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SHORT COMMUNICATION

Trikatu, a Thai Ayurvedic Remedy of *Piper nigrum*, *Piper retrofractum*, and *Zingiber officinale* Promotes Anti-cholangiocarcinoma Cell Proliferation via Cell Cycle Arrest

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Abstract

In the present study, the effects of a Thai Traditional herbal recipe called "trikatu" on a cholangiocarcinoma cell line were explored. This classic Ayurvedic remedy is an herbal blend of dried powdered *Piper nigrum* L., Piperaceae, *Piper retrofractum* Vahl, Piperaceae, and *Zingiber officinale* Roscoe, Zingiberaceae. The HPLC quantitative analysis of the active compounds in the formulation were investigated. Total flavonoid, total phenolic contents, and antioxidant assay were performed. Cytotoxic study was determined using sulforhodamine B assay. Cell cycle arrest and apoptosis were evaluated by propidium iodide and annexin V-FITC/PI staining. Western blot analysis was done to evaluate protein expression. Above all, HPLC analyses revealed that the major active constituent was piperine. The result showed that "trikatu" significantly inhibited the migration of cholangiocarcinoma cell which also induced cell cycle arrest on G2 phase via a decrease in the expression of CDK2 and p53, and upregulation of p21and p27. "Trikatu" showed antioxidant capacity, expressed high piperine content, and had anti-cell viability and anti-proliferation effect on cholangiocarcinoma *in vitro*.

Keywords Amide alkaloid · Bile duct cancer · Black pepper · Cytotoxicity · Ginger · Long pepper

Introduction

Cholangiocarcinoma (CCA) has a very high disease incidence particularly in the northeastern region of Thailand, which relates to the high prevalence of liver fluke or *Opisthorchis viverrini* infection. After a human gets infected with liver fluke, a combination of risk factors linked to chronic biliary inflammation, and nitrosamine consumption results in damage and DNA mutation leading to the pathogenesis of cholangiocarcinoma (Sripa and Pairojkul 2008). Moreover, the treatment of CCA is too difficult; after resection, the < 5-year overall survival in cholangiocarcinoma cases is still very low (Chansitthichok et al. 2020). Furthermore, chemical and radiation treatments have side effects on all patients (Dilalla et al. 2020) and can increase drug resistance.

Therefore, researchers have adopted a goal to find alternative ways for cancer treatment. For this reason, new substances from herbs have been reported to have the potential to prevent or treat cancer and develop new anti-cancer agents especially the group of phenolics, alkaloids, flavonoids, piperine, and curcumin (Kurapati et al. 2012). An Ayurvedic formulation of equal parts of three powdered dried herbs, *Piper nigrum* L., Piperaceae (black pepper), *Piper retrofractum* Vahl, Piperaceae (long pepper), and *Zingiber officinale* Roscoe, Zingiberaceae (ginger), in Thai Traditional medicine called "Trikatu," is believed to strengthen immune system. These herbs also work in synergy to stimulate the digestion in the stomach while promoting proper bile flow, healthy detoxification, and lipid metabolism. In addition, previous studies have also reported that these have been medically used as an anti-arthritic agent, and for the treatment of respiratory tract infections, rheumatoid arthritis, digestive infections, cold, asthma, pruritus, pain,

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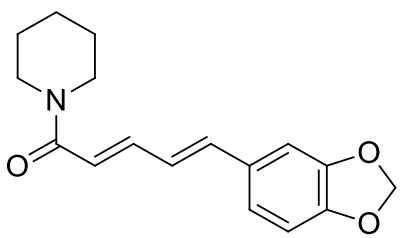
and inflammation (Maenthaisong et al. 2014). The evidence for efficacy for the most common uses for each herb have been described. *Piper nigrum* or black pepper belongs to the family Piperaceae, a rich source of piperine, chavicine, piperamine, piperidine, and some volatile oil and can be used as hepatoprotective, anti-inflammatory, antioxidant, anti-mutagenic, and anticancer agents (de Souza Grinevicius et al. 2016). *Piper retrofractum* fruits were traditionally used only in rainy seasons for carminative, mucolytic, and nausea relief and vomiting. The important substances include piperine, pipernonaline, piperundecalidine, and dehydropipernonaline (Takooree et al. 2019). There are many evidences on their health benefits such as anti-obesity, anti-flatulent, expectorant, anti-tussive, anti-fungal, hepatoprotective, anti-oxidant, and anti-tumor activities (Haq et al. 2021). Ginger rhizomes are composed of various substances and essential oils as 6-gingerol and 6-shogaol (Teng et al. 2019). Ginger rhizomes were commonly used for relief of symptoms such as, nausea, vomiting, stomach discomfort, and reduce cholesterol. In addition, ginger has been reported to have anticancer and chemo-preventive properties (Yusof et al. 2008).

