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Corresponding Author:
Vasanthi V, SRM Dental College,
Bharati Salai, Ramapuram,
Chennai-600089, Tamil Nadu, India
E-mail: drvasanthivinoth@gmail.com

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Antitumoral Efficacy of *Vitex negundo* in Oral Cancer: An *In vitro* Study

Ashwini R[1], Vasanthi V[1], Rajkumar Krishnan[1], Gopikrishna Padmanabhan[1], Shruthi V[1]

[1]SRM Dental College, Bharati Salai, Ramapurum, Tamil Nadu, India

Abstract

Background: Oral squamous cell carcinoma (OSCC) accounts for approximately 90% of all oral cancer cases. Cytotoxic chemotherapeutic drugs interfere with the replication and synthesis of DNA, promoting the death of cancer cells and normal cells as well. Researchers have extracted a number of bioactive substances including volatile oils, flavonoids, lignans, iridoids, and terpenes from the leaves, seeds, and roots of *Vitex negundo*. Anti-inflammatory, antioxidant, anticancer, and antibacterial properties are displayed by these bioactive substances. The present study proposed to establish the cytotoxic, apoptotic activity of crude ethanolic extract of *Vitex negundo* (VN) leaves on an oral cancer cell line.

Methods: The OSCC cell line was exposed to different concentrations of crude ethanolic extract of VN leaves and cisplatin for 24, 48, and 72 hours. The MTT assay was done to check for cell viability. IC_{50} was determined. The AO/PI assay for apoptosis was done at IC50 after 72 hours. Early and late apoptotic changes were observed in VN-treated and cisplatin-treated cells.

Results: In the human OSCC cell line, the percentage of viable cells decreased from 92.33 to 21.08 after 24 hours as the concentration increased from 20 µg/ml to 100 µg/ml. Cell viability decreased from 71.20% to 17.89% after 48 hours and from 61.40% to 14.75% after 72 hours. VN-treated cells were predominantly in the early stage of apoptosis with chromatin changes (yellow-green nucleus) as compared to cisplatin on AO/PI staining.

Conclusions: Crude ethanolic extract of VN leaves had similar cytotoxic and apoptotic efficacy to cisplatin on the OSCC cell line.

Keywords: apoptosis, cytotoxicity, oral squamous cell carcinoma cell line, *Vitex negundo*

Introduction

Head and neck cancer is the seventh most common cancer in the globe with a high incidence in Southeast Asian countries. Over 650,000 cases and 330,000 mortalities worldwide are attributed to HNCs on an annual basis.[1] Oral cancer is regarded as a multifactorial disease caused by environmental, genetic, and epigenetic factors. Oral squamous cell carcinoma accounts for approximately 90% of all oral cancer cases. The incidence and mortality of oral cancer in developing countries is showing a dramatic increase due to habits such as smoking, betel quid chewing, use of smokeless tobacco, and alcohol intake.[2] Oral cancer is the most common cancer predominantly caused by the use of tobacco in middle-aged males in India. Oropharyngeal cancer is reported in patients with human papillomavirus (HPV) infection. India contributes one-third to the global cancer burden, which may be attributed to the fact that the diagnosis is usually made in the advanced stage. West Bengal is leading with the highest number of oral cancer cases, and the lowest number of cases are reported from Kerala.[3]

Chemical and microbial carcinogens dysregulate the cellular signaling pathways, cellular metabolism, cellular differentiation, apoptosis, senescence, angiogenesis, immune response, and inflammation. Molecular upregulation of proto-oncogenes, and downregulation of tumor suppressor genes lead to genetic instability. Besides these genetic causes, certain epigenetic alterations such as DNA methylation, histone modification, and post-translational modification of non-coding RNAs also contribute to carcinogenesis.[4] Oncogenic pathways, namely the mitogen-activated protein kinase (MAPK) pathway, epidermal growth factor receptor (EGFR) pathway, phosphatidylinositol 3 kinase/ mammalian target of rapamycin (PI3K/ mTOR) pathway, Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, and suppressor pathways such as p53/p16/retinoblastoma pathway are deregulated in oral carcinogenesis.[4-6]

Surgery is the most recommended course of treatment for oral cancer, used alone or in combination with radiotherapy and/or chemotherapy. Multimodality therapy with radiotherapy and chemotherapy is advised depending upon the surgical accessibility, positive tumor margin, locally advanced cancer, nodal involvement, and metastasis.[7] All the therapeutic methods have their own advantages and disadvantages. Chemotherapy may be administered before as induction chemotherapy to reduce the size, during definitive treatment as concomitant chemotherapy, or after definitive treatment as adjuvant chemotherapy. Cytotoxic chemotherapeutic drugs interfere with the replication and synthesis of DNA, promoting the death of cancer cells and normal cells as well.[8]

Chemotherapeutic drugs either target the enzymes within the cell or change the metabolism of cells. The concept of chemotherapy initially emerged after systemic mustard gas poisoning damaged the bone marrow and lymphatic tissues. Following this, an animal study using nitrogen mustard to target lymphoma was conducted in mice to prove this hypothesis. Later, nitrogen mustard was administered to a 48-year-old patient at Yale University to treat lymphosarcoma, which paved the way for the chemotherapy regimen. Numerous side effects have been reported from the duration and dosage, as they harm the healthy cells amidst the cancer cells. Bone marrow suppression and hematologic toxicity are a few major adverse effects of chemotherapy.[9]

