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ANTI-CHOLANGIOCARCINOMA CELL GROWTH AND SELECTIVE ABILITY OF BIOACTIVE COMPONENTS OF RIPE WILD MANGO (*Spondias pinnata*) FRUIT EXTRACT

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ABSTRACT

Cholangiocarcinoma is a serious problem of public health in the northeastern region of Thailand. Cancer therapies have many side effects to healthy cells and tissues. Therefore, new anticancer agents are identified from natural resources to decrease side effects and increase the potential of cancer treatments. Ripe wild mango fruit extract which called 6A* was determined the cytotoxic effect in cholangiocarcinoma MO55 cells and a normal epithelial cell of the bile duct MMNK1 cells by using MTT assay. The extract was analyzed total phenolic contents and total flavonoid contents, including screened phytochemical types. In this study, 6A* displayed cytotoxicity in MO55 cells (IC_{50} 0.40 mg/mL) and low cytotoxicity against MMNK1 cells (IC_{50} 2.70 mg/mL). The total phenolic contents and total flavonoid contents of 6A* were 192.14 ± 30.69 mg GAE/g extract, and 73.13 ± 6.34 mg QT/g extract, respectively. The study also found that phytochemicals in 6A* contained tannin, gallic acid, flavonoids, terpenoids, and cardiac glycoside. These molecules could be developed as the new anticancer drugs or cancer prevention agents for treatment cholangiocarcinoma in the future.

Key words: Cancer, bile duct, phytochemical, makok, cytotoxic effect, selective property

INTRODUCTION

According to the report of Gotto *et al*. (2010), the incidence rate of cancer worldwide and the rate of human death are increasing significantly (Gotto *et al*., 2010). Cholangiocarcinoma (CCA) is a kind of malignant tumor that has been indicated the prevalence in Southeast Asia. The highest incidence in the world of cholangiocarcinoma has been reported in the northeastern region of Thailand (Vatanasapt *et al*., 1990; Sripa *et al*., 2005). Therefore, cholangiocarcinoma is an important problem of public health in this area. There are many risk factors that associate with CCA, especially, consumption uncooked freshwater fish which have the parasite; *Opisthorchis viverrini*. Metacercaria of *O. viverrini* can growth in the bile duct as well. The originating of CCA may associate with the toxin, that produced by this parasite.

Moreover, N-nitroso compound and Nitrosamines contained in fermented fish food products can enhance CCA progression for short period, due to people in the northeastern area have a high risk of CCA more than other regions (Khuntikao *et al*., 2005). CCA is difficult to identify because it was recognized and complicated diseases (Wasuworawong *et al*., 2015). Surgery combining radiotherapy and/or chemotherapy is used for cancer treatments. However, these methods have been reported that they have many side effects with healthy cells and tissues (Lin *et al*., 2007). Moreover, the resistance of chemotherapeutic drugs of cancer cells affects cancer treatments (Gatti *et al*., 2005). Thus, the side effects and cancer cell resistance of drugs are the major causes of unsatisfactory in cancer therapy, to date. To improve these problems, new anticancer drugs or the process of cancer preventions have been identified (Hoskin & Ramamoorthy, 2008).

Additionally, cancer preventions have interested to decrease the cancer incidences of humans

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worldwide. Functional foods are the target of cancer-protective sources which are able to reduce free radicals, moderate immune systems, decrease the symptom of diseases and enhance the enzyme functions etc. Plants foods are natural resources of bioactive compounds that have been reported of the pharmaceutical properties. Therefore, vegetables and fruits are promoted to high intake for prevention of cancer (Chahar *et al*., 2011; Galati *et al*., 2000; Middleton *et al*., 2000; Yang *et al*., 2001).

