ANTIPROLIFERATIVE EFFECTS OF *QUERCUS INFECTORIA* GALLS EXTRACT AND ITS MECHANISM OF CELL DEATH ON CERVICAL CANCER (HELA) CELLS

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by

NURAZILA BINTI ZULKIFLY

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LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor 1
ATCC	American Type Culture Collection
AV/PI	Annexin V/propidium iodide
A375	Human melanoma cell line
Bak	Bcl-2 homologous antagonist killer
BCLC	The Barcelona Clinical Liver Cancer
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
CAD	Caspase-activated DNase
CAM	Complementary and alternative medicine
CARD	Caspase recruitment domain
Caov-3	Ovarian cancer cell line
CIN1	Cervical intraepithelial neoplasia 1
CIN2	Cervical intraepithelial neoplasia 1
CIN3	Cervical intraepithelial neoplasia 3
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
DR	Death-receptors
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated death domain

Fas-L	Fas ligand
$FeCl_3$	Iron (III) chloride
FITC	Fluorescein isothiocyanate
GAE	Gallic acid equivalent
GAE-TPC	Total phenolic content was expressed as gallic acid equivalent
GLOBOCAN	Windows based software which provides access to worldwide database of cancer incidence and mortality rates
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HeLa	Cervical cancer cell line
HepG2	Hepatocellular carcinoma cell line
HPV	Human papillomavirus
HPV16	Human papillomavirus strain 16
HPV18	Human papillomavirus strain 18
H_2O	Water
H_2O_2	Hydrogen peroxide
IARC	International Agency for Research on Cancer
ICAD	Inhibitor of caspase-activated DNase
IC_{50}	Extract concentration required to inhibit 50% of cells population
KOH	Potassium hydroxide
L929	Normal fibroblast cell line
MBA	Methylene blue assay
MCF-7	Human breast adenocarcinoma cell line established by Michigan Cancer Foundation-7

$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
Na_2CO_3	Sodium carbonate
NBT	Nitroblutetrazolium
NO	Nitric oxide
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
OD	Optical density
OH	Hydroxyl radical
O_2^-	Superoxide anion
PBS	Phosphate buffer saline
PI	Propidium iodide
PS	Phosphatidylserine
PUMA	p53-upregulated modulator of apoptosis
p53	Tumour protein
QI	*Quercus infectoria*
QIA	*Quercus infectoria* galls aqueous extract
QIE	*Quercus infectoria* galls ethanol extract
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S.E.M	Standard error mean
SMAC	Second mitochondria-derived activator of caspase
SOD	Superoxide dismutase

TCT	Thinprep cytological test
TNF	Tumor necrosis factor
TPC	Total phenolic content
USA	The United States of America
US NCI	National Cancer Institute of United States
Vero	Normal kidney cell line
$V79^{c}$	Oxidant-induced carcinogenesis cells
WHO	World Health Organization
XOD	Xanthine oxidase
X/XOD	Xanthine/xanthine oxidase

LIST OF SYMBOLS

%	Percentage
<	Less than
>	Higher than
±	About
≤	Less and equal to
≥	Higher and equal to
˚C	Degree Celsius
µg	Microgram
µl	Microlitre
µg/ml	Microgram per millilitre
µm	Micrometre
cells/ml	Cells per millilitre
cm	Centimetre
cm^3	Cubic centimetre
g	Gram
kDa	Kilodalton
L	Litre
M	Molar
mg	Milligram
mg/ml	Milligram per millilitre
ml	Millilitre
mm	Millimetre
ng/ml	Nanogram per millilitre

nm	Nanometre
U/ml	Units per millilitre
v/v	Volume per volume
w/v	Weight per volume
xg	Relative centrifugal force

KESAN ANTIPROLIFERASI EKSTRAK *QUERCUS INFECTORIA* DAN MEKANISME KEMATIAN KEPADA SEL KANSER SERVIK (HELA)

ABSTRAK

Kanser merupakan salah satu penyakit yang menjadi punca utama kematian di seluruh dunia. Sehingga kini kebanyakan kaedah rawatan yang sedia ada memudaratkan dan mendatangkan kesan sampingan terhadap pesakit. Oleh kerana itu, pesakit mula mencari penyelesaian alternatif seperti menggunakan ubat-ubatan herba. Sumber-sumber bertulis telah membuktikan *Q. infectoria* (QI) mempunyai pelbagai sifat terapeutik termasuk aktiviti antikanser. Walau bagaimanapun, mekanisme di sebalik aktiviti antikansernya tidak dijelaskan dengan terperinci. Justeru, dalam kajian ini, QI telah dipilih untuk diuji aktiviti antiproliferasi dan mod tindakbalasnya. Aktiviti antiproliferasi ekstrak akuas (QIA) dan ekstrak etanol (QIE) *Q. infectoria* terhadap sel kanser servik (HeLa), sel kanser ovari (Caov-3) dan sel kanser hati (HepG-2) ditentukan melalui kaedah asai metilina biru (MBA). Aktiviti antiproliferasi ekstrak terhadap sel ginjal normal (Vero) dan sel fibroblast normal (L929) juga diperiksa untuk melihat kesan toksik dan keselektifan ekstrak. Di samping itu, sel yang dirawat dengan DMSO berfungsi sebagai kawalan negatif dan sisplatin sebagai kawalan positif. Kemudian, graf dos-tindak balas diplot bagi menentukan nilai IC_{50}. Kemudian, mekanisme kematian sel terawat-QIA diperiksa melalui pewarnaan Hoechst 33258, pewarnaan berganda FITC-annexin V/propidium iodida dan pengesanan protein apoptosis. Selain itu, pemeriksaan kandungan fitokimia, ujian antioksidan serta analisis jumlah kandungan fenolik juga dijalankan untuk melihat hubung kait aktiviti biologi. Keputusan kajian menunjukkan QIA

