



Regulation of estrogen receptor-mediated apoptosis by vitexin in human colorectal carcinoma cells

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ABSTRACT

Colon cancer is the is the third most common type of cancer, causing death, with a survival rate of 5 years usually treated with chemotherapy or surgery. Many chemotherapeutic drugs are tested, but they have limitations such as resistance development, toxicity, limited response in advanced stages, and a lot of adverse side effects. To combat this problem, the current study focuses on vitexin, a phytochemical that has anti-inflammatory, anti-cancer, and anti-oxidant properties, is tested on HCT-116 cell lines as a chemotherapeutic drug for colon cancer. The present study proves that the phyto compound vitexin can upregulate the estrogen receptor genes and downregulate c-myc gene, which indicates that vitexin is a good therapeutic agent and induces the estrogen-mediated pathway on the regulation of cancer cell growth.

1. INTRODUCTION

Cancer is a disease characterized by uncontrolled cell growth, with significant global mortality [1]. In 2023, approximately 609,820 cancer-related deaths occurred in the United States, and in 2024, around 2,001,140 new cases are reported. Lung, stomach, and liver cancers are the most lethal, among major cancer death occurred due to lung cancer [2]. Colon cancer, originating from benign polyps, undergoes malignant transformation over 10-15 years, and its staging involves assessing tumor characteristics, lymph node involvement, and metastasis [3]. Early detection through screening methods such as colonoscopy and AI tools improves polyp identification. Surgical treatment is primary for resectable colorectal cancer (CRC), with chemotherapy, radiation, and immunotherapy used for non-resectable cases, though they can cause significant side effects and drug resistance [4]. Recent research focuses on combining natural phytochemicals, such as carotenoids,

flavonoids, and polyphenols, with synthetic drugs to reduce adverse effects while targeting cancer stem cells and modifying the gut microbiome [5]. Vitexin from *Vitex negundo*, is a glyco flavonoid that possesses good anticarcinogenic activity against different cancers, such as endometrial, breast, gastric, lung, liver, and colon, by inducing apoptosis through various mechanisms. One such mechanism includes estrogen-mediated pathways, in which vitexin can act as a selective estrogen receptor (ER) modulator and induce apoptosis in cancer cells by regulating the ratio of anti-apoptotic and pro-apoptotic gene (Bcl-2 & Bax) expressions [6]. Various studies have proven the binding efficiency of glyco flavanoids on ERs, both ER α and ER β , on the regulation of Alzheimer's disease, reproductive problems, and neurotoxicity [7]. Estrogenic role of glyco flavanoids activates ER α and ER β , leads to intracellular signalling pathways, and is responsible for the changes in cellular behavior [8]. Limited information is available on the protective effect of vitexin in CRC, mediated by ERs, and the complex role of apoptotic and pro-apoptotic genes in colon cancer progression. An experimental trial was carried out to check the estrogenic role of vitexin on ER and its associated role on cancer control in order to highlight the potential therapeutic strategies of vitexin for improving CRC treatment [9].

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2. MATERIALS AND METHODS

2.1. Cell maintenance

The human HCT116 colon adenocarcinoma cells were purchased from the National Centre for Curative Sciences in Pune, India. The cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin maintained at 37°C, 5% CO₂. Once the cells reach 75%-80% confluence, allowed to wash with phosphate buffered saline (PBS) (pH:7.4), after removing the media, harvested with 0.25% Trypsin-EDTA, and seeded to reach the required density under the same incubation condition [10].

2.2. Cytotoxicity assay

Cell cytotoxicity was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The MTT enters into the mitochondria in the viable cells and is reduced to purple-coloured formazan crystals, which are insoluble. The cells were solubilized with dimethyl sulfoxide, and released as solubilized formazan reagent was measured spectrophotometrically at 570nm. Different concentrations (1-25 µg/ml) of vitexin (Natural remedies, Bangalore, India) were dissolved in methanol were treated with HCT116 for cytotoxicity assay. Methanol was used as control. The optical density was measured at 570nm on a microplate reader. The 50% inhibitory concentration (IC₅₀) of the drug was calculated from the dose-response curves [11].

