Chemopreventive Properties *Curcuma heyneana* Rhizome Ethanolic Extract on Hepatocellular Carcinoma Cells, JHH-4

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Abstract

Hepatocellular carcinoma is the most common type of liver cancer. Curcuminoids are natural polyphenol compounds abundant in *Curcuma heyneana* ethanolic extract (CHE) and are known to inhibit breast and cervical cancer cell proliferation. Based on previous research, curcuminoid compounds have been studied to inhibit the growth of the liver cancer cell model, HepG2. This study aims to examine the potential of CHE as a chemopreventive agent in liver cancer using JHH-4 cell as a model. CHE was obtained by maceration method using ethanol which was then identified for its phytochemical profile using thin layer chromatography (TLC). Then TLC results were quantified to calculate the levels of compounds present in the CHE based on spot intensity with ImageJ software. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay was conducted to determine the radical scavenging activity of CHE. Cytotoxic activity of CHE on JHH-4 liver cancer cells was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Extraction produces a yield of 10.2 %w/w. CHE contains 4.52 %w/w curcuminoid compound consisting of 0.49 %w/w curcumin, 3.21 %w/w demethoxycurcumin, and 0.82% w/w bisdemethoxycurcumin. CHE exhibited antioxidant activity with an IC_{50} value of 378.96 µg/mL, meanwhile ascorbic acid as a positive control has an IC_{50} value of 8.49 µg/mL. Cytotoxic activity of CHE on JHH-4 cells is characterized by an IC_{50} value of 16.62 µg/mL which is classified as having strong cytotoxic activity. This study concluded that CHE has the potential to be developed as a chemopreventive agent in liver cancer.

Keywords: *liver cancer, hepatocelullar carcinoma, Chemopreventive, antioxidant,* Curcuma heyneana.

INTRODUCTION

In recent years, liver cancer has become a major global health problem and one of the leading cause of cancer-related death worldwide. According to the World Health Organization (WHO), liver cancer is the fifth most common cancer and the third leading cause of death globally, responsible

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for around 830,000 deaths each year (Asrani, *et al.*, 2019). Hepatocellular carcinoma (HCC) is the most common type of liver cancer, accounting for about 75% of all cases (Duong, *et al*., 2022). HCC is a challenging form of cancer to identify due to its internal nature, as signs of liver cancer only become apparent in the latter stages of the disease. (Deepa, *et al*., 2022).

Hepatocellular carcinoma has been treated using surgical procedures, radiation, organ transplantation, and chemotherapy (Hermawan, *et al*., 2011). Chemotherapy functions by impeding the proliferation of cancer cells, halting the process of cancer cell division and exterminating cancer cells (Liu, *et al*., 2016). Unfortunately chemotherapy has detrimental impacts on the body's health, including hair loss, intestinal issues, anemia, and other adverse reactions. Furthermore, liver cancer patients may face a significant financial burden due to substantial cost associated with treatment. Therefore, additional investigation is required to address liver cancer.

Indonesia is a mega-biodiversity country rich in potential chemopreventive medical plants agents. Chemopreventive is defined as efforts to inhibit, prevent, or suppress the development of cancer by using natural ingredients, synthesis, or a combination of both (Indrasetiawan, *et al*., 2020). Chemopreventive can serve as a remedy for mitigating adverse effects of chemotherapy that detrimentally affect the overall health of the body. Chemopreventive drugs are potent viable replacement for present liver cancer therapy, as it is expensive and has low success rate (Anwanwan, *et al*., 2020).