Presently, there are studies on the use of various crude drugs or herbal extracts against cancer cells, such as the combination of *Curcuma longa* L., Zingiberaceae, with *Z. officinale* which showed significant inhibitory effects on prostate cancer (PC-3 M) cell line (Kurapati et al. 2012). *In vitro* activities of ethanolic extracts of *P. nigrum* inhibited colorectal carcinoma cell line types HCT-116, HCT-15, and HT-29 (IC_{50} values 3.2, 2.9, and 1.9 µg/ml in HCT-15 cells; 4.0, 3.1, and 3.4 µg/ml in HCT-116 cells; and 7.9, 6.1, and 7.4 µg/ml in HT-29 cells at 24, 48, and 72 h, respectively) in time-dependent and dose-dependent manner and increased the cytotoxic efficacy (Prashant et al. 2017). Previous studies reported that *P. longum* concentration at 0.1–0.4 mg/ml had inhibitory activities on lung cancer cells (A549), prostate cancer cells (DU-145), leukemia cancer cells (THP-1), ovarian cancer cells (IGR-OVI-1, A2780, OVCAR3, and SKOV3), and colorectal cancer cells (HT-29, HCT116) (Ovadje et al. 2014). Piperine (**1**), the amide alkaloid responsible for the pungency of black pepper and long pepper, has been shown to possess selective cytotoxic action toward tumor cells. There have been no reports on piperine causing toxicity in mammal cell line. However, it has been reported to have acute toxicity in animals, with LD_{50} values varying depending on the administration route of a single dose of 15–400 mg/kg body wt (Piyachaturawat et al. 1983).



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However, although each herb in the Thai traditional recipe has different bioactive chemicals and properties, there is no evidence supporting the cytotoxicity of "trikatu." The present study aimed to ascertain the effect of this herbal formulation using *in vitro* human cholangiocarcinoma cells. Evaluation of "trikatu" cytotoxicity, anti-migration, cell-cycle arrest, apoptosis, and the expression of proteins regulating cancer cells against cholangiocarcinoma (KKU-214) cell line was conducted. This Ayurvedic herbal formulation may be developed into an anti-cancer drug that can be used in endemic areas of cholangiocarcinoma.

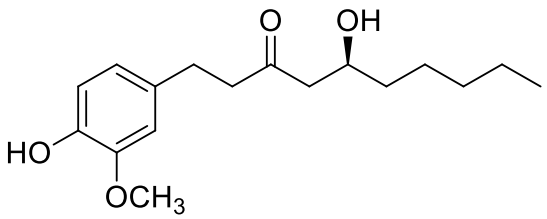
Materials and Methods

To prepare the powder of "trikatu" formulation, *Piper nigrum*, *Piper retrofractum*, and *Zingiber officinale* (Fig. S1) were identified following the Thai Herbal Pharmacopoeia, 2016, by Assist. prof. Pichet Wetwithan. Vouchers are on deposit at the herbarium of the Department of Thai Traditional Medicine, and accession numbers were assigned as follows: flowers of *Piper retrofractum* Vahl., Piperaceae (No. 25620003), fruits of *Piper nigrum* L, Piperaceae (No. 25620002), and rhizomes of *Zingiber officinale* Roscoe, Zingiberaceae (No. 25620001). The ratio of each ingredient was 1:1:1 w/w. The "trikatu" recipe was soaked with 80% ethanol (1:5 w/v) for 24 h at room temperature. The liquid extract was filtered and evaporated under a vacuum using a rotary evaporator (Buchi, Switzerland) and crude extracts were stored at − 20 °C for further analyses.

Cholangiocarcinoma cell line (KKU-214) moderately differentiated adenocarcinoma was established from cholangiocarcinoma at the research institute Khon Kaen University, Thailand. Cell line KKU-214 was grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, an antibiotic mixture of 100 units/ml of penicillin and 100 µg/ml of streptomycin and maintained under incubation in a humidified environment with 5% CO_2 atmosphere at 37 °C.

