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Effect Of Vanillin On Cell Viability, Morphological Characteristics, Apoptosis And Mitochondrial Potential Of Human Oral Cancer Cell Line Kb: An In-Vitro Study

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Abstract
Vanillin is utilized as a flavour ingredient in food, fragrance and pharmaceutical sectors.. It has been linked to a varietyof biological effects and has shown antioxidant, antimicrobial, analgesic, anti-sickling, anti-carcinogenic and anti-mutagenic properties in a variety of in vitro models. This research investigated the anticancer potential of vanillin against KB, ahuman oral cancer cell line. Cell viability assays, acridine orange (AO)-ethidium bromide (EB) double staining, as well as other assays being used, intracellular reactive oxygen species level by using dichloro-dihydro-fluoresceindiacetate (DCFH-DA) assay and the laser dye Rh-123 mitochondrial potential (MMP) assay. Vanillin also has higher cytolytic or cytostatic effect on malignant KB cells than on normal cells. With increase in concentration of drug, the cell viability decreased. The IC_{50} for KB cell lines were 400 µg/ml. Results showed that apoptosis wasinduced by vanillin. Disturbance and loss of mitochondrial potential was detected by green fluorescence imaging ascompared to control for vanillin treatments. In our study, intracellular ROS generation was increased in KB cells. In conclusion vanillin could be a helpful oral cancer preventing agent.

Keywords*: Vanillin, Anticancer, KB cell line, Apoptosis, Cell viability, MTT assay, mitochondrial potential, Reactive oxygen species.*

1. Introduction

Cancer that may impact on every body part is a class of diseases triggered due to the failure of regulation of the cell growth. This is characterized by uncontrolled growth of cells [1]. Oral cancer represents itself as one of the most occurring cancers in humans, with lower survival rate. Almost 100% of oral carcinoma is histo-pathological squamous cell carcinomas [2]. Carcinoma of the mucosa lining accounts for more than 90% of cancers of the buccal mucosa and oropharynx with comparatively uncommon neoplasm's occurring in soft tissues and small salivary glands, resulting numerous indications, such as mucous membrane development and ulceration, discomfort, inflammation, speech trouble, mouth opening, chewing, difficulty in swallowing, bleeding, and weight loss [3]. WHO estimates that the number of patients with oral cancer will continue to rise globally, continuing this pattern well into the next few decades [4]. The occurrence of oral cancer is growing tremendously globally, particularly in progressing countries, included India, this kind of cancer reports for 40–50% of all forms of cancers [5]. Growing the prevalence of mouth carcinoma is strongly aligned with cigarette smoking, areca nut chewing and liquor intake. The average five year survival rate for mouth cancer sufferers hasn't really improved considerably over the last 5-6 centuries but also remains at 50% amid current advancements of oral cancer treatment [6]. Tobacco, betel quid, areca nut, and human papilloma virus have all been linked to the etiology of oral cancer [7].Natural bioactive components perform a key role in the formation of anticancer drugs. Natural anti-tumor molecules reveal excellent pharmacotherapeutic ability [10, 13]. Vanillin, a commonly used vanilla flavouring drug, is said to have many chemo-preventive effects, including antioxidant, antimutagenic and anticarcinogenic effects *in-vivo* [14, 15, 16, and 17]. Vanillin has also been found to be capable of suppressing the metastasis of human lung cancer cells [18].Vanillin pre-treatment increased TRAIL-induced cell death via the apoptotic pathway in HeLa cells. Vanillin pre-treatment decreased TRAIL-induced p65 phosphorylation and NF-κB transcriptional function [19]. we checked the cell viability, morphological characteristics, apoptosis and mitochondrial potential of vanillin on KB cell line

2. Material and Methods

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2.1 Materials

DMEM, Streptomycin, Penicillin-G, L-glutamine, Phosphate Buffered Saline (PBS), 3-(4,5dimethylthiozol-2-yl)-2,5 diphenyltetrazoliumbromide,2'7'diacetyldichlorofluorescein,sodiumdodecylsulfate,trypanblue,trypsin-EDTA, ethylene diamine tetra acetic acid, acridine orange, ethidium bromide, rhodamine-123, triton X-100, ethanol,dimethyl sulfoxide and bovine serum albumin were brought from Sigma Aldrich Chemicals Pvt. Ltd (India). The other chemicals utilized were of analytical quality and purchased from Hi media Laboratories Pvt. Ltd. in India.

