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Curcuma aromatica rhizomes ethanol extract: A potential therapeutic agent against HepG2 Hepatoma cells

Bui Dinh Thach[1], Doan Chinh Chung[1], Tran Thi Linh Giang[1], Trinh Thi Ben[1], Le Thi Dung[2], Le Thi Kim Khanh[1], Vu Quang Dao[1], Le Nguyen Tu Linh[1] & Thien-Hoang Ho[3*]

[1] Institute of Tropical Biology, Vietnam Academy of Science and Technology, 9/621 Hanoi highway, Vietnam

[2]University of Natural Science, Vietnam National University Ho Chi Minh City, No. 227 Nguyen Van Cu Std., Ward 4, District 5, Ho Chi Minh City, Vietnam.

[3]Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, No. 12 Nguyen Van Bao Street, Go Vap District, Ho Chi Minh City, Vietnam

*Email: hothienhoang@iuh.edu.vn

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Abstract

Curcuma aromatica, a Vietnamese medicinal plant, exhibits diverse therapeutic applications including blood loss, blood stasis, bloody urination, bloody vomiting, irregular menstruation, epilepsy, and cancer treatment. *C. aromatica* rhizomes ethanol extract and curcumol content have been shown strong inhibition of cancer growth. HepG2 cells were exposed to different doses of ethanol extract and curcumol extract. The degree of cell growth inhibition was determined using the WST-1 assay, and Hoechst 33258 spectrofluorometric assay, while the rate of cell death by apoptosis was assessed using the annexin-propidium iodide double-staining assay. Flow cytometry was employed to study the cell cycle, while the transwell assay was used to assess cell migration and invasion. The expression of *bax* and *bcl-2* genes was analyzed using real-time reverse transcription-PCR. *C. aromatica* rhizomes ethanol extract (CE) consisted of a total phenolic content value of 15.02±0.18 (mg/g extract) and a curcumol level of 849.8 ± 23.73 (mg/g extract). The growth of HepG2 cells was suppressed in a dose-dependent manner by *C. aromatica* rhizomes ethanol extract. Following a 72-hour exposure to an (CE) concentration of 100 µg/mL, the inhibition rate was measured to be 34.559 ± 0.456 %, the IC50 value was determined to be 44.79 µg/mL and the inhibition of curcumol was 50.961± 0.641 %, and the IC50=93.48. The application of CE induced late apoptosis in HepG2 cells. The CE also induced programmed cell death (apoptosis) in HepG2 cancer cells, effectively suppressing their growth, movement, and invasion by controlling the synthesis of Bcl-2 mRNA. This work gives novel insights into the antihepatoma action of (CE) and establishes a foundation for the development of preliminary anticancer medications.

Keywords

antioxidant activity; *Curcuma aromatica* Salisb.; curcumol; extraction; phytochemical screening

Introduction

Hepatocellular carcinoma (HCC), commonly known as liver cancer, is a substantial worldwide health hazard. The tumor has a high degree of malignancy and a strong ability to invade and spread, making it prone to invasion and metastasis during the development and evolution of the tumor. Tumor cells invade blood arteries, leading to either vascular invasion or the formation of a blood clot within the blood vessels (1). HCC has been

linked to chronic viral hepatitis, mainly caused by infections of hepatitis B and C viruses (HBV and HCV, respectively) (2). Additional variables that contribute to the beginning of HCC include non-viral chronic hepatitis, such as alpha-1-antitrypsin deficiency, hereditary hemochromatosis, and glycogen storage disease. Other risks include alcohol-related steatosis, nonalcoholic steatohepatitis (NASH), exposure to aflatoxin B1, and cigarette smoking (3, 4). Despite significant progress in medical technology, the outlook for HCC remains bleak, with very few effective treatment options available. The identification of highly effective anti-cancer compounds with minimum adverse effects is essential for enhancing patient prognosis (5).

Natural products have emerged as promising candidates for anticancer drug development. One such plant, *Curcuma aromatica* Salisb, known as "Nghe trang" in Vietnamese, or aromatic turmeric, is a perennial tuberous herb characterized by its annulate, fragrant white rhizome, exhibiting an internal orange-yellow hue. The plant mostly grows in the tropical and subtropical regions of South and Southeast Asia (6). The plant has established a reputation for its antibacterial activities, antioxidant, anticancer, anti-inflammatory, and wound healing. It also serves as an antidote for snake venom (7, 8).

