โครงการ

การเรียนการสอนเพื่อเสริมประสบการณ์

ชื่อโครงการ ปริมาณสารแคปไซซินและกิจกรรมการต้านมะเร็งของสารสกัดหยาบจาก *Capsicum annuum* L.

Capsaicin content and anticancer activity of crude extract from *Capsicum annuum* L.

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ปีการศึกษา 2562

คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

Capsaicin content and anticancer activity of crude extract from *Capsicum annuum* L.

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A Project Submitted in Partial Fulfillment of the Requirements for the Bachelor of Science Program in Botany Department of Botany, Faculty of Science, Chulalongkorn University

Academic Year 2019

ปริมาณสารแคปไซซินและกิจกรรมการต้านมะเร็งของสารสกัดหยาบจาก *Capsicum annuum* L.

นาย ปวเรศ ศิวาพรนุกูล

โครงงานวิทยาศาสตร์ฉบับนี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปริญญาวิทยาศาสตรบัณฑิต สาขาพฤกษศาสตร์ ภาควิชาพฤกษศาสตร์
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2562

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Academic Year	2019

Accepted by Department of Botany, Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirement for the Bachelor of Science Program

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Abstract

Capsaicinoid is a secondary metabolite produced in green and red chilies. Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the main capsaicinoids, has been linked with anticancer activities through the mechanisms that are not completely understood. Furthermore, intraspecific variability of capsaicinoids is a hindrance for phamaceutical application. In this study, the dried chili fruits of *Capsicum annuum* L. cv. Jinda and cv. Cayenne were extracted with acetonitrile and the capsaicin contents were determined with high performance liquid chromatography (HPLC). As a result, the capsaicin content of Jinda was 75.145±1.418 mg/g dry weight and Cayenne was 1.267±0.094 mg/g dry weight. Furthermore, the crude extracts (100-500 μM capsaicin equivalent) were applied to cervical cancer cells (SiHa) for anticancer potentiality evaluation. Viability assay showed no anticancer activity when SiHa cells were treated with Jinda crude extracts. This may be that other secondary metabolites presenting in chili crude extract conceal anticancer properties of capsaicin. Thus, further studies, e.g. viability assay using pure capsaicin and antioxidant activity of chili crude extract, are required for better comprehension.

Keywords: Capsaicin, Capsaicinoids, Cervical cancer

ชื่อโครงงาน ปริมาณสารแคปไซซินและกิจกรรมการต้านมะเร็งของสารสกัดหยาบจาก *Capsicum annuum* L.

ชื่อนิสิต ปวเรศ ศิวาพรนุกูล

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บทคัดย่อ

แคปไซซินอยด์เป็นสารเมแทบอไลต์ทุติยภูมิที่สร้างขึ้นในพริกสีเขียวและสีแดง ประกอบด้วยกลุ่มหลัก คือ แคปไซซิน (8-methyl-N-vanillyl-6-nonenamide) ซึ่งมีส่วนเกี่ยวข้องกับกิจกรรมต้านมะเร็ง แต่กลไกนั้นยังไม่เป็นที่เข้าใจอย่างสมบูรณ์ นอกจากนี้ความแปรผันของปริมาณแคปไซซินอยด์เป็นข้อจำกัดหนึ่งในการนำไปใช้ประโยชน์เชิงเภสัชกรรม ในการศึกษานี้ผลแห้งของพริก *Capsicum annuum* L. cv. Jinda และ cv. Cayenne ถูกนำมาสกัดด้วยอะซิโตไนไตรล์และมีการวัดปริมาณแคปไซซินด้วยวิธี high performance liquid chromatography (HPLC) ซึ่งพบว่าพริก Jinda มีปริมาณแคปไซซิน 75.145±1.418 มิลลิกรัมต่อกรัมน้ำหนักแห้ง และพริก Cayenne มีปริมาณแคปไซซิน 1.267±0.094 มิลลิกรัมต่อกรัมน้ำหนักแห้ง นอกจากนี้สารสกัดหยาบ (มีปริมาณแคปไซซินเทียบเท่า 100-500 ไมโครโมลาร์) ได้ถูกนำมาใช้กับเซลล์มะเร็งปากมดลูก (SiHa) เพื่อประเมินศักยภาพในการต้านมะเร็ง จากการทดสอบความมีชีวิต (viability assay) ไม่พบความสามารถในการต้านเซลล์มะเร็ง SiHa ของสารสกัดหยาบของพริก Jinda ซึ่งอาจเกิดจากการมีสารเมแทบอไลต์ทุติยภูมิชนิดอื่น ๆ ในสารสกัดหยาบที่บดบังคุณสมบัติการต้านมะเร็งของแคปไซซิน ดังนั้น การศึกษาเพิ่มเติม เช่น การทดสอบความมีชีวิตโดยใช้แคปไซซินบริสุทธิ์และกิจกรรมการต้านอนุมูลอิสระของสารสกัดหยาบจากพริกเป็นสิ่งจำเป็นต่อการเข้าใจกลไกเหล่านี้มากขึ้น

คำสำคัญ: แคปไซซิน, แคปไซซินอยด์, เซลล์มะเร็งปากมดลูก

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my family for their love, caring and encouragements. My sincere thank goes to my academic advisor, Assistant Professor Dr. Anchalee Chaidee, who has taught me every knowledge as much as she could and given me the great advices since my first year at Chulalongkorn University. Moreover, I would like to thank for the continuous support of the project and her guidance which helped me during the project.

I would like to thank Professor Dr. Wolfgang Pfeiffer who taught me several scientific techniques as clear as possible and for offering me a great opportunity to visit his laboratory at Salzburg University, Austria. I also would like to thank him and his family for the warm welcome, helping and taking good care of me when I was in Austria.

I would like to thank Assistant Professor Dr. Rachaneekorn Tammachote who accepted me to become one of her students under her guidance to carry out the project with great advice.

I would like to thank all members of my senior project committee for their critical perusal and for serving on my examination committee.

I would like to thank Chulalongkorn University and the Development and Promotion of Science and Technology Talents Project (DPST) for the great opportunity.

I would like to thank all staff members of the Department of Botany for their helps.

I would like to express my appreciation and thanks to Mr. Athiwat Tilapornputt for his helps and his suggestions since my first year at Chulalongkorn University.

Finally, I would like to thank Miss Chanatip Thanasarnthungcharoen for her help and encouragements.

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CHAPTER 1

INTRODUCTION

Capsaicin, which is one of predominant structure of capsaicinoids, provides a pungent sensation found in *Capsicum* fruits such as chili pepper fruit (Kozukue et al., 2005; Choi et al., 2006). This pungency substance is produced in the epidermis of placenta and accumulated within specific structure named "blisters" which locates on placenta surface (Suzuki et al., 1980; Stewart et al., 2007).

