RESEARCH ARTICLE

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The Effects of *Vernonia cinerea* Less Extracts on Antioxidant Gene Expression in Colorectal Cancer Cells

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Abstract

Objectives: To investigate the capability of *Vernonia cinerea* extracts to disrupt the intracellular oxidative-antioxidative status in colorectal cancer cells. **Methods:** All experiments were conducted on two colorectal cancer cell lines (SW620 and HT29) with aqueous and ethanol extracts of *Vernonia cinerea* (VC). The cytotoxicity of both extracts was evaluated using MTT assay. Cells were treated for 1, 4, and 7 days with different concentrations of aqueous and ethanol extracts ranging from 100-700 and 10-150 µg/ml respectively. The antioxidant capacity of cell lysates was determined by the 2, 2'-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS), 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activities, and malondialdehyde (MDA) inhibitory effect. The possible action mechanism was also investigated through gene expression of antioxidant enzymes, i.e. superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. **Results:** Both aqueous and ethanol extracts showed dose/time-dependent manners in all assays. Ethanol extract had a higher potency for cytotoxicity with obviously lower IC_{50} and a higher antioxidant capability in cytoplasmic content than aqueous extract, especially at 4-day treatment. Low MDA content and gene expression alteration of four enzymes involved in antioxidant status were found in cells treated with ethanol extract compared to aqueous extract. **Conclusions:** Ethanol VC extracts can cause cytotoxicity to human colorectal cancer cells, possibly be involved in oxidative stress, and/or interfere with oxidative-antioxidative balance by radical scavenging *in vitro*.

Keywords: *Vernonia cinerea*- cytotoxicity- DPPH- ABTS- MDA- antioxidant enzymes

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Introduction

Cancer is one of the major public health problems with a rapidly rising prevalence in people under the age of 50. Colorectal cancer is the third most lethal malignancy in the world. This cancer is caused by multiple factors (Akimoto et al., 2021; Catalano et al., 2021; Vernia et al., 2021). One of the most important mechanisms of the development of colorectal cancer is intracellular oxidative stress caused by an imbalance between the production of reactive oxygen (ROS) and/or nitrogen (RNS) species and the neutralization efficiency of enzymatic and non-enzymatic antioxidants. The exposure to risk factors, such as smoking, stress, alcohol, toxins, and inflammatory processes can cause overproduction of ROS which is closely related to the development of colorectal cancer. Increase of ROS level may lead to redox imbalance and tumor initiation and progression by activation of redox-responsive signaling cascades that promotes abnormal cell growth. It has been confirmed that oxidative stress can be involved in the development of malignant tumors through genetic mutations, initiation of DNA damage, inhibition of apoptosis, promotion of proliferation, differentiation, and migration of malignant cells (Cao et al., 2021; Vecchiotti et al., 2021; Zińczuk et al., 2019). However, little is known about the diagnostic utility of oxidative stress/redox parameters in patients with colorectal cancer (Boakye et al., 2020). Oxidative stress has been a focus point in cancer therapy. There are many reports on colorectal cancer therapy via oxidative stress reduction using natural compounds (Chok et al., 2021; Cruz et al., 2021; Koyuncu et al., 2021; Lau et al., 2021; Wang et al., 2021).

Vernonia cinerea Less. (VC) of Asteraceae Family is a slender stemmed plant, with different leaf shapes and pinkish-purple and numerous flower heads. This plant is known and recommended in Thai traditional medicine, as in other countries, for smoking cessation, asthma, cough, fever, malaria, urinary calculi, arthritis, inflammation, diabetes mellitus, renoprotection, anticancer, antiviral, and antimicrobial activities (Dogra et al., 2014; Puttarak et al., 2018; Toyang et al., 2013; Supabphol et al., 2013). Unfortunately, there is little scientific data available for details of the mentioned effects. Cancer studies

