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Anti-Proliferative Activity of Butenyl Isothiocyanate Against Colon Cancer Cell Line

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Abstract

Background Colorectal cancer (CRC) is the third most common malignancy in both men and women. Rectal cancer and colon cancer are frequently considered to be the same condition due to their shared characteristics. Polyps in the colon are the first sign of the disease and can develop into a malignant tumor. Patients with colon cancer often die of tumor recurrence and metastasis. Butyl isothiocyanate belongs to the isothiocyanate family, which has more attention since they have a lot of biological activity. Additionally, acetylsalicylic acid the non-steroidal anti-inflammatory drug significantly lowers the antiproliferative activity of cancer.

Objective To identify the anti-proliferative activity of butenyl isothiocyanate, acetylsalicylic acid and their combination against colon cancer cell line. In addition to identify the molecular mechanism evaluation of butenyl isothiocyanate by measuring vascular endothelial growth factor (VEGF) gene expression.

Methods Butenyl isothiocyanate and acetylsalicylic acid antiproliferative activity against the CL40 cell line was determined using the (4,5-Dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) test. Butenyl isothiocyanate gene expression levels were studied quantitatively using real-time polymerase chain reaction.

Results Butenyl isothiocyanate, acetylsalicylic acid, and their combination demonstrated dose-dependent significant suppression of proliferation of the colon cancer cell line CL40 ($P < 0.001$). Furthermore, butenyl isothiocyanate showed a fold diminishing in VEGF gene expression of 35.83 % ($P < 0.001$).

Conclusion Butenyl isothiocyanate, acetylsalicylic acid and their combination exhibited a significant antiproliferative effect. Furthermore, butenyl isothiocyanate's molecular mechanism of action was demonstrated to be the down-regulation of VEGF gene expression.

Keywords VEGF, antiproliferation, Butenyl isothiocyanate, Acetylsalicylic acid, MTT, CL-40, colon cancer, gene expression

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List of abbreviations: ANOVA = Analysis of variance, ASA = Acetylsalicylic acid, CL40 = Colon cancer cell line, ELISA = Enzyme-linked immunosorbent assay, FHC = Fetal human cell line, GADPH = Glyceraldehyde 3-phosphate dehydrogenase, IC50 = Half maximal inhibitory concentration, ITC = Isothiocyanate, MTT = (4,5-Dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide, VEGF = Vascular endothelial growth factor

Introduction

Cancer refers to a variety of diseases characterized by the out-of-control growth of cells. If allowed to continue and spread, this proliferation will be fatal. Metastasis, the spread of tumors to new locations, is responsible for about 90% of cancer-related deaths [(1)]. Colon cancer is a common malignant tumor in the digestive

system. In recent years, the incidence rate of colon cancer has gradually increased and the 5-year survival rate is still not high. Patients with colon cancer often die of tumor recurrence and metastasis [2]. Colon cancer, also known as colorectal cancer (CRC), is a deadly disease that is all too common in developed countries. (CRC) is the third most common malignancy in both men and women in the United States. Rectal cancer and colon cancer are frequently considered to be the same condition due to their shared characteristics. Polyps in the colon are the first sign of the disease and can develop into malignant tumors. A colonoscopy is the gold standard for screening for colon cancer and the diagnosis of polyps in the colon [3].

Butenyl isothiocyanate (ITC) is one member of isothiocyanates, which is compounds of the type R-N=C=S, where R is an alkyl or aryl group. Hydrolysis of glucosinolates results in several different byproducts, including ITCs. In the form of the glucosinolate gluconapin, butenyl-ITC is found mostly in the Brassica rapa plant family, which includes pak choi, Chinese cabbage, turnips, turnip greens, and turnip tops, among other foods. Glucosinolates are naturally occurring chemicals that contain sulfur and nitrogen and are produced from glucose and amino acids. When intact, this chemical molecule has little biological activity, but the myrosinase enzyme breaks it down into hydrolytic compounds isothiocyanates that have a lot of biological activity [4,5].

