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Research Article

Vanillic Acid Suppresses Proliferation and Induces Apoptosis of Gastrointestinal Cancer Cells via Inhibition of mTOR Expression

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ABSTRACT

Gastrointestinal cancer is a major global health concern associated with high mortality rates. Adjuvant therapies, including surgery and chemotherapy, are widely used in cancer treatment. However, chemotherapy often causes significant side effects, harming healthy cells and leading to long-term complications. Therefore, alternative therapeutic strategies with fewer adverse effects are urgently needed. This study aimed to investigate the antioxidant and cytotoxic activity of vanillic acid, a phenolic compound on gastrointestinal cancer cell lines, specifically KKU-100 and HepG2 cell lines. The evaluation of antioxidant properties was conducted by the DPPH and FRAP assays, while cytotoxicity assessment was performed through MTT assays. The cell apoptosis rate was analyzed using flow cytometry, and the expression of the mTOR gene was quantified through real-time RT-PCR analysis. Our results demonstrated strong antioxidant activity, with a maximum FRAP value of 235.77±0.11 μgAAE/mg of vanillic acid and a DPPH free radical scavenging rate of 69.33±3.11%, with an effective concentration (EC_{50}) of 2.69 mg/mL. Furthermore, vanillic acid significantly suppressed the proliferation of KKU-100 and HepG2 cell lines, with IC_{50} values of 1508 μg/mL and 634.3 μg/mL, respectively, in a dose-dependent manner. Flow cytometry analysis revealed a slight increase in apoptosis in both cell lines. Notably, vanillic acid downregulated mTOR expression in both KKU-100 and HepG2 cells. The findings suggested that vanillic acid, may serve as a promising therapeutic candidate for the treatment of gastrointestinal cancers.

Keywords: Gastrointestinal cancer, Vanillic acid, Apoptosis, mTOR

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Introduction

Gastrointestinal (GI) cancer indeed poses a public health issue and remains a leading cause of cancer-related mortality worldwide. Several factors have been implicated in the development of gastrointestinal cancer including genetic abnormalities, lifestyle choices, and exposure to pathogens [1]. Several pathogens have been implicated in increasing the risk of gastrointestinal cancer development. For example, chronic infections with *Opisthorchis viverrini* and hepatitis B virus (HBV) induce oxidative stress, contributing to the development of cholangiocarcinoma (CCA) and liver cancer, respectively [2–4]. Currently, the combination of surgery and adjuvant chemotherapy is a well-established treatment for various types of cancer. However, chemotherapy often leads to toxic side effects, including fatigue, nausea, vomiting, alopecia, decreased appetite, and increased susceptibility to infections [5]. While ongoing advancements in technology and medicine are making chemotherapy more effective with fewer side effects, researchers continue to explore novel cancer treatment alternatives.

Medicinal plants and their bioactive compounds have long served as valuable resources in medical research. These plants contain substances with therapeutic properties that can be used for disease treatment, prevention, and health promotion. Compared to conventional treatments, medicinal plants often cause fewer side effects, are more accessible, cost-effective, and support the sustainability of biological resources [6]. Compounds derived from medicinal plants can have multiple therapeutic activities including antioxidant, anti-inflammatory, immune-boosting, and anticancer properties [7]. For instance, mannooligosaccharides derived from coconut meal exhibit cytotoxic effects against HCT116 colorectal cancer cells by inducing caspase activation and ROS generation, which lead to cell cycle arrest and apoptosis [8]. Similarly, compounds from *Adenosma bracteosum* (Bonati) inhibit cells proliferation and inducing apoptosis on liver cancer cell; HepG2 and lung cancer cells; NCI-H460 through caspase-3 activation and ROS production [9]. Thai noni juice (*Morinda citrifolia* L.) ethanol extracts have been shown to exhibit cytotoxic effects on CCA cell lines via upregulate the expression of genes linked to apoptosis, while showed less toxicity to peripheral blood mononuclear cells [10]. Additionally, ferulic acid (FA) significantly inhibits cell proliferation and invasion on Caski and Hela cervical cancer cell lines via inducing a halt in the cell cycle at the G0/G1 phase by modulating cell cycle-related proteins expression such as cyclin D1, p21, and p53 [11]. These findings highlight the potential of bioactive compounds from medicinal plants in the development of cancer treatments.