Chili pepper fruits have been used for various purposes. For food purposes, chili pepper fruits are used as food additives in several cultures specifically Asian cuisines (Perkins et al., 2002). In addition to food purposes, capsaicinoids in chili pepper fruits have been used for medical and pharmaceutical purposes including analgesia, anticancer, anti-inflammatory, antioxidative and anti-obesity activities (Negulesco et al., 1987; Govindarajan and Sathyanarayana, 1991; Liu and Nair, 2010; Luo, Peng, and Li, 2010). It has been reported that capsaicinoids are able to relieve headache, neck pain, oral mucositis, rhinopathy, hyperreflexia and cutaneous pain caused by skin tumors (Hautkappe et al., 1998). Currently, the study of capsaicin in medical field has been reported that capsaicin induced apoptosis in an *in vitro* human-gastric cancer cells (SNU-1) (Kim et al., 1997). In another study, capsaicin reduced the growth of numerous lines of leukemia cells through G_0-G_1 phase of cell cycle arrest and apoptosis in mice which tumor weight was reduced by 50%, after daily injection of 50 mg/kg of capsaicin for 6 days (Ito et al., 2004). Similarly, Mori et al. (2006) reported that capsaicin inhibited the growth of prostate cancer cells without producing any toxicity in mice about 50% by weight when injected 5 mg/kg of capsaicin to the mice 3 days per week for 4 weeks. Sanchez et al. (2006) have also reported that capsaicin induced apoptosis of prostate tumor PC-3 cells. Primarily, the proposed anticancer mechanism of capsaicin involves the induction of cell cycle arrest and apoptosis. However, capsaicin is also known to be carcinogen in some conditions. Therefore, more information on molecular mechanism in different cell systems is necessary. Furthermore, other metabolites in

chili fruits may have mutually concerted anticancer capacities. Therefore, this study was initiated in order to investigate capsaicin content in crude extract of *C. annuum* L. and anticancer activity of the crude extract based on apoptosis induction of cervical cancer cells. Anticancer activity of crude extract can be uncovered by using biochemical and cell biology approaches, e.g. viability, caspase enzyme activity, DNA fragmentation and nuclei staining. Findings obtained in this study will provide basic knowledge necessary for future in-depth study on molecular mechanism of capsaicin and other metabolites in chili fruits, for a potential therapeutic application against cancer.

1.1 Objective

The aim of this study was to determine capsaicin contents and anticancer activity of chili crude extracts.

CHAPTER 2

LITERATURE REVIEWS

2.1. Chemical structure and properties of capsaiciniods

The plants within *Capsicum* genus are able to produce capsaicinoids, which cause pungency taste when the substances interact with sensory receptors. The chemical structure of capsaicinoids is nearly related to alkaloid compounds (Baby and Ranganathan, 2016; Ryu, Kim, Kim, and Rhee, 2017). General structure of capsaicinoids contains a vanillyl group bonded with an amide and an alkyl chain (Figure 2.1). Furthermore, capsaicinoids possess various kinds of the alkyl group as: (i) unsaturated and ramified (i.e. capsaicin), (ii) saturated and ramified (i.e. dihydrocapsaicin or nornordihydrocapsaicin), and (iii) saturated and linear (i.e. nonivamide). As a result, the chemical structure of capsaicinoids provides amphiphilic characteristic with no basicity behavior as in general alkaloid compounds (Alberti et al., 2008; Cheok et al., 2016).

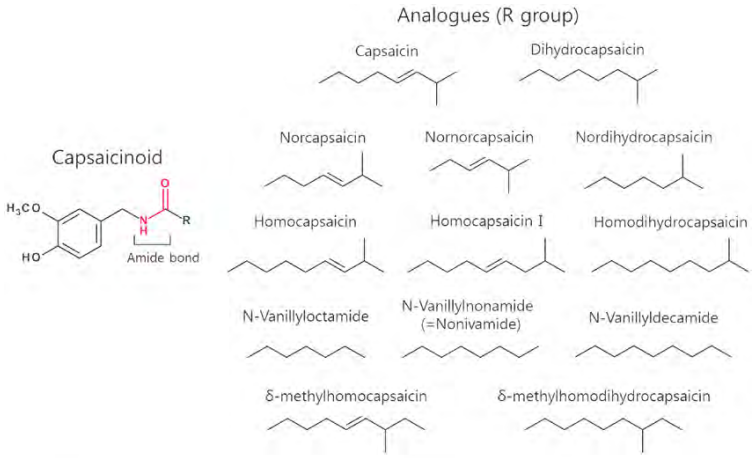

Figure 2.1 General chemical structure of capsaicinoids (Basith, Cui, Hong, and Choi, 2016)

The original concentration of capsaicinoids is reduced over 50% when exposed to temperature above 80 °C; however, they resist to an ionizing radiation. The thermal degradation cleaves carbon-nitrogen at amide position and then generates free vanillyl group and free acyl chain (Schweiggert, Carle, and Schieber, 2006; Alberti et al., 2008; Cheok et al., 2016). It has been reported that capsaicinoids are able to protonate which provide the ability to stabilize radical species and interact with cell membranes, enzymes, and sensory receptors (Mohammad, Ahmad, and Heng, 2013; Loizzo et al., 2015). Thus, capsaicinoid bioactivity is mainly attributed to the presence of vanillyl group in capsaicinoid structure (Hayman and Kam, 2008).

Capsaicinoids interact with the transient receptor potential vanilloid-1 (TRVP1), which is a sensorial receptor accounted for the neural response to several kinds of stimuli such as pain, osmolarity, and temperature by means of producing pain and/or heat as a warning to the body of possible harmful situations (Lu, Ho, and Huang, 2017). The effects of capsaicinoids on TRVP1 are not only the pungent sensation but also providing pain relief and antioxidant properties, including anti-cancer and anti-inflammatory effects (Lu, Ho, and Huang, 2017).

Due to amphiphilic behavior, capsaicinoids are dissolved in low to medium polarity solvents (Hayman and Kam, 2008). For example, appropriate solvents used to extract capsaicinoids are methanol, ethanol, or acetonitrile. Mostly, acetonitrile is used as a solvent due to the highest efficiency to dissolve capsaicinoids (Hayman and Kam, 2008). In addition, when capsaicinoids are exposed to a basic condition in the presence of metal, phenolate ion is formed leading to increasing solubility (Kim et al., 2017).

2.2. Biosynthesis and catabolism of capsaicinoids

Placenta of *Capsicum* fruits contain the largest concentration of vanillylamine. Capsaicinoids are mianly biosynthesized at the placenta by condensation of vanillylamine with an acyl acid precursor through phenylpropanoid pathway as shown in Figure 2.2 (Zewdie and Bosland, 2001; Monforte-Gonzalez, Medina-Lara, Gutierrez-Carbajal, and Vazquez-Flota, 2007; Gonzalez-Zamora et al., 2015; Ananthan, Subhash, and Longvah, 2018).