Acetylsalicylic acid (ASA) is a traditional medication that has been used for more than one century to treat and prevent cardiovascular and cerebrovascular illnesses. It was first derived from willow bark [6]. Acetylsalicylic acid inhibits vascular endothelial growth factor (VEGF)-stimulated endothelial cell migration [7], cancer cells [8], and adenosine diphosphate (ADP) [9]. However, the process by which these impacts occur is complex. The host microenvironment contains a wide variety of endogenous angiogenesis stimulators and inhibitors. Some examples include interleukin-8 (IL-8), fibroblast growth factor-1 (acidic FGF, FGF1), fibroblast growth factor-2 (basic FGF, FGF2), VEGF, vascular permeability factor (VPF), and platelet-derived growth factor (PDGF) [10]. By activating these inhibitors and inhibiting these stimulants, ASA may be able to reduce angiogenesis and slow tumor growth.

This study aimed to identify the anti-proliferative activity of butenyl ITC, ASA and their combination against colon cancer cell line. In addition to identify the molecular mechanism evaluation of ITC by measuring VEGF gene expression.

Methods

(4,5-Dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) protocol

Utilizing 96-well plates, the MTT cell viability experiment was carried out. $1*10^4$ cells per well were used to seed the cell lines. After a gentle shake, the microplate was covered with sterile parafilm and placed in an incubator at 37°C and 5% carbon dioxide for 24 hr. After 24 hr, the media was removed and two-fold serial dilutions of butenyl-ITC and ASA (6.25, 12.5, 25, 50, 100, 200 µg/ml) were added to the wells, allowing the cells to produce a confluent monolayer. Each concentration was employed in triplicate, and a control (cells fed with serum-free media) was included for comparison. The plate was exposed to 5% CO_2 at 37°C for a set period of time (24 hr). Upon removing the media, 10 µl of MTT solution, which had been made by adding 5 mg/ml of phosphate-buffered saline (PBS) to each well, was then added. After 4 hours in an incubator at 37°C and 5% CO_2, the plates were taken out and the top layer has been discarded. The residual crystals in the wells were solubilized after the MTT solution was removed by adding 50 µl of DMSO (Dimethyl Sulphoxide), It was then shaken for 30 minutes while incubating at 37°C.

Absorbance was determined by reading the samples in an enzyme-linked immunosorbent assay (ELISA) reader set to 575 nm and comparing them to a reference sample read at 630 nm. The optical density data were

statistically analyzed to calculate the Half maximal inhibitory concentration (IC50), or the concentration of substance needed to reduce cell viability by 50% for each cell line. The following equation was used to calculate the percentage of cytotoxicity, which is the rate at which cell growth was inhibited. The equation for linear and logarithmic correlation was used to determine IC50 values.

1-(A0-A1)/(A2-A1)
A0 = Absorbance of sample
A1 = Absorbance of blank
A2 = Absorbance of control

Cell viability % = Mean sample absorbance/ mean control absorbance × 100%.

Inhibition % = 1-cell viability %.

The equation for linear and logarithmic correlation was used to determine IC50 values. A 100x magnification microscope field was used to study the cell under an inverted microscope [11,12].

Determination of Cl40 gene expression in colon cancer cell line

Quantitative real-time polymerase chain reaction (RT-qPCR) protocol

This is the key step in the research, which is divided up into two phases; the first phase involves creating cDNA from RNA using a specific primer for the VEGF and housekeeping gene GADPH transcripts, as shown in table (1) and a proto-script cDNA synthesis kit. Geneious Prime software was used to create a new primer, and an oligo analyzer programme was used to verify its specs. The steps of the method have been completed.

