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The phytochemicals present in *Vitex negundo* leaf extract transcriptionally regulates colorectal cancer: An *In vitro* study using HCT-116 cells

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Colorectal cancer (CRC) ranks as the second most common cause of cancer-related deaths. Currently, there is no targeted medication to improve the treatment of colorectal cancer. In this study, we tried to evaluate the anti-cancer properties of *Vitex negundo* (*V. negundo*) using colon cancer cell lines (HCT-116 cells). Flow cytometry and transcriptome analysis were used to determine whether the phytochemicals promote apoptosis in the HCT-116 cells treated with *V. negundo* leaf extract at a concentration of 54 µg/mL. Treatment with *V. negundo* showed 28.6% and 18.5% of cells underwent early and late apoptosis when compared with controls. Further, we extended our studies to investigate the genes related to apoptosis and other signalling pathways using transcriptome analysis with and without treatment of *V. negundo* in HCT-116 cells by employing RNA sequencing. Our results revealed that the identification of 195876 transcripts derived from the datasets, with 44682 genes that are predicted to be expressed differently. To forecast the expression characteristics, we utilized functional enrichment analysis using the GO process, GO function, and GO component. Moreover, we used the functional annotation database of David to pinpoint the biological pathways associated with CRC. Pathway study of signalling pathways associated with colorectal cancer revealed that 133 genes are implicated, 23 of which are specifically involved in WNT signalling pathways regulated in CRC, and 6 high-rank genes are represented by PPI analysis. In conclusion, our studies revealed that the phytochemicals present in *V. negundo* transcriptionally regulate genes related to apoptosis and cell cycle in HCT116 cells.

Keywords: Apoptosis, Colorectal Cancer, Gene ontology, Transcriptome, *Vitex negundo*

Current cancer treatments, including immunotherapy, surgery, chemotherapy, radiotherapy, and anti-cancer medications, often have limited effectiveness due to side effects such as drug resistance, recurrence, and effect on non-targeted cells, as well as other toxicity issues[1]. In the search for efficient and less side effects medication for treating CRC, India's abundant biodiversity and status as a medicinal plant hub are being tapped into by researchers to discover phytochemicals for CRC treatment[2]. Many phytochemicals are effective against various cancers, having advantages over synthetic substances such as lower toxicity and simple extraction methods[3]. The present study is aimed to elucidate the pro-apoptotic activity and transcriptome analysis of *V. negundo* in colon cancer HCT-116 cell lines. Qualitative and quantitative analysis revealed that *V. negundo* leaf extract contains alkaloids, terpenoids, and flavonoids[4]. These secondary metabolites have antiproliferative and anti-metastatic actions in cancer *via* triggering the apoptotic pathway[5]. The study examined by *Giau Van et al* 2022, isolated artemetin, vitexicarpin, and penduletin compounds from *V. negundo*. The compounds were evaluated for the antiproliferative potential against HepG2 and MCF-7 cell lines by cell viability assay[6]. The docking studies and pharmacokinetics revealed that studies on the anti-cancer activity of artemetin, vitexicarpin and penduletin compounds should be taken into consideration as good anticancer candidates for further study. *Akiel et al* (2022) study investigation on the compound viridiflorol from *V. negundo* leaf extract, therapeutic effects of viridiflorol *in vivo* using

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animal models of cancer. Viridiflorol demonstrates cytotoxic and apoptotic ability in three different cancer cell lines (brain, breast and lung)[7].

Transcriptomics is the study of entire set of transcripts present within a cell, and their quantity for specific functional and developmental conditions[8]. The complete set of RNA molecules focus on mRNA's which reflects the genes that are actively expressed in a cell or tissue at a given time or in each situation[9]. Here 95% of mRNAs are not actively translated into proteins. Understanding the transcripts with differential functional elements of the genome is important within the cell. The transcripts include mRNAs, non-coding RNAs and small RNAs that are actively involved in structural, functional, and regulatory characterization within a cell[10].Generally, in human cells contains 20000 to 25000 functional genes are actively involved in protein synthesis with different patterns of gene expressions. By collecting and comparing the transcriptomes of each cell types normally functional or differentially expressed both in healthy and diseases cells[11].

The functions of most genes are unknown. There are several factors that can render the content of transcriptomes that can alter splicing, RNA editing and alternative transcription among others. Our objective of the study is to understand the differential expression of mRNAs that can present in cells. There are several techniques are used to identify the RNA expressions in a normal and disease cell. At beginning of 1990's identification of genes and expression studies are developed using serial analysis of gene expression experiment (SAGE)[12]. After 2000's microarray chip-based method is developed to identify the gene expression, but still the techniques are vastly used techniques to study RNA expression. On 2003 onwards, RNA-Seqtechnique is relatively used to study the small RNA transcripts of both qualitative and quantitative expressions to discover transcripts in a cell[13]. The identification of gene expression of common and rare transcripts is achieved through advanced high-throughput sequencing technology, known as RNA sequencing (RNA-Seq)[14]. The RNA-seq technique also used for identification of abnormal expressions of alternative splicing, novel transcripts, ncRNAs, and fusion genes. In principle, RNA-Seq can be carried out by all next generation sequencing (NGS) platforms, but the library preparation methods are different with each platform[15]. RNA-Seq platform provides far higher coverage and greater resolution of the dynamic nature of the transcriptomes. The elucidation has been made possible by using different RNA-Seq workflows, which include sample preparation, library construction, sequencing, and data analysis of functional complexity of the transcription[16,17].

Cancer genomic research seeks to find gene expressions that can alter the disease condition that leads to cancer. Several genes may exhibit differential expression in both disease and normal situations. The current study is aimed at finding gene expressions, both qualitative and quantitative, that can be addressed with *Vitex negundo* leaf extract as possible therapeutic molecules.

Materials and Methods

Extract preparation

The leaves of *V. negundo* L. were taken from the University of Agriculture Sciences, GKVK, Bangalore, Karnataka, India. A taxonomist from the University of Agricultural Sciences, GKVK, Bangalore, identified and certified the plant species. For *V. negundo* L., a voucher specimen with catalog number 4853 was submitted to the same department's herbarium. The methanolic extract was obtained through the use of a rotary evaporator. Also utilized 2g of the methanol extract, which was dissolved at a concentration of 10 mg/mL in sterile MilliQ water for the biological experiments.

GC-MS analysis

The isolated *V. negundo* extract sample was subjected to GC-MS analysis in Bangalore Analytical Centre. The analysis was carried out using gas chromatography in the Trace-1310 mode, connected to a thermo scientific triple quad mass spectrometer (MS) model TSQ8000. Experimental conditions of GC-MS system were as follows: TR 5-MS capillary standard non-polar column, dimension: 30Mts, ID: 0.25 mm, Film thickness: 0.25µm. Flow rate of mobile phase (carrier gas: He) was set at 1.0 mL/min. In the gas chromatography part, temperature programme (oven temperature) was 40°C raised to 250°C at 5°C/min and injection volume was 1 µL. The sample was injected in a ratio of 1:30 using the split sampling technique. The retention indices (RI) of the compounds were obtained by comparing the retention durations of a series and the identity of each component was confirmed by comparing its retention index with mass in a full scan ranging from 50 to 600 *m/z*.

Cell culture

The HCT116 colon cancer cell lines were supplied by the National Center for Cell Sciences (NCCS) in Pune, India and were grown at MP Biomedicals using Dulbecco's Modified Eagle Medium (DMEM) with Glucose and Insulin (DMEM-HG) that had been supplemented with 10% (v/v) Fetal Bovine Serum (FBS). Cisplatin served as a standard for comparing growth suppression methods. All commonly used drugs and chemicals (analytical grade) were purchased from HiMedia, Mumbai, India, and Sigma Chemical Co, USA.

