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ORIGINAL ARTICLE

Rubus sanctus Schreb. Root Extract Alters the MicroRNA Expression and Inhibits Tumor Activities of Colorectal Cancer Cell Lines

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ABSTRACT

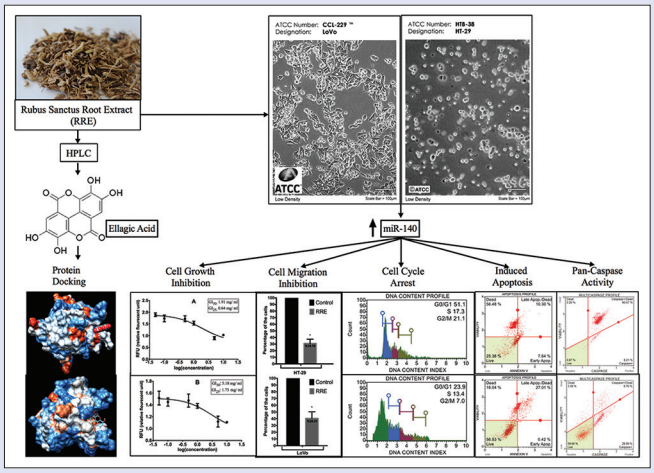
Background: Colorectal cancer (CRC) is one of the most common cancers in the world. Although surgical and screening techniques have vastly improved in the last 30 years, chemotherapeutics have not advanced sufficiently for successful treatment. **Objective:** The aim of this study is to investigate the microRNA (miRNA) expression changes and anticancer agent potential of *Rubus Sanctus* Schreb. root extract (RRE) on LoVo and HT-29 colorectal adenocarcinoma cell lines. **Materials and Methods:** LoVo and HT-29 CRC cell lines treated with different concentrations of RRE to find growth inhibitory effect with WST-1 assay. Fifty percent growth inhibition and 25% growth inhibition concentrations further evaluated with annexin V, total caspase, cell cycle, and migration assays. Real-time polymerase chain reaction was used to investigate the expression differences in miRNA after extract treatment. **Results:** Cell proliferation was reduced 77.98% in HT-29 cells after RRE treatment ($P < 0.05$). In the cell invasion analysis, RRE reduced invasion in both cell lines up to 75.56% ($P < 0.05$). In addition, RRE induced apoptosis in up to 98% of a cell population ($P < 0.05$). Similarly, pan-caspase activity increased to 97.6% and 87.2% in LoVo and HT-29 cell lines, respectively ($P < 0.0001$). After extract treatment, among the nine miRNAs evaluated, only miR-140 expression was significantly increased in both cell lines after RRE treatment ($P < 0.05$). **Conclusion:** Our data show for the first time that RRE has the capability to inhibit CRC cell proliferation and invasion and alter epigenetic mechanisms. Although further studies should be conducted on this topic, RRE is thought to be a potential candidate for the future studies regarding new therapy options.

Key words: Apoptosis, blackberry, colorectal cancer, invasion, microRNA, *Rubus sanctus* Schreb.

SUMMARY

- *Rubus Sanctus* Schreb. root extract (RRE) inhibits cell growth of HT-29 and LoVo colorectal cancer (CRC) cell lines
- RRE increases expression of microRNA-140, which targets many genes, crucial for cancer cell metabolism
- Cell cycle is arrested and cell migration is decreased due to RRE implication
- RRE also induces apoptosis and increases pan-caspase activities in both CRC cell lines.

Abbreviation used: RRE: *Rubus Sanctus* Schreb. root extract; CRC: Colorectal cancer; GI_{50}, GI_{25}: 50% and 25% growth inhibition; miRNA: MicroRNA; MGMT: O-6-Methylguanine-DNA methyltransferase; UTR: Untranslated region; Akt: Protein kinase B; PI3K: Phosphoinositide 3-kinase; NF-κB: Nuclear factor kappaB; iNOS: Inducible nitric oxide synthase; COX-2: cyclooxygenase 2; TNFα: tumor necrosis factor alpha; IL-6: Interleukin 6; DMEM: Dulbecco's Modified Eagle's medium; RPMI-1640: Roswell Park Memorial Institute 1640 medium; HPLC: High-performance liquid chromatography; UV-Vis: Ultraviolet-visible; FBS: Fetal bovine serum; OD: Optical density; RFU: Relative fluorescence unit; PE: Phycoerythrin; 7-AAD: 7-Aminoactinomycin D; PBS: Phosphate-buffered saline; EGFR: Epidermal growth factor receptor; 5-FU: Fluorouracil; MSI: Microsatellite instability; NSCLC: Nonsmall cell lung cancer; TGF-β: Transforming growth factor beta; EMT: Epithelial–mesenchymal transition



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INTRODUCTION

Colorectal cancer (CRC) is one of the most well-studied and well-described cancers in terms of genetics and molecular pathways. During the last few decades, through dramatic advances in molecular techniques, tremendous amounts of data have been collected and used to diversify treatment options. Those studies have also revealed that CRC does not simply arising from one molecular pathway malfunction, but interactions between many pathways contribute to its heterogeneous structure.[1] Recently, the role of epigenetic mechanisms in CRC became a popular

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research area because the effects of DNA methylation on mismatch repair and O-6-methylguanine-DNA methyltransferase (MGMT) genes were clarified. Epigenetic changes can be described as alterations that can regulate gene expression without causing any change in DNA sequence. Some of the epigenetic mechanisms are DNA methylation, posttranslational histone modification, nucleosome positioning, and small noncoding RNA molecules, especially microRNAs (miRNAs).

miRNAs are 17–25 nucleotide long, noncoding, regulatory RNA molecules. They can be found in many organisms including mammals, plants, and even viruses. miRNAs bind the 3' or 5'untranslated region (UTR) or coding region of messenger RNAs, and in this way, they regulate gene expression by directly inhibiting translation or causing RNA degradation. The latest version of miRBase web database includes 35,828 mature miRNA products in 223 species.[2] Since the discovery of miRNA, their roles in disease have been studied intensively. In particular, the differential expression patterns in cancer studies have given rise to a tremendous number of studies. In the first study in 2002, Calin *et al*. demonstrated miRNA expression in chronic lymphatic leukemia.[3] Then, in 2003, Michael *et al*. found reduced miRNA expression in CRC for the first time.[4] miRNAs are thought to be responsible for nearly 60%–70% of all genes. Therefore, it was argued that by affecting cellular mechanisms such as proliferation, apoptosis, cellular differentiation, and invasion, which are crucial for carcinogenesis, miRNAs regulate oncogenes and tumor-suppressor genes in CRC.