Wild mango (*Spondias pinnata* (L.f.) Kurz or *S. mangifera* Wild or *S. acuminate* Roxb.) is a medicinal plant that membered in Genus *Spondias*, family Anacardiaceae. Wild mango can grow in a temperate area and is widely find in Southeast Asia, including Thailand. It is a dietary plant used as the ingredient of traditional foods. Various parts of wild mango have been found the bioactive compounds which display pharmaceutical properties (Sameh *et al*., 2008). Wild mango fruit is used in traditional medicine for the therapy of many illnesses. The phytochemical agents in wild mango fruit show the potential of antioxidant, anti-inflammatory anti-microbial, including anti-cancer in many kinds of cancer cells (Sameh *et al*., 2008). Patathananone (2017) has reported that the biological activities of ripe wild mango fruit extract displayed anticancer activity on oral carcinoma cell lines (KB cells). In addition, these bioactive compounds exhibited a very low cytotoxic effect with normal cells (Vero cells). However, the study on anti-cancer activity in cholangiocarcinoma of ripe wild mango fruit extract has not been reported.

Therefore, the aims of this study are; to determine the cytotoxic effect of phytochemical components in ripe wild mango fruit extract in cholangiocarcinoma cells (MO55) compare with normal epithelial bile duct cell lines (MMNK1) using MTT assay; to do phytochemical screening, including total phenolic and total flavonoid contents analysis and to separate these molecules by thin-layer chromatography (TLC).

MATERIALS AND METHODS

Plant collection and extractions

Ripe wild mango fruits were washed three times with water and cleaned by using 70% v/v ethanol. Next, the plant sample was frozen at -20°C for overnight. After the seed was removed, pericarps and fruits were collected and stayed on the ice. The pericarps and fruits were homogenized by using a blender for 5 min. The homogenate was freeze dried by freeze dryer. The extraction processes were prepared as in the report of Patathananone *et al*. (2019). The extract (10.0 g) of isopropanol solvent was separated by liquid/liquid extraction. The separate funnel was shaken 10 min and stayed at room temperature 30 min for three times. The hexane and methanol layers were kept and concentrated by the evaporator. The methanolic part was called 6A* fraction. This extraction part was placed at -20°C until the next procedures.

Cell viability assay

Cholangiocarcinoma cell lines (MO55 cells) and normal epithelial bile duct cells (MMNK1) were kindly provided by Associate Professor Doctor Banchob Sripa (Khon Kaen University, Khon Kaen, Thailand). These cell lines were maintained and constructed as previously described by Wasuworawong *et al*. (2015). Both cell lines were cultured in Dulbecco's Modified Eagle Medium high-glucose (DMEM) supplemented with 10% FBS, 100 Units/mL penicillin and 100 µg/mL streptomycin in T25 cell culture flask, at 37°C under a 5% CO_2, 95% air. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay (MTT assay) according to Patathananone *et al*. (2019). After cell trypsinization, cells were resuspended by using a complete culture medium. Both cell lines, approximately 6×10^3 cells/well, were seeded in a 96-well tissue culture plate for 24 hr. After this period, cells were treated with various final concentrations of 6A* at 37°C for 24 and 48 hr. The treatment solution was removed and then 0.5 mg/mL of MTT solution (0.5 mg/ml in 1X PBS pH 7.4) was placed into each test well, mixed gently. The plate was incubated in 37°C for 4 hr and transferred MTT solution from each well. After that, 100 µL of DMSO was subjected to dissolve the formazan crystals. The absorbance value was measured at 570 nm by using ELISA plate reader (Verioskan Flash). The percentage of cell viability was calculated according to Patathananone *et al*. (2019).

Total phenolic contents

Bioactive components of 6A* fraction were analyzed total phenolic contents by reacted with folin-ciocaltue assay with a slightly modified method according to Rangsriwong *et al*. (2009). Approximately, 3.5 mL of 2.0% w/v Na_2CO_3 solution was placed into a test tube. After that, 100 µL of different concentration of standard gallic acid and 6A* fraction was added and mixed. Next, 100 µl of Folin reagent, which was diluted for ten folds, was subjected to each condition. All reactions were incubated in the dark at room temperature for 30 min. The absorbance value was detected at 750 nm by spectrophotometer (Thermo Science, U.S.A.). The standard curve of gallic acid was used to calculate total phenolic content in this extracted part.

