

## VANILLIN EXTRACTED FROM PROSO MILLET AND BARNYARD MILLET INDUCE APOPTOSIS IN HT-29 AND MCF-7 CELL LINE THROUGH MITOCHONDRIA MEDIATED PATHWAY

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### ABSTRACT

**Objective:** The main aim of the study was to investigate the bioactive compound vanillin extracted from proso millet (compound 1), and barnyard millet (compound 2) induces apoptotic cell death and whether it is mediated through mitochondrial pathway in HT-29 and MCF-7 cell line.

**Methods:** The cells were treated with 250 µg/ml and 1000 µg/ml concentration of extracted vanillin for 48 hrs. Cytochrome c release and expression level of pro-apoptotic protein Bax and caspase-9 were detected by western blot analysis.

**Results:** The results reveal that extracted compounds increased the release of cytochrome c and upregulating the expression of Bax and caspase-9 as concentration increases in a dose-dependent manner.

**Conclusion:** The study suggests that the vanillin compound extracted from these millets induces apoptotic cell death through a mitochondria-dependent pathway.

**Keywords:** Proso millet, Barnyard millet, Vanillin, HT-29 cells, MCF-7 cells, Cytochrome c, Bax, Caspase-9.

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### INTRODUCTION

Cancer is one of the most leading sources of death in both developing and developed countries and sustains to be a main community health problem in various parts of the world [1]. Cancer is one of the most life-threatening diseases [2] with an uncontrolled growth and spread as atypical cells and related with a programmed cell death, dysregulation of apoptosis [3]. World cancer report released by the International Agency for Research on Cancer States that worldwide there were 7.6 million of deaths and 12.4 million of new cancer cases in both males and females [4]. Nowadays, cancer treatments such as surgery, radiation, and chemotherapeutic agents have not completely effective against the low or high survival incidence of most of the cancers [5]. In the area of cancer chemoprevention, the new anticancer drugs developed from foods or plant sources is one of the most prominent and auspicious approaches and this led to the detection of several novel anticancer drugs [6].

More than thousands of year human have trusted the natural products as the most productive origin of medicines and leads for drug development [7]. In several countries especially in India, China, and Egypt, the traditional medication systems were designed based on the plant-based natural product. In recent years, natural product and imitative from the natural product as a new anticancer drug and antibiotics are permitted by US Food and Drug Administration (FDA) [8-10]. Natural products mostly from plant source have an important basis for discovering new bioactive components with few side effects and high efficacy [11]. Anticancer properties have been revealed against a broad range of cancers by numerous foods, for example, grains, cereals, spices, vegetables, and medicinal plants and their bioactive compounds [12].

Many literature studies have stated that mechanism of action of anticancer drugs is based on apoptosis induction, and it initiated

a new approach for searching anticancer drug [13]. Several cancer chemical drugs have induced apoptosis to neglect cancer cells and in which cells die is due to physiological process [14]. Apoptosis is a programmed cell death; it is important for normal cell mechanism. The relationship between cancer and apoptosis has been highlighted with numerous evidence proposing that correlated the processes of transformation progression and metastasis implicate the modification of normal apoptotic pathways. Apoptosis is severely regulated pathway responsible for damaged cells. It not only signifies an effective mechanism of elimination of harmful cells but it also plays an important role in the growth and maintenance of tissue homeostasis [15]. The main typical feature of the apoptosis pathway was mitochondrial dysfunction [16].

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a naturally occurring bioactive compound found in the pods of *Vanilla* spp., and also found in many plant species consuming various properties. It is one of the most extensively used flavoring agents in food and personal products [17]. Over past 20 years, there has been an increasing attention in using vanillin as an antimutagenic agent [18]. Based on the relationship among antimutagenic and anticarcinogenic activities, it was liable that vanillin presents anticarcinogenic effect [19]. Vanillin has potential antioxidant and antitumor properties [20], and it was reported that its activity was more beneficial for regular health care [21]. Cheng *et al.* found that vanillin significantly alter gene expression in the human hepatocellular carcinoma cell line HepG2 [17] and Lirdprapamongkol *et al.* reported that vanillin suppresses the *in vitro* invasion and *in vivo* migration of mammary adenocarcinoma cancer cells [22]. In our previous studies, it was confirmed that extracted vanillin compound have effectively induced apoptotic cell death. Intrinsic and extrinsic signaling pathways are variable in different types of cells. The exact mechanism of vanillin induced apoptosis in HT-29 and MCF-7 cells is still unknown. Hence, in this study, we investigate the molecular mechanism of apoptosis induced by vanillin compound extracted from

proso and barnyard millet in human colon cancer cell line (HT-29) and human breast cancer cell line (MCF-7).

