



Bio-pharmacological screening on liver-protective and anti-hepatocarcinoma activities of Vietnam natural products

Minh Quan Pham

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Université
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THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par : *l'Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)*

Présentée et soutenue le 30/05/2016 par :

Minh Quan PHAM

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anti-hepatocarcinoma activities of Vietnam natural products**

JURY

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You beat cancer by how you live, why you live, and in the manner in which you live

Stuart Scott

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List of Abbreviations

Aa: Amino acid

BCG: Bacillus Calmette-Guérin immunotherapy

CM: Culture medium

DG-AMMOS: Distance Geometry and Automated Molecular Mechanics Optimization

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

Dox: Doxorubicin

GLOBOCAN: Global burden of cancer study

HBV: Chronic Hepatitis B Virus

HCC: Hepatocellular carcinoma

HCV: Chronic Hepatitis C Virus

HD: High Dose

HA: Hazard Assessment

Hits: Identified potential compounds

HPLC: High-performance liquid chromatography

HTS: High-throughput screening

IC₅₀: Half maximal inhibitory concentration

INPC: Vietnam Institute of Natural Products Chemistry

kDa: Kilodalton

MCF-7: Breast cancer cell line

MOST: Vietnam Ministry of Science and Technology

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NAFLD: Non-alcoholic fatty liver disorders

NBRI: National Botanical Research Institute of South Africa

NCI: United States National Cancer Institute

OAc: CH₃COO

OD: Optical density

PARP: Poly (ADP – ribose) polymerase

PDB: Protein Data Bank

PFT – α : Pifithrin alpha

PI: Propidium iodide

PS: Phospholipid phosphatidylserine

ROS: Reactive oxygen species

SD: Standard Deviation

SDH: Succinate dehydrogenase

SDS-PAGE: Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis

SES: Socio-economic status

SM: Solamargine

SS: Solasonine

t – RNA: Transfer-Ribonucleic acid

U2OS: Osteogenic sarcoma

USA: United States of America

UV: Ultraviolet radiation

VAST: Vietnam Academy of Science and Technology

VMOH: Vietnam Ministry of Health

VS: Virtual screening

WB: Western Blot

A - Introduction

Hepatocellular carcinoma (HCC), which represents more than 80% of all liver cancer cases, is posing a serious threat to the human health, especially in South East Asia. It is estimated that in Vietnam, this disease is increasing dramatically with about 22,000 people affected per year and 95% of them will die. Liver cancer is rarely detected in early stage and once detected, treatment faces a poor prognosis in most cases. The therapeutic approaches against HCC depend on its stage of development, and include surgery, chemotherapy and radiotherapy. Chemotherapy involves the administration of compounds that act on cancerous cells by either killing them or preventing their proliferation. Currently, more than half of cancer chemotherapeutic small molecules used are derived from or inspired by natural products.

Today, the very poor prognosis and a lack of effective treatments fully justifies the search of new molecules and therapeutic strategies against HCC.

Vietnam is a country with favorable natural conditions for a rich and varied biodiversity. It is estimated that there are about 12,000 species of plants and nearly 11,000 marine organisms had been recorded so far until now. Despite this high potential source of original bioactive substances, only a very limited number of studies have been done to explore the Vietnam plants and marine resources for their cytotoxic properties and to isolate and characterize the active molecule.

The present work was conducted on natural products originated from Vietnamese plants with the aim to identify natural compounds with potential anti-hepatocarcinoma activities.

Two experimental screening approaches were used :

- The first approach refers to a pharmacological screening method including the establishment of Vietnamese database of natural products. This database contains compounds isolated (and their structure determined) from medicinal plants that are commonly used in traditional prescriptions for liver protection. This approach allowed the identification of a family of *ent*-kaurane diterpenoids isolated from *Croton kongensis* Gagnep., which exhibit original cytotoxic, apoptotic and sensitization properties on human liver cancer HepG2 and Hep3b cell lines.
- The second approach aimed at developing a “Virtual screening” strategy. Research was carried out on a database which is the collection of all known compounds

isolated or originated from Vietnamese plants using computational tool called Autodock. The objective focused on finding natural molecules that could abrogate the interaction between p53 and Mortalin, an hsp70 chaperone protein also identified as a marker for hepatocarcinoma, expecting that inhibition of mortalin – p53 interaction should result in the activation of the apoptotic process. *In silico* screening of a database of 354 natural compounds allowed the identification of solasonine as a potent inhibitor of p53 – mortalin interactions. Pharmacological studies confirmed that solasonine was able to inhibit mortalin - p53 interaction. This inhibition abrogated cytoplasmic sequestration of p53, allowing its translocation to the nucleus and inducing cell death of human hepatocellular carcinoma cell lines through p53-dependent pathways.

B - Bibliographic Review

1. Cancer and Hepatocellular Carcinoma

1.1. Cancer

1.1.1. Overview of cancer

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. There are more than 100 different types of cancers, most of them are named depending on which tissue or organ they come from, for example: cancer begins in the cells of the large bowel is called colon cancer (Hattersley L., 2013). Cancer types can be grouped into four main categories including:

- Carcinoma: cancer that begins in the skin or in tissue that line or cover internal organs. There are a number of sub-types of carcinoma, including adenocarcinoma, carcinoma, squamous and transitional cell carcinoma.
- Sarcoma: cancer that begin in bone, cartilage, fat, muscle, blood vessels or other connective or supportive tissue.
- Leukaemia: cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- Lymphoma and myeloma: cancer that begins in the cells of the immune system.
- Central nervous system cancers: cancer that begin in the tissues of the brain and spinal cord.

Nowadays, cancer has become a major public health and it is attracting attention from all over the world. It could be caused in one of three ways, namely: incorrect diet, genetic predisposition and via the environment. It is estimated that approximately 95 % of all cancers are caused by life style and may take about 20 to 30 years to develop.

Modern human is confronting with increasing incidence of cancer and cancer deaths annually (Reddy L., 2003). According to the most recent global burden of cancer study (GLOBOCAN) data from the International Agency for Research on Cancer (<http://www.globocan.iarc.fr> - GLOBOCAN, 2012), there were about 8,2 million deaths and

more than 14 million people affected directly by different types of cancer on world scale, the three most common being lung, breast and colorectal cancers (Figures 01 and 02).

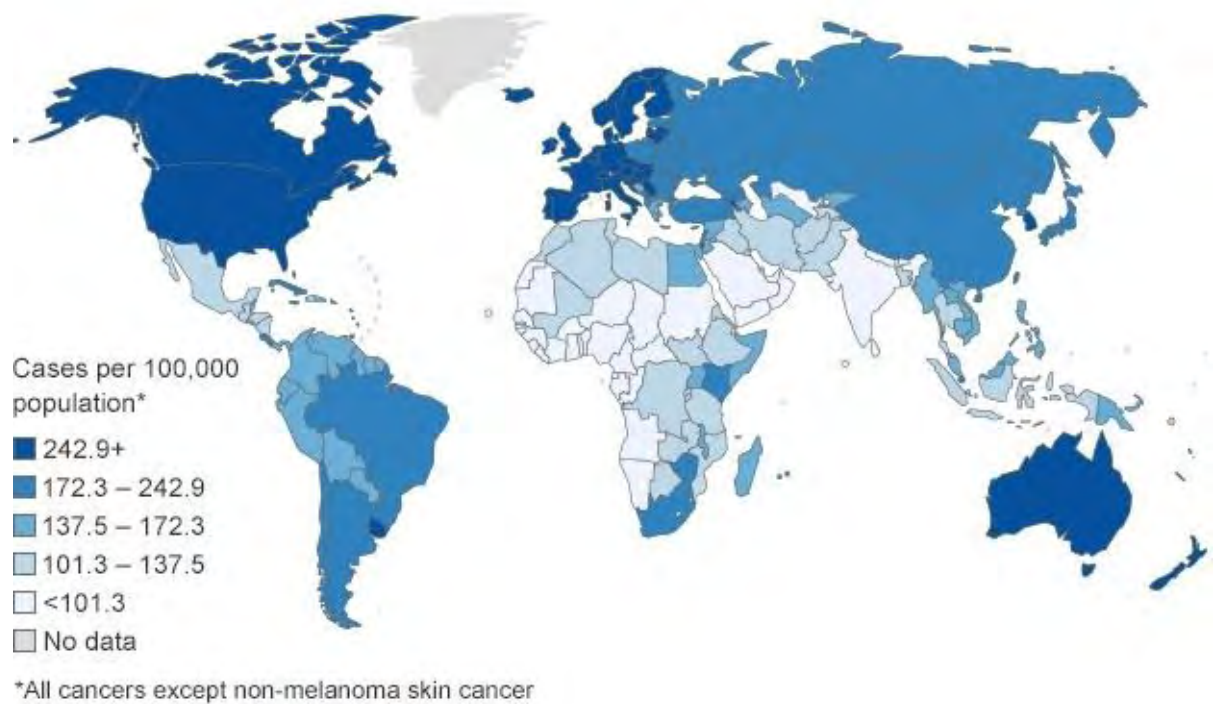


Figure 01 : Cancer incidence statistics worldwide in 2012 (GLOBOCAN 2012)

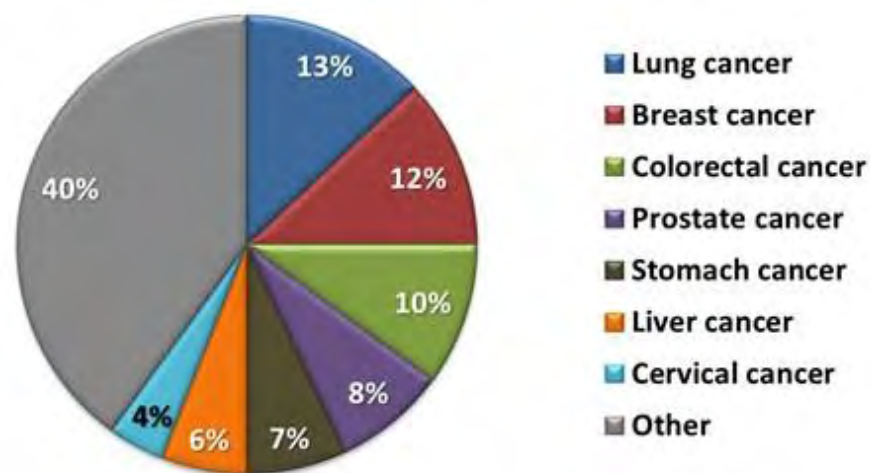


Figure 02 : Most common cancer worldwide in 2012 (GLOBOCAN 2012)

In Vietnam, Ministry of Health has predicted that the 21st century will be the century of cancer, cardiovascular and other noncommunicable diseases due to the process of urbanization, industrialization, plus the sequela of war still exist and threaten human health every day (<http://moh.gov.vn/pages/index.aspx>; Anh P. T. H., 2002). According to the statistics in several oncology hospitals in Hanoi, Ho Chi Minh and some other provinces in 2008, there are approximately 100,000 to 150,000 people suffer different types of cancer and over 73% of them (about 70,000 patients) die annually which makes Vietnam become one of the countries has highest mortal rate in the world and this number tends to increase in the near future. It is predicted by 2020, there will be about 200,000 new cancer cases and 100,000 deaths per year in Vietnam (Vietnam National Cancer Hospital - <http://benhvienk.vn>).

Despite the significant progress made in recent decades in researching treatments for some types of cancer, cancer prevention and cure remain elusive in many countries. The burden of cancer keeps increasing both in economically developed and developing countries as a result of population aging and growth as well as increasingly an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and “westernized” diets (Jermal A., 2011).

1.1.2. Aetiologies of Cancer

The risk factors vary widely depending on different lifestyle, economic, social, climate or environment pollution. It is estimated that tobacco and smoking is the world leading cause followed up by unhealthy diet and obesity (World Cancer Report 2014).

Until now, it is impossible for human to explain why one person develops cancer while another does not. However, many researches have pointed out that some risk factors may increase the rate of cell mutations that favor a person’s chance to develop cancer. They include intrinsic factors such as: age, gender or family history but many causes for this disease come also from external effects. These different factors are shown in Figure 03 and briefly presented below :

a. Modifiable risk factors

- Tobacco and smoking: tobacco use is identified worldwide as the first leading cause of many cancers such as lung, oral cavity, esophagus or pancreas.
- Unhealthy diet: diet is believed to be implicated in about 30% to 40% of all cancers. A diet high in saturated fats, lack of fibre, fruits and vegetables could increase the rate of getting breast, esophagus and prostate cancer.
- Ultraviolet radiation (UV): sunlight is the main source of UV rays, being exposed to the UV is the major risk factor to several types of skin cancer.
- Infectious agents: since the start of 20th century, infections are known to play a role in cancer. Worldwide, they are accounted for 15% to 20% of different types of cancer and especially high in developing countries. Basically, there are three types of infectious agents including: viruses, bacteria and parasites, for example: human papilloma virus, hepatitis B, C viruses.
- Physical inactivity: an unhealthy lifestyle would increase the risk of some cancers such as: breast, colon, esophagus cancer. This factor is closely linked to the unhealthy diet with low nutrition.

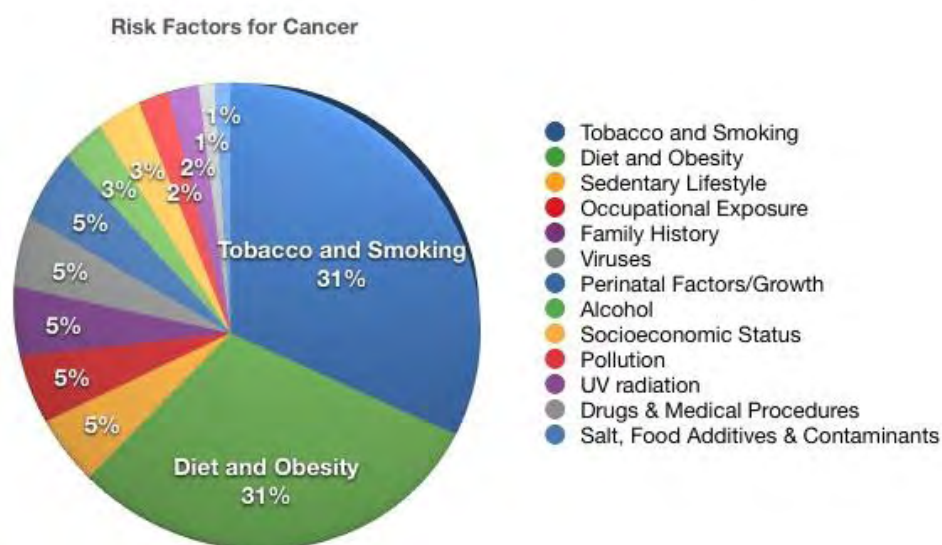


Figure 03 : Main factors causing cancer worldwide (Food, Nutrition, Physical activity, and the prevention of cancer: A global perspective., 2007)

b. Other modifiable factors

- Alcohol: consuming too much alcohol would raise the risk of suffering some cancers such as: oral cavity, mouth, liver and esophagus. It should be noticed that drinking and smoking together even make the situation worse.
- Socio-economic status (SES): SES is closely linked to the risk of developing certain cancers based on a person's income, education level and occupation. For example: low SES is associated with lung, stomach and cervical cancer while high SES is responsible for breast and testicular cancer.
- Occupational exposure: it has been estimated that around 3% to 6% of all cancers worldwide are caused by exposure to carcinogens in the workplace (Driscoll T., 2005; Rushton L., 2012). Some common types of cancer factors a person can suffer include arsenic, asbestos, silica and benzenes.
- Environmental pollution: this has become a serious issue in many developing countries with the pollution of air, water and soil which represents one of the factors causing about 4% of all cancers.
- Food contaminant and obesity: it should be noticed that an unhealthy lifestyle leads to obesity and consumption of certain food contaminants such as aflatoxin, pesticides could pose a carcinogenic risk to human. The common cancers resulting from this field are liver, kidney, breast cancer.
- Ionizing radiation: in daily life, we are all exposed to small amounts of naturally or manufactured radiation, for example the exposure comes from medical X-rays or in some war zones. This issue is also one among factors causing cancers.

c. Non-modifiable factors

- Age: as cancer takes time to develop, most of the cases are diagnosed in people older than 65 years old. This high rate could be explained by the combination of long time exposure to carcinogens and weakening of the body's immune system. However, some cancers such as retinoblastoma, Wilm tumor occur almost exclusively in children because of gene mutations.

- Ethnicity – race: different genetic background between races or ethnicities would be an issue contributing to the risk of suffering cancers, especially in breast cancer. However, in most of the cases, the reason comes from different lifestyle and exposure to carcinogen agents.
- Heredity: a small but significant proportion of cancers are due to inherited conditions. In some families, due to specific gene fault, they could have higher risk of developing certain cancers for example breast, ovarian or bowel cancer.
- Gender: some cancers occur only in one gender due to different anatomy such as prostate in men and uterus in women. Some others occur in both genders but at different rates, for example oral cavity, liver.

d. Other risk factors

- Hormonal and reproductive factors: sex steroid hormones such as estrogens could affect the development of some cancers in women like breast and ovary cancer. In men, testosterone hormone is involved in prostate cancer. However, these factors only contribute a small account to the burden of these cases.
- Immunosuppression: the immune system plays an important role in defending the body against cancer. Certain medical drugs and infections are the most common factors as immunosuppressants. The best known factor for this case is HIV which is associated with Kaposi's sarcoma (Sloan F. A. and Gelband H., 2007).
- Medicinal drugs: in some cases, hormonal drugs can cause cancers while others reduce the risk. It has been reported that certain anti-cancer agents are carcinogen to human which cause another cancer years later (Harris C. C., 1976).

1.1.3. Mechanisms of cancer development

Cancer is, in some aspects, a genetic disease since many cancer types arise from the alteration (mutation) in the deoxyribonucleic acid (DNA) of normal cells. Cancer begins with damage to one or more genes in a single cell. This damage causes cells to proliferate

uncontrollably and to be transformed in abnormal cells. At this stage, if the body's immune system does not recognize and kill these abnormal cells, then they will develop to even more abnormal and finally converted to cancer cells. The characteristics of cancer cells is they divide more rapidly than normal cells and start to pile on top of each other and form malignant tumor, these tumors will invade healthy tissues. The process of a normal cell to become a cancerous tumor usually take years to develop (Figure 04).

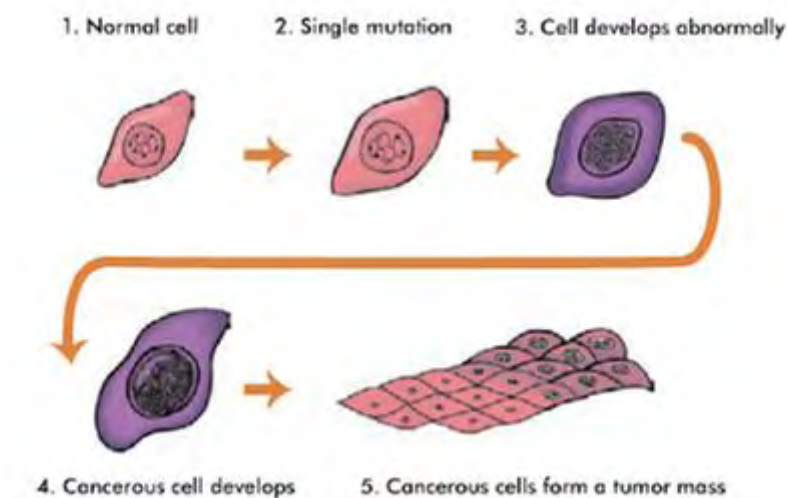


Figure 04 : Development process of cancerous cells. How a tumor develops: Damage to genes in a single cell (mutations) can lead to progressively more abnormal cells. Eventually, abnormal cells may become cancerous cells, which often divide quickly and do not die. (The Metastasis Research Society UK, <http://www.metastasis.icr.ac.uk>)

Several hallmarks of malignant cancer are autonomous growth; invasion and metastasis. Histologically, cancer is characterized by some morphological alterations, including changes in tissue architecture, cytological abnormalities of both the nucleus and cytoplasm and the presence of abnormal mitosis.

In medical field, the term “stage” is used to describes the severity of a person’s cancer. As shown in Figure 05, during early stages, there may be only small cancerous tumor, if they remain localized, then the disease is curable by surgical removal if the tumor is detected in time. However, malignant tumors tend to metastasis. In more advanced stages, the tumors

growth larger and start to break away from parent mass, enter the lymphatic or vascular circulation and move to different sites in the body. This process makes surgical removal of metastasis difficult if not impossible and it is no longer possible to cure the disease.

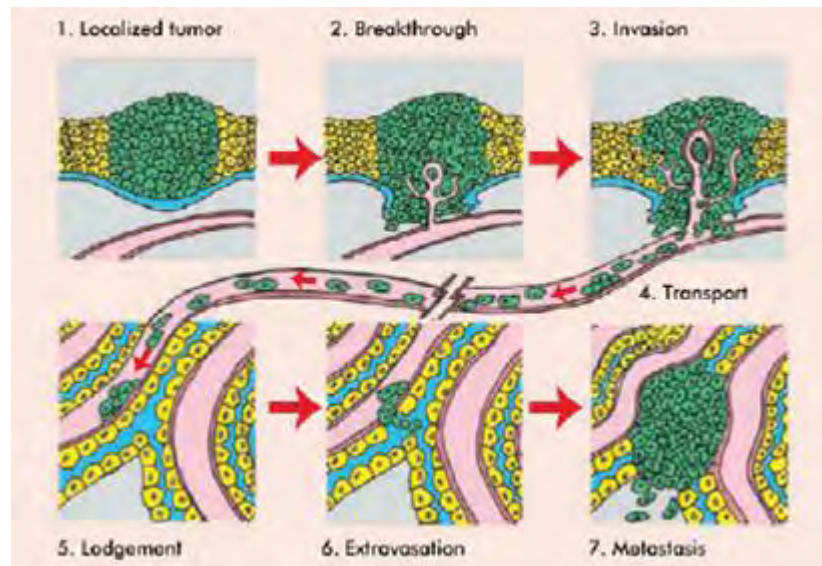


Figure 05 : Metastasis mechanism of cancerous cells. How a tumor spreads: Metastases happen when cancer cells from the original tumor spread to different parts of the body, and eventually form secondary tumors at sites distant from the original tumor. (The Metastasis Research Society UK, <http://www.metastasis.icr.ac.uk>)

1.2. Hepatocellular Carcinoma (HCC)

1.2.1. Overview of Hepatocellular Carcinoma

Liver cancer comprises diverse, histologically distinct primary hepatic neoplasms, which include hepatocellular carcinoma, intrahepatic bile duct carcinoma (cholangiocarcinoma), hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma and epithelioid haemangioendothelioma (Anthony P. P., 2002). Among these, HCC – arises from hepatocytes or hepatocyte progenitors, which are the parenchymal cells of the liver (American Cancer Society, Facts and Figures, 2010) – representing more than 80 % of all cases. Due to the high tolerance of liver, HCC is rarely detected in early stage and once detected, treatment faces a poor prognosis in most cases (Singh R., 2009).

Nowadays, HCC has become the sixth most common cancer and the second leading cause of death from cancer worldwide with estimated 746,000 deaths and 782,000 new cases in 2012 (GLOBOCAN, 2012). More than 80% of these deaths and new cases occur in the less developed regions, in some parts of Asia and Africa, especially in Eastern Asia, the ratio is more than 100/100,000 population, whereas in Europe and North America, it is estimated as 2-4/100,000 population (Bain I., 1997; Duvoux C., 1998) (Figure 06).

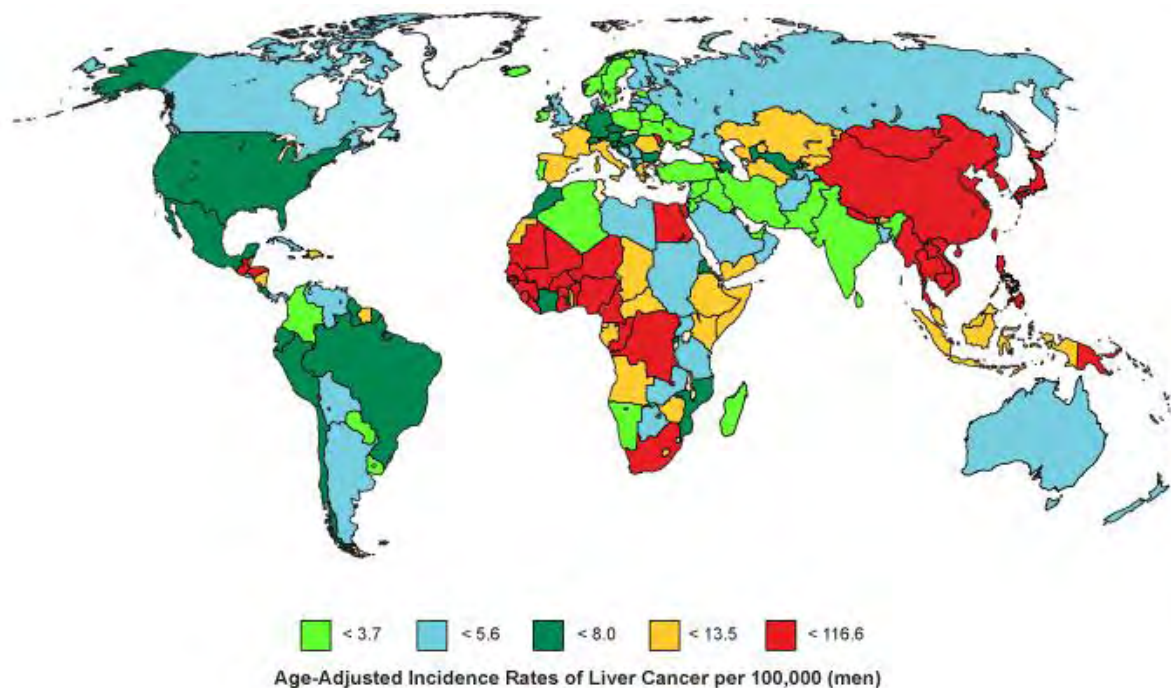


Figure 06 : Worldwide Incidence of Hepatocellular Carcinoma (International agency for research on cancer 2013)

In Vietnam, liver cancer is on the rising trend. It is estimated to have 22,000 liver cancer cases per year, of which 21,000 die, or 95.4% of the total cases - the Hanoi Moi newspaper reported from a seminar jointly held by Bach Mai Hospital and Military 108 Hospital. According to Vice Chairman of Bach Mai Hospital - Mai Trong Khoa, liver cancer has a high disease incidence rate in Vietnam and over the world with 780,000 new cases globally every year and 750,000 of which die. In terms of incidence and mortality, liver cancer ranks second and first in men and third and second in women in 2012 (GLOBOCAN 2012).

In addition, gender can also influence the risk of infection with a clear predominance in males at worldwide scale, ranging from 8:1 in countries with high frequency of HCC to about 2:1 in nations with low frequency. For all the above reasons, it is important to recognize that liver cancer is becoming a major public health problem all over the world.

1.2.2. Aetiologies of Hepatocellular Carcinoma

There are many factors that have been considered to be reasons for hepatocarcinogenesis including chronic alcohol abuse, consumption of contaminated food (Aflatoxin B1), chronic Hepatitis B Virus (HBV) or Hepatitis C Virus (HCV) infections, fatty liver diseases associated with obesity, non-alcoholic fatty liver disorders (NAFLD), diabetes, fungal toxins (aflatoxins), toxic industrial chemicals, environment pollutants and some certain metabolic disorders (Badvie S., 2000; Paraskevi A. F., 2006).

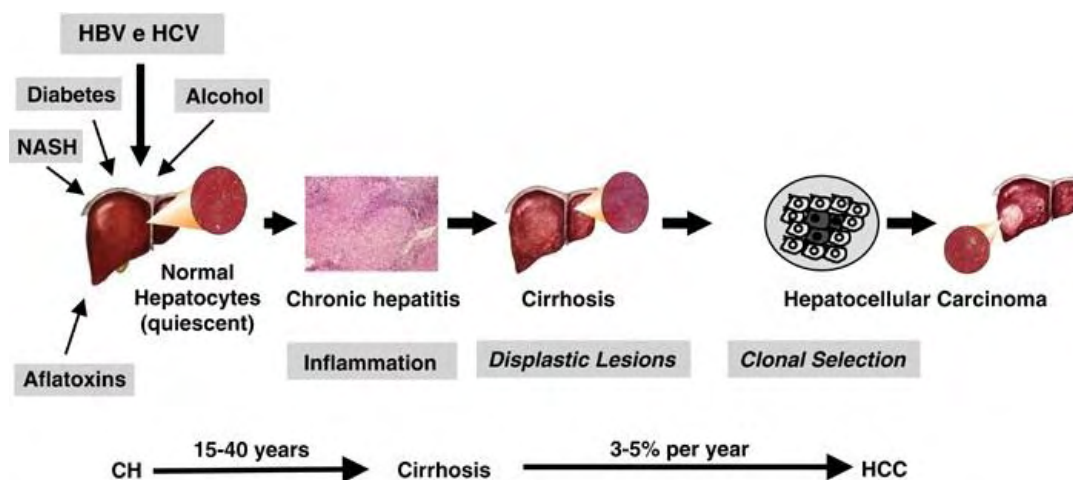


Figure 07 : Hepatocellular carcinoma development process (Levrero M., 2006)

The most prevalent causes of HCC are variable due to regionality, for example HBV and HCV infections are the major risk factors worldwide, whereas these cases constitute less than half in the USA. In most western countries, the major risk factors of hepatocarcinogenesis are alcoholic cirrhosis and obesity-related non-alcoholic fatty liver diseases (American Cancer Society, 2010). In Vietnam, HBV infection is the major cause of chronic liver diseases,

including HCC, which increase constantly and are estimated to burden by 2025 (Nguyen V.T., 2008).

1.2.3. Pathogenesis of HCC development

Mechanisms leading to the development of HCC are abundant according to the variability of HCC aetiologies, which results in a high genetic and phenotypic heterogeneity of these tumors. HCC develops in a multi-step process in chronically injured livers. Liver injury might result from one or more of the major HCC risk factors, such as HBV/HCV infections, alcohol-related cirrhosis, Aflatoxin B1 or genetic disorders. A survey of the diverse hepatocarcinogenesis mechanisms points to common pathogenetic pathways and processes (Figure 08) (Paraskevi A. F., 2006).