2.2 Cell viability

KB cells were grown in DMEM medium adapted with 10% bovine serum, penicillin (100units/ml), and streptomycin (100 mg/ml). The cells were harvested and diluted in DMEM medium to a density of 1×10^4 cells/ml, seeded in 96 well plates for each well, and incubated for 24 hours to enable attachment. The cells were treated with different concentrations of vanillin (100 to 1000µg/ml) and each test was done in triplicates. Kb cells were incubated at 37°C in a humidified 95% air and 5% CO_2 incubator for 24 hours. After incubation, the drug-containing cells were washed with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubation for another 4 hours at 37°C. The purple precipitated formazan formed was dissolved in 100 µl of concentrated DMSO and the absorbance was measured at 540 nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC50) values were calculated and the optimum doses were analyzed at different time period. The IC50 values were determined from the sample vanillin dose responsive curve where inhibition of 50% cytotoxicity compared to vehicle control cells.

2.3 Measurement of apoptotic induction using acridine orange/ethidiumbromide (AO/EB) Dual staining method

The fluorescence microscopic analysis of apoptotic cell death was carried out by the method of Baskic *etal*. 2006 [20]. Kb cells after harvesting were seeded at 5×10^4 cells/well in 6 well plate in a DMEM growth medium and incubated for 24 hours. After incubation KB cells were treated with Van 40 µg/ml in a serum free DMEM medium. At 37°C, the plate was incubated over night in a 5% CO_2 incubator. The amount of 200µL dye mixture was prepared by dissolving 100µL/mg acridine orange and 100µL/mg ethidium bromide in PBS. The cells was separated, rinsed with chilled PBS, but also dyed for 5min using a 1:1 combination of AO (100g ml-1) / EB (100g ml-1). Fluorescence was used to examine the labelled cells at 40x magnification. At the end of treatment, the cells were collected and washed three times with PBS. The count undergoing apoptosis as calculated as a function of the actual count in the area.

2.4 Measurement of reactive oxygen species (ROS)

The dichloro-dihydro-fluorescein diacetate tests were used to calculate the amount of ROS within the cells. DCFH- DA is indeed a cell penetrating lipophilic molecule which has been deacetylated to DCF by cell esterases in the cytoplasm [21]. The radical's hydroxyl, peroxyl, alkoxyl, nitrate, and carbonate subsequently converted dichloro-dihydro-fluorescein diacetate (DCFH-DA) to a fluorescent molecule (excitation 530 nm, emission 485 nm).The hydrogen peroxide or superoxide radicals did not oxidise DCFH-DA.

In 6 well plates, Kb cells were planted (2×10^6 cells/well) prior to exposure in DMEM medium for 24 hours. Cells were treated with vanillin 40µg/ml and were maintained at 37°C at 5% CO_2. After treatment of vanillin, cells was washed by PBS and loaded with 25µM dichloro-dihydro-fluorescein diacetate in DMEM for half hour at 37°C. afterwards, treated cells were rinsed with DMEM, and fluorescence was monitored every 5 minutes for 30 minutes (excitation 485 nm, emission 535 nm) using an spectrofluorometry at 37°C. The mean slope per minute was used to compute ROS generation, which was then standardized to a unexposed control.