mempunyai aktiviti antiproliferasi yang lebih baik berbanding QIE dengan perencatan pertumbuhan paling baik kepada sel HeLa (nilai IC_{50} = 13.64 ± 2.39 μg/ml) dan bersifat sitoselektif. Hasil kajian juga menunjukkan sel HeLa yang dirawat dengan QIA menjalani apoptosis, dibuktikan melalui perubahan pada morfologi nukleus dan kehadiran jasad apoptotik serta peningkatan pada kadar apoptosis. Tambahan, hasil menunjukkan QIA mencetus apoptosis melalui tapak jalan-p53. Peningkatan aras p53 dilihat menurunkan aras Bcl-2 dan meransang pembebasan sitokrom c, seterusnya apoptosis dilaksanakan melalui pengaktifan caspase-3. Walau bagaimanapun, tiada perubahan pada ekspresi Bax dikesan dalam kajian ini. Berdasarkan ujian pengesanan kandungan fitokimia, QIA didapati mengandungi tannin, flavanoid dan alkaloid. Malah, daripada ujian antioksidan QIA mempamerkan aktiviti pemerangkapan radikal DPPH dan superoksida X/XOD yang hebat serta mempunyai jumlah kandungan fenolik yang tinggi. Kesan antiproliferasi QIA mungkin disumbangkan oleh kepelbagaian sebatian yang bertindak sebagai agen antioksidan yang kuat. Secara kesimpulannya, QIA mencetus apoptosis dalam menjalankan aktiviti antiproliferasi yang selektif terhadap sel kanser.

ANTIPROLIFERATIVE EFFECTS OF *QUERCUS INFECTORIA* GALLS EXTRACT AND ITS MECHANISM OF CELL DEATH ON CERVICAL CANCER (HELA) CELLS

ABSTRACT

Cancer is one of the leading causes of death worldwide. To date, most of the available therapies are detrimental and cause side effects to the patients. Therefore, patients turn to alternative treatments by utilizing herbs. Accumulating evidences have shown the wide range of therapeutics properties of *Q. infectoria* (QI) galls including anticancer activity. However, the mechanism of action was not well explained. Therefore, in this study, QI galls were selected for the evaluation of antiproliferative activity and mode of action. The antiproliferative activity of *Q. infectoria* galls aqueous extract (QIA) and ethanol extract (QIE) against cervical cancer (HeLa), ovarian cancer (Caov-3) and liver cancer (HepG-2) cell lines has been accessed by methylene blue assay. The antiproliferative activity towards normal kidney (Vero) and normal fibroblast (L929) cells were also evaluated to determine the toxic effects and selective property of the extracts. In addition, cells treated with DMSO served as negative control and cisplatin as positive control. Dose-response curve were then constructed to determine the IC_{50} values. Then, the mode of cell death in QI-treated cells was determined by Hoechst 33258 staining, FITC-annexin V/propidium iodide double staining and detection of apoptotic proteins. Besides, phytochemicals screening, antioxidant tests and total phenolic content analysis were done to observe the connection of bioactivity. From the results, as compared to QIE, QIA showed better antiproliferative activity with best growth inhibition towards

HeLa cells (IC_{50} value = 13.64 ± 2.39 µg/ml) and exhibit cytoselective property. This current finding also showed that HeLa cells treated with QIA undergone apoptosis, represented by the alteration of nuclear morphology and presence of apoptotic bodies as well increased rate of apoptosis. Moreover, results have shown that QIA induced apoptosis through p53-dependant pathway. Upregulation of p53 has been observed to downregulate Bcl-2 and promoted secretion of cytochrome c, thus facilitated the execution of apoptosis through caspase-3 activation. However, no alteration of Bax expression was detected in this study. Based on the phytochemicals screening, QIA comprises of tannin, flavanoid and alkaloid. Furthermore, the antioxidant profiles showed that QIA exhibited great DPPH radical scavenging and X/XOD superoxide scavenging activities and contains high total phenolic contents. The antiproliferative activity of QIA might be contributed by the diversity of compounds that act as strong antioxidants. From this study, we can conclude that QIA exhibited its selective antiproliferative activity against HeLa cells by induction of apoptotic cell death.

CHAPTER 1

INTRODUCTION

1.1 Study background

Chemotherapy is one of the leading approaches among cancer therapies, which involves the utilization of selection of drugs to control advance stages of malignancies and as a prophylactic against promising metastasis (Raguz and Yague, 2008; Sarkar and Li, 2009; Caley and Jones, 2012). Despite the achievement made in the improvement of effective chemotherapy drugs, their toxicity to normal tissues and adverse side effects in multiple organ systems as well as drug resistance, have remained the main barriers for the successful clinical application (Sak, 2012).

