Anti-proliferation and Anti-migration of Schizophyllum commune extracts on Human Cholangiocarcinoma Cell Line

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Abstract

Objective: To investigate the possible anti-proliferation and anti-migration effect of the crude extracts from *Schizophyllum commune* against human cholangiocarcinoma cell line, KKU-M213.

Methods: In this study, dried fruiting bodies of *S. commune* were extracted with 95% ethanol and water. Antiproliferative activity of the crude extracts from *S. commune* against human cholangiocarcinoma cell line, KKU-M213 was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, diphenyl tetrazolium bromide (MTT) assay. Wound healing assay was used for investigating the effects of the mushrooms crude extracts on KKU-M213 cell migration.

Results: The percentage yields of *S. commune* ethanol and water extracts were 5.42 % and 7.78 %, respectively. The results of MTT assay showed that 200 µg/ml of ethanol crude extracts significantly inhibited proliferation of KKU-M213 to 50% compared to untreated control. While hot water extracts of *S. commune* did not have any effects on KKU-M213 cell proliferation. Wound healing assay result showed that both hot water and ethanolic extracts of *S. commune* suppressed KKU-M213 cell migration.

Conclusion: This is the first report showing the anti-proliferation and anti-migration activity of this mushroom against human cholangiocarcinoma. The active anticancer components of *S. commune* extracts will be further investigated.

Keywords: Cholangiocarcinoma (CCA), *Schizophyllum commune, mushroom extracts, anticancer*

ฤทธิ์ต้านการเจริญเติบโตและเคลื่อนที่ของเซลล์มะเร็งของสารสกัดจากเห็ดแครง Schizophyllum commune ต่อเซลล์มะเร็งท่อน้ำดี

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บทคัดย่อ

วัตถุประสงค์: ศึกษาฤทธิ์ต้านการเจริญเติบโตและการเคลื่อนที่ของสารสกัดเห็ดแครงต่อเซลล์มะเร็งท่อน้ำดี

วิธีดำเนินการวิจัย: นำดอกเห็ดแครงแห้งมาสกัดสารสกัดหยาบโดยใช้ร้อยละ 95 เอธานอล และน้ำเป็นตัวทำละลาย เมื่อได้สารสกัดหยาบจากเห็ดแล้วนำมาศึกษาฤทธิ์ต้านการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดีชนิด KKU-M213 โดยวิธี MTT และศึกษาฤทธิ์ต้านการเคลื่อนที่โดยวิธี wound healing assay

ผลการวิจัย: ผลผลิตร้อยละของการสกัดสารจากเห็ดแครงโดยใช้เอทานอลและน้ำเป็นตัวทำละลาย เท่ากับ ร้อยละ 5.42 และ 7.78 ตามลำดับผลการศึกษาฤทธิ์ต้านการเจริญเติบโตโดยวิธี MTT พบว่า สารสกัดเอทานอลของเห็ดแครงที่ความเข้มข้น 200 mg/ml ยับยั้งการเจริญเติบโตของเซลล์ลงร้อยละ50 เทียบกับกลุ่มควบคุม แต่สารสกัดน้ำไม่มีผลยับยั้งการเจริญเติบโตของเซลล์ KKU-M213 สำหรับฤทธิ์ต้านการเคลื่อนที่ของเซลล์ซึ่งทดสอบโดยวิธี wound healing พบว่าทั้งสารสกัดเอทานอลและน้ำของเห็ดแครงมีผลยับยั้งการเคลื่อนที่ของเซลล์ KKU-M213 อย่างมีนัยสำคัญทางสถิติ

สรุป: จากผลการศึกษานี้แสดงให้เห็นว่าสารสกัดเห็ดแครงมีฤทธิ์ต้านการเจริญเติบโตและการเคลื่อนที่ของมะเร็งท่อน้ำดี โดยในอนาคตจะทำการศึกษาองค์ประกอบสำคัญของสารสกัด เพื่อเพิ่มโอกาสที่จะพัฒนาต่อยอดไปเป็นสารต้านมะเร็ง