2.3. Cell migration assay

Cells grown in DMEM media with 80% confluence in 6 well plate, were used for migration assay. Media was removed, added 250 µl of 5 µg/ml of mitomycin C to each well to control the proliferation. Incubated the plate for 2 hours at 37°C and 5% CO₂. Removed the medium with mitomycin C. After washing the cells with PBS, a scratch was created on monolayer cells with a 10 µl tip across the center of the well, along the axial of tip should be perpendicular to the bottom of the well. The cells were viewed under a phase contrast microscope. About 100,000 cells were used in a 6-well plate was treated with drug at respective concentrations. The culture dish was placed in an incubator, and the image was captured every 12 hours. The images were taken using an inverted microscope at 40X magnifications. The wound images were viewed and analysed by ImageJ software. For wound closure calculation, the scratch width was measured using the software, considering the center of the scratch in every picture, from the scratch wall. The percentage of the wound open area was calculated by dividing the scratch area at 12, 24, and 36 hour time points by the scratch area at 0 hour time point [12].

The wound closure area percentage was calculated using the following formula:

$$\% \text{ Wound Closure} = \frac{A_{t=0h} - A_{t=\Delta h}}{A_{t=0h}} \times 100$$

where A_{t=0h} – Initial wound area; A_{t=Δh} – Wound area at time point.

2.4. Free radical scavenging assay

Different concentrations of vitexin were added with 1 ml of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) dissolved in methanol, kept in the dark for 30 minutes, and the UV/VIS spectrophotometric absorbance was measured at 523 nm. Standard and blank were processed simultaneously [13]. The scavenging activity was calculated using the following formula:

$$\% \text{ Scavenging Activity} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

where A_{sample} - Absorbance of the test sample;
A_{control}-Absorbance of the control.

2.5. Lipid peroxidation

Lipid peroxidation was assessed by thiobarbituric acid (TBA) assay, which measures malondialdehyde levels. Vitexin-treated cells were washed, treated with TCA and centrifuged. The supernatant was mixed with TCA and TBA, incubated at 95°C, and cooled. Butanol was added and separated. Absorbance was measured at 530 nm to evaluate lipid peroxidation [14].

2.6. Total antioxidant capacity

The ferric-reducing antioxidant power (FRAP) assay evaluates total antioxidant capacity (TAC) by reducing Fe³⁺ to Fe²⁺, forming a blue Fe²⁺-(2,4,6-Tri(2-pyridyl)-s-triazine) (TPTZ) complex measured at 593 nm. The FRAP reagent was prepared by mixing a 10:1:1 ratio of acetate buffer, TPTZ, and FeCl₃. Sample solutions, diluted in methanol and water, are incubated with the reagent for 30 minutes [15].

2.7. Apoptotic assay

An acridine orange (AO) - propidium iodide (PI) mixture was used to stain cells, the live cells emit green fluorescence, while the dead cells emit red fluorescence when cells are excited with the proper light source. The cells were seeded with a confluency of 3,000 cells in a 35 mm dish and incubated for 24 hours. The cells were treated with 7.8 µg/ml of vitexin. After 24 hours of drug exposure, the media was removed, and 100 µl of AO/PI stain was added to the plate. The number of viable and non-viable cells at the end of treatment was observed using an inverted fluorescent microscope [16].

2.8. RNA isolation, cDNA synthesis, and quantitative PCR (qPCR) analysis

HCT116 colon cancer cell lines (3 × 10⁵ cells) were treated with vitexin (IC₅₀) for 48 hours. At the end of 48 hours treatment, the RNA extraction was carried out by the trizol method. The quantity and the purity of the RNA were tested in a nanodrop spectrophotometer. Reverse transcription reactions were carried out for the production of cDNA Revert Aid First Strand cDNA Synthesis Kit. Genes specific for estrogen receptor-beta (ER-β), cellular myc (C-myc), interleukin-6 (IL-6), interferon-gamma (INF-γ), and tumor necrosis factor-α (TNF-α) were selected for the expression analysis. Quantitative reverse transcription-polymerase chain reaction was performed with high carboxy rhodamine Amplicon SYBR Green Master Mix and specific primers of target genes. Gene-specific forward and backward primers are designed for

Table 1. The forward and reverse primer to certain genes.

Genes	Forward primers (5'-3')	Reverse primer (5'-3')
ER β	TGGG- CACCTTTCTCCTT	GGTGTGTCTAGCGATCTTG
C-myc	AAGCTGAGGCACA- CAAAGA	GCTTGGACAGGTTAGGAG- TAAA
IL 6	ACCTCGAAACAG- CATCTGAC	AGATGCCGTCGAGGATGTA
IFN- γ	ATGTCCAACGCAAAG- CAATAC	ACCTCGAAACAGCATCTGAC
TNF- α	GAACTTGGAGGGCTAG- GATT	CAGCGAGTCCTTCTCACATT
β -actin	GCTCGTCGTCGACAAC- GGCT	CAAACATGATCTGGGT- CATCTTCTC

the gene amplification reaction. Expression values for qPCR products were processed along with the housekeeping gene β -actin as a reference gene, and ratios relative to non-treated samples were generated. The relative expression fold change was calculated by the $2^{-\Delta\Delta Ct}$ method [17]. The used primer sequences are given in Table 1.

