Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Vanillin an Active Constituent from *Vanilla* Bean Induces Apoptosis and Inhibits Proliferation in Human Colorectal Adenocarcinoma Cell Line

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Submitted: 01-06-2019 Revised: 14-08-2019 Published: 11-02-2020

ABSTRACT

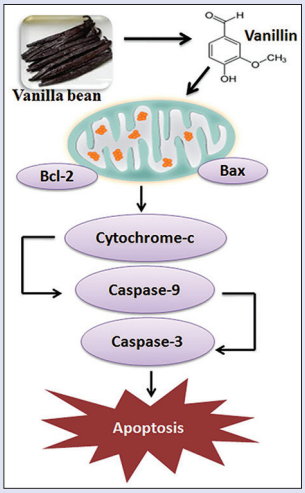
Background: Colorectal cancers global incidence is increasing due to the rapid acquisition of Western lifestyle habits such as high caloric nutrition and sedentary status. Natural bioactive products play critical roles in anticancer drug development. **Aim:** The aim of the study was to investigate the antiproliferative and proapoptotic effects of vanillin (VL) an active constituent from *Vanilla* bean in colon cancer (human colorectal adenocarcinoma cell line [HT-29]) cells. **Methodology:** We demonstrated the cell growth and apoptosis in HT-29 cells. The apoptotic measures were analyzed by analyzing mitochondrial membrane potential (Δψm) level; apoptotic bodies by acridine orange/ethidium bromide and Hoechst staining. **Results:** Our results indicated that VL induces apoptosis as evidenced by cell viability loss, resulting in the altered Δψm in HT-29 cells. Further, the prooxidant role of VL inhibit B-cell lymphoma 2 (Bcl2) expression with the simultaneous upregulation of BCL-2 associated X, Cytochrome c, Capspase-9 and 3 protein expressions in HT-29 cells. **Conclusion:** Thus, in this regard, we have concluded that VL induces apoptosis in HT-29 cells through inducing oxidative damage and modulates apoptotic marker expressions. VL may therefore be used as a therapeutic agent for colon cancer treatment.

Key words: Apoptosis, colon cancer, human colorectal adenocarcinoma cell line, nuclear fragmentation, proliferation, vanillin

SUMMARY

- Natural bioactive products play critical roles in anticancer drug development. Natural molecules with antitumor activity, in fact, reveal excellent potential for pharmacotherapy. Although the Vanilin is reported previously against different cancer cells, following the present study against colon cancer cells. The observation of the present study has clearly demonstrated that in human colorectal adenocarcinoma cell line (HT-29) colon cancer cells, *Vanilln* induces apoptosis and the inhibition of proliferation, as evidenced by cell viability loss, resulting in the altered Δψm in HT-29 cells. Further, the Vanilin inhibit Bcell lymphoma 2 (Bcl-2) expression with the simultaneous up-regulation of BCL-2 associated X, Cytochrome c, Caspase-9 and 3 protein expressions in HT-29 cells. Thus, we have concluded that Vanilin induced apoptosis through oxidative damage and apoptotic marker expressions may be its therapeutic property for colon cancer treatment.

Abbreviations used: VL: Vanillin; AO: Acridine orange; EtBr: Ethidium Bromide; Bcl-2: B-celllymphoma2; Bax: B-celllymphoma2associatedX; HT-29: Human colorectal adenocarcinoma cell line; Δψm: Mitochondrial membrane potential; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; IC_{50}: The half maximal inhibitory concentration; TBST: Tris-buffered saline and Tween-20; EDTA: Ethylenediaminetetra acetic acid; Rh-123: Rodamine-123; FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's Modified Eagle Medium.



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DOI: 10.4103/pm.pm_235_19

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INTRODUCTION

Colorectal cancer is the world's third-most common cancer and the fourth leading cause of cancer-related death, accounting for more than one million new cases per year.[1] Colorectal cancers global incidence is increasing due to the rapid acquisition of Western lifestyle habits such as high caloric nutrition and sedentary status. Colorectal cancer is also increasingly affecting people in newly industrialized countries.[2] Effective colorectal cancer treatment is hindered by low compliance with screening recommendations, late-stage diagnosis of new cases of colorectal cancer, severe toxicity to chemotherapy and radiotherapy, and resistance to therapy and cancer recurrence.[3] These limitations require new approaches to effective colorectal cancer prevention and treatment. In addition to dietary and lifestyle interventions, safe