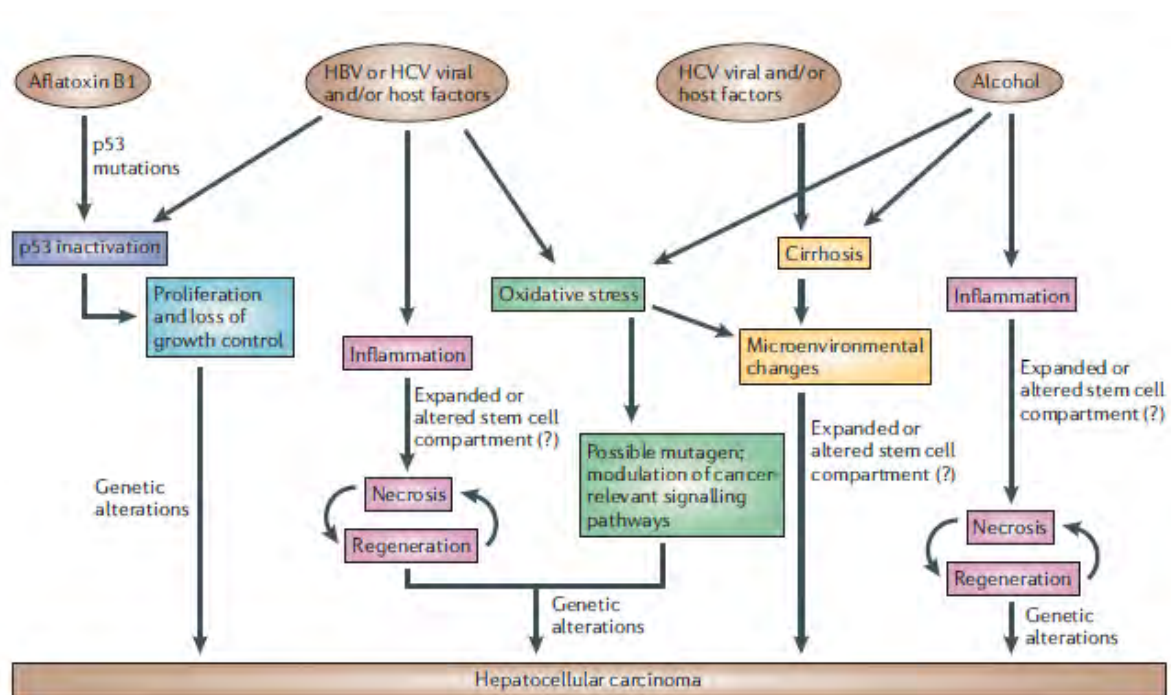


Figure 08 : The suspected mechanisms of hepatocarcinogenesis for various risk factors (Paraskevi A. F., 2006)

The initial liver injury signified itself as necrosis then followed by continuous cycles of proliferation and damage. On the other hand, these continuous cycles of proliferation and

necrosis cause telomere shortening, liver injury provokes activation of stellate cells, which play a role in the synthesis and deposition of collagen. Both of these contribute to the formation of abnormal liver nodules, together with fibrous scar tissue, a state which is called cirrhosis (Paraskevi A. F., 2006; Feo F., 2009). Formation of hyperplastic nodules is the next step in the pathogenesis of HCC, which are still considered as pre-malignant lesions. Together with the accumulation of genetic damage, leading to moderate genomic instability, these pre-malignant lesions progress into dysplastic nodules which have the ability of autonomous growth. Dysplastic nodules are composed of cells with altered characteristics such as increased nuclear to cytoplasmic ratio and they tend to form mild trabecular disarrays (Feo F., 2009).

Figure 09 summarizes the current knowledge of the genetic and genomic events associated with the development of HCC. The HCC neoplastic evolution proceeds through a multi-step histological process that is less well defined than that of other cancer types (Paraskevi A. F., 2006).

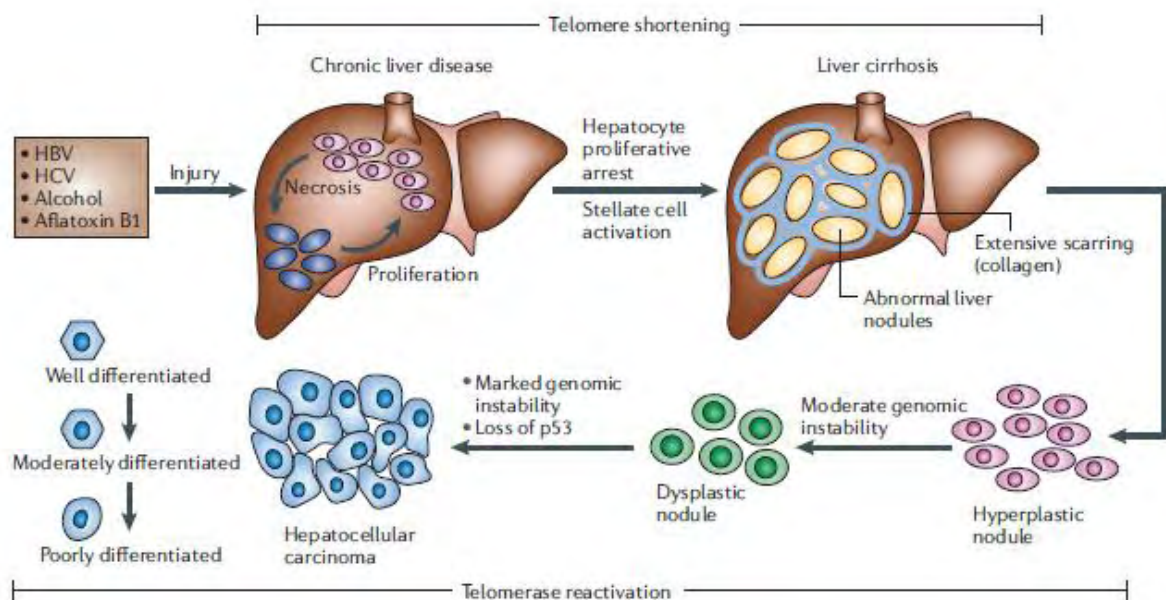


Figure 09 : Histopathological progression and molecular features of HCC (Paraskevi A. F., 2006)

1.3. Treatment against cancer

Currently, many therapies are available. The type of treatment that patients received will depend both on their type of cancer and the advantages/disadvantages of each method. In general, doctors usually use the combination of several methods in order to obtain the best therapeutic effect for curing cancer. Some main types of cancer treatment are described below:

- a. **Surgery:** surgery is one of the most effective method for cancer treatment. Surgery includes two main options: the first is open surgery in which the surgeons make large cut to remove tumors out of patient's body, the second is minimal invasive surgery with small cut and use of laparoscope with special tools to insert inside the body and operate the surgery.
- b. **Chemotherapy:** chemotherapy treatment uses drugs to kill cancer cells, the number and type of drug used depending on the patient's cancer and their metastasis. Chemotherapy is utilized either to cure cancer when the tumor is localized and does not spread or to ease the cancer symptoms by limiting tumor growth and spreading when cancer has reached the later stage.
- c. **Targeted therapy:** this method focuses on the difference between normal and cancerous cells in order to minimize adverse impacts on healthy tissues. There are two main forms of targeted drugs including small-molecule drugs that penetrate cells easily and attack them from inside. The second type is monoclonal antibodies which are the man-made versions of large immune system proteins acting at the outer surface of cancerous cells.
- d. **Radiation therapy:** radiotherapy uses high energy particles or waves to damage cancerous cells. Patients usually receive high doses of radiation to slow cancer cell growth and to ease problems caused by tumors such as breathing trouble or bladder control. Currently radiation is considered to be one of the most common method, however, the side effect is that normal cells can also be harmed during treatment.
- e. **Immunotherapy:** in medicine, doctors also use the immune system to fight against cancer. This is a method that either stimulates or eduquates the patient's immune

system to kill cancerous cells. Some common types of immunotherapy can be named like monoclonal antibodies, vaccines, cytokines, Bacillus Calmette-Guérin (BCG).

- f. Photodynamic therapy: this method uses photosensitizing agents in combination with certain kinds of light to kill cancer cells. The mechanism of this technic consists in injecting the agents either into the bloodstream through a vein or on the skin, after a certain amount of time, cancer cells will absorb the drugs then light will be applied. Under the effect of light, the drugs will form some types of chemical that kill the cells.
- g. Hormone therapy: this method can be applied for some hormone-dependent cancers such as prostate, breast or ovarian cancers. This therapy can be successful to slow or stop tumors from growing by either stopping the production of certain types of hormone or preventing hormones to interact with their receptors.
- h. Laser treatment: the advantage of this method is laying on the thin and focus beam of laser's light, which should therefore not harm normal cells nearby. There are three main types of laser commonly used in treatment including carbon dioxide, argon and neodymium. Doctors usually use laser to destroy the small areas of cancerous cells or to shrink tumors in order to make them easier to remove in surgery. Due to the functions above, laser treatment is a very effective method in support to other cancer treatment therapies.
- i. Stem cell transplant: this method is used in the treatment of some types of cancer such as leukaemia, lymphoma and myeloma. When a patient takes a high dose of chemotherapy or radiation, not only cancerous cells are killed but also stem cells in the bone marrow. Thus, patients need to receive additional stem cells to survive. Normally, stem cells are collected from patient's blood or from other donor then they are transferred through a drip to replace the dead one.

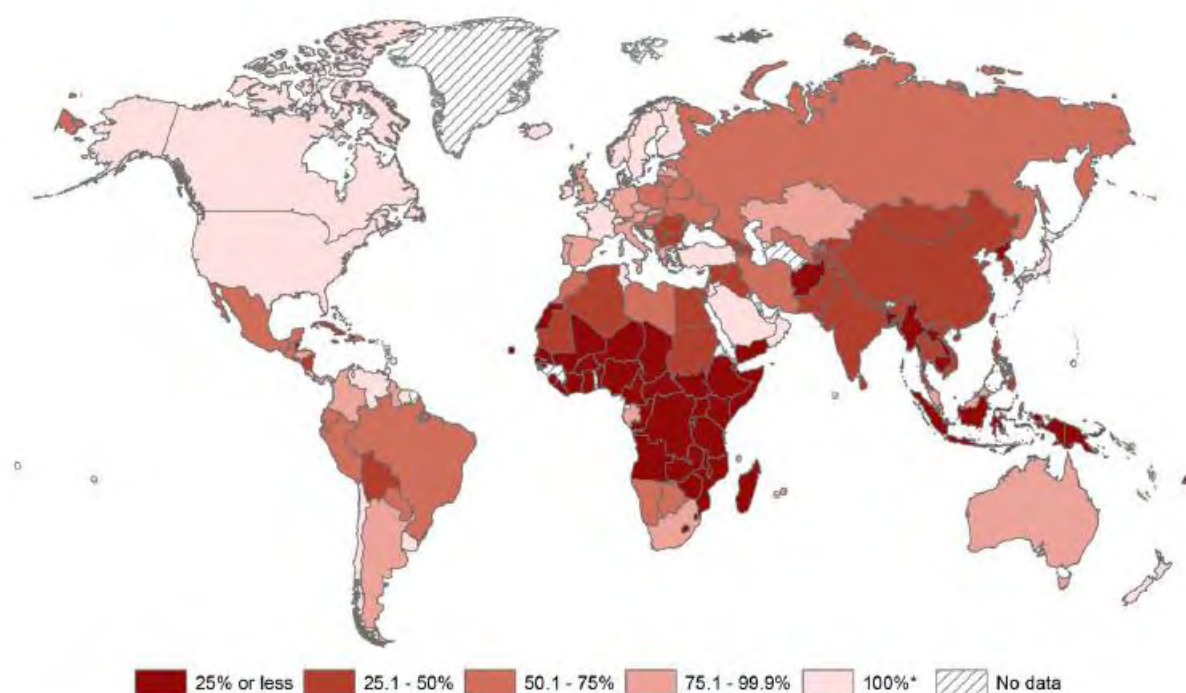


Figure 10 : Estimated percentage of patients able to access radiotherapy in 2013 (The Cancer Atlas, second edition)

Figure 10 presents an example of the differences in medical quality for cancer treatment between developed and developing countries based on the number of patients receiving radiotherapy in which the number of medical centers in developing countries being overloaded seriously. In conclusion, based on the rapid development of medical technologies, there may be a hope for human to cure cancer in the near future, however, it should be noticed that it is always better to keep a healthy lifestyle than suffering diseases, as a quote said: “An ounce of prevention is worth more than a million pounds of cure” (David Agus).

Today, the various chemotherapeutic approaches are mostly described in the frame of clinical trials. In systemic treatment, sorafenib is the only molecule showing some efficacy for unresectable and advanced metastatic HCC, whereas gemcitabine, doxorubicine and capecitabin display only marginal activities.

2. Natural products as sources of anticancer agents

2.1. The origin of medicine

Among all the medicines being provided on the international market, more than 60% of them are derived from or inspired by natural products and only 35% are totally synthesized. It is estimated that the number of drugs made from the isolation of natural sources account for 6% and those coming from derivatives of them is approximately 27% in total (Newman D. J., 2016). Also, about 27% of drugs are natural mimic molecules and synthetic molecules based on the carbon skeleton of natural products.

During the time frame of 34 years covered from 01/1981 to 12/2014, there were total 1211 types of medicine have been registered. Their classifications are shown in Figures 11 and 12.

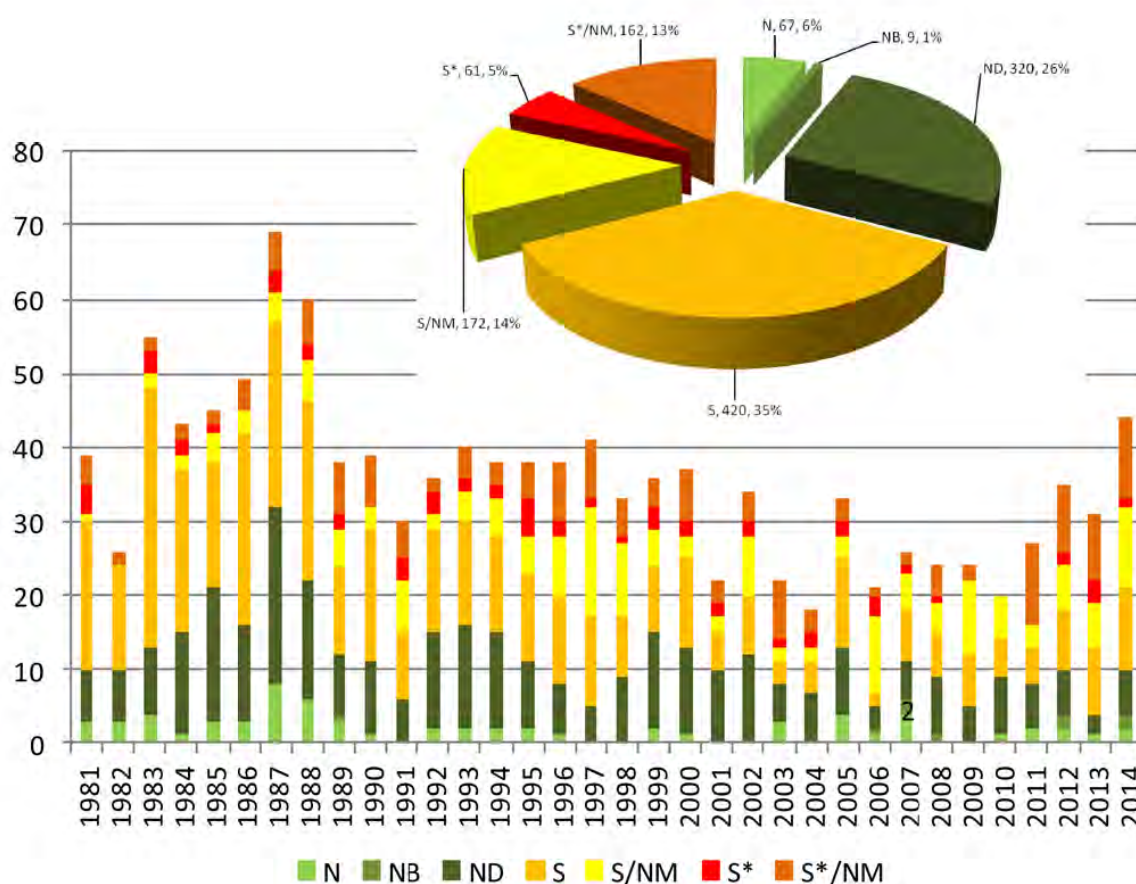
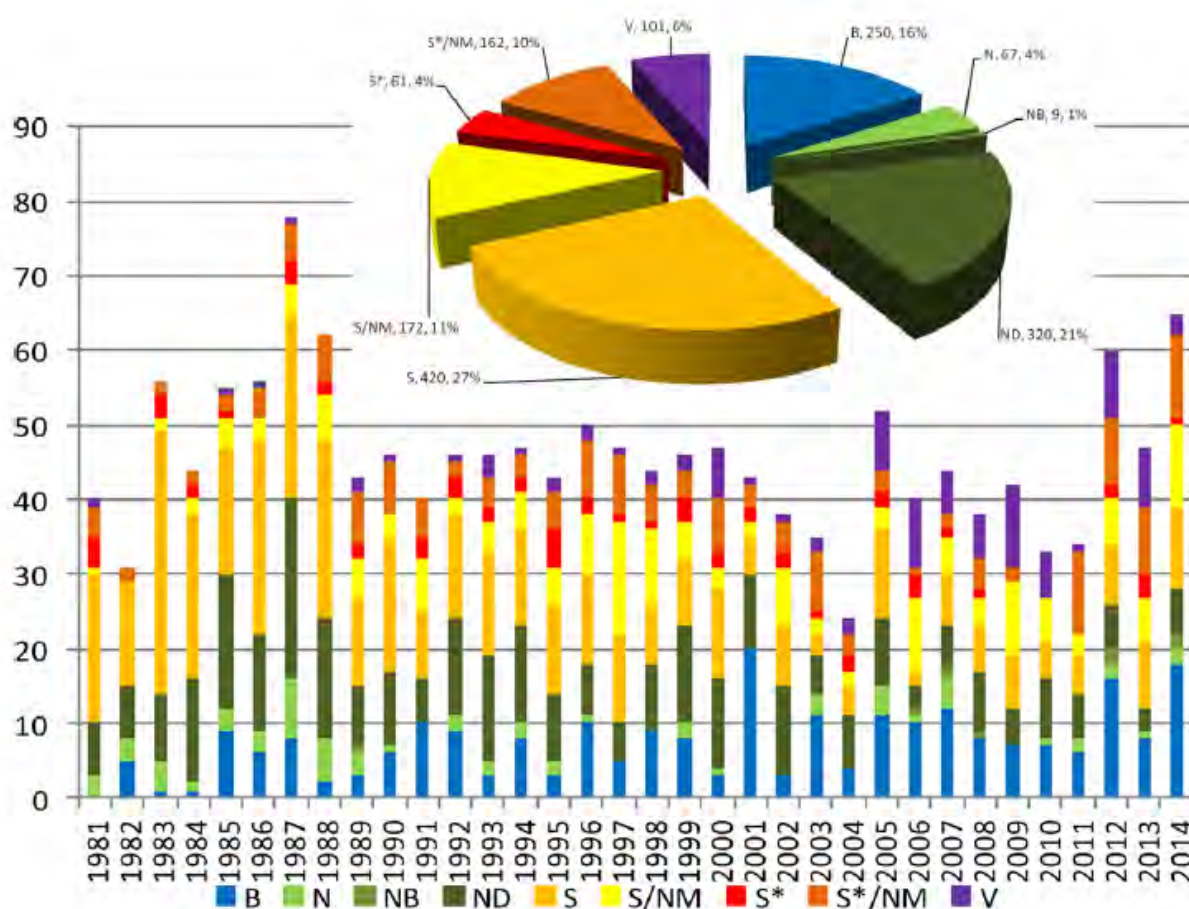


Figure 11 : Classification of 1211 types and number of drugs registered worldwide during period from 1981 to 2014 (Newman D. J., 2016)

As shown in figure 11, which refers only to small molecules, after a significant increase in the discovery of new medicines started from 1981 until 1988 with the peak of nearly 70 new drugs registered in 1987, the numbers of certified drug per year remain stable from close to 40 for most of the 1989 to 2000 time frame (except for 1991) then slightly decline to close to 20 from 2001 to 2010, with the exception of 2002 and 2005, when the figures climbed above 30. In the recent four years (2011 to 2014), the numbers showed a sign of rising again from 28 in 2011 to 44 in 2014 (Newman D. J., 2016).

If we include also vaccines (V) and biological macromolecule (B) in the statistics then the number of approved drugs showed a range from around 20 to just over 50 per year from 1989 to 2014, particularly in 2012 and 2014 the figures reached 60 and 65, respectively. It should be noted that the vaccine data are not complete, so the overall numbers could increase.



Major categories of sources:

N:	Unaltered natural product
B:	Biological macromolecule – usually a large protein or large peptides (> 50 residues)
ND:	Natural product derivative
NB:	Botanical drug
S:	Synthetic drug
S*:	Made by total synthesis, the pharmacophore is from a natural product
S/NM:	Natural product mimic synthetic drug
S*/NM:	Synthetic drugs based on natural product skeleton mimic natural pathway
V:	Vaccine

Figure 12 : Classification of 1562 types and number of drugs registered worldwide during period from 1981 to 2014, including also vaccines (V) and biological macromolecule (B) (Newman D. J., 2016)

2.2. The origin of cancer drugs

As of 2016, there were 246 anticancer agents being provided in the market worldwide in which up to about 80% of them are drugs originated or related to natural products and only 19% being from total synthesis (Figure 13).

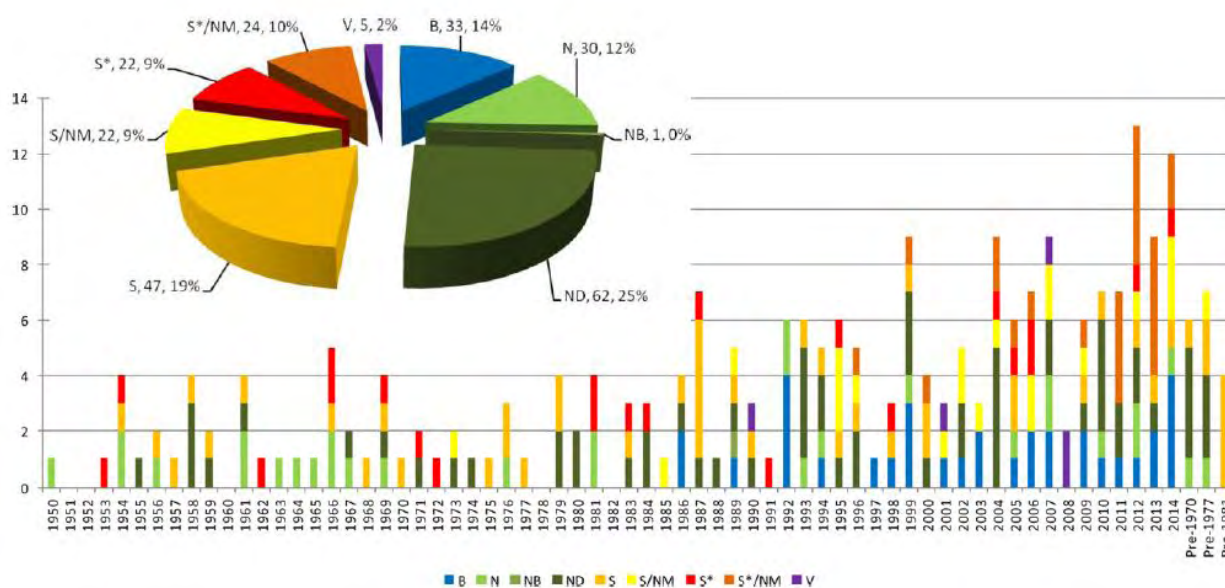


Figure 13 : Classification and number of anticancer agents certified worldwide annually from 1940s until 2014 (Newman D. J., 2016)

From the antitumor area perspective, there was a significant aspect in the recent six years with a constant increase of approved drugs ranged from 6 to over 10 per year from 2009 to 2014 (except for 2013). This number promises more anticancer drugs with stronger effects as well as a broader treatment ability will be developed in the near future.

2.3. Overview of anticancer agents worldwide

2.3.1. Anticancer agents from plant

Some of these agents are shown in Figure 14.

One of the very well-known anticancer agents is the alkaloids isolated from Madagascar periwinkle, the *Catharanthus roseus* G. Don. species including Vinblastine and Vincristine (Guéritte F., Fahy J., 2005), especially, these molecules can also be isolated from the symbiotic fungi on this plant (Yang X., 2004).

Some other compounds with similar structure like Vinorelbin and Vindesin were semi-synthesised. These molecules act through a mechanism that inhibits tubulin polymerization.

Most of the time, other anticancer drugs are used in combination with these molecules in treating different types of cancer such as leukemia, testicular cancer, breast cancer, lung cancer or Kaposi's sarcoma (Cragg G. M., 2009).

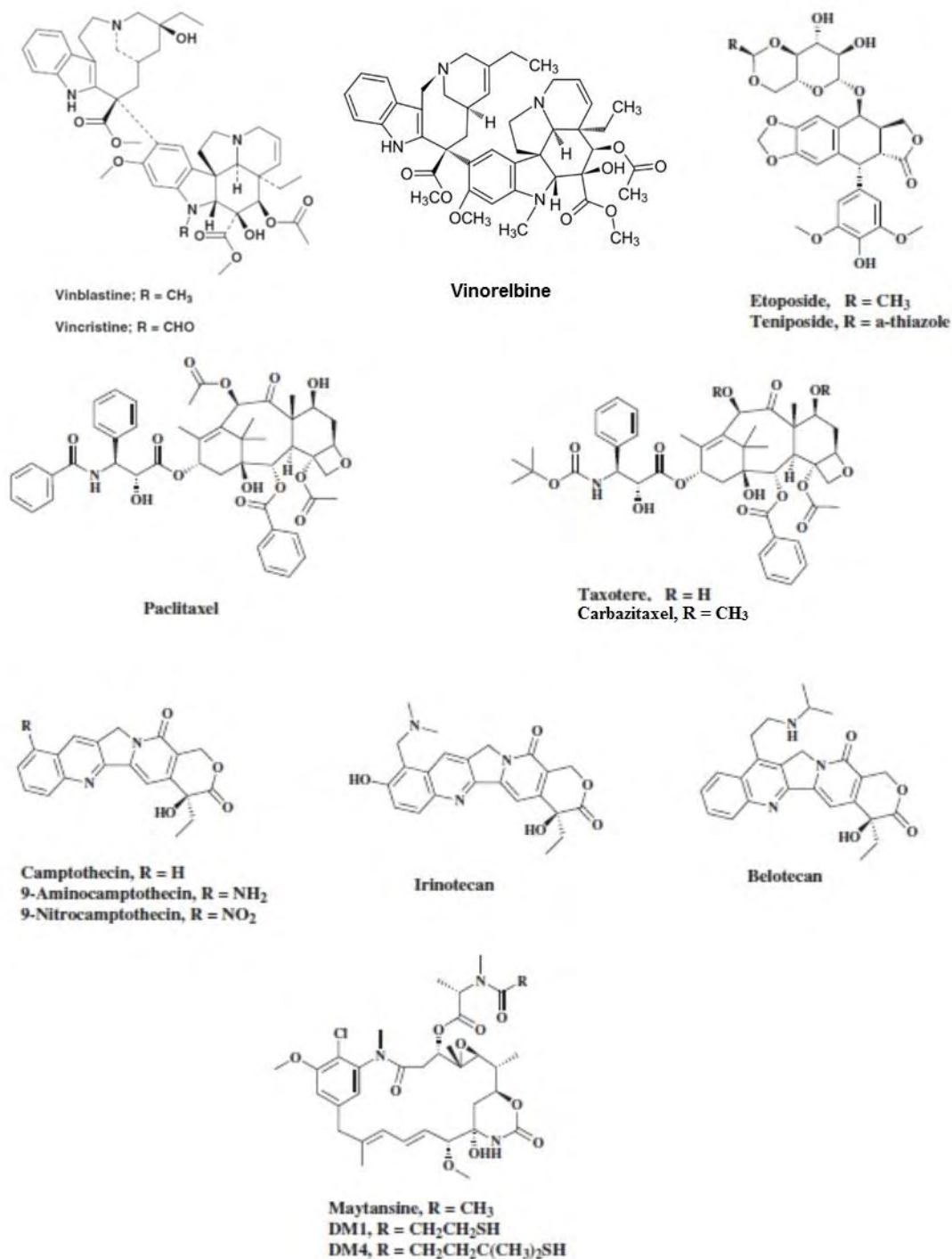


Figure 14 : Examples of plant-derived anticancer agents (Newman D.J., 2016)

Etoposide, etopophos and teniposide are semi-synthetic derivatives of the natural product epipodophyllotoxin (Lee K. H., 2005), an isomer of podophyllotoxin which was isolated as an active antitumor agent from the root of various species of genus *Podophyllum*. Recently, an endophytic fungus isolated from *P.peltatum* has been found to be a source to produce Podophyllotoxin (Eyberger A. L., 2006). Etoposide and Teniposide are effective agents in clinical treatment of lymphomas, bronchial and testicular cancers. The mechanisms of these molecules based on inhibiting topoisomerase II, an enzyme involved in the replication pathway of Deoxyribonucleic acid - DNA during cell cycle progression (Cragg G. M., 2009).

Taxanes and camptothecins are chemotherapeutic agents derived from natural products and have very strong cytotoxic effects. Paclitaxel (Taxol®) was mainly isolated from the bark of the Pacific yew, *Taxus brevifolia* Nutt. (Kingston D. G. I., 2005). This molecule, along with some other key precursors (the baccatins), also exists in the leaves of many different species such as European's and some Vietnam's yews. A significant amount of baccatins were then semi-synthesized to paclitaxel and other bioactive analogues, for example docetaxel (Taxotere®). Notably, Taxol® has also been isolated from many endophytic fungi (Strobel G., 2004), however, these fungi have not been developed to be a sustainable source (Cragg G. M., 2009). Paclitaxel is utilized in the treatment of breast, ovarian, non-small cell lung cancer and Kaposi's sarcoma; meanwhile, docetaxel is mainly used in the treatment of breast and non-small cell lung cancer.

Topotecan (Hycamptin®) and Irinotecan (Camptosar®; CPT-11) are semi-synthetic derivatives from camptothecin which was isolated from Chinese ornamental tree *Camptotheca acuminata* Decne. (Rahier N. J., 2005). Camptothecin also reported to be produced by an endophytic fungus of the family Phycomycetes (Amna T., 2006), this species is isolated from the bark of *Nothapodytes foetida* (Puri S. C., 2005). Although camptothecin has been excluded during clinical stage since 1970s by the United States National Cancer Institute (NCI) due to its toxicity with bladder, the studies on its derivatives have been conducted widely. Two derivatives Topotecan and Irinotecan have stronger effects and exhibit low toxicity. Topotecan is used in the treatment of ovarian and small cell lung cancers, Irinotecan in the treatment of colorectal cancer. The mechanism for these agents of this class is to inhibit topoisomerase I, a key enzyme related to the replication of DNA during cell cycle progression (Cragg G. M., 2009).

Other plant-derived agents are homoharringtonine, flavopiridol and combretastatins. Homoharringtonine was isolated from Chinese plant *Cephalotaxus harringtonia* (Itokawa H., 2005). A racemic mixture of harringtonine and homoharringtonine was tested successfully in treating acute and chronic myelogenous leukemia. Flavopiridol is a total synthesis compound, however, its flavonoid basic structure is based on rohitukine, a flavonoid which has anti-inflammatory and immunomodulatory bioactivities, isolated from species *Dysoxylum binectariferum* Hook. f. (Meliaceae). Flavopiridol has been going through clinical trials for treating different types of cancer such as acute leukemia (Byrd J. C., 2005). The combretastatins (for example: combretastatin A₄) were isolated from the South Africa bush *Combretum caffrum* during 1970s in the collaboration program between NCI with National Botanical Research Institute of South Africa (NBRI) (Pinney K. G., 2005). Combretastatin A₄, a water soluble derivative, could be a promising agent against thyroid cancer.

2.3.2. Anticancer agents from marine organisms

Some of these agents are shown in Figure 15 next page.

Ocean habitat promises to be an abundant source for anticancer agents. Recently, many compounds with significant anti-tumor activity have been detected from ocean such as Ecteinascidin 743 (Et743) and Aplidine which are very well-known anticancer agents and being provided widely in American and European market.