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have shown that VC has cytotoxicity to cancer cells mainly due to the action of apoptosis. The inhibition of multi-drug resistance transporters or drug efflux pump and STAT3-STAT2 phosphorylation has been mentioned as an interesting mechanism (Appadath et al., 2014; Beeran et al., 2020; Khay et al., 2012; Pouyfung et al., 2019). In addition, VC possesses both cytotoxic and antimetastatic effects on lung cancer cells (Maiti et al., 2021; Pratheeshkumar and Kuttan, 2011a). Amuthan et al. (2020) reported the protective effects of cisplatin-induced nephrotoxicity in cancer therapy. VC can ameliorate the immunosuppression and oxidative stress caused by cyclophosphamide and gamma radiation (Pratheeshkumar and Kuttan, 2010; Pratheeshkumar and Kuttan, 2011b). Flavonoids and sesquiterpene, especially vernolide-A, are the bioactive compounds of VC (Pratheeshkumar and Kuttan, 2011c; Pratheeshkumar and Kuttan, 2011d; Pratheeshkumar and Kuttan, 2012; Rajamurugan et al., 2011; Zhang et al., 2019). The aim of this study was to explore the effects of *Vernonia cinerea* extracts on disruption of the intracellular oxidative-antioxidative activities in colorectal cancer cells.

Materials and Methods

Plant extract

The aerial parts of VC were purchased locally from local markets at Bangkok, Thailand in December, 2018 and identified by an expert taxonomist, Prof Wandee Gritsanapun, PhD, Faculty of Pharmacy, Mahidol University, Thailand. A voucher specimen (SWU-PY-1802) was deposited at the Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Thailand after authentication. The identified plant was dried at 45°C and powdered. The powder was extracted twice by hot water at 70-80°C and spray-dried. For ethanol extract, the plant powder was then extracted twice with 95% ethanol for 24 h, and evaporated under reduced pressure at 50°C. The yield of aqueous (VC-HSD) and ethanol (VC-EtOH) extracts were 7.99 and 7.5% wt/wt respectively. The ethanol extract was redissolved in a minimum volume of dimethyl sulfoxide for *in vitro* studies. Both extracts were kept in amber bottles in the refrigerator for further uses.

Cell line and cell culture

Human colorectal cancer cell lines (SW620 and HT29) were donated by the Institute of Biotecnology and Genetic Engineering, Chulalongkorn University, Thailand. The cells were grown in Dulbecco′s Modified Eagle′s Medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained in an atmosphere of 95% air and 5% CO_2 at 37˚C. In addition, the cells were regularly confirmed for mycoplasma contamination every two months.

Chemicals and reagents

Cell culture medium and supplements were procured from Gibco BRL Life Technologies (Grand Island, NY, USA). 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS•+), 2,2–diphenyl–1–picrylhydrazyl (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide and BCA protein assay kit were purchased from Sigma-Aldrich (St Louis, USA). TRIzol reagent was ordered from Thermo Fisher Scientific, Inc., USA and SuperScript™ Reverse Transcriptase kit was obtained from Invitrogen, USA.

Cell viability assay

The cells were seeded at a density of 7×10^4 cells/ml in 24-well plates, maintained for 24 h and treated with aqueous and ethanol extracts of VC for 1, 4 and 7 days at concentrations ranging from 100-700 and 10-150 µg/ml. At the end of incubation, the culture medium was removed and cells were incubated again with 1 mg/ml MTT solution for three hours. The absorbance was then determined at 570 nm using enzyme-linked immunosorbent assay (ELISA) microplate reader (Biotex-synergy-HT). All experiments were performed in triplicate. The cell viability was calculated as a percentage of the control (untreated cells). Inhibitory concentration at 50 % (IC_{50}) growth was used to compare cell viability in the presence and absence of VC extract (Yahayo et al., 2013; Tangjitjaroenkun et al., 2021). IC_{50} of standard anticancer drug, doxorubicin, was used as positive control to confirm invariable status of cancer cells for cell viability assay.

Cell lysates

Cell lysates were prepared as described by the manufacturer (Lipid peroxidation (MDA) assay kit (ab118970); Abcam, UK). Briefly, after approaching 90 % confluence, the treated cells were washed with cold phosphate buffer saline (PBS), homogenized on ice in lysis buffer solution, and centrifuged to remove insoluble content. The supernatant was collected and protein was calibrated by BCA protein assay kit. The cell lysates were continued for DPPH, ABTS, and MDA assays.