Curcuma heyneana is a species of plant belonging to the genus Curcuma, part of the Zingiberaceae family. *Curcuma heyneana* grows in yards with moist soil. On Java Island, *Curcuma heyneana* generally grows in October and produce rhizome to be harvested in May or June, meanwhile a good quality rhizome can only be harvested in July (Astuti, *et al*., 2014). *Curcuma heyneana* is known to have several health benefits and can increase appetite. *Curcuma heyneana* contains curcuminoids, namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Syarifah, *et al*., 2019). Several previous studies has demonstrated that *Curcuma heyneana* as anticancer, antioxidant, and antiviral properties (Fajrin, *et al*., 2023). Curcumin has been shown to up-regulate intracellular reactive oxygen species (ROS) levels above the threshold through binding to several enzymes involved in ROS metabolism, thereby induce apoptosis in tumor cells (Larasati, *et al*., 2018). Based on previous research, *Curcuma heyneana* has antioxidant activity through radical scavenging mechanism based on DPPH assay (Marianne, *et al*., 2018). Cytotoxic effect is reflected on MCF-7 breast cancer cells and Ca-Ski cervical cancer cells (Atun, *et al*., 2010). *Curcuma heyneana* possesses antiviral properties against hepatitis C, a known etiology of liver cancer (Wahyuni, *et al*., 2018). Based on previous research, curcuminoid compounds have been studied to inhibit the growth of the liver cancer cell model, HepG2 (Abdeel-Lateef, *et al*., 2016).. Accordingly, *Curcuma heyneana* has the ability to act as a chemopreventive agent in cancer cells. However, there has been no research on chemopreventive activity of *Curcuma heyneana* against the JHH4 liver cancer cell model.

The purpose of this study was to investigate the potential of *Curcuma heyneana* rhizome as a chemopreventive agent in JHH-4 liver cancer cells. These cells produce alpha fetoprotein and albumin, have low MYCN and NCYM expression, and show absence of p53 protein expression (Qin, *et al*., 2018). *Curcuma heyneana* extract containing curcuminoids is expected to have cytotoxic effect on liver cancer cells and to have antioxidant activity that is beneficial to the health of the body.

MATERIALS AND METHODS

Curcuma heyneana Extract (CHE) Maceration

The rhizome of *Curcuma heyneana* was collected from the yard area of Pondo, Condong Catur, Sleman, Special Region of Yogyakarta,

Indonesia. The plant is five months old and was harvested in April 2023. Subsequently, the rhizome was authenticated in the Phytochemical Laboratory of the Faculty of Pharmacy at UGM with authentication number 13.5.4/UN1/FFA.2/BF/PT/2023. The analysis revealed that the simplicia used in this study originated from the species *Curcuma heyneana* Val & Zijp. Before extraction, *Curcuma heyneana* simplicia was prepared by cutting it horizontally with a thickness of approximately 5 millimeters. Next, the simplicia was subjected to dehydration in a dehydrator at a temperature of 40ºC for a duration of one day and one night. Once the simplicia has dried, it is pulverized in a blender and strained through a 40-mesh filter. The powder that was provided is measured to be 4 grams then mixed with a tube rotator (IWAKI) using 40 mL of ethanol solvent. This mixture is shaken at a continuous speed of 100 rpm for a duration of 24 h. The maceration products were strained via a Buchner funnel that was linked to a vacuum pump (WIPRO). Subsequently, the substance is subjected to a drying process and then evaporated under a fume hood for 48 h to acquire the extract of *Curcuma heyneana* (CHE).

Thin Layer Chromatography (TLC)