Total flavonoid contents

Total flavonoid contents in 6A* were analyzed by using the method according to Olajire *et al*. (2011). Approximately, 1 mL of the extracted sample (6A*) and different concentrations of standard quercetin were placed into tubes that contained 4.0 ml of double-distilled water (DDW).

Next, 0.3 ml of 5% w/v $NaNO_2$ was added into each tube and mixed. All reaction tubes were incubated at room temperature for 5 min. Then, 0.3 mL of 10.0% w/v $AlCl_3$ was added into a volumetric flask, mixed and incubated for 6 min. After that, 2.0 mL of 1.0 M NaOH was subjected and adjusted the volume to 10.0 mL by DDW. These reaction solutions were measured the absorbance value at 510 nm by UV-Visible spectrophotometer (Thermo Science, U.S.A.). These absorbance values of quercetin were plotted as a standard curve for using the calculation of total flavonoid contents.

Separation of the biological components by thin layer chromatography (TLC)

Phytochemical compounds in 6A* fraction were separated by TLC. Gallic acid, quercetin, and tannic acid were spotted on silica sheet for using as the standard agents. The silica sheet was placed in the mobile phase chamber which contained chloroform: methanol+H_2O (1:1:0.05). Then, the separated silica sheets were dried at 105°C for 10 min. The spot of phytochemical compounds was detected at 256 nm by a UV detector lamp, including stained with 0.05% anis-aldehyde in acid-methanol.

Screening phytochemical compounds

The phytochemical compounds screening was studied by modified methods as previously described by Rangsriwong *et al*. (2009).

Saponins test

Approximately 1.0 g of 6A* was placed into a test tube and dissolved by 2.5 mL of 50% v/v ethanol. It was incubated in a water bath at 60°C for 5 min. Then, the tube was shaken vigorously. The positive result was indicated by the appearance of the bubble.

Tannins test

The 1.0 g of extracted sample (6A*) was dissolved by 3.0 mL of Deionized water (DI water). It was incubated in a water bath at 60°C for 5 min. The precipitant was removed and then 200 µL of the supernatant was subjected to the tube. Next, a small volume of 10% w/v ferric chloride was dropped into the tube and mixed. Tannin was indicated by presenting of green color or blue-black color.

Determination flavonol, flavanones by cyanidin reaction

The 1.0 g of sample (6A*) was dissolved in methanol. The sample solution (1.0 mL) was subjected to the tube. Concentrated hydrochloric acid (2.0 mL) was added and mixed gently. Two small pieces of magnesium (Mg) were placed into a tube and then shake. The red or orange color indicated that this extract contained flavonols and flavanones, flavones.

Terpenoids assay

1.0 mL of supernatant (6A*in dichloromethane) was added into the test tube. Next, concentrated H_2SO_4 at 0.5 mL was placed gently into this tube. The appearance of the brown ring between both layers suggested that terpenoids were found.

Detection of steroids

Approximately 1.0 mL of 6A* supernatant was mixed with 0.5 mL of Glacial acetic acid. After that, Conc.H_2SO_4 was added into this reaction tube for five drops. The positive test was demonstrated by the exhibition of blue or blue-green color in this solution.

Cardiac glycosides determination

The extract was dissolved by dichloromethane and collected the supernatant to analyze the cardiac glycosides. The filtrated supernatant of this extract was added into the test tube for 1.0 mL and then 10% w/v $FeCl_3$ has dropped amount 1-2 drops, mixed. Next, Glacial acetic acid was placed for five drops into this reaction tube and mixed gently. After that, 0.5 mL of Conc. H_2SO_4 was added carefully into the tube. The brown ring between the layer of H_2SO_4 and the extract supernatant demonstrated that the cardiac glycosides were found.