Apoptotic assay

From our previous research work has performed cytotoxicity analysis revealed the HCT-116 colon cancer cells were shown the IC_{50} value of leaf extract 54 µg/mL and Cisplatin 50 µg/mL[4]. Additionally, one of the wells remains untreated to serve as a negative control. Briefly, culture cells in a 6-well plate at a density of 3×10^5 cells/2 mL and incubate in a CO_2 incubator overnight at 37°C for 24 hours. Cells were treated with required extract and cisplatin and then incubated for 12-16 hours. Following the treatment, carefully transfer the media from each well into 5mLwash tubes containing 500 µL of PBS after centrifugation[18]. Discard the PBS and substitute it with 200 µL of trypsin-EDTA solution. At 37°C, incubate for 3-4 min, and then transferred to the centrifuge tubes. Centrifuge the tubes at 300 × g for five min at 25°C decant the supernatant and re-suspend the cells to achieve a concentration of 1×10^6 cells/mL using 100 µL of 1X binding buffer in a 5 mL culture tube, resulting in 1×10^5 cells. Incorporate 5µL of AbFlour 488 Annexin V into the solution. Allow the cells to incubate for 15 min at room temperature (25°C) in a dark environment. Subsequently, add 2 µL of propidium iodide (PI) and 400µL of 1X binding buffer to each tube, then gently vortexed the mixture. Analyzed the samples using flow cytometry (Beckman Coulter, USA) immediately following the addition of PI[19].

Total RNA isolation, qualitative and quantitative analysis

The ZR Quick RNA Miniprep Plus Kit (ZYMO Research) was used to isolate total RNA from the treated HCT-116 CRC cell lines with *V. negundo* leaf extract, as instructed by the manufacturer. Nano Drop was used to check the quality and quantity of the isolated RNA samples, and then Agilent Tape Station was used with High Sensitivity RNA Screen Tape to assess their quality and quantity[20, 21].

Illumina 2x150 PE library preparation

PCR-passed RNA samples were used to create the RNA-Seq paired end sequencing libraries through the Illumina TruSeq Stranded mRNA sample Prep kit. Using Poly-T attached magnetic beads, mRNA was enriched from the total RNA, and followed by enzymatic fragmentation in a brief manner, to facilitate RNA dependent synthesis, Super Script II and Act-D mix are used to convert the first strand of cDNA. Synthesizing the first strand of cDNA into the second strand was done using a second-strand mix. AMPure XP beads were used to purify the dscDNA, and then it was ligated with adapters and enriched using a specific number of PCR cycles[22].

Quantity and quality check (QC) of library on Agilent 4200 Tape Station

The 4200 Tape Station system (Agilent Technologies) performed an analysis of PCR-enriched libraries using AMPure XP beads after purifying them using high sensitivity [23].

Cluster generation and sequencing

The Agilent Tape Station profile will be used to obtain the Qubit concentration for the libraries and the mean peak sizes, Cluster generation and sequencing was accomplished by loading the PE Illumina libraries onto the NovaSeq6000 [24]. The NovaSeq6000 has the ability to sequence template fragments in both forward and reverse directions through paired-end sequencing. A pair-end flow cell was used by the kit reagents to attach samples to compatible adapter oligos. The adaptive systems were constructed to permit the selective cleavage of the forward strands when the reverse strand is re-synthesized during sequencing. Sequencing from the opposite end of the fragment was done using the copied reverse strand. Finally, the raw sequence data was taken and done further analysis.

Sequence annotation and alignment (FastQC (version 0.11.9)

FastQC is widely used tool for the analysis of quality of sequence data. It could perform quality of sequencing data and trimming the unwanted data. Ensembl Genome Browser was used to obtain the reference genomic of Homosapiens. The download links for the reference fasta file and the GTF are, respectively. (ftp://ftpensemblorg/pub/grch37/release-87/fasta/homo_sapiens/dna/Homo_sapiens GRCh37 dnatoplevelfagz).The high-quality reads of HCT116-Control (Reference drug (Cisplatin) treated), HCT116-Treated (*V. negundo* leaf extract) and HCT116-Untreated (Positive control) datasets were

linked to the Homo sapiens reference genome. By using Bowtie2 (http://bowtie-biosourceforgenet/ bowtie2/manualshtml). First to create reference genome index using bowtie and the resultant index files to convert into transcriptome indexes using TopHat (https://ccb jhuedu/software/tophat/manual shtml) with default parameters (inner distance 150 and standard deviation 100). After the index creation, the raw sequence data is aligned with reference genome using TopHat. The TopHat initially removes poor quality reads and best reads is mapped with reference genome. The TopHat builds a splice junction to predict potential exons to confirm the mapped reads and the overall aligned scores is predicted in histogram of log2 reads both case and control reads provide gene candidates of selected sequence reads. The TopHat reads high-quality queries of 50-75 bp with best qualities is further used to predict differential expression analysis.

Differential gene expression (DEGs) analysis

Differential Gene Expression analysis serves as a crucial method for detecting genes that exhibit significant variations in expression levels across different experimental conditions or biological samples[25]. This analysis allows us to identify genes that are crucial for phenotypic diversity, disease progression, or treatment responses. Utilizing RNA-seq, we can detect and measure the levels of transcripts, providing insights into the dynamic gene expression patterns present in a cell or tissue[26]. Genes that are differentially expressed (DEGs) show notable variations in their expression levels when comparing different experimental groups. Differentially expressed genes (DEGs) can be detected using various statistical techniques that evaluate both the extent of change and the statistical significance of differences between groups, specifically through measures such as 'fold-change' and 'p-value' (0.05).

Functional enrichment analysis

The functional GO and KEGG enrichment analysis was carried out using the comprehensive tool for annotation visualization and integrated discovery (DAVID, https://david.ncifcrf.gov/)[27]. Analysis was divided into three main categories: biological processes (BP), cellular components (CC), and molecular functions (MF). The analysis and visualization of this tool is based on the *P* values 0.05 data analysis and statistical values enrichment score. Using the shengxin online tool (http://www. bioinformatics.com.cn/)[28], the KEGG bubble diagram was plotted and the GO term data analysis and visualization of three functions frame lines were enhanced.

PPI analysis

The structural and functional protein network analysis tool (STRING 11.0v) is a search engine for retrieving interacting proteins. It aims to provide the greatest features with various data access points and a high coverage score system (https://string-db.org/)[29]. Protein-protein interactions between filtered DEGs are performed using STRING, and a compressive score of >0.4 is regarded as a significant value. A Java-based open-source program called Cytoscape_v3.9.1 (https://cytoscape.org/) is used to visualize intricate network analysis.

qRT-PCR

Total RNA was extracted from both treated and untreated HCT-116 cells utilizing a Trizol reagent, following the guidelines provided by the manufacturer. The synthesis of cDNA necessitated one microgram of total RNA. For the qRT-PCR procedure, SsoFastEvaGreenSupermix (Bio-Rad, CA, USA) was utilized, and each reaction was conducted using a Qiagen Rotor-Gene 6000 real-time PCR device. A summary of the primers used for the amplification of the target gene is provided in (Table 3). The fold change was calculated employing the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

To examine the data, SPSS version 2 was used (Chicago, IL, USA) and one-way ANOVA. All statistical comparisons were regarded as statistically important *(P*< 0.05) among groups, and the data is shown as the mean ± standard deviation of three separate studies.