The physicochemical analysis of the crude powder "trikatu" was carried out following the Thai Herbal Pharmacopoeia. Samples were analyzed using a Shimadzu HPLC system adapted from a THP, 2016. Reverse phase separation was performed at 40 °C using a C18 column (Waters, USA). The mobile phase was adjusted to an isocratic elution methanol and water (4:1 v/v). The HPLC operating parameters were as follows: injection volume, 10 µl; flow rate, 1 ml/min; chromatographic run time, 15 min; PDA spectra

were recording at 254 nm. The standards piperine (**1**) and 6-gingerol (**2**) (Sigma-Aldrich, Switzerland) were diluted with methanol to give concentrations of 10, 20, 40, 80, and 160 µg/ml and 1000 µg/ml of *P. nigrum*, *P. retrofactum*, and *Z. officinale*. The "trikatu" extract was then diluted with the same solvent. All samples were filtered through a 0.45-µm syringe filter before injection. The "trikatu" extracts were identified by comparing their retention times with the standard piperine (**1**, purity ≥ 97%, Sigma-Aldrich) and 6-gingerol (**2**, purity 98%, Sigma-Aldrich), which is found in all members of the Zingiberaceae family.



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Total phenolic contents of all the "trikatu" extracts were evaluated with Folin-Ciocalteu method applied according a previous study (Zulkifli et al. 2020). Total flavonoid content was quantified following the method previously described by Zulkifli et al. (2020). The anti-oxidant analysis was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The measurement was carried out and calculations were performed as previously described (Promraksa et al. 2015). The 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical-scavenging assay was conducted from a previous study (Zulkifli et al. 2020).

The effect of "trikatu" on cholangiocarcinoma cell viability was determined using the sulforhodamine B (SRB) assay. Briefly, the human CCA cell line (KKU-214) was maintained and seeded into 96-well culture plates at a density of 3×10^4 cells/well. After culturing for 24 h, the cells were treated with various concentrations of "trikatu" extracts at 0–1000 µg/ml, the major compounds piperine (**1**) and 6-gingerol (**2**) at 0–50 µM. Gemcitabine 10 µM was used as a positive control. Cells were treated with the various concentrations for 24 h, and then stained with SRB solution under light protection. The absorbance was measured at 510 nm using a microplate reader spectrophotometer and IC_{50} value was calculated. KKU-214 cell cultures were then exposed to 0.5-fold, onefold, and 1.5-fold increase in "trikatu" IC_{50} concentration to final concentrations of 260, 520, and 780 µg/ml. The effect on inhibition of cell migration was determined using the wound healing assay. KKU-214 cells were seeded into 6-well culture plates at a density of 1×10^5 cell and cultured and incubated for 24 h. The cells were then scathed with a sterilized tip, the distance was measured at the beginning, and the samples were continued to be cultured using the "trikatu" treatment described above. The width distance was measured at 0–24 h post-treament by image J program under a microscope.

For cell cycle analysis, KKU-214 cells were seeded into 6-well culture plates at a density of 5×10^5 cell/well and treated with concentrations of 0.5 fold-IC_{50}, onefold-IC_{50}, and 1.5 fold-IC_{50} of "trikatu" extract. Human KKU-214 were grown in 6-well culture plates. The next day, they were treated with "trikatu" at various concentrations for 24 h. They were then harvested with 0.25% trypsin, washed, re-suspend in PBS, and fixed in 70% ethanol at 4 °C. For PI staining, KKU-214 cells were washed with PBS and centrifuged to remove 70% ethanol, and 0.1 mg/ml RNase (BD Biosciences, USA) and 10 µl of propidium iodide (PI) were added. The sample was then incubated for 15 min and analyzed using flow cytometry (FACS-Canto II, BD Biosciences, UK) (Saenglee et al. 2018). Annexin-V/PI staining was used to determine apoptotic and necrotic cells.

