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Research Article

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Hydroxychavicol, a polyphenol from *Piper betle* leaf extract, induces cell cycle arrest and apoptosis in *TP53*-resistant HT-29 colon cancer cells

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Abstract: This study aims to elucidate the antiproliferative mechanism of hydroxychavicol (HC). Its effects on cell cycle, apoptosis, and the expression of c-Jun N-terminal kinase (JNK) and P38 mitogen-activated protein kinase (MAPK) in HT-29 colon cancer cells were investigated. HC was isolated from *Piper betle* leaf (PBL) and verified by high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR), and gas chromatography-mass spectrometry (GC-MS). The cytotoxic effects of the standard drug 5-fluorouracil (5-FU), PBL water extract, and HC on HT-29 cells were measured after 24, 48, and 72 h of treatment. Cell cycle and apoptosis modulation by 5-FU and HC treatments were investigated up to 30 h. Changes in phosphorylated JNK (pJNK) and P38 (pP38) MAPK expression were observed up to 18 h. The half maximal inhibitory concentration (IC_{50}) values of HC (30 μg/mL) and PBL water extract (380 μg/mL) were achieved at 24 h, whereas the IC_{50} of 5-FU (50 μmol/L) was obtained at 72 h. Cell cycle arrest at the G_0/G_1 phase in HC-treated cells was observed from 12 h onwards. Higher apoptotic cell death in HC-treated cells compared to 5-FU-treated cells ($P<0.05$) was observed. High expression of pJNK and pP38 MAPK was observed at 12 h in HC-treated cells, but not in 5-FU-treated HT-29 cells ($P<0.05$). It is concluded that HC induces cell cycle arrest and apoptosis of HT-29 cells, with these actions possibly mediated by JNK and P38 MAPK.

Key words: *Piper betle*; Hydroxychavicol (HC); Cell cycle; Apoptosis; c-Jun N-terminal kinase (JNK); P38 mitogen-activated protein kinase (MAPK)

1 Introduction

Plants and natural products have been investigated extensively for centuries, focusing on drug discovery and development. Anticancer agents such as vincristine, vinblastine, and paclitaxel are examples of drugs derived from plants and natural products (Amin et al., 2009; Bhanot et al., 2011; Batra and Sharma, 2013). *Piper betle* leaves (PBLs) have been used for centuries throughout Asia, and by ayurvedic practitioners for the prevention and treatment of cancers (Garodia et al., 2007). While betel quid (BQ) chewing was found to be carcinogenic and BQ components were shown to induce inflammatory response in oral mucosal cells (Jeng et al., 2000, 2001; Trivedy et al., 2002). PBL was shown to have antioxidant, antimutagenic, anticarcinogenic, antiplatelet, and anti-inflammatory effects (Jeng et al., 2002; Chang et al., 2007; Kumar et al., 2010; Gundala and Aneja, 2014). In the past, various studies revealed the potential cytotoxic effect of PBL extracts on various cancer cells such as colon (Ng et al., 2014), cervical (Widowati et al., 2013), breast (Abrahim et al., 2012), and prostate (Abrahim et al., 2012). Among the many bioactive compounds of PBL, hydroxychavicol (HC) has been the most widely reported for its cytotoxic effect (Kumar et al., 2010; Gundala et al., 2014). Studies have shown its involvement in reactive oxygen species (ROS) generation (Gundala et al., 2014), DNA damage (Chen et al., 2000), cell cycle deregulation and apoptosis (Chang et al., 2002; Jeng et al., 2004;

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Chakraborty et al., 2012; Rahman et al., 2014). Abnormalities in cell proliferation and the evasion of programmed cell death (apoptosis) are the two prominent hallmarks of cancer (Hanahan and Weinberg, 2011). Disruptions in cell signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, play an important role in cancer development and progression (Dhillon et al., 2007). Among the MAPK family proteins, the c-Jun N-terminal kinase (JNK) and P38 MAPK are associated with colon cancer, whereas the extracellular signal-regulated kinase (ERK) is linked with rectal cancer (Slattery et al., 2012). Various fundamental cellular processes involved in cancer progression, such as apoptosis, proliferation, differentiation, motility, stress response, and survival, are controlled by the JNK and P38 MAPK signaling pathways (Wagner and Nebreda, 2009). Many reports associated the disruption of cell cycle and induction of apoptosis in cancer cells by natural products with the signal transduction regulation (Sarkar et al., 2009; Chakraborty et al., 2012; Angulo et al., 2017).

The first-choice chemotherapy drug for colon cancer has been 5-fluorouracil (5-FU); however, it has limited effectiveness due to its short biological half-life (Wigmore et al., 2010). It exerts its effect via tumor protein p53 (*TP53*) activation (Longley et al., 2003), though only approximately 40%–50% of sporadic colorectal cancer cases are associated with *TP53* mutation (Takayama et al., 2006). Drug resistance has been a great challenge in the treatment of colon cancer. Any compounds that can exert an inhibitory effect on *TP53* mutant cells could therefore serve as potential drugs for this purpose. The HT-29 cell line is a model of *TP53*-mutated cells, hence it is an excellent candidate for studying the effects of potential chemotherapeutic agents on colon cancer.

