CANCER PREVENTION AND ANTI-CANCER EFFECT OF ANTHOCYANINS IN CHOLANGIOCARCINOMA

SASIKAMON KHOPHAI

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy in Biochemistry and Biochemistry Technology
Suranaree University of Technology
Academic Year 2024

การป้องกันมะเร็งและผลของการต้านมะเร็งของสารแอนโทไซยานินในมะเร็งท่อน้ำดี

นางสาวศศิกมล ค้อไผ่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเคมีและเทคโนโลยีชีวเคมี
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2567

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a degree of Doctor of Philosophy

Thesis Examining Committee

Sarinya Kongpetch

(Asst. Prof. Dr. Sarinya Kongpetch)
Chairperson

(Assoc. Prof. Dr. Chutima Talabnin)
Member (Thesis Advisor)

(Prof. Dr. James R. Ketudat Cairns)
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(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)
Vice Rector for Academic Affaire
and Quality Assurance

(Prof. Dr. Santi Meansiri)
Dean of Institute of Science

ศศิกมล ค้อไผ่ : การป้องกันมะเร็งและผลของการต้านมะเร็งของสารแอนโทไซยานินในมะเร็งท่อน้ำดี (CANCER PREVENTION AND ANTI-CANCER EFFECT OF ANTHOCYANINS IN CHOLANGIOCARCINOMA). อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.ชุติมา ตลับนิล, 83 หน้า.

คำสำคัญ: ต้านมะเร็ง/ ป้องกันมะเร็ง/ มะเร็งท่อน้ำดี

แอนโทไซยานินเป็นสารในกลุ่มฟลาโวนอยด์ที่มีความสามารถในการละลายน้ำได้และมีหน้าที่สร้างเม็ดสีต่าง ๆ เช่น สีม่วง สีน้ำเงิน พบได้ในผักและผลไม้ เช่น บลูเบอรี่ องุ่น ข้าวไรซ์เบอรี่และข้าวเหนียวดำ เป็นต้น สารแอนโทไซยานินมีความสัมพันธ์กับการป้องกันและต่อต้านมะเร็งด้วยกลไกต่าง ๆ รำข้าวเหนียวดำถือเป็นแหล่งวัตถุดิบของสารแอนโทไซยานิน เช่น ไซยานิดิน 3-กลูโคไซด์ ที่มีฤทธิ์ในการต่อต้านสารอนุมูลอิสระและต่อต้านมะเร็ง อย่างไรก็ตามปัจจุบันยังไม่มีการศึกษาผลของแอนโทไซยานินที่พบในรำข้าวเหนียวดำต่อการป้องกันและต่อต้านมะเร็งในมะเร็งท่อน้ำดี ในการศึกษาครั้งนี้สารแอนโทไซยานินถูกสกัดจากรำข้าวเหนียวชื่อว่า BBR-M-10 ผลการวิเคราะห์องค์ประกอบแสดงให้เห็นว่าสารแอนโทไซยานินหลักใน BBR-M-10 คือ ไซยานิดิน 3-กลูโคไซด์ ในการศึกษาต่อมาผลของสารสกัด BBR-M-10 ต่อการยับยั้งการเกิดสารอนุมูลอิสระและการตายรวมไปถึงความเสียหายต่อสายพันธุกรรมที่เกิดจากการกระตุ้นโดยสารไฮโดรเจนเปอร์ออกไซด์ในเซลล์ท่อน้ำดีปกติของมนุษย์ ผลการศึกษาพบสารสกัด BBR-M-10 ช่วยยับยั้งการเกิดสารอนุมูลอิสระได้และยังสามารถลดผลกระทบที่เป็นพิษและส่งผลต่อการตายของเซลล์ท่อน้ำดีปกติได้ จากการทดลองพบว่าสารสกัด BBR-M-10 ยับยั้งการสารอนุมูลอิสระผ่านการกระตุ้นการแสดงออกของยีนที่เกี่ยวข้องกับเอนไซม์ต่อต้านอนุมูลอิสระ และลดการตายของเซลล์ท่อน้ำดีปกติผ่านการควบคุมการแสดงออกของโปรตีนแคสเปส-3 และเพิ่มการแสดงออกของโปรตีนเซอร์ไววิน นอกจากนี้ความเสียหายของสายพันธุกรรมยังได้ถูกตรวจสอบผ่านโปรตีนแกรมมา H2AX จากการทดลองพบว่าสารสกัด BBR-M-10 ลดการแสดงออกของโปรตีนแกรมมา H2AX ในเซลล์ท่อน้ำดีปกติที่ได้รับการกระตุ้นโดยสารไฮโดรเจนเปอร์ออกไซด์ การค้นพบนี้แสดงให้เห็นว่าแอนโทไซยานินจากรำข้าวเหนียวดำสามารถป้องกันการเกิดความเสียหายต่อสายพันธุกรรมที่เกิดจากสารไฮโดรเจนเปอร์ออกไซด์และส่งผลต่อการตายของเซลล์ท่อน้ำดีปกติผ่านการยับยั้งการสะสมสารอนุมูลอิระภายในเซลล์และการควบคุมการแสดงออกของยีนต่อต้านอนุมูลอิสระ จากผลการศึกษาแสดงให้เห็นว่าแอนโทไซยานินจากรำข้าวเหนียวดำมีน่าจะมีบทบาทในการป้องกันการเกิดมะเร็งท่อน้ำดี

นอกจากนี้ได้มีรายงานเกี่ยวกับกลไกการต่อต้านมะเร็งของแอนโทไซยานินไว้โดยกลไกต่าง ๆ ได้แก่ การลดการแพร่กระจายของเซลล์มะเร็ง การย้ายถิ่นและการบุกรุก และการเหนี่ยวนำให้เกิดการตายของเซลล์มะเร็งต่าง ๆ ในการศึกษาครั้งนี้ยังมีวัตถุประสงค์เพื่อศึกษาผลของสารสกัด BBR-M-10 ต่อการยับยั้งการพัฒนาของมะเร็งท่อน้ำดีในแง่ต่าง ๆ ผลการศึกษาของสารสกัด BBR-M-10 ต่อการยับยั้งการเจริญเติบโต พบสารสกัด BBR-M-10 ไม่มีผลกระทบต่อการเจริญเติบโตของเซลล์มะเร็งท่อน้ำดี เซลล์ท่อน้ำดีปกติ และเซลล์ไฟโบรบลาสปกติ แต่สารสกัด BBR-M-10 ยับยั้งการเคลื่อนที่และการบุกรุกของเซลล์มะเร็งท่อน้ำดีได้ จากนั้นกลไกของปรากฏการณ์ดังกล่าวถูกตรวจสอบและแสดงให้เห็นว่าสารสกัด BBR-M-10 ยับยั้งการเคลื่อนย้ายและการบุกรุกของเซลล์มะเร็งท่อน้ำดีผ่านการกระตุ้นการแสดงออกของของโปรตีนเอฟแอกติน และ คลอดิน-1 และลดการแสดงออกของโปรตีนไวเมนติน นอกจากนี้ยังพบว่ามีการถ่ายโอนสัญญาณผ่านทางตัวกลาง AKT ที่ลดลงในเซลล์มะเร็งท่อน้ำดีที่ถูกกระตุ้นด้วยสารสกัด BBR-M-10 จากการทบทวนวรรณกรรมพบว่าการเกิดกลไกไกลโคซิเลชันบนโปรตีนมีบทบาทสำคัญในการแพร่กระจายของมะเร็ง เพื่อตรวจสอบกลไกดังกล่าวเลคตินจำนวน 16 ชนิดถูกใช้เพื่อระบุความแตกต่างในการแสดงออกของไกลแคนบนผิวและภายในเซลล์มะเร็งท่อน้ำดีหลังการกระตุ้นด้วยสารสกัด BBR-M-10 การเปลี่ยนแปลงของเลคตินที่ลดลงหลังจากการกระตุ้นด้วย สารสกัด BBR-M-10 ของการแสดงออกของไกลแคนนั้นสังเกตได้อย่างชัดเจนในเลคตินชนิด Sambucus nigra lectin (SNA) ซึ่งเป็นเลคตินที่จดจำกรดเซียลิกที่เชื่อมโยงกับโครงสร้างกาแลคโตสในไกลแคน และยังสัมพันธ์กับการแสดงออกของยีนในการสร้างเอนไซม์ที่ใช้ในการสร้างกรดเซียลิกดังกล่าวด้วย ผลการศึกษานี้ชี้ให้เห็นว่าแอนโทไซยานินที่ได้มาจากรำข้าวเหนียวดำมีศักยภาพในการยับยั้งการแพร่และรุกรานของเซลล์มะเร็งท่อน้ำดี

โดยสรุปรำข้าวเหนียวดำถือเป็นแหล่งวัตถุดิบสำคัญของสารแอนโทไซยานินที่มีคุณประโยชน์ด้านสุขภาพ ในแง่มุมของมะเร็งนั้นสารสกัด BBR-M-10 มีศักยภาพเป็นสารป้องกันการเกิดมะเร็งผ่านการยับยั้งอนุมูลอิสระและยับยั้งการทำลายสารพันธุกรรม และสารสกัด BBR-M-10 มีผลยับยั้งการพัฒนาของมะเร็งท่อน้ำดี

สาขาวิชาเคมี
ปีการศึกษา 2567

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____

SASIKAMON KHOPHAI : CANCER PREVENTION AND ANTI-CANCER EFFECT OF ANTHOCYANINS IN CHOLANGIOCARCINOMA. THESIS ADVISOR : ASSOC. PROF. CHUTIMA TALABNIN, Ph.D. 83 PP.

Keyword: Anti-cancer/ Cancer prevention/ Cholangiocarcinoma

Anthocyanins are water soluble flavonoids responsible for the color of fruits and flowers such as grapes, blueberries, rice berry and black sticky rice. The roles of anthocyanins have been associated with anti-cancer and cancer prevention. Black rice bran is rich in anthocyanin pigments especially cyanidin 3-glucoside (C3G) which has high antioxidant and anti-cancer activities. However, the effect of anthocyanins on cholangiocarcinoma (CCA) prevention and progression has never been demonstrated yet. In the present study, black rice bran-derived anthocyanins, namely BBR-M-10 were extracted from the bran of the black pigmented rice (*Oryza sativa L.*). The TLC and HPLC results showed that the major anthocyanins in BBR-M-10 was C3G. First, to demonstrate the effect of BBR-M-10 on CCA prevention, H_2O_2-stimulated cholangiocytes (MMNK1) model was used to investigate the effect of BBR-M-10 on antioxidant activity and DNA damage. The results demonstrated that pretreatment with BBR-M-10 reduced the cytotoxic effect of H_2O_2-mediate MMNK-1 cell death and the reactive oxygen species (ROS) accumulation. The downregulation of cleavage caspase-3 and up-regulation of survivin were also detected in H_2O_2-treated MMNK-1 upon pretreatment with BBR-M-10. Moreover, the expressions of phase II antioxidant genes including *NRF-2* and *NQO-1* were increased upon pretreatment with BBR-M-10. Furthermore, the expression of DNA damage marker, gramma H2AX, was also determined. The immunofluorescent and western blot analysis showed that pretreatment with BBR-M-10 hindered the expression of gramma H2AX in H_2O_2-treated MMNK-1. Our findings show that BBR_M-10 can prevent H_2O_2-indeced DNA damage and cell death in cholangiocytes via suppressing intracellular accumulation of ROS and up-regulation of phase II antioxidant genes. Therefore, the utility of rice bran anthocyanins would be a potential health benefit for CCA prevention.

Next, the anti-cancer effect of anthocyanins has been documented by a variety of mechanisms: reducing cancer cell proliferation, migration and invasion, and induction of apoptosis. In the present study, we also aimed to demonstrate the inhibitory effect of BBR-M-10 on CCA progression. Cell viability test demonstrated that BBR-M-10 has no toxicity to CCA cell lines, cholangiocytes and normal fibroblast cell lines. Wound healing and Transwell assay showed that BBR-M-10 inhibited CCA cell migration and invasion ($P<0.05$). BBR-M-10 attenuated CCA cell migration and invasion evidenced by up-regulation of epithelial markers (F-actin and claudin-1), down-regulation of mesenchymal markers (vimentin); and a decrease in the activation/phosphorylation of AKT in BBR-M-10-treated CCA cell lines. Due to the glycosylation of several proteins, it plays an important role in the epithelial mesenchymal transition (EMT). A panel of 16 lectins was used to identify differences in glycan expression of CCA cell lines after BBR-M-10 treatment. The change of glycan expression in BBR-M10 treated CCA cells was obviously observed by Sambucus nigra lectin (SNA) which is a lectin that recognizes alpha2,6 sialic acid linked to N-acetyl galactosamine or galactose of the glycans structure (SNA binding-glycans). Low expression of SNA binding-glycans was associated with the reduction of alpha2,6 sialyltransferase gene expression (ST6GAL1) in BBR-M-10 treated CCA cell lines. Our findings suggest that BBR-M-10 has potential to be used as an anti-metastatic agent against CCA

In conclusion, black rice brans are a valuable source of anthocyanins that exhibit beneficial health effects in CCA evidenced by 1) preventive effect of BBR-M-10 in H_2O_2-indeced ROS generation and DNA damage and 2) inhibitory effect of BBR-M-10 on CCA progression.

School of Chemistry

Academic Year 2024

Student's Signature _____

Advisor's Signature _____

ACKNOWLEDGEMENT

First, I would like to express my sincere thanks to my advisor, Associate Professor Dr. Chutima Talabnin, for her valuable suggestion, constant encouragement throughout the course and giving me the valuable opportunity to join the research group. It is my greatest chance to be one of her students. The good advice and support of my co-advisors, Professor Dr. James R. Ketudat Cairns, has been invaluable on an academic level, for which I am grateful. I would like to express my deep gratitude to Professor Dr. MD. Okada Seiji for his support, inspiration and valuable suggestions since I was in Japan.

Sincere thanks and appreciation are also addressed to for serving as my external examiners and providing valuable suggestions since proposal examination. Gratitude is expressed to a scholarship from the Thailand Research Fund international research network grant IRN62W0004, Thailand.

I wish to express my sincere thankfulness to CT and KT lab members and all friends at the school of chemistry, institute of science for their friendship and kindness throughout my study. Special thanks to Dr. Kanokwan Lowhalidanon who kindly assisted and valuable suggestions. Sincere appreciation is to all teaching staff in the institute of science. I owe my deepest thankfulness to my parents, lovely sister and my partner for their endless love, cheerfulness and devotion throughout my life. Finally, I would like to thank you for everything that forced me to be here. I am gratitude to know people who stand by my side when times get hard and take me out of trouble. I am honored to surround with people who give me love, motivation and encouragement.

Sasikamon Khophai

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
BBR-M-10	black rice bran-derived anthocyanin
C3G	cyanidin-3-glucoside
CCA	cholangiocarcinoma
CyCl	cyanidin chloride
DPPH	2,2'-Diphenyl-1-picrylhydrazyl radical
γH2AX	phospho-histone H2A.X
H_2O_2	hydrogen peroxide
NQO1	NAD(P)H quinone dehydrogenase 1
Nrf2	nuclear factor erythroid 2
OV	Opisthorchis viverrini
P3G	peonidin-3-glucoside
PARP1	poly (ADP-ribose) polymerases
PeCl	peonidin chloride
ROS	reactive oxygen species
µg	Microgram
mg	milligram
µL	Microliter
mL	Milliliter

CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Pigmented rice with black, purple and red pericarp color is recognized as a potential source of antioxidant compounds such as anthocyanin, (Ghasemzadeh, Karbalaii, Jaafar, and Rahmat, 2018; S. YamuangmornProm, 2021). Generally, a rice milling system is used to remove the husk by the process of grinding brown rice, then to remove the bran layers and endosperm. Pigmented rice bran contains high amounts of nutrients and a large number of biologically active phytochemicals, including tocopherols, tocotrienols, oryzanols, vitamin B complex, phenolic compounds, as well as anthocyanins (Pitija, Muntana, Sriseadka, Vanavichit, and Mahatheeranont, 2013). Up to now, phenolic compounds and anthocyanins are still gaining great attention in the prevention and treatment of many human cancers including leukemia, breast, lung, liver, cervix, stomach and colorectal cancer (M.-H. Chen, Choi, Kozukue, Kim, and Friedman, 2012) (Henderson et al., 2012) (Hudson, Dinh, Kokubun, Simmonds, and Gescher, 2000). According to the above reports, the consumption of phenolic compounds and anthocyanins from pigmented rice has been considered to provide benefits to human health (S. YamuangmornProm, 2021).

Phenolic compounds are known as secondary metabolites of plants consisting of one or more aromatic rings with one or more hydroxyl groups. These compounds are involved in defense against ultraviolet radiation or aggression by pathogens (Vasantha Rupasinghe, Nair, and Robinson, 2014). Polyphenols are very important among phenolic compounds due to their high amount in nature and their role in the prevention of different diseases such as cardiovascular, neurodegenerative diseases and cancer (Scalbert, Manach, Morand, Remesy, and Jimenez, 2005). Based on the differences in chemical structure, polyphenols can be divided into two classes:

flavonoids and non-flavonoids (Rentzsch, Wilkens, and Winterhalter, 2009) (Terrier, Poncet-Legrand, and Cheynier, 2009).

Flavonoids are a group of natural substances with variable phenolic structures. Flavonoids are found in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine. Flavonoids are considered as a choice in a variety of pharmaceutical, medicinal and cosmetic applications. These natural products are well known for their health-promoting effects, such as anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties, as well as their capacity to modulate cellular enzyme function. Ferrandiz and co-workers demonstrated that flavonoids inhibit inflammation in arachidonic acid metabolism through the enzyme pathway regulation (Panche, Diwan, and Chandra, 2016) (AlcarazFerrándiz, 1987). In addition, flavonoids inhibit the oncogenic signaling pathway and induce transcription and translation of tumor suppressor genes in several cancer cell lines (Sambantham et al., 2013) (H. Zhang et al., 2016) (Shirakami, Sakai, Kochi, Seishima, and Shimizu, 2016) (Bao et al., 2016). Flavonoids can be classified into different subgroups depending on their structures which are: flavones, flavonols, flavanones, flavanonols, flavanols or catechins, chalcones and anthocyanins (Terrier et al., 2009).

Anthocyanins are water-soluble pigments which belong to the phenolic group with red to blue color (MonagasBartolomé, 2009). They occur mainly as glycoside form of anthocyanidins such as cyanidin, delphinidin, peonidin, pelargonidin, petunidin and malvidin (He et al., 2010) (Aza-González, Nuñez-Palenius, and Ochoa-Alejo, 2012). The most common anthocyanin in foods is cyanidin-3-glucoside. The important role of this compound is as an antioxidant (reducing agent) (Hock Eng Khoo, Azrina Azlan, Sou Teng Tang, and See Meng Lim, 2017). They promote cardiovascular health and also have anti-cancer activity and anti-inflammatory properties (C. Hui et al., 2010) (Kruger, Davies, Myburgh, and Lecour, 2014) (Wallace, 2004). The anticarcinogenic activity of anthocyanins was revealed in both in vitro and in vivo studies. Several study have shown radical scavenging activity, stimulation of phase II detoxifying enzymes, reduced cell proliferation, inflammation, angiogenesis and invasiveness, and induction of apoptosis (Vasantha Rupasinghe et al., 2014). Anthocyanins occur generally in fruits, such as grapes (He et al., 2010) and berries (SlimestadSolheim, 2002). Moreover, there

are several studies that pigmented grains and rices as a promising source of anthocyanin (FrancavillaJoye, 2020), (Supapohn YamuangmornProm-U-Thai, 2021).

Cholangiocarcinoma (CCA) is an aggressive cancer arising from the bile duct epithelial cells, which has the highest incidence in the world in northeastern, Thailand (SripaPairojkul, 2008). Liver fluke (*Opisthorchis viverrini*, Ov) infection is defined as a major risk factor for CCA development in this area. The molecular mechanisms by which the liver fluke induce chronic inflammation related to CCA development have been described (Yongvanit, Pinlaor, and Bartsch, 2012). Chronic inflammation induced by Ov disturbs the homeostasis of oxidants/antioxidants and DNA repair enzymes by increased levels of proinflammatory cytokines and nuclear factor κB (NF-κB) that control COX-2 and inducible nitric oxide activities. These oxidative and nitrative stresses can induce production of reactive oxygen and nitrogen species in inflamed target cells, resulting in high levels of oxidized DNA and DNA bases modified by lipid peroxidation products in animal and human tissues. The above information indicated that Ov infection causing chronic inflammation may lead to failure of the immune response, which contributes to the progression of cholangiocarcinogenesis. The therapies for its malignant tumors are based on chemotherapeutic drugs which provide the cytotoxic effects and cause death of tumor cells by direct damage to DNA or by inhibition of cell division. Unfortunately, these drugs are mostly unspecific, therefore, their administration often affects normal tissue (Astolfi et al., 2013). Among the other adverse effects, constitutional symptoms included hyposthenia and asthenia, fever, weight loss and appetite loss. Cisplatin myelosuppression caused hematological toxic effects, such as anemia, leukopenia, neutropenia and thrombocytopenia. The dermatological disorders included alopecia, itchiness, skin rash, edema, arm phlebitis and mucositis. Neurotoxicity mainly affects the peripheral system, in comparison to the central nervous system. The most common symptoms were paraesthesia, followed by cephalalgia, speech impairments, aphasia, agnosia, lipothymia (near-fainting syndrome), convulsions, panic and transient ischemic attacks (mini-strokes), visual failure, sensory-motor deficits and motor coordination impairments (Astolfi et al., 2013).