Cite this article as: Xie Y, Zhang J, Wang C, Fan Q, Zhang Y. Vanillin an active constituent from *Vanilla* bean induces apoptosis and inhibits proliferation in human colorectal adenocarcinoma cell line. Phcog Mag 2020;16:197-200.

preventive drugs, phytotherapeutics, or tailor-made food supplements are needed to combat colorectal cancer epidemics and metabolic disorders.[4]

Natural bioactive products play critical roles in anticancer drug development. Natural molecules with antitumor activity, in fact, reveal excellent potential for pharmacotherapy.[5] In many of these molecules, especially the phenolic substances, anti-oxidant activity is commonly found. Considering the involvement of reactive species as a source of different cancer types, diets and/or drug therapies involving bioactive substances with anti-oxidant activity may well be a preventive treatment approach to maintain the patient's well-being. In various pharmaceutical, nutritional and cosmetic products, some of these natural substances are present. For example, vanillin (VL), a secondary plant metabolite and the main component of *Vanilla*, is a carbonic structure derivative of phenolic phenylpropane C6-C1.[6] It acts as an important component used worldwide for flavor and aroma. VL is found in several essential plant oils, mainly *Vanilla planifolia*, *Vanilla tahitensis,* and *Vanilla pompon*; it is frequently found in processed foods, drinks, and pharmaceutical products as well as in perfumery.[7] Although the VL is reported previously against different cancer cells, the present study will be the first study against colon cancer cells. In this study, we show that in human colorectal adenocarcinoma cell line (HT-29) colon cancer cells, VL an active constituent of *Vanilla* bean induces apoptosis and the inhibition of proliferation.

MATERIALS AND METHODS

Chemicals and reagents

VL and Panitumumab were purchased from Sigma Chemical (St.-Louis, USA). Acridine orange (AO), Hoechst stain, Ethidium Bromide (EtBr), and Rhodamine-123 (Rh-123) were purchased from Himedia. Cell culture chemicals such as heat inactivated fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium medium, glutamine, penicillin-streptomycin, Ethylenediaminetetra acetic acid, trypsin, phosphate-buffered saline (PBS), low melting agarose, normal melting agarose; B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X (Bax), caspase-9, caspase-3, and Cytochrome c monoclonal antibodies were purchased from Sigma chemical Co., St. Louis, USA. All other chemicals and solvents were obtained from Fisher Inorg., Aromatic Limited.

Cell culture

Human colon cancer cells (HT-29), were purchased from Tangdu Hospital, China. The cells were maintained in medium with 1% glutamine, 10% FBS, and 100 U/ml antibiotic in CO_2 incubator.

Proliferation assay

Cellular proliferation of -29 were determined by 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay with some minor modifications.[8] After treatment, cells (1×10^5/well) were plated in 96-well plates. The cells were rinsed thrice with PBS to remove the residual drug from each well. Next, 50 µl of MTT (5 mg/ml) was added. The plates were incubated for 4 h in 5% CO_2 incubator. The formazan crystals were dissolved with 100 µl of dimethyl sulfoxide. Percent cell viability was determined by measuring increase in the absorbance values (OD 570 nm). The The half maximal inhibitory concentration (IC_{50}) value was determined from the graph. Panitumumab was used as a positive control (Data not shown).

Detection of apoptotic nuclei

The apoptosis was observed by using AO and Hoechst stains as previously observed by Muzaffer *et al.*[9] The cells were stained with different stains (10 µg/mL) for 30 min and were observed at 330/380 nm under excitation and at 440 nm under barrier filters by using fluorescence microscope with ×40. Four hundred cells were counted and visualized per sample. While observation the cells having condensed/fragmented nuclei were considered as percentage apoptotic cells.

Determination of the membrane potential of mitochondria

Rh-123 was used to determine the mitochondrial membrane potential (Δψm). The cells, both treated and untreated were stained with 5 µM of Rh-123 stain for 15 min to review the Δψm.[10] The cells were then visualized at 450–490 nm under a fluorescence microscope.

Western blot analysis

The isolated protein concentration was calculated by using Nanodrop. The protein (50 µg) isolated from each group were fractionated and transferred on 10% sodium dodecyl sulfate- polyacrylamide gel electrophoresis gel and nitrocellulose acetate-membrane, respectively. The membrane was then positioned in a 5% BSA at 4°C for 12 h. After blocking, the membrane was PBS washed and placed in a solution of primary antibodies (1:1000) followed by Tris-buffered saline and Tween-20 wash and secondary antibody (conjugated with horseradish peroxidase, 1:2000) incubation for 1 h. Finally, the membrane development and visualization of protein bands was performed using LI-COR.