For the western blot analysis, the following concentrations at 260, 520, and 780 µg/ml of "trikatu" were used on KKU-214 cells. Cells lysed were prepared using radio-immuno precipitation buffer (RIPA). After estimating protein concentrations (using Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific), equal amounts of proteins of each group were separated in a 12% SDS–polyacrylamide gel and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline containing 0.1% Tween-20 and 5% skimmed milk at 37 °C for 1 h, the membranes

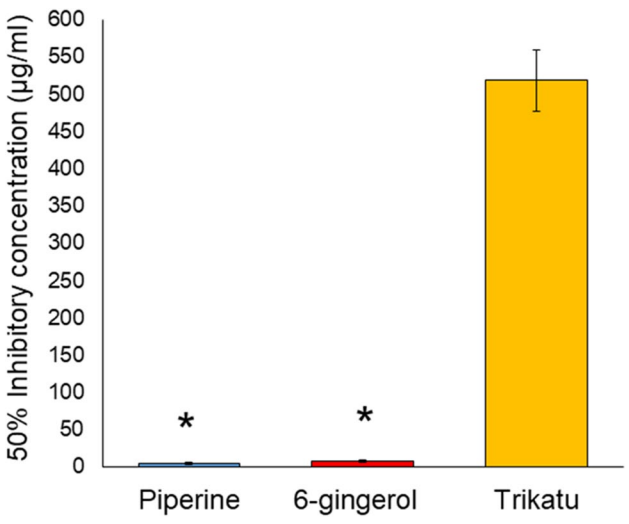

Fig. 1 IC_{50} values of the standard piperine **(1)**, 6-gingerol **(2),** and "trikatu" extracts at 24 h. * indicates a significant impact (*$p < 0.05$) compared to the herbal formulation extracts

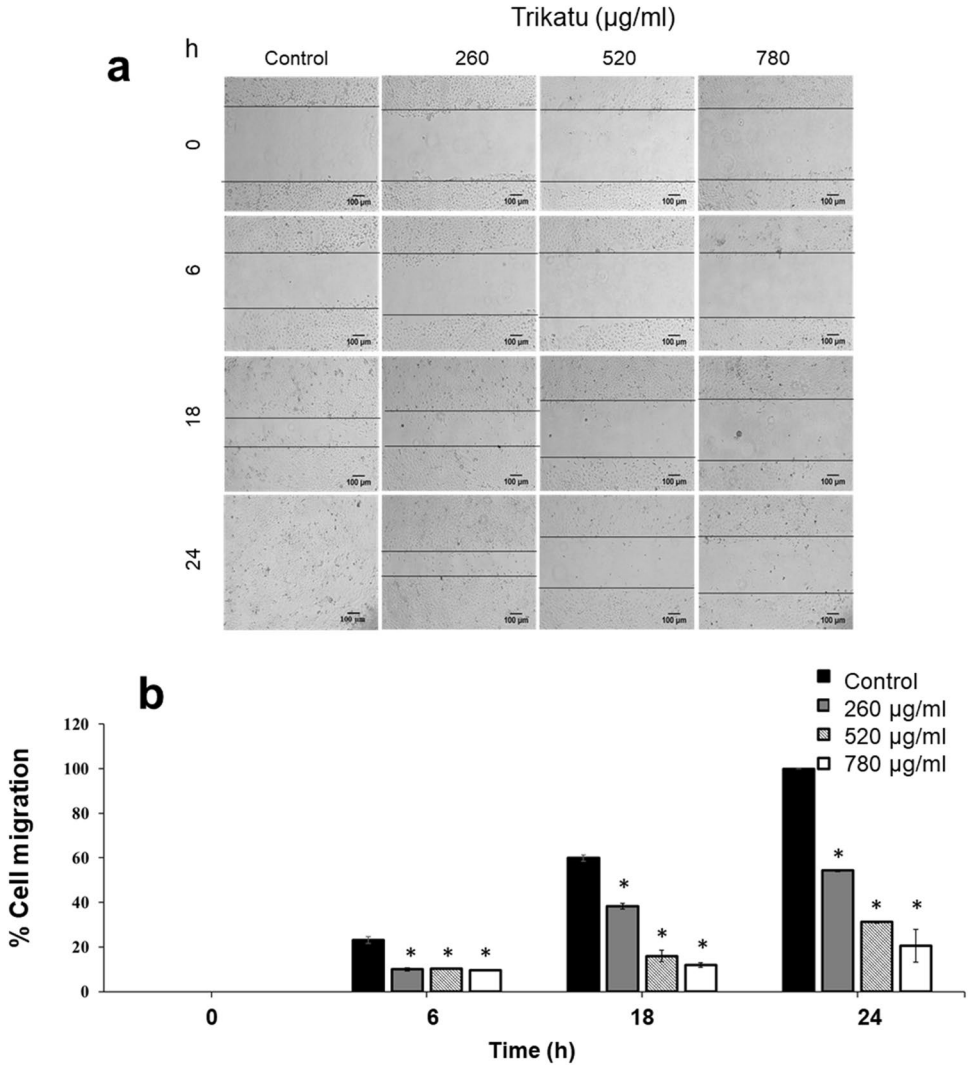

Fig. 2 **a** Wound healing assay on KKU-214 cells exposed to "trikatu" with 260, 520, and 780 μg/ml at 0–24 h; **b** quantification of wound healing represented % of cell migration. * indicates a significant impact (*$p < 0.05$) compared to the control