As a result, cancer remains a major contributor to mortality worldwide (American Cancer Society, 2011). According to GLOBOCAN (2008), cervical and liver cancers are among the two most common types of cancer diagnosed and two most leading cancer deaths in men and women in several continents of the world (American Cancer Society, 2011). Meanwhile, ovarian cancer which listed as fifth most common cause of cancer death in women represents as the most deadly gynecologic cancer in 2012 (Hoffman *et al.,* 2012). In 2008, these types (cervical, ovarian and liver) of cancer have been estimated as top ten most death causing cancer in developing countries (American Cancer Society, 2011).

This worrying phenomenon has encouraged many researchers to search for new anticancer agent. In this attempt, natural sources get extra attention by

pharmacologists and biochemists in the development of potential chemotherapeutic agents (Abad and Bermejo, 2001; Gurib-Fakim, 2006). All over the ages, nature has become important sources in providing basic needs for human daily life (Gurib-Fakim, 2006). The consumption of nature not only limited as a source of food even more than that, it also plays a role in the preparations of traditional medicines for thousand years to alleviate diseases and for health maintenance (Gurib-Fakim, 2006; McChesney *et al.,* 2007).

Plant derived natural sources have a long history usage in traditional medicinal systems, which served as integral elements in the preparation of remedies by traditional practitioners of ayuverda, traditional Chinese medicine, siddha and ancient Egypt system to treat various ailments (Cai *et al.,* 2004; Wilson *et al.,* 2007; Meena *et al.,* 2009; Aboelsoud, 2010). Even in 21st century, the therapeutic values of plants still treasured by modern people, with 80% of world population rely on plant-based therapy for their primary healthcare (Farnsworth, 1985; Meena *et al.,* 2009).

As a rich sources of therapeutic agents for the treatment of diseases and ailments, plants has been accepted as complementary and alternative medicine (CAM) to cure several illness including cancer (Yin *et al.,* 2013). In United States of America (USA), plants constitute 30-75% of used CAM by most cancer patients (Richardson, 2001). According to epidemiologist, daily intake of plant rich phytochemicals can reduce the prevalence of certain types of cancers (Sporn and Suh, 2002; Surh, 2003; D'Incalci *et al.,* 2005; Russo *et al.,* 2005). By acting as antioxidants, several phytochemicals present in plants such as vitamins (A, C, E, K), carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes

and minerals protect the body cells from being damaged by free radicals leading to the anticancer activity (Heber, 2004; Madhuri and Pandey, 2009).

The traditional and folk practices have provided clues and knowledges which lead to the discovery and emergence of plant-derived chemotherapeutic drugs. This nutritional approach has brought a new direction to raise cancer therapy by means of reducing the adverse side effects and improve the effectiveness of chemotherapeutic drugs. For example, drugs like vinblastine, vincristine, taxol, and camptothecin isolated from different species of plants have improved the chemotherapy of some cancers (Newman *et al.,* 2003).

Since the introduction of *in vitro* prescreening programme in 1986, research area has been witnessed with the growing number of plant-based anticancer agent in preclinical development, but, none of them have yet entered the stage of general use (Cragg and Newman, 2005). In response to this current need, the utilization of plants as one of the most valuable sources is considered as a significant approach for the discovery of effective and safe anticancer agents.

Therefore, in this current investigation, a plant namely, *Quercus infectoria* (QI) galls has been selected due to its wide usage in traditional practices worldwide, particularly among Malaysian women. In addition, several scientific evidences have demonstrated the broad range of therapeutic properties associated with this plant including anti-inflammatory, antioxidant, antidiabetic, larvicidal, antibacterial, anti-fungal and anticancer properties (Redwane *et al.,* 2002; Kaur *et al.*, 2004; Basri and Fan, 2005; Yamunarani *et al.,* 2005; Kaur *et al.,* 2008; Hasmah *et al.,* 2010; Saini

and Patil, 2012). Based on the literatures available, there are limited informations and studies that reported the potential of this plant as anticancer agent. Moreover, mechanisms of action rely behind its anticancer activity is not well described by previous investigators.

The ability of chemotherapeutic drugs to inhibit proliferation of cancer cells via apoptosis has been suggested as an important feature in the development of new cancer therapy (D'Agostini *et al.,* 2005). Apoptosis is a form of cell death, where cells commit to suicide in natural way without affecting their surrounding cells to remove damage, senescent and unwanted cells (Huppertz and Herrler, 2005; Exbrayat *et al.,* 2012). The acquired ability of cancer cells to evade apoptosis represents a main causative factor in the growth and progression of cancer (Hanahan and Weinberg, 2000; Zivny *et al.,* 2010). Although abundant of anticancer agents targeting apoptosis under studies, majority of them are still in preclinical development due to low specificity and susceptibility to drug resistance (Russo *et al.,* 2006). Therefore, it is a reliable and promising strategy to target apoptosis as a mechanism for cancer therapy.

Hence, the potential of QI galls as anticancer agent against cervical cancer (HeLa), ovarian cancer (Caov-3) and liver cancer (HepG-2) cell lines via apoptosis was studied. In this present study, phytochemicals and antioxidant activity related to its anticancer effects were also elucidated.

1.2 General objective

The aim of this study is to determine the ability of QI galls aqueous (QIA) and ethanol (QIE) extracts to selectively inhibit the proliferation of cervical cancer (HeLa), ovarian cancer (Caov-3) and liver cancer (HepG-2) cell lines without causing harmful effects towards normal cells. The most potent extract then chosen for the determination of mode of cell death (apoptosis), phytochemical screenings and antioxidant activity.

