ผลของสารสกัดจากยอดมะม่วงหิมพานต์ในการปกป้องเซลล์จากภาวะเครียดออกซิเดชันที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์ในเซลล์เยื่อบุลำไส้ Caco-2

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บทคัดย่อ

ภาวะเครียดออกซิเดชันมีความสัมพันธ์กับการเกิดโรคเรื้อรังของระบบทางเดินอาหารหลายชนิด ยอดมะม่วงหิมพานต์มีสารต้านอนุมูลอิสระ ต้านการอักเสบ และมีฤทธิ์ต้านความเป็นพิษต่อเซลล์ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดสอบผลของสารสกัดจากยอดมะม่วงหิมพานต์ในการปกป้องเซลล์จากภาวะเครียดออกซิเดชันที่เหนี่ยวนำด้วยสารไฮโดรเจนเปอร์ออกไซด์ (H_2O_2) ในเซลล์เยื่อบุลำไส้ Caco-2 โดยให้ H_2O_2 หรือ H_2O_2 ร่วมกับสารสกัดที่ความเข้มข้นต่างๆ และใช้เควอซิทินเป็น positive control วัดเปอร์เซ็นต์เซลล์ที่มีชีวิตด้วยวิธี MTT assay ระดับอนุมูลอิสระของออกซิเจน (ROS) ภายในเซลล์จากปริมาณฟลูออเรสเซนต์ของสาร 2′,7′-dichlorofluorescein (DCF) การเปลี่ยนแปลงค่าความต่างศักย์ของเมมเบรนไมโทคอนเดรีย (MMP)โดยใช้ Rhodamine123 (Rh123) รวมทั้งการทำงานของ caspase-3 โดยใช้ fluorometric assay kit ผลการศึกษาพบว่า เซลล์เยื่อบุไส้ Caco-2 หลังได้รับสาร H_2O_2 มีการเพิ่มขึ้นของ ROS รบกวนการทำงานของไมโทคอนเดรีย เพิ่มการทำงานของเอ็นไซม์ Caspase-3 และลดเปอร์เซ็นต์เซลล์มีชีวิต ส่วนหนึ่งอาจเกิดจากการตายของเซลล์แบบ apoptosis สารสกัดและเควอซิทินสามารถลดระดับ ROS ในเซลล์ เพิ่มความต่างศักย์ของเมมเบรนไมโทคอนเดรีย ยับยั้งการทำงานของเอ็นไซม์ Caspase-3 และส่งผลให้มีการเพิ่มขึ้นของเปอร์เซ็นต์เซลล์มีชีวิตอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับเซลล์ที่ได้รับ H_2O_2 อย่างเดียว โดยสารสกัดลดการสร้าง ROS ได้มากกว่าเควอซิทินที่ความเข้มข้นเดียวกัน ผลการศึกษาแสดงให้เห็นถึงฤทธิ์ปกป้องเซลล์เยื่อบุลำไส้ Caco-2 จากภาวะเครียดออกซิเดชันที่เกิดจากสาร H_2O_2 ของสารสกัดจากยอดมะม่วงหิมพานต์

คำสำคัญ: ยอดมะม่วงหิมพานต์ เควอซิทิน เซลล์เยื่อบุลำไส้ Caco-2 ภาวะเครียดออกซิเดชัน

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Protective Effects of Cashew (*Anacardium Occidentale*) Leaf Extract Against Hydrogen Peroxide-Induced Oxidative Stress in Intestinal Caco-2 Cells

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Abstract

Oxidative stress with the overproduction of free radicals results in several chronic disorders of the gastrointestinal tract. The cashew (*Anacardium occidentale*) leaves contain high levels of various antioxidants, anti-inflammatory agents and exhibit anticytotoxic properties. This study was aimed to investigate the protective effects of cashew leaf extract against hydrogen peroxide (H_2O_2) induced oxidative stress in Caco-2 cells. Cells were treated with H_2O_2 with and without various concentration of cashew leaf extract (CLE) or quercetin that was used as positive control. Cell viability was assessed by the MTT assay. Intracellular reactive oxygen species (ROS) was evaluated using 2′,7′-dichlorofluorescein (DCF) fluorescence. Mitochondrial membrane potential (MMP) was monitored using rhodamine123 (Rh123) and caspase-3 activity was measured using a fluorometric assay kit. The results showed that exposure of Caco-2 cells to H_2O_2 increased ROS generation, disrupted MMP, activated caspase-3 activity and decreased cell viability that partly resulted from apoptosis. Treatment with CLE or quercetin significantly reduced the level of ROS, improved MMP, inhibited caspase-3 activation and increased cell viability compared to H_2O_2 treatment alone. The greater suppressing H_2O_2-induced ROS generation were observed with CLE treatment compared to quercetin at the same concentrations. These results demonstrated the protective activities of CLE against H_2O_2 induced oxidative stress in intestinal Caco-2 cells.