Traditional medicine has been proven to have medicinal value with fewer side effects as compared to allopathic drugs. It has been reported that more than 10,000 studies from India are related to the antioxidant and antidiabetic efficacy of medicinal plants.[10] Natural extracts were considered to have a chemotherapeutic effect as they interfered with the formation of microtubules and were able to disrupt microtubule assembly. In the late 1950s, antimitotic extract from *Catharanthus roseus* was introduced as vinca alkaloids.[11] Phytochemicals have been reported to exert targeting the molecular mechanisms. Few studies have demonstrated that the traditional herbs exert antitumor efficacy by inducing cytotoxicity, promoting apoptosis, regulating epigenetic modifications, and inhibiting metastasis along with their antioxidant and anti-inflammatory activity.[12]

Different species of *Vitex*, such as *Vitex agnus-castus, Vitex rotundifolia, Vitex trifolia,* and *Vitex negundo,* have been documented to have medicinal values. *Vitex negundo* (VN), also called Nirgundi or five-leaved chaste tree, is a member of the Verbenaceae family. VN is a tenuous shrub or small tree that can grow to a maximum height of 5 meters with quadrangular branches. Leaves of VN are lanceolate with three to five leaflets, fruits are black, and flowers are bluish-purple. The entire plant has therapeutic properties and is used in Ayurveda, Chinese, Siddha, and

Unani to treat multiple ailments as a sarvaroganivarani for all diseases. Leaves have been reported to have antibacterial, anti-inflammatory, antihistaminic, antidiabetic, antioxidant, and anticancer action. Crude extracts of VN and bioactive compounds were proven to induce apoptosis, inhibit angiogenesis, and induce cytotoxicity by targeting molecular pathways in lung cancer, breast cancer, ovarian cancer, and colon cancer cell lines.[13] The present study aimed to evaluate the cytotoxic and apoptotic activities of crude ethanolic extract of VN leaves on an oral cancer cell line.

Methodology

Ethical approval

The study was approved by the Institutional Review Board (SRMDC/IRB/2021/MDS/NO.605)

Identification of the species and procurement of VN leaves

Fresh, mature leaves of VN, authenticated from Pandit Jawaharlal Nehru College of Agriculture and Research Institute, Karaikal, were used.

Preparation of crude ethanolic extract of VN leaves

Procured leaves were shade-dried for a period of two weeks and coarsely powdered. The extraction process was done using a Soxhlet apparatus. 50 mg of the powder was weighed and packed into a thimble made from filter paper. 200 ml of 90% ethanol was added to the extraction flask, and the temperature was set at 80°C, as the boiling point of ethanol is approximately 78°C-79°C. The process of extraction was repeated for 24 hours. The solvent was removed using a rotary vacuum evaporator, and the concentrate was stored at 4°C for further studies.[14]

Cell culture

The human oral keratinocyte cell line and the human oral squamous cell carcinoma cell line were procured from NCCS, Pune. Cells were cultured using DMEM supplemented with 10% FBS and antibiotics (penicillin, streptomycin, amphotericin B) in humidified 5% carbon dioxide at 37°C until 80% confluent.

Cell viability assay—MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay—oral cancer cells

The MTT assay was done to determine the viability of cells after treatment with different concentrations of crude ethanolic extract of VN leaves. 1.5×10^4 cells/well were seeded into 96-well plates. These seeded cells were treated with five concentrations of crude ethanolic extract of VN leaves (20, 40, 60, 80, and 100 µg/ml) at 37°C for 24, 48, and 72 hours. At the end of each incubation period, a working solution of 20 µl of MTT reagent was added to each well and further incubated for 4 hours. 100 µl of DMSO was then added to each well, and the absorbance was read using an ELISA reader at 570 nm. The experiment was done in triplicate. Cisplatin (2, 4, 6, 8, 10 µg/ml) was used as a positive control. Untreated cells were used as a negative control. Percentage of viable cells was calculated using the formula[15],

$$\text{Cell viability \%} = \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

Cell viability assay—MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay—human oral keratinocytes

The percentage of viability of human oral keratinocyte cells was also identified after treatment with five concentrations of crude ethanolic extract of VN leaves (25, 50, 100, 150, and 200 µg/ml) at 37°C for 24, 48, and 72 hours. This was done to prove that the crude ethanolic extract of VN leaves was not cytotoxic to normal cells.

Inhibitory concentration (IC_{50}) identification by standard curve method

The IC_{50} value (half-maximal concentration 50%) was calculated for crude ethanolic extract of VN leaves and cisplatin by the standard curve method. A scatter plot was drawn using concentration on the x-axis and cell viability on the y-axis. A linear trendline was drawn, and the R-squared value with equation was noted. IC_{50} was determined by four parametric logistic equations for 24 hours, 48 hours, and 72 hours for crude ethanolic extract of VN leaves and cisplatin.[16-18]

Acridine Orange/Propidium Iodide (AO/PI) dual staining—assessment of apoptotic activity

AO/PI dual staining was done to evaluate the apoptotic activity of crude ethanolic extract of VN leaves. Wells containing the test and control group at IC_{50} concentration were washed with PBS and stained with 16 µl of dual fluorescent staining solution containing 100 µg/ml AO and 100 µg/ml PI (AO/PI, Sigma, St. Louis, MO). The mix was allowed to diffuse and enter into the cells for 5 min. The stained cells were viewed under a fluorescence microscope (Invitrogen EVOS FL imaging; 40X magnification). Both the viable and dead cells were permeated by AO, and the nuclei appeared green under a fluorescent microscope. The nuclei of dead cells whose cytoplasmic integrity was lost appeared red from PI staining. Thus, nuclei of viable cells showed green fluorescence, early apoptotic cells with fragmented chromatin showed yellow-green fluorescence, late apoptotic cells showed orange fluorescence, and dead cells fluoresced red.[19,20]

Statistical analysis

The results were analyzed for statistical significance using Statistical Package for Social Sciences (SPSS) software version 16. Statistical significance was set at $p<0.05$. Mean and standard deviation were calculated for the triplicate absorbance values of the MTT assay. The percentage of viable cells was calculated from the mean absorbance value. Mann-Whitney U test was done to compare the mean of cell viability between the VN and cisplatin-treated cells. The Friedman Kruskal-Wallis test was done to compare the difference in mean for each concentration after 24 hours, 48 hours, and 72 hours.