Vanillic acid (VA), 4-hydroxy-3-methoxybenzoic acid, is classified as an aromatic phenolic acid and is recognized as an oxidized derivative of vanillin. It emits a pleasant odor and has been extracted from various medicinal plants, with particularly high concentrations found in the roots of *Angelica sinensis*. VA exhibits antibacterial, anti-inflammatory, and anticancer properties [12]. Previous studies have demonstrated its anticancer effects in various cancers, including in MCF-7 breast cancer cell lines, VA has been shown to suppress cell growth in a dose-dependent, likely due to the overproduction of ROS and the induction of apoptosis [13]. In hepatocellular carcinoma, VA inhibits cell proliferation by downregulating cyclin-D1 expression and inducing apoptosis through the upregulation of pro-apoptotic genes Caspase-3 and Bad, while suppressing the expression of anti-apoptotic gene Bcl-2 [14]. Similarly,

in HCT116 human colon cancer cell lines, where VA significantly decreases HIF-1α protein synthesis through the inhibition of the mTOR/p70S6K/4E-BP1 pathways [15]. The mTOR signaling pathway plays a significant role in cellular growth and proliferation. Its dysregulation is a hallmark of various cancers and has become a focus of medical research and therapeutic intervention [16, 17].

These findings underscore the potential of vanillic acid as a promising alternative therapy for cancer via the mTOR pathway. This study aimed to explore the antioxidant properties and potential anticancer effects of vanillic acid on gastrointestinal cancer, specifically targeting liver cancer and CCA cell lines. Additionally, the study investigated vanillic acid's potential as a treatment option for gastrointestinal cancer.

Materials and Methods

Cell lines and reagent

This study utilized two human cancer cell lines: the HepG2 represents as liver cancer cell line and the KKU-100 represents as cholangiocarcinoma (CCA) cell line. HepG2 cell line was kindly supplied by Assoc. Prof. Dr. Porntip Pinlaor. KKU-100 cell line (JCRB1568) was obtained from the Japanese Collection of Research Bioresources and currently stored at the Cholangiocarcinoma Research Institute (CARI), Khon Kaen University. Both cells were cultured in Ham's F-12 medium enriched with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin and were incubated at 37 °C in a 5% CO_2 incubator. Vanillic acid (#94770, HPLC) was purchased from Sigma-Aldrich, USA.

Ferric reducing antioxidant power (FRAP) assay

The experiment was adapted based on previous studies [18]. Ascorbic acid was diluted to concentrations range 10 to 500 μg/mL to establish the standard calibration curve. FRAP reagent was freshly prepared by combining 0.25 M of acetate buffer (pH 3.6), 20 mM ferric chloride (Merck KGaA, Germany), 10 mM 2,4,6-tripyridyltriazine (TPTZ; Sigma-Aldrich, USA), in a 10:1:1 ratio. A total of 18 μL of vanillic acid solution was mixed with 182 μL of FRAP reagent in a 96-well plate and incubated at a temperature of 37 °C in the dark. After 30 minutes of incubation, absorbance was quantified at 593 nm using a microplate reader (Tecan/Sunrise Microplate Reader, Switzerland). The mean±SD was used to show the results. Micrograms of ascorbic acid equivalent per milligram of vanillic acid (μg AAE/mg vanillic acid) was used to measure the ferric reducing antioxidant capacity.