คำสำคัญ: มะเร็งท่อน้ำดี, เห็ดแครง *Schizophyllum commune,* สารสกัดเห็ด, ต้านมะเร็ง

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor of biliary epithelium associated with high metastatic and mortality rates. Incidence of this cancer has increased worldwide; in Thailand, the highest incidence is in the northeastern region, where *Opisthorchis viverrini* infection is also prevalent [1-2]. Nowadays, surgery is the only possible curative treatment in the early stage of disease. However, most CCA patients cannot be cured by surgery because of locally advanced or metastatic disease. Furthermore, there is a limitation of effective chemotherapy and radiotherapy for this cancer. Treatment of cholangiocarcinoma with gemcitabine or gemcitabine plus cisplatin, the standard chemotherapy regimen, is mostly ineffective with low clinical response rate [3]. Because of the poor response to systemic chemotherapy, searching for new and effective bioactive compounds from medicinal plants and the use of traditional medicine might be the alternative way to treat cholangiocarcinoma patients.

Mushroom has long been used as a nutritional and herbal medicine resource. Medicinal mushrooms possessing therapeutic properties have been used in traditional medicine for their anti-cancer, antioxidant, immunomodulating, anti-inflammatory properties [4]. Biologically active compounds exerting anti-cancer properties from various mushrooms, including *Ganodama Lucidum*, *Cordyceps militaris*, *Hericium erinaceus*, *Phellinus linteus*, *Lentinula edodes*, *Schizophyllum commune*, have been assessed [4-6].

Schizophyllum commune, commonly known as split gill mushroom, a bacidomycete white-rot fan-shaped fungus, can be found in all areas of Thailand and worldwide. Polysaccharide extracts from it have been reported to inhibit Sarcoma 180 tumor in mice. However, studies in human cancers have shown that Schizophyllan increases overall survival of head and neck cancer patients[7-8]. The phenolic compounds such as phenyl benzoate and phenol have been identified from *Schizophyllum commune* extracts. The antioxidant activity *of S. commune* extracts has been shown to be directly proportional to total phenolic content of the extracts [9]. Moreover, N-acetyl-D-galactosmine-specific lectins have been characterized and purified from *Schizophyllum commune* cultivated in Thailand. The cytotoxicity against cancer cell line of these purified lectins has also been reported [10]. The iminolactones, Schizine A and Schizine B, from ethanol extracts of *S. commune* exhibited the growth inhibition of several cancer cell lines [11].

To our knowledge, the anti-cancer activity of *S. commune* extracts against cholangiocarcinoma has not been studied before. Therefore, in the present study, the possible anti-proliferative and anti-migratory activity of the crude extracts of *S. commune* against human cholangiocarcinoma cell line, KKU-M213 were investigated.

Methods

Cell line and culture conditions

Cholangiocarcinoma cell line KKU-M213 was kindly provided by Prof. Sripa B (Khon Kean University, Khon Kean, Thailand). Cells were cultured in HAM/F12 medium (GIBCO, Life Technologies Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (GIBCO, Life Technologies Grand Island, NY), 100 U/ml Penicillin G sodium, 100µg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B at 37 °C in a humidified incubator with 5% carbon dioxide (CO_2).

Mushroom Extracts

Fruiting bodies of *Schizophyllum commune* were generously provided by Asst. Prof. Rattapon Sornprasert (Chandrakasem Rajabhat University, Bangkok, Thailand). Crude mushroom extraction was performed as follows. Mushroom was air dried and ground to a fine powder. For ethanol extraction, 150 g of *S. commune* powder was macerated twice with 400 ml of 95% (v/v) ethanol with stirring at 40 °C for 48 h. For hot water extraction, 150 g of *S. commune* powder was soaked twice with 400 ml of boiled distilled deionized water in a shaking water bath at 100 °C for 4 h. Extracts were pooled together and sediment removed by using gauze

cloth; they were then filtered through Whatman No. 4 filter paper. The solvents, ethanol and water were removed from extracts using a rotary vacuum evaporator. The percentage yield of the crude extracts was calculated as: [(weight of crude mushroom extract (gram) /weight of dry mushroom (gram)) × 100%]. The stock solution of *S. commune* water extracts was prepared by dissolving extracts in sterile water while ethanol extracts were dissolved in DMSO to a concentration of 1 mg/ml. The various working concentrations of mushroom extracts were prepared by filtered 1 mg/ml extracts through 0.22 mm filter and diluted by culture medium.

