoptotic dead cell nuclei which emitted bright pink on condensed and fragmented chromatin [24].

Most of the chemotherapeutic agents used in cancer treatment induce a gene regulated phenomenon called apoptosis which in turn induces in chromatin condensation, nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation [28,29]. The dye acridine orange surpasses into all the cells and makes the nuclei appear green, whereas ethidium bromide stains the nuclei red only when the integrity of cytoplasmic boundary is lost [30]. Thus only live cells display normal green nuclei. Early apoptotic cells with fragmented or condensed chromatin revealed bright green nuclei while late apoptotic cells appeared condensed orange chromatin fragments, whereas cells that have died from direct necrosis display themselves with normal orange nucleus [31]. The results of AO/EB staining also exhibited a high number of apoptotic cells on treatment with MVL, NMVL compared with control cells. The increased ROS levels and depletion in ATP changes were reported by Zorov et al. [32]. Another major add on factor could be NMVL constitutes heavy metals such as mercury and sulfur in its ingredients which could have initiated sustained release, exerting a higher percentage of apoptotic cells [33,34].

Conclusion

Thus, it can be concluded that in this preliminary study by research analysis, the use of MVL and NMVL has caused a remarkable effect in oral cancer lines within short duration (24 hours treatment) and minimal IC50 concentrations. Therefore, both MVL and NMVL have naturally occurring antioxidants which has the ability to induce apoptosis possibly through caspase-3 intrinsic signaling pathway. Further, MVL, NMVL holds great promise for the use in chemopreventive and chemotherapeutic strategies. Nevertheless, further investigations are necessary to validate its therapeutic claims to determine its effects in in-vivo models also.

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