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Vitexin modifying the expression of *APC, p53, KRAS* genes with metabolome profile changes in HT-29 colorectal cancer cell line

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ABSTRACT

Background & Aim: *Colorectal cancer* is a clinically heterogeneous disease resulting from metabolome pattern alterations of many metabolites and their genetic factor interactions in man. Chemotherapy in colorectal cancer is generally followed by multiple side effects, including drug resistance; It is well established that herbal medicines are gaining worldwide interest in treating many cancers. Vitexin is an apigenin flavone glycoside present in hawthorn has exhibited therapeutic properties. This study was performed to assess the antitumor properties of Vitexin on the expression of *p53, KRAS,* and *APC* genes and the metabolome profile alterations associated with these genetic modifications.

Experimental: Cells were treated with different concentrations of Vitexin, and toxicity and cell growth inhibition were ascertained *in vitro* using the MTT assay method. Cells were treated with Vitexin, and gene expression was determined. Following metabolome 1HNMR spectroscopy with 1D NOESY protocol and the resulting spectra scrutinized to classify differentiated metabolites and their biochemical pathways. Integrative systems biology analysis software examined the metabolites and the genes, and the main pathways modulated by gene expression were identified.

Results: Our finding revealed that a 50% inhibitory concentration for Vitexin was 16.32 µM, while the relative expression of tumor suppressor genes *APC* and *p53* in treated cells enhanced and the expression of the *KRAS* oncogene gene decreased significantly compared to the control group. The crucial changes in convergent metabolic phenotype with genes were identified in this investigation.

Recommended applications/industries: Our findings revealed that Vitexin exhibits antitumor properties by targeting a specific biochemical pathway in the cell's metabolome profile due to changes in genes involved in colon cancer.

1. Introduction

Colorectal cancer (CRC), a global health dilemma, is one of the most common cancers known, and despite significant advances in its treatment, such as chemotherapy, radiotherapy, and advanced surgery, it is still the third leading cause of cancer-related deaths worldwide (Ogunwobi *et al.*, 2020). CRC is a complex disease caused by genetic and epigenetic factors and usually results from several genetic mutations in tumor suppressor genes and oncogenes such as *APC, p53,* and *KRAS* in the colon mucosa cells as the cancer adenoma sequence (Ng *et al.*, 2019). One of the treatment dilemmas for CRC is the different responses of patients to therapy despite the same histopathological features, which is probably due to heterogeneous molecular

changes both at the level of gene expression and downstream metabolic changes. These include the resistance of cancer cells to chemotherapy and the recurrence of cancer cells. To increase the effectiveness of treatment outcomes, we must fully understand the mechanism of the disease and new drug goals based on this, introduce new treatment protocols to improve and increase patient survival (Wang *et al.*, 2013). In recent years, traditional medicine and especially herbal medicine and its active compounds as a cheap and accessible source for the general population or supplementation of the treatment or prevention of diseases, have received much attention (Aiello *et al.*, 2019).

Vitexin (4′,5,7-Trihydroxyflavone 8-C-glucoside, Apigenin 8-C-glucoside, 8-C-Glucosylapigenin, Orientoside) is a flavonoid glycoside of apigenin ($C_{21}H_{20}O_{10}$) (Figure 1).

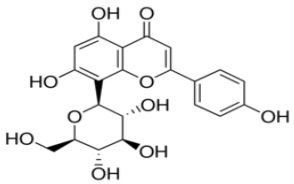

Figure 1. Vitexin Structure

It is present in some traditional herbs, such as hawthorn, otter flowers, bamboo leaves, and pearl millet (Babaei *et al.*, 2020), with anti-inflammatory, antioxidant, analgesic, and neuroprotective properties and improves ischemic heart disease (Peng *et al.*, 2021). Its anti-cancer and anti-metastatic effects have manifested in various cancers such as leukemia, glioblastoma, choriocarcinoma, liver, colon, ovary, prostate, and nasopharyngeal cancer (Ganesan and Xu, 2017).

Cancer cell metabolism is significantly dissimilar from normal cells because they require significant metabolic changes to endure the growth and progression of cancer, mainly related to changes in gene expression patterns in cancer cells. Exploring the metabolome profiles of cancer cells by metabolomics is one of the most efficient ways to diagnose cancer function as identification of altered pathways identifies altered metabolites that are effective in the development, progression, and inhibition of cancer (Schiliro and Firestein, 2021; Li and Deng, 2017).

One of the high throughput technologies employed in metabolomics is nuclear magnetic resonance spectroscopy (1HNMR), which is a non-destructive and non-invasive method compared to other possible approaches. Following NMR spectroscopy, bigdata classification analysis such as partial least discrimination Analysis (PLS-DA) is generally applied to classify the differentiating metabolites in NMR spectra (Li and Deng, 2017). This study aimed to focus on the anti-cancer effects of vitexin and investigate the influence of this substance on metabolome profile reprogramming for changes in the expression of genes involved in colorectal cancer carcinogenesis on HT-29 cells.

2. Materials and Methods

2.1. Reagents

Reagents: Vitexin (CAS No.3681-93-4) with molecular weight of 432.38 and ≥95.0% purity and trypan blue solution, 3-(4,5-di-methyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Sigma-Aldrich, USA). RNX plus (Sinagen, Iran), the 2-step real-time-PCR kit (Aldrich-sigma, UK), Dulbecco's Modified Eagle's Medium (DMEM), and the Roswell Park Memorial Institute (RPMI) medium were purchased from Merck KGaA© (Darmstadt, Germany).