Recent studies highlight its promise in combating cancer, notably its capacity to prevent cell proliferation and migration. Nevertheless, the fundamental mechanisms behind its anti-hepatoma action have not been extensively investigated. Curcumol, which belongs to the Sesquiterpenoid group, has been isolated from many plant species of the Zingiberaceae family. Curcumol has demonstrated notable inhibitory effects on human lung adenocarcinoma ASTC-a-1 cells via a caspases-independent mitochondrial pathway. Furthermore, it has an exceptional capacity to inhibit the spread of breast cancer cells by blocking MMP-9 through the JNK1/2 and Akt-dependent NF-κB signaling pathways. Moreover, curcumol has been observed to induce cell cycle arrest and apoptosis in human nasopharyngeal carcinoma CNE-2 cells via IGF-1R/PI3K/Akt signaling pathway (9-11). The investigations provided evidence that curcumol displayed notable anticancer properties. Nevertheless, the impact and mechanisms of its action against liver cancer remain mostly unclear. In this study, we investigated the ethanol extract *Curcuma aromatica* rhizomes ethanol extract (CE) and its constituent curcumol against the human hepatoma cell line HepG2. The results of our study provide new and valuable information on how *C. aromatica* combats hepatoma, which may be used to build efficient and natural therapy approaches for liver cancer.

Materials and Methods

Materials

Curcuma aromatica were grown in Cau Ngang, Tra Vinh, Viet Nam (9°48′10″B 106°26′54″Đ) from April 2022 to December 2022 (rainy season). After this time, the products of photosynthesis synthesized in the leaves and pseudostems will be transported to the rhizome and storage roots for accumulation. *Curcuma aromatica* rhizomes are harvested, washed, and dried to 15% humidity. After drying, the rhizomes are crushed through a 0.25mm sieve to make powder. The powder samples were used to extract. The voucher specimens (VST 2022-168) were deposited in the Department of Bio-active Compounds at the Institute of Tropical Biology. Comparative morphology was used to identify the scientific names of studied species based on the prior publications (12), and DNA Barcoding (data not shown by our study).

Methods

Extraction and determination of the Curcumol compound

Place 0.5 g of dry powder sample into a 50 mL test tube with a cap (each concentration repeated three times). Utilize the soaking method with 60% ethanol for 24 hours at 50 °C. Filter the solution to collect the extract. Store the extracted solution in a 50 mL test tube, add the corresponding solvent to achieve a uniform volume of 50 mL for ease of calculation, and close the lid. Preserve the extract at -20°C for stability in determining the biologically active ingredients. Analyze the curcumol content in the extract using High-Performance Liquid Chromatography (HPLC)

Determination of the phenolic content of the extract

0.5 mL of the sample was mixed with 2.5 mL of Folin-Ciocalteu reagent, take the mixture stabilized for 6 minutes. Next, add 2 mL of a 7.5% (w/v) sodium carbonate, and incubate in the dark at room temperature for 1 hour. Utilize a UV-Vis spectrophotometer (Jasco V730 UV-VIS) to measure the absorbance of the solution at 765 nm, with gallic acid as the reference standard. Quantify the Total phenolic content by measuring it in milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW) (13).

Determination of the curcumol content of the extract

Determination of curcumol content by HPLC method (14), Curcumol standard solution (5-100ppm), HPLC analysis was performed on a 2995 system (Water, USA), connected to a DAD 2996 detector. Chromatographic separation used a C18 column (250 × 4.6 mm, 5 μm), mobile phase solvent includes a mixture of acetonitrile: methanol: H_2O: phosphoric acid ratio 550:225:225:1. Flow rate was 1 mL/min, injection sample volume was 10 µL. Results were recorded at 210 nm wavelength.

Cell culture

HepG2 and Chang's Liver cells were obtained from the American Type Cell Culture Collection (ATCC, Maryland, USA). The cells were cultivated in DMEM (Delbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (GIBCO BRL) and 1% penicillin-streptomycin. The incubation settings consisted of a temperature of 37°C in a humidified incubator with a gas composition of 5% CO_2 and 95% air. The cultures were

monitored by doing regular microscopic inspections using an inverted microscope.

Cell viability assay

HepG2 cells were placed onto 24-well plates at a density of 5×10^4 cells/well and incubated at 37°C. CDEM was added to each well after 1 day and 3 days. Cell viability was assessed following each period using the WST-1 test (EZ3000, Daeil Lab Service Co., Seoul, Korea). Each well was rinsed with PBS. Then, 200 µL of the WST-1 agent, diluted at a ratio of 1:10 with the culture media, was added and the cells were cultivated for one hour. Next, 100 µL from each well was transferred to a 96-well plate and read using an ELISA plate reader (SUNRISE, TECAN, Grödig, Austria) at a wavelength of 450 nm.