Statistical analysis of experimental data

The analyses of all experiments were carried out using one-way analysis of variance, followed by Duncan's Multiple Range Test. The data that were statistically significant at $P < 0.05$ was considered. The statistical data were expressed as mean ± SD ($n = 5$).

RESULTS

Effect of vanillin on the proliferation of human colorectal adenocarcinoma cell line cells

The results obtained from this study showed clearly the decreased level of cell viability in VL treated cells [Figure 1a]. The inhibition of HT-29 cell viability with IC_{50} values 20 µM indicates the remarkable potential of VL [Figure 1b]. We observed maximum cell viability at 20 µM of VL treatment in normal cells and HT-29 cells treated with higher concentration (5–20 µM) of VL showed decreased cell viability.

Effect of vanillin on mitochondrial membrane potential

In this study, control HT-29 cells showed increased fluorescence intensity (2342.48 ± 31.13). On the other hand, treatment with VL significantly decreased Δψm (639.14 ± 20.5) as evidenced by decreased fluorescence [Figure 2A and B].

Effect of VL on apoptosis

The apoptotic nuclear fragmentation evaluated by Hoechst [Figure 3] and AO/EtBr [Figure 4A and B] staining shows the significant recovery by VL treatment when compared to control groups. Very few nuclear fragmentations were detected in control for both Hoechst as well as for AO/EtBr staining.

Effect of vanillin on apoptotic protein expression levels

The effect of VL was observed on apoptotic markers such as Bcl-2, Bax, Caspase-9, Caspase-3, and Cytochrome-c expressions [Figure 5A and B]. The expression level of proapoptotic proteins such

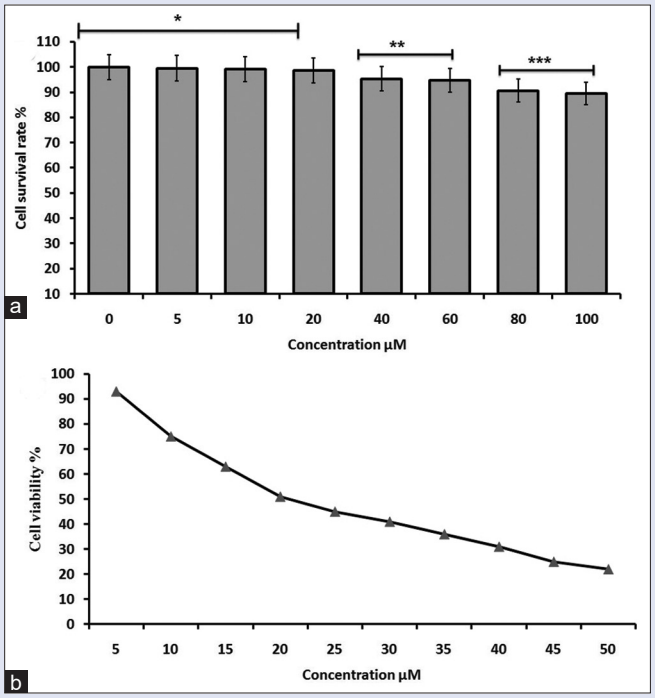

Figure 1: **Significancant at $P<0.01$; ***Significant at $P<0.05$. The cytotoxicity effect of Vanillin on human colorectal adenocarcinoma cell line and normal HGF-1 cells measured by 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide assay. (a) The effect of Vanillin on the cell proliferation of HGF-1 cells. (b) Cell proliferation inhibition effect of Vanillin in human colorectal adenocarcinoma cell line cells. The statistical analysis was carried out using one-way ANOVA. Values are represented mean standard deviation of three experiments. $P < 0.05$ was significantly different from the control sample