## METHODS

### Plant material and extraction of compounds

Dried seeds of Proso millet (PM) (CO(PV)5) and Barnyard millet (BM) (CO(KV)2) were purchased and authenticated from the Department of Millet, Tamil Nadu Agriculture University, Coimbatore, Tamil Nadu, India. Bioactive compounds were extracted by supercritical fluid extraction system. The structure of the extracted bioactive compounds from PM and BM were elucidated using spectroscopic methods (ultraviolet, infrared [IR], liquid chromatography-mass spectrometry [LC-MS]), and <sup>1</sup>H- and <sup>13</sup>C-nuclear magnetic resonance [NMR]).

### Cell culture

HT-29 human colon cancer cells and MCF-7 human breast cancer cell line were purchased from National Centre for Cell Sciences, Pune, India. The cells were cultured and maintained in McCoy's 5A medium for HT-29 and Dulbecco's modified Eagle's medium for MCF-7 supplemented with 10% heat-inactivated fetal bovine serum and 10,000 IU/ml penicillin G, 10 mg/ml streptomycin, and 25 µg/ml amphotericin. The cells were maintained at 37°C in a humid of 5% CO<sub>2</sub> incubator. The experiments were performed when the cells reach 80% confluence. For *in vitro* molecular studies, cells were seeded in a 100 mm sterile plate for Western blotting and incubated in 5% CO<sub>2</sub> incubator for 24 hrs at 37°C. The cells were treated with 250 µg/ml and 1000 µg/ml concentration of extracted compounds and incubated for 48 hrs, and analysis was performed. The dimethyl sulfoxide treated cells served as control.

### Preparation of cell lysate

HT-29 cells and MCF-7 cells were seeded at a density of 1×10<sup>7</sup> cells/100 mm Petri dish and grown for 24 hrs. The cells were treated with 250 µg/ml and 1000 µg/ml concentration of extracted compounds (1 and 2) from PM and BM and incubated for 48 hrs. After 48 hrs treatment, the medium was aspirated, and cell culture plate was placed on the ice and washed twice with ice-cold phosphate buffer saline (PBS). Drained the PBS and the cells were lysed by added 250 µl lysis buffer (NP-40 buffer-150 mM NaCl, 50 mM Tris, pH 8.0, and 1% NP-40) containing protein inhibitor cocktail. The cells were scraped from the plate using cold plastic cell scraper and gently transferred the cell suspension into the pre-cooled centrifuge tubes. The tubes were kept on ice for 30 minutes with constant agitation. The lysates were centrifuged at 12,000 rpm for 20 minutes at 4°C and collected the supernatant to the new tubes and stored at 4°C until sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis [23].

### Lactate dehydrogenase (LDH) assay

LDH catalyzes the oxidation of lactate to pyruvate in the presence of coenzyme NAD<sup>+</sup>. The measurement of the amount of LDH released from cells is one of the key methods to evaluate the cytotoxic cell death [24]. After treating the cells with 250 µg/ml and 1000 µg/ml concentration of extracted compounds for 48 hrs. The medium was aspirated and centrifuged at 300 g for 5 minutes, and the supernatant was collected to measure the LDH activity. Briefly, 50 µl of cell culture medium was added to 2 ml of Tris-EDTA-NADH buffer and incubated at 37°C for 15 minutes. After incubation, 200 µl of pyruvate was added, and immediately optical density was measured at 339 nm for 3 minutes at a time interval of 15 seconds.