Results

MTT cell viability assay

The cytotoxic efficacy of crude ethanolic extract of VN leaves after 24 hours, 48 hours, and 72 hours was determined at different concentrations in the human oral keratinocyte cell line and the human oral squamous cell carcinoma cell line. In the human oral keratinocyte cell line, the cell viability after 72 hours was 88.59% at a concentration of 200 µg/ml of VN leaves (Figure 1). Thus, the cytotoxic effects were tumor-specific.

In the human OSCC cell line, the cell viability decreased from 92.33% to 21.08% after 24 hours as the concentration increased from 20 µg/ml to 100 µg/ml. The cell viability decreased from 71.20% to 17.89% after 48 hours and from 61.40% to 14.75% after 72 hours (Figure 2).

The cell viability was also determined with different concentrations of cisplatin after 24 hours, 48 hours, and 72 hours. The percentage of viable cells decreased from 90.81% to 20.36% after 24 hours, 85.68% to 17.18% after 48 hours, and 72.43% to 14.09% after 72 hours (Figure 3). As described in Table 1 & Table 2, at a concentration of 20 µg/ml of the crude ethanolic extract of VN leaves, cell viability was 92.33%, 71.2%, and 61.4% after 24, 48, and 72 hours, respectively. The difference was not statistically significant (p=0.241) (N=3). 74.47%, 65.04%, and 52.37% were the percentages of viable cells at a concentration of 40 µg/ml of VN after 24, 48, and 72 hours. The difference did not show any statistical significance (p=0.142) (N=3). Similarly, at 60 µg/ml, 51.7%, 47.23%, and 40.67% percent of cells were viable after exposure to VN leaves after 24, 48, and 72 hours with statistical significance (p=0.001) (N=3). Again at 80 µg/ml, the difference was statistically significant (p=0.002) (N=3) with 31.725, 28.93%, and 26.84% percent of viable cells. The difference was also statistically significant (p=0.002) (N=3) at 100 µg/ml after 24, 48, and 72 hours, with 21.08%, 17.89%, and 14.75% percent of viable cells, respectively.

This concentration-dependent cytotoxicity was also assessed for cisplatin for comparison (Table 3 & Table 4). At a concentration of 2 µg/ml, the percentage of viable cells was 90.81%, 85.68%, and 72.43% after 24, 48, and 72 hours, respectively. On comparison, the difference was not statistically significant (p=0.15) (N=3). At 4 µg/ml, 72.21%, 69.21%, and 41.12% were the viability percentages with a statistically significant difference (p=0.02) (N=3). Similarly, a significant difference (p=0.02) (N=3) was found at 6 µg/ml, with viability of 40.1%, 39.22%, and 33.92% after 24, 48, and 72 hours. At 8 µg/ml, the viability was 26.94%, 25.13%, and 22.79% with a significant difference (p=0.01) (N=3). At the final concentration of 10 µg/ml also, the difference was significant (p=0.002) (N=3) with viability of 20.36%, 17.18%, and 14.09%.

To prove the time-dependent cytotoxic efficacy, the viability was also compared after 24, 48, and 72 hours between different concentrations of VN and cisplatin (Figure 5). The first concentration was 20 µg/ml of VN leaves crude ethanolic extract and 2 µg/ml of cisplatin.

The second concentration was 40 µg/ml of VN and 4 µg/ml of cisplatin. The third concentration was 60 µg/ml of VN and 6 µg/ml of cisplatin. The fourth concentration was 80 µg/ml of VN and 8 µg/ml of cisplatin. The fifth concentration was 100 µg/ml of VN and 10 µg/ml of cisplatin. The difference was significant for the 3rd (p=0.04) (N=3) and 4th (p=0.03) (N=3) concentrations among 5 different concentrations of VN and cisplatin after 24 hours. After 48 hours, the 1st (p=0.03), 2nd (p=0.02), and 3rd (p=0.02) (N=3) concentrations of VN and cisplatin showed statistical significance. After 72 hours, again, 1st (p=0.01), 2nd (p=0.01), and 3rd (p=0.04) (N=3) were statistically significant (Table 5).

Inhibitory Concentration (IC_{50})

The IC_{50} value was determined for VN and cisplatin after 24, 48, and 72 hours. For VN, IC_{50} was 64.83 µg/ml, 54.47 µg/ml, and 41.829 µg/ml after 24, 48, and 72 hours, respectively. Similarly, 5.65 µg/ml, 5.77 µg/ml, and 4.05 µg/ml were the IC_{50} values of cisplatin after 24, 48, and 72 hours, respectively.

Apoptotic assay - AO/PI dual staining

The cells at IC_{50} were treated with AO/PI stain to detect the morphological change of apoptosis. Cells treated with control did not show any apoptotic change (green) (Figure 4a). Most of the cisplatin-treated cells were either in the early (yellow-green) or late stage (orange) of apoptosis (Figure 4b). VN-treated cells were predominantly in the early stage of apoptosis with chromatin changes (yellow-green nucleus) as compared to cisplatin (Figure 4c).