The biosynthesis of capsaicinoids has an acyl as a precursor; in which each type of capsaicinoids has a unique acyl precursor such as 8-methyl-trans-6-nonenoic acid (capsaicin), 8-methylnonanoic acid (dihydrocapsaicin), 7-methylnonanoic acid (nordihydrocapsaicin), 9-methyldecanoic acid (homodihydrocapsaicin) and 9-methyldec-trans-7-enoic acid (homocapsaicin) (Aza-Gonzalez, Nunez-Palenius, and Ochoa-Alejo, 2011). The acyl precursor, C9-C11 branched chain, is synthesized by serial enzymatic reactions which start from an amino acid and present reactions of transamination, decarboxylation, elongation and reduction (Monforte-Gonzalez, Medina-Lara, Gutierrez-Carbajal, and Vazquez-Flota, 2007). In the elongation step, the process is promoted by malonyl-CoA and the cycle might occur variously from two to four cycles depending on types of capsaicinoids being synthesized (Monforte-Gonzalez et al., 2007).

The final step of capsaicinoid synthesis involves the reduction of ketone to alcohol and the dehydration of alcohol to provide the double bond to fatty acid chain or to generate saturation of carbon chain. However, the synthesis of capsaicinoids is limited by either the concentration of vanillylamine or the acyl precursor (Steewart et al., 2005; Mazourek et al., 2009).

The biosynthesis of capsaicinoids has high activity within 30-50 days after flowering. In this period, the pungency level is normally high in whole fruit and capsaicinoid concentrations naturally decrease after the period due to plant metabolism (Dıaz, Pomar, Bernal, and Merino, 2004).

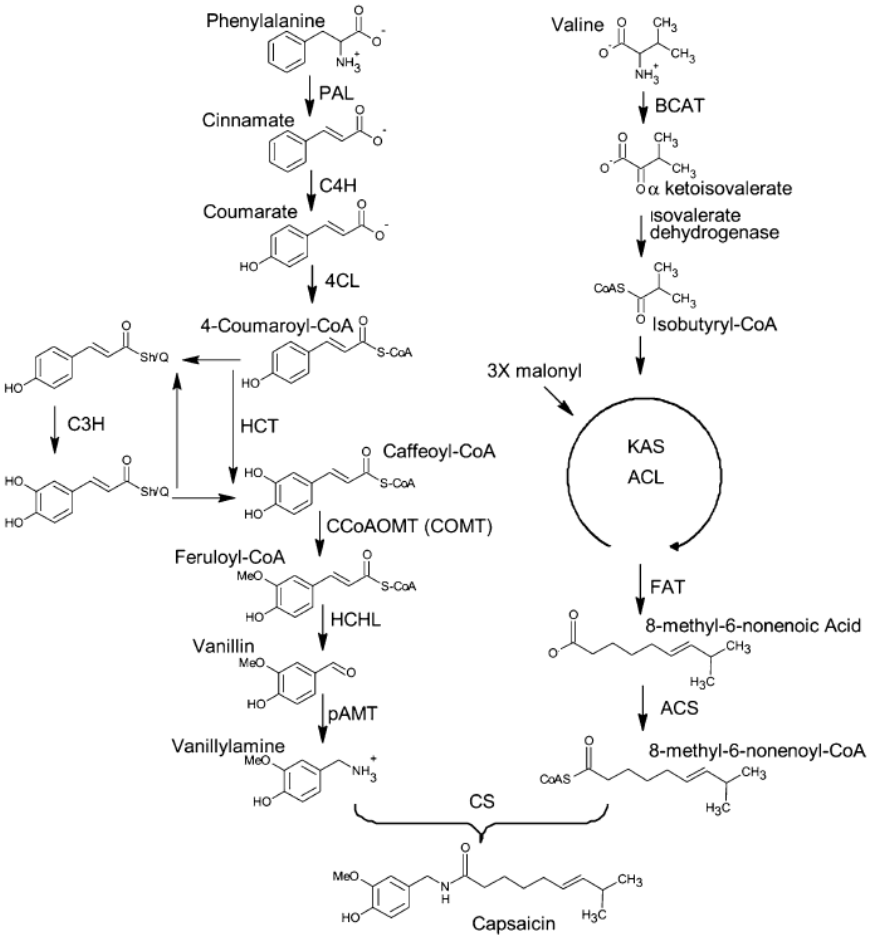

Figure 2.2 Capsaicinoid biosynthetic pathway (represented by capsaicin) via phenylpropanoid pathway*

*Abbreviation: PAL phenylalanine ammonia lyase, C4H cinnamate 4-hydroxylase, 4CL 4-coumaroyl-CoA ligase, HCT hydroxycinnamoyl transferase, C3H coumaroyl shikimate/quinate 3-hydroxylase, CCoAOMT caffeoyl-CoA 3-Omethyltransferase, COMT caffeic acid O-methyl transferase, HCHL hydroxycinnamoyl-CoA hydratase/lyase, pAMT putative aminotransferase, BCAT branched-chain amino acid transferase, KAS ketoacyl-ACP synthase, ACL acyl carrier protein, FAT acyl-ACP thioesterase, ACS acyl-CoA synthetase, CS capsaicin or capsaicinoid synthase (Steewart et al., 2005; Mazourek et al., 2009)

2.3. Capsaicinoid content in chili fruits

There are over 20 capsaicinoids in chili fruits, which are categorized into four major classes based on their molecular structures: (i) capsaicin group (70%), (ii) dihydrocapsaicin group (22%), (iii) nordihydrocapsaicin group (7%) and (iv) homocapsaicin and homodihydrocapsaicin analogue group (1%) (Conforti, Statti, and Menichini, 2007; Koleva-Gudeva, Mitrev, Maksimova, and Spasov, 2013).

Most of capsaicinoids are synthesized in placenta tissues; however, other tissues also synthesize capsicinoids in small quantity (Suzuki et al., 1980; Stewart et al., 2007). After being synthesized, the capsaicinoids are translocated to different parts of fruit and plant organs (Golubkina et al., 2014; Ananthan, Subhash, and Longvah, 2018). Table 2.1 shows capsaicinoid contents of various chili cultivars of *C. annumn*, in which only major capsaicinoid contents we shown. There is high variation in capsaicinoid contents; for instance, the values of capsaicin range from 54 to 2,480 μg/g dry weight, and the values of dihydrocapsaicin range from 40 to 15,360 μg/g dry weight.

The pungency level is expressed in Scoville Heat Unit, SHU (Scovlile, 1912). The SHU is a method to express pungency level by means of organoleptic test. Briefly, alcohol extracted capsaicin from dried chili is diluted with a solution of sugar and water until a panel of taste-testers cannot detect pungency (Scovlile, 1912). Nowadays, the SHU method has been replaced by chromatographic methods which are more reliable and accurate (Thomas, Schreiber, and Weisskopf, 1998).

Table 2.1 Capsaicinoid contents in various cultivars of *C. annuum* L.