Thin layer chromatography was performed on a 10 cm x 3 cm silica gel f 254 plate (Merck, Darmstadt, Germany) as the stationary phase. Then the plate was activated by heating in a dehydrator at 105ºC for 30 minutes (Sari, *et al*., 2022). Sample was prepared by dissolving 1 mg dry extract to 0.2 mL methanol (Merck) then vortexed to form a 5 mg/mL CHE solution. A solution of 95% curcuminoid extract was used as a standard. Prior to elution, the chamber was saturated by observing the elution on a filter paper. Mobile phase used was chloroform and methanol in a ratio of 19:1 (Kemenkes., 2017). Plate was observed on visible light, 245 nM UV light, 366 nM UV light to determine the Rf value of the spots gained. Intensity of the spot was calculated using ImageJ software to analyze the proportion of curcuminoid content in CHE (Sowers, *et al*., 2022).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The antioxidant activity of CHE was analyzed through the ability of CHE to turn radical DPPH into a stable form which absorbed light at 517 nm. Ascorbic acid was prepared as a positive control in the concentration of 1000 µg/mL. Then, a series of multilevel dilution was used in a range of 1 µg/mL-20 µg/mL with triple replication and pipetted in 160 µg/well. Sample of CHE was prepared as well in the concentration of 5000 µg/mL in 96% ethanol and made into a series of multilevel dilution with concentration's range of 150 µg/mL-1000 µg/mL and replicated three times. DPPH was added as much as 40 µg/well and ethanol solvent, a control solution, was added equally as much for each well. Plate then was incubated in a dark environment for 30 minutes, then read in 517 nM wavelength (BSN, 2018). The data obtained from absorbance was converted into a DPPH reduction percentage and analyzed using the linear regression method. DPPH reduction showed the antioxidant activity of CHE. Antioxidant activity is classified into 5 categories, namely very strong antioxidant activity (IC_{50}<50 µg/mL), strong (IC_{50} 50-100 µg/mL), medium (IC_{50} 101-250 µg/mL), weak (IC_{50} 250-500 µg/mL), and very weak (IC_{50}>500 µg/mL) (Marjoni, *et al*., 2017).

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

JHH-4 cells, a liver cancer cell model of hepatocellular carcinoma type taken by a 51-year-old Japanese man with a similar morphology to epithelial cells, were 80% confluent before grown in 96 well plates at a rate of 2,500 cells/well. Cells were incubated 1x24 h and treated with various concentrations of CHE (1, 10, 25,

50, 100, 250, and 500 µg/mL) in triple replication. Then the cells were incubated in a CO_2 incubator for 1x24 h. After incubation, the test solution was discarded, washed with PBS and added 100 µl/well reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 0.5 mg/mL. MTT assay is colorimetric method uses MTT reagent where the tetrazolium salt is broken down into formazan crystals by succinate tetrazolium reductase which is present in the cell respiration pathway in active mitochondria of living cells. Formazan crystal gave a purple colour in an absorbance of 595 nm using an ELISA reader (BSN, 2018; Nurcholis, *et al*., 2017). After incubation for 4 h, 10% SDS stopper was added in 0.01 N HCl as much as 100 µl/well and left overnight in the dark (Hasumura, *et al*., 1988; Jenie, *et al*., 2021). The data obtained in the form of absorbance was converted into a percentage of live cells and analyzed using the linear regression method. According to the National Cancer Institute (NCI), a compound is considered to have strong cytotoxic activity if the IC_{50} value is <20 µg/mL, moderate cytotoxic activity if the IC_{50} ranges from 21-200 µg/mL, weak cytotoxic activity if the IC_{50} ranges from 201-500 µg/mL, and has no cytotoxic activity if the IC_{50} value is >500 µg/mL (Bukowski, *et al*., 2022).

RESULTS

Curcuma heyneana Extract (CHE) Maceration

Curcuma heyneana extract (CHE) preparation was done by drying the simplicia (Figure 1A) in a dehydrator for 24 h. After the simplicia is dry, the simplicia is blended into a blender then sieved using a 40-mesh sieve (Figure 1B), 4 grams of the passed powder is weighed and macerated in 40 mL ethanol p.a. for 24 h. The maceration results were filtered and concentrated (Figure 1C). The extract obtained was 408 mg or 10% w/w of dry simplicia (Figure 1D).