Table 1. Primer sequences used in this study

Genes	Forward primer	Revers primer
VEGF	CCCACTGAGGAGTCCAACAT	CATACCTCCCCTGTGCAACT
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

1. An aliquot of 18 µl was added from each sample of isolated total RNA into the fresh PCR tube.
2. Ten µl of the ProtoScript reaction mix were added, which contained deoxynucleotide (dNTPs), buffer, and other necessary ingredients, to each sample.
3. MuLV Enzyme was then introduced as 2 µl of each sample to the reaction.
4. An aliquot of 2 µl hexamer was added.
5. Using the thermocycler, this combination was incubated for an hour as shown in table (2).

Table 2. Thermal cycler steps of conditions cDNA Reverse Transcription

Condition	Step 1	Step 2	Step 3	Step 4
Temperature	15°C	50°C	60°C	4°C
Time	20 sec	4 min	20 sec	-----

It was carried out 12 times, and then the enzyme was inactivated at 95°C (Relative quantitative PCR). The second step of this technique was selecting cDNA samples with controls at the same run that contained 200 g/ml of butenyl ITC. For VEGF and the housekeeping gene, there were two PCR tubes per sample, one for each gene. The fluorescent power of SyberGreen was used for quantity detection. The components in the reaction mix were listed in the table (3) with their corresponding quantities below.

After quickly spinning PCR tubes for one minute at 2000 rpm to eliminate bubbles and collect liquid, the programme for Real-Time PCR was set up using the specified thermocycling technique, as given in table (4).

Table 3. Components of quantitative real-time PCR used in genes expression experiment

Component	Volume per 20 µl Reaction
qPCR Master Mix, (SYBR)	10 µl
Forward primer	1 µl
Reverse primer	1 µl
cDNA Template	5 µl
Nuclease-Free Water	to 20 µl

Table 4. RT-PCR cycling program

Loop's steps	Temperature	Time	Number of cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	20 sec	
Annealing	55°C	20 sec	40
Extension	72°C	20 sec	
Melt Curve	55-95°C		1

Delta delta Ct method

The delta delta Ct (ΔΔCt) method is the quickest and most straight forward approach because it involves only a comparison of the Ct values of the target gene and the reference gene. The ΔCt between the target gene and the reference gene was calculated as declared in the following formula: ΔCt = Ct target gene - Ct reference gene Thus, the difference between the ΔCt of the unknown and the ΔCt of the calibrator is determined, giving the ΔΔCt value,

ΔΔCt = (Ct target - Ct reference) sample - (Ct target- Ct reference) Calibrator Calibrator as shown in table (5).

The value 2−ΔΔCt is thus the normalised target quantity in the sample, to evaluate variations in gene expression between samples [13].

By utilising the (2−ΔΔCt) comparative threshold (CT) method, we were able to determine the relative differences in mRNA expression levels.

Table 5. Calculations for the Delta Delta Ct method

Control untreated cells		Treated cells		Control untreated cells - Treated cells	mRNA increase or decrease
HKG Ct	GOI Ct	HKG Ct	GOI Ct	ΔCt control- ΔCt treated	2^-(ΔΔCt)

HKG: Housekeeping gene GOI: Gene of interest Ct: Cycle threshold

Statistical analysis

The statistical design for this study was presented as mean±SD (standard deviation). Group values were compared by one-way analysis of variance (ANOVA) and then by the Tukey Post-hoc test (t-test) and considered significant at P <0.0001 by using the GraphPad Prism software, version 8 (Graph Pad Software Inc., La Jolla, Co). The combination index (CI) value was calculated by using the Chou–Talalay method, to provide information on the nature of drug interaction between butenyl ICT and ASA in the MTT assay.