Although isolated at very low quantity from the ascidian *Ecteinascidia turbinata* (Henriquez R., 2005), the yield of ET743 is enough for pre-clinical and clinical research then can be harvested through large-scale aquaculture the species *E. turbinata*, but later, supplies were obtained by a 21-step semi-synthetic conversion from cyanosafracin B, a metabolic compound isolated from the marine microbe *Pseudomonas fluorescens*. Ecteinascidin 743 has been approved in Europe for sarcoma treatment since the end of 2007, and has also been under clinical trial in treating ovarian, soft tissue sarcoma, breast, endometrial, prostate, non-small cell lung and pediatric cancer (Cragg G. M., 2009). Ecteinascidin 743 is the first compound of a novel class of DNA-binding agents which has transcription-targeted mechanism (Henriquez R., 2005).

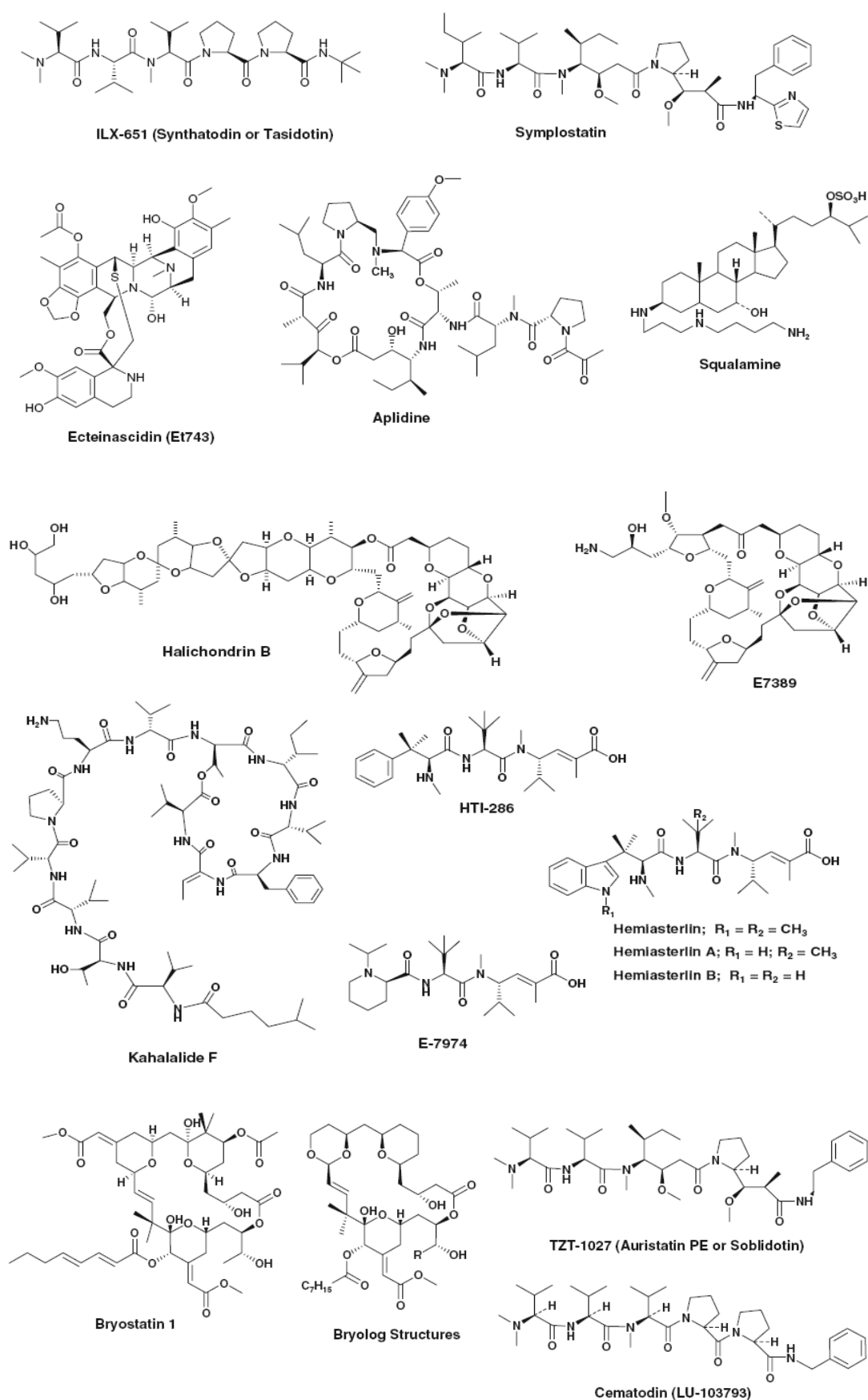


Figure 15 : Some marine organisms-derived anticancer agents (Cragg G. M., 2009)

Aplidine (dehydrodidemnin B) is isolated from the Mediterranean tunicate *Aplidium albicans* and has been going through clinical trial phase II in some cancer treatment such as melanoma, pancreatic, small cell, non-small cell lung, prostate cancer and acute lymphoblastic leukemia (Henriquez R., 2005). The mechanism of this molecule remains unclear until now. Halichondrin B is isolated from several sponges, for example: *Halichodria okadai* from Japan (Yu M. J., 2005). The yield is enough for pre-clinical and clinical trials and it is obtained through the isolation progress from species *Lissodendoryx* sp., which is aquacultured at large-scale in some deep water regions of New Zealand. The successful strategy to synthesize Halichondrin B and norhalichondrin B was utilized to synthesize other analogs with simpler structure which are more stable. Among those, E7389 was selected for drug development and has been in pre-clinical trial phase III against refractory breast cancer (Yu M. J., 2005).

Bryostatins are a series of macrocyclic lactones originally isolated from the bryozoan *Bugula neritina* (Hale K. J., 2002; Newman D. J., 2005). Due to some side effects when used as single agent, bryostatins are expected to get better when used in combination with some other anticancer agents such as alkaloids isolated from periwinkle. Many extensive studies have been conducted to synthesize simpler analogs with better bioactivity. These efforts have resulted in the synthesis of bryologs, this series have even better bioactivities than bryostatin 1 in *in vitro* tests (Wender P. A., 1998, 1999, 2002, 2003).

While clinical trials of dolastatin 10 had been cancelled, several derivatives of dolastatin were progressed through clinical development (Flahive E., 2005). The synthetic compound TZT-1027 (auristatin PE or soblidotin) has been going through clinical phase II (Shimoyama T., 2006). The analog cematodin (LU-103793) was in clinical trial phase II against malignant melanoma, metastatic breast cancer and non-small cell lung cancer and still show no side effect (Flahive E., 2005). Clinical studies phase II against breast and non-small cell lung cancer are also conducted with ILX651 (synthadotin and tasidotin), a third derivative generation of dolastatin. Interestingly, dolastatins are proved to be origin from micro-organisms with the proof is the isolation of symplostatin 1 from the marine cyanobacterium *Symploca hynoides* (Flahive E., 2005) following by the reports of peptides with similar structure of dolastatins from the ubiquitous cyanophyte *Lyngbya majuscula* (Flahive E., 2005).

2.3.3. Anticancer agents from microbial sources

Antitumor antibiotics include members of anthracyclin and bleomycin which are very important anticancer agents. Some of these agents are shown in Figure 16.

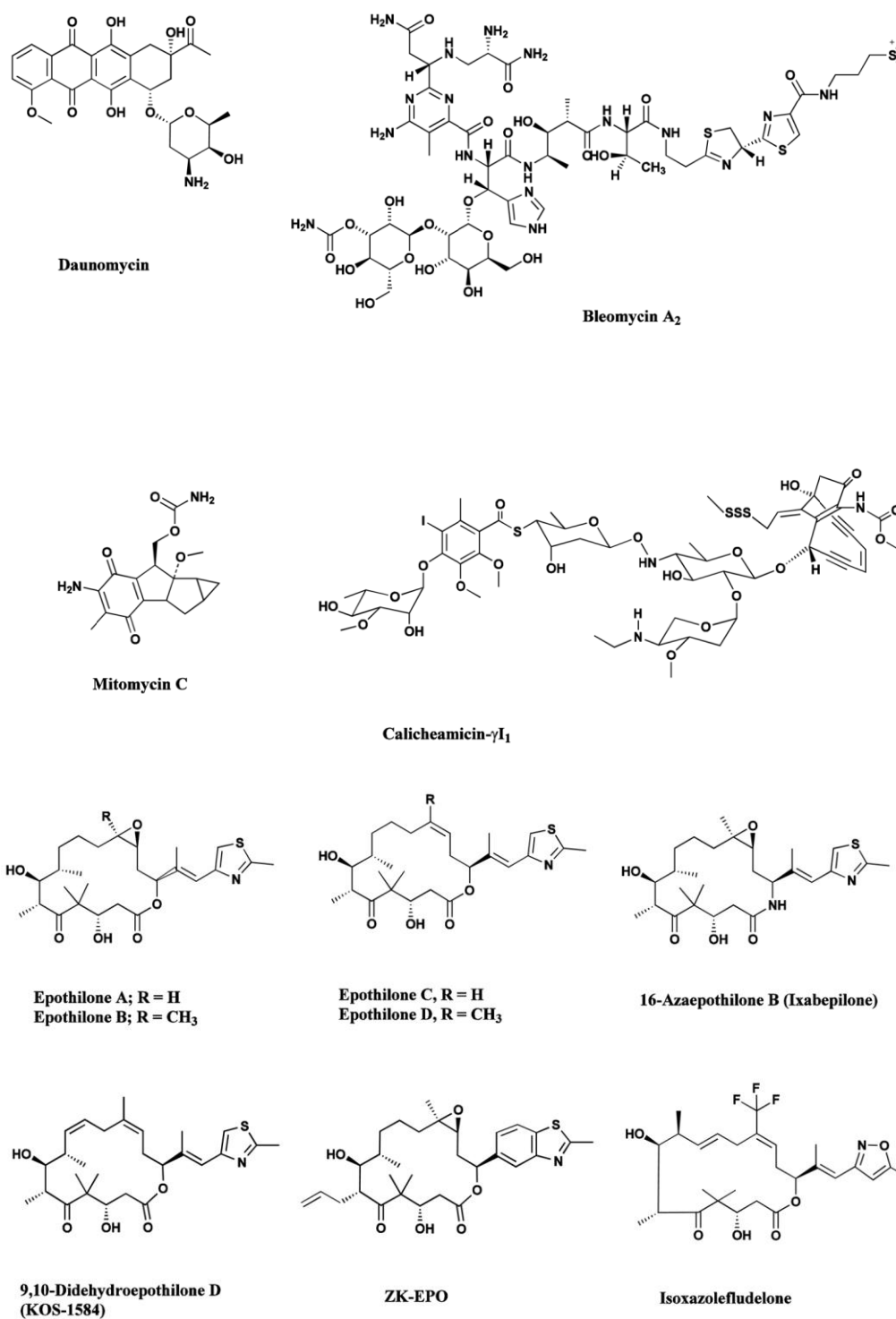


Figure 16 : Some microbial-derived anticancer agents (Cragg G.M.; Newman D.J., 2013)

Anthracyclin is an important class of microbial-derived agents, therein daunorubicin and its analogs doxorubicin (adriamycin) are effective anticancer agents used, among others, in breast cancer treatment (Arcamone F., 2005). The analogs of doxorubicin such as epirubicin, idarubicin, pirarubicin and valrubicin are certified for clinical use and the extent effect of doxorubicin is being studied through targeted delivery techniques (Arcamone F., 2005). Another important class is the glycopeptolide antibiotics known as bleomycin (for example bleomycin A₂, Blenoxane[®]) (Hecht S. M., 2005). Bleomycin is believed to impact on DNA cleavage but recently researches show that the major mechanism of action could be the inhibition of t-RNA (Transfer-Ribonucleic acid). The major reason for epothilone researches is that these epothilones were active against paclitaxel-resistant cell lines. Ixabepilone is synthesized by Bristol-Myers and approved in 2007. Epothione B (patupilone), epothilone D (KOS-862) and ZK-EPO have been under clinical trials (Cragg G. M., 2009).

Until now, the difficulty of culturing naturally occurring micro-organisms is the major obstacle in studying biology and chemistry of micro-organisms. It has been estimated that less than 1% of micro-organisms seen microscopically have been cultivated. However, the number of highly effective agents isolated from them is very impressive. It confirms the potential of an enormous untapped resource for drug discovery (Cragg G. M., 2009).

2.4. Vision of the development of natural-derived anticancer agents

Naturally-derived compounds may have selective bioactivities and may act on specific targets. Among the different mechanisms of naturally-derived anticancer agents, interaction with tubulin has been one of the most common, more than 25% of anticancer agents being considered to be related with it. Examples of different targets of several anticancer drugs in the cell cycle are shown in Figure 17.

In previous natural products researches, new compounds were mainly isolated randomly or through biological activity screening such as antibiotic or cytotoxicity. Nowadays, developed countries have created new drug generations that are discovered and isolated through powerful genetic screening and biochemistry methods which allow scientists to identify more accurately compounds containing the desired bioactivities in different extracts. Further, these tests can provide precious preliminary data on the mechanism of action of these molecules need to validate the early steps of the drug discovery process.

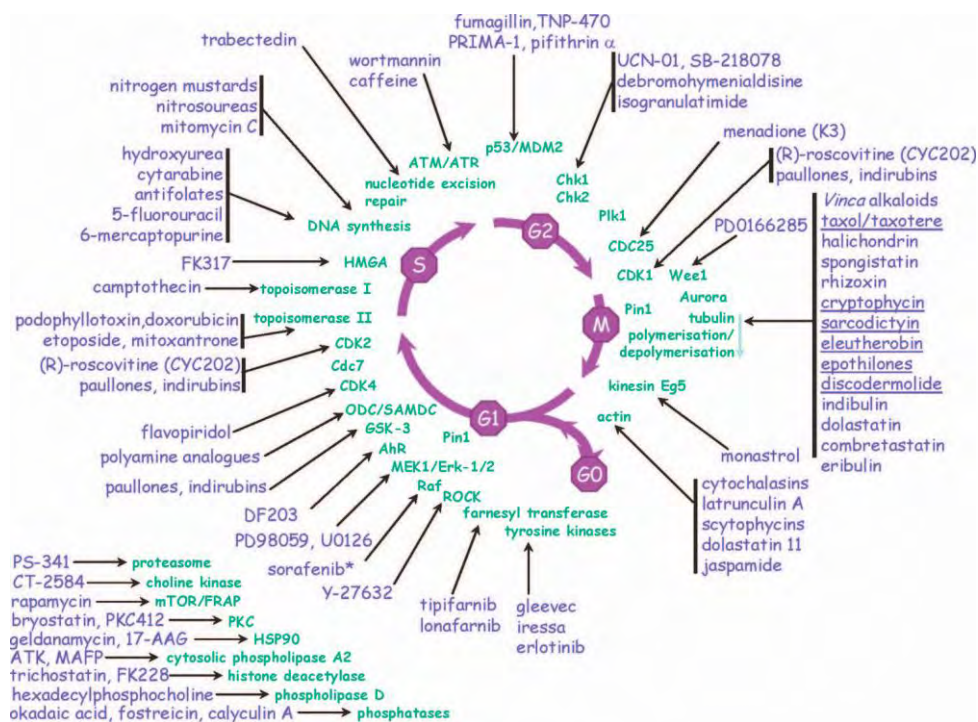


Figure 17 : Cell cycle targets of some natural-derived anticancer agents (Meijer L., 2003)

In addition to the “real” biological screening, a new method called “virtual” *in silico* screening has recently occurred. This method utilizes the advance in informatic technology to screen virtually new compounds expected to have high bioactivities from a huge database.

The advantage of this approach is to minimize the cost and time in drug discovery and development process. Normally, it consumes 800 million euros and 10 to 15 years to put a new drug into circulation with conventional methods, meanwhile, with the modern computer systems (for example: computer network – grid), millions of structures could be virtually screened just within a few weeks. WISDOM (World-wide In Silico Docking on Malaria) could be named as a representative example for this method, the project uses grid for virtual screening and anti-malarial drug development on the computer network worldwide. In the 3 years of the project from 2007 to 2010, hundreds of millions of compounds had been screened and dozens of them were processed to test *in vitro* and then *in vivo*.

3. Vietnamese natural products as sources of anticancer agents

3.1. Potential of Vietnamese plant sources

Vietnam territory is located in the tropical monsoon climate, up to three fourths of its area are forests and mountains and terrain dissected, therefore the climate condition is varied with many characteristic sub-climates. These factors have made the flora of Vietnam become very diverse, rich and colorful. According to Professor La Dinh Moi, the number of vascular plants in Vietnam is ranging from 12,000 to 13,000 species. In 1998, Phan Ke Loc had estimated total 10,386 species (including 733 species only found in cultivation) belonged to 2257 genera and 305 families. By 2002, there were additional 13 genera, 222 species and 30 taxons discovered and described.

Statistics showed that, within 50 years the number of plants used for medicinal purpose has increased rapidly (Moi L. D., 2005), for example:

- In 1952: only 1,350 medicinal plants were identified in all three Indochina countries.
- In 1986: there were 1,836 medicinal plants were counted in Vietnam.
- In 1996: the number raised up to 3,200 species.
- By 2005: it is estimated about 4,000 species were discovered.

For a long time ago, ethnic communities of Vietnam, especially the residents living on the high mountain – remote areas, have known to use herbs as medicines and health care for themselves. Many remedies are being circulated among the mountainous ethnic communities and each ethnic has its own special prescriptions and herbs. Until now, there are still many medicinal plants that modern science does not yet known. According to the preliminary statistics of Vietnam Ministry of Health, there were about 39,813 prescriptions and traditional remedies identified in Vietnam until 2005 (<http://moh.gov.vn/pages/index.aspx>; Moi L. D., 2005).

The advantage of traditional medicine (oriental medicine) is to strengthen immunity, harmonize yin and yang, viscera. However, its ability to inhibit tumor is still limited, in comparison with modern medical approaches. The modern medicine with surgery, radiotherapy, chemotherapy can have rapid and significant effects on tumors, however, it contains many side effects. Thus, the combination of Eastern and Western medicine will

promote strengths to overcome weakness in cancer treatments better than themselves alone. The interest of combining traditional medicines with modern medicine in cancer treatment has been highlighted through the following points:

- The use of traditional medicines before and after tumor removal surgery increase health recovery process.
- Traditional medicines can increase the susceptibility of the tumors to chemotherapy and radiotherapy and also reduce side effects of these methods.
- Oriental medicines can strengthen the immunity, reduce complications, the risk of metastasis and improve the life quality of cancer patients.
- Some prescriptions can directly inhibit the growth of cancerous cells.

From the factors above, it can be concluded that the treasure of medicinal plants in Vietnam is very abundant and diverse. It is worthwhile to carry out serious researches to find out bioactive compounds from remedies and their mechanisms. For example, according to a collaboration between scientists in Vietnam Academy of Science and Technology (VAST) and The Ohio State University during period 2008 to 2011, there were 824 plants corresponding to 398 species studied, from which 17 new and 42 known compounds were found to have *in vitro* activity in one or more bioassays, exhibiting promising IC₅₀ values (Perez L. B., 2014).

However, these types of study are still limited and there are many opportunities for scientists to conduct more researches in this field. The obtained results will provide a scientific insight and clarity about oriental medicines, contribute to the development of drugs in serving for cancer treatment.

3.2. Potential of Vietnamese marine sources

Vietnam's sea belongs to the Pacific Ocean, where the biodiversity resources are abundant. With about 3,260 km of coastline and more than one million km² area of the sea, Vietnam owns one of the largest natural asset in the world. There are approximately 11,000 species that have been recorded so far in Vietnam's marine and coastal including 2,458 species of fish, 653 species of sponges, 650 seaweeds and so on. Besides providing a huge

source of organisms, Vietnam's sea offers also endless natural compounds for research and development of new drugs.

In recent decades, there have been several intensive researches on marine natural compounds carried out by Vietnamese scientists including national projects and especially some international collaboration programs with Russia, South Korea, Italia. As a result, there were a lot of marine natural compounds isolated and structure determined, some of them were recorded as new compounds. Based on the biological activity oriental method, samples which were likely to contain bioactive substances, have been selected for further studies and many of them yielded positive results, such as:

- From KC09.15 National research project: 405 samples belonging to 134 marine species along the Vietnam's coastal were collected and conducted to chemical and biological researches. The results obtained showed that 4 new compounds were discovered and 105 samples exhibited cytotoxicity in which 50 of them were cytotoxic to multi-cancer cell lines. One functional food named SALAMIN was developed in supporting for cancer treatment (Long P. Q., 2009).
- From KC09.09/06-10 National research project: 101 marine samples were collected focus on coral, sponge and echinoderm species: 43 samples showed cytotoxicity on cancerous cells, and 59 compounds isolated in which 12 were new substances (Long P. Q., 2009).

However, due to difficulties in collecting samples, preservation and the very small content of bioactive compounds obtained, the study of screening and isolating bioactive substances from marine organisms is still very limited and not worth the resources potential.

3.3. Potential of Vietnamese microbial sources

Study on microbiology in Vietnam have been started from 1960 at some leading institutions and universities including National Institute of Science and Technology, Hanoi University, Food Industry Research Institute, University of Agriculture and Hanoi University of Technology. The yield of these studies is very diverse and covers several main topics such as production of biomass and microbial spores, enzymes (native and recombinant), bioactive

compounds and microbial biodiversity for various applications in agriculture, industry, healthcare and environment.

As an agriculture country, since 1993, Vietnam government has implemented priority policy for science and technology development in which biotechnology is one of the key fields. In this case, the microbial gene management policy was requested. In 1997, Vietnam Ministry of Science and Technology (MOST) issued Art 2117 for bio-resource management (including plants, animals and microbes). Up to date, there are 8 culture collections of microbes maintaining 18,000 microbes mainly isolated from Vietnam (fungi, yeasts, bacteria, actinomycetes, micro algae and virus). According to statistics from several international collaboration and national projects conducted by Vietnam Academy of Science and Technology with Germany, Russia ..., there were some bioactive compounds which exhibit cytotoxicity to cancerous cells discovered during the researches, for example:

- In the framework of the international collaboration project between Vietnam and Russia (2006 - 2008): 5 bioactive compounds were isolated from fungi *Basidiomycetes*, located in the mountainous areas, these substances exhibited both anti-oxidant, antibiotic and cytotoxic activities (Long P. Q., 2009).
- From the national project on fundamental research carried out by Institute of Natural Products Chemistry (INPC) during the period 2007 – 2008: 104 parasitic strains were isolated from six medicinal plants, 5 compounds had their structure determined and showed positive results on cytotoxic test on several cancer cells (Long P. Q., 2009).

In conclusion, the results obtained above are still very limited and represent a first step for the research on compounds of microbial origine in Vietnam. Therefore, this field remains full of potential for scientists to conducted more researches in searching for anti-cancer compounds in the future.

4. Targeting mortalin – p53 interactions as an anti-cancer strategy

Among the innovative approaches developped in the past decade in drug discovery, targeting protein-protein interactions has emerged as a potent strategy in oncology (Ivanov A.

A., 2013). In this field, efforts have been made to search for small molecules which inhibit the interaction between p53 and proteins which negatively the tumor suppression functions of p53. Promising results have been obtained with inhibitors of the interaction between p53 and the ubiquitin ligase MDM2 which are currently tested in phase I trials (Andreeff M., 2016)

With a similar aim, finding inhibitors of chaperone proteins of p53 represents a challenging approach. Mortalin is a stress chaperone of Hsp70 family of proteins that performs various functions related to proliferation, mitochondrial biogenesis, chaperoning and stress response (Renu W., 2002; Sunil C. K., 2007). Mortalin binds to p53 tumor suppressor protein and sequesters it in the cytoplasm, resulting in an inhibition of the transcriptional activation and control of centrosome duplication functions of p53, thus causing lifespan extension of normal human cells and increase malignant properties of human cancer cells (Kaul S. C., 2005; Lu W-J., 2011; Vaishnavi K., 2012; Grover A., 2012). It is expected that the abrogation of mortalin-p53 interaction will reactivate p53 function. This could represent an original anticancer therapeutic approach.

In recent studies, small molecules, some of natural origin, have been identified as inhibitors of p53-mortalin interactions (Utomo D. H., 2012; Grover A., 2012). Further, it has been demonstrated that mortalin is associated with liver cancer for which it could be used as a marker to predict its early tumor recurrence (Xin Y., 2008).

We thus wished to answer to the questions : 1) could Vietnamese natural products be identified as inhibitors of mortalin – p53 interaction ?, and – 2) if yes, could these molecules display pharmacological activity against hepatocellular carcinoma.

5. Overview of virtual screening in drug discovery

It is a fact that the traditional drug discovery is a time-consuming and very expensive process. It has been estimated that for a drug cycle from literature through clinical trials and then to the market will need from 10 – 15 years and around 800 million US dollars (Song C.M., 2009). In the early 1990s, there were some efforts to find new technologies in the field of combinatorial chemistry and high-throughput screening (HTS) in order to shorten the drug discovery process by enabling huge libraries of compounds to be synthesized and screened in short periods of time. However, these inventions did not give as many positive results as

expected. Many of identified potential compounds (hits) were reported to fail in the optimization process due to absorption, distribution, metabolism, excretion and toxicity (ADME/Tox) deficiencies (Kennedy T., 1997; Venkatesh S., 2000; Hou T., 2004). Therefore, searching for new strategies that could help to choose appropriate compounds and limit the use of a significant amount of resources is an important objective for the scientists.

The first time the term “Virtual Screening” (VS) appeared in a peer-reviewed publication was in 1997 and since then it has developed rapidly in pharmaceutical research (Lavecchia, 2013). Virtual screening is a powerful technique for identifying hit molecules as starting points for medicinal chemistry. It is usually described as a step by step method with a cascade of sequential filters able to narrow down and choose a set of lead-like hits with potential biological activity against intended drug targets. The compounds studied do not necessarily exist, and their "testing" does not consume valuable substance material. Taken to its extreme, any molecule can, in theory, be evaluated by VS. Depending on the intended follow-up, databases for VS contain up to 10 million available compounds and this number can be handled in one VS experiment.

Starting from the first algorithm program namely UCSF Dock using computational method (Irwin D. K., 1982), many other systems have been introduced, including: Gold (Gareth J., 1997), Dock (Ewing T. J., 2001), Glide (Thomas A. H., 2004; Richard A. F., 2004), FlexX (Bernd K., 1999), AutoDock (Oleksandr V. B., 2002).

Software	Free for Academia	Website
AutoDock	Yes	http://autodock.scripps.edu/
Dock	Yes	http://dock.compbio.ucsf.edu/
FlexX	No	http://www.biosolveit.de/flexx/
Glide	No	http://www.schrodinger.com/
Gold	No	http://www.ccdc.cam.ac.uk/products/life_sciences/gold/
EADock		http://lausanne.isb-sib.ch/agrosdid/projects/eadock/eadock_dss.php

Surflex	No	http://www.tripos.com/index.php
ICM	No	http://www.molsoft.com/docking.html
LigandFit	No	http://accelrys.com/products/discovery-studio
eHiTS	No	http://www.simbiosys.ca/ehits/index.html
SLIDE	Yes on demand	http://www.bch.msu.edu/~kuhn/software/slide/index.html
ROSETTA_DOCK	Yes on demand	http://rosettadock.graylab.jhu.edu/
Virtual Docker	No	http://www.molegro.com/mvd-product.php
Ligand_Receptor Docking	No	http://www.chemcomp.com/software-sbd.htm
FRED	Yes on demand	http://www.eyesopen.com/oedocking
ZDOCK	Yes	http://zlab.umassmed.edu/zdock/

Table 01 : Example of some commonly used Docking Software (Lavecchia A., 2013)

In the field of molecular modelling, docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex (Figure 18). AutoDock is a script driven flexible automated and random search docking technique operated by altering the ligand or a subset of ligand with several rotatable bonds to predict the binding interaction between small molecules to the known receptor 3D structure.

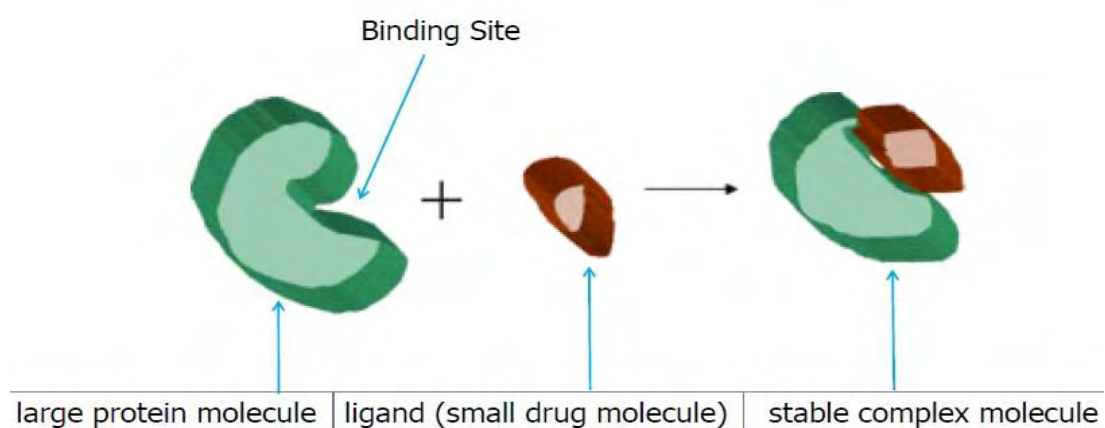


Figure 18 : Simulation of a docking between ligand and protein (Khamis M. A., 2015)

AutoDock uses the Lamarckian Genetic Algorithm and empirical free energy scoring function (Morris G. M., 1999) to provide reproducible results for ligands with approximately 10 flexible bonds.

C - Thesis Objectives

The objective of this thesis was defined to identify Vietnamese natural compounds endowed with antiproliferative properties against HCC. As described above, Vietnam possesses an enormous potential of sources of natural products together with a long history of traditional medicine based on plants. In Vietnam, research on natural products in plant has been far more developed than in the marine and microorganism sectors which have attracted the attention of scientists only recently. To date, collection and availability of molecules from Vietnamese plant remain much higher and richer than those from marine or microorganisms. In this context, we therefore found coherent to focus this thesis on the study of natural products originated from plants.

For our purpose, we used the well characterized human cell lines HepG2 and Hep3b represent cellular reference models for *in vitro* pharmacological studies, proven to be well adapted for the study of new molecules active on HCC (Qiu G. H., 2015).

In a first approach of pharmacological screening, we first aim to collect a group of Vietnamese natural compounds which are isolated from plants commonly used in traditional remedies known for their liver protective properties. The next stage consisted in biochemical studies (such as cytotoxicity assays, subG1 and annexin V tests) conducted on the natural compounds selected from the primary screening. In addition, because it has been shown that natural products can enhance the sensitivity of human HCC cells to the cytotoxic effect of doxorubicin, an anthracycline used in HCC treatment (Weiss R. B., 1992; Tam K., 2013), we further explored the ability of the potential molecules to enhance doxorubicin cytotoxicity in the HepG2 and Hep3b model cell lines.

In the second approach, we developed a virtual screening strategy to identify potential compounds that could abrogate the Mortalin – p53 interaction. The initial step was to create a database of all natural compounds isolated/originated from Vietnamese plants and then to carry out *in silico* analysis using computational tools. In the second step, more detailed pharmacological studies were conducted on the natural compound identified in order to validate it as a potent inhibitor of p53 – mortalin interaction active on HCC.

D - Materials and Methods

1. Plant Material

All the Vietnamese natural compounds used in this thesis were isolated and their structure determined in Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology in Hanoi, with a purity of over 97% as measured using HPLC (High-performance liquid chromatography).

All purified compounds were initially dissolved in dimethyl sulfoxide (DMSO) to adjust their concentration for use in pharmacological experiments and stored at -20°C.

1.1. Natural compounds from medicinal plants of Vietnam

The selection and collection of natural compounds to be screened is based on their origine from Vietnamese medicinal plants, in particular, from those which are used popularly as liver protective or curing hepatocellular carcinoma diseases. According to this criteria, we have collected 26 compounds which were divided into 7 separate groups.