Statistical analysis

The data were analyzed according to the statistic method shown in Patathananone *et al*. (2016). The absorbance value of each condition was analyzed by using one-way ANOVA. Post-hoc Duncan test is a statistical method used to compare the absorbance value between the groups. The data showed a mean ± SD of the percentage cell viability.

RESULTS AND DISCUSSION

Anti- cholangiocarcinoma

The 6A* fraction was determined the cytotoxic effect of cholangiocarcinoma (MO55 cells) and compared with MMNK 1 cells which is a kind of

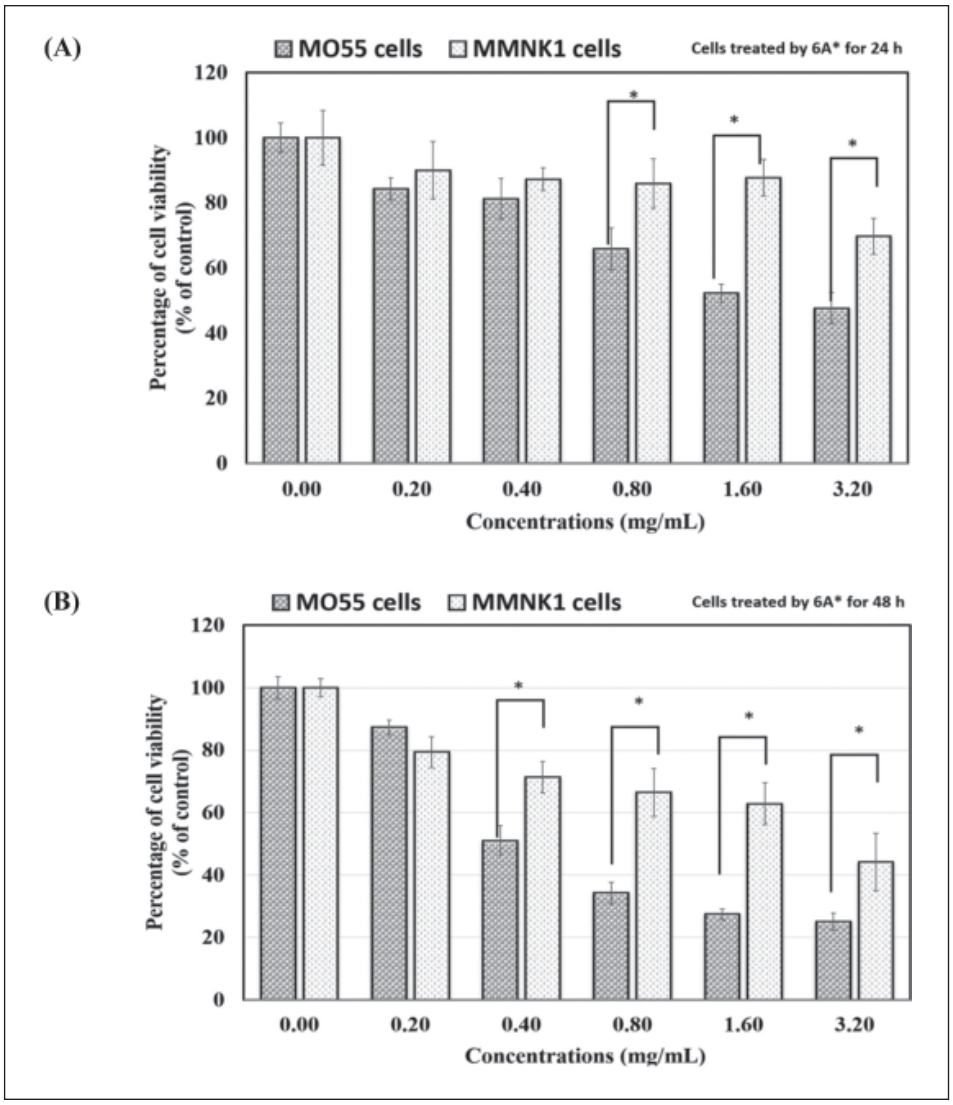

Fig. 1. The comparison of the cytotoxic effect of the bioactive components from 6A* in MO55 and MMNK1 cells incubated for 24 and 48 hr Fig. 1A and 1B. The symbol * displayed significant data from the control, $p < 0.05$.