were incubated at 4 °C overnight with primary antibodies against p53, p21, p27, and CDK2, and β-actin (total cell lysate) and subsequently incubated with the appropriate secondary antibody. The secondary antibody binding was visualized using an enhanced chemiluminescence kit (Pierce Biotechnology), quantified by densitometry (ImageQuant LAS 4000, GE Healthcare, Piscataway, NY, USA), and analyzed using the program Scion Image (Scion Corp., Frederick, MD, USA). Relative intensity was determined and normalized to β-actin.

All data were expressed as means ± standard error of triplicate measurements. Standard deviation (SD) did not exceed 5% for most of the values obtained. The treatment means were subjected to a one-way analysis of variance (ANOVA) test by SPSS Software version 16. The half-maximal inhibitory concentration (IC_{50}) was subjected to a Calcusyn (version 3.0). Results were considered statistically significant when a p-value < 0.05.

Results and Discussions

The quality control standardization of "trikatu" is shown in Table S1. The loss on drying of the herbal formulation was 10.22% w/w, and total ash content and acid insoluble contents were 5.86 and 0.56% w/w, respectively. The microbial limit test as such as total aerobic microbial count and total combined yeasts and molds count of "trikatu" recipes was 2,200,000 and less than 10 per gram. The bacteria species, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeuruginosa*, and *Salmonella* spp., were not detected in "trikatu."

The compound profiling was determined by HPLC. The identification of individual compounds was carried out by comparing the retention times of signals in the samples with signals for the standard quantitative analysis of each marker at different wavelengths, for piperine at 340 nm and 6-gingerol at 210 nm. The R^2 value for piperine

