

APOPTOSIS MEDIATED CYTOTOXIC EFFECTS OF PURIFIED VITEXIN FROM *VITEX NEGUNDO* AGAINST HCT 116 COLON CANCER CELLS

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ABSTRACT

Objective : Colorectal cancer leads to highest rate of morbidity due to the failure in the conventional treatment with severe side effects, drug resistance, and toxicity. There is emergence for identifying safer and more effective phytomedicine induces apoptosis as a therapeutic strategy in colon cancer treatment. The aim of the present work to explore the cytotoxic effects of purified vitexin against colon cancer through *in vitro* analysis.

Materials and Methods : Study focuses on purification of phyto compound from medicinal plant and testing the cytotoxic potential against colon cancer through *in vitro* studies using HCT 116 cell line model. The methanolic leaf extract was subjected to GC-MS analysis, column chromatography, thin layer chromatography and HPLC analysis to confirm the purification of vitexin. Purified vitexin was tested for the cytotoxicity, radical scavenging and antioxidant role against HCT 116 colon cancer cell lines. The study further extended with the anti-inflammatory, migration inhibition and apoptosis induction analysis. Chi-square test was used for finding the associations between the treated and untreated groups. Independent t test was applied to differentiate the groups.

Results : Presence of various bioactive phyto compounds were confirmed by qualitative analysis in which, the polyphenols and flavonoids exist in higher quantity. The purified vitexin fractions showed good radical scavenging role against free radical generation and lipid peroxidation. And further the results confirm the cytotoxic and apoptotic potential of vitexin against HCT 116 colon cancer cells.

Conclusions : The bioactive vitexin effectively respond on inflammation, oxidative damage, thereby contributing to wound healing in cancer cells, and induces apoptosis mediated cytotoxicity may act as an effective alternative chemotherapeutic agent against colon cancer cells.

KEYWORDS: Vitexin, *V.negundo*, GC-MS, HPLC, HCT 116, Apoptosis, Cytotoxicity, Health Care.

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1. INTRODUCTION

Herbal medicines are also known as "phytomedicines" as they are mostly made from plants using eco-friendly processes (Lippert and Renner, 2022). Herbal medicine or phytomedicine research in healthcare is on the rise as a result of the growing acceptance of natural and herbal remedies because of its availability, affordability, and reduced side effects (Bhattacharya *et al.*, 2021). Since ancient times, herbal medicines have been an important and widely used source in the field of medicine. The use of plants in medicine has progressively changed throughout time as natural products rich in bioactive chemicals that originate from plants or animals have been shown to boost immunity, protect against disease, and have therapeutic benefits (Neha *et al.*, 2021; Koirala *et al.*, 2020). Phytomedicine is recently known to be widely studied and used to treat various conditions due to reduced adverse effects and improved therapeutic potential. Various plant components, such as powdered roots, bark, or other parts, are frequently used to aid in the treatment of illnesses. The efficacy of the medications and the patient's condition will determine how long the treatment takes, and it may take months or years. To guarantee the efficacy of herbal medications, targeted research is essential (Adarshan *et al.*, 2022; Babaei *et al.*, 2020). Cancer is a disorder when certain body cells proliferate out of control and invade other organ systems. It is a major global health issue, with various treatment options varying based on the type and stage of the disease (Nyam weya *et al.*, 2023; Rowles and Erdman, 2020). Radiotherapy, chemotherapy, surgery, and palliative care are the main forms of treatment. Cancer causes inflammatory responses, weakened immune systems, lowered cell viability, and DNA damage (Hamdi *et al.*, 2020; Dwivedi *et al.*, 2021). Free radical and oxidative stress based

cellular damage causing a range of physiological and metabolic diseases that are detrimental to human health. The elderly exhibit this imbalance because of a decline in endogenous antioxidant pathways and an increase in reactive oxygen species (ROS) brought on by the activation of ROS-producing enzymes (Nadeem *et al.*, 2020; Preetha *et al.*, 2021). This can result in adverse drug reactions, cognitive impairment, increased risk of hospitalization, and death (Shafie *et al.*, 2020). Thus, as an alternative nutritional therapy is necessarily needed to improve good quality of life. The phytochemistry utilization improved nowadays, which are now a viable tool for treating illnesses and disorders associated with cancer due to advancements in phytochemical delivery. New food products that meet the demands of the elderly population can be created by incorporating these natural leaves into daily routine. Therefore, it is essential to create long-term, effective approaches to cancer prevention and control. Plant based secondary metabolites shows significant protective role against several malignancies. Several plant extracts have been evaluated for possible use in the treatment of cancer (Angmo *et al.*, 2023; Woottisin *et al.*, 2020; Tawfeeq *et al.*, 2023; Duan *et al.*, 2022). *Vitex negundo* (*V. negundo*) is a widely studied and used plant, its parts, especially the extracts from the leaves and bark, are well renowned for their great therapeutic potential. Vitexin an apigenin flavonoid glucoside is the main phytochemical found in the plant exhibits antioxidant role, protects against lipid peroxidation and reactive oxygen species (Aly Motawa *et al.*, 2020; Babaei *et al.*, 2020; Huang *et al.*, 2022). Along with that volatile oil, flavonoids, polyphenols, iridoids, glycosides, and steroids are among the plant's numerous phytochemical constituents that are popularly known for their disease curing effects against various pathological conditions and gene expression related to stress. Leaf extracts taken orally have anti-inflammatory, analgesic, antidiabetic, and antihistamine qualities, while *V. negundo* fruits are well-known for their ability to treat piles diseases, arthritic joints, and inflamed eyes (Kaiser *et al.*, 2021; Weiskirchen *et al.*, 2023; Menon *et al.*, 2024). Vitexin, activates neutrophils and macrophages during inflammation and inhibits their anti-inflammatory role. Many studies have examined the potential cardiovascular protective effects of its bioactive components, which include ursolic acid, betulinic acid, corosolic acid, etc. *Vitex negundo* regulates apoptosis in tumorigenesis, a key process in cancer signaling (Nwachukwu *et al.*, 2021; Xiang *et al.*, 2020; Shaikh and Patil, 2020). An attempt was made to focus on the biochemical importance of purified vitexin from *V. negundo* against the cancer migration and cancer progression on HCT 116 colon cancer.

2.0. MATERIALS AND METHODS

2.1 Extraction of *V. negundo*

The crude extraction of leaf, fruit and root of *V. negundo* was done by Soxhlet extraction method using organic and aqueous solvents. The samples were collected, shade dried and powdered. 10g of the powdered sample was extracted with 100ml of the solvents (Acetone, Chloroform, Ethanol, Diethyl ether, Methanol and Water). Different evaporation temperature was set to collect the extractions. The collected aliquots were maintained in 4°C refrigeration.

2.2 Qualitative Phytochemical Analysis

Presence of phytoconstituents were carried out using all the three extracts to check the presence of carbohydrates, proteins, amino acids, saponins, tannins, carotenoids, polyphenols, flavonoids and glycosides using standard protocols (Bhuyan *et al.*, 2024).