Advanced screening and surgical methods have paved the way for increased survival of CRC patients. On the other hand, chemotherapeutic options are far behind those technologies, and thus, recently, plant extracts have become more important in drug discovery studies. Blackberries have been used for centuries for daily consumption and for their benefits in many diseases. They are rich in phenolic compounds and have direct contact with digestive tract organs, especially with colon mucosa, since they are poorly absorbed through small intestine and metabolized by colonic microbiota.[5]

Rubus sanctus Schreb. (blackberry) belongs to the *Rosaceae* family, and it is rich in vitamins, minerals, organic acids, and many phenolic compounds that are known to have beneficial effects for human health.[6] They are consumed fresh and in jam, and the leaves and roots have been used for tea in Anatolia.[7] For centuries in various societies, products and extracts of different species of *Rubus* have been used for the ulcer, sterility (human), diuretic, kidney stones, hemorrhoids, and many other treatments in folk medicine.[8-10] Because the *Rubus* species (black raspberry, Marion grape, blackberry, red raspberry, and strawberry) contains a high amount of anthocyanin and ellagic acid, extracts of these plants have been evaluated for their anticancer effects. Anthocyanin and ellagic acid have been shown to have important roles in the scavenging capabilities of free radicals, activating and regulating genes responsible for xenobiotic metabolism, altering cellular signals, and inducing apoptosis.[11,12] Ellagic acid, one of the primary phenolic compounds in *R. sanctus* Schreb. root extract (RRE), has been studied in the context of CRC for the past few years. Umesalma and Sudhandiran found that ellagic acid can inhibit tumor growth in a 1,2 dimethylhydrazine-induced rat colon cancer model by repressing the protein kinase B-phosphoinositide 3-kinase pathway.[13] In a similar *in vivo* study, it was shown that a nanocapsule formulation of ellagic acid also has inhibitory effects on inflammation-related genes, such as nuclear factor kappaB (NF-kB), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), tumor necrosis factor alpha (TNFα), and interleukin-6 (IL-6).[14] Although RRE consists of similar amounts of organic compounds as other parts of the plant, as far as we know, there has not been any study on CRC or other cancers. In this study, the potential anticancer effects of RRE on CRC cell lines were investigated by examining cell proliferation, invasion, apoptosis, and miRNA expression. Therefore, the aim of this study was to evaluate the anticancer potential of *R. sanctus* against CRC cell lines. The importance of this study is that root extract of *R. sanctus* plant has not been previously tested as an anticancer agent. Hence, the novelty of our research is that we have found not only anticancer effects of extract but also miRNA expression changes and the docking properties of ellagic acid against cancer-related proteins, which has not been studied in the past.

MATERIALS AND METHODS

Plant collection and extraction

R. sanctus Schreb. roots were collected from Uludag University Campus and identified by Prof. Dr. Hulusi Malyer, a botanist and plant systematics expert, Uludag University, Turkey. Residual soil was washed from the roots, and they were air-dried at room temperature in the dark for approximately for 1 month. After homogenization with a Warren blender, 100 g of pulverized root was extracted in ethanol with continuous shaking at room temperature. After extraction, the sample was evaporated and stored at −20°C. Before use, a stock solution of 250 mg/ml RRE was prepared in Dulbecco's Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI-1640) medium, filtered through a 0.2-μm syringe filter, and stored at −20°C.

High-performance liquid chromatography

An Agilent 1200 high-performance liquid chromatography (HPLC) system (Waldbronn, Germany) was used for analysis of the RRE extract. Chromatographic separation was performed using an XBridge C18 (4.6 mm × 250 mm, 3.5 μm) column from Waters (USA). The mobile phase consisted of 1% formic acid in water (solvent A) and acetonitrile (solvent B). Gradient conditions were as follows: 0–10 min 13% B, 10–20 min 41.5% B, 20–25 min 70% B, 25–35 min 10% B, and the total run time was 35 min. The column was equilibrated for 10 min before each analysis. The flow rate was 0.5 ml/min, and the injection volume was 10 ml. Data acquisition and preprocessing were performed with Chemstation for LC (Agilent). The monitoring wavelength was 280 nm for ellagic acid. Peaks were identified based on comparison of retention time and ultraviolet-visible spectra with standard of ellagic acid.

Cell culture

The colorectal adenocarcinoma cell lines HT-29 (ATCC® HTB-38™) and LoVo (ATCC® CCL-229™) and a normal epithelium HUVEC (ATCC® CRL-1730™) cell line were purchased from ATCC (Teddington, Middlesex, UK) and grown in DMEM and RPMI-1640 culture medium, respectively. Both mediums were supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. All cells were grown at 37°C in a humidified incubator with 5% CO_2. All cell culture reagents were purchased from Lonza (Basel, Switzerland).

Cell growth assays

For the WST-1 assay, the HT-29 cells, LoVo cells, and HUVECs were seeded in 96-well plates at a density of 10^4 cells/well and allowed to attach to the wells overnight at 37°C. The next day, the cells were treated with eight different dilutions of the RRE (100–0.05 mg/ml) for 24 h. At the end of the incubation, the medium was exchanged with 10 μl WST-1 reagent and 100 μl of fresh medium, and the cells were incubated at 37°C for an additional hour. The absorbance of each well was read at 480–620 nm in an ELISA reader (Tecan, Sweden). Medium with WST-1 reagent was used as a blank. Each assay was performed in triplicate, and the experiment was repeated at least three times. The growth inhibitory percentage (GI%) for each concentration of the extract was calculated as follows:

$$GI\% = \frac{\text{Mean absorbance of threated cells}}{\text{Mean absorbance of unthreated cells}} \times 100$$

The concentration of RRE that reduced cell growth by 50% and 25% (50% and 25% growth inhibition [GI^{50} and GI^{25}]) was calculated from the dose–response relationship by nonlinear regression analysis using GraphPad Prism software version 7.00 for Mac, GraphPad Software (La Jolla, CA, USA).

Cell invasion assay

Cell invasion was evaluated using a Chemicon QCM™ 24-Well Collagen-Based Cell Invasion Assay (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. In brief, 0.3 ml of serum-free medium was added into the interior of each insert to rehydrate the collagen layer for 30 min at room temperature, and then, the medium was replaced with 0.3 ml of prepared serum-free cell suspension containing 0.5×10^6 cells with the GI_{50} concentration of RRE and/or a negative control. Then, 0.5 ml of medium containing 10% FBS was added to the lower chamber. Cells were incubated for 24 h at 37°C. After 24 h, all of the noninvaded cells were removed from the interior of the insert, and the invaded cells were stained with CyQUANT® GR stain. The stained cells were dissolved in extraction buffer, and 200 µl cell solutions were transferred to a 96-well culture plate for colorimetric reading of optical density at 560 nm. The invasion assay was conducted in triplicate. The relative fluorescence unit value represents the invasive ability. Statistical evaluations were performed using Student's *t*-test and calculated with GraphPad Prism software version 7.00 for Mac (La Jolla, CA, USA).