Cultivar/Variety	Extraction method	Capsaicin μg/gD.W.	Dihydrocapsaicin μg/gD.W.	Reference
Cayenne cv. CA408	Ultrasound assisted extraction	54	40	Bae et al., 2014
Serrano cv. Tuxtlas	Ultrasound assisted extraction	81	88	Bae et al., 2014
Jeromin*	Microwave-assisted extraction	104	87	Barbero et al., 2016
Guajilla cv. San Luis	Ultrasound assisted extraction	120	170	Gonzalez-Zamora et al., 2013
Jariza*	Microwave-assisted extraction	154	106	Barbero et al., 2016
Jalapeno cv. Ixtapa	Ultrasound assisted extraction	169	114	Bae et al., 2014
Espelette	Ultrasound assisted extraction	211	203	Duelund and Mouritsen, 2016
Cayenne cv. Mesilla	Ultrasound assisted extraction	212	115	Bae et al., 2014
Jalapeno	Solvent extraction	214	147	Schmidt et al., 2017
Ancho cv. Don Matias	Ultrasound assisted extraction	250	290	Gonzalez-Zamora et al., 2013
Toftegaard Hot Banana	Ultrasound assisted extraction	382	314	Duelund and Mouritsen, 2016
Serrano cv. Don Diego	Ultrasound assisted extraction	530	1520	Gonzalez-Zamora et al., 2013
Puya	Ultrasound assisted extraction	550	1180	Gonzalez-Zamora et al., 2013
Mokho 2	Ultrasound assisted extraction	614	609	Sricharoen et al., 2016
Serrano	Solvent extraction	707	527	Giuffrida et al., 2012
Sinpezon	Solvent extraction	716	308	Giuffrida et al., 2012
Cayenne*	Ultrasound assisted extraction	717	779	Barbero et al., 2013
Bola*	Microwave-assisted extraction	975	90	Barbero et al., 2016
De arbol	Ultrasound assisted extraction	1070	5220	Gonzalez-Zamora et al., 2013
Jalapeno	Solvent extraction	1101	843	Giuffrida et al., 2012
Serrano	Ultrasound assisted extraction	1103	707	Duelund and Mouritsen, 2016
Huai Si Thon Kanlapaphruek	Ultrasound assisted extraction	1644	1089	Sricharoen et al., 2016
Yot Son Khem 80	Ultrasound assisted extraction	1826	1009	Sricharoen et al., 2016
Chiltepin	Ultrasound assisted extraction	2170	15360	Gonzalez-Zamora et al., 2013
Jalapeno cv. Don Julio	Ultrasound assisted extraction	2480	8030	Gonzalez-Zamora et al., 2013

*values are presented as μg/gF.W.

2.4. Anticancer properties of capsaicin

In spite of the fact that there are many advanced approaches against cancer, it is still the major cause of morbidity and mortality worldwide (Wiseman, 2008). In contrast to most healthy cells, cancer cells have abilities to resist growth-inhibitory signals, proliferate independently from growth-stimulatory factors, replicate without limit, evade apoptosis, and acquire both invasive and angiogenesis properties (Hanahan and Weinberg, 2000).

As invasiveness, toxicity, and ineffectiveness of current therapeutic approaches, using natural products and dietary compounds has become more intense to prevent and remedy cancers (Reddy, Odhav, and Bhoola, 2003). Several epidemiological researches have shown that consumption of fruits and vegetables significantly decreased the risks of developing cancers. Moreover, some phytochemical compounds are able to suppress at any stages of cancer from initiation stage to metastasis stage (Reddy, Odhav, and Bhoola, 2003).

Capsaicin has been used for many medicinal purposes because it has analgesic, antioxidant, anti-inflammatory, and anti-obesity properties (Simone, Baumann, and LaMotte, 1989; Kim et al., 2003; and Kang et al., 2007 Galano and Martinez, 2012; Brederson, Kym, and Szallasi, 2013). The anticancer mechanism of capsaicin has been proposed that it increased apoptosis and cell-cycle arrest; nevertheless, the exact cellular mechanisms are still not completely comprehended (Ito et al., 2004).

2.4.1 Capsaicin and apoptosis

One of mechanisms which can eliminate many types of cancer is apoptosis. However, several types of cancer acquire the ability to disrupt apoptotic pathways and improve anti-apoptotic mechanism (Hanahan and Weinberg, 2011). Many researches show that capsaicin has the ability to induce apoptosis in over 40 distinct cancer cell lines such as lung, colonic, leukemia, skin, prostatic, pancreatic, and endothelial cells (Ito et al., 2004; Lee et al., 2004; Min et al., 2004; Mori et al., 2006; Athanasiou et al.,

2007; Kim et al., 2007; Pramanik, Srinivas, and Srivastava, 2011); however, capsaicin does not induce apoptosis in normal cells (Surh, 2002; Ito et al., 2004; Pramanik, Srinivas, and Srivastava, 2011; Bley et al., 2012).

Capsaicin targets several proteins which are involved in the initiation of mitochondrial death pathway leading to apoptosis in various types of cancer cell lines (Amantini et al., 2009). For example, capsaicin activates the cluster of differentiation 95 (CD95)-mediated intrinsic and extrinsic apoptosis pathways (Amantini et al., 2009) and suppresses expression of anti-apoptotic protein, B-cell lymphoma 2, leading to caspase-9 and caspase-3 activation, loss of mitochondrial membrane potential, and increase in cytochrome c release (Kim, Trudel, and Wogan, 2009).

The capsaicin receptors, transient receptor potential vanilloids (TRPVs), promote Ca^{2+}-mediated mitochondrial damage and cytochrome c release (Amantini et al., 2007; Amantini et al., 2009). In many types of cancer, TRPV1 mediates the proapoptotic activity of capsaicin (Kim, Kim, Oh, and Jin, 2006; Amantini et al., 2007; Amantini et al., 2009; Caprodossi et al., 2011; Zheng et al., 2016).

The p53-mediated tumor suppression is a mechanism against cancer (Michael and Oren, 2002). The anticancer mechanism of capsaicin was found that it induces p53 phosphorylation at the Ser-15 residue (Ito et al., 2004) and p53 acetylation through down-regulation of sirtuin 1 (Lee, Chen, Su, and Chueh, 2015). The experiment which treated urothelial cells with capsaicin shows that capsaicin increases the expression of p53 and phosphorylation at Ser-15, Ser-20, and Ser-392 residues, leading to increased apoptosis (Amantini et al., 2009). These results might be the clues that p53 is a target of the anticancer activity of capsaicin.

In several types of cancer cells, beta-catenin is constitutively active (Lee, Richardson, Dashwood, and Baek, 2012). The deregulation of beta-catenin-dependent signaling leads to the development of malignancies. The study of colorectal cancer cells found that capsaicin induced down-regulated beta-catenin transcription and

reduces beta-catenin stability resulting in apoptosis (Zewdie and Bosland, 2001). In addition, capsaicin also increased apoptosis via the activation of novel pro-apopotic gene, NSAID-activated gene-1, and the activation might be a novel pathway associated with phosphorylation of CCAAT/enhancer binding beta protein by glycogen synthase kinase 3β and protein kinase C (Lee, Krisanapun, and Baek, 2010).

2.4.2 Capsaicin and cell cycle arrest

Cell cycle is divided into G_0/G_1, S and G_2/M phases with DNA checkpoints each phase to assure the DNA integrity. Alteration of cell cycle pathway dramatically increases risk of cancer. Thus, the cell cycle and growth arrest are considered as one of mechanism against cancer (Kastan and Bartek, 2004).