Real- time quantittive (qrt-PCR)

HepG2 cells were harvested with a concentration of 5×10^4 cells. Total RNA was extracted Qiagen RNeasy kit. cDNAs were synthesized using the Goscript™ Reverse Transcription System Kit (Promega, Japan). The qrt-PCR was run in CFX96 Touch Real-time PCR System Biorad). The cDNA was measured using the SYBR Green Realtime PCR Master Mix (Toyobo, Japan) following the directions provided by the manufacturer. The primers utilized for the studies are outlined in Table 1.

Table 1. The primers used for the Real-time PCR

Target gene	Primer sequence
GAPDH	F: 5'-GAAGGTCGGAGTCAACGGATTT-3'
	R: 5'-CTGGAAGATGGTGATGGGATTTC-3'
Bcl-2	F: 5'-TTCTTTGAGTTCGGTGGGG-3'
	R: 5'-CAGGAGAAATCAAACAGAGGC-3'
Bax	F: 5'-CTTTTGCTTCAGGGTTTCATC-3'
	R: 5'-CACTCGCTCAGCTTCTTGGT-3'
	R: 5'-CCAAATCCTCCAGAACCAAT-3'

Spectrofluorometric assay

A spectrofluorometric assay was carried out to detect nuclear condensation and fragmentation in intact cells (15). HepG2 cells were cultivated in a 96-well plate at a temperature of 37°C. The cells were treated with CE at concentrations of 50mg/mL and 100mg/mL Analysis of the cells was conducted at 4, 24, and 48 hours by centrifugation (8000g) at room temperature for 5 minutes. Subsequently, 70 mL of the supernatant was replaced with warm phosphate-buffered saline (PBS), and then 10 µL of H33258 solution was added to each well, with the final concentration ranging from 0.1 to 5 µg/mL, for 60 minutes. Spectrofluorometric measurements were then performed at an excitation/emission wavelength of 352/461 nm using a fluorescent microplate reader (Tecan Infinite F200, Switzerland).

Annexin V-FITC Apoptosis Assay

The quantification of apoptosis in HepG2 cells was performed through a Flow cytometry Annexin V-FITC/PI staining assay (16). The collected cells were washed with cold PBS and stained with 5 µL of FITC-Annexin V solution and 5 µL of PI solution. After gentle vortexing, the cells were incubated for 15 minutes at room temperature in the dark. Subsequently, 400 µL of 1X binding buffer was added to the samples and the analysis was performed using FACS (FACS Aria II from BD Biosciences, USA) within 1 hour.

Statistics

The data were presented as mean ± SD (n = 3). The statistical analysis was conducted using Student's t-test and One-way ANOVA with SigmaPlot version 11.0.

Results and Discussion

Extraction and determination of the Curcumol compound

Various extraction methods were employed to obtain extracts from *Curcuma aromatica* powder (data not shown). For this study, we utilized a 60% ethanol concentration at 50°C for 24 hours to extract polyphenol and curcumol. The total phenolic and curcumol contents were 20.44 ± 0.78 mgGAE/g and 970.27 ± 15.71 mg/g extract, respectively. The HPLC quantification of curcumol is illustrated in Figure 1.

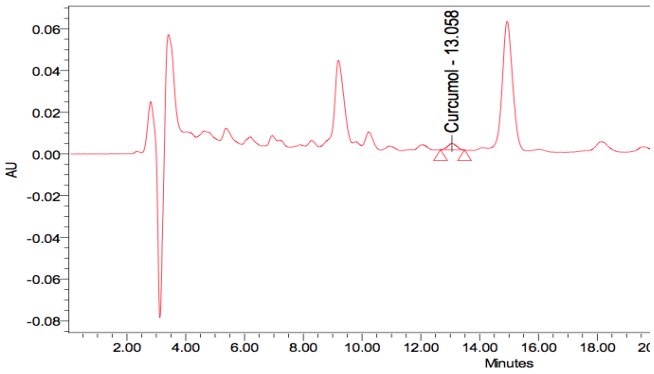

Figure 1. HPLC results of *Curcuma aromatica* root extract sample

Cytotoxicity test

HepG2 cells and Chang's cells were treated with CE and curcumol at concentrations (3.125; 6.25; 12.5; 25; 50; 75; and 100 µg/mL) and 0 µg/mL were used as a blank control for 24, 48, and 72 hours respectively. The ability to inhibit cell proliferation was tested by the WST-1 method. The results showed that CE and curcumol both caused secondary cell death. Depending on the concentration and treatment time, especially at 100 µg/mL and cultured for 72 h. At 72 h, all samples of CE and curcumol (100 µg/mL) reduced cell viability, with the survival rate of HepG2 cells being 34.559 ± 0.456 % (*Curcuma aromatica*), and 50.961± 0.641 %, (curcumol).