### Western blot analysis

The total protein concentration was estimated using Bradford method with bovine serum albumin (BSA) used as a standard. The equal amount of proteins (75 µg/ml) from each sample was reconstituted with 3× Laemmli sample buffer (0.1875M Tris-HCl, 6% SDS, 15% 2-mercaptoethanol, 30% glycerol, 0.006% bromophenol blue, and pH 6.8) and the mixture was boiled at 90°C for 5 minutes. The denatured proteins were separated on 15% SDS-PAGE followed by transferring the protein in the gel onto the polyvinylidene difluoride membranes by wet blotting transfer at 80 V, 200 mA for 2.5 hrs. The membranes were

washed and blocked with 5% BSA in tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 hrs at room temperature. Moreover, membranes were incubated with primary antibodies anti-Bax (1:2000 dilution), caspase-9 (1:1000 dilution), and cytochrome c (1:1000 dilution) for an overnight on a rocker at 4°C. Then, the membrane washed thrice in TBST for 5 minutes each, followed by incubating the membrane in horseradish peroxidase-conjugated secondary antibody (1:5000 dilution) for 1.5 hrs at room temperature. After incubation over, the membrane was washed thrice in TBST. Then, the membrane was developed using Bio-Rad Clarity Max™ western ECL blotting substrate and visualized in Chemidoc (Bio-Rad Molecular Imager ChemiDoc XRS + Imaging System, California, USA). Then, the blot was stripped using stripping buffer (Tris-HCl, 10% SDS, 2-mercaptoethanol) at 50°C for 30 minutes. After washing with TBST thrice, the same blot was used to probe for the next protein [25].

### Statistical analysis

All the experiments were done in triplicates and mean data values are expressed as their deviation as mean±SD, and all data were analyzed by two-way analysis of variance (ANOVA) using GraphPad Prism 5 software to determine the significant differences between the groups. p<0.05 was considered to be significant, \*indicates a significant result when compared with control (p<0.05).

## RESULTS AND DISCUSSION

The bioactive compounds extracted from PM and BM using supercritical fluid extraction method. The extracted compounds structure was elucidated using spectroscopic methods (LC-MS, IR, <sup>1</sup>H- and <sup>13</sup>C-NMR). Based on these spectroscopic results it was identified that bioactive component extracted from PM (compound 1) and BM (compound 2) was found to be a phenolic aldehyde-vanillin (4-hydroxy-3-methoxybenzaldehyde) (data not shown). LDH assay results confirm that extracted compound vanillin efficiently induces non-cytotoxic cell death which implies that it might be intermediated through induction of apoptosis in HT-29 and MCF-7 cells (data not shown).

Apoptosis plays a key role in the treatment of cancer and oncogenesis. It removes extra unwanted or damaged cells and is a regulated pathway mainly for maintaining homeostasis in multicellular organisms. It can be triggered by numerous internal and external signals. These signaling pathways are controlled by the expression of apoptosis-related proteins [26]. There are two groups of proteins involved in regulation of apoptosis are anti-apoptotic and pro-apoptotic proteins [27]. In our previous study, it was confirmed that compound 1 and 2 induce apoptotic cell death at the concentration of 250 µg/ml and 1000 µg/ml for 48 hrs. In this study, we explicate the possible mechanism through which extracted compounds induce apoptotic cell death in HT-29 and MCF-7 cells. The cells were treated with 250 µg/ml and 1000 µg/ml concentration of extracted compound from PM and BM for 48 hrs. The changes in the expression level of the protein that might be involved in compounds induce apoptotic cell death was observed in western blotting.

### Effect of extracted compound vanillin from PM and BM on cytochrome c release

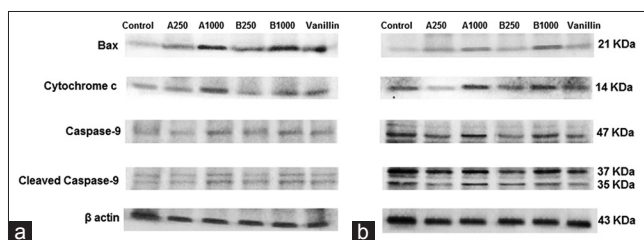
Cytochrome c is main signaling molecule of apoptosis which translocates from mitochondria into the cytosol of many cell types undergoing mitochondria-dependent apoptosis [25]. Cytochrome c is the pro-apoptotic signal molecules which can initiate the reaction of the caspase cascade and induce the apoptosis in the end [28]. After treating HT-29 and MCF-7 cells for 48 hrs with a concentration of 250 µg/ml and 1000 µg/ml of compound 1 and 2. It enhances the release of cytochrome c from mitochondria. The expression level of cytochrome c release was significantly increased in both the cell lines in a dose-dependent manner when compared with control (Figs. 1a and b, 2b).