Keywords: *Anacardium occidentale* (cashew) leaves, Quercetin, Intestinal Caco-2 cells, Oxidative stress

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Introduction

The ingested materials and pathogens can cause inflammation by activating the gastrointestinal epithelium and immune cells to produce inflammatory cytokines and other mediators that contribute further to oxidative stress[1]. The gastrointestinal tract is one of the major sites of interaction between ROS and dietary antioxidants[2]. ROS generated exceed antioxidant defense system can result in the pathogenesis of gastrointestinal tract diseases such as gastritis, enteritis, colitis and associated colorectal cancer[3]. Chronic uncontrolled immune response coupled with the generation and release ROS involve in inflammatory bowel disease (IBD) that characterized by perpetuating intestinal inflammation and tissue destruction[4]. The environmental oxidative stressors such as high fat diet, alcohol and xenobiotic contaminants can enhance the production of colonocyte H_2O_2 and finally ulcerative colitis (UC), a major form of IBD which has symptoms of large intestine inflammation[5]. Additionally, it has shown that ROS-mediated genetic alteration, lipid peroxidation and protein cysteine residues involved in the development of colorectal cancer (CRC) and colitis-associated-cancer (CAC)[6-7]. Therefore, the natural compounds which can suppress ROS production become the one of rational choices for prevention and mitigation of oxidative stress-induced gastrointestinal disorders. The cashew tree (*Anacadium occidentale* L.) is widely distributed in many tropical regions such as Kenya, Indonesia, Vietnam and the south of Thailand. The young and tender cashew leaves can be eaten raw as a side dish or condiment of the local diet[8]. Cashew plants have been used as folk medicine for centuries because its biological properties; antimicrobial, antioxidant, antiulcerogenic, and anti-inflammatory effects from leaf and shoot, fruit, nut and other parts of the plant[9-10]. The cashew leaves exhibited relatively high antioxidant activities assessed by ferric reducing antioxidant power (FRAP), DPPH radical scavenging activity (DPPH), and oxygen radical absorbance capacity (ORAC) assays[9,11]. As a rich source of polyphenols, several polyphenols such as quercetin, gallic acid, kaempferol, catechin, chlorogenic acid, cyanidin and peonidin have been found in cashew leaves [11-13]. *A. occidentale* leaf extract was shown to have oxidative stress resistance and antiaging properties in the *Caenorhabditis elegans* model[14]. Recently, the cashew extract demonstrated a protective effect against glutamate/H_2O_2-mediated oxidative stress-induced neuronal cell toxicity[15]. A previous study also showed that ethanolic extract from cashew leaves inhibited HCl/ethanol induced-gastric lesions in female rats[16]. However, there is a lack of evidence to

support whether or not cashew leaf extract has any inhibitory effect on oxidative damage in intestinal epithelial cells. Meanwhile, a causal role for H_2O_2 in the pathogenesis of gastrointestinal mucosal diseases has been proposed. Therefore, in the current study, the effects of an ethanolic extract of the cashew leaves on H_2O_2 - induced oxidative stress and cytotoxicity in human intestinal cells were investigated.

Materials and Methods

Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), sodium pyruvate, glutamax, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), non-essential amino acids, penicillin/streptomycin, fetal bovine serum (FBS), phosphate-buffered saline (PBS) and trypsin-EDTA (0.05%) were from Thermo Fisher Scientific (Waltham, MA USA). Hydrogen peroxide (30% H_2O_2), dimethyl sulfoxide (DMSO) and ethanol were purchased from Merck (Darmstadt, Germany). Quercetin, 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluo-rescin diacetate (DCFH-DA), Rh123, and caspase-3 assay kit were from Sigma (St. Louis, MO, USA).

Preparation and extraction of plant material

1. Plant material

The fresh cashew leaves were collected from Nakhon Si Thammarat province. Samples were purchased from market and transported on ice in a cool box to the laboratory within 24 hours. The fresh cashew leaves were identified and classified by Plants of Thailand Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University. The voucher specimen number is 015923 (BCU).

2. Plant extraction

The freshly collected cashew leaves were washed, chopped and homogenized before freeze-drying. One hundred gram of freeze-dried cashew leaves was soaked in 900 mL of 70% ethanol (1:9 w/v). The mixture was allowed to stand at room temperature with stirring for 72 hours. The extract solution was vacuum filtered through a Buchner funnel and then, evaporated on the rotary evaporator before freeze-drying. Cashew leaf extract (CLE) was stored at -20°C until further use.

3. Determination of total phenolic content [17]

The Folin-Ciocalteu method was used to determine the total phenolic content. In brief, 0.2 mL of CLE (0.2 mg/mL) was mixed with 0.2 mL of Folin-Ciocalteu phenol reagent. After 20 min incubation in the dark, 1 mL of 8.5% of sodium carbonate (Na_2CO_3) was added to the mixture and incubated for 90 min. The absorbance of the developed color was measured at 765 nm. The total content of phenolic compounds

was determined by a standard curve prepared with gallic acid. The total phenolic content was expressed as mg gallic acid equivalents (GAE).