normal bile duct cells. The cytotoxic effect of 6A* fraction was shown in Figure 1. The bioactive agents in 6A* fraction also presented the cytotoxic effect in MO55 cells. The percentage of cell viability was decreased according to dose-dependence. The IC_{50} value was indicated in Table 1. On the other hand, MMNK1 cells represented the percentage of cell viability higher than MO55 cells. The IC_{50} dose was shown in Table 1. The cytotoxic effect comparison of 6A* was shown in Figure 1(A) and 1(B). These results indicated that phytochemical components in 6A* displayed the anticancer potential in MO55 cells but showed the very low cytotoxic effect with MMNK1 cells. The morphological changing of MO55 and MMNK1 cells were represented in Figure 2(A) and 2(B), respectively. In Figure 3(A), both cells were treated with 6A* fraction at different concentrations, which showed 0, 1.6 and 3.2 mg/mL and incubated for 24 and 48 hr. Negative control or untreated (0 mg/mL) of both cells were indicated at the top of the column. The results showed that the morphological of MO55 cells changed after incubated with the phytochemicals in 6A* fraction (indicated by arrow). The number of dead cells presented higher than the control condition, which indicated that bioactive agents in 6A* displayed the cytotoxicity with MO55 cells. Moreover, the density of cells confluence also decreased after treated with this sample. These data related to the percentage of cell viability (Fig. 1(A), 1(B)). On the

Table 1. IC_{50} value of 6A* in MO55 and MMNK1 cells

Cell Types	IC_{50} value (mg/ml) of 6A*	
	24 hr	48 hr
MO55 cells	1.80	0.40
MMNK1 cells	> 3.20	2.70