1.2. Natural compounds from virtual screening

Another approach to select and collect samples is based on the creation and availability of a Vietnamese plant compounds database which can be screened in a virtual screening for a given biological activity (inhibition of mortalin – p53 interaction in the frame of this study). Solasonine which was identified as the hit with the highest score among the 354 screened molecules was successfully isolated and purified in Vietnam.

2. Virtual Screening

2.1. Database of Vietnamese natural compounds

Within the framework of this thesis, we focus on natural compounds which are originated or isolated from Vietnamese plants. This database, therefore, was constituted from two main sources: Vietnamese literatures including information of more than 100 theses in the field of

natural products and the second is Dictionary of natural products edition 2010 using Microsoft Access (Microsoft® Office 2010) (Figure 19).

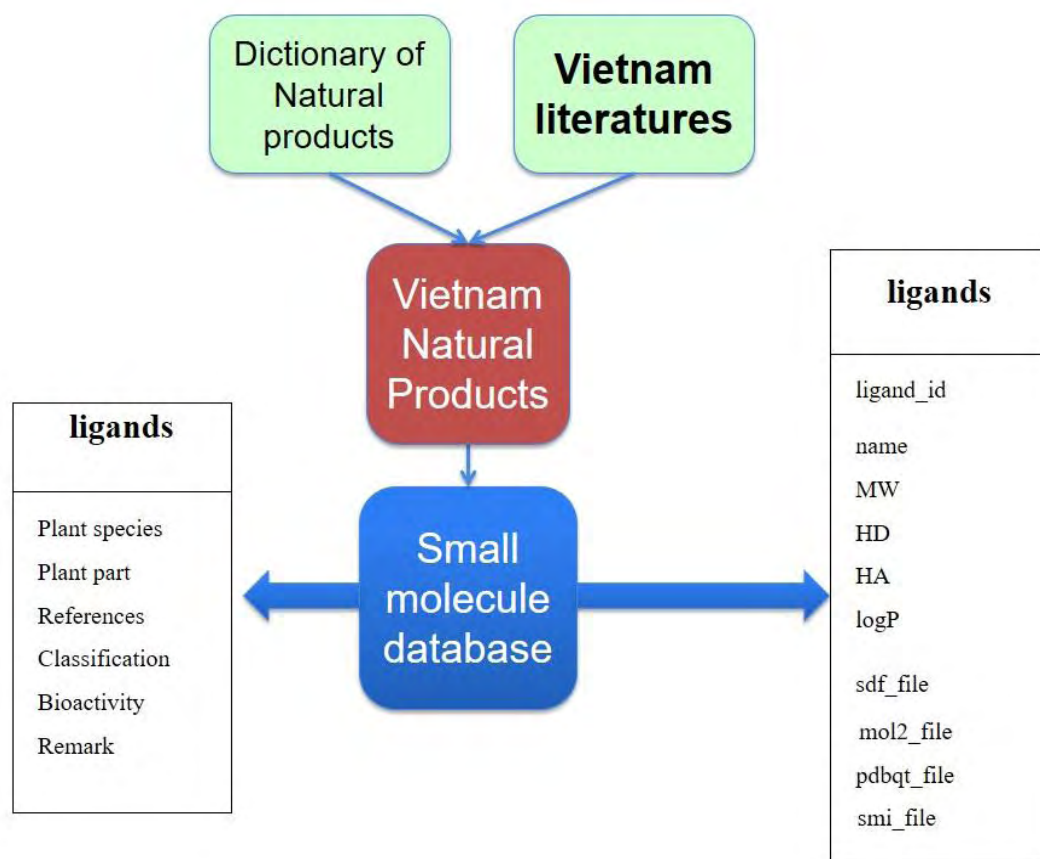


Figure 19 : Organization of Vietnamese database of natural compounds

This process has resulted in a database consist of 354 natural molecules and most of them have been tested and shown to possess at least one biological activity. For a given compound, various informations are available, as exemplified in Figure 20.

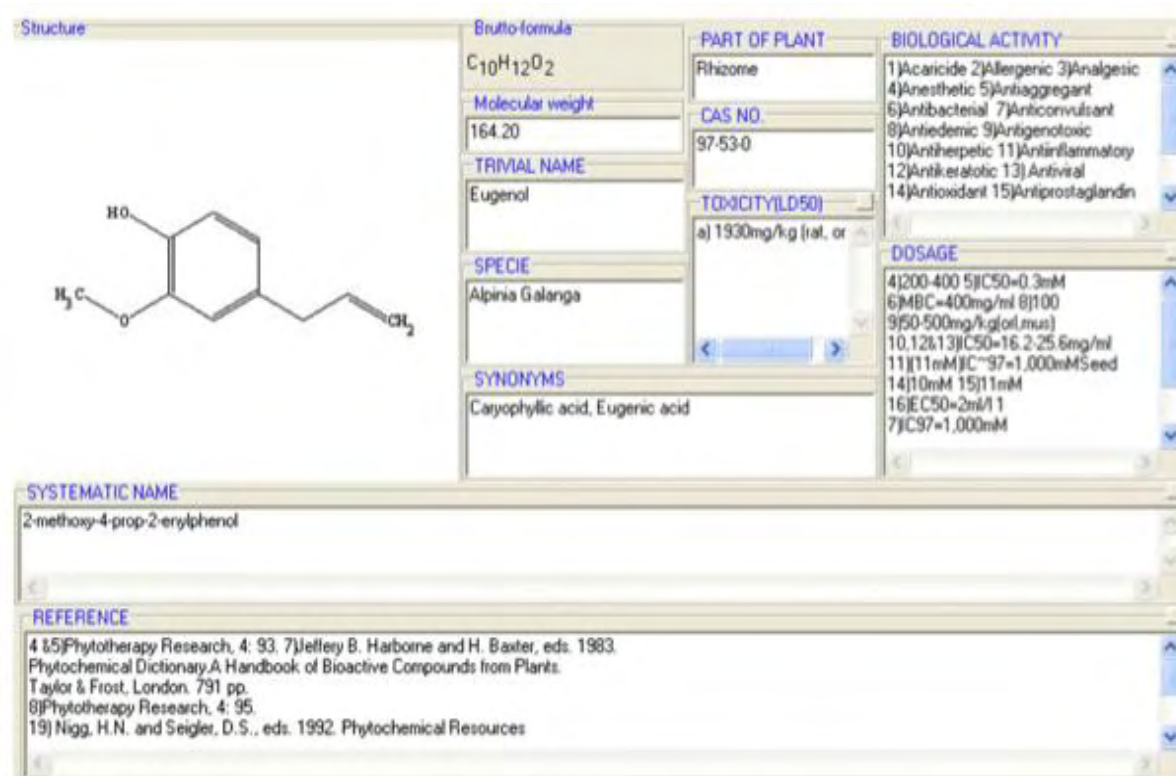


Figure 20 : Example of one compound's information presented in the database

In the next stage, based on the requirement of AutoDock tool, all the molecules underwent a preparation process with multiple steps to get ready for virtual screening. Examples of the procedure are described in figure 21.

Firstly, the molecules were manually draw in the form of two – dimension (cdx format) then transformed into three – dimension structure (mol2 format) using ChemBioDraw and ChemBio3D Ultra version 14.0 (PerkinElmer®). The reason for choosing mol2 as primary file type used for docking is this format has the advantage of storing all the necessary information for atom features, position and connectivity. Besides, it is also a standardized format that many modelling program nowadays can read.

In the following stage, this database in mol2 format was processed through DG-AMMOS program to generate 3D conformations based on distance geometry and optimize their structures. The final output of this procedure is in mol2 file types also and from this point, the molecules are ready for docking. DG-AMMOS is a new tool developed by Maria A. M. et al. in 2009 to produce 3D conformations of small molecules using Distance Geometry and

Automated Molecular Mechanics Optimization for *in silico* screening. The advantage of this program is that the source code is entirely available to users and it can be applied to libraries with a large number of compounds (David L., 2009).

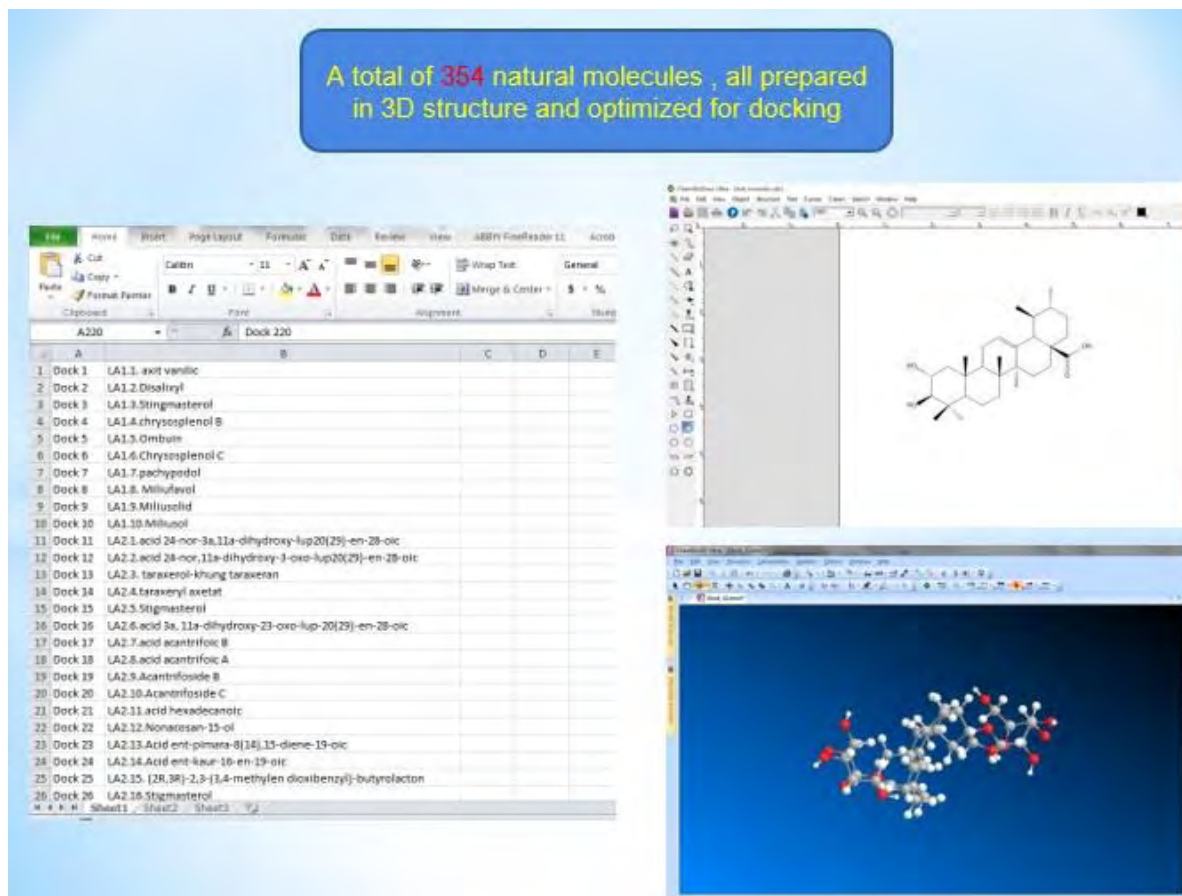


Figure 21 : Example of the preparation of Vietnamese database

2.2. Protein preparation

According to literatures, Mortalin binds to p53 tumor suppressor protein and sequesters it in the cytoplasm. This results in an inhibition of the transcriptional activation and control of centrosome duplication functions of p53, thus causing lifespan extension of normal human cells and increase malignant properties of human cancer cells (Kaul S. C., 2005; Lu W-J., 2011; Vaishnavi K., 2012; Grover A., 2012). It is expected that the abrogation of Mortalin-p53 interaction will reactivate p53 function and could become a proposition for cancer therapy.

2.2.1. Primary Mortalin protein model – PDB ID: 3N8E

Mortalin is a stress chaperone of Hsp70 family of proteins that performs various functions related to proliferation, mitochondrial biogenesis, chaperoning and stress response (Renu W., 2002; Sunil C. K., 2007). In the previous studies, it has been demonstrated that Mortalin associates with liver cancer metastasis and can be used as a marker to predict early tumor recurrence (Xin Y., 2008). Some researchers had identified the p53-binding site of mortalin resides in the peptide binding domain which matched with 3N8E model (residues 439 – 597; downloaded from the Protein Data Bank (PDB) archive). Therefore, we choose this model for docking studies (Iosefson O., 2010; Utomo D. H., 2012).

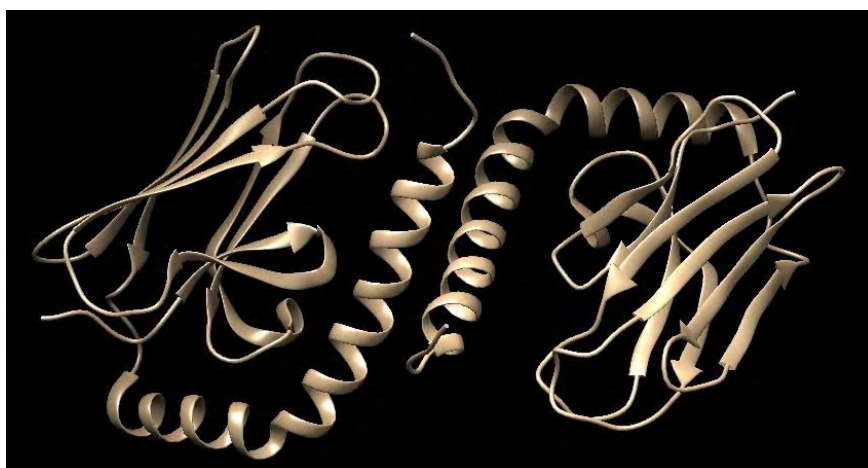


Figure 22 : Visualization of Mortalin protein model 3N8E (residues 439 – 597)

2.2.2. Secondary Mortalin protein model – PDB ID: 4KBO

Besides the p53-binding site of Mortalin as presented above, also we found out that in some other publications, there is another possibility that p53 interacts with Mortalin within the residues 253 – 282 domain (Kaul S. C., 2001; Kaul S. C., 2003; Grover A., 2012; Nigam N., 2015). There is one Mortalin protein model could be the appropriated candidate for our study available on the Protein Data Bank, it has the PDB ID: 4KBO. This model contains amino acids sequence 54 – 429 therefore we believe it is suitable for carrying out docking research with Vietnamese natural compounds database.



Figure 23 : Visualization of Mortalin protein model 4KBO (residues 54 – 429)

2.2.3. Primary p53 protein model – PDB ID: 1AIE

p53 – 53 kDa, the “guardian of the genome”, is a key tumor suppressor protein that regulates cell cycle, prevents cancer by inducing apoptosis, activates DNA repair mechanism and enhances the activity of p21 (Vaishnavi K., 2012). Loss of p53 function is one of the early events in immortalization of human cells (Levine, 1997; Duncan, 2000). It has been proven that the mortalin-binding site of p53 located in the carboxyl terminus 312 – 352 residues, especially, residues 323 – 327 play an important role in the interaction between Mortalin – p53 protein (Kaul S. C., 2005; Iosefson O., 2010). For the reasons above, we decided to use p53 model – 1AIE: residues 326 – 356 (Protein Data Bank) to be the most appropriate target for docking studies.

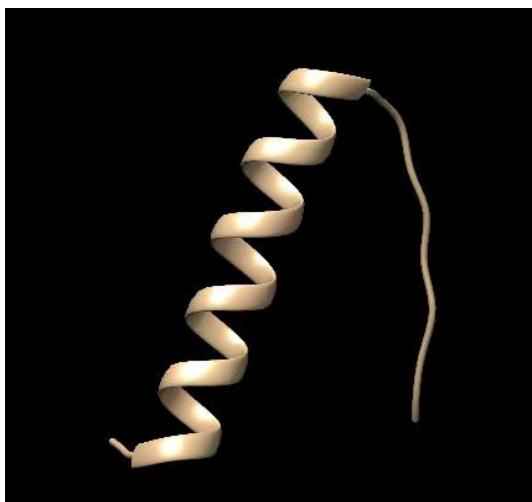


Figure 24 : Visualization of p53 protein model 1AIE (residues 326 – 356)

2.2.4. Secondary p53 protein model – PDB ID: 3D09

According to a research conducted by Vaishnavi et al. in 2012, there is a possibility the interaction between Mortalin – p53 protein could be affected by the binding of compounds with two amino acids (aa) Arg 282 and Leu 111 in p53 protein and therefore, contribute to the reactivation of p53 functions (Vaishnavi K., 2012). For this reason, we took model 3D09 which contains residues 96 – 290 (Protein Data Bank) as the second target for researching p53 model.

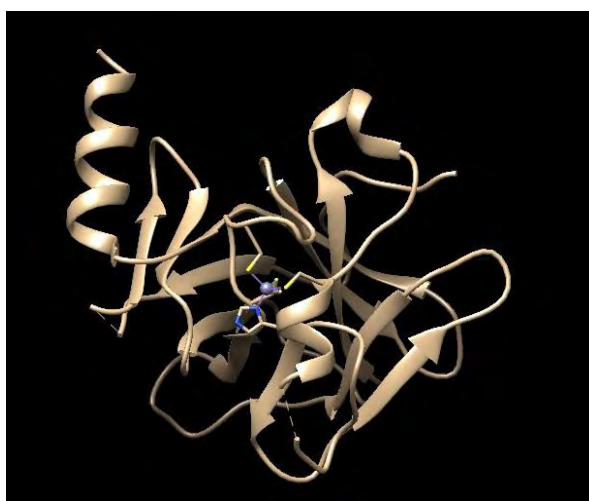


Figure 25 : Visualization of p53 protein model 3D09 (residues 96 – 290)

2.3. Docking using AutoDock

The organization of our *in silico* screening study of 354 natural products from Vietnamese plants database is shown in Figure 26.

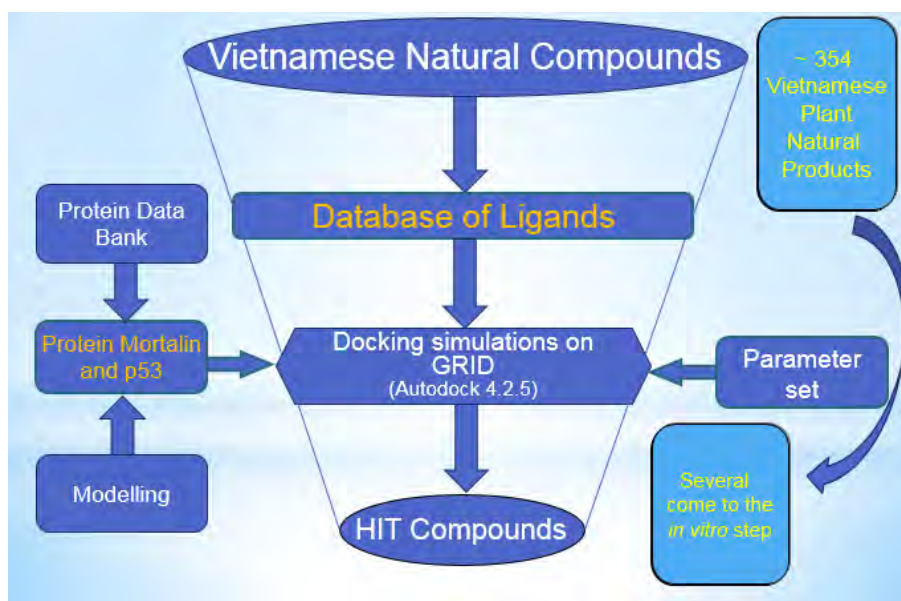


Figure 26 : Scheme of virtual screening of Vietnamese natural compounds database on targeted protein models for Mortalin (3N8E; 4KBO) and p53 (1AIE, 3D09)

The docking results obtained using AutoDock 4.2.5 and the Graphical User Interface program “AutoDock Tools” were used to prepare, run, and analyze the docking simulations. The procedure of an Autodock modelling is described below (see Table 02)

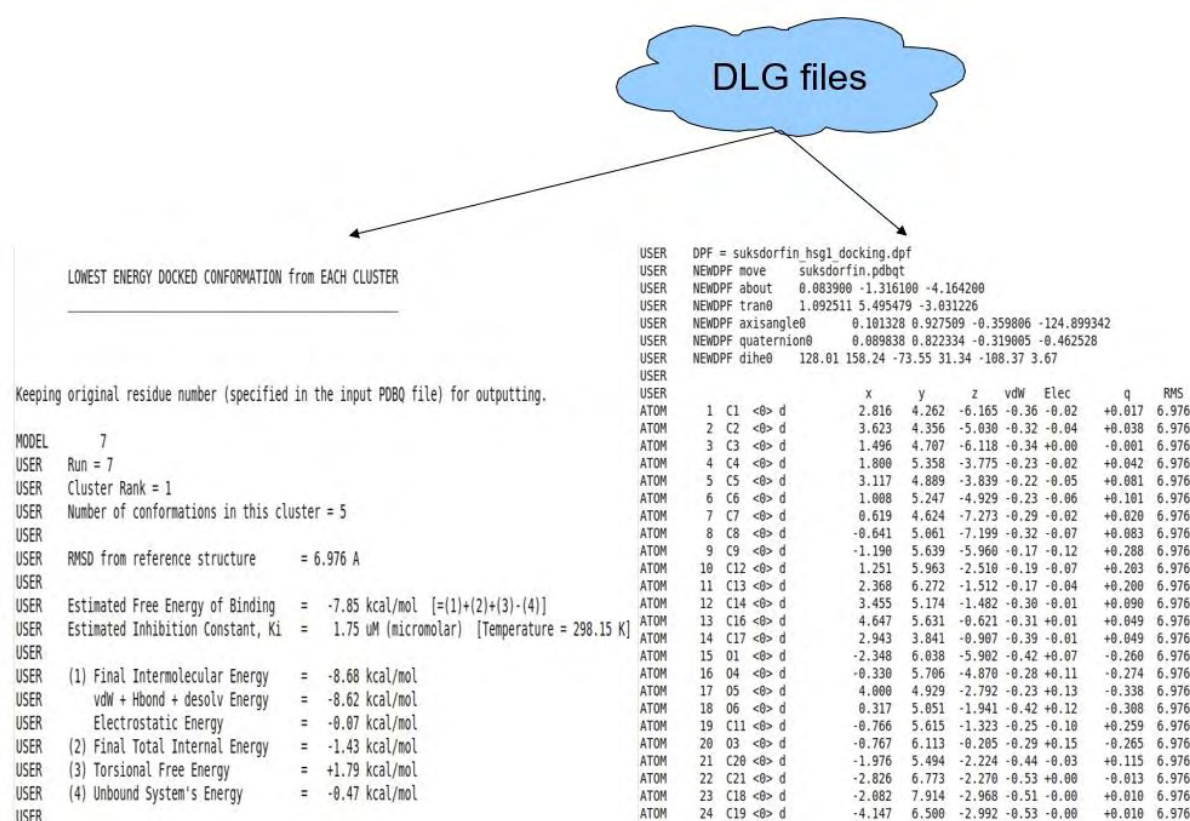
Proteins (3N8E, 4KBO, 1AIE and 3D09) were prepared for docking simulations by assigning of partial charges, solvation parameters and hydrogens to the receptor molecule. Water molecules were removed from the protein molecule to make it a free receptor. Since ligands are not peptides, Gasteiger charge was assigned and then nonpolar hydrogens were merged. AutoDock assigns the rigid roots to the ligand automatically saving time as compared to manual picking. Atomic solvation parameters were assigned to the receptor using default parameters.

The grid box was generated around possible ligand-binding site. All the AutoDock docking runs were performed in Intel Core™ i3-2330 CPU @ 2.20 GHz of Sony origin with 6 GB DDR RAM.

Step	Input	Output	Content
Files preparation	R.pdb	R_rigid.pdbqt	Rigid part of the receptor
		R_flex.pdbqt	Flexible part of the receptor
	L.pdb	L.pdbqt	
AutoGrid	R_rigid.pdbqt		
	R.gpf		Grid parameters
		R.glg	Grid log file
		R.*.map	Atom-specific affinity maps
		R.maps.fld	Grid_data_file
		R.d.map	Desolvation map
		R.e.map	Electrostatic map
Docking preparation	R_rigid.pdbqt	L.pdf	Docking parameters
	R_flex.pdbqt		
	L.pdbqt		
Docking	R_rigid.pdbqt	L.dlg	Log + coordinates + energies
	R_flex.pdbqt		
	L.pdbqt		
	L.pdf		
	R.*.map		
	R.maps.fld		
	R.d.map		
	R.e.map		

Table 02 : Procedure model of the Autodock visualization (R = Receptor; L = Ligand)

After the docking process complete, the final step is to analyze output results (L.dlg files) in order to select potential compounds which have the best binding score and their optimal interact positions with proteins. The binding energy was used as the criteria for filtering the best candidates. As presented in figure 27, the detail information of the molecules including three – dimensional coordinates and docked energies were fully listed in the obtained log file.



Major categories of energy:

Binding energy :	The total energy has released during the process of docking
Final intermolecular energy :	The energy formed by the interaction between ligands and protein
Torsional free energy :	The energy in which the molecule loses during being rotated
Final Internal energy :	The internal energy of molecule when changing from unbound state to bound state
Inhibition constant, K_i :	Theoretical inhibition constant value of the compound

Figure 27 : Example of a docking result output

The final internal energy of a molecule is determined by taking total internal energy exclude for unbound system's energy. Then, the binding energy (kcal/mol) is calculated according to the formula below:

$$\text{Binding energy} = \text{Final intermolecular energy} + \text{Torsional free energy} + \text{Final internal energy}$$

3. Biological Studies

3.1. Cell lines and cell culture

3.1.1 Cell lines

All biological assays were tested on two human hepatocellular carcinoma cell lines HepG2 (ECACC[®] Catalogue No. 85011430) and Hep3b (ATCC[®] HB – 8064[™]):

+ HepG2: cell type derived from liver hepatocellular carcinoma of a 15-year-old Caucasian male. According to literature, HepG2 is Mortalin and wild type p53 positive.

+ Hep3b: cell type derived from liver hepatocellular carcinoma of a 8-year-old Black male. Hep3b is Mortalin positive but does not contain p53 protein.

T75 flasks were used for Hep3b and T25 for HepG2 cell line (Thermo Fisher Scientific). Both of them were cultured using Dulbecco's Modified Eagle's Medium (DMEM 1X) + Glutamax[™] – Low Glucose (Gibco Industries Inc.) supplemented with 10% fetal bovine serum (Hyclone) and 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma) at 37°C in a humidified atmosphere with 5% CO₂, 95% air and complete humidity. The culture medium is renewed every 2 to 3 days. In these conditions, cell doubling time was around 30 hours.

3.1.2. Cell culture

Once reached about 80% confluency, cells were washed with PBS 1X (Gibco Industries Inc.) and detached using 0.05% trypsin/EDTA (Gibco Industries Inc.) using Primo Vert microscope (Zeiss) to observe the dispersion of cell layer. Then, the dye exclusion method was utilized to measure number of cell in the flask.

In the last step, sufficient amount of complete growth medium was added with appropriate aliquot of cells suspension into new culture vessels to reach the following cultivation ratio:

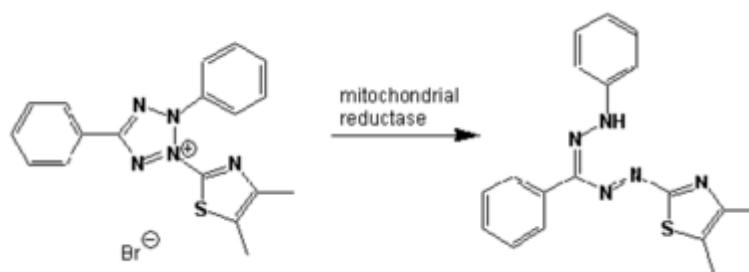
- For HepG2 cell line: the sub-cultivation ratio is 1:10
- For Hep3b cell line: the sub-cultivation ratio is 1:20

3.2. Cell viability Assay

3.2.1. Principle of the MTT test

The MTT test or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma Co.) was used in this study. The reason for this choice is that MTT assay is one of the most used method for measuring cell viability due to its simple and rapid technique. This colorimetric test is based on the activity of the mitochondrial enzyme: succinate dehydrogenase (SDH) which results in the cleavage of the yellow MTT into the purple formazan crystal.

SDH is a membrane-bound protein that forms part of the citric acid cycle in which it plays an important role in the oxidation of succinate into fumarate. It simultaneously forms Complex II in the electron transport chain in the mitochondria and with the reduction equivalent FADH₂ produced from the succinate oxidation reduces ubiquinone into ubiquinol which then moves further to Complex III.



The dye penetrates through cell membrane and accumulates in intracellular level, yellow MTT is oxidized by mitochondrial succinate dehydrogenase into purple formazan crystals. These crystals are dissolved under the action of DMSO, producing a coloured solution whose absorption can be determined using EON™ microplate spectrophotometers (Biotek). This reduction only exists in metabolically active cells, therefore, conversion can be directly related to the number of living cells. When the agent is compared with the amount of formazan produced by control cells, the effectiveness of the agent in causing death of cells can be deduced through the production of a concentration-response curve.

3.2.2. Technique

The MTT assay was performed according to the method described by Mosmann, 1983. For HepG2 cells: 2×10^5 cells/mL and Hep3b: $1,5 \times 10^5$ cells/mL were seeded into 96 well plate (SARSTEDT, sterile) to 100 μ L per well. The plates were incubated at 37°C for 48 hours to obtain about 80% confluent cells. Following this phase, compounds prepared at different concentrations were filled into the plate. Each compound was tested three times and each time in triplicate to reduce the risks of errors.

μ M	Compound 1			Compound 2			Compound 3			Compound 4		
500												
100												
20												
4												
0,8												
0,16												
0,032												
	CM+5% DMSO	CM+5% DMSO	CM+5% DMSO	DMSO	DMSO	DMSO	CM	CM	CM	CM	CM	CM

Table 03 : Sample organization of a 96-well plate for MTT assay (CM: Culture medium)

Measure in presence of DMSO 100% was used for measuring 0% cell viability. Measure in presence of culture medium only was used for determining the viability in absence of compound, taken as the reference (100%). Depending on each type of experiment, different incubation time will be selected. After the incubation, aspirated culture medium in the plate and washed cell with PBS 1X, then, 20 μ L of MTT (MTT solution was prepared at 5mg/mL in sterile PBS 1X and store away from light) plus 100 μ L of DMEM without phenol red were added into each well. The plate is shaken (350 rpm) for 20 minutes at room temperature then incubated for 3 hours at 37°C in 5% CO₂, 95% air and complete humidity conditions. After the incubation, add 100 μ L of DMSO into each well to dissolve the formazan crystals which then take a violet color. The plates were further incubated for 5 minutes at room temperature, and the optical density (OD) of the wells was determined using EON™ microplate spectrophotometers at a test wavelength of 590 nm and a reference wavelength of 630 nm.

The viability of cells is calculated according to formula below:

$$\text{Viability (\%)} = (\text{OD of exposed cells} : \text{OD of cell unexposed}) \times 100$$

The half maximal inhibitory concentrations (IC₅₀) were determined using the Graphpad Prism software (Graphpad Software, Inc. San Diego, CA, USA).

3.3. Quantitative detection of apoptosis rate

3.3.1. Principle of the SubG1 test

This is a method used to detect cells that have lost some of their DNA in late stage of apoptosis process following endonucleases activity. Endonucleases degrade DNA in small fragments of about 180 bp, which accumulate in the cell. After ethanol fixation and wash with a phosphatecitrate buffer, these DNA oligimeres leak out of the cell decreasing the DNA content. Using the nucleic acid stain propidium iodide (PI), the number of hypodiploid cells undergoing this process can be counted in subG1 region of PI histogram.