4. High performance liquid chromatography (HPLC) analysis of quercetin[18]

The CLE was filtered through a 0.22 μm nylon syringe filter and analyzed by HPLC equipped with ZORBAX Eclipse XDB-C18 column (4.6 × 250-mm, 5-μm, Agilent, USA). The mobile phases were binary mixture of methanol and 0.5% acetic acid in ultrapure water 60:40 v/v for quercetin analysis. The measurements were conducted with 10 μL injection volume and 1 mL/min flow rate at 25 °C. Quercetin was detected at 258 nm and its amounts was quantified by comparing peak area with standard curve.

Cell culture and treatment

Human Caco-2 cells were grown in DMEM, supplemented with 10% FBS, 1% sodium pyruvate, 1% glutamax, 1% HEPES, 1% non-essential amino acids and 1% penicillin/streptomycin. Cells were maintained in a cell incubator under humidified 5% CO_2 and 95% air at 37ºC.

Caco-2 cells were seeded in a 96-well plate at a density of 5×10^4 cells per well and grown for 24 h to allow attachment to the culture plate prior to commencing each experiment. Then, cells were treated with CLE and quercetin with or without H_2O_2 for 24 h to determine the effects of CLE on H_2O_2 induced oxidative damage in cells. Quercetin was selected as a positive control in this study. Stock solutions of CLE and quercetin were dissolved in 1% and 2% DMSO, respectively. These solutions were diluted at least 1,000 times in serum free medium before being added to the cells, resulting in a maximum of 0.002% of DMSO.

Determination of cell viability

Cell viability was assessed by the MTT method[19]. After treatment, the medium was removed from each well, after that 200 μL of serum free medium was added. Then 10 μL of MTT reagent was added to each well and incubated at 37°C for 2 h. The medium with MTT was aspirated from each well and 100 μL of DMSO were added to dissolve the formazan crystals. The absorbance of the samples was read at the measure wavelength of 570 nm and reference wavelength 630 nm. The results were expressed as percentage of cell viability compared with the control. % Cell viability = (Absorbance of treated cells / Absorbance of control cells) x 100

Measurement of intracellular reactive oxygen species

Intracellular ROS was evaluated using the fluorescent probe 2′, 7′-dichlorofluorescein diacetate (DCFH-

DA)[20]. Briefly, the cultured Caco-2 cells were seeded in a black 96-well plate at a density of 1×10^5 cells/well for 24 h then incubated with 20 µM DCFH-DA in the loading medium at 37 °C for 30 min. Cells were washed with PBS and treated with CLE and quercetin with or without H_2O_2 for 24 h. After treatment, the fluorescence intensity was captured using a microplate reader with an excitation wavelength of 485 nm and emission wavelength of 535 nm. The results were expressed as a percentage of fluorescence intensity in treated cells relative to control, calculated as follows: % ROS production = (Fluorescence intensity of treated cells / Fluorescence intensity of control cells) x 100

Determination of mitochondrial membrane potential (MMP)

Monitoring MMP was performed using a fluorescent dye Rh123 [21]. Briefly, cells were seeded in a black 96-well plate at a density of 1×10^5 cells/well and incubated at 37°C, 5% CO_2 for 24 h. The cells were treated with CLE and quercetin with or without H_2O_2 for 24 h. After treatment, the cells were incubated with 10 µg/mL Rh123 in the loading medium at 37 °C for 30 min. Then, cells were washed twice with PBS and analyzed by microplate reader with an excitation wavelength of 485 nm and emission wavelength of 530 nm. The results were expressed as a percentage of fluorescence intensity in treated cells relative to control, calculated as follows: % Rh123 fluorescence intensity = (Fluorescence intensity of treated cells / Fluorescence intensity of control cells) x 100

Determination of caspase-3 activity

Caspase-3 activity was measured using a fluorimetric assay kit (Sigma)[22]. Briefly, Cells were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated at 37°C, 5% CO_2 for 24 h. The cells were treated with CLE and quercetin with or without H_2O_2 for 24 h. After that, the plate containing the treated cells was kept on ice. The cells in each well were added with 25 µL lysis buffer and incubated on ice for 20 min. The 200 µL assay buffer containing substrate was added and mixed well by pipetting. Then, 200 µL of cell lysate was transferred to a fluorimeter 96 well plate. Finally, the fluorescence intensity was captured using a microplate reader with an excitation wavelength of 360 nm and emission wavelength of 460 nm. The results were calculated using a 7-Amino-4-methylcoumarin (AMC) standard curve. Protein expression was measured using a Bicinchoninic Acid (BCA) protein assay. The results were expressed as activity of caspase-3 (nmol product/ mg protein).

Statistical Analysis

All values were presented as mean ± SD. The level of significant was $p < 0.05$. The results were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests to compare differences among groups by using GraphPad Prism software, version 5.00.

Results

Percentage yield and phytochemical constituents of CLE

The extract yield of CLE prepared by 72 h soaking in 70% ethanol was 13.45 % (13.45 g per 100 g dried leaf powder). The total phenolic content of CLE in the present study expressed as milligram gallic acid equivalent per gram of dry matter extract was 437.57 ± 2.34 mg GAE/g DM. The reverse phase HPLC analysis confirmed the presence of quercetin with a value of 168.1± 4.3 µg/g DM.