Annexin V assay

The annexin V assay was performed according to the manufacturer's instructions. Briefly, 1×10^6 cells were seeded in a 6-well plate in triplicate and treated with three different concentrations of RRE for 24 h. In addition, 10 mM H_2O_2-treated cells were used as a positive control, and untreated cells were used as a negative control. Then, cells were analyzed with a Muse™ Cell Analyzer (Merck Millipore, Germany) according to the manufacturer's instructions using Muse™ Annexin-V and Dead Cell Reagent (Merck Millipore, Germany). Apoptosis and necrosis were analyzed with quadrant statistics on annexin V-phycoerythrin (PE)-positive cells and 7-amino actinomycin D (7-AAD)-positive cells, respectively.

Multi-caspase assay

The caspase assay was performed as instructed by the Muse Multi-Caspase Kit (Merck Millipore) protocol. 2×10^5 cells incubated with three different RRE concentrations, including negative and positive controls, were then harvested to quantitatively measure caspase activation. Cell samples in 1X caspase buffer with 50 µl of Muse Multi-Caspase reagent working solution were incubated at 37°C for 30 min at final concentration between 1×10^5 and 5×10^6 cells/ml. Then, 150 µl of working solution with 7-AAD which can detect dead cells was added to each triplicate sample, and the samples were analyzed with the Muse Cell Analyzer.

Cell cycle analysis

Cell cycle analysis was carried out with Muse™ Cell Cycle Kit (Merck Millipore) according to the manufacturer's instructions. To determine the effect of RRE on the cell cycle, cells were seeded in 6-well plates at a density of 1×10^5 cells/ml for 24 h. After incubation, cells were treated with three different RRE concentrations for additional 24 h. After treatment periods, cells were collected, washed, and fixed overnight in 70% ethanol in phosphate-buffered saline at −20°C. Fixed cells were pelleted and stained with cell cycle analysis reagent which includes the nuclear DNA intercalating stain propidium iodide and RNase A in a proprietary formulation and then incubated for 30 min at 37°C in the dark according to the manufacturer's instruction. Then, approximately 5000 events were analyzed on a Muse™ Cell Analyzer.

MicroRNA expression analysis

Previously calculated GI_{25} and GI_{50} concentrations of RRE for LoVo and HT-29 cell lines were applied for 24 h. After the RRE treatments, miRNA extraction was performed using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Extraction success was evaluated using a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) at 260 and 280 nm. A reverse transcription reaction was performed using a miScript II RT Kit (QIAGEN, Valencia, CA). Then, nine miRNAs were chosen for their antitumoral and invasive properties in CRC; U48 snRNA was used as an internal control. Expression changes in the selected miRNAs [Table 1] were measured with a miScript Primer Assay and miScript SYBR® Green PCR Kit RT-qPCR method (Qiagen, Valencia, CA, USA) performed on a LightCycler 480II system (Roche Diagnostics, USA). All samples were run in triplicate, and the manufacturer's instructions were followed throughout the experiments. U48 snRNA was used as an internal control for normalization, and fold-change expression was calculated by the $2^{-\Delta\Delta CT}$ method.

In silico analysis

Protein targets of ellagic acid, which is the main ingredient and the active reagent of RRE, were evaluated with SwissTargetPrediction servers.[15] Among the targeted proteins [Table 2], Braf, K-ras, Her-2, p53, and epidermal growth factor receptor (EGFR) were used for docking analysis. Since, they play direct roles in migration, cell proliferation, or apoptosis. Docking analysis was performed using SwissDock[16] and Chimera programs.[17]

RESULTS

Determination of ellagic acid in *Rubus sanctus* Schreb. root extract

Ellagic acid was determined in RRE by HPLC–diode array chromatography. 250 mg/ml RRE stock solution was used for analysis, and the amount of ellagic acid was calculated as 0.16 mg ellagic acid per g of plant.

Rubus sanctus Schreb. root extract inhibits cell growth in HT-29 and LoVo colorectal cancer cell lines

Eight different RRE concentrations ranging between 100 and 0.05 mg/ml were applied to a single-cell layer of LoVo and HT-29 CRC cell lines to determine the cell growth inhibition pattern. RRE inhibited cell growth in CRC cell lines in a concentration-dependent manner [Figure 1]. RRE was found to inhibit cell growth by up to 52.56% in LoVo and 77.89% in HT-29 cell lines [Table 3]. GI_{50} concentrations were calculated as 1.903 and 5.176 mg/ml for LoVo and HT-29 cells, respectively. From GI_{50} values, the LoVo cell line was found to be more sensitive than to RRE than the HT-29 cell line. HUVECs were also treated with the highest GI_{50} concentration of RRE (5.176 mg/ml), and only 7.33% growth inhibition was detected. Thus, the GI_{50} concentration was determined to be harmless for healthy cells (not shown in Figures).

Fifty percent growth inhibition concentrations of *Rubus sanctus* Schreb. root extract inhibit cell invasion

A CHEMICON cell invasion assay depends on Boyden chamber

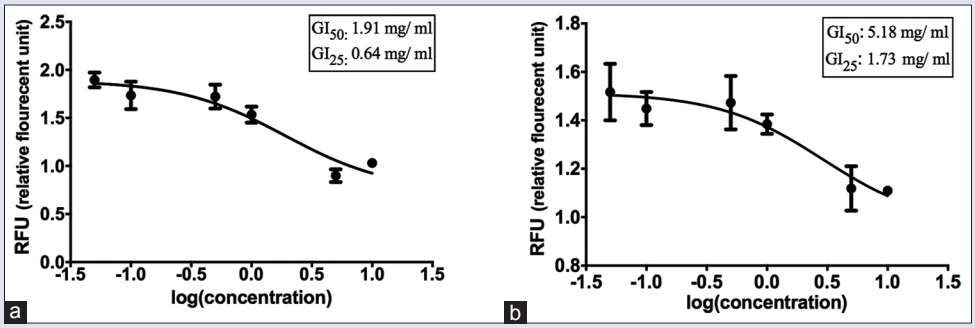

Figure 1: (a) LoVo and (b) HT-29 cell lines proliferation inhibitions. Cells were treated with 8 different concentrations (100–0.05 mg/ml) of *Rubus sanctus* Schreb. root extract. Twenty-five percent and 50% growth inhibitory were calculated from the dose–response relationship by nonlinear regression analysis