Discussion

Oral cancer has been reported to be the ninth cause of mortality from cancer on a global basis. Independent risk factors for oral and oropharyngeal cancer include smoking forms of tobacco, smokeless forms of tobacco, and HPV infection. The incidence of oral cancer has increased from 4.28/100,000 to 4.52/100,000 in the last two decades at the global level. Among the Southeast Asian countries, India was the top ranker with the highest burden of oral cancer, as per data from the International Agency for Research on Cancer (IARC). Unfortunately, most of the patients are diagnosed at their advanced stage with only a 5-year survival period. Various time intervals, such as patient interval, diagnostic interval, pre-treatment, and treatment interval, have been reported in the diagnosis and management of oral cancer. The mortality rate from oral cancer can be considerably decreased by significantly reducing the time period of these intervals.[21-23] Cancer therapeutics such as surgery, radiotherapy, and chemotherapy also contribute to the morbidity, indirectly affecting the quality of life and survival rate.[24]

Surgical management is the primary treatment of choice for oral cancer; however, radiotherapy remains the standard treatment for inaccessible tumor sites and surgically unfit patients. Literature suggests that chemo-

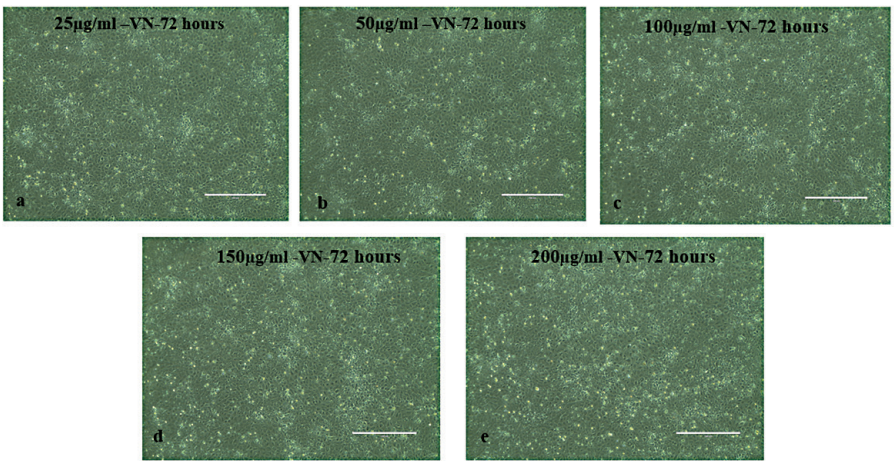

Figure 1: (a), Human oral keratinocyte cell line treated with 25 µg/ml of VN: (b), Human oral keratinocyte cell line treated with 50 µg/ml of VN: (c), Human oral keratinocyte cell line treated with 100 µg/ml of VN: (d), Human oral keratinocyte cell line treated with 150 µg/ml of VN: (e), Human oral keratinocyte cell line treated with 200 µg/ml of VN (scale bar - 100 µm).

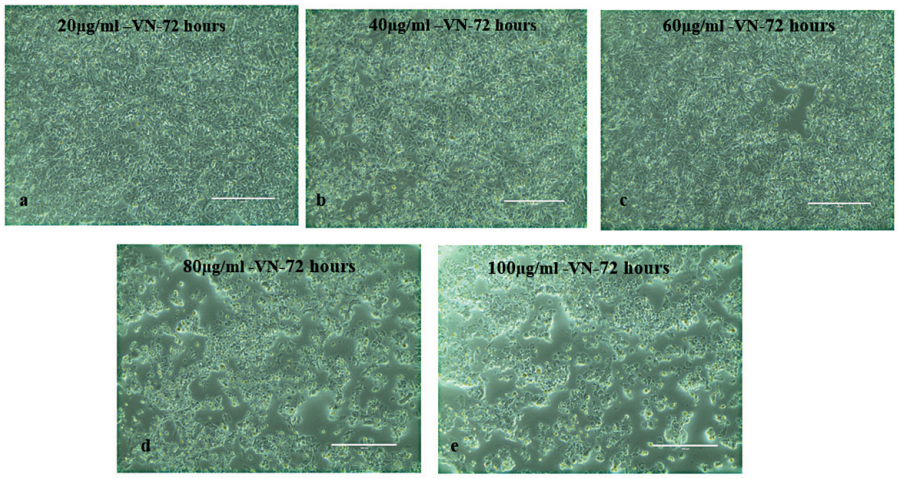

Figure 2: (a), Human oral squamous cell carcinoma cell line treated with 20 µg/ml of VN: (b), Human oral squamous cell carcinoma cell line treated with 40 µg/ml of VN: (c), Human oral squamous cell carcinoma cell line treated with 60 µg/ml of VN: (d), Human oral squamous cell carcinoma cell line treated with 80 µg/ml of VN: (e), Human oral squamous cell carcinoma cell line treated with 100 µg/ml of VN (scale bar - 100 µm).

