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Original Article

Enhancement of apoptosis in HCT116 and HepG2 cells by *Coix lacryma-jobi* var. *lacryma-jobi* seed extract in combination with sorafenib

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ABSTRACT

Objective: *Coix lacryma-jobi*, a highly regarded Asian herb widely used in traditional Chinese medicine, is recognized for its dual benefits in promoting overall health and treating various diseases. While it exhibits moderate anticancer efficacy when used alone, this study investigated the enhanced anticancer potential of raw and cooked *Coix lacryma-jobi* var. *lacryma-jobi* (CL) seed extracts in combination with sorafenib against HCT116 and HepG2 cancer cell lines. The combination of sorafenib with other anticancer agents, including natural extracts, has garnered significant attention as a promising strategy for developing more effective cancer therapies.

Methods: Dry powders of raw (R) and cooked (C) CL seeds, obtained from a local commercial source in Thailand, were extracted and fractionated using ethanol (E), dichloromethane (D), ethyl acetate (A), and water (W) to produce eight fractions: CLRE, CLCE, CLRD, CLCD, CLRA, CLCA, CLRW, and CLCW. The coixol content in raw and cooked seed extracts was quantified and expressed as μg of coixol per gram of extract. The cytotoxic effects of these fractions were evaluated against HCT116 and HepG2 cells using the MTT assay. Fractions demonstrating the most significant cytotoxic responses were combined with sorafenib to evaluate their synergistic effects. Apoptosis induction and mitochondrial membrane potential (MMP) were assessed, and the underlying mechanism of apoptosis was explored by analyzing reactive oxygen species (ROS) generation and antioxidant protein expression levels. Additionally, the combination treatment's effect on the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathway was investigated.

Results: One gram of CLCE and CLCD extracts contained higher coixol levels (7.02 μg and 9.69 μg, respectively) compared to CLRE and CLRD (2.66 μg and 5.96 μg, respectively). Coixol content in CLRA, CLRW, and CLCW fractions was undetectable under the study conditions. All extract fractions exhibited IC_{50} values exceeding 1 mg/mL after 24- and 48-hour incubations with HCT116 and HepG2 cells, indicating limited cytotoxicity when used independently. CLRD and CLCD fractions were selected for combination studies at a concentration of 1 mg/mL, combined with sub-IC_{50} concentrations of sorafenib to minimize its side effects. This combination significantly increased cytotoxicity, inducing apoptosis in HCT116 and HepG2 cells by elevating ROS levels and reducing the expression of superoxide dismutase 2 and catalase. Furthermore, the combination treatment downregulated the PI3K/AKT/mTOR pathway, indicating a targeted anticancer mechanism.

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Conclusion: The combination of CLCD with sorafenib demonstrates significant potential as a strategy for future anticancer therapies. This CL seed extract, cultivated and commercially available in Thailand, shows promise as a natural supplement to enhance the efficacy of chemotherapy in upcoming clinical anticancer applications.

1. Introduction

Numerous compounds obtained from plant extracts have shown anticancer properties while causing minimal side effects on normal cells. As a result, the use of plant extracts in combination with traditional chemotherapy drugs at lower dosages is being considered as a promising approach to enhance anticancer effectiveness and reduce damage to healthy cells. *Coix lacryma-jobi* L. (*Coix*), commonly known as adlay or Job's tears, is a native Asian grass crop classified within the Poaceae family. Among the four recognized varieties of *Coix*, *Coix lacryma-jobi* var. *lacryma-jobi* (CL) is referred to as "薏苡 (*yì yǐ*)" in traditional Chinese medicine (TCM), where it is regarded as a top-grade herb (Flora of China, 2006; Kuo, Chen, & Chiang, 2012). This classification is also acknowledged by *the World Flora Online (WFO) Plant List* (WFO Plant List, 2024). It has been reported to promote numerous health benefits, including nourishing life, enhancing vital energy, revitalizing the body, and extending one's lifespan. The dehulled and polished endosperm of *Coix* seeds has been used for millennia as both a grain with therapeutic properties and a nutrient-rich food with many health advantages for treating a variety of illnesses (Huang et al., 2021a; Kang et al., 2020; Kuo, Chen, & Chiang, 2012). *Coicis Semen* (Yiyiren in Chinese), derived from the dry, mature seeds of another variety, *C. lacryma-jobi* var. *ma-yuen* (Rom. Caill.) Stapf (CM), is classified in the 2015 edition of the *Chinese Pharmacopoeia*. It is not only a commonly prescribed TCM, but also a highly valued dietary ingredient, recognized forits potential therapeutic effects in alleviating various ailments, including cancer (Chiang et al., 2022; Son et al., 2019; Zhu et al., 2020). The Chinese herbal mixture, Qu-Yu-Jie-Du Decoction (QYJD), which contains 20.8% *Coix* seed, has a long history of being effective in treating colorectal cancer cells (Fang et al., 2018). Studies have shown that QYJD can alleviate colitis in mice by reducing inflammation, specifically by decreasing neutrophil and macrophage infiltration in colon tissues (Zhao et al., 2022). Additionally, QYJD Granules, widely used in TCM for their anticancer properties, have been shown to improve outcomes in patients with unresectable hepatocellular carcinoma (HCC) when combined with transarterial chemoembolization. This combination therapy is also more cost-effective compared to using chemotherapy drugs alone (Zhao et al., 2017). Therefore, the use of *Coix* in TCM suggests its potential as a candidate for future cancer therapeutic strategies.

Coix seeds exhibit the highest ratios of carbohydrates and proteins compared to other cereal crops, containing 67% carbohydrates and 20% proteins. The predominant protein found in *Coix* seeds is coixin, which belongs to the seed storage prolamin category. In addition, numerous secondary metabolites are identified, including benzoxazinoids, lactams, phytosterols, and coixol (6-methoxybenzoxazolinone) (Kang et al., 2020). Compounds derived from *Coix* seeds have been shown to have several pharmacological properties. Out of the ten chemicals extracted from *Coix* seeds, only the coixol and 2-*O*-*β*-glucopyranosyl-7-methoxy-*2H*-1,4-benzoxazin-3(4*H*)-one demonstrates a more potent anti-melanin formation compared to the positive control arbutin, while not impacting cell survival (Amen et al., 2017). It has been suggested that 3-*O*-(*trans*-4-feruloyl)-*β*-sitostanol, 3-*O*-(*cis*-4-feruloyl)-*β*-sitostanol, and *β*-sitostanol, together with linoleic acid and oleic acid, from the whole seed extracts of CM contribute to the anti-dyslipidemic and hypercholesterolemic activities in hyperlipidemic hamsters fed a high-fat diet (Huang et al., 2021a). The administration of six hydroxy unsaturated fatty acids derived from acetone and ethanolic seed extracts of CM resulted in enhanced insulin sensitivity in individuals with diabetes, leading to reductions in both glucose and lipid levels, all while avoiding significant adverse reactions (Yokoi, Mizukami, Nagatsu, Tanabe, & Inoue, 2010). Three of the ten phenolic acids found in CM seeds: ferulic acids, *p*-coumaric acids, and sinapinic acids, improved the gut microbiome homeostasis to ameliorate abnormal serum lipid profiles in high-cholesterol diet-fed rats (Wang et al., 2015).

The seed extracts of *Coix* demonstrated specific anticancer properties without affecting normal cells (Amen et al., 2018; Jiang, Li, Zhang, Wang, & Li, 2024; Kuo, Chen, & Chiang, 2012; Meng et al., 2023; Sainakham, Manosroi, Abe, Manosroi, & Manosroi, 2016; Yin et al., 2023). Additionally, the sprout extract of CM exerted antiproliferative apoptotic effect in HeLa cells (Son et al., 2019), HCT116, and CCD-18Co colorectal cancer cells (Son et al., 2017). The ethanolic extracts from the husk and seed coat of the CM exhibited growth-inhibiting activity through apoptotic activation in breast cancer MCF-7 and cervical cancer HeLa cells (Chiang et al., 2022). CM, a medicinal material of the Qingyihuaji Decoction (QYHJ) formula in TCM, consists of 68 bioactive compounds that are recognized for their ability to suppress inflammation and apoptosis in pancreatic cancer cells (Yang et al., 2022b). Phenolic compounds, flavonoids, steroids, and fatty acids found in ethanol extracts of the testa portion of seeds of CM have been reported to exert cytotoxic effects on endometrial cancer cells (Huang et al., 2021b). Additionally, extracts from CM seed, which are abundant in storage proteins, coixins, vitamin E, squalene, and phytosterols, have shown strong antioxidant properties against chronic diseases, such as cancer (Kang et al., 2018). In the same manner, extracts from *Coix* seeds, rich in proteins, lipids, carbohydrates, amino acids, vitamins, and inorganic salts, along with the polysaccharide CP-1, demonstrated apoptotic effects on human lung cancer cells (Lu et al., 2013). Three types of *Coix* originating from the Loei area in Thailand, namely Thai Black Loei, Laos Black Loei, and Laos White Loei, have exhibited anticancer properties, which could be linked to the existence of lactams found in their extract fractions (Manosroi et al., 2016; Manosroi et al., 2019). Kanglaite, an anticancer drug derived from *Coix* seed oil extract, has been approved by Food and Drug Administration for clinical trials in the United States (Normile, 2003; Xi et al., 2016). It has been reported to alleviate adverse effects associated with radiochemotherapy in pancreatic cancer patients (Liu, Yu, and Ding, 2019) and is currently being investigated for its potential synergistic effects when combined with first-line chemotherapy for advanced cancer cells (Gao et al., 2021; Lu et al., 2022). As *Coix* has been widely recognized for its potent anticancer properties, several studies have demonstrated its efficacy when used alone. Importantly, *Coix* extracts exhibited no harmful effects on normal cells, suggesting their potential as a safe and effective natural extract (Ni et al., 2021; Son et al., 2017, 2019). Therefore, further investigation into the therapeutic potential of CL in cancer treatment is warranted, particularly in combination with chemotherapeutic agents.

Sorafenib, a multi-targeted kinase inhibitor, is commonly recognized as the standard treatment for advanced hepatocellular carcinoma (HCC) and various other cancers. However, its clinical efficacy is often compromised by significant toxicity, numerous

side effects, and the development of drug resistance, particularly with prolonged use (Rodríguez-Hernández et al., 2020; Yu et al., 2024). As a result, combination therapies involving sorafenib and other anticancer agents have gained attention as a promising strategy, showing improved therapeutic outcomes and reduced resistance (Donne & Lujambio, 2023; Fulgenzi et al., 2023). In particular, combining sorafenib with plant-based or herbal extracts has been shown to improve its anticancer efficacy significantly (Abdu, Juaid, Amin, Moulay, & Miled, 2022; Cohen, Mukerji, Timmermann, Samadi, & Cohen, 2012; Ghanem et al., 2023; Li et al., 2024; Lu, Fei, & Zhang, 2018; Ma, Wang, Liu, Chen, & Wang, 2020; Pal et al., 2015; Parhira et al., 2025; Sumorek-Wiadro et al., 2020; Zhu et al., 2023). Therefore, exploring cancer treatment strategies that combine sorafenib with herbal extracts may improve therapeutic responses and mitigate drug resistance.

Despite the limited research on CL seed extracts cultivated and commercialized in Thailand for their anticancer activities, this study aimed to investigate the mechanisms underlying their anticancer activity. In particular, raw and cooked CL seed extracts were separated into fractions using ethanol (EtOH), dichloromethane (DCM), ethyl acetate (EtOAc), and water (W), and their impact on HCT116 and HepG2 cells was assessed. Additionally, the possible synergistic effects of CL seed extracts as a food supplement in conjunction with low doses of chemotherapy sorafenib could boost their anti-cancer effectiveness and mitigate the treatment constraints linked to chemotherapy-induced side effects. This indicates a hopeful path for upcoming studies and practical applications in cancer therapy.

