https://doi.org/10.48048/tis.2025.9111

Pharmacological and *In Vitro* Studies on Quercetin-Rich Onion Peel Extract (*Allium cepa*) and N-Acetylcysteine Enhancing Apoptosis in Colorectal Cancer

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Received: 7 October 2024, Revised: 11 November 2024, Accepted: 18 November 2024, Published: 30 January 2025

Abstract

Colorectal cancer (CRC) is one of the most common types of cancer globally with the second highest cancer-related mortality, having increased by almost 40 % over the past 40 years. Considering quercetin's limited bioavailability, N-acetylcysteine (NAC) has previously emerged as a promising candidate for treatment as it has been shown to stabilize quercetin and enhance its anti-cancer effectiveness. Therefore, the present study aims to investigate the effectiveness of onion peel extract and NAC in inducing apoptosis in CRC. Extracts were taken from onion peel using 60 % v/v ethanol and identified using High-Performance Liquid Chromatography (HPLC). Furthermore, the micronucleus assay was utilized to investigate the genotoxicity of the extract. Pharmacological network analysis was employed to elucidate the mechanism underlying the combined action of quercetin and NAC on CRC, with KEGG enrichment analysis also used to study key signaling pathways. Additionally, *in vitro* studies were conducted using MTT assay, apoptosis assay via flow cytometry, and western blot analysis in HT-29 and HCT-116 cells. Quercetin is a major compound in onion peel extract and showed no genotoxic effects. Pharmacological network analysis identified potential targets for quercetin and NAC combination therapy, revealing 363 overlapping target genes of quercetin and NAC in CRC, indicating their involvement in inducing CRC apoptosis. The KEGG pathway analysis revealed the top 10 pathways and showed that a majority of the genes were involved in MAPK pathways. *In vitro* studies demonstrated that the combined treatment significantly increased cytotoxicity and apoptosis induction, evidenced by upregulated expression of caspases 3, 7, 8, and 9 in HT-29 and HCT-116 cells. These findings emphasize the therapeutic potential of quercetin-rich onion peel extraction, characterized by the absence of genotoxicity, when used in combination with NAC therapy to enhance apoptosis efficacy in CRC. This approach holds promise as a candidate for alternative medicine in the treatment of human CRC.

Keywords: Onion peel extract, Quercetin, Genotoxicity, Pharmacological network, CRC, Apoptosis

Introduction

Colorectal carcinoma (CRC) is one of the most prevalent malignant tumors, accounting for approximately 1. 9 million new cancer cases and 900,000 deaths worldwide in 2020 [1]. CRC is a malignancy of the large intestine or rectum. In most cases, it starts with dysplastic adenomatous polyps which can progress to CRC [2]. Recently, oxidative stress has been firmly related to all aspects of cancer, from carcinogenesis to the tumor-bearing state [3]. The damage from oxidative stress causes cell injury and uncontrolled, abnormal cell growth, which eventually progresses to cancer [4]. Despite the availability of

chemotherapeutic agents for CRC, patients often experience significant adverse effects such as hematologic toxicity, gastrointestinal toxicity, and neuropathy [5]. Natural products have been considered as a source of therapeutic agents [6]. Their diverse chemical structures and mechanisms of action present a promising avenue for developing treatments that are safer [7]. Furthermore, reducing the toxic side effects of current chemotherapeutic agents would significantly improve patient quality of life and treatment efficacy. Therefore, identifying natural products that exhibit anticancer activity without associated toxicity is crucial for potential future CRC treatments.

Epidemiological and dietary intervention studies have suggested that diet-derived flavonoids can make a helpful contribution to cancer treatment, principally due to their pro-apoptotic or anti-angiogenic activities [8]. In addition, quercetin is a ubiquitous flavonoid commonly found in nature, such as in some vegetables, tea, fruit and wine [9,10]. In particular, the biological effects of quercetin have been published in a significant number of scientific studies which have shown it to have anti-oxidant, anti-inflammatory, immune-protective, and even anti-carcinogenic effects, causing apoptosis of prostate, liver, breast, pancreatic, and lung cancer cells [11-14]. Although the anti-apoptotic effects in various cancers are clearly evidenced, quercetin has demonstrated low bioavailability [15]. Hence, various additive compounds have been studied Zhou *et al.* [16] to improve its bioavailability, with the most promising being N-acetylcysteine (NAC), which has been shown to preserve the stability of quercetin and increase its anti-cancer activity [17].

Recently, we demonstrated that the combination of quercetin-rich onion extract and NAC improved migration and invasion in CRC cell lines through iNOS suppression [18]. The present study aims to investigate the anti-apoptotic activity of quercetin-rich onion extract and NAC to enhance apoptosis in HCT116 and HT-29 colorectal cancer cell lines. A pharmacological network analysis was employed to review the potential mechanisms and associated signaling pathways, with the results confirmed by *in vitro* studies. The findings from this study may inform future research aimed at developing alternative treatments to inhibit cancer growth by promoting apoptosis in colorectal cancer.

Materials and methods

Quercetin extraction

The extract was obtained from Detox (Thailand) Co., Ltd., Chiangmai, Thailand. Briefly, onion peel was dried and ground, with 60 % v/v ethanol then used as the extracting solvent. The extract was then evaporated and freeze-dried. The extract was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C.

Determination of quercetin by high-performance liquid chromatography (HPLC)

The fingerprinting of quercetin in the extract was analyzed by the reversed-phase HPLC technique in accordance with a modification in a previous study [19]. The mobile phase solution comprised 0.5 % formic acid in distilled water (A) to maintain a low pH, stabilizing flavonoids in their desired form, and absolute methanol (B). This highlights the importance of pH control for optimal separation and accurate analysis. The gradient elution conditions were composed of (solvent A; solvent B; %v/v) 40:60, 60:40, 0:100 and 40:60 with respective periods of 0 - 5, 5 - 6, 6 - 9 and 9 - 13 min, respectively. The flow rate was controlled at 1 mL/min under an ACE® C18 column (Advanced Chromatography Technologies, Scotland) (250 mm × 4.6 mm; 5 µm). The fingerprints of quercetin were detected by a UV detector at 200 - 600 nm. The quercetin standard was used for the identification.