Cytotoxicity of CLE and quercetin on Caco-2 cells

The cytotoxicity of CLE and quercetin in Caco-2 cells was assessed to determine the appropriate treatment concentration for subsequent experiments. The concentrations not higher than 5 µg/mL and 100 µg/mL for CLE and quercetin, respectively, exhibited no significant difference in cell viability when compared to control group (Figure 1A, B). These concentration ranges of CLE and quercetin were considered non-toxic and were selected for use in subsequent experiments.

Hydrogen peroxide- induced cytotoxicity and oxidative stress in Caco-2 cells

Caco-2 cells were treated with 400 to 1600 µM H_2O_2 for 24 h. After treatment, the cell viability was determined using MTT assay. The viability of H_2O_2 treated cells was reduced in a dose-dependent manner with significant decreases at the H_2O_2 concentrations ≥ 600 µM. To determine the induction of intracellular ROS by H_2O_2, cells were exposed to 400, 600, 800 and 1000 µM of H_2O_2 for 24 h. H_2O_2 at concentrations ranging 600 to 1000 µM showed dose-dependently enhancing ROS generation. However, intracellular ROS level was significantly increased only in the presence of 1000 µM H_2O_2 that lowered Caco-2 viability to 71.85%, (Figure 2A, B). Therefore**,** 1000 µM H_2O_2 was chosen to be used to induce oxidative stress in further experiments.

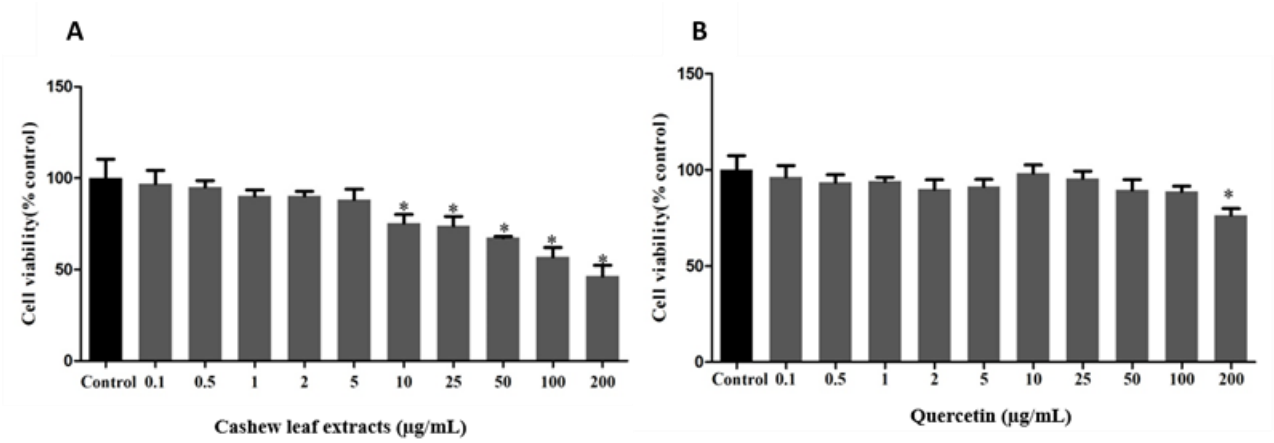

Figure 1. Cytotoxicity of CLE and quercetin in Caco-2 cells. Cells were treated with different concentrations of CLE (A) and quercetin (B) for 24 h before measuring cell viability by MTT assay. The results are expressed as the means ± SD (n=3); $^{*}p<0.05$ compared to the untreated control