2.2. Cell culture

The colorectal cancer cell, HT-29 cell line, code NCBI C154 were purchased from Pasteur Institute of Iran Cell bank. After counting and determining the percentage of survival they were cultured in tissue flask with DMEM medium containing 10% fetal bovine serum (FBS) and antibiotics streptomycin (100 µg/mL), penicillin 100 U/mL in an incubator at 37 °C, with 5% CO2 and 95% moisture. The flasks medium changed every 48 h.

2.3. MTT assay

After counting and evaluating the viability of cells using trypan blue, 1×10^4 cells were trypsinized, then re-cultured in 96-cell well plates and incubated for 24 h. After ensuring that the cells adhered to the base of the plate, they were treated with 150 µL of diluted vitexin with DMSO at various doses (0, 10, 15, 20, 25, 30

µM). After 24, 48, and 72 hours, 20 µL of MTT (5 mg/mL) reagent was added to all wells and incubated for 4 h in an incubator at 37°C. After dissolving the formazan precipitates with DMSO, the absorbance with a wavelength of 570 nm and reference length wave of 630 nm was read. Cell growth inhibitory and cytotoxicity were correlated with the control sample along with IC_{50} determination. The experiment was performed in three replications (Kumar *et al.*, 2018).

2.4. Expression profiles of APC, KRAS, and P53 genes after using vitexin

Extraction of total RNA was done by using Gene All kit from control and treated cells which treated with IC_{50} of vitexin in 48 h according to the relevant protocol. The purity and concentration of RNA samples were evaluated using a Nano-Drop spectrophotometer (ND-1000TM, Thermo Scientific) and integrity with 1% gel electrophoresis. The amount of 1 µL of RNA samples, the results of which had an optical absorption ratio of 280/260 nm between 1.8-2, was performed for DNA synthesis using a 2-step PCR-RT kit (Aldrich-sigma, UK). The primers were designed using Gene Runner software, and the correct binding of the primers to the relevant sequence was investigated, and the binding of the primers to other sequences was investigated by the Blast program at NCBI and prepared by Sina Clone Company. The primers were synthesized as follows: p53 (forward, 5'-ACAAGGTTGATGTGACCTGGA-3', reverse, 5'-TGTAGACTCGTGAATTTCGCC-3'); APC (forward, 5'- GACTGGTATTACGCTCAACTTCA -3', reverse, 5'- CAATTGCCTTCTGGTCATATCTG -3'); *KRAS* (forward, 5'-AGCGTCACTGGCACTTTCAAA-3', reverse, 5'-CACCCACATAGAAGACCTGGT-3'); GAPDH (forward, 5'-AGGGCTGCTTTTAACTCTGG-3', reverse, 5'-CCCCACTTGATTTTGGAGGG -3'). After investigating the melting curve and evaluating the efficiency of the primers, and drawing the standard curve, the expression of *p53*, *KRAS*, and *APC* genes was examined, and the raw data obtained from quantitative real-time polymerase chain reaction (qRT-PCR) were analyzed using software (Green *et al.*, 2018). After amplification, CT (threshold) of the samples were identified and also determined (PCR efficiency mean) and based on Relative Quantification (RQ) method and $2^{-\Delta\Delta Ct}$ formulation [$\Delta\Delta CT= (Ct^{Target}-Ct^{GAPDH})- (Ct^{Control}-Ct^{GAPDH})$], it was multiplied by the expression of *p53*, *KRAS*, and *APC* genes and compared with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) internal gene and interpreted in the presence of balanced normalization.

2.5. Metabolites extraction

HT-29 cells (8×10^6 cells number) treated with IC_{50} concentration (16.32 µM) of vitexin for 48 hours and (8×10^6 cells number) were considered as control. The cells were then trypsinized and washed twice with cold PBS, centrifuged at 4000 rpm for 10 min at 4ºC, and then supernatant discarded. To extract the metabolites by two-phase hydrophilic and lipophilic extraction methods we resuspended cells in DMEM without FBS, centrifuged at 15000 rpm for 15 min at 4 ºC, and washed twice with PBS. 1.0 mL of chilled chloroform and methanol (1: 2 ratio) followed by chloroform and water (1:1ratio) was added to the cell suspension, vortexed, and sonicated for 5 min at 4 ºC followed by centrifugation at 12000 rpm at 4°C for 10 min. The pH of the supernatant was adjusted to 6.8 and then was centrifuged again as above. Both hydrophilic and lipophilic samples were lyophilized prior to NMR spectroscopy (Saborano *et al.*, 2019).

2.6. ^{1}HNMR spectroscopy

To the lyophilized hydrophilic samples (n=5), at a ratio of 10% by weight of each sample, D_2O was added to eliminate the interference of the water, 50 µl of 1 mMol 3-(Trimethyl silyl) propionic-2,2,3,3 acid sodium (TSP) was used to record the zero point of the spectrum and imidazol (2 m Mol) was used as pH indicator. Also, to the lipophilic samples (n=5), 10% by weight deuterated chloroform was added and the samples were scan in NMR Broker MHZ 500 with 1D NOESY protocol with 0.05 S mixing time, 2 S relaxation delay, spectral width of 6410.256 HZ and number of scans 500 (Saborano *et al.*, 2019).