Micronucleus assay

V79 Chinese hamster lung fibroblasts (ATCC® CCL-93™) were kindly provided by the Department of Pathobiology, Faculty of Science, Mahidol University, Thailand. The assay was performed in accordance with the slightly modified method of the previous study [20]. Briefly, the micronucleus assay is a well-established cytogenetic technique used to assess genotoxicity, or the potential of a substance to induce damage to the genetic material within cells. This assay identifies the formation of micronuclei, which are small, extranuclear bodies that arise when chromosome fragments or entire chromosomes fail to integrate into the main nucleus during cell division. V79 cells were plated into 6 well plates and incubated at 37 °C in a humidified atmosphere that was supplied with 5 % CO_2 for 24 h. After incubation, the cells were treated with quercetin

and DMSO as a vehicle control, and then incubated at 37 °C with 5 % CO_2 for 3 h. After treatment, the cells were washed with fresh serum-free DMEM 3 times and Cytochalasin B (Sigma-Aldrich, USA) was added to block cytokinesis at 18 h. Subsequently, the cells were deposited onto glass slides by cytocentrifugation and fixed in ice-cold 90 % methanol for 15 min. The slides were then dried and stained with Giemsa staining. Finally, the slides were observed under a light microscope at 400X magnification to determine the effect on the micronuclei (MN) of the treated samples in the V79 cells. The evaluation of MN involved analyzing at least 1,000 bi-nucleated cells in accordance with the criteria of Countryman and Heddle [21]. The MN were characterized by being enclosed in a nuclear membrane, occupying an area less than one-third the size of the main nucleus, located within the cytoplasm, and not connected to the main nucleus by nucleoplasmic bridges.

Target gene prediction

Pharmacological targets of quercetin and NAC were retrieved from 3 online databases, namely, the Swiss Target Prediction Database (www.swisstargetprediction.ch/), Super-PRED (https://prediction.charite.de/), and Sea Search Server (https://sea.bkslab.org/). The colorectal cancer-related targets were found from the GeneCards (www.genecards.org/), DisGeNET (www.disgenet.org/), and OMIM (www.omim.org). Then, the quercetin combination with NAC and cancer-related targets were mapped in a VennDiagram v2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) to obtain overlapping targets (22).

Protein interaction network

To obtain the hub targets of quercetin and NAC for treating colorectal cancer, the overlapping targets were imported into the STRING platform (https://string-db.org/) to construct a protein–protein interaction (PPI) network. This network provides insights into the biological mechanisms underlying target-related pathogenesis at the protein level. The protein type was set as Homo sapiens. The interaction network between overlapping targets was visualized by Cytoscape 3.10.1 software, and topology analysis was performed using the CytoHubba plug-in to identify the hub genes in terms of degree [23].

KEGG enrichment analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a key public database for validating pathways. Pathway enrichment analysis uses KEGG pathways and a hypergeometric test to identify those significantly enriched in a specific gene set versus the genome background, based on upregulated and downregulated genes [24]. KEGG pathway enrichment analysis was conducted utilizing the web interface available at https://www.bioinformatics.com.cn. Target gene names were input for the enrichment analysis. A significance threshold was established at a *p*-value of < 0.05, and the 10 pathways with the lowest *p*-values were identified as the top 10 pathways.

Cell culture and treatment

The human colorectal cell lines HT-29 and HCT-116, considering their widespread use, exhibit epigenetic differences. Mutations in PIK3CA, BRAF, KRAS, and TP53 across different cell lines make them an ideal choice for studying the mechanisms of colon cancer, as demonstrated in previous studies [25]. HT-29 cells (ATCC® HTB-38™, Virginia, USA), and HCT-116 (ATCC® CCL-247™, Virginia, USA) — were routinely maintained in McCoy's 5A media supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % L-glutamine, 1 % penicillin-streptomycin and non-essential amino acid using a humidified incubator (37 °C and 5 % CO_2)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay is commonly used in cytotoxicity testing to evaluate the effectiveness of drugs, toxins, or other compounds in inhibiting the growth or inducing cell death in cancer cells or other cell types, as demonstrated in previous experiments [26]. The HT-29 and HCT-116 cells were seeded in 96-well plates for 24 h. Then the culture media were replaced by samples of various concentrations. After 24 h, the cells were incubated with 100 µl of MTT solution for 3 h followed by DMSO. The results were analyzed with a microplate reader (EMax® Plus Microplate Reader, Molecular devices) at the optical density (OD) of 570 nm.

Flow cytometry

Flow cytometry is a quantitative technique that distinguishes between live, early apoptotic, late apoptotic, and necrotic cells by assessing phosphatidylserine exposure and membrane integrity. In early apoptosis, phosphatidylserine translocates to the outer leaflet of the plasma membrane, where it binds to Annexin V conjugated with fluorescein isothiocyanate (FITC). Propidium iodide (PI) is a DNA-binding dye that penetrates cells with compromised membranes, staining late apoptotic and necrotic cells. [27]. To detect the apoptotic cell population, the cells were stained with Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide staining using an Apoptosis Detection Kit (Sigma- Aldrich, USA). Briefly, the HT- 29 and HCT- 116 cells were seeded in 6- well plates and incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 48 h. After incubation, the culture media was removed and replaced with the media-containing sample and then incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 24 h. Next, the cells were trypsinized and washed with PBS buffer. Afterward, the solution was centrifuged, and the pallet was resuspended with binding buffer mixed with FITC- conjugated annexin V and phycoerythrin- conjugated PI. The cells were then washed with PBS buffer and fixed with 1 % paraformaldehyde. The resuspended cells were incubated at room temperature in the dark for 15 min. The labeled cells were determined by using FACScan (Becton Dickinson, USA).