DPPH assay

Free radical scavenging activity was determined using DPPH assay. The DPPH (0.1 mM) was prepared in 100% ethanol and cell lysates were added into the DPPH solution. The reaction mixture was shaken thoroughly and left at room temperature for 30 min. The decrease in absorbance of the resulting solution was monitored at 517 nm using ELISA microplate reader. The control was done by adding the distilled water into the DPPH solution instead of cell lysate. Butylated hydroxy toluene was used as reference. The percentage of DPPH decolorization of cell lysates was calculated as DPPH radical scavenging activity (difference absorbance of control and extract (or standard) x100) / absorbance of control.

ABTS assay

The ABTS cation radical (ABTS•+) was obtained by mixing the stock solution of 7 mM ABTS solution and 2.4 mM potassium persulphate in equal quantities and leaving the mixture in the dark for at 14 hours at room temperature before use. The ABTS•+ solution was then diluted to an absorbance 0.7 ± 0.01 at 736 nm. Cell lysates were allowed to react with ABTS solution for seven minutes. Absorbance was then monitored at 736

nm using ELISA microplate reader. The percentage of ABTS decolorization of the sample was calculated like that of DPPH assay.

Lipid peroxidation assay

The measurement of thiobarbituric acid-reactive substances (TBARS) is a well-established method for detecting lipid peroxidation. This study used the TBARS assay for the rapid detection of the thiobarbituric acid-malondialdehyde (TBA-MDA) adduct at 532 nm using Lipid peroxidation (MDA) assay kit (ab118970, Abcam, UK) following the manufacturer's instructions.

Real-time quantitative PCR

After treatment with VC extracts, cells were washed with PBS, harvested, and total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc., USA) in accordance with the manufacturer's instructions. The purity and integrity of total RNA was checked at 260/280 nm ratio. cDNA was synthesized from 1.0 µg of total RNA using a SuperScriptTM Reverse Transcriptase kit (Invitrogen, USA). Gene expression of four antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx) was quantified and compared with an internal control (GAPDH) using quantitative real-time PCR in CFX96 Touch Real-Time PCR Detection System (BIO-RAD, USA) (Yahayo et al., 2013; Tangjitjaroenkun et al., 2021).

Statistical analysis. All experiments were performed at least three times with water as the negative control. All data including IC_{50} values were expressed as the mean and standard deviation (SD). One-way ANOVA and Student's t-test were used to test the significant differences between groups (SPSS IBM Singapore Pte Ltd; Registration No.1975-01566-C) (P-value<0.05).

Results

Cell viability assay

Two human colorectal cancer cell lines, SW620 and HT29, were exposed to serial dilutions of aqueous and ethanol VC extracts through MTT assay as described previously. Dose-response curves (% survival and at least five concentrations of VC extracts) of three independent sample preparations were used to evaluate average IC_{50}

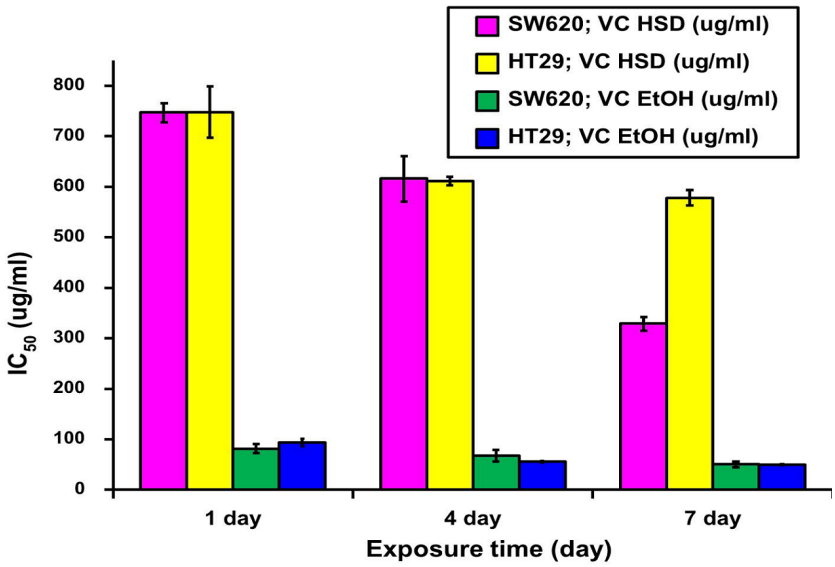

Figure 1. IC_{50} from Cytotoxicity of Aqueous and Ethanol VC Extracts in SW620 and HT29 after Receiving Treatment for 1, 4 and 7 Days. Data were expressed in mean ± SD of three independent experiments.