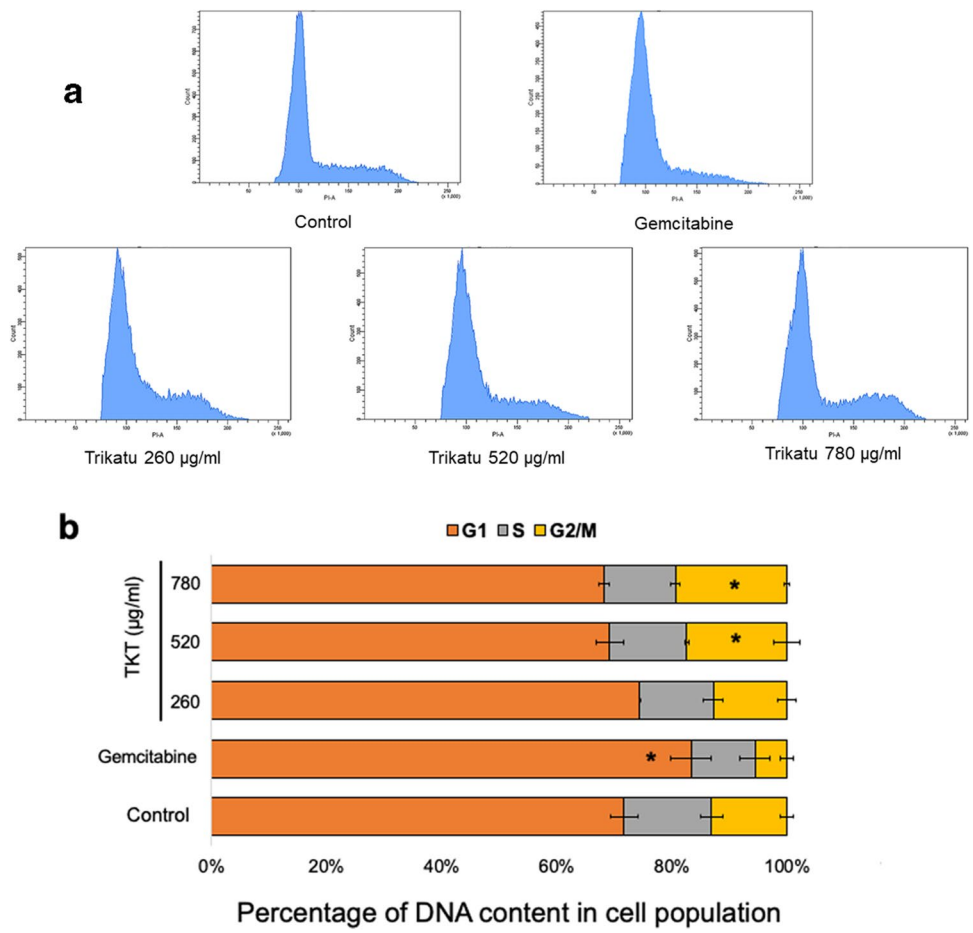

Fig. 3 Histogram profiles of cell cycle distribution exposed to "trikatu" and Gemcitabine on KKU-214 cells, cell distribution histogram (**a**), and percentage on each stage of cell population (**b**). * indicates a significant impact ($*p < 0.05$) compared to the control