This study was designed to explore the antiproliferative potential of PBL water extract and its active compound HC on HT-29 cells. The modulation effects of HC on the cancer cell cycle, apoptosis, as well as JNK and P38 MAPK protein expression, were further investigated.

2 Materials and methods

2.1 Sample collection

A total of 10 kg of PBL was collected in Simpang Morib, Selangor, Malaysia. Samples of leaves and fruits were sent to the Plant and Herbs Herbarium at the Forest Research Institute of Malaysia (FRIM) for verification. Leaves were subjected to further processing. They were washed and dried in oven (Memmert, Schwabach, Germany) overnight at 60 °C until the moisture level was below 10%. The dried PBLs were ground, sealed, and stored in an airtight container at 25 °C until further use.

2.2 Water extraction of PBL

Water extraction from PBL was performed with a 30-L pilot extractor for herbs (Technoniaga Sdn Bhd, Kuala Lumpur, Malaysia). Extraction was carried out and monitored at 60 °C. The ratio of dried PBL to water was 1: 20 (mass: volume, kg/L) with extraction running for 1 h. Thereafter, the water extract was filtered and filtrates were freeze-dried in a freeze drier (Virtist Genesis, New York, USA). Freeze-dried samples (porous powder) were stored at −20 °C.

2.3 HC isolation

The freeze-dried crude PBL water extract (10.0 mg) was dissolved in 20 mL of water and 30 mL of methanol (analytically pure (AR) grade), and then filtered on 140 g of prepacked MCI-Gel® column (Mitsubishi Chemical Corporation, Tokyo, Japan) conditioned with 50% water. The eluent used was 100 mL of 50%–100% methanol in distilled water at various concentrations. Each fraction of about 50 mL was collected and simultaneously spotted on a thin layer chromatography (TLC) plate along with the HC standard. The solvent system used for TLC was hexane (AR grade) and ethyl acetate (AR grade) in the volume ratio of 7: 3. The fractions containing HC were pooled and dried using a rotary evaporator (Hahn Shin Science, Gyeonggi-do, Korea). Thereafter, column chromatography, with silica gel as the stationary phase, was performed to further isolate HC. The dried sample was loaded evenly onto the column containing silica gel, and a fraction collector (Advantec, Southampton, UK) was attached to the column. The volume ratio of eluent used was 8: 2 of hexane (AR grade) and ethyl acetate (AR grade). As the fractions were collected, TLC was performed for each one. Fractions with HC were pooled and further dried using a rotary evaporator. Part of the isolated HC was sent for validation, while the remaining amount was stored at −80 °C.

2.4 HPLC

Isolated HC was injected into high-performance liquid chromatography (HPLC) to determine the chemical profile and to quantify concentration. Standards of HC were prepared with methanol in concentrations ranging from 20 to 2 000 ppm (part per million). The injection volume was 10 μL. The HPLC analyses were carried out using a Waters 600E System Controller (Waters Corporation, Massachusetts, USA) coupled with a Waters 996 Photo iodide Array Detector (Waters Corporation). The stationary phase was Phenomenex Luna C18 100 A (250 mm×4.6 mm, 5 μm particle size). Elution was performed using a gradient system consisting of a mixture of 0.1% formic acid in Milli-Q water and 100% acetonitrile. The flow rate of the mobile phase was set at 1 mL/min at room temperature. Chromatograms were acquired at 280 nm for 20 min.

2.5 NMR

The nuclear magnetic resonance (NMR) spectra were obtained from a JEOL ECX500 FT NMR Spectrometer system (JEOL Ltd., Tokyo, Japan). Deuterated chloroform ($CDCl_3$) was used to dissolve HC. The chemical shifts from the spectra were recorded in terms of ppm, and coupling constants (*J*) were given in Hertz (Hz).

2.6 GC-MS

Mass spectra were recorded with electron ionization mass spectrometry (EIMS) using a direct injection probe on a Shimadzu gas chromatography-mass spectrometry (GC-MS) QP 5050A Spectrometer (Shimadzu Corporation, Kyoto, Japan).

2.7 Cell line and cell culture maintenance

The HT-29 cells were purchased from American Tissue Culture Collection (ATCC, Manassas, USA). They were cultured in 5% carbon dioxide at 37 °C in McCoy's 5A medium modified with L-glutamine supplemented with 10% fetal bovine serum (FBS). During passages, cells were washed with phosphate-buffered saline (PBS) and trypsinised with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) for 3 min. Following the detachment of cells, culture medium was added to neutralize the trypsin-EDTA. Cells were then centrifuged (4 °C, 1 000 r/min, 5 min), the supernatant was discarded and the pellet was resuspended in culture medium. Cell enumeration was performed in a hemocytometer via the trypan blue exclusion method. An appropriate number of cells were then seeded for either propagation or treatment.