Results

GC-MS analysis

The presence of phytochemicals in the *V. negundo* (L) extract is confirmed by GC-MS analysis. The following phytocompounds were identified based on their retention time. The identified compounds are 1,4-DI-O-Benzylidene-DL-ribitol, Dibenz[a,c] cycloheptan-7-amine,1,2,3-trimethoxy,4-(3-methylanilino)-4-oxobutanoic acid, Ethyl (E,2S)-2-[3-[tert-butyl(dimethyl)silyl]oxypropyl]-2-cyano-7-phenylhept-3-enoate, 9-[4,5-dihydroxy-6 (hydroxymethyl)oxan-2-yl]-1H-purin-6-one,alpha-Ethynyl-5,6,7,8-tetrahydro-5,5,8,8-tetra methyl-

2naphthalenemethanol,4-Tert-Octylphenol,3-hydroxy-5-methyl-2-propan-2-ylcyclohexa-2,5-diene-1,4-dione,Ethyl4-(3,7,12-trihydroxy-10,13dimethyl 2,3,4,5,6,7,8,9,11, 12,14,15,16,17 tetradecahydro-1H-cyclopenta[a] phenanthren-17-yl) pentanoate, (E)-5-(5,5,8a-trimethyl-2-methylidene3,4,4a,6,7,8 hexahydro-1H-naphthalen-1-yl)-3-methylpent-2-enoicacid and2-(4a-methyl-8-methylidene1,2,3,4,5,6,7,8a octahydronaphthalen-2-yl)propan-2-ol (Table 1 & Fig.1).

Apoptosis assay

The Earlier research indicated that phytochemicals possess anti-cancer properties by facilitating apoptosis and suppressing the cell cycle [30]. Therefore, in this study, we examined through flow cytometry whether the phytocompounds found in *V. negundo* demonstrate comparable effects. Flow cytometry analysis indicated a notable increase in AbFlour 488 fluorescence in the control group. Cells treated with *V. negundo* leaf extract at a concentration of 54 µg/mL exhibited 28.6% and 18.5% of cells undergoing early apoptosis (Fig. 2). Cisplatin (50 µg/mL) served as a positive control, demonstrating a significant enhancement in apoptotic activity, with 18.4% of cells undergoing early apoptosis and 10.52% in late apoptosis. The proportion of cells that

Table 1 — List of compounds from GC-MS analysis of *V. negundo* leaf extract

Sl. No	RT (min)	Compound Name	m/z (g/mol)	Mol. formula	PubChem CID
1	8.49	1,4-DI-O-Benzylidene-DL-ribitol	328.4	$C_{19}H_{20}O_5$	219700
2	20.74	Dibenz[a,c]cycloheptan-7-amine,1,2,3-trimethoxy	299.4	$C_{18}H_{21}NO_3$	627054
3	27.06	4-(3-methylanilino)-4-oxobutanoic acid	207.23	$C_{11}H_{13}NO_3$	763244
4	27.82	Ethyl (E,2S)-2-[3-[tert-butyl(dimethyl)silyl] oxypropyl]-2-cyano-7-phenylhept-3-enoate	429.7	$C_{25}H_{39}NO_3Si$	154732574
5	32.43	9-[4,5-dihydroxy-6-(hydroxymethyl) oxan-2-yl]-1H-purin-6-one	297.27	$C_{11}H_{15}N_5O_5$	136023352
6	33.52	alpha-Ethynyl-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenemethanol	242.36	$C_{17}H_{22}O$	11817362
7	36.42	4-Tert-Octylphenol	206.32	$C_{14}H_{22}O$	8814
8	43.02	Ethyl4-(3,7,12-trihydroxy-10,13 dimethyl2,3,4,5,6,7,8,9,11,12,14,15,16,17 tetradecahydro-1H-cyclopenta[a] phenanthren-17-yl) pentanoate	436.6	$C_{26}H_{44}O_5$	536919
9	47.01	2-(4a-methyl-8-methylidene1,2,3,4,5,6,7,8a-octahydronaphthalen-2-yl) propan-2-ol	222.37	$C_{15}H_{26}O$	521215
10	55.03	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	424.2	$C_{23}H_{32}O$	5363101
11	69.02	Ethyl iso-allocholate	436.65	$C_{26}H_{44}O_5$	6452096
12	93.00	7,8-Epoxylanostan-11-ol, 3-acetoxy	502.23	$C_{32}H_{54}O_4$	541562
13	120.97	1-Heptatriacotanol	536.1	$C_{37}H_{76}O$	537071

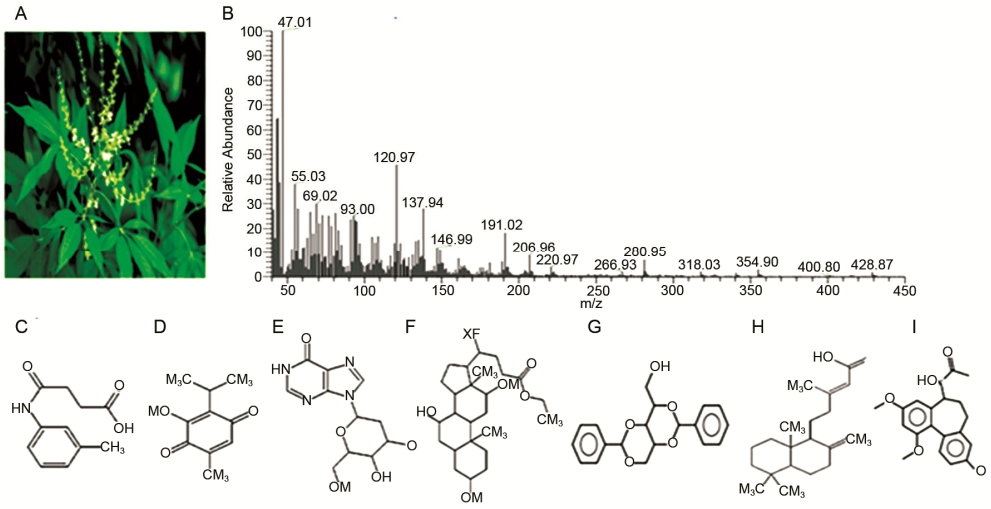

Fig. 1 — (A) *V. negundo* leaf extract; (B) GC-MS analysis; (C)1,4-DI-O-Benzylidene-DL-ribitol; (D) Dibenz[a,c]cycloheptan-7-amine,1,2,3-trimethoxy; (E) 4-(3-methylanilino)-4-oxobutanoic acid; (F) Ethyl (E,2S)-2-[3-[tert-butyl(dimethyl)silyl] oxypropyl]-2-cyano-7-phenylhept-3-enoate; (G) alpha-Ethynyl-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenemethanol; (H) 4-Tert-Octylphenol; and (I) 2-(4a-methyl-8-methylidene1,2,3,4,5,6,7,8a-octahydronaphthalen-2-yl) propan-2-ol