Free radical scavenging activity using DPPH assay

This experiment was modified from previous studies [18]. Stock solutions of vanillic acid (10 mg/mL in DMSO) were diluted to 0.5 to 10 mg/ml. 20 μl of each vanillic acid solution was combined with 180 μL of a freshly prepared 0.1 mM DPPH reagent which prepared by dissolving 0.001 g of DPPH (Sigma-Aldrich, USA) in 25 mL of absolute ethanol. After incubating the mixture at room temperature in the dark for 30 minutes, absorbance was recorded at 517 nm using a microplate reader. Results were presented as the mean±SD. EC_{50} (effective concentration) values were determined from

curves generated by correlating the percentage of DPPH scavenging activity with the concentration of vanillic acid. The percentage of DPPH scavenging activity was determined using the following formula:

$$\text{DPPH Scavenging (\%)} = \frac{\text{Abscontrol-Abssample}}{\text{Abscontrol}} \times 100$$

Abs control refers to the absorbance measured for the control sample, and *Abs sample* refers to the absorbance measured for the test sample.

Cytotoxicity test

The evaluation of vanillic acid's cytotoxicity was conducted through the MTT colorimetric assay. HepG2 and KKU-100 cell lines were cultured in 96-well plates at a density of 3000 cells/well and allowed to incubate for 24 hours at 37°C within a 5% CO_2 incubator. Stock solutions of vanillic acid (400 mg/mL in DMSO) were diluted at concentrations range 500-2000 µg/ml and a maximum DMSO percentage of 0.5%. Then, cells were treated with vanillic acid for durations of 48 and 72 hours. The cells were rinsed with 1X PBS after each treatment phase and incubated for two hours at 37°C with MTT reagent. After removing the MTT solution, DMSO was added. A microplate reader was used to measure absorbance at 540 nm. The mean±SD was used to express the results. The half-maximal inhibitory concentration (IC_{50}) of vanillic acid was determined, based on the correlation between cell viability and vanillic acid concentrations. Cell viability (%) was calculated as:

$$\text{Cell viability (\%)} = \frac{\text{MeanOD540 sample}}{\text{MeanOD540 control}} \times 100$$

Where *Mean OD540 sample* is the average optical density at 540 nm of the treated sample, and *Mean OD540 control* is the average optical density of the untreated control.

Apoptosis assay using flow cytometry with annexin V/PI staining

KKU-100 and HepG2 cell lines were seeded at a density of 2 x 10^5 cells/well. The cells were treated to apoptosis induction by concentrations of vanillic acid at 1000, 1500, and 2,000 µg/mL for KKU-100, and concentrations of 300, 600, and 1,200 µg/mL for HepG2. Following treatment, the cells were centrifuged at 252 x g for 5 minutes after being washed with 1X PBS. The supernatant was removed, and the cells were resuspended in 50 µl of annexin V binding buffer. Next, 1 µL of propidium iodide (PI) and 2.5 µL of annexin V-FITC (V) were added to the suspension, and the mixture was incubated for 15 minutes at room temperature in the dark. After the incubation period, 400 µL of 1X annexin V binding buffer was added. Flow cytometry (BD FACSCanto II, Becton Dickinson) was then employed to analyze the samples.

Real-time reverse transcription-PCR (Real-time RT-PCR)

RNA extraction was performed using Trizol (Life Technologies™). A volume of one milliliter of Trizol was added to the cell pellet in the tube and mixed using a vortex. Following adding 200 µL of chloroform, the mixture was incubated for 3 minutes at room temperature. Subsequently, centrifugation

at 4°C, 14,575 x g for a duration of 15 minutes. The aqueous phase was transferred to a new tube, and 500 µL of isopropanol was added. The solution was inverted and allowed to incubate overnight at -20°C. Following centrifugation for 20 minutes at 4°C, 14,575 x g then the supernatant was discarded, and 1 mL of 70% ethanol was added. The mixture was centrifuged again, after that the RNA pellet was allowed to dry at room temperature before being dissolved in 20-50 µl of DEPC-free water. The RNA was incubated overnight at 4°C and preserved at -80°C until use. The concentration of RNA was determined using a nanodrop spectrophotometer. cDNA synthesis was performed from total RNA through the process of reverse transcription. Real-time RT-PCR was conducted using SYBR® Green for Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 58°C for 30 sec. Specific primers for mTOR (NM_001386500) and β-actin (NM_001101) (Integrated DNA Technologies, Inc.) were utilized in the experiment shown in Table 1.