Table 1: MicroRNAs used for expression analysis

miRNA	Accession code	Mature sequence accession code	miRNA seguence
hsa-miR-19a	MI0000073	MIMAT0004490	14-aguuuugcauaguugcacuaca-35
hsa-miR-21	MI0000077	MIMAT0000076	8-uagcuuaucagacugauguuga-29
hsa-miR-140	MI0000456	MIMAT0000431	23-cagugguuuuacccuaugguag-44
hsa-miR-106a	MI0000113	MIMAT0000103	13-aaaagugcuuacagugcagguag-35
hsa-miR-124-1	MI0000443	MIMAT0004591	14-cguguucacagcggaccuugau-5
hsa-miR-181a-1	MI0000289	MIMAT0000256	24-aacauucaacgcugucggugagu-46
hsa-miR-182	MI0000272	MIMAT0000259	23-uuuggcaaugguagaacucacacu-46
hsa-miR-195	MI0000489	MIMAT0000461	15-uagcagcacagaaauauuggc-35
hsa-miR-451a	MI0001729	MIMAT0001631	17-aaaccguuaccauuacugaguu-38

miRNA: MicroRNA

Table 2: Protein targets of ellagic acid

ChEMBL number	Target protein	Abbreviation	Score
ChEMBL5619	DNA-(apurinic or apyrimidinic site) lyase	APEX1	1.000
ChEMBL4096	Cellular tumor antigen *p53*	p53	1.000
ChEMBL203	Epidermal growth factor receptor	EGFR	1.000
ChEMBL1293	G-protein coupled receptor 35	GPR35	0.999
ChEMBL2231	Cytochrome P450 1A1	CYP1A1	0.994
ChEMBL5990	Breast cancer type 1 susceptibility protein	BRCA1	0.985
ChEMBL1824	Receptor tyrosine-protein kinase erbB-2	ERBB2	0.975
ChEMBL4899	Mitogen-activated protein kinase 8	MAP3K8	0.852
ChEMBL4860	Apoptosis regulator Bcl-2	BCL	0.693
ChEMBL3142	DNA-dependent protein kinase	PRKDC	0.532
ChEMBL2916	Telomerase reverse transcriptase	TERT	0.516

principles. Treatment of LoVo and HT-29 cells with RRE for 24 h caused a reduction of 72.06 and 75.56%, respectively, in the cell motility [Figure 2]. The similar invasion inhibition percentages may be due to similar mechanisms since ellagic acid targets the EGFR protein.

Rubus sanctus Schreb. root extract induces apoptosis in both cell lines

A Muse™ Annexin V and Dead Cell Kit, which is a combination of annexin V-PE and 7-ADD assays, was used to evaluate how RRE inhibits cell growth in HT-29 and LoVo cell lines. The previously calculated GI_{25} and GI_{50} values and the maximum effective concentrations of RRE were applied to cells. The assay results can be grouped into four classes. First, the lower-left quadrant contains viable cells that are annexin V-PE (–) and 7-ADD (–), and second, the lower-right represents cells in the early stages of apoptosis that are annexin V-PE (+) and 7-ADD (–). Third, the upper-right quadrant contains cells that are positive for both markers and are in the late stages of apoptosis or dead by apoptotic mechanisms, and at last, the upper-left quadrant contains cells that have died via necrosis rather than through the apoptotic pathway and are only 7-ADD marker positive. The live cell quadrant showed similar results in both cell lines and was consistent with the previous WST-1 assay in a dose-dependent manner [Figure 3]. We combined the early and late apoptotic classes to assess the total apoptotic effect of RRE. The percentage of apoptotic cells increased with the RRE concentration. In the LoVo cell line, the GI_{50} and maximum growth inhibition (GI_{max}) concentrations of RRE induced apoptosis by 26.11% and 97.96%, respectively. Similarly, in the HT-29 cell line, RRE caused 27.53% apoptosis with the GI_{50} concentration and 83.23% with the GI_{max}. The apoptosis induction in LoVo cells was much higher than in HT-29 cells.

Pan-caspase activation increases with *Rubus sanctus* Schreb. root extract concentration

RRE-treated cells showed a significant percentage of apoptosis and cell growth inhibition. To determine the caspase activity of those apoptotic profiles, we used a Muse Multi-caspase assay. This assay counts and determines the percentage of cells with multiple caspases such as caspases 1, 3, 4, 5, 6, 7, 8, and 9. There was a significant dose-dependent increase in multiple caspase activities observed in treated cells, according to the increase in the concentration of RRE [Figure 4]. After 24 h of treatment with different concentrations of RRE, elevated pan-caspase activity in LoVo and HT-29 cells was observed to be up to 98.58% and 87.2%, respectively. In addition, live cell concentrations were found to be similar to those previously collected WST-1 and annexin V results.

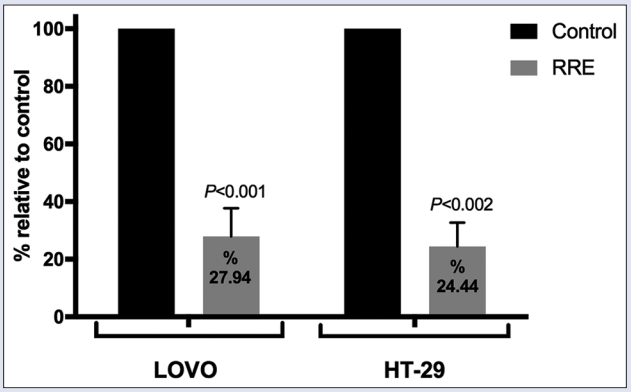

Figure 2: A significant decrease in cell migration was observed after *Rubus sanctus* Schreb. root extract treatment. Columns of the bar graphs show higher than 70% decrease in cell migration of both cell lines

Rubus sanctus Schreb. root extract causes DNA fragmentation and cell cycle arrest

RRE treatment led to cell cycle arrest at the G0/GI phase in HT-29 cells but not in LoVo cells. Apoptotic DNA fragments, which are known as sub-GI peaks, were elevated in both cell lines relative to control groups. The percentage of DNA fragmentation increased in HT-29 cells from 3.7% to 7.25%, but this increase not statistically significant. The sub-G1 cell population changed significantly in LoVo cells treated with the GI_{50} concentration of RRE. While it was 8.5% in control cells, a nearly 6.5-fold increase occurred after treatment with the GI_{50} concentration of RRE. This DNA fragmentation also caused overall decrease in percentage of cells in G0/GI, S, and G2/M phases [Figure 5].

The microRNA-140 expression significantly increases in both cell lines

GI_{25} and GI_{50} concentrations that were determined with the WST-1 analysis were applied to LoVo and HT-29 cell lines for 24 h. After miRNA extraction, the expression profiles of nine previously evaluated miRNAs were analyzed with a miScript assay [Table 4]. miR-140 regulates many cellular mechanisms and genes and is found to have elevated expression in both cell lines [Table 5]. This dose-dependent increase in LoVo and HT-29 cell lines was found to be up to 5.74- and 95.1-fold, respectively, ($P < 0.05$). miR-19a expression rose 63.41-fold only in the HT-29 cell line. The expression levels of the other miRNAs did not change significantly or the change with respect to the control group was not dose dependent.

In silico analysis showed interaction of ellagic acid and many cellular pathways

Protein targets of ellagic acid were determined by SwissTargetPrediction tools and confirmed with the ChEMBL database [Table 2]. Docking models were analyzed with the SwissDock and Chimera platforms.[15,16] p53, EGFR, HER-2, BCL-2, BRAF (wt and V600E mutant), and KRAS docking models were further evaluated because they had the highest ChEMBL scores and play important roles in apoptosis and invasion[17] [Figure 6]. Protein data bank codes, FullFitness, Estimated ΔG scores in kcal/mol, and further information are listed in Supplementary Table 1.