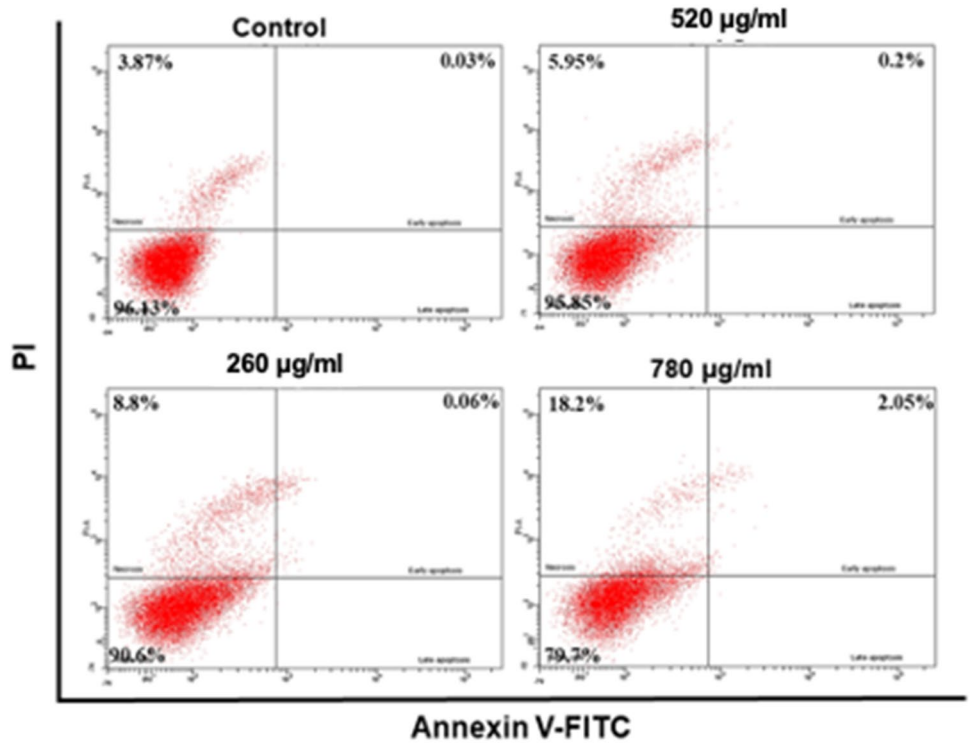

Fig. 4 Effect of "trikatu" extracts on induction of apoptosis

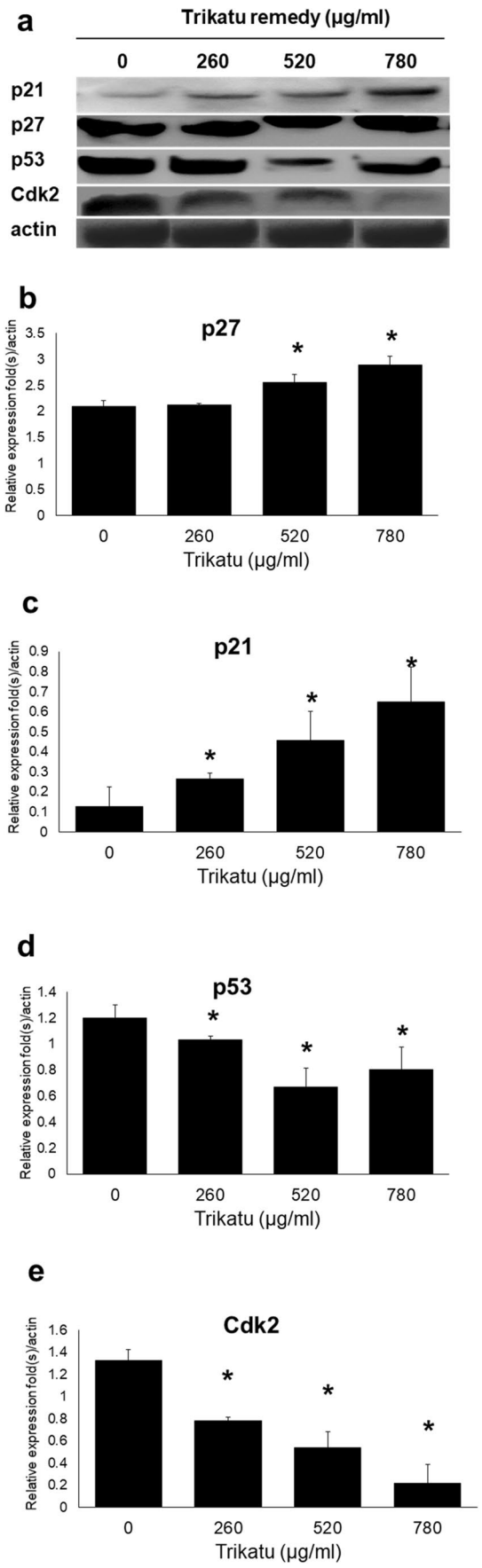

Fig. 5 Western blot analysis on the effect of "trikatu" on G2 cell cycle arrest when assessed in whole cell lysate in KKU-214 CCA cells; β-actin was used as the control. * indicates a significant impact (*$p < 0.05$) compared to the control

(**1**) was 0.9992 (y = 2,217,552.95x + 10,868.61) which showed at retention time of 5.85 min (Fig. S2), and *P. nigrum*, *P. retrofactum*, and "trikatu" which showed at retention time (tR) 5.935, 5.848, and 5.862 min, respectively (Fig. S2). Piperine was observed in chromatograms obtained from all samples. The piperine contents for *P. retrofactum*, *P. nigrum*, and "trikatu" were 268.42 ± 10.19, 199.25 ± 2.10, and 7.44 ± 1.59 µg/mg, respectively, and similar to previous reports (Liu et al. 2015). The R^2 value for 6-gingerol (**2**) was 0.9995 ($y = 932684x + 1247.4$) which showed at retention time 4.30 min and detected in *Z. officinale* (Fig. S2). The compound 6-gingerol was observed in chromatograms obtained from *Z. officinale* extracts but was not observed in the herbal mixture (Fig. S2). The present study found 6-gingerol contents of *Z. officinale* to be 3.27 ± 0.16 µg/mg. However, the active chemical principle responsible for the pharmacological effects of the "trikatu" formulation remains unclear, but could be the result of synergy of its major components with additional compounds (Tripatara et al. 2012).

The total phenolic content of "trikatu" was found to be 76.32 ± 1.60 mg GAE/g DW extract while the flavonoid content was found to be 4.16 ± 0.56 mg QE/g DW extract. The antioxidant activity found in the "trikatu" by DPPH and ABTS assay. Antioxidant properties of "trikatu" using DPPH and ABTS were found as 50% free radical inhibition 216.91 ± 5.37 and 9.65 ± 0.28 µg/ml, respectively.

The proliferation of KKU-214 cells was significantly inhibited by "trikatu" in a dose-dependent manner as compared to the control cells. Concentrations of extracts required to inhibit cell proliferation by 50% were determined. The IC_{50} values of "trikatu" were 518.36 ± 41.38 µg/ml at 24 h which decreased cell viability in a dose-dependent manner (gemcitabine IC_{50} values was 10 µM at 24 h). For major compounds, IC_{50} values of piperine was 15 ± 2.58 µM (4.28 µg/ml) and 26 ± 1.62 µM (7.65 µg/ml) for 6-gingerol at 24 h and significantly higher than "trikatu" formulation extract (Fig. 1).