Table 1 Primers used in Real-time RT-PCR.

Genes	primer sequences
mTOR	F: GCCGCGCGAATATTAAAGGA R: CTGGTTTCCTCATTCCGGCT
β-actin	F: CTTAGTTGCGTTACACCCTTTCTTG R: CTGTCACCTTCACCGTTCCAGTTT

Real-time RT-PCR analysis

Data analysis was conducted using the Comparative ΔΔCt Method. The Ct values (ΔCt) of the target genes were normalized based on the expression levels of β-actin. The calculation for relative quantification was performed using the following formula: ΔCt for each gene = $(Ct_{\text{of target gene}})-(Ct_{\text{of }\beta\text{-actin}})$, and Relative Quantification of the target gene using the equation $2^{-(\Delta Ct \text{ of target gene})}$. Then, calculate the fold change of gene expression in a liver cancer cell line (HepG2) and a CCA cell line (KKU-100) treated with a series of concentrations of vanillic acid.

Statistical analysis

GraphPad Prism v 8.0.1 was used for statistical analysis, which included EC_{50} and IC_{50} value computation. The mean±standard deviation of two separate experiments is used to represent the results. One-way ANOVA was used to detect the statistical significance among various groups. P-values below 0.05 were regarded as statistically significant.

Results and Discussion

The abundance of bioactive compounds found in medicinal plants, such as polyphenols, flavonoids, carotenoids, and phenolic acids, is well-documented. These substances play a significant role in enhancing overall well- being by promoting cellular health, supporting immune function, and preventing degenerative diseases. Their importance extends beyond therapeutic properties, as they exhibit notable anti-inflammatory, anti-cancer, and antioxidant activities. Among these compounds, vanillic acid, an aromatic phenolic acid derived from various medicinal plants, has been extensively studied for its potential bioactive effects, including its anti-inflammatory, anti-cancer, and antioxidant properties [12].

In this study, we focused on vanillic acid' s antioxidant properties and its anti- cancer effects in gastrointestinal (GI) cancer. Oxidative stress, driven by excess free radical production, often creates a microenvironment conducive to tumor development. Inflammatory cells may contribute to cancer progression by releasing free radicals, which cause DNA damage. Antioxidants, like vanillic acid, play a crucial role in cancer prevention by neutralizing oxidative stress, thereby protecting cells from mutagenic damage that can lead to tumor formation. Previous studies have demonstrated vanillic acid's potent ability to scavenge DPPH free radicals [13].

Antioxidant activity of vanillic acid using FRAP assay

The antioxidant properties of vanillic acid were evaluated using the FRAP assay, which measures the ability of compounds to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) through antioxidant activity. The maximum antioxidant activity observed for vanillic acid was 235.77±0.11 μgAAE/mg of vanillic acid.

Free radical scavenging activity of vanillic acid using DPPH assay

The ability of vanillic acid to scavenge free radicals was determined using the DPPH assay, which evaluates antioxidant capacity based on the compound's ability to neutralize DPPH free radicals. Vanillic acid exhibited the highest scavenging activity at 69.3±3.11% for DPPH free radicals (Figure 1). The EC_{50} value for the highest DPPH scavenging activity was found to be 2.690 mg/mL.