### Effect of extracted compound vanillin from PM and BM on expression of Bax and caspase-9

In the mitochondrial pathway of apoptosis, several signaling molecules can cause the alterations in mitochondrial membrane permeability

transition and mitochondrial membrane potential resulting in the release of cytochrome c [29]. Mitochondrial initiated cytochrome c release is directly assisted by Bax [30]. Bax is a pro-apoptotic member of Bcl-2 family important for apoptosis. It could be responsible for these mitochondrial changes and activation of caspase both *in vitro* and *in vivo* during apoptosis [31]. The changes in the expression of the pro-apoptotic protein Bax that might be involved in extracted compound induce cell death was detected by western blotting. As

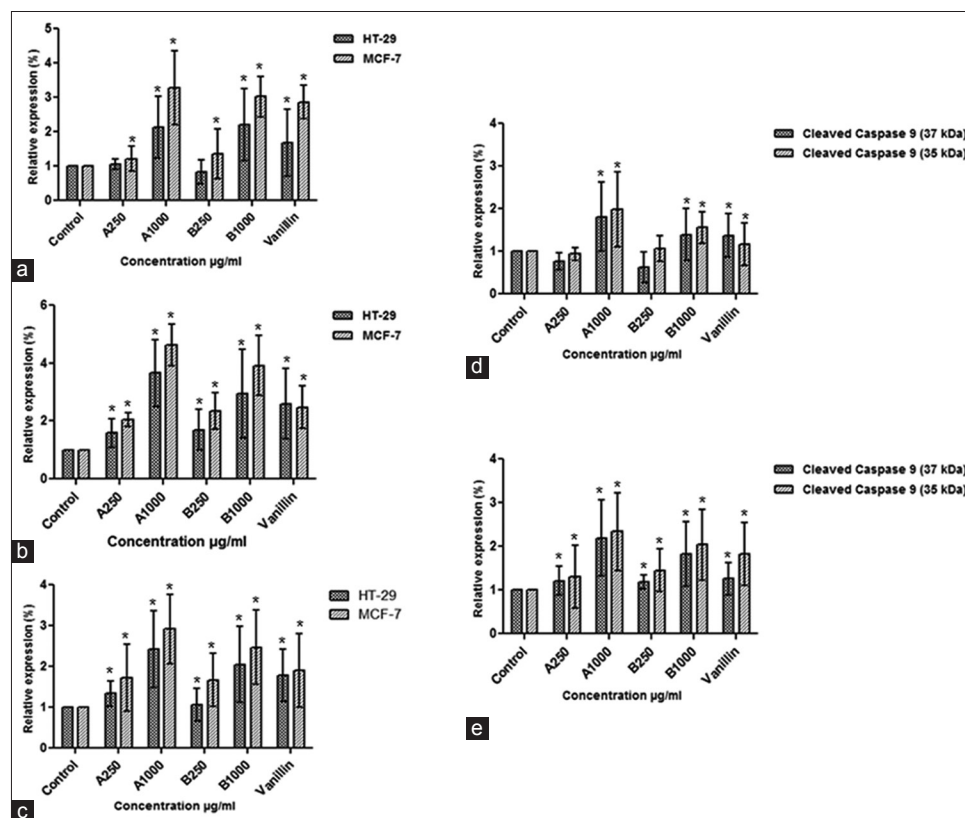
shown in Figs. 1a and 2b the result indicates that on the 48 hrs treatment of extracted compound 1 and 2 in HT-29 and MCF-7 cell line, the expression level of Bax was dose-dependently up-regulated in colon cancer cell line. Similarly in MCF-7 cell line expression level of Bax was also up-regulated (Figs. 1b and 2a). Therefore, the effect of compounds (1 and 2) on inducing apoptosis in HT-29 and MCF-7 cells might be related to the upregulation of the Bcl-2 family proteins.