3.3.2. Technique

In the framework of this research, to investigate effect of *ent*-kaurane family on the apoptotic rate, HepG2 and Hep3b cell types were incubated with compound 5 at various concentrations for 48 hours (0-2-4-8-16 µM). Cells were seeded at a density of 4 x 10⁵/mL into 6-well plates (SARSTEDT, sterile) for Hep3b cell type and 6 x 10⁵/mL into 24-well plates (SARSTEDT, sterile) for HepG2. The plates were incubated at 37°C for 48 hours to obtain about 80% confluent cells. According to CycleTest™ Plus DNA Reagent kit (BD Biosciences), the procedure is carried out in the following steps:

a. Cell suspensions

+ Cells were washed with PBS 1X and dissociated with Gibco® Cell Dissociation Buffer, enzyme free PBS. Next, sufficient amount of complete growth medium were added to each

well and brought to centrifuge (500 rpm) at room temperature (20°C - 25°C). Cells were kept on ice from this step.

b. Staining

In this step, cells were stained respectively with Solution A, B and C in accordance to the protocol of BDScience before analyzed using the LSRII flow cytometer (BD Biosciences) within 1 hour after the staining procedure end.

3.4. Measurement of cell apoptosis using flow cytometry for Annexin V

3.4.1. Principle of the method

Apoptosis, or programmed cell death, is a normal physiologic process for removal of unwanted cells. The process is characterized by specific morphologic features, including loss of plasma membrane asymmetry and attachment, plasma membrane blebbing, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA.

Loss of plasma membrane asymmetry is one of the earliest features of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.

Annexin V, a 35-36 kDa Ca^{2+} -dependent phospholipid-binding protein, has high affinity for PS, and fluorochrome-labeled Annexin V can be used for the detection of exposed PS using flow cytometry. Thus, it can be used for the detection of apoptosis. Also the differentiation between apoptotic and necrotic cells can be performed by simultaneous staining with Propidium Iodide, the cell membrane integrity excludes PI in viable and apoptotic cells, whereas necrotic cells are permeable to PI. Therefore, cells that are considered viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive.

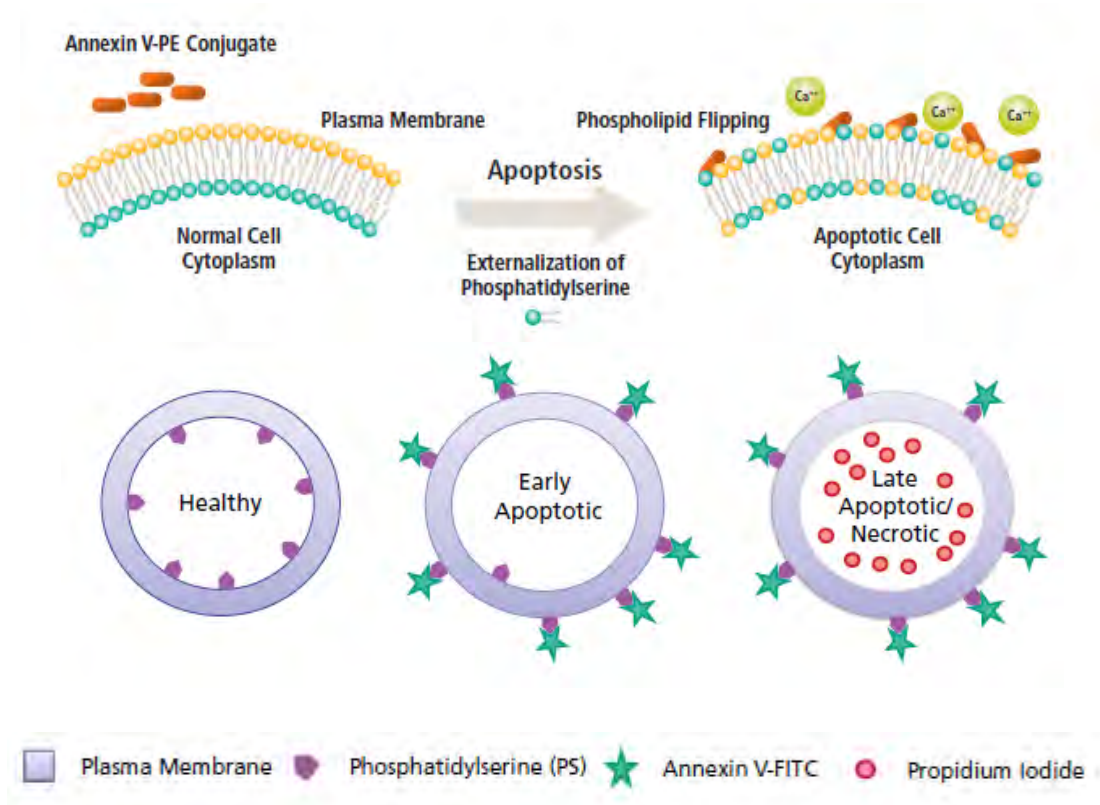


Figure 28 : Diagram showing healthy and apoptotic cells with markers for detection of apoptosis (from BD Reagents Apoptosis Brochure)

3.4.2. Technique

The apoptosis process was evaluated using the BD Pharmingen™ Annexin V: FITC Apoptosis Detection Kit I. Cells were seeded at a density of $4 \times 10^5/\text{mL}$ into 6-well plates for Hep3b cell type and $6 \times 10^5/\text{mL}$ into 24-well plates for HepG2. The plates were incubated at 37°C for 48 hours to obtain about 80% confluent cells then incubated with various compounds as described below:

- Compound 5 (*ent*-kaurane family): tested on both HepG2 and Hep3b cell lines at five concentrations (0-2-4-8-16 μM) for 48 hours of incubation.
- solasonine: tested at concentrations of 0-5-7,5-10 μM on HepG2 and 0-2,5-5-7,5 for Hep3b at 24 hours of incubation.
- 3 controls (without compounds) were prepared for this assay including:

- Cells without PI and FITC Annexin V solution staining
- Cells staining with PI solution only
- Cells staining with FITC Annexin V solution only

According to the manufacturer's instructions, the procedure is carried out in the following steps:

a. Cell suspensions

- After the incubation, cells were washed with PBS 1X and dissociated with Gibco® Cell Dissociation Buffer, enzyme free PBS. Added sufficient amount of complete growth medium to each well and brought to centrifuge (500 rpm) at room temperature (20°C - 25°C). Cells were kept on ice from this step. Next, washed cell with cold PBS 1X and centrifuged two times before process to staining procedure.

b. Staining

In this step, cell were stained respectively with Annexin V Binding buffer, FITC Annexin V and PI solution in accordance to the protocol of BDScience before analyzed using the LSRII flow cytometer (BD Biosciences) within 1 hour after the staining procedure end.

3.5. Sensitization assay of Doxorubicin and ent-kaurane compounds to hepatocarcinoma cell lines

3.5.1. Principle of the assay

To date, HCC is still a major challenge for medical oncologists due to its highly chemoresistant nature and causing a lot of side effects to normal tissues (Llovet J. M., 2005). Hence, alternative treatment strategies are urgently needed. Doxorubicin (Dox) is an anthracycline antibiotic isolated from *Streptomyces peucetius* that has been used effectively for the treatment of several cancers including: hepatocarcinoma, ovarian, breast and prostate (Yang T. S., 2002; Fong M. Y., 2012). Nevertheless, the use of Doxorubicin is limited by

severe dose-dependent side effects such as acute nausea, stomatitis, neurological, cardiotoxicity and bone aplasia (Fong M. Y., 2012; Fan C. 2014). Due to those problems, single-agent chemotherapy is no longer favored for treating HCC and many efforts have been made until now to identify chemo-sensitizers which are agents that are able to augment efficiency of anticancer drugs and simultaneously overcome multi-drug resistance and side effects.

Recently, studies of combination therapy with Doxorubicin has gained attention (Yang T. S., 2002; Kaur K., 2007; Gambari R., 2013; Fan C. 2014). For example: Kaur K. et al. proved that Withaferin A enhances the cytotoxic effect of Doxorubicin in an osteogenic sarcoma (U2OS) and in breast cancer cell line (MCF-7) using a cell proliferation assay.

In our study, we evaluated the ability of *ent*-kaurane compounds to enhance the cytotoxic effects of Doxorubicin on HCC cell lines.

3.5.2. Technique

Both HepG2 and Hep3b cell lines were used to test for the potentiation effects. Cells were seeded at concentration 2×10^5 cells/mL for HepG2 and $1,5 \times 10^5$ cells/mL for Hep3b cell type into 96 well plate (SARSTEDT, sterile) to 100 μ L per well. The plates were incubated at 37°C for 48 hours to obtain about 80% confluent cells before incubated with compounds.

a. Potentiation effects of *ent*-kaurane compounds with Doxorubicin

Doxorubicin was used at 4 μ M. Cells were incubated with Doxorubicin either alone or in combination with compounds 1 to 7 at three concentrations: 2, 5 and 10 μ M (except compound 3 tested at 20, 50 and 100 μ M). Next step, incubated for 24 hours then measured using cell viability assay (MTT assay).

	Dox alone (4 μ M)			Dox + Compound			Compound alone		
2 μ M									
5 μ M									
10 μ M									
	CM+5% DMSO	CM+5% DMSO	CM+5% DMSO	DMSO	DMSO	DMSO	CM	CM	CM

Table 04 : Sample organization of the potentiation experiment of Doxorubicin with *ent*-kaurane compounds (CM: Culture medium)

b. Potentiation effect of compound 5 with Doxorubicin

Cells were incubated with Doxorubicin at various concentrations starting at 500 μ M followed by serial of 5-fold dilutions down to 0.032 μ M either alone or combined with compound 5 (CP5), the representative molecule of the *ent*-kaurane family, at 3 different concentrations: 2, 5 and 10 μ M. Cells were incubated for 6, 12, 24 and 48h of incubation before measured for cell viability using cell viability assay (MTT assay).

μ M	Dox alone			Dox + CP5 (2 μ M)			Dox + CP5 (5 μ M)			Dox + CP5 (10 μ M)		
500												
100												
20												
4												
0,8												
0,16												
0,032												
	CM+5% DMSO	CM+5% DMSO	CM+5% DMSO	DMSO	DMSO	DMSO	CM	CM	CM	CM	CM	CM

Table 05 : Sample organization of the potentiation experiment of Doxorubicin with compound 5 (CM: Culture medium)

3.6. Pifithrin alpha assay

3.6.1. Principle of the assay

Pifithrin alpha (PFT- α) [2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-*p*-tolylethanone hydrobromide] is a synthetic, water-soluble and stable compound. It has been considered to be a specific inhibitor of p53 and useful for reducing the side effects of cancer therapy due to the blockage of p53-dependent transcriptional activation and apoptosis

(Komarov P. G., 1999). PFT- α is thus used experimentally in studies aiming at understanding p53-dependent apoptotic processes.

3.6.2. Technique

Cytotoxicity was evaluated using the MTT assay. PFT- α (Sigma) was purchased in white crystalline powder and was made up as stock solutions in DMSO (54,4 mmol/L) prior to dilution into medium to the required final concentration. Cells were seeded at 2×10^5 cells/mL for HepG2 and $1,5 \times 10^5$ cells/mL for Hep3b cell lines into 96 well plate (SARSTEDT, sterile) at the volume 100 μ L per well. Plates were incubated at 37°C for 48 hours to obtain about 80% confluent cells. We performed the experiments at two different incubation times (12 and 24 hours) with various concentrations (2.5, 5, 7.5 μ M) of solasonine alone or in combination with PFT- α at 10, 20, 30 μ M. Cells were pre-incubated with PFT- α for 1 hour prior adding solasonine.

μ M	solasonine alone			solasonine + 10 μ M PFT- α			solasonine + 20 μ M PFT- α			solasonine + 30 μ M PFT- α		
7,5												
5												
2,5												
				10 μ M PFT- α alone			20 μ M PFT- α alone			30 μ M PFT- α alone		
	CM+5% DMSO	CM+5% DMSO	CM+5% DMSO	DMSO	DMSO	DMSO	CM*	CM	CM	CM	CM	CM

Table 06 : Sample organisation of a 96-well plate for PFT- α experiment

After incubation, plates were processed to measure the half maximal inhibitory concentration (IC_{50}) value according to the MTT assay protocol presented above.

3.7. Western Blot

3.7.1. Principle of the method

Western Blot - WB (immunoblotting or protein blotting) is a powerful technique used in cell and molecular biology for detecting specific proteins in a sample of tissue homogenate or

extract that are of low abundance. The term “*Western Blot*” was given to the technique of Burnette (Burnette W. N., 1981) which is a procedure slightly modified from the method originated in the laboratory of Harry Towbin (Towbin H., 1979).

This method uses SDS-PAGE (Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis) to separate native proteins by three-dimension structure or denatured proteins by the length of the polypeptide. Then, the proteins are transferred to an adsorbent membranes made of nitrocellulose or polyvinylidene difluoride (PVDF) where they are stained with antibodies specific to the target proteins (Towbin H., 1979; Renart J., 1979).

In the framework of this research, this technique is utilized to prove the existence of Mortalin protein in both HepG2 and Hep3b and to investigate the possible mechanisms of action of solasonine in HCC cell lines.

3.7.2. Technique

Cells were seeded at a density of $4 \times 10^5/\text{mL}$ into 6-well plates for Hep3b cell type and $6 \times 10^5/\text{mL}$ into 24-well plates for HepG2. Plates were incubated at 37°C for 48 hours to obtain about 80% confluent cells before incubated with solasonine at different concentrations: 0 – 2,5 – 5 – 7,5 μM for 24 hours. After incubation, cells were washed with PBS 1X and detached using 0.05% trypsin/EDTA (Gibco Industries Inc.): add 1mL/ well for 6-well plate and 0,2 mL/well for 24-well plate, then incubate at 37°C until cells are dispersed from the flasks (usually within 5 to 15 minutes). Add sufficient amount of complete growth medium into each well and resuspend carefully then counting cells using hemocytometer in order to transfer one million cells into each 15 mL tube.

a. Determining the presence of Mortalin protein

Total cell lysates of both HepG2 and Hep3b cell lines were collected using lysis buffer (NET with 0,5% Triton 100X). Protein concentrations were measured using Pierce[®] BCA Protein Assay Kit (Thermo Scientific) in accordance with the manufacturer’s instructions then the cell lysate proteins (50 μg) were separated by 12,5% SDS – PAGE and transferred onto nitrocellulose membranes (GE Healthcare) using a semidry Trans-Blot[®] Turbo[™] Transfer System (Bio-RAD). The blotting membranes were blocked with PBS 1X/0,1% Tween-20

containing 10% skim milk for one night at 4°C. Primary antibodies specific for Mortalin (Cell Signaling) was diluted at ratio 1:1000 as indicated by the manufacturer and reacted with membranes at 4°C for 4 hours. Monoclonal antibody to β -actin (diluted ratio 1:1000; Cell Signaling) also used as an internal control. Then, membranes were washed three times with PBS Tween Milk and followed by incubation with secondary antibodies anti-rabbit (diluted at ratio 1:2000; Cell Signaling) for one hour before washed with PBS Tween. The protein levels were analyzed by enhanced chemiluminescence with Supersignal® West Dura Extended Duration Substrate Kit (Thermo Scientific).

b. Investigating the apoptotic mechanisms induced by solasonine

In this assay, we carried out experiment with cell incubated with solasonine at different concentrations. Cell lysates including: cytosolic, nuclear soluble and nuclear insoluble fractions of both HepG2 and Hep3b cell lines were collected using lysis buffer (Schreiber E., 1990). Protein concentrations were measured using Pierce® BCA Protein Assay Kit (Thermo Scientific) in accordance with the manufacturer's instructions then the cell lysate proteins (400 μ g) were separated by 12,5% SDS – PAGE and transferred onto nitrocellulose membranes (GE Healthcare) using a semidry Trans-Blot® Turbo™ Transfer System (Bio-RAD). The blotting membranes were blocked with PBS 1X/0,1% Tween-20 containing 10% skim milk for one night at 4°C.

Primary antibodies specific for p53 (diluted ratio 1:800; Life Technology) and p21 (diluted ratio 1:2000; Cell Signaling) were incubated with membranes at 4°C for 4 hours. Then, membranes were washed three times with PBS Tween Milk and followed by incubation with secondary antibodies anti-mouse (diluted at ratio 1:2000; Cell Signaling) for one hour before washed with PBS Tween. The protein levels were analyzed by enhanced chemiluminescence with Supersignal® West Dura Extended Duration Substrate Kit (Thermo Scientific). Monoclonal antibody to β -actin (diluted ratio 1:1000; Cell Signaling) also used as an internal control for cytosolic fraction, for nuclear soluble and nuclear insoluble fractions were PARP (diluted ratio 1:1000; Cell Signaling) and Lamin B1 (diluted ratio 1:1000; Cell Signaling) respectively.

3.8. Statistical analysis

All experiments were performed at least three times. Value were expressed as mean $\pm$ SD. Differences were analyzed using the Student's t-test. Differences with $p < 0.05$ (*) or $p < 0.001$ (***) were considered statistically significant.

E - Results and Discussion

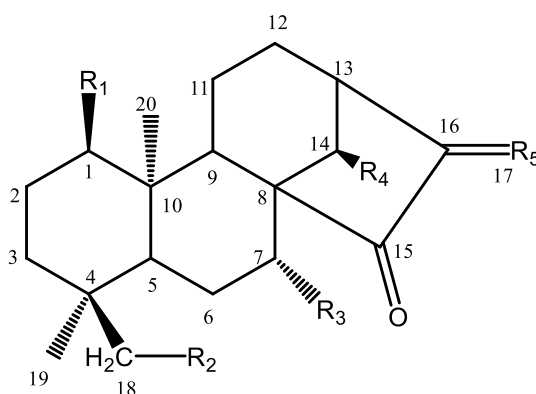
1. Pharmacological screening of Vietnamese plants database

1.1. Collecting Vietnamese natural compounds from medicinal plants resources

The collection consisted of 26 natural compounds originated from medicinal plants commonly used in traditional prescriptions for liver protective and liver cancer treatment in Vietnam. All the compounds were isolated and structure determined in Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology with a purity over 97% as measured using HPLC (High-performance liquid chromatography). These compounds were classified into 7 groups based on their original plants.

1.1.1. *Ent*-kaurane compounds from *Croton kongensis* Gagnep.

This group includes 7 diterpenoids. *Croton kongensis* Gagnep belongs to Euphorbiaceae family, commonly named in Vietnamese as “Kho sam Bac Bo”, is a tropical shrub native to Northern Vietnam (Giang P. M., 2003). It has been used commonly in traditional prescription to treat leprosy, psoriasis, malaria and genital organ prolapse (Chi V. C., 1997; Giang P. M., 2003). *Ent*-kaurane-type diterpenoids were isolated from the leaves of *Croton kongensis* Gagnep. and their structure determined from phytochemical investigations (Minh P. T., 2003; Giang P. M., 2004; Kuo P. C., 2007). For some of them, the cytotoxic properties have been tested against breast MCF-7, lung A549 (Kuo P. C., 2007), liver SK-HEP1 (Sul Y. H., 2013), intestinal Caco-2 and colon LS180 (Thuong P. T., 2014) cancer cell lines. The structure of 7 studied ent-kauranes are shown in Table 07.



No.	Name	R ₁	R ₂	R ₃	R ₄	R ₅	MW (g/mol)
1	<i>ent</i> -18-acetoxy-7 β -hydroxykaur-16-en-15-one (C ₂₂ H ₃₂ O ₄)	H	OAc	OH	H	=CH ₂	360
2	<i>ent</i> -1 α -acetoxy-7 β ,14 α -dihydroxykaur-16-en-15-one (C ₂₂ H ₃₂ O ₅)	OAc	H	OH	OH	=CH ₂	376
3	<i>ent</i> -16(S)-18-acetoxy-7 β -hydroxykaur-15-one (C ₂₂ H ₃₄ O ₄)	H	OAc	H	OH	-CH ₃	362
4	<i>ent</i> -7 β ,14 α -dihydroxykaur-16-en-15-one (C ₂₀ H ₃₀ O ₃)	H	H	OH	OH	=CH ₂	318
5	<i>ent</i> -18 α -acetoxy-7 α ,14 β -dihydroxykaur-16-en-15-one (C ₂₂ H ₃₂ O ₅)	H	OAc	OH	OH	=CH ₂	376
6	<i>ent</i> -1 α ,14 α -diacetoxy-7 β -hydroxykaur-16-en-15-one (C ₂₄ H ₃₄ O ₆)	OAc	H	OH	OAc	=CH ₂	418
7	<i>ent</i> -1 α ,7 β -diacetoxy-14 α -hydroxykaur-16-en-15-one (C ₂₄ H ₃₄ O ₆)	OAc	H	OAc	OH	=CH ₂	418

Table 07 : Structure of diterpenoids isolated from *Croton kongensis* Gagnep.

1.1.2. Triterpenoids from *Syzygium formosum* (Wall.) Masam.

This plant belongs to Myrtaceae family and it has been used for long time as medicine for some significant bioactivities such as: liver protective, antibacterial, anti-tumor... (Fujioka T.,

1994; Huong V. N., 2003; Thanh H. V., 2008; Jaki B. U., 2008) We chose 6 compounds isolated from leaves for bioactivities study. Their structures are shown in Table 08.

No.	Name	Structure	MW (g/mol)
8	Betulinic acid (3β -hydroxy-lup-20(29)-en-28-oic acid) ($C_{30}H_{48}O_3$)		456
9	2α,3β-dihydroxy-lup-20(29)-en-28-oic acid ($C_{30}H_{48}O_4$)		472
10	Corosolic acid ($2\alpha,3\beta$ -dihydroxy-urs-12-en-28-oic acid) ($C_{30}H_{48}O_4$)		472
11	Asiatic acid ($2\alpha,3\beta,23$ -trihydroxy-urs-12-en-28-oic acid) ($C_{30}H_{48}O_5$)		488

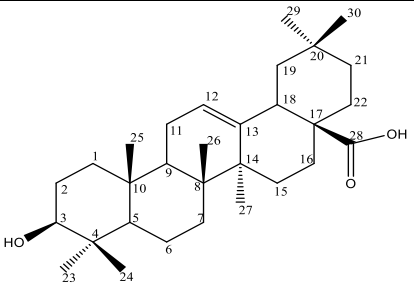
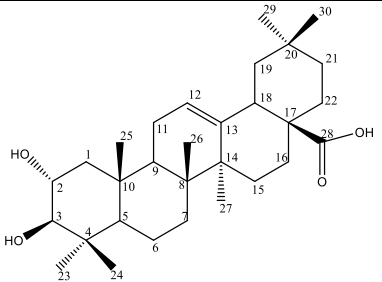
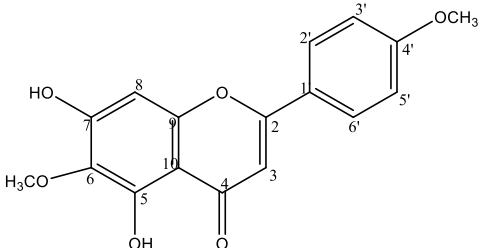
12	Oleanolic acid (3 β -hydroxy- olean-12-en-28-oic acid) (C ₃₀ H ₄₈ O ₃)		456
13	2α,3β-dihydroxy- olean-12-en-28-oic acid (C₃₀H₄₈O₄)		472

Table 08 : Structure of triterpenoids isolated from *Syzygium formosum* (Wall.) Masam.

1.1.3. Flavonoids from *Clerodendrum indicum* (L.) Kuntze

Clerodendrum indicum (L.) Kuntze belongs to Verbenaceae family. It is popularly used in fever, atrophy, consumption, cough, bronchitis and blindness. Leaves are used as vermifuge and tonic. The root is considered useful in asthma, cough and scrofulous affection (Rehman A. U., 1997; Rahman M. Z. A., 2000; Shrivastava N., 2007; Raihan S. Z., 2012). The structure of 2 compounds Pectolinarigenin and Hispidulin isolated from flower and leave are described below (Table 09).

No.	Name	Structure	MW (g/mol)
14	Pectolinarigenin (5,7-dihydroxy-6,4'- dimethoxyflavone) (C ₁₇ H ₁₄ O ₆)		314

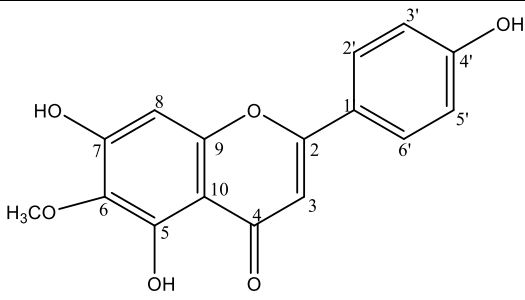
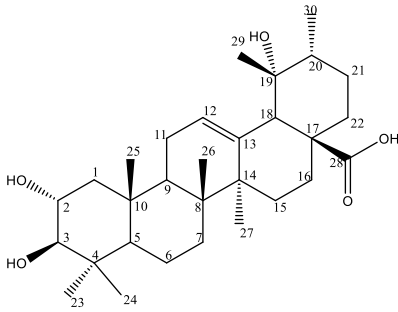
15	Hispidulin (5,7,4'-trihydroxy-6-methoxyflavone) (C ₁₆ H ₁₂ O ₆)		300
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Table 09 : Structure of compounds isolated from *Clerodendrum indicum* (L.) Kuntze

1.1.4. Compounds isolated from *Rubus alceifolius* Poir.

Rubus alceifolius Poir. belong to Rosaceae family commonly found in many provinces in Vietnam and other Asia countries. It is a well-known medicinal plant for hepatoprotective activity and also efficiency in anti-oxidant, treatment of urologic diseases, diabetes, kidney stone (Hong Z., 2010; Zhao J., 2013, 2014, 2015). Table 10 presents the structure of 3 compounds isolated from root of this plant.

No.	Name	Structure	MW (g/mol)
16	Euscaphic acid (2 α ,3 α ,19-trihydroxyurs-12-en-28-oic acid) (C ₃₀ H ₄₈ O ₅)		488

17	Suavissimoside R1 (28-O- β -D-glucopyranosyl ester of 2 α ,3 β ,19-trihydroxyurs-12ene-23,28-dioic acid) (C ₃₆ H ₅₆ O ₁₂)		680
18	Trachelosperoside A1 (28-O- β -D-glucopyranosyl ester of 2 α ,3 β ,19-trihydroxyurs-12ene-24,28-dioic acid) (C ₃₆ H ₅₆ O ₁₂)		680

Table 10 : Structure of compounds isolated from *Rubus alceifolius* Poir.

1.1.5. Compounds isolated from *Smilax glabra* Roxb.

Smilax glabra Roxb. belong to Smilacaceae family distributed widely in the territory of Vietnam and many other Asia countries. *In vitro* and animal studies have shown that this botanical has antioxidant, antiviral, renoprotective, immunostimulatory, anti-inflammatory, hepatoprotective and anticancer properties (Kuo Y. H., 2005; Ban J. Y., 2006; Chu K. T., 2006; Ooi L. S., 2008; Xia D., 2013). Table 11 presents the structure of 2 compounds isolated from root of this plant that we have collected.

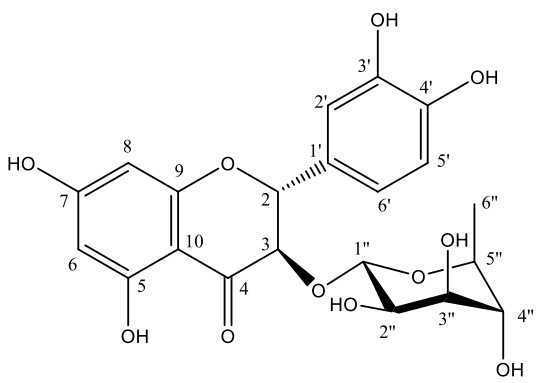
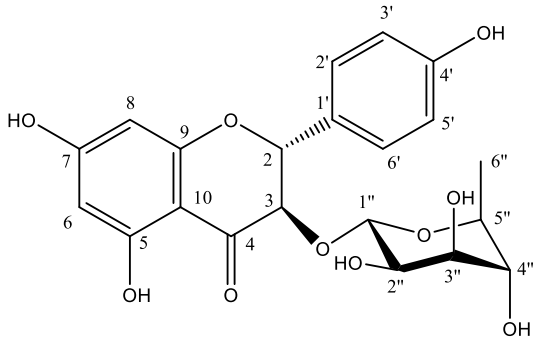
No.	Name	Structure	MW (g/mol)
19	Astilbin (2 <i>R</i> ,3 <i>S</i>)-3,3',4',5,5,7-pentahydroxyflavanone-3-O-L-rhamnopyranoside (C ₂₁ H ₂₂ O ₁₁)		450
20	Engeletin (2 <i>R</i> ,3 <i>S</i>)-3,5,7,4'-tetrahydroxyflavanone-3-O-L-rhamnopyranoside (C ₂₂ H ₂₂ O ₁₀)		434

Table 11 : Structure of compounds isolated from *Smilax glabra* Roxb.

1.1.6. Compounds isolated from *Sarcandra glabra* (Thunb.) Nakai

Sarcandra glabra (Thunb.) Nakai belongs to Chloranthaceae family is a species distributed widely in many regions in Vietnam and South East Asia countries. Laboratory studies have shown that it can stop cancer division, reduce side effects of cancer treatment, control bleeding disorders and used in traditional Chinese medicine for bone fractures and arthritis (Hocking G. M., 1997; Li Y., 2006; Li W. Y., 2007; He X. F., 2010). The structure of 2 compounds isolated from root of this plant are described in Table 12.

No.	Name	Structure	MW (g/mol)
21	2',6'-dihydroxy-3',4'-dimethoxychalcon (C ₁₇ H ₁₆ O ₅)		300
22	(2R)-5-hydroxy-6,7-dimethoxyflavanon (C ₁₇ H ₁₆ O ₅)		300

Table 12 : Structure of compounds isolated from *Sarcandra glabra* (Thunb.) Nakai

1.1.7. Compounds isolated from *Hydrangea macrophylla* (Thunb.) Ser.

Hydrangea macrophylla (Thunb.) Ser. is a species of flowering plant in the family Hydrangeaceae. Many studies have proven this plant has anti-allergic, anti-malarial and anti-microbial properties and also hepatoprotective activity by suppression of D-galactosamine-induced liver injury (Yoshikawa M., 1996; Kamei K., 2000; Nakagiri R., 2003; Zhang H., 2009). Table 13 (next page) shows 4 compounds isolated from different parts of this plant.

No.	Name	Structure	MW (g/mol)
23	Phylodulcin (3 <i>R</i>)-3-(3'-hydroxy-4'-methoxyphenyl)-8-hydroxy-dihydroisocoumarin (C ₁₆ H ₁₄ O ₅)		331
24	Thunberginol I (3 <i>R</i>)-3-(3-methoxy-4-hydroxyphenyl)-8-hydroxydihydroisocoumarin (C ₁₆ H ₁₄ O ₅)		286
25	Hydrangenol (3 <i>S</i>)-3-(4-hydroxyphenyl)-8-hydroxydihydroisocoumarin (C ₁₅ H ₁₂ O ₄)		301
26	(+) -Hydrangenol 4'-O-Glucoside (3 <i>S</i>)-3-(4-hydroxyphenyl)-8-hydroxydihydroisocoumarin-4'-O-Glucoside (C ₂₁ H ₂₃ O ₉)		463

Table 13 : Structure of compounds isolated from *Hydrangea macrophylla* (Thunb.) Ser.