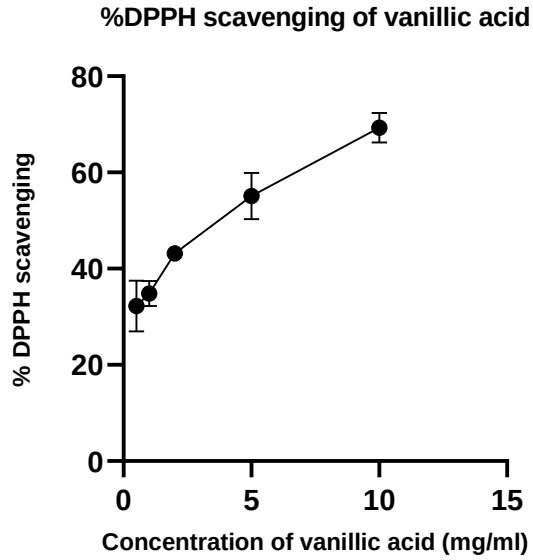

Figure 1 The percentage of DPPH radical scavenging activity by series concentration of vanillic acid.

Cytotoxicity of vanillic acid on gastrointestinal cancer cell lines using MTT assay

HepG2 and KKU-100 cells were exposed to treatment with a range of vanillic acid concentrations (500-2000 μg/mL) to evaluate cytotoxic effect of vanillic acid on the cancer cells proliferation. The findings indicated that vanillic acid effectively suppressed cell proliferation in dose dependent. For KKU-100 cells, the IC_{50} was 1978 μg/mL at 48 hours and 1508 μg/mL at 72 hours. Similarly, for HepG2 cells, the IC_{50} values were 634.3 μg/mL at 48 hours and 1195 μg/mL at 72 hours (Figure 2).

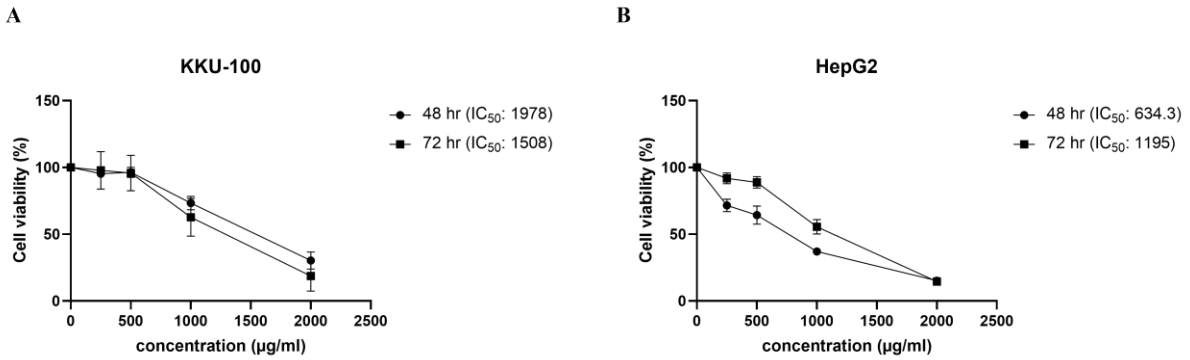

Figure 2 Vanillic acid reduced cell viability of KKU-100 and HepG2 cell lines. Cells were treated with a concentration of vanillic acid range of 500-2000 μg/mL. The percentage of cell viability A) KKU-100 and B) HepG2.

Effect of vanillic acid on apoptosis pathway

Annexin V/PI staining was performed to assess the effect of vanillic acid on apoptosis induction in HepG2 and KKU-100 cells. Flow cytometry analysis showed an increase in total apoptosis rates of

HepG2 cells treated with vanillic acid with 1.10±0.42%, 1.50±1.13%, and 4.30±1.41% at concentrations of 300, 600, and 1200 μg/mL for 48 hours, respectively. Similarly, KKU-100 cells treated with vanillic acid for 72 hours exhibited increased total apoptosis rates of 1.95±0.07%, 3.1±0.57%, and 6.9±0.71% at concentrations of 1000, 1500, and 2000 μg/mL, respectively (Figure 3).