Western blot analysis

In this study, western blot analysis was applied to investigate the molecular mechanisms by analyzing protein expression levels [28]. The method can confirm apoptosis- related proteins- level changes in colorectal cells treated with quercetin extract combination with NAC. The HT- 29 and HCT- 116 cells were placed into 6- well plates for 24 h and then incubated with samples in various concentrations. After incubation, the cells were harvested and washed with ice- cold PBS buffer, pH 7.4. Then, the cells were re-suspended in lysis buffer containing RIPA lysis buffer (Merck, Germany) and complete protease inhibitor (Sigma-Aldrich, USA). The cell lysates were homogenized by a homogenizer (Vibro cell, Sonics, USA) and centrifuged at 8,000 rpm at 4 °C for 10 min to collect the supernatant. The total protein was determined using protein kits (Merck, Germany). Proteins from each sample were separated by 12 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Germany). After blocking with TBST, the membrane was incubated with a primary antibody at 4 °C overnight followed by a secondary antibody. The bound antibodies from each sample were visualized using Luminata Forte western HRP substrate (Merck, Germany). Finally, the protein was determined using a Gel documentation analyzer (ImageQuant LAS 500, UK), and the intensity was examined with Image J software.

Statistical analysis

All data presented are representative of 3 independent experiments. The data were analyzed as mean ± standard deviation (mean ± SD) and compared by ANOVA (one-way analysis of variance) followed by Tukey's multiple comparisons test in GraphPad Prism version 9 (GraphPad Software Inc., CA, USA). *p*-values < 0.05 were considered significantly different.

Results and discussion

Fingerprint analysis and genotoxicity of onion peel extract

The identification of quercetin from onion peel extract was performed by using a High- Performance Liquid Chromatography (HPLC) assay. The results showed the fingerprint of standard quercetin at a retention time of 3.476 min with 0.1856 AU (**Figure 1(A)**). As expected, onion peel extract presented a dominant peak of quercetin at a retention time of 3.455 min and 0.1716 AU with 92.03 % of total area (**Figure 1(B)**). A spike peak of standard quercetin and onion peel extract showed a predominant peak at a retention time of 3.457 min and 0.3108 AU with 97.88 % total area (**Figure 1(C)**). This result indicated that the quercetin was a major compound in the onion peel extract.

The genotoxicity of the onion peel extract was investigated for evaluating the potential genotoxicity by detecting chromosomal damage induced during cell division using micronucleus assay. The genotoxicity was determined by counting the micronucleus per 1,000 bi- nucleated cells (**Figure 1(D)**), which then indicates the chromosomal damage. The results showed that quercetin 100 and 200 µg/mL did not significantly affect the formation of MN, as shown in **Figure 1(E)**, when

compared with the vehicle control. These results indicate that the onion peel extract demonstrated no genotoxicity and is safe.

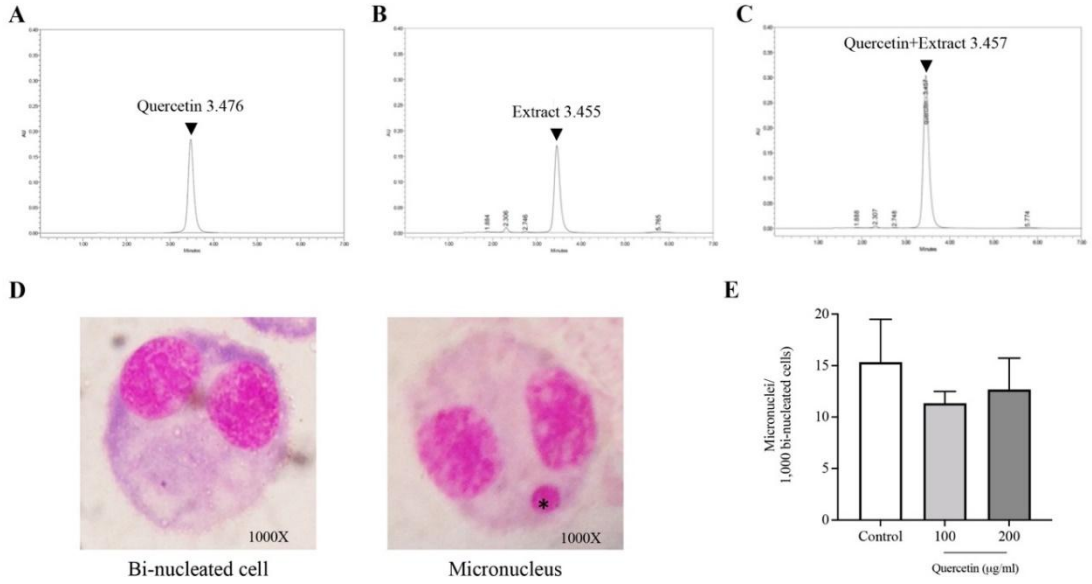

Figure 1 The identification of onion peel extract and the testing of genotoxicity using micronucleus assay.

Quercetin fingerprints of onion peel extract and the value of retention time. Standard quercetin (A), onion peel extract (B), and spike peak of standard quercetin and onion peel extract (C) were analyzed for their phytochemical profiles by the HPLC technique. The genotoxic effects of quercetin were investigated in V79 cells and determined by observing the number of micronucleus (**Figure 1(D)**; asterisk) under a light microscope. Results were expressed in a bar graph as MN per 1,000 Bi-nucleated cells (E). The results were expressed as mean ± SD.

Prediction of quercetin and NAC targets in CRC through pharmacological network analysis

The study employed network pharmacology to elucidate the mechanism underlying the combined action of quercetin and NAC on CRC. A Venn diagram revealed 363 shared target genes implicated in quercetin and NAC with CRC (**Figure 2(A)**). These overlapping targets were further analyzed to construct a protein-protein interaction (PPI) network, leading to the identification of the top 10 hub genes, ranked using the MCC algorithm (**Figure 2(B)**). Nodes with high MCC scores were highlighted in red, indicating their significance, while yellow nodes represented lower MCC scores.