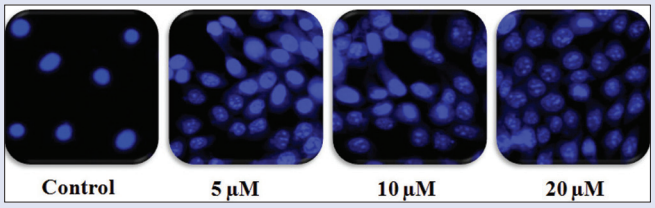

Figure 3: Shows apoptotic morphological changes (Hochest staining) were observed in control and Vanillin treated cells (Conrtol). Untreated control cells (Compact nuclei), (5–20 µM) The different concentration of Vanillin-treated human colorectal adenocarcinoma cell line cells shows increased fragmented nuclei and membrane blebbing

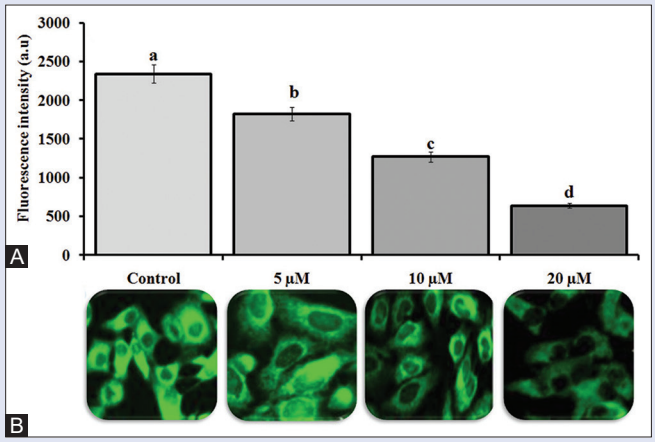

Figure 2: (A) Untreated human colorectal adenocarcinoma cell line cells shows high fluorescence indicate polarized mitochondria membrane (5–20 µM) shows human colorectal adenocarcinoma cell line cells were treated with different concentration of Vanillin for 24 h and fluorescence intensity was decreased as indicate collapsed mitochondria matrix. The images were acquired by floid cell imaging station. (B) fluorescence intensity was detected by spectrofluorometer. All experiments were performed in triplicate and all values were expressed as mean standard deviation of the mean. The (a-d) asterisks indicate significant difference from control ($P < 0.05$)

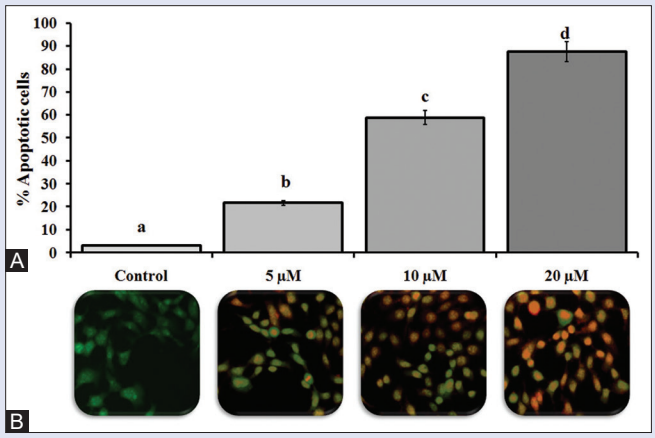

Figure 4: Fluorescence microscopy images of apoptotic morphology by dual staining (Acridine orange/Ethidium Bromide) (Control). untreated human colorectal adenocarcinoma cell line cells, (A) (5–20 µM) shows Vanillin treated human colorectal adenocarcinoma cell line cells shows increased % of apoptotic cells in a concentration dependent manner. (B) Percentage apoptotic cells were calculated. Data are expressed as mean standard deviation from three independent experiments. $P < 0.05$ is significantly different from the untreated cells

as Caspase-9, Caspase-3, and Bax were downregulated in control group as compared to treated groups. Conversely, VL treated cells were observed over-expressed of proapoptotic proteins. While in control group, Bcl-2 expression was up-regulated and down-regulated by VL treatment.

DISCUSSION

Phytochemicals are attracted to chemotherapeutic drug development within the scientific community. Epidemiological studies suggest that the incidence of cancer progression is minimized by regular consumption of natural phytonutrients. For instance, more than half of anti-cancer preparations are developed from natural sources.[11] The present study is designed to observe the apoptotic signaling and the effect of VL on the cell viability and apoptotic signaling in HT-29 cells. The observation of the present study clearly indicates that VL can effectively inhibit the cell proliferation in concentration-dependent manner [Figure 1a], with IC_{50} value of 20 µM [Figure 1b]. These findings clearly indicate that treatment with VL, resulted in complete colon carcinoma cells death when compared to the reference drugs. Hence, it may be a valuable candidate for cancer chemotherapy.