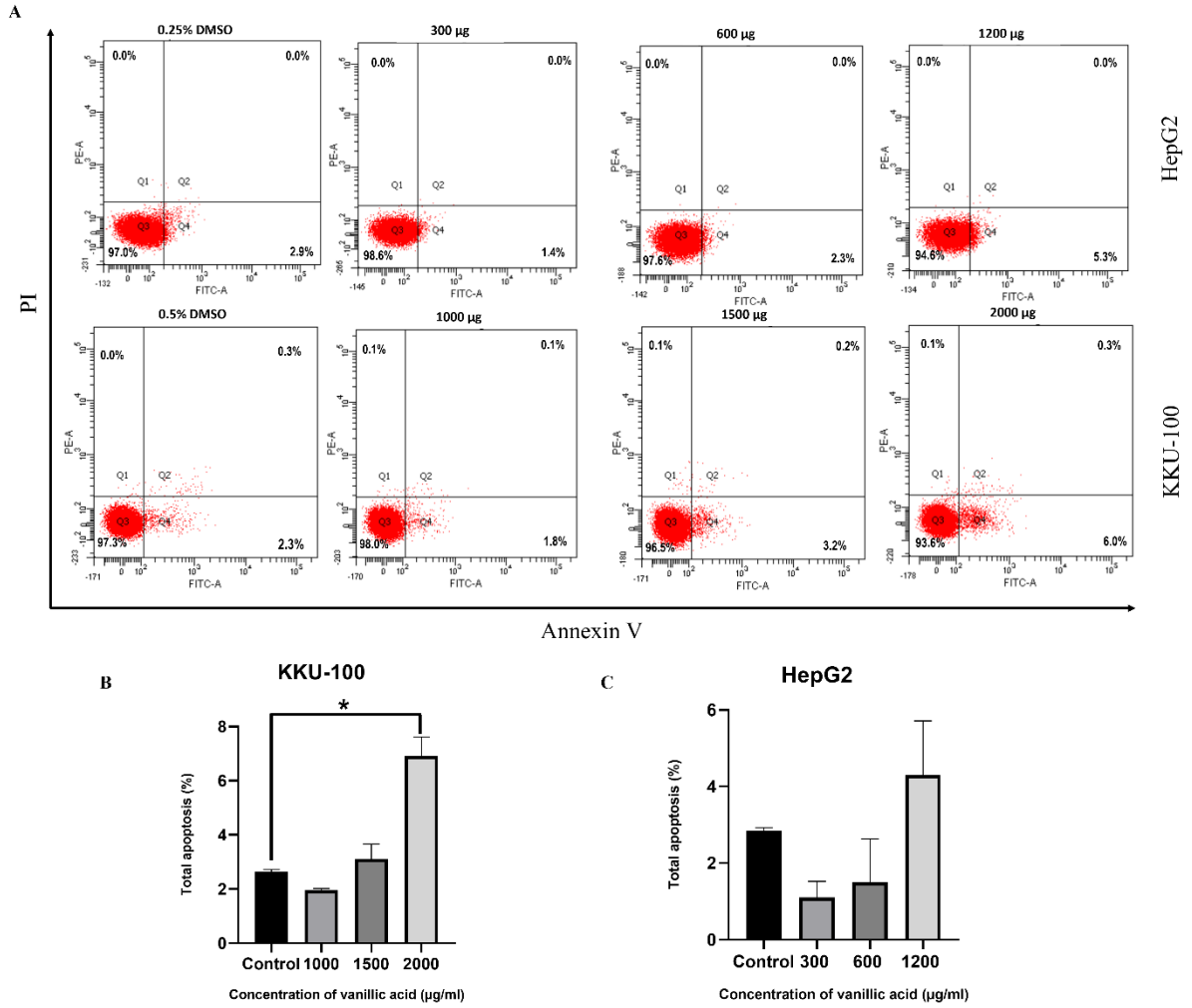

Figure 3 Vanillic acid-induced gastrointestinal cancer cell apoptosis. Flow cytometry analysis of Annexin V and PI was performed on HepG2 and KKU-100 cells treated with vanillic acid. A) Overall cell apoptosis of KKU-100 and HepG2, B) Total apoptosis rate of KKU-100, and C) Total apoptosis rate of HepG2. Statistical significance (*P < 0.05).