2. Materials and methods

2.1. Plant materials

The seeds of CL weighing 30 kg were acquired from a local commercial company in Thailand known as Raitip company, Thanya Farm Co., Ltd., located in Nonthaburi, Thailand, with lot number 22100008. These seeds were verified by a taxonomist and a dry plant specimen (No. 005348) was deposited as a reference at the PNU Herbarium, Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand. The Department of Agriculture, Ministry of Agricultural and Cooperatives, Thailand, granted approval for the collection and utilization of this plant in accordance with the national guideline for research purposes [as per Plant Varieties Protect Act B.E. 2542 (1999) section 53] under permission number 0284. The raw, dried powdered CL seeds were produced by grinding the raw, dried seeds. The CL seeds were cooked by combining 500 g of raw seeds and 1.2 L of water in a rice cooker (Phillips, Bangkok, Thailand), then cooking using an automatic program. After cooking, the CL seeds were dried on a stainless-steel rack in a hot air oven at 50 °C for 48 h until completely dry, and then powdered using a blender. The dry powder of raw and cooked seeds of CL was stored in plastic bags and airtight plastic containers in a dry place at room temperature (27 ± 5) °C until extraction.

2.2. Sample preparation

The dry powder of the raw (10 kg) and cooked (10 kg) seed of CL were extracted by using 95% ethanol 5 L (Commercial grade, Liquor distillery organization, Chachoengsao, Thailand), then put into the ultrasonic cleaning equipment (Shenzhen Jietai Ultrasonic Cleaning Equipment Co., Ltd., Guangdong, China,), room temperature for 30 min. The alcoholic filtrate was separated from the plant residues and evaporated at 45 °C with a rotary evaporator (Buchi, Flawil, Switzerland) to yield the EtOH fractions of raw (CLRE) (875 g, 8.75% yield of dry raw seed) and cooked (CLCE) (405 g, 4.05% yield of dry cooked seed). The extract's percentage yield at 100 g of CL dry powder was calculated. The 100 g of the CLRE and CLCE were suspended in 200 mL of water, then fractionated by dichloromethane (DCM) (400 mL × 3 times), followed by removal of the solvent to get CLRD (44.99 g, 44.99% yield of CLRE) and CLCD (75.72 g, 75.72% yield of CLCE). The left water layers were then partitioned with EtOAc (400 mL × 3 times) followed by evaporated by rotary evaporator to gain the CLRA (1.26 g, 1.26% yield of CLRE) and CLCA (0.52 g, 0.52% yield of CLCE). The rest of the water layers of CLRW and CLCW were frozen and dried to obtain the water fractions of raw (CLRW) (12.16 g, 12.16% yield of CLRE) and cooked (CLCW) CL seeds (3.69 g, 3.69% yield of CLCE). All the extracts were kept in tight amber glass bottles and refrigerator (3 ± 2) °C until used.

2.3. High-performance liquid chromatographic (HPLC) analysis

The coixol contents of eight fractions CLRE, CLCE, CLRD, CLCD, CLRA, CLCA, CLRW, and CLCW were determined by following the protocols suggested by Zhang, et al. with slight modification (Zhang, Ma, Xu, Tian, & Jia, 2010). The parameters of the HPLC system (Shimadzu pump LC-10ATvp, Kyoto, Japan) used to analyze coixol in the samples (5 mg/mL in methanol, 20 μL) were as follows: C_{18} column (ACE® Excel 5 C_{18}, 250 mm × 4.6 mm, 5 μm), mobile phase consisting of acetonitrile (HPLC grade, Labscan, Bangkok, Thailand) and 0.1% phosphoric acid (AR grade, Sigma Aldrich, St Louis MO, USA) in water (25:75, volume percentage) with a flow rate of 0.8 mL/min for 20 min, detection wavelength was set at 230 nm, and column temperature was maintained at 25 °C. The content of coxiol (98.78% purity, MedChemExpress, NJ, USA) in raw and cooked seeds extracts were calculated by using the coixol standard curve (0.01–1.00 μg/mL, $Y = 89\,912X - 610$, $R^2 = 0.997\,7$, where Y represented the peak area response in the HPLC chromatograms at the retention time of (13.465 ± 0.021) min and X was a concentration of coixol (μg/mL). The coixol contents in each extract were expressed as μg of coixol per one gram of extract (mean ± SD) from three separate experiments (Zhang, Ma, Xu, Tian, & Jia, 2010).

2.4. Cell culture

Human cell lines, colorectal carcinoma (CRC): HCT116 [RCB2979, RIKEN BioResource Research Center (BRC) Cell Bank, Ibaraki, Japan], hepatocellular carcinoma (HCC): HepG2 [JCRB1054, Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan], and normal fibroblast IMR-90 (JCRB9054, JCRB Cell Bank, Osaka, Japan) were grown in complete growth medium [Dulbecco's modified Eagle medium (DMEM) (Corning, NY, USA)] supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, MA, USA) and 1% penicillin and streptomycin (Gibco, Thermo Fisher Scientific, MA, USA), and the incubation condition was 37 °C humidified with 5% CO_2.

2.5. Antiproliferative activity of extracts

Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and incubated for 24 h. Following incubation, the cells were treated with extract fractions at varying concentrations, as well as with combinations of the extracts and sorafenib, for 24 and 48 h at 37 °C in a 5% CO_2 incubator. Subsequently, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Merck KGaA, Darmstadt, Germany) was applied to stain cells to assess cell viability through the reduction of yellow-colored MTT to purple formazan crystals by the mitochondrial reductase enzyme activity. The formazan crystals were dissolved in DMSO, and the optical density (OD) was measured at 595 nm using a microplate reader (BioTek Synergy, Agilent, CA, USA).

2.6. Apoptosis assay by flow cytometry

Cells were seeded at a density of 2×10^5 cells/mL in a 12-well plate and incubated for 24 h. Following incubation, the cells were

exposed to the extracts for the designated time periods. Apoptosis was assessed by harvesting the cells and staining with the dual dyes, Annexin V/FITC (Invitrogen, Thermo Fisher Scientific, MA, USA) and propidium iodide (PI, Invitrogen, Thermo Fisher Scientific, MA, USA), for flow cytometric analysis. Single Annexin-V/FITC staining indicates externalization of phosphatidylserine presented in the early stages of apoptosis, whereas dual staining with Annexin-V/FITC and PI indicated cells in the late stages of apoptosis. Cell apoptosis was evaluated using CytoFLEX flow cytometry and CytExpert (Version 2.4.0.28) software (Beckman Coulter, CA, USA).

2.7. Analysis for mitochondrial membrane potential (MMP) by flow cytometry

Cells were seeded in a 12-well plate at a density of 2×10^5 cells per well and incubated for 24 h. Following exposure to the extracts for the designated time periods, the cells were harvested and stained with MMP probe: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolocarbocyanine iodide (JC-1) dye (Invitrogen, Thermo Fisher Scientific, MA, USA). Strong red fluorescence was emitted by JC-1 aggregation in the healthy mitochondria, while MMP in apoptotic cells emitted a monomeric green fluorescence. MMP was evaluated using CytoFLEX flow cytometry and CytExpert (Version 2.4.0.28) software.

2.8. MMP detection using fluorescence microscopy

Cells were seeded at a density of 5×10^5 cells/2 mL on a glass coverslip in a 35 mm culture dish and incubated for 24 h. After exposure to the extracts for the designated time periods, the cells were fixed with 10% formaldehyde for 15 min at room temperature. MMP was detected using JC-1 dye, and nuclear staining was performed with 4′,6-diamidino-2-phenylindole dihydrochloride Hoechst 33342 (Invitrogen, Thermo Fisher Scientific, MA, USA). Fluorescence microscopy was used to examine the MMP of the stained cells (BX53F2, Olympus Corporation, Tokyo, Japan).

2.9. Detection of intracellular reactive oxygen species (ROS) levels by flow cytometry

Cells were seeded in a 12-well plate at a density of 2×10^5 cells per well and incubated for 24 h. After exposure to the extracts for the designated time periods, cellular ROS levels were measured using the cellular permeable fluorescent dye 2′,7′-dichlorodihydro fluorescein diacetate (H2DCFDA, D399, Invitrogen, Thermo Fisher Scientific, MA, USA). The oxidized 2′,7′-dichlorofluorescein (DCF), indicative ROS production, was quantitated using CytoFLEX flow cytometry and CytExpert (Version 2.4.0.28) software.

2.10. Determination of protein expression by Western blotting

Cells were incubated at a density of 2×10^6 cells/4 mL in a 60 mm culture dish for 24 h. After exposure to the extracts for the designated time periods, total intracellular protein was extracted using a mammalian protein extraction reagent (M-PER) (Thermo Fisher Scientific, MA, USA) supplemented with a proteinase inhibitor (HiMedia Laboratories Private Limited, Maharashtra, India). A bicinchoninic acid assay reagent (Thermo Fisher Scientific, MA, USA) was used to measure protein concentration. Protein was then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Life Science, Bio-Rad Laboratories Ltd., Bangkok, Thailand). After blocking nonspecific protein, the target protein was probed with primary antibodies, including rabbit polyclonal superoxide dismutase 2 (SOD2)/manganese superoxide dismutase (MnSOD) (AF5144, Affinity bioscience, OH, USA), rabbit polyclonal catalase (DF7545, Affinity bioscience, OH, USA), phosphatidylinositol 3-kinase (PI3K) antibody, p110β (09-482, Merck KGaA, Darmstadt, Germany), protein kinase B (AKT) pan monoclonal antibody (E.32.10) (MA5-14999, Invitrogen, Thermo Fisher Scientific, MA USA), mammalian target of rapamycin (mTOR) antibody (ab32028, Abcam Singapore Pte Ltd., The Metropolis Tower Two, Singapore), and mouse monoclonal β-actin antibody (8H10D10, Cell Signaling Technology Inc., MA, USA). The membrane was then probed with secondary antibodies including horseradish peroxidase (HRP)-conjugated goat anti-rabbit (65-6120, Invitrogen, Thermo Fisher Scientific, MA, USA) or anti-mouse (A28177, Invitrogen, Thermo Fisher Scientific, MA, USA) antibodies. Luminata Forte Western HRP substrate (Merck KGaA, Darmstadt, Germany) was then applied and chemiluminescence (Image Quant LAS 4000; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for the detection of protein bands as relative protein/β-actin levels. The levels of proteins were calculated using ImageJ version 1.46.

2.11. Statistical analysis

Statistically significant differences between the experimental groups and the vehicle group from the results of three independent experiments with triplicated performance were determined using One-way analysis of variance (ANOVA) or Student's *t*-test with Tukey's post hoc test (HSD) at $P < 0.05$. GraphPad Prism version 9 (GraphPad Software, MA, USA) was utilized for data analysis. Data presented were calculated as the mean ± SD values.

3. Results

3.1. Coixol contents of CL seed extracts

The HPLC chromatograms of the blank, standard coixol (0.05 μg/mL), the 5 mg/mL eight fractions of CL seeds were shown in Fig. S1. The coixol contents in each extract were expressed as μg of coixol per one gram of extract (Fig. 1) from three separate experiments (Zhang, Ma, Xu, Tian, & Jia, 2010). In the analysis, it was discovered that 1 g of CLCE and CLCD had coixol levels of 7.02 and 9.69 μg, respectively, surpassing the amounts found in CLRE and CLRD by approximately 2.64 (2.66 μg) and 1.65 (5.96 μg) times, respectively. The CLCA contained 2.33 μg coixol/g extract. The content of coixol in CLRA and CLRW together with CLCW, could not be detected in this condition.