1.3 Specific objectives

The specific objectives of this study are:

1) To extract QI galls using water and ethanol.
2) To determine the antiproliferative activity of cells treated with QI galls extracts.
3) To study the mode of cell death in cells treated with the most potent QI galls extract.
4) To evaluate the antioxidant activity and phytochemicals groups of the most potent QI gall extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is a disease formed from uncontrolled growth and progression of abnormal cells beyond their normal boundaries (Rieger, 2004). This disease is caused by several factors which are classified into external and internal factors, may act together to initiate and promote carcinogenesis. Tobacco, chemicals, radiation, and infectious organisms are among the identified external factors, meanwhile, internal factors include inherited mutations, hormones, immune conditions, and mutations that occur from metabolism (Rieger, 2004).

Primary cancer derived as tumour mass that developed at the origin where the conversion of normal cells into tumour cells occurs (Oppenheimer, 2006). Frequently, tumours or also known as neoplasms are benign and slow-growing masses that pressure rather than invade neighbouring tissues (Oppenheimer, 2006). However, if left untreated, benign tumours can become malignant in which the cells can detach from their origin and set up metastasis (secondary cancer) (Kainsa *et al.,* 2012). Generally, malignant tumours possess the ability to grow faster, invade adjacent tissue and colonize distant organs (Kainsa *et al.,* 2012). Further comparisons of benign and malignant tumours are summarized in table 2.1.

Table 2.1: Comparisons between benign and malignant tumours (Walsh, 2002).

	Benign	**Malignant**
Cells	Relatively normal and mature	Little resembles to normal, poorly differentiated, atypical in size and shape, non-uniform and immature
Growth	Slow and restricted. Non-invasive of surrounding tissue, expansive and pushing aside normal tissue.	Rapid and unrestricted. Invasive of surrounding tissue.
Recurrence	Rare	Frequent
Spread	Localized and encapsulated.	Metastasize via blood and lymph stream.
Threat to host	Prognosis favorable. The effects depend on size and location. May cause pressure on vital organs and obstruct a passageway, which is usually corrected by surgical excision of neoplasms.	Threatens life due to its local vicious proliferation and formation of secondary neoplasms in other structures. Prognosis more favorable with early diagnosis and treatment when cells show less departure from the normal and there is no metastasis.

2.1.1 Incidences of cancer

Cancer is a major public health burden in both developed and developing countries. According to International Agency for Research on Cancer (IARC) about 12.7 million new cancer incidences in 2008 have been reported worldwide, of which 5.6 million cases attributed by economically developed countries and 7.1 million in economically developing countries. This situation contributes to the 7.6 million of total deaths due to cancer which is about 21 000 cancer deaths a day. This global burden is expected to raise up to 21.4 million new cancer cases and 13.2 million cancer deaths by 2030 (American Cancer Society, 2011).

In Malaysia, cancer was listed as third most deadly diseases after heart diseases and diseases of pulmonary circulation, and septicaemia, which account 11.28% of overall causes of diseases. Based on the National Cancer Registry, out of total 18 219 cancer cases, the incidence was higher in female population with 10 096 cases (55.4%) compared to 8, 123 cases (44.6%) have been diagnosed in male population (Zainal Ariffin and Nor Saleha, 2011).

In this present study, three types of cancer originated from cervix, liver and ovary were focused due to their high mortality rate among male and female populations especially those in the developing regions.

2.1.1.1 Cervical cancer

Cervical cancer is the third most frequent cancer in women and has been documented as second leading cause of fatality for 242 000 women in developing countries (American Cancer Society, 2011; Jemal *et al.,* 2011). In Malaysia, cervical cancer has been reported as second most common cancer in female. Annual report demonstrated that 2145 of new cervical cancer cases has been diagnosed which contributed to 621 new cancer deaths among Malaysian women in 2012 (Teo, 2015).

Cervical cancer is characterized as sexual transmitted disease which arises from human papillomaviruses (HPV) where 92.9% of reported incidences in human bearing HPV DNA (Bosch *et al.*, 1995). So far, HPVs are classified into low-risk and high-risk of all 73 types based on their relationship with pre-neoplastic and malignant cervical lesions (De Villiers, 1989; De Villiers, 1994; Zur Hausen and De Villiers, 1994). Low-risk HPVs constitute of types 6 and 11 mostly observed in *Condyloma accuminata* and low-grade cervical intraepithelial neoplasia 1 (CIN1) (Rattray *et al.,* 1996; Chan and Berek, 2007). On the other hand, high-risk HPVs such as types 16, 18, 31, 33, 35, 39, 56 and 58 frequently infect CIN2, CIN3 and invasive cervical carcinomas (Rattray *et al.*, 1996).

The transmission of HPV viruses can be reduced through sexual barrier by decreasing sexual partners and the consistently use of condom. In addition, cervical cancer is preventable by early detection and taking vaccination (Cain *et al.,* 2009). Vaccines are recommended as an effective tool for primary prevention to avoid up to 70% of HPV infections (Chan and Berek, 2007). Nonetheless, vaccines utilization is

costly and require appropriate infrastructure to preserve the vaccines (Cain *et al.,* 2009).