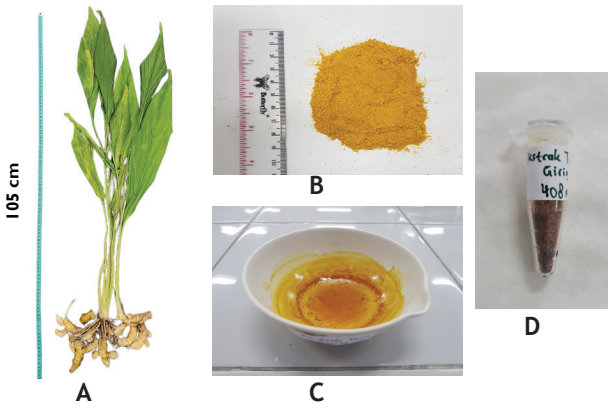

Figure 1. Extraction of *C. heyneana* rhizome. As much as 4 grams of *Curcuma heyneana* simplicia powder was macerated with ethanol *p.a* and stirred using a tube rotator for 24 h then concentrated and dried for 24 h. As much as 408 mg of *Curcuma heyneana* macerate extract was produced. *Curcuma heyneana* simplicia(A); *Curcuma heyneana* simplicia powder passes 40 mesh (B); *Curcuma heyneana* extract (CHE) 10.2% w/w (C,D).

Phytochemical Constituent of *Curcuma heyneana* Extract (CHE)

Phytochemical analysis to determine the bioactive compounds of the CHE was carried out by Thin Layer Chromatography (TLC) with an eluent of chloroform and methanol in 19:1 ratio. The spots of CHE was identified at Rf 0.85, 0.56, and 0.4 which parallel to standard 95% curcuminoids extract at Rf 0.84, 0.59, 0.35 in visible light visualization, UV 254 nM, and UV 366 nM (Figure 2). This result proved the presence of curcumin, demothxycurcumin, and bisdemotheoxycurcumin in CHE. Visually, the spots of demethoxycurcumin looked thicker than those of curcumin and bisdemethoxycurcumin on visualization of UV 366 nM.

The 366 nM UV light spot intensity was measured by ImageJ software. The result showed speckled areas of curcumin, demethoxycurcumin, and bisdemethoxycurcumin on standard curcumin, standard 95% curcuminoid extract and CHE

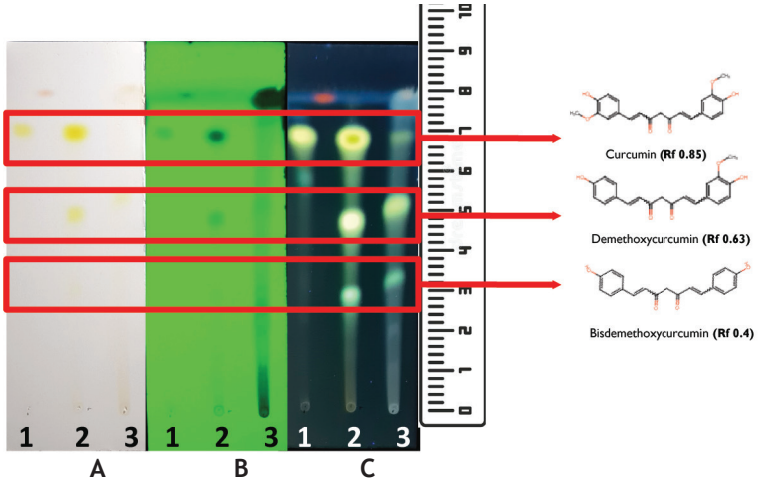

Figure 2. Chromatogram Profile. Standard curcumin 1 mg/mL, curcuminoid standard 1 mg/mL, and 15 mg/mL *Curcuma heyneana* extract (CHE) were eluted in a mixture of chloroform and methanol (19:1) with silica gel plate stationary phase F254 (Merck). The confirmed extract contains curcumin at Rf 0.85, demethoxycurcumin with Rf 0.63, and bisdemethoxycurcumin with Rf 0.4 as seen in the visualization of A. visible light; B. uv 254 nM, C. uv 366 nM. 1. standard curcumin 1 mg/mL; 2. standard curcuminoids 1 mg/mL, and 3. CHE 15 mg/mL.

(Figure 3). The area obtained the levels of curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Demethoxycurcumin has the highest concentration in CHE of 3.21 %w/w, while curcumin compounds in CHE have concentrations of 0.49 %w/w and bisdemethoxycurcumin 0.82 %w/w (Table 1).

