EFFECTS OF *PIPER LONGUM* EXTRACTS ON LIVER CANCER CELL MIGRATION AND INVASION

ALISA JAIPETCH

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR MASTER DEGREE OF SCIENCE
IN MEDICAL SCIENCE
FACULTY OF ALLIED HEALTH SCIENCES
BURAPHA UNIVERSITY
2022

ฤทธิ์ของสารสกัดดีปลีต่อการแพร่กระจายและการลุกลามของเซลล์มะเร็งตับ

อลิษา ใจเพ็ชร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต
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The Thesis of Alisa Jaipetch has been approved by the examining committee to be partial fulfillment of the requirements for the Master Degree of Science in Medical Science of Burapha University

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ALISA JAIPETCH : EFFECTS OF *PIPER LONGUM* EXTRACTS ON LIVER CANCER CELL MIGRATION AND INVASION. ADVISORY COMMITTEE: NARIN CHANGKLUNGMOA, Ph.D., PORNANAN KUEAKHAI, Ph.D. WIPAPHORN JAIKUA, Ph.D. 2022.

Liver cancer is the most common type of cancer which is an accident leading to death worldwide. In present, chemotherapy is the most common type of conventional cancer therapy. However, it has some limitations due to tumor metastasis, medication-induced side effects and drug resistance. Alternative therapies are needed for solving this problem. As a result, natural products are interesting as a new strategy to cure cancer. Black pepper is a plant in which some anti-cancer effects have been reported. Therefore, we were interested in the potential of *Piper longum*, a plant in the same family as black pepper, as a new anticancer therapeutic drug. The aim of this study is to determine the cytotoxicity, anti-migration, anti-invasion and colony formation of *P. longum* extract on liver cancer cell line (HepG2) and normal liver cell line (FL83B) by using MTT assay, scratch plate assay, transwell assay and colony formation assay. The result showed that the IC50 values of *P. longum* extract were more than 1,000 µg/ml on both cells. Moreover, *P. longum* extract from 400 µg/ml at 24 h and 600 µg/ml onwards significantly inhibited the cancer migration. The anti-invasion result showed that 400 and 600 µg/ml significantly decreased cell invasion. The colony formation assay was used to test, the percentages of colony number and size were showed significantly decreased by *P. longum* extract from 100 µg/ml onward inhibited the colony formation at both 24 and 48 h. However, the size of colony formation was showed significantly decreased from 200 µg/ml onward at 48 h. In conclusion, *P. longum* extract is safe because it has the potential to inhibit the growth of liver cancer cells. The *P. longum* extract should be considered as a candidate to be developed as a new therapeutic drug, adjunctive treatment or food supplement for liver cancer in the future.

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Alisa Jaipetch

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CHAPTER I
INTRODUCTION

1.1 Introduction

Liver cancer is the ninth most common cancer in females and the fifth in males worldwide (WHO, 2018), in which a hepatocyte undergoes a genetic transformation into a cancer cell. Furthermore, liver cancer is a public health problem which the accident and mortality rate of cancer is increasing every year worldwide. Conventional cancer therapy is in many ways such as surgical, radiotherapy and the most common is chemotherapy. However, chemotherapy is limited by having severe side effects and drug resistance (McWhirter et al., 2013). Additional therapies are therefore needed to adjust the problems. Natural products are consulted to have anticancer activity with high effectiveness and less toxicity. In present, more than 60% of drugs currently use for cancer treatment, have been isolated from natural products and obtained from plants (Gordaliza, 2007). This leads to greater interest in a natural product to use for new anticancer therapeutic drugs. In current studies, *Prunus persica* has indicated about effects of cell death through the reduction of the proliferation and migration of cells on HepG2 cell lines. Furthermore, a previous study has investigated gastric cancer cell lines by treated with *Annonaceous acetogenins* extract indicated about induction of the proliferation of cells.

Piper longum is a well-known long pepper that is a climber plant and belonging to the *Piperaceae* family. It's widely distributed in Indonesia, Vietnam, China, and Philippines (Zaveri et al, 2010). Long pepper haves to use in cooking for spicy seem like pepper (Manoj et al., 2004). In traditional medicine, *P. longum* is a used component of medicines in the treatment of sleeping problems, menstrual pain, chronic gut-related pain, etc. (Sunila & Kuttan, 2004). Furthermore, numerous studies have reported the presence of different biologically active compounds in *P. longum* such as alkaloids, saponins, amygdalin, essential oils, piperanine and piperine (Li et al., 2013). It was reported that water extract of *P. longum* showed good antioxidant activity (Sunila & Kuttan, 2004) and their ability to anti-inflammatory and anti-tumor activity of the supercritical extract in SKOV-3, RAW 264.7, HeLa,

and HepG2 cells (Guo et al., 2019). However, the effects on biological activity and active substance of *P. longum* were still unclear.

This assumption, together with the need for novel therapeutic drugs for liver cancer led us to focus anti-cancer effect and cytotoxicity of *P. longum* extract on liver cancer cell lines by MTT assay and anti-migration, anti-invasion, and colony formation on liver cell lines was investigated.

1.2 Objectives of this study

1. To extract and determined the phytochemical composition of ***P. longum***
2. To evaluate cell viability of *P. longum* extract by MTT assay on liver cancer cell lines
3. To determine the anti-migration, anti-invasion and colony formation of ***P. longum*** extract on the liver cancer cell lines

CHAPTER II
LITURATURE REVIEW

2.1 Cancer

2.1.1 Carcinogenesis

Cancer is one of the diseases that affected without control division of the body's cells and almost all body tissues are beginning to cancer. Although cancer can begin everywhere in the body but mechanisms and each type of cancer have their unique features. Furthermore, the basic system of cancer has similarities in all forms of the ailment. Start with abnormal cells caused by many factors develops to hyperplasia and begin to proliferate quickly and aggressively. Cells will be invaded and become cancer in the end (Figure 1).

2.1.2 Incidence of cancer

Cancer is a major public health concern in the world and the second leading cause of death worldwide. According to the World Health Organization, in 2018 there were 9.6 million cancer cases worldwide. The most common cancers are Lung (2.09 million cases), breast (2.09 million cases), colorectal (1.80 million cases), Prostate (1.28 million cases), Skin cancer (non-melanoma) (1.04 million cases), and Stomach (1.03 million cases) (World Health Organization, 2018). Furthermore, people who die from cancer are involved in behavior and food intake as low fruit and vegetable intake, high body mass, tobacco, and alcohol used. Tobacco used is the most important risk factor of cancer deaths (Collaborators, 2016) . However, the main causing cancer death is tobacco but another factor is important too. Infection causing was reported as one of the risk factors for cancer such as hepatitis B virus, etc. its reports that 25% of cancer cases of low- and middle-income countries have infections developed and turned into cancer (Plummer et al., 2016).

Incidence of cancer in Thailand, According to WHO in 2020 have a new case of cancer patients about 190,636 people of both sex and all ages. The most common cancers in Thailand are liver cancer about 27,394 people (14.4%) following by Lung 12.4%, Breast cancer 11.6%, Colorectal cancer 11.1%, Cervix uteri 4.8%, and other cancer 45.7%, respectively (The Global Cancer Observatory, 2020) (Figure.2).

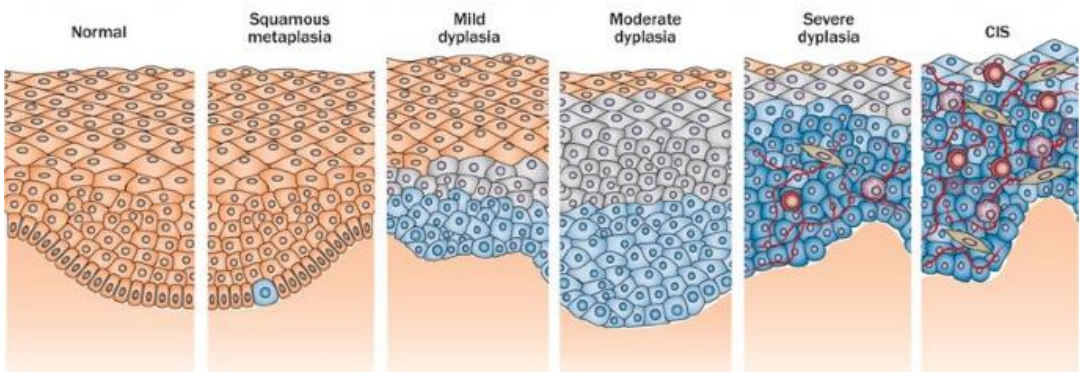

Figure.1 Development of cancer

(Keith & Miller, 2013)

In characteristic of cancer is abnormal cell growth with the potential to invade and migrate to nearby tissue or organ through the blood and lymph node systems. The formation of cancer involves three stages such as initiation, promotion, and progression. Initiation, Cancer begins from cells that have an abnormal system in cell division such as mutations, transitions, and deletions in DNA, the main cause of immature or abnormal cells is an escape. As mentioned previously, there is still another cause that can arise is epigenetics. Epigenetic may be generated of cancer progenitor cells and carcinogenesis initiated (Sarkar et al. , 2013) . After that, the promotion stage. This step related to activation of oncogenes or abnormal tumor suppressor genes lead to uncontrolled cell cycle division and apoptotic mechanisms are inactivated to affected inhibition apoptosis. The next step is progression, the step of induction of angiogenesis for invasion and metastasis. (Figure.2)

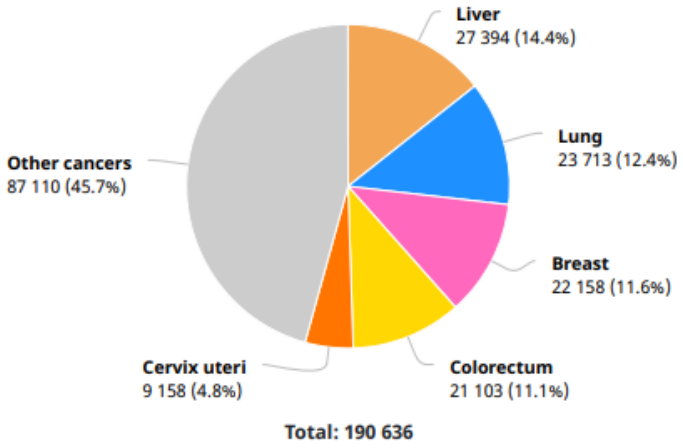

Figure.2 Incidence of cancer in Thailand

(The Global Cancer Observatory, 2020)

In addition, proteins target related to carcinogenesis were demonstrated. The important or main protein that causes cancer is p53 because p53 is responsible for cell division in checkpoint and p53 is a tumor suppressor gene that imperative for G1 phase arrest after DNA damage. Mutations at the p53 are the most frequent genetic found with cancer in humans. Other protein involved in cell division such as p21, p16, and p27 all above is cell cycle regulators that regulation about inactivates cyclin E-Cdk2, cyclin A-Cdk2, and cyclins D1-, D2-, and D3-Cdk4 complexes for inhibiting pRB phosphorylation is related to preventing progression of the cell cycle beyond G1 (David E. Malarkey, 2013).