2.5 Measurement of mitochondrial membrane potential(MMP)

MMP were determined by employing Bhosle *et al*. 2005 [22] modified method. And for its mitochondrial selecti-vity and luminous characteristics, the laser dye Rh-123 has been shown to be a specific probe for the localization of mitochondria in living cells. Rh-123 enters into mitochondrial matrix and causes photo-luminescent quenching, dependent on mitochondria transmembrane potential. MMP depleted the Rh-123 released from the mitochondria and emits florescence. Higher florescence value indicates MMP description. Kb cells were placed into six well plateswith cover slip then subjected to various concentration of vanillin 400 µg/ml. The cells has been stained using the Rh-123 dye, 15 minutes of incubation. PBS was used to rinse these cells twice and fixed. The fluroscence intensity was measured at 535 nm and the percentage of Kb cells reflecting pathological changes were calculated.

2.6 Statistical analysis

For data analysis, SPSS software was utilized and the data was expressed as mean±S.D. Significant was evaluated as a value of less than 0.05 or less than 0.01.

Results

2.1 Cellviability

The KB cells have been exposed to multiple concentrations of vanillin to assess therole of different concentrations on cell viability. The graph was plotted as % cell viability against the concentration of drug treated. The changes in cell viability and morphology can be seen in Fig. 1. It was observed that with the increase in concentration of drug, the cell viability decreased (Fig. 2). The IC50 values were determined from the sample vanillin dose responsive curve where inhibition of 50% cytotoxicity compared to vehicle control cells. All tests were carried out in duplicate at least three times.

The MTT assay revealed that vanillin has a stronger cytolytic and cytostatic impact on cancer cell than itdoes on human cells. Appropriately, the IC50 obtained in this analysis (400 mg/ml) is very close with the onerecorded on breast cancer line by Lirdprapamongkol et al. [23]. Vanillin is designated as a cytotoxic agent against numerous cell lines, including murine fibroblast 3T3 cells, at high doses (mM range) [24, 25], A2780-SC1 human ovarian cancer cells [26], HT-29 [27], HepG2 cells [28], HeLa cells [29], and SW480 human colorectal cancer cells [30].Although, few studies have been conducted to investigate vanillin's cytotoxic mode of action.

3.2. Measurement of apoptotic induction using acridine orange/ethidium bromide(AO/EB)

The apoptotic and necrotic indices were analyzed using EtBr/Ao dual staining. Fluorescence microscopic research demonstrated that apoptosis was most prevalent following vanillin (400 /ml and 600 g/ml) treatments for 24 hours. The normal cells appeared green in color while the vanillin treated cells showed color changes as, early apoptotic cells exhibit a yellow-greenish nucleus with compressed/fragmented chromatin, whereas late apoptotic cells have compressed but also broken chromatin of orange yellowish colour; and cells that are being perished from immediate necrosis have a bright orange red nucleus with red colour fluorescence (Fig. 3).Thus, the cell death due to apoptosis was significantly observed in KB cells treated with 400 µg/ml and 600 µg/ml vanillin for24 hours.