Taking the above information together, we hypothesized that the rice anthocyanins may exert CCA prevention and anti-cancer effects in CCA. Additionally, the anthocyanin extracts from rice bran of pigmented rice would provide an excellent resource for the large-scale production of anthocyanin for supporting various beneficial food applications. To prove this hypothesis, we aim to investigate 1) the effect of rice anthocyanin treatment on H_2O_2-induced MMNK-1 cell death and 2) effect of rice anthocyanin on CCA cell growth and CCA progression.

1.2 Objectives of the study

1.2.1 To investigate the effect of black rice bran-derived anthocyanins on H_2O_2-induced DNA damage in cholangiocytes and their relevant molecular mechanisms.

1.2.2 To investigate the inhibitory effect of black rice bran-derived anthocyanins on CCA cell growth and progression and their relevant molecular mechanisms.

1.3 Research hypothesis

1.3.1 Black rice bran-derived anthocyanins can prevent H_2O_2-induced DNA damage.

1.3.2 Black rice bran-derived anthocyanins can suppress malignant phenotypes in CCA cell lines.

CHAPTER II

LITERATURE REVIEWS

2.1 Phenolic compounds

There are many phenolic compounds that can be found in both non pigmented rice and pigmented rice. Several studies showed that high of total anthocyanin content (TAC) and antioxidant activity are found in all black rice components including endosperm, and rice bran of in black rice (*Oryza sativa* L.) (Ilmi, Pratiwi, and Purwestri, 2018). Moreover, C3G was found to be predominant compounds in the purple rice bran (Das, Goud, and Das, 2017). Pigmented rice bran is found to contain high amounts of nutrients and a large number of biologically active phytochemicals, such as those in groups of anthocyanin (Pitija et al., 2013). Chen et al. (M.-H. Chen et al., 2012) have demonstrated that red bran extracts had antitumor activity against leukemia, cervical cancer and freckle cancer.

2.1.1 Phenolic compounds

Phenolic compounds are a group of secondary metabolites of plant metabolism that contribute little to the physiological functions of the plant and are characterized by the presence of more than one phenol unit in molecule (Marzag, Warnault, Bougrin, Martinet, and Benhida, 2014). Phenolic compounds can be classified into several classes that depend on the number of phenol rings that they contain and the structural elements that link these rings to one another as shown in Figure 2.1 (StevensonHurst, 2007). They are divided into three major groups: (1) flavonoids, allied phenolic, and polyphenolic compounds; (2) terpenoids, and (3) nitrogen-containing alkaloids and sulfur-containing compounds. Phenolic compounds are found in berries, grapes/wine, tea, chocolate/cocoa, coffee, soybeans, and other fruits and vegetables (Manach, Scalbert, Morand, Rémésy, and Jiménez, 2004). Nowadays, the beneficial health effects of these phenolic compounds are an interesting point, because these compounds have the role in antiaging, antioxidant, anti-inflammatory, and anti-

activities. There is increasing evidence has provided that dietary phenolic compounds may express their chemo preventive effects by modulating various mechanisms of the epigenetics in humans. Therefore, phenolic compounds could be a promising agent for treating cancers and other diseases in humans.

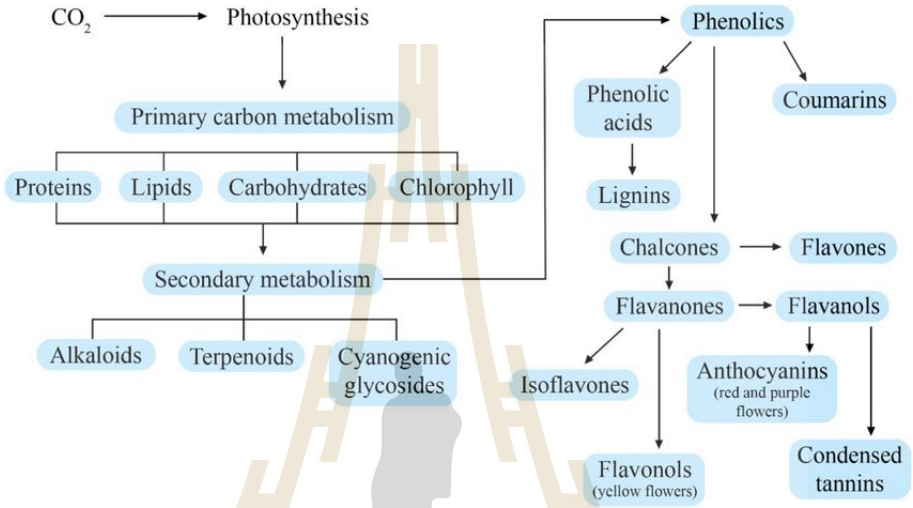


Figure 2.1 Primary and secondary metabolism linkage in plants. (Vuolo, Lima, and Junior, 2019)

Phenolic compounds are widely produced in plant tissues, especially contributing color, flavor, and astringency to fruits. Phenolic compounds are considered as secondary metabolites of plant metabolism that contribute to the physiological or ecological functions of the plant (Swanson, 2003). Phenolic compounds are by-products of the metabolism of the aromatic amino acid phenylalanine. A major group of water-soluble phenolic compounds, the anthocyanins, contribute colors to fruits. Phenolic compounds consists of an aromatic ring, containing one or more hydroxyl substituents (Tsao, 2010). Phenolic compounds have been studied for the ability to neutralize oxidative stress of chronic oxidative stress, which is linked to several metabolism disorders and a range of pathologies, such as obesity, diabetes, and cancers (Furukawa et al., 2004) (Reuter, Gupta, Chaturvedi, and Aggarwal, 2010). When natural antioxidant system defenses are destroyed by an excessive reactive oxygen species (ROS), oxidative stress occurs, leading to damage to cellular and extracellular macromolecules (BurtonJauniaux, 2011). Antioxidant

properties of phenolic compounds can express their reducing properties as hydrogen- or electron-donating agents, which can act as free-radical scavengers (antioxidants) as well as the ability of metal chelation, which can suppress metal-catalyzed free radical formation. The structure of phenolic compounds is related to their radical-scavenging and metal-chelating activity. The number of hydroxyl groups and their position in relation to the carboxyl functional group influences the antioxidant activity of phenolic compounds (Balasundram, Sundram, and Samman, 2006).

Flavonoids are the largest group of phenolic compounds. They comprise several thousand compounds which are found in plants. Flavonoid structures are basically constituted of two aromatic rings joined by a three-carbon bridge, frequently in the form of a heterocyclic ring as shown in Figure 2.2. Their substitution patterns in the rings, which can be oxygenation, alkylation, glycosylation, sulfation, and acylation, lead to the major flavonoid classes, such as flavonols, flavones, flavanones, flavanols, isoflavones, flavanonols, and anthocyanidins. (Tungmunnithum, Thongboonyou, Pholboon, and Yangsabai, 2018) (BirtJeffery, 2013).

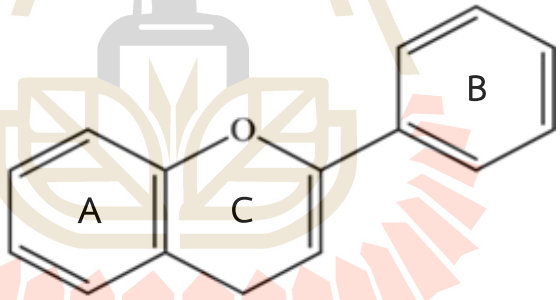


Figure 2.2 Structure of a flavonoid molecule. (Vuolo et al., 2019)

2.1.2 Anthocyanins

Anthocyanins are the pigments that impart the attractive red, blue, purple, violet, and intermediate red-purple fruits, vegetables, and grains (Mazza, 2018) (Seeram, 2001) (WuPrior, 2005). Anthocyanins occur naturally in fruits and vegetables as glycosides. The de-glycosylated form of anthocyanins are known as anthocyanidins (Seeram, Bourquin, and Nair, 2001). Anthocyanins are known in several types varying in the basic anthocyanidin skeleton. The most common anthocyanidins are cyanidin, delphinidin, pelargonidin, malvidin, petunidin, and peonidin (SeeramNair, 2002;

Seeram, Zhang, and Nair, 2003). The most common glycoside sugars encountered on anthocyanidins are glucose, galactose, rhamnose, and arabinose usually as 3-glycosides or as 3,5-diglycosides. However, rutinosides (6-O-L-rhamnosyl-D-glucosides), sophorosides (2-O-D-glucosyl-D-glucosides), and sambubiosides (2-O-D-xylosyl-D-glucosides) are also common, as are some 3,7-diglycosides and 3-triosides (Clifford, 2000).

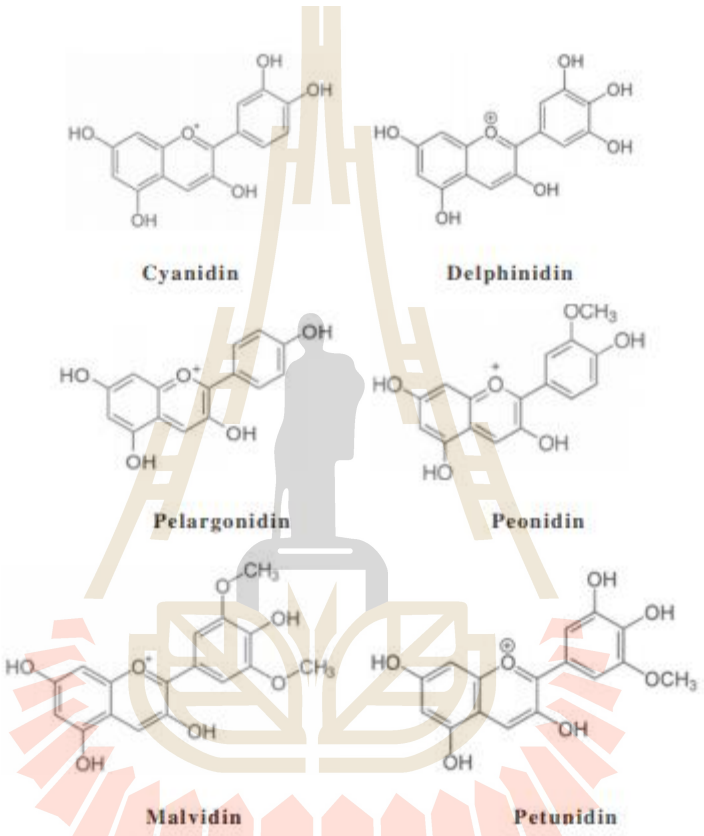


Figure 2.3 Major anthocyanidins in plants.

Anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin) (Figure 2.3) are found in fruits, vegetables and vegetable products, nuts and seeds, beverages, legumes and legume products, and rice (Faggio et al., 2017).

Several studies show the activity of anthocyanin as both anti- cancer and cancer prevention. Amararathna M. et al. (Amararathna, Hoskin, and Rupasinghe, 2020) showed that anthocyanin-rich extracts are able to reduce carcinogen-induced DNA damage in lung epithelial cells. In addition, the study of Chen L. and team found that the antioxidant effect of cyanidin 3-O-glucoside gave remarkable protection against the

cell damage or oxidative damage in HepG2 cells through Keap1/Nrf2-dependent signaling pathway. The study of C. Hui, (Chang Hui et al., 2010) investigated the anticancer effects of an anthocyanin-rich extract from black rice (AEBR) on breast cancer cells in vitro and in vivo. AEBR can reduce the cell proliferation of breast cancer cell lines and induced apoptosis via the intrinsic pathway in vitro by activating the caspase cascade, depolarizing mitochondrial membrane potential, and releasing cytochrome C. Therefore, anthocyanin extracts could be a potential dietary supplement for suppression or prevention of carcer.

2.1.3 Anthocyanins and DNA repairing system

The DNA repair system is essential for maintaining genomic stability by correcting various forms of DNA damage. The related pathways include base excision repair (BER): fixes small, non-helix-distorting base lesions. Nucleotide excision repair (NER): removes bulky DNA adducts and UV-induced damage. Mismatch repair (MMR): corrects errors that occur during DNA replication. Homologous recombination (HR): Repairs double-strand breaks using a sister chromatid as a template. Non-homologous end joining (NHEJ): Joins broken DNA ends directly, often in response to double-strand breaks. These mechanisms are crucial for preventing mutations that can lead to cancer and other genetic disorders, highlighting the importance of DNA repair in cellular health and longevity (ChatterjeeWalker, 2017).

Anthocyanins have been shown to play a protective role in DNA repair mechanisms. They possess antioxidant properties that help reduce oxidative stress, thereby minimizing DNA damage (Tena, Martín, and Asuero, 2020). Some studies suggest that anthocyanins can enhance the expression of genes involved in DNA repair processes, such as Gadd45 and MDM2 (Liu et al., 2013). By promoting cellular repair mechanisms and reducing mutation rates, anthocyanins may contribute to cancer prevention and overall genomic stability, making them a valuable component of a healthy diet.

2.2 Cancer

Cancer is a group of diseases that occur by dividing abnormal cells without growth control that can invade nearby or other parts of the body through the lymph node systems and blood stream. Cancer can develop through the process called carcinogenesis by accumulation of genetic alteration in cells and the process of promotion or progression for tumors (KinzlerVogelstein, 1996). The characteristics of cancer can be modeled in several ways which are called the "hallmarks" of cancer (HanahanWeinberg, 2000). Cancer development requires the acquisition of six fundamental properties: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (KinzlerVogelstein, 1996). There are several main types of cancer. Sarcoma is a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is a cancer that begins in blood-forming tissue, such as the bone marrow, and causes too many abnormal blood cells to be made. Lymphoma and multiple myeloma are cancers that begin in the cells of the immune system. Carcinoma is a cancer that begins in the skin or in tissues that line or cover internal organs (Mitra, Ganguli, and Chakrabarti, 2018).

2.2.1 Cholangiocarcinoma (CCA)

Cholangiocacinoma (CCA) is a malignant tumor that originates from the epithelium of the bile duct which can be classified into 2 types including intrahepatic and extrahepatic CCA that depend on their anatomic location. Intrahepatic CCA arises from the bile duct epithelial cell inside the liver whereas extrahepatic CCA arises from bile duct epithelium outside the liver, except the ampulla of vater and gallbladder. Intrahepatic and extrahepatic CCA account for 5-10% and 80-90% respectively (Khan, Thomas, Davidson, and Taylor-Robinson, 2005). Extrahepatic CCA can be classified into distal and perihilar CCA. Moreover, extrahepatic CCA can be classified according to Bismuth classification into types I to IV (BlechaczGores, 2008) (Figure 2.4). In addition, CCA can be classified according to its macroscopic growth pattern. The macroscopic classification of intrahepatic and extrahepatic CCA includes mass-forming, periductal-infiltrating, intraductal growth, as show in figure 2.5 (Sripa et al., 2007). Intrahepatic CCA

also can be classified as well differentiated, moderately differentiated and poorly differentiated (BlechaczGores, 2008; Sirica, 2008).

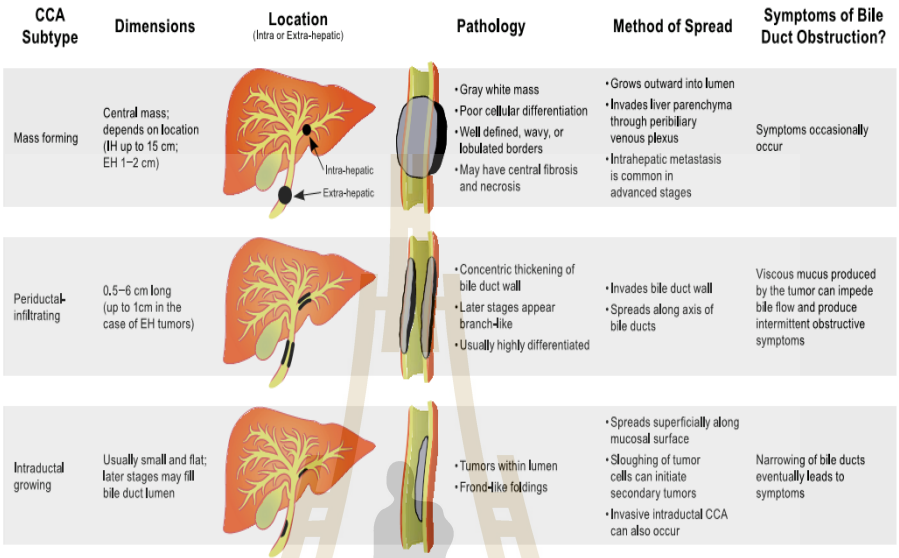


Figure 2.4 Classification of CCA. Intrahepatic and extrahepatic CCA, classified into types I to IV.

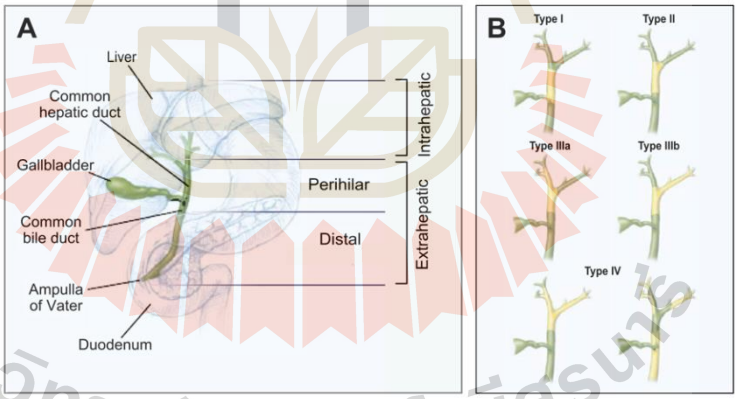


Figure 2.5 Classification of CCA. Intrahepatic and extrahepatic CCA, classified to mass forming, periductal-infiltrating and intraductal growth (Sripa et al., 2007).

2.2.2 Incidence, etiology and epidemiology

CCA is a carcinoma accounting for 10 to 15% of primary liver cancer after hepatocellular carcinoma (HCC) with age-standardized incidence rates (ASRs) between 0.3 and less than 1.5 per 100,000 in western countries (BlechaczGores, 2008;

UstundagBayraktar, 2008). Even though the incidence of CCA is rare worldwide, the incidence was high in East Asia. Moreover, CCA is commonly seen in Thailand, especially in the northeastern area (Parkin, Ohshima, Srivatanakul, and Vatanasapt, 1993). The highest incidence has been reported in Khon Kaen with ASRs at 94.8 and 39.4 per 100,000 in the year 1998–91 in males and females, respectively (SripaPairojkul, 2008). Nowadays, the new high incidence rate in the other provinces in the northeastern area is also reported. Therefore, CCA is recognized as a significant health problem in Thailand, especially in the northeastern area.

Even though the etiology of CCA remains obscure, several risk factors have been identified. The risk factors of CCA include primary sclerosing cholangitis, chronic intraductal gall stones, Caroli's disease, choledochal cysts or liver fluke infection etc (Khan et al., 2002). In Asia, the major risk factor of CCA is linked with liver fluke infection, *Opisthochis viverini* (Ov) and *Clonorchis sinensis*. Ov infection is a major risk factor of CCA in Thailand, Cambodia and Lao PDR, whereas *C. sinensis* is found in China, Korea, Vietnam, Taiwan and previously in Japan (Sithithaworn, Yongvanit, Duenngai, Kiatsopit, and Pairojkul, 2014). The association between Ov infection and CCA has been reported in both experimental and epidemiological studies. Ov infection is acquired by eating raw or undercooked cyprinoid fish that contaminate with metacercaria of Ov (Sithithaworn et al., 2014). After eating, the metacercaria excysts into the gastrointestinal system, duodenum and migrates to intrahepatic bile duct. The repeated or prolong infection causes chronic inflammation resulting in oxidative and nitrative DNA damages along with bile duct proliferation and dysplasia (Pinlaor et al., 2003). Finally, CCA could develop.

2.2.3 Carcinogenesis of Ov-associated cholangiocarcinoma

Chronic inflammation is one risk factor for many human cancers, including CCA. One of the mechanistic links between cancer and inflammation is reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are generated via immune cells that infiltrate to inflammatory site. The chronic inflammation induced by Ov infection is involved with the generating of nitric oxide (NO) and other reactive oxygen and reactive nitrogen species (ROS and RNS). These Oxidative/nitrative stress can cause

damage to many cellular biomolecules, including proteins, lipids and DNA.(Ohshima, Tatemichi, and Sawa, 2003). Production of endogenous NO is catalyzed by inducible nitric oxide synthase (iNOS), the enzyme mainly produced by inflammatory cells, especially macrophages that are induced by inflammatory cytokines (MayerHemmens, 1997). There is evidence demonstrating that, the activation of iNOS since the response to inflammatory cytokines causes over-production of NO, resulting in DNA damage and inactivate the enzymes that are involved in the DNA repair process (Jaiswal, LaRusso, Burgart, and Gores, 2000). Furthermore, Pinlaor demonstrated that, Ov-infection can cause oxidative and nitrosative DNA damage in hamster, this result was supported by the presence of 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-nitroguanine, which are biomarkers for the damage of DNA in the liver of hamsters infected with Ov. The nitrative and oxidative DNA damage and the expression of iNOS which is induced via the infection of Ov may participate in CCA carcinogenesis (Pinlaor et al., 2004). Furthermore, the high of 8-oxodG level was seen in liver tissues of CCA patients and the level of 8-oxodG was seen in urine and leukocytes of Ov-infected patients higher than healthy subjects (Thanan et al., 2008). Normally, genotoxic events caused by DNA damage can lead to the repair mechanism, which is DNA mismatched repair or, if the damage is beyond repair or cell death through apoptosis, then these mutated cells are permitted to survive and can transform to malignant cells. All these events are described in Figure 2.6.