Results from Figure 2 indicated that the liver cancer cell line HepG2: CE exhibited an IC50 value of 50-75 µg/mL, whereas curcumol has an IC50 near 100µg/mL. CE demonstrates a greater ability to inhibit inhibits proliferation of cancer cells compared to curcumol.

Figure 3 shows that, for the liver cell lines (Chang's liver), the CE and curcumol have minimal effects (less toxic), and toxicity is only observed at 100 µg/mL after 72 hours of treatment (Figure 3). Therefore, the CE demonstrates the ability to inhibit the proliferation of cancer cells (HepG2) with little impact on normal cells.

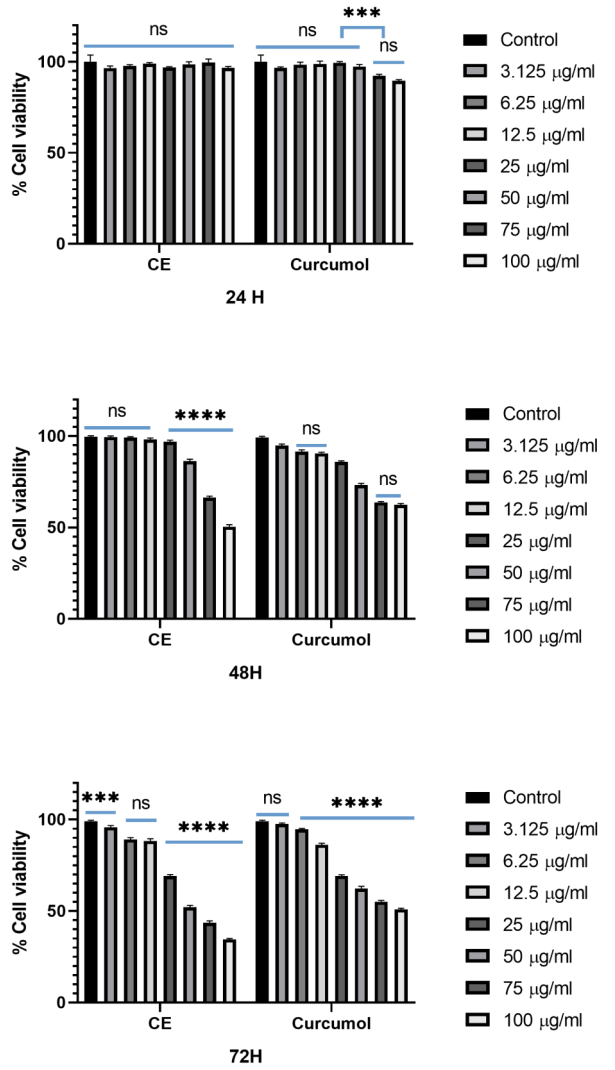

Figure 2. Effects of *Curcuma aromatica* extract and curcumol on the proliferation of HepG2 cells using the WST-1 method. A) **%** Cell viability, relative to the initial number at time 24h with the indicated nontoxin except for concentrations of curcumol 75 mg/mL and 100 mg/mL. B) Exposure for 48 hours to CE concentrations of 50-100 µg/mL, and curcumol concentrations of 6.25-100 µg/mL exhibited toxicity. C) Exposure for 72 hours to CE and curcumol concentrations of 12.5-100 µg/mL exhibited toxicity. Data represent means±SD of three independent experiments (ns: Nonsignificant, ***P<0.05,****P<0.0001)