In human esophageal carcinoma cells, capsaicin induced G_0/G_1 phase arrest increasing p21 and decreasing cyclin-dependent kinases 4 (CDK4), cyclin-dependent kinases 6 (CDK6), and cyclin E (Wu et al., 2006; Chen et al., 2012). Moreover, treatment with capsaicin on human cancer KB cells inhibited proliferation and induced cell-cycle arrest (Lin et al., 2013). In *in vitro* study of colon cancer cell lines, capsaicin induced dose-dependent reduction in cyclin D1 (Lee, Richardson, Dashwood, and Baek, 2012).

2.5. Cervical cancer

The forth rank of common cancer in women and the seventh rank of common cancer in the world is cervical cancer. This cancer has found in many parts of the world. The major incidence of cervical cancer was found in less developed area (Vaccarella, Laversanne, Ferlay, and Bray, 2017). In 2012, there were five countries having the highest incidence of cervical cancer cases including India (122,844 cases), China (61,619 cases), Indonesia (20,928 cases), Brazil (18,503 cases), and the Russian Federation (15,342 cases) (Momenimovahed et al., 2017).

2.5.1 Characteristics of SiHa cells

The SiHa cells are squamous epithelial cells (Figure 2.3) (Friedl, Kimura, Osato, and Ito, 1970) containing human papillomavirus 16 (HPV 16). Under an electron microscope, the cells contain typical desmosomes at the cell junctions and an abundance of intermediate filaments, namely tonofilaments, in the cytoplasm (Figure 2.4) (Friedl et al., 1970).

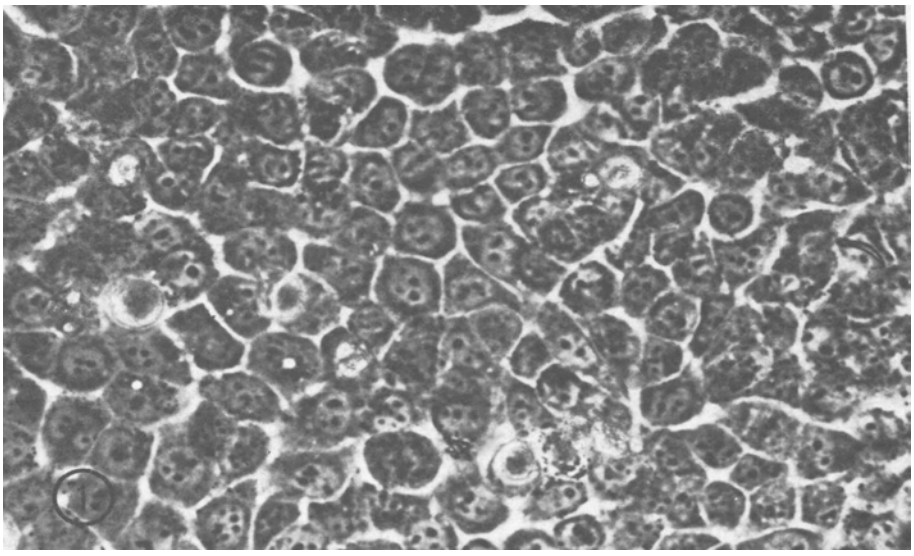

Figure 2.3 A monolayer culture of SiHa cells in 20th cell passage (Friedl et al., 1970)

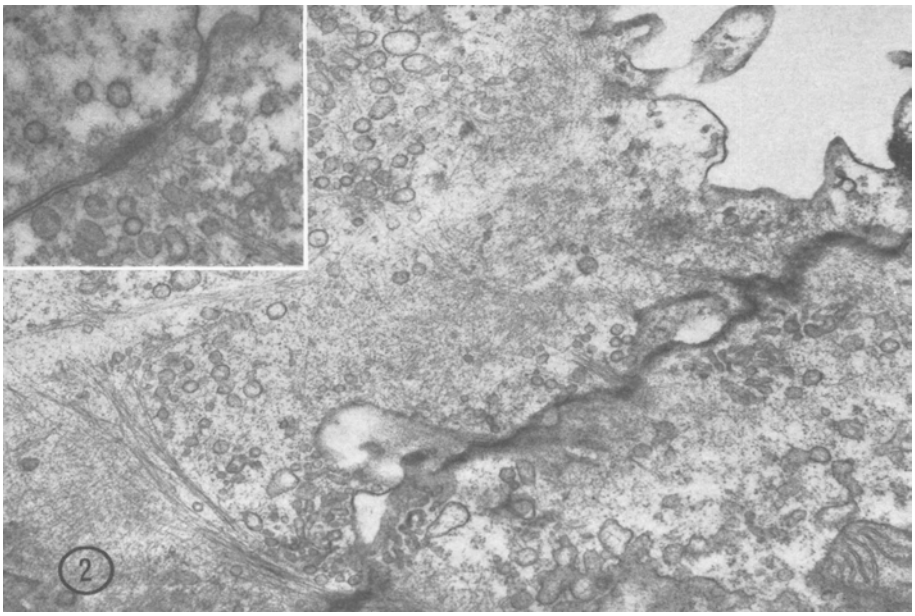

Figure 2.4 The electron micrograph of SiHa cells with many desmosomes and tonofilaments (Friedl et al., 1970)

CHAPTER 3

MATERIALS AND METHODS

3.1. Collection of plant materials

Fresh chilies (*Capsicum annuum* L. cv. Jinda and cv. Cayenne) purchased from markets were dried at 60°C for 72 hours in an oven. Then the dried chili fruits were ground into smaller size of about 0.75 mm using a grinder (Schmidt et al., 2017). Ground chili powder was kept in a desiccator for future analysis.

3.2. Extraction of chili fruits

In this process, the extraction of chili fruits was conducted using the same procedures as Schmidt et al. (2017) with some modifications. Briefly, 0.5 g of the dried and ground chili fruits was extracted by incubating with 12.5 ml of acetonitrile/water (80:20 v/v) for 60 min at 150 rpm on an orbital shaker. Then, the mixture was centrifuged at 3000 rpm, 10 °C, for 15 minutes. The supernatant was taken and kept in a container, and the residue was extracted again using the same protocol as previously described. The supernatant was brought to a volume of 25 ml with 80% (v/v) acetonitrile. Subsequently, an aliquot of 1.5 ml was solvent evaporated using a vacuum centrifuge for 4 hours. The obtained dried residues were stored at −18 °C until further analysis.

3.3. Capsaicinoid analysis

The dried residues of each cultivar, Jinda and Cayenne pepper, were submitted to Food Research and Testing Laboratory (FRTL), Faculty of Science, Chulalongkorn University to determine the capsaicin contents by using high performance liquid chromatography (HPLC) analysis. The residues (extracted from 0.03 g DW chili sample) were dissolved with 2 ml of acetonitrile and filtered before having been injected to the HPLC system (Waters Associates model ALC/GPC equipped with an M.6000A pump, a U6K injection). The column was eclipse XDB-C18 (5 µm, 46 x 150 mm); the mobile

phase was 0.02 M NaH_2PO_4 with a flow rate 1.000 ml/min and an injection volume of 60 µl. The UV detection was at 280 nm. Capsaicin content was determined based on a calibration curve. Capsaicin (≥50 %, Roth, Germany) was used as a standard. The standard capsaicin was dissolved with HPLC grade acetonitrile. A standard curve was prepared with serial dilutions of 50, 100, 200, 300 and 500 ppm. Two sub-samples were measured for both chili extract and the standard.