Effect of vanillic acid on gene expression related to mTOR signaling pathway

Real-time RT-PCR analysis was used to investigate the effect of vanillic acid on the expression of the mTOR gene in both KKU-100 and HepG2 cells. In KKU-100 cells, the fold change in mTOR expression was reduced by 0.0136±0.01 at a vanillic acid concentration of 2000 μg/mL compared to the 0.5% DMSO control. In HepG2 cells, a reduction in mTOR expression by 0.0607±0.05 was observed at a concentration of 1200 μg/mL compared to the 0.25% DMSO control (Figure 4).

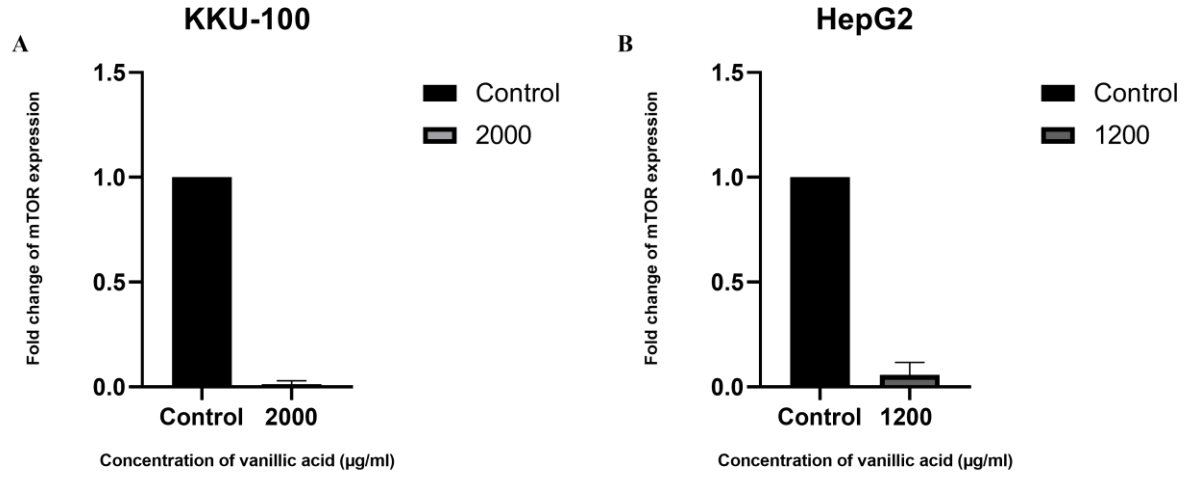

Figure 4 Vanillic acid suppressed mTOR expression in gastrointestinal cancer cell lines. The fold change of mTOR expression on A) KKU-100 and B) HepG2.