2.7.Data analysis

The spectra obtained were uploaded to the Matlab software (ver. R2020) and used the ProMetab (v.3_3) code to preprocessed the spectra for baseline correction, normalization and binning (0.005) and finally to convert to metrics data sheet. The data was analyzed by using partial least squares discrimination analysis (PLS-DA), and the differentiating chemical

shifts obtained were identified by using the Human Metabolome Database (HMDB). The identified metabolites and the genes were then examined at the MetaboAnalyst software through joint pathway analysis. The most important metabolic pathways and altered metabolites in each pathway were identified as integrated or convergent with the studied genes.

2.8. Statistical analysis

Data obtained from 3 separate IC_{50} studies and gene expression were reported as mean ± standard deviation (SD). Student t-test was used to compare groups in experiments, and p-value<0.05 was statistically significant.

3. Results and discussion

Evaluation of toxicity and cell inhibition of HT-29 cells by using vitexin at different concentrations in three-time intervals of 24, 48, and 72 hours and three independent experiments was shown in Figure 1. Vitexin increased cell growth inhibition in a dose- and time-dependent manner. The IC_{50} or dose that increases cell growth inhibition by up to 50% is presented in Figure 2 at three-time intervals.

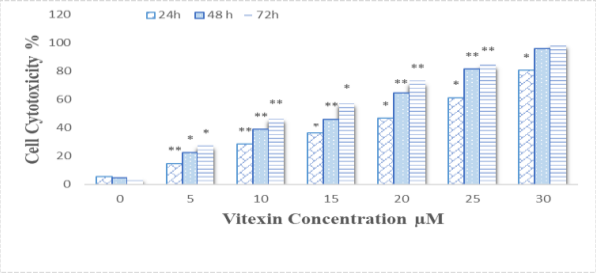

Figure 2. Cell growth inhibition of HT-29 class colorectal cancer cells against different concentrations of vitexin in three time periods of 24, 48, and 72 hours. Samples were reported as growth inhibition percentages compared to control samples. This experiment was performed in three replications. Data are presented as mean ± SD of three independent experiments. * Represent $P < 0.05$, **represent $P < 0.01$ compared with untreated cells.

Cancer cells need principal genetic changes to maintain their survival, energy supply, and proliferation and the metabolic changes that result from them (Long *et al.*, 2020). In this study, the effect of reducing the viability of vitexin on colorectal cancer cells of the HT-29 class was first evaluated by the MTT method. The results showed that treatment with a concentration of 5 and 30 µM with vitexin for 24 h reduced the increased the growth inhibition of HT-29 cancer cells by 2.74 and 5.8 folds compared to control cells, respectively, indicating that this process has a direct relationship to increase the growth inhibition of cells depending on its concentration**.** On the other hand, it was shown that the effect of vitexin on increasing cell growth inhibition is also time dependent, because treatment with 5 µM concentrations of vitexin in 24 and 72 hours, respectively, increased by 2.74 and 15.5-fold inhibits cell growth. Numerous other studies have been performed to investigate the effects of toxicity and reduced viability of vitexin and its isomers on tumors and different categories of cancer cells. In a similar study, the effect of vitexin in different concentrations (2.5-50 µM) increased the growth inhibitory of various colorectal cancer cells types (HCT-116, HCT-116DR, HT-29, and KM12C), human cervical cancer (HeLa), cancer (Bhardwaj *et al.*, 2017).

Based on the data obtained from the MTT assay test, a concentration of 16.32 µM was used to evaluate gene expression. Treatment with 16.32 µM concentration of vitexin increased 9.38 and 9.12 tumor suppressor genes *p53* and *APC*, respectively, and decreased the *KRAS* oncogene gene by 0.12-fold compared to control cells (Figure 3).

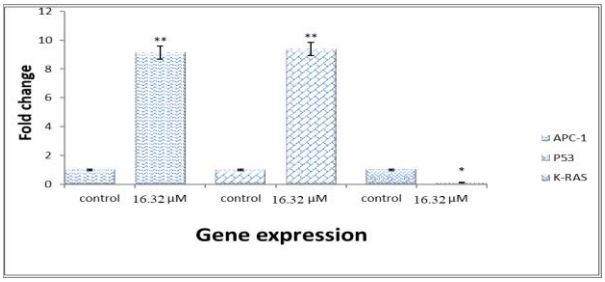

Figure 3. Expression rate of *APC*, *KRAS*, and *P53* genes in HT-29 colorectal cancer cell line treated with 16.32 µM vitexin for 48 hours using qRT-PCR. Data are expressed as mean ± SD and three independent experiments. * Represent $P < 0.05$, **represent $P < 0.01$ compared with untreated cells.

Colorectal cancer is a heterogeneous disease affected by multiple epigenetic factors and genetic cascade changes in the *APC*, *KRAS*, *TP53* genes, followed by disruption of the signaling pathways, which causes the transition from normal clone cells to adenomas and the onset and progression of colorectal cancer. Any mutation in the *APC* tumor suppressor gene activates the β-catenin WNT pathway and initiates adenoma cancer cells and the development of polyps.

