ผลของแอสปาร์เทมต่อการเคลื่อนที่และการเพิ่มจำนวนของเซลล์เยื่อบุผิวลำไส้มนุษย์

นางสาวธวิวรรน์ สวัสดิ์โสภานนท์

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EFFECT OF ASPARTAME ON MIGRATION AND PROLIFERATION OF HUMAN INTESTINAL EPITHELIAL CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Food Chemistry and Medical Nutrition
Department of Food and Pharmaceutical Chemistry
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Thesis Title	EFFECT OF ASPARTAME ON MIGRATION AND PROLIFERATION OF HUMAN INTESTINAL EPITHELIAL CELLS
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ธวิวรรน์ สวัสดิ์โสภานนท์ : ผลของแอสปาร์เทมต่อการเคลื่อนที่และการเพิ่มจำนวนของเซลล์เยื่อบุผิวลำไส้มนุษย์ (EFFECT OF ASPARTAME ON MIGRATION AND PROLIFERATION OF HUMAN INTESTINAL EPITHELIAL CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภญ. ดร.กุลวรา เมฆสวรรค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภก. ดร.ปิติ จันทร์วรโชติ, 90 หน้า.

แอสปาร์เทมเป็นสารให้ความหวานสังเคราะห์ที่ใช้กันอย่างแพร่หลายในอาหารหลายประเภท ถึงแม้ว่าจะมีการศึกษาหลายการศึกษาเกี่ยวกับความเป็นพิษของแอสปาร์เทม แต่ยังไม่มีการศึกษาถึงผลของแอสปาร์เทมต่อการสมานแผลของเซลล์เยื่อบุผิวลำไส้มนุษย์ เนื่องจากกระบวนการเคลื่อนที่ (migration) และแบ่งตัวเพิ่มจำนวน (proliferation) ของเซลล์เยื่อบุผิวในทางเดินอาหาร เป็นกระบวนการที่สำคัญต่อการสมานแผลเมื่อเกิดบาดแผล เพื่อคงสภาพความแข็งแรงของแนวเซลล์เยื่อบุผิวให้สามารถทำหน้าที่เป็นด่านป้องกันต่อไปได้ ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของแอสปาร์เทมต่อการเคลื่อนที่และการเพิ่มจำนวนของเซลล์เยื่อบุผิวลำไส้มนุษย์ และศึกษากลไกที่เกี่ยวข้องที่ทำให้เกิดผลดังกล่าว การศึกษานี้เป็นการศึกษาแรกที่พบว่าแอสปาร์เทมมีผลยับยั้งการเคลื่อนที่ของเซลล์เยื่อบุผิวลำไส้อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อการศึกษาด้วยวิธี wound healing assay และ Boyden chamber cell migration assay แต่ไม่มีผลต่อการแบ่งตัวเพิ่มจำนวนของเซลล์เยื่อบุผิวลำไส้ นอกจากนี้ยังพบว่า แอสปาร์เทมมีผลลดการสร้าง lamellipodia ของเซลล์ในระหว่างการเคลื่อนที่อย่างมีนัยสำคัญ ($p < 0.05$) สำหรับกลไกในการยับยั้งการเคลื่อนที่ของเซลล์เยื่อบุผิวลำไส้มนุษย์พบว่า แอสปาร์เทมลดปริมาณโปรตีนที่ควบคุมการเคลื่อนที่ของเซลล์ คือ phosphorylated FAK (pFAK), phosphorylated Akt (pAkt), caveolin-1 (Cav-1), Rac1-GTP และ RhoA-GTP การศึกษานี้แสดงให้เห็นว่า แอสปาร์เทมมีผลต่อการสมานแผลโดยยับยั้งการเคลื่อนที่ของเซลล์เยื่อบุผิวลำไส้มนุษย์ ซึ่งข้อมูลที่ได้จะเป็นประโยชน์ต่อผู้ป่วยที่มีภาวะแผลในทางเดินอาหารให้ระมัดระวังการบริโภคผลิตภัณฑ์ที่มีแอสปาร์เทมเป็นส่วนประกอบ เพื่อไม่ให้เกิดผลเสียต่อกระบวนการสมานแผล

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TAWIWAN SAWADSOPANON: EFFECT OF ASPARTAME ON MIGRATION AND PROLIFERATION OF HUMAN INTESTINAL EPITHELIAL CELLS. ADVISOR: ASST. PROF. KULWARA MEKSAWAN, Ph.D., CO-ADVISOR: ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D., 90 pp.

Aspartame is the most common artificial sweeteners in various food products. Although many studies about toxicity of aspartame are conducted, there are no studies about the effect of aspartame on intestinal wound healing. As migration and proliferation of the epithelial cells along gastro-intestinal tract (GI) are important processes for wound healing to maintain the intestinal barrier, the purpose of this study was therefore to investigate the effect of aspartame on migration and proliferation of human intestinal epithelial cells. Possible mechanism involved in these processes was also examined. The present study revealed for the first time that aspartame at non-toxic concentrations significantly inhibited intestinal epithelial cell migration determined by both wound healing and Boyden chamber cell migration assays ($p < 0.05$) but had no effect on proliferation of the cells. Furthermore, the numbers of lamellipodia per cell significantly reduced in aspartame-treated cells ($p < 0.05$). Regarding molecular mechanisms, it was found that aspartame suppressed the cellular levels of the migration regulatory proteins namely phosphorylated FAK, phosphorylated Akt, Cav-1, Rac1-GTP, and RhoA-GTP. Altogether, this study indicated that aspartame might affect the intestinal wound healing through suppression of cell migration. This information may be beneficial to patients with GI ulcer in considering consumption of aspartame-containing products to prevent the negative effect on wound healing process.

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LIST OF ABBREVIATIONS

ANOVA	one-way analysis of variance
ADI	acceptable daily intake
Akt	protein kinase B
APT	aspartame
AQPs	aquaporins
BSA	bovine serum albumin
Cells/ml	cells per milliliter
Cav-1	caveolin-1
CO_2	carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dsDNA	double stranded DNA
ERK	extracellular signal-regulated kinase
et al.	et alibi, and others
FAK	focal adhesion kinase
FBS	fetal bovine serum
GI	gastro-intestinal
Glu	glutamine
h	hour, hours

IBD	inflammatory bowel disease
JNK	Jun N-terminus kinase
kcal/g	kilocalorie per gram
MAPK	mitogen-activated protein kinase
mg/day	milligram per day
mg/mL	milligram per milliliter
mg/kg BW/day	milligram per kilogram body weight per day
min	minute, minutes
mM	millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSAIDs	nonsteroidal anti-inflammatory drugs
NEAA	non-essential amino acids
OD	optical intensity
p-Akt	phosphorylated Akt
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
p-ERK	phosphorylated ERK
p-FAK	phosphorylated FAK
PI	propidium iodide
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PI3K	phosphatidylinositol 3-kinase

PI3K/Akt	phosphatidylinositol 3-kinase/protein kinase B
p	*p* value
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
Ser	serine
TBST	Tris-buffred saline with 0.1% Tween
Thr	threonine
Tyr	tyrosine
%	percentage
°C	degree Celsius
µg/ml	microgram per milliliter
µL	microliter
µm	micrometer
µM	micromolar

CHAPTER I
INTRODUCTION

1.1 Background and Rationale

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is a low-caloric artificial sweetener which currently presents in various products consumed by lots of people around the world (Ogata and Hayes, 2012). It was discovered in 1965 and approved for an ordinary use by the United States Food and Drug Administration (FDA) in 1996 (Kroger, Meister and Kava, 2006). Aspartame provides energy about 4 kcal/g. It is approximately 160 to 200 times sweeter than sucrose; therefore, only small amounts with negligible calories are incorporated into sweeten foods or beverages (Shankar, Ahuja and Sriram, 2013). This sweetener has been used in various food products including tabletop sweeteners, dairy products, beverages, pharmaceuticals, and medical foods (Whitehouse, Boullata and McCauley, 2008). The acceptable daily intake (ADI) of aspartame has been set at 40 mg/kg BW/day in the European Union (SCF, 1985) and 50 mg/kg BW/day in the United States for both children and adolescents (FDA, 1984). According to the study in the United States, the average consumption of aspartame is about 4.85 mg/kg BW/day (Marinovich et al., 2013).

Nowadays, aspartame is authorized to use in variety of food products (Yang, 2010). Although it is approved to use in the food products, the studies on its safety are still continuously conducted. No evidences showed genotoxicity and

carcinogenicity of aspartame at the concentration below the established ADI (Kirkland and Gatehouse, 2015). The human epidemological study found no association between aspartame and the risk of common cancers (Chattopadhyay, Raychaudhuri and Chakraborty, 2014). However, increased risk of lymphoma and leukemia in people consuming beverages added with aspartame as an artificial sweetener were reported (Schernhammer et al., 2012).

Aspartame is a methyl ester of the two amino acids, aspartic acid and phenylalanine. Under digestion, aspartame is efficiently hydrolyzed by the digestive enzyme to phenylalanine, aspartic acid and a small amount of methanol. It is absorbed into the circulatory system in the form of these amino acids in the similar way to other dietary protein consumption. Therefore, the intact form of aspartame was not found in the human blood system (Whitehouse et al., 2008). Considering GI biology and aspartame chemical structure, the epithelial cell lining of GI tract has a considerable chance to expose to aspartame. These observations lead to the present research question that aspartame may have an impact on epithelial cell behaviors.

The gastrointestinal tract involves many organs and endocrine glands which have coordination function of several major physiological processes during normal digestion (Pandol, Raybould and Yee, 2009). The gastrointestinal canal is lined with epithelial cells which provide a surface layer for transportation of products or nutrients from digestion and water as well as electrolytes across the epithelial cells to the blood or lymph system. The epithelial lining can be wounded in many conditions such as

exposure of chemicals and pharmaceutical products, bacterial infection, as well as spontaneous injury occurring during normal digestion (Rubin, 2009). The intestinal wound makes the epithelial lining loss its protective barrier; therefore, the recovery process of intestinal epithelium barrier is necessary to maintain its integrity.

Wound healing is a complex biological process involving the activation and balance of cell migration and proliferation processes (Iizuka and Konno, 2011). Cell migration is the process that epithelial cells surrounding the wound border start to migrate from the areas adjacent to the denuded area, and cell proliferation is the process that cells divide to increase the cell number in order to complete the barrier integrity (Sturm and Dignass, 2008). Epithelium restitution occurs rapidly by migration of epithelial cell within minutes to hours to prevent severity of mucosal damage. Following that, the regeneration through cell proliferation is the latter process at a few days to complete cell recovery (Göke and Podolsky, 1996; Silen and Ito, 1985). All of these processes are associated with various regulatory proteins.

The migration of epithelial cells is a complex biological mechanism which requires the integration of various cell responses (Ortega-Carrion, Feo-Lucas and Vicente-Manzanares, 2016). Cell migration process is associated with the change of cell cytoskeleton and focal adhesion forming through the function of various signaling molecules such as integrins, focal adhesion kinase (FAK), protein kinase B (Akt), caveolin-1 (Cav-1), Rac1-GTP, and RhoA-GTP (Huttenlocher, Sandborg and Horwitz, 1995; Navarro, Anand-apte and Parat, 2004). Many studies have indicated that FAK

signaling regulates the formation of focal adhesions in cells through several pathways, including induction of phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt)-dependent pathway. The PI3K/Akt-dependent pathway is associated with Akt phosphorylation and the regulation of the small GTPases, Rho-GTP and Rac-GTP (Burridge and Chrzanowska-Wodnicka, 1996; Larsen, Tremblay and Yamada, 2003; Mitra, Hanson and Schlaepfer, 2005). In addition, activation of Cav-1 is found to promote cell migration via PI3K/Akt pathway and activation of Rho family (Chanvorachote, Chunhacha and Pongrakhananon, 2014). The movement of cell is motivated by actin organization in response to cellular signals. The Rho family, mainly Rac1-GTP and RhoA-GTP plays a role in cell motility in promoting organization of the cytoskeleton (Ridley, 2001).

Following the movement of cells into the denuded area, cell proliferation occurs to replace the lost epithelial cell populations. Cell proliferation is a process which increases the number of cells (Iizuka and Konno, 2011). Several proteins including extracellular signal-regulated kinase (ERK), a member of mitogen-activated protein kinase (MAPK) family and Akt were found to be associated with increased cell population (Huang, Jacobson and Schaller, 2004; Larsen et al., 2003). MAPK signaling pathways are associated with ERK1/2 activation which may be regulated by PI3K/Akt pathway in order to mediate cell growth (Mebratu and Tesfaigzi, 2009).

As the intestinal epithelium lining in the digestive tract can contact with nutrients and food components, these factors may have an influence on the process

of wound healing and alter the normal defensive barrier. Isoflavones, a major component in soy beans was found to have an inhibitory effect on intestinal cell proliferation (Booth et al., 1999), while fermentable dietary fiber showed a stimulating effect on intestinal epithelial cell proliferation (Goodlad et al., 1989). In addition, vitamin D ($1,25(OH)_2D_3$) was found to have a stimulating effect on Caco-2 cell migration in culture (Kong et al., 2008). However, there is no evidence available for the effect of aspartame, an artificial sweetener generally used in many types of food product on wound healing of intestinal epithelial cells. Therefore, the present study was proposed to evaluate the effect of aspartame on cellular migration and proliferation in human intestinal epithelial cells. The knowledge gain from this study may add on the information about aspartame.

1.2 Objectives of the study

To study the effect of aspartame on migration and proliferation of human intestinal epithelial cells and possible mechanism involved in these processes

1.3 Benefits of the study

This study provides the information on the role of aspartame in intestinal epithelial cell migration and proliferation. The results of the study may be beneficial for individuals with GI injury to be aware of aspartame-sweetened product consumption.