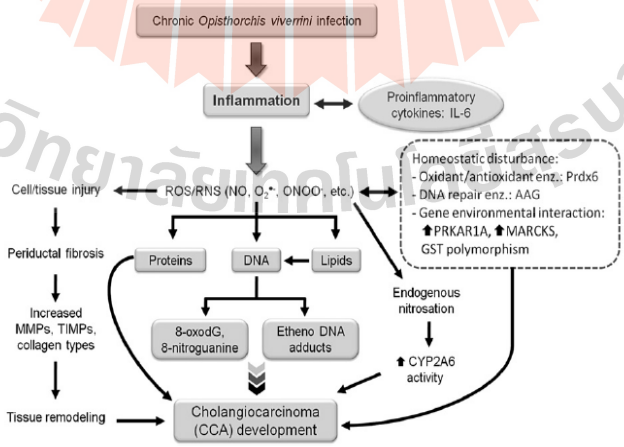


Figure 2.6 A possible mechanism of liver fluke-induced inflammation in the association to cholangiocarcinogenesis (Yongvanit et al., 2012).

2.2.4 Cholangiocarcinoma treatment

There are different types of standard treatments for patients with bile duct cancer, such as surgery, radiation therapy and chemotherapy (Ilyas, Khan, Hallemeier, Kelley, and Gores, 2018). Surgical resection remains the main potential therapy for CCA (Cillo et al., 2019). There are 2 general types of surgery for bile duct cancer, including potentially curative surgery (resectable and unresectable) (Ilyas et al., 2018) and palliative surgery which is done to relieve symptoms such as blockage of the bile ducts (S. Y. Park et al., 2011). For patients with advanced-stage or unresectable surgery, radiation therapy and systemic chemotherapeutics are the primary treatment options of cholangiocarcinoma (Ilyas et al., 2018). Radiation therapy or adjuvant therapy uses high-energy rays or particles to destroy cancer cells. Chemotherapy (chemo) is treatment with cancer-killing drugs that are usually given into a vein (IV) or taken by mouth. The drugs used most often to treat bile duct cancer include 5-fluorouracil (5-FU), Gemcitabine (Gemzar®), Cisplatin (Platinol®), Capecitabine (Xeloda®) and Oxaliplatin (Eloxatin®) (Valle, 2017). In some cases, 2 or more drugs are combined to make them more effective. For example, combining gemcitabine and cisplatin may help people live longer than just gemcitabine alone (von der Maase, 2002). Gemcitabine and cisplatin in combination show a synergistically effect and is associated with longer overall survival and greater response in biliary cancer (J. O. Park et al., 2015). There are several clinical studies that found evidence of the potential effects of gemcitabine-cisplatin on survival and tumor response in patients with biliary tract cancer. The evidence suggest that gemcitabine-cisplatin extends overall survival approximately 5 to 12 months compared with mono therapeutic drug (2.5 to 4.5 months) (Astolfi et al., 2013) (Valle et al., 2010). However, the mechanism of these drugs is by entering the bloodstream going to all areas of the body and attacking cells that are dividing quickly, as well as other cells in the body, such as bone marrow, intestines, and hair follicles, as well as the targets of chemotherapy. These cells can be affected by chemo, which can lead to side effects, including hair loss, loss of appetite, nausea and vomiting, diarrhea, nerve damage (neuropathy), etc. (Binder et al., 2007). Nowadays, researchers try to find new types of treatment, which are being tested in clinical trials, including immunotherapy and targeted therapy, as well as

treatment with natural phenolic compounds to reduce the side effects of chemotherapeutic drugs and provide the potential improvement of cancer killing.

CHAPTER III

RESERCH METHODOLOGY

3.1 Materials

3.1.1 Human cell lines and cell culture

CCA cell lines established from CCA patients including KKU-213A, and KKU-055 and an immortalized cholangiocyte cell line, MMNK-1, were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan. KKU-213A and KKU-055 were characteristic the histological type as adenosquamous and moderately differentiated adenosquamous respectively (Dokduang et al., 2010). The cell lines were cultured in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with inactivated 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, $NaHCO_3$ in a humidified atmosphere containing 5% CO_2. Human fibroblast cell line (IMR-90) was obtained from the American Type Culture Collection and cultured in EMEM medium supplemented with inactivated 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin. All cell lines were incubated at 37 °C in a humidified incubator containing 5% CO_2. Lack of mycoplasma contamination was verified in all cell lines by PCR assay.

3.1.2 Chemical and Reagents

Cyanidn chloride (cat. no. 80022), peonidin chloride (cat. no. 80085), Cyanidin-3-glucoside (cat.no. 89616) and peonidin-3-glucoside (cat. no. 89754) were purchased from PhytoLab GmbH & Co. (Vestenbergsgreuth, Germany). Sulforhodamine B (SRB; cat. no. S1402), TCA (cat. no. T0699) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; cat. no. D6883) were purchased from Sigma-Aldrich (Merck, KGaA). TRIzol reagent (cat. no. 15596026) and BCA Protein Assay Kit (cat. no. 23225) were obtained from Thermo Fisher Scientific, Inc. SensiFAST cDNA Synthesis Kit (cat. no. BIO-65053) was purchased from Bioline, Meridian. LightCycler® 480 SYBR Green I Master mix (cat.

no. 04707516001) was purchased from Roche Molecular Systems, Inc. Cell culture reagents including DMEM (cat.no. 12100-046), penicillin-streptomycin (cat. no. 15140-122), and fetal bovine serum (cat. no. 10270-098) were purchased from Gibco; Thermo Fisher Scientific, Inc. Primary antibodies against caspase-3 (#9662), cleaved caspase-3 (#9661), survivin (#2808) and gamma H2AX (#80312) and Anti-mouse IgG-Alexa^{TM} 647 conjugate (#4410S) were purchased from Cell Signaling Technology. Anti-PARP1 (cat no. 13371-1) was obtained from Proteintech. Primary antibodies against, pAKT (cat. no. 4060S) and total AKT (cat. no. 4685S) were purchased from Cell Signaling Technology. Anti claudin-1 (cat. no. sc-81796), vimentin (cat. no. sc-6260), slug (cat. no. sc-166476) antibodies were purchased from Santa Cruz Biotechnology, Inc. HRP-conjugated secondary antibodies for anti-goat (cat. no. A15999) was purchased from Invitrogen; Thermo Fisher Scientific, Inc. Anti β-actin (cat. no. sc-47778) was purchased from Santa Cruz Biotechnology, Inc. HRP-conjugated secondary antibodies (cat. no. NXA931 and NA934), ECL prime blocking reagent (RPN415V) and ECL prime western blot detection (RPN2236) were obtained from Cytiva. Hoechst 33258 (cat. no. H3569) was obtained from Thermo Fisher Scientific, Inc. Sixteen biotinylated lectins and ABC-peroxidase solution (cat. no. PK-4000) were purchased from Vector Laboratories, Inc. SignalStain® DAB substrate kit (cat. No. 8059) was obtained from Cell Signaling Technology, Inc. Alexa fluor 488 conjugated streptavidin (1:400) was purchased from Thermo Fisher Scientific, Inc.

3.2 Methods

3.2.1 Black rice bran-derived anthocyanins extraction

Rice anthocyanin extraction has been prepared by Chokchaisiri S. and coworkers. The powdered bran of black sticky rice (*Oryza sativa* L.) was obtained from Phimai. The powdered bran rice (5 kg) was extracted successively with n-hexane followed by 0.1% HCl in MeOH at room temperature. The MeOH extracts were filtered and concentrated to dryness under reduced pressure. The MeOH extract (385 g) was subjected to column chromatography (CC) using a gradient solvent system of hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH to give 8 fractions (BBR-M1– BBR-M8).

Then, BBR-M7 was further purified in CC of sephadex LH20 by using MeOH to give BBR-M-10 fractions. The BBR-M-10 was concentrated to dryness under reduced pressure and further investigated the biological effect in cholangiocyte and CCA cell lines.

3.2.2 Thin-layer chromatography (TLC)

The commercial standard and product mixture (10 μl of each) were loaded on a pre-coated silica gel (5 × 5 cm) and developed with a solvent system consisting of n-butanol/acetic acid/water, 3:1:1 (v/v). The developed plates were stained by painted in 10% sulfuric acid reagent and then heated by dryer.

3.2.3 High-performance liquid chromatography (HPLC)

The HPLC system used was an Agilent technologies 1260 Infinity (Spectra system thermo Finnigan, San Jose, CA, USA). Compound separation was carried out on a ZORBAX SB-C18 StableBond Analytical 4.6x250mm 3.5-Micron. Mobile phases consist of 0.1% trifluoroacetic acid (A) and acetonitrile (B) with the following gradients: isocratic 10% B for 5 min, and the linear increase to 15% B in the following 15 min, hold 15% B for 5 min, then increased from 15 to 18% B during next 5 min and from 18 to 35% B over 20 min. The wavelength for detection was 520 nm. The column temperature was set at 35°C. The flow rate was 1 mL/min, and the injection volume was 20 μL. All experiments were performed in triplicate detection (Hou, Qin, Zhang, Cui, and Ren, 2013).

3.2.4 Antioxidant activity by DPPH assay

The DPPH assay will be done by using Brand-Williams et al. (1995) method (Brand-Williams, 1995). The 0.1 mM DPPH solution will be prepared by dissolving 2 mg DPPH with 50 mL methanol and then stored at -20°C. The anthocyanin compounds and anthocyanin rice extracts (10 μl) as well as ascorbic acid (standard antioxidant) are soluble in methanol. The anthocyanin compounds and anthocyanin rice extracts and ascorbic acid will be added to react with 190 μl of the DPPH solution for 30 min in the dark. Then the absorbance will be taken at 517 nm. The standard curve should be linear between 25 and 800 μg of ascorbic acid. The scavenging effect (%) will be calculated by the formula as below.

DPPH scavenging effect (%) = $\frac{A_0 - A_1}{A_0} \times 100$

; A_0 as the absorbance of control and A_1 as the absorbance of sample.

3.2.5 ABTS radical scavenging activity

The solution of 7 mM ABTS with 2.45 mM potassium persulphate or ABTS free radical will be made and diluted with methanol before using. The anthocyanin compounds and anthocyanin rice extracts will be prepared at various concentrations in methanol and 20 µL of test solutions will be added to 180 µL of ABTS free radical solution. The absorbance will be measured at 750 nm after 20 min incubation. Ascorbic acid will be used as positive control. The ABTS free radical scavenging activity will be calculated using the formula: as below.

ABTS scavenging effect (%) = $\frac{A_0 - A_1}{A_0} \times 100$

; A_0 as the absorbance of control and A_1 as the absorbance of sample.

3.2.6 Anthocyanin and BBR-M-10 treatment in cholangiocyte and CCA cell lines

Sulforhodamine B (SRB) assay was used to determine cell proliferation in both cholangiocyte (MMNK-1) and CCA cell lines (KKU-213A, KKU-055). Cholangiocyte at the exponential phase were seeded 7×10^3 cells/well into 96 well-plates in triplicate in 96 well plates and incubated overnight. Then MMNK-1 cells were incubated in control medium or medium containing of C3G, P3G or BBR-M10, whereas combination condition between pretreated with C3G or BBR-M10 for 24 hr followed by H_2O_2 for 3 and 6 hr were performed at 37°C in a humidified 5% CO_2 atmosphere. Moreover, CCA cell lines including KKU-055, KKU-213A and normal fibroblast IMR-90 cells were seeded at 7×10^3 cells/well into 96 well-plates and incubated overnight. Then, the cells were treated with various concentrations of Cy, C3G, Pe, P3G and BBR-M-10 and incubated for 24 h. Then the fixation was performed by adding TCA in each well. The plates were then incubated at 4°C for overnight. TCA-treated cells were stained with 0.4% (w/v) SRB in 1% (v/v) acetic acid for 30 min, and subsequently washed five times with 1% (v/v) acetic acid to remove the unbound stain. The plates were left to dry, and the

protein-bound stain will be solubilized with 100 µl of 10 mM Tris base (pH 10.5) for 30 min on a shaker. Absorbance was measured at 564 nm using a microplate reader (Thermo Fisher scientific Oy, Finland). The half-maximum inhibitory concentration (IC_{50}) value was calculated with GraphPad Prism 8 (GraphPad Software, Inc.).

3.2.7 Measurement of intracellular ROS accumulation in MMNK-1 cells

MMNK-1 cells were seeded at 3.5×10^5 cells per well into 6 well plates and incubated at 37°C, 5% CO_2 overnight. After 24 h seeding, the cells were pretreated with C3G at 200 µM or BBR-M-10 at 200 or 400 µg/ml for 24 h and then treated with H_2O_2 at 200 µM for another 3 or 6 h at 37°C in a humidified 5% CO_2 atmosphere. Then, intracellular ROS accumulation was detected using the DCFH-DA fluorescence assay, as previously described (Talabnin, Talabnin, and Wongkham, 2020). In brief, the cells were stained with 20 mM DCF-DA reagent for 30 min. After washing with PBS, the stained cells were trypsinized and re-suspended in PBS. The fluorescent signal from DCF-DA was determined in a flow cytometer.

3.2.8 RNA extraction and qPCR in cholangiocyte and CCA cell lines

MMNK-1 cells were seeded at 3.5×10^5 cells per well into 6 well plates and incubated at 37°C, 5% CO_2 overnight. After 24 h seeding, the cells were pretreated with BBR-M-10 at 200 or 400 µg/mL for 24 h and then treated with H_2O_2 at 200 µM for another 1 h at 37°C in a humidified 5% CO_2 atmosphere. In addition, KKU-055 and KKU-213A cells were seeded at 3.5×10^5 cells/well into 6 well plate. After 24 h, the cells were treated with BBR-M-10 at 200 µg/ml for 24 h. According to the manufacturer's instructions, total RNA was extracted from the MMNK-1 cells with TRIzol reagent. The RNA quality and quantitation were measured by a NanoDrop 2000 spectrophotometer (Wilmington, USA) and agarose gel electrophoresis. A SensiFAST cDNA Synthesis Kit was used to synthesize cDNA from the RNA. Quantitative PCR using a LightCycler® 480 SYBR Green I Master Mix was performed to investigate the expression level. The Primers used for quantitative RT-PCR (Table 3.1) were described by Talabnin et al. (Talabnin et al., 2020). Gene amplification was performed by initial denaturation at 95°C for 5 min, followed by denaturation at 95°C for 10 s, annealing at 50-60°C for 10 s and extension at 72°C for 10 s, for 40 cycles. The β-actin gene was used as an internal control.

Relative mRNA levels of each gene were normalized with β-actin and calculated by the $2^{-\Delta\Delta}$CT method (LivakSchmittgen, 2001).

Table 3.1 Primer sequences used for quantitative PCR.

Assay	Primer	Sequence	Annealing temperature (°C)
Antioxidant genes	AKR1C1 F	5'- CAT GCC TGT CCT GGG ATT T -3'	60.0
	AKR1C1 R	5'- AGA ATC AAT ATG GCG GAA GC -3'	
	AKR1C3 F	5- CAT TGG GGT GTC AAA CTT CA -3'	60.0
	AKR1C3 R	5'- CCG GTT GAA ATA CGG ATG AC -3'	
	NRF2 F	5'- TAC TCC CAG GTT GCC CÁC A -3'	60.0
	NRF2 R	5'- CAT CTA CAA ACG GGA ATG TCT GC -3'	
	GCLC F	5'- ATG CCA TGG GAT TTG GAA T-3'	60.0
	GCLC R	5'- AGA TAT ACT GCA GGC TTG GAA TG -3'	
	GCLM F	5'- GAC AAA ACA CAG TTG GAA CAGC -3'	60.0
	GCLM R	5'- CAG TCA AAT CTG GTG GCA TC -3'	
Glycan-enzyme genes	ST3GAL1-F	5'- CAC ATA CTG GCA CTC ATC TAA -3'	50.0
	ST3GAL1-R	5'- TCT CCA GCA TAG GGT CCA -3'	
	ST3GAL3-F	5'- GTA TGA TCG GTT GGG CTT C-3'	60.0
	ST3GAL3-R	5'- CGC TCG TAC TGC TCA GG - 3'	
	ST3GAL4-F	5'- GTC AGC AAG TCC CGC T-3'	60.0
	ST3GAL4-R	5'- CTT GTT GAT GGC ATC TCC C -3'	
	ST3GAL6-F	5'- GGT ATC TTG TGG CCA TAT TCC - 3'	60.0
	ST3GAL6-R	5'- CTC CAT TAC CAA CCA CCA C- 3'	
	ST6GAL1-F	5'- CTT GTT TTC CTG CTC AGA -3'	50.0
	ST6GAL1-F	5'- GCA AAC AGA AGA AAG ACC A -3'	
Internal control	Actin-F	5'- GAT CAG CAA GCA GGA GTA TGA CG -3'	50-63
	Actin-R	5'- AAG GGT GTA ACG CAA CTA AGT CAT AG -3'	

F: Forward; R: Reverse.

3.2.9 Wound-healing assay

KKU-055 and KKU-213A cells were seeded at 3 × 10^5 cells/well into a 24 well plate. After 24 h growth to 90% cell confluence, a vertical wound was scratched through the cell monolayer by a sterile 200 µL plastic micropipette tip. Cell debris was removed and replaced with BBR-M-10 at 50, 100 and 200 µg/ml in serum free-DMEM medium. The migration of cells during the wound healing process was observed and digitally photographed under a microscope at 100× magnification at 0, 12, 18 and 24 h (magnification, ×100). The wound area was evaluated in Image J software (version 1.53a; National Institutes of Health) and calculated as below

$$\text{Migration area} = \frac{\text{Area of original wound - Area of wound during healing}}{\text{Area of original wound}}$$

3.2.10 Transwell migration and invasion assay

KKU-055 and KKU-213A cells at a density of 1 × 10^5 cells in 200 µL of FBS-free medium with or without BBR-M-10 at 50, 100, 200 µg/ml were seeded into transwell inserts for the migration assay or 200 µg/ml matrigel-coated transwell inserts for the invasion assay. Six hundred microliters of 10% FBS-containing medium was loaded into the lower chamber to create a chemotactic gradient. After 12 h, the transwell inserts were removed from the plate and the cells on the upper side of the membranes were removed by a cotton-tipped applicator. Migrated cancer cells on the bottom side of the membranes were fixed with 10% TCA overnight, stained with SRB for 30 min, then washed with 10% acetic acid, and allowed to dry. Images of migrated cells were taken under the microscope and counted (presented values are averages of 5 fields at 100× magnification).

3.2.11 Lectin cytochemistry

KKU-055 and KKU-213A cells were seeded at a density of 3 × 10^4 cells/well into a 24-well plate and incubated overnight. The cells were treated at 0 and 200 µg/ml of BBR-M-10 for 24 h. Then, the cells were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBST for 10 min. The

endogenous hydrogen peroxide-generating activity was blocked by 0.3% of hydrogen peroxide for 30 min. Then, nonspecific binding was blocked with 3% bovine serum albumin (BSA) for 30 min. A 1:500 dilution of biotinylated lectins (Table 1) was added and incubated at 4°C overnight, on a shaker. Then, ABC-Peroxidase Solution was used for 1 h at room temperature as the secondary antibody to determine the lectin signal. Then, a SignalStain® DAB substrate kit was used to visualize the signal under an inverted microscope at a magnification of 100×.

3.2.12 Lectin staining by flow cytometry.

KKU-055 and KKU-213A cells were seeded at a density of 3×10^4 cells/well into a 6-well plate. After 24 seeding, the cells were treated at 0 and 200 µg/ml of BBR-M-10 for 24 h. Then, the cell pellet was harvested and washed with 2% FBS in PBS. After that, the cell pellet was fixed by 4% paraformaldehyde in PBS for 15 min and nonspecific binding was blocked with 3% BSA for 30 min on ice. Then, the cell pellet was incubated for 1 h on ice with biotinylated SNA lectins at 1:500 dilution. Alexa fluor 488 conjugated streptavidin (1:400) was added for 1 h to determine the lectin signal. Then, cell pellets were resuspended in 2% FBS in PBS and analyzed by flow cytometry.

3.2.13 Immunofluorescent analysis in cholangiocyte and CCA cell lines

MMNK-1 cells were seeded at 4×10^4 cells per well into 8-well cell culture slides and incubated at 37°C, 5% CO_2 overnight. After 24 h seeding, the cells were pretreated with BBR-M-10 at 200 or 400 µg/mL for 24 h and then treated with H_2O_2 at 200 µM for another 3 h at 37°C in a humidified 5% CO_2 atmosphere. Moreover, KKU-055 and KKU-213A cells were seeded at a density of 2.5×10^4 cells/well into 8-well cell culture slides and incubated at 37°C, 5% CO_2 overnight. After 24 h seeding, the cells were treated with 0 and 200 µg/ml BBR-M-10 for 24 h. Then, the cells were washed by PBS and fixed with 4% paraformaldehyde. The fixed cells were permeabilized with 0.1%Triton-X100 in PBS for 10 min on ice. Blocking solution (2% bovine serum albumin in 0.1% PBST) was added into the cells for 30 min on ice and then the MMNK-1 cells were incubated at 4°C overnight with anti-γ-H2AX (1:100) and CCA cells were incubated with Phalloidin (1:50). After washing with PBS, Alexa™ 647 conjugated anti-mouse IgG (1:500) was used as secondary antibodies for visualizing γ-

H2AX and incubated for 30 min in a dark area. Nuclear counterstain with Hoechst 33258 solution (1:1,000) was applied for 10 min. The stained cells were visualized on the stained cells were visualized on a confocal microscope (Nikon, Tokyo, Japan).