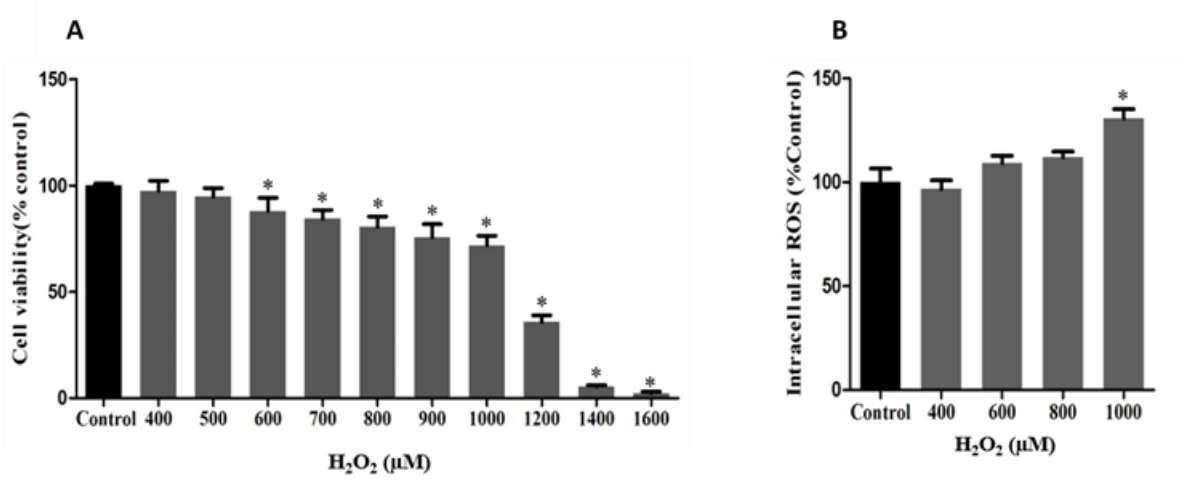

Figure 2. H_2O_2- induced cytotoxicity and oxidative stress in Caco-2 cells. Cells were treated with different concentrations of H_2O_2 for 24 h before measuring cell viability by MTT assay (A) and assessing intracellular ROS using DCFH-DA assay (B). The results are expressed as the means ± SD (n=3); $^{*}p<0.05$ compared to the untreated control.

Effects of CLE and quercetin on H_2O_2 - induced reduction of Caco-2 cells viability

H_2O_2 at 1000 µM significantly reduced cell viability to the level of 74.7% of control. Co-treatments with CLE (0.1 to 5 µg/mL) or quercetin (0.1 to 10 µg/mL) with 1000 µM H_2O_2 resulted in % cell viability that were significantly higher than that of H_2O_2 alone (90-103% for CLE and 83-85% for quercetin. At the same dose of 2 µg/mL co-treatments with CLE or quercetin, the percent cell viabilities were increased up to 16.8% (Figure 3A) and 13.3 % (Figure 3B) compared to H_2O_2 treatment.

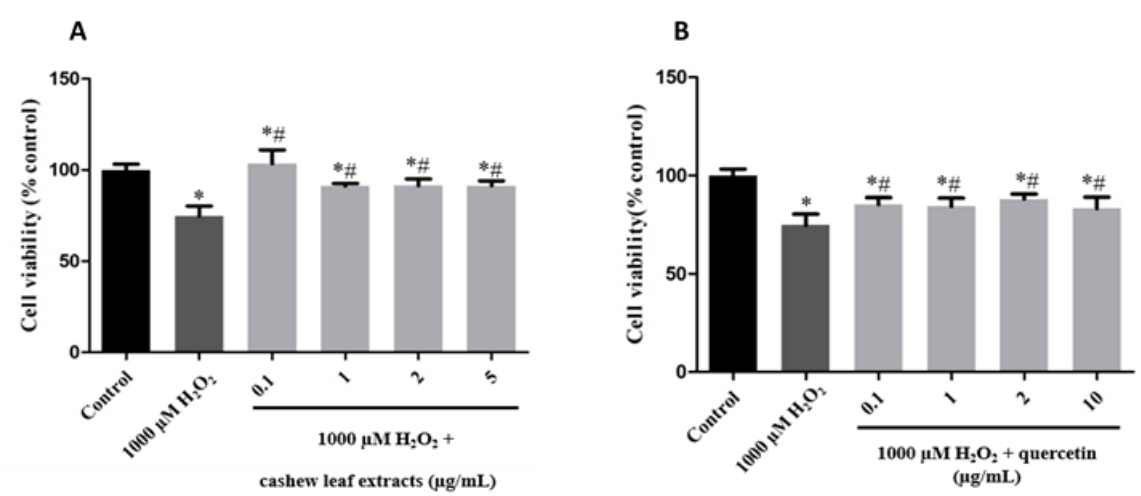

Figure 3. Cell viability of Caco-2 cells co-treated with 1000 µM H_2O_2 and various concentrations of CLE (A) or quercetin (B) for 24 h. The results are expressed as the means ± SD (n=6); $^{*}p<0.05$ compared to the untreated control, $^{\#}p<0.05$ compared to H_2O_2 treatment alone.

Effects of CLE and quercetin on H_2O_2 - induced intracellular ROS generation in Caco-2 cells

ROS levels were increased significantly when the cells were exposed to 1000 µM H_2O_2 for 24 h (122.6% of control). Co-treatment H_2O_2 with non-toxic concentrations of CLE or quercetin were significantly dose-dependent suppressed intracellular oxidative stress induced by H_2O_2 (Figure 4A, B). CLE and quercetin could scavenge and inhibit H_2O_2 -induced ROS generation in Caco-2 cells. At the same concentration, CLE showed the stronger antioxidant activity than quercetin. It was noted that all doses of CLE suppressed the ROS level to lower than H_2O_2 treatment and control whereas quercetin did not.

Effects of CLE and quercetin on H_2O_2 - induced loss of MMP and increase in caspase activity in Caco-2 cells

Mitochondrial function is a key indicator of cell health. It has reported that intracellular ROS and apoptotic cell death are associated with MMP collapse. This study assessed the effects of CLE and quercetin on the MMP decline by using the RH123 fluorescent dye. Rh123 is lipophilic, it normally accumulates in the mitochondrial membrane. An intact MMP is associated with the cellular uptake of Rh123 and high fluorescence. As shown in Figure 5A, 1000 µM H_2O_2 treatment for 24 h decreased the MMP evidenced by the reduction of mean Rh123 fluorescence to 65.48 % relative to the untreated control. CLE or quercetin at 5 µg/mL raised the mean MMP 10%, and 5%, respectively, higher than that of H_2O_2 treatment alone.