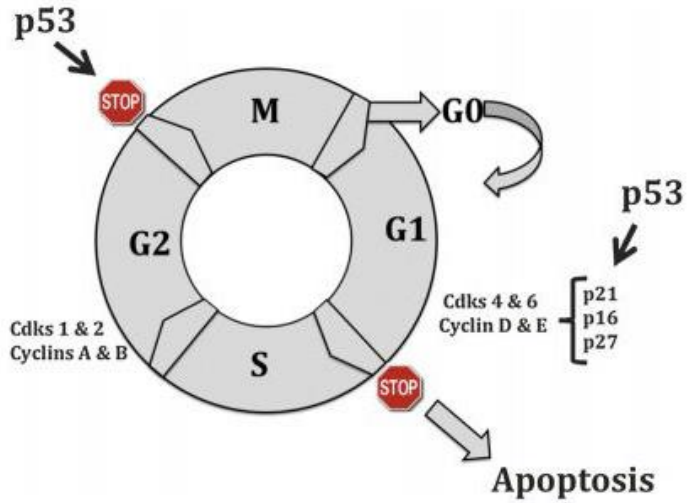

Figure.3 p53 involved cell division

(David et al., 2013)

According to, the main reason for cancer's virulence is the progression of the cancer cells following by the metastatic. Metastasis is a process that includes migration and invasion of cancer cells. When cancer has spread, there have many factors involved, Describe the dosages when abnormal cells or cancer cells have occurred. In currently studies with cultured fibroblast have indicated that a few factors related to the formation of cancer are involved in integrin protein because integrin protein acts on cell adhesion with extracellular matrix (ECM) and integrin protein is the main cause of the formation of "focal contacts" (Zamir et al., 2000), which cause to degrade of ECM. When contact with ECM ligand, integrin will clusterization on the membrane and adapter protein or signaling protein is a carrier to pass through the intracellular, that leads to phosphorylation and dephosphorylation within the cells. In

2002, partway and factors involved in integrin protein were investigated. The formation of focal contact is related to many pathways such as phosphatidylinositol 3-phosphate (PI3K), the protein kinase C (PKC), and Rho family GTPases (Degani et al., 2002).

In addition, have a study investigating the epithelial-mesenchymal transition (EMT) is a common event in the progression of various types of cancer and appearances loss of expression of E-cadherin, which is adhesion molecules and regulated by the activity of Rho GTPase (Bozzuto et al., 2010). The invadopodia are also related to metastasis, that are cellular protrusions including actin that can mediate the proteolysis of ECM (Stylli et al., 2008) and The role of invadopodia in specifically aggressive tumors such as gliomas, breast carcinomas, and melanomas have been recently discussed(Stylli, Kaye, & Lock, 2008). Then, the marker of invadopodia was investigated and including F-actin (filamentous actin form), Arp2/3, N-WASP, and cortactin, SH3-proteins involved in various tumors (Linder, 2007). Moreover, numerous studies have indicated that the ERK1/2 factors involved the cell motility (Webb et al., 2000) and ERK may regulate the focal contact. When Ras, Raf, and MEK1/2 activated, followed by the phosphorylation of ERK consisting of MLCK, calpain, paxillin, and FAK. Currently studies have shown p38 plays a key role in the migration of cancers, which is a main protein for the formation of stress fiber related to stimulating the directional migration of tumors (Hannigan et al., 2001). Furthermore, c-Jun N-terminal kinase 1/2 (JNK1/2) is also regulated in migration cell about the formation of stress fibers, that leads to the migration. According mentioned above, factors related to migration and invasion such as p38, ERK1/2, PI3K pathway, and JNK1/2 regulate MMPs activated (Figure.4).

MMPs is regulation of transcriptional and post-transcriptional level including cytokines, growth factors, hormones, oncogenes and tumor promoters (Birkedal-Hansen et al., 1993). In addition, MMPs more activated leads to ECM degradation. The activity of MMPs is regulated by the family of tissue inhibitors of metalloproteinases (TIMPs) such as TIMP1 and TIMP2. TIMP1 acts on inhibits the activation of proMMP-9 and TIMP2 is regulated activation of pro-MMP2 by at the low concentration of stimulation, TIMP2 promotes the complex of pro-MMP2 and activates of MMP2. The expression of MMPs and TIMPs changes during cancer

formation. Thus, the imbalance of MMPs and other factors is an important role in the invasion and migration of cancer.

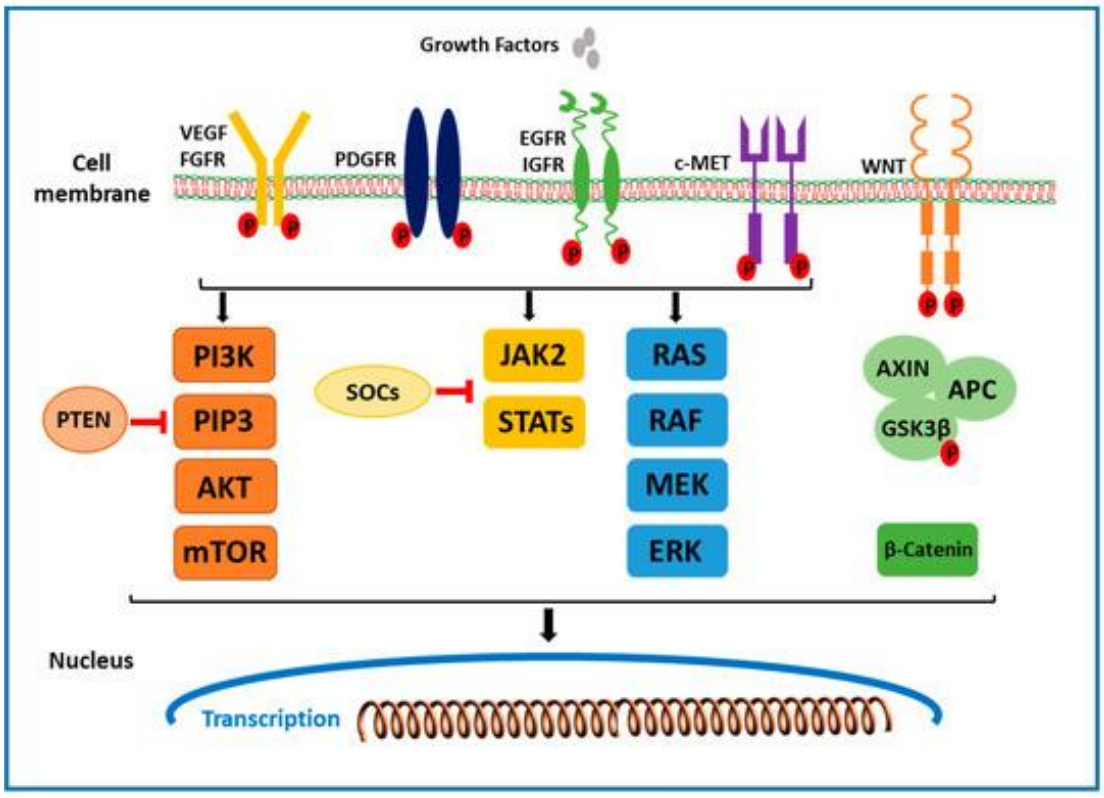

Figure.4 Major signaling pathways involved in hepatocellular carcinoma (Dimri et al., 2020)

2.1.3 Liver cancer

The rate of death from liver cancer increased by 43% (from 7.2 to 10.3 deaths per 100,000) between 2000 and 2016 worldwide (Xu, 2018) and is The most common cancer found in Thailand in 2020 is liver cancer about 27,394 of 190,636 people or 14.4% from other types of cancers (WHO, 2022). Liver cancer is a disease in which a hepatocyte undergoes a genetic transformation into a cancer cell and liver cancer is the ninth most common cancer in females and the fifth in males worldwide. Liver cancer has 2 types are hepatocellular carcinoma and cholangiocarcinoma. There are differences in position and cell type. Hepatocellular carcinoma develops by the transformation of hepatocytes and Cholangiocarcinoma caused by intrahepatic bile duct epithelium.

Carcinogenesis of liver cancer has various risk factors such as hepatitis B or C infectious, fatty liver, obesity, alcohol addicted and medication harmful to the liver (Jutaghokiat, 2020). Furthermore, one of the causes of cancer is liver inflammation. Chronic liver inflammation and hepatocarcinogenesis pathways are related to the inflammation cancer transformation during the development of primary liver cancer (Chen. et al., 2018). Cancer is associated with inflammation because in 2002 there was a research that 15% of humans cancer caused by inflammation and chronic infection (Coussens & Werb, 2002). Hence, inflammation factors are related to cancer such as chemokine and cytokines including PI3K/ AKT/ mTOR, VEGF, Wnt, and MAPK pathway, that consistent with the previous study which related to identification on the basis of specimens from patients at early cancer stages have investigated on a biomarker of hepatocellular carcinoma, that showed high serum levels of alpha-fetoprotein, poor cell differentiation, chromosomal instability, TP53 mutations, and activation of oncogenic pathways e.g., RAS–mitogen-activated protein kinase (MAPK), AKT–mammalian target of rapamycin (mTOR), and MET (a hepatocyte growth factor receptor) (Zucman-Rossi., 2015). All factors mentioned before playing a role in the proliferation and mutation of liver cells. In previous studies, WNT pathway is involved hepatocyte differentiation that causes by WNT signaling pathway related to hepatocellular carcinoma (Chen et al., 2016).

Liver cancer has 4 stages, stages range from stage I through IV. As a rule, the lower the number, the less cancer has spread such as stage IV is spread more. And all stages are shown in Figure.5

- In stage I, tumors size id least than 2 cm or smaller that hasn't grown into blood vessels.
- In stage II, tumors size larger than 2 cm but that has grown into blood vessels.
- In stage III, more than one tumor but that has spread into lymph node.
- In stage IV, tumors are spread to nearby lymph nodes

Normal **I** **II** **III** **IV**

Figure.5 Stage of liver cancer.

(Fujiwara et al., 2018)

2.1.3.1 Treatment of liver cancer

Current treatment is maintained according to the stage of cancer. Which primary liver cancer, hepatocellular carcinoma is most common. The treatment of cancer is one of the most difficult cancers to treat because liver cancer is often diagnosed at an advanced stage and treatment with chemotherapies is low efficient for liver cancer patients (Verslype et al., 2009). For patients with early stages of liver cancer suitable for surgery or liver transplantation more than other procedures. Furthermore, the drug has been used as well. The drug is called "Sorafenib", which is a treatment for primary liver cancer by inhibiting the activity of Raf-1 and other tyrosine kinases, such as vascular endothelial growth factor receptor 2 (VEGFR-2), VEGFR-3, Fms-like tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor 1 (FGFR-1) (Zhu et al., 2009). However, sorafenib is used for treatment but liver cancer is considered to be a chemotherapy-refractory tumor. In addition, chemotherapy is resistant in some patients. Hence, in the present which artificial new drugs are being develop.

2.1.3.2 Phytochemical treatment

Numerous studies indicate that more than 60% of drugs, currently in use for cancer treatment from natural products that obtained from the plant (Gordaliza, 2007). In previous studies, there have been studies looking for a drug to treat liver cancer. *Prunus persica* was tested on HepG2 cell lines about cell migration and cell viability assay and shown it works that it can reduce the proliferation and migration of cells (Shen et al., 2017). Curcumin also has studied the treatment of cancer in 2015 has to research that effectiveness of curcumin have induction of cell death through inhibits NF-κB signaling pathway (Marquardt et al., 2015). Moreover, there are also many plants that have the effect of inhibition on liver cancer cell lines.

One of them is *Piper sarmentosum* shown results that reducing apoptosis by apoptotic morphological changes in HepG2 cells were observed and a typical intrinsic apoptotic characterization, which included fragmentation of nuclear DNA in ethanol extract-treated HepG2 cells (Ariffin et al., 2009). In addition to extraction using solvent, other extracts have been studied that can inhibit the growth of liver cancer such as the uses of supercritical fluid extract of *Piper longum* showed an effect of cytotoxicity against liver cancer cell lines (HepG2) (Guo et al., 2019).

2.2 *Piper longum*, history and traditional medicines

Piper longum known as " Long pepper" (Figure. 6) is a climber plant distribute in Indonesia, Sri Lanka, Philippines, Vietnam, and China (Zaveri et al, 2010). In Thailand distribute at Chanaburi, Nakorn Pathom, and Kanchanaburi. It was belonging to *Piperales* order, *Piperaceae* family, and *Piper* genus. It's distributed in hot and humid weather. Long pepper is called in Europe, there is also another name as Pippali in Sanskrit language or Pibo in Chinese (Yadav et al., 2020). The fruit has green and when it ripe is orange, the surface of the fruit is rough and composite (Peter, 2012). When broken, the surface shows a central axis, and 6-12 fruit arranged around an axis (Joshi, 1944).