Radical Scavenging Activity of *Curcuma heyneana* Extract (CHE)

Radical scavenging activity of ascorbic acid and CHE were obtained by DPPH as a radical agent. Radical scavenging activity of vitamin C as a positive control resulted in IC_{50} of 8.49 µg/mL

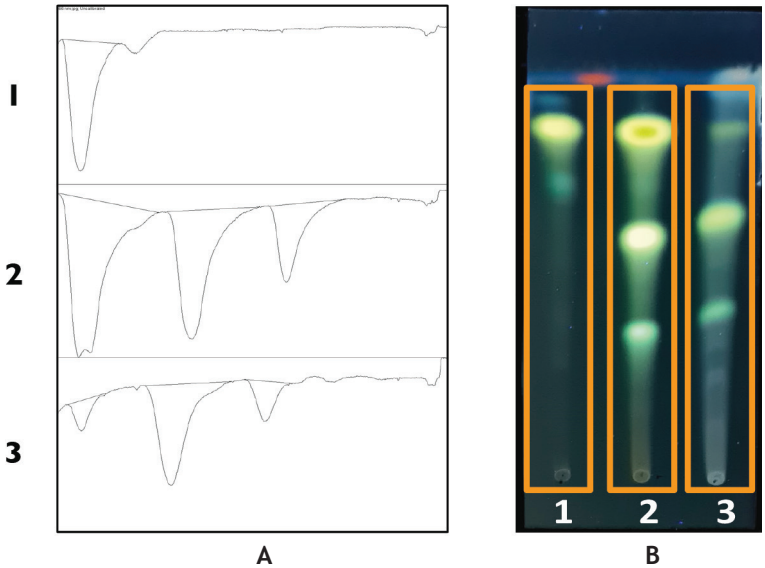

Figure 3. Intensity band curve from TLC results at 366 nM UV light. TLC results on 366 nM UV light were measured on the intensity band using the ImageJ software by measuring spot area on standard curcumin 1 mg/mL, standard curcuminoid extract 95% 1 mg/mL, and CHE 15 mg/mL. A. Intensity band curve from TLC results; B. TLC results at 366 nM UV light.

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Table 1. %Content of curcumin, demethoxycurcumin, and bisdemethoxycurcumin in curcumin standard, curcuminoid extract 95% standard and Curcuma heyneana extract.

Compound	Sample	Area	Content (%w/w)
Curcumin	Curcumin Standard	30470.21	100
	Curcuminoid Extract 95% Standard	58253.81	6.37
	Curcuma heyneana Extract	4492.46	0.49
Demethoxycurcumin	Curcuminoid Extract 95% Standard	39517.85	4.32
	Curcuma heyneana Extract	29342.63	3.21
Bisdemethoxycurcumin	Curcuminoid Extract 95% Standard	17907.79	1.96
	Curcuma heyneana Extract	7464.1	0.82

and classified as strong antioxidant (Figure 4A). The R-value of 0.99 indicates a good correlation between the data at the 95% level of confidence (R>0.81). Radical scavenging activity of CHE resulted in IC_{50} of 378.96 µg/mL and classified as weak antioxidant (Figure 4B). The R-value of 0.98 indicates a good correlation between the data at the 95% level of confidence (R>0.88).

Cytotoxic Activity of *Curcuma heyneana* Extract (CHE) in JHH-4 Cells

MTT assay was carried out by treating JHH-4 cells with each treatment of CHE. The higher the capitalized concentration, efficiently lower viability of living cells (Figure 5B). This corresponds to the IC_{50} obtained, 16.62 µg/mL (Figure 5A) and was classified as strong cytotoxic (Haryanti, *et al*., 2018).