2.9. Docking analysis

The autodock was performed using estrogen receptor- α (ER- α) (B subunit), ER- β (B subunit), and G-protein coupled estrogen receptor (GPER) as a protein target and vitexin as a ligand. The protein structure file was downloaded from databases like the Protein Data Bank, and the protein structure was prepared. After removing water molecules, the hydrogen atoms were added. The prepared protein structures were saved in PDBQT file after assigning Gasteiger charges. The ligand vitexin structure was prepared using CHEMSKETCH tool. The grid was set up with the measurement of $40 \times 40 \times 40$ Å³ centred on the active site of protein for better facilitation of ligand binding. The docking input files for docking procedure include the protein, the ligand, and the grid were used for the active docking in AutoDock 4.2. The Lamarckian Genetic Algorithm was repeated for 100 times for obtaining good results. The ideal ligand interactions with the lowest binding energy (ΔG) were selected from the clusters and used for further analysis. Best binding poses were analysed with receptor-ligand interaction using Discovery Studios Visualizer 2024 (Dassault Systemes BIOVIA®, San Diego, CA). The bonding affinity was calculated, and the results were confirmed with re-docking protocol in Autodock [18].

3. RESULTS

3.1. Cell cytotoxicity assay

The MTT assay used to determine cellular viability. Only viable cells will have active metabolism of converting MTT to purple formazan crystals, which was represented as the quantitative and sensitive detection towards cell proliferation, measuring the growth of cells with a linear relationship to cell activity and absorbance rate. The percentage cytotoxicity was calculated using the mean of triplicate and SE, which was

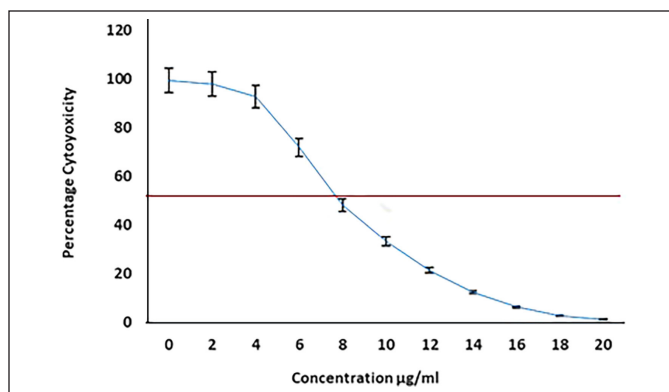


Figure 1. The HCT 116 cells were treated with vitexin with different concentrations for 24 hours, and growth inhibition was determined by MTT.

plotted against the concentration of vitexin. From the graph, the IC_{50} of vitexin was calculated on the treatment of vitexin at different concentrations based on serial dilution. Methanol was considered as a baseline control that has low cytotoxicity effects in the cells compared with the vitexin-treated groups. While evaluating the cytotoxicity, the vitexin-treated group shows a significant effect compared with control. The results of cytotoxicity showed that the cell death is concentration-dependent. The increased concentration of vitexin decreases the proliferation of HCT116 cells. The 50% growth inhibitory concentration of vitexin was found to be 7.8 µg/ml (Fig. 1).

3.2. Cell migration assay

The migration was analyzed on HCT116 cell lines treated with different concentrations of vitexin in time intervals of 0, 24, and 36 hours by scratch wound assay viewed under an inverted phase contrast microscope. Compared with the control the cell migration was observed in the treated group, but fast and maximum migration was observed in the control group over the period. In the test group, the different concentrations of vitexin were treated and examined under a microscope, and the migration rate was calculated at the end of 24 and 36 hrs intervals. The images clearly showed the fast migration of the cancer cell lines in the lower-concentration treated groups. Almost all cells cover $80\% \pm 3\%$ of the scratched area, indicating the concentration used is not enough to stop the migration of cancer cells. Unlike this result, the IC_{50} value of vitexin proves a better inhibitory role against the cancer cell migration; only about $10\% \pm 2\%$ of cells were seen on the scratched area (Figs. 2 and 3). The image shows cell migration at different time intervals, and the cell migration is inhibited at 36 hours, but it is not inhibited at 24 hours. So, vitexin at this concentration cannot inhibit cell motility. By comparing images, it is clear that cell migration is inhibited in 7.8 and 15 µg/ml of vitexin treated on HCT116 cell lines observed in the time intervals of 0, 24, and 36 hours.