Figure.6 Long pepper

Furthermore, *P. longum* has been widely used in traditional medicines, that many properties and previous studies to indicating the various effects such as

Table.1 Pharmacological activities of *P. longum*

(Yadav et al., 2020)

Activity	Extract	Part	Model	Major finding	References
Anti-inflammatory	Chloroform	Fruit	*In vitro*, Human umbilical vein endothelial Cells	• inhibits adhesion of neutrophils to endothelial monolayer • Block the TNF-α-induced expression of CAM, • Inhibits the NADPH	Singh et al., 2008
	Oil	Fruit	Carrageenan-induced right hind paw edema model	72% reduction of edema induced by carrageenan (Standard drug-Ibuprofen)	Kumar et al., 2009
	Aqueous	Seed	Freund's complete adjuvant induced arthritis rats	Reduced paw swelling (Standard drug-Diclofenac)	Yende et al., 2010
Anti-diabetic	Essential oil	Fruit	Streptozotocin Induced diabetes	Treatment results in anti-diabetic action	Kumar et al., 2013
Antioxidant	Ethanol	Seed	DPPH assay, Scavenging of Super oxide, Nitric oxide, and Hydroxyl radicals	Treatment shows potential antioxidant action (Standard drug- Ascorbic acid)	Ramesh et al., 2011
	Petroleum ether	Root	Isoproterenol Induced myocardium ischemia	Potential antioxidant action with protection in myocardial ischemic	Jagdale et al., 2009

Hepato-protective	Ethanol	Fruit	CCl4 induced liver fibrosis	Anti-fibrotic action was observed due to the flavonoid content of *P. longum*	Christina et al., 2006
	Aqueous	Fruit	$AlCl_3$ induced hepatotoxicity	Decrease levels of serum biochemical markers	Sharma et al., 2014
Anti-cancer	Ethanol	Fruit	G-361, HT-29 ,HCT116, OVCAR-3, BxPC-3, NCM460 cell lines	Treatment leads to selective cell death in leukemia, pancreatic and colon cancer while normal cell was unaffected, Treatment induces caspase-independent apoptosis in cancer cells, without affecting non-cancerous cells, also targeting the mitochondria, leading to dissipation of the mitochondrial membrane potential and increase in ROS production	Ovadje et al., 2014
	Hexane, Benzene, Chloroform, Ethyl-acetate, Alcohol, Aqueous	Fruit	Prostate (DU 145), Lung (A549), Leukemia (THP-1),ovary (IGR-OVI-1) and breast (MCF -7) cancer cell lines	Cytotoxicity with effect on sub G_1 phase	Sharma et al., 2014

2.3 *Piper longum*, chemical compound

P. longum produces various chemical compounds and various components. In previous studies indicated that the main component in *P. longum* is piperine (Figure 7) which about 3-5% in the plant following by essential oil, steroids, coumarin, alkaloid, sterol, saponins, amygdalin, piperanine, and pipereicosalidine (Li et al., 2013).

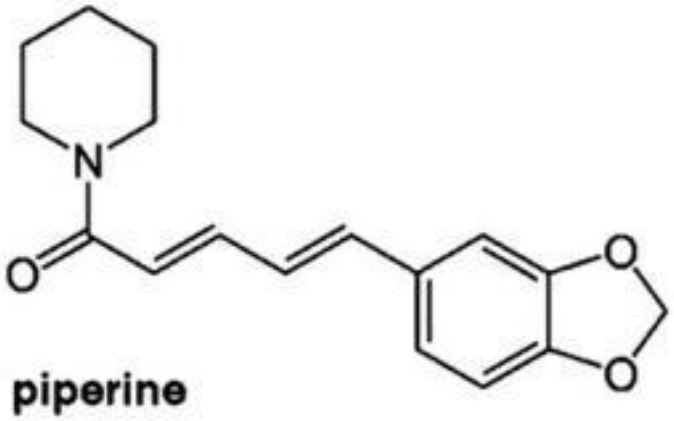

Figure.7 Main chemical compounds in *P. longum*

(Derosa et al., 2016)

In previous studies, the main compound of *P. longum* is piperine and piperine has a medical effect such as antitumor and apoptosis-inducing effect on human melanoma cells (Yoo et al., 2019). These studies have shown the effect of piperine for treats on A375SM that melanoma cell lines and show that good effect in inducing apoptosis. Furthermore, in current studies have shown the role of piperine in the prevention and progressive cancer, which show high potential chemopreventive properties to induction of cell cycle arrest, increased autophagy, and apoptosis (Zadorozhna et al., 2019). Which related to previous studies about piperine is acts on modulating cell cycle progression as a part of their chemopreventive mechanism (Meeran & Katiyar, 2008). In addition, the effect of piperine on anticancer was investigated in many studies such as table 2. Hence, piperine has traditional medicine, and numerous studies shown good properties of pure piperine in both *In vitro* and *In vivo*.

Table.2 Effects of piperine on type of cancers

(Zadorozhna, Tataranni, & Mangieri, 2019)

Type of cancer	Source of piperine	Function on piperine	Reference
Breast cancer	Purchased from Fluka (St Louis, MO, USA).	Arrest of cell cycle at different checkpoint in relation to cancer cell lines; unbalance of ROS homeostasis and induction of apoptosis	Lai et al., 2012
Melanoma	Obtained from LKT Laboratories (St. Paul, MN)	Arrest of cell cycle at G1 phase through downregulation of cyclin D1, activation of CDK (p21/WAF1) and unbalance of ROS homeostasis	Fofaria et al., 2014
Ovarian cancer	Purchased from Sigma Chemical (St. Louis, MO)	Activation of intrinsic pathway of apoptosis after the release of mitochondrial cytochrome c to cytosol, the activation of caspase-3 and -9, the PARP cleavage and the inactivation of p38/MAPK and JNK	Si et al., 2018
Lung cancer	Purchased from Sigma chemical company, USA.	Reduction of oxidative stress mediated by mitochondrial activities and enhancement of both enzymatic and non-enzymatic defence systems	Selvendiran et al., 2006
Oral squamous carcinoma	Purchased from Sigma-Aldrich, USA	Mediation of mitochondrial pathway of apoptosis.	Siddiqui et al., 2017

Hepato-cellular carcinoma	Obtained from Sigma-Aldrich chemicals (USA)	Interaction with CYP1A1 enzyme, mediating deficiency of benzo(a)pyrene (BP) metabolism and consequently abolishing cancer aggressiveness	Gunasekaran et al., 2017
Fibro-sarcoma	Purchased from Sigma Chemical (St. Louis, MO).	Inhibition of PKCα and ERK1/2 phosphorylation and reduction of NF-κB and AP-1 nuclear translocation, so leading to down-regulation of MMP-9 expression	Hwang et al., 2011

2.4 Cytotoxicity

Cytotoxicity is an assay to the action of chemotherapeutic agents on living cells and this assay for determining cell viability involves dyes such as Trypan Blue or Coomassie blue. The other methods of cytotoxicity assay include dehydrogenase-based assay, the MTT method, XTT assays, and WST assay. Furthermore, Cytotoxicity is assay to screen before *In vivo* experiments for studies about the toxic substance that causes cell death. However, not always assays will be correct 100% , requiring additional checks by *In vivo* or other methods. In addition, the procedures are found in ISO10993-5.

2.4.1 MTT assay

The MTT assay is a colorimetric assay for measuring cell metabolic activity and wildly to evaluated cell viability by using the principle of enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl] -2,5-diphenyltetrazolium bromide (MTT) (Figure. 8) to MTT-formazan crystal is catalyzed by mitochondrial succinate dehydrogenase. In MTT assay that cell monolayers common should be used because easily to measured and preparing.

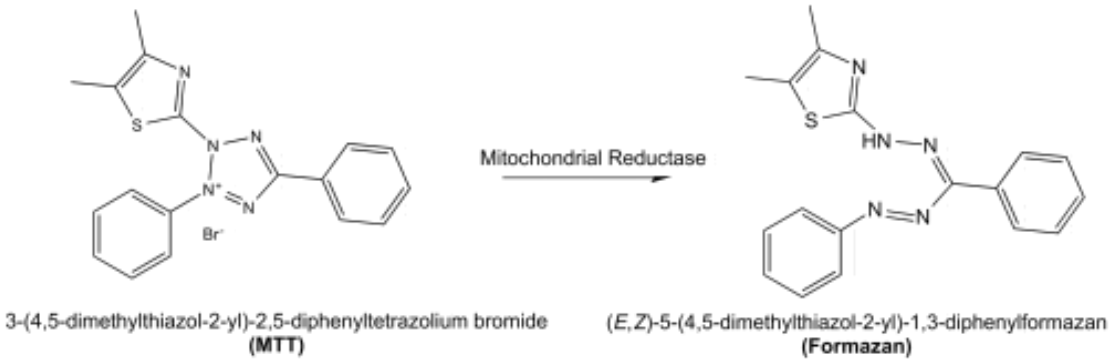

Figure.8 Formazan formation of MTT assay

2.5 Migration and invasion assay

The migration and invasion assay are a method to measuring of cell viability in cancer research to look at the features of the migration of cancer cells**.** Migration assays are commonly used as wound healing assays **(** Scratch assay**)** and Transwell assay**.**

2.5.1 Wound healing assay (Scratch plate assay)

Wound healing assay or scratch assay is wildly commonly used for measuring cell migration *in vitro***.** This method is an easy to be preparing cell and low-cost method**.** The monolayer cell was used in this method for scratch in a plate **(**Figure 9**).** Furthermore, a wound healing assay has a study on the effects of cell-matrix and cell-cell interactions on cell migration (Liang et al., 2007)**.**

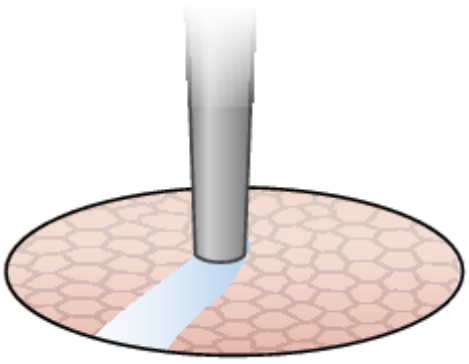

Figure.9 Wound healing assay

(Vedula et al., 2013)

2.5.2 Transwell assay

Another way to look at the migration of cells is the Transwell assay. Transwell assay is a commonly and classical In vitro method to be used to study the proliferation and migratory response of cell movement and ability of cell migration and cell invasion. This method using the principle of cell–cell adhesion by cell movement through intermediary as 3D gels. Matrigel™, collagen, and fibrin (Berens et al., 2015) (Figure 10) . In advantages, when the cell using the different cell as human cell or animal cell testing on cytotoxicity may be shown different result. However, the method has a limitation as to when using monolayer cell may be cell damaged more than cells in an organized tissue because this cell will be reduced damage from toxic.

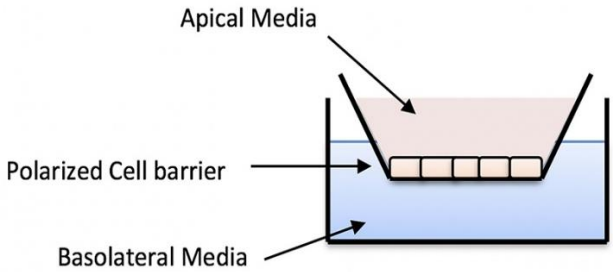

Figure.10 Transwell assay

(Garcia-Castillo et al., 2018)

2.6 Colony formation assay

Colony formation is technique that investigating cell proliferation and measuring the ability of a cell to divide and form a colony. This assay is gold standard for determined of cell sensitivity to radiation and efficiency drug of cancer. However, colony formation assay is basic technique independent of the way how the cells are dead (Kabakov & Gabai, 2018). Furthermore, after cultured cell and treated with drug or extract that analyzed by crystal violet staining for cell counted.

2.7 OECD guideline for testing of chemicals and cell culture

2.7.1 Cell lines

2.7.1.1 Normal liver cell lines (FL83B)

FL83B is a hepatocyte cell lines or non-disease liver cells, obtained from mouse' s mus musculus organ from 16-day-old fetal mouse and grows in a

medium that included glucocorticoid. This cell type has epithelial morphology form and growth properties is an adherent cell. FL83B has a reported these cells is similar parenchymal liver cells. Furthermore, Thorbecke' s experimentation has shown that cells can generate proteins (Hochwald et al., 1961) including albumin and HDL. Another characteristic of FL83B cell is these cells are less differentiated cells. In the cultured, FL83B cultured by DMEM medium with 10 FBS and 1% P/S antibiotic in an incubation with 5% CO_2 and 37ºC.