Notably, several hub genes such as TP53, CASP3, and CASP8 have been found to play crucial roles in the cellular response to reactive oxygen species (ROS) and the regulation of apoptosis. TP53, a tumor suppressor protein, is a major regulator of apoptosis in response to DNA damage and oxidative stress. CASP3 and CASP8 are key executors of apoptosis, with CASP3 acting as the main executioner caspase and CASP8 initiating the extrinsic apoptotic pathway. These hub genes hold promise as potential targets modulated by quercetin combined with NAC in the treatment of CRC.

The KEGG pathway analysis was conducted to elucidate the key signaling pathways associated with the anti-colorectal cancer efficacy of quercetin and NAC. The top 10 pathways with the most significant gene ratios in the enrichment results were represented using a bubble plot (**Figure 3(A)**), which revealed that a majority of the genes were involved in MAPK pathways. The specific target genes modulated by quercetin and NAC within the MAPK signaling pathway are illustrated in **Figure 3(B)**. This suggests that MAPK signaling pathways respond to stress

conditions (e.g., ROS) and regulate various cellular processes, such as proliferation, differentiation, and apoptosis, through classical MAPK, JNK, and p38 MAPK pathways. These findings suggest that the main targets of the quercetin and NAC combination are involved in inducing apoptosis in CRC.

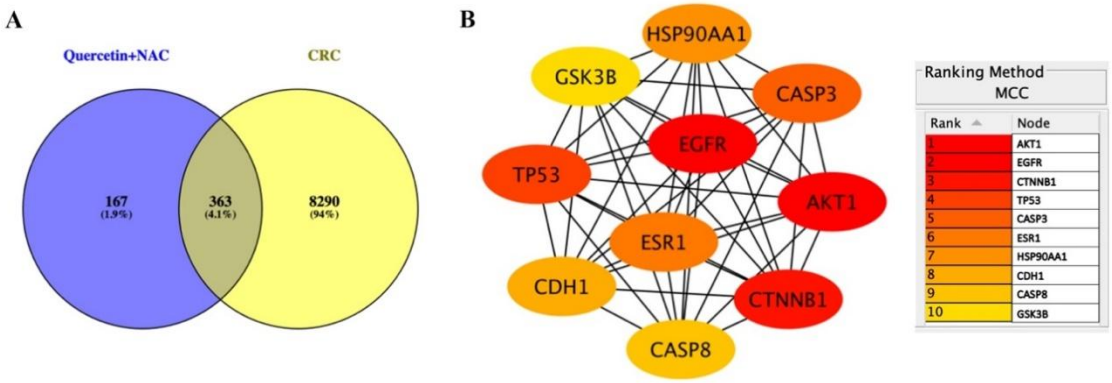

Figure 2 Venn diagram and PPI networks of overlapping target genes of quercetin and NAC in CRC.

Venn diagram identified 363 overlapping target genes of quercetin and NAC in CRC (A). The top 10 hub genes within the macro-module were identified using the CytoHubba plugin in Cytoscape (B). The image depicts the relative importance of these hub genes, with a color gradient from red to yellow, with red being the most essential and yellow being the least significant. NAC = N-acetylcysteine.

Table 1 Top 10 genes ranked by MCC method.

Rank	Gene symbol	Gene name	MCC score
1	AKT1	RAC-alpha serine/threonine-protein kinase	3.3809E+19
2	EGFR	Epidermal growth factor receptor	3.3803E+19
3	CTNNB1	Catenin beta-1	3.3798E+19
4	TP53	Cellular tumor antigen p53	3.3788E+19
5	CASP3	Caspase 3	3.3784E + 19
6	ESR1	Estrogen receptor	3.3784E + 19
7	HSP90AA1	heat shock protein 90 alpha family class A member 1	3.3780E+19
8	CDH1	Cadherin-1	3.3756E + 19
9	CASP8	Caspase 8	3.3507E + 19
10	GSK3B	Glycogen synthase kinase-3 beta	3.3297E + 19