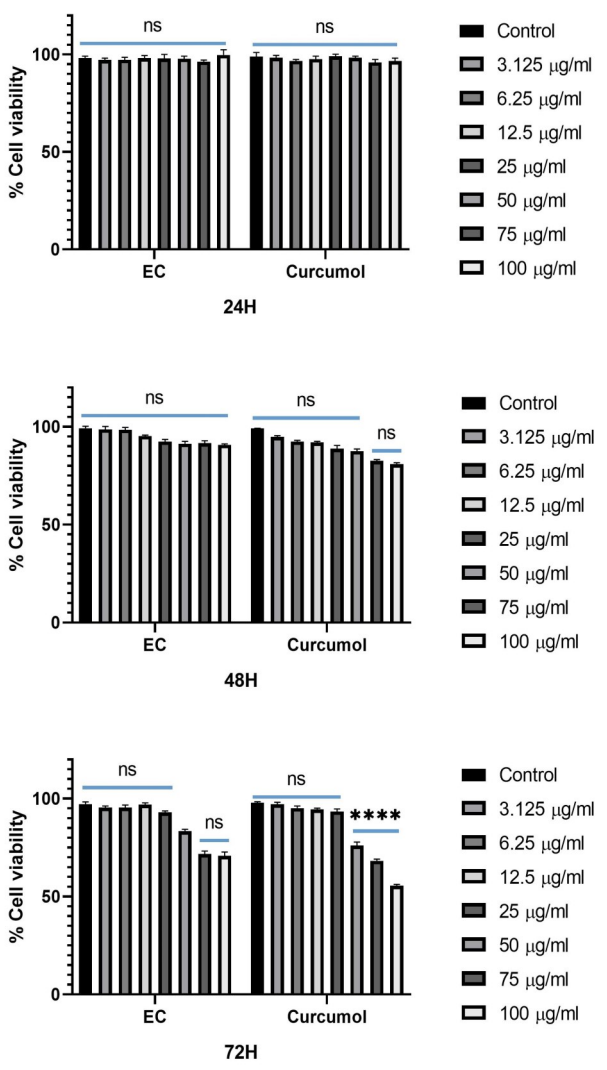

Figure 3. Effects of *Curcuma aromatica* extract and curcumol on the proliferation of Chang's cells using the WST-1 method after 24h, 48h, and 72h. A) **%** Cell viability, relative to the initial number at time 24h with the indicated nontoxin. B) Exposure for 48 hours to CE exhibited nontoxicity except for concentrations of curcumol concentrations of 75-100 µg/mL exhibited less toxicity. C) Exposure for 72 hours to CE concentrations of 75- 100 µg/mL, and curcumol concentrations of 50-100 µg/mL exhibited mild toxicity. Data represent means±SD of three independent experiments (ns: Nonsignificant, ****P<0.0001)

Evaluation of cell nuclear fragmentation

Results show that through light wavelengths and electron microscopy, it is possible to identify the phenotypic changes that occur during apoptosis. At an early stage, chromosomes will clump together into blocks (pyknosis) and this is a characteristic sign of apoptosis. Following the pyknosis stage, the cell membrane will undergo cleavage to form cell debris from the apoptotic body called the "budding" process. Then, the cells will enter the stage of programmed death (apoptosis).

At first, we examined the effect of CE on HepG2 cells by observing their capacity to undergo apoptosis. We used two different doses of the extract (50 and 100 µg/mL) and compared the results to a control group. The observations were conducted at time intervals of 24, 48, and 72 hours. Following 24 hours, the extract treatment group exhibited alterations in the cell membrane and the presence of apoptotic bodies, but the control group did not. The findings indicated that the cell morphology in the control group exhibited clarity and consistency with the dye. Nevertheless, among the treatment groups of the extract, certain regions exhibited a higher level of darkness, whereas certain locations were encompassed by diminutive zones of intense light (Figure 4). The degree of fragmentation of the HepG2 cell nucleus increased proportionally with longer treatment durations and larger concentrations of the treatment extract.

The results presented in (Figure 5) and (Figure 6) reveal a statistically significant difference in the proportion of apoptotic cell groups between samples treated with CE and the control group at three-time points: 24, 48, and 72 hours. With the increase in treatment time, the percentage of HepG2 cells entering apoptosis also rises. Thus, CE can induce HepG2 cells to enter apoptosis at a concentration of 50 µg/mL after 24 hours of treatment.