CHAPTER II
LITERATURE REVIEW

2.1 Sweeteners

Food additives are any substances or ingredients originating from both natural and synthetic sources. They are not normally consumed as a food even if it has nutritive value. They are incorporated into food products during a preparation process or a technological purpose in the manufacture or sensory function (Griffiths and Borzelleca, 2014). Food additives play a variety of roles in the modern food supply and are necessary to be safe and suitable for their intended use (Bearth, Cousin and Siegrist, 2014). Because all of food additives are never considered completely safe, the ADI is set to prevent overconsumption (Saltmarsh and Insall, 2013).

Sweeteners are a member of food additives that give a sweet taste. The sweeteners can be classified as nutritive sweeteners and non-nutritive sweeteners depending on the providing calories (Saltmarsh and Insall, 2013). Nutritive sweeteners or caloric sweeteners including sugar (monosaccharides or disaccharides) and sugar alcohol (polyols) provide their sweetness with energy. Non-nutritive sweeteners or artificial sweeteners are high-intensity sweeteners which are used in small amount to mimic the sweetness effect of sugar or energy-containing sweeteners. They provide sweetness like a sugar without energy and do not have properties such as browning, crystallization, or microbial inhibition similar to sugar (Ogata and Hayes, 2012).

Nowadays, the incidents of obesity and diabetes increase and become a major health problem. These results from an imbalance between food consumption and energy expenditure, especially the accelerating consumption of sugar (Flegal et al., 2010). Therefore, diet behavior and lifestyle have been changed in a tendency to low-caloric products. Sugar-free products are popular because of their less calories with sweetness. Food industries use artificial sweeteners which can offer the taste of sweetness to consumers with low caloric content instead of high caloric sugar (Das and Chakraborty, 2016). The tendency of manufacturing products containing artificial sweeteners and the number of population consuming artificial sweeteners have increased since in 1990 (Figure 1) (Sylvetsky et al., 2012). The sweetener-added products may be beneficial in reducing the risk of dental caries and help patients with obesity or prediabetes and diabetes manage their calories and glucose status (Gardner et al., 2012). The examples of artificial sweeteners approved for use in the United States are acesulfame-K, neotame, saccharin, sucralose, and aspartame (Whitehouse et al., 2008).

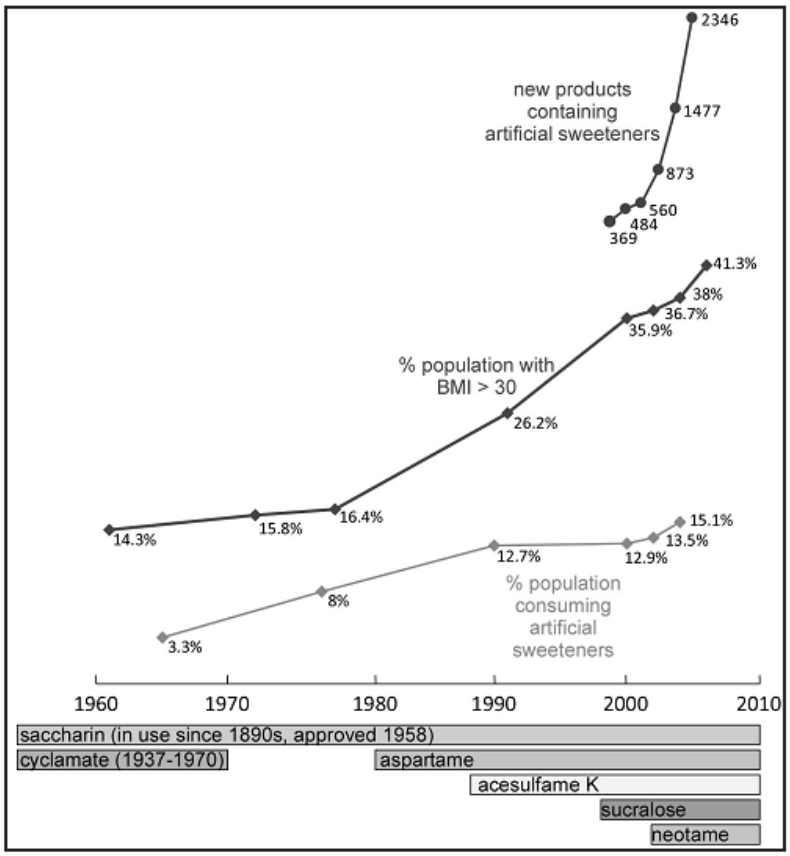


Figure 1 Timeline of artificial sweeteners usage trends in the United States (Yang, 2010)

2.2 Aspartame

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is an artificial sweetener which was accidentally discovered by James Schlatter in 1965 during the research for a new anti-ulcer drug (Mazur et al., 1970). Aspartame is approximately 160 to 200 times sweeter than sugar and provides energy about 4 kcal/g (Ogata and Hayes, 2012). It has been used as a low-caloric sweetener in more than 6,000 various types of products and consumed by millions of people all around the world. It was first approved by the FDA in 1981 for specific dry foods and in 1996 for a general-purpose sweetener in all

foods and drinks. The European Union has set the ADI of aspartame at 40 mg/kg BW/day (SCF, 1985) and the United States has set at 50 mg/kg BW/day (FDA, 1984).

Aspartame is one of the most common artificial sweeteners found in various low-caloric foods and beverages such as tabletop sweeteners, carbonated soft drinks, chewing gum, breakfast cereal, dairy products, and other dry products (Table 1). It is also used as a sweetener in some pharmaceutical products (e.g. medical foods, multi-vitamins and sugar-free cough drops) (Chattopadhyay et al., 2014). Although numerous products containing aspartame have been released in the market, the average consumption of the highest use have remained below the ADI (Kroger et al., 2006). In the United States, the estimated daily intake of aspartame has been found about 1/10 of the ADI and does not exceed 30% of the ADI (Butchko et al., 2002). The dietary intake survey of aspartame consumption in 9701 individuals in the United States demonstrated that the mean of aspartame consumption was 4.85 mg/kg BW/day, and the 90th percentile of aspartame consumption was 10.43 mg/kg BW/day (Magnuson et al., 2007).

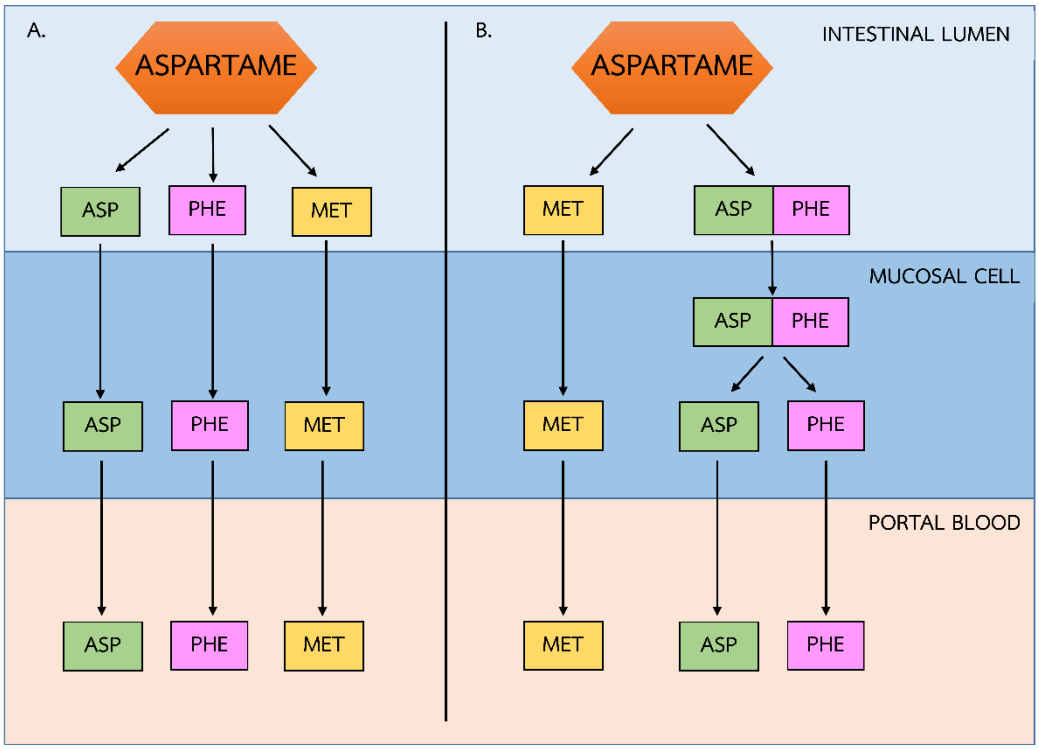


Figure 2 Hydrolysis of aspartame in the intestinal lumen, mucosal cells and portal blood (Stegink, 1987) (A) Aspartame is hydrolyzed in the intestinal lumen to aspartate (ASP), phenylalanine (PHE) and methanol (MET). (B) Aspartame is hydrolyzed in the intestinal lumen to MET and dipeptide aspartyl-phenylalanine which is further hydrolyzed to ASP and PHE in mucosal cells.

2.2.1 Metabolism of aspartame

Aspartame is a dipeptide of the amino acids aspartic acid and phenylalanine which esterified with methyl group of methanol. Upon the ingestion, aspartame is hydrolyzed by the enzyme in both the intestinal lumen and mucosal cells into natural elements, aspartic acid, phenylalanine and methanol with a few breakdown products such as formaldehyde, formic acid and diketopiperazine (Figure 2) (Stegink, 1987). The two amino acid components are metabolized in the similar way as they are derived

from other common sources of dietary protein; therefore, they do not accumulate in the body (Whitehouse et al., 2008).

Because of its metabolites, the FDA has specified the warning label on the aspartame-containing package for the patients with phenylketonuria, a genetic disorder that patients lacks or has insufficient amounts of the enzyme phenylalanine hydroxylase which metabolizes phenylalanine to tyrosine (Chattopadhyay et al., 2014). Although the aspartame intake should be considered by patients with phenylketonuria, the amount of phenylalanine digested from aspartame at lower than ADI level may not have the serious adverse effects for these patients (EFSA FIP (Food Ingredients and Packaging), 2013; Mackey and Berlin, 1992). The safety of methanol consumption is also concerned. However, the research about estimated methanol in food indicated that the levels of methanol from aspartame-sweetened foods and beverages are less than those from fruits and vegetables (Magnuson et al., 2007). Moreover, the Scientific Committee on Food (SCF) and European Food Safety Authority (EFSA) also report that methanol derived from aspartame is not genotoxic and carcinogenic, and the consumption of aspartame at below the ADI level is safe (EFSA FIP (Food Ingredients and Packaging), 2013; SCF, 2002).

2.2.2 Aspartame toxicity

The use of aspartame is still controversial due to the concern of its toxicity. Although it was approved to use in the food products by the FDA, the study on its safety still continues. Acute toxicity and sub-chronic toxicity of aspartame has been tested in various animal models, and the results found that the toxicity was very low (Magnuson et al., 2007). In addition, there is no evidence about gene mutations and genotoxicity of aspartame when tested in microbial, cell culture and animal models. The *in vitro* and *in vivo* studies indicate that aspartame does not have genotoxic effect at the concentration below ADI, and the available data show no genotoxic concern. (Jeffrey and Williams, 2000; Kirkland and Gatehouse, 2015; Rencüzoğulları et al., 2004; Sasaki et al., 2002).

Carcinogenicity of aspartame intake was considered, and the carcinogenic effect of aspartame and other low-caloric sweeteners was not found in most animal studies (NTP (US National Toxicology Program), 2005). Aspartame did not increase the cancer risk at several common sites, and only significant increased risk of laryngeal cancer was observed in humans (Bao et al., 2008; Bosetti et al., 2009; Gallus et al., 2007). However, aspartame at doses close to ADI significantly increased incidence of lymphoma and leukemia in male and female rats (Soffritti et al., 2006; Soffritti et al., 2007). The incidence of leukemia and lymphoma increased with high consumption of aspartame-sweetened soda (Schernhammer et al., 2012). Additional concern regarding to brain tumor was also evaluated. The results suggested that the increased incidence

of brain cancer may be associated with aspartame consumption (Olney et al., 1996). However, the animal studies and the further human epidemiological studies on aspartame and brain cancer did not show the increased brain cancer risk by aspartame (Butchko and Stargel, 2001; Lim et al., 2006). The SCF concluded that aspartame was not associated with the increased incidence of brain tumor (SCF, 2002), and the FDA expert committee on food additives suggested that aspartame is safe for human consumption (Magnuson et al., 2007).

2.3 Wound healing of gastrointestinal tract

The gastrointestinal tract consists of many major organs which have coordination function associating with glandular organs for physiological processes of food intake including motility, secretion, digestion, absorption, and elimination of the food (Pandol et al., 2009). The surface of the gastrointestinal tract is covered with epithelial cells, a monolayer of columnar cells. Epithelial cells serve as the largest and most important barrier of GI tract. Their functions include transporting the nutrients from digestion and regulating the exchange between electrolytes and water across them into the blood or lymph (Turner, 2009). In addition, the epithelial cells are physical and biochemical barrier protecting mammalian hosts from the harmful substances including foreign matters, microorganisms and toxins produced by certain microorganisms (Laukoetter, Nava and Nusrat, 2008).

The intestinal epithelial cells are susceptible to several challenges such as diseases, pharmaceutical products, chemical components, infection, radiation and injury occurring during normal digestion which can disturb their integrity (Rubin, 2009). Direct contact of some irritants or drugs and cytotoxic agents can cause the damage of epithelial cells and make them loss of their barrier function (Turner, 2009). *Helicobacter pylori* (*H. pylori*) infection and nonsteroidal anti-inflammatory drugs (NSAIDs) which are the most common causes of duodenal and gastric ulcers interfere the wound healing ability of mucosal cells (Stewart and Ackroyd, 2008; Tabel et al., 2003). The damage and impairment of the epithelial barrier was found to be associated with various gastrointestinal diseases such as inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis (Peyrin-Biroulet et al., 2011).