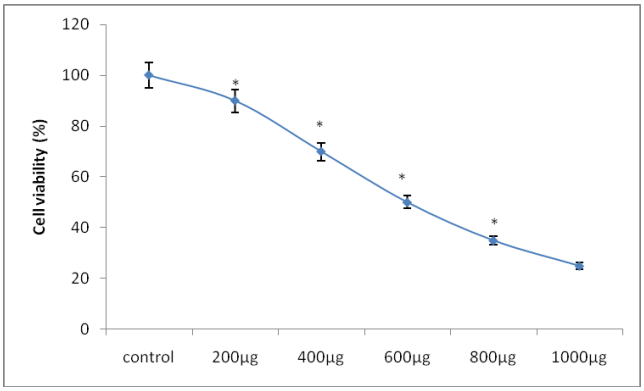
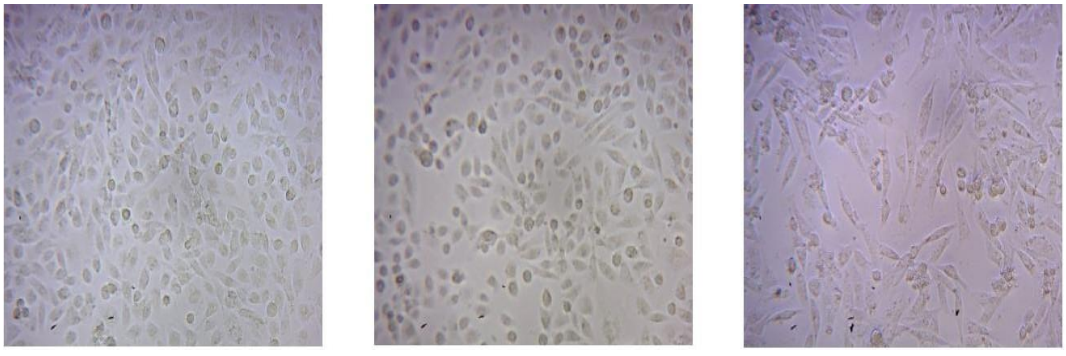
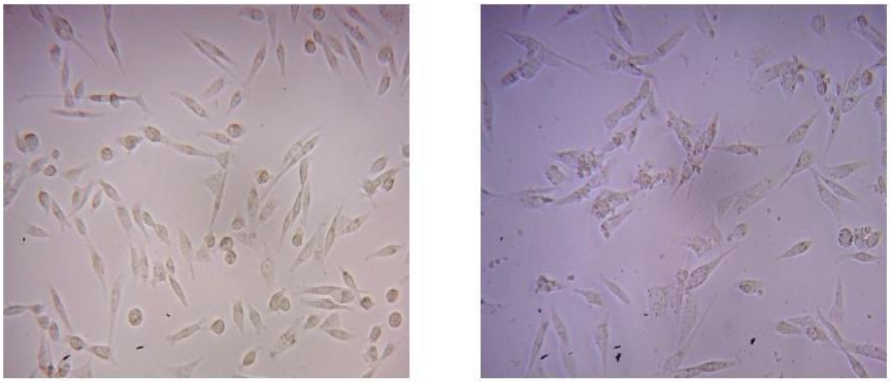


Fig.1: Effect of vanillinon cell viability and morphological characteristics of KB cells assessed by MTT assay.

The KB cells treated with escalating doses of V (200-1000 µg /ml) for 24 hours, and the findings are shown as a cell cytotoxicity ratio for KB cells using MTT assay. The data was given as mean ± SD asterisks indicates statistical difference compared to control.



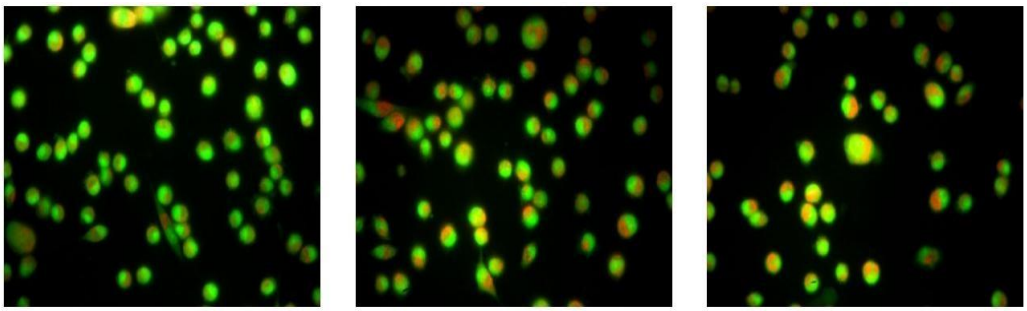
Control **200µg/ml** **400µg/ml**



600µg/ml **800µg/ml**

Fig.2: Physical changes in control and vanillin treated human oral cancer KB cells for 24h.

A photograph under a microscope (20xs) depicts the physical alterations in KB cells caused by vanillin treatment (200, 400,600, and 800 g/ml for 24 h) as opposed to the control. The photographs of the controls, which displayed standard intact cell morphology, were captured using a light microscope.