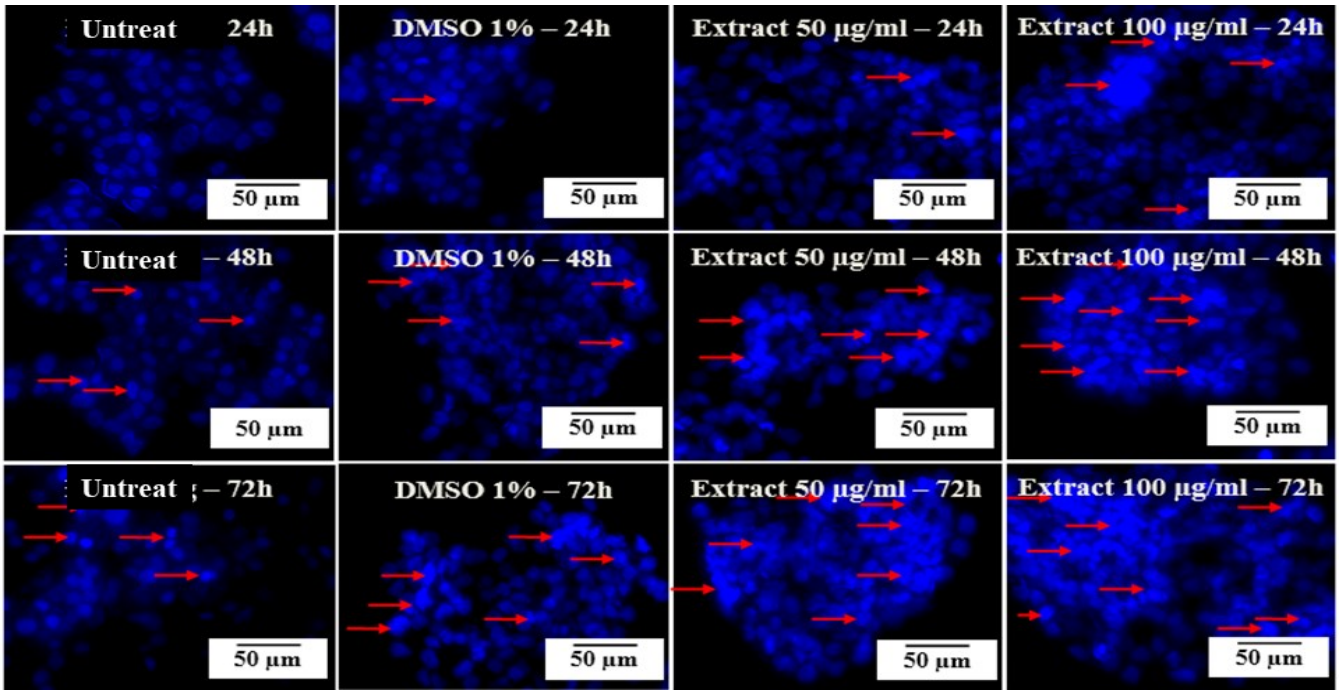

Figure 4. Image of HepG2 cells stained with Hoechst 33342 under the influence of *Curcuma aromatica* extract, Arrows indicate condensed and fragmented nuclei.