2.3.1 Wound healing process

The intestinal epithelium can be injured by toxic substances, inflammation, microbes, oxidative stress, and pharmaceutical products. In IBD patients, the promotion of mucosal healing is suggested to be a major therapeutic strategy, and the specific factors involving the healing process are needed to be identified to improve the intestinal epithelium wound healing process and manage the clinical outcome (Groschwitz and Hogan, 2009). Therefore, rapid healing of the epithelial barrier following the damage is essential for maintenance of normal intestinal homeostasis. The intestinal epithelium lining is a greatly dynamic tissue which has the ability to

regenerate rapidly, and cellular turnover is completed within a few days. After the intestinal epithelium injury, the wound healing process will quickly begin (Iizuka and Konno, 2011; Sturm and Dignass, 2008).

This repairing process involves the balance of cellular migration and proliferation processes (Neurath and Travis, 2012). The migration process occurs very rapidly. Cells surrounding the wound lose their columnar shape and change to flat morphology in order to migrate into the wound to close the gap and restore the mucosal integrity. The migration of cells adjacent to the damaged area following injury has been termed as epithelial restitution. This process occurs within minutes to hours, and it is independent from proliferation process. Then, epithelium cell proliferation is required to increase the pool of cells in order to complete the barrier integrity. The proliferation process begins within hours or days after the injury (Göke and Podolsky, 1996; Silen and Ito, 1985).

2.3.2 Regulatory proteins involving wound healing

Cell migration and proliferation are essential for many physiological and pathological processes, especially in wound repair (Iizuka and Konno, 2011). Cell motility is a complex biological process involving the coordination of four general steps, plasma membrane protrusion at the leading edge, adhesion formation and stabilization, detachment at the cell rear, and cell body movement (Ananthakrishnan

and Ehrlicher, 2007; Lauffenburger and Horwitz, 1996) (Figure 3). During these processes, there are many proteins involved.

Extracellular matrix (ECM) is a structural support which is important for intrinsic biological processes. The process of cell migration involves the dynamic interaction between a cell and ECM which the cell migrates and attaches to. The linkage between the actin filament of cell and ECM serves as focal adhesion at the leading edge (Holly, Larson and Parise, 2000). Moreover, actin, the fundamental cytoskeletal molecule also has a role in regulation of cell migration. The organization of the actin filament is necessary to stress fibers and lamellipodia formation during the cell movement (Le Clainche and Carlier, 2008). There are various protein signaling molecules which play a role in this process such as integrins, focal adhesion kinase (FAK), protein kinase B (Akt), and Rho family proteins (Burridge and Chrzanowska-Wodnicka, 1996; Huttenlocher et al., 1995; Larsen et al., 2003).

In addition to cell migration, the epithelial cells proliferate to increase the pool of cell to replace the wound (Wong and Wright, 1999). The proliferation of cells plays an important role in the maintenance of epithelial barrier. The process involves many proteins signaling through the cell cycle such as Akt and extracellular signal-regulated kinase (ERK), a member of mitogen-activated protein kinase (MAPK) family (Larsen et al., 2003; Zhang and Liu, 2002).

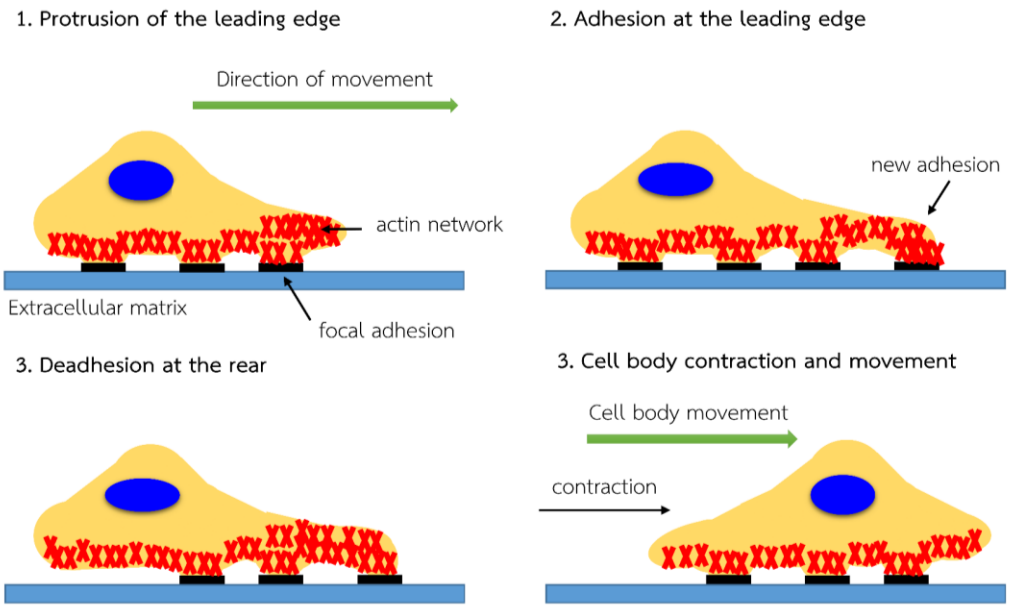


Figure 3 Schematic of cell movement (Ananthakrishnan and Ehrlicher, 2007)

2.3.2.1 Integrin family

Integrins are a huge family of glycoprotein transmembrane receptors which are essential for regulation of cell migration and other cellular processes. They are implicated in cell migration as cellular adhesion molecules mediate the attachment of cell and ECM in many contexts such as the movement of cell during the repairing process (Huttenlocher and Horwitz, 2011). The association of integrin cluster with adaptor proteins promote the assembly of actin cytoskeletons to form focal adhesion, the cell-matrix adhesion (Giancotti and Ruoslahti, 1999). They have α and β heterodimers which can combine together in different ways to form at least 24 heterodimers. Each heterodimer consists of a large domain which links to other signaling proteins in order to mediate the signaling pathways including the actin cytoskeleton and intracellular signaling of cell function. The specificity of binding

depends on the recognization of extracellular domain of integrins with matrix ligands (Srichai and Zent, 2010).

Integrin β1 is a member of integrin family which has a critical role in cellular migration. It can bind to multiple signaling proteins during the tissue repair (Srichai and Zent, 2010). The study in mice containing a fibroblast with integrin β1-deficient gene exhibited the impairment of cutaneous wound closure (Liu et al., 2010). In addition, knockdown of integrin β1 subunit gene in mouse embryonic stem cells showed the reduction of cellular migration (Fiissler et al., 1995).

2.3.2.2 Focal adhesion kinase (FAK)

Focal adhesion kinase (FAK) is a 125-kDa cytoplasmic tyrosine kinase which plays an important role in cytoskeleton organization and cell adhesion formation. It composes of three domains including a central kinase domain, a N-terminal FERM domain and a C-terminal focal adhesion targeting domain. The N-terminal domain has the tyrosine 397 (Tyr397) site which can be phosphorylated and interact with other signaling proteins to regulate cell migration. The Tyr397 site is the only position that can be autophosphorylated by FAK itself (Zhao and Guan, 2011). FAK was first discovered in 1992 by Steve Hanks as a tyrosine phosphorylated protein that localized within integrin-enriched cell adhesion sites in normal cells. This protein was named as FAK based on its function to recruit and activate other proteins at the focal adhesion site (Parsons, 2003).

A number of studies showed that defect of FAK inhibited cell motility response (Mitra et al., 2005; Schlaepfer, Mitra and Ilic, 2004). Upregulated expression of FAK was observed in the migration process of keratinocytes in epidermal wound healing (Gates et al., 1994) and in the migration process of human umbilical vein endothelial cell healing (Romer et al., 1994). Moreover, it was found that L-arginine, a nutritional supplement promoted intestinal wound repair by enhancing intestinal cell migration through FAK activation (Rhoads et al., 2004).

FAK has binding sites for many signaling molecules that regulate FAK function (Schlaepfer et al., 2004). During cell migration, FAK is activated by integrin. The phosphorylation of FAK produces a FAK–Src signaling complex formation which regulates multiple downstream signaling pathways (Zhao and Guan, 2011). The FAK-Src complex promotes the phosphorylation of proteins such as p130Cas which leads to the Cas/Crk complex formation in order to regulate membrane ruffling and facilitate the activation of Rho family proteins (Zhao and Guan, 2011). Moreover, the interaction of FAK with phosphatidylinositol 3-kinase (PI3K) can stimulate cell migration through many downstream effectors for the Rho family proteins. Several downstream signalings of FAK pathways have been associated in mediating FAK activation which result in cytoskeletal organization to promote cell migration. The Rho family of small GTPases are a key regulator of actin organization and lamellipodia formation in cell migration (Tomar and Schlaepfer, 2009).

2.3.2.3 Protein kinase B (Akt)

PI3K is a lipid kinase family which requires for several cell functions such as cell proliferation, survival and migration of many different cell types through direct and indirect binding of proteins crosslinking with other pathways such as Rho family signaling pathway (Cain and Ridley, 2009). The PI3K/Akt pathway is one of the signaling pathway in cell migration and proliferation which was identified in the early 1980s through the insulin signaling pathway (Hemmings and Restuccia, 2012). The activation of PI3K by phosphorylation at hydroxyl group in inositol ring of inositol phospholipid generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3), the second messenger, which then recruits other signaling proteins including Akt (Cantrell, 2001).

Akt or protein kinase B, a 60 kDa protein, is a serine/threonine (Ser/Thr) kinase which was characterized to three members, Akt1, Akt2 and Akt3. Akt 1 and Akt2 are expressed in almost all tissues, while Akt3 is found in neuronal tissue (Bellacosa et al., 2005). All isoforms of Akt require phosphorylation at Ser/Thr residue which are mediated by PI3K signaling through PIP_3. PIP_3 generated at the plasma membrane mediates membrane translocation of Akt. Akt is interacted with PIP_3 which leads to alteration of its conformation allowing phosphorylation and activation (Fresno Vara et al., 2004). The active Akt mediates multicellular signal transduction pathways especially in cell migration and proliferation (Manning and Cantley, 2007). In cell migration, the activation of Akt has an influence on cell motility in mammalian fibroblasts and endothelial cells. The study of vascular endothelial growth factor in

endothelial cells demonstrated that endothelial cell motility was associated with Akt activation (Higuchi et al., 2001; Morales-Ruiz et al., 2000).

In addition to the role in promoting cell migration, the activation of Akt can also stimulate proliferation through multiple downstream protein signalings. The activation pathway involves the cell cycle regulation either by direct phosphorylation of the target proteins or by indirect regulation of protein expression levels (Testa and Tsichlis, 2007). PI3K/Akt signaling was found to play a role in regulation of proliferative signals in intestinal epithelial cells (Sheng et al., 2003), and activated Akt can stimulate cell proliferation by increasing translocation of transcription proteins (Bellacosa et al., 2005).

2.3.2.4 Caveolin-1 (Cav-1)

Caveolin-1 (Cav-1) is a major protein of caveolae membrane representing a subset of membrane lipid domain. The caveolin family in mammals consists of three principle members, Cav-1, Cav-2 and Cav-3. Co-expression of Cav-1 and Cav-2 are often found in a variety of cells and tissues such as endothelial cells and fibroblasts, while expression of Cav-3 is specifically found in muscle cells (Navarro et al., 2004). All three caveolins have different N and C terminal structures. Based on the length of N terminal, there are two isoforms (α and β) of Cav-1. Cav-1 contains a specific motif called scaffolding domain which plays a role in recruiting proteins to caveolae and interacting with other proteins (Núñez-Wehinger et al., 2014).

Cav-1 involves in calcium signaling, angiogenesis, anoikis, cholesterol transport, and cell migration. It has been proposed that expression of Cav-1 is required for cell motility, especially wounding a cell monolayer by controlling polarization of signaling molecules (Isshiki et al., 2002; Shaul and Anderson, 1998). The phosphorylation of Cav-1 at Tyr14 residue is required for cell polarization (Parat, Anand-Apte and Fox, 2003). In the study of endothelial cell migration, Cav-1 seems to be released from caveolar structure and associated with focal complex formation at the leading edge (Beardsley et al., 2005). This association can also be induced by wound healing (Navarro et al., 2004). Depletion of Cav-1 in cells exhibit impaired muscle wound healing (Volonte, Liu and Galbiati, 2004). Moreover, Cav-1 has a connection with the cytoskeleton. Cav-1 is linked to the actin cytoskeleton through actin crosslinking protein filamin (Stahlhut and van Deurs, 2000). During cell movement, Cav-1 regulation pathway involves Akt, Rho and Rac activation. The expression of Cav-1 appears to be mediated by PI3K/Akt pathway (Navarro et al., 2004). In Cav-1–deficient cells, the decrease in Rho protein level was found (Grande-García et al., 2007). In addition, regulation of Rac1-GTP activity by Cav-1 was also shown (Shao et al., 2013).

2.3.2.5 Rho and Rac proteins

Cell migration is a multistep process which requires an actin cytoskeleton organization in response to extracellular signals to regulate the protrusion and the FA formation at the leading edge as well as contraction of cell body (Le Clainche and

Carlier, 2008). Actin is the most functional abundant intracellular protein of most cells which polymerizes to form actin filaments. The actin cytoskeleton composes of actin filaments crosslinking into networks and bundles. There are various proteins regulating the assembly and disassembly of actin filaments. Most of these proteins are essential components of the actin cytoskeleton (Dominguez and Holmes, 2011). The actin organization and the contraction of actin cytoskeleton are important processes for cell migration. The actin organization results in stress fiber and lamellipodia formation at the leading edge of migrating cells (Iizuka and Konno, 2011). The organization of actin is suggested to be involved with the function of the Rho family of small GTPases (Sturm and Dignass, 2008).