2.8 MTS assay

There were three treatment groups: (1) 5-FU (25−200 μmol/L), (2) crude PBL water extract (50−800 μg/mL), and (3) HC (10−50 μg/mL). Each treatment solution was freshly prepared on the day of treatment; treatment effect was observed up to 72 h. A total of 1×10^4 cells/well were seeded in a 96-well plate. The plate was incubated at 37 °C for 24 h. Thereafter, cells were treated with respective treatments. The duration of treatment was set at 24, 48, and 72 h for (1) 5-FU, and 24 and 48 h for (2) crude PBL water extract and (3) HC. At the end of each respective treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) solution was added to each well, wells were gently shaken and incubated at 37 °C for 1 h. Subsequently, the MTS product was measured at 490 nm using a microplate reader (BioTek, Winooski, USA). Cell viability was calculated based on the equation below: cell viability=(absorbance of treated cells−absorbance of treatment alone)/(absorbance of untreated cells−absorbance of media alone)×100%.

2.9 Cell cycle analysis

The seeding of HT-29 cells was performed in a 6-well plate at a concentration of 3×10^5 cells/well for 24 h. To observe the cell cycle event, cells were treated with the respective half maximal inhibitory concentration (IC_{50}) values of HC (30 μg/mL) and 5-FU (50 μmol/L) for 12, 18, 24, and 30 h. Non-treated (NT) controls and treated cells were then harvested. Cells were centrifuged, the supernatant was discarded, and the cell pellet was washed with cold PBS twice and fixed with ice-cold 70% ethanol solution. The fixed cells were centrifuged, washed twice with cold PBS, and resuspended in 0.5 mL of 10 μg/mL RNase solution, followed by incubation for 30 min in the dark at 4 °C. Thereafter, 0.5 mL of 20 μg/mL propidium iodide (PI) solution was added and incubated in the dark at 4 °C for 30 min. Samples were then vortexed gently for the measurement of fluorescence excitation of PI (FL-2 signal) using the

Accuri C6 flow cytometer (BD Biosciences, New Jersey, USA). The percentages of cell population in the different phases (G_0/G_1, S, and G_2/M) of cell cycle were determined using the ModFit LT Version 4 software (Verity Software House, Topsham, USA).

2.10 Apoptosis assay

HT-29 cells were seeded in a 6-well plate at a concentration of 3×10^5 cells/well for 24 h. Cells were then treated with HC (30 μg/mL) and 5-FU (50 μmol/L) for 12, 18, 24, and 30 h. Samples were prepared as per manufacturer's instructions (BD Biosciences). Briefly, control and treated cells were trypsinized, and cell pellets were resuspended with 1× Annexin-binding buffer (BD Biosciences) to a cell concentration below 1×10^6 cells/mL. Five microliters of Annexin V-fluorescein isothiocyanate (V-FITC) (1× solution provided in the kit; BD Biosciences) and 5 μL of 20 μg/mL PI (BD Biosciences) were then added to a tube containing a total of 1×10^5 cells. Following the incubation of tubes at room temperature for 15 min, 400 μL of Annexin-binding buffer was added to each sample tube. Thereafter, cells were analyzed within an hour by flow cytometer using the FL-1 signal (V-FITC signal) and FL-2 signal (PI) in the Accuri C6 flow cytometer (BD Biosciences). A minimum of 1×10^4 events was collected for each sample, and the population of cells was characterized into different quadrants (dot plot) using the CFlow Plus analysis software (BD Biosciences).

2.11 Protein extraction and quantification

A total of 3×10^5 cells per well were seeded in a 6-well plate for 24 h, followed by treatments with HC (30 μg/mL) and 5-FU (50 μmol/L). The cells were harvested at 12 and 18 h. The cell pellet was washed twice with cold PBS. Subsequently, 60 μL of radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor (5%) and phosphatase inhibitor (5%) was used to lyse the cells. Cells were incubated for 30 min on ice followed by centrifugation (4 °C, 12 000 r/min) for 10 min. The extracted protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, USA).