Proliferation assay

To assay anti-proliferation effect of *S. commune* crude extracts, KKU-M213 cell was seeded in 96 well plates ($5x10^3$ cells/ well) in a final volume of 100 µl and allowed to attach overnight in HAM/F-12 medium supplemented with 10% FBS. The medium was then removed, and replaced with either medium with *S. commune* extracts in various concentrations (0-200 µg/ml) for 0, 24, 48, and 72 hours at 37 °C. Viability cell was evaluated by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test by replacing medium containing *S. commune* extracts with the mixing of 5 mg/ml MTT solution with normal culture media and then incubated for another 4 hours. The MTT was then converted to formazan crystal by mitochondrial enzyme in viable cells. After aspirating the medium, insoluble fomazan crystals were solubilized with 200 µl DMSO. Viability cell was assessed by measuring absorbance at 540 nm in a Microplate Reader (MultiskanEX, Thermolabsystems).

Wound healing assay

The effect of *S. commune* crude extracts on cell migration was investigated by wound healing assay. KKU-M213 cells ($1x10^5$ cell/ well) were seeded in a 24-well plate. When reaching 95% confluence, wound was made at the center of the well by scraping the cell monolayer with a 200 µl pipette tip. Cells were washed 3 times with PBS and incubated with 0, 100, and 200 µg/ ml of *S. commune* crude extracts. The cell images were captured immediately (time zero) under a microscope (40X magnification) and then captured again after 6 hours of incubation. The area of wound was determined by Image J software (http://rsb.info.nih.gov). The degree of cell migration was expressed as percentage of wound closure:

$$\text{Wound closure} = [(A_{0h} - A_{6h}) / A_{0h}] \times 100\ (\%)$$

Where A_{0h} is the area of the wound measured immediately after scratching and A_{6h} is the area of the wound measured 6 h after scratching.

Statistical analysis

Statistical analysis of the results was performed using SPSS software version 17.0. Data from at least three experiments were presented as the mean ±standard error of mean [12]. The comparison between the groups was carried out by one-way ANOVA (LSD). The results were considered significant at a value of $P < 0.05$.

Results

Extract yield of S. commune.

The extraction of dry fruiting bodies of *S. commune* was performed with hot water and ethanol. The percentage yield of the crude extracts is summarized in Table 1. In our results, the water extracts of *S. commune* showed a higher percentage yield (7.78 %) than that of ethanol extracts (5.42 %). This could possibly have been due to the higher polarity of water and also the higher extraction temperature used in water extraction procedure.

Table 1:

Yield of the crude extracts of *S. commune* fruiting bodies prepared using different solvents

Solvent	Yield (% w/w)
water	7.78
95% ethanol	5.42

The anti-proliferative activity of S. commune extracts

The effect of *S. commune* extracts against cholangiocarcinoma cell proliferation is shown in Figure 1. KKU-M213 cell treated with various concentrations of the *S. commune* water extract (50, 100, 200 µg/ml) for 48 and 72 h showed a significant increase in cell proliferation in dose dependent manner. In contrast, cell proliferation was not significantly changed with 10 µg/ml of extract for 24-72h (Figure 1a). Whereas, the ethanol extracts of *S. commune* appeared to have potent anti-proliferation properties with up to 50% reduction (Figure 1b). KKU-M213 cell proliferation was reduced to 85 % of control after treating cell with 200 µg/ml of ethanol extracts for 48 h. At 72 h exposure time, KKU-M213 cell proliferation was significantly reduced to 72 and 50 % (P<0.01) at 100 and 200µg/ml of ethanol extracts, respectively. However, increased in cell proliferation was observed when treatment of KKU-M213 cell with ethanol extracts at lower dose (10-100 µg/ml) for 48h.