The Rho family of small GTPases most likely consists of Rho and Rac which are suggested to control the organization and remodeling of the actin cytoskeleton. Rho family were first revealed a role in mediating actin formation containing structure 20 years ago by mediating the actin formation containing structure. Most of Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) to bind with GTP which can stimulate their downstream targets. They are inactivated by GTPase-activating proteins (GAPs) which hydrolyze GTP on Rho proteins to GDP (Ridley, 2015). This family can be further divided into many sub-groups including the Rho (isoforms A, B, and C) and Rac (isoforms 1, 2, 3) (Hall, 2012). Rho regulates the actin filaments contraction, while Rac regulates the actin polymerization to form lamellipodial protrusions (Nobes and Hall, 1995).

Rac is required for regulation of actin polymerization at the front of the cell and membrane protrusion. Lamellipodium, the extension of flat membrane protrusion, is cytoskeletal protein projection at the front of the cell. Rac-GTP induces membrane extension and lamellipodia formation at the leading edge by regulating actin polymerization. It stimulates Arp2/3 complex in order to further initiate actin polymerization and the formation of actin branches to move the cell forward (Ridley, 2001).

According to the migration mechanism, cell requires the contraction of cell body to move forward. Rho-GTP plays a role in cell body contraction and cell retraction at the rear. Rho regulates stress fibers formation and actin contraction via Rho-associated protein kinase (ROCKs) which is a Rho-kinase regulating the phosphorylation of myosin light chain (MLC). Reduction of Rho activity causes the decrease in cell migration by inhibiting cell body contraction (Amano, Fukata and Kaibuchi, 2000). Previous studies have reported that Rho and Rac were regulated by various intracellular signaling molecules. The engagement of integrins at the leading edge involves the activation pathway of Rho and Rac (Huttenlocher and Horwitz, 2011; Raftopoulou and Hall, 2004). Moreover, the activation of Rho and Rac depends on PI3K activity (Higuchi et al., 2001).

2.3.2.6 Extracellular signal-regulated kinase (ERK)

Mitogen-activated protein kinases (MAPKs) family are protein kinase that are associated with a variety of complex cellular responses including cell proliferation (Kim and Choi, 2010). They can be divided into three subgroups which are extracellular signal-regulated protein kinase (ERK), p38 and Jun N-terminus kinase (JNK), depending on the amino acid sequence motif. It is well known that MAPKs plays roles in cell proliferation process. The absence of ERK protein components demonstrated the significant inhibition of cell proliferation (Huang et al., 2004; Pagès et al., 1993).

ERK is a subfamily of MAPKs which exists in two isoforms, p44 (ERK1) and p42 (ERK2). ERK contains a Thr-Glu-Tyr motif with the kinase domain that can be stimulated by a variety of signaling pathways. The activation of ERK requires the signaling of MEK1/2 and Raf-1 molecule. The MEK1/2 signaling recruits the Raf protein to the membrane which then phosphorylates and activates the MEK1/2. The MEK1/2 molecule is the upstream protein of ERK which can phosphorylate the Thr and Tyr residues of ERK (Seger and Krebs, 1995). The activated ERK is translocated to the nucleus to mediate cell growth by activating transcription factors during the entire G0-G1 cell cycle (Zhang and Liu, 2002). Interference of ERK protein signaling affects the cellular proliferation, while the activation of ERK stimulates proliferation of the intestinal cells (Rhoads et al., 1997; Waseem et al., 2014). Moreover, PI3K/Akt seems to be an upstream signaling molecules to activate downstream MAP kinases such as ERK (Laprise et al., 2004).

2.4 Effects of foods, nutrients and other food components on wound healing

Direct contact between substances consumed into the digestive tract and intestinal epithelial cells can affect the wound healing process. There are many researches about the effects of foods, nutrients and other food components on intestinal wound healing process. The study of on the effect of vitamin A and chronic corticosteroids on small and large intestinal wound healing in rats found that vitamin A at high-dose (10,000 IU/kg/day) given preoperatively or only postoperatively for two weeks significantly reversed the inhibitory effect of steroid on wound healing (Phillips et al., 1992). The oral administration of honey at 10 mg/kg/day in rats with obstructive jaundice after abdominal operation showed the significant increase in hydroxyproline, a marker of collagen synthesis (Ergul and Ergul, 2010).

The migration and proliferation process of epithelial cell in wound healing may be modulated by nutrients or food components which direct contact to epithelial lining along the GI tract. Refeeding starved rats with dietary fibers can stimulate intestinal epithelial cell proliferation (Goodlad et al., 1989). L-Glutamine was found to have the stimulating effect on rat crypt cell proliferation (Rhoads et al., 1997), and arginine showed a role in stimulating intestinal cell migration via nitric oxide production (Rhoads et al., 2008). Moreover, supplementation of n-3 and n-6 fatty acids may increase the density of cells migrating across the wound (Ruthig and Meckling-Gill, 1999), and zinc at physiological concentrations significantly enhanced intestinal epithelial cell migration (Cario et al., 2000). In contrast, genistein, a plant isoflavone in

soy beans was found an inhibitory effect on intestinal cell proliferation (Booth et al., 1999).

Although artificial sweeteners are generally considered metabolically inert, the available data indicated that they have physiological effects that may interfere with the gastrointestinal tract function. It was found that aspartame (400 mg) administered 60 min before the meal may have an inhibitory effect on food intake (Rogers et al., 1995). In addition, aspartame (400 mg) preloaded 60 min before the liquid meal suppressed plasma glucagon-like peptide-1 (GLP-1) (Hall et al., 2003), while the levels of postprandial glucose and insulin were not affected by aspartame (Anton et al., 2010). However, the effect of aspartame on intestinal cell migration and proliferation, the important process in the intestinal wound healing has never been observed.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell culture

The colorectal adenocarcinoma Caco-2 cells widely characterized and used as intestinal-like cells (Sambruy et al., 2001) were obtained from American Type Culture Collection (Manassas, VA, USA). Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 2 mM L-glutamine, and 100 units/mL penicillin and streptomycin (Gibco, Gaitherburg, MA, USA). Cells were incubated in a humidified incubator containing 5% CO_2 at 37 °C.

3.1.2 Reagents

Aspartame (L-aspartyl-L-phenylalanine methyl ester), trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, propidium iodide (PI), dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibodies for integrin β1, pFAK, FAK, pAkt, Akt, pERK, ERK, Cav-1, Rac1-GTP, RhoA-GTP, and β-actin and anti-mouse and anti-rabbit secondary antibodies were obtained from Cell Signaling (Danvers, MA, USA).

3.2 Experimental design

The study design is shown in Figure 4. At first, to investigate the cytotoxic effect of aspartame on colorectal Caco-2 cells, cells were treated with aspartame at various concentrations (0, 1, 5, 10, 25, 50, 100, 250, and 500 μM) for 24 h at 37°C, and cell survival was determined by MTT colorimetric assay. To confirm the cytotoxicity of aspartame on Caco-2 cells, mode of cell death in response to aspartame treatment was determined. Cells were treated with aspartame at these indicated concentrations and then apoptotic and necrotic cell death were detected by nuclear staining assay. Non-toxic concentrations of aspartame related to the average concentrations of aspartame usually consumed were used in the further experiments.

To determine the effects of aspartame on Caco-2 cell migration, a monolayer of cells was cultured and a wound was made. The cell monolayers were treated with aspartame at non-toxic concentrations, and cell migration was examined by wound healing assay. To confirm the effects of aspartame on Caco-2 cell migration, Boyden chamber cell migration assay was also performed. Cells were seeded into the upper chamber of a transwell and allowed to migrate through the pore of the chamber. Cell morphology characterization during cell migration was determined as well. Cells treated with aspartame were incubated with 1:100 phalloidin-rhodamine and photographed their morphology by a fluorescence microscope. To investigate the effects of aspartame on proliferation of Caco-2 cells, cells were treated with aspartame at non-toxic concentrations, and cell viability was measured by MTT assay. Finally,

proteins involving in mechanisms of aspartame effects on cell migration and cell proliferation were evaluated by Western blot analysis.

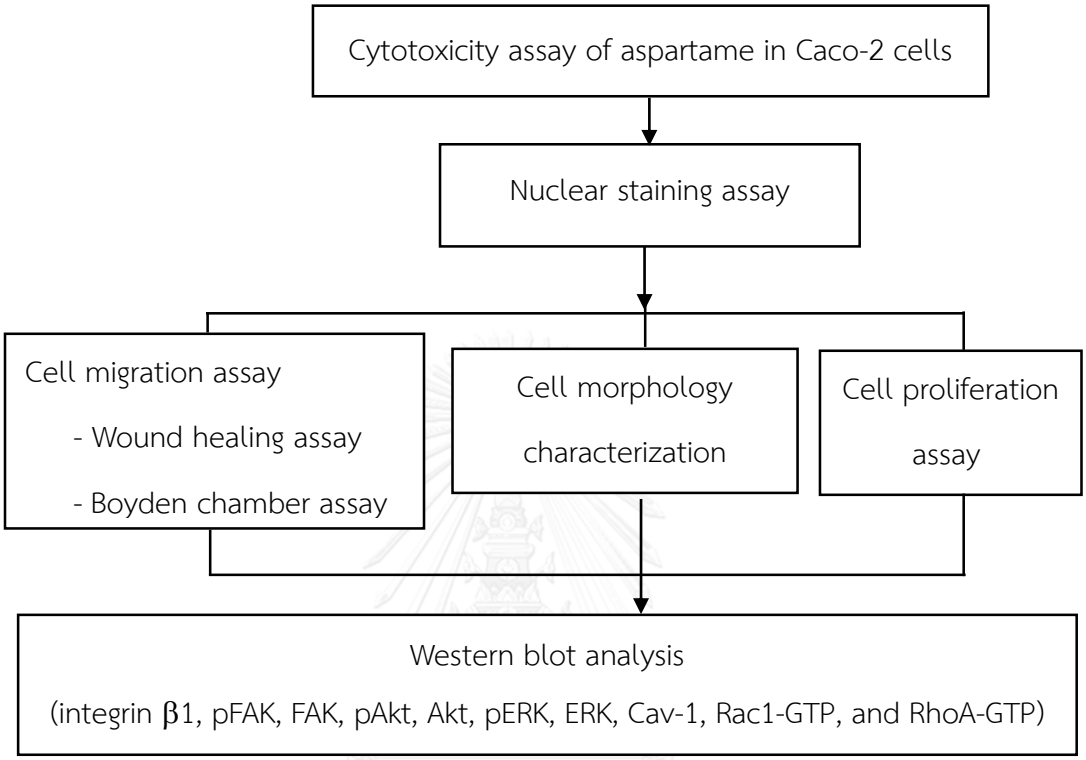


Figure 4 Experimental design of the study

3.3 Methods

3.3.1 Sample preparation

To investigate the effects of aspartame on Caco-2 cells, aspartame was dissolved in PBS. Then, aspartame solution was diluted with DMEM medium, as described by Alleva et al. (2011) to prepare aspartame concentrations ranging from 0 to 500 μM.

3.3.2 Cytotoxicity assay

To investigate the cytotoxicity of aspartame on Caco-2 cells, cell viability was determined by MTT colorimetric assay (van Meerloo, Kaspers and Cloos, 2011). Cells at the density of 1 x 10^5 cells/mL were seeded in 96-well plate and incubated for 24 h at 37°C. Then, the cells were treated with various concentrations of aspartame (0, 1, 5, 10, 25, 50, 100, 250, and 500 µM) for 24 h at 37°C. After aspartame treatment, 100 µL per well of MTT solution (0.4 mg/mL) was added and incubated for 4 h at 37°C. Then, the MTT solution was removed, and 100 µL of DMSO was added to dissolve the formazan crystal formed, giving purple color. The optical intensity (OD) of MTT product was spectrophotometrically measured at 570 nm using a microplate reader (Wallac Model 1420, USA). The viable cells can convert yellow MTT solution to formazan product, a purple color substance dissolved in DMSO, by mitochondria dehydrogenase enzyme. The maximum absorbance of formazan solution is about 570 nm, and it is referred to the amount of viable cells. The OD ratio of treated and non-treated control cells was calculated and presented as a percentage of cell viability as follows:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treatment}}{\text{Absorbance of control}} \times 100$$

3.3.3 Nuclear staining assay

Apoptotic and necrotic cell death were investigated by Hoechst 33342/PI co-staining (Freshney, 2010a). Hoechst 33342 dye is a cell-permeant nucleic acid stain which emits blue florescence when binds to dsDNA. Fluorogenic PI dye is, on the other hand, a membrane impermeant dye used to exclude from live and apoptotic cells. PI dye is incorporated into necrotic cells when membrane integrity is destroyed. The blue Hoechst 33342 detects apoptotic cells, while the red PI detects dead necrotic cells. After 24-h treatment with various concentrations of aspartame (0, 1, 5, 10, 25, 50, 100, 250, and 500 μM), cells were stained with 10 μM of the Hoechst 33342 and 5 μg/mL of PI for 30 min at 37°C in the dark. The apoptotic cells having condensed chromatin and/or fragmented nuclei were stained by Hoechst 33342, whereas the DNAs of necrotic cells having membrane damage were stained by PI. Fluorescence in the cells was visualized and scored under a fluorescence microscope (Olympus IX51 with DP70) using blue filter for Hoechst 33342 and red filter for PI. The percentages of apoptosis and necrosis were calculated by comparing with the untreated cells as follow:

$$\% \text{ Apoptosis} = \frac{\text{Apoptotic cells}}{\text{Total cell number}} \times 100$$

$$\% \text{ Necrosis} = \frac{\text{Necrotic cells}}{\text{Total cell number}} \times 100$$