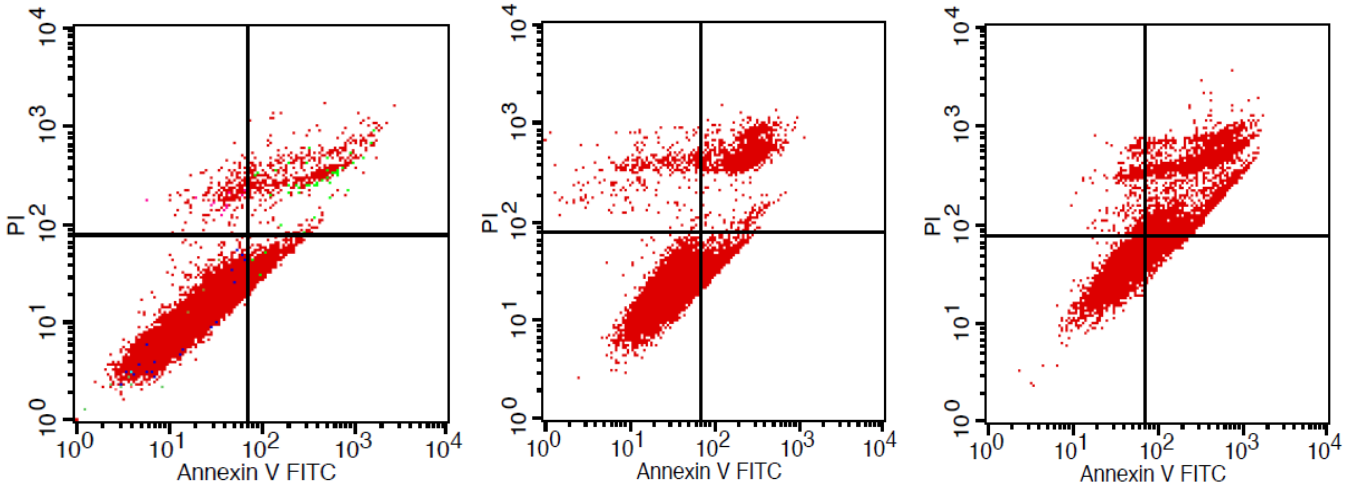

Figure 5. The impact of time exposure to a concentration of 100 µg/mL of CE on apoptosis in HepG2 cells was assessed using flow cytometry with Annexin V-FITC/PI staining. The lower left quadrant represents viable cells, the lower right quadrant shows early apoptotic cells, the upper right quadrant signifies late apoptotic cells, and the upper left quadrant indicates dead cells.

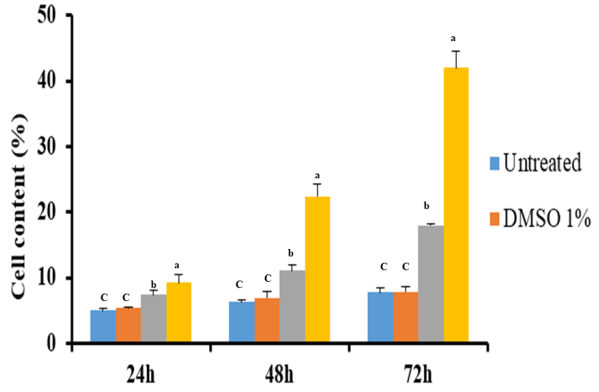

Figure 6. Percentage of HepG2 cells entering apoptosis after treatment with *Curcuma aromatica* extract for 24h,48h and72h (a, b, c: statistically significant difference, $p < 0.05$).

CE and curcumol inhibited Bcl-2 expression

In this study, we determined the ability to inhibit liver carcinoma cells (HepG2) through Bcl-2 expression. The HepG2 cells were treated with CE at two concentrations (50 and 100 µg/mL) after 48 hours, using 1% DMSO as a control. Figure 7 indicates a difference between the control group and the 100 µg/mL treatment, but no difference was observed with the 50 µg/mL treatment. The expression of Bcl-2 decreased by 40% in the batch treated with 100 µg/mL extract after 48 hours and remained unchanged in the batch treated with 50 µg/mL extract. Although there was a decrease in expression, it was not statistically significant.

This provides evidence of a reduction in the expression of the Bcl-2 gene in the nuclei of cell batches treated with CE at a concentration of 100 µg/mL after 48 hours

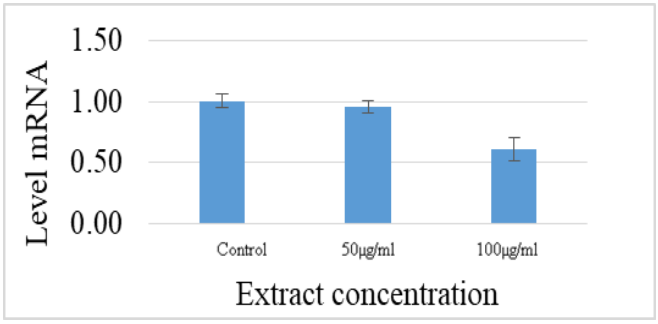

Figure 7. Effect of *Curcuma aromatica* extract on Bcl-2 expression in HepG2 cells.

Bax expression

We also assessed Bax expression in HepG2. According to the chart in Figure 8, there is no statistical difference between the control group and the treatments of 50 µg/mL and 100 µg/mL. This indicates that the expression of the Bax gene in the nuclei of cell batches treated with CE did not change after 48 hours.

In the control group, there was a balanced expression of Bcl-2 and Bax mRNA, preventing cells from entering apoptosis. Conversely, in groups of cells treated with CE at concentrations of 100 µg/mL, the balance in the expression of Bcl-2 and Bax mRNA shifted downward. This alteration in Bcl-2 mRNA expression at the transcriptional level leads to the induction of these cells into apoptosis, exhibiting characteristic features of apoptosis.

The RT-PCR analysis results above align with the assessments of cytoplasmic fragmentation, chromatin condensation, and nuclear fragmentation. The process of reducing the expression of transcription products of the anti-apoptotic gene Bcl-2 and maintaining the expression of the pro-apoptotic gene Bax corresponds to morphological changes in the apoptosis process, such as more pronounced cell fragmentation, cytoplasmic changes, chromatin condensation, and nuclear fragmentation.