3.2.14 SDS-PAGE and western blot analysis

Total protein was extracted from MMNK-1 with and without treatment and measured by a BCA assay kit. 30 μg of total proteins from whole cell lysate of each sample was separated by 10 or 15% SDS-PAGE. The separated proteins were transferred onto nitrocellulose blotting membranes. The nonspecific binding was blocked with 5% ECL prime blocking reagent containing 0.05% Tween 20 at room temperature for 1 h. Then, the membranes were incubated at 4°C overnight with primary antibody at dilutions of 1:1,000 for caspase3, cleaved caspase 3, survivin, and gamma H2AX; 1:2,000 for β-actin or 1:5,000 for PARP1 in PBS containing 0.05% Tween 20 (PBST). After that, the membranes were washed with PBST and incubated with HRP-conjugated secondary antibody at a dilution of 1:2,000 for 1 hour at room temperature. Finally, the target proteins were detected and visualized by ECL prime western blot detection. The density of each target protein was determined with Image J software (version 1.53a; National Institutes of Health) and normalized to β-actin.

For BBR-M-10 treated CCA cell lines, forty micrograms of protein extracts were separated by 10–15% SDS-PAGE and transferred to nitrocellulose membrane (Cytiva, Little Chalfont, UK). Nonspecific binding was blocked with 5% ECL prime blocking reagent containing 0.05% Tween 20 at room temperature for 1 h. Then, the membranes were probed with primary antibody at dilutions of 1:1,000 for vimentin, claudin-1, pAKT and AKT; and 1:500 for slug and ST6GAL1 in PBS containing 0.05% Tween 20 (PBST). at 4°C overnight. The membranes were further incubated with secondary antibody at room temperature for 1 h. The target protein signals, and the density of each target protein were determined by description as above.

3.2.15 Statistical analysis

Results are expressed as the mean ± SD from three independent experiments. Differences of functional experiments such as cell proliferation and migration in between groups were compared by using of the student-t test in GraphPad Prism 8 (GraphPad Software, Inc.). A P value of < 0.05 was considered significant.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Characteristics of black rice bran extract

Upon fractionation of black rice bran extract, the fraction that appeared to contain the highest concentration of anthocyanins based on coloration was designated BBR-M-10. Total anthocyanin content in BBR-M-10 was 108.75 mg CGE/100 g dw (100.37 mg/L). The percentage yield of anthocyanin extraction was 0.11%. Then BBR-M-10 was analyzed by TLC and HPLC techniques to determine its anthocyanin composition. TLC analysis suggested that the main anthocyanin components in BBR-M-10 were cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) (Figure 4.1A). This result was consistent with HPLC analysis, in which C3G was found in BBR-M-10 in the highest amount of 94.54 mg/L whereas P3G and cyanidin concentrations were 7.28 and 1.432.47 mg/L respectively (Figure 4.1B). Next, DPPH and ABTS assays demonstrated that the radical scavenging activity of BBR-M-10 was increased in a dose-dependent manner. At a concentration of 400 µg/mL, the radical scavenging activity in the DPPH and ABTS assay of BBR-M-10 were 80.71+1.22% and 79.3+1.23% respectively whereas at the same concentration, the radical scavenging activity of standard ascorbic acid were 86.50+0.94% for DPPH and 92.37+1.68% for ABTS. Additionally, The IC50 values in the DPPH and ABTS assays for BBR-M-10 were 128 and 100 µg/mL whereas IC50 values of ascorbic acid in the DPPH and ABTS assays were 90.8 and 66.0 µg/mL respectively (Figure 4.2A and 4.2B). These results show that the antioxidant capacity of BBR-M-10 is comparable with that of ascorbic acid.

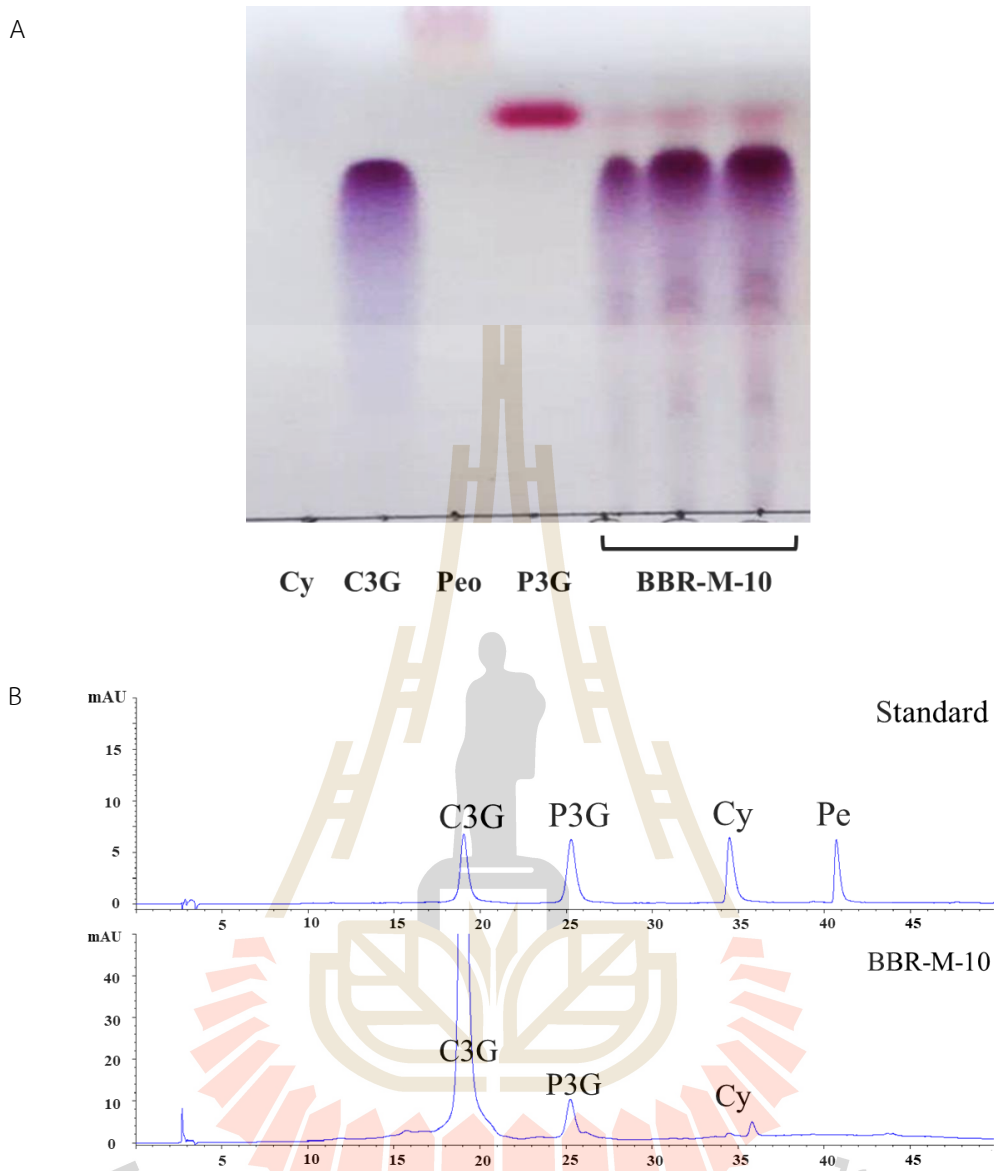


Figure 4.1 Identification and quantitative analysis of anthocyanin composition in black rice bran extract (BBR-M-10). (A) Thin layer chromatography of BBR-M-10 and standard anthocyanins including cyanidin (Cy), cyanidin-3-glucoside (C3G), peonidin (Peo) and peonidin-3-glucoside (P3G). (B) High-performance liquid chromatography of BBR-M-10.

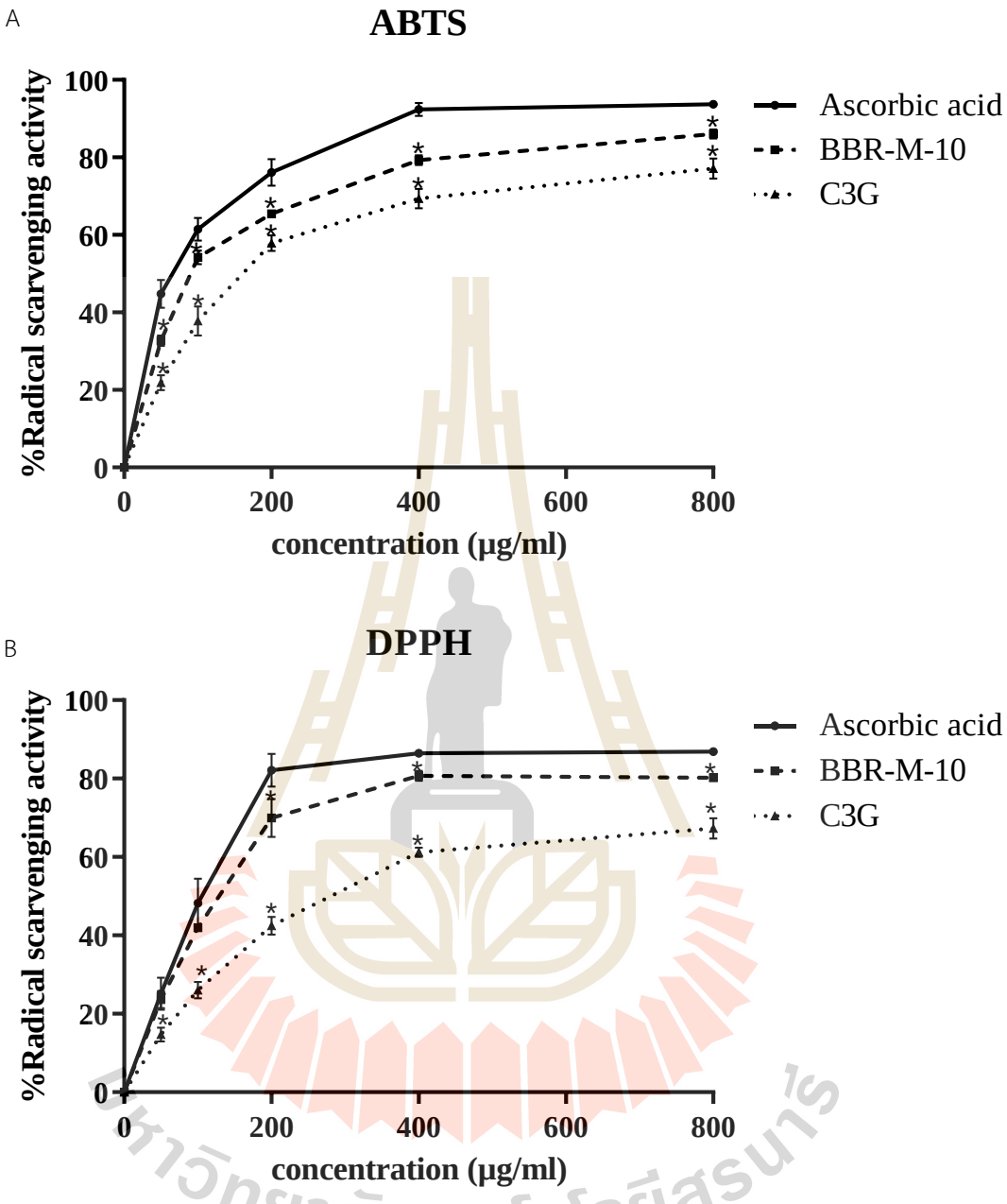


Figure 4.2 Antioxidant capacity of BBR-M-10 (A and B) antioxidant activity of BBR-M-10 using ABTS (A) and DPPH assay (B). Values are expressed as mean + standard deviation of three independent experiments. *$p<0.05$ versus ascorbic acid. $p<0.05$ was defined as statistically significant.

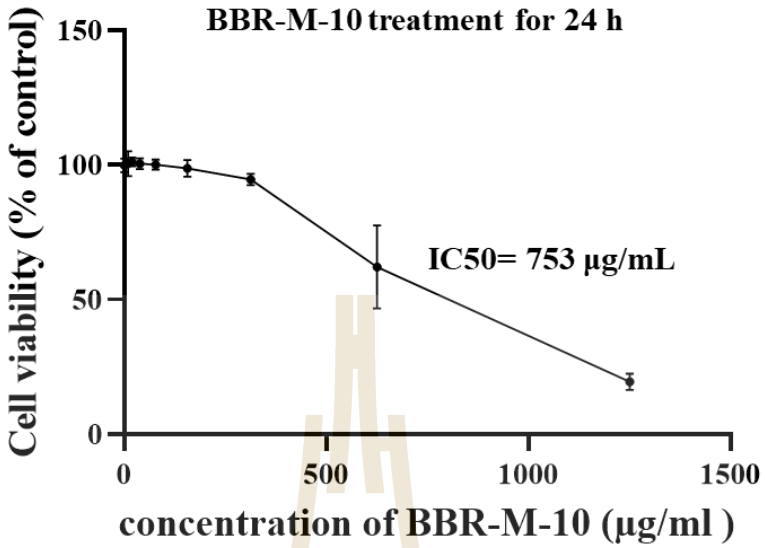
4.2 BBR-M-10 prevent H_2O_2-induced oxidative stress and DNA damage in cholangiocytes through activation of the Nrf2-NQO1 axis.

4.2.1 BBR-M-10 attenuates oxidative stress-induced cell death in H_2O_2-stimulated cholangiocyte cells

Liver fluke-induced chronic inflammation of bile duct epithelium is a major risk factor for promoting oxidative stress-mediated development of cholangiocarcinoma (SripaPairojkul, 2008). Additionally, chronic treatment of cholangiocytes with H_2O_2 has demonstrated that the cells can adapt to oxidative stress and demonstrates malignant phenotypes, such as increased cell growth and the loss of cell-to-cell adhesion (Thanan et al., 2015). In this study, H_2O_2-stimulated cholangiocyte (MMNK-1) model was used to investigate the effect of BBR-M-10 on cancer prevention. Firstly, the cytotoxic effect of BBR-M-10 on MMNK-1 cells was determined. MMNK-1 cells were treated with BBR-M-10 at various concentrations (0-1,250 µg/mL) and incubated for 24 h. A cytotoxic effect of BBR-M-10 was observed at the concentration more than 312.5 µg/mL but the IC50 value for MMNK-1 was 753 µg/mL (Figure 4.3A). Therefore, BBR-M-10 concentrations at 200 and 400 µg/ml were selected to use in subsequent experiments since those concentrations are considered as non-cytotoxicity, evidenced by percentage of cell viability above 80%. Then, the effect of H_2O_2 on MMNK-1 cell viability was also examined. MMNK-1 cells were treated with H_2O_2 at various concentrations (0-1,000 µM) and incubated for 6 h. Cell viability decreased in a dose-dependent manner and the IC50 value was 155 µM (Figure 4.3B). As a result, H_2O_2 at 200 µM was used to induce oxidative stress in MMNK-1 cholangiocytes. Next, cell viability was used to investigate whether BBR-M-10 attenuates oxidative stress-induced cell death in H_2O_2-stimulated MMNK-1 cells. Pretreatment with BBR-M-10 at 200 and 400 µg/mL for 24 h enhanced the viability of MMNK-1 cells stimulated by H_2O_2 (Figure 4.4A). Similar preventive effects were also observed in H_2O_2-stimulated MMNK-1 cells with C3G pretreatment (Figure 4.4B). Furthermore, western blot analysis demonstrated that up-regulation of survivin and down-regulation of cleaved caspase 3 and cleaved PARP1 were clearly observed in H_2O_2-stimulated MMNK-1 cells with BBR-M-10

pretreatment (Figure 4.5). These findings indicate that C3G in BBR-M-10 may play a role in decreasing the toxic effect of H_2O_2 on MMNK-1 cells.

A



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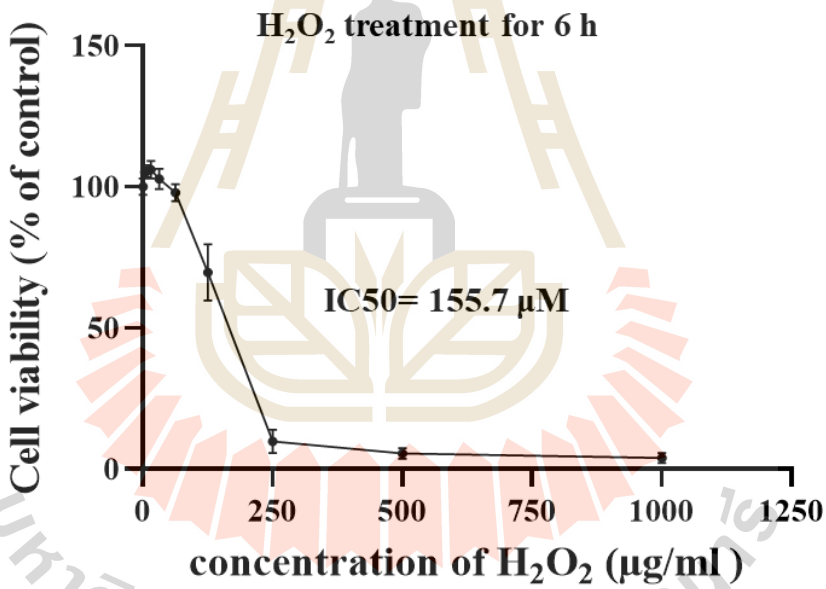
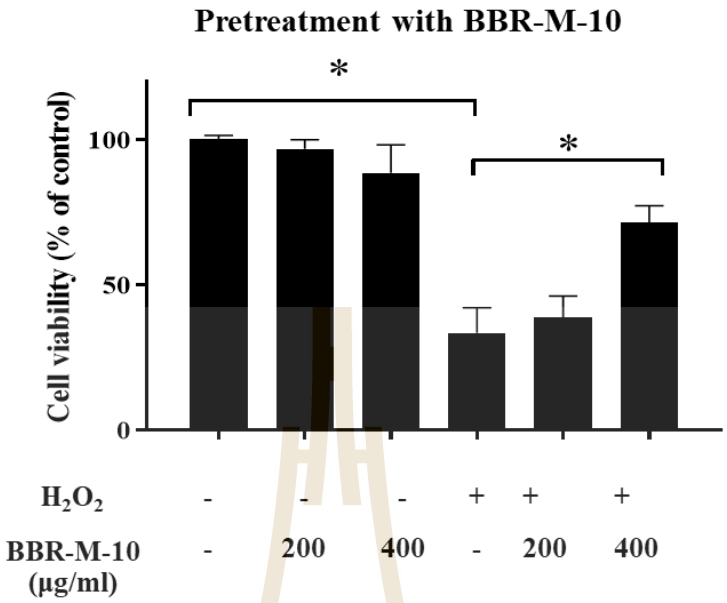


Figure 4.3 Cytotoxic effect of BBR -M10 and H_2O_2 in MMNK-1 cells for 24 h (A) Cytotoxic effect of BBR -M10 in MMNK-1 cells for 24 h and (B) cytotoxic effect of H_2O_2 in MMNK-1 cells for 6 h was determined by SRB assay. Representative data from three independent experiments are shown. Values are presented as mean ± standard deviation. *, $p<0.05$ versus H_2O_2-stimulated MMNK-1 without pretreatment. $p<0.05$ was defined as statistically significant.

A



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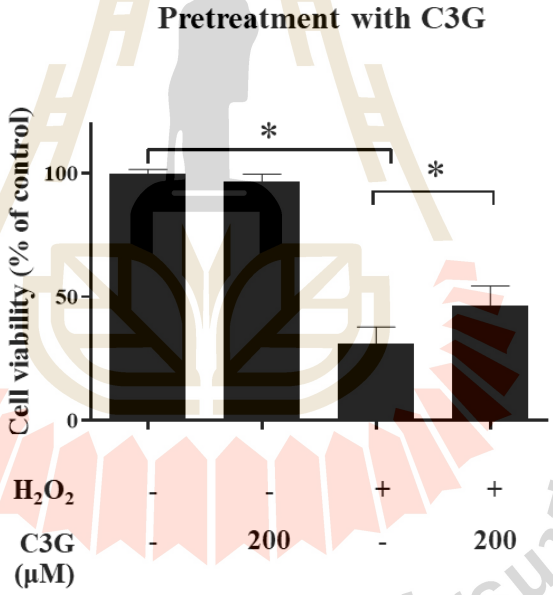

Figure 4.4 Effect of BBR-M-10 on oxidative stress-induced cell death. Cell viability of H_2O_2-stimulated MMNK-1 cells with pretreatment with BBR-M-10 (A) or C3G (B) for 24 h. Representative data from three independent experiments are shown. Values are presented as mean ± standard deviation. *, $p<0.05$ versus H_2O_2-stimulated MMNK-1 without pretreatment. $p<0.05$ was defined as statistically significant.