3.3.4 Cell migration assay

Effect of aspartame on Caco-2 cell migration was determined by wound healing and Boyden chamber cell migration assays (Liang, Park and Guan, 2007). For the wound healing assay, cells at a density of 3×10^4 cells/well were seeded for a confluent monolayer in a 96-well plate, and then a wound space was made by scraping a center straight line with a 1-mm-wide tip. The well was then rinsed gently with PBS to remove the debris and replaced with fresh DMEM medium supplemented with 10% FBS. After that, the cell monolayers were treated with aspartame at non-toxic concentrations and allowed to migrate for 12, 24 and 48 h at 37°C. Micrographs were taken under a phase contrast microscope (Olympus DP70, Melville, NY), and the wound spaces were measured using Olympus DP controller software. The average wound space from those random fields of view was calculated in term of migration level and represented as relative migration level. The relative migration level was also calculated by dividing the change in the wound space of treated cells by that of the non-treated control cells in each experiment as follows:

$$\text{Migration level} = \frac{\text{(Average space at time 0 h) - (Average space at each time)}}{\text{(Average space at time 0 h)}}$$

$$\text{Relative migration level} = \frac{\text{Migration level of treatment}}{\text{Migration level of control}}$$

For Boyden chamber cell migration assay (Brown and Bicknell, 2001), cell migration is performed in two medium containing chambers separated by a porous membrane. Cells at a density of 3×10^4 cells/well were seeded into the upper chamber of a transwell with 8 μm pore size membrane in a 24-well plate in serum-free medium and incubated with non-toxic concentrations of aspartame. Complete DMEM medium containing 10% FBS was added to the lower chamber of the unit. This allowed the cells in upper chamber of the transwell to migrate through the pores of the membrane into the medium containing serum in lower compartment. Following the 48-h incubation, the non-migrated cells in the upper chamber were removed by cotton-swab wiping, and the cells that migrated to the underside of the membrane were stained with 10 μg/mL of Hoechst 33342 for 10 min. The cells were then visualized and scored under a fluorescence microscope (Olympus IX51 with DP70). The percentage of number of stained cells per field was calculated by comparing with the non-treated control cells in each experiment and represented as the average percentage of number of stained cell per field as follows:

$$\% \text{ Number of cells/field} = \frac{\text{Number of stained cells of treatment}}{\text{Number of stained cells of control}} \times 100$$

3.3.5 Cell proliferation assay

Cell proliferative effect of aspartame was determined. Cells at a density of 2 x 10^3 cells/well were seeded in 96-well plate for 24 h at 37°C. After that, the cells were treated with aspartame at non-toxic concentrations for 0, 1, 2, 3 and 5 days at 37°C. Cell proliferation was measured by MTT assay as described in 3.3.2. The OD ratio of treated and non-treated control cells at the indicated time was calculated and presented in terms of relative cell viability as follows:

$$\text{Relative cell viability} = \frac{\text{Absorbance of treatment}}{\text{Absorbance of control}}$$

3.3.6 Cell morphology characterization

Cell morphology was investigated by phalloidin-rhodamine assay (Maiuthed and Chanvorachote, 2014). Phalloidin specifically binds with high affinity to the polymerized actin. After treated with aspartame at non-toxic concentrations for 24 h, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.25% Triton-X100 in PBS for 5 min, rinsed with PBS, and then blocked with 4% BSA in PBS for 60 min. Following that, the fixed cells were incubated with 1:100 phalloidin-rhodamine in PBS for 20 min at room temperature, then rinsed with PBS and mounted with 50% glycerol in PBS. The cell morphology was then imaged by a fluorescence microscope (Olympus IX51 with DP70). The relative number of lamellipodia, flat sheet-like structures, per cell was calculated by comparing with the

non-treated control cells and represented as the average relative number of lamellipodia as follows:

$$\text{Relative number of lamellipodia/cell} = \frac{\text{Number of lamellipodia/cell of treatment}}{\text{Number of lamellipodia/cell of control}}$$

3.3.7 Western blot analysis

The expression of proteins associated in migration and proliferation process (integrin β1, pFAK, FAK, pAkt, Akt, pERK, ERK, Cav-1, Rac1-GTP, and RhoA-GTP) was determined by Western blot analysis (Mahmood and Yang, 2012). After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCL (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Molecular Biochemicals, Basel, Switzerland) for 60 min on ice. Cell lysates were collected, and the protein content was determined using the BSA protein assay kit (Pierce, Rockford, USA). Proteins of each sample were denatured by heating at 95°C for 5 min with Laemmli loading buffer and subsequently loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred onto nitrocellulose membranes (Bio-Rad, USA). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM sodium chloride and 0.05% Tween 20) at room temperature and then incubated with appropriate primary antibodies at 4 °C overnight.

Membranes were washed three times with TBST for 10 min and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The immune complexes were detected by chemiluminescence (SuperSignal West Pico; Pierce, Rockford, USA), and the levels of proteins were quantified using the densitometry software (Image J software, National Institute of Health, USA). Beta-actin was used as a loading control in each treatment.

3.3.8 Statistical analysis

All data are expressed as the means ± the standard error of mean (SEM) of independent triplicate experiments and normalized with the non-treated control. Multiple comparisons were used to examine for significant differences of multiple groups using one-way analysis of variance (ANOVA), followed by individual comparisons with the Tukey's post-hoc test. Statistical significance level was set at $p < 0.05$.

CHAPTER IV
RESULTS

4.1 Cytotoxic effect of aspartame on colorectal Caco-2 cells

To determine the non-toxic concentration of aspartame, cells were treated with aspartame at the concentrations of 0, 1, 5, 10, 25, 50, 100, 250, and 500 μM for 24 h at 37°C, and cell viability was determined by MTT assay. The result showed that aspartame at all indicated concentrations had no significant effect on cell viability (Figure 5). Therefore, these concentrations of aspartame (0-500 μM) were considered non-toxic.

To confirm non-toxic effect of aspartame at these concentrations, cell death response to aspartame was investigated by Hoechst 33342/PI co-staining assay. Cells were treated with various concentrations of aspartame (0, 1, 5, 10, 25, 50, 100, 250, and 500 μM) for 24 h at 37°C, and then stained with Hoechst 33342 and PI. The results indicated that when Caco-2 cells were treated with aspartame at the concentrations up to 500 μM, neither apoptotic nor necrotic cells were observed (Figure 6). In consequence, this information may use to clarify the cytotoxic effect of aspartame on cell migration and proliferation response. Aspartame at the concentrations of 0, 10, 25, 50, and 100 μM were chosen for further experiments according to the average concentration of aspartame consumed in the United States.

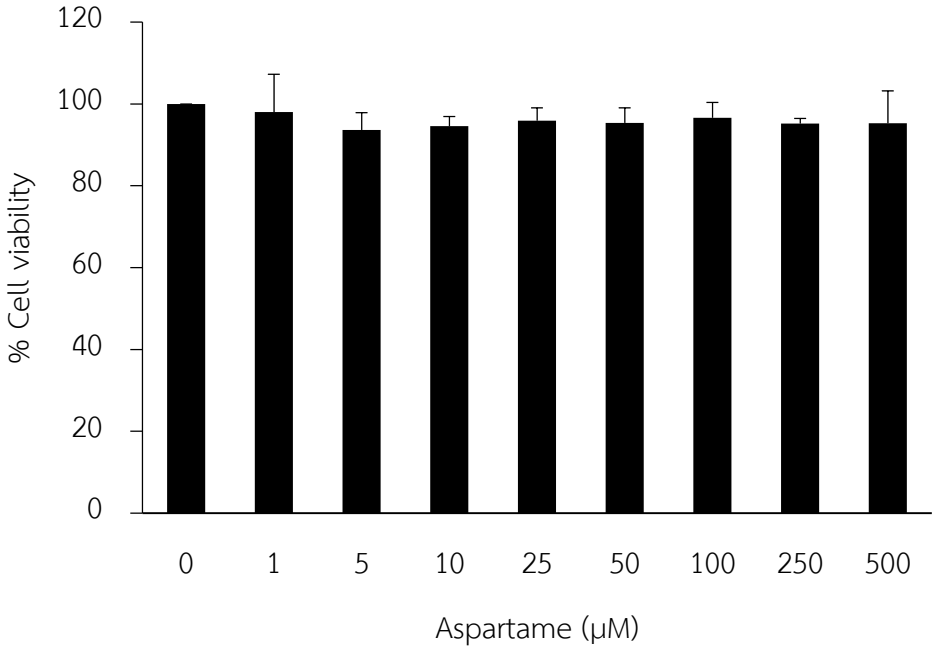
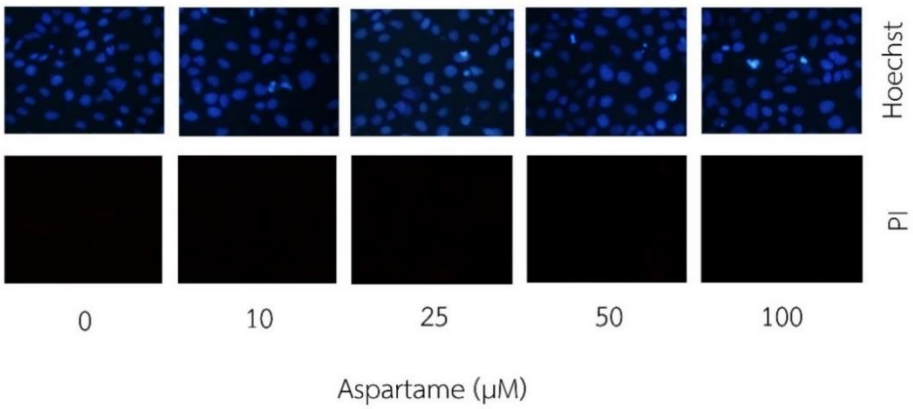


Figure 5 Cytotoxic effect of aspartame on colorectal Caco-2 cells

A.



B.

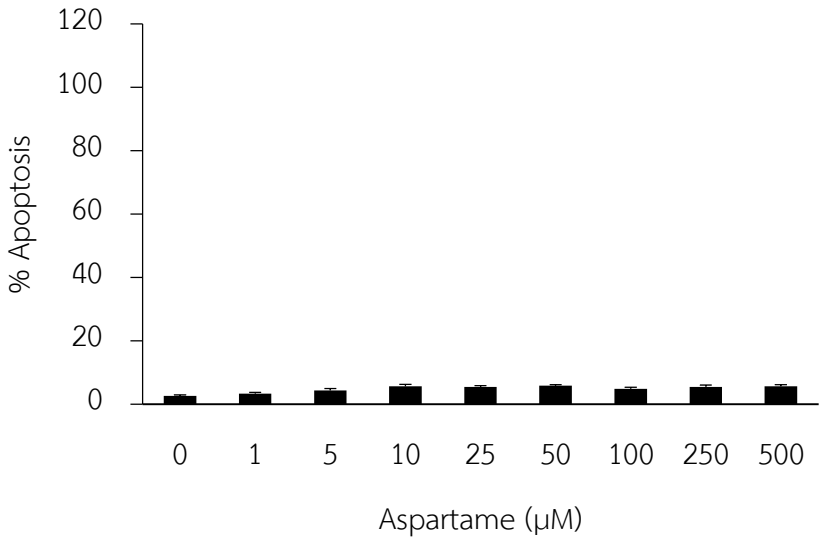


Figure 6 Effect of aspartame on mode of cell death of colorectal Caco-2 cells (A) Nucleus of the cells determined by Hoechst 33342/PI co-staining (B) The percentage of apoptotic cells

4.2 Effect of aspartame on migration of colorectal Caco-2 cells

As cell motility is essential for the wound healing of epithelial cells in digestive system, the effect of aspartame on cell migration was evaluated by wound healing assay. The monolayer of cells were scratched and treated with aspartame at non-toxic concentrations (0, 10, 25, 50, and 100 μM). The migratory behavior was determined at 12, 24 and 48 h after wounding. The relative migration levels and photographs indicated that aspartame had significant inhibitory effect on Caco-2 cell migration. The results showed that aspartame significantly inhibited the motility of cells across the wound space at the concentrations of 25, 50 and 100 μM, especially at the concentration of 100 μM (approximately 10%, 20% and 30% reduction in cell motility, respectively) (Figure 7).

To confirm the effect of aspartame on Caco-2 cells migration, Boyden chamber cell migration assay was performed. Cells were added to the upper chamber of a transwell (8-μm pore size) in a 24-well plate and treated with the indicated concentrations of aspartame. The results showed that aspartame inhibited the migration of cells through the membrane at the concentrations of 25, 50 and 100 μM (approximately 25%, 40% and 50% reduction in cell migration, respectively) when incubated for 48 h (Figure 8). This assay supported the inhibitory effect of aspartame on cell migration, especially at the concentration of 100 μM.