3.4. Scoville Heat Unit (SHU) conversion

The quantity of capsaicin in each cultivar, Jinda and Cayenne pepper, was used to calculate the Scoville Heat Unit (SHU) as an equation: SHU= 1.6×10^7 x (capsaicin in g/g D.W.) (Todd, Bensinger, and Bifu, 1977).

3.5. Cell culture

The human cervical cancer SiHa cells (American Type Culture Collection), which were kindly provided by Associate Professor Dr. Pattamawadee Yanatatsaneejit, were routinely grown in Dulbecco's Modified Eagle Medium (DMEM) with 10 % (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic-antimycotic. All cell cultures were incubated at 37°C in humidified air containing 5% CO_2 under sterile condition.

3.6. Viability assay

To examine the effects of crude extracts on SiHa, exponentially growing cells were seeded at 10^4 cells/well in 96-well plates. After 48 hours of incubation, when a partial monolayer was formed, the medium was removed and 220 µl of the medium containing chili extract or solvent (DMSO) was added. The crude extracts applied to SiHa cells were prepared by dissolving with DMSO to final concentrations of 100, 200, 300, 400, and 500 µM capsaicin equivalent. All crude extract solution contained 0.1% DMSO; therefore 0.1% DMSO was used as a solvent control. After adding crude extract solution, SiHa cells were re-incubated at 37°C and 5% CO_2 for 24 hours. Then, the medium was removed and 100 µl of 0.1% (w/v) alamarBlue was added to each well and the plate were incubated at 37°C for 1 hour. Then the fluorescence of the test

reagent (resazurin) was measured using SpectraMax® M3 Microplate Reader with the excitation/emission wavelengths set at 570/600 nm. The absorbance of a pink-fluorescent product, resorufin, measured in control cells was set to 100% viability.

3.7 Statistical analysis

Data analysis was performed using SPSS software for Windows (SPSS Statistics 22, SPSS Inc., Chicago, IL, USA). Statistical comparisons among groups of viability assay were conducted using the one-way analysis of variance (ANOVA). Viability data were tested for normality distribution. The level of significance tests was set at $p < 0.05$.

CHAPTER 4

RESULTS

4.1. Chili crude extract preparation

Ground chili fruits (0.5 grams) of Jinda and Cayenne were extracted using acetonitrile which yielded approximately 0.03 grams of chili crude extract. The crude extract was seen as orange oily liquid mixed with solid residues (Figure 4.1).

Figure 4.1 Chili fruits and ground chili powder (A) and chili crude extract (B)

4.2. Capsaicinoid analysis

Major capsaicinoids presenting in the chili extracts were identified as shown in the high performance liquid chromatography (HPLC) chromatograms (Figure 4.2). According to the standard HPLC chromatogram (Figure A1, Appendix) and Johnson, Majors, Werum, and Reiche (1979), peaks 1 and 2 detected at the retention times of 3.743 and 4.786 min were indicated as capsaicin and dihydrocapsaicin, respectively (Figure 1A). Then, capsaicinoid contents were calculated from relative integration signal areas, compared to standard capsaicinoid signal areas. The capsaicin content of Jinda chili was 75.145±1.418 mg/g dry weight and Cayenne was 1.267±0.094 mg/g dry weight (Table 4.1). In addition, Jinda chili had dihydrocapsaicin of about 97.460±1.254 mg/g dry weight, which was in a similar range as capsaicin. For Cayenne, dihydrocapsaicin content was 0.633±0.330 mg/g dry weight, which was approximately a half fold of its capsaicin content (Table 4.1).

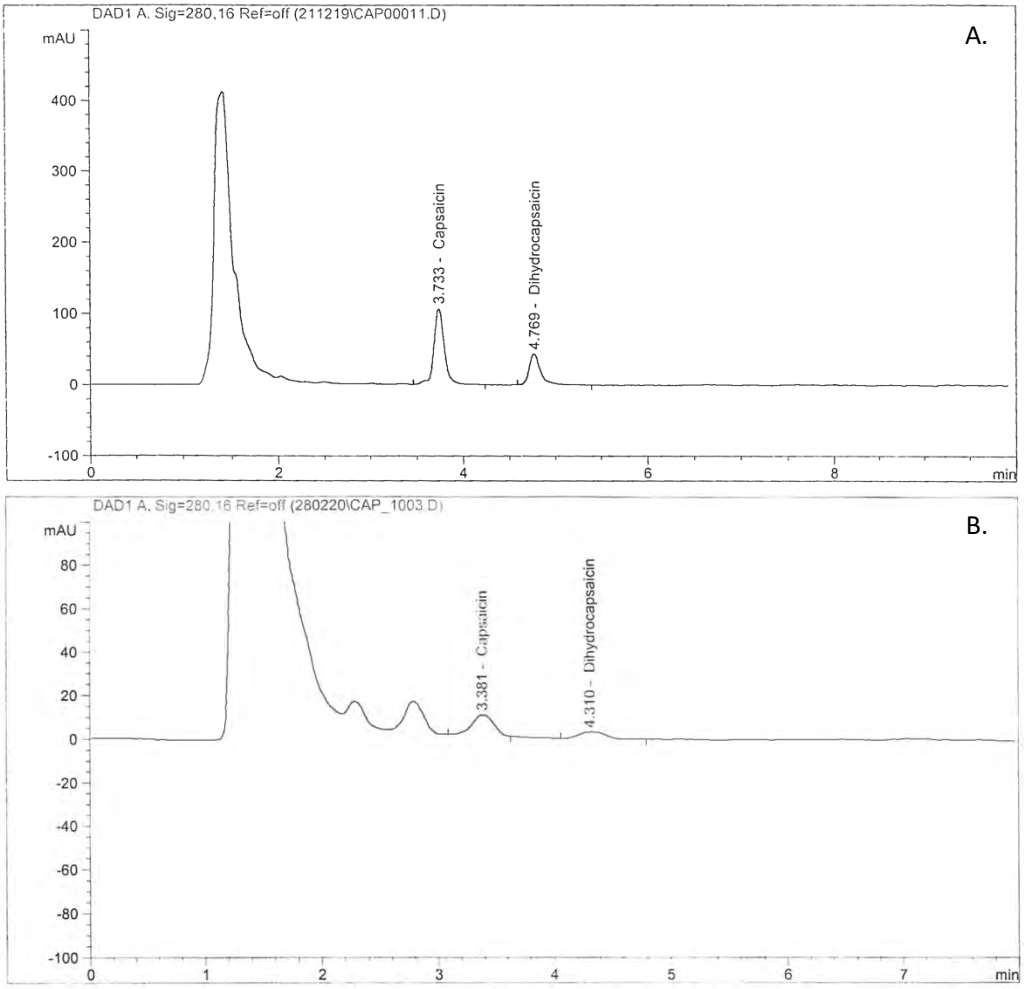

Figure 4.2 HPLC chromatograms of capsaicinoid in Jinda (A) and Cayenne (B) chili crude extract

Table 4.1 Capsaicinoid contents and Scoville Heat Unit of dried chili

Chili cultivar	Capsaicin (mg/g DW)[a]	Dihydrocapsaicin (mg/g DW)[a]	Scoville Heat Unit (SHU)
Jinda	75.145±1.418	97.460±1.254	1202318 ± 22689
Cayenne	1.267±0.094	0.633±0.330	20267±1508

[a] values in mg/g DW ± SD, n = 2

4.3. Scoville Heat Unit (SHU) conversion

The Scoville Heat Unit (SHU) of Jinda chili was 1202318 ± 22689 SHU, which was 40 folds higher than the SHU of Cayenne chili (20267±1508 SHU) (Table 4.1).