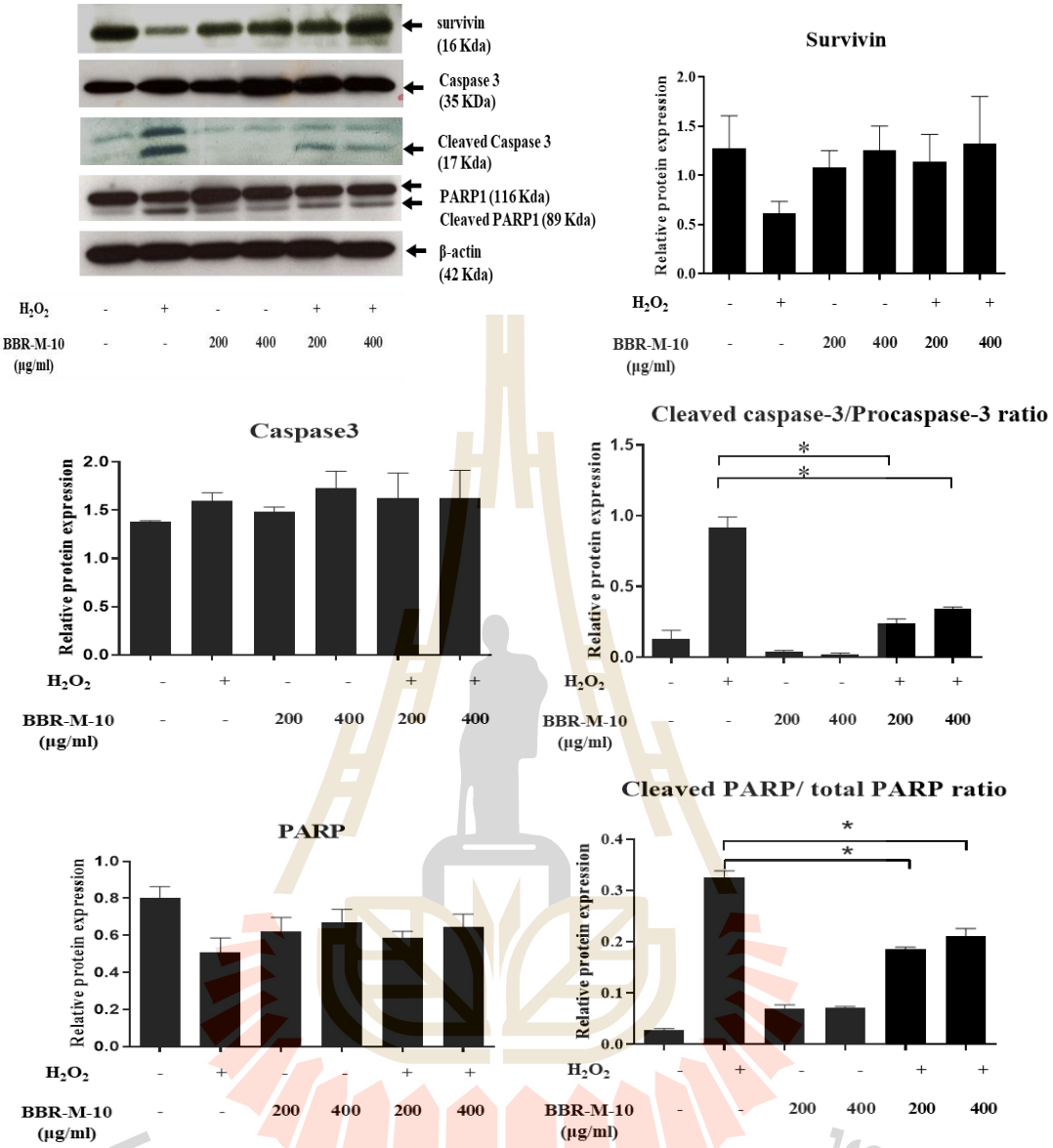

Figure 4.5 The protein expression of apoptotic proteins (caspase 3, cleaved caspase 3 and PARP1) and survivin were determined in H_2O_2-stimulated MMNK-1 cells with pretreatment with BBR-M-10 and control cells by Western blot assay. Representative data from three independent experiments are shown. Values are presented as mean ± standard deviation. *, $p<0.05$ versus H_2O_2-stimulated MMNK-1 without pretreatment. $p<0.05$ was defined as statistically significant.

4.2.2 BBR-M-10 alleviates oxidative stress in H_2O_2-stimulated cholangiocyte cells by reducing ROS accumulation and up-regulation of NQO1

The intracellular ROS accumulation and expression of phase II antioxidant genes were measured to investigate the mechanism of BBR-M-10 reduction of oxidative stress in H_2O_2-stimulated MMNK-1 cells. The intracellular ROS accumulation was evaluated by DCF-DA staining and analyzed by flow cytometry. The result showed that H_2O_2 induced intracellular ROS accumulation in a time-dependent manner. However, pretreatment with BBR-M-10 or C3G significantly decreased intracellular ROS accumulation in H_2O_2-stimulated MMNK-1 cells at 3 and 6 h (Figures 4.6A and 4.6B). Next, the expression levels of 8 phase II antioxidant genes were investigated by quantitative RT-PCR. The result showed that mRNA expression levels of phase II antioxidant genes, including Nuclear Factor Erythroid-2-Related Factor 2 (Nrf2), glutamate-cysteine ligase modifier subunit (GCLM), NAD(P)H quinone dehydrogenase 1 (NQO1), glutathione S-transferase P (GSTP-1) and superoxide dismutase 2 (SOD2) were significantly increased in BBR-M-10 treated MMNK-1 compared with those of control cells (Figure 4.7). Moreover, NQO1 was most highly expressed in BBR-M-10 treated MMNK-1 cells among all phase II antioxidant genes (Figure 4.7). As a result, the mRNA expression levels of Nrf2 and NQO1 were determined to investigate whether BBR-M-10 diminishes ROS accumulation in H_2O_2-stimulated MMNK-1 cells by activating the Nrf2 pathway. The gene expression experiments demonstrated that the mRNA expression levels of Nrf2 and NQO1 were significantly increased in H_2O_2-stimulated MMNK-1 cells with BBR-M-10 pretreatment (Figures 4.8A and 4.8B). These findings suggest that BBR-M-10 has an antioxidant effect through activation of the Nrf2-NQO1 axis.

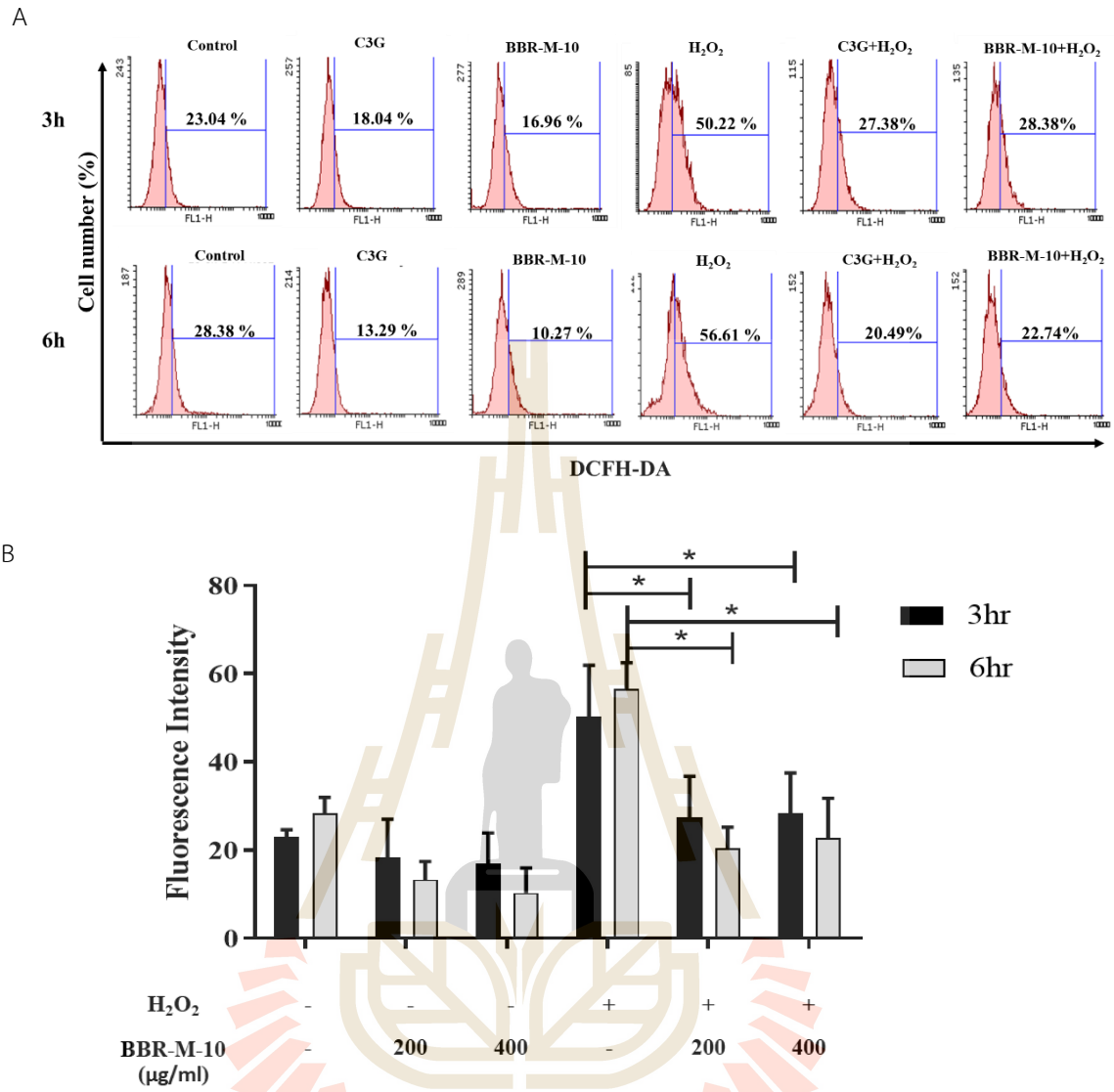

Figure 4.6 BBR-M-10 promotes antioxidant activity. (A and B) ROS accumulation of H_2O_2-stimulated MMNK-1 cells with pretreatment with BBR-M-10 or C3G for 3 and 6 h. Values are presented as mean ± standard deviation of three independent experiments. *, $p<0.05$ versus H_2O_2-stimulated MMNK-1 without pretreatment. $p<0.05$ was defined as statistically significant.

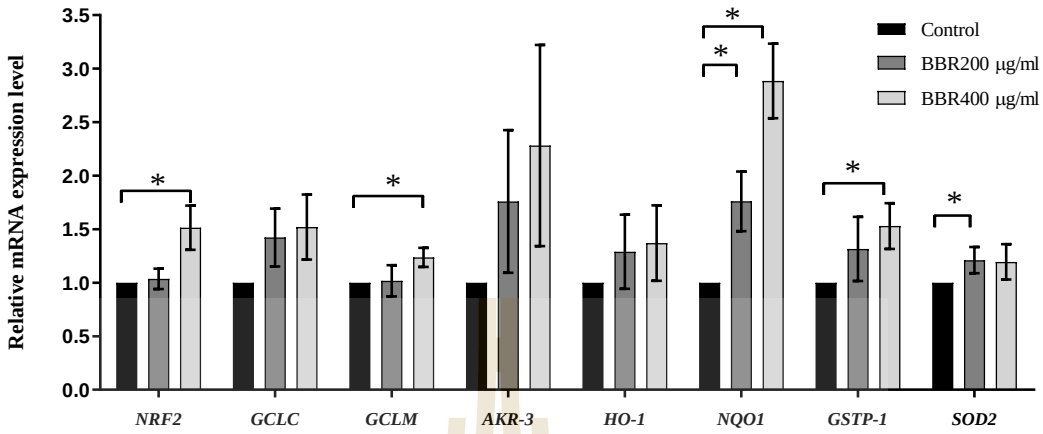

Figure 4.7 The mRNA expressions of 8 phase II antioxidant genes determined in BBR-M-10 treated MMNK-1 cells and control cells by quantitative RT-PCR. Values are presented as mean ± standard deviation of three independent experiments. *, $p<0.05$ versus H_2O_2-stimulated MMNK-1 without pretreatment. $p<0.05$ was defined as statistically significant.

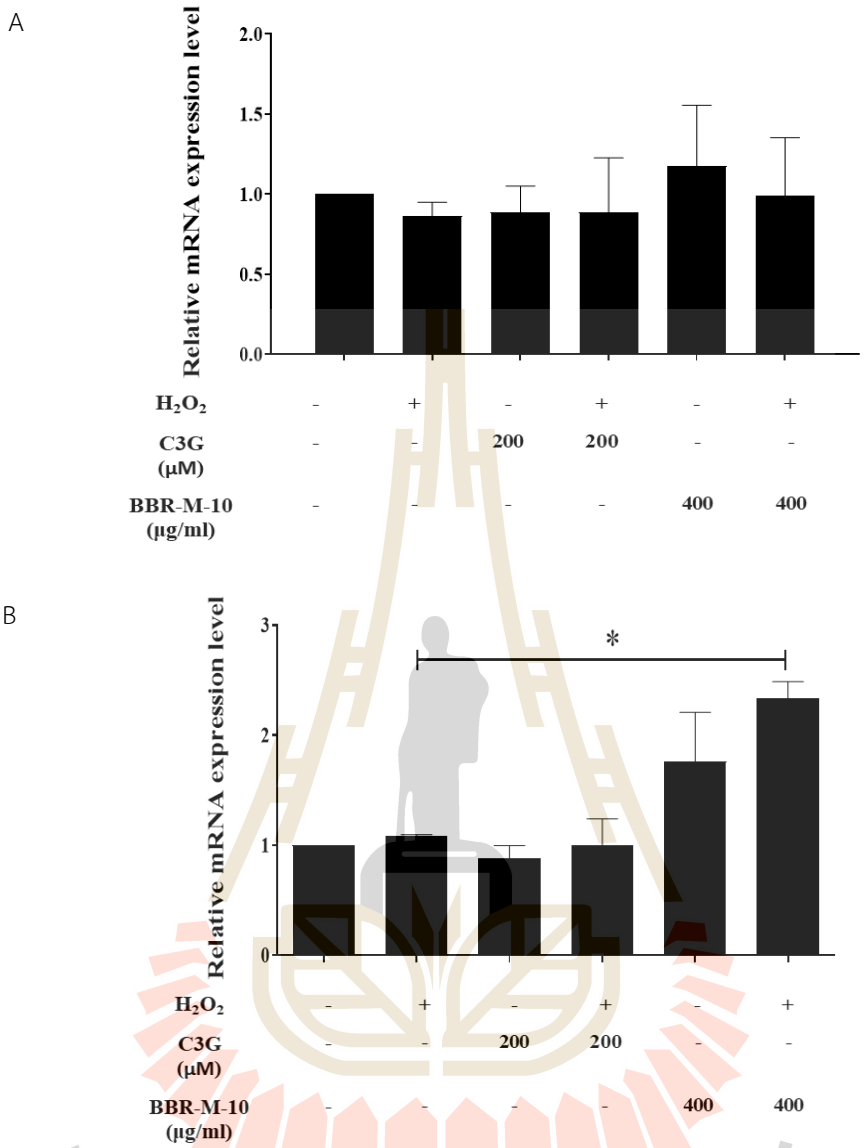

Figure 4.8 The mRNA expression of Nrf2 and NQO1 in H_2O_2-stimulated MMNK-1 with pretreatment of BBR-M-10 or C3G. Nrf2 (A) and NQO1 (B) were investigated in H_2O_2-stimulated MMNK-1 with pretreatment of BBR-M-10 or C3G and control cells. Values are presented as mean ± standard deviation of three independent experiments. *, $p<0.05$ versus H_2O_2-stimulated MMNK-1 without pretreatment. $p<0.05$ was defined as statistically significant.

4.2.3 BBR-M-10 reduces H_2O_2-induced DNA damage in cholangiocyte cells

To evaluate the preventive effect of BBR-M-10 against oxidative stress-induced DNA damage, Phospho-histone H2A.X (γH2AX), a marker for DNA double-strand breaks (DSBs), was examined in H_2O_2-stimulated MMNK-1 cells with or without BBR-M-10 pretreatment. Immunofluorescent analysis showed that γH2AX expression was increased in H_2O_2-stimulated MMNK-1 cells. Nevertheless, pretreatment with BBR-M-10 at 200 and 400 µg/mL for 24 h decreased the γH2AX signal, suggesting reduced DNA damage of MMNK-1 cells stimulated by H_2O_2 (Figure 4.9). Moreover, western blot analysis confirmed that BBR-M-10 pretreatment down-regulated γH2AX in H2O2-stimulated MMNK-1 cells (Figure 4.10). These results show that BBR-M-10 possesses cytoprotective properties against oxidative stress through the reduction of ROS accumulation and DNA damage in MMNK-1.

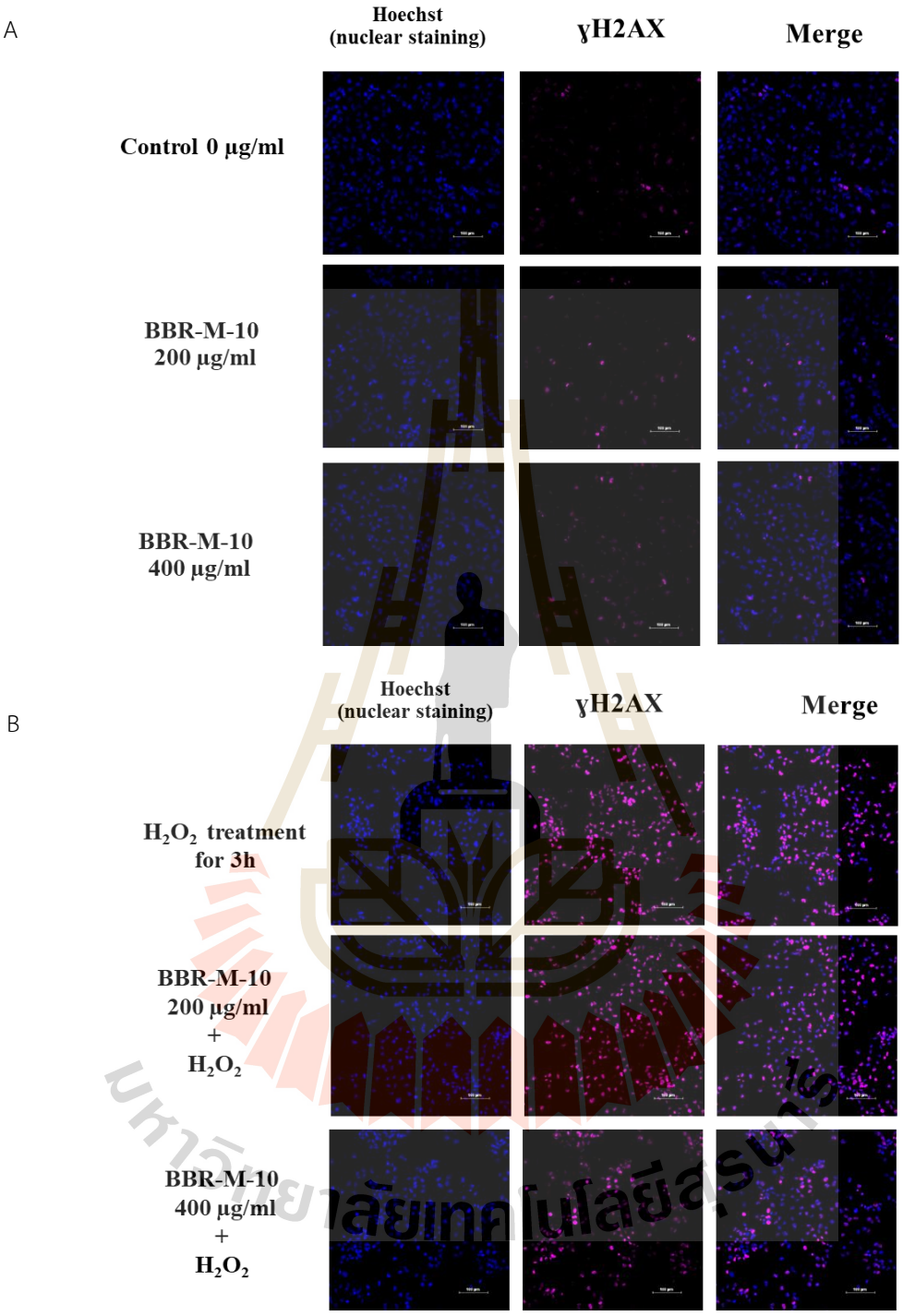

Figure 4.9 Effect of BBR-M-10 on oxidative stress-induced DNA damage by immunofluorescent staining. MMNK-1 cells were pretreated with BBR-M-10 and then treated with H_2O_2 at 200 µM for another 3 h.