Table 1. Primers Used for Real–time PCR Amplification

Genes	Primer sequence (5' ® 3')		Product size (bp)
SOD	Forward primer:	GAGACTTGGGCAATGTGACTG	201
	Reverse primer:	TTACACCACAAGCCAAACGA	
CAT	Forward primer:	CCTGGAGCACAGCATCCAAT	85
	Reverse primer:	GAATGCCCGCACCTGAGTAA	
GPx	Forward primer:	TGGACATCAGGAGAACTGTCAGA	134
	Reverse primer:	AGACAGGATGCTCGTTCTGCC	
GRx	Forward primer:	AAGCGGGATGCCTATGTGAG	107
	Reverse primer:	TTGGGATCACTCGTGAAGGC	
GAPDH	Forward primer:	AGTCCACTGGCGTCTTCACC	119
	Reverse primer:	GTTCACACCCATGACGAACATG	

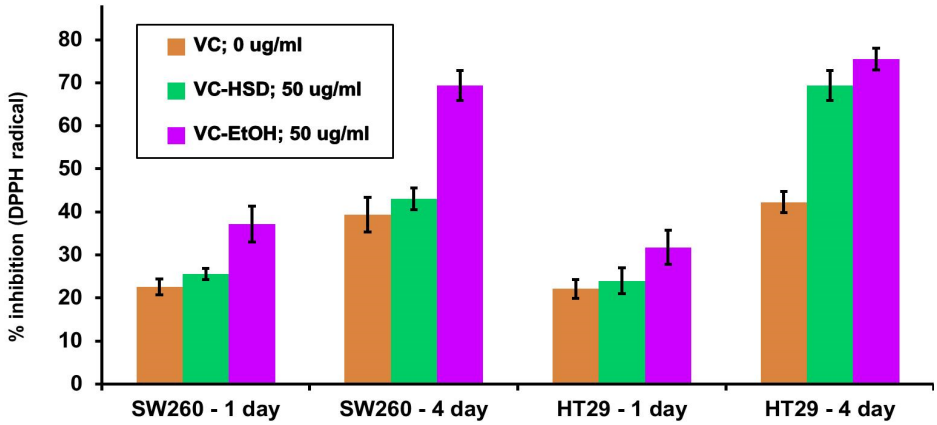

Figure 2. % Inhibition of DPPH Radical of Cell Lysates in SW620 and HT29 Grown in Aqueous and Ethanol VC Extracts for 1 and 4 Days at Concentration of 50 µg/ml. Data were expressed in mean±SD of three independent experiments.

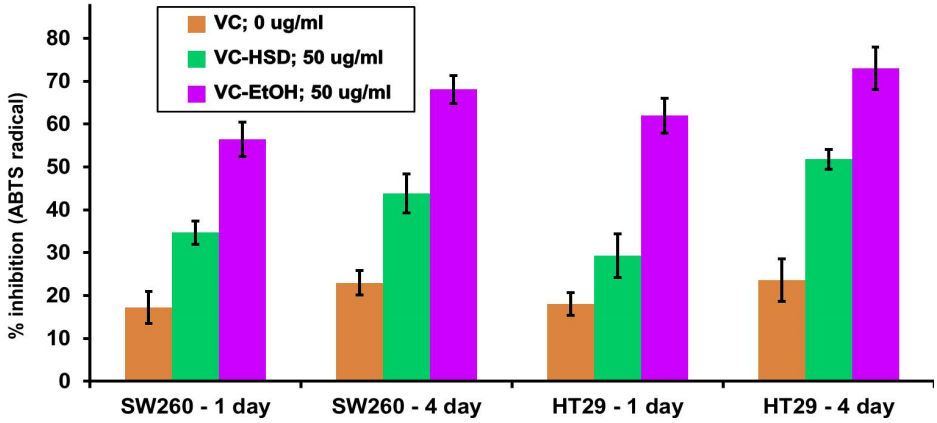

Figure 3. % Inhibition of ABTS Radical of Cell Lysates in SW620 and HT29 Grown in Aqueous and Ethanol VC Extracts for 1 and 4 Days at Concentration of 50 µg/ml. Data were expressed in mean±SD of three independent experiments.