Control **400µg/ml** **600µg/ml**

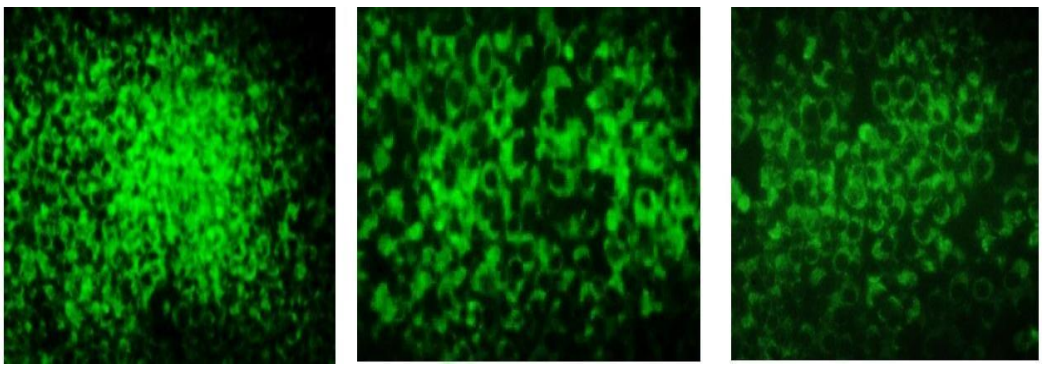
Fig.3: The impact of vanillin on the apoptotic incidence in KB cells.

Cells treated with Vanillin (400 & 600 µg/ml) for 24h, Fluorescence microscopy was used to examine samples dyed with the dual dye AO/EB. Living cells shows fluorescence in green with normal nucleus. Early apoptotic cells with fragmented nucleus shows yellow fluorescence with constricted chromatin. Mature apoptotic cells show orange fluorescence due to chromatin condensation / fragmentation (evenly red/orange stained cell nuclei).

3.3 Measurement of mitochondrial membrane potential (MMP)

To know whether apoptosis is transduced through the mitochondrial pathway, we assessed mitochondrial membrane using Rh-123. Disturbance in mitochondrial function had been observed to be required in apoptotic cascade. Our result had shown that both treatments of vanillin (400µg/ml and 600µg/ml) for 24 hours induced depolarization in mitochondrial membrane which resulted in decreased green fluorescence as compared to untreated cells with bright green fluorescence. The effect of vanillin treatments on mitochondrial membrane potential in KB cells is shown by Fig 4.

In our experiment, loss of potential of the mitochondrial membrane was detected by green fluorescence imaging as compared to control for vanillin treatments.



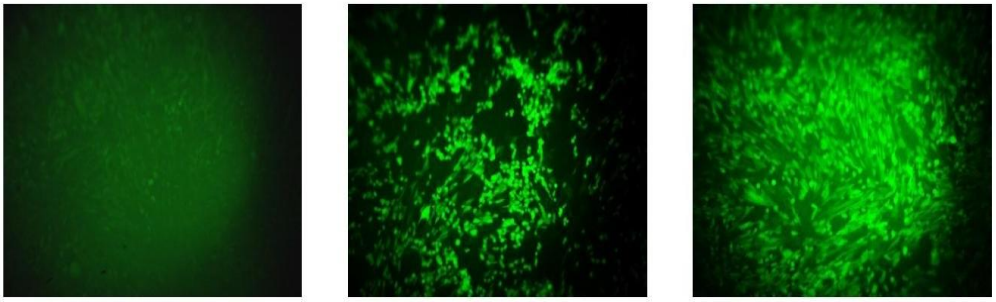
Control **400µg/ml** **600µg/ml**

Fig.4: Effects of vanillin on the MMP of KB cells.

The progressive decrease in green fluorescence in vanillin (400 & 600 µg/ml) treated KB cells suggests a reduction in MMP. The results were analyzed by fluorescent microscope (Labomed, USA) at 40x magnification. The fluorescent image shows control (Rhaccumulation) and vanillin 400 & 600 µg/ml (No Rhaccumulation).