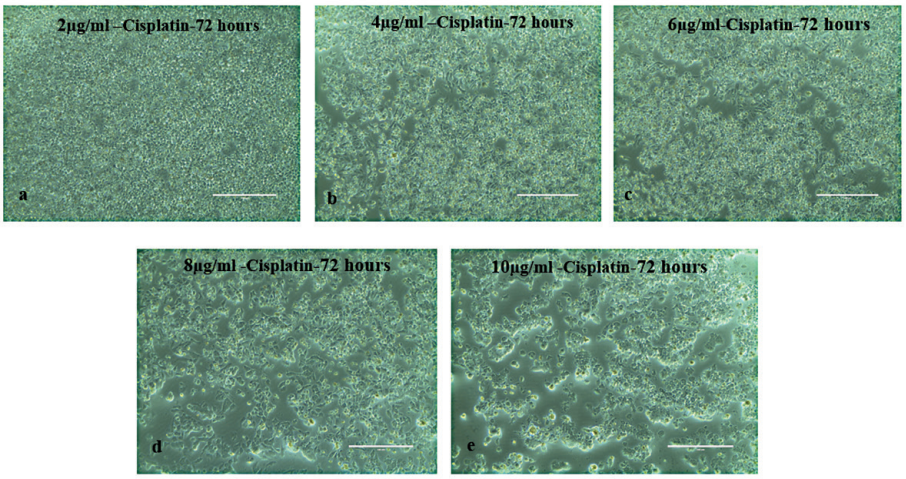

Figure 3: (a), Human oral squamous cell carcinoma cell line treated with 2 µg/ml of cisplatin: (b), Human oral squamous cell carcinoma cell line treated with 4 µg/ml of cisplatin: (c), Human oral squamous cell carcinoma cell line treated with 6 µg/ml of cisplatin: (d), Human oral squamous cell carcinoma cell line treated with 8 µg/ml of cisplatin: (e), Human oral squamous cell carcinoma cell line treated with 10 µg/ml of cisplatin (scale bar - 100 µm).

radiotherapy can be considered for advanced stages of locoregional head and neck cancer.[25] A study by Sarma *et al*., compared the complications of management of oral cancer with and without neoadjuvant chemotherapy. The study reported that patients who received two to three cycles of neoadjuvant chemotherapy prior to surgical resection for tumor shrinkage had better outcomes than the control group.[26] Though chemotherapy is a blessing for patients with advanced cancer, systemic adverse effects affect the quality of life of the patients, thus prompting the need for less toxic, effective phytochemical alternatives for cancer management. Traditional herbs are being studied in recent days for anticancer activity.

VN has been proven in literature to have potent antimicrobial, antioxidant, anti-inflammatory, anti-osteoporotic, anti-androgenic, anti-diabetic, anti-tumoral, and hepatoprotective efficacy. Flavonoids, terpenoids, steroids, and lignans have been reported to be the four major classes of compounds from VN. Almost 120 compounds have been isolated from VN belonging to these four major classes.[27] Vitexin, vitexicarpin, triterpenoids, lagundinin, nishindaside, betulinic acid, negun-

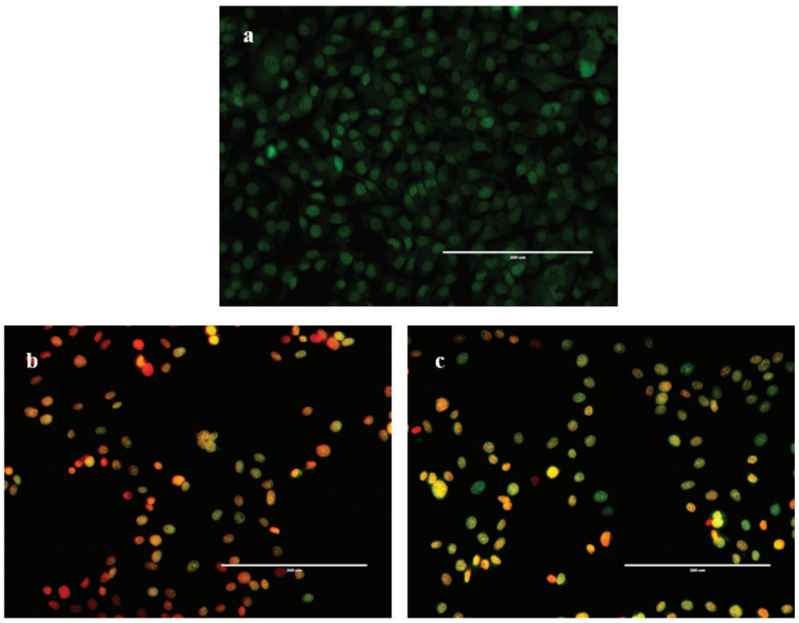

Figure 4: (a), Cells treated with control did not show any apoptotic change (green): (b), Cisplatin-treated cells either in the early (yellow-green) or late stage (orange) of apoptosis: (c), VN-treated cells in the early stage of apoptosis (yellow-green nucleus) (scale bar - 200 µm).

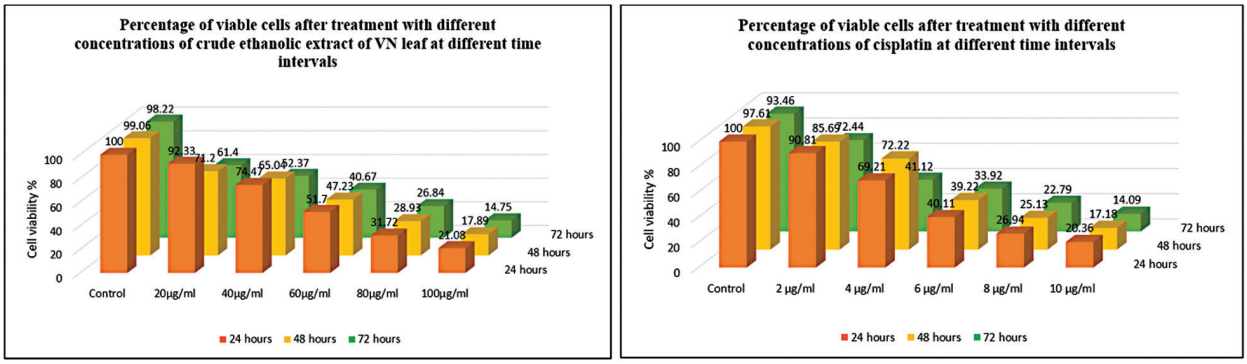

Figure 5: Cell viability following treatment with different concentrations of crude ethanolic extract of VN leaf after 24 hours, 48 hours, and 72 hours; Cell viability following treatment with different concentrations of cisplatin after 24 hours, 48 hours, and 72 hours.