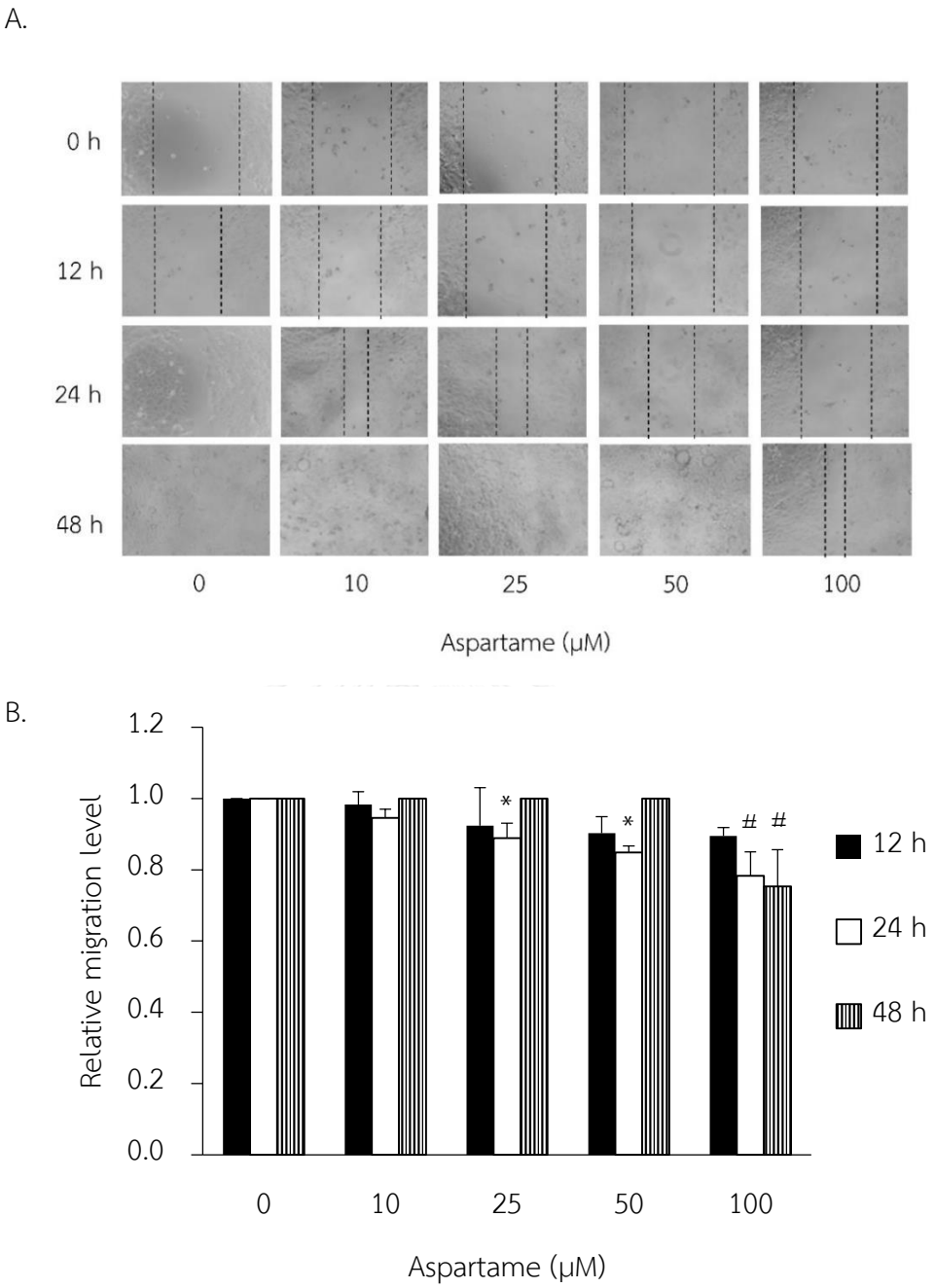
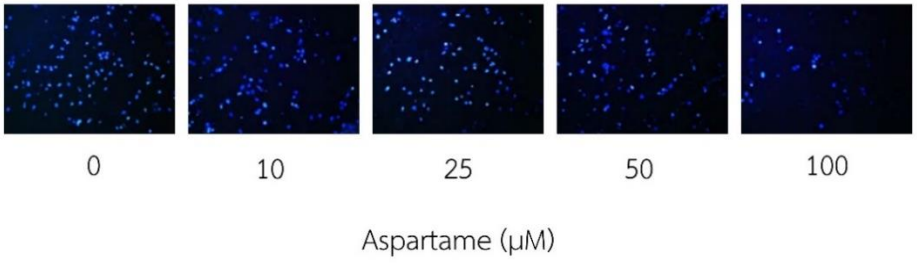


Figure 7 Effect of aspartame on colorectal Caco-2 cell migration determined by wound healing assay (A) The photographs of the wound space (B) Relative migration level of Caco-2 cells

*$p < 0.05$ and $^{\#}p < 0.001$ versus non-treated control

A.



B.

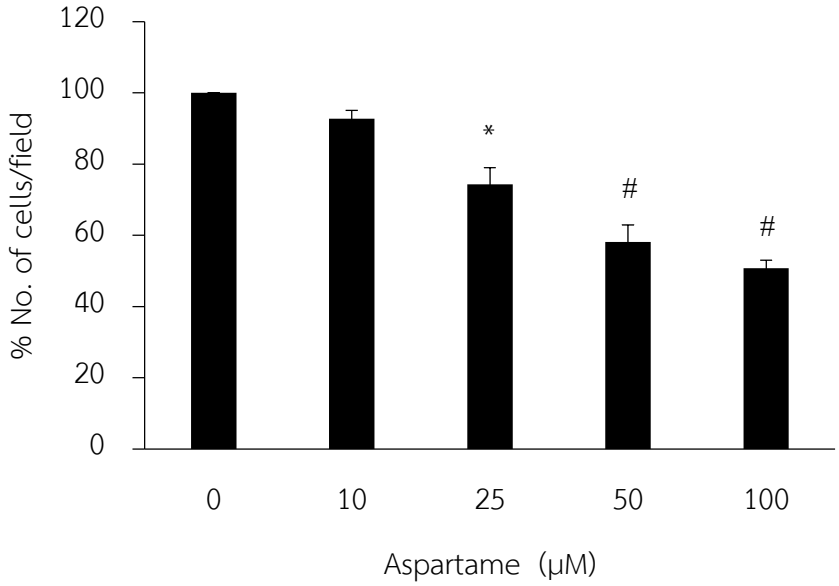


Figure 8 Effect of aspartame on colorectal Caco-2 cell migration determined by Boyden chamber cell migration assay (A) Migratory cells determined by Boyden chamber cell migration assay (B) The percentage of number of cells/field

*$p < 0.05$ and $^{\#}p < 0.001$ versus non-treated control

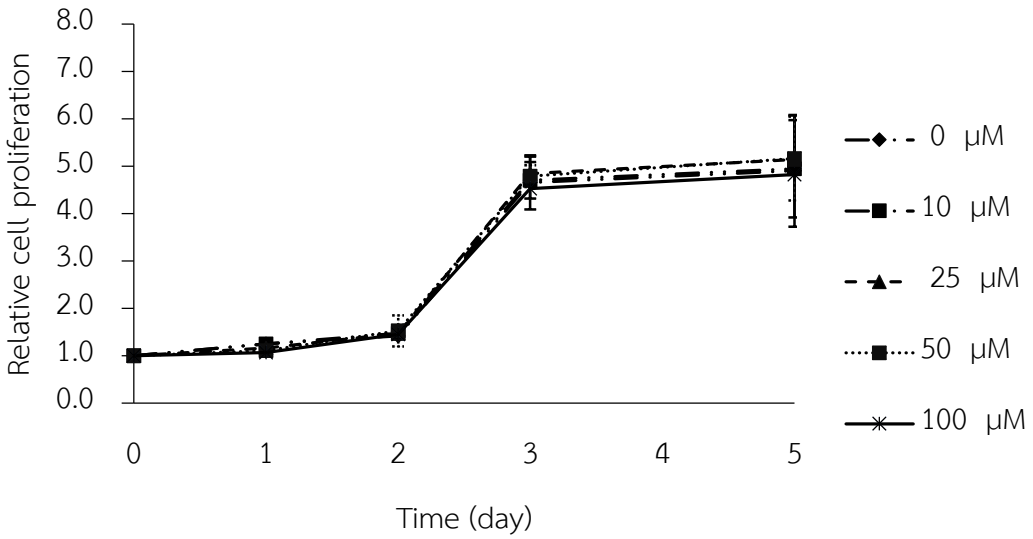


Figure 9 Proliferative effect of aspartame on colorectal Caco-2 cells

4.3 Effect of aspartame on proliferation of colorectal Caco-2 cells

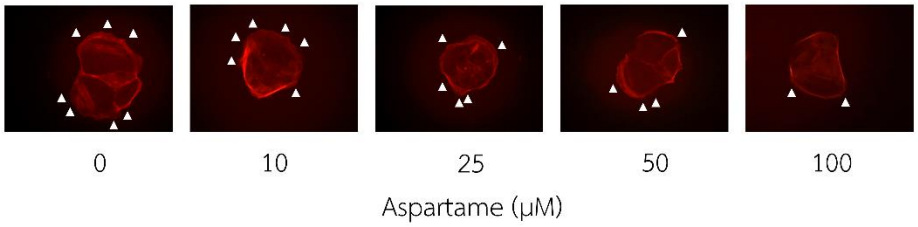
The proliferative effect of aspartame on Caco-2 cells was determined by MTT assay. The cells were treated with aspartame at non-toxic concentrations (0, 10, 25, 50, and 100 μM) for 1, 2, 3, and 5 days. The results showed that aspartame at these concentrations did not significantly affect cell proliferation (Figure 9).

4.4 Effect of aspartame on lamellipodia formation of colorectal Caco-2 cells

Cell motility is associated with cell protrusions and lamellipodia formation. Lamellipodium is a thin sheet cell protrusion at the leading edge of the cell enriched with actin cytoskeleton. Lamellipodia formation plays an important role in cell movement. The former results in wound healing and Boyden chamber cell migration assay showed that aspartame had suppressive effect on Caco-2 cell motility. The effect

of aspartame on lamellipodia formation was then further investigated by phalloidin-rhodamine staining assay. Cells were treated with aspartame at non-toxic concentrations (0, 10, 25, 50, and 100 μM) for 24 h and incubated with phalloidin-rhodamine to identify cell morphology. The results demonstrated that aspartame significantly reduced the number of lamellipodia per cell at the concentrations of 25, 50 and 100 μM (approximately 40%, 60% and 80% reduction, respectively) in comparison to that of non-treated control (Figure 10).

A.



B.

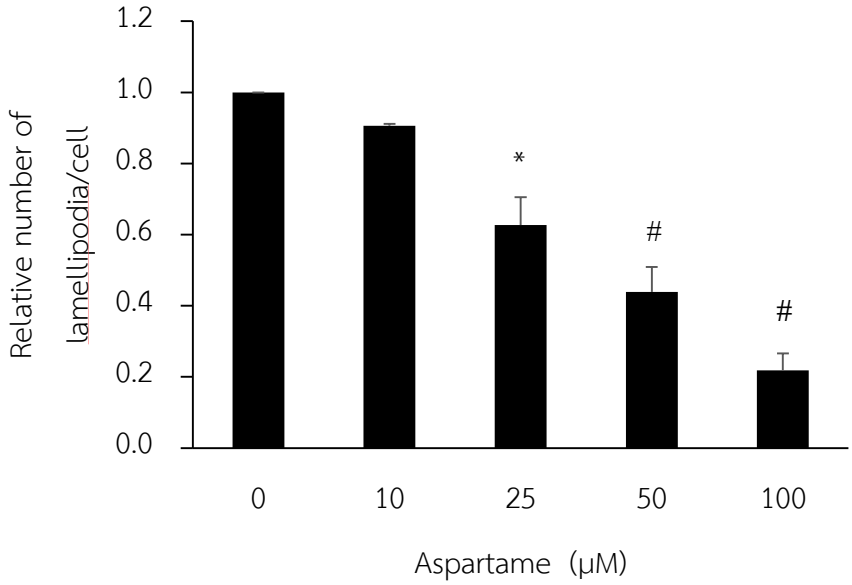


Figure 10 Effect of aspartame on lamellipodia formation (A) The cell morphology visualized under fluorescence microscopy (B) The relative number of lamellipodia/cell *$p < 0.05$ and $^{\#}p < 0.001$ versus non-treated control

4.5 Effect of aspartame on expression of proteins involving cell migration and proliferation process in colorectal Caco-2 cells

To explain the possible underlying mechanism of aspartame on cell migration and proliferation, the expression of regulating proteins in Caco-2 cells was determined by Western blotting. Cells were treated with aspartame at non-toxic concentrations (0, 10, 25, 50, and 100 μM) for 24 h, and then the expression levels of regulating proteins including integrin β1, pFAK/FAK, pAkt/Akt, pERK/ERK, Cav-1, Rac1-GTP, and RhoA-GTP were evaluated. Figure 11A-11D showed that aspartame at the concentrations of 25, 50 and 100 μM significantly decreased the levels of activated FAK, activated Akt, Cav-1, Rac1-GTP, and RhoA-GTP as compared to the non-treated control. In addition, the integrin β1 level was significantly decreased in the cell treated with aspartame at the concentration of 50 and 100 μM. However, this study found that aspartame at the indicated concentrations had no significant effect on pERK/ERK level (Figure 12A and 12B). These results suggested that aspartame inhibited cell migration through down-regulation of p-FAK, p-Akt, Cav-1, Rac1-GTP, and RhoA-GTP.