Subsequently, a change in the *KRAS* oncogene gene activates the EGF signaling pathway, which causes cancer to progress from early stages to the adenomatous stage, eventually leading to a mutation in the *P53* tumor suppressor gene entering the malignant stage (Ping *et al.*, 2020). On the other hand, these multiple molecular interactions in colorectal cancer cause the emergence of different metabolite phenotypes, and these resulting metabolic changes may also precede and cause several genetic and molecular changes in these cells. There is a need to recognize these changes and therefore review to discover different markers and treatments. Metabolomics reflects the relationship between genotype and phenotype in various diseases and drug responses as an evolving discipline for diagnosing cancer, drug responses, and represents downstream products in the cell cascade (Long *et al.*, 2020).

Our findings revealed that vitexin significantly enhanced the *APC* tumor suppressor gene expression by about 9.12 folds, increased 9.38 folds in the *p53* tumor suppressor gene, and diminished *KRAS* oncogene expression 0.12-fold as compared to control cells. A comparative investigation using HCT-116 and LoVo colorectal cells enhanced the *p53* gene and initiated cell apoptosis. Additionally, applying vitexin for 14 days reduced colorectal cancer tumors in mice and enhanced the expression of the *p53* gene in tumors (Chen *et al.*, 2018). Vitexin also proved its antitumor effect in three human malignant melanoma cell lines by increasing *p53* gene expression (Liu *et al.*, 2018).

Examination of 1D ^{1}HNMR spectra obtained from the two groups shows principal metabolic changes after treatment with vitexin**.** Figure 4A shows the score plot of PLS-DA of the control group and treatment with vitexin in the hydrophilic phase, and Figure 4B shows the overlapping spectra of these two groups in the lipophilic phase. In this study, PLS-DA two-dimensional score plots of metabolites extracted from cells in two groups in the hydrophilic phase showed that the metabolites were separated in two distinct clusters with small overlap so that the separation was 97.3 % and 1.1% of the variance showed the total data (Figure 4A). In the lipophilic phase, this isolation was performed entirely and showed a difference of 88.6% and 7.9% of the total variance of the data. (Figure 4B).

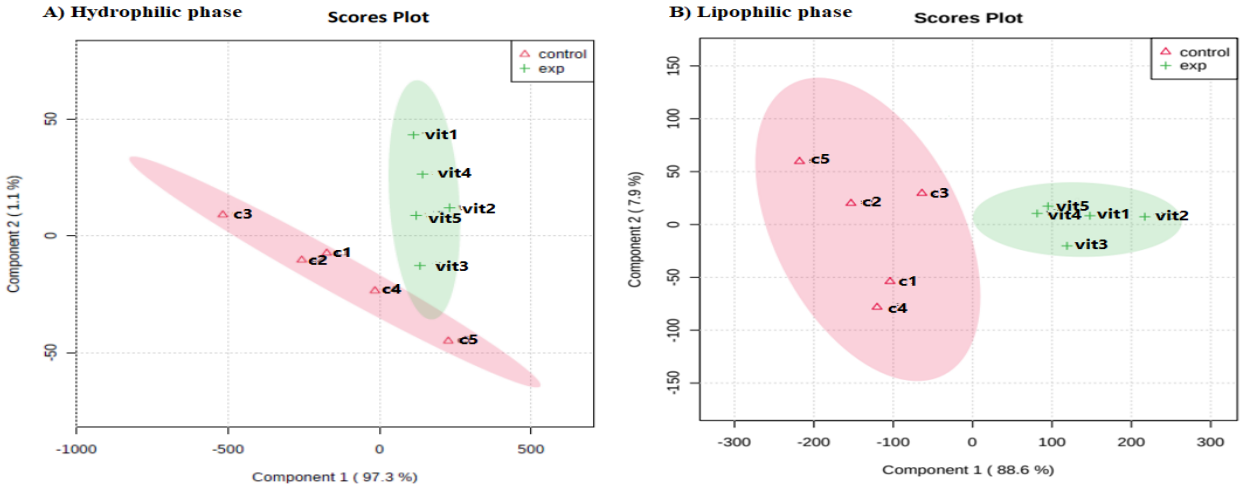

Figure 4. Score plot of PLS-DA in metabolites of the control group and treated with vitexin treated with eugenol (pink color) and control group (green color) in two phases (A) hydrophilic and (B) lipophilic. The diagrams show a well-defined cluster differentiation between the two groups in both phases.

Figures 5A and 5B show the Variable Important Projection (VIPs) and show the scores of the separated metabolite profiles related to the hydrophilic and lipophilic phases of the treated samples compared to the control. The spots in the graph indicate the chemical shift levels of the differentiating metabolites with their VIP score of the measurement of the importance of the variable (chemical changes) in the PLS-DA model. The Y-axis represents the VIP scores for each variable on the X-axis (greater than 1). The colored boxes on the right show the relative concentrations of the corresponding metabolite in the experimental and control cell groups.