3.2. Cytotoxic effects of CL seed extracts on HCT116 and HepG2 cells

The cytotoxicity of eight fractions, CLRE, CLRD, CLRA, CLRW, CLCE, CLCD, CLCA, and CLCW was investigated in HCT116 and HepG2 cells over 24 and 48 h using the MTT assay. The IC_{50} values for HCT116 and HepG2 cells after both 24 and 48 h of incubation were greater than 2 000 μg/mL in all fractions, with the except of CLRD, CLRA, CLCD, and CLCA in HCT116, which had IC_{50} values ranging from 1 500–1 800 μg/mL during both time points (data not shown). Consistent with our findings, previous studies have demonstrated that semi-purified extracts of *n*-hexane, ethyl acetate, and *n*-butanol of CL seed extracts exhibited greater antiproliferative effects than the methanolic extract (Manosroi et al., 2016).

Based on the results of the current research, it was found that CL seed extracts alone did not show significant anticancer properties. Nevertheless, it was suggested that the anticancer efficacy of the seed extracts might be improved by combining them with the chemotherapy drug sorafenib on HCT116 and HepG2 cells. Based on the findings of the current research and additional literature, sorafenib demonstrates IC_{50} values ranging from 5–18 and 13–20 μmol/L after a 24- and 48-hour incubation period in HCT116 and HepG2 cells, respectively (Alsulaimany et al., 2023;

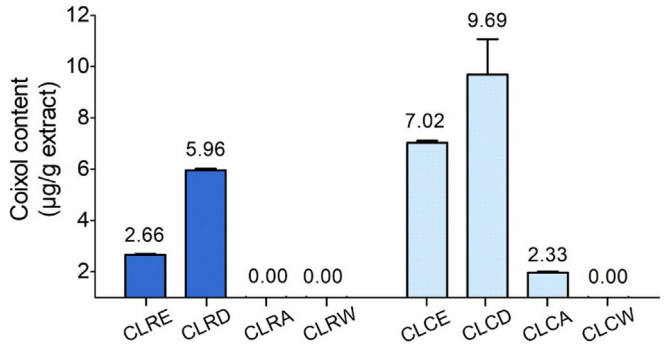

Fig. 1. Coixol contents in eight fractions of CL seed extracts (CLRE, CLRD, CLRA, CLRW, CLCE, CLCD, CLCA and CLCW).

Elwan et al., 2022; Hsu et al., 2016; Hu, Chang, Hsieh, & Huang, 2022; Ismail et al., 2023; Taha, Aboulwafa, Zedan, & Helmy, 2022; Talezadeh Shirazi et al., 2022; Walker et al., 2009; Yao, He, Chen, Sun, Fang, & Xu, 2012; Zhang et al., 2019a). Therefore, further investigation was conducted using CLRD, CLRA, CLCD, and CLCA in combination with sorafenib at doses below the IC_{50} level on HCT116 and HepG2 cells. All four fractions were chosen at concentrations of 1 000 μg/mL in combination with sorafenib at concentrations of 2 μmol/L and 6 μmol/L for application in HCT116 cells. Sorafenib at concentrations of 1 μmol/L and 4 μmol/L was selected for application in HepG2 cells, with 24- and 48-hour incubation periods.

The result demonstrated that after 48 h of incubation, a combination of CLRA with 2 μmol/L or 6 μmol/L sorafenib had a significantly greater cytotoxic effect on HCT116 cells compared to either the vehicle or a single treatment. In a 48-hour incubation treatment, CLCD in combination with sorafenib at 6 μmol/L and CLCA in combination with sorafenib at 2 μmol/L showed a significant cytotoxic effect in HCT116 cells. Incubation for 24 h showed that CLCA in combination with sorafenib at 2 and 6 μmol/L had significantly higher cytotoxicity on HCT116 cells compared to either the vehicle or a single treatment (Fig. 2A). After 24 h of incubation, only a combination of CLRA with 4 μmol/L sorafenib had a significantly greater cytotoxic effect on HepG2 cells. However, after 48 h of incubation, combinations of CLRA with 1 and 4 μmol/L sorafenib showed a significantly greater cytotoxic compared to either the vehicle or a single treatment. Additionally, after 48 h, the combination of CLCD and CLCA with sorafenib at 1 μmol/L exhibited significant cytotoxic effects compared to either the vehicle or a single treatment (Fig. 2B).

Notably, while the combination treatments showed significant differences compared to the vehicle control and individual components (extracts or sorafenib at their respective doses), no significant differences were observed among the various combination groups for each cell line treated with each extract.

Consequently, this finding suggests that at 48 h of incubation with CLCD extracts in both HCT116 and HepG2 cells, considering their coixol content and the results of cytotoxic tests, is an optimal condition for further investigation of the incubation effect on cancer treatment using a combination regimen. Our research aligns with previous studies, which have demonstrated that heating process enhances the biological activity of extract fractions from Thai CL seed cultivars, resulting in higher free radical scavenging activity than raw extracts (Manosroi et al., 2016). In HCT116 cells, the optimal combination was CLCD at 1 000 μg/mL with sorafenib at 6 μmol/L, while in HepG2 cells, it was also CLCD at 1 000 μg/mL with sorafenib at 1 μmol/L. Further investigations aimed to evaluate the mechanism of cytotoxic effect via apoptosis induction in these two types of cancer cells.

To verify the safety of combination therapy for normal cells prior to its potential use against cancers, normal fibroblast IMR-90 cells were treated for 24 and 48 h with CL seed extracts at 1 000 μg/mL of CLCD, and with 1, 2, 4, and 6 μmol/L of sorafenib (Fig. 3). After 24 h, the cellular response to the combination did not differ from that of the vehicle control or the single treatment. Following a 48-hour of incubation period, it was observed that the combination of CLCD and sorafenib resulted in a statistically significant cytotoxic effect, distinct from both the vehicle group and individual treatments. The impact of this combination on IMR-90 cells was less pronounced in comparison to HCT116 and HepG2 cells, indicating that the combination of CLCD and sorafenib, each administered at concentrations below their respective IC_{50}, displayed a more targeted anticancer effect than either treatment alone, while causing minimal cytotoxicity to normal cells. As a result, the safe application of CLCD in combination with sorafenib, which selectively targets cancerous cells, holds promise for future cancer therapy. Our results align with previous research demonstrating notably higher specific cytotoxicity against human uterine sarcoma cells treated with the hexane extract fraction of CL testa than normal uterine smooth muscle cells (Chang et al., 2018). Furthermore, the methanolic extract of CL seed exhibited a more focused inhibitory effect on human lung cancer A549 cells than on MRC-5 normal lung fibroblasts (Chang, Huang, Chiang, & Hsia, 2003).

3.3. Combination of CLCD and sorafenib enhanced apoptosis in HCT116 and HepG2 cells for 48 h incubation

The efficiency of apoptosis-inducing activity of a combination of CLCD and sorafenib was evaluated by flow cytometry. The result demonstrated that after 48 h of incubation, CLCD at 1 000 μg/mL in combination with 6 μmol/L sorafenib significantly induced more apoptosis in HCT116 cells than the vehicle control or single therapy (Fig. 4A and B). Similarly, HepG2 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and 1 μmol/L sorafenib exhibited a statistically significant apoptotic effect (Fig. 4C and D). Therefore, the anticancer activity of CLCD in combination with the chemotherapeutic sorafenib in HCT116 and HepG2 cells was enhanced through increased apoptosis induction.

3.4. Combination of CLCD and sorafenib triggered apoptosis in HCT116 and HepG2 cells by inducing dissipation of MMP during a 48-hour incubation period

The mechanism of the apoptosis-inducing effect of a combination of CLCD and sorafenib via the dissipation of MMP was analyzed using flow cytometry. After 48 h of incubation, CLCD at 1 000 μg/mL in combination with 6 μmol/L sorafenib effectively decreased MMP in HCT116 cells compared to the vehicle control and treatments with CLCD or sorafenib alone (Fig. 5A and B). Similarly, HepG2 cells treated for 48 h with 1 000 μg/mL of CLCD and 1 μmol/L sorafenib exhibited a statistically significant reduction in MMP (Fig. 5C and D). Thus, the apoptosis-inducing effect of CL extracts in combination with the chemotherapeutic sorafenib in both HCT116 and HepG2 cells was attributed to the MMP-dependent pathway. Staining with JC-1 dye and observing MMP changes under a fluorescence microscope confirmed the enhanced apoptotic effect of the CLCD and sorafenib combination in both cell lines (Fig. 6).

3.5. Combination of CLCD and sorafenib-activated apoptosis in HCT116 and HepG2 cells correlated with increased ROS formation and suppressed antioxidant enzyme expressions during a 48-hour incubation period

ROS levels and antioxidant markers such as superoxide dismutase (SOD) and glutathione (GSH) are known to contribute to the antioxidant effect of CL extracts (Du et al., 2020). Coix seed oil