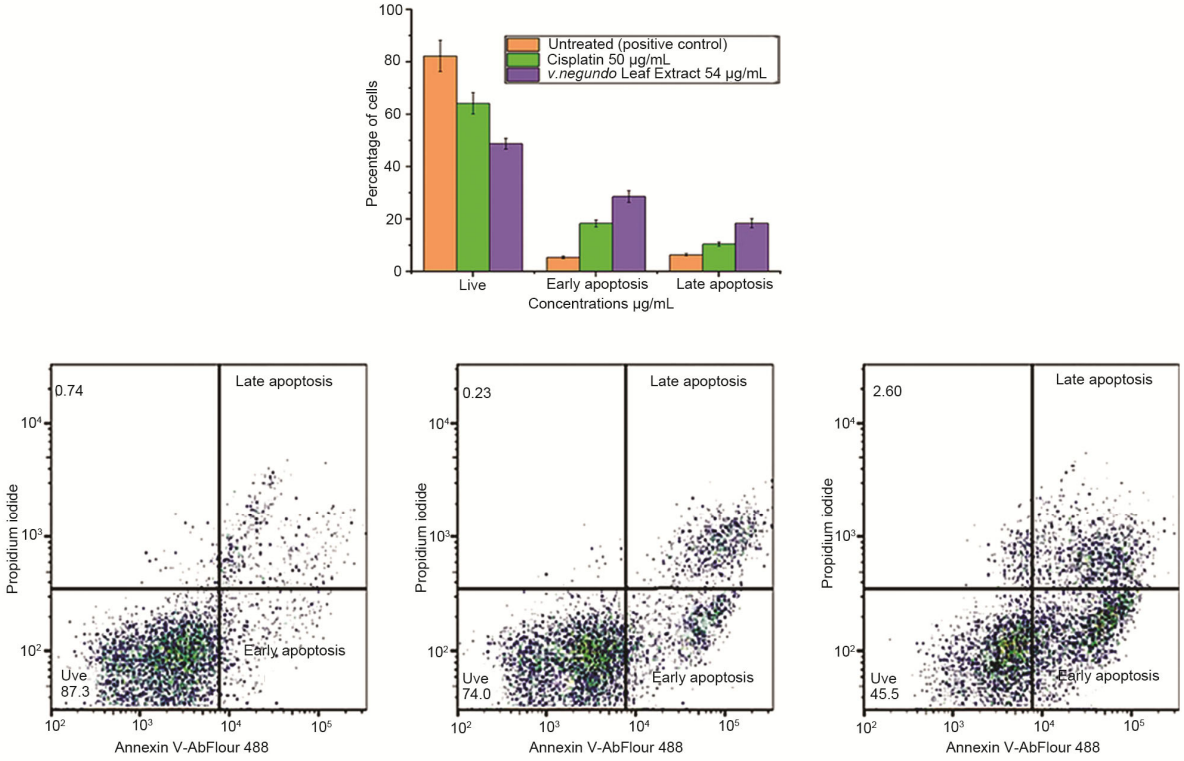

Fig. 2 — The impact of *V. negundo* leaf extract on apoptosis in the HCT-116 colon cancer cell line was evaluated. The results are expressed as mean ± SD from three separate experiments, as outlined in the methods section. $P<0.05$ is considered as a statistically significant

were positive for AbFlour 488-Annexin V and negative for propidium iodide (PI) increased in correlation with the number of early apoptotic cells in the treated samples. Additionally, as the number of cells rises, the extract triggers apoptotic signalling proteins[31]. In the current study, a rise in early apoptosis was noted, which may be attributed to the synergistic action of the phytocompounds found in *V. negundo* that activate the apoptosis signalling pathway.

QC of RNA samples on Agilent TapeStation

ZR Quick RNA Miniprep Plus Kit (ZYMO Research) to isolate total RNA from the human cell samples that were received, following the manufacturer's instructions. The RNA samples were assessed for both quality and quantity using NanoDrop and then again using Agilent TapeStation with High Sensitivity RNA ScreenTape to ensure accurate results. The QC passed RNA samples were processed for PE library preparation using TruSeq stranded mRNA Library Prep Kit as per the kit protocol. The quality of the prepared libraries passed in our QC step and processed for sequencing on Illumina platform using 2 × 150 bp chemistry[32].

FastQC (version 0.11.9)

The Raw Data of transcriptome-seq data is used to predict quality using FASTQC software. The FASTQC tool can read the data with several parameters that can influence the prediction of the quality of raw data. The parameters such as total base quality, per tile quality, GC content and N-base pair distribution, to find sequencing errors, look for adaptors, overrepresented k-mers and duplicate reads. The sequenced raw data of HCT116-Control (Reference drug (Cisplatin) treated), HCT116-Treated (*V. negundo* leaf extract) and HCT116-Untreated (Positive control) datasets. Address these issues v 0.39 was used to remove adaptor sequence data, uncertain reads (reads with more over 5% unknown nucleotides "N") and low-quality sequence data (reads with much more and over 10% creation of a high (QV) 20 phred score) in order to generate quality clean reads a minimum size of 150 nt (nucleotide) was used after snipping. High quality readings for the HCT-116 test were produced after the converter and low-quality sequences were removed from of the raw data these excellent standard (QV>20) paired-end amplicons have been used for referenced based read modelling. Further analysis for

an ideal RNA-Seq library, this metric should be greater than or equal to 90 %. While alignment rates close to 70 % may still be acceptable depending on the quality of the RNA input and the reference genome used, in this studies Sequence annotation and alignment was done by using reference genomic of Homo sapiens has GRCh37 and the result represent (Table 2).

Differential gene expression (DEGs)analysis

In the present study, the predicted diffentially regulated genes by the transcriptome analysis using RNA sequence. The result showed that 195876 transcripts been identified from the datasets, in that 44682 genes are predicted for differential gene expression (Fig. 3 & Table 3).

Functional enrichment analysis

Gene ontology is an important tool in bioinformatics for annotating genes and their products, classifying genes into three primary ontologies: Biological Process [BP], Molecular Function [MF], and Cellular Component [CC][33]. In total HCT-116 cells (Control) regulated genes are 14991 and segregated like up regulated 11281 genes and down regulated genes are 3710, for HCT-116 cells treated with *V. Negundo* leaf extract, regulated genes are 15451 up regulated 12762 and down regulated 2686 and HCT-116 cells treated with Cisplatin total regulated genes are 14991 the up regulated genes 12762 and down regulated genes are 2686.From expressed genes top 50 regulated genes in in HCT-116 cells control, *V. negundo* and Cisplatin represented in (Fig. 4).

In the present study, we have predicted diffentially regulated genes by the transcriptome analysis using RNA sequence. The result showed that 195876 transcripts been identified from the datasets, based on the prediction, there are 44682 genes for differential gene expression (Fig. 5). After analyzing the differential gene expression, 14991 genes are found in reference drug Cisplatin, while 14095 genes are observed in the untreated condition, and 15451 genes are detected in the *V. negundo* leaf extract treated condition. After identifying upregulated and down-regulated genes with a significance of $P< 0.05$ (Table 4), we ultimately selected 773 genes for functional enrichment analysis. Of these 773 genes, 301 are involved in protein synthesis.