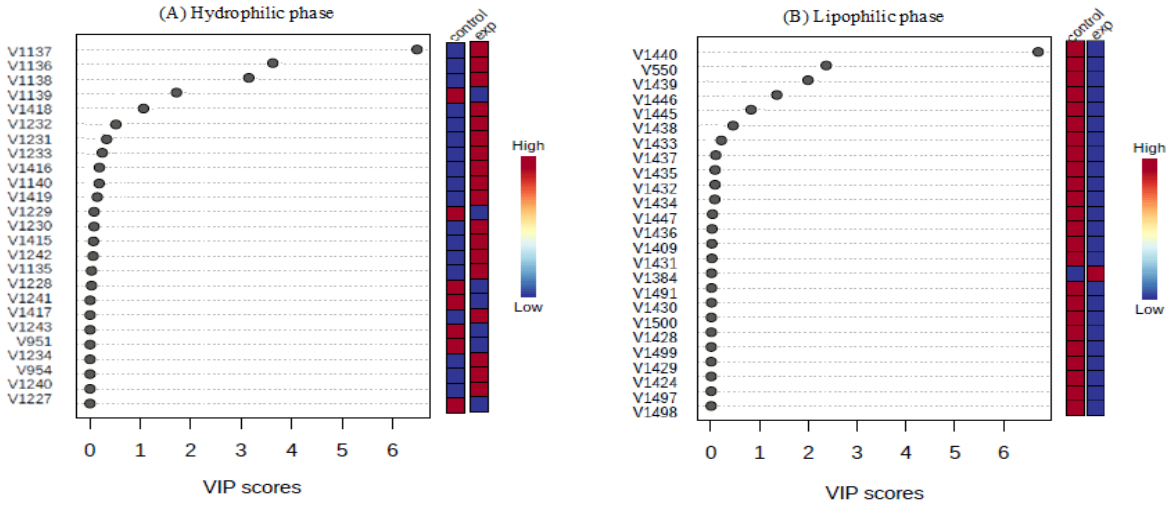

Figure 5. Important metabolites identified in PLS-DA by VIP scoring method. The boxes to the right of the VIP chart show the abundance ratio and concentration of metabolites.

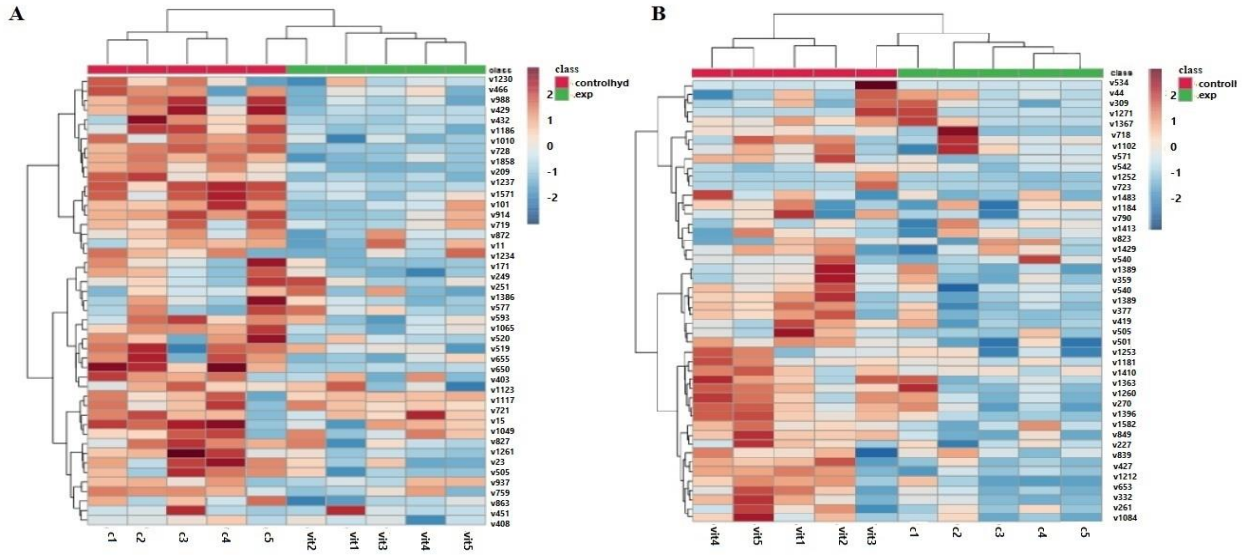

Figure 6. Heat map showing the variations in the control (red) and experimental groups (green). Regression values of variations indicated by color and the number of differentiating variations is shown on the right.

The chemical shifts of the metabolites are identified from their variable numbers and using the HMDB database. To evaluate and analyze metabolome profiles that are regulated by the genes, *APC*, *K-RAS*, and *P53* genes ID and the metabolites outliers were enrolled in the pathway analysis software, and modified pathways were discovered. Biochemical pathways that Vitexin has modified are shown in Figure 7. Most of the altered metabolic pathways and the altered metabolites in these pathways and the association of these alterations with potential genetic alterations related to the genes studied in HT-29 cancer cells affected by vitexin treatment are shown in Table 1.

Table 1. The most important metabolic pathways and identification of altered metabolites and the relationship between these changes and potential genetic alterations related to the genes studied in Het-29 cancer cells that have been affected by vitexin treatment.