**Fig. 1:** Effect of extracted vanillin compounds on expression of pro-apoptotic protein Bax, caspase-9 and cytochrome c release in HT-29 cells and MCF-7 cells. (a) HT-29 cells were treated with 250 µg/ml, and 1000 µg/ml for 48 hrs and protein expression level was detected. (b) MCF-7 cells were treated with 250 µg/ml, and 1000 µg/ml for 48 hrs and protein expression level was detected. Protein (75 µg/ml) from each sample was resolved on 15% sodium dodecyl sulfate-polyacrylamide gel and western blot performed. β actin is a housekeeping protein used as a positive loading control in all experiments. The result from three independent experiments is shown. Where, A - proso millet (A250, A1000) and B - barnyard millet (B250, B1000)

Caspase-9 is one of the key initiator caspases and an important member of the caspase (cysteine-aspartic acid protease) family. Caspase-9 was activated during programmed cell death. On apoptotic activation, cytochrome c released from mitochondria linked with the pro-caspase and facilitate stimulation of caspase 9 (47 kDa) involves in proteolytic processing resulting into two subunits at 35 and 37 kDa. These subunits attend to increase the apoptotic response. In addition, the cleaved caspase-9 processes other members of caspase (caspase-3 and caspase-7) to initiate a caspase cascades which leads to the apoptosis. To study, whether the extracted compounds initiate the apoptosis through a caspase-dependent pathway. HT-29 and MCF-7 cell lines were treated for 48 hrs with 250 µg/ml and 1000 µg/ml concentration of compound 1 and 2. Fig. 1a and b show that the expression levels of caspase-9 were significantly up-regulated (Fig. 2c) and activated the cleaved caspase-9 (p35 and p37 subunit) as concentration increases in a dose-dependent manner (Figs. 1a and b, 2d and e). On various cancer cell lines that might prompt apoptosis through translocation of Bax that activate caspase to induce apoptosis [32].

In previous studies, vanillin considered as a safe compound for use in food and drug by the FDA based on the LD50 oral vanillin concentration in rats (1.58-2.8 g/kg) [33]. Ho *et al.* (2009) stated that profile of gene expression was assessed in vanillin treated HepG2 cell line.



**Fig. 2:** Bar chart is representing the expression level of apoptotic protein Bax, caspase-9 and cytochrome c quantified by densitometric analysis. (a) The expression level of Bax in HT-29 and MCF-7 cell line. (b) The expression level of cytochrome c release in HT-29 and MCF-7 cell line. (c) The expression level of caspase-9 in HT-29 and MCF-7 cell line. (d and e) The expression level of cleaved caspase-9 in HT-29 and MCF-7 cell line, respectively. Where, A - proso millet (A250, A1000) and B - barnyard millet (B250, B1000). The expressions of all the protein were quantified with respect to the expression of β actin. Values are expressed as a mean ± standard deviation from three independent experiments. \*Indicates a significant result when compared with control (p<0.05)



Downregulating genes by vanillin are categories in three groups they are cell cycle, regulation of cellular process and cell death. Most of the down-regulated genes were related to cancer development. Vanillin inhibits AP-1 protein activity by decreasing the phosphorylation of ERK (extracellular signal-regulated protein kinase), this specifying that vanillin-regulated AP-1 activity through the ERK pathway [17]. In addition, Deb *et al.* (2011) postulated that vanillin enhanced tumor necrosis factor-related apoptosis-inducing ligand inducing cell death through NF- $\kappa$ B activation [22]. In HT-29 cells vanillin induce both cytolytic and cytostatic effects which led to cell death through apoptosis pathway [18]. Our results reported for the first time that vanillin compound extracted from PM and BM induced apoptotic cell death through mitochondria mediated pathway. The expression of Bax, caspase 9 protein and cytochrome c release was up-regulated in HT-29 and MCF-7 cells in a concentration dependent manner through apoptotic signaling pathway.

## CONCLUSION

Our results reveal for the first time that molecular mechanism of extracted compound vanillin from PM and BM significantly induces apoptotic cell death in human colon cancer cell line (HT-29) and human breast cancer cell line (MCF-7) through apoptotic signaling pathway. During apoptosis processes, extracted compounds increased the release of cytochrome c and upregulating the expression level of Bax and caspase-9 as concentration increases in a dose-dependent manner. From these outcomes, it was concluded that vanillin compound induces apoptosis in HT-29 and MCF-7 cells show that vanillin can be used as an effective chemotherapeutic agent. Further, it is necessary to identify whether this millet consumption will have an effect on colon and breast cancer control in an *in vivo* animal model.

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