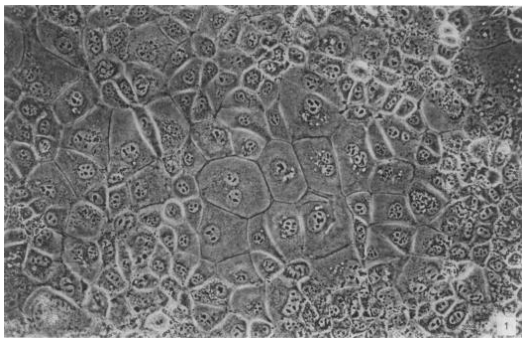

Figure. 11 FL83B cell lines in phase contrast

(Breslow et al., 2020)

2.7.1.2 Liver cancer cell lines (HepG2)

HepG2 (Figure.11) is consists of human liver carcinoma cells, derived from the liver tissue of a 15-year-old Caucasian male and transiently transfected with the human and an androgen-responsive reporter. HepG2 has some residual P450 activity. These cells are commonly used in toxicology *In vitro* studies. In the culture, HepG2 was cultivated in DMEM with 2.2 mg/mL sodium bicarbonate ($NaHCO_3$), and 1% sodium pyruvate, supplemented with 10% FBS and 1% antibiotic (P/S), in an incubator at 37°C, 5% CO_2

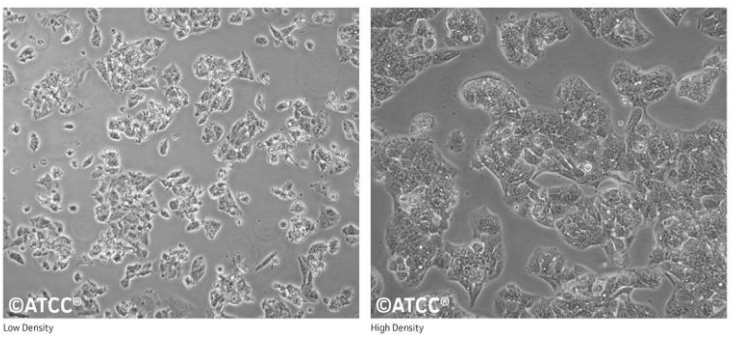

Figure. 12 HepG2 cell lines

(**ATCC, 2020**)

CHAPTER III
MATERIALS AND METHODS

3.1 Crude extract of *Piper longum*

Dried fruit of *Piper longum* in this study was obtained from Thailand. Briefly, *Piper longum* dried fruit was blended by Grinder machine for powder form. Then, 1 kg of the powder were extracted with 3L of ethanol at room temperature for 7 days. The extract was separated with filter of Whatman No.1 for 2 time. Extract was evaporated by Rotary evaporation at 53º C. Then, the obtained solid part of extract was extracted with 200 mL of water for 1 day. The obtained water extract was evaporated by Rotary evaporation at 58 º C. The crude extract stored in 4 ºC until used. Extract was dissolved in 100% DMSO to dilution for 1 mg/ mL for stock solution and stored at -20ºC.

3.2 Testing of chemical compound

3.2.1 Determination of total phenolic content (TPC)

The total phenolic content was determined by Folin–Ciocalteu colorimetric method as described in previously study (Athipornchai & Klangmanee, 2021) The extract (20 µL) was mixed with 10% Folin-Ciocalteu's phenol reagent (80 µL) in a 96-well plate. The solutions were shook and pre-incubated at room temperature for 5 min. 2.5% of Na_2CO_3 solution (100 µL) was added and kept for 30 minutes in the dark. The absorbance was measured at 760 nm by using microplate reader (EPOCH-2, BioTek, USA). Gallic acid (0.005 to 0.08 mg/mL) was used as a standard. The total phenolic content in each extract was compared with the standard curve and expressed as mg of gallic acid equivalents per 1 mg of solid crude extract (mg GAE/mg solid crude). The procedure was repeated in triplicate.

3.2.2 Determination of total flavonoid content (TFC)

Total flavonoid contents were determined according to the aluminum chloride colorimetric assay modified from previously study (Athipornchai & Jullapo, 2018). Quercetin was used as a standard at concentrations of 0.005 to 0.08 mg/mL. For determination, 20 µL of extract was mixed with 180 µL of 1% (w/v) aluminum chloride ($AlCl_3$) methanolic solution in a 96-well plate. The absorbance of the reaction mixture was measured at 440 nm by a microplate reader (EPOCH-2, BioTek,

USA), after incubation at room temperature for 10 minutes. The total flavonoid contents in the extract were determined using a standard curve established with quercetin and the results expressed as mg of total flavonoid contents per 1.0 g of dry extract as quercetin equivalents (QE). All tests were conducted in triplicate and averaged.

Table.3 Color change reaction

Methods	Color change
Folin-Ciocalteu reagent	Colorless ⟶ Dark blue
Aluminium trichloride reagent	Colorless ⟶ Yellow

3.3 Cell lines and cell culture

Hepatocarcinoma cell lines (HepG2) and normal hepatocyte cell lines (FL83B) were used in this study. HepG2 and FL83B cell lines were purchased from American Type Culture Collection (ATCC) . Briefly, both cells were cultured Dulbecco's Modified Eagle Medium (DMEM) supplemented with Penicillin G (10 U/mL), Streptomycin (10 µg/mL), and 10% heat-inactivated fetal bovine serum (cell culture medium). Cells were incubated at 37º C in a humidified atmosphere of 95% air and 5% of CO_2 incubator.

3.3.1 Sub culturing

The cells were subcultured every other day. Start with removed culture medium by 10 mL seropipette and then trypsinization by 1 mL 0.05% trypsin solution for no more than 5 minutes. Stop reaction by culture medium and the cell resuspended or pipetted in flask for cell coming off from the flask. Then the cells were put on the 15 mL tube for centrifuged at 1,600 rpm for 5 minutes. Complete medium was discarded supernatant and added for the cell into the newly 25 cm^2 T-flask in a 37º C in a humidified atmosphere of 95% air and 5% of a CO_2 incubator.

3.3.2 Cell counting and dilution

Cells were trypsinized and pipetted to 15 mL tube for centrifuged at 1,600 rpm for 5 minutes. Complete medium was discarded supernatant and added for 5 mL for diluted and resuspended until the cells break down and pipetted some cell 20 µl to microcentrifuge tube for count cell. Cells were added Tryphan blue 20 µl (ratio 1:1) in microcentrifuge tube. Count on hemocytometer showed 9 grid (Figure.12) and cells were calculated using the following equation:

$$\text{Cell density (cell/mL)} = \frac{\text{total cell count}}{5} \times \text{dilution factor x } 10^4$$

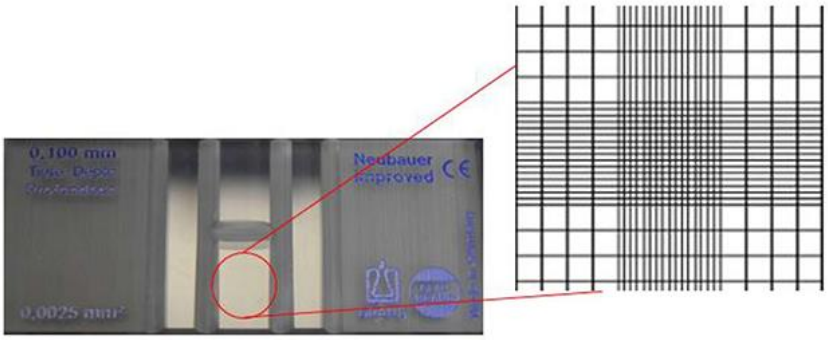

Figure.13 Grid of hemocytometry

(Vembadi et al., 2019)

3.4 Cytotoxicity

3.4.1 Cell viability by MTT assay

The cell viability was measured by MTT assay. HepG2 and FL83B cells were counted and diluted into 96 well plate with complete medium at $1x10^4$ per well. 96 well plate within cells was incubated for overnight at 37º C in a humidified atmosphere of 95% air and 5% of CO_2 incubator. After that, remove complete medium and adding *P. longum* extract in 25, 50, 100, 200, 400, 800 and 1000 µg/mL into 96 well plate within the cells and incubating for 24 and 48 h. For MTT testing, Diluting MTT for 0.5 µg/mL. Remove extract every well and adding MTT solution 100 µl every well in 96 well plate. Incubating for 1-2 h. And then removed MTT solution before clearing cells by DMSO. Now a purple formazan crystal was appeared. Taken to measuring the absorbance at 570-690 nm.

Cells were calculated percentage of cell viability and calculated the concentration of 50% cytotoxicity (IC50) by equation:

$$\text{The \% of cell viability} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \text{ x } 100$$

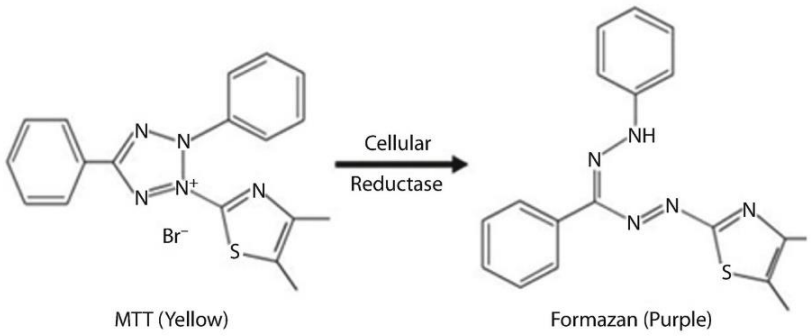

Figure.14 Formazan crystal

3.5 Cell migration by scratch plate assay

Cells were prepared for $1x10^6$ in a well of a 6-well plate and incubated for overnight or 24 h or cells were growth 90**%** of plate**.** After that, was removed complete medium and used clearing 200 µl of pipette scratch on basement for 5 mm**.** Do not used to much force because too many cells to falling off from plate**.** Then, cells were cleared by 1X phosphate buffer saline **(**PBS**).** Extract was added 100 µl with concentration 100, 200, 400 and 600 µg/mL and incubation for overnight again**.** Cells were captured by camera at 0, 24, 48 h and were analyzed the results of pictures by light microscopy, measured the distance of the wound compare with camera capture in 0 h**.**

$$\textbf{\%}\ \text{Wound closure} = \frac{A_{t=0h} - A_{t=\Delta h}}{A_{t=0h}} \times 100\textbf{\%}$$

$A_{t=0\,h}$; Length of scratch area at 0 h

$A_{t=\triangle\,h}$; Length of scratch area at 24 and 48 h

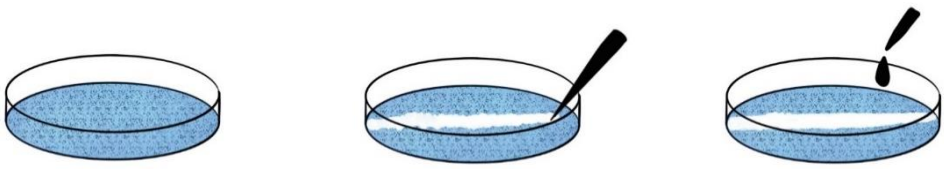

Figure.15 Scratch plate

3.6 Cell invasion by transwell assay

Start with place Transwell (Corning®) was inserted into a 24-well tissue culture plate and coated chamber with 50 µl Matrigel®, incubated 2 h-1 week. HepG2

cells were added for $5x10^4$ with *P. longum* extract for dilution any concentration 100, 400 and 600 µg/mL in 250 µl into Transwell (Figure. 15) and well under inserted chamber were added 800 µl of 20% FBS complete medium. Incubation for overnight at 37º C in a humidified atmosphere of 95% air and 5% of CO_2 incubator. After that, Get rid of the *P. longum* extract and fixation cells with methanol for 15 seconds, rinse Transwell with PBS. Cells were stained with crystal violet (dissolved with methanol) at room temperature for 15 sec. After that Transwell and cells were removed by cotton bud. The invaded cells were stained and obtained under light microscope.