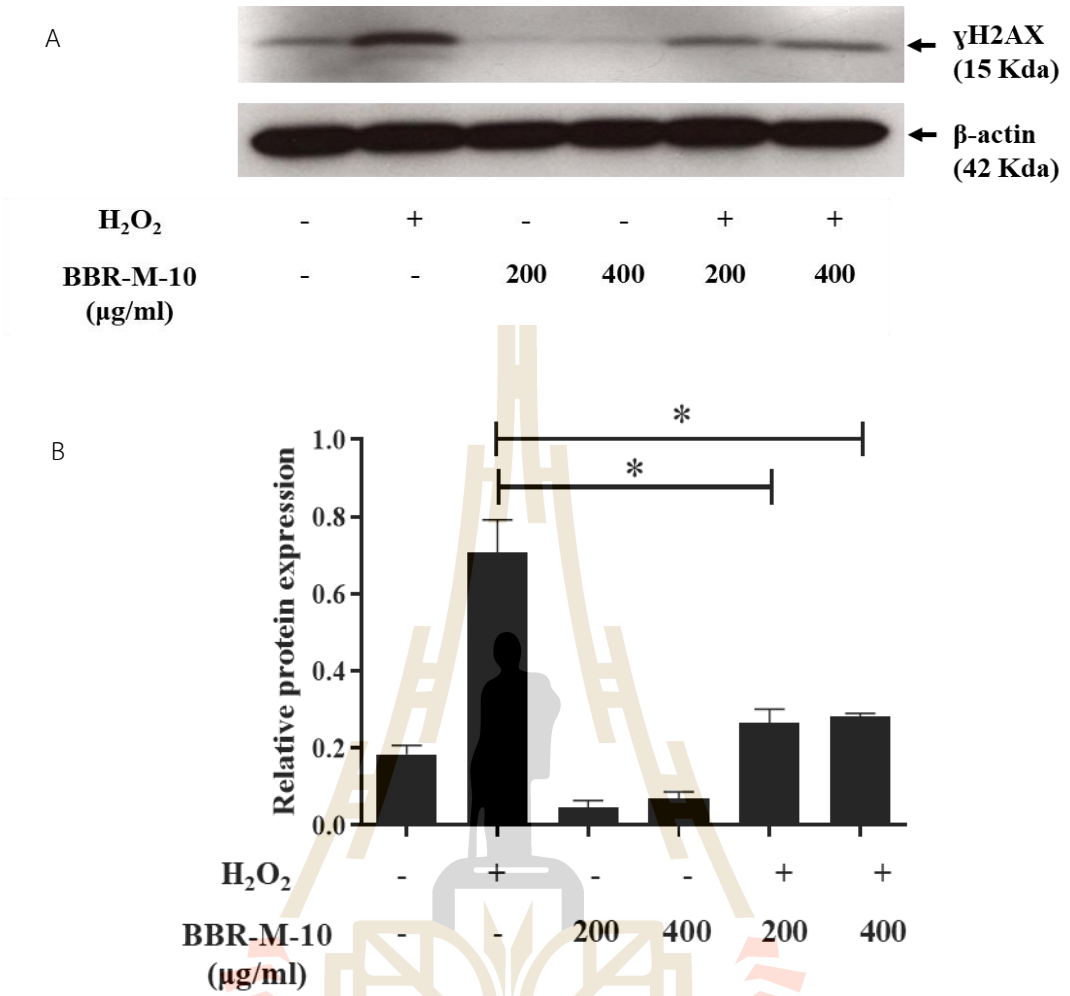

Figure 4.10 Effect of BBR-M-10 on oxidative stress-induced DNA damage by Western blot analysis. The expression of DNA damage response (γ-H2AX) was observed by immunofluorescent staining (A; x200 magnification, scale bar, 100 μm) Representative data from three independent experiments are shown. Values are presented as mean ± standard deviation. *, $p<0.05$ versus H_2O_2-stimulated MMNK-1 without pretreatment. $p<0.05$ was defined as statistically significant.

4.3 BBR-M-10 attenuate cholangiocarcinoma cell migration via the alteration of epithelial-mesenchymal transition and sialylation

4.3.1 Effect of BBR-M-10 on CCA cell viability

To evaluate the effect of BBR-M-10 on CCA cell viability, KKU-055 and KKU-213A cells were treated with BBR-M-10 at various concentrations (0-5,000 µg/ml) and incubated for 24 h. Normal fibroblast cell line IMR-90 was included for comparison to normal cells. A cell viability test demonstrated that KKU-055, KKU213A and IMR-90 cells were less sensitive to BBR-M-10. The respective IC50 values of BBR-M-10 were 2.94, and 3.47 for CCA cell lines KKU-055 and KKU-213A, and 4.30 mg/ml for IMR-90 (Figure 4.11). Our previous study demonstrated that the main anthocyanin components in BBR-M-10 are cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G). Thus, the effects of C3G and P3G on CCA cell viability were investigated in both CCA cell lines including KKU-055 and KKU-213A. The result showed that CCA cell viability was not affected upon C3G or P3G treatment at 0-500 µM (Figure 4.12A and 4.12B). Thus, low doses of BBR-M10 at 50, 100 and 200 µg/ml were selected to assess the effect of BBR-M-10 on CCA cell migration and invasion.

In addition, aglycons of the anthocyanins (anthocyanidins) including, cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin are mixed with glycoside form of anthocyanins but it was less than its glycosides in nature (H. E. Khoo, A. Azlan, S. T. Tang, and S. M. Lim, 2017) (Y. Zhang, Vareed, and Nair, 2005). In this study, the cytotoxic effects of aglycones, cyanidin chloride (CyCl) and peonidin chloride (PeCl) were investigated. A cell viability test demonstrated that KKU-055, KKU213A were sensitive to CyCl and PeCl. The respective the IC50 values of CyCl were 121 and 250 µM for KKU-055 and KKU-213A (Figure 4.13A) and IC50 values of PeCl were 67, and 245 µM for KKU-055, and KKU-213A respectively (Figure 4.13B). However, CyCl and PeCl were non-toxic to IMR-90 cell lines.

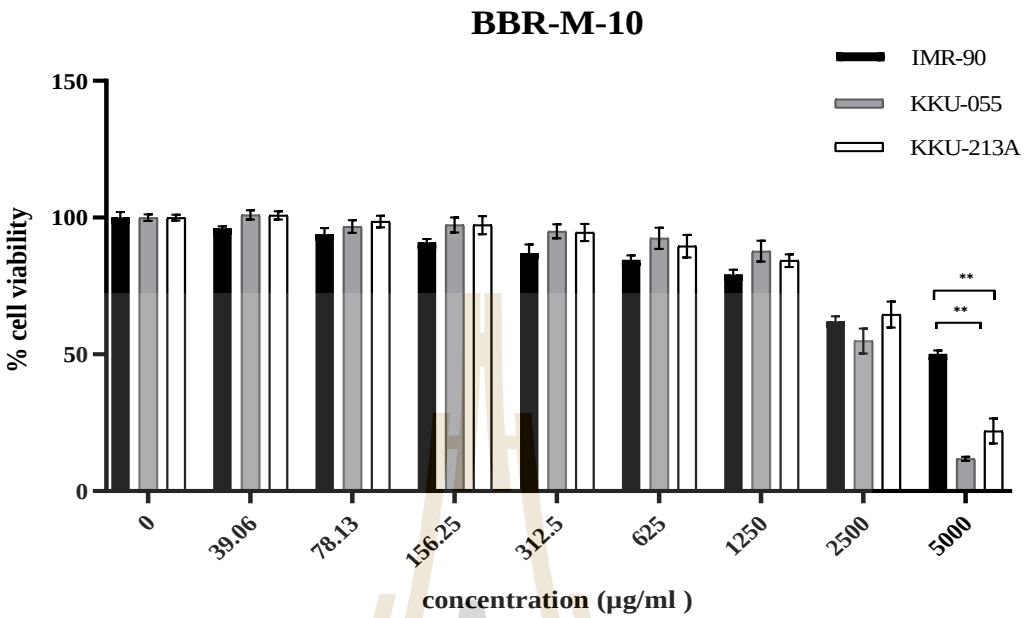

Figure 4.11 Anti-proliferative effect of BBR-M-10 in CCA cell lines. BBR-M-10 treatment in CCA cell lines and Normal lung fibroblast. Representative data from three independent experiments are shown. Values are presented as mean ± standard deviation. *, $p<0.05$; **, $P<0.01$ were defined as statistically significant.

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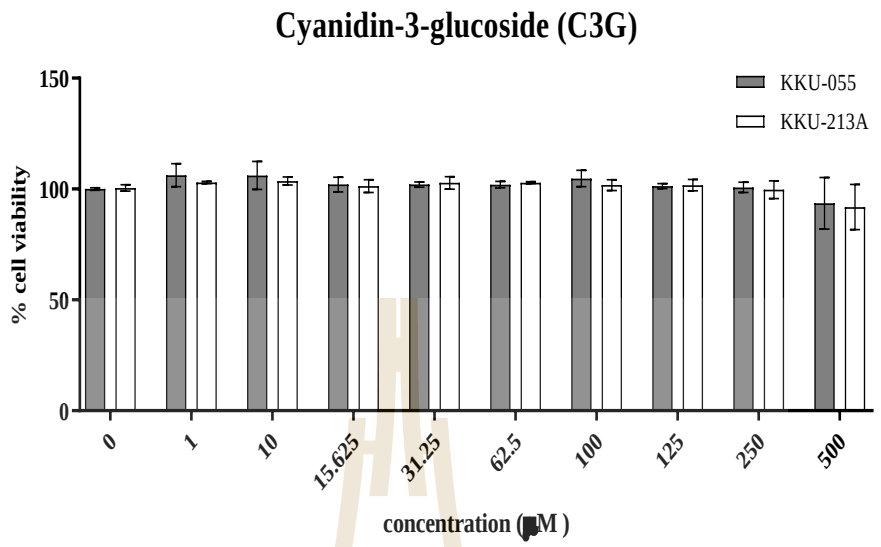

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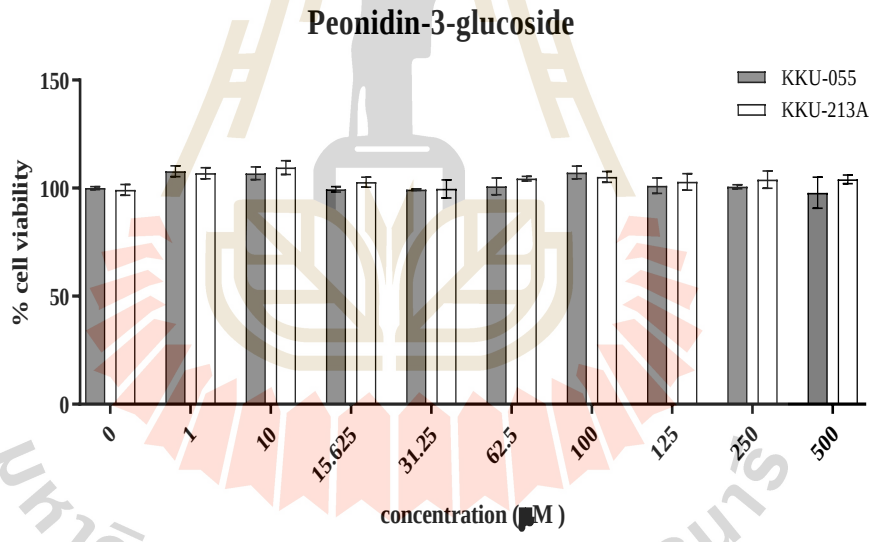

Figure 4.12 Anti-proliferative effect of anthocyanins in CCA cell lines (A) cyanidin-3-glucoside (B) peonidin-3-glucoside. Representative data from three independent experiments are shown. Values are presented as mean ± standard deviation. *, $p<0.05$; **, $P<0.01$ were defined as statistically significant.

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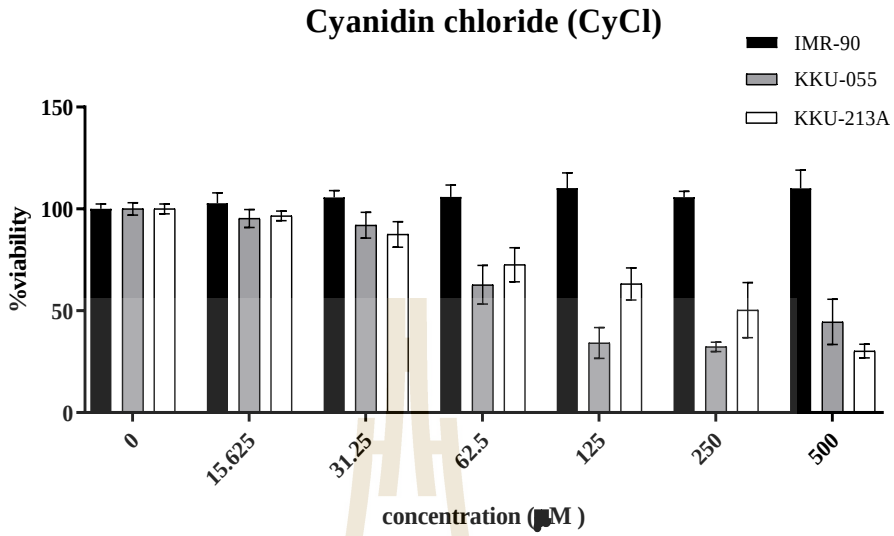

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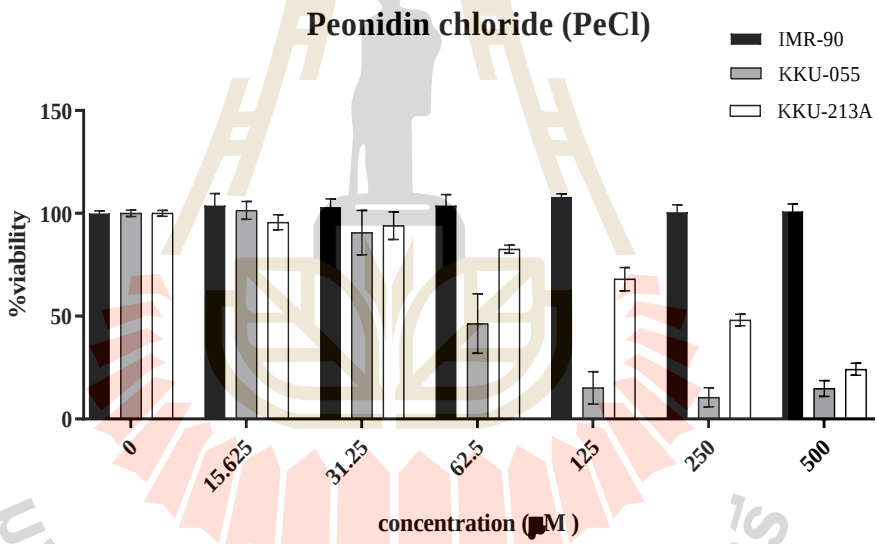


Figure 4.13 Anti-proliferative effect of anthocyanidins in CCA cell lines (A) Cyanidin chloride and (B) Peonidin chloride treatment in CCA cell lines and Normal lung fibroblast. Representative data from three independent experiments are shown. Values are presented as mean ± standard deviation. *, $p<0.05$; **, $P<0.01$ were defined as statistically significant.

4.3.2 BBR-M-10 inhibits CCA cell migration and invasion

Effects of anthocyanins on crucial cancer activities including cell proliferation, migration and invasion have been documented in various cancers (P. N. Chen et al., 2006). To address whether BBR-M-10 affects cancer activities, the migration and invasion properties of CCA cell lines upon BBR-M-10 treatment were assessed by wound healing and transwell migration and invasion assays. KKU-055 and KKU213A cells were treated with BBR-M-10 at 0, 50, 100 and 200 µg/ml. The wound healing experiment demonstrated a dose-dependent decrease in migration ability in both KKU-055 and KKU-213A cells at all observation periods (12, 18, and 24 h) ($P<0.01$) (Figure 4.14 and 4.15). Transwell migration assays confirmed that BBR-M-10 reduced the migration ability of both KKU-055 and KKU-213A cells (Figure 4.16A and 4.16B). Moreover, BBR-M-10 treatment at 200 µg/ml significantly decreased the cell invasion ability of KKU-055 and KKU-213A cells (Figure 4.17A and 4.17B). These findings suggested to us that BBR-M-10 may hinder the signaling pathway for promoting CCA cell migration and invasion.

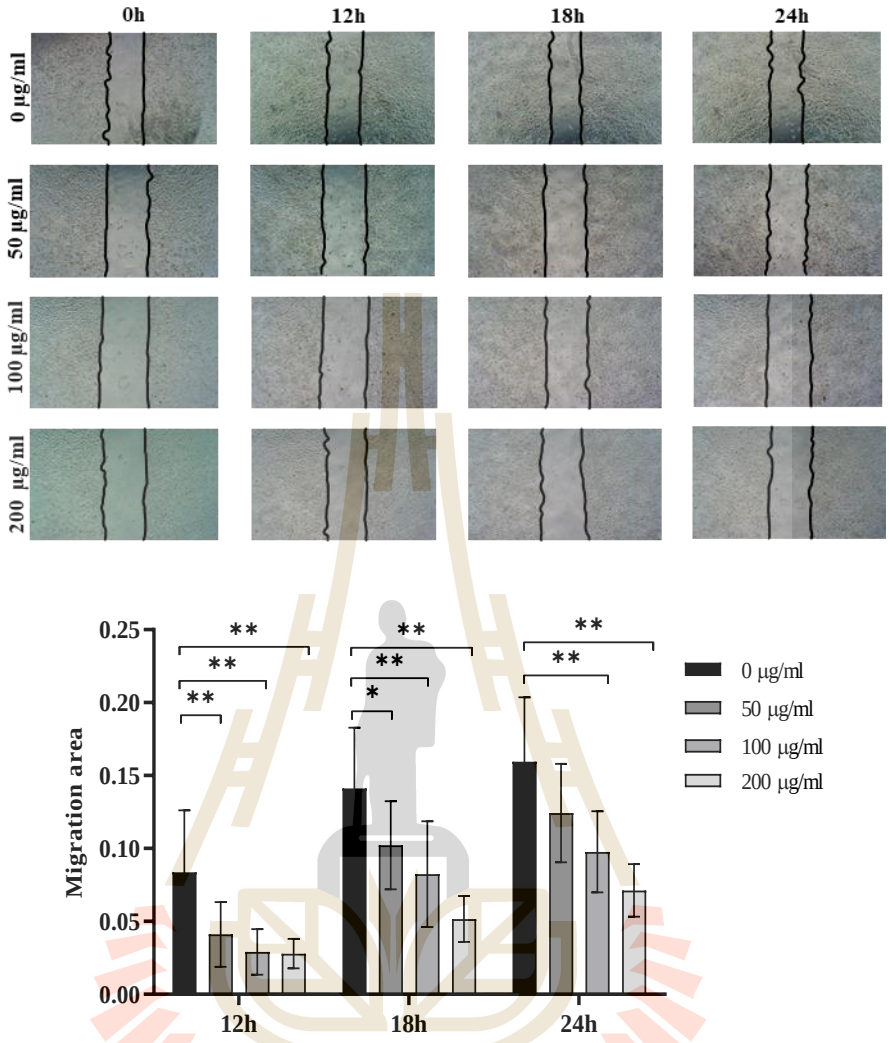


Figure 4.14 Effect of BBR-M-10 on migration of KKU-055 cell lines. Wound healing assay was performed to evaluated the migration of KKU-055 cell lines upon BBR-M-10 treatment at 50, 100, 200 µg/ml for 0, 12, 18 and 24 h. Values were obtained from three independent experiments and presented as mean ± standard deviation (*P,<0.05; **P,<0.01). (magnification, ×200).

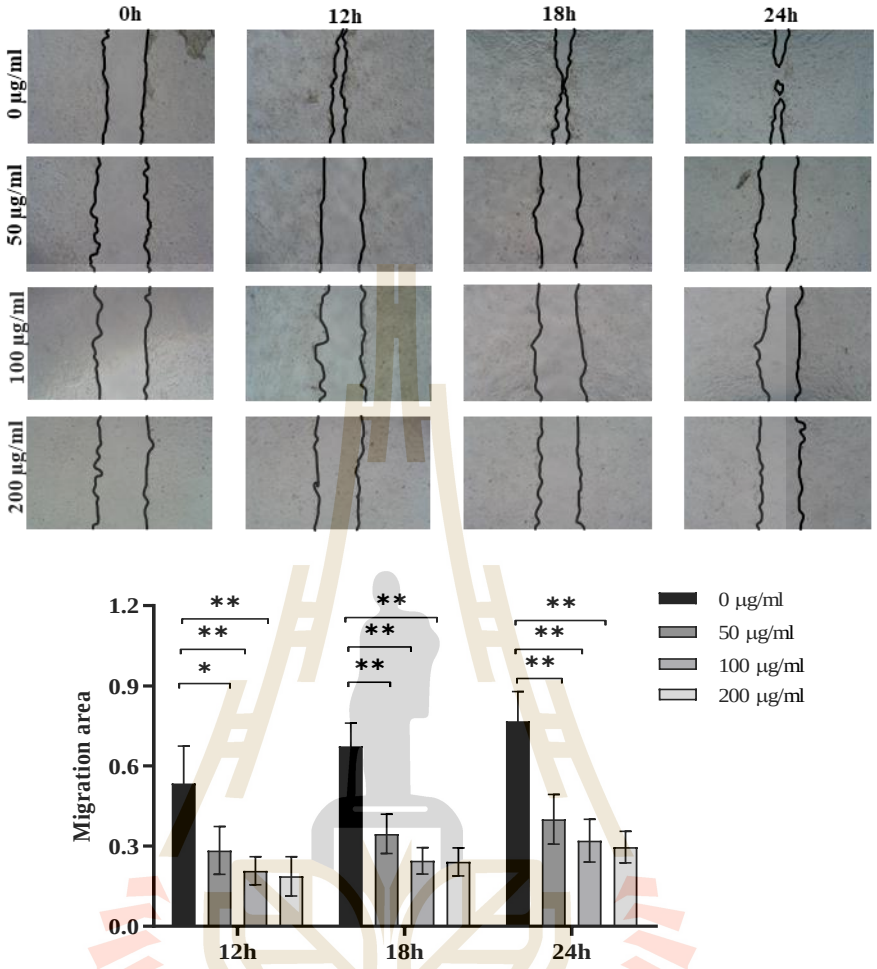


Figure 4.15 Effect of BBR-M-10 on migration of KKU-213A cell lines. Wound healing assay was performed to evaluated the migration of KKU-213A cell lines upon BBR-M-10 treatment at 50, 100, 200 µg/ml for 0, 12, 18 and 24 h. Values were obtained from three independent experiments and presented as mean ± standard deviation (*P,<0.05; **P,<0.01). (magnification, ×200).