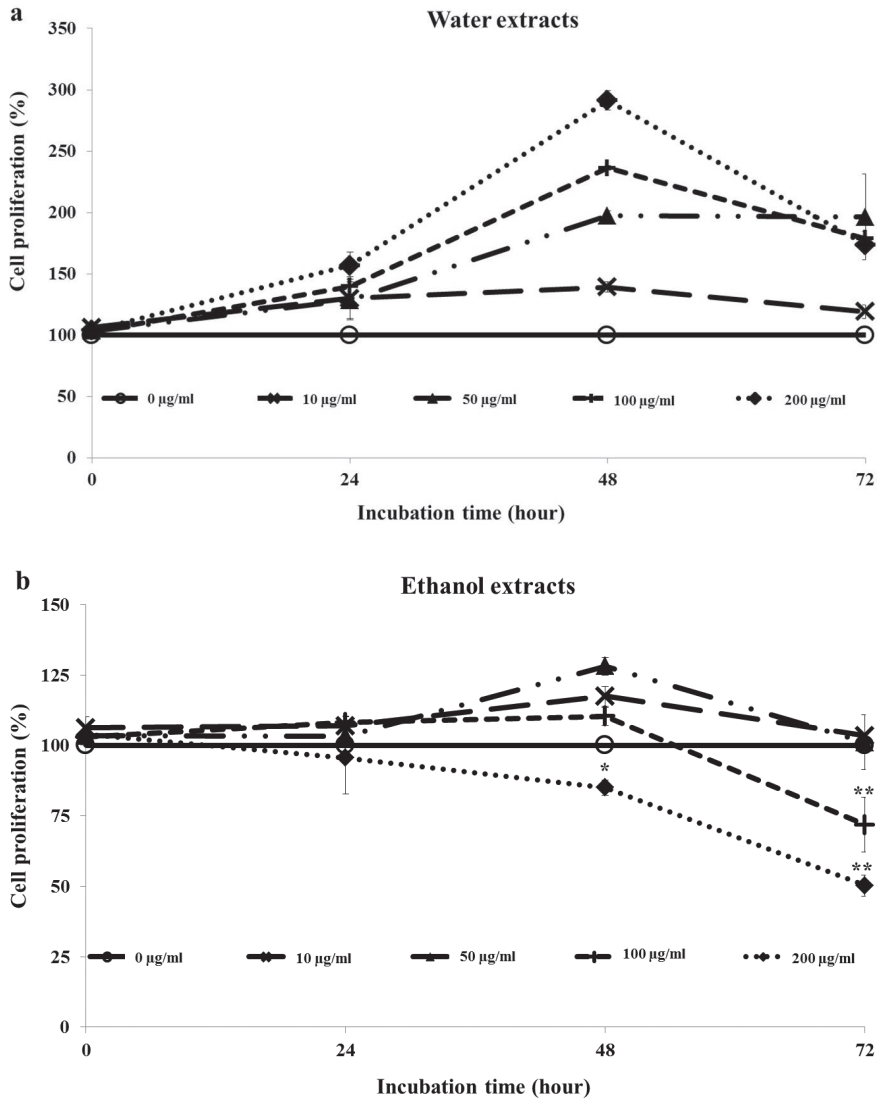

Figure 1: Effect of hot water extracts (a) and ethanol extracts (b) on cellular proliferation in KKU-M213 human cholangiocarcinoma cells incubated for 24, 48, and 72 h. Proliferation was determined by MTT test. Data are expressed as percentage of control ± SEM.* $P < 0.05$, ** $P < 0.01$

The anti-migration activity of *S. commune* extracts

To assess the anti-migration potential of *S. commune* against KKU-M213 human cholangiocarcinoma cell line, wound healing assay was performed in the presence of various concentrations of extracts. The 95% confluent KKU-M213 cell was wounded using pipette tip. After 6 h of incubation, the wound closure was observed and imaged under inverted microscope. Migration of KKU-M213 cell line treated with *S. commune* water extracts was significantly inhibited (Figure 2) with the percentage of wound closure reduced to 32% and 2% at 100 and 200 µg/ml, respectively. Ethanol extracts of *S. commune* also inhibited KKU-M213 cell migration (Figure 3) to 21 and 6 % at 100 and 200 µg/ml respectively. At 100 and 200 µg/ml of *S. commune* extracts, no cytotoxic effects were observed as confirmed by MTT assay at 6h (Figure 4).