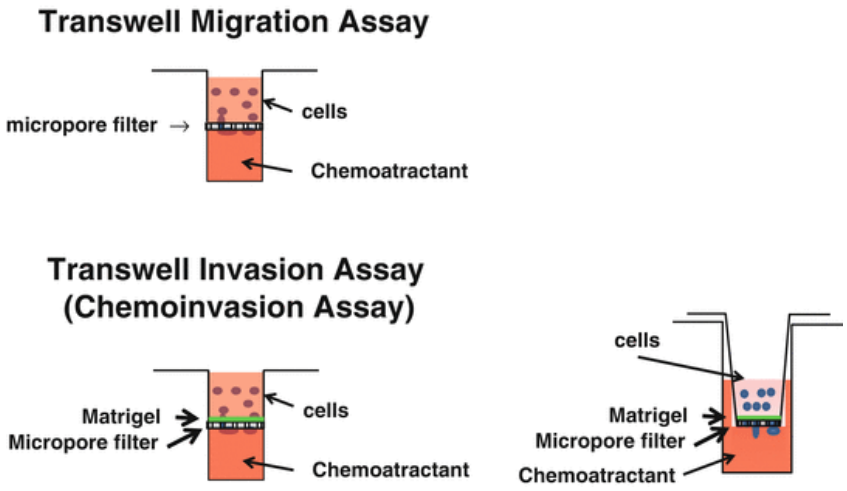

Figure.16 Transwell composition

(Hieda et al., 2015)

3.7 Colony formation

HepG2 cells were added to 6-well plate for $2x10^3$ cells/well and incubation for 24 h. After that, cells were treated with *P. longum* extract with concentration 100, 200, 400 and 600 µg/mL and incubated for 24 and 48 h. Cells were removed extract before added 10% FBS DMEM for cells. Incubation for 1-2 weeks for analyzed by crystal violet stained and results were kept by light microscopic. Cells were analyzed for percentage of size of colony and colony number by equation:

$$\text{The \% of size of colony} = \frac{\text{Lenght of colony of treated cells}}{\text{Lenght of colony of control cells}} \times 100$$

$$\text{The \% of colony number} = \frac{\text{Number of colony of treated cells}}{\text{Number of colony of control cells}} \times 100$$

3.8 Statistical analysis

Statistical analysis was performed using a one-way ANOVA for the analysis of the test results, Duncan analysis and Graphpad Prism version. 7. 0 statistical software was used analyzed of data. Furthermore, the data were shown significant on *P* value < 0.001 and < 0.05.

CHAPTER IV
RESULTS

4.1 Extraction of *Piper longum*

The crude extract was extracted by ethanol and water. In the present study, the extract was evaporated that final product is crude extract. %Yield was equaled and that was 0.319%. After that, total phenolic and flavonoid compound was measured. The total phenolic compound of *P. longum* extract was showed in figure.17 and 18

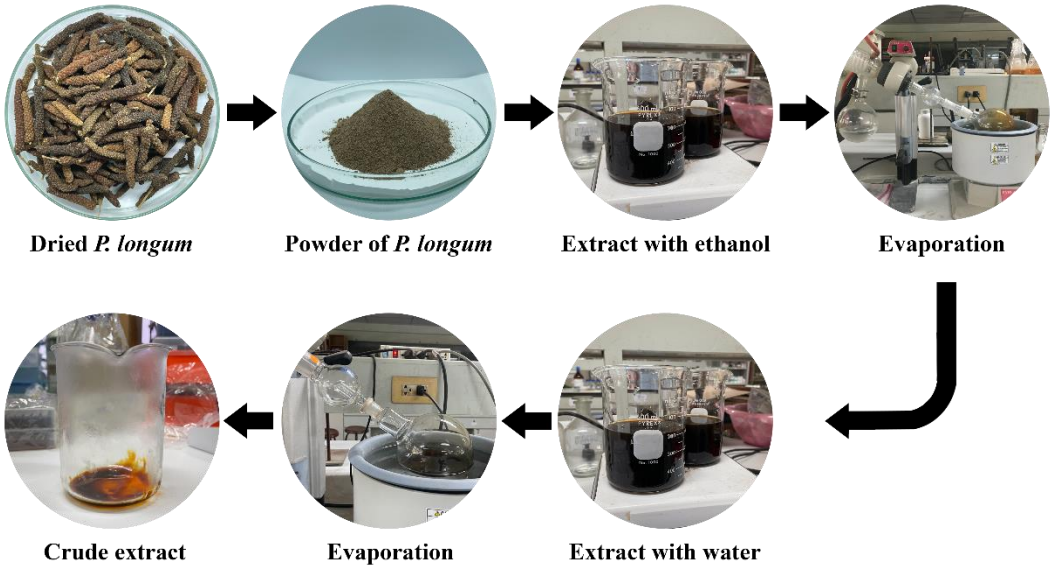

Figure.17 Overall of crude extract

The picture was showed extraction method that crude extract was final product in this study. %Yield was equaled and showed 0.319% (3.19 g per 1 kg of *P. longum*).

4.2 Total phenolic and total flavonoid contents

Polyphenolic and flavonoid compounds are commonly found in medicinal plants, vegetables and fruits and have been reported to have multiple biological activities (Kähkönen et al., 1999). Total phenolic contents in the ethanol and water extract of *P. longum* were determined by Folin–Ciocalteu method using gallic acid as the standard. The absorbance values were obtained at different concentrations of gallic acid were used for the construction of calibration curve. Total phenolic content of the ethanol and water extract was calculated from the regression equation of

calibration curve (y = 31.176x + 0.0076, R2 = 0.9977) and expressed as mg gallic acid equivalents (Hannigan et al.) per gram of sample in dry weight (mg/g). The present study found that the total phenolic contents of the ethanol and water extract was 14.45 ± 0.01 mgGAE/g. In addition, the total flavonoid content of the extract was calculated from the regression equation of the calibration curve (y = 30.988x + 0.0056, R2 = 0.9998) and expressed as mg quercetin equivalents (QE) per gram of sample in dry weight (mg/g). The result found that the total flavonoid content of the ethanol and water extract was 1.61 ± 0.03 mgQE/g.

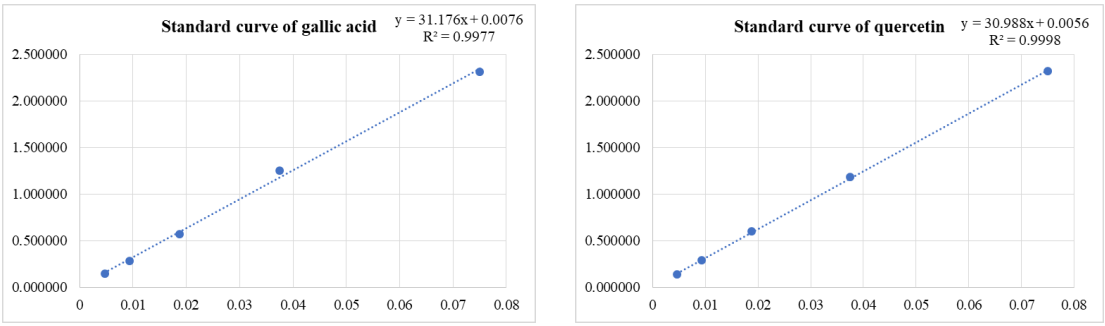

Figure.18 Standard curve of gallic acid and quercetin

The pictures were showed paragraph of standard curve of gallic acid (left) and quercetin (right) for compared that equaled total phenolic and flavonoid

Table.4 Total phenolic and flavonoid content

Extract	Total phenolic content (mgGAE/g)	Total flavonoid content (mgQE/g)
P. longum	14.45 ± 0.01	1.61 ± 0.03

4.3 Cytotoxicity assay of *Piper longum* extract by MTT assay

The viability of HepG2 cells by *P. longum* extract was investigated by MTT assay that assay commonly used in determine survivor of living cells by principle of colorimetric assay from formazan crystal. In this study, cells were used 2 cells such as liver cancer cell lines (HepG2) and normal liver cell lines (FL83B) that was evaluated cytotoxicity from *P. longum* extract at 24 h and 48 h. Cell density was used $1x10^4$ cells per well both 24 and 48 h.

4.3.1 Cell viability evaluation on normal liver cell lines (FL83B)

All results (Figure.19-20) were compared with 1 % DMSO (Control). This study, *P. longum* extract was significantly reduced the percentage of cell

viability on FL83B at 400 and 600 µg/mL (*P*-value < 0.05) at 24 h. Moreover, the concentration of 800 and 1,000 µg/mL was reduced the percentages of cell viability at 24 h on *P*-value < 0.001. IC50 was calculated and showed that cell viability at 24 h was more than 1,000 µg/mL. In addition, cell viability of *P. longum* extract on FL83B at 48 h was similar to 24 h but at 48 h that the concentration of 800 and 1,000 µg/mL were significantly reduced the percentages of cell viability on *P*-value <0.05 and <0.001 respectively. IC50 of effective at 48 h was more than 1,000 µg/mL. Therefore, *P. longum* extract rather safety for cell or non-toxicity due to this extract was reduced cell viability but IC50 was related high concentration to inhibits growth of cell.

4.3.2 Cell viability evaluation on liver cancer cell lines (HepG2)

P. longum extract was determined that effective in against cancer that cause examined on cancer cell**.** This study, liver cancer cell lines or HepG2 was investigated with *P. longum* extract**.** HepG2 cells were treated similar concentration of *P. longum* on FL83B such as 25, 50, 100, 200, 400, 800, 1,000 µg/mL for 24 and 48 h. 1**%**DMSO was used control**.** The percentages of cell viability of *P. longum* extract on HepG2 cell lines were showed in Figure**.** 19, 20**.** Cells were treated at 24 h and showed quite high that survival or HepG2 cells were no significantly decreasing**.** However, at 48 h HepG2 cells were significant increased cell viability on concentration 800 and 1,000 µg/mL **(***P*-value <0**.**05**).** After that, IC50 was calculated and showed the IC50 of cell viability of *P. longum* extract on HepG2 cell lines was more than 1,000 µg/mL both 24 and 48 h**.**

Table.5 %Cell viability ± SD of FL83B cell

Extract	Time (h)	%Cell viability ± SD (FL83B cells)								IC50 (µg/ml)
		25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	600 µg/ml	800 µg/ml	1000 µg/ml	
PL-AQ	24	116.36 ± 2.39	107.11 ± 0.96	106.67 ± 5.75	107.03 ± 5.40	88.77 ± 3.36	78.55 ± 0.05	72.06 ± 9.04	69.83 ± 4.13	>1,000
	48	126.37 ± 1.56	125.96 ± 4.90	134.71 ± 4.13	125.34 ± 3.68	116.25 ± 2.80	97.88 ± 0.20	89.29 ± 1.85	60.98 ± 3.60	>1,000

%Cell viability by MTT assay of FL83B cells at 24 and 48 h in concentration 25, 50, 100, 200, 400, 600, 800 and 1,000 µg/mL were showed in this table and was represented of SD. The IC50 of *P. longum* extract that was inhibited cell viability of FL83B cell was showed. Furthermore, 1%DMSO was used to control.

Table.6 %Cell viability ± SD of HepG2 cell

Extract	Time (h)	%Cell viability ± SD (HepG2 cells)								IC50 (µg/ml)
		25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	600 µg/ml	800 µg/ml	1000 µg/ml	
PL-AQ	24	103.16 ± 1.61	106.03 ± 1.19	108.55 ± 0.78	106.62 ± 1.15	106.44 ± 3.10	100.07 ± 2.54	108.89 ± 4.20	104.44 ± 3.26	>1,000
	48	112.14 ± 4.61	115.61 ± 0.95	119.93 ± 7.30	109.72 ± 8.20	106.42 ± 5.89	94.36 ± 3.66	87.46 ± 2.30	78.90 ± 4.19	>1,000

%Cell viability by MTT assay of HepG2 cells at 24 and 48 h in concentration 25, 50, 100, 200, 400, 600, 800 and 1,000 µg/mL were showed in this table and was represented of SD. The IC50 of *P. longum* extract that was inhibited cell viability of HepG2 cells was showed. Furthermore, 1%DMSO was used to control.