Results

Assessment of cancer cell and normal cell lines proliferation

The result in the figure (1) showed that dose-dependent viability of the cancer cell line growth and the normal cell line after exposure to butenyl-ITC by using six serial dilutions as following 200, 100, 50, 25, 12.5, 6.25 µg/ml and their respective viability percentage± SD for CL40 cell line was 52.55±2.01%, 64.74±2.95%, 75.72±4.88%, 91.82±0.93%, 95.22±0.77% and 96.14±0.59%, while their respective viability percentage± SD for FHC cell line was 69.56±4.56%, 75.95±2.45%, 83.36 ±7.35%, 87.35±2.38%, 95.52±0.96%, and 95.99±0.68%.

Whereas butenyl-ITC exhibited significant antiproliferative activity (P <0.0001) against the CL40 cells line in a dose-dependent pattern with concentrations of 25, 50, 100, and 200 µg/ml, related to the FHC cell line, only butenyl-ITC treatments at concentrations of 6.25 µg/ml and 12.5 µg/ml showed no significant differences in CL40 cell line inhibition rate.

As indicated in table (6), the IC50 values for butenyl-ITC for the CL40 cell line were 64.13 g/ml, while the IC50 values for the FHC cell line were 70.07 g/ml. These values were derived from the logarithmic equation. Where Y equals the viability percentage and X equals the Log concentration.

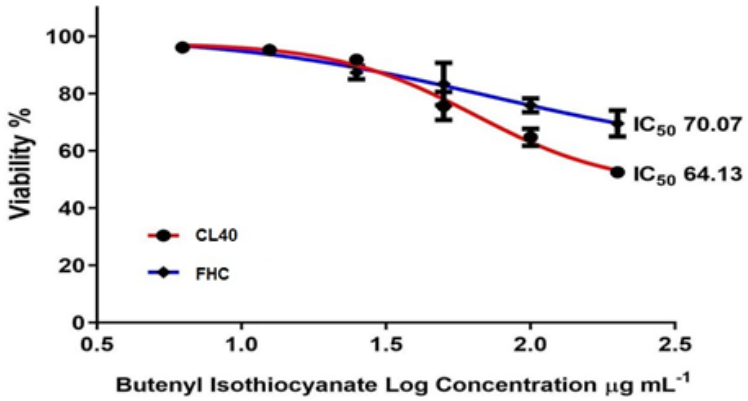

Figure 1. Cell survival curve (mean± SD%) of CL40 and FHC cell lines after treatment with Butenyl-ITC using MTT in vitro assay

Table 6. The IC50 value for each Butenyl-ITC, ASA and combination of Butenyl-ITC and ASA in colon cancer (CL40) cell line and normal (FHC) cell line

Type of sample	IC 50 in CL40 cell line	IC50 in FHC cell line
Butenyl-ITC	64.13	70.07
ASA	94.95	193
Combination of Butenyl-ITC and ASA	55.37	203.9

Growth of both the cancer cell line and the normal cell line were shown to be dose-dependent, as depicted in figure (2) after exposure to ASA by using six serial dilutions as following 200, 100, 50, 25, 12.5, 6,25 µg/ml and their respective viability percentage±SD for CL40 cell line was 61.11±6.49%, 74.54±1.16%, 94.83±0.52%, 94.83±2.05%, 94.91±0.69%, and 95.33±0.27%, while their respective viability percentage±SD for FHC cell line was 78.13+4.07%, 88.27+2.61%, 93.60+2.10%, 94.17+1.57%, 95.22+0.82%, and 95.56+1.38%. Whereas ASA exhibited significant antiproliferative activity (P <0.0001) against the CL40 cells line in a dose-dependent pattern with concentrations of 100, and 200 µg/ml, related to the FHC cell line, only ASA treatments at concentrations of 6.25, 12.5, 25 and 50 µg/ml showed no significant differences in CL40 cell line inhibition rate.

Inhibition concentrations (IC50) for ASA were calculated using a logarithmic equation, and the results were 94.95 g/ml for the CL40 cell line and 193.0 g/ml for the FHC cell line table (6). Where Y equals the viability percentage and X equals the Log concentration.