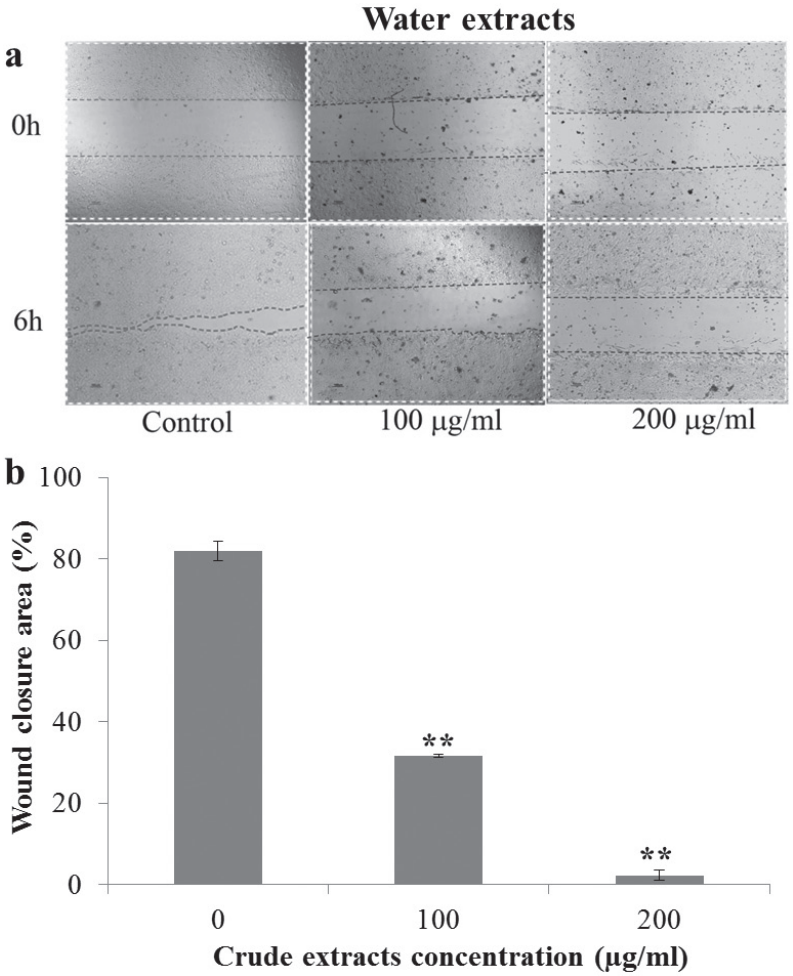

Figure 2: Effect of *S. commune* hot water extracts on cellular migration in KKU-M213 human cholangiocarcinoma cells determined by wound healing assay. Image of wound and cell migration closing the wound was taken at time zero and 6 h after wounding (a). Cell migration was plotted as percentage of wound closure (b). Data are expressed as mean ± SEM.* $P < 0.05$, ** $P < 0.01$.