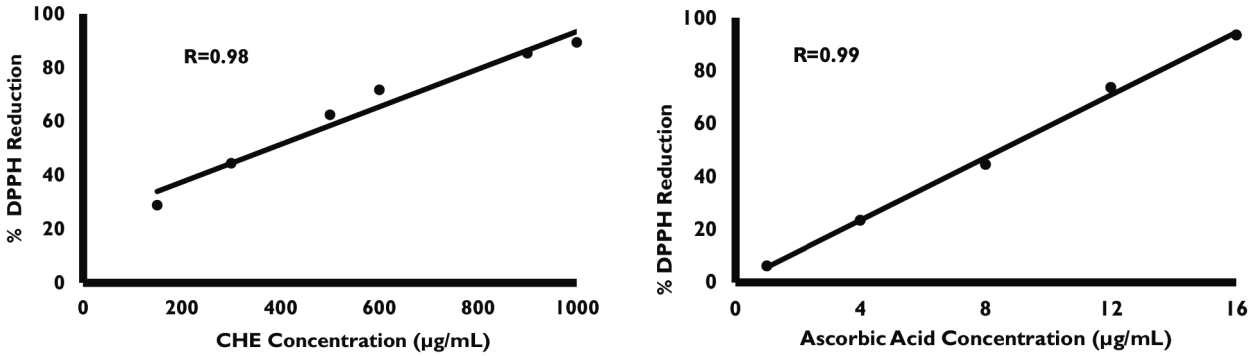

Figure. 4. Antioxidant profile of ascorbic acid and *Curcuma heyneana* extract (CHE). (a) Ascorbic Acid has antioxidant activity against DPPH treatment with IC_{50} of 8.49 µg/mL obtained from a linear regression calculation of ascorbic acid concentration vs. % free radical reduction of DPPH. Meanwhile (b) CHE has antioxidant activity against DPPH treatment with IC_{50} of 378.96 µg/mL.

DISCUSSION

C. heyneana is a plant belonging to the rhizome group of the genus Curcuma which has been widely used by the public because it is known for its properties. This research reveals the potential of *Curcuma heyneana* Val & Zijp rhizome as a chemopreventive agent, especially in liver cancer with the JHH-4 cell model, seen from cytotoxic and antioxidant activity. These two activities

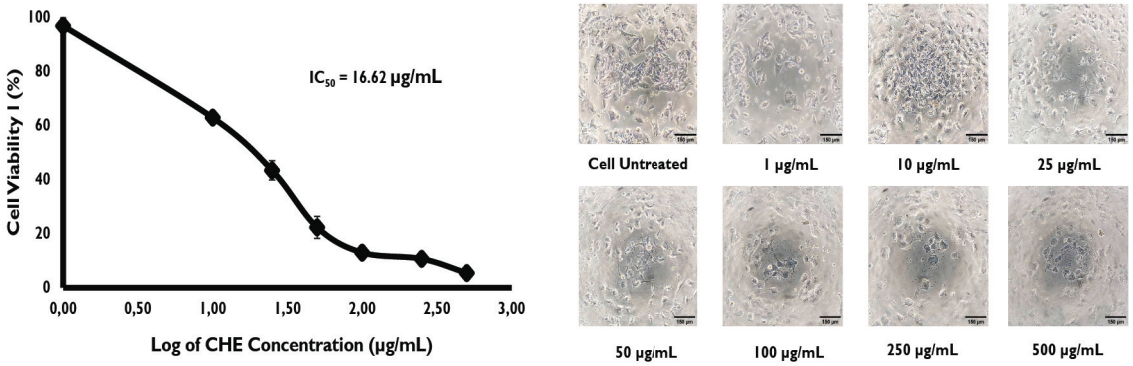

Figure 5. CHE Cytotoxicity Profile on JHH-4 cells. 2.500 of JHH4 cells were seeded in each well on a 96-well plate and treated with serial concentrations of CHE (1-500 µg/mL) for 24 h. Cytotoxic test was carried out using the MTT Assay method. (a) Viability profile of JHH4 cells due to CHE treatment and obtaining IC_{50} of 16.62 µg/mL; (b) JHH-4 cells morphology after being treated.

are two important aspects in the mechanism of chemopreventive agents. CHE has the ability to capture free radicals and inhibit cancer cell proliferation. The cell model used in this study was the JHH-4 cell, a strong proliferative hepatocellular carcinoma model (Hasumura, *et al*., 1998).