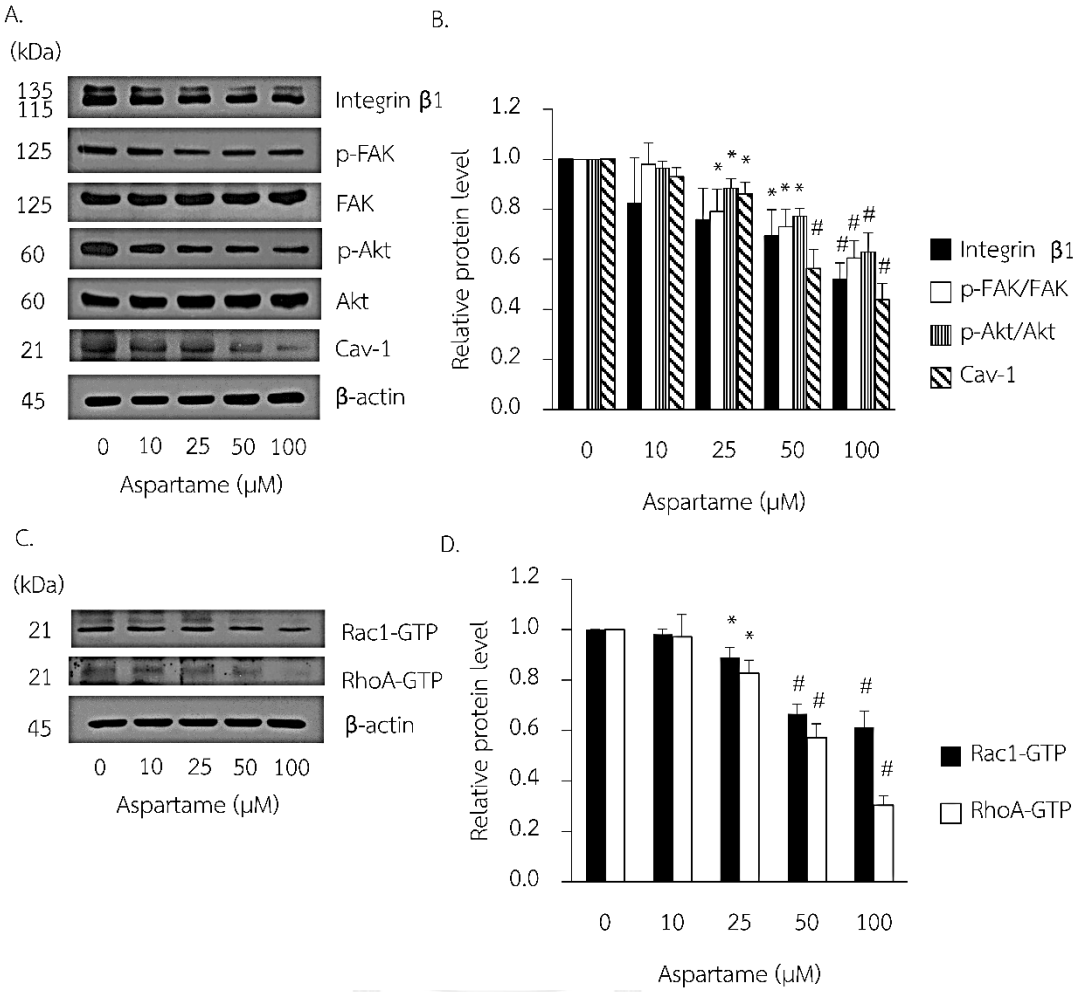


Figure 11 Effect of aspartame on migration regulatory proteins (A, C) The expression of integrin β1, pFAK, FAK, pAkt, Akt, Cav-1, Rac1-GTP, and RhoA-GTP (B, D) The immunoblot signals quantified by densitometry

*$p < 0.05$ and $^{\#}p < 0.001$ versus non-treated control

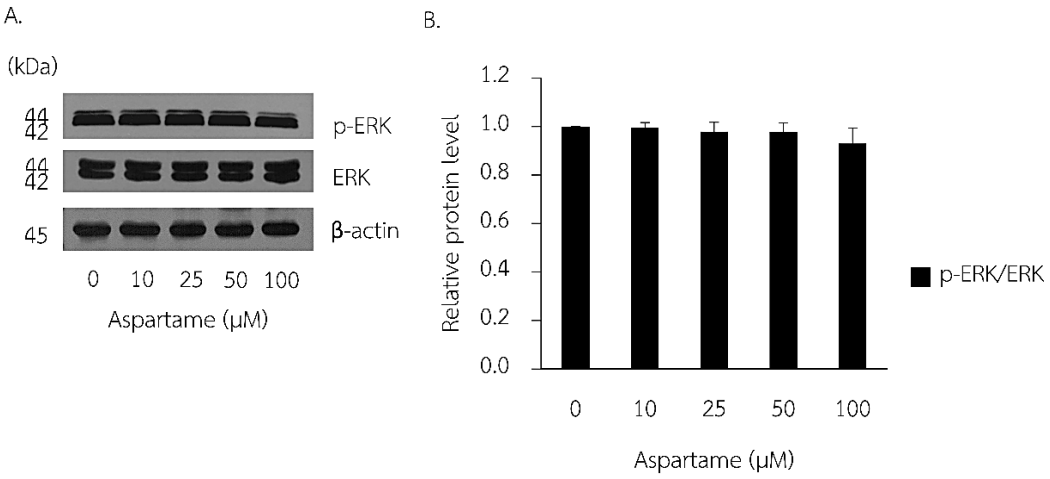


Figure 12 Effect of aspartame on proliferation regulatory proteins (A) The expression of pERK and ERK (B) The immunoblot signals quantified by densitometry

CHAPTER V
DISCUSSION

The present study was proposed to investigate the effect of aspartame on migration and proliferation in human intestinal epithelial Caco-2 cells. The expressions of proteins related to migration and proliferation process including integrin β1, pFAK/FAK, pAkt/Akt, pERK/ERK, Cav-1, Rac1-GTP, and RhoA-GTP were also determined.

5.1 Effect of aspartame on the viability of colorectal Caco-2 cells

Aspartame is a methyl ester of the dipeptide of the amino acids aspartic acid and phenylalanine which is metabolized in the digestive tract into the amino acids (Shankar et al., 2013). With normal digestion, aspartame can quickly interact with epithelial cell lining along the digestive tract before absorption. Therefore, it may play a role in the wound healing process. The present study demonstrated for the first time that the aspartame could inhibit the migration of injured human intestinal epithelial cells and thus delay wound healing process.

In this study, the effect of aspartame on wound healing was examined in the Caco-2 cells. This cell line has been widely accepted as a model for intestinal absorption. The study of hydrolysable drug and ester prodrug absorption into Caco-2 cells was demonstrated the hydrolyzing activity of Caco-2 cells (He et al., 2004; Imai et al., 2005). The intact form of aspartame may have a chance to contact with intestinal epithelial Caco-2 cells before hydrolysis reaction. Moreover, Caco-2 cells are a well-

established in vitro model which have characteristics and morphologies similar to the normal human intestinal epithelial cell (Sambruy et al., 2001). This cell model has been widely used for various aspects including wound healing study (Seltana, Basora and Beaulieu, 2011). The studies about the role of vitamin D in migration process (Kong et al., 2008) and the effect of ghrelin on proliferation process of intestinal mucosal barrier during the wound healing (Waseem et al., 2014) are the examples of the studies that used Caco-2 cell line as a model. Therefore, Caco-2 cells were used as a model for the present intestinal epithelial cell wound healing study.

The non-toxic concentrations of aspartame were determined. The result showed that all indicated concentrations of aspartame (0, 1, 5, 10, 25, 50, 100, 250, and 500 μM) were not toxic to cells. Moreover, the result from Hoechst 33342/PI co-staining confirmed no cytotoxic effect of aspartame at the concentration up to 500 μM on Caco-2 cells. This supported the result of previous studies in human umbilical vein endothelial cells (HUVEC) and human cervical carcinoma cells (HeLa) that aspartame at concentration up to 100 μM had no effect on cell viability (Alleva et al., 2011; Pandurangan, Enkhtaivan and Kim, 2016). For further experiment in this study, aspartame at the concentration up to 100 μM was thus chosen as this range was non-toxic and closed to current average consumption levels of aspartame in the United States (Marinovich et al., 2013).

5.2 Effect of aspartame on migration of colorectal Caco-2 cells

The intestinal epithelium lining is an important barrier performing with complex systems to maintain the intestinal homeostasis. This epithelial lining can be wounded in many cases such as by contacting with some chemicals and pharmaceutical products, bacterial infection as well as spontaneous injury during normal digestion process. When intestinal epithelial cell is injured, its barrier function will be impaired (Rubin, 2009). Therefore, the rapid wound repair of epithelial cell surface following injury is a necessary process to preserve normal intestinal homeostasis (Roda et al., 2010; Turner, 2006).

The balance of cell migration and proliferation processes is a critical step in restoring barrier function of the wounds, and the disturbance of this step may delay the process of wound healing (Iizuka and Konno, 2011; Sturm and Dignass, 2008). Cell migration and cell proliferation appear to be affected by some components consumed that directly contact intestinal epithelial cells. Linolelaidic acid, a fatty acid supplementation showed significant inhibitory effect on intestinal epithelial cell growth at concentration above 250 μmol/L (Ruthig and Meckling-Gill, 1999), and arginine supplementation was found to increase rate of intestinal cell migration through FAK activation signaling (Amano et al., 2000). The interference with gastric wound healing was also found with NSAIDs use (Pai et al., 2001). Moreover, loss of some components of cells may affect this healing process. The suppression of cell migration during wound

repair was found in gastric epithelial cells with knockdown of aquaporins (AQPs), a water channel protein (Hayashi et al., 2009).

The study of underlying association between artificial sweetener consumption and adverse metabolic outcome demonstrated that artificial sweeteners may cause the unexpected disturbance along the gastro-intestinal (GI) tract (Pepino, 2015). The low-caloric sweeteners from soda drink may have an interaction with sweet taste receptor in the gut and interfere microbiological function in the GI tract (Meyer-Gerspach, Wölnerhanssen and Beglinger, 2016). As aspartame can quickly interact with epithelial cell lining along the digestive tract before absorption, it may play a role in wound healing.

In the present study, we found that aspartame at indicated concentrations (0, 10, 25, 50, and 100 μM) inhibited Caco-2 cell migration. Moreover, the cell characterization assay also showed the decrease in cell migration by the reduction of lamellipodia formation in a dose-dependent manner. However, when the cells were treated with aspartame at the concentrations of 25 and 50 μM for 48 h, the significant inhibitory effect on cell migration determined by wound healing assay was not found, but the significant inhibitory effect on cell migration was found when it was determined by Boyden chamber cell migration assay. The mucosal healing becomes a clinical marker for determination of recovery after IBD therapy, and it is associated with the risk assessment for future surgeries (Neurath and Travis, 2012). The study in patients with IBD revealed that mucosal healing was associated with better symptoms and

suggested that factors affecting cell migration and proliferation process in the wound healing should be investigated to prevent the development of disease (Peyrin-Biroulet et al., 2011; Rieder et al., 2012). The present finding may raise the concern of aspartame use in patients with intestinal injury.

The underlying mechanism of inhibitory effect of aspartame on cell migration in this study was found to be associated with significant down-regulation of integrin β1, activated FAK and activated Akt. Cell motility in the process of wound healing is a multiple process involving the change of cell cytoskeleton and membrane protrusion. Previous studies demonstrated important roles of various cellular signaling proteins including integrins, FAK and Akt in cell migration process (Holly et al., 2000; Huttenlocher et al., 1995; Navarro et al., 2004; Xue and Hemmings, 2013). During cell movement, integrins play an essential role in the signaling process to form focal adhesion complex. This process composes of integrin cluster and adaptor protein to provide a structural framework (Holly et al., 2000).

Integrins are a family of glycoprotein receptors that bind to ECM ligands. The integrin-dependent signaling induces a conformational change in the integrin resulting in FAK autophosphorylation at Tyr397 position, which will further incorporate with other signaling proteins required for cell spreading (Huttenlocher and Horwitz, 2011; Tomar and Schlaepfer, 2009). The autophosphorylation at Tyr397 of FAK has been proved to be a binding site for other signaling proteins, and the mutation at Tyr397 site of FAK suppressed its ability to stimulate cell migration (Cary, Chang and Guan,

1996). In integrin-mediating cell migration, FAK regulates contraction of cell with the ECM at focal adhesion site (Hauck, Hsia and Schlaepfer, 2002; Mitra and Schlaepfer, 2006). The reduction of cell motility with no significant change in integrin-β1 distribution was found in cells derived from FAK-deficient mice embryos indicating the important role of FAK for integrin-β1 signal generating (llic et al., 1995).

The phosphorylated FAK at position Tyr397 was reported in the regulation of cell movement associating with Akt phosphorylation (Turečková et al., 2009). The activated FAK recruits the Src-homology domain of PI3K which regulates various signaling pathways (Sieg, Hauck and Schlaepfer, 1999). It was found that the inhibition of PI3K activation was able to inhibit FAK-promoted migration. PI3K signaling mediates phosphorylation of Akt through PIP_3, and this pathway has been reported as a key signaling pathway modulated by activated FAK promotes cell migration (Chen et al., 1996; Mitra et al., 2005). Therefore, FAK-Akt association pathway is required to promote cell migration (Meng et al., 2009). The present study found that the protein levels of integrins, pFAK and pAkt were decreased in the cell treated with aspartame. The reduction of activated Akt may be a downstream response to the reduction of phosphorylated FAK.

The FAK-Akt mediated signaling results in the activation of downstream Rho family proteins to promote the protrusion formation of cell motility (Hanks et al., 2003). PI3K/Akt activation by receptor protein tyrosine kinase is an important upstream for the activation of Rho family which regulates cell movement (Higuchi et al., 2001; Liao

et al., 2006). These observations suggested that Akt activation has an impact on cell motility through the modulation of actin. The increased active levels of Rac and Rho were detected during the actin polymerization and lamellipodia formation at the front of migrating cells (Raftopoulou and Hall, 2004; Zhao and Guan, 2011). The activation of Rac promotes the formation of plasma membrane protrusion and lamellipodia, while the active Rho regulates the stress fiber and focal adhesion formation (Nobes and Hall, 1995).