2.12 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

A handcast gel was prepared with 15% resolving and 6% stacking gel (1%=0.01 g/mL). The lysed proteins were denatured at 95 °C for 5 min, and 15 μg of protein was loaded into each well with 4 μL of Page Ruler™ Prestained Protein Ladder (Thermo Scientific) and resolved at 150 V for 1 h. The proteins were transferred to the polyvinylidene fluoride (PVDF)-trifluoroethylene membrane (GE Healthcare, Amersham, UK) using semi-dry transfer (15 V, 1 h). Membranes were blocked for 2 h with 3% bovine serum albumin (BSA) in Tris-buffered saline with Tween® 20 (TBST) using gentle agitation. The membrane was then washed (four times for 5 min each), followed by optimized diluted primary antibody incubation. phosphorylated JNK (pJNK) and P38 (pP38) MAPK (Cell Signaling Technology, Danvers, USA) were incubated overnight at 4 °C, whereas β-actin (Santa Cruz Biotechnology, Santa Cruz, USA) was incubated for 1 h at room temperature. Membranes were then washed followed by secondary antibody conjugated horseradish peroxidase (Santa Cruz Biotechnology) incubation for an hour with gentle agitation. Finally, the membrane was washed and the protein of interest was developed using Western Bright ECL Select (Advansta, San Jose, USA). The signal was captured by the BioSpectrum 600 Imaging System (UVP, Upland, USA).

2.13 Quantification of protein bands

The density of protein bands was analyzed using ImageJ quantification software (National Institutes of Health, Sacaton, USA). The relative density of bands was normalized against the respective β-actin relative density. The sample to control density ratio percentage was calculated as per the formula below: density ratio percentage= (normalized sample/normalized control)×100%.

2.14 Statistical analysis

Data processing for statistical analysis was performed for cell cycle, apoptosis, and western blotting results. Data are presented as mean±standard deviation (SD) of three independent experiments. Means were compared by one-way analysis of variance (ANOVA) and post-hoc least significant difference (LSD) tests using SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, USA). In each analysis, HC- and 5-FU-treated cells were compared against NT cells. Data are considered to be significant when $P<0.05$.

3 Results

3.1 Isolation of HC from crude PBL water extract

The sample collected was confirmed as *Piper betle* Linn. by the Medicinal and Aromatic Plant Department at FRIM. Isolated HC was initially verified and quantified using HPLC (Figs. S1 and S2). Based on the equation of the standard curve, the concentration of isolated HC was determined as 1125 ppm (1.125×10^6 μg/mL). Subsequently, NMR and GCMS confirmed the isolated product as HC (Table S1, Figs. S3 and S4).

3.2 Greater cytotoxic effect of HC on HT-29 cells than crude PBL water extract

To investigate the potential cytotoxic effect of crude PBL water extract and HC on HT-29 cells, proliferation analysis via MTS assay of these treatments was conducted at various time points with results compared to the standard drug, 5-FU. The latter exerts its cytotoxic effect on HT-29 cells after 72 h of treatment, with IC_{50} determined at 50 μmol/L (Fig. 1a). The cytotoxic effect of 5-FU did not continue to rise with increasing 5-FU concentration (>50 μmol/L). Crude PBL water extract and HC demonstrated cytotoxic effect on HT-29 cells as early as 24 h (Figs. 1b and 1c). The IC_{50} values of PBL extract were determined at 380 μg/mL (24 h) and 220 μg/mL (48 h), respectively (Fig. 1b). The IC_{50} of HC was found to be lower concentration compared to the PBL extract, namely, 30 μg/mL at 24 and 48 h (Fig. 1c). Crude PBL water extract and HC exert their cytotoxic effect in a time- and dose-dependent manner. Since the IC_{50} values for both these treatments were determined earlier at 24 and 48 h, respectively, the cytotoxic effects of crude PBL water extract or HC at 72 h were not investigated.

3.3 HC induced G_0/G_1 cell cycle arrest in HT-29 cells

The effects of 5-FU and HC on the cell cycle of HT-29 cells were established by cell cycle analysis using a flow cytometer. The 50 μmol/L treatment of 5-FU induced cell cycle arrest at the G_1/S phase from 12 to 30 h, which was deduced by the accumulation of cells at the S phase and the decreased population of cells in the G_2/M phase (Fig. 2). Treatment with HC (30 μg/mL) induced a G_0/G_1 phase cell cycle arrest from 12 to 24 h (Fig. 2). This was evidenced by the accumulation of cell population at the G_0/G_1 phase and a decline in cell population at the S phase when compared to the NT cells. Interestingly, there was also a significant increase of cell population at the G_2/M phase at 12 h of HC treatment when compared to the NT cells. Cell cycle arrest was observed at the G_0/G_1 and also the G_2/M phases at 12 h of HC treatment. The G_2/M phase cell population decreased at 18 and 24 h when compared to the NT cells, which implies a lack of cell cycle progression. However, there was a significant (P<0.05) decrease in the G_0/G_1 cell population, an increase in number of cells in the S phase, and a decrease in the G_2/M phase cell population at 30 h.