BP-regulated DEGs were observed to be involved in processes such as ATP metabolism, SRP-dependent membrane-targeting co-translational protein, membrane-targeting co-translational protein, membrane-targeting protein it directs to the ER, the catabolic process of nuclear transcribed mRNA, establishes the

Table 2 — RNA-Seq quality statistics and alignment

Sample	Mapping %
HCT116-Control (Cisplatin)	90.2%
HCT116-Treated (VNLE)	92.8%
HCT116-Untreated (control)	96.1%

Table 3 — Differential Gene Expression with significance difference based on *p*-value

Conditions	Total Genes	Up-regulated ($P<0.05$)	Down-regulated ($P<0.05$)
HCT-116 cells (Control)	14095	10980	3115
HCT-116 cells treated with Cisplatin	14991	11281	3710
HCT-116 cells treated with *V.negundo* (VNLE)	15451	12762	2686

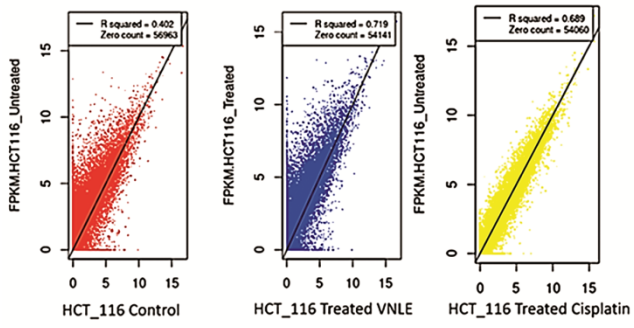

Fig. 3 — Scatter diagram of differential genes expressed in HCT-116 cell line in control, *V. negundo* leaf extract and Cisplatin

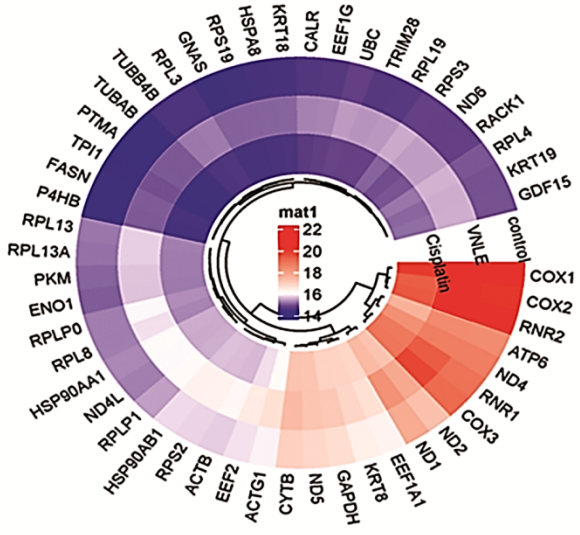

Fig. 4 — Cluster heatmap for top 50 genes differentially expressed in HCT-116 cells control, *V. negundo* and Cisplatin

Table 4 — Functional enrichment analysis of regulated DEGs involved in pathway

Term	Count	%	P-Value	Fold Enrichment	FDR
WNT signaling pathway	133	64.25121	1.02E-223	34.89348	8.24E-222
Melanogenesis	54	26.08696	2.27E-58	19.55084	9.20E-57
Basal cell carcinoma	42	20.28986	8.59E-55	28.15965	2.32E-53
Pathways in cancer	76	36.71498	1.50E-45	7.001527	3.04E-44
Proteoglycans in cancer	49	23.6715	3.91E-33	8.870289	4.52E-32
Signaling pathways regulating pluripotency of stem cells	40	19.32367	1.58E-29	10.34436	1.60E-28
Colorectal cancer	23	11.11111	1.68E-19	13.43098	1.51E-18
Oxytocin signaling pathway	32	15.45894	2.42E-19	7.723789	1.96E-18
Inflammatory mediator regulation of TRP channels	18	8.695652	9.20E-10	6.649946	4.14E-09
Endometrial cancer	13	6.280193	1.03E-08	9.051316	4.17E-08
Insulin secretion	15	7.246377	5.93E-08	6.389164	2.09E-07
MAPK signaling pathway	25	12.07729	8.54E-08	3.577595	2.77E-07
Renin secretion	13	6.280193	1.23E-07	7.354194	3.83E-07
Endocrine and other factor-regulated calcium reabsorption	11	5.31401	2.67E-07	8.850175	7.73E-07
cAMP signaling pathway	20	9.661836	1.76E-06	3.657097	3.85E-06
Cholinergic synapse	14	6.763285	9.56E-06	4.56643	1.94E-05
Estrogen signaling pathway	13	6.280193	1.49E-05	4.754226	2.68E-05
Retrograde endocannabinoid signaling	13	6.280193	1.83E-05	4.660083	3.23E-05
Cell cycle	14	6.763285	3.19E-05	4.087691	5.50E-05
Axon guidance	14	6.763285	4.12E-05	3.991131	6.95E-05
cGMP-PKG signaling pathway	15	7.246377	1.03E-04	3.437209	1.46E-04
Natural killer cell mediated cytotoxicity	13	6.280193	1.21E-04	3.857938	1.66E-04
Notch signaling pathway	8	3.864734	3.00E-04	6.034211	3.92E-04
TGF-beta signaling pathway	10	4.830918	4.59E-04	4.31015	5.54E-04
Oxidative phosphorylation	11	5.31401	0.00349	2.99442	0.00349
MicroRNAs in cancer	16	7.729469	0.011917	2.025469	0.011917
Ras signaling pathway	13	6.280193	0.021488	2.082604	0.021488
p53 signaling pathway	6	2.898551	0.036376	3.242262	0.036376
Phosphatidylinositol signaling system	7	3.381643	0.05227	2.58609	0.05227
GABAergic synapse	6	2.898551	0.083897	2.555666	0.083897
Insulin signaling pathway	8	3.864734	0.085146	2.098856	0.085146

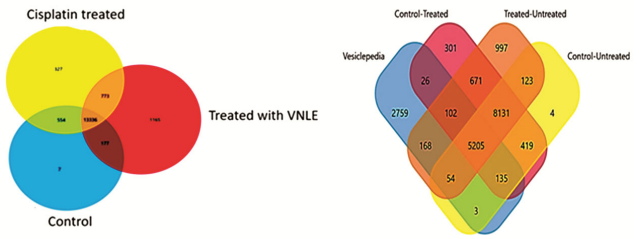

Fig. 5 — Vesiclepedia diagram represents the comparative differential genes associated with protein expression based on three different conditions

localization of proteins in the endoplasmic reticulum, viral transcription and translational initiation. The regulated DEGs in MF, observed the involvement in processes such as structural constituent of ribosome, NADH dehydrogenase activity, cadherin binding, oxidoreductase activity, MHC class ll protein complex binding, oxidoreductase activity, MHC protein complex binding and rRNA binding. The regulated DEGs in CC cytosolic ribosome, ribosomal subunit, focal adhesion, cell-substrate junction, cytosolic large ribosomal subunit, respirasome, respiratory chain complex, large ribosomal subunit, oxidoreductase complex. The functional enrichment analysis of in KEEG pathway regulated are involves DEGs like WNT signaling pathway, Melanogenesis, Basal cell carcinoma, Pathways in cancer, Proteoglycans in cancer, Signaling pathways regulating pluripotency of stem cells, Colorectal cancer, Oxytocin signaling pathway, Inflammatory

mediator regulation of TRP channels, Endometrial cancer, Insulin secretion, MAPK signaling pathway, Renin secretion, Endocrine and other factor-regulated calcium reabsorption, cAMP signaling pathway, Cholinergic synapse, Estrogen signaling pathway, Retrograde endocannabinoid signaling, Cell cycle, Axon guidance, cGMP-PKG signaling pathway, Natural killer cell mediated cytotoxicity, Notch signaling pathway, TGF-β signaling pathway, Oxidative phosphorylation, MicroRNAs in cancer, Ras signaling pathway, p53 signaling pathway, Phosphatidylinositol signaling system, GABAergic synapse and Insulin signaling pathway (Fig. 6).

Pathway analysis identified 133 genes involved in WNT signaling pathways. We also identified 23 genes specifically up-regulated in colorectal cancer. Based on the analysis of signaling pathways related to CRC and related genes, information is provided in (Table 5).