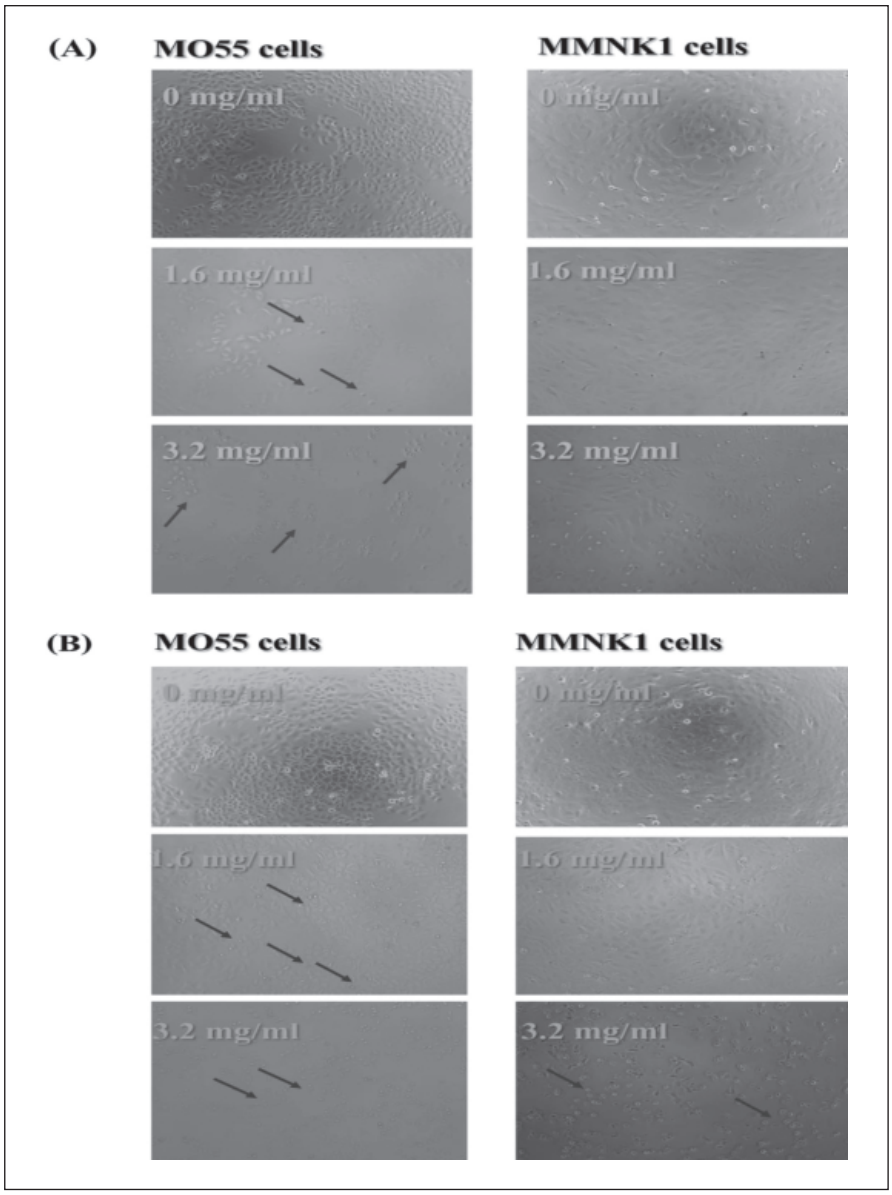

Fig. 2. The morphological changed of MO55 cells (Left column) and MMNK1 cells (Right column) after incubated with 0, 1.6 and 3.2 mg/mL of 6A* fraction for 24 and 48 hr. Photography was observed and taken pictures under the inverted microscope that used magnify power 20×.

other hand, MMNK1 showed more morphological of healthy cells more than MO55 cells at the same treatment concentrations. In this study, it was the first time that we indicated the cytotoxicity of ripe wild mango fruit extracts in MO55 cells. Interestingly, this extract showed a low cytotoxic effect in the normal epithelial bile duct (MMNK1) cell lines. The result indicated that IC_{50} value was 2.70 mg/mL for MMNK1 cell at 48 hr. This IC_{50} dose was 6-fold higher than the IC_{50} value that exhibited in MO55 cells. These results suggested that the phytochemical compounds in 6A* may have more affinity binding with cancer cells than normal cells. It is possible that these bioactive agents may have the selective ability, leading to the appearance of cytotoxicity in cancer cells higher than normal cells. This anti-cancer activity of the phytochemicals in 6A* related to the report described by Patathananone *et al*. (2019). These bioactive agents in 6A* exhibited the cytotoxicity in an oral carcinoma cell line (KB cells). The IC_{50} value showed 1.40 mg/mL for KB cells, which was incubated by 6A* for 48 hr. Importantly, the phytochemicals in 6A* displayed low cytotoxicity in normal fibroblast cells (NHDF cells). The percentage of cell viability of NHDF cells presented higher than 80% for treated concentrations. IC_{50} value for NHDF cells showed higher than 1.6 mg/mL that was greater than the IC_{50} dose in KB cells.