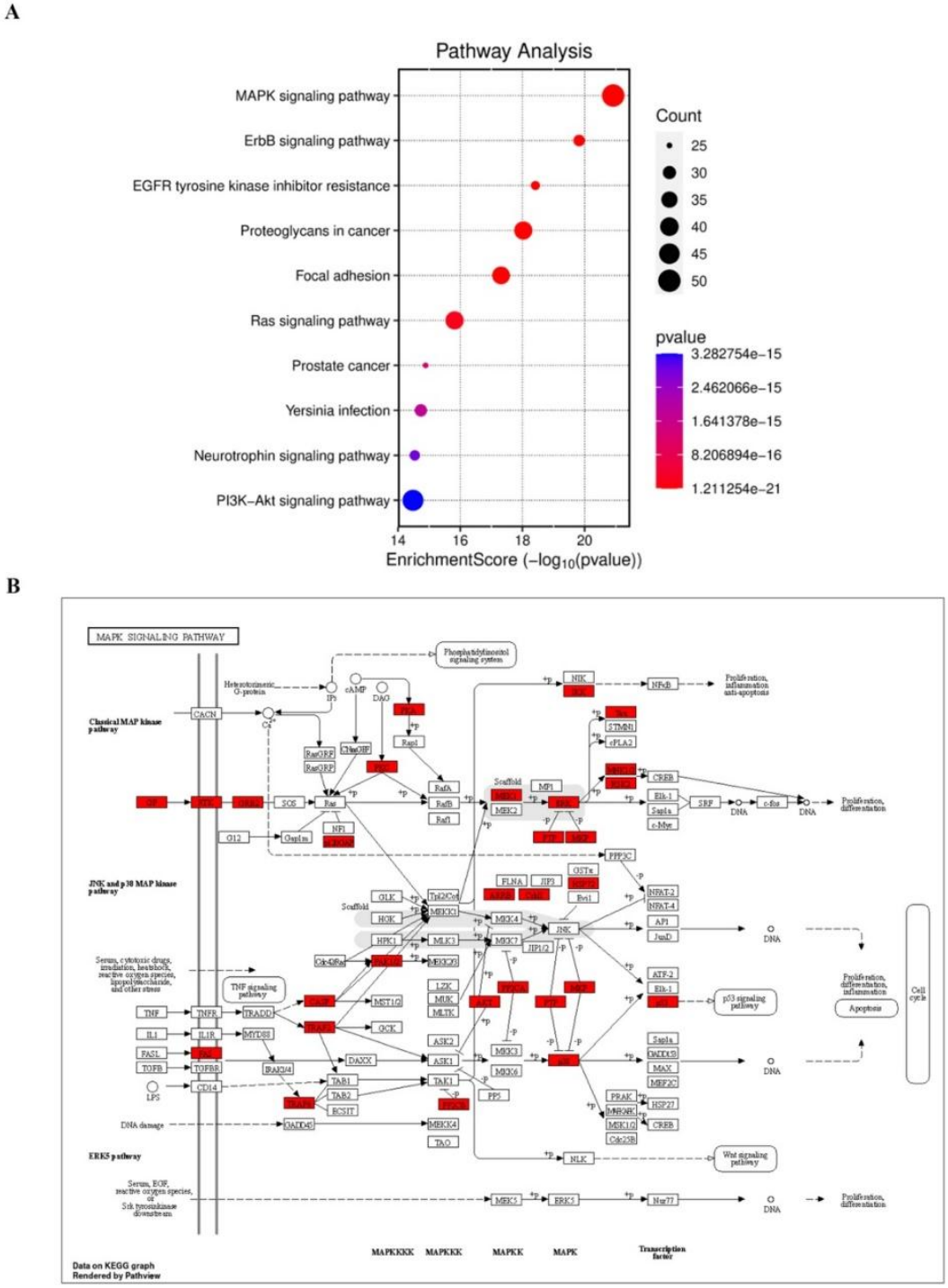

Figure 3 KEGG pathway enrichment analysis.

Bubble chart showing the top 10 KEGG pathway enrichment results (A). A higher gene ratio represents a higher level of enrichment. The size of the dot indicates the number of target genes in the pathway, and the color of the dot reflects the *p*-value range. (B) The highest enrichment pathway, MAPK signaling. Red boxes represent the targets related to the core component-target-pathway network.

The cellular toxicity of quercetin and NAC combination treatment on CRC cells

Pharmacological network analysis revealed the potential effect of quercetin and NAC on the apoptosis pathway. Therefore, we conducted further *in vitro* investigation of the combined effect. The cell viability of combination treatment was investigated by MTT assay. The effects of combined treatment on HT-29

CRC cells showed that the cytotoxic doses of quercetin significantly induced toxicity, reducing cell viability to 61.83 ± 5.46 % at 50 µg/mL and 47.67 ± 5.09 % at 100 µg/mL compared with the control (100 %) at $p < 0.01$. Notably, the quercetin extract combined with 5 mM NAC dramatically inhibited cell viability to 38.33 ± 3.98 % at 50 µg/mL and 28.50 ± 2.35 % at 100 µg/mL compared with the control (100 %), as shown in **Figure 4(A)**. The HCT-116 cells treated with the quercetin extract revealed a significantly inhibited cell viability at 64.33 ± 9.33 % (100 µg/mL), and 43.17 ± 4.96% (200 µg/mL) compared with the control (100 %) at $p < 0.01$. Notably, the combined treatment dramatically inhibited cell viability to 40.83 ± 3.31 % at 100 µg/mL and 25.00 ± 3.90 % at 100 µg/mL compared with the control (100 %), as shown in **Figure 4(B)**. The results suggest that the combination of quercetin and NAC significantly enhances the cytotoxic effect on CRC cells compared to using quercetin alone.

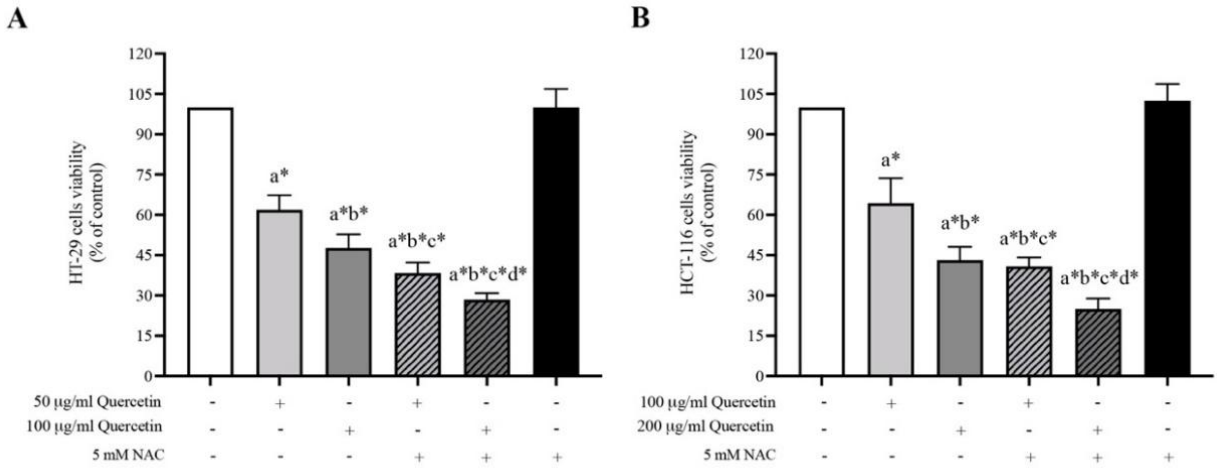

Figure 4 The cytotoxic effects of the quercetin and NAC combination treatment on CRC cells.