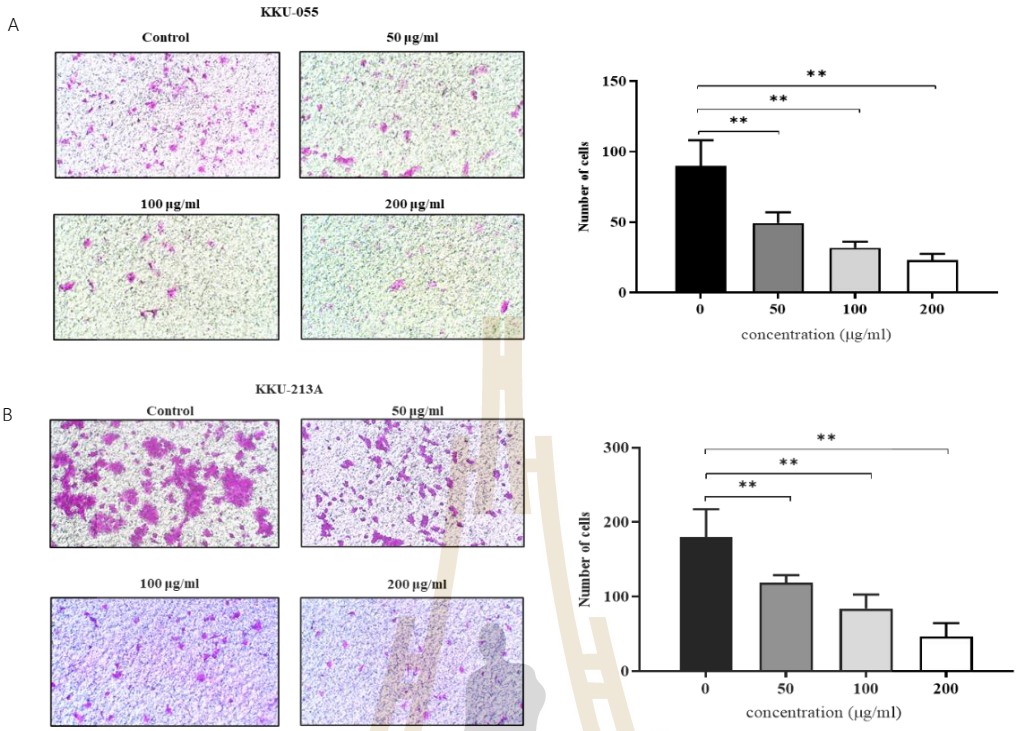


Figure 4.16 Effect of BBR-M-10 on migration of CCA cell lines. Transwell migration assay (A and B) was performed to evaluated the migration of KKU-055 and KKU-213A cell lines upon BBR-M-10 treatment at 50, 100, 200 µg/ml for 0, 12, 18 and 24 h. Values were obtained from three independent experiments and presented as mean ± standard deviation (*P,<0.05; **P,<0.01). (magnification, ×200).

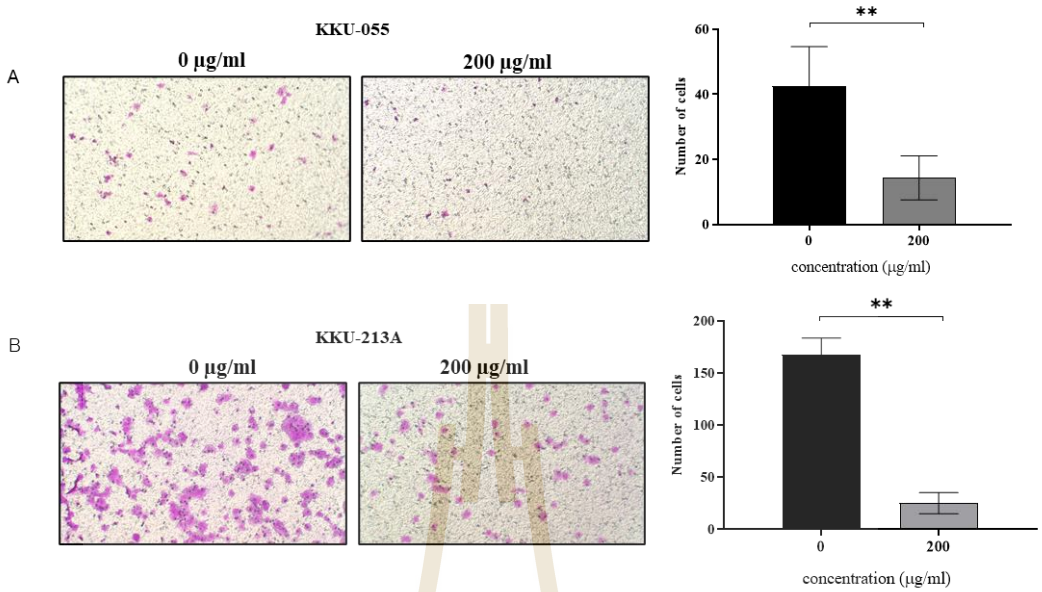


Figure 4.17 Effect of BBR-M-10 on invasion of CCA cell lines. Transwell invasion assay (A and B) was performed to evaluated the invasion of KKU-055 cells and KKU-213A cells upon BBR-M-10 treatment at 200 µg/ml for 12 hr. Values were obtained from three independent experiments (*P,<0.05; **P,<0.01). (magnification, ×200).

4.3.3 BBR-M-10 attenuates epithelial-mesenchymal transition in CCA via the AKT pathway

Actin polymerization plays a crucial role in regulating cell structure in cancer cell migration and invasion (Izdebska, Zielinska, Grzanka, and Gagat, 2018). To address the effect of BBR-M-10 in the regulation of lamellipodium formation, KKU-055 and KKU213A cells were treated with BBR-M-10 at 0, 100 and 200 µg/ml. Filamentous actin (F-actin) was detected by phalloidin staining and visualized by confocal microscopy. BBR-M-10 reduced actin cytoskeleton dynamics in both KKU-055 and KKU-213 cells (Figure 4.18). Epithelial-to-mesenchymal transition (EMT) is a key initiating step in cancer invasion and metastasis which is modulated via multiple signaling pathways including AKT signaling. Several EMT markers, including claudin-1, slug and vimentin, are regulated via this pathway (Karimi Roshan et al., 2019). The effect of BBR-M-10 on CCA cell migration and invasion via EMT was investigated. Western blot analysis demonstrated that BBR-M-10 induced the expression of claudin-1 but suppressed the expression of vimentin in both CCA cell lines. Additionally, reductions in the ratio of phosphorylated to non-phosphorylated AKT (pAKT/AKT) were observed in both CCA cell lines upon BBR-M-10 treatment (Figure 4.19).

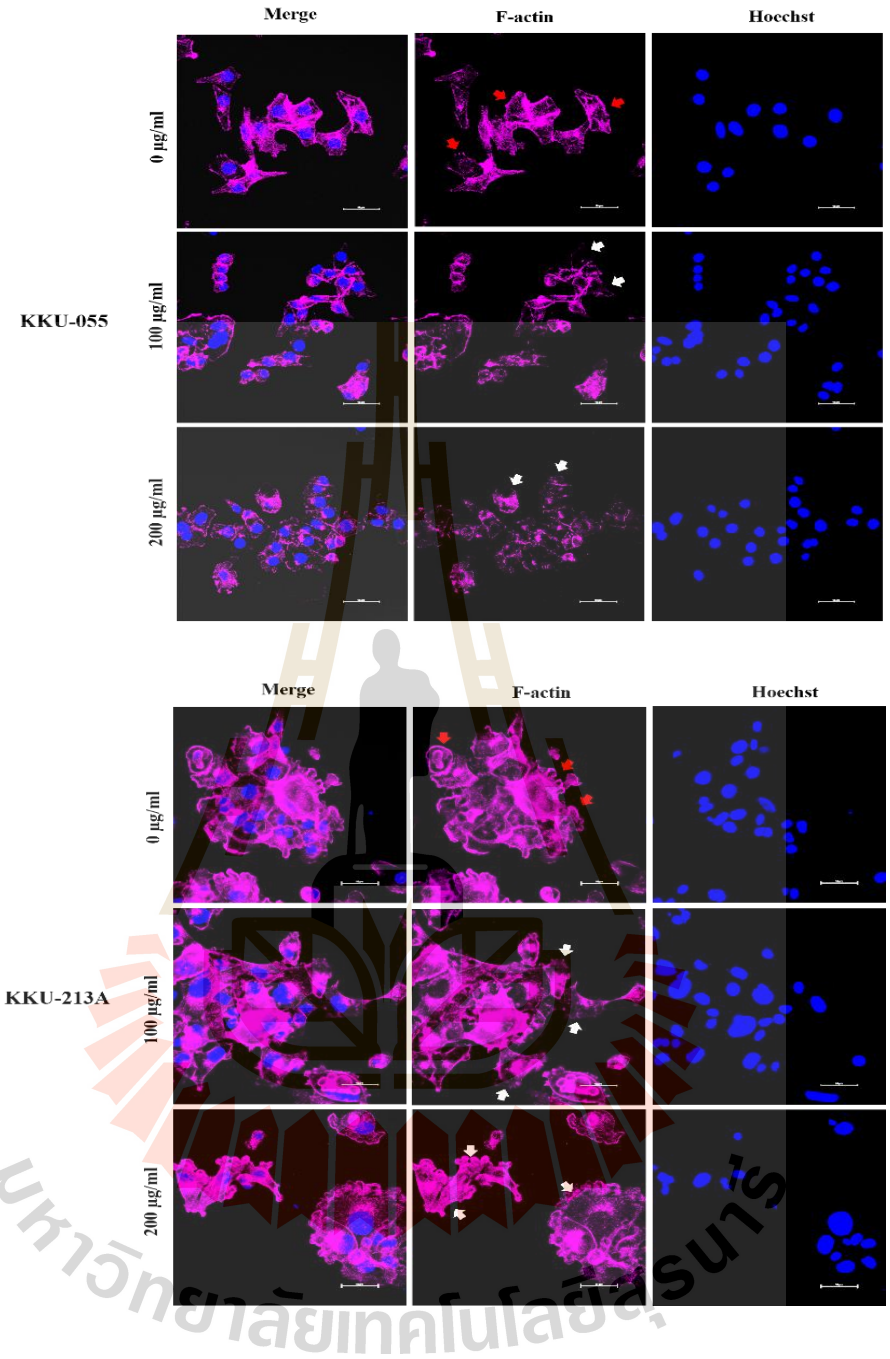


Figure 4.18 BBR-M-10 reduces the accumulation of actin filament. KKU-055 cells and KKU-213A cells were treated with BBR-M-10 treatment at 100, 200 µg/ml for 24 h. Detection of F-actin was performed Phalloidin staining.

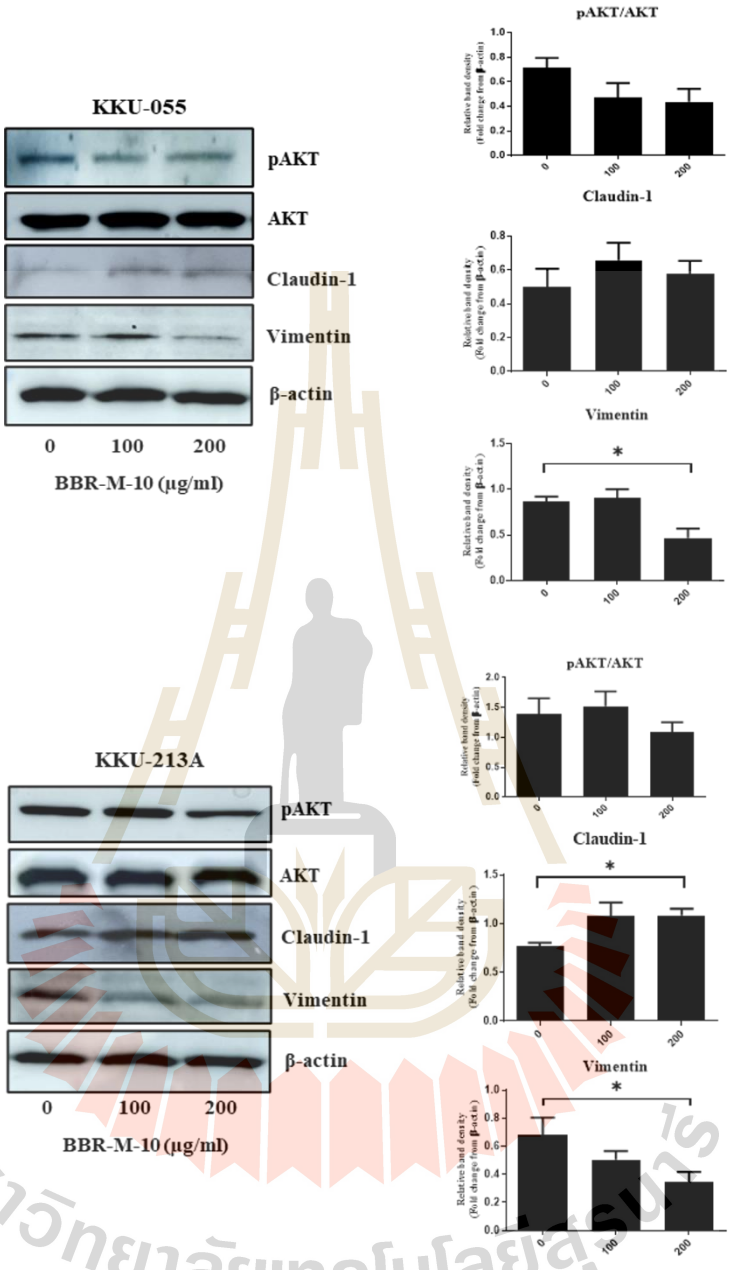


Figure 4.19 BBR-M-10 reduces the epithelial-mesenchymal transition via AKT pathway. KKU-055 cells and KKU-213A cells were treated with BBR-M-10 treatment at 100, 200 µg/ml for 24 h. Western blot analysis of pAKT, AKT, Claudin-1, Vimentin expressions in KKU-055 and KKU-213A cells. The relative expressions of proteins were obtained from three independent experiments (*P, <0.05; **P, <0.01).

4.3.4 BBR-M-10 alters sialylation in CCA

Aberrant glycosylation is a hallmark of cancer and is associated with crucial cancer behaviors including EMT (Lucena et al., 2016). To investigate whether BBR-M-10 induces glycosylation change in CCA cell lines, we used a panel of sixteen lectins to identify any differences of glycan expression between BBR-M-10-treated and control CCA cells (Table 4.1). Lectin cytochemistry demonstrated that low expression of SNA binding α2,6-sialylated glycan was observed in only BBR-M-10-treated KKU-213A cells (Figure 4.20A). Additionally, flow cytometry analysis confirmed that surface SNA binding α2,6-sialylated glycan had low expression in BBR-M-10-treated KKU-213 cells lines (Figure 4.20B). As a result, we further investigated whether BBR-M-10 alters the expression of sialyltransferase genes, including alpha2,3 sialyltransferase genes *ST3GAL1, 2, 3, 4*, and *6* and alpha2,6 sialyltransferase gene *ST6GAL1*. The gene expression experiment demonstrated that the mRNA expression of *ST3GAL4 and ST6GAL1* was reduced after BBR-M-10 treatment in KKU-213 cells (Figure 4.21B). Moreover, protein expression of ST6GAL1 was also reduced after BBR-M-10 treatment in KKU-213 cells (Figure 4.21B and 4.21C). It should be noted that altered expression of α2,3-sialylated glycans in both CCA cell lines were not detected via MAL II Lectin staining after BBR-M-10 treatment. Therefore, protein expression of *ST3GAL4* was not included in the present study. Taken together, our findings suggested that BBR-M-10 may affect the progression of CCA via the reduction of glycoprotein sialylation.

Table 4.1 Glycan expression in CCA cell lines evaluated.

Lectin	Major sugar specific	KKU-055		KKU-213A	
		0 µg/ml	200 µg/ml	0 µg/ml	200 µg/ml
PNA	Galactose	++	++	+	+
SBA	N-acetylgalactosamine	+	+	+	+
UEAI	Fucose	++	++	+++	++
WGA	N-acetylglucosamine	+++	+++	+++	+++
DBA	N-acetylgalactosamine	+++	+++	+++	+++
RCA	N-acetylgalactosamine	+++	+++	+++	++
ConA	Mannose	+++	+++	+++	+++
LEL	N-acetylglucosamine	+++	+++	++	++
ECL	Galactose	+++	+++	+++	+++
STL	N-acetylglucosamine	+	+	+	+
Jacalin	Galactose	+	+	+	+
DSL	N-acetylglucosamine	++	++	++	++
VVL	N-acetylgalactosamine	+	+	++	++
GSLII	N-acetylglucosamine	++	++	++	++
SNA	(α2,6) linked sialic acid	+++	+++	+++	++
MALII	(α2,3) linked sialic acid	-	-	+	+

Note:

PNA, *Arachis hypogaea* (peanut) agglutinin; SBA, *Glycine max* (soybean); UEA I, Ulex europaeus agglutinin I; WGA, *Triticum vulgaris* (wheat germ); DBA, *Dolichos biflorus* agglutinin; RCA_{120}, *Ricinus communis* agglutinin; Con A, Concanavalin A; LEL, *Lycopersicon esculentum* (tomato) lectin; ECL, *Erythrina cristagalli* lectin; STL, *Solanum tuberosum* (potatoe) lectin; DSL, *Datura Stramonium* lectin; VVL, *Vicia villosa* agglutinin; Jacalin; GSL II, *Griffonia (Bandeiraea) simplicifolia* lectin II; SNA, *Sambucus nigra* lectin; MAL II, *Maackia Amurensis* Lectin II.

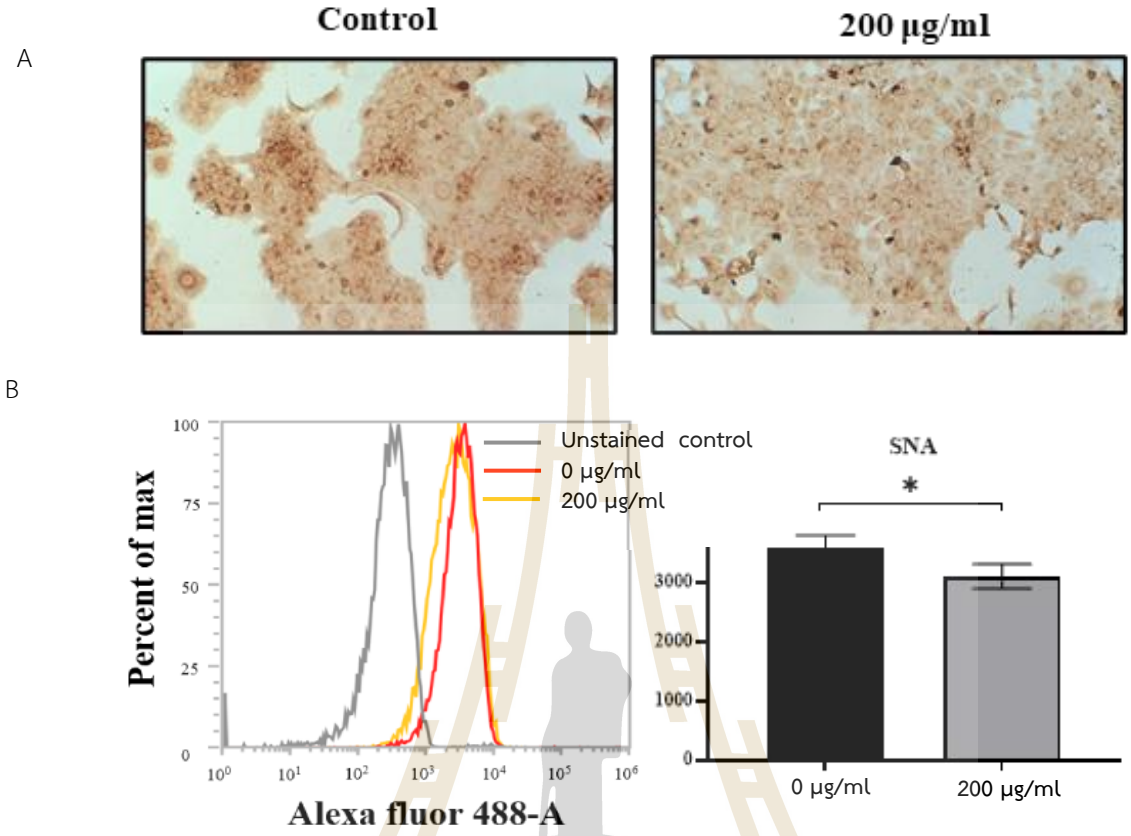
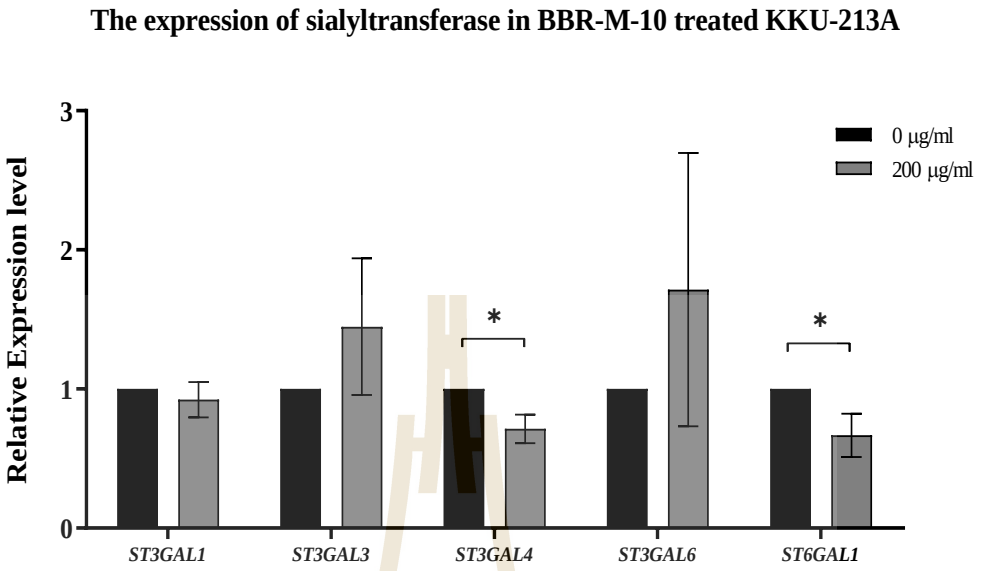


Figure 4.20 BBR-M-10 alters sialyation in CCA lines. KKU-055 cells and KKU-213A cells were treated with BBR-M-10 treatment at 200 µg/ml for 24 h. (A) Cytochemistry of α 2,6sialylated glycan was depicted with SNA lectin in KKU-213A. (B) Intensity of α 2,6sialylated glycan was depicted with SNA lectin in KKU-213A and analyzed by flow cytometer. Histogram overlays of the negative control (gray), untreated group (red) and BBR-M-10 treated group (yellow). Data was obtained from three independent experiments (*P, <0.05; **P, <0.01).