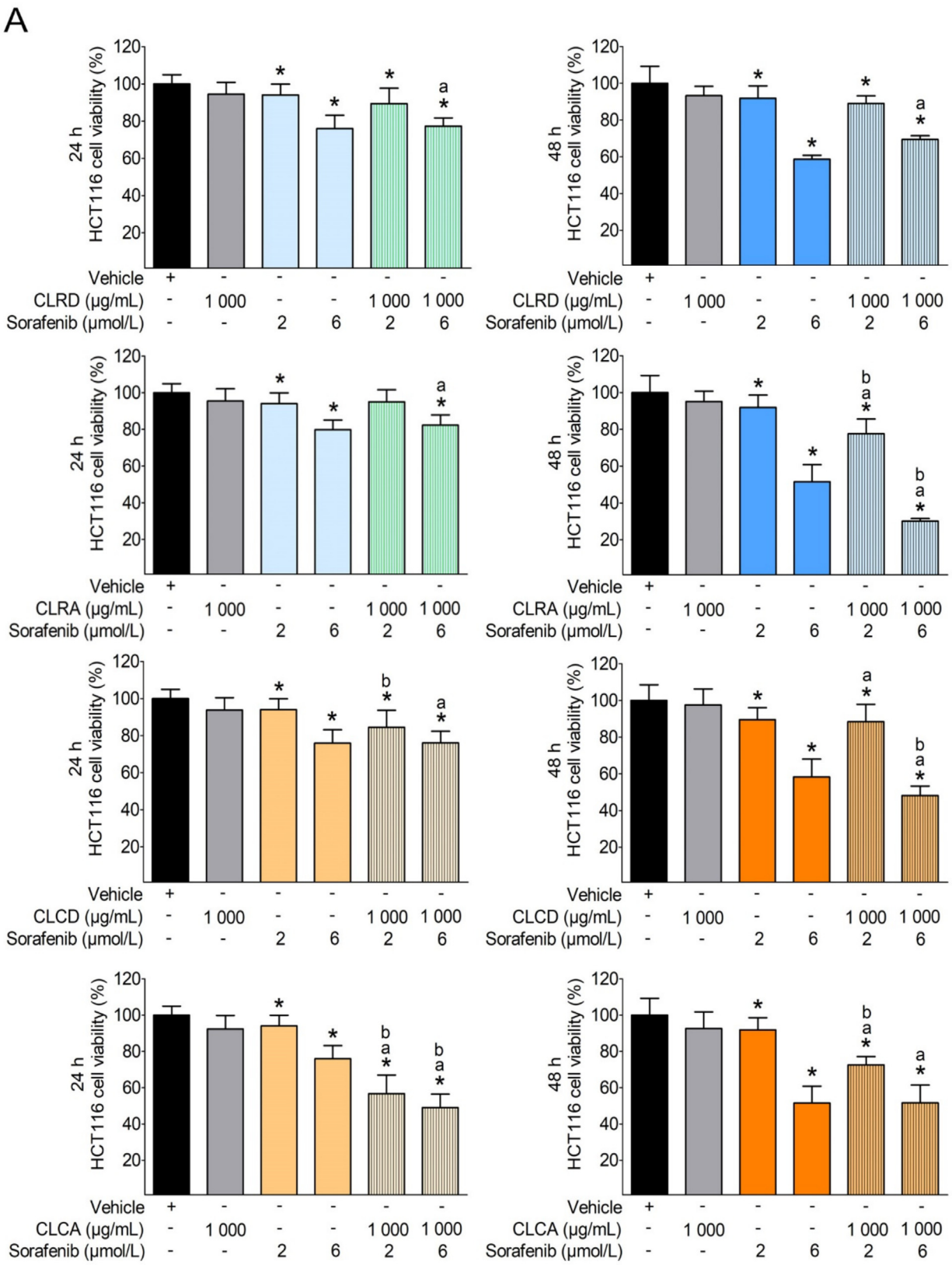

Fig. 2. Cytotoxic effect of a combination of CL seed extracts of raw and cooked dichloromethane and ethyl acetate extract fractions (CLRD, CLRA, CLCD, and CLCA, respectively) and sorafenib on HCT116 and HepG2 cells. (A) A combination extracts at 1 000 μg/mL and sorafenib at 2 μmol/L and 6 μmol/L in HCT116 cells. (B) A combination of extracts at 1 000 μg/mL and sorafenib at 1 μmol/L and 4 μmol/L in HepG2 cells. The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* a single extract fraction treatment, $^{b}P < 0.05$ *vs* a single sorafenib treatment at its same concentration.

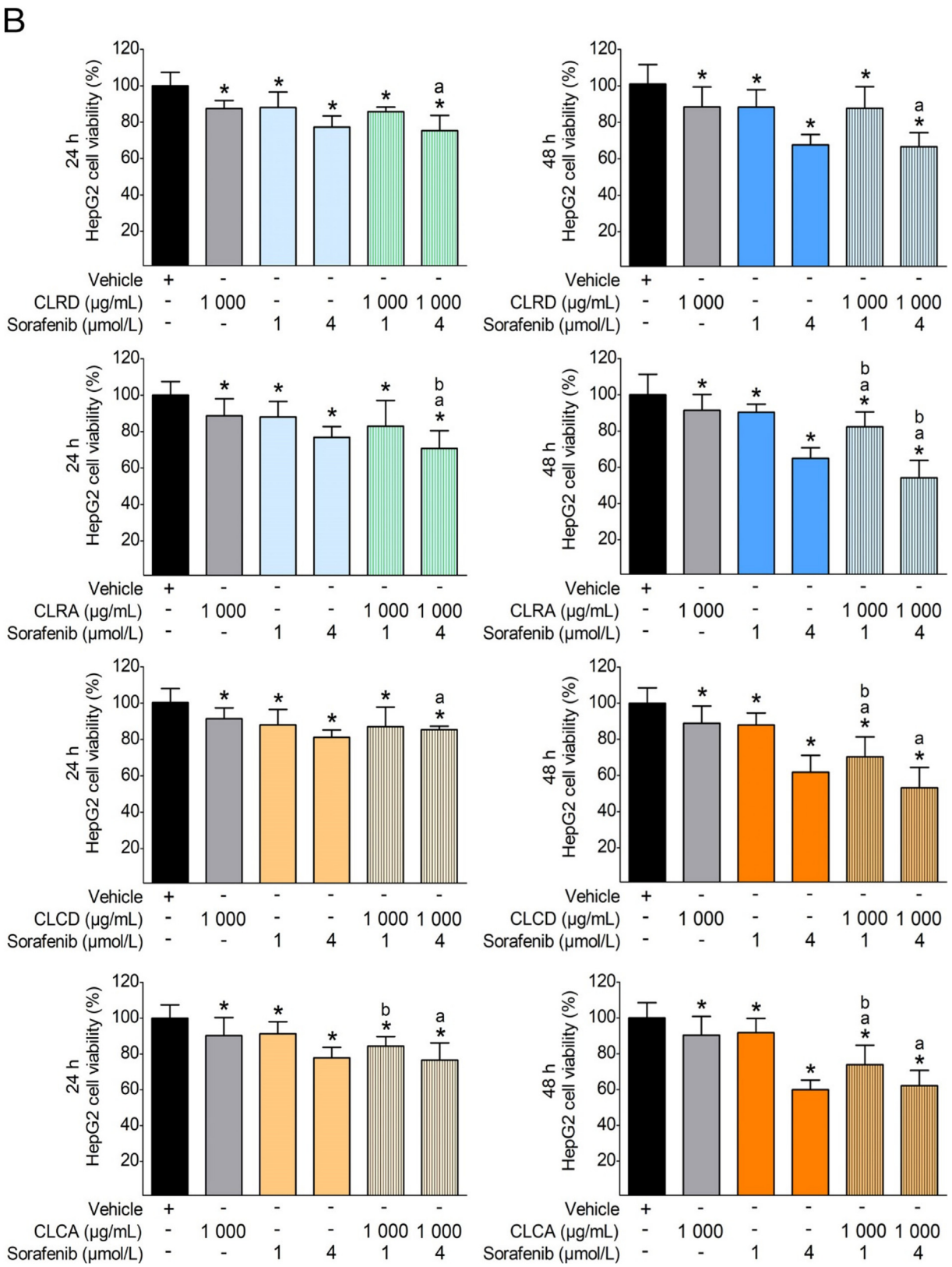

Fig. 2 (*continued*)

has been shown to induce mitochondrial functional damage associated with ROS production in cancer cells (Yang et al., 2022a). However, there have been fewer scientific reports on ROS-mediated apoptosis in cancer cells following CL seed extracts. After 48 h of treatment with CLCD at 1 000 μg/mL in combination with 6 μmol/L sorafenib in HCT116 cells, and CLCD at 1 000 μg/mL in combination with 1 μmol/L sorafenib in HepG2 cells, ROS levels increased more effectively than with vehicle control, CLCD, or sorafenib therapy alone (Fig. 7). As depicted in Fig. 8 (with original uncropped Western blot images provided in Fig. S2), a significant

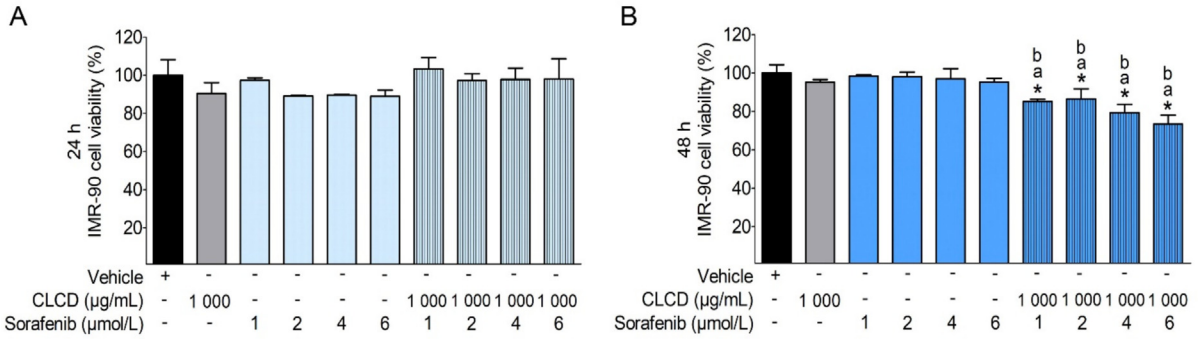

Fig. 3. Cytotoxic effect of a combination of CLCD and sorafenib on IMR-90 cells. The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* a single CLCD fraction treatment, and $^{b}P < 0.05$ *vs* a single sorafenib treatment at its same concentration.

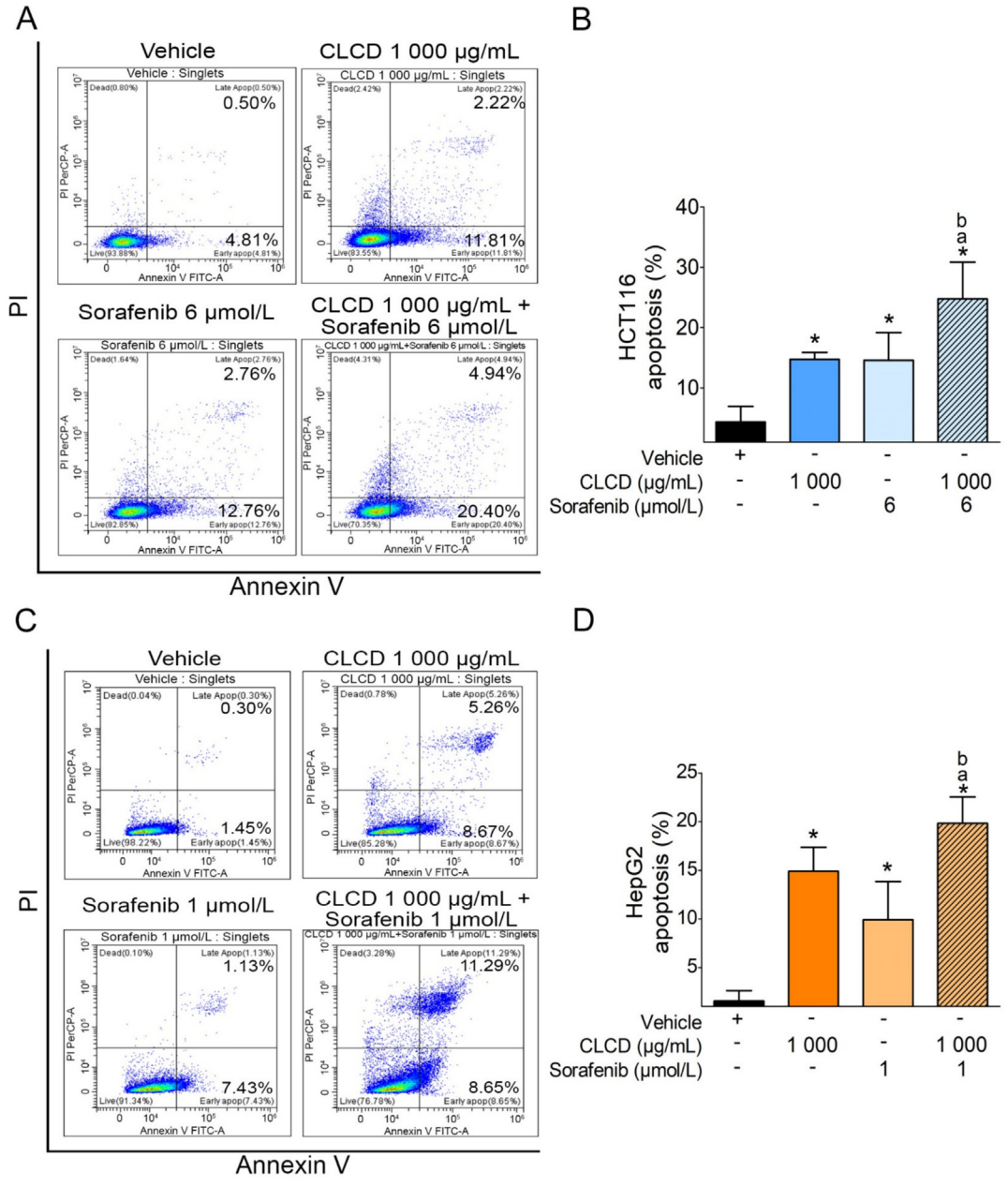

Fig. 4. Combination of CLCD and sorafenib triggered apoptosis in HCT116 and HepG2 cells. (A) HCT116 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 6 μmol/L. (B) Histogram depicting percentage of apoptotic HCT116 cells. (C) HepG2 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 1 μmol/L. (D) Histogram illustrating proportion of apoptotic HepG2 cells. The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* CLCD fraction treatment, $^{b}P < 0.05$ *vs* sorafenib treatment.