2.3 Quantitative Phytochemical Analysis

2.3.1 Total Polyphenol Content (TPC) : The phenolic quantity was assessed to evaluate the redox and antioxidant characteristics, of the test sample. 0.5ml of the sample were added with 8ml of distilled water and added 0.5ml of the 1N Folin Ciocalteu reagent, kept in 4°C for 10 mins. After adding 1ml of 20% sodium carbonate, the mixture was left in the dark for an hour. A blue colour development is indicative of the phenol presence compared with standard curve, of gallic acid equivalent was used to calculate the total phenolic content in the extract (Bhuyan *et al.*, 2024).

2.3.2 Total Flavonoid content (TFC) : Flavonoids are antioxidant secondary metabolites they can deactivate the free radicals. The diluted sample, treated with 0.3ml of 5% sodium nitrate. After 5min all the tubes were further added with 0.3 ml of 10% aluminium chloride. The mixture was mixed with 2ml of 1M sodium hydroxide in sixth minute. The reaction mixture's contents were immediately diluted with 2.4ml of distilled water and properly mixed and absorbance measured at 510nm. The standard gallic acid was processed in the same way to quantify total flavonoids (Bhuyan *et al.*, 2024).

2.4 GC-MS analysis

The methanolic extract was used to perform gas chromatography-mass spectrum (GC-MS) analysis of on a GC clarus 500 Perkin Elmer system, consists of an AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer, using helium (99.999%) as the carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI (split ratio of 10:1), the Elite-1 fused silica capillary column, operates in electron impact mode at 70 eV. The temperature set for injector and io-source was 250°C and 280°C. The oven temperature was set to start at 110°C (isothermal for 2 min), increase by 10°C/min to 200°C, then increase by 5°C/min to 280°C, and finally end at 280°C for a 9 min isothermal (Mohanasundaram *et al.*, 2019).

2.5 Purification of Vitexin by column chromatography

Column chromatography method of purification utilizes the mixture of compounds were separated into distinct components by using the solutes being adsorbed with the help of a stationary phase. Chromatography was performed with 10ml crude methanolic extract using silica gel 60-120. The mobile phase with the combinations

of hexane:ethyl acetate (10:90), with increasing polarity were used for the elution process. The fractions were collected in aliquots of 10ml for further use (Balwinder Singh *et al.*, 2016).

2.6 Separation of Vitexin by Thin layer chromatography

The column chromatography separated aliquots were subjected to thin layer chromatographic (TLC) separation using a suitable solvent system. TLC plates were made by preparing a slurry using silica gel G and a thin layer was coated onto a glass slide and allowed to dry. A drop of the column aliquots fractions and the standard vitexin were loaded on the dried TLC plates. The plates were placed in chromatographic chamber containing the solvent mixture ethyl acetate:methanol:water (5:1:5) as mobile phase. The chamber was kept closed to prevent solvent evaporation. The migration of the solvent was facilitated by the capillary action. At the end of the run the plates were left to dry. They were then sprayed with boric acid:oxalic acid mixture, and the compound separation was detected under UV-transilluminator. The separated fractions on TLC plates exhibiting various spots were compared. The presence of vitexin was indicated by the light yellow colour fractions compared with the standard vitexin. The travelling distance of solute and solvent were measured and the Rf values were calculated using the following formula

$$R_f = \frac{\text{Distance travelled by the compound (cm)}}{\text{Distance travelled by the solvent front (cm)}}$$

$$\% \text{ Scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.7 HPLC Analysis

High Performance Liquid Chromatographic (HPLC) analysis of the TLC identified methanolic leaf fractions were carried out to further confirm the existence of vitexin isolation. Using 0.1% acetic acid (solvent A) and methanol (solvent B) as the mobile phase in gradient mode, the analysis was performed on an RP-18 column. A gradient elution profile of the following was used: 0 min, 30% B; 5 min, 40% B; 20 min, 40% B; 25 min, 50% B; 30 min, 70% B; 40 min, 70% B; 42 min, 95%; 60 min, 95% B; 65 min, 30% B; 70 min, 30% B. Before the subsequent injection, 30% B was used to equilibrate the column for five minutes. The HPLC was filled with 10µL of standards and sample at a flow rate of 1mL/min. All analyses were performed in a column oven at 30°C. The compounds were identified at 330nm, the components were distinguished (Madhuranga and Samarakoon, 2023).

2.8 In vitro radical scavenging assay

The measurement of the radical scavenging potential of vitexin was carried out in order to check the antioxidant capacity of the test compound. A set of test tubes, with 0.1ml of the crude extracts and the purified vitexin samples from both leaf and root were taken. 2,2-diphenyl-1-picrylhydrazyl (DPPH) added, and kept in dark for 30 mins to 1 h at 517nm, the colour was measured (Karolina and Kulesza, 2020). The difference on optical density between the DPPH solution alone and in combination with the sample was estimated. The antioxidant capacity of sample was calculated using the formula.

2.9. Cell lines

2.9.1 Cell culture and maintenance

The human HCT 116 colon adenocarcinoma cell lines were obtained from the National Centre for Curative Sciences (NCCS), Pune. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), with antibiotic and antimycotic components. Cell viability was determined using trypan blue and used for the analysis. Ethical approval was obtained from the Institutional Ethical Committee to conduct the cell line related work (CSP/24/FEB/143/59 and CSP/24/FEB/143/60).

2.9.2 Cell lysate preparation

One lakh cell was trypsinized and the cells were transferred to an eppendorf tube and centrifuged for 5min at 4°C, the supernatant was discarded, the pellet was resuspended with 5ml ice-cold PBS and centrifuged. The pellet obtained was suspended with cell lysis buffer, mixed by pipetting it slowly for 8 -10 times and then it was kept in the water bath for 5 min.

2.10 Cell Cytotoxicity Assay

The seeded cells were treated with the purified vitexin fractions and standard drug capecitabine. The purified vitexin was used for serial dilution to obtain the different concentration ranges from 10-100µg/ml was used for the cytotoxicity assay. Different concentrations of vitexin were treated and incubated for 48 h, then stained with

$$\% \text{ Inhibition} = \frac{[(\text{OD (untreated)}) - \text{OD (treated)}]}{\text{OD (untreated)}} \times 100$$

MTT solution. The formed formazan crystals were dissolved and measured at 570nm on microplate reader

2.11 FRAP assay

The antioxidant capacity is the ability of the cell's survival in the free radical environment. Cells were exposed with methanolic leaf extract and vitexin at the concentration 10-100 µg/ml. The cell lysate was prepared from different samples were used for the assay. The assay mixture consists 300µl working reagent containing 4.16ml acetate buffer (pH : 3.6), 416µl 2,4,6-tripyridyl-s-triazine (TPTZ) solution, and 416µl ferrous sulphate heptahydrate were added with 10µl cell lysate. Working reagent and water were taken as blank and absorbance was read at 593nm (Gonfa *et al.*, 2023).

$$\text{Antioxidant Capacity} = \frac{(\text{Absorbance of Sample} - \text{Absorbance of Blank})}{\text{Slope}}$$