The study of epithelial wound healing found that epithelial cell migration was associated with function of Rho family proteins in focal adhesion formation and lamellipodia extension (Lotz, Rabinovitz and Mercurio, 2000). In the present study, aspartame was found to decrease the number of lamellipodia. This study also found the decreased expression of Rac1-GTP and RhoA-GTP proteins in response to aspartame treatment. The reduction of Rac1-GTP and RhoA-GTP levels in this study was in correlation with the reduction of FAK/Akt expression.

In the present study, aspartame significantly decreased the level of Cav-1 protein, which is a major protein of caveolae membranes playing a role in cell migration process (Núñez-Wehinger et al., 2014). Cav1-null fibroblasts showed the decrease in migration and Rho-GTPase family protein level indicating the role of Cav-1 in promoting migration of normal cells (Grande-García et al., 2007). Previous study indicated that Cav-1 was associated with cell movement through a PI3K/Akt-dependent mechanism (Chanvorachote et al., 2014). Down-regulation of Cav-1

expression leads to the attenuation of Akt phosphorylation and then inhibits actin filament network through the Rho family (Navarro et al., 2004; Núñez-Wehinger et al., 2014). It is possible that aspartame decreases cell migration through down-regulating Cav-1 protein level with subsequent decrease in the levels of Rho-GTPase family proteins. Therefore, aspartame may have an effect on cellular migration by suppression of the protein levels of pFAK, pAkt, Cav-1, Rac1-GTP, and RhoA-GTP.

5.3 Effect of aspartame on proliferation of colorectal Caco-2 cells

Cell proliferation is an essential process in the wound healing to maintain epithelial cell homeostasis and integrity. Various cellular signaling proteins including Akt and ERK play important roles in cell proliferation process (Huang et al., 2004; Larsen et al., 2003). The study of platelet-derived growth factor (PDGF)-induced rat hepatic stellate cell proliferation found that FAK was an upstream of PI3K signaling protein in the proliferative response (Reif et al., 2003), and the proliferative signals by PI3K through PI3K/Akt pathway in intestinal epithelial cells was required for intestinal epithelial proliferation (Sheng et al., 2003). ERK is essential for stimulation of intestinal cell proliferation (Aliaga et al., 1999), and the factors disturbing the ERK signaling also affect the cell proliferation process (Rhoads et al., 1997). The role of Erk1/2 in regulation of cell proliferation was found to be associated with Akt signaling pathway (Zhang and Liu, 2002), and PI3K/Akt-dependent inhibition of ERK was demonstrated in different cell types (Laprise et al., 2004).

The present study found that aspartame had no significant effect on cell proliferation. Significant decrease in Akt protein level was found without significant decrease in pERK protein level. Previous study indicated that both PI3K and Akt were required in endothelial cell proliferation and migration with different potential signalings below Akt (Steinle et al., 2002), and Akt and ERK activation may be independent from each other (Peng et al., 2005). Moreover, the effect of some nutrients consumed on intestinal epithelial cell migration was found without the effect on cell proliferation (Cario et al., 2000). For these reasons, it may be possible that aspartame has significantly effect on intestinal migration with no effect on intestinal epithelial cell proliferation via ERK-independent pathway.

This study demonstrated that aspartame inhibited migration of intestinal epithelial cells with no effect on cell proliferation. However, such results were possibly due to the effects of its metabolites, phenylalanine and aspartic acid. It was found that phenylalanine at high dose (1mM and 10mM) and at the concentration of 0.1mM decreased migration and proliferation of human fibroblasts, respectively (Vaughan, Alkadhem and Seagraves, 2016). Nowadays, there is no study on the effect of phenylalanine and aspartic acid on migration and proliferation of intestinal epithelial cell. Therefore, this issue requires further studies to clarify what exactly affect wound healing of GI tract.

CHAPTER VI
CONCLUSION

The present study demonstrated that aspartame at the concentrations of up to 500 μM was relatively non-toxic. When chosen aspartame at the concentrations of up to 100 μM which closed to the average concentration of aspartame consumed in the Unites States for experiments, it was found that aspartame at the concentrations of 25, 50 and 100 μM significantly suppressed migratory behavior of intestinal epithelial cells and decreased the number of lamellipodia per cell. However, aspartame at indicated concentrations had no significant effect on epithelial cell proliferation. This study also demonstrated that aspartame inhibited the motility of cells through FAK/Akt and Cav-1-dependent pathways (Figure 13).

The inhibition of epithelial cell migration may delay the healing process and lead to other disorders. As the migration of epithelial cell has an important impact on the wound healing, the information gained from this study may initiate further investigations about the new caution of aspartame-sweetened product consumption in people with intestinal injury and may be useful to individuals with GI ulcer in choosing appropriate foods. However, further experiments may be required to investigate the effect of aspartame at increasing doses on wound healing and the other possible underlying mechanisms for more information. The additional clinical studies

may be needed to further characterize the effects of aspartame in foods or beverages on GI wound in patients with GI injury.

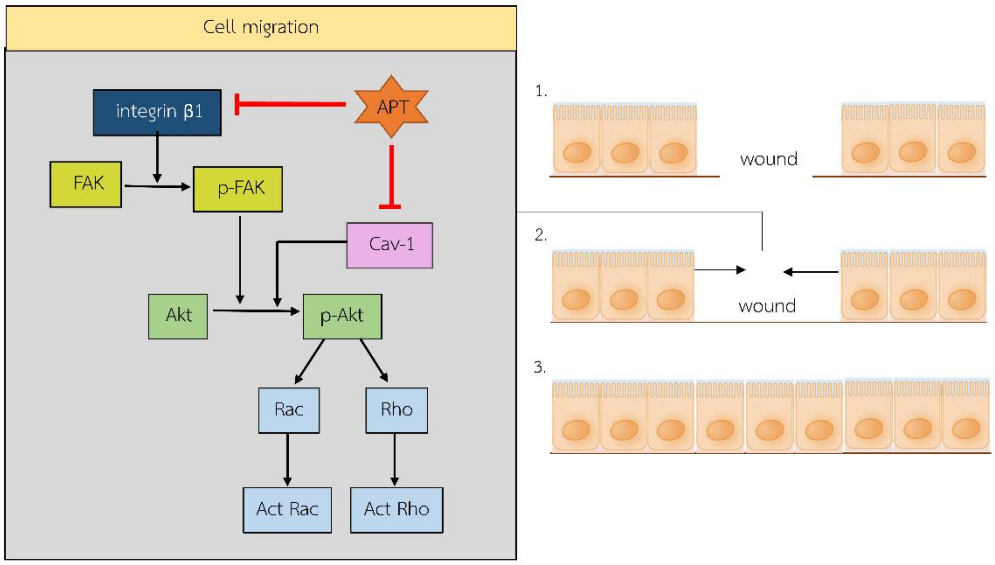


Figure 13 Schematic diagram summarizing the effect of aspartame (APT) on intestinal epithelial cell migration. When the epithelial cells are injured (1), the wound healing process involving the balance of cell migration and proliferation occurs. Cells around the wound will migrate and proliferate to close the gap area. This study found that aspartame inhibited intestinal epithelial cell migration through down-regulation of integrin β1, pFAK, pAkt, Cav-1, Rac1-GTP, and RhoA-GTP (2). After the repairing process, the wound will be completely healed (3).

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APPENDIX

TABLE OF EXPERIMENTAL RESULTS

Table A-1 The percentage of Caco-2 cell viability for each experiment determined by MTT assay after treatment with various concentrations of aspartame

Aspartame (μM)	% Cell viability		
	N1	N2	N3
0	100.00 ± 0.02	100.00 ± 0.04	100.00 ± 0.11
1	99.57 ± 0.01	88.21 ± 0.01	106.40 ± 0.02
5	90.14 ± 0.01	92.59 ± 0.01	98.32 ± 0.01
10	91.97 ± 0.01	96.50 ± 0.01	95.28 ± 0.02
25	92.47 ± 0.03	98.47 ± 0.05	96.89 ± 0.03
50	99.60 ± 0.02	93.63 ± 0.04	92.86 ± 0.03
100	94.81 ± 0.01	94.05 ± 0.05	100.95 ± 0.01
250	94.67 ± 0.02	96.61 ± 0.01	94.27 ± 0.01
500	102.93 ± 0.02	87.18 ± 0.01	95.81 ± 0.01

Table A-2 The percentage of apoptotic Caco-2 cell detected by Hoechst 33342/PI after treatment with various concentrations of aspartame

Aspartame (μM)	% Apoptosis		
	N1	N2	N3
0	2.38	2.46	2.89
1	3.17	2.98	3.78
5	4.52	3.50	4.78
10	5.76	4.91	6.09
25	5.13	5.91	5.21
50	5.36	6.10	5.89
100	4.34	5.10	5.10
250	5.66	5.90	4.79
500	5.56	5.21	6.21

Table A-3 The relative cell migration of Caco-2 cells determined by wound healing assay and analyzed under a phase contrast microscope after treatment with non-toxic concentrations of aspartame at different time

Aspartame (μM)	Relative cell migration								
	12 h			24 h			48 h		
	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
10	0.97	0.95	1.02	0.92	0.96	0.96	1.00	1.00	1.00
25	0.94	0.81	1.02	0.93	0.85	0.89	1.00	1.00	1.00
50	0.95	0.91	0.85	0.83	0.87	0.85	1.00	1.00	1.00
100	0.90	0.91	0.87	0.81	0.83	0.71	0.81	0.81	0.64

Table A-4 The percentage of number cells/field of Caco-2 cells determined by Borden chamber cell migration assay and analyzed under a fluorescence microscope after treatment with non-toxic concentrations of aspartame at 48h

Aspartame (μM)	The percentage of number cells/field		
	N1	N2	N3
0	100.00	100.00	100.00
10	95.24	92.42	90.67
25	69.39	75.00	78.67
50	61.22	60.61	52.67
100	48.98	50.00	53.33

Table A-5 The relative number of lamellipodia/cell of Caco-2 cells determined by wound healing assay and analyzed under a fluorescence microscope after treatment with non-toxic concentrations of aspartame at 24h

Aspartame (μM)	Relative number of lamellipodia/cell		
	N1	N2	N3
0	1.00	1.00	1.00
10	0.91	0.90	0.91
25	0.64	0.70	0.55
50	0.45	0.50	0.36
100	0.18	0.20	0.27

Table A-6 The relative levels of cell viability of Caco-2 cells determined by MTT assay after treatment with non-toxic concentrations of aspartame

Aspartame (μM)	Relative cell viability														
	Day 0			Day 1			Day 2			Day 3			Day 5		
	N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	1.00	1.00	1.00	1.36	1.26	1.12	1.45	1.43	1.36	4.16	4.72	5.09	3.88	4.61	6.22
10	1.00	1.00	1.00	1.35	1.29	1.10	1.38	1.59	1.43	4.15	4.89	5.07	3.75	4.84	6.25
25	1.00	1.00	1.00	1.34	1.01	1.13	1.36	1.50	1.55	4.55	5.04	4.95	3.78	5.38	6.27
50	1.00	1.00	1.00	1.24	1.04	1.03	1.54	1.55	1.48	4.45	4.90	5.02	4.28	4.84	6.37
100	1.00	1.00	1.00	1.08	1.07	1.04	1.05	1.61	1.69	3.61	4.79	5.20	3.65	4.73	6.10

Table A-7 The relative levels of migration-associated proteins in Caco-2 cells determined by Western blot analysis after treatment with non-toxic concentrations of aspartame at 24h

Aspartame (μM)	Relative protein level								
	integrin β1			pFAK/FAK			Cav-1		
	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
10	0.98	0.87	0.62	1.07	0.97	0.90	0.97	0.91	0.91
25	0.90	0.66	0.72	0.69	0.83	0.86	0.87	0.81	0.90
50	0.78	0.72	0.58	0.67	0.71	0.81	0.56	0.64	0.49
100	0.59	0.51	0.46	0.56	0.68	0.58	0.50	0.37	0.44

Table A-8 The relative levels of proteins involved with actin organization in Caco-2 cells determined by Western blot analysis after treatment with non-toxic concentrations of aspartame at 24h

Aspartame (μM)	Relative protein level								
	pAkt/Akt			RhoA-GTP			Rac1-GTP		
	N1	N2	N3	N1	N2	N3	N1	N2	N3
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
10	0.96	0.94	0.99	0.98	1.05	0.88	0.97	0.97	1.00
25	0.85	0.92	0.88	0.77	0.86	0.86	0.89	0.85	0.93
50	0.79	0.74	0.79	0.52	0.63	0.56	0.71	0.63	0.66
100	0.71	0.61	0.56	0.29	0.27	0.34	0.54	0.64	0.66

Table A-9 The relative levels of proliferation-associated proteins in Caco-2 cells determined by Western blot analysis after treatment with non-toxic concentrations of aspartame at 24h

Aspartame (μM)	Relative protein level		
	pERK/ERK		
	N1	N2	N3
0	1.00	1.00	1.00
10	1.00	0.93	1.05
25	1.10	0.91	0.92
50	1.03	0.88	1.02
100	1.01	0.92	0.86

VITA

Miss Tawiwan Sawadsopanon was born on June 11, 1988 in Bangkok, Thailand. She received her Bachelor of Science in Pharmacy from the Faculty of Pharmaceutical Sciences, Chulalongkorn University in 2011. After graduation, she worked as a researcher in the Research and Development Institute of the Government Pharmaceutical Organization in 2011-2013. Her responsibilities as a researcher included development and improvement of formulation in solution and injection dosage form, analyzing the preliminary formulation and providing the formulation and analytical information for registration. In addition, she also worked as a part-time pharmacist in the P&F drugstore in 2011-2013. Her responsibilities included dispensing prescription, selling medicines and advising patients of any adverse side-effects of medicines.