Diagnosis and treatment at early stages provide a proficient treatment and longer survival times (Levi *et al.*, 2000). There are numerous techniques offered for the diagnosis of cervical cancer based on the cytological and histological alterations namely invasive tissue biopsies, pap smear, the thin-prep cytological test (TCT), naked-eye visual inspection of the cervix with acetic acid, colposcopy and cervicography (Janicek and Averrete, 2001; Kesic, 2006).

The risk of death due to cervical cancer however can be reduced through suitable treatment and follow up (Cain *et al.*, 2009). Hysterectomy has remained as standard therapy for cervical cancer. Radical hysterectomy with lymph node dissection or pelvic radiotherapy provide 5-years survival of early stage cervical cancer (Cain *et al.*, 2009). In constrast, chemoradiotherapy has serve as standard mechanisms for advance stages of cervical cancer with 5-years survival (Cain *et al.,* 2009).

2.1.1.2 Ovarian cancer

Ovarian cancer can be categorized into three subgroups based on aetiologies and clinical behaviour, known as:

i. Epithelial cell tumour: is the most common ovarian cancer which make up over than 85% of all cases (Agarwal and Kaye, 2003).
ii. Germ cell tumour: is rare which presents only 1% of all ovarian cancer and more frequent among 26 years old women (Norris and Jensen, 1972; Quirk and Natarajan, 2005).
iii. Stromal cell tumour: rare and diagnosed in 50 years old patients (Quirk and Natarajan, 2005).

Ovarian cancer is identified by its high mortality rate and has been reported as main cause of death in developed countries (Fasching *et al.,* 2009). According to the World Health Organization GLOBOCAN, in year 2012 about 238 719 patients throughout the world has been diagnosed with ovarian cancer and has been recorded as fourth most regular cancer in Malaysia, make up 6.5% of all cancers in Malaysian women (Zainal Ariffin and Nor Saleha, 2011; Ferlay *et al*., 2012).

Detection at early stages is complicated since this disease often does not show any symptoms until become highly developed, making prognosis for ovarian cancer is poor with 70% of patients are usually diagnosed with advanced stages (Kirwan *et al.,* 2002).

Generally, ovarian cancer is treated by surgery and chemotherapy (Chan *et al.,* 2007). Initial surgery can reduce or remove the tumour mass, however, if tumour is

larger than 2 cm, the potential to survive for 12 to 16 months is lower compared to those with tumour less than 2 cm that have 40 to 45 months of survival (Mutch, 2002). For over three decades, the requirement for more efficient and less toxic chemotherapeutic agent for ovarian cancer has brought first-line chemotherapy to undergo a series of evolutions (Agarwal and Kaye, 2003). Recently, intravenous-platinum and taxane-based chemotherapy has been applied in clinical practices as a standard patients' care following surgery (Ozols *et al.,* 2003; Thigpen *et al.,* 2005).

Although combination treatment of surgery and chemotherapy have showed advantages in controlling the progression of ovarian cancer, recurrence and ultimate succumbing of this disease forces many researches to investigate for new approaches to improve the available treatment (Onda and Yoshikawa, 2011).

2.1.1.3 Liver cancer

Globally, liver cancer is fifth most frequent cancer and second leading cause of mortality in male, with 70% to 85% of total liver cancer burden accounted by hepatocellular carcinoma (HCC) (Jemal *et al.,* 2011; Ferlay *et al.,* 2012). HCC has been reported as a leading cause of liver cancer mortality all over the world and the incidence is expected to further increase in future (Venook *et al.,* 2010). It has been reported that 85% of liver cancer cases occur in developing parts of the world, where East and Southeast Asia are among the highest rates of liver cancer (American Cancer Society, 2011).

Chronic infections of hepatitis B or hepatitis C viruses are the main cause of liver cancer, accounting for 80% of hepatocellular carcinoma (HCC) (Rosen, 2011;

Arzumanyan *et al.,* 2013). HBV has been found as major risk factor of more than 50% global HCC incidences including Malaysia (Nguyen *et al.,* 2009; Norsa'adah and Nurhazalani-Zayani, 2013). In Malaysia, HCC is ranked as eighth most regular cancer among male with incidence rate is 3.6 per 100 000 persons (Tirunagari and Shaik, 2013). Other possible factors such as long-term exposure to some dietary carcinogens, obesity, alcoholism, diabetes, cirrhosis and tobacco smoking may be contributed to the development of this disease (Perz *et al.,* 2006; Chuang *et al.,* 2009).

Current days, various treatment options are accessible to address HCC patients depending on the stage of the disease. According to The Barcelona Clinic Liver Cancer (BCLC) staging system, HCC are categorized into four main categories known as early stage (stage 0-A), intermediate stage (stage B), advance stage (stage C) and end-stage (stage D) disease (Forner *et al.,* 2012).

Patients with early stage HCC (stage A-0) are those diagnosed with solitary lesions or up to three lesions < 3cm. Possible curative treatments for early stage HCC may include resection, liver transplantation, or ablation with 5-year survival of 50% to 70%. Patients with early tumour are introduced to the surgical resection or liver transplantation for their treatment. While, patients bearing single asymptomatic HCC < 2 cm and lack of vascular invasion or satellites with functional liver (Child-Pugh class A) are classified under subgroup of very early HCC (stage 0). An excellent outcome with almost no risk of reccurence is feasible, if patients are subjected to ablation or resection (Forner *et al.,* 2012).