Metabolic pathway	Participating Metabolites	Total	Hits	Pathway impact value	Raw p	Change in gene expression		
						APC 9.12-fold>+	*P53* 9.38-fold>+	*KRAS* 0.12 fold<-
Valine, leucine, and isoleucine biosynthesis	L-Threonine L-Isoleucine L-Valine	12	3	0.27273	5.9507E-5	✓	✓	✓
Aminoacyl-tRNA biosynthesis	L-Glutamate L-Valine L-Isoleucine L-Lysine	74	5	0.068493	1.0431E-4	-	-	✓
Biotin metabolism	L-Lysine Biotin	21	2	0.3	0.0088784	-	✓	✓
Steroid biosynthesis	Lathosterol Cholesterol Cholesterol ester	82	3	0.16049	0.0177	-	✓	✓
Glycerolipid metabolism	Phosphatidate Triacylglycerol	35	2	0.20588	0.023757	-	✓	-
Nitrogen metabolism	L-Glutamate	10	1	0.22222	0.057649	-	✓	-
D-Glutamine and D-glutamate metabolism	L-Glutamate	10	1	0.33333	0.057649	✓	✓	✓
Purine metabolism	dGTP Deoxyguanosine dGMP	166	3	0.10303	0.1041	-	✓	-

Total, the total number of compounds in the pathway; Hits, the actual matched number from the user uploaded data; Raw p, the original p-value calculated from the enrichment analysis, using MetaboAnalyst database.

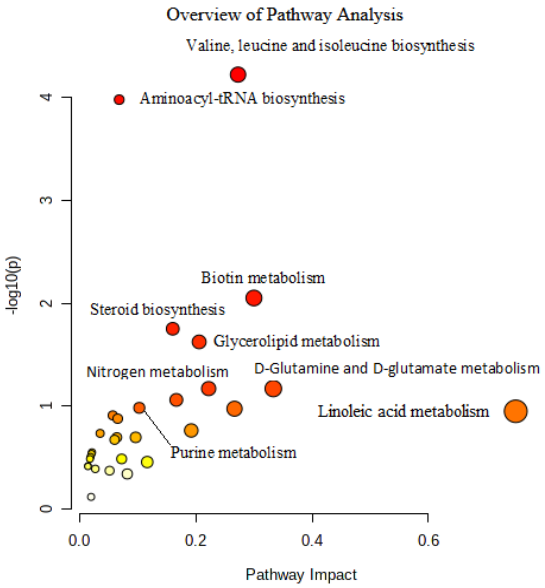

Figure 7. Analysis and visualization of metabolic pathways converging with the studied gene, which is rapidly altered under the influence of vitexin treatment. Each point represents a metabolic pathway, and the point size positively correlates with the effect of the metabolic pathway and degree of centrality test. The x-axis is based on topological analysis and shows the effect of the path. The y-axis is arranged based on the scores of the enrichment analysis.

According to the results, the most critical interconnected cycles of importance include biosynthesis of branched-chain amino acids valine, leucine, and isoleucine, biosynthesis of t-RNA, nitrogen metabolism, glutamate and glutamine metabolism, and altered metabolites in these pathways, including L - threonine, L-isoleucine, L-valine, L-glutamate, and L-lysine.

The high need for amino acids, especially the metabolism of branched-chain amino acids in cancer cells, is critical for reprogramming for the high proliferation of these cells, providing energy, and participating in nucleotide synthesis. On the other hand, branched-chain amino acids are the main source of nitrogen for alanine and glutamine production. Apart from the supportive role of branched-chain amino acids in biosynthesis and energy supply, these amino acids, including leucine, are involved in essential signals such as mTOR, a protected serine/threonine kinase in the PI3K / Akt / mTOR cell pathway that promotes cancer by controlling cell growth, proliferation and migration (Nie *et al.*, 2018). On the other hand, increasing the expression of cytoplasmic branched-chain amino acid aminotransferase enzyme (BCAT), especially

leucine, in glioblastomas and T cells has increased the proliferation and migration of these cancer cells (Zheng *et al.*, 2009). The amount of amino acids in colorectal cancer tissues as well as in non-polyp tissue of APCmin/+ mice is higher than in abnormal tissues (Yoshie *et al*., 2012). Since the first step in the onset of colorectal cancer is a mutation in the *APC* gene, the high amino acid increase is likely due to a mutation in the *APC* gene and activation of the Wnt /Bcatnin pathway, and activation of the autophagy pathway and increase in amino acids (Yoshie *et al*., 2012; Soto-Pantoja *et al.*, 2016).

Amino acyl tRNA synthetase (ARS), in addition to the second principal cause of amino acid binding in protein translation, have other domains such as glutathione S-transferase and endothelial monocyte activating polypeptide II domains, which under normal conditions cause abnormal functions of these enzymes; the onset and progression of cancers such as colorectal, lung, breast, leukemia, ovarian, liver, pituitary adenoma, bronchial lymphoma, stomach, and pancreatic cancer. Some of these (ARS), such as methionyl-tRNA synthetase (MRS), are known as cancer markers in some cancers, such as lung cancer and most metastases, and are also high in colorectal cancer. Some, such as isoleucyl-tRNA synthetase 2 (IARS2), asparaginyl-tRNA synthetase (NRS), and lysyl-tRNA synthetase (KRS), have been linked to cancer progression. In cancers, the expression of a number of these ARSs undergoes alterations, such as isoleucyl-tRNA synthetase (IRS), the promoter of which has been modified in non-polyposis colon cancer (HNPCC) (Zhou *et al.*, 2020). Most of these changes are influenced by the expression of multiple genes; for example, this process of carcinogenesis and progression is regulated by the aminoacyl tRNA synthetase and AIMP by the *p53* gene (Wang *et al.*, 2018; Choi *et al.*, 2011). Increasing the level of MARS through specific binding to Rad3-related protein (ATR) by regulating *p53* gene expression reduced apoptosis due to DNA damage in colorectal cells and increased carcinogenicity in colorectal cancer (Soto-Pantoja et al. 2016). In lung cancer, specific AIMP2 binding by intermittent binding to *p53* reduces apoptotic function and reduced survival in these patients (Choi *et al.*, 2011). In leukemia, IARS2 may be involved in the development of acute myeloid leukemia by regulating *p53* / p21 / PCNA / eIF4E (Li *et al.*, 2019).