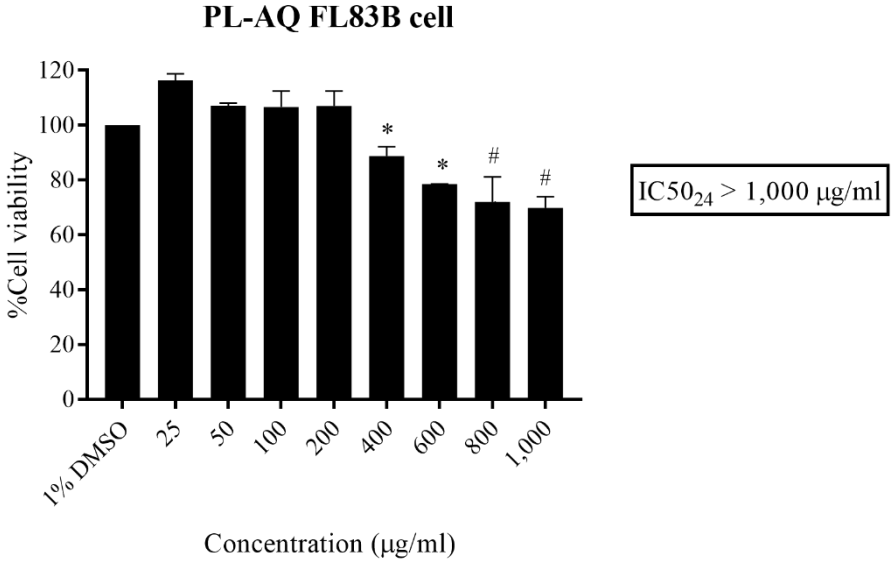

Figure.19 The percentage of cell viability on FL83B at 24 h

The percentages of cell viability on FL83B cells were showed in paragraph in concentration 25, 50, 100, 200, 400, 600, 800 and 1,000 µg/mL at 24 h. The mean±SD and IC50 were showed in result. (*, <0.001, #, <0.05 compared with 1%DMSO)

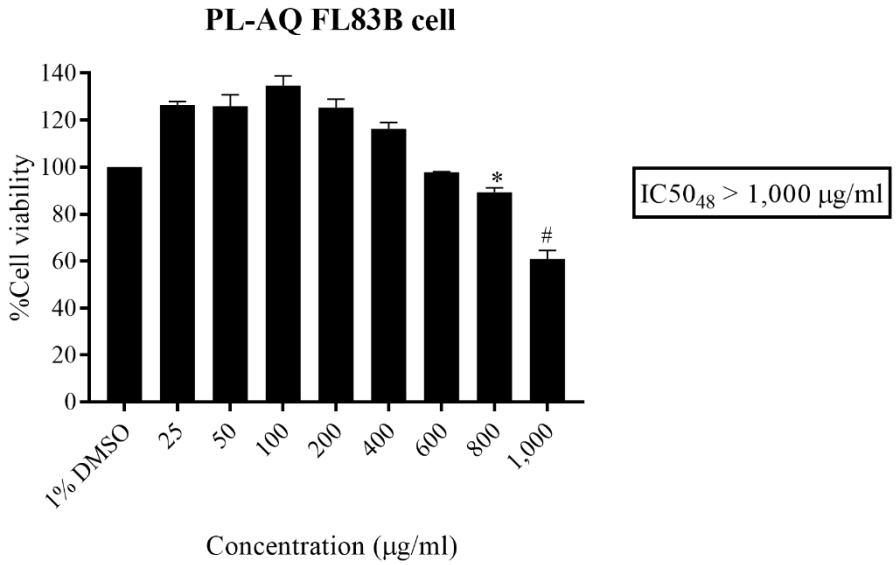

Figure.20 The percentage of cell viability on FL83B at 48 h

The percentage of cell viability on FL83B cells were showed in paragraph in concentration 25, 50, 100, 200, 400, 600, 800 and 1,000 µg/mL at 48 h. The mean±SD and IC50 were showed in result. (*, <0.001, #, <0.05 compared with 1%DMSO)

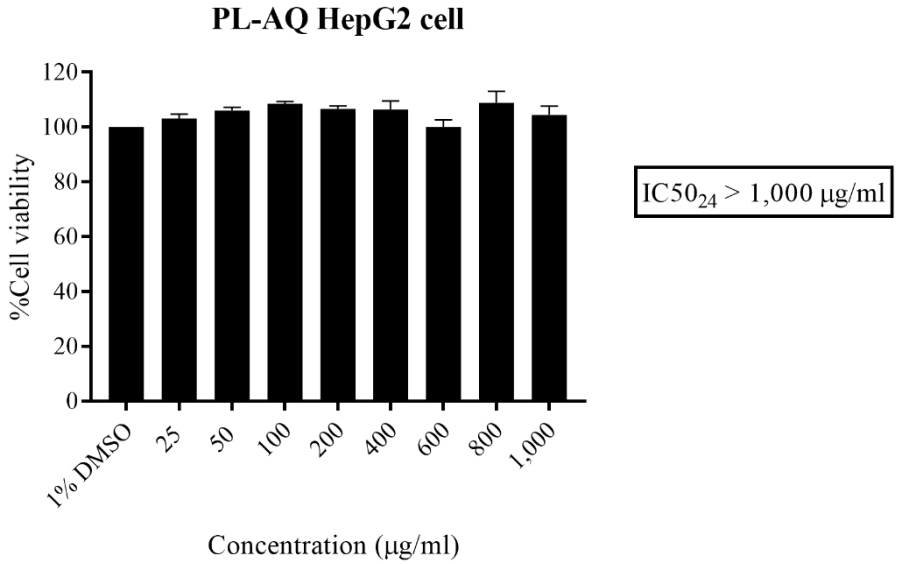

Figure.21 The percentage of cell viability on HepG2 at 24 h

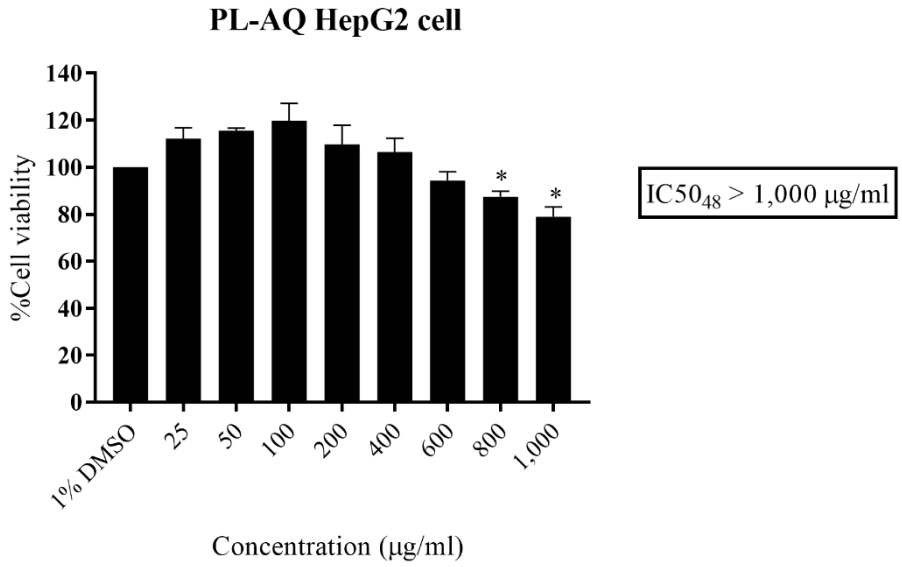

Figure.22 The percentage of cell viability on HepG2 at 48 h

Figure 21 and 22, %cell viability of HepG2 cells were showed in result at 24 and 48 h in concentration 25, 50, 100, 200, 400, 600, 800 and 1,000 µg/mL that compared with 1%DMSO. This paragraph was showed mean±SD and IC50 of ability of *P. longum* extract on HepG2 cells.

4.4 The anti-migration effect of *P. longum* extract on HepG2 cell lines

Subsequently, HepG2 cells were determined the effective of migration with Scratch plate assay. This result was showed movement of HepG2 cell. *P. longum* extract was used in concentration 100, 200, 400, and 600 µg/mL. The percentages of wound closure were showed in figure.23-25. Concentration 400 and 600 µg/mL at 24 h were significant decreased with (P-value<0.05) in %wound closure 15.02% and 14.20% respectively and concentration 100, 200 and 400 µg/mL were not significant decreased in % wound closure. Furthermore, the concentration of 600 µg/mL was significant decreased in %wound closure 30.90% when compared with cell untreated *P. longum* extract in 48 h (P-value<0.05) (Figure. 24). Space that passed the scratch line by pipette tip of HepG2 cells was showed in figure 23. Cells were moved into empty space approach to the other side of cells, which one of these was called metastasis that one of cause to death from cancer. However, Cell proliferation and movement of HepG2 cells were inhibited with *P. longum* extract that showed evidence by scratch test. Cells were not into the scratch region compared with

untreated extract. Effective of anti-migration of HepG2 in a dose-dependent manner was appeared by *P. longum* extract.

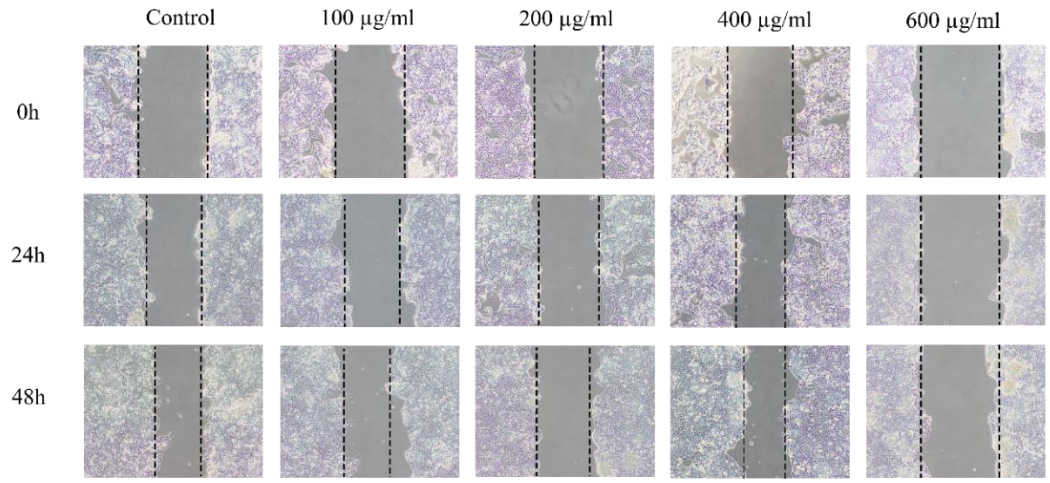

Figure.23 Migration ability of HepG2 cells

The result of scratch plate assay was showed in figure.21 that was represented ability of migration of HepG2 cells. HepG2 cells were treated *P. longum* extract in concentration 100, 200, 400, and 600 µg/mL at 0, 24 and 48 h. Result was explained movement of HepG2 cells into empty space of 6-well plate. All the pictures were captured by light microscopy.