values. As indicated in Figure 1, both aqueous and ethanol extract graphs showed dose- and time-dependent manner. Ethanol extract showed remarkably more effectiveness with clearly lower IC_{50} in both cell lines. Aqueous extract had IC_{50} values ranging from 300 to 800 µg/mL which was more than 3-8 folds higher than that of ethanol extract with IC_{50} values lower than 100 µg/mL.

DPPH assay

Intracellular free radical scavenging capability of cells treated with aqueous and ethanol VC extracts was tested by DPPH radical scavenging assay. VC concentration at 50 µg/ml of both extracts were chosen in this study to compare the antioxidant power. Cell lysates from cells exposed to ethanol extract showed a higher antioxidant capability than aqueous extract, especially in the 4-day treatment group (Figure 2).

ABTS assay

ABTS assay was also used to assess intracellular free radical scavenging capability of cells treated with aqueous and ethanol VC extracts with the same VC concentration at 50 µg/ml. The same result was found with higher antioxidant capability of ethanol extract (Figure 3). Figures 2 and 3 show the antioxidant power of cytoplasmic content of SW620 and HT29 in time.

Lipid peroxidation assay

TBARS assay is a simple, precise, sensitive, and reproducible spectrophotometric method for quantitative MDA determination as a marker for lipid peroxidation. This method was used to determine MDA level in cytoplasmic content of human colorectal cancer cell lines, SW620, and HT29 when exposed to different concentrations of aqueous and ethanol VC extracts. Figure 4 shows a dose-dependent manner of MDA reduction in SW620 and HT29. The obvious point is the different concentrations of extracts so that aqueous extract concentrations are 8-fold higher than that of ethanol extract.

Expression of antioxidant genes

Gene expression was assessed through quantitative real-time RT-PCR, normalized with GAPDH, and expressed as the mean score of fold change compared to control condition. The four antioxidant genes were down-regulated in human colorectal cancer cells (SW620) then treated with aqueous and ethanol VC extracts for four days. In contrast, upregulation was obviously found in HT29 grown in aqueous VC extract for four days, while

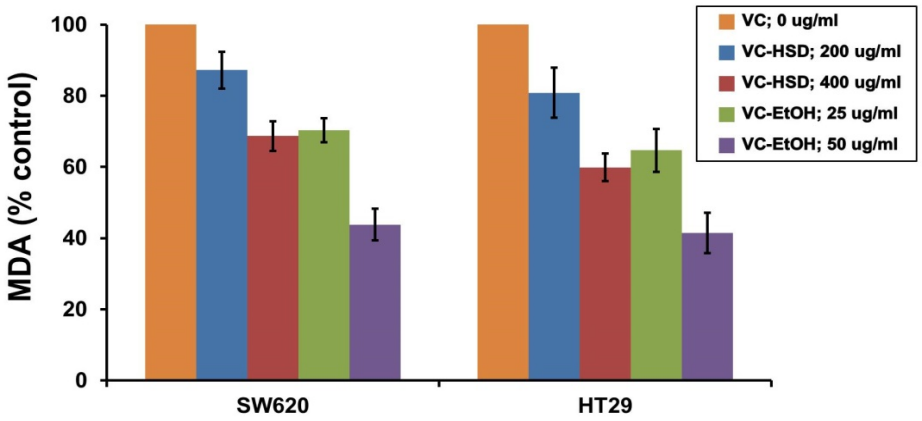

Figure 4. MDA Content from TBARS Assay of Cytoplasmic Content of Human Colorectal Cancer Cell Lines (SW620 and HT29) Grown in Aqueous and Ethanol VC Extracts for 4 Days. Data are expressed in mean ± SD of three independent experiments.