The CRC cells were treated with quercetin alone or with a combination of quercetin and NAC for 24 h using the MTT assay. The results were expressed as mean ± SD. * Statistical significance showed $p < 0.01$; a*: the data compared with control, b*: the data compared with 50 or 100 µg/mL quercetin, c*: the data compared with 100 or 200 µg/mL quercetin, and d*: the data compared with 50 or 100 µg/mL quercetin combined with 5 mM NAC treatment, respectively. NAC = N-acetylcysteine.

The cellular apoptosis effect of quercetin and NAC combination treatment on CRC cells

Flow cytometry was used to quantify the population of apoptotic cells using the Annexin V-FITC/PI staining assay. HT-29 cells treated with quercetin alone and quercetin combined with NAC resulted in a significant increase in total apoptotic cells compared with the control. Quercetin increased the population of total apoptotic cells in HT-29 cells to 17.53 ± 2.09 % at 50 µg/mL and 32.70 ± 2.96 % at 100 µg/mL. The combination treatment dramatically increased the population of total apoptotic cells to 53.33±11.59% at 50 µg/mL and 68.34 ± 2.41% at 100 µg/mL (**Figures 5(A)** and **5(B)**). In the HCT-116 cells, quercetin increased the population of total apoptotic cells to 17.79 ± 3.05 % at 100 µg/mL and 26.40 ± 1.52 % at 200 µg/mL. The combination treatment dramatically increased the population of apoptotic cells to 36.05 ± 1.53 % at 100 µg/mL and 43.96 ± 1.25 % at 200 µg/mL (**Figures 5(C)** and **5(D)**). The results indicate that the combination therapy is more effective, with the combination of quercetin and NAC inducing apoptosis more effectively than quercetin alone.

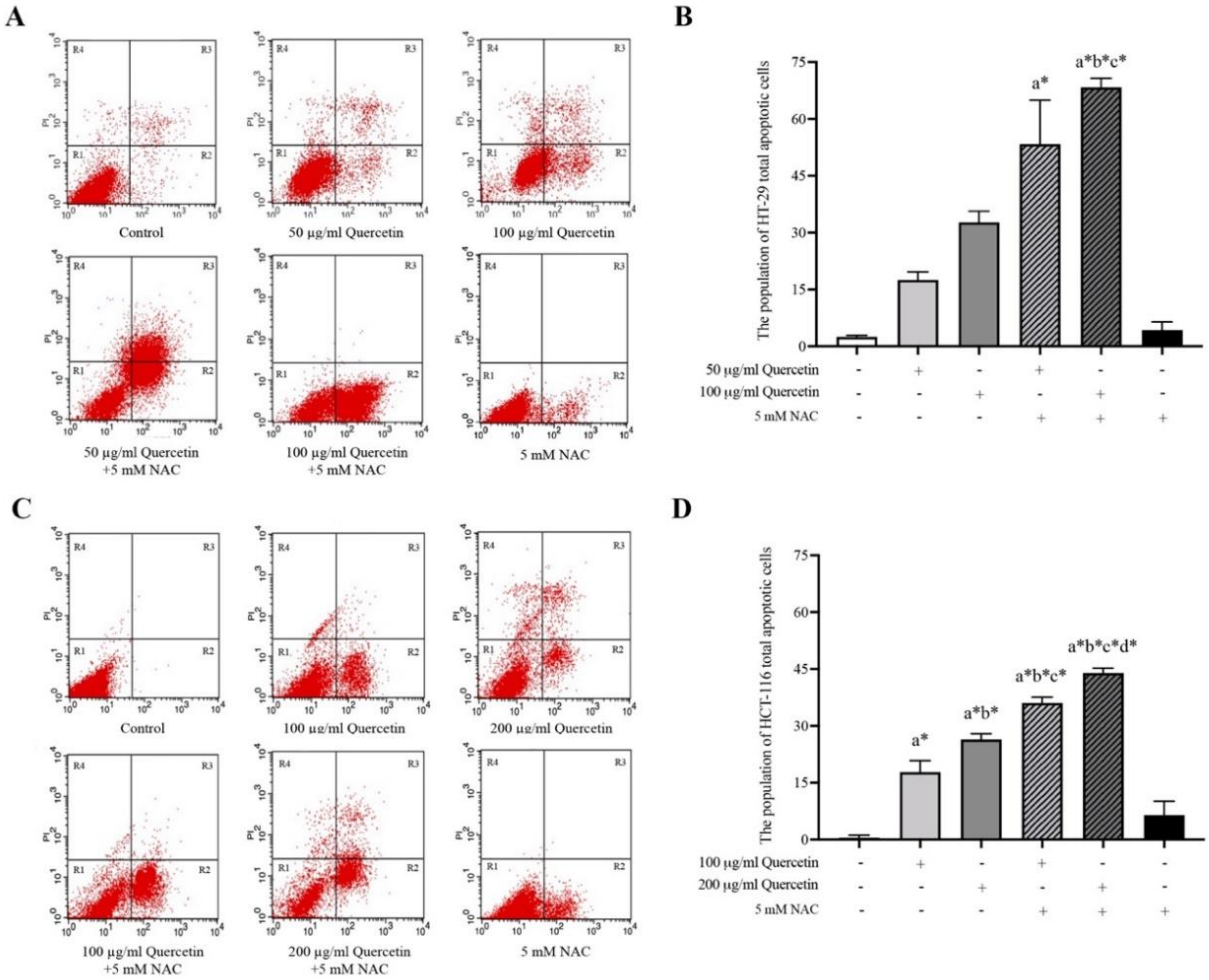

Figure 5 The apoptotic effect of quercetin alone and the combination of quercetin and NAC in CRC cells.