The investigation yielded conclusions regarding the amount of curcuminoids in CHE. Analysis using TLC showed the presence of curcuminoids in CHE, specifically curcumin, demethoxycurcumin, and bisdemethoxycurcumin. The distinguishing feature of CHE, compared to other plants in the Curcumin genus, is the significantly higher quantity of demethoxycurcumin (Table 1). Demethoxycurcumin antioxidant activity is known to be more potent than curcumin (Arrue, *et al*., 2017). In previous research, demethoxycurcumin had cytotoxic activity on gastric adenocarcinoma (AGS), colorectal adenocarcinoma (SW-620), and hepatocellular carcinoma (HepG2) cell lines (Araya-Sibaja, *et al*., 2024).

The antioxidant activity of the ethanolic extract of *C. heyneana* obtained in this study was compared with the results of the previous DPPH method antioxidant assay (Table 2). IC_{50} value of the ethanol extract of *C. heyneana* in this study was higher than previous studies, 378.96 µg/mL. Antioxidant activity is classified into 5 categories, namely a compound very strong antioxidant activity (IC_{50}<50 µg/mL), strong (IC_{50} 50-100 µg/mL), medium (IC_{50} 101-250 µg/mL), weak (IC_{50} 250-500 µg/mL), and very weak (IC_{50}>500 µg/mL) (Marjoni, *et al*., 2017). The results of this study indicate that the antioxidant activity of CHE is relatively weak, confirmed by study from Kusumawati (2018), obtaining IC_{50} of 338.18 µg/mL might due to the use of juvenile rhizomes of *C. heyneana* (Astuti, *et al*., 2014).

CHE exhibited strong cytotoxic activity on JHH-4 cells using MTT Assay method (Bukowski, *et al*., 2022). JHH-4 cells have strong proliferative characteristics due to overexpression of alpha fetoprotein, a crucial proliferation regulator of hepatocarcinoma cancer cell (Sauzay, *et al*., 2016). The IC_{50} results obtained in this study was lower compared to previous study by acquiring IC_{50} of 16.62 µg/mL. These results indicate that CHE has stronger cytotoxic activity than the *Curcuma longa* methanolic extract (49 µg/mL) and curcumin compounds isolated from *Curcuma longa* extract (90 µg/mL) on another liver cancer cell model, HepG2 (Abdeel-Lateef, *et al*., 2016). In addition,

the curcumin compound has been shown to increase intracellular ROS levels above the threshold through binding to several enzymes involved in ROS metabolism, thereby triggering apoptosis in tumor cells (Larasati, *et al*., 2018). These results may indicate the potential of CHE to be developed as chemopreventive agent in liver cancer model.

CONCLUSION

The curcuminoid portion of CHE is found to be dominated by demethoxycurcumin. *Curcuma heyneana* Extract (CHE) has strong cytotoxicity against JHH-4 hepatocellular carcinoma cells. The indifference of ideal harvested period of simplicia is hypothesized in contributing to weak radical scavenging activity result of CHE to DPPH. In previous study, curcumin able to increase intracellular ROS levels, resulting in tumor cells growth inhibition.Thus, CHE exhibits the capacity to act as a chemopreventive agent.

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AUTHOR CONTRIBUTION

CFS conducted the experiment, data analysis, and manuscript preparation. DM supported the manuscript preparation. MRPA supported the experiment and data analysis. DRR and NN supported the *in vitro* experiments. AH supervised the experiment and manuscript. EM designed of the study, supervised the experiments, and finished the manuscript. All the authors declair to have not had conflict in interest.

CONFLICT INTERESTS

The authors declare no competing or potential conflicts of interest concerning the research and publication of this article.

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