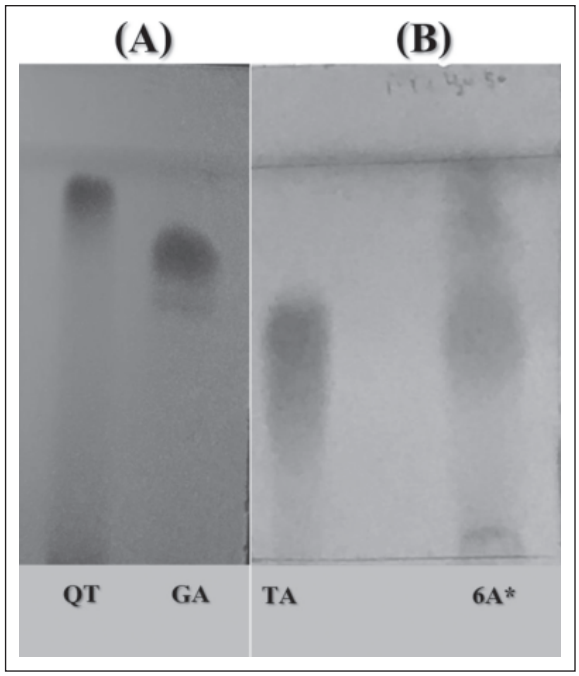

Fig. 3. Thin layer chromatography (TLC) of 6A* fraction and standard agents; gallic acid (GA), quercetin (QT), tannic acid (TA). The separation of substances was detected by UV lamp at 256 nm (Fig. 3A), while Fig. 3(B) was developed by 0.05% anis-aldehyde in acid-methanol.

Table 2. Phytochemical compounds screening in 6A* fraction

Screening compounds	Results
Polyphenolic/phenolic compound	+
Tannin	+
Saponin	–
Flavonoids	+
Flavonols	–
Flavanones	–
Flavone	+
Terpenoids	+
Cardiac glycoside	+
Steroid	–

"+" means present.
"–" means absent.

Total phenolic contents/total flavonoid contents

Polyphenolic compounds are the major secondary metabolites of plants that have been found more than 8,000 different kinds of polyphenols. These phytochemicals are classified into ten various general subclasses (Bravo, 1998). Polyphenolic compounds have been reported to have the spectrum of biological activities such as antioxidant, antimutagenic, anticarcinogenic, anti-allergic, anti-inflammatory, and modulation of enzymatic activities (Chahar *et al*., 2011; Galati *et al*., 2000; Middleton *et al*., 2000; Yang *et al*., 2001). These bioactive agents may have benefits for the healthcare of humans. Especially, some special molecules of polyphenols which can develop to be as chemo-preventive or therapeutic agents against cancer (Birt *et al.,* 2001). Therefore, in this report, the total phenolic content in 6A* fraction was analyzed by folin reagent. Evaluation was base on standard curve of gallic acid $y = 0.0435x - 0.0202$, $R^2 = 0.99$. The total phenolic contents of 6A* were 192.14 ± 30.69 mg GAE/g extract. Moreover, flavonoids are phytochemical compounds that have reported the anticancer property *in vitro* and *in vivo*. In this study, total flavonoid contents were calculated by quercetin equivalent, that represented, $y = 0.0134x + 0.0125$, $R^2 = 0.9998$. Total flavonoid contents were indicated to be 73.13 ± 6.34 mg QT/g extract. This result suggested that flavonoids are a big group of phytochemical agents in 6A* fraction.

Screening bioactive components in 6A* fraction

Qualitative phytochemical screening of 6A* fraction was observed by different screening methods. The results were shown in Table 2. Phenolic, flavonoids, tannin, terpenoids, cardiac glycoside were found in 6A* fraction. The anti-cancer property that displayed in 6A* fraction also related to these molecules. However, these data also exhibited the screening results of some phyto-chemicals to explain the possibility of the function of these compounds. It is necessary to analyze the bioactive compounds with other techniques.

Thin-layer chromatography

The data which appeared on the silica sheet was represented in Figure 3. Gallic acid (GA), quercetin (QT) and tannic acid (TA) were used as the standard agents. The band of the phytochemical compound in 6A* fraction showed a similar R_f value with gallic acid and tannic acid. Tannins are classified into two groups such as (1) condense tannins and (2) hydrolysate tannins which are water-soluble polyphenols. The extracted process that used the aqueous methanol due to tannins was dissolved and found in 6A* fraction. This result related to the result of phytochemicals screening which shows in Table 2. However, the bioactive agents in 6A* fraction will be analysed and determined biological activities, including their structures in the future work.