The study of the transcriptome can forecast the recognized anti-cancer properties of *V. negundo*, leading to an emphasis on pathways pertinent to tumor development and the advancement of cancer. Therefore, both the upstream signaling components of the pathways and the subsequent changes in gene expression were reduced when cells were exposed to the extract[34]. In addition to the WNT/β-catenin and TGF-β signaling pathways, a decrease in the activity of the spliceosome pathway also indicates a potential anti-cancer effect. The down-regulation in activity of these pathways suggests that the known anti-cancer effects of the extract might be accurately foreseen through the analysis of RNA sequencing[35].

PPI analysis

The PPI network analysis results as available a total of 25 genes were considered as number for hub genes (Table 6 & Fig. 7). It showed the analysis of following number of nodes:23, number of edges:154, average node degree:13.4, avg. local clustering coefficient:0.823, expected number of edges:50, PPI enrichment *p*-value:< 1.0e-16. The using Cytohubba plugin in Cytoscape was used to identify 6 hub genes from the protein-protein interaction network using the most recent maximal clique centrality approach

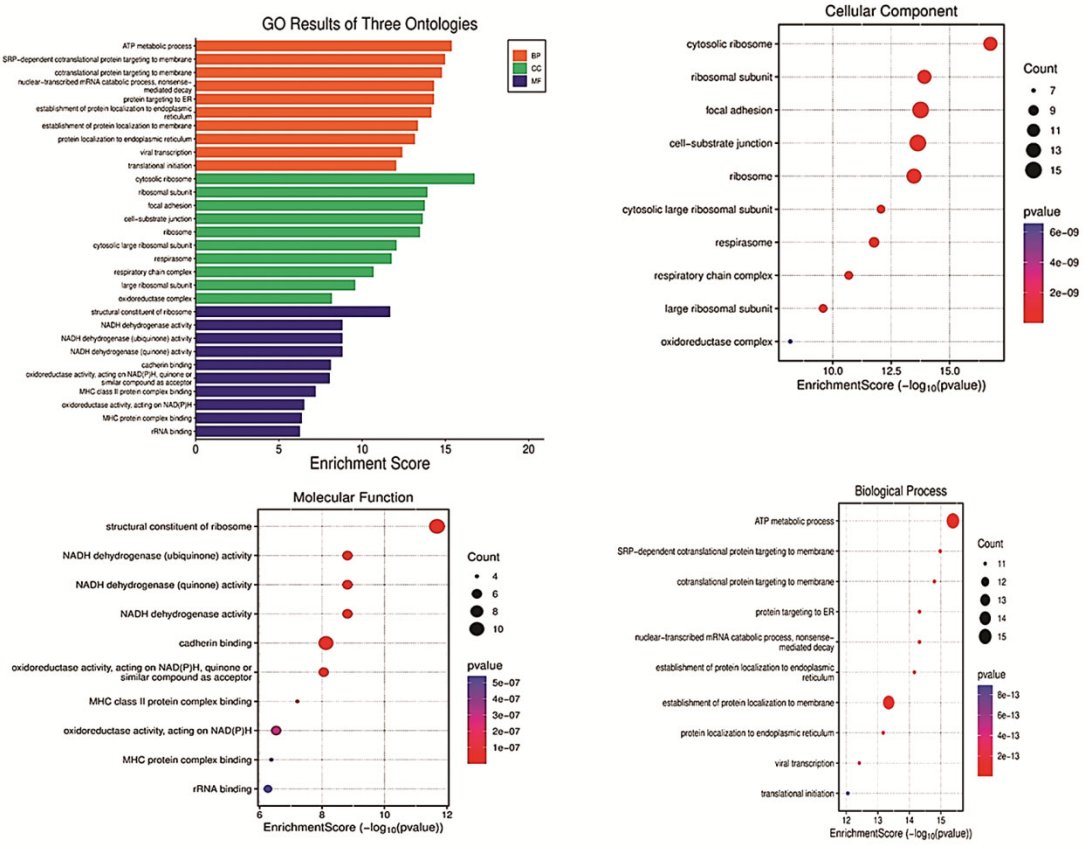

Fig. 6 — Gene Ontology enrichment analysis highlighting functional categories significantly represented. The regulated genes for cellular component, molecular function and biological process

Table 5 — Signaling pathways associated with colorectal cancer

Description	Genes
WNT signaling pathway	FZD10, FRAT1, CCND3, FRAT2, CCND2, SOX17, CCND1, MYC, PRKACG, RUVBL1, EP300, PRKACA, BTRC, PRKACB, MAP3K7, SKP1, PRKCG, WNT5B, CSNK2A1, PRKCB, FBXW11, WNT5A, CTNNBIP1, CSNK2A2, PRKCA, CSNK1E, DKK1, DKK2, RBX1, DKK4, SFRP4, SFRP1, SFRP2, DAAM1, TBL1XR1, DAAM2, SFRP5, CSNK2B, TP53, CTBP2, CTBP1, TCF7, CUL1, PSEN1, NLK, WNT6, PORCN, PPP3R1, PPP3R2, DVL1, DVL2, DVL3, WNT1, WNT2, WNT3, WNT4, FZD1, WNT10B, FZD3, CREBBP, JUN, WNT10A, SMAD4, FZD2, FZD5, FZD4, WNT3A, FZD7, SIAH1, FZD6, FZD9, NFATC3, FZD8, NFATC2, NFATC1, NFATC4, FOSL1, VANGL1, VANGL2, BAMBI, GSK3B, WNT2B, CHD8, PPP3CA, PPP3CB, PPP3CC, RAC2, RAC3, RAC1, APC2, MMP7, WNT9B, AXIN1, CACYBP, WNT9A, AXIN2, WNT16, SENP2, RHOA, PLCB3, PLCB4, PLCB1, PLCB2, PPARD, CAMK2B, CAMK2D, ROCK2, CAMK2A, LEF1, PRICKLE2, LRP5, WNT8B, PRICKLE1, CXXC4, NKD1, NKD2, LRP6, MAPK9, MAPK8, WNT11, WIF1, TBL1Y, GPC4, TBL1X, CAMK2G, TCF7L2, TCF7L1, CSNK1A1, WNT7B, WNT7A, MAPK10, APC, CTNNB1
Melanogenesis	GSK3B, WNT2B, FZD10, PRKACG, EP300, PRKACA, PRKACB, PRKCG, WNT5B, PRKCB, WNT5A, WNT9B, PRKCA, WNT9A, WNT16, PLCB3, PLCB4, PLCB1, PLCB2, CAMK2B, CAMK2D, TCF7, CAMK2A, LEF1, WNT8B, WNT6, WNT11, DVL1, DVL2, DVL3, WNT1, WNT2, CAMK2G, WNT3, WNT4, FZD1, TCF7L2, WNT10B, FZD3, WNT10A, CREBBP, TCF7L1, FZD2, FZD5, FZD4, WNT3A, FZD7, WNT7B, FZD6, FZD9, FZD8, WNT7A, GNAS, CTNNB1
Colorectal cancer	APC2, GSK3B, TCF7L2, JUN, TCF7L1, SMAD4, SMAD3, LEF1, TCF7, AXIN1, AXIN2, RHOA, MAPK10, MAPK9, MAPK8, APC, CCND1, MYC, RAC2, RAC3, CTNNB1, RAC1, TP53
TGF-β pathway	SMAD4, CREBBP, SMAD3, BAMBI, MYC, CUL1, EP300, RHOA, SKP1, RBX1
Oxidative phosphorylation	ND6, ATP6, COX3, COX2, COX1, ND1, ND4L, CYTB, ND2, ND5, ND4
p53 signaling pathway	CCND3, CDKN1A, CCND2, CCND1, SIAH1, TP53