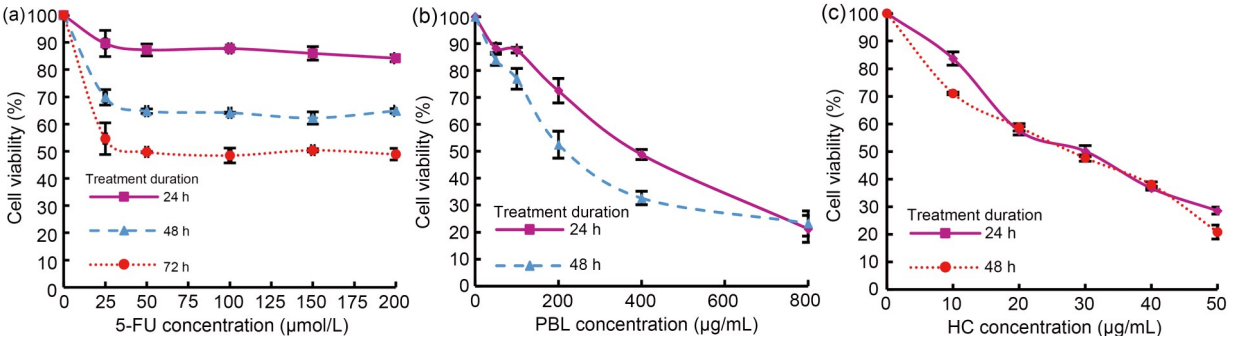

Fig. 1 Cytotoxic effects of 5-FU (a), PBL water extract (b), and HC (c) on HT-29 cells at various time points. MTS assay was used to measure the percentages of cell proliferation. (a) Cell viability of HT-29 cells treated with 5-FU (25−200 μmol/L) at 24, 48, and 72 h. The IC_{50} of 5-FU treatment was 50 μmol/L at 72 h. (b) Cell viability of HT-29 cells treated with crude PBL water extract (50−800 μg/mL) at 24 and 48 h. The IC_{50} values of crude PBL water extract were 380 μg/mL at 24 h and 220 μg/mL at 48 h. (c) Cell viability of HT-29 cells treated with HC (10−50 μg/mL) at 24 and 48 h. The IC_{50} of HC treatment was 30 μg/mL at 24 and 48 h. Data are presented as mean±standard deviation (SD) of three independent experiments. 5-FU, 5-fluorouracil; PBL, *Piper betle* leaf; HC, hydroxychavicol; IC_{50}, half maximal inhibitory concentration.

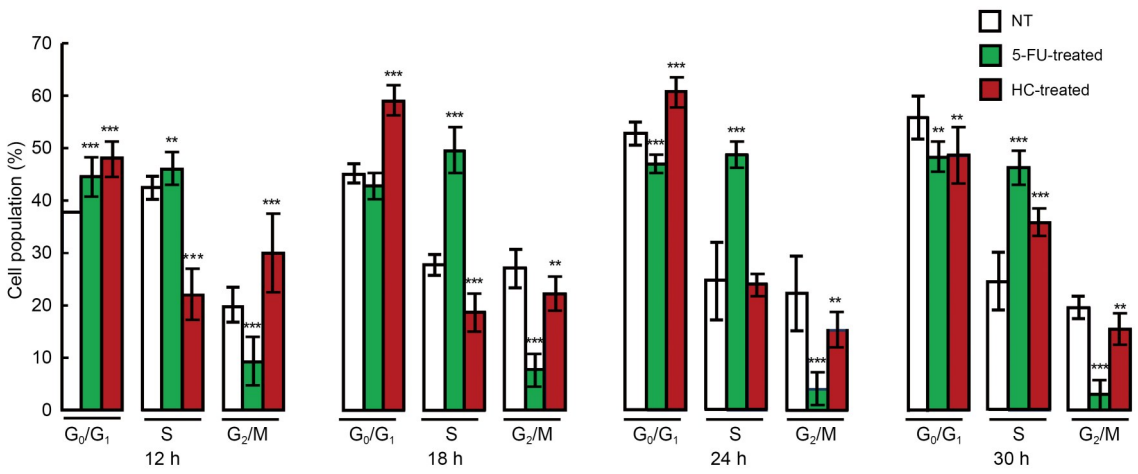

Fig. 2 Cell cycle profiles of NT, 5-FU (50 μmol/L) and HC (30 μg/mL)-treated HT-29 cells after 12, 18, 24, and 30 h of treatment. 5-FU treatment induced cell cycle arrest at G_1/S phase from 12 to 30 h in HT-29 cells. Treatment by HC of HT-29 cells induced G_0/G_1 cell cycle arrest from 12 to 24 h. Data are presented as mean±standard deviation (SD) of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared to the NT cells at each cell cycle phase. 5-FU, 5-fluorouracil; HC, hydroxychavicol; NT, non-treated.