Tannins or tannic acids are large complexed polyphenol. Tannins are exhibited in many dietary plants. They are used to protect the plant infection from the plant pathogens, since they can be found in many parts of plants, including fruits. Tannins in green tea leave have been suggested as an anticarcinogenic. Some derivative molecules of tannins have been exhibited to decrease the mutagenic activity of a number of mutagens.

Moreover, gallic acid is a hydrolysate tannin which has been presented many biological properties such as antioxidant, antimicrobial, anti-inflammatory, including anticancer activity in many kinds of cancer cell lines. It is used as a food additive and applied to cancer therapeutic agents (Chung *et al*., 1998). Therefore, the inhibitory cholangiocarcinoma cell growth may associate with these tannin molecules.

Furthermore, flavonoids are the group of phytochemicals in 6A* fraction which has total flavonoids contents around 73.13 ± 6.34 mg QT/g extract. Previous reports have been indicated that flavonoids are a class of polyphenols that have been found more than 4,000 categories (Chahar *et al*., 2011). Some flavonoids in tea have shown the properties of cancer prevention, including induction apoptotic cell death. Flavonol and flavones are sub-sub classes of flavonoids which have been suggested that they can prevent the mutation of the specific enzyme (Kandaswami *et al*., 2005). Hirano *et al*. (1994) reported the anti-cancer ability of 28 flavonoids on human acute myeloid leukemia cell line HL-60. Genistein, honokiol, machilin A, matairesinol, and arctigenin had the strongest effects on this cell line (Chahar *et al*., 2011; Hirano *et al*., 1994). Therefore, anticancer activity on cholangiocarcinoma could be related to the effects of these flavonoids. It is possible that some flavonoid molecules in this report could be applied as the cancer prevention agents or combination used with the chemotherapeutic substances for cancer treatment.

Moreover, terpenoids are biological agents which presented in 6A* fraction. These molecules may be able to inhibit cholangiocarcinoma cell growth *in vitro*. Terpenoids are major food constituents derived from vegetables, fruits, and different plant food species. The structures of terpenoids have 4 structure-wise such as monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and tetraterpenoids. The most anticancer property has been found in triterpenoids. However, the mode of action of terpenoids for anticancer remains unclear (Thoppil, and Bishayee, 2011; Salminena *et al*., 2008; Huang *et al*., 2012). The future work, bioactive molecules of terpenoids in ripe wild mango fruit extracts which presented anticancer ability will be analyzing the molecular mechanism.

In addition, cardiac glycosides are a group of phytochemical components represented in 6A* fraction. Cardiac diseases have been treated by cardiac glycosides for more than 200 years. Previous reports have been suggested that there are some kinds of cardiac glycoside that may display anticancer activity. Moreover, three cardiac glycosides have been applied for the therapy of cancer. Interestingly, they were studied about dose-limiting toxicities, including maximum tolerated dose in phase 1 clinical trial, to date (Slingerland *et al*., 2013). Therefore, the anticancer activity in cholangiocarcinoma of 6A* fraction may associate with the appearance of cardiac glycosides. However, this data will be supported by future studies.

Overall, the bioactive compounds in ripe wild mango fruit extract presented anticancer activity in cholangiocarcinoma. However, they showed a very low cytotoxic effect with normal (MMNK1) cell lines. Phytochemical compounds which showed the selective ability between cancer cells and normal cells is the goal target for safe applications. Bioactive agents have been reported of the varieties of anticancer mechanism. The bioactive components which contained in 6A* fraction will be investigated of the structure and mode of action in cholangiocarcinoma. These compounds could be developed as a food supplement, anticancer drugs to prevent and treat cancer progression.

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