The CRC cells were treated with quercetin alone or with the combination of quercetin and NAC for 24 h using flow cytometry. The histogram of flow cytometric analysis in HT-29 (A) and HCT-116 (B). Results were expressed in a bar graph as the population of total apoptotic cells in HT-29 (C) and HCT-116 (D). The results were expressed as mean ± SD. * Statistical significance at $p \leq 0.05$; a*: the data compared with control, b*: the data compared with 50 or 100 µg/mL quercetin, c*: the data compared with 100 or 200 µg/mL quercetin, and d*: the data compared with 50 or 100 µg/mL quercetin combined with 5 mM NAC treatment, respectively. NAC = N-acetylcysteine.

The effects of quercetin and NAC combination treatment on the expression of apoptosis-related proteins

As combining quercetin with NAC significantly increased the apoptosis efficiency of quercetin, we further investigated the apoptosis pathway using western blot analysis. In HT-29 cells, quercetin at 100 µg/mL significantly induced protein expressions of caspases 3 and 7. Notably, quercetin combined with NAC showed significantly higher protein expression, including caspases 3, 7, 8, and 9, when compared with quercetin alone, as presented in the bar graphs (**Figure 6(C)**). In the HCT-116 cells, quercetin-treated cells at 200 µg/mL potentially induced protein expression of caspases 9. The combined treatment with NAC also showed significantly induced levels of protein expressions in caspases 3, 7, 8 and 9 when compared with quercetin alone, as presented in the bar graphs (**Figure 6(D)**). Our results showed that the combination of quercetin and NAC enhances apoptosis in cancer cells by activating both intrinsic and extrinsic apoptotic pathways. This programmed cell death is mediated by a family of proteases called caspases. Caspases are classified into initiator caspases (e.g., caspase-8 and

caspase-9) and executioner caspases (e.g., caspase-3 and caspase- 7) [29]. The results suggest that the combination of quercetin and NAC enhances apoptosis efficiency, as evidenced by the increased protein expressions of caspases 3, 7, 8, and 9 in both HT-29 and HCT-116 cells.

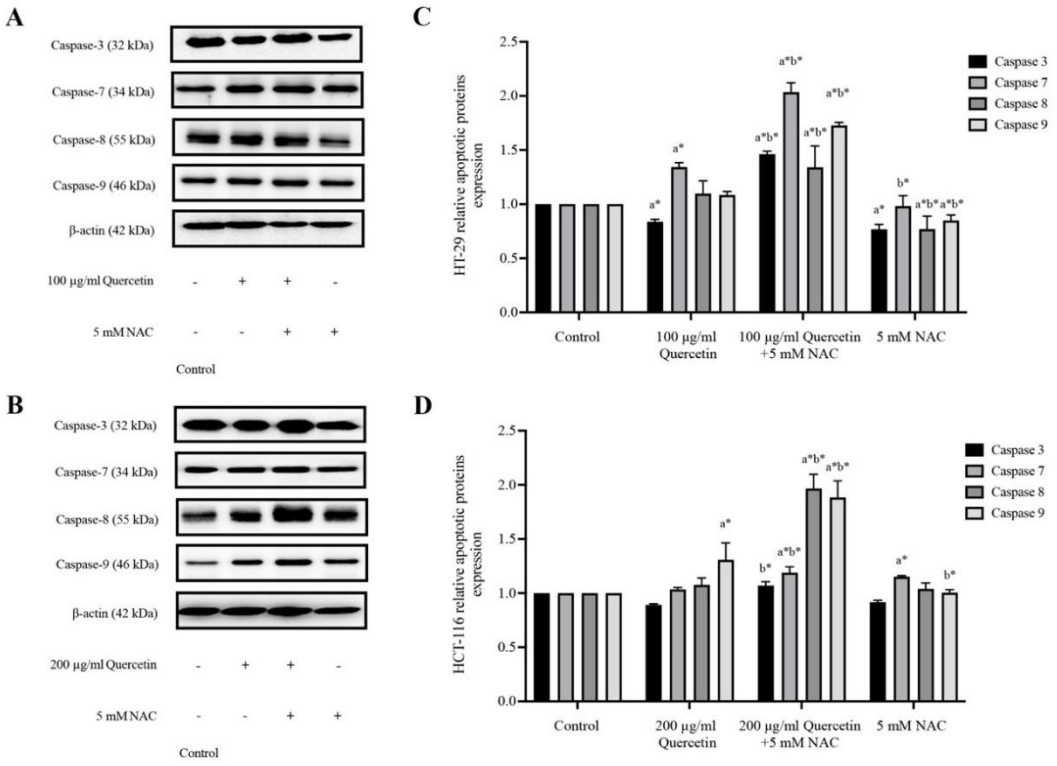

Figure 6 The apoptosis-related protein expression in CRC cells after treatment with quercetin and the combination of quercetin and NAC.

The expressions of apoptotic protein markers-caspases 3, 7, 8 and 9 were investigated by western blot analysis after treatment for 24 h in HT-29 (A) and HCT-116 (B). The β-actin housekeeping protein was used as an internal control. The band intensities of the proteins were analyzed by the ImageJ program and the results expressed as relative intensity of β-actin in HT-29 (C) and HCT-116 (B). * Statistical significance was at $p \leq 0.05$; a*: the data compared with the control and b*: the data compared with 100 or 200 µg/ mL quercetin, respectively. NAC = N-acetylcysteine.

Discussion

Quercetin is a flavonoid found in many fruits, vegetables, leaves, and grains [30]. It possesses potent antioxidant properties, which help protect cells from oxidative damage. Quercetin is the primary compound in the skin of red onions, while quercetin-4-glucoside is the main compound in its bulb [31]. This study used HPLC analysis to identify quercetin extracted from onion peel compared with standard quercetin. The results revealed the single spike peak of standard quercetin and onion peel extract at the quercetin peak. In agreement with previous reports, we found that quercetin and its derivatives are critical dominant constituents of onions [32].