1.2. Biochemical study of Vietnamese natural compounds

1.2.1. Cytotoxic effects of 26 natural compounds on hepatocarcinoma HepG2 and Hep3b cell lines

All 26 natural compounds in our database have been tested on two hepatocarcinoma HepG2 and Hep3b cell lines to check for their cytotoxicity effects. The IC₅₀ results are shown in Table 14.

Compound	24 hrs		48 hrs		72 hrs	
	HepG2	Hep3b	HepG2	Hep3b	HepG2	Hep3b
1	8.3 ± 1.5	8.0 ± 0.6	3.6 ± 1.5	8.6 ± 0.4	3.8 ± 0.8	3.4 ± 0.2
2	11.2 ± 5.1	9.9 ± 1.2	9.6 ± 3.1	8.5 ± 0.7	5.8 ± 0.9	4.8 ± 0.4
3	98.6 ± 25.4	116.2 ± 41.6	85.2 ± 32.5	69.6 ± 13.9	116.9 ± 18.9	99.4 ± 10.2
4	8.4 ± 1.8	7.8 ± 2.3	3.9 ± 0.3	6.1 ± 0.5	3.9 ± 0.1	3.8 ± 0.2
5	3.1 ± 0.5	9.0 ± 0.4	3.5 ± 0.8	3.7 ± 0.1	3.2 ± 0.2	3.7 ± 0.4
6	5.7 ± 1.2	9.5 ± 0.5	5.0 ± 1.2	6.1 ± 0.5	4.2 ± 0.1	5.3 ± 0.5
7	12.1 ± 3.9	16.1 ± 5.8	13.7 ± 1.3	9.7 ± 0.1	14.1 ± 2.2	15.5 ± 0.6
8	97.6 ± 26.2	168.9 ± 20.8	104.0 ± 25.8	67.9 ± 30.5	25.6 ± 14.2	31.2 ± 0.8
9	33.1 ± 8.1	59.9 ± 13.0	24.0 ± 3.3	39.1 ± 12.5	25.9 ± 2.8	25.9 ± 0.8
10	39.3 ± 4.3	52.3 ± 1.3	47.2 ± 4.1	42.7 ± 4.8	50.3 ± 3.2	44.9 ± 1.5
11	29.6 ± 5.9	52.0 ± 1.8	23.4 ± 6.0	32.2 ± 8.3	20.7 ± 4.3	21.5 ± 3.5
12	> 500	123.3 ± 8.6	192.4 ± 65.0	118.3 ± 20.8	230.9 ± 57.3	120.8 ± 4.7
13	101.3 ± 24.3	57.9 ± 22.1	105.0 ± 18.0	83.3 ± 11.2	87.3 ± 12.8	50.9 ± 2.1

14	94.2 ± 40.1	108.4 ± 22.3	171.15 ± 43.3	113.4 ± 4.5	95.0 ± 51.1	56.5 ± 3.3
15	215 ± 24.6	90.9 ± 15.9	106.8 ± 24.6	67.2 ± 12.9	129.4 ± 18.1	84.4 ± 3.5
16	> 500	> 500	204.2 ± 62.7	> 500	247.8 ± 72.2	285.3 ± 5.6
17	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
18	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
19	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
20	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
21	49.3 ± 9.5	93.3 ± 18.9	49.5 ± 3.1	70.1 ± 12.8	48.1 ± 3.5	60.6 ± 7.0
22	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
23	> 500	> 500	> 500	> 500	> 500	> 500
24	> 500	> 500	> 500	> 500	> 500	> 500
25	> 500	> 1000	> 1000	> 1000	> 1000	> 1000
26	> 500	> 1000	> 1000	> 1000	> 1000	> 1000
Doxo	50.8 ± 3.3	49.3 ± 17.3	26.5 ± 1.8	25.1 ± 2.1	29.0 ± 1.8	28.1 ± 1.4

Table 14 : Cytotoxic activity (IC₅₀, μM) of 26 Vietnamese natural compounds on human HCC HepG2 and Hep3b cell lines after 24 – 48 – 72 hours of incubation. Doxorubicin was used as positive control. Results are expressed as the mean ± SD values from three independent experiments.

Among the 26 tested compounds, 17 compounds exhibited a cytotoxic effect (IC₅₀ < 500 μM) against HepG2 and Hep3b cell lines with no significant difference between these two cell types. We divided the bioactive molecules into 3 groups depending on their activity (IC₅₀) and their chemical structures. Firstly, the diterpenoids family (compounds 1 to 7) was identified as the strongest cytotoxic group with most of the IC₅₀ values lower than 10 μM except compound 3 with the toxicity 8 to 10 fold lower due to structure different (see detailed

analysis in following paragraph 1.2.2.). The second group included compounds 8 to 13 and 16 classified into two main skeletons: Lupane and Ursane (Figure 29)

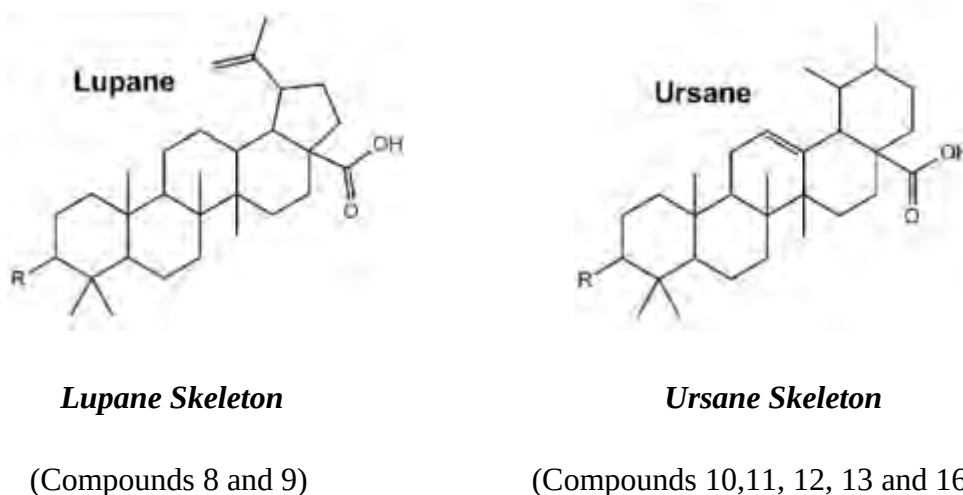


Figure 29 : Lupane and Ursane skeletons

Most of the cytotoxicity values of these compounds ranged within 50 – 100 μ M values (except compound 16), depending on the incubation time. Within a given group of compounds, the difference observed in their activity could be correlated with their structural difference. When comparing compounds 8 and 9, compound 9 exhibited a higher cytotoxicity due to the OH group at position 2. This group helps in increasing the bioactivity and also making the compound more soluble. Regarding compounds 10, 11, 12, 13 and 16, the higher cytotoxicity of compound 11 may be explained by the presence of an OH group at position 23, making the molecule more reactive and more soluble than the other related compounds.

The three other active compounds 14, 15 and 21 were arranged into a third group (the flavonoids) in which it was difficult to establish a correlation between their activity and their structure.

Of the 26 compounds tested, the *ent*-kaurane family (compounds 1 – 7) isolated from *Croton kongensis* Gagnep. clearly exhibited the most interesting preliminary data and was therefore selected for further and more detailed pharmacological investigations.

1.2.2. Cytotoxicity effects of *ent*-kaurane diterpenoids on hepatocarcinoma cell lines

As mentioned above, all the *ent*-kaurane compounds elicited a cytotoxic activity against both HepG2 and Hep3b cell lines without marked difference between the two cell lines. The fact that no significant difference was observed in the cytotoxic effect against HepG2 and Hep3b despite the distinct pharmacological properties of the two cell lines (Qiu G. H., 2015) suggested a cell line-independent mechanism of action of the compounds. Indeed, it is accepted that the cytotoxicity of *ent*-kaurane diterpenoids results from the formation of reactive oxygen species (ROS) through Michael reaction with regulatory thiol containing enzymes or coenzymes (Santagata S., 2012; Liu C. X., 2012; Lin Z., 2015), likely to be shared by the two HepG2 and Hep3b cell lines and others.

The activity remained similar or weakly enhanced from 24 to 72 hours of incubation, with most of the IC₅₀ values ranging from 3 to 10 μ M. Cytotoxicity depended on the structure of the compound. Compound **5**, for which the cytotoxic properties have not been tested in the literature, was the most active, with IC₅₀ values around 3 μ M, followed by compound **1**. Cytotoxicity of compound **1** on HepG2 and Hep3b cell lines was in the same range as that measured on the SK-HEP1 cell line for the identical *ent*-kaurane diterpenoid named CrT1 (Sul Y. H., 2013). Activity of compounds **1**, **2** and **4** against HCC HepG2 and Hep3b cell lines was comparable to the activity of identical molecules described against other tumor cell lines such as A549, MCF-7 or KB (Kuo P. C., 2007). Compounds **1**, **2**, **6** and **7** were also found active in the brine shrimp lethality assay (Phan M. G., 2005).

Interestingly, all the compounds except compound **3**, share the O=C–C=CH₂ system, described to be responsible of the cytotoxicity of sesquiterpene antitumor agents (Lee K. H., 1971; Lee K. H., 1977), and possess the 16-en-15-one basic skeleton shown to play an important role in the cytotoxic activity of *ent*-kaurane diterpenoids (Kuo P. C., 2007; Thuong P. T., 2014). Conversely, absence of a hydroxyl group at C7 position may contribute to a decreased activity (Kuo P. C., 2007). Consistent with this observation, compound **7**, bearing the 16-en-15-one structure but lacking the hydroxyl group at the C7 position, exhibited, to a lesser extend, a decreased activity with IC₅₀ values above 10 μ M. Further, our observation correlates with the weak cytotoxic activity measured for compound **7** in the brine shrimp lethality assay (Phan M. G., 2005). Compound **3** which lacks both the 16-en-15-one basic skeleton and a hydroxyl group at C7 position, displayed the weakest activity, with IC₅₀ values in the 100 μ M range, i.e. 10 to 30 times higher than those of compound **5**. Interestingly, it

must be noticed that the R5 methyl group which results in a well-known hyperconjugation effect (Figure 30), is only present in compound 3.

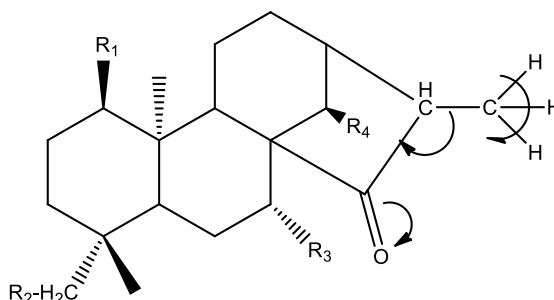


Figure 30 : Structure with arrows illustrates the hyperconjugation effect for compound 3

Hyperconjugation leads to a more stable structure and, consequently, to a less reactive functionality, which may affect the biological properties of *ent*-kaurane diterpenoids. In accord with our observation, an *ent*-kaurane diterpenoid identical to compound 3 showed no cytotoxicity in the brine shrimp lethality assay (Phan M. G., 2005) and was inactive in other biological assays such as inhibition of Silent information regulator two ortholog (SIRT1) (Dao T. T., 2010) or anti-staphylococcal activity (Giang P. M., 2006).

1.2.3. Morphological changes induced by *ent*-kaurane diterpenoids on hepatocarcinoma cell lines

Beside cytotoxicity assay, change in the morphology of the cell exposed to compounds may give pertinent information on the cellular processes underlying cell death. As a part of morphological investigation, the modification in the cell shape was therefore observed under light microscope. Figure 31 describes the morphology of both HepG2 and Hep3b cell lines after 48 hours of incubation with an *ent*-kaurane diterpenoid. As shown, both of the two cell types got rounded and shrinked apart.

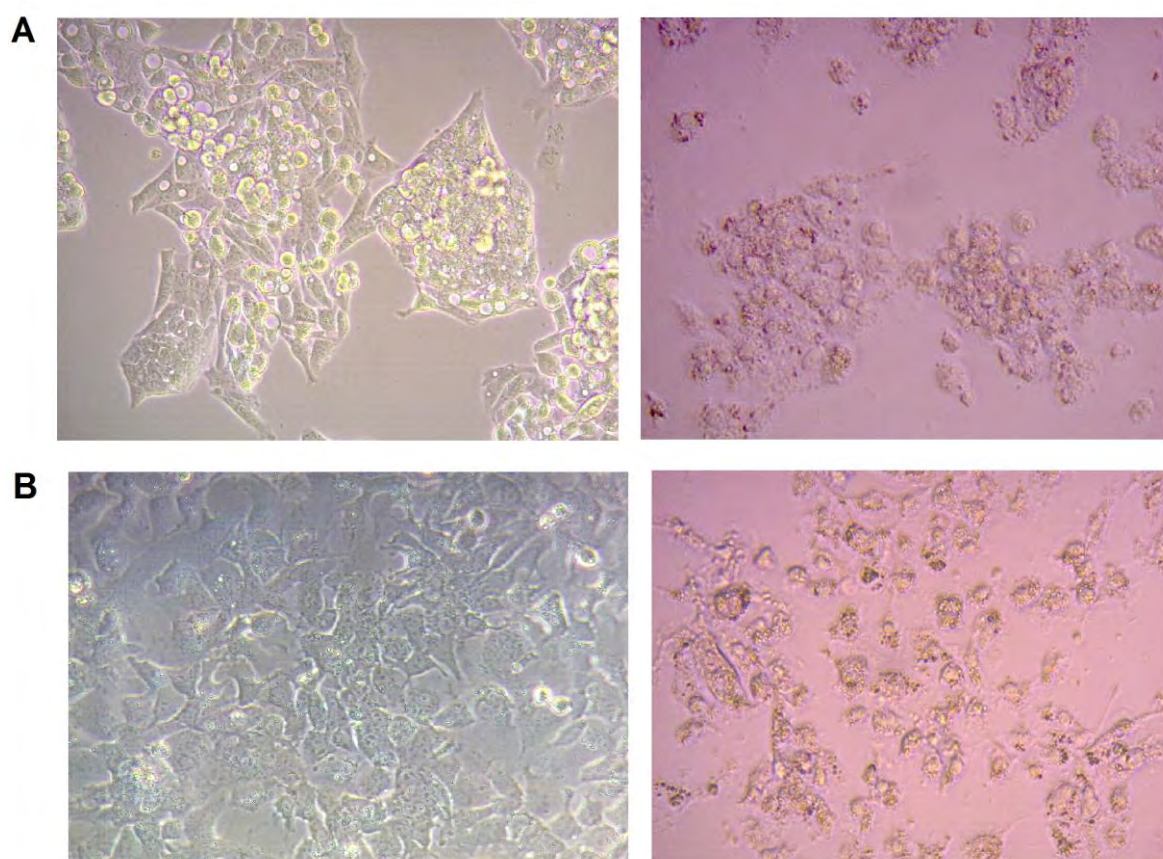


Figure 31 : Morphology of HepG2 (A) and Hep3b (B) hepatocarcinoma cell lines incubated (right panels) or not (left panels) with Vietnamese *ent*-kaurane diterpenoid compound 5 for 48 hours.

To further explore the cellular mechanisms underlying the observed cytotoxic effects, we used compound 5 as the representative molecule of our series of compounds.

1.2.4. Concentration-dependent induction of subG1 phase by *ent*-kaurane diterpenoid compound 5 on human HCC HepG2 and Hep3b cell lines

Cytometry analysis of the cell population in the SubG1 phase is commonly used to evaluate the pro-apoptotic properties of anti-tumor molecules. We tested compound 5 in a range of concentrations framing its IC₅₀ values. Results are shown in Figure 32.

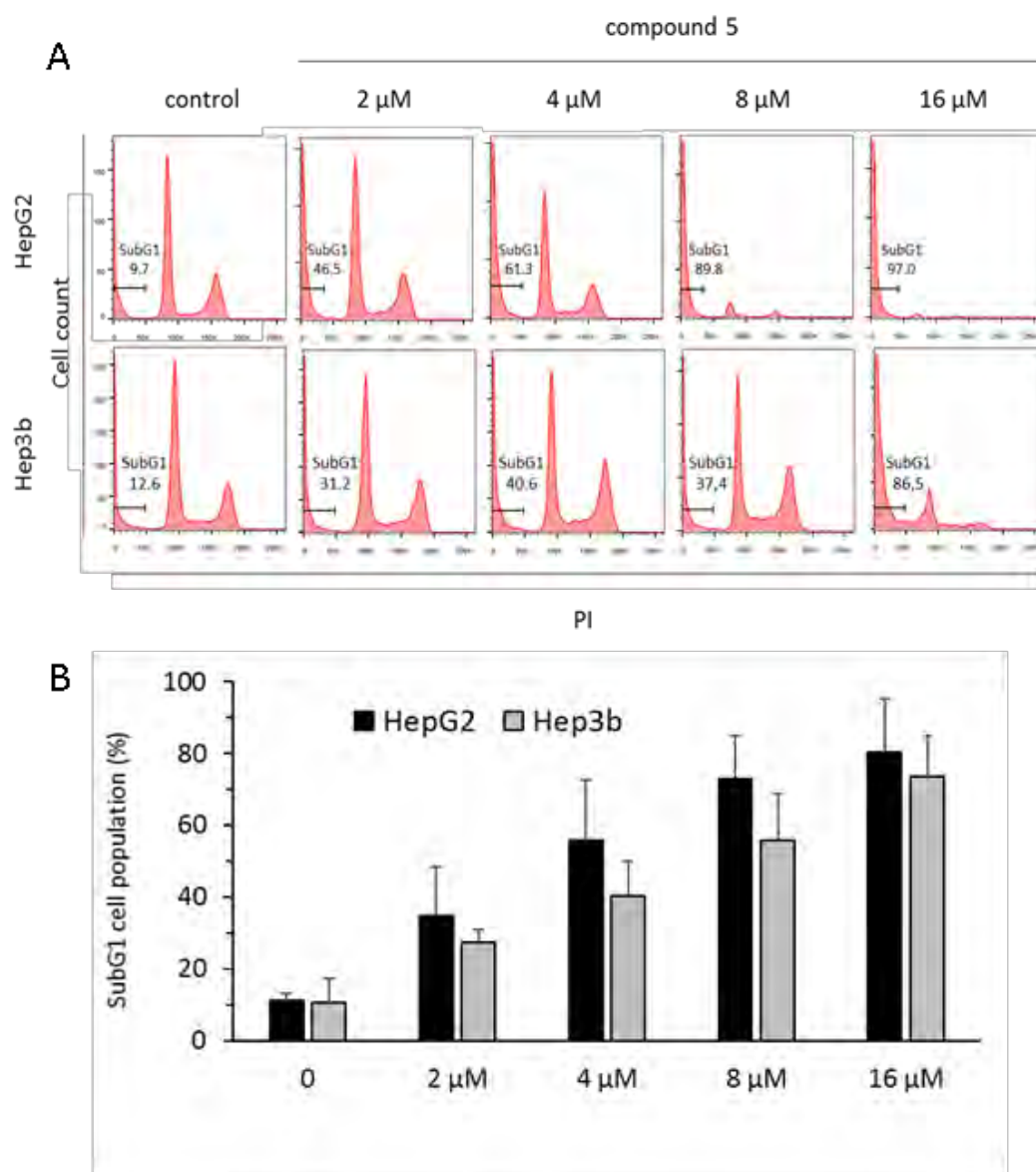


Figure 32 : Pro-apoptotic effect of *ent*-kaurane-type diterpenoid compound 5 on human hepatocarcinoma cell lines HepG2 and Hep3b. **A** – Hep G2 (upper panels) and Hep3b (lower panels) cells were incubated with no (control) or increasing concentrations (2 to 16 μ M) of compound 5 for 48 hours followed by staining with propidium iodide. DNA content was analyzed by flow cytometry. Number of cells (Cell count) is represented as a function of DNA content (PI). The % of cells in subG1 is indicated. Data presented are from one representative experiment. **B** – Histograms showing the % of cells in subG1 phase as a function of increasing concentrations of compound 5. Columns and bars are the mean and SD from 3 independent experiments.

Panel A illustrates the cell population observed in absence or presence of increasing concentrations of compound **5** in representative experiments performed on HepG2 (upper panels) and Hep3b (lower panels) cell lines. Panel B shows that in absence of compound **5**, the subpopulation of both HepG2 and Hep3b cell lines in the subG1 phase was low, around 10% (11.2 ± 2.1 for HepG2 and 10.6 ± 6.9 for Hep3b). In presence of compound **5**, the % of cells in the subG1 phase increased in a concentration-dependent manner, from around 30% (35.0 ± 13.3 for HepG2 and 27.5 ± 3.3 for Hep3b) at 2 μM of compound **5** to around 80% (80.3 ± 15.0 for HepG2 and 73.7 ± 11.1 for Hep3b) at 16 μM of compound **5**. This observation evidenced the pro-apoptotic effect of compound **5**.

Although the difference was not statistically different, the % of subG1 population in presence of compound **5** was repeatedly and consistently higher for HepG2 than Hep3b at a given concentration of compound **5**, suggesting a different sensitivity between the two cell lines. At the concentration of 4 μM , close to the IC_{50} values determined in the cytotoxicity assay at 48 hours (see Table 14), about half of the cell population was in the subG1 phase (56.0 ± 12.3 for HepG2 and 40.4 ± 9.7 for Hep3b). This is in favor of a correlation between the cytotoxic and pro-apoptotic properties of compound **5**. Interestingly, the % of subG1 population of HepG2 and Hep3b cell lines induced by compound **5** in our study are quite comparable to those obtained with CrT1 (identical to compound **1**) on the SK-HEP1 cell line (Sul Y. H., 2013).

These findings, observed on three different HCC cell lines, suggest that *ent*-kaurane diterpenoids might share common or closely related pro-apoptotic mechanisms of action leading to apoptosis in human HCC cell lines.

1.2.5. Concentration-dependent induction of apoptosis by *ent*-kaurane diterpenoid compound **5 on human HCC HepG2 and Hep3b cell lines**

Concomittent analysis of annexin V and propidium iodide by flow cytometry allowed the identification of early and late apoptotic stages and therefore brought a more detailed insight on *ent*-kaurane diterpenoid-induced apoptosis. Results are presented in Figure 33.

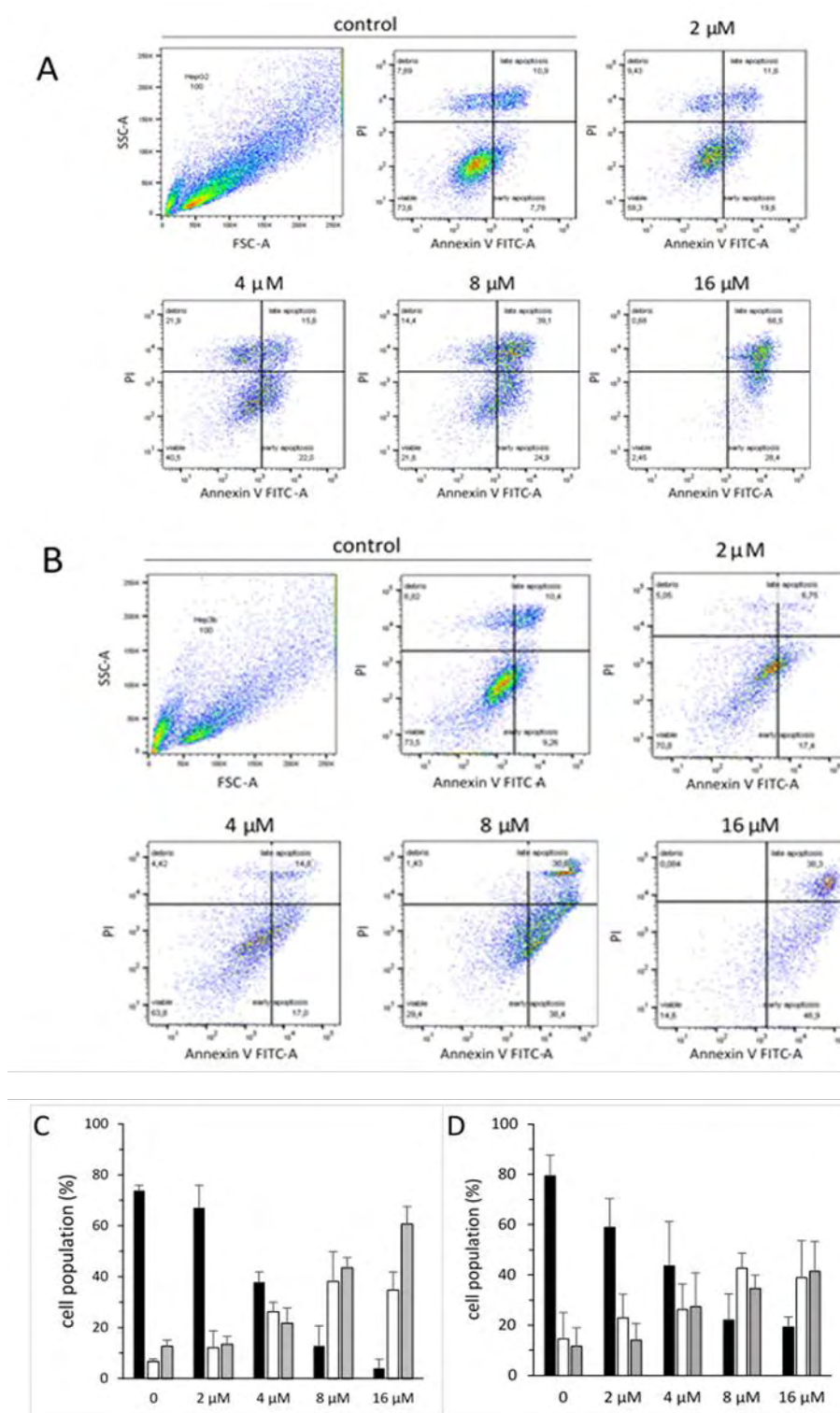


Figure 33 : Induction of apoptosis of human hepatocarcinoma cell lines HepG2 and Hep3b by *ent*-kaurane-type diterpenoid compound 5. HepG2 and Hep3b cells were incubated with no (control) or increasing concentrations (2 to 16 μ M) of compound 5 for 48 hours followed by staining with Annexin V FITC and propidium iodide. DNA content and Annexin V expression were analyzed by flow cytometry. **A** and **B** - Data presented are from one

representative experiment performed on HepG2 (**A**) and Hep3b (**B**) cell lines. In the upper left panel, the light scattered properties of control cells (FSC-A, SSC-A) are shown. In each other panel, viable, early apoptotic and late apoptotic cells are gated in lower left, lower right and upper right quarters respectively (debris are shown in upper left quarter). **C** and **D** – Percentage (%) of viable, early apoptotic and late apoptotic (black, white and grey columns respectively) HepG2 (**C**) and Hep3b (**D**) cells as a function of increasing concentrations of compound 5. Columns and bars are the mean and SD from 3 independent experiments.

Panel A illustrates the observed cell population in absence or presence of increasing concentrations of compound 5 in a representative experiment performed on HepG2 cell line. A similar pattern was obtained with the Hep3b cell line (Panel B). As shown in panels C (HepG2) and D (Hep3b), a high proportion (%) of cells was viable and non-apoptotic (73.8 ± 2.1 for HepG2 and 79.4 ± 8.3 for Hep3b) in absence of compound 5.

When incubated with compound 5, the % of viable cells declined in a concentration-dependent manner. In good agreement with the IC_{50} values determined in the cytotoxicity assay and the observations made in subG1 experiments, the population of viable cells at $4 \mu\text{M}$ was slightly below 50% (37.9 ± 4.1 for HepG2 and 43.5 ± 17.7 for Hep3b). This further supported the link between the cytotoxic and apoptotic properties of compound 5. At the highest concentration tested, viability of HepG2 cells was dramatically affected (4.1 ± 3.5) whereas that of Hep3b cells remained rather stable (19.3 ± 4.1), suggesting, as noted before, a different sensitivity of the two cell lines. In parallel, there was an increase in early apoptotic cell populations which reached a plateau around 40% for both cell lines at the highest concentration of compound 5 (34.7 ± 7.2 for HepG2 and 38.9 ± 14.7 for Hep3b). The double positive cell population (which contains late apoptotic and/or necrotic cells) also increased with a marked difference between the two cell lines, increase being continuous for HepG2 (60.6 ± 3.5) but stabilized around 40% for Hep3b (41.4 ± 11.9) at $16 \mu\text{M}$ of compound 5. In HepG2 cells, the high % of late apoptotic cells correlated with the low % of viable cells. In striking contrast with our observation, late apoptosis was barely detectable in HepG2 cells incubated with longikaurin A, a natural *ent*-kaurane (Liao Y. J., 2014).

Two additional and complementary observations sustained evidence of apoptosis in both HepG2 and Hep3b cell lines. First, under light microscope, we observed rounded and

shrank cells upon exposure of both cell lines to compound 5. Second, in correlation with these morphological changes, cells showed altered flow cytometry light scatter properties, Figure 34 illustrates a decrease in forward scatter (FSC-A, X axis) and an increase in side scatter (SSC-A, Y axis), which resulted from cell shrinking and apoptotic vesicles formation respectively for HepG2 (Panel A) and Hep3b (Panel B).

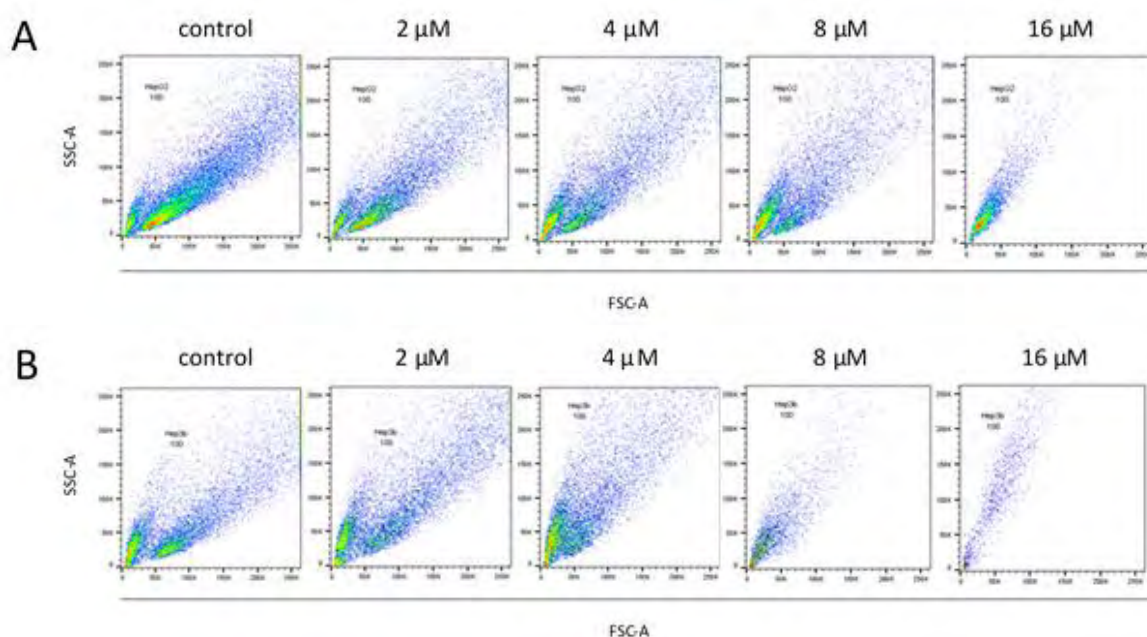


Figure 34 : Flow cytometry light scatter properties of human hepatocarcinoma cell lines HepG2 and Hep3b incubated with *ent*-kaurane-type diterpenoid compound 5. HepG2 and Hep3b cells were incubated with no (control) or increasing concentrations (2 to 16 μ M) of compound 5 for 48 hours followed by staining with Annexin V FITC and propidium iodide. Data presented the light scattered properties (FSC-A, SSC-A) from one representative experiment performed on HepG2 (**Panel A**) and Hep 3b (**Panel B**) cell lines.