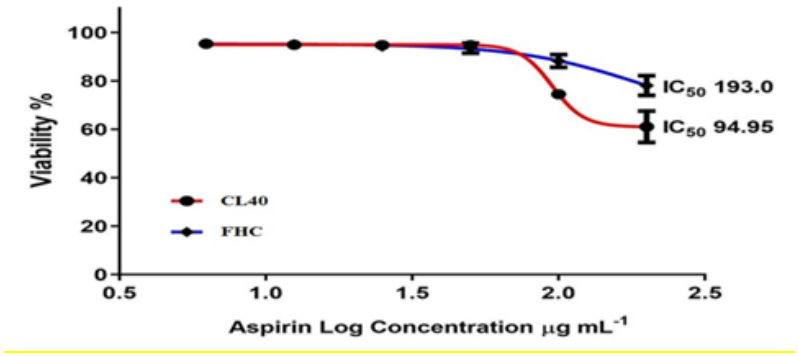

Figure 2. Cell survival curve (mean± SD%) of CL40 and FHC cell lines after treatment with Acetyl salicylic acid using MTT in vitro assay

The result in the figure (3) showed that dose-dependent viability of the cancer cell line growth and the normal cell line after exposure to a combination of butenyl-ITC and ASA by using six serial dilutions as follows 200, 100, 50, 25, 12.5, 6.25 µg/ml and their respective viability percentage±SD for CL40 cell line was 37.81±4.04%, 48.88±5.17%, 68.60±4.62%, 89.08±4.29%, 95.72±0.81%, and 95.18±1.28%, while their respective viability percentage± SD for FHC cell line was 76.50±4.85%, 87.92±3.58%, 92.21±2.78%, 96.49±1.29%, 96.95±1.14% and 94.29±2.98%.

Whereas the combination of butenyl-ITC and ASA exhibited significant (P <0.0001) antiproliferative activity against the CL40 cell line in a dose-dependent pattern with concentrations of 25, 50, 100, and 200 µg/ml, by comparing with the FHC cell line, only the combination of butenyl-ITC and ASA treatments at concentrations of 6.25 µg/ mL and 12.5 µg/ml showed no significant differences in CL40 cell line inhibition rate.

Logarithmic equation analysis revealed that the IC50 values for the butenyl-ITC and ASA combination were 55. 37 g/ml for the CL40 cell line and 203.9 g/ml for the FHC cell line, respectively table (6). Where Y equals the viability percentage and X equal the Log concentration.

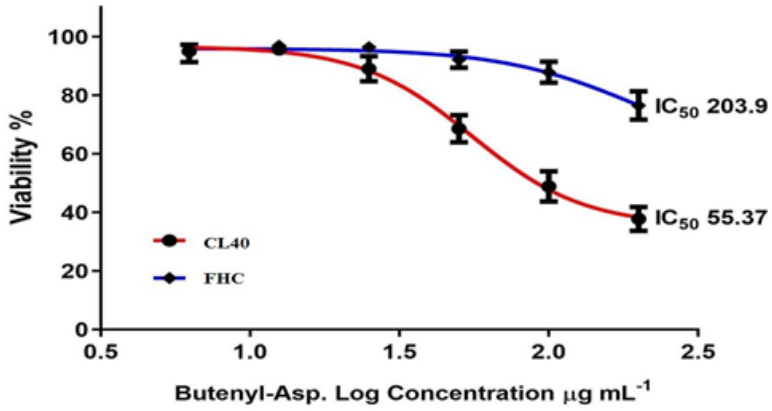

Figure 3. Cell survival curve (mean± SD%) of CL40 and FHC cell lines after treatment with a combination of Butenyl-ITC and ASA using MTT in vitro assay

Determination of VEGF gene expression in colon cancer CL40 cell line

The Ct values of both the HKG and the GOI obtained from the real-time RT-PCR are represented in table (7). The diminution in VEGF gene expression was represented in figure (4). The type of assay is relative quantification and the fluorescent dye that was used was Cyber Green dye.