The CLE showed significant and stronger increased MMP level in H_2O_2 induced oxidative stress in cells compared to quercetin.

As shown in Figure 5B, following 24 h exposure to 1000 µM H_2O_2, there was increase in caspase-3 activity to 246.6%, compared to untreated cells. It was shown that 5 µg/mL CLE or quercetin exerted significant suppression on H_2O_2, induced caspase-3 activity, to 166.9 and 178.2 %, respectively.

Discussion

Ingested xenobiotics and microbial infections can induce oxidative injury and gastrointestinal (GI) inflammatory responses involving the pathogenesis of various GI diseases[23-24]. Various foods and dietary components possessed antioxidant activities will offer as a promising way to prevent and treat the GI diseases caused by the excessive ROS[25]. *A. occidentale* or cashew leaves are consumed as one of Thai indigenous vegetables. It has been reported to be good sources of antioxidants and has been shown to possess antioxidant properties[11]. In this study, the total phenolic and quercetin contents of CLE was 437.57 ± 2.34 mg GAE/g DM and 168.1± 4.3 µg/g DM, respectively. A recent study by Sassi et al. (2022) found lesser amounts of total phenolic content and quercetin in the extract of freeze-dried *A. occidentale* leaves with the values of 12.28±2.97 mg GAE/g dry weight and 25.59±1.32 µg/g dry weight, respectively[13]. Absolute methanol was used as extraction solvent with the solvent to sample ratio similar to our study. However, the extraction was carried out by the shorter extraction time (two times of 1 h shaking). Cashew leaves were assessed to have the highest antioxidant properties among the leaves of 10 species of ulam herbs[12]. Andarwulan et al. (2012) reported the three flavonoids, quercetin, kaempferol, and myricetin content in cashew leaves and quercetin accounted for 87% of total measured flavonoids[26]. The pharmacological properties of cashew leaves as antioxidant, anticancer, hypoglycaemic, hypolipidemic, anti-cholesterolemic, anti-ulcerogenic, anti-hypertensive, analgesic and anti-inflammatory activities were demonstrated[12]. Despite the protective roles of cashew leaves has been reported in the different aspects, the underlying molecular mechanisms regarding antioxidant and gastrointestinal cytoprotective effects have not been fully revealed. In addition, it has been proposed that the natural combination of phytochemicals in plant-based foods is responsible for their potent antioxidant activity. The single antioxidant may not be able to replace the combination of natural phytochemicals in fruit and vegetables for deriving the health benefits[27]. Here, the

protective effects of CLE against H_2O_2 induced oxidative toxicity in human intestinal cells were investigated using its major flavonoid quercetin as positive control in all experiments.

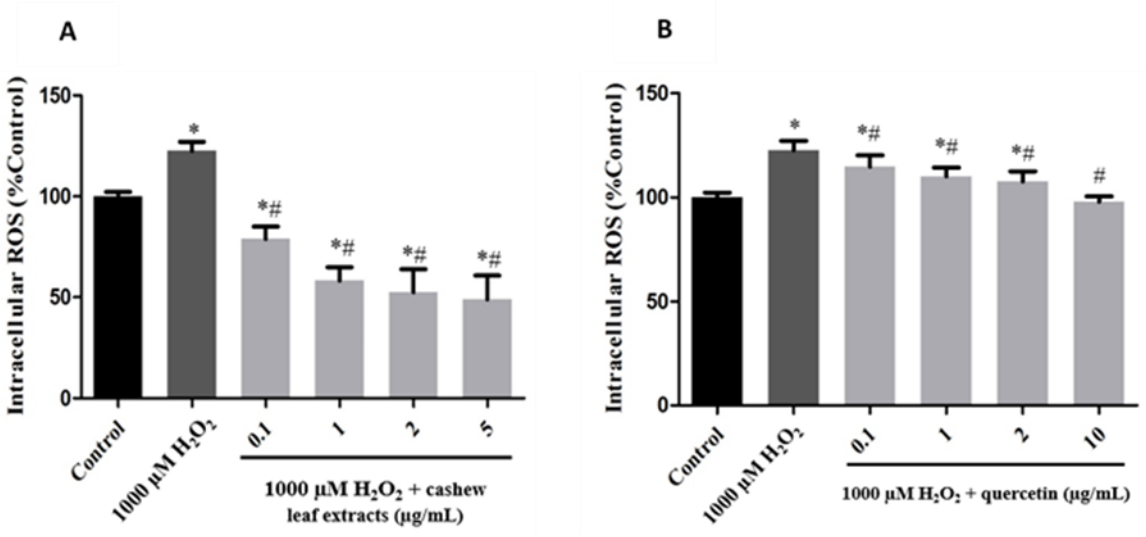

Figure 4. Intracellular ROS generation in Caco-2 cells co-treated with 1000 µM H_2O_2 and various concentrations of CLE (A) or quercetin (B) for 24 h. The results are expressed as the means ± SD of (n=6); $^{*}p<0.05$ compared to the untreated control, $^{\#}p<0.05$ compared to H_2O_2 treatment alone.