3.3. Free radicals scavenging assay

Vitexin showed the strongest percentage of inhibition and radical scavenging activity compared with the reference standard gallic acid. In the DPPH assay, the vitexin at 15 µg/

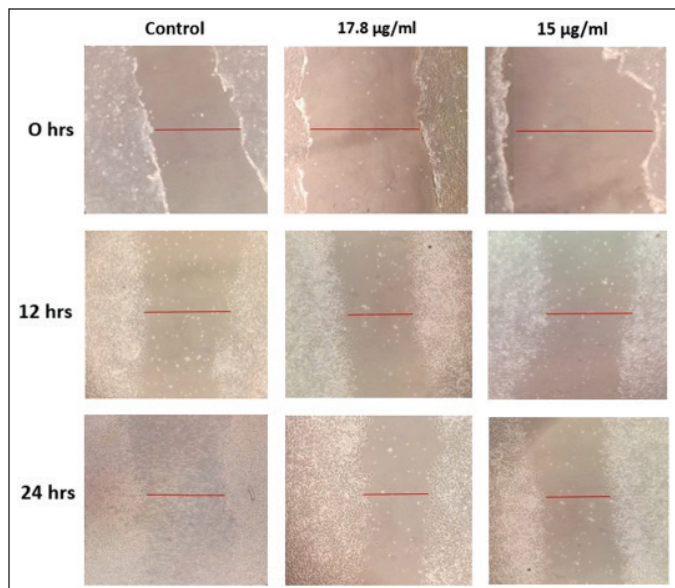


Figure 2. Cell migration assay.

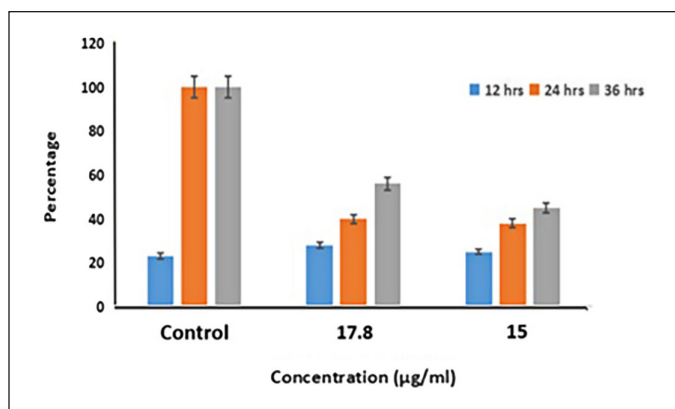


Figure 3. Cell migration assay.

ml shows more than 60% of the scavenging activity. The results of the hydroxyl free radical scavenging assay showed 98% scavenging activity at the concentration of 20 µg/ml by vitexin, which is almost equal to the potential standard free radical scavenger gallic acid. The HCT116 cancer cells were further tested with the radical generation, and the scavenging role of vitexin treated with different concentrations were also tested with the above-mentioned assay. The results showed that the decreased percentage may be due to the continuous radical development by the cancer cells, which was also significantly inhibited by vitexin at higher concentrations above 15 µg/ml (Fig. 4).

3.4. Lipid peroxidation assay

Lipid peroxidation activity on HCT116 cells was measured using thio barbituric acid reactive substances, which are used for lipid oxidation and antioxidant activity in physiological systems. They play a crucial role in the disruption of redox homeostasis, contribute to the development of various diseases, and also play a vital role

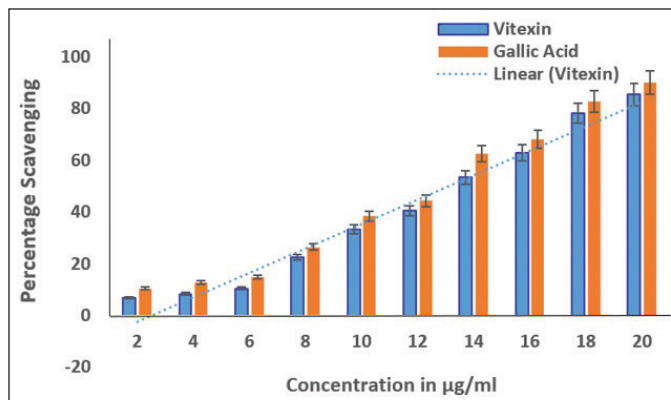


Figure 4. Free radical scavenging activity.