2.12 In vitro anti-inflammatory assay

The anti-inflammatory activity of crude leaf extract, purified vitexin and standard drug capecitabine were evaluated using a protein denaturation inhibition assay. The 5mL of the reaction mixture consisted of 0.2mL of 1% bovine albumin, 4.78mL of phosphate-buffered saline (PBS, pH 6.4), and 0.02mL of sample, and the mixture were incubated in a water bath (37°C) for 15 min, and then the reaction mixture was heated at 70°C for 5 min, the turbidity was read at 660 nm using a UV-VIS spectrometer. PBS was taken as a control for this assay. The protein denaturation inhibition was calculated using the formula below

2.13 Lipid Peroxidation assay

The one lakh cells were exposed to different concentrations of vitexin. The media were removed, and cells were then washed with PBS. To the cells, 1ml of 2.5% tri chloroacetic acid (TCA) was added and the cells were scrapped out using a scrapper. Then the contents were centrifuged and the supernatant was separated into new eppendorf tubes (500µl to each). 400µl 2.5% TCA was taken as blank and in all other tubes 500µl of 2.5% TCA and 800µl of TBA were added, incubated at 95°C, 3 ml of butanol was added to all the tubes and mixed well using

$$\% \text{ Inhibition of denaturation} = \frac{(1 - \text{Absorption of Test}) \times 100}{\text{Absorption of Control}}$$

vortex. The aqueous solution was separated, and measured in UV spectrophotometer (Pijuan *et al.*, 2019).

2.14 Migration Assay

For migration assay 80% confluence as a monolayered cells 1 lakh cells were used in a 6 well plate and incubated for 12 hrs in 5% CO₂ incubator at 37°C. The monolayer was scratched with 10µl tip across the center of the well, along axial of tip should be perpendicular to bottom of well. The cells were viewed under phase contrast microscope. The cells were treated with drug at respective concentrations. The culture dish was placed in incubator and images were captured every 12 hrs once. The images were seen in phase contrast microscope and pictures were captured of each well and marked as 0 hr, 12 hrs, 24 hrs, 36 hrs respectively (Adhya *et al.*, 2024). The wound closure area percentage was calculated using the formula

$$\% \text{ Area (t)} = A(t) / A(0) \times 100$$

Where A(t) - Wound area at time t, A(0) - Initial wound area

2.15 Apoptosis Assay

The apoptosis assay was carried out by staining the cells with acridine orange (AO) - propidium iodide (PI) mixture, 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 hrs. The cells were treated with IC₅₀ values of methanol extract and vitexin. After 24 hrs of drug exposure, the media was removed, and 100µl of AO/PI stain was added to the plate. The viable and non-viable cells at the end of treatment were observed using an inverted fluorescent microscope.

2.16 Data Analysis

Statistical analysis was carried out with SPSS 16.0 software. Chi-square test was used for finding the associations between the treated and untreated groups. Independent t test was applied to differentiate the groups. Statistical significance was accepted if p < 0.05.

3.03.0 RESULTS

3.13.1 Qualitative Phytochemical Analysis

The crude extracts were tested for the presence of carotenoids, polyphenols, flavonoids, glycosides, saponins, tannins, carbohydrates, proteins and amino acids. The polyphenols, flavonoids and glycosides were present in acetone, chloroform, ethanol and methanol extracts of leaves, flowers and root. Tannins and saponins were absent in all the tested solvents. Carbohydrates, proteins and amino acids are present in acetone, chloroform and diethyl ether extract. Aqueous extract shows negative results for all the tests. Out of the tested sample the root of *V. negundo* contains more the amounts of polyphenols and flavonoids compared with leaf and flower extract. In the root crude extracts the presence of proteins, flavonoids, glycosides and polyphenols were observed in methanol and ethanol extracts. The presence of proteins and glycosides were observed in the acetone extract and the chloroform extract shows the existence of flavonoids and glycosides. The results obtained for the qualitative phytochemical analysis were indicated in **Table 1**.

Table 1: Qualitative Phytochemical analysis of *V. negundo*

Phytochemicals	Acetone			Chloroform			Diethyl ether			Ethanol			Methanol			Water		
	L	F	R	L	F	R	L	F	R	L	F	R	L	F	R	L	F	R
Carbohydrates	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	-
Proteins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Amino acids	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-

Saponins	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-
Tannins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carotenoids	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-
Flvonoids	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-
Polyphenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Glycosides	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-

L – Leaf, F – Flower, R – Root ; (+) Present, (-) Absent

3.2 Quantitative phytochemical Analysis

The results of the qualitative analysis confirmed the presence of polyphenols and flavonoids in methanol, ethanol, chloroform, acetone and di-ethyl ether leaf extracts and methanol, ethanol and chloroform root extracts. The quantity of the phenolic content in these extracts was assessed and compared with the standard gallic acid. While comparing all the solvents acetone, and methanol leaf and root extracts had higher concentration of phenolic content. Similarly higher concentration of the flavonoid content was observed in methanol, ethanol, chloroform and acetone leaf extracts and chloroform, methanol, ethanol root extracts compared with diethyl ether extract (Table 2).

Table 2: Quantitative phytochemical analysis of *V. negundo*

Extract	Total Phenolic Content (mg/ml)		Total Flavonoid Content (mg/ml)	
	Leaf	Root	Leaf	Root
Acetone	1.50 ± 0.02	0.20 ± 0.04	1.40 ± 0.05	0.20 ± 0.03
Chloroform	1.10 ± 0.03	0.10 ± 0.03	0.50 ± 0.02	0.70 ± 0.04
Diethyl Ether	0.15 ± 0.04	0.40 ± 0.04	0.30 ± 0.05	0.30 ± 0.04
Ethanol	1.30 ± 0.03	0.50 ± 0.50	1.00 ± 0.03	1.20 ± 0.03
Methanol	2.50 ± 0.04	1.50 ± 0.05	2.30 ± 0.50	1.40 ± 0.04

Values are Mean ± SEM of triplicates (N=3)

3.3. GC-MS analysis of methanol extract

The GC-MS chromatogram analysis of methanolic leaf extract shows 35 different components. These compounds were identified based on the retention time, percentage of area covered, percentage of height, etc. Among these components the frequently recognized compounds dodecane, nonadecane, eicosane, hexadecane, triacontane, tetracontane etc., were also identified. (Figure 1; Table 3). Most of the identified compounds listed in the GC-MS belongs to long chain hydrocarbons, fatty acid derivatives, phenolic components and plant secondary metabolites.