doside, diterpene, β-caryophyllene, vitexoside, vitexdoin, vitegnoside, ursolic acid, and vitelignin are a few active phytoconstituents of VN leaves.[27-30]

Species of Vitex have been documented to target the PI3K/Akt pathway, JAK/STAT pathway, Wnt/β-catenin pathway, MAPK pathway, and NF-κB pathway. The extracts were also stated to activate caspases, activate pro-apoptotic proteins, inhibit anti-apoptotic proteins, upregulate cytochrome c, downregulate MMP-9, induce cell cycle arrest, induce apoptosis, inhibit viability of cells, inhibit epithelial mesenchymal transition, and prevent invasion and migration. Vitexicarpin/casticin from VN has been proven through *in vitro* studies to exert antitumorigenic effects on breast cancer, bladder cancer, cervical cancer, colon cancer, hepatocellular cancer, leukemia, esophageal cancer, lung cancer, ovarian cancer, prostate cancer, and melanoma.[31-32]

Xin *et al*., reported that lignans from VN augmented histone phosphorylation, Cdk1 phosphorylation, increased expression of cyclin B1, induced cell cycle arrest at G2/M phase, and induced apoptosis in various cancer cell lines.[33] Vo *et al*., explored the anti-tumorigenic and apoptotic

Table 1: Percentage of viable oral squamous cell carcinoma cells following treatment with different concentrations of crude ethanolic extract of VN leaf after 24 hours, 48 hours and 72 hours.

24 hrs	Control	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	100 µg/ml
	0.759	0.698	0.568	0.395	0.243	0.164
	0.752	0.697	0.561	0.392	0.235	0.152
	0.746	0.689	0.552	0.38	0.238	0.16
Mean	0.752333	0.694667	0.560333	0.389	0.238667	0.158667
SD	0.006506	0.004933	0.008021	0.007937	0.004041	0.00611
Cell Viability (%)	**100**	**92.33**	**74.47**	**51.70**	**31.72**	**21.08**
48hrs	Control	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	100 µg/ml
	0.746	0.535	0.489	0.356	0.218	0.136
	0.745	0.537	0.489	0.356	0.217	0.133
	0.745	0.535	0.49	0.354	0.218	0.135
Mean	0.745333	0.535667	0.489333	0.355333	0.217667	0.134667
SD	0.000577	0.001155	0.000577	0.001155	0.000577	0.001528
Cell Viability (%)	**99.06**	**71.20**	**65.04**	**47.23**	**28.93**	**17.89**
72hrs	Control	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	100 µg/ml
	0.738	0.463	0.394	0.305	0.202	0.112
	0.738	0.463	0.393	0.307	0.201	0.113
	0.739	0.462	0.394	0.306	0.202	0.111
Mean	0.738333	0.462667	0.393667	0.306	0.201667	0.112
SD	0.000577	0.000577	0.000577	0.001	0.000577	0.001
Cell Viability (%)	**98.22**	**61.40**	**52.37**	**40.67**	**26.84**	**14.75**

Table 2: Comparison of mean viability of oral squamous cell carcinoma cells with different concentrations of crude ethanolic extract of VN leaf after 24 hours, 48 hours, and 72 hours.

Concentration of crude ethanolic extract of VN leaf	Time interval	Percentage of viable cells (%)	*p*-value
Control	24 hours	100	0.312
	48 hours	99.06	
	72 hours	98.22	
20µg/ml	24 hours	92.33	0.241
	48 hours	71.2	
	72 hours	61.4	
40µg/ml	24 hours	74.47	0.142
	48 hours	65.04	
	72 hours	52.37	
60µg/ml	24 hours	51.7	0.001*
	48 hours	47.23	
	72 hours	40.67	
80µg/ml	24 hours	31.72	0.002*
	48 hours	28.93	
	72 hours	26.84	
100µg/ml	24 hours	21.08	0.002*
	48 hours	17.89	
	72 hours	14.75	

Friedman Kruskal-Wallis test - *statistically significant (*p*-value less than 0.05).