Table 6 — The hub node gene analysis of differentially expressed genes

Hub node 1	Hub node 2	Node 1 accession	Node 2 accession	Score
APC2	AXIN2	9606.ENSP00000257430	9606.ENSP00000302625	0.999
GSK3B	LEF1	9606.ENSP00000324806	9606.ENSP00000265165	0.824
TCF7L2	TCF7	9606.ENSP00000340347	9606.ENSP00000486891	0.943
JUN	RAC2	9606.ENSP00000360266	9606.ENSP00000249071	0.453
TCF7L1	SMAD3	9606.ENSP00000332973	9606.ENSP00000282111	0.563
SMAD4	TP53	9606.ENSP00000341551	9606.ENSP00000269305	0.875
SMAD3	TP53	9606.ENSP00000332973	9606.ENSP00000269305	0.963
LEF1	APC	9606.ENSP00000257430	9606.ENSP00000265165	0.848
TCF7	APC2	9606.ENSP00000442954	9606.ENSP00000340347	0.639
AXIN1	CCND1	9606.ENSP00000262320	9606.ENSP00000227507	0.779
AXIN2	CCND1	9606.ENSP00000302625	9606.ENSP00000227507	0.774
RHOA	CCND1	9606.ENSP00000227507	9606.ENSP00000400175	0.589
MAPK9	CTNNB1	9606.ENSP00000495360	9606.ENSP00000394560	0.613
MAPK8	RAC2	9606.ENSP00000378974	9606.ENSP00000249071	0.953
APC	CCND1	9606.ENSP00000257430	9606.ENSP00000227507	0.595
CCND1	TCF7	9606.ENSP00000227507	9606.ENSP00000340347	0.457
MYC	APC	9606.ENSP00000257430	9606.ENSP00000478887	0.613
RAC2	RHOA	9606.ENSP00000249071	9606.ENSP00000400175	0.94
RAC3	RHOA	9606.ENSP00000304283	9606.ENSP00000400175	0.924
CTNNB1	RAC2	9606.ENSP00000495360	9606.ENSP00000249071	0.468
RAC2	JUN	9606.ENSP00000360266	9606.ENSP00000249071	0.453
TP53	LEF1	9606.ENSP00000265165	9606.ENSP00000269305	0.551

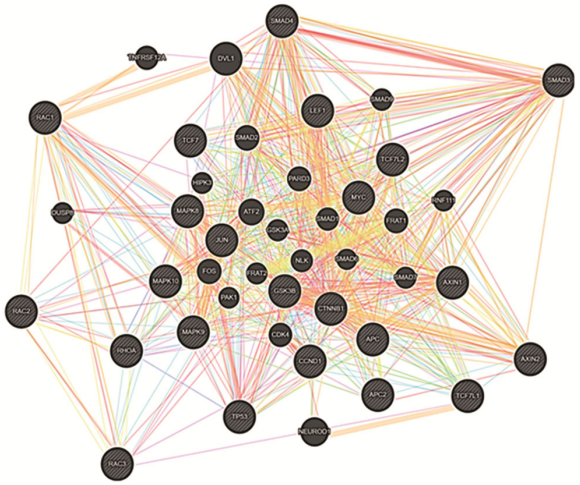

Fig. 7 — Hub genes analysis of regulated 25 genes related CRC from PPI analysis

Table 7 — Top 6 in network Sheet1 ranked by MCC method

Rank	Name	Score	Number of nodes	Number of edges	p-value
1	CTNNB1	1.67E+08	11	37	0.000356
2	MYC	1.67E+08	11	36	0.0295
2	SMAD4	1.67E+08	11	48	1.74e-12
3	AXIN1	1.67E+08	11	55	< 1.0e-16
4	LEF1	1.67E+08	11	44	3.21e-06
4	TP53	1.67E+08	11	35	0.0582

(MCM) (Table 7)[36]. The results represent 6 top genes and their interactions with other genes are represented by the nodes and highlighted in red and yellow (Fig. 8).

Protein-drug interaction network analysis was performed. The genes identified by MMC those genes are CTNNB1, MYC, SMAD4, AXIN1, LEF1 and TP53. CTNNB1 number of nodes 11 and *p*-value is 0.000356, MYC number of nodes 11 and *p*-value is 0.0295, SMAD4 number of nodes 11 and p-value is 1.74e-12, AXIN1 number of nodes 11 and *p*-value is < 1.0e-16, LEF1 number of nodes 11 and *p*-value 3.21e-06 and TP53 number of nodes 11 and *p*-value 0.0582.

qRT-PCR

The expression of critical genes that govern cancer progression was examined using qRT-PCR. The transcription factors CTNNB1, MYC, SMAD4, AXIN1, LEF1, and TP53 play a regulatory role in various genes associated with cancer metabolism. The findings indicated that the mRNA expression levels of CTNNB1, MYC, SMAD4, AXIN1, LEF1, and TP53 were significantly lower in the HCT-116 cell lines treated with *V.negundo* extract when compared to the

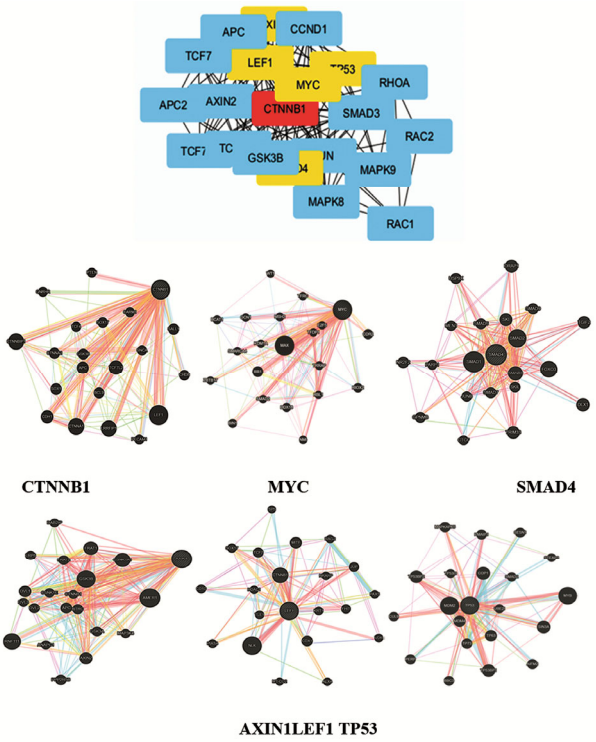

Fig. 8 — Representation of top six rank regulated genes from analysis of Cytohubba plugin in Cytoscape and hub gene PPI analysis of all regulated 6 genes

control groups, as illustrated in (Fig. 9). The primers utilized for the mRNA expression analysis are listed in (Table 8).

Discussion

V. negundo is known for its medicinal properties. Our previous qualitative and quantitative analysis of extract revealed the presence several phytochemicals, which is further confirmed by GC-MS analysis [5]. Moreover, our studies revealed the cytotoxicity efficacy of *V. negundo* leaf extracts in HCT-116 cell lines. The extract demonstrated the IC_{50} value of cytotoxicity at a concentration of 54 µg/mL. Cell cycle checkpoints are critical for preventing cells from progressing to the next phase of the cell cycle. In *V. negundo*-treated cell cultures, flow cytometry data revealed a progressive reduction in apoptotic levels throughout the early and late stages of treatment. In particular, apoptosis rates reduced from 28.6% in the early stage to 18.5% in the late stage, in contrast to the reference drug-treated cell cultures, which had apoptosis rates of 18.4% in the early stage and 10.52% in the late stage.