DISCUSSION

The effects of RRE on two colorectal adenocarcinoma cell lines were evaluated mainly in terms of apoptosis and miRNA expression. The

Table 3: The maximum concentrations and the maximum inhibitions with the WST-1 assay after *Rubus Sanctus* Schreb. root extract treatment

Cell line	RRE concentration (mg/ml)	Percentage inhibition
HT-29	10	77.98
LoVo	5	52.56
HUVEC	10	7.33

RRE: *Rubus sanctus* Schreb. root extract

Table 4: MicroRNA expression results in fold-change

miRNA	LoVo		HT-29	
	GI_{25}	GI_{50}	GI_{25}	GI_{50}
U48 snRNA	1	1	1	1
miR-124	−3.153	−3.371	1.062	2.066
miR-195	−5.697	−1.811	−2.822	25.75
miR-21	1.382	4.248	1.753	64.893
miR-106a	−1.968	−4.277	3.991	81.008
miR-19a	−4.037	−1.421	−1.807	63.411*
miR-182a	−1.519	1.481	−4.367*	−1.120
miR-181a	−1.457	2.154	−18.464	−1.251
miR-451	−3.131	3.450	−9.126	37.186
miR-140	2.351*	5.736*	−1.414	95.100*

*$P<0.05$. GI_{50}: 50% growth inhibitory; GI_{25}: 25% growth inhibitory; miRNA: MicroRNA

Table 5: Cellular pathways that regulated by miR-140 targeted genes

KEGG pathway name and accession code	*P*	Number of miR-140 targeted genes
Pathways in cancer (hsa05200)	0.048	29
Proteoglycans in cancer (hsa05205)	0.001	22
Hippo signaling pathway (hsa04390)	0.001	20
Regulation of actin cytoskeleton (hsa04810)	0.004	19
Cell cycle (hsa04110)	0.013	15
Signaling pathways regulating pluripotency of stem cells (hsa04550)	0.019	13
p53 signaling pathway (hsa04115)	0.002	11
Renal cell carcinoma (hsa05211)	0.002	9
Chronic myeloid leukemia (hsa05220)	0.048	9
Pancreatic cancer (hsa05212)	0.048	9
Central carbon metabolism in cancer (hsa05230)	0.009	8
TGF-beta signaling pathway (hsa04350)	0.016	8
Colorectal cancer (hsa05210)	0.028	8
ECM-receptor interaction (hsa04512)	0.001	7

TGF-β: Transforming growth factor beta; ECM: Extracellular matrix

HPLC analysis results showed that the amount of ellagic acid in RRE is 0.0406 mg/ml (0.16 mg/g per sample). This amount is higher than the *Rubus* fruit ellagic acid content (0.1 mg/g).[18] These data encouraged us to investigate the effect of the root instead of other parts of the plant. Although there have been many studies investigating the ethnopharmacy of *Rubus* species, nearly all of them have focused on *Rubus* fruits. As far as we know, there has not been any other study regarding RRE and its effects on CRC. There are three studies of the effects of RRE on breast cancer cell lines and microorganisms. In the first study, Riaz *et al.* showed that RRE has the highest antimicrobial activity compared to other parts of the plant.[19] The other study demonstrates that RRE inhibits DNA topoisomerase I and induces apoptosis in MCF-7 breast cancer cell lines.[20] Another study conducted on microorganisms concluded that biofilm produced by *Staphylococcus aureus* is inhibited by RRE, and this give rise to increased sensitivity against antibiotics.[21] In our study, the WST-1 results showed that RRE inhibits LoVo and HT-29