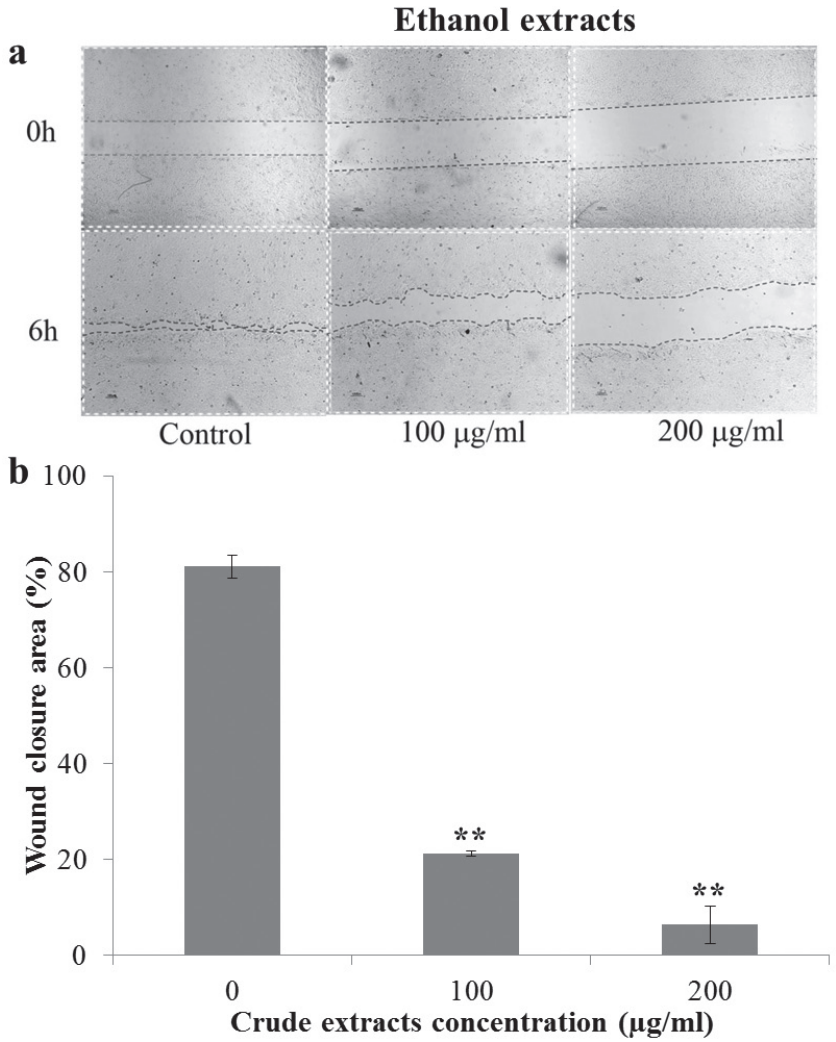

Figure 3: Effect of *S. commune* ethanol extracts on cellular migration in KKU-M213 human cholangiocarcinoma cells determined by wound healing assay. Image of wound and cell migration to close the wound was taken at time zero and at 6 h after wounding (a). Cell migration was plotted as percentage of wound closure (b). Data are expressed as mean ± SEM.* $P < 0.05$, ** $P < 0.01$

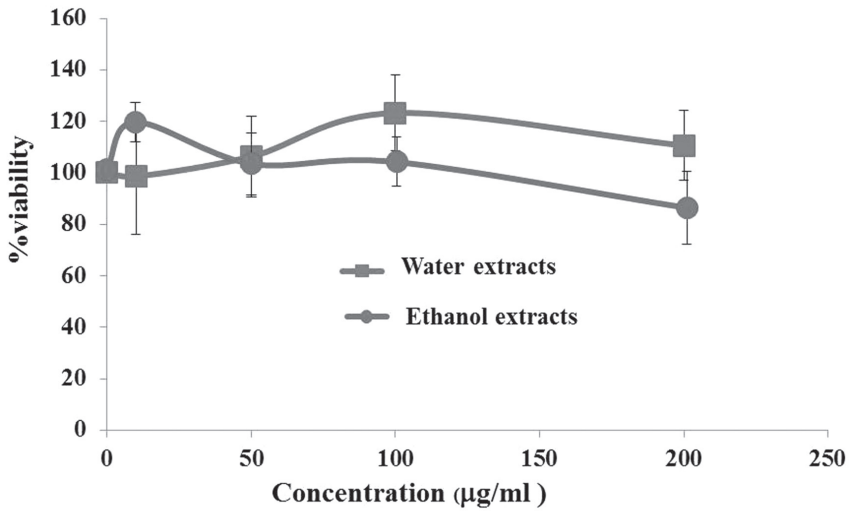

Figure 4: Effect of hot water extracts and ethanol extracts on cytotoxicity in KKU-M213 human cholangiocarcinoma 6 h. Cell viability was determined by MTT test. Data are expressed as percentage of control ± SEM.* $P < 0.05$, ** $P < 0.01$.