3.4 Apoptotic cell death induced by HC as early as 12 h after treatment

Flow cytometry analysis by Annexin V-FITC/PI staining enabled the study of the mode of cell death; apoptosis and necrosis were induced by 5-FU and HC. The time-dependent study allowed cell populations to be analyzed over a period of time, observing their progression leading to cell death. Treatment by HC of HT-29 cells led to significant cell death from 12 to 30 h in comparison to the NT cells (Fig. 3). The apoptotic effect of HC were more prominent than apoptotic cell death triggered by 5-FU treatment. It began to induce apoptosis significantly after 18 h of 5-FU treatment. Nonetheless, a significant increase in cell death through necrosis was observed for HC-treated cells from 24 h onwards in comparison to the NT cells.

3.5 Activation of JNK and P38 MAPK in HT-29 cells after HC treatment

The expression of pJNK and pP38 MAPK protein in 5-FU- and HC-treated HT-29 cells was studied using western blot analysis at 12 and 18 h. There was a significant ($P<0.05$) increase in pJNK and pP38 MAPK in HC-treated HT-29 cells at 12 h (Fig. 4). However, no significant difference was observed in the expression of pJNK or pP38 between NT and 5-FU-treated HT-29 cells at 12 or 18 h.

4 Discussion

The *TP53* gene is a classical tumor suppressor. In response to cellular stress, such as DNA damage, JNK and P38 regulate cell proliferation and apoptosis through the activation of *TP53*. Activated *TP53* promotes cell growth arrest, DNA repair, and apoptosis.

Cells of the HT-29 line bear *TP53* mutation (Sui et al., 2014) and display chemoresistant features. Mutant *TP53* possesses dominant-negative inhibition toward wild-type *TP53*. However, the intracellular balance between the expression levels of wild-type and mutant *TP53* may play a role in determining cell fate in response to DNA damage (Ozaki and Nakagawara, 2011).

Our results showed that 5-FU treatment induced G_1/S cell cycle arrest and a late onset (72 h) of cell death. These observations could be explained by the 5-FU mechanism and the *TP53* mutation of HT-29 cells, respectively. 5-FU is a cell cycle-specific, S phase-dependent fluorinated pyrimidine analogue that interferes with thymidylate synthesis. It inhibits thymidylate synthase (TS) in a *TP53*-dependent manner (Sui et al., 2014). Its metabolite, 5-fluorouridine 5'-triphosphate (FUTP), is extensively incorporated into RNA disrupting normal pyrimidine synthesis, subsequently leading to delayed cell cycle progression (Zhang et al., 2008). This causes cells to exhibit prolonged DNA synthesis (S phase), and the cells are hence arrested at the G_1/S phase. Cells with unrepairable

