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In-vitro Anti-Proliferative Effects of Ethanolic Extract of *Vanilla planifolia* Leaf Extract against A431 Human Epidermoid Carcinoma Cells

K. Vijaybabu and K. Punnagai

[1]Professor and Head of the Department of Pharmacology, Annapoorna Medical College and Hospital, Salem 636302, Tamil Nadu, India.
[2]Professor and Head of the Department of Pharmacology, Sri Ramachandra Medical College and Hospital Sri Ramachandra Institute of Higher education and Research(Deemed to be University), Porur Chennai Tamil Nadu, India.
*Corresponding author E-mail:punnagaiguna@hotmail.com

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Squamous cell carcinoma is the second largest among skin cancer diseases. The aim of the present study is to reveal the antiproliferative property of vanilla leaf extract against A431 cells. Antiproliferative property was assessed by MTT assay. Apoptotic property was assessed by DNA fragmentation assay. Antiproliferative property of extract was revealed in a dose dependent manner. IC50 of the extract against A431 cells was revealed at 31.2μg/ml. This study revealed the cancer suppression capability of vanilla leaf extract in skin cancer cell lines.

Keywords: A431 cell lines, Vanilla leaf extract, MTT assay, DNA fragmentation.

Cancer is the major and common among various diseases worldwide. Among various types of cancer, skin cancer is the common type. Squamous cell carcinoma is the second largest among skin cancer diseases[1-3]. The treatment for this disease include surgical management and medical management including topical and systemic drug therapies[4]. Current available medical management include doxorubicin, vincristine, cisplatin, 5-fluorouracil and bleomycin. But these drugs cause more side effects due to lack of specificity in drug action[5]. Many herbal extracts have been shown to possess anticancerous effects with less side effects against skin cancer in the recent researches[6-8]. *Vanilla planifolia* (vanilla)is a well known plant which is extensively used as flavouring agent in various food and medicinal products. Because of growing interest for natural products or phytochemicals and their medicinal values, recent studies had shown many medicinal properties of V. *planifolia*. The flavouring agent was obtained majorly from beans of vanilla. This included vanillin, vanillic acid which comprised 250 compounds altogether[9,10]. Another study revealed that out of 200 compounds, 26 compounds were at a concentration of 1mg[11-13]. Vanilla was first discovered by Aztecs who were Mexicans during 1300's. Aztecs were the first to use it as flavouring agent in drinks. This crop was native to Mexico, due to absence of natural pollinator

outside Mexico as stated by the botanist Charles Morren (1836) and this led to the discovery of vanilla's artificial pollination[14]. With this it has explored to other parts of world. Use of vanilla plant as health food agent had ethnical variation. Aztecs used for its stimulant, carminative and aphrodisiac properties. In Venezuela it is used for treating fever and spasm. Argentinian used it for spasms and sexual dysfunctions. Palauans used this plant for treating dysmenorrhoea, fever and hysteria[15]. Recently Sophie et al. 2003 has revealed its protective properties against free radicals in skin[16]. The leaf extracts of vanilla species were revealed to possess compounds that have mosquito larvicidal properties at a dose of 0.1-0.2mg/ml[17]. A431 cells were reported to have tumor forming cells similar to squamous cell carcinoma and they were profoundly used in skin cancer research[18]. Till date vanilla plant extract has never been tried to explore its anticancerous effects in these cell lines. This study aimed to investigate the anticancer effects of vanilla leaf extract against these cell lines which may benefit the patients with skin cancer.

MATERIALS AND METHODS

Cell line and culture

A431 cells were bought from National Centre for Cell Sciences, Pune. The cultured cells were maintained in cell culture media, Minimal Essential Medium (MEM) composed with antibiotics like streptomycin (100 ìg/ml) and penicillin (100 U/ml), 10% fetal bovine serum (FBS) in a suitable temperature (37 °C) and atmosphere(5% CO_2).

Reagents

FBS and MEM were purchased from Cistron laboratories (Chennai, India) and Hi Media Laboratories (Mumbai, India) respectively. Dimethyl sulfoxide (DMSO), methyl thiazolyl diphenyl- tetrazolium bromide (MTT) and Trypsin were obtained from Sisco research laboratory chemicals (Mumbai, India). Other reagents and chemicals were provided by Sigma Aldrich (Mumbai).

Extract Procedure

Vanilla leaves were collected from yercaud and authenticated by Dr. A. Balasubramanian, Botanist and Siddha Research Consultant (AYUSH). The leaves were shade dried and powdered. The powdered was further treated with ethanol to obtain ethanolic extract. The procedure followed Sun et al. 2001, with slight modifications [17]. Two hundred grams of powdered extract was treated with 500 ml of ethyl alcohol (98%) and allowed to stand for 2 days. Then the procedure was repeated twice with the precipitate. The obtained alcohol was freezed and filtered. The obtained solution was lyophilized to produce powdered extract of vanilla leaves.