Discussion

Bioactive compounds isolated from mushrooms have recently been highlighted for their therapeutic potential as anticancer agents. In the present study, we demonstrated for the first time that ethanol extracts of *S. commune* inhibits the growth of cholangiocarcinoma cells, while both hot water and ethanol extracts suppress cell migration. The elucidation of anti-proliferative and anti-migratory activity of *S. commune* extracts may increase the pharmacological value of this medicinal mushroom; further investigations on bioactive components identification and mechanisms of action against cholangiocarcinoma are needed.

Water and ethanol were the most commonly used solvents for mushroom extraction because of their safety and low cost. Different extraction protocols, polarity of solvent, time and temperature may determine the extraction efficiency as well as the potential to extract bioactive components from mushroom. In the present study, the water extracts of *S. commune* exhibited higher extractions yield than that of ethanol extracts. The extraction yield indicated that *S. commune* contains higher amounts of hydrophilic substances than that of lipophilic ones. These results agree with the study of Hu *et al.* who reported that the extraction yield of hot water extracts of the mushroom *Inonotus obliquus* is 10 times higher than the yield from ethanol extracts [13]. Vaskovsky *et al* (1998) reported that mushrooms contain a lot of polar substances, which can dissolve more in higher polar solvent [14]. This may support our finding that the extraction efficiency of water was higher than ethanol. Since the polarity of water is higher than that of the organic solvent, ethanol.

Polysaccharides are major active components from mushroom extracts with potent anticancer activity. The well-identified mushroom-derived polysaccharides with antitumor and immunomodulating properties include: lentinan from fruiting bodies of *Lentinula edodes*, and schizophyllan, an exo-polysaccharide from the liquid cultured broth product of *Schizophyllum commune* [15-16]. These two well-known polysaccharides have passed clinical trials in Japan, China and the USA [6]. Beside polysaccharides of mushroom extracts, other secondary metabolites such as polyphenol compounds have been suggested to have anticancer activity [17]. Methanol extracts of *S. commune* fruiting

bodies showed high content of phenolic compounds that may contribute to high antioxidant activity. While the water extracts of *S. commune* have low antioxidant activity, which may be explained by the low phenolic content of the extracts [18]. Several studies have reported that mushroom water extracts contain high amounts of polysaccharides, while ethanol extracts are high in phenolic compounds [13].

The anti-proliferative activity of substances might be considered as the indicator of anticancer properties since uncontrolled cell proliferation of cancer resulted in cancer progression. In the present study, ethanol extracts of *S. commune* were shown to be more effective than the water extracts in antiproliferative activity against cholangiocarcinoma cell lines. This data is in agreement with Hu et.al, who reported that the anti-proliferative effect of the ethanol extracts of *Inonotus obliquus* was much stronger than that of water extracts [13]. The strong anti-proliferative activity of ethanol extracts implied that water and ethanol extracts of *S. commune* contained different bioactive substances. Many studies of mushroom extracts have previously indicated that the antioxidant activity of extracts could be responsible for the inhibition of cancer proliferation [19], thus supporting the suggestion that the antiproliferative activity may result from antioxidative components in ethanol extracts of S. commune. Since phenolic compounds of mushroom extracts have been suggested to be the major component playing an important role in its antioxidant activity, the correlation between phenolic contents and antioxidant activity of S. commune extracts has been reported [20]. Moreover, the exopolysaccharide extracted from mycelial cultivation of *S.commune* have no effect on murine machrophage cell (RAW 264.7) proliferation but exhibited the anti-inflamatory effect [21].