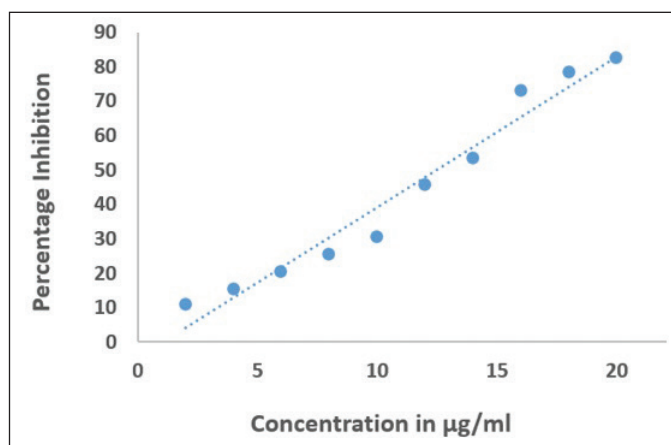


Figure 5. Lipid peroxidation assay.

in maintaining cellular homeostasis through signalling representation and cell death properties. HCT cell lines of control and treated groups were taken under different concentrations of vitexin, which was measured at an absorbance of 570 nm shows the lipid peroxidation activity of vitexin at different concentrations (2-20 µg/ml). The lipid peroxidation was tested with lower to higher concentration (2-20 µg/ml) in order to understand the dose response of vitexin at different concentrations against free radical generation, and from the result, it was evident that, in the tested concentration of vitexin showed a linear relationship with scavenging activity (Fig. 5).

3.5. Total antioxidant capacity

The antioxidant capacity of vitexin taken in different concentrations on HCT116 cell lines was carried out by FRAP assay. The lipid peroxidation and TAC were tested with lower to higher concentration (2-20 µg/ml) in order to understand the dose response of vitexin at different concentrations against free radical generation, and from the result, it was evident that in the tested range of concentration shown a linear relationship on scavenging activity. The TAC was identified as increased by increasing the concentration of vitexin. This shows that a lower concentration of vitexin

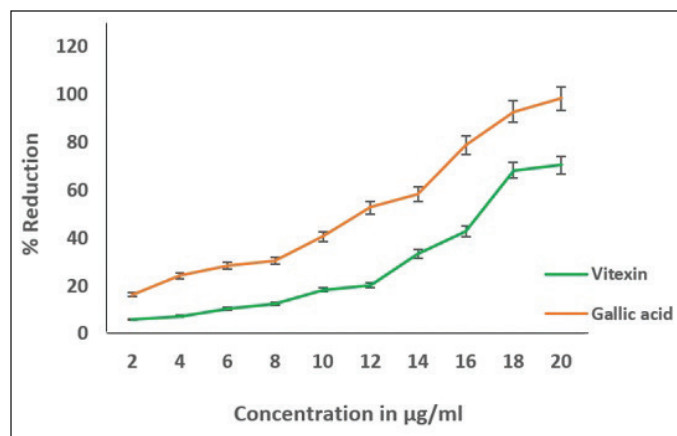


Figure 6. Total antioxidant capacity assay.

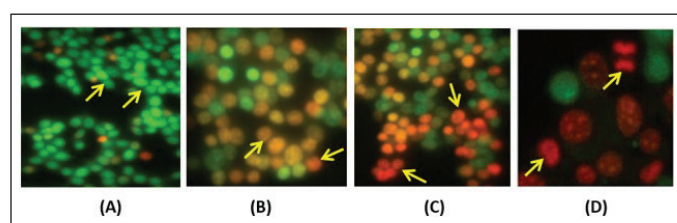


Figure 7. Apoptosis assay. Apoptotic characteristics on treatment of vitexin in HCT 116 colon cancer were observed on 24 hours of treatment (A) - Control viable cells indicated by green fluorescence. (B) and (C) - Early apoptotic features, namely, blebbing and chromatin condensation as well as late apoptotic cells were detected after 24 hours of treatment with vitexin indicated by orange fluorescence. (D) Late apoptosis and cell necrosis were observed after 24 hours of vitexin shown by red fluorescence.

can improve the antioxidant capacity, as proved in HCT116 colon cancer cell lines (Fig. 6).

3.6. Apoptosis assay

The apoptotic assay was done with double staining of AO/PI, in which the live and dead cells were identified based on the emission of green and orange fluorescence. As shown in Figure 7, the untreated HCT116 cancer cells appeared as healthy green cells with intact nuclei. By treating with IC_{50} concentration of vitexin at 24 hours of incubation, a greater number of dead cells were observed under a fluorescent microscope. The apoptotic results indicate that the vitexin-treated cells showed more late apoptotic actions with a reddish-orange appearance, which is due to the PI-positive band of denatured DNA within 12–24 hours and can also induce cancer cell apoptosis through the relatively early apoptotic appearance of yellowish-orange fluorescence. The extended prolonged incubation induces the cellular necrosis and death observed in the HCT116 by AO/PI-double-stained cells.