The common use of natural products or isolated compounds has provided a strong rationale for investigating their short- and long-term toxicological effects [33]. Genetic toxicology refers to the study of chemically or physically induced changes to DNA and chromosomes, and the assessment of genotoxicity is a regulatory requirement during pharmaceutical development to evaluate potential carcinogenic risk [34]. Genotoxicity can manifest in a variety of ways, including mutations, chromosomal aberrations, and

changes in chromosome number, all of which can induce carcinogenesis by increasing genomic instability [35]. The genotoxicity of quercetin was also tested in this study by using *in vitro* micronucleus assay**.** From our findings, genotoxicity was observed in the form of DNA damage causing micronucleus formation, which represents a hallmark of chromosome breakage [36]. Quercetin at a high concentration showed no genotoxicity, which did not affect the significant formation of micronuclei**.** This is in accordance with Erlund *et al.*, who studied the pharmacokinetic characteristics of oral administration of 8, 20, and 500 mg of quercetin aglycone in healthy volunteers [37]. The results suggest that the extracted quercetin did not demonstrate significant genotoxicity effects and thus could be classified as a low-risk and useful substances for drug development.

The combined effect of quercetin and NAC on colorectal cancer was investigated by utilized network pharmacology. A total of 363 target genes shared between Quercetin combined with NAC and CRC were identified, leading to the discovery of top hub genes. Key hub genes like TP53, CASP3, and CASP8 play crucial roles in cellular response to reactive oxygen species and apoptosis regulation. Although quercetin is well known for acting as an antioxidant under normal conditions, it can act as a pro-oxidant in cancer, leading to the generation of ROS [38]. Also, in response to high ROS levels, TP53 is activated and functions as a transcription factor, regulating the expression of various genes involved in cell cycle arrest, DNA repair, and apoptosis (B-C) [39,40]. CASP3 and CASP8 are 2 different members of the caspase family of proteases, which play essential roles in apoptosis, or programmed cell death. CASP3 is a key executioner caspase involved in the final stages of apoptosis. It is activated downstream of both intrinsic and extrinsic apoptotic pathways (D) [41]. CASP8 is an initiator caspase primarily involved in the extrinsic apoptotic pathway triggered by death receptors (E) [42].

KEGG pathway analysis revealed that most of the genes were involved in the MAPK pathways. The MAPK pathways were implicated in the induction of apoptosis, with high levels of ROS activating the JNK and p38 MAPK pathways. Correlated with the study of CRC cells, ROS can induce apoptosis through ROS-mediated JNK/p38-MAPK signaling pathways (F) [43]. This mechanism suggests that pro-oxidant therapies may induce cell death in cancer cells. Consequently, quercetin and NAC likely target these pathways to induce apoptosis through their pro-oxidant effects in CRC.

The effects of quercetin-rich onion peel extract and its combination with NAC were further examined *in vitro* on CRC cells. Quercetin exhibits antioxidant and anti-cancer properties, but its therapeutic efficacy is limited due to poor bioavailability and rapid systemic degradation. NAC, known for its antioxidant capabilities and as a precursor to glutathione, has been proposed to enhance the effects of other therapeutic agents by modulating oxidative stress in cancer cells [44]. While NAC offers protective effects, its direct anti-cancer efficacy is limited when used alone [45]. This study aims to enhance the therapeutic efficacy of quercetin against CRC cells, building on previous findings that demonstrated its anti-cancer potential.

In this study, the combination treatment enhanced the apoptotic effect, as evidenced by an increased population of apoptotic cells. These effects were mediated through the activation of apoptotic caspases consisting of upstream initiators, caspases 8 and 9, and downstream effectors, caspases 3 and 7. These results can be supported by previous findings which show that the expression of apoptotic caspases can be activated through an extrinsic pathway involving transmembrane death receptors, leading to the activation of caspases 8 [46]. On the other side, ROS activates mitochondrial, leading to loss of the inner mitochondrial membrane's permeability, resulting in activation of caspases 9 together with caspases 8 leading to directly activating caspases 3/6/7 and triggering apoptosis [47]. Consequently, the treatment with quercetin, particularly in combination with NAC, induced the apoptosis pathway, specifically through the activation of caspases 3, 7, 8, and 9. This study, utilizing both pharmacological and *in vitro* analyses, highlights the potential of quercetin-rich onion peel extract combined with NAC to enhance apoptotic induction in CRC cells.

Conclusions

In conclusion, quercetin constitutes a major compound of onion peel extract. Furthermore, the assessment of its genotoxicity using the micronucleus assay indicated that onion peel had no genotoxic effects.

Pharmacological network analysis identified potential targets affected by the combination of quercetin and NAC, particularly implicating their involvement in inducing apoptosis in CRC. Additionally, *in vitro* studies demonstrated that the combined treatment significantly enhanced the cytotoxic effect and exhibited greater efficacy in inducing apoptosis, as evidenced by increased protein expressions of caspases 3, 7, 8, and 9 in both HT-29 and HCT-116 cells. These findings highlight the potential therapeutic benefits of quercetin-rich onion peel extraction, along with its safety profile, and of NAC combination therapy in enhancing apoptosis efficiency in CRC, representing a potential candidate for alternative medicine in the treatment of human CRC. Further studies, including *in vivo* experiments, are essential to confirm the biological effects observed in animal models. This step will validate the findings in a complex physiological context, provide more robust data for potential clinical applications, and position this approach as a promising candidate for alternative therapies in the treatment of human CRC.

Acknowledgements

The authors are sincerely grateful to the Department of Pathobiology, Faculty of Science, Mahidol University, Thailand, for providing their facilities and laboratory space and Dr. Nasapon Povichit from Detox (Thailand) Co, Ltd. for providing the onion peel extract. This research was funded by the Thailand Science Research and Innovation (TSRI) and co-funded by Detox (Thailand) Co., Ltd, grant number Ph. D. 60I0063.

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