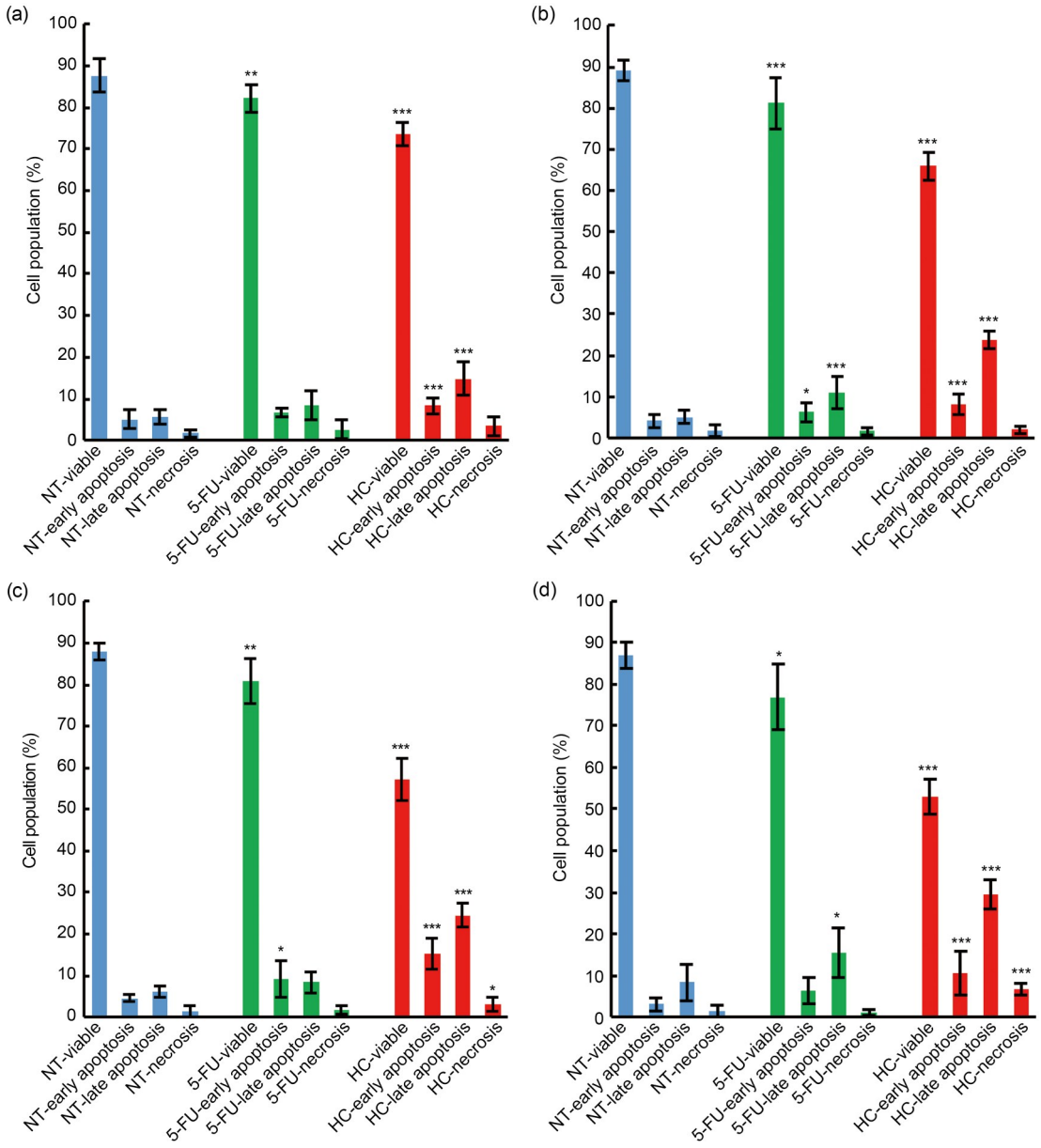

Fig. 3 Apoptotic profiles of NT, 5-FU- and HC-treated HT-29 cells at 12 (a), 18 (b), 24 (c), and 30 h (d). NT cells together with 50 μmol/L 5-FU-treated and 30 μg/mL HC-treated HT-29 cells for the indicated period were harvested and stained with Annexin V-FITC and PI before being subjected to flow cytometry analysis. HC treatment significantly induced apoptosis from 12 h onwards, and 5-FU treatment led to significant apoptosis induction after 18 h. The percentage of apoptotic cell death from HC treatment was higher compared to that of 5-FU treatment. Data are presented as mean±standard deviation (SD) of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, correspond to treatment versus NT cells for each cell population. 5-FU, 5-fluorouracil; HC, hydroxychavicol; NT, non-treated; V-FITC, V-fluorescein isothiocyanate; PI, propidium iodide.

damage will then proceed to programmed cell death, i.e., apoptosis. Apoptosis induced by 5-FU is controlled by the TP53 protein; the mutation of *TP53* gene is associated with 5-FU resistance due to a reduction in pro-apoptotic protein expression (Boyer et al., 2004). In addition, a repression effect of *TP53* on pyrimidine catabolic enzyme, dihydropyrimidine dehydrogenase (DPD), has also been reported (Gokare et al., 2017). Functional *TP53* enables the inhibition of both pyrimidine synthesis and 5-FU catabolism. In the treatment of 5-FU, the lack of functional *TP53* is highly undesirable. Cell death triggered by 5-FU was observed

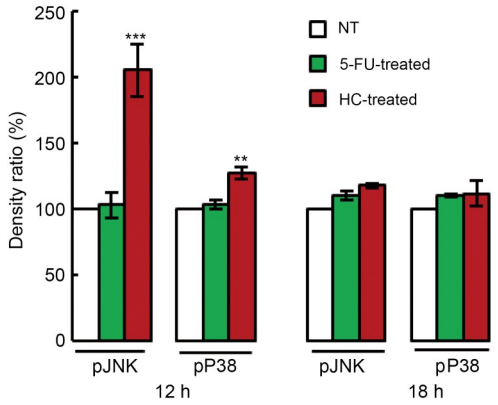

Fig. 4 Expression of pJNK and pP38 in HT-29 cells treated with 5-FU and HC. HT-29 cells were treated with 50 μmol/L 5-FU and 30 μg/mL HC for 12 and 18 h. NT cells acted as control. Following the respective duration of treatment, cells were harvested, lysed, and examined for the expression of pJNK and pP38 using SDS-PAGE and western blotting. Increased expression of pJNK and pP38 was observed in HC-treated cells after 12 h of treatment. Data are presented as mean±standerd deviation (SD) of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ refer to treatment compared to the NT cells. pJNK, phosphorylated c-Jun N-terminal kinase; 5-FU, 5-fluorouracil; HC, hydroxychavicol; NT, non-treated; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

in this study, although this occurred at late onset after 72 h of treatment.