Table 3: Percentage of viable oral squamous cell carcinoma cells following treatment with different concentrations of cisplatin after 24 hours, 48 hours and 72 hours

24hrs	Control	2 µg/ml	4 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml
	0.756	0.687	0.553	0.301	0.203	0.152
	0.749	0.686	0.542	0.298	0.205	0.157
	0.759	0.683	0.54	0.309	0.202	0.152
Mean	0.75467	0.68533	0.545	0.30267	0.20333	0.15367
SD	0.00513	0.00208	0.007	0.00569	0.00153	0.00289
Cell Viability (%)	**100**	**90.81**	**72.21**	**40.10**	**26.94**	**20.36**
48hrs	Control	2 µg/ml	4 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml
	0.725	0.647	0.526	0.297	0.192	0.134
	0.738	0.641	0.521	0.295	0.181	0.126
	0.747	0.652	0.52	0.296	0.196	0.129
Mean	0.73667	0.64667	0.52233	0.296	0.18967	0.12967
SD	0.01106	0.00551	0.00321	0.001	0.00777	0.00404
Cell Viability (%)	**97.61**	**85.68**	**69.21**	**39.22**	**25.13**	**17.18**
72hrs	Control	2 µg/ml	4 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml
	0.706	0.547	0.304	0.267	0.172	0.104
	0.705	0.541	0.312	0.245	0.176	0.106
	0.705	0.552	0.315	0.256	0.168	0.109
Mean	0.70533	0.54667	0.31033	0.256	0.172	0.10633
SD	0.00058	0.00551	0.00569	0.011	0.004	0.00252
Cell Viability (%)	**93.46**	**72.43**	**41.12**	**33.92**	**22.79**	**14.09**

Table 4: Comparison of mean viability of oral squamous cell carcinoma cells with different concentrations of cisplatin after 24 hours, 48 hours, and 72 hours.

Concentration of cisplatin	Time interval	Percentage of viable cells (%)	*p*-value
Control	24 hours	100	0.11
	48 hours	97.61	
	72 hours	93.46	
2µg/ml	24 hours	90.81	0.15
	48 hours	85.68	
	72 hours	72.43	
4µg/ml	24 hours	72.21	0.02*
	48 hours	69.21	
	72 hours	41.12	
6µg/ml	24 hours	40.1	0.02*
	48 hours	39.22	
	72 hours	33.92	
8µg/ml	24 hours	26.94	0.01*
	48 hours	25.13	
	72 hours	22.79	
10µg/ml	24 hours	20.36	0.002*
	48 hours	17.18	
	72 hours	14.09	

Friedman Kruskal-Wallis test - *statistically significant (*p*-value less than 0.05).

Table 5: Comparison of time-dependant cytotoxic efficacy of crude ethanolic extract of VN and cisplatin .

Time interval	Concentration	Cell viability %	*p*-value
24 hrs	20µg/ml of VN leaf crude ethanolic extract	92.33	0.29
	2µg/ml of cisplatin	90.81	
	40µg/ml of VN leaf crude ethanolic extract	74.47	0.06
	4µg/ml of cisplatin	72.21	
	60µg/ml of VN leaf crude ethanolic extract	51.7	0.04*
	6µg/ml of cisplatin	26.94	
	80µg/ml of VN leaf crude ethanolic extract	31.72	0.03*
	8µg/ml of cisplatin	26.94	
	100µg/ml of VN leaf crude ethanolic extract	21.08	0.26
	10µg/ml of cisplatin	20.36	
48 hrs	20µg/ml of VN leaf crude ethanolic extract	71.2	0.03*
	2µg/ml of cisplatin	85.68	
	40µg/ml of VN leaf crude ethanolic extract	65.04	0.02*
	4µg/ml of cisplatin	69.21	
	60µg/ml of VN leaf crude ethanolic extract	47.23	0.02*
	6µg/ml of cisplatin	39.22	
	80µg/ml of VN leaf crude ethanolic extract	28.93	0.13
	8µg/ml of cisplatin	25.13	
	100µg/ml of VN leaf crude ethanolic extract	17.89	0.32
	10µg/ml of cisplatin	17.18	
72 hrs	20µg/ml of VN leaf crude ethanolic extract	61.4	0.01*
	2µg/ml of cisplatin	72.43	
	40µg/ml of VN leaf crude ethanolic extract	52.37	0.01*
	4µg/ml of cisplatin	41.12	
	60µg/ml of VN leaf crude ethanolic extract	40.67	0.04*
	6µg/ml of cisplatin	33.92	
	80µg/ml of VN leaf crude ethanolic extract	26.84	0.07
	8µg/ml of cisplatin	22.79	
	100µg/ml of VN leaf crude ethanolic extract	14.75	0.12
	10µg/ml of cisplatin	14.09	

Mann-Whitney U test - *statistically significant (*p*-value less than 0.05).

efficacy of compounds from VN (vitexicarpin, penduletin, and artemetin) in human breast cancer and liver cancer cell lines.[34] Awale *et al*., tested the cytotoxic efficacy of chrysoplenetin, the active constituent of VN, against a panel of 39 human cancer cell lines and the ethanolic extract of VN against a human pancreatic cell line. This study reported that the chrysoplenetin compound from VN induced apoptosis and cytotoxicity in the cell lines.[35]

The cytotoxic efficacy of leaves of *Vitex rotundifolia* was analyzed by Chaudhry *et al*.,[36] in a breast cancer cell line. Crude methanolic extract and seven fractions were prepared from the leaves, and an MTS assay was done in their study. Seven different concentrations of extract and fractions were evaluated for cytotoxic efficacy after 24 hours, 48 hours, and 72 hours. The Annexin V/PI staining assay was done to prove the apoptotic change in morphology of the cells induced by *Vitex*. IC_{50} after 72 hours was found to be 79.43 µg/ml, and at this concentration apoptotic changes were also noticed. Aslanturk *et al*.,[37] evaluated the cytotoxic, apoptotic, and antioxidant activity of crude extract from seeds of *V. agnus-castus* on the MCF-7 breast cancer cell line by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. Methanol, diethyl ether, petroleum ether, ethyl acetate, and aqueous extracts were compared for potent anticancer activity. Methanol and aqueous extracts had better antioxidant activity than other