4.4. Viability assay

For anticancer potentiality evaluation, the effects of crude extract (Jinda) on the viability of SiHa cells was assessed using alamarBlue (Figure 4.3). Based on HPLC results, chili crude extract was dissolved with 0.1% (v/v) DMSO to the final concentrations of 0, 100, 200, 300, 400, and 500 μM equivalent concentrations of capsaicin. The viability of SiHa cells treated with each concentration was expressed as a relative value to the control group treated with 0.1% (v/v) DMSO. It was found that there was no statistically significant difference of viability among each concentration including the control. In addition, viability data were normally distributed (Figure A2, Appendix). However, it was observed that SiHa cells treated with the chili crude extract at low concentrations tended to have increasing viabilities; at 200 μM capsaicin equivalent the viability was 128%. In conclusion, Jinda chili crude extracts showed no cytotoxicity effect in SiHa cell line.

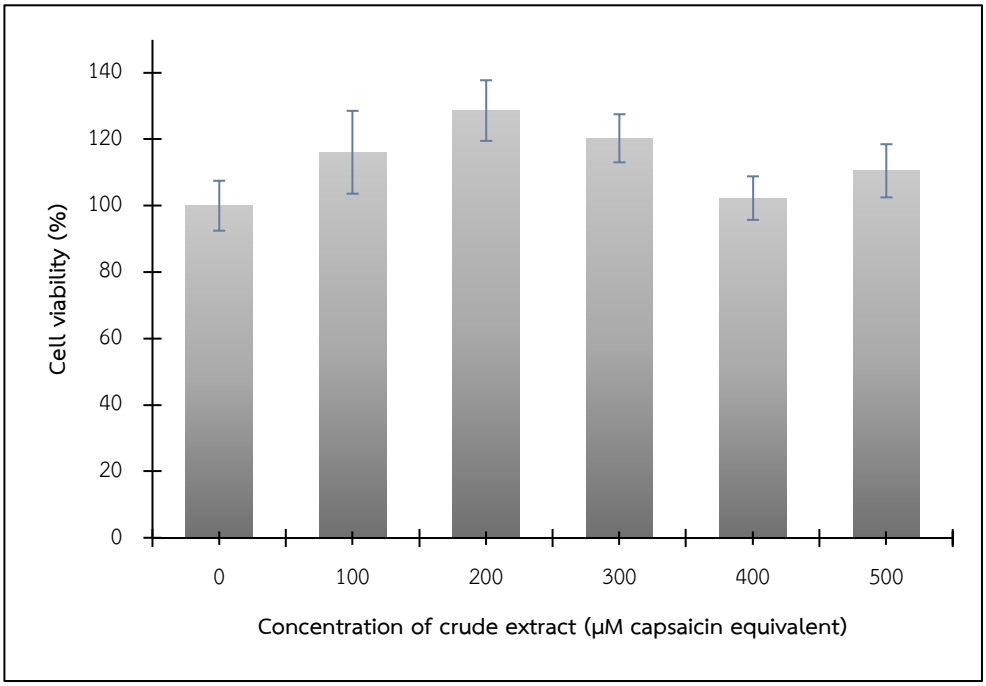

Figure 4.3 Dose response curve of viability of SiHa cells after being treated with crude extract of Jinda chili for 24 hours. The concentration of crude extract is shown as capsaicin equivalent. Fluorescent signal in control cells (treated with 0.1% DMSO) was set to 100% of viability. Mean± SE (n = 9). There was no significant mean difference according to ANOVA ($p \leq 0.05$).

In addition, a boxplot presented a high variation of SiHa cell responses to Jinda chili crude extract (Figure 4.4). Control cells (n= 6) treated with DMSO, having median viability of 107.25%, showed the lowest of variation. In contrast, SiHa cells (n=9) highly varied in the viability when exposed to Jinda chili crude extract. Although the median values were rather similar among 5 concentrations of crude extract treatments, whisker length and box width indicated heterogeneity of effect within SiHa cell population, especially at low concentration of crude extract. Thus, SiHa cellular responses to standard capsaicin are required for comprehensive interpretation.

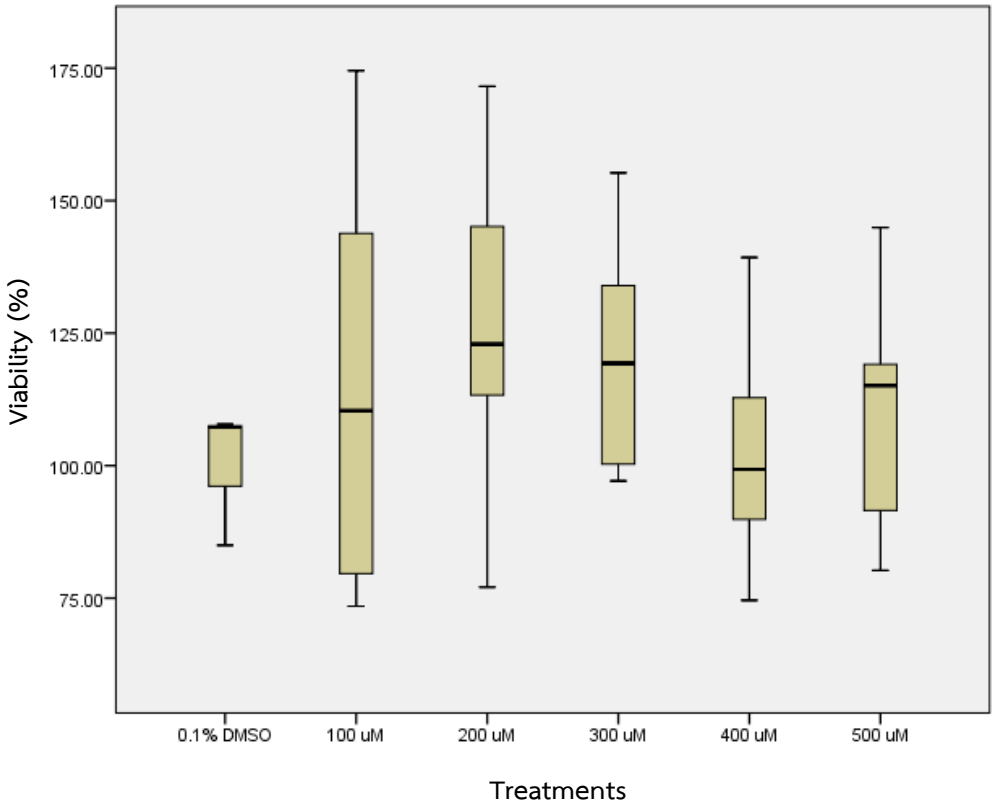

Figure 4.4 Boxplot representing the viability of SiHa cells when exposed to different Jinda chili crude extract for 24 hours; for 100-500 μM capsaicin equivalent treatments (n = 9) and 0.1% DMSO treatment (n = 6).