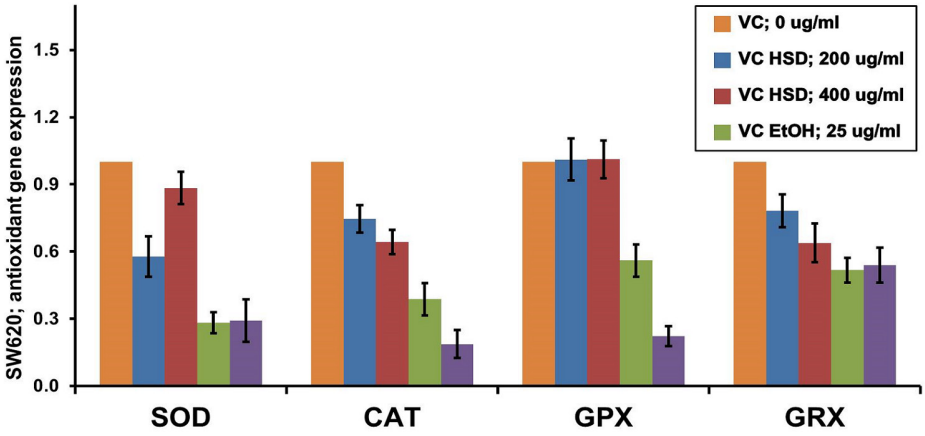

Figure 5. Down Regulation of Antioxidant Gene Expression in Human Colorectal Cancer Cells (SW620) Treated with Aqueous and Ethanol VC Extracts for 4 Days. Data are expressed in mean ± SD of three independent experiments.

upregulation and downregulation were found in HT29 grown in ethanol VC extract.

Discussion

The safety and protective effect to normal cells and endothelial cells of aqueous VC extracts have been studied previously (Supabhol et al., 2013). This study showed a significantly higher IC_{50} of aqueous VC extract compared to that of ethanol VC extracts in both human colorectal cancer cell lines. It was found that extraction conducted using ethanol can extract compounds or ingredients containing more cytotoxic effects. This highlights the influence of solvent and extraction methods on the cytotoxic activity of VC extract. It is the reason why extracts may display differential cytotoxic effects

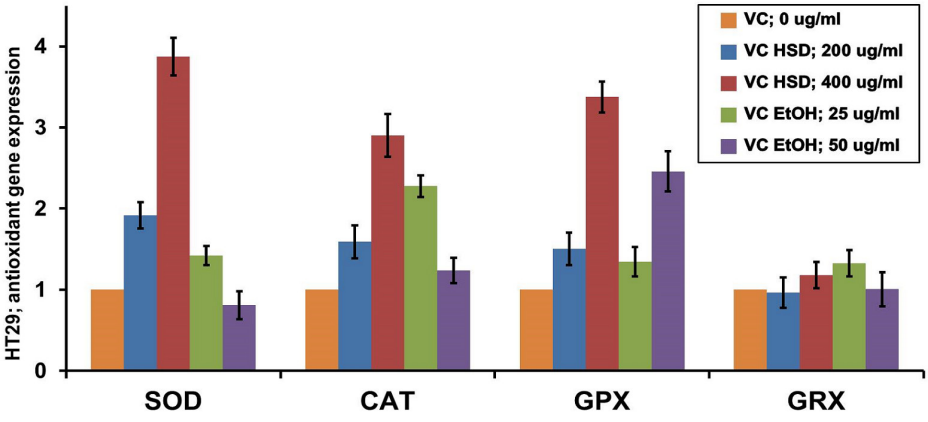

Figure 6. Upregulation and Downregulation of Antioxidant Gene Expression in Human Colorectal Cancer Cells (HT29) Treated with Aqueous and Ethanol VC Extracts for 4 days. Data are expressed in mean ± SD of three independent experiments.

depending on the extraction solvent. Ethanol is known as a good solvent for a number of compounds such as polyphenols. A key advantage of ethanol is that it is safe for human consumption (Kowalska et al., 2021; Traversier et al., 2020).