3.7. Gene expression analysis

The real-time PCR analysis was carried out to analyse the ER genes, transcription regulatory genes, and inflammatory genes, and the changes observed under the treatment of IC_{50}

Table 2. Gene expression analysis.

Genes	Δ CT control	Δ CT treated	$\Delta\Delta$ CT	Fold change	Regulation
ER β	33.30	31.62	-1.686	3.219	Upregulated
C-myc	29.43	33.13	3.703	0.076	Downregulated
IL6	29.42	32.00	2.578	0.167	Downregulated
INF γ	29.20	34.34	5.133	0.028	Downregulated
TNF α	30.67	33.78	3.109	0.115	Downregulated

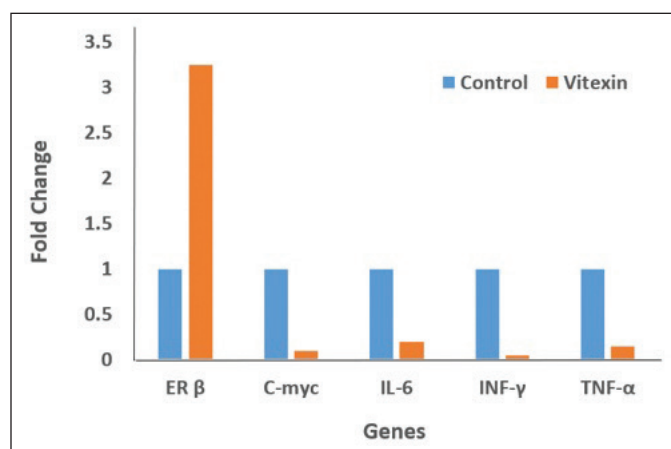


Figure 8. Gene expression analysis.

Table 3. Binding profile of the estrogen receptors.

Binding profile	ER- α	ER- β	GPER
Binding energy	-6.81	-8.93	-0.73
Ligand efficiency	-0.19	-0.26	-0.02
Inhibitory constant	10.22	284.71	293.88
Intermolecular energy	-10.39	-12.51	-6.63
Vander Waals dissolving energy	-10.39	-12.51	-6.69
Electrostatic energy	0.0	0.0	0.0
Total internal energy	29.71	29.71	1,510.0
Torsional energy	3.58	3.58	5.97
Unbound energy	29.71	29.71	1,510.0

dose of vitexin on HCT116 cancer cells. The evaluated gene expression profile was done specifically with apoptosis induction in cancer cells and showed the relative expression during 12 hours of vitexin treatment. The gene expression analysis shows the variations of tested genes compared with untreated control. The fold change values indicate that C-myc, IL-6, INF- γ , and TNF- α are downregulated while ER- β is upregulated in treated samples compared to control samples (Table 2, Fig. 8).

3.8. Docking analysis

In the docking analysis, genetic algorithms are used for checking the conformational search. Molecular docking was performed using the Autodock tools 4.2. graphical user interface, which generates different confirmations of protein