CHAPTER 5

DISCUSSION AND CONCLUSION

In this study, we used the chili crude extracts to preliminarily investigate the anticancer activity of capsaicin on cervical cancer cell line. However, the capsaicin content of the crude extracts was highly varied based on the extraction procedure. To optimize the yield, we used dried chili fruits since this kind of fruit gave the highest yield of capsaicin concentration. According to Padilla and Yahia (1998), dried fruits gave the highest yield of capsaicin and total capsaicinoids but the lowest dihydrocapsaicin concentration when compared to fresh and frozen fruits (Padilla and Yahia, 1998). In term of solvent, Nagoth, Preetam Raj, and Antoine's study (2014) concluded that acetonitrile and acetone gave higher yield of capsaicin concentration than other solvents such as methanol, hexane, and dimethylsulfoxide (DMSO). In addition, extraction with acetonitrile had higher purity of capsaicin than acetone and other solvents (Nagoth, Preetam Raj, and Antoine, 2014). Thus, we used acetonitrile as a solvent for capsaicin extraction.

By applying solvent extraction method with acetronitrile on dried chili fruits, our results show that different cultivars of *C. annuum*, Cayenne and Jinda, highly varied in capsaicin (and dihydrocapsaicin) contents. In this study, capsaicin content in Cayenne chili was 1267 µg/g dry weight, which is comparable to the values reported previously (Barbero et al., 2013; Bae et al., 2014). Furthermore, Jinda chili which is known for its high pungency level contains higher quantity of capsaicin (75145 µg/g dry weight) than Cayenne. In addition, the SHU of dried Jinda chili fruits reported here is approximately 30 folds higher than the SHU of the fresh fruit reported as 28050-40200 SHU (Kaewprasit and Koonngien, 2004). In total, the results affirm intraspecific variation of capsaicin contents in *C. annuum*. In addition, we suggest *C. annuum* cv. Jinda as a potential source of capsaicinoids for in-depth research on pharmaceutical potentiality.

Based on viability assay, there was no anticancer activity of Jinda crude extracts in SiHa cell line. The concentration of chili crude extract applied in this study (up to 500 μM capsaicin equivalent) are in the range of capsaicin concentration that highly reduced cell viability (Chang et al., 2011). Several publications described the effect of hyper-osmotic stress on cancer cell which resulted in the decrease of both cell migration and cell proliferation (Jung, Park, Jeon, and Kwon, 2011; La Porta et al., 2015; Miermont, Lee, Adriani, and Kamm, 2019). Thus, we avoided the effect of hyper-osmotic stress on the cells during viability assay by which the capsaicin equivalent concentrations of the chili crude extracts were not exceeded 500 μM. However, the pure capsaicin (500 μM) showed the capacity to decrease cell viability through cell apoptosis induction in various types of cancers such as prostate cancer and breast cancer (Chow, et al., 2007; Chang et al., 2011). There are other kinds of secondary metabolites, i.e. flavonoids and carotenoids, in chili fruits (Bae et al., 2014; Kim et al., 2016), the antioxidant and anti-inflammation properties of these compounds may cover up the cytotoxicity effects of capsaicin. In addition, a tendency of having increasing viability (e.g. 128% viability with 500 μM capsaicin equivalent) raises a question whether the chili crude extract enhances the proliferation of cancer cell line. To affirm this speculation, pure capsaicin should be applied to SiHa cells for comparison, with cell viability assay and cellular morphological change of cervical cancer cells investigated.

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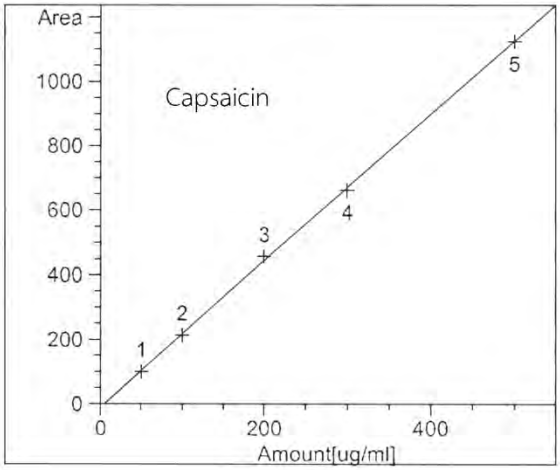
Appendix



Capsaicin at exp. RT: 3.787

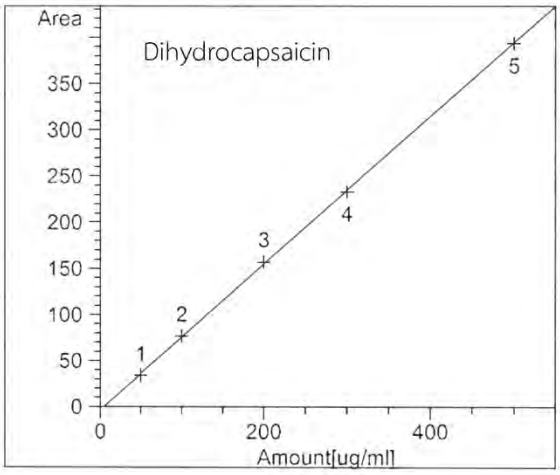
DAD1 A, Sig = 280, 16 Ref = off

Correlation: 0.99981 Residual Std. Dev.: 9.20718

Formula: y = mx + b

m : 2.26589 b : -10.50312

x : Amount [µg/mL] y : Area



Dihydrocapsaicin at exp. RT: 4.839

DAD1 A, Sig = 280, 16 Ref = off

Correlation: 0.99993 Residual Std. Dev.: 1.93669

Formula: y = mx + b

m : 7.96457e-1 b : -4.26518

x : Amount [µg/mL] y : Area

Figure A1 Calibration curves of standard capsaicin and dihydrocapsaicin

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	groups	Statistic	df	Sig.	Statistic	df	Sig.
treatments	0.1% DMSO	.341	6	.028	.802	6	.061
	100 uM	.168	9	.200*	.915	9	.355
	200 uM	.172	9	.200*	.963	9	.827
	300 uM	.228	9	.193	.897	9	.238
	400 uM	.144	9	.200*	.969	9	.890
	500 uM	.214	9	.200*	.899	9	.246

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure A2 Normality test of viability of SiHa cells when exposed to different Jinda chili crude extract for 24 hours; Treatment groups: 100-500 μM capsaicin equivalent (n = 9) and 0.1% DMSO (n = 6).