Figure 1: GC-MS analysis of methanolic leaf extracts of *V. negundo*

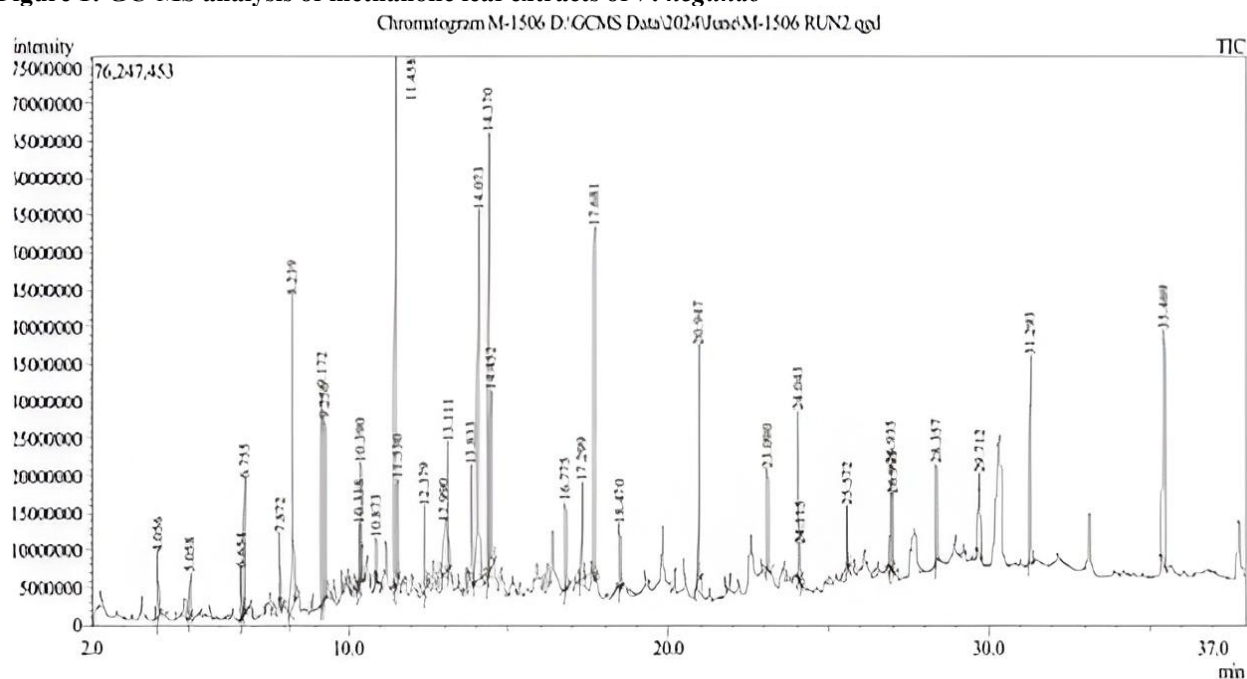


Table 3: GC-MS analysis of methanolic leaf extracts of *V. negundo*

Peak No.	Retention Time	Area	Area %	Height	Height %	A/H	Compound Name
1	4.056	21498630	1.07	8475905	1.19	2.54	Dodecane
2	5.058	12301898	0.61	5889157	0.83	2.09	Dodecane, 2,6,11-trimethyl-
3	6.654	12835500	0.64	6470474	0.91	1.98	1-Tridecene
4	6.755	34986287	1.74	18108735	2.55	1.93	Tetradecane
5	7.872	17010436	0.85	9378746	1.32	1.81	Eicosane
6	8.239	122000000	6.07	42149291	5.99	2.89	Phenol, 2,5-bis(1,1-dimethylethyl)-
7	9.172	51022160	2.54	28990710	4.08	1.76	1-Pentadecene
8	9.256	43095846	2.14	24459073	3.44	1.76	Hexadecane
9	10.338	15342512	0.76	8447161	1.19	1.82	Carbonic acid, octadecyl vinyl ester
10	10.39	36868112	1.83	16432933	2.31	2.24	Eicosane
11	10.873	8293294	0.41	5242553	0.74	1.58	Eicosane
12	11.458	130000000	6.48	70909052	9.98	1.84	1-Nonadecene
13	11.53	22643674	1.13	14069458	1.98	1.61	Nonadecane
14	12.379	25563264	1.27	11163446	1.57	2.29	Dibutyl phthalate
15	12.99	42502502	2.11	6542135	0.92	6.5	Eicosane
16	13.111	50934802	2.53	17310890	2.44	2.94	Germanicol acetate
17	13.833	48385476	2.41	14900804	2.09	3.25	Dibutyl phthalate
18	14.073	234000000	11.64	49664414	6.98	4.71	n-Hexadecanoic acid
19	14.37	159000000	7.91	59594845	8.38	2.67	1-Nonadecene
20	14.452	75205018	3.74	23947547	3.37	3.14	3,5-di-tert-Butyl-4-hydroxyhydrocinnamic acid
21	16.775	31163762	1.55	11785406	1.66	2.64	Fumaric acid, 2-ethylhexyl octyl ester
22	17.299	4121555	2.05	13152068	1.85	3.13	l(+)-Ascorbic acid 2,6-dihexadecanoate
23	17.681	121000000	6.01	46900277	6.59	2.58	1-Nonadecene
24	18.47	23441541	1.17	8478973	1.19	2.76	Hexadecanoic acid, 2-hydroxyethyl ester
25	20.947	83755140	4.17	31808366	4.47	2.63	Behenic alcohol
26	23.09	38369910	1.91	13436308	1.89	2.86	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester
27	24.043	60025538	2.99	22540291	3.17	2.66	1-Heptacosanol
28	24.115	14858046	0.74	5257422	0.74	2.83	Triacontane
29	25.572	21836717	1.09	8251091	1.16	2.65	Tetracontane
30	26.935	40029502	1.99	13868870	1.95	2.89	Bacteriochlorophyll-c-stearyl
31	26.995	25900058	1.29	10316577	1.45	2.51	Heneicosane
32	28.357	34475748	1.71	13306700	1.87	2.59	Tetracontane
33	29.712	49287366	2.45	10879916	1.53	4.53	Tetracontane
34	31.293	101299036	5.04	27820393	3.91	3.64	Tetracontane
35	35.469	160197331	7.97	31350881	4.4	5.12	Dotriacontane
Total		2010751284	100	711342697	100		

3.4 Purification and Separation of Vitexin

Column chromatography was performed to isolate vitexin from methanolic leaf and root crude extracts, as it is having high phenolic and flavonoid contents. Adsorption of solutes from the solution was done using silica gel as stationary phase and the solutes were eluted with hexane: ethyl acetate solvent mixture. For methanol leaf crude extract upon column chromatography, greenish - yellow and pale-yellow fractions were collected. The pale-yellow fractions obtained were pooled together and as they were indicative of the presence of vitexin. From the root crude extract yellowish brown, yellowish fractions were collected and the yellowish fractions were pooled together. The both columns separated fractions were subjected to thin layer chromatography to confirm the presence of vitexin along with standard vitexin. Differential affinities between the two phases allowed the components to be split into a stationary phase and a mobile phase. The resulting fractions were contrasted with the reference vitexin under UV detection and R_f values, the column separated pooled yellow fractions confirms the presence of vitexin. From the crude methanolic leaf extract and only fractions 1 to 4 out of 6 eluted fractions showed the presence of vitexin. The 4 different root fractions, vitexin were identified in 3-5 fractions out of 6 fractions. The distance travelled by the solvent front, standard vitexin and the fractions were noted and their R_f values were calculated using the formula. (Table 4).