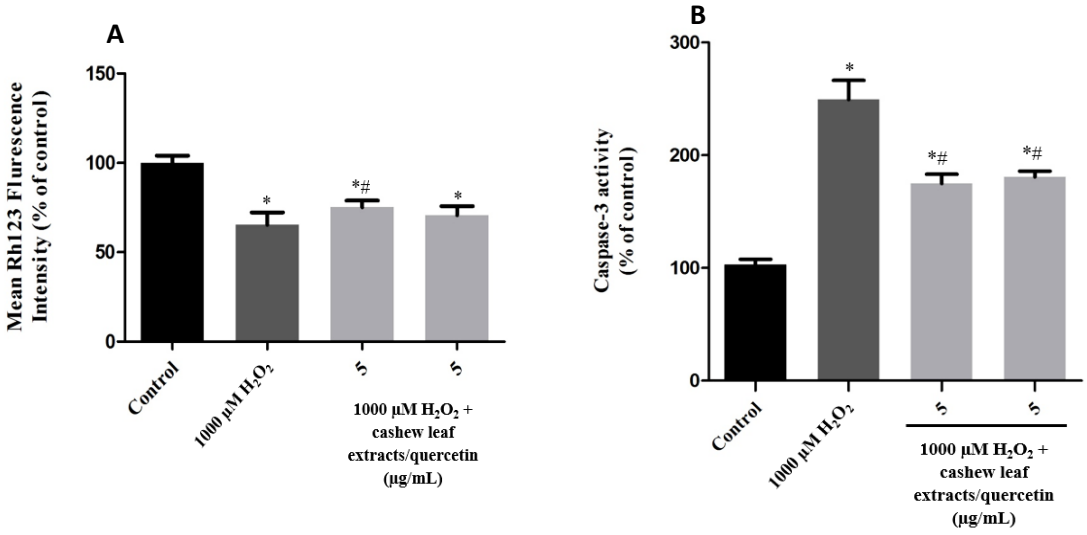

Figure 5. (A) Mitochondrial membrane potential in Caco-2 cells co-treated with 1000 µM H_2O_2 and 5 µg/mL CLE or 5 µg/mL quercetin for 24 h. (B) Caspase-3 activity in Caco-2 cells co-treated with 1000 µM H_2O_2 and 5 µg/mL CLE or 5 µg/mL quercetin for 24 h. The results are expressed as the means ± SD (n=6); * $p<0.05$ compared to the untreated control, $^{\#}$ $p<0.05$ compared to H_2O_2 treatment alone.

Because of the similar morphology, marker enzyme, microvillar structure, tight junction and permeability as small intestinal epithelial cells, Caco-2, originating from human epithelial colorectal adenocarcinoma cells was used as representative of the small intestinal epithelium[28]. Caco-2 is one of the cell antioxidant models established to study the antioxidant activity of active substances that reflect by indicators such as intracellular ROS level, degree of cell damage and cell survival rate[29]. In the present study H_2O_2 treatment was used to produce oxidative stress induced intestinal epithelial cell damage due to its solubility in both lipid and aqueous environments, freely diffusing through cell membranes before reacting with specific molecular targets, modulating essential cellular functions or triggering cell death by apoptosis or necrosis with time and concentration dependent[30]. The oxidative degradation of membrane lipids, loss of membrane integrity and increased permeability was found in Caco-2 cells exposed to 1 mM of H_2O_2 for 24 h. These results were demonstrated by staining with 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent stain that could penetrate cell membranes, bind to DNA of dead or dying cells[31-33]. At high concentrations H_2O_2 can damage energy-transforming cell systems. The reaction between iron and H_2O_2 (Fenton reaction) generated by donating an electron from soluble Fe (II) to an H_2O_2 molecule, results in H_2O_2 decomposition into hydroxyl radicals which aggressively attack and oxidize virtually all organic molecule[34]. Role of mitochondria in the pathogenesis of apoptosis has been well defined. A previous study found that exposure intestinal epithelial cell line SW-480 to 4 mM H_2O_2 increased the percentage of apoptotic cells from 10 to 69 within 24 h. The appearance of decreased MMP and release of cytochrome c suggested mitochondrial dysfunction might be the key event in the development of apoptosis[35]. Apoptosis mainly consists of two main pathways, the extrinsic pathway triggered by external stimuli or ligand molecules that involve death receptors (DRs) and the intrinsic mitochondrial mediated pathway. Both extrinsic and intrinsic pathways converge at the same point of final executioner caspases; caspase-3, caspase-6 and caspase-7[36].