Table 7. Cycles threshold (Ct) of the housekeeping gene (GAPDH) and gene of interest (VEGF)

Gene Name	Ct Control untreated cells	Ct Cells treated with a tested substance	ΔCt	ΔΔ Ct	Fold increment In VEGF gene expression
GAPDH	17.88	18.98	0.55 (control)	0.64	0.641713
VEGF	18.43	20.17	1.19 (treatment)		

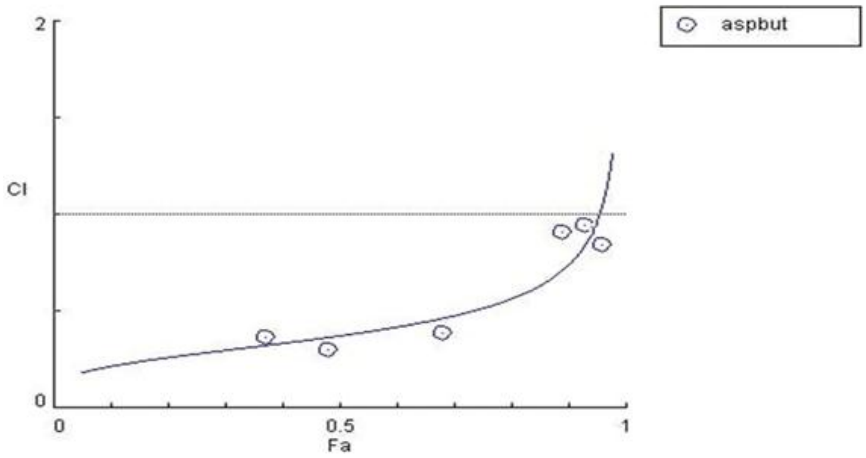

Figure 4. Combination index plot of butenyl isothiocyanate and aspirin using MTT in vitro assay

Data analysis of the real-time RT-PCR results revealed a reduction in transcript levels of the VEGF gene in CL40 colon cancer cell line culture exposed to the butenyl-ITC as shown in figure (5). the concentrations of 200 µg/ml diminished transcription of VEGF gene to 35.83% (P <0.0001).

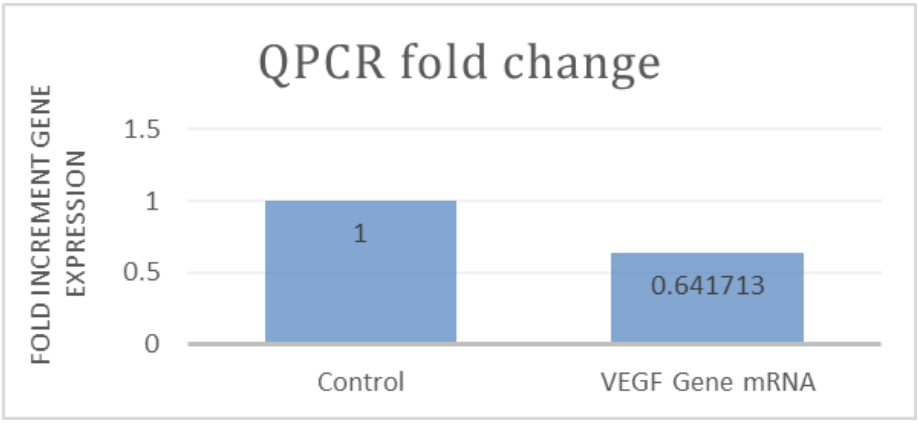

Figure 5. Fold diminution in VEGF gene as a result of treating the colon cancer cell line CL40 with 200 µg/ mL Butenyl-ITC compared with the control

Discussion

The MTT assay is one of many in vitro tests that was first described by Mosmann; this assay's many benefits include its simplicity, low cost, safety, and good reproducibility when determining both cell viability and cytotoxicity, as well as its automation potential [14].