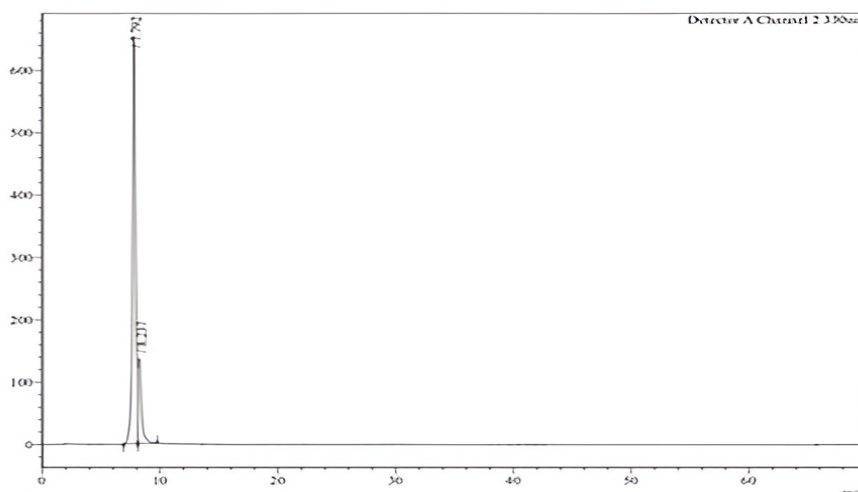
Table 4: Thin layer separation of vitexin from methanolic extracts

Fractions	Leaf		Root	
	Distance (cm)	Rf Value	Distance (cm)	Rf Value
Standard Vitexin	4.2	0.35	4.2	0.35
Fraction 1	4.0	0.33	3.2	0.26
Fraction 2	4.2	0.35	3.3	0.27
Fraction 3	4.2	0.35	4.2	0.35
Fraction 4	4.2	0.35	4.2	0.35
Fraction 5	4.2	0.35	4.2	0.35
Fraction 6	3.9	0.32	3.0	0.25

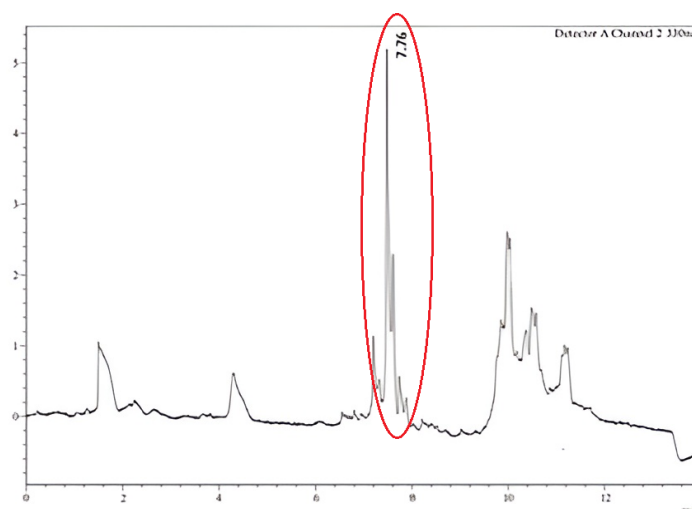
3.5 HPLC Analysis

In the HPLC analysis, the fractions collected from the column chromatography were allowed to run to check the presence of vitexin, and compared with the standard vitexin, which was run in the same chromatographic conditions. The results showed within 10 min of the running time vitexin peak was observed at 7.79 retention time. The column collected fractions of methanolic crude extracts, were showed major and minor peaks. While comparing with the standard peak, the major peak identified at 7.76 the retention time was correlated and identified as vitexin (**Figure 2**).

Figure 2: HPLC analysis of methanolic fractions of *V. negundo* leave



(A) Standard Vitexin



B) Methanolic fractions

3.6 Radical Scavenging Assay

The radical scavenging potential of the crude extract of *V.negundo* and the purified fractions were carried out, to check the antioxidant ability of the leaf and root samples. The chloroform, ethanol and methanol leaf and root extracts showed good radical scavenging activity, slight variation with in the solvents was also observed. Methanol leaf crude extract exhibited higher radical scavenging activity up to 65% and 67% in the leaf and root extracts. The purified vitexin fractions also tested for the radical scavenging potentiality, which also exhibits good in scavenging activity up to the level of above 70% (Table 5).

Table 5: Radical scavenging activity

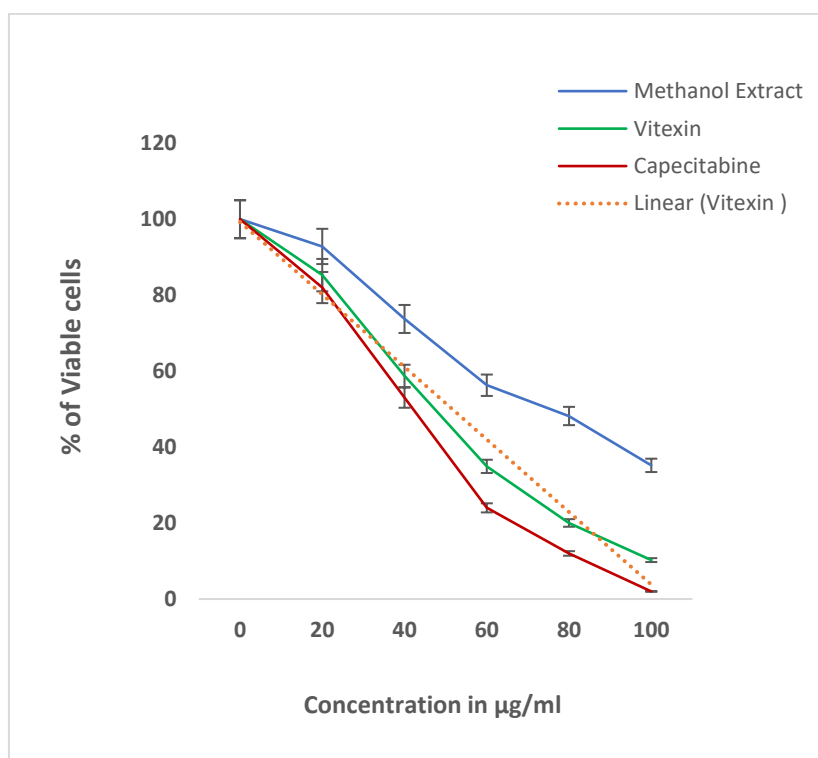
Extract	Leaf	Root
Chloroform	41.4 ± 2.38	55.8 ± 3.84
Ethanol	24.4 ± 3.94	52.9 ± 1.83
Methanol	65.5 ± 3.58	67.3 ± 3.48
Vitexin Fractions	78.8 ± 2.84	72.2 ± 4.28

Values are Mean ± SEM of triplicates (N=3)

3.7 Cell Cytotoxicity Assay

The cytotoxic activity of the methanolic crude extract and the purified vitexin fractions were carried out to determine the fifty percent inhibitory concentration (IC₅₀) on HCT 116 colon cancer cell lines. The standard drug capecitabine was also treated for comparison. The results of the purified vitexin clearly showed the concentration dependent cytotoxic effect on colon cancer cell lines. On treatment of increased vitexin concentration from 10-100 µg/ml the cell viability of the colon cancer cells was decreased gradually. The linear regression line was obtained to calculate the IC₅₀ values and calculated as 80µg for methanol extract 48µg for vitexin and 42µg for capecitabine (Figure 3).