The results of current study showed that the treatment with H_2O_2 at 1000 µM caused significant reduced cell survival, increased intracellular ROS, declined MMP, and enhanced caspase-3 activity compared to control. Co-treatment of H_2O_2 with various concentrations of CLE or quercetin were able to prevent a decline in cell viability compared to H_2O_2 treatment alone. The high level of ROS lead to

irreversible damage of macromolecules, thus eventually leading to disruption of cellular functions[37]. The intracellular generation of ROS has the great impact on cell function and survival. Cells normally need antioxidative mechanisms that fine-tune ROS levels, ensuring normal cellular functioning. ROS regulates different transcription factors, enzymes, and/or other proteins inducing signaling pathways that assure proper function. This redox signaling comprises oxidative eustress or physiological oxidative stress[38]. The measurement of intracellular ROS was done as index of the overall oxidative stress in living cells. Cells exposed to H_2O_2 had the increased levels of ROS concomitantly with decrease in cell viability. CLE or quercetin co-treatment with 1000 µM of H_2O_2, showed significantly inhibitory effect on intracellular ROS. The results demonstrated that CLE and quercetin could act as scavengers of the ROS in Caco-2 cells. However, the property of enhancing the cellular antioxidant defense system could not be excluded. At the same concentration range 0.1- 2 µg/mL, CLE could suppress H_2O_2 enhanced overproduction of ROS to the level lower than that of control while quercetin could not.

Regarding to MMP and caspase-3 activity, ROS leads to the opening of mitochondrial PT pore delay following by decrease in MMP, release of cytochrome c, and caspase cascade reaction, eventually leading to apoptosis[39]. The mean Rh123 fluorescence of Caco-2 cells decreased to 65.48% by 1000 µM H_2O_2 exposure. The results suggested that H_2O_2 induced cell death partly from the disruption of mitochondria mediated apoptosis pathway. CLE at concentrations 5 µg/mL significantly improved the MMP decreasing while quercetin at the same concentration showed an increasing trend but not statistically significant. Caspases regulate apoptotic cell death via cleavage of numerous target proteins. Among all executioner caspases, caspase-3 is the most important because the convergence of the extrinsic and intrinsic pathways occurs at the proteolytic activation of caspase-3. Once caspase-3 is activated, the further activation of the caspase 3-activated DNase (CAD) and protease lead to degradation of nucleus DNA, cytoskeletal proteins, chromatin condensation and finally apoptotic cell death[36]. Our results showed that 1000 µM H_2O_2 significantly increased caspase-3 activity in Caco-2 cells. Both 5 µg/mL CLE or quercetin co- treatment significantly reduced caspase-3 activity to the levels lower than H_2O_2 alone. These results may be explained by antioxidant contents and their activities that were found in cashew leaves[9-13]. In addition, the protective effects against oxidative toxicity

of CLE in neuronal cells, C. elegans and in experimental animals were reported[14-16]. Recently, quercetin, a major flavonoid in cashew leaves was shown to improve the proliferation and apoptosis status in Caco-2 cells under H_2O_2-induced oxidative stress by increasing intracellular glutathione (GSH). In the same study, quercetin was also demonstrated the ability to ameliorate dextran sulfate sodium (DSS) -induced colitis in mice[40].

A number of evidence suggests the health benefits of fruits, vegetables, whole grains, and other plant foods are attributed to the synergy or interactions of bioactive compounds and other nutrients in whole foods. It has shown that fruits and vegetable phytochemical extracts exhibit strong antioxidant and antiproliferative activities and the major part of total antioxidant activity is from the combination of phytochemicals[41]. For this study, 2 µg/mL or 5 µg/mL of CLE that contained quercetin only 0.34 ng/mL and 0.84 ng/mL, respectively showed the protective effects against oxidative stress induced cytotoxicity in intestinal Caco-2 cells that were comparable to that of quercetin at the same concentrations. The results from the present study suggest that the synergistic interactions of bioactive compounds in CLE may provide the protective effects against the oxidative induced intestinal epithelium damage. In addition to quercetin, the phytocomponents presented in cashew leaves such as cyanidin, β-carotene, myricetin and chlorogenic acid had been shown to exhibit the bioactivity toward alleviating oxidative stress in Caco-2 cell model[42-45].

Conclusion

The present study investigated the protective effects of CLE against H_2O_2 induced oxidative toxicity in intestinal Caco-2 cells. The antioxidative and anti-cytotoxic effects were examined by measurements of the bioactive compounds and the effects on cell viability, intracellular ROS production, MMP and caspase-3 activity. Exposure of Caco-2 cells to 1000 µM H_2O_2 decreased cell viability and mitochondria membrane potential while increased intracellular ROS and caspase-3 activity. Treatment with CLE and quercetin that was a positive control, significantly increased cell viability, improved MMP, reduced the level of ROS and inhibited caspase-3 activation. These results suggest cashew leaves has antioxidative and anti-apoptotic activities against H_2O_2 induced oxidative stress and toxicity in intestinal Caco-2 cells. Moreover, CLE exerted the similar protective ability against oxidative stress induced cytotoxicity in the human intestinal cells as quercetin at the same concentrations. These results may be explained by the additive and synergistic

effects of phytochemicals in CLE. However, the other bioactive compounds in addition to quercetin in CLE were not determined. It cannot conclude that what the main bioactive compounds were responsible for the bioactivity of CLE. The further study should be carried out for determining the ROS signaling pathway responsible for the protective effects of CLE in oxidative induced intestinal cell damage.

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Conflict of interest

The authors declare no conflict of interest.

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