However, FACS test results and nuclear staining showed that the CE extract can induce HepG2 cells to enter apoptosis at a concentration of 50 µg/mL after 24 hours. Nevertheless, our results did not show significant differences in Bax and Bcl-2 gene expression at this time point compared to the control. To examine changes at the molecular level, further observation of mRNA expression levels of p53 and caspase family genes was necessary.

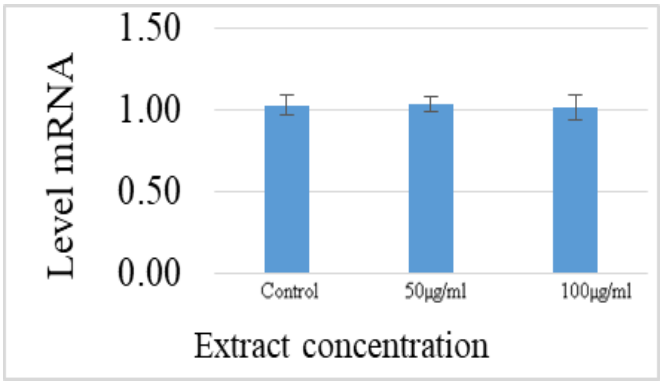

Figure 8. Effect of *Curcuma aromatica* extract on Bax expression in HepG2 cells. There is no statistical difference with $p > 0.05$.

Discussion

Supawadee Burapan (2020) studied the ethanol extracts obtained from various Curcuma species. The results indicated that the total phenolic content varied depending on the species, ranging from 0.4 ± 0.1 to 22.3 ± 2.4 mg GAE/g. The highest total phenolic content was observed in C. longa (22.3 ± 2.4 mg GAE/g), while *Curcuma aromatica* exhibited a phenolic content of 11.0 ± 0.2 mg GAE/g (17). Our results for the ethanol extract of *Curcuma aromatica* growth in Tra Vinh (Viet Nam) showed a total phenolic content twice as high as the aforementioned study.

Curcuma aromatica is rich in essential oils and secondary metabolites, including zederone, curcumol, curcumin, germacrone, camphor, curzerenone, 7-methanoazulene, 1,8-cineole, β-elemene, linalool, 9-oxo-neoprocurcumenol, neoprocurcumenol, β-curcumene, ar-curcumene, and xanthorrhizol (18, 19). However, the extraction of curcumol for anticancer properties using ethanol extract in *Curcuma aromatica* has not been previously performed. Huang et al. (2000) only researched the isolation of curcumol but did not evaluate the concentration of curcumol in *Curcuma aromatica*. Our study discovered curcumol in the ethanol extract of *Curcuma aromatica* and determined its content to be 970.27 ± 15.71 µg/g extract by HPLC. This study is significant because most research indicates that curcumol is present in essential oil solutions at low concentrations (20).

Curcuma aromatica and curcumol are recognized for their anticancer properties, being identified as apoptosis inducers (21). Our investigation revealed that both CE and curcumol effectively inhibited the growth of HepG2 cells in a time- and concentration-dependent manner. This study demonstrates that CE and curcumol induce apoptosis in human hepatoma HepG2 cells by downregulating Bcl-2 expression. The apoptotic-inducing is primarily affected by the equilibrium between Bcl-2 and Bax, rather than only by the amount of Bcl-2 (22). This equilibrium also influences cell growth. Cell viability is constant while the levels of Bcl-2 and Bax are in equilibrium. Increased levels of Bcl-2 expression result in the suppression of cell apoptosis (23). Our investigation reveals the involvement of Bax and Bcl-2 in the induction of HepG2 cell apoptosis by CE and curcumol. The mRNA expression of Bcl-2 was decreased, and Bax was balanced, resulting in a subsequent increase in the Bax/Bcl-2 ratio, which induced apoptosome formation.

Conclusion

Based on the findings, it was deduced that the chosen extraction conditions for *Curcuma aromatica* rhizomes induce apoptosis in HepG2 cells, hindering cancer cell division. The observed inhibitory effect was attributed to a reduction in the expression of the anti-apoptotic gene, Bcl-2, and an elevation in the expression of the proapoptotic gene, Bax.

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Authors' contributions

This study was conceptualized and executed by BDT, who also authored the manuscript. All authors participated in the literature review, and manuscript revision, and provided valuable insights. THH finalized the manuscript and coordinated submission to the journal.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare

Ethical issues: None

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