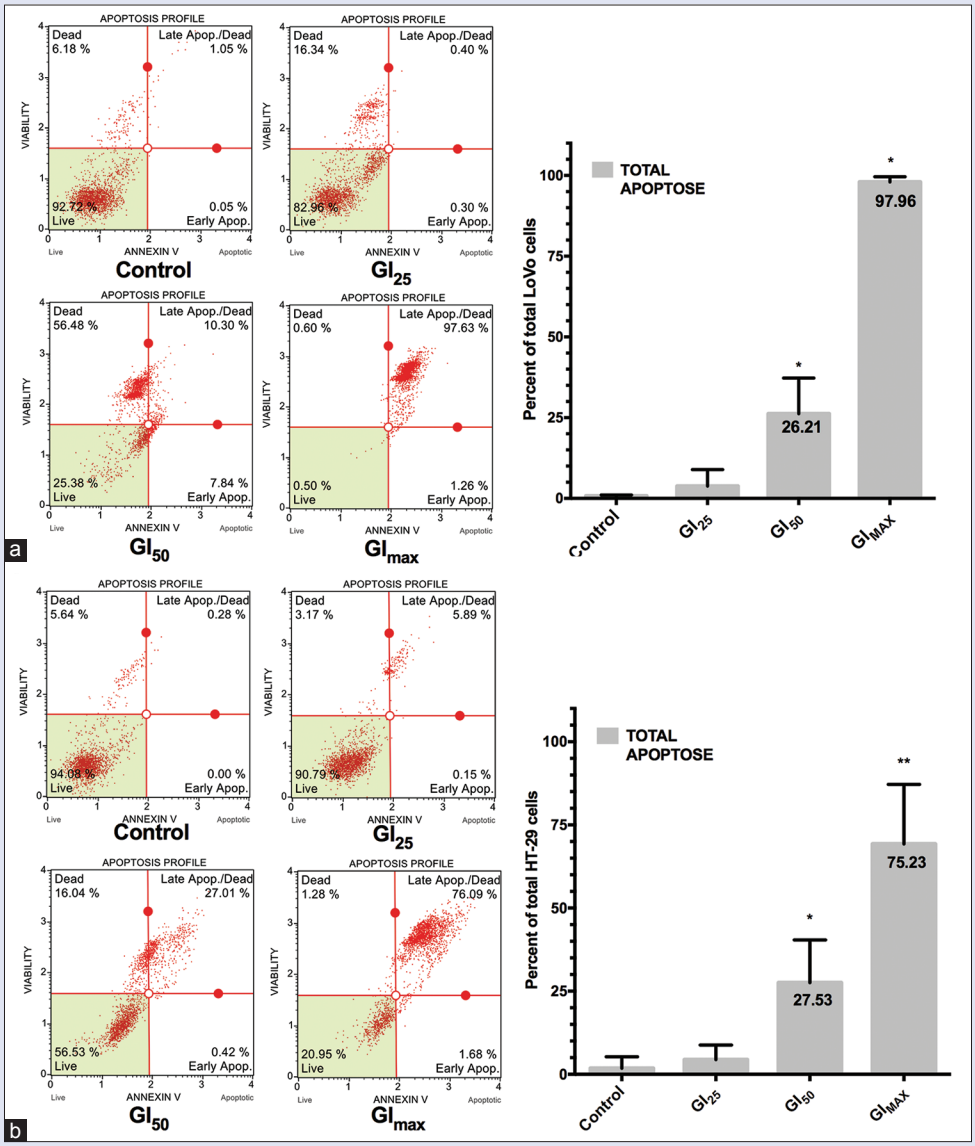

Figure 3: Assessment of apoptosis in (a) LoVo and (b) HT-29 cell lines treated with the *Rubus sanctus* Schreb. root extract for 24 h. The cells cultured either in DMEM or in RPMI media were used as control. The apoptotic cells were detected by annexin V-PI dual staining. Upper-left quadrant (annexin V–, PI+) represented dead cells; upper-right quadrant (annexin V+, PI+) represented late apoptotic cells; lower-right quadrant (annexin V+, PI–) represented early apoptotic cells; lower-left quadrant (annexin V–, PI–) represented live cells. The percentage of total apoptotic cells (right quadrants) was calculated and shown in the bar graphs. The extent of apoptosis was significantly high both in cell lines. *$P \leq 0.05$, **$P \leq 0.01$

cell proliferation up to 52.56% and 77.98%, respectively [Table 3]. No other studies have been published previously revealing the impact of RRE on the viability of LoVo and HT-29 cell lines. Inhibition of the cell proliferation of the HUVEC endothelial cell line, which was used as a healthy cell control group, remained limited, with only 7.33% inhibition even at the highest (10 mg/ml) concentrations of RRE. Lee *et al.* found similar results in 2000, on the MCF-7 breast cancer cell line. Although different cell lines were used for cell proliferation assays, they showed that *Rubus crataegifolius* root inhibits cell proliferation at concentrations between 0.2 and 1.0 mg/ml in a dose-dependent manner.[20] *Rubus fairholmianus*, a species closely related to *R. sanctus*, was studied recently on melanoma and lung cancer cell lines. Root extract of the plant inhibited cell proliferation through the caspase-dependent apoptotic pathway.[22] The same group of researchers found similar cell growth inhibition (up to 59%) in a human CRC (Caco-2) cell line.[23] Other *Rubus* species have also been found to be effective in terms of antitumor activities. The extracts of *Rubus jamaicensis*, *Rubus rosifolius*, *Rubus racemosus*, *Rubus acuminatus*, and *Rubus idaeus* inhibited cancer cell growth, including colon, breast, lung, and gastric human tumor cells.[24]

Metastasis, the spread of cancer to other tissues, is important, especially in late-stage CRC. The effect of RRE on cancer cell motility has not been studied yet. RRE at GI_{50} concentrations significantly reduced the invasiveness of LoVo and HT-29 cells by 72.06% and 75.56%, respectively [Figure 2]. In a similar study, "colon-available" raspberry (a close relative of *R. sanctus*) extract was found to inhibit the invasive ability of HT115 colon cancer cells.[25]

Apoptosis is a tightly controlled and an important mechanism of programmed cell death. The annexin V assay was used to evaluate both cell lines to ascertain whether the observed reduction in viable cell number was due to induction of apoptosis. Different *Rubus* sp. extracts

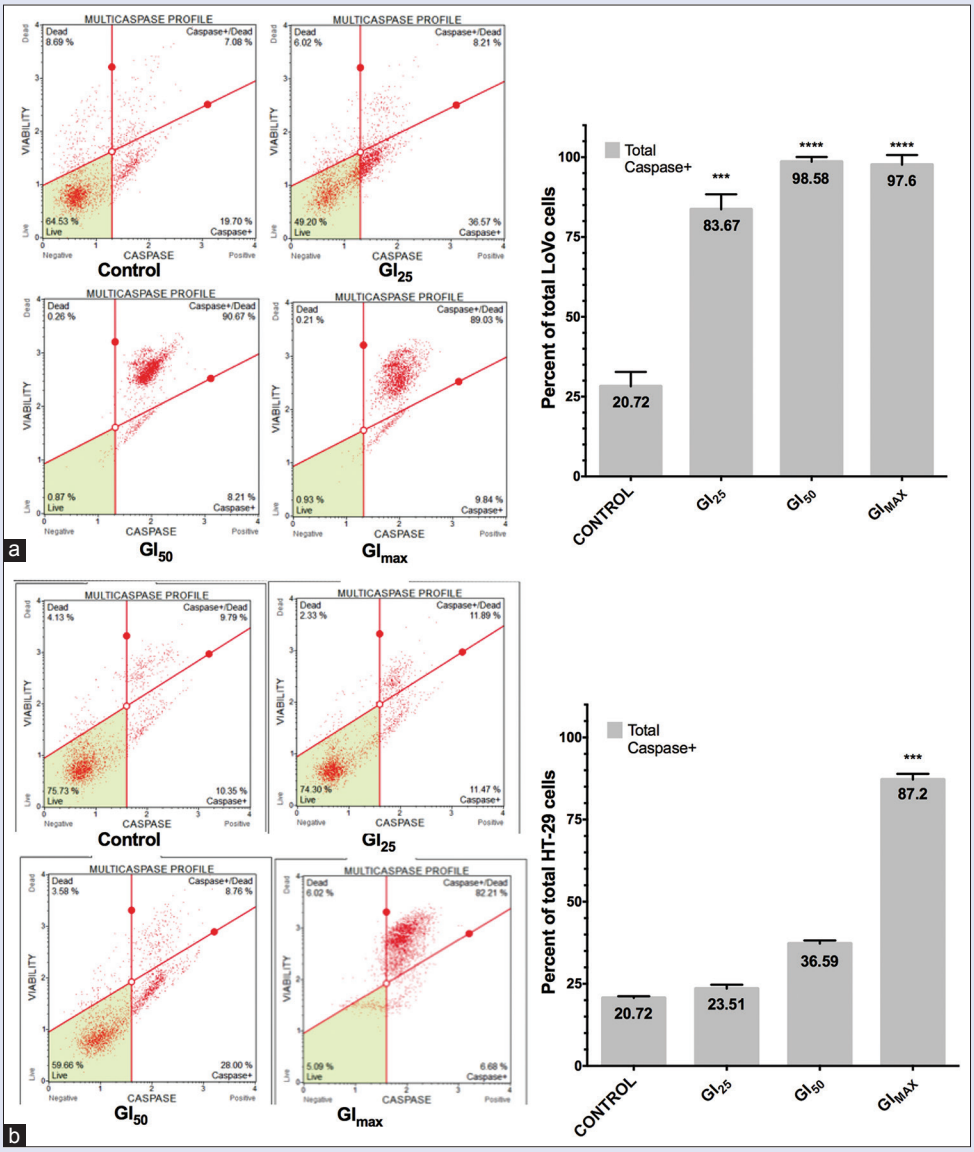

Figure 4: *Rubus sanctus* Schreb. root extract mediated apoptosis of (a) LoVo and (b) HT-29 cell lines. After 24-h treatment with 25%, 50%, and maximum growth inhibition concentrations of *Rubus sanctus* Schreb. root extract, apoptosis was assessed using the Muse™ Cell Analyzer and the Muse™ Multi-caspase Kit. Hydrogen peroxide (10 mM, 3 h) were used as positive control. Representative multi-caspase profiles (dot plots) are presented. The percentage of total caspase-induced cells (right quadrants) was calculated and shown in the bar graphs. ****$P \leq 0.0001$, ***$P \leq 0.0013$