While mitochondrion the major cell organ involved in apoptosis shows the permeability transition by stress in its membrane potential, respiration, and the release of cytochrome-c via outer membrane channels into the cytosol, followed by the activation of different caspases,

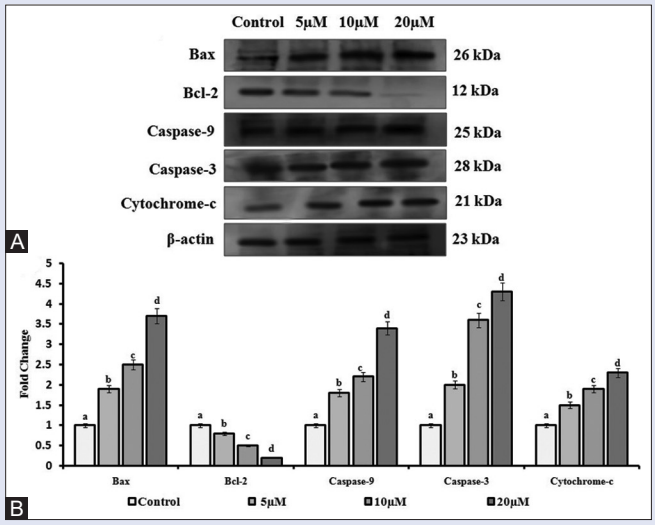

Figure 5: Effects of Vanillin on apoptotic protein B-cell lymphoma 2 associated X, B-cell lymphoma 2, cytochrome c, caspase-9, and caspase-3 in human colorectal adenocarcinoma cell line cells. (A) Shows the band intensities were quantified by densitometry and normalized to respective β-actin loading control. (B) The representative graph shows the relative protein expression of fold changes in western blots. Values are expressed as mean standard deviation for six experiments. Values that do not share a common superscript letter (a-d) between groups differs significantly at $P < 0.05$ (Duncan's Multiple Range Test)

mainly responsible for apoptosis.[12] The observation of the present report has observed the variation of Δψm by VL in concentration-dependent manner [Figure 2A and B]. This could be associated with VL's anticancer property. On the other hand, the Hoechst [Figure 3A and B] and Ao/EtBr [Figure 4A and B] staining assessed the nuclear fragmentation of apoptosis. VL treatment significantly increased the level of apoptotic cells and nuclear fragmentation. This VL property shows the increased rate of cell death and thus indicates a higher rate of apoptosis in HT-29 cells.

Apoptosis is cell-intrinsic machinery that has been mainly regulated *via* Bcl-2 family, helping to maintain the environment of cells. While the deregulation of Bax/Bcl-2 give rise to cancer by skipping the apoptosis.[13] Previously, it has been reported that the loss of Δψm results in apoptosis through proapoptotic gene activation.[13,14] Accordingly, the observation of the present study clearly indicate that VL-induced apoptosis was go together with by altering the Δψm, by suppressing the Bcl-2 and through up regulating proapoptotic markers, which clearly induced apoptosis through mitochondrial pathway. The VL treatment showed over-expression of Caspase-9, Caspase-3, and Bax when compared with nontreated/control, which is down-regulated. While, Bcl-2 protein expressions is down regulated by VL and up-regulated in control cells. Apoptotic marker regulation was significantly comparable with the previous studies.[15] These findings clearly indicates that VL may be a promising therapy against colon cancer cells. The future directions of this study are focusing on preclinical *in vivo* as well as clinical effects of VL on colon cancer prevention.

CONCLUSION

We suggest that VL, an active constituent of *Vanilla* bean, by means of mitochondrial mediated apoptosis, apoptotic nuclear blubbing, and nuclear fragmentation, inhibits cell proliferation in HT-29 cells. In addition, VL's pro-oxidant role modulates apoptotic protein expression such as decreased Bcl-2, increased Bax leads to mitochondrial release of Cytochrome-c, resulting in Caspase-9 and-3 activation. Overall the possible mechanisms of action of VL-induced mitochondrial-mediated apoptotic signaling pathways are under investigation. However, the findings of the present study indicate that VL may be used as a novel therapeutic agent to treat and/or prevent colon cancer.

Acknowledgements

The authors are thankful to the Department of Oncology, Tangdu Hospital for laboratory facilities.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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