The use of transcriptome analysis has become prevalent in predicting the biological effects of various

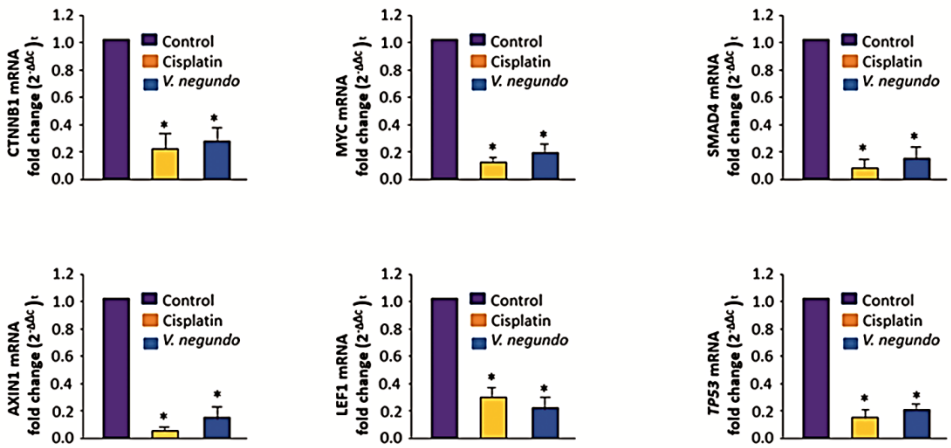

Fig. 9 — Effects of the *V. negundo* HCT-116 cancer cell lines' expressions of CTNNB1, MYC, SMAD4, AXIN1, LEF1, and TP53 are examined in an extract. For duration of 24 hours, cells were subjected to either the extract at 54 µg/mL or the vehicle (0.1% DMSO). The CTNNB1, MYC, SMAD4, AXIN1, LEF1, and TP53 gene expression relative fold regulation by qRT-PCR, *P< 0.01

Table 8 — The list of primers used in mRNA expression analysis

Sl. No	Gene Name	Sequence	
		Forward primer (5'−3')	Reverse primer (3'−5')
1	CTNNB1	GGTAGGGTGGGAGTGGTTTA	GCTTGGTTAGTGTGTCAGGC
2	MYC	CCTCCAGCCTGATCTTTTGG	GCGTTATTCCCCAGACCACT
3	SMAD4	CCTTCAAGCTGCCCTATTGT	AATGGCTTCTGTCCTGTGGA
4	AXIN1	GCTCATTGTTCCTTGACGCA	CAGTTCTCCCTCCTCACCAG
5	LEF1	AGTTGCCTGATCCTTCCCT	AAGGAGGTGGTGATTGAGGG
6	TP53	GTTACCTCCTCTCGTAGCCC	CTTCCTCTGCTTCCTCCTCC

active compounds. Nevertheless, the analysis of plant extracts presents difficulties due to their chemical complexity, as these extracts may contain hundreds of biologically active compounds[37]. The present work aimed to obtain a comprehensive understanding of the biological effects of a plant extract by utilizing gene set analysis. An analysis of this nature could indicate potential health impacts of the plant, which involves correlating biological pathways with specific diseases[38]. Prior investigations have aimed to explore the effects of certain plant extracts on the transcriptomes of specific cells or tissues. However, there has been no initiative to predict the biological or pharmacological functions of these extracts based on the findings from these studies[39]. This RNAseq analysis plays a crucial role in enhancing our comprehension of the mechanisms underlying the anticancer effects of the extract[40].

The transcriptome is the full range of mRNA expressed by an individual or a population of cells. In the present investigation, we have studied the influence of *V. negundo* on the transcriptome in HCT-116 cells using RNA sequence. The results demonstrated the identification of 195,876 transcripts from the datasets, from which 44,682 genes were predicted to exhibit differential gene expression. Based on differential gene expression, 14991 genes are observed in cisplatin-treated, 14095 genes in untreated, and 15451 genes from *V. negundo*-treated HCT-116 cell cultures. Further, we predicted the up-regulated and down-regulated genes using FASTQC software. In total, 773 genes were identified for functional enrichment analysis. Of these, 301 genes play a role in protein synthesis. The purpose of the functional enrichment analysis is to anticipate expression characteristics based on the Gene Ontology (GO) process, function, and component. Subsequently, we utilized the DAVID functional annotation database to identify the biological pathways associated with colorectal cancer (CRC). Our pathway analysis revealed that 133 genes are implicated in WNT signaling pathways, with 23 of these genes being specifically regulated in the context of colorectal cancer. The Cytohubba plugin in Cytoscape was used to identify six (CTNNB1, MYC, SMAD4, AXIN1, LEF1, and TP53) hub genes from

the protein-protein interaction network by employing the latest maximal clique centrality method (MCM).

The gene CTNNB1 is responsible for producing the β-catenin protein, which acts as a transcriptional factor controlling genes related to cell growth, viability, and movement. Research suggests that the Wnt/β-catenin signaling pathway plays a crucial role in driving the development of colorectal cancer[41]. The proto-oncogene MYC is crucial in the progression of colorectal cancer, as it facilitates stress adaptation, metabolic reprogramming, and immune evasion, all of which contribute to the development of CRC and resistance to treatment[42]. The lung squamous cell carcinoma involves SMAD4 signaling, CRC progression is linked to reduced SMAD4 expression[43]. AXIN1 plays a key role in the Wntpathway by assembling the β-catenin destruction complex, which leads to the suppression of Wntand β-catenin-dependent target genes. The abnormal activation of the Wnt pathway is widely believed to be a major factor driving colorectal cancer[44]. LEF1, a transcription factor, is highly expressed in CRC cells in comparison to normal intestinal cells. Its overexpression is linked to a negative prognosis and contributes to tumorigenesis, as well as the proliferation, migration, and invasion of cancer cells[45]. The mutation of the TP53 tumor suppressor gene is believed to be a significant factor in the advancement of CRC and could serve as a valuable prognostic indicator. The incidence of TP53 mutations in CRC is estimated to be around 40% to 50%[46]. The phytochemicals present in extract significantly altered genes related to different cancer signaling pathways. Besides, the strong antioxidant activity also helps to reduce the oxidative stress burden in cancer cells. All together phytochemicals present in extract efficiently prevented the proliferation of CRC cells.

Conclusion

In conclusion, reduced oxidative stress, suppressed cell proliferation, caused programmed cell death, prevented angiogenesis activity and cell cycle arrest are the mechanisms that phytochemicals used to stop or decrease cancer growth. Numerous phytochemicals are being investigated as potential anti-cancer medications. When creating anti-cancer medications, one or more proteins or pathways involved in the progression of cancer must be blocked. The induction of apoptosis in 28.6% and 18.5% of cells in early and late apoptosis, respectively, and the regulation of 44682 genes, including 133 genes implicated in CRC signaling pathways, suggest that *V. negundo* phytochemicals could target specific mechanisms involved in colorectal cancer progression. With 23 genes specifically involved in WNT signaling pathways, this natural compound may offer a new avenue for developing targeted therapies, particularly for patients with limited treatment options. Based on our results the molecular pathways implicated in the prevention of colon cancer by methanolic leaf extract of *V. negundo*. This study, examined transcriptome analysis of extract treated HCT-116 cells demonstrated to have anti-cancer capabilities *via* promoting apoptosis and cell cycle arrest.

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Conflict of interest

Authors declare that there is no conflict of interest.

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