Our study confirmed the antioxidant activity of vanillic acid, particularly through the FRAP and DPPH assays. The highest FRAP value recorded in our study was 235.77 µgAAE/mg of vanillic acid, while DPPH scavenging activity reached 69.33%, The EC_{50} value was found to be 2.69 mg/mL. These findings underscore its antioxidant properties and suggest that vanillic acid may contribute to cancer prevention by mitigating oxidative stress and safeguarding cellular DNA from mutations, consistent with existing literature [19, 20]. Previous reports suggest vanillic acid has anti-proliferative effects that have been observed against various cancer cell types. For instance, human breast cancer, non-small cell lung cancer, and colon cancer [13, 21-22]. So, further analysis demonstrated the cytotoxicity and anti-proliferative effects of vanillic acid on GI cancer cell lines, KKU-100 and HepG2. Our MTT assay revealed that vanillic acid has the potential to inhibit the proliferation in both KKU-100 and HepG2 cells depend on the dose-dependent manner. Our results showed that the effective IC_{50} value of HepG2 was 634.3 µg/mL or 0.6 mg/mL, which was better than the IC_{50} value of *Pulicaria undulata* ($2.7x10^4$ µg/mL or 27.7 mg/mL) [23]. In KKU-100, our results show IC_{50} was calculated to 1508 µg/mL or 1.50 mg/mL which is in line with IC_{50} of Thai noni juice Chiangrai (TNJ-Cr) ethanolic extract (1.15±0.02 mg/mL) [10]. Ho et al. reported that vanillic acid which is a major metabolite from vanillin in humans did not show an antiproliferative effect on HT-29 colon cancer cells while vanillin shows effective antiproliferative on HT-29 cells with IC_{50} equal to 400 µg/mL [24]. It is intriguing that the evidence reports indicate that the varying IC_{50} values observed in individual cancer cell types suggest that the cytotoxic effects of vanillic acid are influenced by the specific types of cells targeted, as well as by other derivatives of vanillic acid or its structurally related compounds. Apoptosis, also known as programmed cell death, plays a pivotal role in the elimination of cancer cells and the inhibition of cancer cell proliferation.

Apoptosis was regulated through the extrinsic death receptor pathway and the intrinsic mitochondrial pathway [25]. Our findings indicated that cell apoptosis was slightly elevated in both

HepG2 and KKU-100 cells when exposed to vanillic acid at all concentrations. Despite its cytotoxic effects, vanillic acid did not induce apoptosis as well in KKU-100 and HepG2 cells, as indicated by flow cytometry. Therefore, it implies that vanillic acid may not inhibit the proliferation of gastrointestinal cancer cells through apoptosis. Instead, we investigated its influence on the mTOR signaling pathway. The mTOR signaling pathway primarily controls the proliferation and metabolism of cells, both of which are key factors in cancer progression [26]. The mTOR signaling pathways are widely recognized for their role in protein synthesis through mTOR phosphorylation and its downstream effectors, including p70S6K, 4E-BP1, and eIF4E [27]. Furthermore, mTOR plays a crucial role in the modulation of autophagy, which is widely considered an inhibitory mechanism against cancer development. a cellular mechanism responsible for breaking down and reusing cytoplasmic elements when nutrients and energy are deficient [28].

Our study demonstrated that vanillic acid downregulates mTOR expression in both KKU-100 and HepG2 cells. Consistent with findings from Gong, et al., where vanillic acid suppressed phosphorylation of mTOR in HCT116 colon cancer cells [13]. This suggests that vanillic acid's anticancer properties may be mediated through the inhibition of the mTOR signaling pathway, leading to reduced cell proliferation. These findings suggest that vanillic acid warrants further investigation as a potential agent for combination therapies in cancer treatment. Its ability to modulate key cancer-related pathways, such as mTOR, positions vanillic acid as a promising compound in the development of novel anticancer therapies.

Conclusions

This study highlights the therapeutic potential of vanillic acid, a phenolic compound commonly found in medicinal plants, for gastrointestinal cancer treatment. Vanillic acid exhibits strong antioxidant activity, effectively scavenging free radicals and reducing oxidative stress, a key factor in cancer progression. Moreover, vanillic acid demonstrated cytotoxic and antiproliferative effects on GI cancer cells in a dose-dependent manner. Although its influence on apoptosis was limited, vanillic acid's anticancer properties may be attributed to the suppression of the mTOR signaling pathway, which regulates cell growth and cancer development.

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