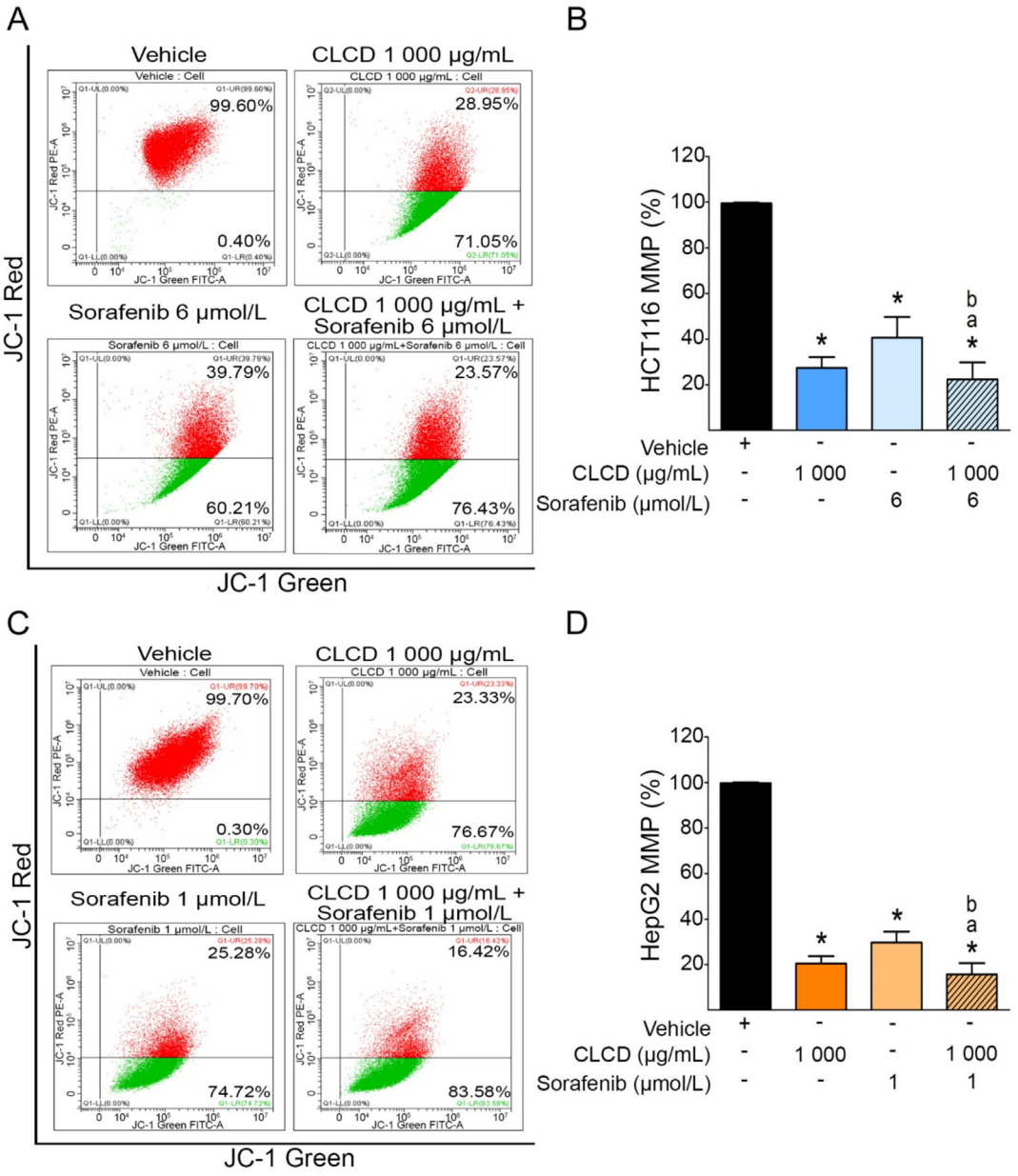

Fig. 5. Combination of CLCD and sorafenib triggered dissipation of MMP-dependent apoptosis in HCT116 and HepG2 cells. Flow cytometric measurement of MMP staining was done with JC-1 dye in (A) HCT116 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 6 μmol/L. (B) Histogram displaying percentage of red fluorescence intensity of HCT116 cells. (C) HepG2 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 1 μmol/L. (D) Histogram displaying percentage of red fluorescence intensity of HepG2 cells. The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* CLCD fraction treatment. $^{b}P < 0.05$ *vs* sorafenib treatment.

suppression of catalase expression in HCT116 and SOD2 expression in HepG2 was observed after 48 h of treatment with CLCD and sorafenib combination, compared to the vehicle control, CLCD, or sorafenib alone. However, SOD2 expression in HCT116 cells and catalase expression in HepG2 cells did not exhibit a statistically significant decrease but exhibited a declining trend.

To evaluate the significant role of ROS in inducing apoptosis, we utilized *N*-acetyl-l-cysteine (NAC) at 10 μmol/L to inhibit ROS formation in HCT116 cells treated with CLCD at 1 000 μg/mL in combination with 6 μmol/L sorafenib, and in HepG2 cells treated with CLCD at 1 000 μg/mL in combination with 1 μmol/L sorafenib over a 48-hour period. Pre-incubation with NAC for 2 h alleviated the apoptotic effects of the treatments in both cells (Fig. 9). Overall, the results demonstrate that combining CLCD with the chemotherapeutic sorafenib at doses below their respective IC_{50} values in HCT116 and HepG2 cells produced an enhanced apoptotic MMP-dependent effect associated with increased oxidative stress response compared to each treatment individually.

3.6. Combination of CLCD and sorafenib induced apoptosis in HCT116 and HepG2 cells, potentially involving inhibition of PI3K/AKT/mTOR pathway during a 48-hour incubation period

The phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway has been reported to regulate apoptosis in cancer cells (Rong et al., 2020; Yang, Pi, & Wang, 2018; Zhou, Jiang, Chen, Wu, & Zhang, 2020). In comparison to the vehicle control and their respective single treatment groups, HCT116 cells treated with CLCD at 1 000 μg/mL in combination with 6 μmol/L sorafenib, and HepG2

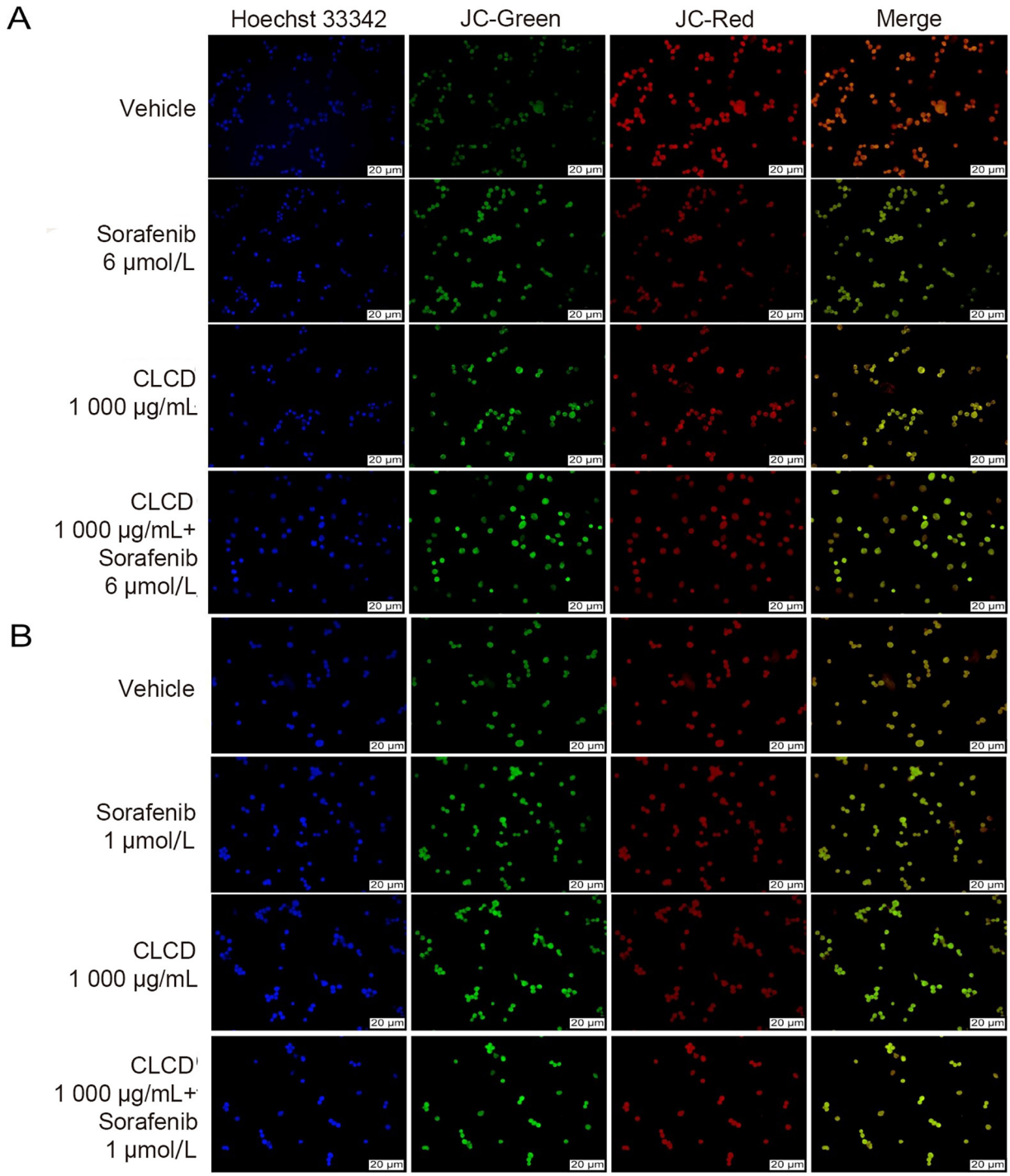

Fig. 6. Combination of CLCD and sorafenib triggered dissipation of MMP-dependent apoptosis in HCT116 and HepG2 cells assessed by fluorescence microscopy. Representative fluorescent images of (A) HCT116 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 6 μmol/L, and (B) HepG2 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 1 μmol/L. Hoechst 33342 was used to stain apoptotic cell nuclei with DNA fragmentation. The control vehicle comprised 0.8% DMSO. Scale bar = 20 μm, under × 40 magnification, visualized by fluorescence microscopy.

cells treated with CLCD at 1 000 μg/mL in combination with 1 μmol/L sorafenib over a 48-hour period, demonstrated a significant reduction in the protein expression of PI3K, AKT, and mTOR [Fig. 10 (original uncropped western blot images shown in Fig. S3)]. However, the expression of PI3K in HCT116 cells did not show a significant decrease but exhibited a downward trend. Therefore, it is hypothesized that the inhibition of PI3K, AKT, and mTOR may be associated with the apoptotic mechanism induced by the combination of CLCD and sorafenib treatment in cancer cells.