Another metabolic pathway that changes in cancer is nitrogen metabolism. The prominent donor of nitrogen is glutamine, which enters the Krebs cycle through the anaplerotic pathway and participates in the synthesis of nucleic acids. Nitrogen access is controlled by transaminases and the amines, especially glutaminase and phosphoribosyl pyrophosphate amido transferase, and an imbalance between the two causes more carcinogenicity and cancer cell growth. The renewability of nitrogen sources by various enzymes such as carbamoyl phosphate synthetase (CPS), glutamate dehydrogenase (GDH), and glutamine synthetase (GS) is very important for the anabolic processes of cancer cells (Kurmi and Haigis, 2020; Kodama *et al.*, 2020).

One of the most important metabolic pathways in cancer is the metabolism of glutamate and glutamine. Cancer cells consume glutamine as the most abundant amino acid in the body due to a lack of energy and nutrients. This amino acid is anaplerotic and participates in the biosynthesis of nucleic bases, fatty acids, and also through glutathione synthesis, causes antioxidant defense and maintains the oxidation balance. It is also involved in mitochondrial metabolism and energy production by entering the TCA cycle as a carbon source (Kodama *et al.*, 2020; Choi and Park, 2018). Glutamate pathway metabolism is mediated via the *P53* gene by regulating glutaminase 2 (GLS2) expression (Choi and Park, 2018; Zhang *et al.*, 2013). The *p53* gene also compensates for glutamine deficiency in the Krebs cycle by activating and increasing the expression of aspartate/glutamate transporter (SLC1A3) in pancreatic cancer (PDCA) and continues to provide energy and raw materials for cancer cells to proliferate and maintain redox potential (Dufour *et al.*, 2012). In addition, in colorectal cancer, a mutation in the *KRAS* gene from the PI3K-AKT-mTOR signaling pathway counteracts glutamine deficiency and activates the expression of human asparagine synthetase ASNS, causing the production of aspartame from aspartate and the continuation of cell growth (Toda *et al.*, 2016).

Altered metabolites in these pathways include L-threonine, L-isoleucine, L-valine, L-glutamate, and L-lysine. One of the main sources of cancer cells for mono carbon metabolism other than glucose is amino acids such as glycine and serine, threonine. However, due to mutations in the enzyme threonine dehydratase (Tdh) in humans, this enzyme is inactivated, and this

has caused a change in the metabolism of mono carbon amino acids in humans compared to other organisms. Mice embryonic stem cells (ES) desperately need this enzyme to convert threonine to acetyl coenzyme A and glycine to perform carbon metabolic processes. Threonine is also required for the methylation of histones in mice and the promotion of carbon metabolism (Wang, Alexander and McKnight, 2011). The addition of threonine deaminase also reduced threonine deficiency in leukemia cell activity (Greenfield and Wellner, 1977). L-isoleucine and L-valine are other altered metabolites in these pathways and are branched-chain amino acids. The amino acids valine, leucine, and isoleucine are high in all four stages of colorectal cancer. Since these amino acids act as anaplerotic in some tissues, especially several cancerous tissues, and can meet the needs of these cells for energy and continuous proliferation (Yusof *et al.*, 2018). One of the hallmarks of pancreatic duct cancer is an increase in the level of these amino acids in the blood, which may occur due to the induction of *KRAS* gene expression (Zeitouni *et al.*, 2016).

In a study on cancer stem cells (CSCs), colorectal cancer cells with CD110 (thrombin receptor) markers were colonized in liver cells containing large amounts of thrombin. These cells were shown to regulate the catabolic pathway of lysine from two pathways. One is the production of coenzyme acetyl, which causes the acetylation of Low-density lipoprotein receptor-related (LRP6) receptors, which in turn activates the *APC*-Wnt B-catenin signaling pathway and restores it. On the other hand, it causes the production of glutathione and the preservation of redox oxidation potential, and the continuation of the carcinogenic process in colorectal cancer (Gao *et al.*, 2013; Wu *et al.*, 2015).