The cellular components involved in apoptosis of HepG2 and Hep3b cells lines induced by compound 5 remain to be identified. Along this line, two different, but not exclusive, apoptotic cellular pathways triggered by *ent*-kaurane diterpenoids in two different HCC cells have been reported recently: the regulation of AMP-activated protein kinase by CrT1, isolated from *Croton kongensis* in SK-Hep1 cells (Sul Y. H., 2013), and the activation of ROS/JNK/c-

Jun pathway by longikaurin A, isolated from *Isodon ternifolius*, in SMMC-7721 cells (Liao Y. J., 2014).

1.2.6. *Ent*-kaurane diterpenoids potentiate the cytotoxic activity of doxorubicin on human HCC cell lines

1.2.6.1. Sub-toxic concentration of *ent*-kaurane diterpenoids enhances the cytotoxicity of doxorubicin in human HCC HepG2 and Hep3b cell lines

Doxorubicin is one of the most commonly used chemotherapeutic agent against various cancers including HCC (Weiss R. B., 1992; Tam K., 2013). However, as a single agent, efficacy of doxorubicin against HCC remains limited, mainly due to chemoresistance and severe side effects (Carvalho C., 2009). Combined therapy has emerged as a strategy for improving the therapeutic effects of doxorubicin in HCC (Cervello M., 2012; Finn R. S., 2013). In this field, few natural molecules have been successfully used to enhance the cytotoxic effect of doxorubicin in ovarian (Fong M. Y., 2012) or liver (Lee W. Y., 2010; Fan C., 2014; Gambari R., 2013) cancer cells. In the framework of further researches on *ent*-kaurane diterpenoids, compound 1 to 7 were used to study whether they can potentiate the cytotoxic effects of doxorubicin on human HCC HepG2 and Hep3b cell lines.

	Cytotoxic activity (%)			
	HepG2		Hep3b	
doxo alone	9.3 ± 6.3	-	8.5 ± 4.5	-
doxo + cpd 1	38.5 ± 3.7	(0.3 ± 5.5)	81.8 ± 7.4	(1.7 ± 1.2)
doxo + cpd 2	37.3 ± 5.3	(2.3 ± 2.1)	63.0 ± 11.2	(0.3 ± 2.3)
doxo + cpd 3	24.3 ± 2.5	(2.3 ± 4.0)	55.5 ± 14.3	(0.3 ± 1.5)
doxo + cpd 4	39.5 ± 3.9	(3.0 ± 4.6)	72.8 ± 11.3	(1.3 ± 3.8)
doxo + cpd 5	49.8 ± 2.1	(2.0 ± 4.4)	79.0 ± 10.1	(2.0 ± 3.6)
doxo + cpd 6	39.3 ± 6.0	(4.0 ± 3.6)	59.0 ± 3.4	(2.7 ± 1.2)
doxo + cpd 7	25.5 ± 7.0	(2.7 ± 5.5)	42.5 ± 6.5	(2.0 ± 2.6)

Table 15 : Cytotoxic activity (%) of doxorubicin alone or in presence of *ent*-kaurane diterpenoids (compounds 1 to 7) on human HepG2 and Hep3b hepatocarcinoma cell lines after 24 hours of incubation. Results are expressed as the mean ± SD values from three independent experiments. Doxorubicin (Dox) was used at 4 µM. Compounds (cpd) 1 to 7 were used at subtoxic concentrations (i.e. inducing less than 5 % cytotoxicity). Activity of compounds 1 to 7 alone are shown in parentheses.

As shown in Table 15, doxorubicin at a concentration of 4 µM displayed a weak cytotoxicity (9.3 ± 6.3 % for HepG2 and 8.5 ± 4.5 % for Hep3b cell lines) when used alone. When *ent*-kaurane diterpenoids were added at sub-toxic concentrations (less than 5% cytotoxicity), a dramatic potentiation effect was observed in both HepG2 and Hep3b cell lines. Whatever the compound, sensitivity to doxorubicin was much higher for Hep3b than HepG2 cell lines. For example: with compound 1, the percentage of cell death was 38.5 ± 3.7 % for HepG2 and 81.8 ± 7.4 % for Hep3b.

Since no significant difference was observed between the seven compounds, compound 5 was chosen as the representative compound for a further detailed investigation to clarify the mechanism of potentiation induced by *ent*-kaurane diterpenoids.

1.2.6.2 Sub-toxic concentration of *ent*-kaurane compound 5 strongly enhances the cytotoxicity of doxorubicin in human HCC cell lines

As shown in Table 16 and Figure 35, a low, subtoxic concentration of compound 5 efficiently sensitized both HepG2 and Hep3b cell lines to doxorubicin cytotoxicity in a time-dependent manner. Cytotoxicity of doxorubicin alone increased regularly with IC₅₀ values ranging from 109.3 ± 7.7 µM to 26.5 ± 1.8 µM for HepG2 and from 86.9 ± 7.3 µM to 25.1 ± 2.1 µM for Hep3b between 6 hours and 48 hours of incubation.

Incubation time (hrs)	IC ₅₀ (µM)			
	HepG2		Hep3b	
	Dox	Dox + cpd 5	Dox	Dox + cpd 5
6	109.3 ± 7.7	79.4 ± 6.0 (1.4)	86.9 ± 7.3	64.5 ± 3.8 (1.3)
12	66.6 ± 13.4	9.2 ± 0.9 (7.9)	56.2 ± 3.0	6.2 ± 0.5 (9.1)
24	50.8 ± 3.3	4.8 ± 0.6 (11)	49.3 ± 17.3	5.1 ± 0.3 (9.7)
48	26.5 ± 1.8	3.2 ± 0.6 (8.3)	25.1 ± 2.1	2.3 ± 0.3 (11)

Table 16 : Cytotoxic activity (IC₅₀, µM) of doxorubicin (Dox) in absence or in presence of *ent*-kaurane diterpenoid compound 5 (cpd 5) on human HepG2 and Hep3b hepatocellular carcinoma cell lines after 6, 12, 24 and 48 hours of incubation. Results are expressed as the mean ± SD values from three independent experiments. For a given time, the ratio of the IC₅₀ values of doxorubicin in absence / in presence of compound 5 is shown in parentheses.

As shown in Figure 35 from 12 to 48 hours, compound 5 dramatically enhanced the cytotoxicity of 20 µM doxorubicin, cell viability decreasing to less than 5% at 48 hours (compared to 60 % for doxorubicin alone). Accordingly, a significant - 8 to 11 fold – decrease was measured for the IC₅₀ values (Table 16). Conversely however, at 6 hours of incubation, compound 5 had only a weak to moderate effect on the IC₅₀ values (IC₅₀ ratio < 2, Table 16) and did not enhance the cytotoxic effect of 20 µM doxorubicin (Figure 35) in both cell lines. This 6 hour-delay ruled out the hypothesis of an immediate, non-specific action of compound

5, such as disruption of the cellular membrane integrity, which would allow doxorubicin to better penetrate the cell. It indicated that the sensitization effect of compound 5 necessitated triggering of intracellular pathways, which remain to be characterized.

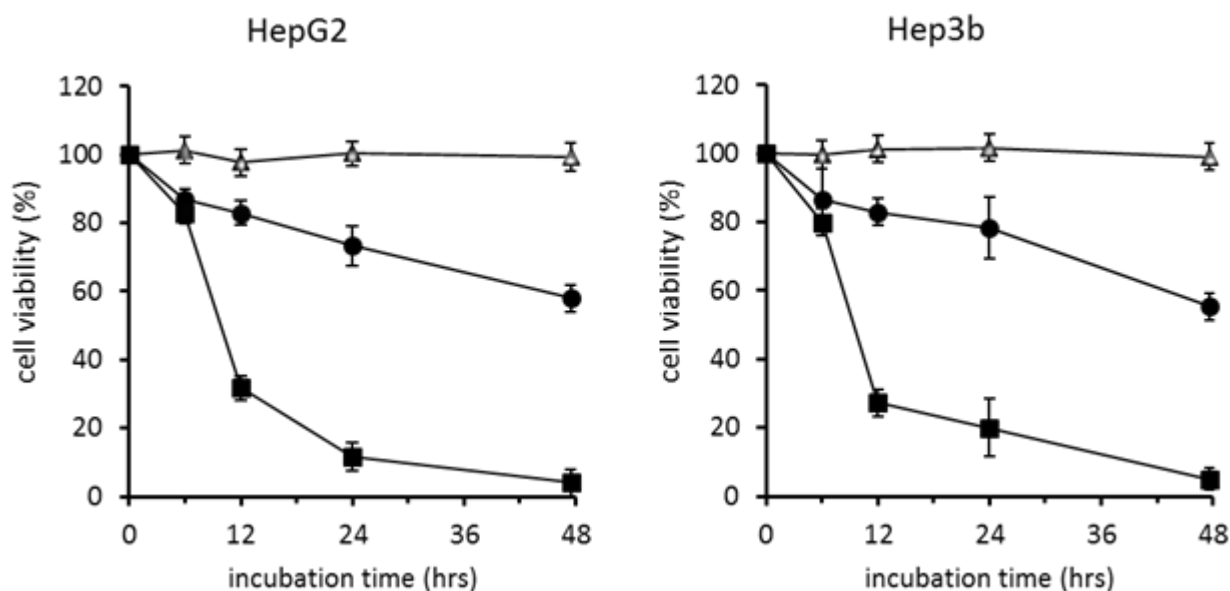


Figure 35 : Sensitization of human hepatocarcinoma cell lines HepG2 and Hep3b to doxorubicin cytotoxicity by *ent*-kaurane-type diterpenoid compound 5. Cell viability was analyzed as a function of time and measured using a MTT assay. HepG2 (left panel) and Hep3b (right panel) cells were incubated during 6, 12, 24 or 48 hours with 20 μ M doxorubicin alone (full circle) or in combination to compound 5 at a subtoxic concentration (1 μ M, full square) of compound 5. As control, compound 5 alone at 1 μ M (open triangle) exhibited no significant cytotoxicity. Values shown are the mean $\pm$ SD from three independent experiments. For a given time, p value < 0.05 (*) or < 0.001 (***) indicated statistically significant differences between measures done with doxorubicin alone and in combination to compound 5.

Enhanced ROS production has been associated to the cumulative effects between doxorubicin and withaferin A in ovarian cancer (Fong M. Y., 2012) or selenocystine in HCC (Fan C., 2014). Interestingly, as discussed above, *ent*-kaurane diterpenoid-induced formation of ROS also mediates cytotoxicity (Lin Z., 2015) and apoptosis (Thuong P. T., 2014). The fact that ROS generation is also one of the mechanisms of action involved in doxorubicin

antitumor activity (Gewirtz DA., 1999; Minotti G., 2004) may be a possible explanation of the observed additive effects between compound 5 and doxorubicin. To complete our work, further biochemical studies are necessary to confirm this hypothesis. All together, these data suggest that *ent*-kaurane diterpenoid-induced ROS formation may play a dual role in cancer cells: sensitization at low subtoxic concentrations on one hand, apoptosis-mediated cytotoxicity at higher concentrations on the other.

2. Virtual Screening research and Biochemical studies of solasonine

2.1. Results of Virtual Screening database of Vietnamese plants

2.1.1. *In silico* screening of natural products library isolated from Vietnamese plants

The 354 molecules were screened for their ability to interact both with mortalin on one side and p53 on the other using the models 3N8E and 1AIE for mortalin and p53 respectively.

As shown in Figure 36, most of the molecules exhibited a moderate or weak binding score. A set of 11 molecules out of 354 (3.1 %) with a high binding score emerged, among which a subset of 5 with values higher than 200 (Table 17).

With the lowest binding energy of mortalin interaction (-15.62 kcal/mol) and the fourth lowest binding energy of p53 interaction (-14.53 kcal/mol), solasonine, a steroidal alkaloid glycoside (Figure 37) isolated from the Vietnamese plant *Solanum xanthocarpum* displayed the highest combined score. Very interestingly, solamargine, a closely related molecule, exhibited the fourth highest combined score.

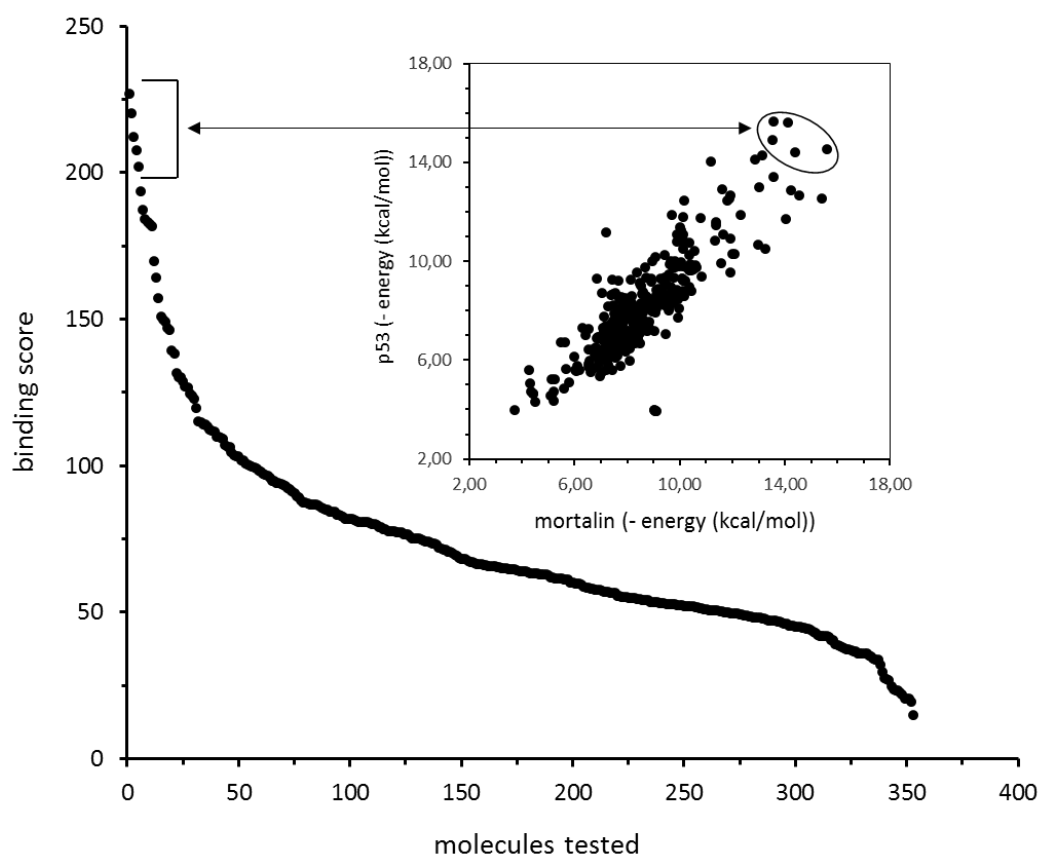


Figure 36 : Binding score of the 354 natural compounds from the Vietnamese plants database. For a given compound, the binding score was defined as the product of its respective binding energies to mortalin and p53. The insert shows the 2D distribution (p53 vs mortalin) of the binding energies of the 354 compounds (1 dot represents 1 compound). The 5 compounds with the highest binding scores (values > 200) are circled in the insert.

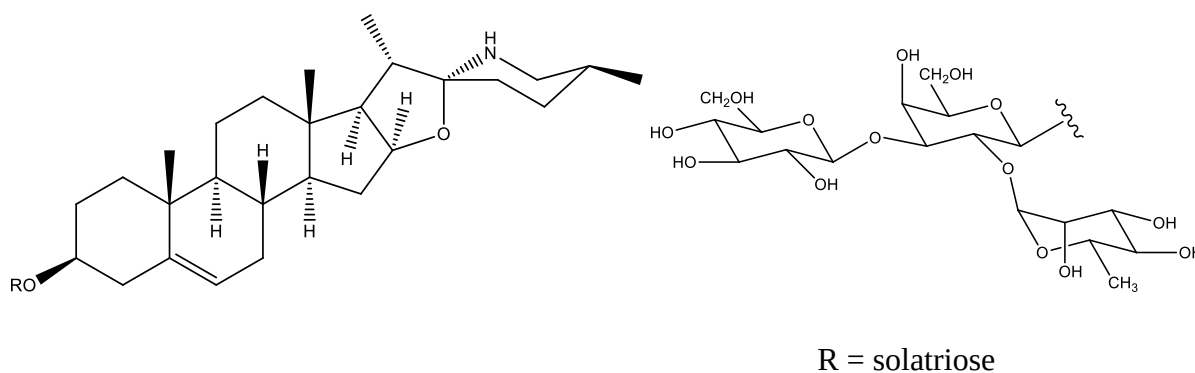


Figure 37 : solasonine structure

Rank	Compound	Binding score	Binding energy (kcal/mol)	
			Mortalin	p53
1	solasonine	226,96	- 15,62	- 14,53
2	acantрифосиде C	220,27	- 14,12	- 15,60
3	acantрифосиде B	212,08	- 13,56	- 15,64
4	solamargine	207,50	- 14,42	- 14,39
5	quercetin-3,7-dimethylether-3'-O- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- β -D-glucopyranosid	202,00	- 13,53	- 14,93
	<i>withanone (control)</i>	<i>106.29</i>	<i>- 10.40</i>	<i>- 10.20</i>

Table 17 : The five natural compounds with the highest binding score. The binding score (middle column) was defined as the product of the binding energies to mortalin and p53 (two right columns). Withanone is shown as a control.

Solamargine and solasonine have been described to exert antiproliferative activity in different tumor cell lines (Munari C. C., 2014). Further, solamargine has been shown to trigger apoptosis in human Hep3b (Kuo K. W., 2000), SMMC-7721 and HepG2 (Ding X., 2012; Xie X., 2015) hepatoma or osteosarcoma U2OS (Li X., 2011) cell lines. Withanone, a withanolide isolated from *Withania somnifera*, shown to bind to mortalin and to abrogate mortalin – p53 interactions (Grover A., 2012; Vaishnavi K., 2012) was screened as a control. In our assay, withanone was ranked at the 34th position, interacting with mortalin (PDB-ID: 3N8E) with a binding energy (-10.4 kcal/mol) comparable to that previously described using the same model (Vaishnavi K., 2012).

In additional experiments, we measured the binding energy of solasonine, solamargine and withanone in two other structural models 4KBO and 3D09 of mortalin and p53 respectively: model 4KBO focuses on domain aa 253-282 of mortalin also described as a possible site of interaction with p53 (Grover A., 2012) and p53 model 3D09 representing the p53 sequence aa 96-290 which is suggested to affect its interaction with mortalin (Vaishnavi

K., 2012). In these two models, solasonine and withanone showed the lowest and highest binding energy respectively (Table 18).

Compound	Mortalin		p53	
	3N8E	4KBO	1AIE	3D09
solasonine	- 15.62	- 9.63	- 14.53	- 14.14
solamargine	- 14.42	- 9.15	- 14.39	- 13.63
withanone	- 10.40	- 7.11	- 10.22	- 8.36

Table 18 : Binding energies of solasonine and solamargine to mortalin and p53. Binding energy (kcal/mol) was calculated in two different models of mortalin (3N8E and 4KBO) and in two different models of p53 (1AIE and 3D09). Withanone was used as the control.

Also, all the three compounds bound with a better binding energy to aa 439-597 sequence (model 3N8E) than to aa 253-282 sequence (model 4KBO) of mortalin and with a better binding energy to aa 312-352 sequence (model 1AIE) than to aa 96-290 sequence of p53. One can thus hypothesize that the preferential binding sites of these compounds on either mortalin and/or p53 will be those of respective lowest binding energy.

2.1.2. Docking of solasonine into mortalin and p53

Solasonine was then docked into mortalin and p53 using the models 3N8E and 1AIE respectively, for which solasonine presented the better interactions. Docking is illustrated in Figure 38 and hydrogen bond interactions are reported in Table 19.

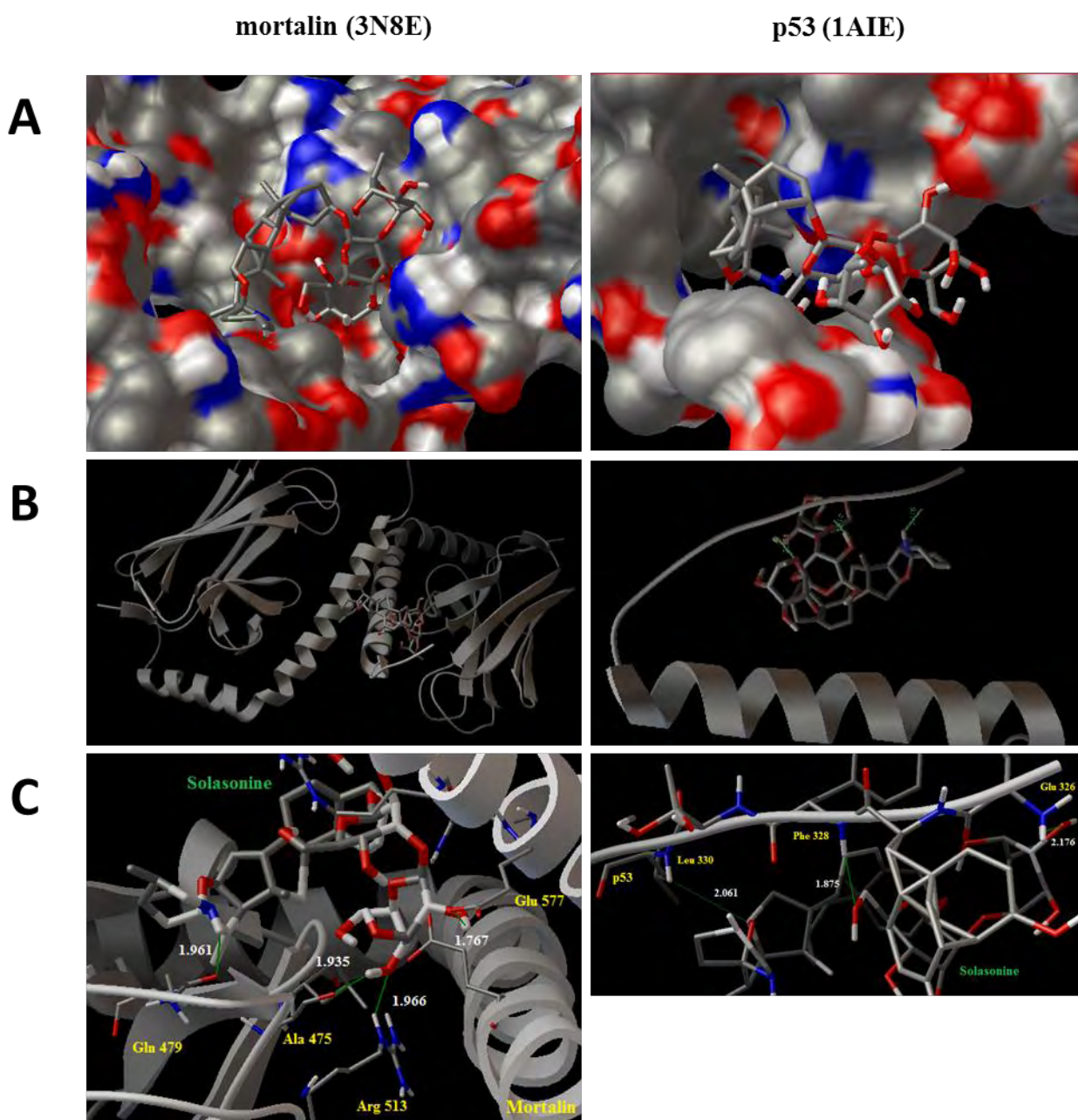


Figure 38 : Interactions of docked solasonine with mortalin (left panels) and p53 (right panels). **A** - Solasonine occupying the mortalin and p53 spaces that are crucial for their interaction. Nitrogen, oxygen and aliphatic carbon atoms of the proteins are colored in blue, red and grey respectively. **B** - Multi-template homology modeled 3D structures. **C** - Closer views showing the hydrogen bonds between solasonine and mortalin or p53. The interacting residues of mortalin or p53 are shown in yellow.

Protein	Hydrogen bond interactions	Distance (Å)	Binding energy (kcal/mol)
Mortalin (3N8E)	Ala (475) <u>O</u> ... H	1.935	- 15.62
	Gln (479) <u>OE1</u> ... H	1.961	
	Arg (513) <u>HE</u> ... O	1.966	
	Glu (577) <u>OE2</u> ... H	1.767	
p53 (1AIE)	Glu (326) <u>HN2</u> ... O	2.176	- 14.53
	Phe (328) <u>HN</u> ... O	1.875	
	Leu (330) <u>O</u> ... H	2.061	

Table 19 : Hydrogen bond interactions between solasonine and mortalin or p53. The protein models used are 3N8E for mortalin and 1AIE for p53. The residues involved in an hydrogen bond with solasonine are mentioned and their interacting atoms are underlined. Calculated distance (Å) is indicated for each hydrogen bond. The binding energy (kcal/mol) of solasonine to each protein is recalled.

As shown, solasonine bound efficiently to mortalin with a low binding energy (-15.62 kcal/mol) by forming strong hydrogen bonds (with distances shorter than 2 Å) with 4 residues (Ala 475, Gln 479, Arg 513 and Glu 577) located in the substrate binding domain of mortalin. Interestingly, one of these residues, Arg 513, has been described as the “latch” between the “lid and cleft regions” of mortalin, thereby playing an important role in the chaperone function of this protein (Kaul S. C., 2007). Withanone and the related withanolide molecule withaferin-A have also been shown to interact with Mortalin Arg 513 (Vaishnavi K, 2012). Solasonine also bound to p53 but less efficiently than to mortalin both in terms of binding energy (- 14.53 kcal/mol) and affinity by engaging only 3 hydrogen bonds formed with 3 residues (Glu 326, Phe 328 and Leu 330). On the basis of these interactions with residues located within the “hot spot” of mortalin – p53 interactions, one may thus hypothesize that solasonine will behave as a potent inhibitor of these interactions by binding with high affinity to mortalin and/or, to a lesser extend, p53.

2.2. Pharmacological studies of solasonine in HepG2 and Hep3b cell lines

2.2.1. Cytotoxic activity of solasonine in HepG2 and Hep3b cell lines

The inhibitory effects of solasonine on HepG2 and Hep3b cell viability was first measured at 24 hours and 48 hours of incubation and compared with those of solamargine and withanone (Table 20).

	IC ₅₀ (μM)			
	24hrs		48 hrs	
	HepG2	Hep3b	HepG2	Hep3b
solasonine	7.1 ± 0.4	4.5 ± 0.3	4.7 ± 0.2	4.0 ± 0.1
solamargine	7.6 ± 0.4	3.9 ± 0.1	5.5 ± 0.5	3.8 ± 0.2
withanone	12.3 ± 1.4	8.2 ± 0.4	5.1 ± 0.3	4.4 ± 0.1

Table 20 : Cytotoxic activity of solasonine and solamargine in HepG2 and Hep3b cell lines. IC₅₀ (μM) were measured at 24 and 48 hours of incubation in presence of increasing concentrations of solasonine (0.032 - 500 μM) in HepG2 and Hep3b cell lines. Withanone was used as a positive control. Data are the mean ± SD of three independent experiments.

For each time and in each cell line, solasonine and solamargine exhibited a similar activity. At 24 hours, solasonine and solamargine showed higher IC₅₀ values in HepG2 (7.1 ± 0.3 μM and 7.6 ± 0.4 μM respectively) than in Hep3b (4.5 ± 0.3 μM and 3.9 ± 0.1 μM respectively). At 48 hours, the IC₅₀ values observed were lowered in HepG2 (4.7 ± 0.2 μM and 5.5 ± 0.5 μM respectively), being closer and comparable to those observed in Hep3b, which remained unchanged (4.0 ± 0.1 μM and 3.9 ± 0.2 μM respectively). In a second time, the antiproliferative activity of solasonine was measured as a function of time (Figure 39). HepG2 cells were less sensitive than Hep3b cells to solasonine. As shown in Figure 39 A, in presence of 20 μM solasonine, HepG2 cell viability decreased in a time-dependent manner,

reaching 50% after 6 hours and remaining stable around 20% after 24 hours. In contrast to the observation made in HepG2, Hep3b cell viability decreased dramatically within the first 1.5 hour, reaching around 25% after 3 hours and being less than 5% after 12 hours.

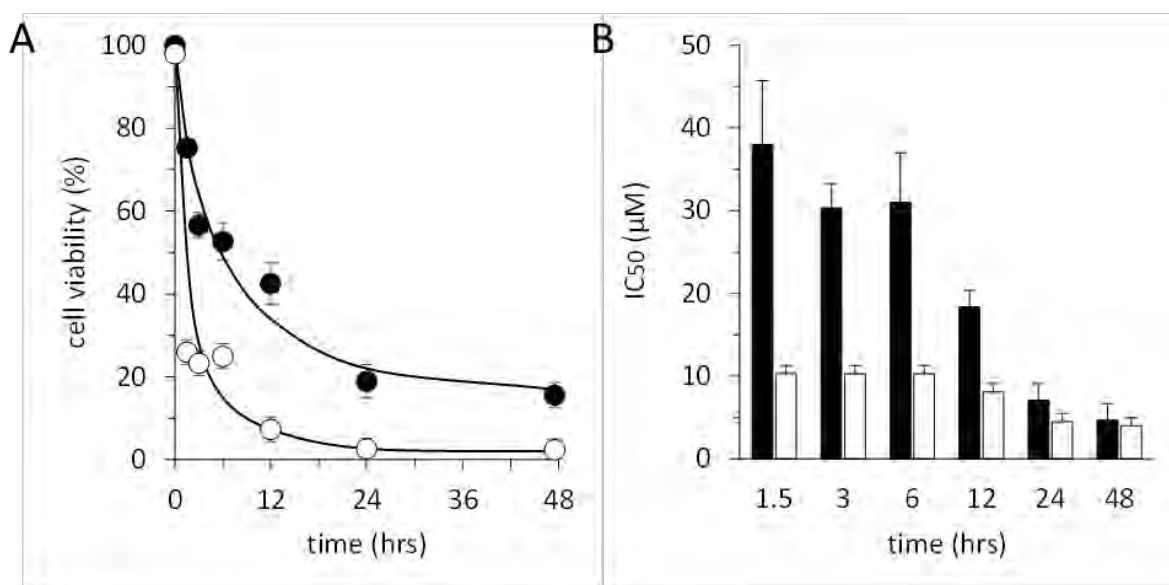


Figure 39 : Cytotoxic activity of solasonine in HepG2 and Hep3b cell lines. **A** – Cell viability was measured as a function of time of incubation in presence of solasonine (20 µM) in HepG2 (black circles) and Hep3b (white circles) cell lines. **B** – IC₅₀ (µM) were measured as a function of time of incubation in presence of increasing concentrations of solasonine (0.032 - 500 µM) in HepG2 (black histogram) and Hep3b (white histogram) cell lines. In **A** and **B**, data are the mean ± SD of three independent experiments.