4. Discussion

Our results underscore the significance of the findings, in line with earlier studies, which showed a markedly higher specific cytotoxic effect on human cancer cells in the CLCD seed extracts as opposed to normal cells (Chang, Huang, Chiang, & Hsia, 2018; Chang, Huang, & Hung, 2003). The substantial influence of dietary promotion on general well-being is illustrated by the adlay seed extracts, which play a role in their efficacy in multi-target cancer prevention and therapeutic approaches (Kuo, Chen, & Chiang,

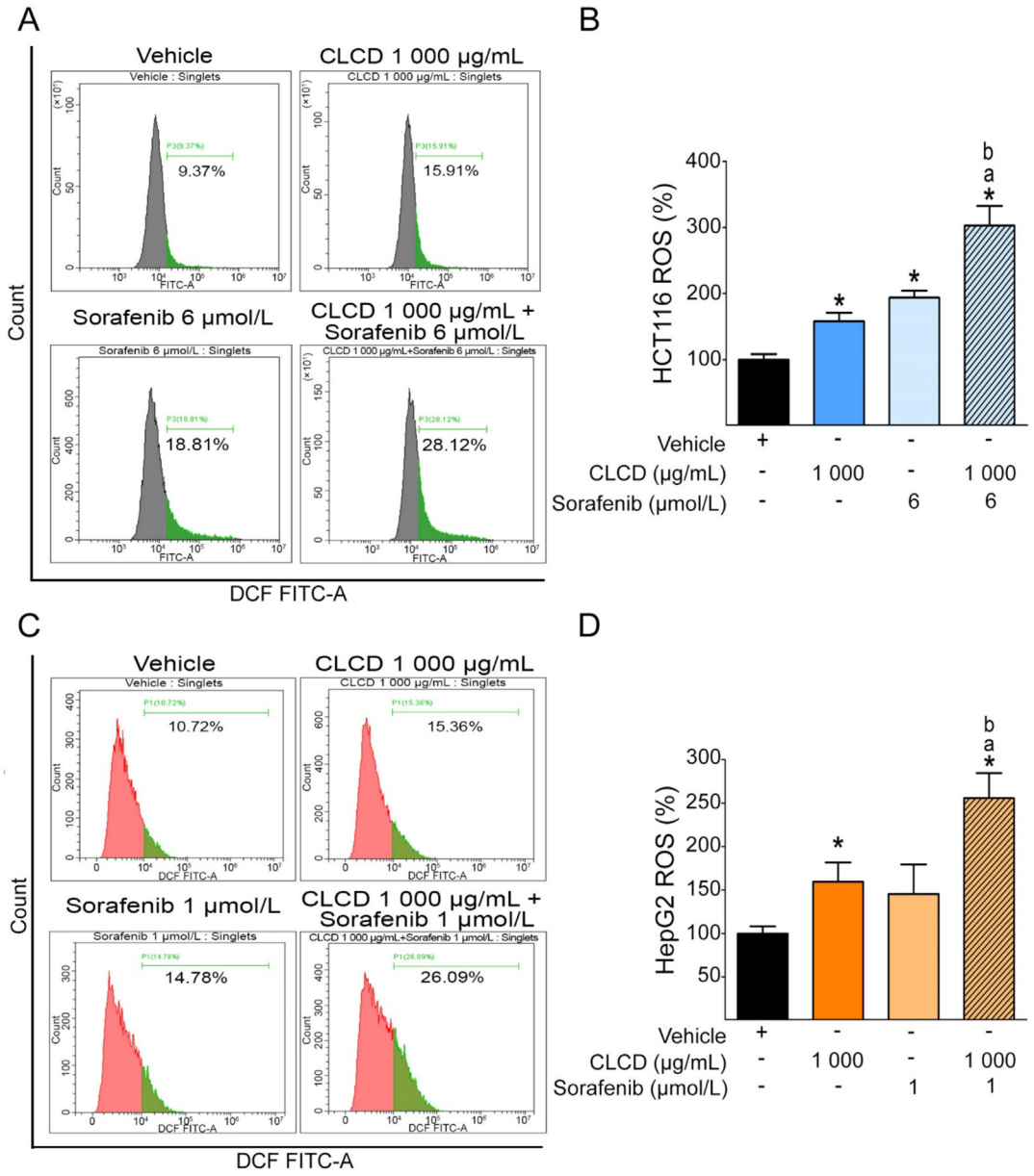

Fig. 7. Combination of CLCD and sorafenib increased ROS formation in HCT116 and HepG2 cells by flow cytometry following. HCT116 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 6 μmol/L (A–B). HepG2 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 1 μmol/L (C–D). The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* CLCD fraction treatment. $^{b}P < 0.05$ *vs* sorafenib treatment.

2012; Zeng et al., 2022). Consequently, seed extracts of CL, which selectively target cancer cells, are considered safe for future cancer treatment applications. In recent times, there has been a growing emphasis on utilizing a combination of two or more anticancer agents to enhance anticancer treatment. Furthermore, the utilization of seed extracts of CL in isolation has shown limited effectiveness in treating cancer. Nevertheless, when administered at minimal levels to induce cytotoxicity in cancer cells and combined with a low concentration of sorafenib, a synergistic outcome was noted. Therefore, combination therapy has proven to be the most effective strategy for cancer treatment.

Coix seeds are widely recognized as an exceptional functional food due to their rich content of key functional components, surpassing other parts of the adlay plant. These components encompass protein, polysaccharides, resistant starch, amino acids, palmitate, and flavonoids, each contributing significantly to their pharmacological properties (Zeng et al., 2022). Bioactive components discovered in *Coix* seed extracts exerted cytotoxicity against cancer cells. Coixol, an important active compound with anticancer potential, is notably present in "Kanglaite", a formulation containing *Coix* seed oil widely used in China. Enhancing the water solubility of coixol using β-cyclodextrin polymer to form an inclusion compound has demonstrated anticancer effects on non-small cell lung cancer (Wang et al., 2024). CM grain extracts contain phenolics and flavonoids, with flavonoids being one of the major phytochemicals that inhibited HepG2 cell proliferation (Wang, Chen, Xie, Ju, & Liu, 2013). In the husk and seed coat ethyl acetate subfraction layer of CM, numerous phenolic compounds were identified, and quercetin was found to be primarily responsible for apoptotic activity in breast cancer MCF-7 cells and cervical cancer HeLa cells (Chiang et al., 2022).

However, it has been reported that it is not a single bioactive compound, but rather the combination of flavonoids, phenolics, steroids, and fatty acids, including palmitic acid and linoleic acid,

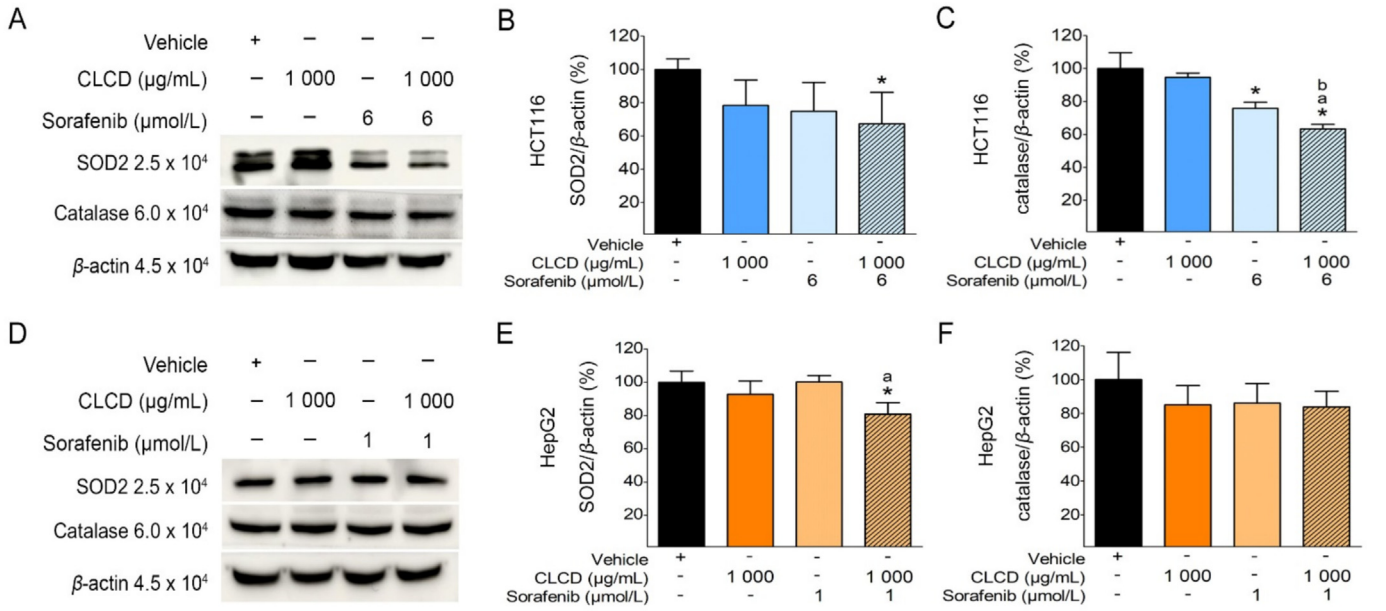

Fig. 8. Combination of CLCD and sorafenib decreased SOD2 and catalase expression in HCT116 and HepG2 cells by Western blotting. Relative expression levels compared to β-actin of HCT116 (A–C) and HepG2 (D–F) cells. The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* CLCD fraction treatment. $^{b}P < 0.05$ *vs* sorafenib treatment.

from the ethyl acetate fraction of CM testa that produces cytotoxic effects against cancer cells (Chen et al., 2011; Chung et al., 2010; Chung et al., 2011a; Huang et al., 2021b; Manosroi et al., 2016; Numata, Yamamoto, Moribayashi, & Yamada, 1994; Xi et al., 2016). The dietary phytosterol compounds, β-sitosterol and campesterol presented in hexane fraction of ethanolic testa *Coix* extracts (Chang, Huang, Chiang, & Hsia, 2018) and β-sitosterol and stigmasterol in ethyl acetate fraction of hull extract from CM (Lin, Shih, Yen, Chiang, & Hsia, 2019) play roles in anticancer effects. In addition, crude polysaccharides of bran seed from *Coix* (Li et al., 2019) and the polysaccharide fraction CP-1 extracted from *Coix* seeds (Lu et al., 2013) induced cancer cell apoptosis. The effect of lactam derivatives such as coixspirolactam A, coixspirolactam B, coixspirolactam C, coixlactam B, and methyl dioxindole-3-acetate in hexane subfraction of the ethyl acetate fraction of CM bran (Lee, Lin, Cheng, Chiang, & Kuo, 2008), two amides with a spermidine structure, coixdine A and coixdine B, in addition to six unidentified compounds extracted from ethyl acetate subfractions of CL seed (Yin et al., 2023) has been reported to possess anticancer activity. Similarly, spirolactams and spiroenone may contribute to the anticancer activity of the ethyl acetate fraction of *Coix* bran extract (Chung et al., 2011b).

Coix seed extracts demonstrated various anticancer mechanisms of action across numerous cancer cell types. *Coix* sprout extrcats inhibited the PI3K/AKT signaling pathway and the expression of cell cycle regulators, leading to apoptosis in HeLa cells (Son et al., 2019). The polyphenol content of *Coix* seed extracts decreased the viability of HepG2 cells by blocking the accumulation of intracellular triglyceride. This inhibition was achieved by activating the AMP-activated protein kinase (AMPK)/acetyl-CoA carboxylase (ACC) pathway, resulting in the suppression of the SREBP1c/FAS pathway and an increase in the expression of peroxisome proliferator-activated receptor γ (PPARγ) (Ma et al., 2022). Hydroxy unsaturated fatty acids from *Coix* seed extract act as PPARγ agonists to treat inflammatory diseases (Yokoi et al., 2009). In tumor tissues of human lung cancer implanted in BALB/c-nu nude mice, the methanolic extract of *Coix* seeds reduced tumor progression by suppressing cyclooxygenases, specifically COX-2, which regulates prostaglandin PGE2 (Chang, Huang, Chiang, & Hsia, 2018), A similar effect was observed in rats bearing colorectal cancer cells treated with dehulled seeds of CM extract (Shih, Chiang, & Kuo, 2004). Kanglaite in *Coix* seed oil inhibited tumor necrosis factor-alpha (TNF-α) activation of nuclear factor-kappa B (NF-κB) signaling pathway for tumor angiogenesis and metastasis in HCT106, HCT116, and LoVo cells. It also reduced the carcinogenic effect of TNF-α in CT26 tumor-bearing animals (Shi et al., 2017).