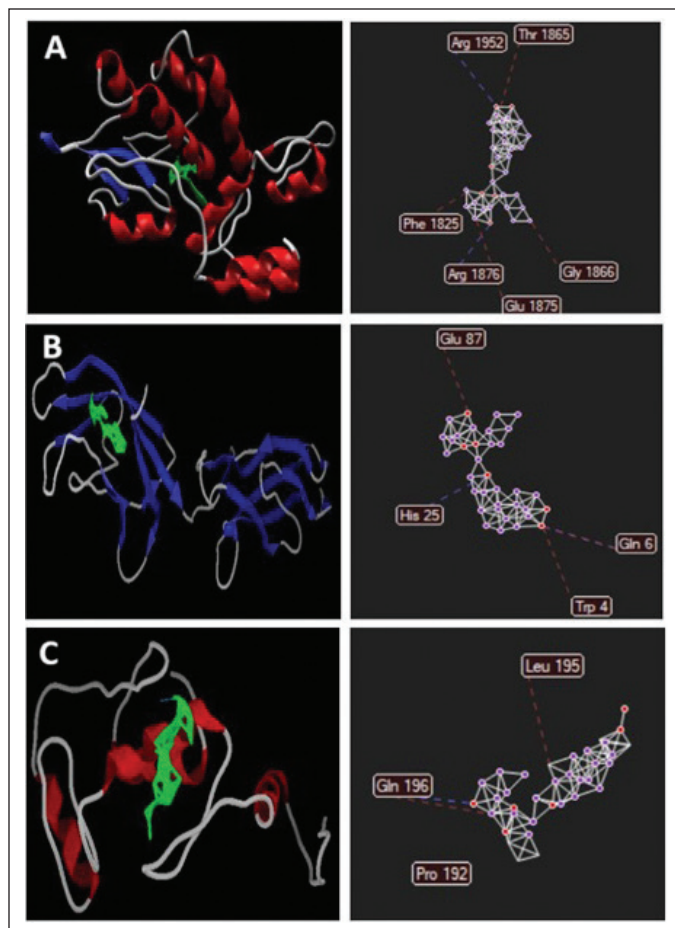


Figure 9. Molecular docking analysis of vitexin and the ER (A)-ER- α - B subunit, (B) ER- β - B subunit, (C) GPER shows the 3D image and interactions of amino acid residues with vitexin. The dotted lines indicates hydrogen bonds.

ligand complex customized in order from lowest to highest binding free energy (ΔG). Docking studies of vitexin on ER- α (B subunit), ER- β (B subunit), and GPER receptors revealed distinct binding profiles. On ER- α , vitexin interacts with amino acids at positions 4, 6, 25, and 87, with a binding energy of -6.81 . For ER- β , binding at positions 1,825, 1,865, 1,875, 1,876, 1,866, and 1,952 showed a binding energy of -8.93 . GPER binding involves positions 192, 195, and 196, with a binding energy of -0.73 . The binding profile of the ERs were depicted in Table 3; Figure 9.

4. DISCUSSION

Colon cancer mortality becomes most common in cancer-related death [19]. Sporadic CRC is the common type of cancer, but it can also be inherited. Initially, it is caused by the formation of polyps, which later turn malignant by the activation of C-myc, KRAS, APC oncogenes, and the inactivation of tumor suppressor genes like DCC and p53 [20]. CRC risk factors include a family history of CRC, history of polyps, obesity, and high-fat diet. Increased consumption of red meat is also correlated to colon cancer. CRC is generally treated by surgery and chemotherapy [21]. Surgery is most effective at stages 0,

I, and II of CRC because the cancerous growth is localized and not spread to other parts of the body. Whereas in stages III and IV, the cancers have the ability to metastasize to other parts of the body. At this stage, surgery will not be effective, so a combination of therapies like chemotherapy, radiation, and immune therapies is necessary [22]. Chemotherapies have always been useful in treating CRC, but it comes with lots of side effects. Also, chemotherapy has been proven to reduce the quality of life of patients [23]. To combat this problem, phytomedicine has come into play as a treatment alternative to different diseases, including CRC. Phytomedicines are comprised of bioactive compounds derived from plant sources that have therapeutic potential, has lesser side effects, and are proven to increase the quality of life in patients [24]. The current study focused on one such phyto compound, vitexin obtained from *V. negundo*, commonly known as the chaste tree. Vitexin, a flavonoid, is commonly used in the treatment of arthritis, inflammatory bowel diseases, Alzheimer's, Parkinson's disease, and cancer [25]. Estrogen has three receptors - ER- α , ER- β , and GPER. To check the binding affinity of ER with vitexin, the docking analysis was performed [26]. The results of docking analysis showed the binding energy for ER- β is less than that for GPER and ER- α with vitexin. Less binding energy indicates an increase in binding affinity of vitexin towards the receptor ER- β [27]. To study the beneficiary role of vitexin, the antioxidant, anti-cancer, and anti-inflammatory properties on HCT116 cell lines were tested with different concentrations of vitexin under a certain half-proximal rate of inhibitory concentration. The IC_{50} of vitexin was found to be $7.8 \mu\text{g/ml}$. Initially, cell motility was checked with different concentrations of vitexin, and the results showed that at 7.8 and $15.0 \mu\text{g/ml}$, the cell migration and motility were inhibited. This indicates that, at higher concentrations, the cells did not respond to the treatment. So, the results inferred that higher concentrations of vitexin may inhibit cell migration on higher animal models than cell lines. Literature survey shows that phyto compounds act on certain dose-response relationships, target interactions, and bioavailability absorption, distribution, metabolism, and excretion actions exhibiting either beneficial effects enhancing cellular antioxidant promoting activities, or exerting toxic cellular damage occurs in higher concentrations, and target interaction makes a complete inhibition towards lower dosage of concentration [28]. Additionally, vitexin has increased lipid peroxidation activity at lower concentrations of vitexin treatment. This shows that at IC_{50} and higher concentration has positive effects on the cell lines. A decrease in lipid peroxidation reduces the reactive oxygen species (ROS) levels in the cells, thereby reducing oxidative stress [29]. Vitexin was proven to have antioxidant capacity, which was confirmed by the FRAP assay where the ferric content was decreased at a $7.8 \mu\text{g/ml}$ level. Antioxidant capacity reduces the ROS levels in the cells [30], which was also proved by the present study on the free radicals that were also measured by DPPH assay in the vitexin-treated HCT116 cells. Percentage inhibition is increased on vitexin-treated cell lines, which was directly proportional to the concentration. These exhibit that vitexin has antioxidant and anticancer properties [31]. Although CRC is not a hormone-induced cancer, clinical studies and randomized clinical trials