Figure 3 : Cytotoxicity assay on HCT 116 Colon Cancer cell lines

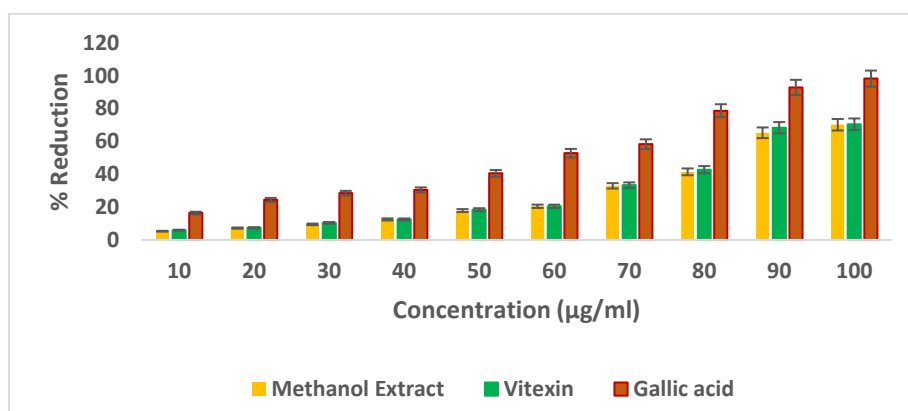


Values are Mean ± SEM of triplicates (N=3)

3.8 Total Antioxidant Capacity

The total antioxidant capacity of the purified vitexin was tested in the HCT 116 colon cancer cell lines using different concentrations by FRAP assay method. The concentrations (10-100µg/ml) used for the cytotoxic assay were also used for determination of total antioxidant capacity. The results showed there was linear increase on the antioxidant status on increasing the concentration of vitexin was observed in the cell lysate on the treatment of vitexin (Figure 4).

Figure 4 : Total Antioxidant capacity of Vitexin on HCT 116 Colon Cancer cell lines



Values are Mean $\pm$ SEM of triplicates (N=3)

3.9 *In vitro* anti-inflammatory Assay

The inhibitory effect of methanolic crude extract, purified vitexin and the standard drug capecitabine on protein denaturation was observed, using bovine serum albumin as a standard protein. Both the methanolic extract and the purified fractions showed good protein inhibitory effect on protein denaturation, on comparison vitexin shows at the maximum 78% of inhibition whereas the methanol extract shows 72% and the drug capecitabine shows 85% inhibitory effect protein denaturation (**Table 6**).

Table 6: Percentage Inhibition of protein denaturation

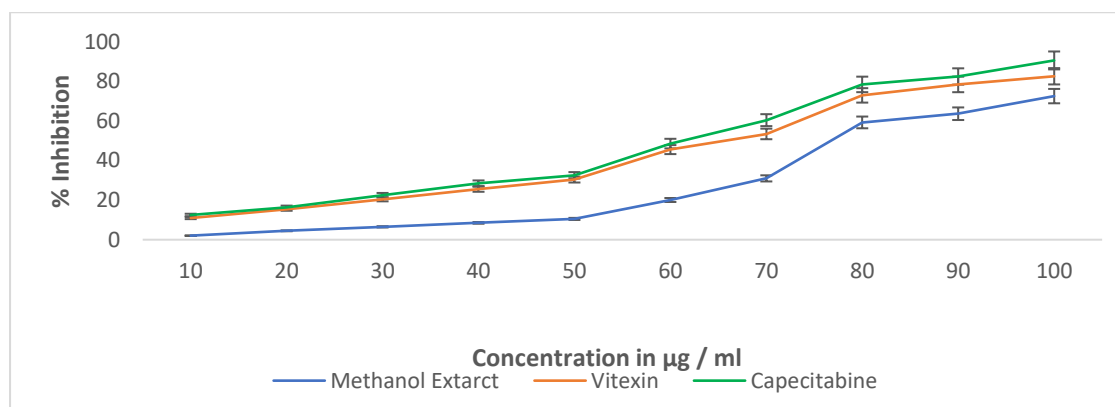
Concentration (µg/ml)	20	40	60	80	100
Methanol Extract	11.93 $\pm$ 0.83	20.84 $\pm$ 0.89	33.49 $\pm$ 1.48	58.29 $\pm$ 1.48	71.62 $\pm$ 1.85
Vitexin	15.92 $\pm$ 0.39	30.49 $\pm$ 0.94	52.90 $\pm$ 1.49	64.20 $\pm$ 1.95	78.89 $\pm$ 1.95
Capecitabine	28.48 $\pm$ 0.84	41.40 $\pm$ 0.39	62.49 $\pm$ 1.95	72.39 $\pm$ 1.59	84.84 $\pm$ 1.20

Values are Mean $\pm$ SEM of triplicates (N=3)

3.10 Lipid peroxidation Assay

Status of lipid peroxidation on HCT116 cells was assessed to check the free radical scavenging capacity of methanol extract and vitexin along with the standard drug. The lipid peroxidation assay was carried out by measuring the malondialdehyde product by treating samples with different concentrations ranging from 10-100µg/ml, in order to understand the dose response of tested sample against free radical generation, from the results, a linear relationship with scavenging activity was observed with significant increase in the inhibition of lipid peroxidation in all the treated group. Methanol extract and the vitexin group showed almost equal level of peroxidation inhibition (80-90%) where as the standard drug shows 70% of inhibition. (**Figure 5**).

Figure 5 : Percent Inhibition of Lipid Peroxidation



3.11 Migration Assay

The migration was assessed with HCT116 cell lines treated with IC₅₀ values of methanolic extract and vitexin in the time intervals of 0 hrs, 12 hrs, and 24 hrs by scratch wound assay viewed under inverted phase contrast microscope. Comparison was done with the untreated control groups which shows fast and maximum migration. Among the treated groups, the vitexin significantly inhibits the cell movement indicated by the lowest level of wound closure than the methanolic extract, and the migration rate was calculated at the end of 12 hrs and 24 hrs intervals. Both the IC₅₀ value of vitexin proves a better inhibitory role against the cancer cell migration, only about 10±2% of cells were seen on the scratched area. Treatment with methanolic crude extracts inhibiting the migration of cells was compared to the vitexin almost nearly 75-80% of cell migration inhibition were observed (**Figure 6 and 7**).