Recent reports have emerged elucidating the enhanced anticancer effects with minimized unspecific side effects from cancer treatment using combinations of natural food-derived extracts with chemotherapeutic agents. For instance, manuka honey combined with 5-fluorouracil (5-FU) has shown a synergistic effect in inducing apoptosis in colorectal cancer cells, mediated by increased intracellular ROS accumulation, without exerting any cytotoxic effects on healthy colon cells (Afrin et al., 2018). Similarly, strawberry tree (*Arbutus unedo* L.) honey exhibited a synergistic effect with 5-FU in inducing apoptosis in colorectal cancer cells by increasing ROS generation, enhancing the expression of p-p38 MAPK and p-ERK1/2, and decreasing mitochondrial respiration and the glycolysis pathway (Afrin et al., 2021). Several studies have demonstrated an integrated anticancer effect when *Coix* seed extracts are combined with conventional anticancer therapies. A plant-based medicinal food (PBMS) composed of a mixture of edible medicinal plants *Coix* seed, *Lentinula edodes*, *Asparagus officinalis* L., *Houttuynia cordata* Thunb., *Taraxacum mongolicum* Hand.-Mazz., and *Grifola frondosa*, has been proposed to induce apoptosis and inhibit migration and invasion of cancer metastasis in mice with SGC-7901 gastric carcinoma (Chen et al., 2021b), as well as in mice with postoperative recurrent tumors and lung metastasis from gastric carcinoma (Tian, Chen, Cao, Zhang, & Chen, 2022). The hexane and ethyl acetate fractions of *Coix* testa extracts synergistically induced cell death when combined with doxorubicin, particularly in drug-resistant human uterine sarcoma cells (Chang, Huang, Chiang, & Hsia, 2018). Thus, *Coix* extracts are proposed to enhance the anticancer potential of drug therapy.

Sorafenib is the primary choice for conventional targeted therapy in advanced HCC and other cancers. However, its high toxicity and numerous side effects limit its effectiveness, especially when used frequently for cancer recurrence and progression. Additionally, extensive drug resistance has been observed, resulting in effective survival extension following sorafenib treatment for only

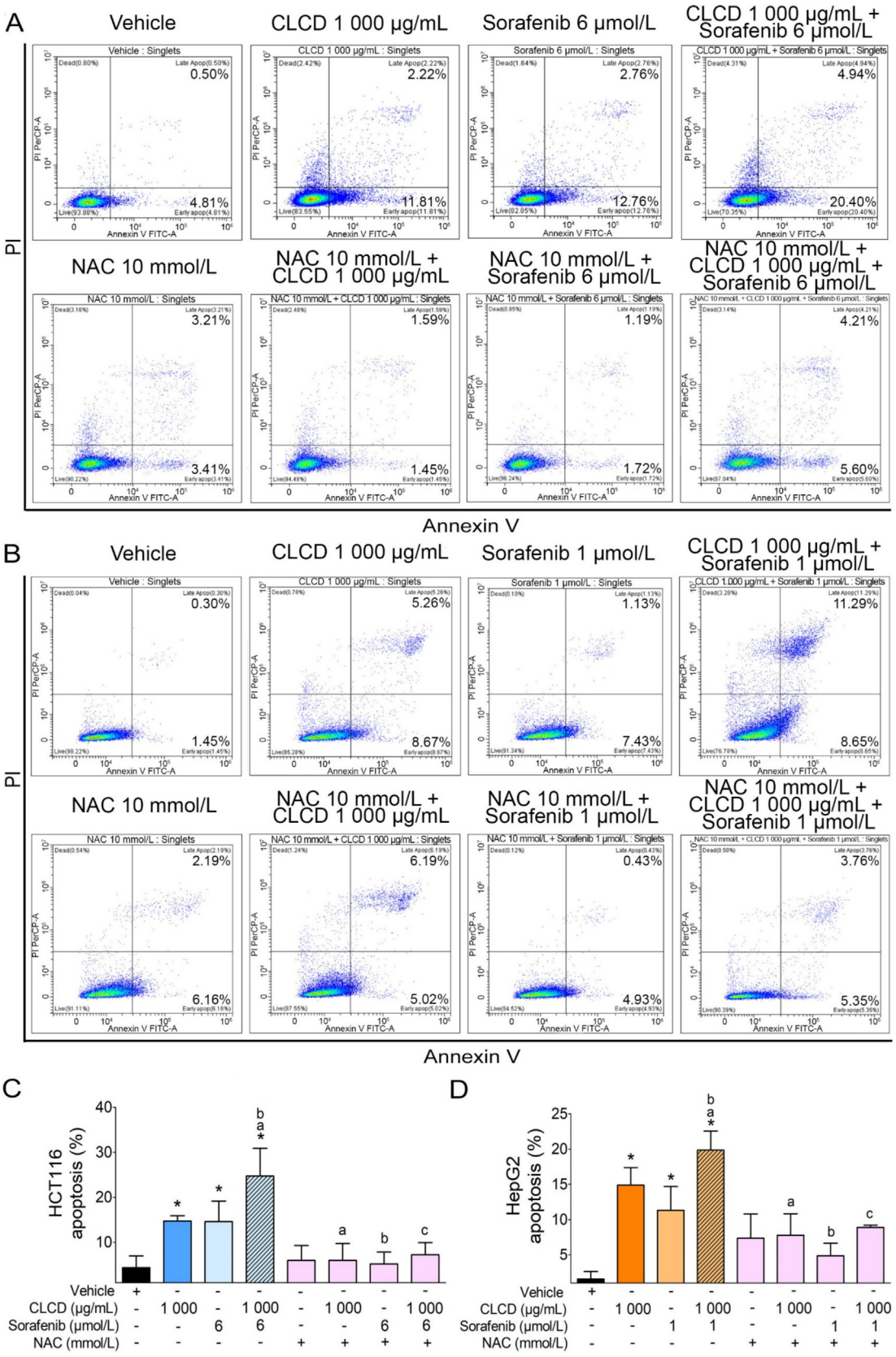

Fig. 9. Significant role of ROS formation in inducing apoptosis in HCT116 and HepG2 cells treated with a combination of CLCD and sorafenib analyzed using flow cytometry after a 2-hour pre-treatment with 10 mmol/L *N*-acetylcysteine (NAC). (A and C) HCT116 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 6 μmol/L, (B and D) HepG2 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 1 μmol/L. The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* CLCD treatment. $^{b}P < 0.05$ *vs* sorafenib treatment, $^{c}P < 0.05$ *vs* a combination of CLCD and sorafenib treatment.

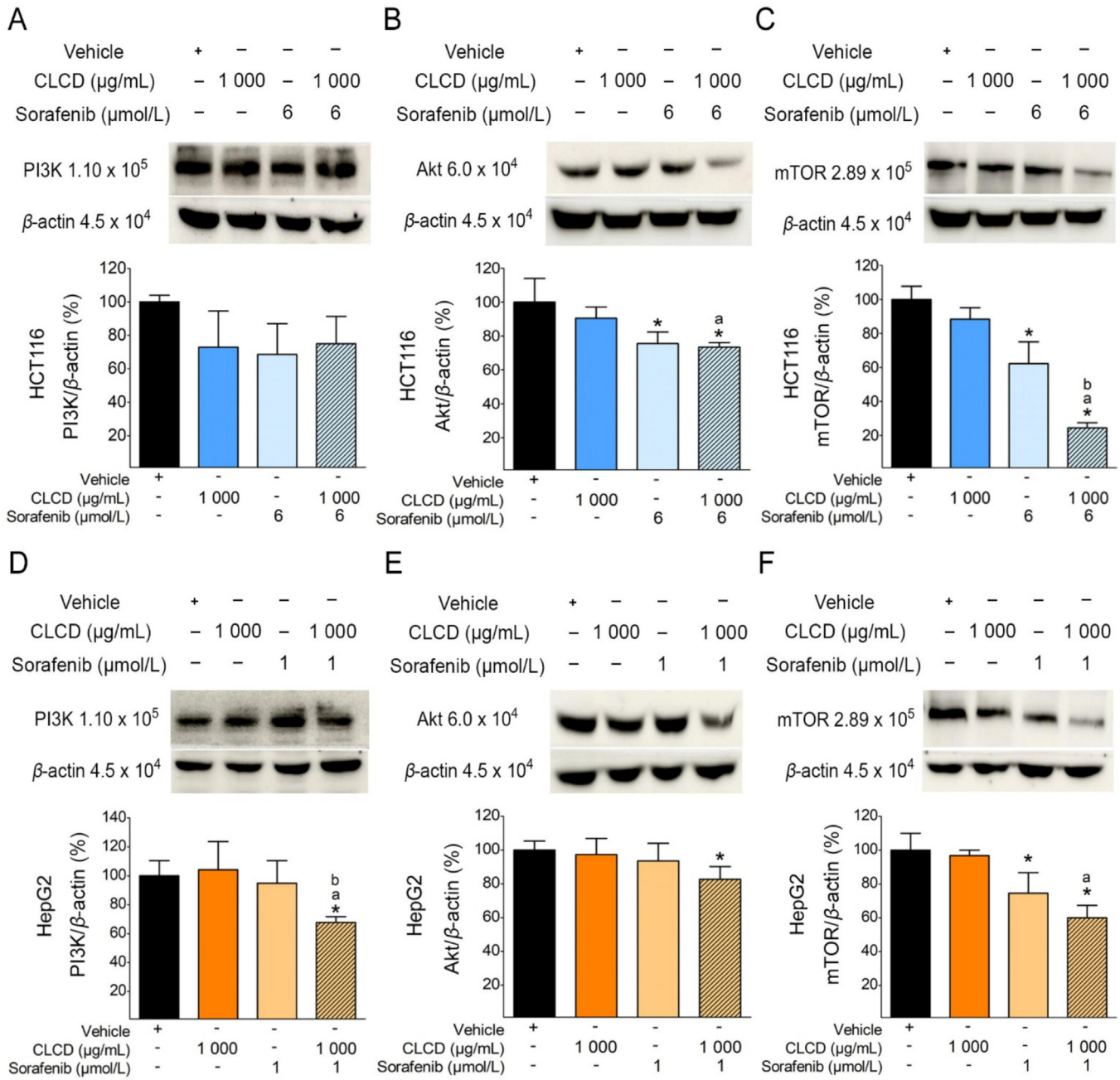

Fig. 10. Combination of CLCD and sorafenib involved downregulation of expression of phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway in HCT116 and HepG2 cells. Representative Western blotting images and bar graphs of relative expression levels of PI3K, Akt, and mTOR proteins following (A–C) HCT116 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 6 μmol/L and (D–F) HepG2 cells treated for 48 h with a combination of CLCD at 1 000 μg/mL and sorafenib at 1 μmol/L. The control vehicle comprised 0.8% DMSO. The analysis of mean ± SD from at least three different experiments was assessed with One-way ANOVA using Tukey's HSD test. $^{*}P < 0.05$ *vs* vehicle control, $^{a}P < 0.05$ *vs* CLCD treatment. $^{b}P < 0.05$ *vs* sorafenib treatment.

a small number of patients (Rodríguez-Hernández et al., 2020). Sorafenib exhibits several mechanisms of action within HCC cells, including the reduction of ATP levels and mitochondrial oxygen consumption, leading to the activation of AMPK and the downregulation of the mTOR signaling pathway (Garten et al., 2019). Furthermore, it inhibits RAF kinase, which controls the mitogen activated protein kinase (MAPK)/Ras/Raf/MAPK (MEK)/extracellular signal-regulated kinase ½ (ERK) cascade pathway in both HCC cells and tumor xenografts in mice (Liu et al., 2006). Sorafenib also plays a role in suppressing the expression of epidermal growth factor receptor and mesenchymal-epithelial transition receptor proteins, resulting in mitochondrial dysfunction and the suppression of the glycolysis pathway (Rodríguez-Hernández et al., 2020). Moreover, it activates hypoxia-inducible factor-1 (HIF-1) and the mTOR/p70 ribosomal S6 kinase (p70S6K) signaling pathways (Yang et al., 2021) while inhibiting the Ras-Raf (pMEK) and AKT pathway (Li et al., 2020; Wehler et al., 2013; Zhang & Yu, 2013). Additionally, the activity of sorafenib involves the production of ROS and disruption of mitochondrial biogenesis and respiration-related protein expression (Hu, Chang, Hsieh, & Huang, 2022).