Patients with single large asymptomatic HCCs without vascular invasion or extrahepatic spread are categorized as stage B. The survival among stage B patients are varies depending on the confounding factors with expected median survival is 16 months and some of them may surpass 50 months median survival (Beaugrand *et al.,* 2003; Cabibbo *et al.,* 2010; Burrel *et al.,* 2012; Malagari *et al.,* 2012). Chemoembolization is recommended as a suitable treatment for stage B HCC (Bruix and Sherman, 2011).

Stage C applies to HCC patients that present vascular invasion or extrahepatic spread and the symptoms begin to appear in stage C patients. Around four to eight months of median survival is expected in patients without treatment and it has been shown that treatment with sorafenib may improve survival (Beaugrand *et al.,* 2003; Llovet *et al.,* 2008; Cheng *et al.,* 2009; Cabibbo *et al.,* 2010).

Patients diagnosed with stage D HCC is regarded as terminal stage where prognosis is dismal and expected survival is less than three months. Patients within this stage normally offered with the best palliative care (Forner *et al.,* 2012). BCLC staging system and available treatment strategy for HCC patients are summarized in Figure 2.1.

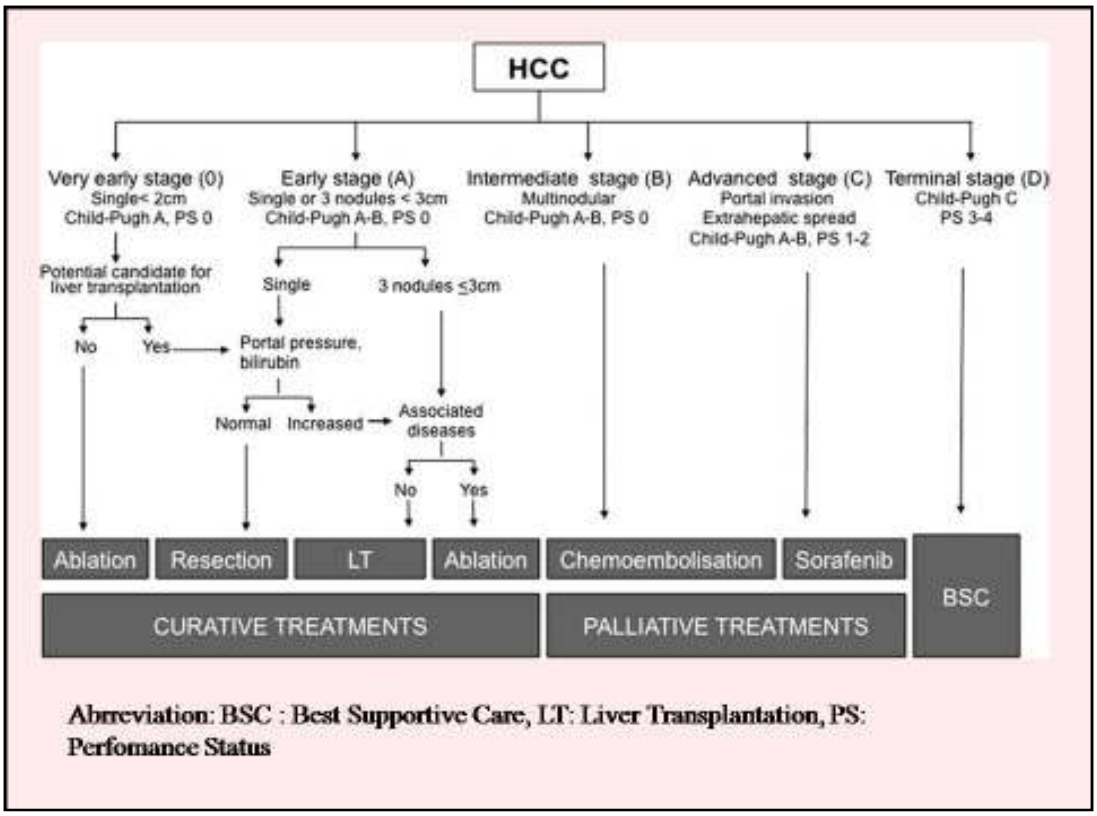

Figure 2.1: BCLC staging system and treatment strategy for HCC patients (adapted from Forner *et al.,* 2012).

2.1.2 Cancer chemotherapy

The application of chemotherapy in the cancer treatment begins in the late 1940s to treat advanced lymphoma (Gilman, 1963). Since then, over 50 licensed drugs have been introduced in the clinical use for the management of cancer (Brighton and Wood, 2005). Chemotherapy is the most frequent used in advanced cancer and usually adopted as an adjunct to surgery for the treatment of primary cancer in order to improve cure rates (Corrie, 2007; Sakuramoto *et al.,* 2007). Chemotherapy following surgical resection which is regularly introduced to the patients with high risk of recurrence is called adjuvant chemotherapy (Corrie, 2007). Meanwhile, neoadjuvant chemotherapy is normally used for the treatment of localized cancer before planned curative surgery (Corrie, 2007). Sometimes, chemotherapy as well used in combination with radiotherapy (Corrie, 2007).

Generally, chemotherapy drugs are classified according to their biochemical properties or cell cycle effects. Those categorized in the same biochemical class which include alkylating agents, antimetabolites, antitumour antibiotics, topoisomerase inhibitors and tubulin-binding drugs share similar mechanism of action (see Table 2.2) (Caley and Jones, 2012). While classification based on the cell cycle specificity is influenced by how drugs are scheduled and combined. Certain chemotherapy agents like antibiotics, antimetabolites, taxoids, vinca alkaloids from different class, except for alkylating agents have phase-specific activity on the cell cycle (see Figure 2.2) (Caley and Jones, 2012).