Figure 6 : Cell migration assay

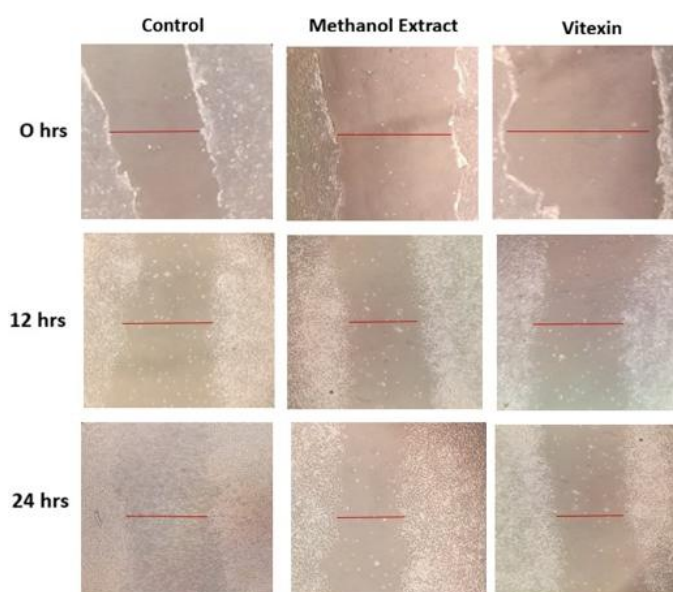
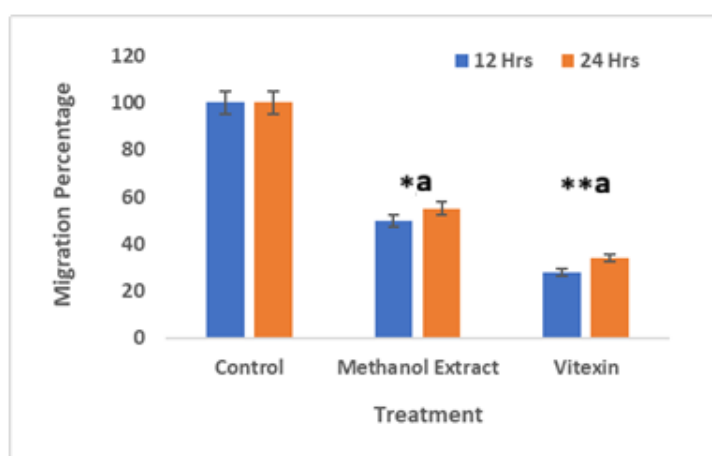


Figure 7 : Percent inhibition of cell migration assay



Values are Mean ± SEM of triplicates (N=3)

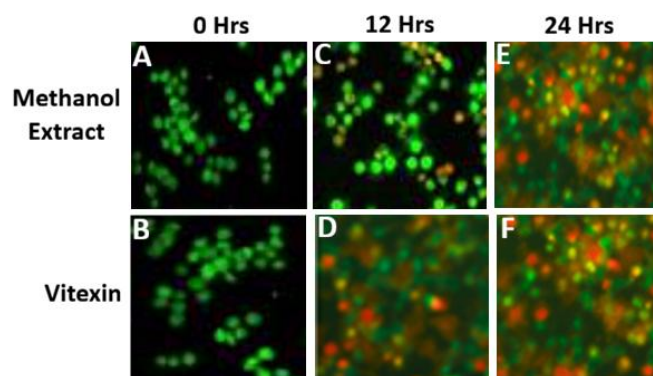
*P<0.01, **P<0.001 - Statistically significant, a – Compared with control

3.12 Apoptotic 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 8**, the untreated HCT 116 cancer cells appeared as healthy green cells with intact nuclei. By treating with IC₅₀ concentration of methanolic extract and vitexin at 24 hrs of incubation, a greater number of dead cells were observed under a fluorescent microscope. Both the treatments equally cause the cell death. The 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 hrs 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 HCT 116 by AO/PI double-stained cells

Figure 8 : Apoptotic assay



A & B - Viable cells indicated by green fluorescence , C& D - Early apoptosis features, namely, blebbing and chromatin condensation as well as late apoptotic cells were detected after 24 hours of treatment with Vitexin indicated, E & F - More intense red fluorescence, indicating extensive cellular damage, necrosis and dead cells.

4. DISCUSSION

Colorectal cancer (CRC) is a multifactorial disease characterized by a progression from epithelial hyperplasia through various stages of atypical hyperplasia (mild, moderate, severe) and adenoma formation, eventually leading to carcinoma. The process is typically initiated by carcinogenic factors causing DNA structural changes, resulting in malignant cell transformation and eventually leading to some morphological changes. Colon cancer is predominantly sporadic, influenced by factors such as age, race, diet, and inflammatory bowel disease. Screening colonoscopy is crucial for detecting premalignant and early-stage lesions. Surgery is the primary curative treatment, with chemotherapy as a necessary adjunct. Radiation therapy is essential for rectal cancer treatment but is less commonly used for colon cancer. Based on the studies done, currently available treatment and medications have limitations, resulting in a low diagnosis rate and curing rate. Thus, developing safe, compliant, highly sensitive, specific, and economically beneficial compound have significant value for improving colorectal cancer treatment (Singh *et al.*, 2024). Present research work aims to check the potentiality of vitexin purified from *V.negundo* and tested against colorectal cancer. According to earlier research, *Vitex negundo* may be able to treat a variety of illnesses due to the presence of its primary and secondary metabolites. Therefore, it may be crucial to support *V.negundo's* traditional medicinal uses and medicinal qualities with the current results. While flavonoids show antioxidant, antimicrobial, and anti-allergic qualities, the presence of phenolic compounds suggests that plants may have antiviral, antibacterial, and wound-healing activity (Tewari *et al.*, 2022).

Qualitative phytochemical screening *V.negundo* of leaf, flower and root extracts revealed the presence of carotenoids, polyphenols, flavonoids, carbohydrates, proteins, and glycosides in methanol, ethanol, and chloroform extracts. The acetone extract contains flavonoids, polyphenols, glycosides, proteins, and carbohydrates, while the di-ethyl ether extract shows the presence of polyphenols, proteins, and carbohydrates. The root crude extracts contained proteins, flavonoids, glycosides, and polyphenols. The acetone extract showed proteins and glycosides, while the chloroform extract showed flavonoids and glycosides. Di-ethyl ether only contained glycosides. Aqueous extract of all the three sample shows negative results for tannin, saponin, flavonoids and polyphenols. The results of qualitative screening proves that the organic solvents are capable of extracting phytochemicals from *V.negundo* than aqueous extract. The methanol, ethanol, and chloroform extracts were observed to be the best solvents used collectively. Similar reports were identified from studies conducted by Adhya *et al.*, (2024) and Vishal *et al.*, (2024), supports that the organic solvents are good enough to extract bioactive compounds from *V.negundo* (Hussein *et al.*, 2025; Zhao *et al.*, 2025).

The quantity of total phenolics and flavonoids content in the crude extracts of the leaves and roots was ascertained through quantitative analysis. According to the findings, good quantity of phenolic and flavonoid contents were found in the leaf and root extracts of acetone, chloroform, diethyl ether, ethanol, methanol. Methanol and ethanol leaf and root extracts had similar concentration ranges, but the total phenolic content in leaf extracts varies from

0.15 to 2.4 mg/ml and in root extracts from 0.2 to 1.4 mg/ml, respectively. The total flavonoid content varied between 0.5 and 2.3 mg/ml in leaf extracts and between 0.2 and 1.4 mg/ml in root extracts. Comparatively, the concentration range of the crude extracts of methanol and ethanol from leaves and roots were similar.