Table.7 %Wound closure ± SD of HepG2 cell

Extract	Time (h)	%Wound closure ± SD				
		Untreated	100 µg/ml	200 µg/ml	400 µg/ml	600 µg/ml
PL-AQ	24	29.20 ± 2.91	28.83 ± 7.21	26.75 ± 3.79	15.02 ± 4.48	14.20 ± 1.38
	48	47.48 ± 5.23	45.03 ± 5.93	43.51 ± 1.57	39.23 ± 1.17	30.90 ± 1.56

The percentages of wound closure from scratch plate assay were showed in table at 24 and 48 h. The concentrations of *P. longum* extract were used 100, 200, 400 and 600 µg/mL. Then, the %wound closure result was showed mean±SD

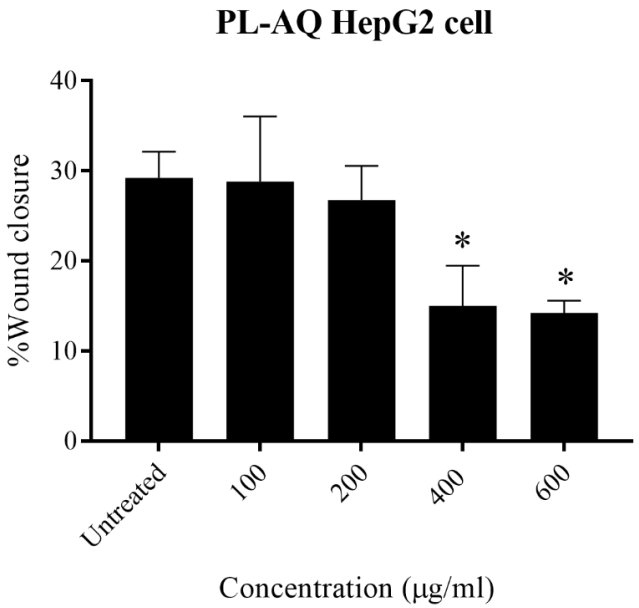

Figure.24 The percentages of wound closure of HepG2 cells at 24 h

The percentages of wound closure by scratch plate assay were showed in figure.22 that mean±SD. The concentrations were used in 100, 200, 400, and 600 µg/mL at 24 h on HepG2 cells (*, <0.05 compared with untreated)

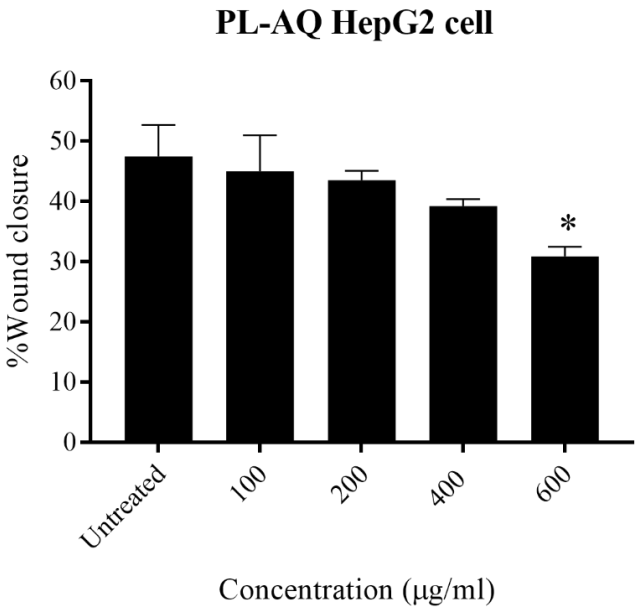

Figure.25 The percentages of wound closure of HepG2 cells at 48 h

The percentages of wound closure by scratch plate assay were showed in figure.22 that mean±SD. The concentrations were used in 100, 200, 400, and 600 µg/mL at 48 h on HepG2 cells (*, <0.05 compared with untreated)

4.5 Effect of *P. longum* extract on invasion assay in HepG2 cell lines

HepG2 cell lines were investigated cell invasion by Transwell assay. Transwell was coated with Matrigel for 2 h at 37ºC. Matrigel is collagenase EMT for cell pass through the pore. Cell density was started at $5x10^4$ cells/mL with *P. longum* extract on the concentration of 100, 200, 400 and 600 µg/ mL. The picture of microscopy was showed and stained with crystal violet in figure. 26 that the concentration of 600 µg/ mL was changed wherewith decreased and was showed in figure. 27 in paragraph of %invasion. The concentration of 400 and 600 µg/mL were significant decreased in %invasion to 95.00 and 55.52% respectively. This result was compared with untreated of *P. longum* extract (*P*-value<0.05).

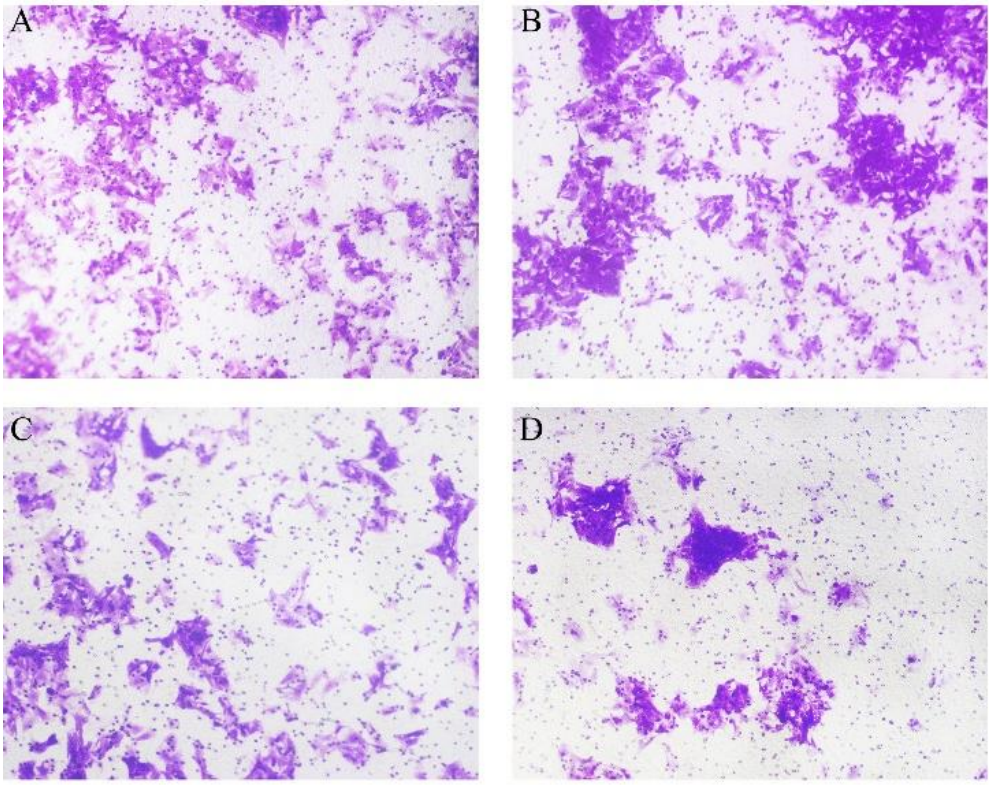

Figure.26 Cell invasion by light microscopy

The figure showed picture that was captured by light microscopic at 10x. A was showed control that cells were untreated with P. longum extract and B, C and D was showed cell with 100, 400 and 600 µg/mL respectively of P. longum extract. Cells were showed ability of invaded cell through the Matrigel pore by Transwell assay.

Table.8 %Invasion ± SD of HepG2 cell

Extract	%Invasion ± SD		
	100 µg/ml	400 µg/ml	600 µg/ml
PL-AQ	95.45 ± 2.34	95.00 ± 3.30	55.52 ± 0.63

Ability of invasion on HepG2 cells was represented mean±SD of %invasion in this table. The concentrations were used 100, 400, and 600 µg/mL of *P. longum* extract

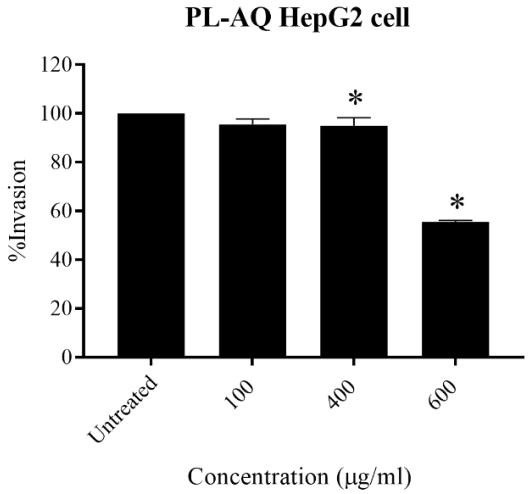

Figure.27 %Invasion of P. longum extract on HepG2 cell lines

Ability of invasion on HepG2 cells was showed in paragraph. *P.longum* extract was used to concentrations such as 100, 400, and 600 µg/mL. *, <0.05 compared with untreated.

4.6 Colony formation effect of *P. longum* on HepG2 cell lines

4.6.1 Number of colony formation

This study, colony number and size of colony were observed with *P. longum* extract on HepG2 cell lines. The assay was measured ability of cancer cell to survivor and growth to form colony after cytotoxic treated. The concentration was started 100, 200, 400 and 600 µg/mL and treated on HepG2 density $2x10^3$ cell/mL at 24 and 48 h. Results were showed picture by light microscopy and stained with crystal violet. The number of colonies was clearly decreased compare with untreated cell. The percentages of colony number were showed in figure.29 that the concentration of 100 (*P*-value<0.05), 200, 400 and 600 µg/mL (*P*-value<0.001) was significant decreased at 24 h. In addition, number of colony formation by dose-

dependent manner (100, 200, 400 and 600 µg/mL) was inhibited by *P. longum* extract at 48 h (Figure. 30).

4.6.2 Size of colony formation

The growth of tumor cell to form colony and size was determined in this assay. The percentage of size of colony was significant constricted on concentration of 100, 200, 400 and 600 µg/mL (*P*-value<0.001) at 24 h and showed in figure.32. Furthermore, at 48 h cells were significant constricted on 200, 400 and 600 µg/mL and also showed in figure.33 that picture by light microscopy of size colony. The colony was stained with crystal violet and the result showed size of colony was clearly constricted.

Table.9 %Colony number ± SD of HepG2 cell

Extract	Time (h)	%Colony number ± SD			
		100 µg/ml	200 µg/ml	400 µg/ml	600 µg/ml
PL-AQ	24	80.82 ± 7.57	77.39 ± 6.04	56.31 ± 4.74	47.36 ± 5.24
	48	80.30 ± 5.57	58.40 ± 7.21	43.73 ± 5.96	28.39 ± 2.88

The result was showed mean±SD in the table. %Colony formation of colony number showed at 24 and 48 h. *P. longum* extract was used in 100, 200, 400, and 600 µg/mL.

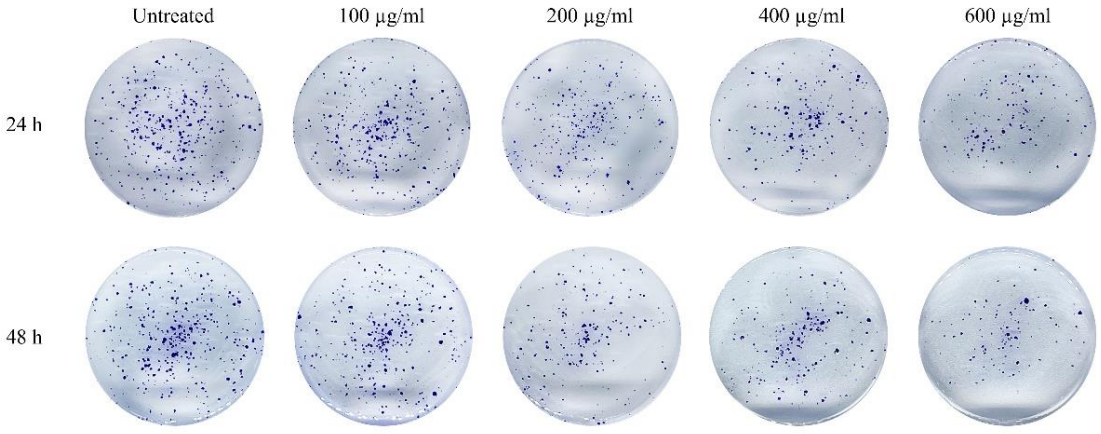

Figure.28 Picture of colony number on HepG2 cell lines by light microscopy

Colony number on HepG2 cells was showed in the picture in concentration 100, 200, 400, and 600 µg/mL at 24 and 48 h in 6-well plate. HepG2 cells were fixed by methanol and stained by crystal violet and captured by light microscopic.