Cytotoxicity Assay

The cytotoxicity assay included 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl—tetrazolium bromide (MTT) assay. Culture media specific to this assay was prepared as suggested by previous literature[19]. A431 cells were treated with MTT and sample with a dose concentration of 1000µg/ml to 7.8µg/ml dilution. This was compared with control cells. The reaction absorbance was observed by spectrophotometer

Table 1. Anticancer effect of *Vanilla planifolia* leaf extract on A431 cells by MTT Assay

S.No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.184	13.77
2	500	1:1	0.273	20.43
3	250	1:2	0.388	29.04
4	125	1:4	0.516	38.62
5	62.5	1:8	0.592	44.31
6	31.2	1:16	0.668	50.00
7	15.6	1:32	0.783	58.60
8	7.8	1:64	0.927	69.38
9	Cell control	-	1.336	100

at 570nm. The cell viability was calculated as per standard formula[20].

DNA fragmentation assay

The assay method was performed as described by Wyllie et al. (1980)[21]. UV transilluminator (Uvitec, England) was used for analysing the DNA fragmentation. The A431 cells were plated and incubated. Then DNA was extracted using manufacturer's protocol. The final DNA sample was mixed with Tris-Taps-EDTA buffer and processed for electrophoresis. DNA was visualized using UV Transilluminator.

RESULTS

Cytotoxicity of vanilla leaf extract on A431 cells

Vanilla leaf extract has shown cytotoxicity to cancer cells in a dose dependent manner. With increase in dose of the extract from 7.8µg/ml, 15.6, 31.2, 62.5, 125, 250, 500, 1000µg/ml there was significant decrease in the percentage of cell viability of A431 cells in the culture starting from (69.38%, 58.60, 50.00, 44.31, 38.62, 29.04, 20.43, 13.77% respectively). The IC_{50} of the plant extract was calculated to be 31.2µg/ml.

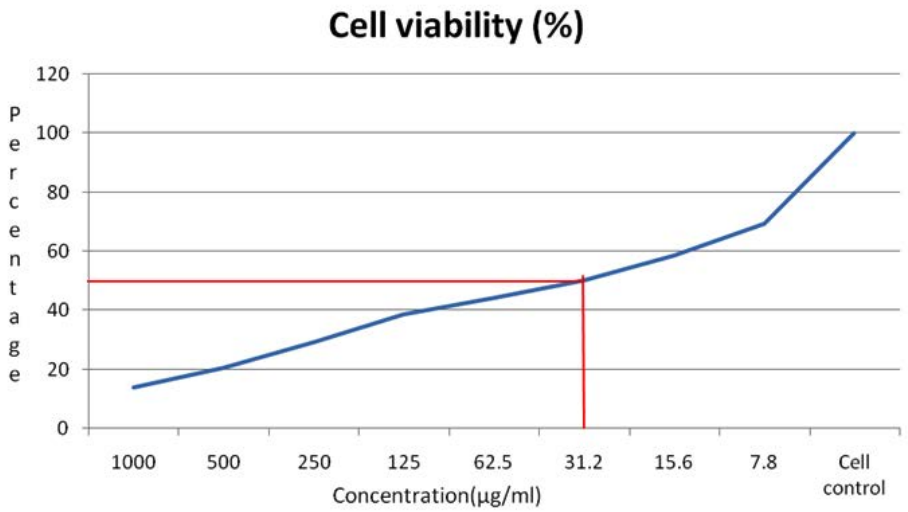

Fig. 1. Cell viability assay of A431 cells

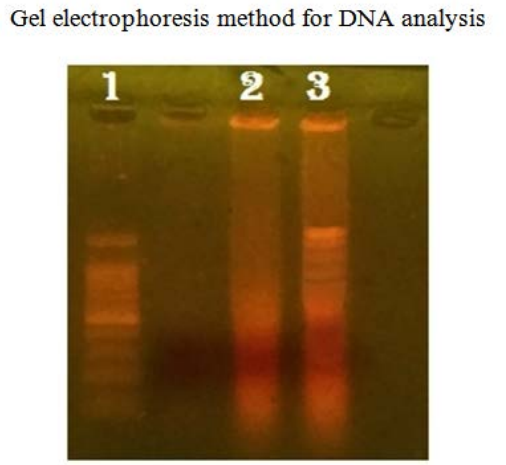

Fig. 2. Apoptotic effects of vanilla leaf extract on A431 cells

Apoptotic effects of vanilla leaf extract on A431 cells

The DNA fragmentation assay with gel electrophoresis method has revealed the DNA fragmentation which was not seen in control A431 cells. But in the cell line (A431) treated with vanilla leaves, the DNA is damaged because of the activity of the sample, the DNA was fragmented.