We compared the cytotoxic effect of PBL and HC to that of 5-FU, and found that PBL water extract and HC treatment exhibited cytotoxic effect on HT-29 cells at 24 h, which is earlier than the cytotoxic effect exerted by 5-FU (72 h). The cytotoxic effect of HC was more potent than that of PBL water extract, which was evident from the lower IC_{50} value for HC. Many studies reported that active compounds are more potent than crude extracts in inhibiting cancer cell growth. One such study demonstrated that HC has a greater effect than crude PBL water extract on HT-29 cells (Ng et al., 2014). Bio-guided fractioning revealed that the fraction containing about 95% of total HC, in comparison to other fractions of PBL extract, exerted the highest cytotoxic effect on prostate cancer cells (Paranjpe et al., 2013). Polyphenols such as curcumin, gingerols, and capsaicin are active compounds from turmeric, ginger, and red chili, respectively, and these compounds have entered clinical trials as prospective chemotherapeutic agents (Sung et al., 2012).

We also analyzed and compared the cell cycle and apoptotic profiles of 5-FU and HC in a time-dependent manner. As early as 12 h after treatment, HC triggered cell cycle arrest primarily at the G_0/G_1 and G_2/M phases, which subsequently led to significantly higher apoptotic cell death of HT-29 cells when compared to 5-FU treatment. This result is in accordance with past studies, where HC-induced apoptosis was observed in consequence of G_0/G_1 (Gundala et al., 2014) and G_2/M arrest (Chakraborty et al., 2012; Majumdar and Subramanian, 2019). At 12 h of HC treatment, we found HC-induced phosphorylation of JNK and P38, on which 5-FU did not have any effect. HC has been reported as one of the ROS-induced natural products (Chakraborty et al., 2012). The JNK and P38 MAPK proteins are expressed in response to various stress stimuli. Induction by oxidative stress activates these proteins, and subsequently leads to the initiation of signaling cascades. In response to oxidative stress, cells can either repair their damage, or cells with irrepairable damage proceed to apoptosis. Therefore, the cell cycle arrest triggered by HC may play an essential role in contributing to the induction of apoptosis.

Both JNK and P38 MAPK are involved in apoptosis through the activation of TP53 or B cell lymphoma-2 (Bcl-2) family proteins (Dhanasekaran and Reddy, 2008; Thornton and Rincon, 2009). Upon activation by P38, phosphorylated TP53 regulates both the G_2/M and G_1/S cell cycle checkpoints through the negative regulation of cell division cycle protein 2 (CDC2) and cyclin-dependent kinase 2 (CDK2), respectively (Thornton and Rincon, 2009). The JNK regulates *TP53* stability (Dhillon et al., 2007) and is involved in (ERK1/2) activation, which subsequently leads to apoptosis induction (Chowdhury et al., 2013). It also phosphorylates c-Jun, which then leads to the formation of activator protein-1 (AP-1). The JNK-AP-1 pathway induces the expression of pro-apoptotic genes such as tumor necrosis factor-α (*TNF-α*), Fas-ligand (*Fas-L*), and Bcl-2 homologous antagonist/killer (*Bak*) (Dhanasekaran and Reddy, 2008). However, HT-29 used in our study possesses mutant *TP53*. Our results evidenced that mutant *TP53* did not suppress HC-induced apoptosis. Both JNK and P38 activation are essential for apoptotic response to HC-induced cell death, and may be independent of *TP53* activity. Nevertheless, a reactivation of mutant *TP53* is possible and provide clues in killing cancer cells bearing the *TP53* mutation. Several small molecules (Wiman, 2010; Brown et al., 2011; Zawacka-Pankau

and Selivanova, 2015) and natural products (Kitagishi et al., 2012; Garufi et al., 2016) have been tested for *TP53* reactivation in mutant *TP53*-carrying cells. A study by Garufi et al. (2016) found that capsaicin, the major constituent of peppers, reduces mutant *TP53* levels and reactivates the wild-type TP53 protein in mutant *TP53*-carrying cells. The clearance of *TP53* mutation has been reported to reduce tumor malignancy and increase the apoptotic response to drugs (Bossi et al., 2006; Martins et al., 2006).

Taken together, we have demonstrated that HC possesses more potent cytotoxic effect than crude PBL water extract or 5-FU on HT-29 cells. Our study provides information on the possible involvement of JNK and P38 MAPK in HC-induced cell cycle arrest and apoptotic cell death of HT-29 cells, which may be independent of *TP53* activity. Nevertheless, the reactivation of mutant *TP53* is still possible. These findings have given more insight into the potential mechanism of action of HC.

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Author contributions

Aiysvariyah RAJEDADRAM, Kar Yong PIN, Sui Kiong LING, and See Wan YAN performed the experimental research and data analysis. Mee Lee LOOI contributed to the study design and data analysis. Mee Lee LOOI and Aiysvariyah RAJEDADRAM contributed to the writing and editing of the manuscript. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Aiysvariyah RAJEDADRAM, Kar Yong PIN, Sui Kiong LING, See Wan YAN, and Mee Lee LOOI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Table S1; Figs. S1–S4