Another important pathway is the biotin metabolism pathway, and its altered metabolites are L-lysine and biotin. Biotin is an essential vitamin in mammals and acts as an active carrier of CO2 and an essential factor in carboxylase reactions and therefore plays a vital role in the metabolism of glucose, fatty acids, and amino acids. On the other hand, its consumption in cancer cells is higher than normal cells due to growth, proliferation, and high energy consumption. In most cancer cells, the expression of biotin receptors, especially sodium-dependent multivitamin receptors (SMVT), is very high and is characteristic of cancer cells (Fam Collot and Klymchenko, 2020). also, biotin regulates the expression of some genes, especially genes encoding inflammatory cytokines, genes involved in glucose metabolism, and genes expressing the propionyl-CoA carboxylase receptor and acyl glycoproteins through pathways such as histone biotinylating and modification. Their covalent vascularization, activation of guanylate cyclase, and nuclear translocation of NF-B are involved in response to biotin deficiency. These pathways are affected by tumor suppressor genes and oncogenes such as *p53* and *KRAS* (Ritchie *et al.*, 2017; Rodriguez-Melendeza and Zempleni, 2003).

Glycerolipid metabolism is another important metabolic pathway that has changed along this pathway. Metabolites that have been altered in this pathway include phosphatidate and triacylglycerol. Based on the presence of keto acyl and isoprene groups, which determine their properties and function, we divide lipids into eight groups, of which glycerolipids are one of these groups. Reprogramming of lipid metabolism, like glucose and amino acid metabolism, is a hallmark of cancer cells, especially colorectal, lung, bladder, pancreas, liver, and ovarian cancer cells, including the synthesis and oxidation of fatty acids mitochondria, elongation, and saturation. It causes many changes, such as changes in the membrane and changes in gene expression, which can be more effective in various aspects of tumorigenesis, such as cell growth and proliferation (Pakiet *et al.*, 2019). In glucose deficiency, the *p53* gene controls lipid metabolism by controlling and inducing two enzymes, Guanidinoacetate methyltransferase (GAMT) and lipin 1 (Lipin1). GAMT regulates fatty acid oxidation and also provides the energy needed for *p53*-induced apoptosis. The enzyme lipin-1 is induced by *p53* in response to starvation and then interacts with fatty acid oxidation in interaction with a complex containing peroxisome proliferator (PPAR) α receptor and PPAR γ-coactivator-1α (PGC-1α). Also, as a phosphatidate enzyme, phosphatase breaks down phosphatidate (PA), a precursor of membrane phospholipids and a regulatory role in lipid metabolism, to diacylglycerol. These two enzymes enter the Krebs cycle by producing acetyl coenzyme A and maintain oxidative phosphorylation (Liang *et al.*, 2013).

Purine metabolism is another metabolic pathway involved in this pathway, and the altered metabolites in this pathway are deoxyguanosine monophosphate (dGMP) and Deoxyguanosine triphosphate (dGTP). In

addition to being the main structural elements of nucleotides, which are the main elements of cell growth and proliferation, Purines also provide the cofactor and energy needed in proliferative and carcinogenic processes. Purines also participate in immune responses and tumor-host interactions by releasing cytokines. Purine and its derivatives, such as inosine, are very important in the proliferation of cancer cells and have been shown to increase the proliferation of melanoma cancer cells (Yin *et al.*, 2018). Also, the ratio of adenosine to inosine in cancer cells causes growth change, invasion, and metastasis. The level and activity of adenosine deaminase, a key purine-degrading enzyme, is known as a marker of cancer diagnosis (Shoshan *et al.*, 2015). Numerous substrates such as glutamine, glycine, aspartate, and a single carbon precursor increase the potency of carcinogens. Purine metabolism is mediated by downstream pathways of some carcinogenic signaling pathways. The *p53* gene controls the metabolism of purines in the HCT116 colorectal cancer cell via the *p53*-miR-34a-IMPDH pathway, and subsequently, inhibition of IMPDH disrupts the GTP-dependent Ras signaling pathway (Kim *et al.*, 2012). The *p53* gene inhibits two enzymes, Guanosine monophosphate synthetase (GMPS) and Inosine-5′-monophosphate dehydrogenase (IMPDH), two key enzymes in the renewal of purine bases (Kim *et al.*, 2012; Holzer *et al.*, 2017). It was also found that the *p53* gene inhibits the synthesis of nucleotides, including purines, and leads to cell death (Huang *et al.*, 2018).

Vitexin inhibited the signaling pathway of PI3K / Akt / mTOR, induced apoptosis, and inhibited the growth of small cell lung cancer cells A549 (Liu *et al.*, 2019). In addition, vitexin can prevent cancer progression by causing oxidative stress and by the formation of autophagosomes in colorectal and hematoma cancer cells (Bhardwaj *et al.*, 2017).

4. Conclusion

One of the most important ways to prevent and treat cancer and other regular treatments is to use appropriate pharmacological agents to inhibit or delay cancer or reversal of malignancy by focusing on various biological and molecular processes, including genomic profiles and related changes, including metabolic changes. although data analysis predicted more metabolites and pathways, but awe selected the pathways according to p values and their relationship to the mentioned genes. In this study, treatment of HT-29 cells with vitexin altered the expression of genes involved in the tumorigenesis process of colorectal cancer and, consequently, the metabolic profile of colorectal cancer cell lines HT-29. It was found that vitexin caused significant metabolic pathways and cancer inhibition by inhibiting tumor suppressor genes *APC* and *p53* and the oncogene gene *KRAS*. However, for further confirmation, further investigation is required to expand these findings' validity and explore the signaling pathways associated with these changes.

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