have been shown to have apoptotic effects in human cancer cells. In HT-29 and HCT-116 cells, blackberry extracts inhibited cell proliferation and induced apoptosis at different concentrations.[26] In another study, Wang *et al.* found that black raspberry-derived anthocyanins suppressed cell proliferation and increased apoptosis in HCT-116, Caco-2, and SW480 cells through regulation of β-*catenin* and *c-Myc* genes.[27] In our study, RRE induced apoptosis in both cell lines with varying degrees of potency. As reported earlier, genetic differences, for instance p53 status, influences the apoptosis potential of cell lines.[28] Two CRC cell lines with microsatellite instability (MSI) CO115 (p53-wt) and HCT15 (p53-mutated) were evaluated for their tolerance for fluorouracil. The results showed that wild-type p53 CO115 cells are more sensitive than p53-mutated HCT15 cells.[29] Similarly, in our annexin V experiment, wild-type p53 LoVo cells demonstrated an enhanced apoptotic activity compared to mutant-p53 HT-29 cells [Figure 3]. Another difference between our cell lines is the MSI status. While LoVo cells are highly instable (MSI highly instable [MSI-H]), HT-29 cells are stable (MSI stable [MSS]). Michael-Robinson *et al.* stated that MSI-H CRC samples have a higher apoptotic index, and consistent with this, LoVo cells, which exhibit an MSI-H status, had a greater number of total apoptotic and caspase+ cells than HT-29 cells.[30] To determine whether or not RRE induces caspase activation, we used a multi-caspase activity assay to detect cleaved fragments of caspases. In 2005, Kim *et al.* found that aqueous extract of Korean black raspberry increased the activity of caspase-3,-7, and -9, in the HT-29 cell line.[31] In the current study, RRE induced multiple caspases in both cell lines. Especially, the total caspase+ cells were 98.58% in the LoVo cells treated with the GI_{50} concentration of RRE [Figure 4]. Caspases cleave many important cellular proteins during apoptosis and give rise to cell shrinkage, chromatin condensation, membrane blebbing, and DNA fragmentation.[32]

Cell division in normal cells is regulated at the cell cycle checkpoints G1/S and G2/M. In colon cancer, regulatory mechanisms at these checkpoints are defective, and cells undergo uncontrolled growth and

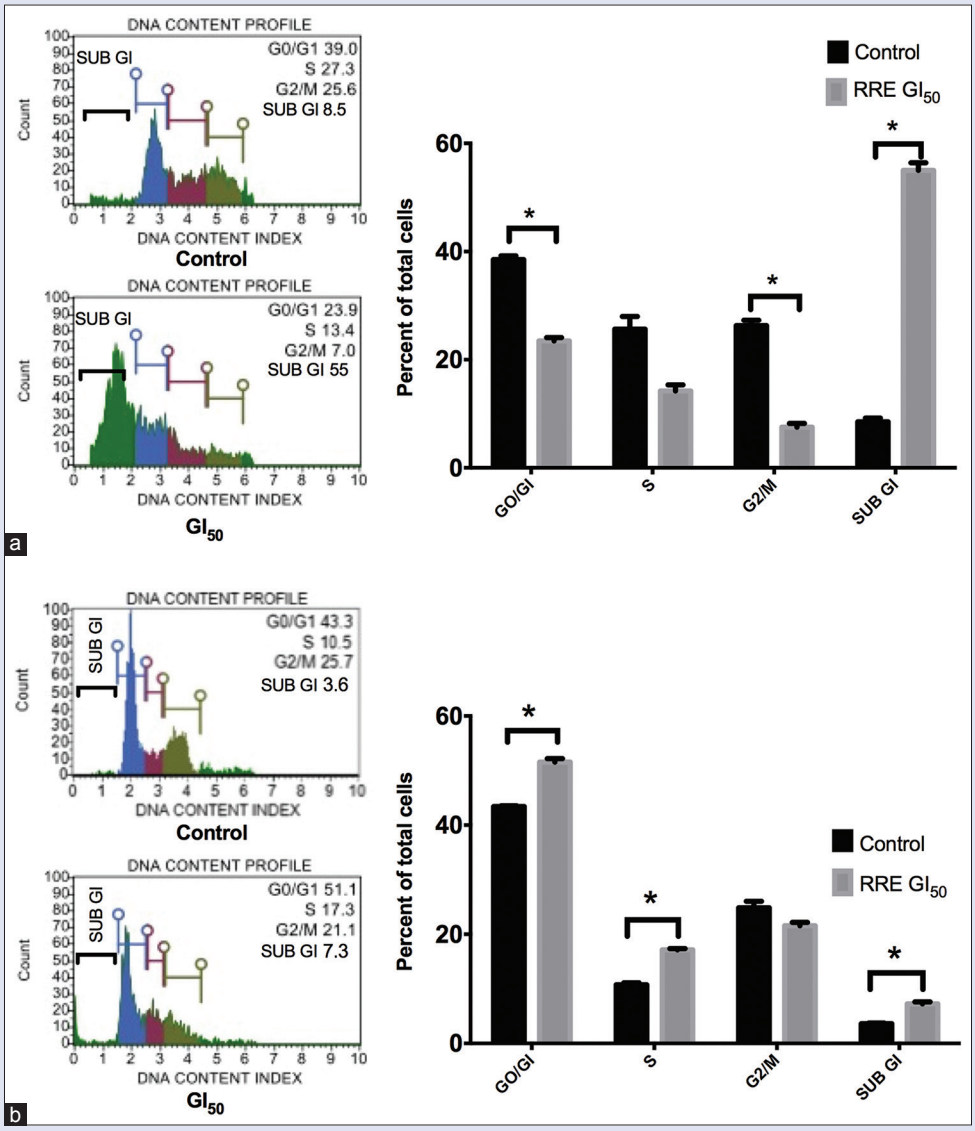

Figure 5: Analysis of cell cycle arrest in *Rubus sanctus* Schreb. root extract treated (a) LoVo and (b) HT-29 cell lines. Cells were treated with 50% growth inhibition concentration 1.903 mg/ml for LoVo and 5.176 mg/ml for HT-29. A significant sub-G1 enrichment was observed in LoVo-treated cell lines $^*P \leq 0.05$

division. Therefore, we investigated the effect of RRE on cell cycle distribution and sub-G1 phase. Significant G0/G1 arrest was found only in HT-29 cells. Besides that, increase in the proportion of sub-G1 cells occurred in both cell lines, but significant increase only found in LoVo cells [Figure 5]. This increase in DNA fragmentation was considered the direct proof of caspase-driven apoptosis due to RRE administration. Similar cell cycle arrest results were reported by Boivin *et al.* in 2007. They found that, different berry juices induce downregulation of cdk4, cdk6, cyclin D1, and cyclin D3 expression in PC-3 prostate cancer cells, suggesting cell-cycle arrest in G1 phase of the cell cycle.[33]