The combination of sorafenib with plants or herbal extracts enhances its anticancer effectiveness (Abdu, Juaid, Amin, Moulay, & Miled, 2022; Cohen, Mukerji, Timmermann, Samadi, & Cohen,

2012; Lu, Fei, & Zhang, 2018; Ma, Wang, Liu, Chen, & Wang, 2020; Pal et al., 2015; Sumorek-Wiadro et al., 2020; Zhu et al., 2023). When sorafenib is combined with bufalin, a steroid from the parotid glands and skin of the Chinese toad, it significantly increases apoptosis in non-small cell lung cancer cells. This effect is triggered by ROS-oxidative stress production and results in the loss of MMP (Kuo et al., 2022). Similarly, a combination of sorafenib with ursolic acid, a natural pentacyclic triterpene, induces selective apoptotic death in human hepatoma cells by substantially increasing intracellular ROS levels (Li et al., 2022). However, there have been no research studies demonstrating the anticancer potential of *Coix* seed extract combined with sorafenib as an alternative candidate to improve cancer treatment regimens.

This study reports the selectivity of CL seed extracts and their ability to enhance the anticancer effect of sorafenib when used in combination, indicating low cytotoxicity and their suitability as anticancer agents. The anticancer effect of a single treatment of CLCD seed extract fraction was found to be less potent, as indicated by an IC_{50} greater than 1 mg/ml during a 24–48-hour incubation period in cancer cells. Nevertheless, when combined with sorafenib at a low dosage, the extract exhibited enhanced effectiveness, indicating potentially beneficial applications in cancer therapy. The mechanism underlying this combination involves the induction of ROS-induced oxidative stress, leading to apoptosis in cancer cells, specifically HCT116 and HepG2 cells. While there are no reports demonstrating the specific mechanism behind the combination of the extract with sorafenib.

It has been reported that *Coix* seed oil, a major component in food and medicine in China, is associated with MMP damage, leading to the release of ROS. Subsequently, the released ROS further stimulates the induction of MMP damage, resulting in the phenomenon of ROS-induced ROS release in cancer cells (Yang et al., 2022a). The generation of ROS is an important mediator of lipid peroxidation and the depletion of antioxidant molecules, which are considered activators of cellular oxidative stress damage. ROS production promotes endoplasmic reticulum (ER) stress, leading to increased calcium release into the cytoplasm, ultimately contributing to mitochondrial damage-mediated apoptosis (Yao et al., 2020). Therefore, targeting ROS production in cancer cells for therapy is considered a potential and promising approach for cancer treatment (Bo et al., 2021).

The mechanism of apoptotic induction in cancer cells following treatment with a combination of the CLCD seed extract and sorafenib was observed to correlate with the suppression of the PI3K/AKT/mTOR signaling pathway. This pathway plays an important role in numerous cellular functions and signaling processes, governing both cell proliferation and apoptosis (Glaviano et al., 2023; Lin et al., 2024; Yao et al., 2020). Several studies have demonstrated the response of the PI3K/AKT/mTOR signaling pathway to treatment with *Coix* extracts. This treatment subsequently regulates apoptosis in cancer cells. The Qingyihuaji Decoction, composed of seven herbs including CM, has exhibited anticancer activity through various pathways such as inflammation, oxidative stress, and apoptosis, all of which involve the regulation of the PI3K/AKT/mTOR pathway (Yang et al., 2022b). Kanglaite, primarily consisting of *Coix* seed oil, has been found to inhibit the expression levels of p-AKT and p-mTOR in the PI3K/AKT/mTOR signaling pathway, which is associated with the apoptotic pathway. However, it has been noted to produce significant non-tumor toxicity in tumor-bearing mice (Liu, Zhang, Wang, & Liu, 2014). Treatment with *Coix* seed oil significantly downregulated the expression of PI3K/AKT at the gene level, as well as the levels of phosphorylated forms of PI3K/AKT, which play roles in the regulation of apoptosis, Bcl-2, Bax, and Caspase-3 expression (Ni et al., 2021). The inhibition of expression of phosphorylated PI3K/AKT by *Coix* seed oil was achieved through the upregulated expression of the tumor suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) mRNA levels. Consequently, the PTEN/PI3K/AKT pathway regulated the mitochondrial apoptosis process (Yang et al., 2022a). CM sprout extract has been shown to induce apoptosis through the inactivation of PI3K/AKT in cancer cells (Son et al., 2019). Furthermore, water extracts of the dried CM sprout extract suggested the suppression of HCT116 proliferation and invasion through the inactivation of the ERK1/2 and AKT pathways under hypoxic conditions (Son et al., 2017).

ROS serves as a critical tumor suppressor agent that acts as the upstream mediator of the PI3K/AKT/mTOR pathway in cancer cells (Zhang et al., 2019b). Several natural compounds and extracts have demonstrated the role of ROS accumulation in inducing apoptosis in various cancer cells, mediated by the inhibition of the PI3K/AKT/mTOR pathway (Chen et al., 2021a; Lin et al., 2023; Liu et al., 2020; Song et al., 2017; Yuan et al., 2022). Additionally, it has been suggested that ROS contributes to a negative regulatory effect on the PI3K/AKT/mTOR signaling pathway, leading to apoptosis induction in cancer cells (Zhang et al., 2023). Thus, ROS serves as the specific upstream mediator for the inhibition of PI3K/AKT signaling and the induction of apoptosis in cancer cells (Liu et al., 2021; Wang et al., 2022; Yao et al., 2020).

Several compounds in *Coix* seed extracts have been considered to play important roles in targeting multiple mechanisms of action, potentially enhancing anticancer efficacy when used in combination with chemotherapy drug sorafenib. Active compounds identified in *Coix* seed extracts include phenolic acids, coumarins, phenolic aldehydes, and flavonoids identified in *Coix* bran (Huang et al., 2021b; Lin, Yang, Zhao, & Zhao, 2018). Phenolic compounds, when combined with sorafenib, exerted apoptosis induction through a decrease in cell cycle regulatory protein, p27, in cancer cells (Bahman, Abaza, Khoushiash, & Al-Attiyah, 2018; Huang et al., 2021b). Similarly, the polyphenolic compound curcumin when combined with sorafenib, demonstrated a stronger antitumor effect than single-agent treatment by reducing the expression of p-ERK and p-AKT proteins (Zhang, Yu, Xie, Chen, & Lv, 2016). Additionally, resveratrol combined with sorafenib activated apoptosis, with ROS generation playing a crucial role in the modulation (Mondal & Bennett, 2016). Coumarins combined with sorafenib exhibited a synergistic effect on apoptosis induction in cancer cells through the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways, without toxic effects on normal cells (Sumorek-Wiadro et al., 2020). Osthole, a natural coumarin, significantly enhanced the antitumor effect when combined with sorafenib by suppressing the expression of sterol regulatory element binding protein-2c, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), and low-density lipoprotein receptor proteins in tumor tissue (Fan et al., 2022), the combination of sorafenib with new 3-thiazolhydrazinyl coumarins demonstrated effective anticancer potential through inhibition of vascular endothelial growth factor receptor-2 (Abolibda, Fathalla, Farag, Zaki, & Gomha, 2023). Flavonoid glycoside showed a synergistic effect with sorafenib on cancer cell cytotoxicity by degrading nuclear factor erythroid 2-related factor 2, which regulates lipid peroxidation-mediated cell death (Yang et al., 2023). Flavonoids combined with sorafenib also reduced epithelial-mesenchymal transition by downregulating the BRAF/MEK/ERK (MAPK) and PI3K signaling pathways, leading to apoptosis in cancer cells (Pal et al., 2016).

However, our study did not determine the direct association of ROS formation with the inhibition of PI3K/AKT/mTOR expression. This suggests that the extract of CL seeds may contribute to apoptotic induction mediated by ROS accumulation, possibly correlated with the inhibition of PI3K/AKT/mTOR expression in cancer cells. Further investigation into the direct role of ROS in modulating the expression of the PI3K/AKT/mTOR signaling pathway underlying apoptotic cancer cell death induction is needed. Additionally, exploring further applications such as the animal testing models of combination treatments using the extracts and chemotherapeu-

tic agents may provide more advantageous considerations for practical clinical applications.

Therefore, we suggest that combining CL seed extracts with other chemotherapeutic agents may lead to targeted therapies that produce a synergistic anticancer effect. This approach could help overcome single-drug resistance and minimize undesirable side effects by enabling the use of lower drug concentrations. Further studies are necessary to investigate the underlying mechanisms and assess these effects *in vivo*.

5. Conclusion

Combining lower doses of sorafenib with less toxic extracts from potentially less efficient anticancer edible plants not only enhances sorafenib's cancer therapeutic efficacy but also reduces the unpleasant side effects associated with anticancer drug treatment. Our results suggest the potential for sorafenib to stimulate apoptosis in HCT116 and HCC HepG2 cells when used in combination with the CLCD polished seed extract cultivated and commercialized in Thailand. Additional research is required to elucidate the mechanism responsible for the apoptotic effect of the CLCD fraction in potential anticancer therapies in combination with chemotherapy drugs. Furthermore, to progress in developing anticancer agents from edible plants for patient care, further investigations are essential to pinpoint the active anticancer components in these extracts. It is also imperative to investigate other types of cancer cells to confirm their efficacy and safety for future patient treatments.

CRediT authorship contribution statement

Supawadee Parhira: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Guoyuan Zhu:** Resources, Writing – review & editing. **Apirath Wangteeraprasert:** Writing – review & editing. **Suphunwadee Sawong:** Formal analysis, Investigation, Methodology. **Pennapha Suknoppakit:** Formal analysis, Investigation, Methodology. **Julintorn Somran:** Writing – review & editing. **Naphat Kaewpaeng:** Formal analysis, Investigation, Methodology. **Khemmachat Pansooksan:** Formal analysis, Investigation, Methodology. **Dumrongsak Pekthong:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Writing – original draft, Writing – review & editing. **Piyarat Srisawang:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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This manuscript has been proofread and edited by Mr. Olalekan Israel Aiikulola, Lecturer (Special Knowledge and Abilities), Faculty of Medical Science, Naresuan University, Thailand.

Appendix A. Supplementary material

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