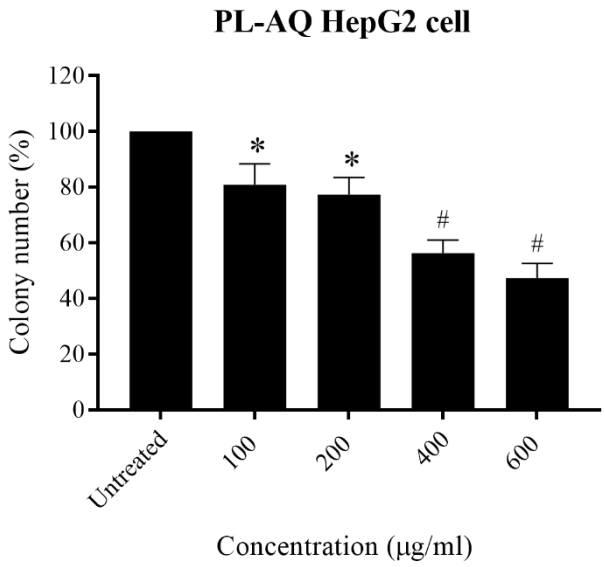

Figure.29 The percentage of colony number on HepG2 cell lines at 24 h

The percentages of colony number on HepG2 cells were showed in concentration of *P. longum* extract 100, 200, 400, and 600 µg/mL at 24 h. Ability of colony formation was represented the growth of HepG2 cells. *, <0.001, #, <0.05 compared with untreated.

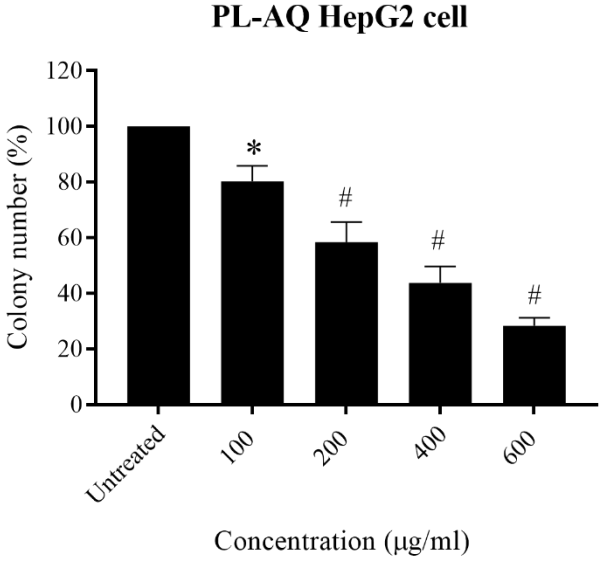

Figure.30 The percentage of colony number on HepG2 cell lines at 48 h

The percentages of colony number on HepG2 cells were showed in concentration of *P. longum* extract 100, 200, 400, and 600 µg/mL at 48 h. Ability of colony formation was represented the growth of HepG2 cells. *, <0.001, #, <0.05 compared with untreated.

Table.10 %Colony size ± SD of HepG2 cell

Extract	Time (h)	%Colony size ± SD			
		100 µg/ml	200 µg/ml	400 µg/ml	600 µg/ml
PL-AQ	24	59.87 ± 8.37	54.97 ± 4.94	45.64 ± 4.70	39.66 ± 3.15
	48	89.45 ± 3.56	71.06 ± 8.06	53.83 ± 2.56	46.59 ± 7.76

The percentages of colony size on HepG2 cells were showed mean±SD in concentration of *P. longum* 100, 200, 400, and 600 µg/mL at 24 and 48 h.

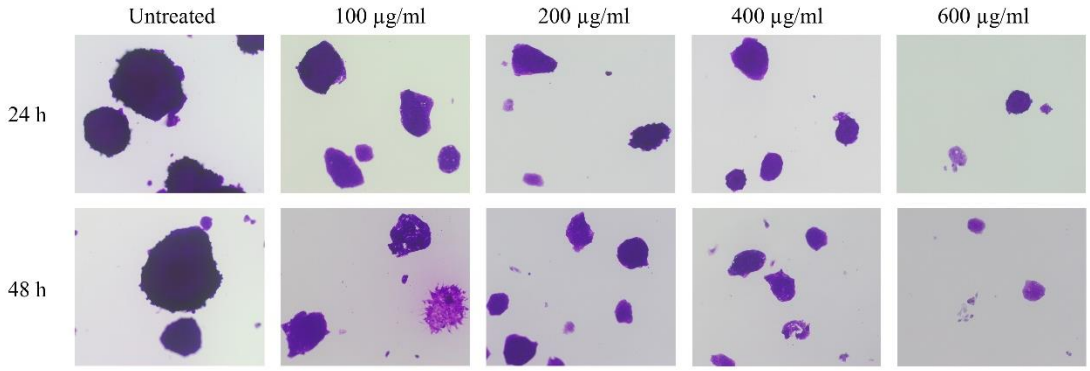

Figure.31 Picture of size of colony in HepG2 cell lines by light microscopy

HepG2 cells were used treated with *P. longum* extract in the concentration 100, 200, 400, and 600 µg/mL at 24 and 48 h. cells were fixed with methanol and stained with crystal violet. The pictures were captured and analyzed by light microscopic 40x and measured with ImageJ. The result was compared with untreated cells.

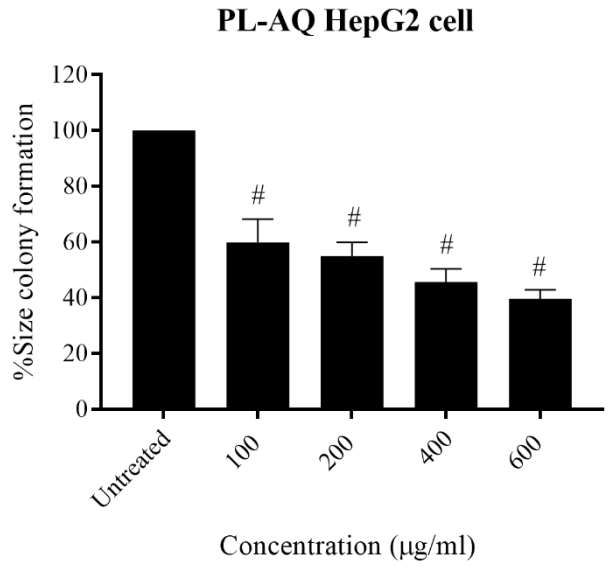

Figure.32 Size of colony of HepG2 cell lines at 24 h

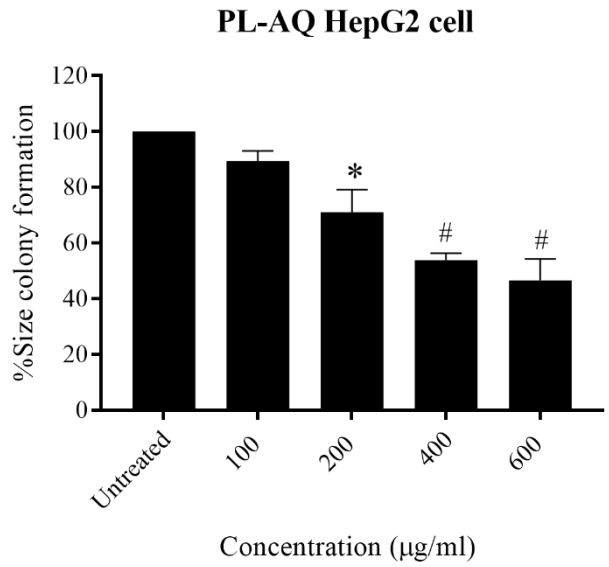

Figure.33 Size of colony of HepG2 cell lines at 48 h

The percentages of colony size on HepG2 cells were showed in concentration of *P. longum* extract 100, 200, 400, and 600 µg/mL at 24 and 48 h. Ability of colony formation was represented the growth of HepG2 cells. *, <0.001, #, <0.05 compared with untreated.

CHAPTER V
DISCUSSION

Liver cancer is the ninth most common cancer in females and the fifth most common in males worldwide. In 2020, liver cancer is the most common cancer in Thailand. However, the most common is chemotherapy which is the conventional therapy due to medication-induced side effects in some patients and developed drug-resistant, new treatments or new effective drugs are needed for liver cancer treatment. Natural products are interesting as a new strategy to cure liver cancer.

Piper longum is a plant of piperaceae family similar to black pepper was reported in a recent study that effective in anti-cancer (Ariffin et al., 2009). *P. longum* is used in traditional medicine such as gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain, and arthritic conditions (Singh, 1992). Crude extract in this study was extracted by ethanol and obtained the solid part was extracted with aqueous and evaporated for crude extract. In this study, we demonstrated the cytotoxicity, anti-migration and anti-invasion effects on the liver cancer cell lines (HepG2) compared with normal liver cells (FL83B). The result of cytotoxicity showed the extract significantly decreased cell viability of HepG2 and FL83B cells (Figure.19-22) showed effective in decreased cell proliferation which is related to the chemical compounds of piperaceae family such as long pepper and black pepper showed piperine effective to anti-cancer in a recent study (Yoo et al., 2019). The chemical compound in *P. longum* extract was investigated by Folin-Ciocalteu reagent for total phenolic tested and aluminium trichloride reagent for total flavonoid tested. However, the extract of *P.* longum is non-toxic in liver cancer referable by National Cancer Institute (NCI) set the criteria of IC50 of crude extract is lower than 30 µg/mL (Hostettmann, 1991) but our result showed IC50 is 1,000 µg/mL both cell at 24 and 48 h.

Migration is an important step become to advance cancer or metastasis cancer, that is one of step we determined in this study. HepG2 cell was demonstrated by the *P. longum* extract in the concentration of cell death is no more than 5% and growth no more than 5% in anti-migration tested. Scratch plate assay is the technique of wound healing observe. We found that the extract was decreased cell proliferation

and migration. According to a recent study, pure piperine effectively inhibits the growth of HepG2 cells (Gunasekaran et al., 2017) and *P. longum* is including piperine that the main component of about 3-5% (Yadav, Krishnan, & Vohora, 2020). Furthermore, Guo et al. in 2017 was reported that *P. longum* extract was inhibits cell proliferation and migration in ovarian cancer. Our result showed the extract was effective anti-migration in a dose-dependent manner. Moreover, the one of aims in this study is to find the anti-invasion on HepG2 cells. Cell invasion and metastasis are participates to the progression of disease such as cancer (Yamaguchi & Condeelis, 2007). Therefore, in this study was necessary to determine the anti-invasion effect by transwell assay. The *P. longum* in our study was inhibited the growth of HepG2 cells in transwell assay. In transwell experiment has been coated with collagen to mimic the typical extracellular matrix. Recently study, it is reported that piperine substances in *P. longum* and other plants in piperaceae family have the ability to reduced metastasis in osteosarcoma cells by suppressing MMP-2/-9 expression (Zhang et al., 2015). For this reason, piperine may be play important role in inhibits cell proliferation, cell migration and cell invasion. The last one, present study was investigated colony formation showed figure. 26-28 that colony numbers and figure.29-31. These findings demonstrated that the extract inhibited colony formation because results showed *P. longum* extract was clearly reduced numbers and size of colonies according to a recent study that *P. longum* extract was reduced colony formation on SKOV-3 cells by superficial fluid extract. This study showed our results that have ability to inhibit cell proliferation, cell growth, cell survival, cell migration and cell invasion which implicated chemical compound that piperine. In addition, *P. longum* extract is effective to anti-cancer but the mechanism is unclear and less research worldwide. At the same time, *P. longum* extract has been tested for finding active components of anti-cancer such as piperine, phenolic and piperlongumine etc. In this study, piperine is the main component which may be piperine is an active compound. Thus, *P. longum* extract could be used in combination with traditional cancer treatment is a possible or adjunctive treatment to lessening the disease's severity.

CHAPTER VI
CONCLUSION

Dried fruit of *P. longum* extract was investigated by cytotoxicity, scratch plate assay, transwell assay and colony formation assay. The extract has ability to inhibit cell proliferation, cell migration and cell invasion. In addition, the extract was less toxic and safety for cancer therapy such as drug for treatment, adjunctive treatment or food supplement for liver cancer protection. Furthermore, this study is a part of enlargement to study other effective of *P. longum* extract and mechanism of *P. longum* is finding more.

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