Although it is known that plant extracts may influence miRNA expression, to date, there have not been any studies of the effects of *Rubus* species on cancer cells. Hence, many plant extracts investigated have been shown to alter miRNA expression in different cancer cell lines.[34,35] RRE significantly increased miR-19a expression by 63.41-fold in HT-29 with respect to the control group [Table 4]. Higher miR-19a expression is often found in CRC. Compared to healthy cells and tissues, miR-19a expression has been shown to be higher in CRC cells and tissue samples.[36,37] Similarly, it has been found that miR-17-92 family miRNAs, including miR-19a, are expressed at higher levels in 19 different lung cancer cell lines.[38] Therefore, the elevated expression of miR-19a can be considered a natural increase of this miRNA in cancer cells. On the other hand, miR-140 expression is increased in both cell lines, with the increasing concentration of RRE [Table 4]. miR-140 targets 537 human genes.[39] Among those genes, 85 are experimentally validated targets, and the rest of them are predicted targets.[40] Many of these genes are have important roles in cellular pathways [Table 5]. Our results indicate that especially apoptosis-related pathways are affected by RRE treatment. Twenty-nine genes that are targeted by miR-140 are found in cancer-related pathways; 15 are found in cell cycle pathways; and 11 are found in p53 signaling pathways.[41] In the literature, it has been reported that miR-140 directly targets *Smad2*. Overexpression of miR-140 in CRC cell lines, HCT116, RKO, and SW480, decreases *Smad2* expression levels, leading to decreased cell invasion and proliferation and increased cell cycle arrest.[42] Another recent study argues that miR-140 inhibits migratory and invasive capacities of CRC cell lines (HCT116 and RKO), at least part by downregulating the expression of ADAMTS5 and IGFBP5. Other than CRC, in a high-throughput

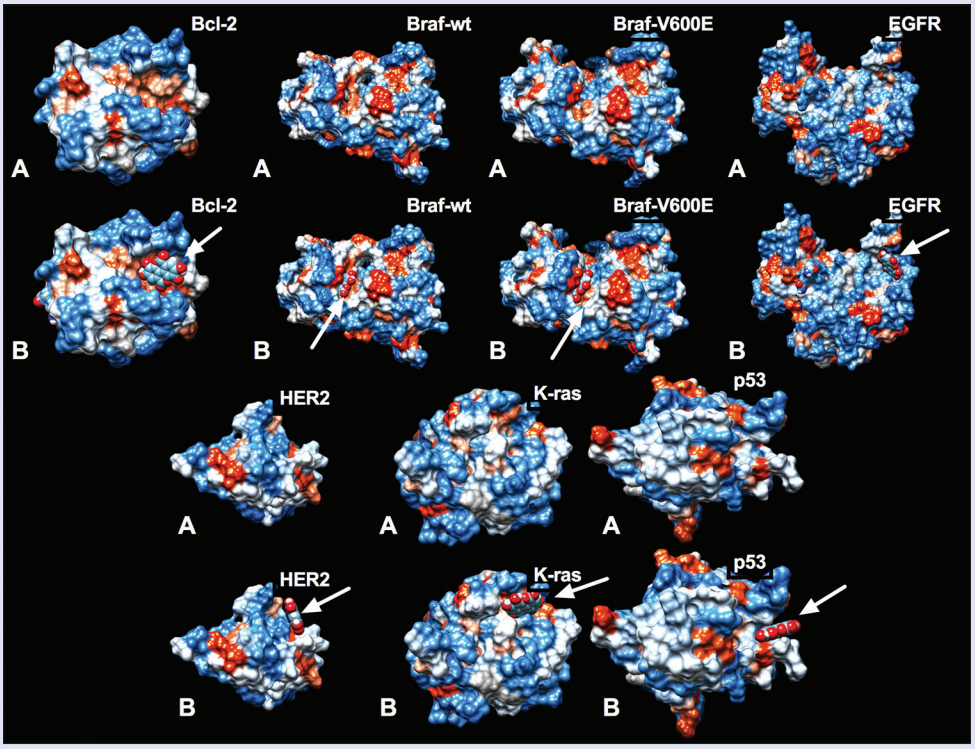

Figure 6: Docking model of ellagic acid with epidermal growth factor receptor, Braf V600E mutated, Bcl-2, K-ras, HER2, and p53 proteins. Protein models show (A) before and (B) after docking. Ellagic acid is indicated by arrow

miRNA expression analysis study, Song *et al*. found that overexpression of miR-140 inhibited cell proliferation in both osteosarcoma U-2 OS (wt-p53) and colon cancer HCT 116 (wt-p53) cell lines.[43] miR-140 has been demonstrated to be effective in tumorigenesis in other cancer types. For instance, in nonsmall cell lung cancer (NSCLC), Yuan *et al*. found that miR-140 is significantly downregulated in tissues and cell lines, and both gain-of-function and loss-of-function studies showed that miR-140 suppresses NSCLC cell proliferation, migration, and invasion *in vitro*.[44] miR-140 downregulation was also found in hepatocellular carcinoma tissue samples with poor prognosis. In this study, the researchers found that miR-140 suppresses cell proliferation and metastasis by inhibiting transforming growth factor beta (TGF-β) and MAPK/ERK signaling.[45] On the other hand, downregulation of miR-140 induces epithelial–mesenchymal transition and promotes invasion by targeting the *Slug* gene in esophageal cancer.[46] Although we found an inhibitory effect of miR-140 in CRC cell lines, previous studies on different cancer cells are compatible with our results. Therefore, we can conclude that RRE causes a significant increase in miR-140 expression, which in turn suppresses migration and cell proliferation.

In summary, we study provides experimental evidence that RRE suppresses CRC proliferation and metastasis through apoptosis, a key player in cancer progression. We found a novel mechanism for RRE as a critical regulator of apoptosis in a colorectal cell line. As a result of this activity, increased expression of miR-140 in CRC cell lines disrupts the survival of CRC cells.

CONCLUSION

The study showed that RRE induces apoptosis in CRC through the increased expression of miR-140. Cell cycle and migration patterns of CRC cell lines are also disrupted by RRE. As the study of plants extract-based therapeutics is a current trend in the literature, our results suggest that RRE may offer a novel therapeutic strategy for treating CRC patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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