3.4 Measurement of reactive oxygen species production

To assess whether apoptosis induced by vanillin is ROS mediated, we examined the production of ROS within cells using the DCFH-DA staining. During our treatment bright green fluorescence were analyzed in treated cells as compared to dull green in control, which signifies the role of ROS generation in apoptosis in KB cells. Fig. 5showsthe ROS production in control and vanillin treated (400 µg/ml and 600µg/ml)KBcellsfor24 hours. In our study, the formation of intracellular ROS in KB cells increased following treatment with vanillin at the concentration of 400 and 600µg/ml for 24h and stained with DCFH-DA in which control exhibited dull green florescence and treatment depicted bright DCF stained green florescence.



Control **400µg/ml** **600µg/ml**

Fig. 5: The effects of vanillin on ROS within the cell formation in KB-cells using the DCFH-DA assay. KB cells were treated with vanillin (400 µg& 500 µg/ml) for 24 h, stained with DCFH-DA staining. Control (dull green florescence) and vanillin (400 & 600 µg/ml) treatment showed bright DCF stained greenish florescence in KB cells.

3. Discussion

In recent years, the scientific community has been fascinated by the remarkable properties that natural substances possess, especially by many features that make them anti-cancer substances. Vanillin shows promising results. As cancer is a diseases closely related to genomic mutation, vanillin does have anti-cancer property. Besides, the low toxicity of vanillin also prompted the use of vanillin as anti-cancer agent to reduce the side effect during treatment (31).

In this study we studied that cell viability, mitochondrial membrane potential, and the apoptotic incidence and ROS generation in kb cell line. The cell viability assay showed that the vanillin exerts more cytolytic and cytostatic effect on cancerous cells compared to normal cells. Interestingly, the IC50 obtained in this study (400 mg/ml) is very similar to the one reported by Lirdprapamongkol *et a*l. on breast cancer line [32].

Apoptosis is a dynamical, biological mechanism of cell death that is subject to a complicated regulatory mechanism, and it is a key factor in preventing carcinogenesis. In this approach, novel techniques capable of triggering apoptosis in cancer cells, altering the susceptibility of tumour and normal cells to anticancer drugs via an apoptotic regulatory system. Apoptosis has been demonstrated to have a great importance in determining cellular toxicity [33]) and to be a dominant mode of cell death after cytotoxic drug treatment [34]). The apoptotic incidence in KB cells dyed with dual dye AO/EB and then evaluated by fluorescence exhibited normal nucleus. Early apoptotic cells with fragmented nucleus shows yellow fluorescence late apoptotic cells exhibit orange fluorescence with compacted chromatin or fragmentations (equally red/orange-stained cell nuclei).

Mitochondria play essential role in many cellular events such as, cellular metabolism, differentiation, cell communication and apoptosis [35].Mitochondrial instability results in alterations in mitochondrial structure, disturbance of mitochondrial membrane potential, instability of electron transport processes, overproduction of reactive oxygen species (ROS), activation of caspase cascades, and the commencement of the death pathway [36,37]. Apoptosis is initiated by loss of mitochondrial membrane potential and over production of reactive oxygen species. In our experiment, loss of potential of the mitochondrial membrane was detected by green fluorescence imaging as compared to control for vanillin treatments.

Reactive oxygen species are vital in cell development and metabolism. Extreme quantities of reactive oxygen species cause oxidative damage to proteins, lipids, RNA, and DNA, resulting in apoptosis [38].In our study, the formation of intracellular ROS in KB cells increased following treatment with vanillin at the concentration

of 400 and 600 µg/ml for 24 h and stained with DCFH-DA in which control exhibited dull green florescence and treatment depicted bright DCF stained green florescence.

Conclusion

As present research has been based on in-vitro evaluation of vanillin impact on kb cell line, further researchis also carried out on vanillin by us on Golden Syrian hamsters. Although vanillin is extensively taken during meals, we may expect it to be effective in arresting and cure of oral carcinoma.

Conflicts of interest

As such nothing is in conflict.

Authors Declaration

The authors thus state that the work represented in this article is original and that any obligation for claims pertaining to the content of this article will be handled by them.

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