Cytoplasmic content of SW620 and HT29 were tested using DPPH and ABTS assays to investigate intracellular antioxidant capacity. These two assays are the most popular methods frequently used to detect the antioxidant capability of several plant extracts. DPPH assay was used to screen the antioxidant activity based on the reduction of the purple DPPH to yellow DPPH-H. DPPH is a radical dissolved in organic solvent, methanol or ethanol. Aqueous alcoholic system is normally used to keep this radical soluble. ABTS assay can be used to detect the ability of hydrophilic and lipophilic antioxidants to scavenge the blue/green cation ABTS generated and dissolved in aqueous and organic solvents (Munteanu and Apetrei C, 2021; Wołosiak et al., 2021). These two assays were used to detect the antioxidant property from lipophobic and hydrophobic molecules in cytoplasmic content of cells grown in culture media with aqueous and ethanol VC extracts. The cytoplasmic content from cells grown in ethanol VC extract expressed a higher antioxidant capability than that in aqueous VC extract. This is consistent with dose- and time-dependent manner of intracellular MDA reduction in Figure 4 when SW620 and HT29 are cultured in aqueous or ethanol VC extracts. Free radicals were the causes of lipid peroxidation and involved in damaging mechanisms of several diseases. MDA is one of the end products in lipid peroxidation and its overproduction is considered as a biomarker of oxidative stress (Tsikas, 2017). Thus, aqueous and ethanol VC extracts may have the ability to scavenge free radicals in both SW620 and HT29. Antioxidant modulation has a higher chance to participate in this cellular event. It is reported that VC extract possesses antioxidant and antimicrobial activities (Rajamurugan et al., 2011; Sonibare et al., 2016). This shows the protective activity in 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-treated blood via the inhibition of MDA formation (Ketsuwan et al., 2017) and reduction of lipid peroxide in blood and liver of mice in carrageenan induced paw edema model (Kumar and Kuttan, 2009).

The possibility of modulating effect through the intracellular antioxidative system was further investigated using gene expression of antioxidant enzymes, SOD, CAT, GPx and GRx. These are the key enzymatic antioxidants to scavenge or deactivate free radicals before cellular components are attacked. In carrageenan induced paw edema mice, VC extract can significantly increase the levels of CAT, SOD, GPx and glutathione-S transferase in blood and liver (Kumar and Kuttan, 2009). Lupeol isolated from VC was found effective in treating cataract formation in Sprague Dawley rats via these antioxidant enzymes (Asha et al., 2016). Enhancing these antioxidant enzymes plays an important role in the protective effect of VC against oxidative stress from gamma radiation in mice (Pratheeshkumar and Kuttan, 2011b). In this study, both aqueous and ethanol VC extracts seemed to reduce gene expression of these four antioxidant enzymes in SW620. Gene expression of antioxidant enzymes in HT29 did not show the same pattern found in SW620. VC extracts may not function in the same pattern in different colorectal cancer cells. However our result showed the effect of VC extracts via the interference of antioxidative balance. Four antioxidative enzymes were changed after VC extract treatment.

In conclusions, VC-EtOH extract exhibited cytotoxicity to both colorectal cancer cell lines. Sesquiterpene lactone, especially vernolide-A, might be one of the bioactive compounds against cancer cells which is better extracted by ethanol. VC extracts were capable of changing the oxidative-antioxidative balance via MDA production or free radical neutralization. This led to the alteration of antioxidative power inside the cancer cells. Suitable concentrations of VC-EtOH extract (25-50 μg/mL) can be used in the future in vivo studies and pharmaceutical development. Protein expression of antioxidant enzymes, i.e. catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase should be further investigated for the explanation of VC in cellular mechanisms.

Author Contribution Statement

R Supabphol was responsible for the overall experimental design and supervision of the experiments and preparation of the final article. W. Yahayo performed most of the experiments and data analysis. J Pakpisutkul, J Suwapraphan, N Sripayak, N Sitkhuntod and S Loyrat helped for experimental design and performed part of the experiments.

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Conflicts of Interest

The authors do not have anything to declare and have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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