The GC-MS analysis was performed to check the existing flavonoids and polyphenolics in the methanolic leaf extract, as it showed good polyphenol and flavonoid content in qualitative assay. Among the total identified 35 compounds most of the compounds were polyphenols basics which possess biomedical importance. The GC-MS analysis of *V. negundo* leaves in a previous study identified a number of phytochemicals, such as 3-hydroxybenzoic acid, Ledol, 9,12,15-Octadecatrienoic acid, (Z, Z, Z)-, Vitamin E, and additional components like Caryophyllene and n-Hexadecenoic acid. Another study conducted on *Oxyria digyna* leaves found that the sample contained about 60 different components (Lu *et al.*, 2025). Overall GC-MS analysis proved the existence of several flavonoids and other phytochemicals in *V. negundo* which possess therapeutic benefits. The GC-MS identified compounds dodecane, nonadecane, eicosane and hexadecane supports plants anti-inflammatory, anti-oxidant, anti-diabetic, anti-microbial and anti-allergic properties. This suggests that the plant's profile is extremely complex, with multiple components and phytochemicals supporting the plant's anti-inflammatory, anti-oxidant, anti-diabetic, anti-microbial, and anti-allergic effects, among other therapeutic benefits. The HPLC analysis the column fractions showed a peak at a retention time of 7 to 9 min, compared with the standard vitexin, the fractions also showed highest peak in the same retention time, which confirms the presence of vitexin in the purified fractions.

In overall the qualitative analysis, quantitative assay and GC-MS analysis confirms that the methanolic extract effectively extracts polyphenols and flavonoids, and were further subjected to column chromatographic separation of vitexin. In the column separation, pale yellow fractions were obtained by eluting with the mixture of hexane and ethyl acetate. The column obtained yellow fractions were further confirmed with vitexin or apigenin 8-C-glucoside by thin layer chromatography, through R_f values of standard vitexin. Vitexin plays a significant role in cancer treatment worthwhile, which is cost-effective natural medicinal product for cancer recovery. Vitexin was extracted from commercial herbal formulations of *Passiflora foetida* using the HPTLC method in a previous study (Zhou *et al.*, 2025).

The separated fractions of vitexin exhibits almost double the capacity of radical scavenging activity against free radical generation. This may be due to, the vitexin can able to interact specific biomolecules and induce the defence mechanism to fight against free radicals. Similar research findings were carried out by another comparative study with *Vitex* species proves the potency against free radicals (Yang *et al.*, 2025). The results of phytochemical screening, the leaves and roots of *V. negundo* have good availability of phyto constituents. These results were correlated with the results of cytotoxicity assay. The results showed that the purified vitexin have better effect on colon cancer compared with methanolic crude extract, identified from linear graph. The IC₅₀ dose also relatively high for methanol extract and reduced concentration of vitexin possess the ability to kill the colon cancer cells within 24 hrs of the treatment. Studies conducted by Hussein *et al.*, (2025) supports the present work on antiproliferative activity of vitexin against colon cancer (Wu *et al.*, 2023). Another report correlates with the present investigations, says that the vitexin and its derivatives possess synergetic effect with other phytochemicals tested on the expression with CDK1/Cyclin B proteins (Spiegel and Russo, 2025). The antioxidant status of methanolic extract and the vitexin fractions good in improving antioxidant status of the treated cells, which was evident from the present investigations, the concentrations dependent improvement was proved by the present investigations. Similar studies conducted earlier correlated with the present work stating that intracellular improvement of antioxidant on vitexin treatment on CaCo2 colon cancer cells (Zhou *et al.*, 2025).

In cancer cells the free radical generation will be induced due to the multiple carcinogenesis mechanism, leads to progressive denaturation of proteins and lipids causes lipid peroxidation and related tissue damage, such a cellular damaged condition was also observed in the present study. The phytomedicine vitexin fractions can revert the protein denaturation and lipid peroxidation status of colon cancer cells. The mechanism behind this may be due to the molecular interaction of vitexin against specific protein involved in cancer progression and cancer metastasis, recent review on protective role of vitexin against oxidative stress induced damages such as inflammation, cancer neurological disorder and cardiac problems, supports our present findings. Another report, conforms that the induced molecular mechanism expressed by vitexin is due to the vitexin structure with distinctive apigenin backbone with C-8 Glycosidic bond, and modulates critical cellular process such as cell cycle, apoptosis, cancer metastasis, epigenetic modifications (Zhou *et al.*, 2025).

When the cancer cells are active, the cellular communication among the cancer cells are fast, which leads to have high metastatic properties, and rapid migration was observed. The cancer cell migration was tested with methanol extract and vitexin treatment, which shows significant inhibition of cell migration within 12 hrs and 24 hrs and effectively prevents the cancer spreading in HCT 116 colon cancer cells. This may be due to the phytochemicals present in the methanol extract and purified fractions of vitexin may be interacted with the membrane bound proteins, and alters the signaling pathways, which requires for cellular migration. *In vitro* studies conducted with gastric cancer, confirms the metastatic inhibition through altering epithelial -mesenchymal transition, and another finding also correlates the present investigation, stating the vitexin possess antimigratory activity, through inhibiting HMGB1 protein expression leading to PI3K/AKT/HIF-1 α pathway in liver cancer cells (Yang *et al.*, 2025).

In the apoptosis assay both methanol extract and purified vitexin fractions, can be show, more apoptotic cells, within 12 hrs and 24 hrs of treatment. The results of apoptosis clearly shows early and late apoptotic cells compared with control. This may be due to the phytochemical vitexin can able to intercalate the cell membrane, and leads to the nuclear alteration and there by modulate the normal structural and functional behavior of cancer cells. Studies conducted by **Wu *et al.*, (2023)**, reports that vitex can stimulate heat stress induced apoptosis in colon cancer cells. Research findings further support with the present work indicates apoptosis may also induced by mitochondrial dysfunction, cell cycle arrest, autophagy induction and blocking cell signaling pathways and explore antiproliferative effect (**Spiegel and Russo, 2025**).

5. CONCLUSION

Findings from the present work are helpful in the hunt for efficient cures for a variety of ailments. Medicinal plants are the main source of potent bioactive compounds. The medicinal plant *V.negundo* possess various bioactive primary and secondary metabolites were found high in methanolic, ethanolic, and chloroform extracts of both in leaves and roots compared with other solvents. The quantity of the phenolics and flavonoids contents were also found to be higher in methanolic and ethanolic leaf and root extracts. GC-MS and HPLC analysis were used for the characterization of bioactive compound, and the results showed the presence of several phytochemicals with potential medical uses. Following the successful isolation of the vitexin fraction via column chromatography, further confirmed by TLC through comparison of the fraction with a standard vitexin. The results of the present study clearly demonstrates that the methanolic crude extract and purified vitexin showed good cytotoxic effect against colon cancer, in addition it possesses radical scavenging activity, antioxidant power and good inhibitory effect against lipid peroxidation. Further analysis proved that the vitexin can effectively stops cancer cell migration and induces apoptosis. The overall findings of the present study confirm that vitexin can be considered as a good phytomedicine comparatively with standard drugs reduced side effects against colon cancer.

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