extracts. The IC_{50} was 83.47 µg/ml with methanol extract. All the extracts had similar cytotoxic and apoptotic activity on the MCF-7 breast cancer cell line. Ibrahim *et al*. evaluated the antioxidant, antitumoral, and anti-inflammatory efficacy of *V. agnus-castus* fruit extract. The inhibition of COX-2 was dose-dependent, and the anti-inflammatory activity was correlated to the anti-tumorigenic efficacy owing to the decreased prostaglandin production.[38] Similarly, Ilhan *et al*. studied the apoptotic activity of essential oil from leaves of *V. agnus-castus* on a multi-drug resistant lung carcinoma cell line and normal human cells also to prove the specific action on tumor cells. The study concluded that both intrinsic and extrinsic apoptotic pathways were activated and the expression of Bcl-2, Bax, Bad, caspases, and TRAIL were modulated by *V. agnus-castus*.[39] Gong *et al*.,[40] isolated rotundifuran from the fruits of *V. trifolia* and found that the proliferation of cervical cancer cells was suppressed by induction of apoptosis. The study also evaluated the molecular mechanisms associated with the development of cervical cancer. CYR61 was identified as a potential target as the relative gene expression was 2.963. The study concluded that *rotundifuran* could upregulate CYR61 expression and can be considered as a potential therapeutic herb in the management of cervical cancer.

The antiproliferative and antioxidant activity of VN was evaluated on a hepatoma cell line by Kadir *et al*. The MTT assay was done to evaluate the antiproliferative activity, and the IC_{50} value after 24 hours, 48 hours, and 72 hours was found to be 66.46 µg/ml, 57.36 µg/ml and 65.12 µg/ml.[15] Mohammad *et al*. isolated the bioactive compound artemetin from VN and evaluated the anticancer and antioxidant activity of the ethyl acetate leaf extract on the MCF-7 breast cancer cell line by the MTT assay. The study reported that the maximum activity was reported at a concentration of 200 µg/ml.[41] In our study, MTT assay was done to analyze the cell viability of OSCC cells after five different concentrations of crude ethanolic extract of VN leaves after 24 hours, 48 hours, and 72 hours, and IC_{50} was found to be 41.83 µg/ml after 72 hours. PI/AO dual staining was done in our study to evaluate the early and late apoptotic changes at IC_{50} concentration. The viability of OSCC cells was found to reduce with increasing dose and time interval. Thus, effects were observed to be dependent on dose and time.

Similarly, Gowthami *et al*.,[42] performed an in-silico modeling study with AutoDock Vina to identify the compounds from the leaves of VN to target the Wnt signaling pathway. Wnt signaling proteins, namely, axin, β-catenin, GSK-3β, APC, and Dishevelled, were selected for the molecular docking study. The binding energy was found to be maximum for luteolin, chrysophanol, and isoorientin. Thus, the study concluded that these compounds can be used as potential targets for the Wnt signaling pathway in colorectal cancer. Vitexin was isolated from the leaves of VN, and its anticancer, apoptotic activity on human malignant melanoma cell lines was studied by Liu *et al*.[43] A cytotoxicity assay was done, and IC_{50} was calculated after 48 hours following exposure to different concentrations of vitexin from VN leaves. Apoptosis was studied with Annexin V/PI staining. RT-PCR was done to explore the expression of p21, PUMA, CDK1, MCM6, CYCE, CDK6, and CYCA, as these were involved in the regulation of the cell cycle. The IC_{50} was found to range between 5 and 15 µM, and the apoptotic activity was around 17% at 5 µM, 39% at 20 µM, indicating the time- and dose-dependent increase in anti-tumorigenic efficacy. Vitexin was also found to induce cell cycle arrest at the G2/M phase. The cytotoxic activity was also determined in a normal human skin keratinocyte cell line, and the IC_{50} was found to be around 80 µM, suggesting the selective cytotoxic activity of vitexin on melanoma cells.[43] These findings are in concordance with our study, as the cell viability of the normal human oral keratinocyte was 88.59% after 72 hours of exposure to 200 µg/ml of crude ethanolic extract of VN leaves, clearly demonstrating the selective cytotoxic action on OSCC cells.

The limitation of the study is that active compounds of the VN leaves were not investigated for anticancer activity. Secondly, the quality of the extract was not assessed before and after storage by phytochemical profiling, or thin layer chromatography, or high-performance liquid chromatography. Thirdly, only crude ethanolic extract was studied; other solvents were not compared for analysis. Fourthly, only VN was considered for the study; other species belonging to the family of Verbenaceae, such as *Vitex agnus-castus*, *Vitex rotundifolia,* and *Vitex trifolia,* were not added, though the literature documented potent anticancer activity. In addition to these, the antioxidant activity of VN was also not included in the study. Further research with isolation and characterization of active compounds accompanied by comparative analysis with

other solvents and in-silico molecular docking studies in the future might fortify the establishment of cost-effective, less toxic phytochemicals in the field of therapeutic oncology. Similar to the multimodality treatment methods, combinations of multiple herbs similar to the study by Nazhvani *et al*.,[44] could also be considered for the development of alternative traditional therapeutic strategies for oral cancer.

Conclusions

Our study attempted to evaluate the cytotoxic and apoptotic efficacy of crude ethanolic extract of VN leaves on an oral squamous cell carcinoma cell line in comparison with cisplatin. After 72 hours of exposure, the cell viability was 14.75% with VN and 14.09% with cisplatin. Thus, the cytotoxic efficacy of VN was almost similar to cisplatin. Future clinical trials would be considered to include VN as an alternative therapeutic regimen in the management of oral cancer.

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Nil

Declaration of Interest

None

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