Chemotherapy exerts their effects by interfering with the biochemical process that is implicated or devoted to cellular replication and cause selective cell death (Osiecki, 2002). Within this process, the rapidly dividing and fast growing cells, by which cancer cells are differentiated from normal cells, are recognized by chemotherapeutic drugs to be executed by apoptosis (Caley and Jones, 2012; Sak, 2012). Unfortunately, the rapidly growing normal cells including blood forming cells and epithelial cells, and tissue with metabolic functions and substantial blood flow such as liver and kidney as well targeted by the non-selective action of chemotherapy drugs, leading to undesirable side effects which become a major clinical problem, whereas toxicity often restricts the efficacy of anticancer agents (Johnstone *et al.,* 2002; Kovacic, 2007).

Table 2.2: Biochemical classification of chemotherapy drugs (Caley and Jones, 2012).

Drug class	**Mechanism of action**	**Examples**
Alkylating agents	Have the ability to form covalent bond with molecules including protein, DNA and RNA.	Cisplatin, carboplatin, chlorambucil, cyclophosphamide, ifosfamide.
Anti-metabolites	Impede DNA or RNA synthesis by either blocking enzyme required for DNA synthesis or become incorporated into DNA or RNA.	5-Fluorouracil, Methotrexate, Pemetrexed, Mercaptopurine, Gemcitabine.
Anti-tumor antibiotics	Intercalate DNA at specific sequences and producing free radicals which results strand breakage.	Bleomycin, Anthracyclines (doxorubicin, epirubicin).
Topoisomerase inhibitors	Uncoiling DNA during replication.	Topoisomerase I (irinotecan, tapotecan) Topoisomerase II (etoposide).
Tubulin-binding drugs	Bind to tubilin to prevent the formation or disassembly of microtubules.	Vinca alkaloids (vincristine, vinorelbine), Taxoids (docetaxel, paclitaxel)

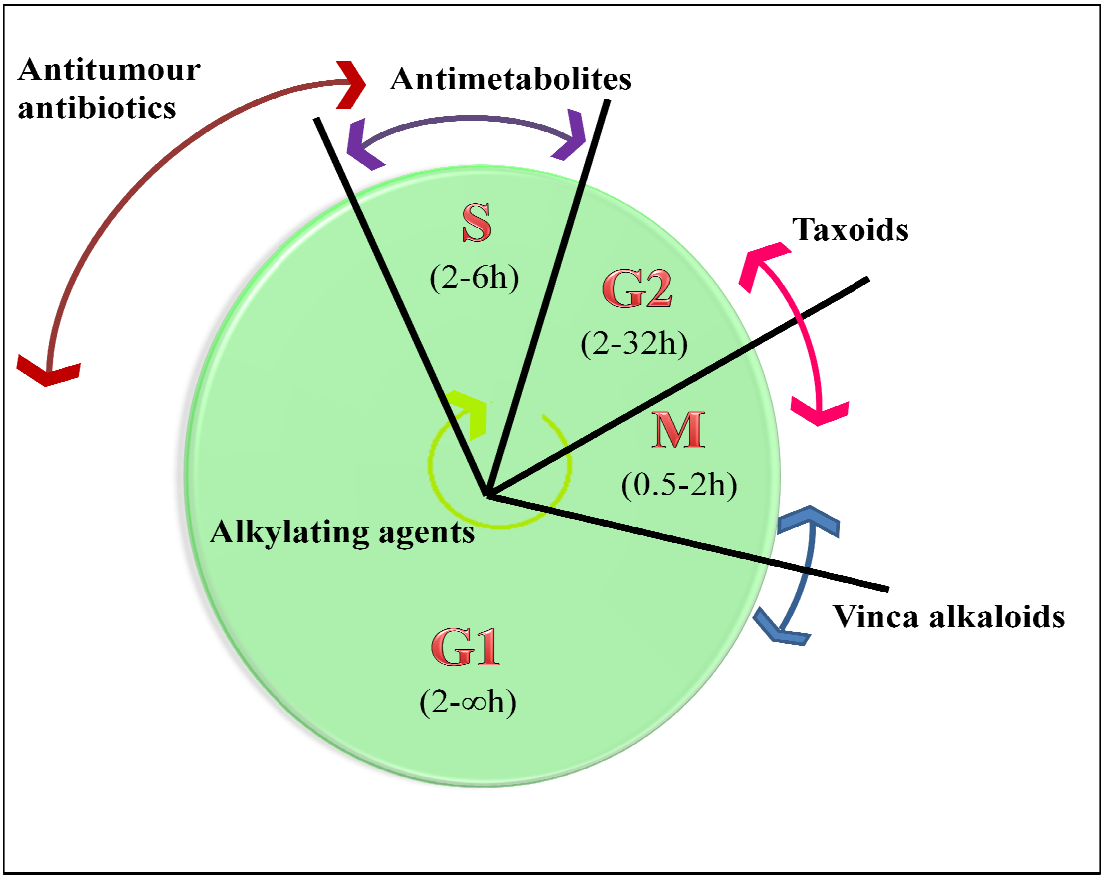

Figure 2.2: The action of certain chemotherapy agents from different classes on the specific phases (G1, S, G2 and M) of the cell cycle, with the action of alkylating agent is non-specific to any phases (adapted from Caley and Jones, 2012).