The anti-proliferative response of ethanol extracts was time-dependent, and dose-dependent. Cell proliferation was increased in KKU-M213 cells treated with the lower doses (10-100 µg/ml) for 48 h, while it was reduced at the higher dose (200 µg/ml). However, cell proliferation was declined in dose-dependent manner at 72h treatment. The biphasic effect on cell growth is commonly found in antitumor agent treating cancer cells, which the cell proliferation was induced at low doses and inhibited proliferation at high doses [22]. Moreover, the dietary phytochemicals such as epigallocatechin-3-gallate (phenolic compound from green tea), curcumin (active component from Curcuma longa) have been classified as hormotic compounds since they have stimulatory effect at low concentration and toxic effect at high concentration[23]. However the underlining cause and mechanism of this biphasic response remain to be determined.

KKU-M213 cell proliferation showed significant improvement after treated with 50-200 µg/ml of *S. commune* water extracts for 48 and 72h. In contrast, at low concentration of water extract (10 µg/ml), no significant change in cell proliferation was observed at all three time points. The difference in cell proliferation in response to low and high dose of water extracts may need further investigation to determine its causes. We hypothesized that the concentration we used in this study (ranging from 0-200µg/ml) may induced adaptive response that resulted in induction of cell proliferation. Increasing concentration range of *S. commune* water extract and its anti-proliferation effect will need to be confirmed. However, the observation that mushroom extracts enhance cancer cell proliferation has previous been reported, *Pleutorus abalonus* extracts significantly induced U937 (human lymphoma cell line) proliferation [24].

Metastasis is the leading cause of death in cancer patients. Cancer cell migration is one of the important steps in cancer invasion and metastasis. The present data showed that water and ethanol extracts of *S. commune* significantly decreased the migratory ability of KKU-M213 cells in a dose dependent manner. The antimigratory effect of *S. commune* extracts did not resulted from cytotoxicity as indicated by MTT assay at 6 h. Several studies have reported the antimigratory effect of mushroom extracts, for example, ethanol extracts of *Antrodia cinamomea* inhibited migration of CL-10 lung cancer cells by regulated Focal Adhesion Kinase

signaling pathway [25]. Moreover, a study of *Ganoderma lucidum* extracts, reported that triterpines from extracts inhibited migration of DU-145 prostate cancer cell lines [26]. Treatment of *Phellinus linteus* extracts resulted in inhibition of the SW 180 colon cancer cell line by reduction of β-catenin expression[27]. The present study did not identify the mechanisms underlying the antimigratory effect of *S. commune* extracts, which requires further investigation.

In this study, we also found the selective effect of water extracts, which significantly inhibited CCA cell migration but not proliferation, whereas ethanol extracts inhibited both. This might be partially explained by different components in water and ethanol extracts. Further study will be needed for investigating the biologically active components in *S. commune* extract.

Many studies have proposed the mechanisms for the anticancer activity of mushrooms including cell cycle arrest, induction of apoptosis and cell signaling inhibition. For example, *Pleurotus ostreatus* extract inhibited growth of MCF-7 cell that was associated with cycle arrest at G0/G1 phase [28]. Moreover, a study by Youn MJ *et.al*., [29] showed that extracts of *Innotus obliquus* inhibited HepG2 cell proliferation, by inducing cell cycle arrest at G0/G1 phase, and induction of cell apoptosis. Ethanol extracts of *Antrodia cinamomea* inhibited PI3K pathway resulting in decreased cell migration [30]. The possible mechanisms underlying antiproliferation and antimigration of *S. commune* are still being studied.

Conclusion

The results of this present study demonstrated the anticancer effect of *S. commune* extracts. Treatment of *S. commune* water extracts inhibited KKU-M213 CCA cell migration whereas ethanol extracts inhibited proliferation and migration. Further study of the active components of extracts and detailed signaling mechanisms are required to support the potential anticancer properties of *S. commune*.

Conflict of interest

We have no financial relationships to disclose.

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