DISCUSSION

A431 cells has provided a better match to squamous cell carcinoma cells for research in skin cancer[18]. A431 cells were characterized by many studies. Epidermal growth factor (EGF) was related to proliferation of A431 cells and their presence was reported by many studies. Kawamoto et al. (1983)

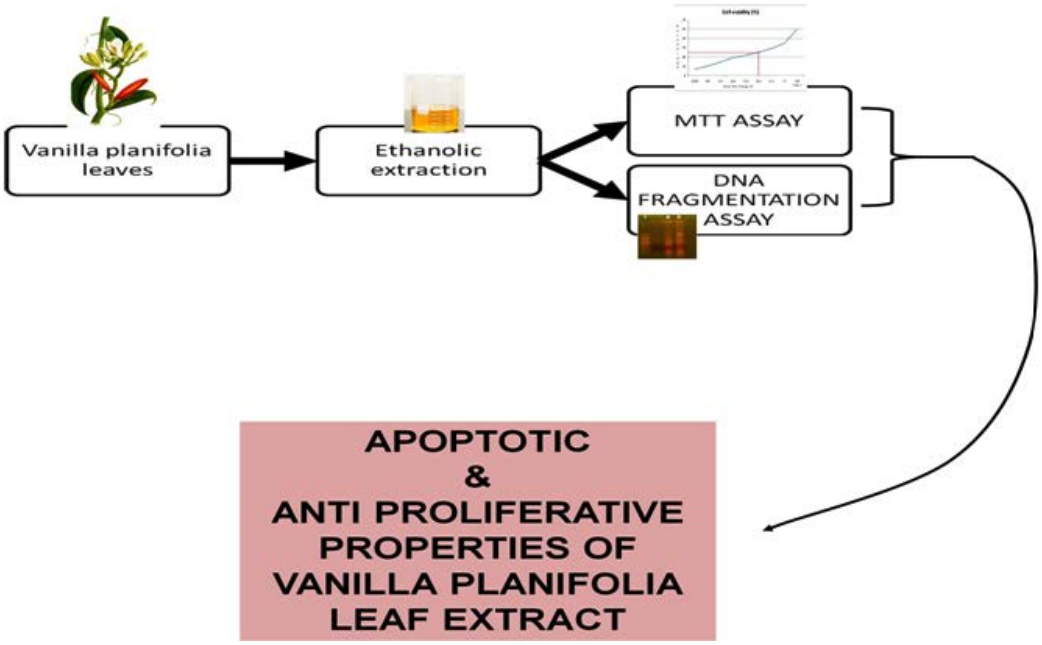

Fig. 3. Graphical Abstract

showed that high affinity EGF receptors were involved in proliferation of A431 cells[22]. The same was revealed by Xu et al. (1984)[23]. Parker et al. (1984) revealed A431 cells as immunogens which can produce anti-receptor monoclonal antibodies that are expressed only in this cells[24]. Walker et al. (1988) revealed that stimulation of EGF was related to phosphotidylinositol kinase [25]. Using this highly characterized cancer cells, we studied the anticancerous screening of vanilla leaf extract. The results of this study revealed the benefits of vanilla leaf extracts against skin cancer type. DNA fragmentation assay using gel electrophoresis method demonstrated the apoptotic effects of the vanilla leaf extract. The anti-proliferative properties of the vanilla leaf extract was shown in this study using cytotoxicity assay. MTT assay showed effective anti-cancerous activity of extract against A431 cells. The assay detects the reduction of MTT salt to blue formazan product by mitochondrial dehydrogenase, which indicates the cell viability[26]. The cell viability of A431 cells decreased with increase of extract dose confirming the anti-cancerous property of the extract. Many studies have revealed the anticancerous properties of various plant extracts using A431 cells[27-30]. Wu et al. (2011) revealed the reduction of migration and invasiveness of A431 cells through inhibition of ezrin by Bacalein. Ezrin protein was reported to enhance tumor metastasis and this protein is highly expressed in skin cancer[31-37]. *Rabdosia rubescens* plant extract was shown to possess apoptotic activity through tyrosine kinase pathway in A431 cells. Oridonin was revealed as the active compound in *Rabdosia rubesecens*[38-42]. The results of the present study may be helpful to characterize the vanilla leaf extract for screening anticancerous property against skin cancer in future studies.

CONCLUSION

The present study concludes the antiproliferative and apoptotic effects of vanilla leaf extracts against A431 cells [Figure 3]. The antiproliferative effects of vanilla leaf extract was shown in a dose dependent manner. Vanilla leaf extract might contain the lead molecule which may be developed as chemotherapeutic agent for treating skin cancer of squamous cell carcinoma type. Further research is required to characterize and understand the molecular aspects of the anticancerous effects of this extract.

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