The effects of "trikatu" on the wound healing of KKU-214 cells are shown in Fig. 2. Crude extract concentrations at 260, 520, and 780 µg/ml caused a significant delay in the migration of KKU-214 cells into the wound area, the percentage of cell migrations at 24 h were 54.30 ± 0.52, 31.28 ± 0.48 and, 16.62 ± 3.97 compared with the control group migration at 100 ± 0. Consistent with our results, piperine had inhibitory effects against the migration, invasion, and metastasis of breast cancer cells (Greenshields

et al. 2015) and 6-gingerol inhibited the migration of HGC-27 gastric cancer cells (Luo et al. 2019) and renal cell carcinoma (786-O and ACHN cells) (Xu et al. 2020).

Cell cycle analysis was also investigated in this study through annexin-V/PI for apoptotic and necrotic cell; PI stained the nucleus, while annexin-V stained the cytoplasm of the cell. The results revealed that the KKU-214 cells treated with "trikatu" extract arrested G2% populations (a 10% increase) and the percentage of DNA content increased at all concentrations (Fig. 3). Conversely, gemcitabine (positive control) significantly inhibited the cell cycle progression by arresting cells in G1 phase via inhibiting the expression of cyclins and cyclin-dependent kinases, such as cyclin D, CDK4, and CDK6. In cell cycle arrest, the development of G2 phase is controlled by a complex network of pathways. Even so, cell cycle progression is primarily regulated by activation of Cdks, activation requires cyclin binding, and phosphorylation of conserved threonine residue by Cdk-activating kinase (Yan et al. 2007). The effects of cell cycle arrest by "trikatu" extract were demonstrated and it showed that this herbal blend could inhibit cell proliferation and induction population of G2 cell cycle arrest in KKU-214 cells. Herein, the results of the KKU-214 cells in the G2/M checkpoint of the cell cycle were similar with those piperine treatment which disrupts the response for G2/M transcription and regulates both CDC and cyclin family of cancer cell resulting in G2 arrest (Zhang et al. 2015). Additionally, no significant changes were found in apoptotic population in KKU-214 cells by "trikatu" extract at 260, 520, and 780 µg/ml which were equal to 0.06, 0.2, and 2.05% of apoptosis as shown in Fig. 4.

The effect of "trikatu" extract on the cell cycle progression by arresting the cells in G2-phase on this pathway was confirmed by using western blot analysis. The expression levels of G2 transition regulators included CDK2, p53, p21, and p27. The upregulation of the protein levels of p21 and p27 was observed while CDK2 and p53 were down regulated after the treatment (Fig. 5) compared with untreated control. The results showed that "trikatu" extract caused a decrease in the expression of p53 and CDK2 and upregulation of p21 and p27 in a concentration-dependent manner. The interaction of cyclin/CDK complexes can inhibit p27 activities, and cyclin-dependent kinase (CDK) inhibitor of p21 is induced by the tumor activities associated with Cdc and CDK family inhibitory activity (Payne et al. 2008). The 6-gingerol can decrease cyclin B1, cyclin A2, and CDC2 expression and increase the mRNA expression of p27 (Luo et al. 2019).

Normally, p53 is present in cells and detectable at lower levels. In abnormal conditions, cell exposure to exogenous stress including inflammation, DNA damage, and the p53 can be activated resulting in DNA repair and apoptosis of impaired cells. The downstream activations of p53 after KKU-214 treatment with "trikatu" extracts also decreased as similar to *Rhus coriaria* L., Anacardiaceae, decreased mutant in p53 which occurred crucial of autophagy activation (El Hasasna et al. 2015).

In conclusion, it was found that "trikatu," an herbal blend of dried powdered *P. nigrum*, *P. retrofractum*, and *Z. officinale*, caused cell arrest in KKU-214 cholangiocarcinoma cell in G2/M phase via upregulation of p21 and p27 and downregulation of p53 and CDK2 in the cancer cell. In addition, "trikatu" demonstrated to contain high amounts of piperine, suggestive of the potent anticancer effects of this herbal formulation which can be used as an alternative herb remedy in combination with anti-cancer clinical agents to avoid drug resistance and decrease side effects of chemotherapy treatment for bile duct cancer.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s43450-022-00339-6.

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Author Contribution KN: data collection, data analysis; PS and JP: data analysis and interpretation; and TB; critical revision of the article; RA: conception and design of the work, and drafting the article.

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