have shown a protective action of estrogen on colon cancer [32]. This was because men are at a higher risk for CRC than women. Though estrogen has three receptors ER- α , ER- β , and GPER, by docking analysis it was found that ER- β has more binding affinity with vitexin than the other two receptors. Also, ER- β has been proven to show a more protective role than the other two receptors. The current study focuses on treating CRC with vitexin through an estrogen-mediated pathway [33]. CRC is associated with inflammatory processes and elevated levels of the inflammatory markers TNF- α , IFN- γ , and IL-6. Stromal fibroblasts release IL6, which is then used by immune cells to trigger STAT3 signalling, thereby promoting tumor growth [34]. The macrophages produced proinflammatory cytokines - TNF- α increased its expression in CRC tissues encouraging the development, invasion, and metastasis of tumors [35]. Furthermore, tumor cell's immune response is also stimulated by IFN- γ , which causes the tumor cells, monocytes, endothelial cells, and fibroblasts to activate chemokine receptors CXCL7 and CXCL11, which promote angiogenesis and the development of tumors [36]. By gene expression analysis, it was found that ER- β was upregulated in the vitexin-treated sample and the inflammatory genes IFN- γ , TNF- α , and IL-6 were downregulated [37]. It is also noteworthy that the study revealed a reduction in the expression of C-myc gene. C-myc is an oncogene that is also estrogen-induced and lacks an estrogen-responsive region in its promoter [38]. Various signalling pathways, including Wnt/ β -catenin, PI3K-Akt, Notch, Ras-MAPK, and NF- κ B, are implicated in the maintenance of cellular homeostasis and the activation of c-myc [39]. These pathways can be modulated by ER- β . The reduction in C-myc levels is a consequence of the inhibition of these pathways by ER- β . The binding of ER- β to the gene regulatory region is necessary for the downregulation of the levels of VEGFA, PDGFA, and ANGPTL4 mRNA. HIF1A expression is impacted by ER- β at both the transcriptional and translational stages [40]. These findings demonstrate that vitexin has phytotherapeutic effects on HCT116 cell lines and that CRC can be treated via an estrogen-mediated mechanism. The present investigations were correlated with the previous proven findings with ERs, through animal and human studies states that the reduction in the expression of ER- β leads to the induction of apoptosis in colon cancer [41]. Another interesting finding also supported the present study, stating that estrogen-mediated colon cancer control can able to cause significant modification in the cell cycle progression and apoptotic cancer prevention [42]. Phytoestrogens are widely distributed in a variety of natural sources, as they possess close structural similarity with estrogen, they can easily bind with ERs and do effective modulations in cancer control without causing any secondary side effects on cancer treatment. There is no previous studies have been conducted on the role of vitexin in estrogen-mediated regulatory pathway in colon cancer, so the findings of the present work would be helpful to identify the molecular mechanism of ER-mediated apoptosis in colon cancer.

5. CONCLUSION

The present proposed work was planned to check the effectiveness of vitexin on estrogen-mediated regulatory pathway in colon cancer cells. As a preliminary analysis,

the work was planned with *in vitro* analysis, and the relative general parameters were checked. Estrogenic role of glyco flavonoids modulates ER α and ER β leads to intracellular signalling pathways and is responsible for the changes in cellular behavior. No previous studies were conducted on the role of vitexin in estrogen-mediated regulatory pathway in colon cancer; therefore, the findings of the present work would be helpful to identify the molecular mechanism of ER-mediated apoptosis in colon cancer. Based on the obtained positive results, further molecular mechanisms will be explored using *in vivo* model with respect to ER-mediated cascade pathway on colon cancer control.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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