A



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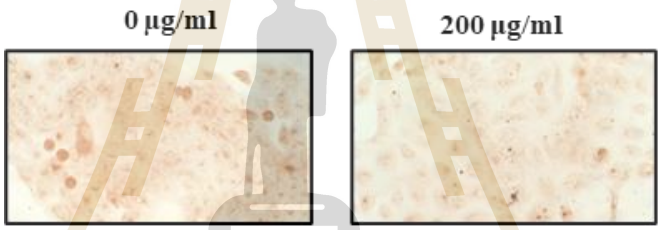

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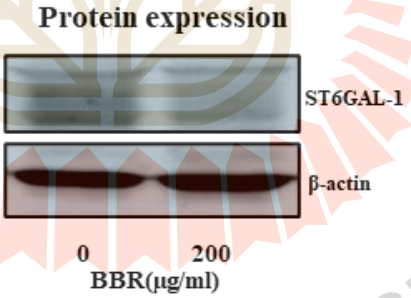

Figure 4.21 BBR-M-10 alters sialyltransferases expression in CCA lines. (A) Relative mRNA expression and protein expression level of sialyltransferases in BBR-M-10-treated CCA cell lines and control. The ST6GAL1 protein expression in BBR-M-10 treated KKU-213A and detected by cytochemistry (B) and western blot (C). Data were obtained from three independent experiments (*P, <0.05; **P, <0.01).

4.4 DISCUSSION

Pigmented rice is a functional food high in phytochemicals, such as polyphenols and anthocyanins. Anthocyanins are mainly found in pigmented rice bran, which consists of two major components including the pericarp and aleurone layers of the grains. The highest anthocyanin content is observed in black rice bran compared to red and brown rice bran (Shao, Xu, Sun, Bao, and Beta, 2014). Anthocyanins exhibit several biological activities, including antioxidants, anti-cancer, anti-apoptosis, and anti-inflammation. (Olivas-Aguirre et al., 2016; Rahman, Mathew, Nair, Ramadan, and Vazhappilly, 2021). In the present study, we demonstrated that black rice bran extract (BBR-M-10) contained C3G as the major anthocyanin and had a high antioxidant capacity associated with a decrease in oxidative stress-induced cell death and DNA damage in H_2O_2-stimulated cholangiocyte cells.

Overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is a major cause of cholangiocarcinoma development due to long-term exposure to inflammation from liver fluke infection (*O. viverrini*). As a result, high levels of etheno-DNA adduct, 8-nitroguanine and 8-oxodG, are formed during CCA development (Dechakhamphu et al., 2008; Pinlaor et al., 2004). Many studies have shown that anthocyanins, especially C3G modulate oxidative stress against DNA damage and apoptosis (Rahman et al., 2021). In the present study, the pretreatment with BBR-M-10 significantly decreased intracellular ROS accumulation in H_2O_2-stimulated MMNK-1 cells, leading to enhanced viability of the MMNK-1cells. This could be due to their chemical structure, especially the 3′ and 4′ hydroxyl groups on the B-ring which are the active site for scavenging free radicals (Ali, Almagribi, and Al-Rashidi, 2016), thereby decreasing the intracellular ROS accumulation in H_2O_2-stimulated cholangiocyte cells.

Several studies demonstrated that C3G modulates oxidative stress through the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant-responsive element (ARE) pathway (Rahman et al., 2021). A similar observation was made for BBR-M-10, since *Nrf2*, *GCLM*, *NQO1*, *GSTP-1* and *SOD2* were highly expressed in BBR-M-10 treated

MMNK-1 cells. Furthermore, mRNA expression of *Nrf2* and *NQO1* was significantly increased in H_2O_2-stimulated MMNK-1 cells with BBR-M-10 pretreatment. The results of the present study suggest that C3G in BBR-M-10 regulates the transcriptional levels of *Nrf2* target genes to reduce ROS accumulation in H_2O_2-stimulated MMNK-1 cells. These findings agree with the studies on H_2O_2-treated HepG2 cells and glutamate-treated HT22 hippocampal neuronal cells, in which pretreatment with C3G protects against cell death from ROS-mediated stress through the increase of the expression of *Nrf2* and *Nrf2*-target genes such as *HO* and *NQO1* expression (Yu et al., 2020) (Sukprasansap, Chanvorachote, and Tencomnao, 2020). Moreover, chronic inflammation-mediated DNA lesions have been demonstrated in liver fluke-induced cholangiocarcinoma through the formation of high levels of 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxodG) (Dechakhamphu et al., 2008; Pinlaor et al., 2004). Excessive amounts of ROS form the 8-oxodG, and a high level of 8-oxodG in CCA tissues is associated with poor prognosis (Thanan et al., 2014). Additionally, high levels of 8-oxodG are detected in cancer stem-like cells of CCA (CD133 and Oct3/4-positive cells) and associated with high expression of DNA damage response (indicated by the presence of γ-H2AX) (Thanan et al., 2013). In the present study, γH2AX expression was increased in H_2O_2-stimulated MMNK-1 cells. Pretreatment with BBR-M-10 in MMNK-1 cells stimulated by H_2O_2 reduced γH2AX expression. These observations suggest that BBR-M-10 exhibits cytoprotective properties against oxidative stress through the reduction of ROS accumulation, leading to the decrease of DNA damage in MMNK-1 cells. However, further studies on 8-oxodG formation will be required to support the utility of BRR-M-10 in CCA prevention. Verification of the preventive effect of BBR-M-10 in *in vivo* will be examined further.

Cancer is a deadly disease characterized by typical cancer properties, including cell proliferation, migration invasion, metastasis and drug resistance (Brown et al., 2023). Despite the approval of various chemotherapies for cancer treatment, success in improving the survival of patients with metastatic cancer remains limited (Anderson

et al., 2019). In liver fluke-associated CCA, the presence of cell migration to lymph nodes and distant metastasis play a crucial factor affecting poor prognosis and shortened survival in CCA patients (Chansitthichok et al., 2020) (Sarkhampee, Ouransatien, Lertsawatvicha, Chansitthichok, and Wattanarath, 2023). Therefore, there is a need for appropriate therapeutic drugs in the prevention and treatment of CCA. In this study, we focused on the potential role of black rice bran-derived anthocyanins (BBR-M-10) in reducing the progression of CCA. Our results demonstrated that BBR-M-10 did not show significant cytotoxic effect on CCA cell lines. This result is consistent with the previous studies in hepatocellular carcinoma, breast and prostate cancer (P. N. Chen et al., 2006) (Zhou et al., 2017) (Jongsomchai, Leardkamolkarn, and Mahatheeranont, 2020). It should be noted that the anti-proliferative effects of an aglycons of anthocyanins (CyCl and PeCl) were observed in CCA cell lines. Several studies investigated the inhibitory effect of aglycons of anthocyanins in cancer properties. Aglycons of anthocyanins induce cancer cell apoptosis effectively than its glycosides (Y. Zhang et al., 2005) (Kang, Seeram, Nair, and Bourquin, 2003). Kang, S.-Y at el. showed the respective IC50 values of cyanidin were 85 and 63 mM for human colorectal cancer cell lines, HCT 116 and HT 29, respectively, whereas those for glycoside's mixture were 260 and 585 mM for HCT 116 and HT 29 cells, respectively (Kang et al., 2003). It is indicated that there are an interesting underlying mechanism making this phenotype differently. Moreover, the tyrosine kinase activity of the epidermal growth-factor receptor (EGFR) was inactivated by aglycone of cyanidin and delphinidin rather than its glycoside (Meiers et al., 2001). This study indicated that cyanidin and delphinidin are potent inhibitors of the EGFR. In addition, Wang, H et al. found that cyanidin inhibit the activities of cyclooxygenase (COX) enzymes in vitro compared to its glucoside's mixture (Wang et al., 1999). Taken together, the aglycons of the anthocyanins have anti-cancer activities. Thus, further investigations of the aglycons of the anthocyanins in CCA are required. Nonetheless, BBR-M-10 significantly decreased the migration and invasion of CCA cell lines via the reductions of lamellipodium formation and epithelial-mesenchymal transition. Thus, BBR-M-10

treatment might represent an alternative agent to supplement treatments for metastatic CCA.

Epithelial-mesenchymal transition (EMT) plays a critical role in facilitating metastasis in various cancers (Huang, Hong, and Wei, 2022) (Lai et al., 2020). EMT triggers a phenotypic shift in epithelial cancer cells, transforming them into mesenchymal cells and ultimately leading to cancer cell metastasis (Huang et al., 2022). Mesenchymal cells exhibit a loss of cell-to-cell interactions regulated by a decrease in epithelial cell markers and increase in the expression of mesenchymal cell markers (Lamouille, Xu, and Derynck, 2014). Numerous studies have highlighted the effect of anthocyanins on cancer invasion. Anthocyanins derived from plants and fruits exhibit anti-metastatic effects in various cancers, including hepatocellular carcinoma, breast and prostate cancer (Huang et al., 2022) (Lai et al., 2020). This is consistent with our present study, in which BBR-M-10 significantly reduced the migration and invasion of CCA cell lines. Actin cytoskeleton plays a critical role in regulating cell structure and motility, allowing cells to migrate and invade (Izdebska et al., 2018). The dynamic polymerization and depolymerization of actin filaments (F-actin) are regulated by actin-binding proteins which usually stabilize the polymerization of F-actin, driving the protrusion of the cell membrane (Izdebska et al., 2018) (Lorente, Syriani, and Morales, 2014). An alteration and accumulation of F-actin at the cell edges or in a lamellipodium have been demonstrated to contribute to the aggressiveness of cancer cell invasion through the extracellular matrix in several cancers (M. Chen et al., 2021; Chung et al., 2022). The reduction of F-actin in lamellipodia formation has been associated with a decrease in cancer invasiveness (OlsonSahai, 2009). Previous studies reported the effects of flavonoids and anthocyanins on the disruption of F-actin formation in diabetic kidney and prostate cancer cells (Jongsomchai et al., 2020) (Lee et al., 2019). In the present study BBR-M-10 reduced F-actin accumulation in CCA cell lines. These findings suggest that BBR-M-10 prevents CCA cell migration and invasion by modulating F-actin formation. Moreover, BBR-M-10 altered the expression of EMT

genes via the up-regulation of epithelial markers (claudin-1) but down-regulation of mesenchymal markers (vimentin). These may be due to the role of vimentin in providing flexibility to cells and promoting cell motility in various cancers (Berr et al., 2023) (Usman et al., 2021). In addition, claudin-1 is recognized as a tight junction protein. Down-regulation of claudin-1 was associated with cancer invasion in various cancers, e.g. CCA (Lamouille et al., 2014) (RaoSamak, 2013). However, in some cancers, claudin-1 has the opposite role in that its high expression suggests involvement in the progression of cancers, such as colon cancers (Bhat et al., 2020). Taken together, our findings suggest that BBR-M-10 attenuated CCA cell migration and invasion via the decrease of EMT.

The PI3K/AKT pathway has frequently been reported as a driver of cancer progression e.g., cell proliferation and metastasis (MaharatiMoghbeli, 2023). The increase of the activation of PI3K/AKT signaling was correlated with metastasis in CCA (Yothaisong et al., 2013). Several EMT markers, including claudin-1, slug and vimentin, are regulated via this pathway (Karimi Roshan et al., 2019). In the present study, BBR-M-10 diminished the phosphorylation of AKT in CCA cell lines. This finding is consistent with studies in breast cancer in which anthocyanins extracted from cherry reduce invasion via downregulation of AKT expression (Layosa et al., 2021).

Glycosylation, a major post-translational modification, usually acts as a “fine tuner” of cellular and molecular interactions (Gabius, 2018). Glycosylation changes are a hallmark of cancer that play an important role in several aspects of malignancy, including proliferation, invasion, and metastasis. EMT is a critical step of metastasis which is believed to be associated with glycosylation changes, evidenced by: i) N-glycan branching; ii) O-glycan truncation; iii) terminal sialylation and, iv) terminal fucosylation during EMT (Pucci, Malagolini, and Dall'Olio, 2021). In the present study, the decrease of terminal α 2,6-sialylated glycans in both CCA cell lines was detected via SNA Lectin staining after BBR-M-10 treatment. However, BBR-M-10 alters the expression of sialyltransferases only in KKU-213A, especially down-regulation of

alpha2,6 sialyltransferase (ST6GAL1) which is the main enzyme responsible for α 2,6 sialylation. These findings suggested that BBR-M-10-altered sialylation is cell type specific, since KKU-213A has high invasion capacity (Figure 4.9B). This is consistent with a study that showed that metastatic CCA patients having low expression of ST6GAL1 have shorter overall survival than metastatic CCA patients having high expression of ST6GAL1 (D. D. Park et al., 2024). Here is the first time to demonstrate the effect of anthocyanin on cancer-associated glycosylation changes.

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Black rice bran-derived anthocyanins (BBR-M-10) protected MMNK-1 cells from H_2O_2-induced oxidative stress and DNA damage. BBR-M-10 decreased ROS accumulation through the increase of the transcription of antioxidant genes (*Nrf2* and *NQO1*) in MMNK-1 cells exposed to H_2O_2. Therefore, our findings suggest the utility of black rice bran-derived anthocyanins in preventing carcinogenic toxicity during CCA development. In addition, black rice bran-derived anthocyanins also diminished metastatic phenotypes, including reduction of epithelial-mesenchymal transition and decreased sialylation. It appeared that the AKT pathway plays a vital role in this inhibitory effect. Taken together, our findings suggest that BBR-M-10 has potential for use as a supplemental treatment for CCA prevention and metastatic CCA.

5.2 RECOMMENDATION

The present study could provide the supportive data of black rice bran-derived anthocyanins in preventing and suppressing CCA in a particular pathway. Thus, combination treatment between BBR-M-10 and chemotherapeutic drugs would be interesting point for further study. In addition, growth inhibitory effects of CyCl and PeCl were detected in CCA cell lines. Therefore, further studies on the underlying mechanism of CyCl and PeCl-modulated the CCA death are required.

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breast cancer epithelial-mesenchymal transition-mediated metastasis in vitro by suppressing FAK signaling. *Int J Mol Med, 40*(6), 1649-1656.

APPENDIX

APPENDIX

PUBLICATION AND PRESENTATION

A.1 List of publications

Khophai, S., Chokchaisiri, S., Talabnin, K., Ketudat Cairns, J. R., & Talabnin, C. (2024). Black rice bran-derived anthocyanins prevent H2O2-induced oxidative stress and DNA damage in cholangiocytes through activation of the Nrf2-NQO1 axis. ScienceAsia.

Khophai, S., Chokchaisiri, S., Talabnin, K., Ketudat Cairns, J. R., & Talabnin, C. (2024). Black rice bran-derived anthocyanins attenuate cholangiocarcinoma cell migration via the alteration of epithelial-mesenchymal transition and sialylation. Biomed Rep.

A.2 List of presentations

Khophai S, Ketudat Cairns JR and Talabnin C. Role of Rice Bran Anthocyanins in Cholangiocarcinoma Prevention. The 17th International Symposium of the Protein Society of Thailand. Chiang Mai University, Chiang Mai, Thailand. Nov 9-11, 2022.

Khophai S, Ketudat Cairns JR and Talabnin C. Role of Rice Bran Anthocyanins in Cholangiocarcinoma migration and invasion. The 30th FAOBMB & 8th BMB Conference, Bangkok, 22-25 November 2023

Abstract submitted to The Pure and Applied Chemistry International Conference (PACCON 2022)

Abstract submitted to the 17th International Symposium of the Protein Society of Thailand (2022)

PST2022 The 17th International Symposium Of The Protein Society of Thailand

ABSTRACT- 20220054

Role of Rice Bran Anthocyanins in Cholangiocarcinoma Prevention

Sasikamon Khophai[1], James R.Ketudat-Cairns[2], Chutima Talabnin[1*]

[1] Biochemistry and Molecular Biology of Cholangiocarcinoma, School of Chemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
[2] Center for Biomolecular Structure, Function and Application, School of Chemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
*Email: sasikamon.sk@gmail.com

Abstract

Anthocyanins are water-soluble flavonoids responsible for the color of fruits and flowers. The roles of anthocyanins have been associated with cancer prevention. Black rice bran is rich in anthocyanin pigments especially cyanidin 3-glucoside (C3G) and have high antioxidant activity. However, the effect of rice bran anthocyanins on cholangiocarcinoma (CCA) prevention has never been demonstrated yet. In present study, we aimed to demonstrate the effect of rice bran anthocyanins, namely BBR-M10, against H_2O_2-induced reactive oxygen species (ROS) generation and DNA damage in immortalized human cholangiocyte cell lines (MMNK-1). TLC and HPLC results indicated that the major anthocyanin in BBR-M10 was C3G. The biological functions of BBR-M10 extracts were performed. The results demonstrated that pretreatment with BBR-M10 reduced the cytotoxic effect of H_2O_2-mediate MMNK-1 cell death and ROS accumulation. The downregulation of cleavage caspase-3 and upregulation of surviving were also detected in H_2O_2-treated MMNK-1 upon pretreatment with BBR-M10. Moreover, the expressions of phase II antioxidant genes were increased upon pretreatment with BBR-M10. Furthermore, the expression of DNA damage marker, gramma H2AX, was also determined. The immunofluorescent and western blot analysis showed that pretreatment with BBR-M10 hindered the expression of gramma H2AX in H_2O_2-treated MMNK-1. Our findings show that rice bran anthocyanins from black rice can prevent H_2O_2-induced DNA damage and cell death in cholangiocytes via suppressing intracellular accumulation of ROS and up-regulation of phase II antioxidant genes. Therefore, utility of rice bran anthocyanins would be a potential health benefit for CCA prevention.

Grant/Support by: International Research Network Program (IRN)

Abstract submitted to the 30th FAOBMB & 8th BMB Conference (2023)

The 30th FAOBMB & 8th BMB Conference, Bangkok
22-25 November 2023

ROLE OF RICE BRAN ANTHOCYANINS IN CHOLANGIOCARCINOMA MIGRATION AND INVASION

Sasikamon Khophai[1,2*], Chutima Talabnin[1,2] and James R. Ketudat-Cairns[3]
[1]*School of Chemistry, Institute of Science, Suranaree University of Technology, 111 University Avenue, Muang, Nakhon Ratchasima, 30000, Thailand.*
[2]*Biochemistry and Molecular Biology of Cholangiocarcinoma, School of Chemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[3]*Center for Biomolecular Structure, Function and Application, School of Chemistry, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*

Cholangiocarcinoma (CCA) is a bile duct cancer that has high incidence in northeast region of Thailand. Liver fluke infection (*Opisthorchis viverrini*, Ov) is defined as a major risk factor for CCA development. Pigmented rice bran from black rice is recognized as a potential source of biologically active phytochemicals, especially anthocyanins compounds. The anti-carcinogenic effect of anthocyanins has been documented by a variety of mechanisms: reducing cancer cell proliferation, migration and invasion, and induction of apoptosis. In the present study, we aimed to demonstrate the effect of black rice bran anthocyanins, namely BBR-M-10, against CCA cell migration and invasion. TLC and HPLC results demonstrated that cyanidin-3-glucoside (C3G) was the major anthocyanins in BBR-M-10. Wound healing and Transwell assay showed that BBR-M-10 inhibited CCA cell migration and invasion ($P<0.05$). Due to the glycosylation of several proteins plays an important role in the epithelial mesenchymal transition (EMT). A panel of 16 lectins was used to identify differences in glycan expression of CCA cell lines after BBR-M-10 treatment. The change of glycan expression in BBR-M10 treated CCA cells was obviously observed by *Sambucus nigra* lectin (SNA) which is a lectin that recognizes sialic acid linked to N-acetylgalactosamine or galactose of the glycans structure (SNA binding-glycans). Low expression of SNA binding-glycans was detected in BBR-M-10 treated KKU-213A. Taken together, our findings suggest that BBR-M-10 has an anti-metastatic effect on CCA cell lines through the alteration of glycosylation. However, further studies on the mechanism of BBR-M-10-inhibited CCA metastasis are required.

Keywords: Anthocyanin; Cholangiocarcinoma; Sialic acid; Cell migration; Cell invasion

CURRICULUM VITAE

Name	Ms. Sasiakmon Khophai
Date of Birth	19 August 1992
Education Background	
2011-2015	Bachelor's degree of Science in Medical Technology, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand
2015-2018	Master's degree of Science in Medical Biochemistry and Molcular Biology, Faculty of Medicine, Khon Kaen University, Thailand.
2022-present	Doctor of Philosophy of Science in Biochemistry and Biochemistry technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand.
Grant received	Thailand Research Fund international research network grant IRN62W0004