The current study found that all tested concentrations of butenyl ITC, ASA and their combination significantly reduced the viability of the CL40 cell line in a dose-dependent method in comparison to the negative control. However, when the butenyl-ITC concentration used increases, the antiproliferative effect of the CL40 cell line increases, but approaches that of normal cells (FHC). Therefore, it's crucial to carefully choose the best dose while choosing the dose and dosing schedule [15].

The significant antiproliferative effect of butenyl-ITC as a member of the ITCs compound on the CL40 colon cancer cell line may be due to it being considered stress and survival-

regulating factor. When cells are exposed to butenyl-ITC, stress levels rapidly increase, and death may result. It also induces a delayed activation of antioxidative, and anticarcinogenic enzymes and the cell's ability to detoxify [16].

Butenyl-ITCs are electrophiles due to a -N=C= S group, which can react with multiple nucleophiles, especially S-based (thiocarbamoylation) It reacts vigorously with cellular thiols, mainly glutathione (GSH) (the most frequent intracellular thiol) [17]. Membrane efflux pumps remove butenyl-ITC and efficiently reduce GSH. GSH-deficient cells are more susceptible to stress-induced damage and apoptosis [18]. Furthermore, butenyl-ITC intracellular metabolites (GS-ITCs) caused mitochondrial damage, suggesting that these conjugates may be the critical component causing mitochondrial damage in butenyl-ITC-treated cells [16]. Since reactive oxygen species (ROS) are formed in the mitochondria and may leak out of damaged mitochondria, an increase in intracellular ROS levels may be responsible for butenyl-ITC's antiproliferative activity [19,20]. ROS appear to be essential for apoptosis, while their overproduction probably leads to non-specific cytotoxicity [21]. and cell cycle arrest [22].

In the current study, the inhibitory effect of butenyl isothiocyanate combined with ASA were investigated. Depending on the IC50 value, the combination exhibited a lower IC50 value than the IC50 value of both substances alone. Therefore, the butenyl-ITC and ASA combination may have a synergistic effect while the effect on normal cells became more save than that of each of butenyl-ITC and ASA alone. The combination index (CI) value was calculated by using the Chou–Talalay method, which showed synergistic effect (CI<1), where CI<1, =1, and >1 indicate synergism, additive effect and antagonism, respectively as shown in figure (4) [23]. This synergistic effect may rely on the targeted tumor's kinetics and the butenyl-ITC and ASA's ability to target more than one mechanism and the dose and dosing schedule.

Real-time PCR is a widely used and increasingly popular approach for the study of gene expression. due to its great sensitivity, excellent reproducibility, and broad quantification range [24].

Ishigami et al. established a correlation between colon cancer tumour growth, including metastasis, and VEGF mRNA expression [25], and discovered evidence suggesting VEGF expression in adenomas and premalignant human colorectal cancer was partially linked to tumour angiogenesis, which occurred concurrently with tumour formation [26].

In the current investigation, 200 µg/ml of butenyl-ITC concentration was used for the real-time RT-PCR, as it gave higher antiproliferative activity than other concentrations. The results showed diminished transcription of VEGF of 35.83%. This study showed the molecular mechanism responsible for the antiproliferative activity of butenyl-ITC against CL40 through the down-regulation of VEGF.

In conclusion, butenyl-ITC and ASA exhibited significant antiproliferative activity against the CL40. There is a significant additive antiproliferative activity of butenyl-ITC and ASA combination against CL40. The molecular mechanism of action that is responsible for the antiproliferative activity of butenyl-ITC against CL40 was probably mediated by the down-regulation of VEGF.

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Author contribution

Mohammed: conducted the practical aspects of the research, collected and analyzed the data, and prepared the initial draft of the manuscript. Dr. Sahib: supervised the research process, provided academic guidance throughout the study, and contributed to the review and refinement of the final version of the manuscript. Both authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this research.

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