2.1.2.1 Cisplatin as standard chemotherapeutic drugs

Cisplatin or also known as *cis*-diamminedichloroplatinum (II), or *cis*-DDP (Figure 2.3) is one of the most potent chemotherapy drugs that have wide application against various types of cancer including testicular, ovarian, cervical, head and neck, esophageal, and non-small-cell lung cancers (Einhorn, 2002; Scagliotti *et al.,* 2008; Gupta *et al.,* 2009; Helm and States, 2009; Lee *et al.,* 2009; Florea and Büsselberg, 2011). This subtype of alkylating agent exerts its antitumour activity through its interaction with DNA. Cisplatin covalently bind at the N7 position of purine bases of the DNA to form primarily 1,2-intrastrand adducts between neighbouring guanosine residues, and a small number of interstrand and monofunctional Pt-DNA adducts. This results to DNA damage which eventually disturbs the normal cellular process such as transcription and replication. As the cell arrest occurs, the Pt lesions are either removed by nucleotide excision repair or apoptosis is triggered (Woźniak and Błasiak, 2002).

Many of cancer patients bearing different forms of cancer have been successfully treated by cisplatin with better prognosis and reduced life threatening including sarcoma cancers, cancers of soft tissue, bones, muscles, and blood vessels (Florea and Büsselberg, 2011). It was found to be the most effective against testicular cancer with cure rates greater than 90% and approaching nearly 100% in early stage cases (Einhorn, 2002). Positive impact following cisplatin administration, though accompanied by toxic side effects and tumor resistance which have limits its usage. The toxic side effects related to the administration of cisplatin identified as nephrotoxicity (kidney damage), emetogenesis (vomiting and nausea), myelosuppression (bone marrow suppression), ototoxicity (hearing loss),

immunosuppression (decreased response to infection) and neurotoxicity (neuron damage) (Desoize and Madoulet, 2002; Florea and Büsselberg, 2006; Günes *et al.,* 2009; Shah and Dizon, 2009; Tsang *et al.,* 2009).

Dealing with these problems, many considerable efforts have been made to improve the efficacy, increase sensitivity and reduce the toxicity implicated with the chemotherapeutic drugs. Discovery of new anticancer agent from natural sources has become a trending strategy. Among them, plants provide a valuable source for the development of effective and safer anticancer agent.

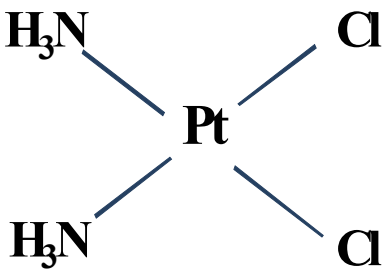

Figure 2.3: Molecular structure of cisplatin (adapted from Woźniak and Błasiak, 2002).

2.2 Plant as source of anticancer agent

Recently, alternative medicine from plant sources have been accepted and acknowledged by modern people. Not only used as part of healthcare regimes, plant also served as natural-based therapy for patients with chronic diseases such as diabetes, hypertension and cancer. For example, more than 60% of cancer patients consume vitamins or herbs as an alternative treatment (Sivalokanathan *et al.,* 2005; Madhuri and Pandey, 2008).

Abundant of scientific articles have reported the wide spectrum of therapeutic effects associated with plant especially in treating cancer. For example, eurycomanone, a plant compound isolated from *Eurycoma longifolia* Jack inhibit the proliferation of hepatocellular carcinoma (HepG-2) cell line via apoptosis (Zakaria *et al.,* 2009).

Until now, in the major amount of potential anticancer compounds originated from plants, only 1-10% of 250 000 to 500 000 of plant species in this world have been chemically and pharmacologically studied for their potential therapeutic values (Verpoorte, 2000). Therefore, to maximize the possibility of discovering effective drugs for the treatment of cancer, the utilization of plant in this present study is realistic and fulfills the current requirement towards new chemotherapeutic agent that should be (Hoekman *et al.,* 1999):

i. Effective
ii. Highly selective for cancer cells (no harmful effects towards normal cells)
iii. Less or no toxicity effects

iv. No negative impact on anticancer therapy

v. Minimal side effects

2.2.1 *Quercus infectoria* galls

There are about 600 species of oak trees of the Family Fagaceae distributed in a variety of habitats such as seacoast, mountain slopes and wet lowlands of the North Temperate Zone (Khouzami *et al.,* 2009).

Quercus infectoria Olivier (QI) of the family Fagaceae is a small tree (4 to 6 feet tall) local to the Greece, Asia Minor, Syria and Iran (Basri and Fan, 2005; Olfat and Pourtahmasi, 2010). The tree shoot bearing galls, an abnormal growth formed from the attack of the gall-wasp called *Cypnis gallae-tinctoria*. The galls which are commonly known as manjakani in Malaysia is rounded hard body with 1-1.5 cm in diameter. The numerous horny protuberances on smooth external surface giving it's a rough touch. Appear as greyish-brown to brownish-black on its external and yellow in colour of the inner surface. The present of pores on it's uneven surface indicates infection (Figure 2.4) (Shrestha *et al.,* 2014).

Figure 2.4: The physical morphology of QI galls.