This latest finding was similar to that observed previously with solamargine in the same Hep3b cell line (Kuo K. W., 2000). Further experiments done in presence of increasing concentrations of solasonine allowed the measurement of IC₅₀ values as a function of time (Figure 39 B). The IC₅₀ values at 24 hours and 48 hours have been reported above (see Table 20). For shorter times, a marked difference was observed between the two cell lines. From 12 hours to 1.5 hour, the IC₅₀ values increased gradually in HepG2 (from 18.4 ± 0.9 µM to 38.0 ± 8.2 µM) whereas they remained stable in Hep3b in the 10 µM range (from 8.1 ± 0.2 µM to 10.5 ± 1.0 µM).

Analyzing the molecular or cellular parameters explaining such a difference in the cytotoxic activity in the two cell lines at short incubation times (below 12 hours) falls out of the scope of the present work but remains to be explored in further studies. However, one possible explanation could come from the morphology and membrane structure which differ between the two cell lines, the thinner cellular membrane of the Hep3b cells allowing a rapid penetration of solasonine within the cell and thus increasing its cytotoxicity.

2.2.2. Effect of p53 inhibitor pifithrin-alpha (PFT- α) on solasonine-induced cytotoxicity

Cytotoxic properties of solasonine were further tested in absence or presence of PFT- α , a small molecule inhibitor of p53 function (Komarov P. G., 1999). Solasonine was used at concentrations around the IC₅₀ values determined previously at 12 hours and 24 hours of incubation. As shown in Figure 40, PFT- α restored viability of HepG2 cells in a concentration-dependent manner at both time of incubation.

At 12 hours, the effect was statistically significant with a marked increased in cell viability, from 53.9 ± 3.1 % in absence of PFT- α to 83.6 ± 7.2 % in presence of 30 μ M PFT- α . The same significant effect, but less marked, was also observed at 24h, cell viability increasing from 51.4 ± 1.6 % in absence of PFT- α to 67.4 ± 0.7 % in presence of 30 μ M PFT- α . In the same conditions, viability of Hep3b cell was unaffected by exposure to PFT- α (from 55.7 ± 4.7 % in absence of PFT- α to 57.3 ± 0.7 % in presence of 30 μ M PFT- α at 12 hours and from 51.9 ± 4.1 % in absence of PFT- α to 52.8 ± 1.6 % in presence of 30 μ M PFT- α at 24 hours).

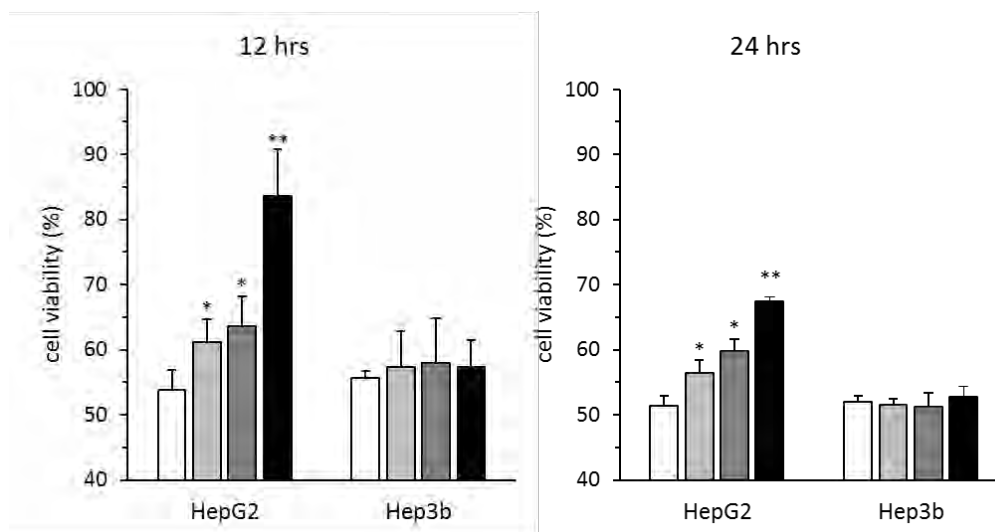


Figure 40 : Effect of PFT- α on solasonine-induced cytotoxicity. Viability of HepG2 and Hep3b cells was measured after 12 hours (left panel) and 24 hours (right panel) of incubation in presence of solasonine at a concentration close to its respective IC_{50} values for each cell line (see figure 39 B), in absence (white histograms) or presence of 10, 20 or 30 μ M of PFT- α (light grey, grey and black histograms respectively). Histograms and bars are the means $\pm$ SD of three independent experiments.

At 12 hours, an almost complete reversion of cell viability could be obtained by blocking p53 function. However, PFT- α has been reported to mediated this effect regardless of the presence or absence of p53 (Sohn D., 2009). In our hands, effect of PFT- α on cell viability was clearly and only observed in p53 expressing HepG2 and not at all in the p53 deficient Hep3b cell line. This suggests that the cytotoxic effect of solasonine may be either p53-dependent and/or p53-independent in these two hepatoma cell lines.

2.2.3. Apoptotic properties of solasonine in HepG2 and Hep3b cell lines

For each cell line, the concentrations of solasonine were chosen around the respective IC_{50} values determined at 24 hours in the cytotoxicity assay (i.e. 7.1 ± 0.3 μ M for HepG2 and 4.5 ± 0.3 μ M for Hep3b, see Table 20 above). Results are presented in Figure 41.

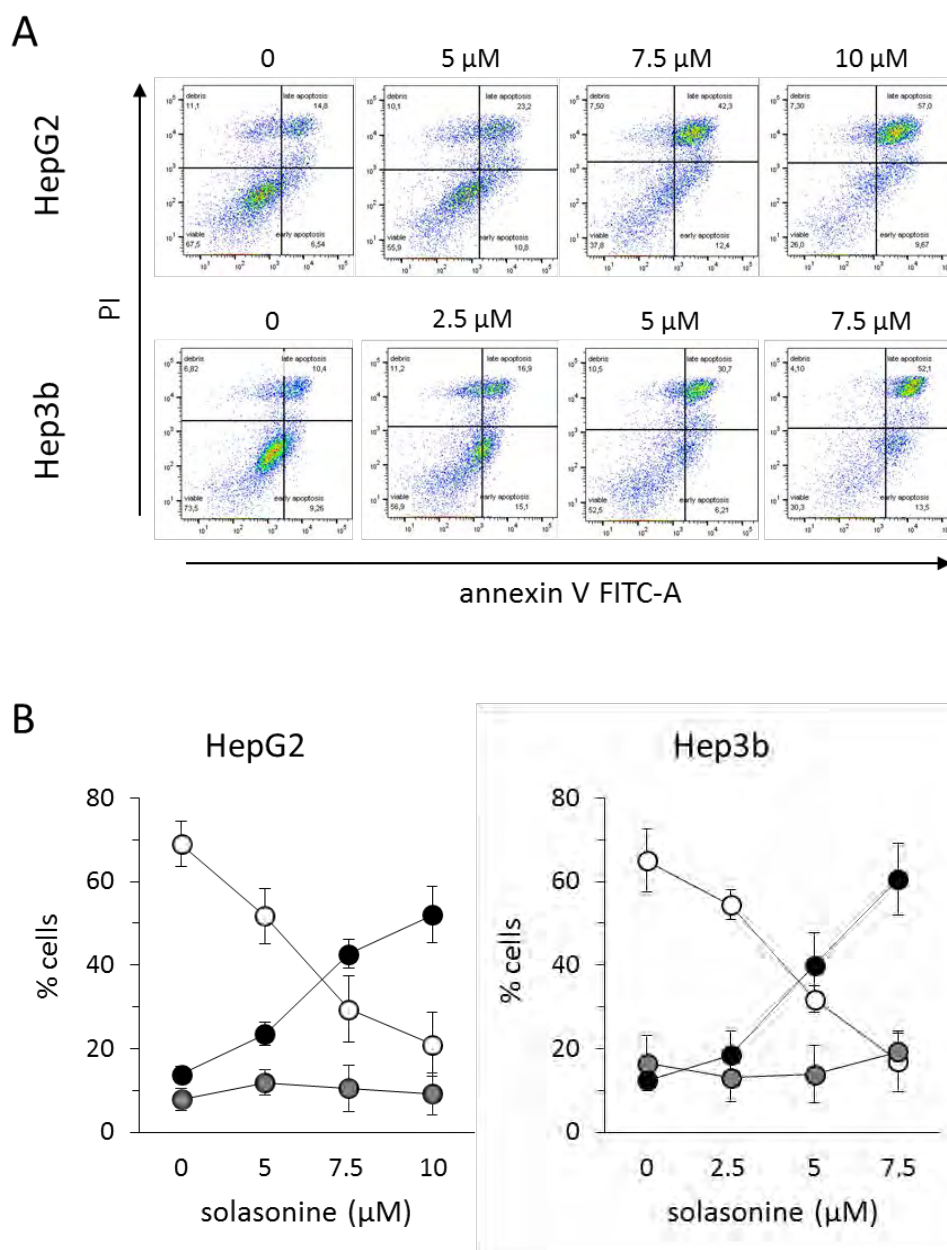


Figure 41 : Induction of apoptosis of human hepatocarcinoma cell lines HepG2 and Hep3b by solasonine. HepG2 and Hep3b cells were incubated with no (control) or increasing concentrations of solasonine for 24 hours followed by staining with Annexin V FITC and propidium iodide. DNA content and Annexin V expression were analyzed by flow cytometry. **A** - Data presented are from one representative experiment performed on HepG2 and Hep3b cell lines. In each panel, viable, early apoptotic and late apoptotic cells are gated in lower left, lower right and upper right quarters respectively (debris are shown in upper left quarter). **B** – Percentage (%) of viable, early apoptotic and late apoptotic (white, grey and black circles respectively) HepG2 and Hep3b cells as a function of increasing concentrations of solasonine. Data are the mean and SD from 3 independent experiments.

Panel A illustrates the observed cell populations in absence or presence of increasing concentrations of solasonine in a representative experiment performed on HepG2 or Hep3b cell lines. As shown in panel B, when both cell lines were incubated for 24 hours with solasonine, the % of viable cells declined in a concentration-dependent manner, dropping from about 70% in absence of solasonine to about 20% at the highest concentration of solasonine tested. In parallel, the number of cells in the double positive cell populations (which contain late apoptotic and/or necrotic cells) increased in a continuous manner for HepG2 and Hep3b cell lines from about 10% in absence of solasonine to about 50-60 % at the highest concentration of solasonine tested. In contrast, the % of early apoptotic cell populations was rather stable and remained below 15-20% even at the highest concentration of solasonine tested.

2.2.4. Subcellular localization of p53 upon exposure to solasonine in HepG2 cell line

As a first control experiment, we analyzed the presence of p53 and mortalin in each HCC cell line. As shown in Figure 42 A, mortalin was present in both HepG2 and Hep3b cell lines, as previously reported (Xin Y., 2008; Chen J., 2014). In contrast, as expected, p53 was present only in HepG2 cell line and not detectable in Hep3b cell line, as previously reported (Lu W. J., 2011).

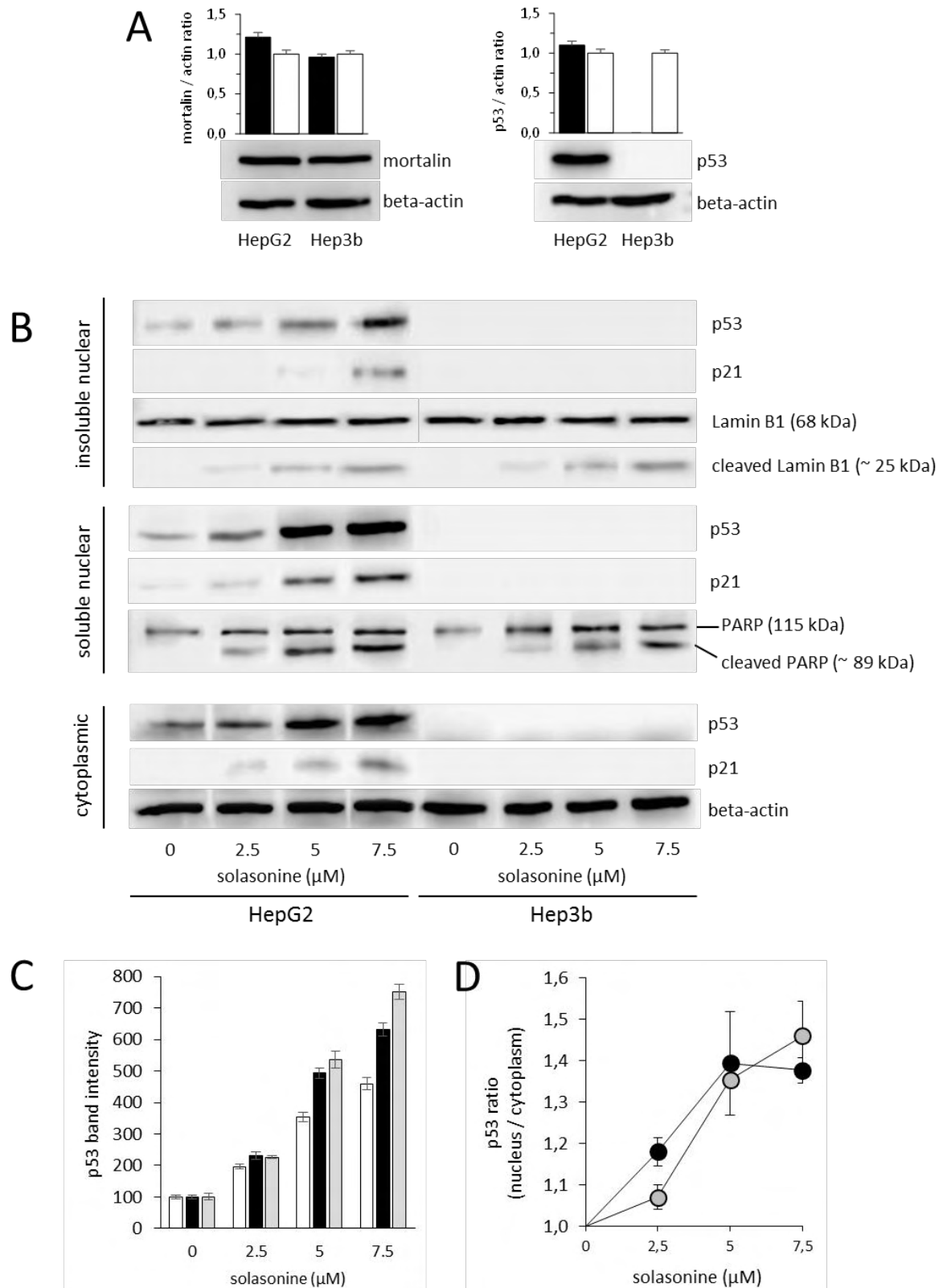


Figure 42 : A – Western-blot detection of mortalin and p53 in HepG2 and Hep3b cell lines. Beta actin was used as the loading control. Results are expressed as the ratio between band intensity of mortalin and beta actin (black and white histograms, left figure) or p53 and beta actin (black and white histograms, right figure). **B** - Western-blot detection of p53 and p21 in cytoplasmic, soluble nuclear and insoluble nuclear fractions of HepG2 and Hep3b cell lines in absence or presence of increasing concentrations of solasonine. Beta actin was used as the loading control in cytoplasmic fractions. Cleaved PARP and cleaved Lamin B1 were used as apoptosis markers in soluble nuclear and insoluble nuclear fractions respectively. **C**- p53 band intensity measured as a function of solasonine concentration in the cytoplasmic, soluble nuclear and insoluble nuclear fractions (white, black and grey histograms respectively). For each fraction, band intensity in absence of solasonine was used as the reference value (100). **D** – p53 band intensity ratio measured between soluble nuclear and cytoplasmic fractions (black circles) and between insoluble nuclear and cytoplasmic fractions (grey circles) as a function of solasonine concentration. The ratio measured in absence of solasonine was used as the reference ratio (= 1). In panels **A**, **C** and **D**, data are the mean $\pm$ SD of 3 independent experiments.

Subcellular localization of p53 in HepG2 cells was then analyzed in absence or presence increasing concentrations of solasonine (Figure 42 B, C). In absence of solasonine, p53 was present mainly in the cytoplasmic fraction and, to a lesser extend, in the soluble nuclear fraction, while being barely detectable in the insoluble nuclear fraction.

In presence of solasonine, the amount of p53 increased in a concentration-dependent manner in all the three fractions (Figure 42 B, C). The increased amount of p53 was more important in the nucleus (both soluble and insoluble fractions) than in the cytoplasm (Figure 42 C, D), reflecting or resulting of a translocation of p53 from cytoplasmic to the nucleus.

One can reasonably hypothesize that this is the consequence of the inhibition of p53 - mortalin interaction by solasonine and the abrogation of the cytoplasmic sequestration of p53 by mortalin.

2.2.5. Activation of signaling pathways upon exposure to solasonine in HepG2 and Hep3b cell lines

Accumulation of p53 protein in the nucleus leads to the activation of numerous target genes that mediate its tumor suppressor functions, among which p21Waf1/Cip1, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors (Harris S. L., 2005). In addition to its cell-cycle inhibitor function, p21 has been reported to affect, either negatively or positively, p53 induction of apoptosis (Abbas T., 2009). As shown in Figure 42 B, in unexposed HepG2 cells, as expected, p21 was absent (cytosolic and insoluble nuclear fractions) or barely detectable (soluble nuclear fraction). In HepG2 cells exposed to solasonine, concomitant with p53 accumulation in the nucleus, p21 expression also increased in a concentration-dependent manner, predominantly in the soluble nuclear fraction. The role played by p21 in HepG2 cells incubated by solasonine remains to be defined in further studies. In p53 negative Hep3b cells, no p21 expression was observed neither in the cytoplasmic nor the nuclear fractions, as expected.

To further assess the apoptotic properties of solasonine, we analyzed the ability of solasonine to induce the cleavage of nuclear lamin B1 and poly (ADP-ribose) polymerase PARP proteins. Cleavage products resulting from the enzymatic action of caspase on these two proteins represent markers for apoptosis. As shown in Figure 42 B, incubation of HepG2 cells with increasing amount of solasonine resulted in a concentration-dependent formation of cleaved PARP and cleaved Lamin B1 as detected in the soluble and insoluble nuclear fractions respectively. Interestingly, these two cleaved products were also observed in nuclear fractions of Hep3b cells.

This observation indicated that solasonine does induce apoptosis in both p53-expressing and p53-deficient cell lines, in accordance with the observations made in the annexin V – PI experiments (Figure 41) discussed above via p53-dependent in HepG2 cells and p53-independent mechanism in Hep3b cells. In these later ones, the mechanism by which solasonine induces apoptosis remains to be elucidated. Since mortalin is present in Hep3b cells (Figure 42 A, Xin Y., 2008; Chen J., 2014), a possible hypothesis is that solasonine is also able to inhibit the interaction between mortalin, and a transcription factor implicated in p53-independent apoptosis pathways, then allowing it to translocate to the nucleus. Further studies are needed to confirm this hypothesis.

F - Conclusion and Perspective

Until now, despite all the efforts made in the search for effective treatments of hepatocellular carcinoma, none of the approaches have been satisfactory and have not yet resulted in highly efficient treatments. Therefore, the objective of finding new bioactive compounds and/or new therapeutic strategies must not be abandoned. Keeping this in mind, two approaches have been followed in this thesis focused on the study of Vietnamese natural compounds originated from plants.

The first part of this thesis was focused on the pharmacological screening of a database of 26 natural compounds. As a results, 7 diterpenoids (the *ent*-kaurane family) were identified to be potential bioactive against hepatocarcinoma, through a classical cytotoxicity assay. The mechanism of action of these compounds has been then explored in deeper details.

Our study not only showed that *ent*-kaurane diterpenoids displayed a structure-related, concentration-dependent, apoptosis-induced cytotoxicity against two human HCC model cell lines but also revealed that they were active at sub-toxic concentration, by sensitizing HCC cells to the anticancer agent doxorubicin. As previously proposed for other natural products (Lee W. Y., 2010; Fong M. Y., 2012; Fan C., 2014; Gambari R., 2014), interaction of *ent*-kaurane diterpenoids with doxorubicin may be viewed as a possible way to enhance the antitumor therapeutic effects of doxorubicin. Conversely, ROS accumulation is involved in the cardiotoxic side effects of doxorubicin (Minotti G., 2004; Hequet O., 2014) and cumulative ROS generation could then result in an unwished potentiation of cardiotoxicity.

Taken all together, our data bring strong support to the potential interest of *ent*-kaurane diterpenoids in the cancer field and more precisely against HCC. Eventhough virtually no acute toxicity has been observed *in vivo* (Zou Q. F., 2013), the side effects of *ent*-kaurane diterpenoids remain poorly documented to date and their careful evaluation should be undertaken, in a perspective of future clinical applicability and development (Wang L., 2011). In parallel, one may expect that the intrinsic low aqueous solubility and weak bioavailability of these natural products (Xu W., 2006) could be successfully overcome through, for example, *ent*-kaurane-based design of synthetic molecules (Ding C., 2014).

In the second approach, 354 compounds originated from Vietnamese plants were virtually screened using AutoDock program in an effort to find appropriate molecules that can abrogate the interaction between Mortalin and p53. So doing, solasonine was identified as the most potential compound. Pharmacological studies demonstrated that solasonine inhibited

Mortalin – p53 interaction, allowing p53 to translocate from cytoplasm into nucleus and then inducing cell death of human hepatocellular carcinoma cell lines through a p53-dependent pathways. Solasonine can exert its action by interacting with high affinity to either mortalin or p53. Interestingly enough, one may note that none of the numerous and critical mutations affecting p53 in cancer cells (Tornesello M. L. 2013; <http://www-p53.iarc.fr>) lies in the p53 sequence to which binds solasonine. With no doubt, this represents an added value to the original pharmacologic properties of solasonine. In parallel to its p53-dependent apoptotic properties in p53-expressing hepatoma cells, we also evidenced powerful p53-independent apoptotic effects of solasonine in p53-deficient hepatoma cells. Eventhough this mechanism remains to be fully elucidated, this dual and complementary properties may bring a further additional interest to solasonine by making wider the panel of cancer cells sensitive to solasonine. Additional studies must be undertaken to support the preliminary results obtained in this thesis. In particular, the interactions between mortalin and p53 should be analyzed at the molecular and biochemical levels on purified proteins or cell extracts and at the biological level by using immunostaining and microscope analysis.

In terms of perspective, two complementary axis of research can logically be proposed. First, on the “pharmacological side”, more studies are, indeed, needed to validate as “promising leads” the natural compounds identified in this study. In particular, if we keep in mind the ultimate goal of finding molecules of therapeutic interest, studies should be undertaken to determine the activity profile of the compounds on a large panel of cell lines from cancerous and normal origins, and the possible mechanisms of resistance, in addition to *in vivo* studies performed in animal models. Second, on the “chemical side”, we will keep updating and expanding the database of Vietnam natural compounds not only from plant and marine resources but also from micro-organisms. One may expect or hope that this will be a mean to increase the chances to discover a “yet hidden” natural compound of highest therapeutic interest, not only in cancer but also in any deadly disease affecting our modern societies, whatever their level of development.

G - References

- Abbas T, Dutta A. (2009). p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer* 9: 400–414
- Amna T., Puri S. C., Verma V., Sharma J. P., Khajuria R. K., Musarrat J., Spiteller M., Qazi G. N., 2005. Bioreactor studies on the endophytic fungus *Entrophospora infrequens* for the production of an anticancer alkaloid camptothecin. *Canadian Journal of Microbiology*, Vol 52, P.189 – 196.
- Andreeff M., Kelly K. R., Yee K., Assouline S., Strair R., Popplewell L., Bowen D., Martinelli G., Drummond M. W., Vyas P., Kirschbaum M., Iyer S. P., Ruvolo V., González G. M., Huang X., Chen G., Graves B., Blotner S., Bridge P., Jukofsky L., Middleton S., Reckner M., Rueger R., Zhi J., Nichols G., Kojima K., 2016. Results of the Phase I Trial of RG7112, a Small-Molecule MDM2 Antagonist in Leukemia. *Clinical Cancer Research*, Vol 22, P. 868 - 876.
- Anh P. T. H., Duc N. B., 2002. The situation with cancer control in Vietnam. *Japanese Journal of Clinical Oncology*, Vol 32, Supplement 1, P.92 – 97.
- Anthony P. P., 2002. Tumors and tumor-like lesions of the liver and biliary tract: etiology, epidemiology and pathology. *Pathology of the Liver*, 4th edition. Edited by: Macsween R., Burt A., Portmann B., Ishak K., Scheuer P., Anthony P. London, New York, Sydney, Toronto: Churchill Livingstone, P.711 – 775.
- Arcamone F., 2005. Anthracyclines. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. Anticancer agents from natural products. *CRC Press, Taylor and Francis Grop, Boca Raton*, P.299 – 320.
- Badvie S., 2000. Hepatocellular Carcinoma. *Postgraduate Medical Journal*, Vol 76, P.4 – 11.
- Bain I., McMaster P., 1997. Benign and malignant liver tumors. *Surgery*, Vol 15, P.169 – 174.
- Ban J. Y., Cho S. O., Koh S. B., Song K. S., Bae K., Seong Y. H., 2006. Protection of amyloid β protein (25 – 35)-induced neurotoxicity by methanol extract of *Smilacis chinae* rhizome in cultured rat cortical neurons. *Journal of Ethnopharmacology*, Vol 106, P.230 – 237.
- Bernd K., Matthias R., Thomas L., 1999. Evaluation of the FlexX incremental construction algorithm for protein-ligand docking. *Proteins*, Vol 37, P.228 – 241.
- Burnette W. N., 1981. “Western Blotting”: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry*, Vol 112, Issue 2, P.195 – 203.

- Butler M. S., 2008. Natural products to drugs: natural product-derived compounds in clinical trials. *Natural Product Report*, Vol 25, P.475 – 516.
- Byrd J. C., Peterson B. L., Gabrilove J., Odenike O. M., Grever M. R., Rai K., Larson R. A., Cancer and Leukemia Group B, 2005. Treatment of relapsed chronic lymphocytic leukemia by 72-hour continuous infusion or 1-hour bolus infusion of flavopiridol: results from Cancer and Leukemia Group B study 19805. *Clinical Cancer Research*, Vol 11, P.4176 – 4181.
- Carvalho C., Santos R. X., Cardoso S., Correia S., Oliveira P. J., Santos M. S., Moreira P. I., 2009. Doxorubicin: the good, the bad and the ugly effect. *Current Medicinal Chemistry*, Vol 16, Issue 25, P.3267 – 3285.
- Cervello M., McCubrey J. A., Cusimano A., Lampiasi N., Azzolina A., Montalto G., 2012. Targeted therapy for hepatocellular carcinoma: novel agents on the horizon. *Oncotarget*, Vol 3, No. 3, P.236 – 260.
- Chen J., Liu W.B., Jia W.D., Xu G.L., Ma J.L., Huang M., Deng Y.R., Li J.S., 2014. Overexpression of Mortalin in hepatocellular carcinoma and its relationship with angiogenesis and epithelial to mesenchymal transition. *International Journal of Oncology*, Vol 44, P. 247-255.
- Chi V. C., 1997. Dictionary of Vietnamese Medicinal Plants. *Medicine Publishing House: Ho Chi Minh City*, P.622 – 623.
- Chu K. T., Ng T. B., 2006. Smilaxin, a novel protein with immunostimulatory, antiproliferative, and HIV-1-reverse transcriptase inhibitory activities from fresh *Smilax glabra* rhizomes. *Biochemical and Biophysical Research Communications*, Vol 340, P.118 – 240.
- Cragg G. M., Newman D. J., 2009. Nature: A vital source of leads for anticancer drug development. *Phytochemistry Reviews*, Vol 8, Issue 2, P.313 – 331.
- Cragg G. M., Newman D. J., 2013. Natural Products: A continuing source of novel drug leads. *Biochimica et Biophysica Acta*, Vol 1830, P.3670 – 3695.
- Dao T. T., Le T. V., Nguyen P. H., Thuong P. T., Minh P. T., Woo E. R., Lee K. Y., Oh W. K., 2010. SIRT 1 inhibitory diterpenoids from the Vietnamese medicinal plant *Croton tonkinensis*. *Planta Medica*, Vol 76, Issue 10, P.1011 – 1014.
- David L., Tania P., Bruno O V., Maria A M., 2009. DG-AMMOS: A new tool to generate 3D conformation of small molecules using distance geometry and automated molecular mechanics optimization for in silico screening. *BMC Chemical Biology*, Vol 9:6.

- Ding X., Zhu F.S., Li M., Gao S.G., 2012. Induction of apoptosis in human hepatoma SMMC-7721 cells by solamargine from *Solanum nigrum* L. *Journal of Ethnopharmacol.* Vol 139, P.599 - 604.
- Ding C., Wang L., Chen H., Wild C., Ye N., Ding Y., Wang T., White M. A., Shen Q., Zhou J., 2014. *Ent*-kaurane-based region- and stereoselective inverse electron demand hetero-Diels-Alder reactions: synthesis of dihydropyran-fused diterpenoids. *Organic and Biomolecular Chemistry*, Vol 12, Issue 42, P.8442 – 8452.
- Driscoll T., Takala J., Steenland K., Corvalan C., Fingerhut M., 2005. Review of estimates of the global burden of injury and illness due to occupational exposures. *American Journal of Industrial Medicine*, Vol 48, P.491 – 502.
- Duncan E. L., Wadha R., Kaul S. C., 2000. Senescence and immortalization of human cells. *Biogerontology*, Vol 1, Issue 2, P.103 – 121.
- Duvoux C., 1998. Epidemiology and diagnosis of HCC in cirrhosis. *Annales de Chirurgie*, Vol 52, Issue 6, P.511 – 517.
- Ewing T. J., Shingo M., Skillman A. G., Irwin D. K., 2001. Dock 4.0: Search strategies for automated molecular docking of flexible molecule databases. *Journal of Computer-aided Molecular Design*, Vol 15, P.411 – 428.
- Eyberger A. L., Dondapati R., Porter J. R., 2006. Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *Journal of Natural Products*, Vol 69, No. 8, P.1121 – 1124.
- Fan C., Zheng W., Fu X., Li X., Wong Y. S., Chen T., 2014. Strategy to enhance the therapeutic effect of doxorubicin in human hepatocellular carcinoma by selenocystine, a synergistic agent that regulate the ROS-mediated signaling. *Oncotarget*, Vol 5, No. 9, P.2853 – 2863.
- Feo F., Frau M., Tomasi M. L., Brozzetti S., Pascale R. M., 2009. Genetic and epigenetic control of molecular alterations in hepatocellular carcinoma. *Experimental Biology and Medicine*, Vol 234, No. 7, P.726 – 736.
- Finn R. S., 2013. Emerging targeted strategies in advanced hepatocellular carcinoma. *Seminars in liver disease*. Vol 33, S. 01, P.11 – 19.
- Flahive E., Srirangam J., 2005. The dolastatins: novel antitumor agents from *Dolabella auricularia*. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. Anticancer agents from natural products. *CRC Press, Taylor and Francis Group, Boca Raton*, P.191 – 214.

- Fong M. Y., Jin S., Rane M., Singh R. K., Gupta R., Kakar S. S., 2012. Withaferin A synergizes the therapeutic effect of doxorubicin through ROS-mediated autophagy in ovarian cancer. *Plos ONE*, Vol 7, Issue 7: e42265. Doi:10.1371/journal.pone.0042265.
- Fujioka T., Kashiwada Y., 1994. Anti-aids agents, 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzigium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. *Journal of Natural Products*, Vol 57, Issue 2, P.243 – 247.
- Gambari R., Hau D. K., Wong W. Y., Chui C. H., 2013. Sensitization of Hep3B hepatoma cells to cisplatin and doxorubicin by corilagin. *Phytotherapy Research*, Vol 28, P.781 – 783.
- Gareth J., Peter W., Robert C. G., Andrew R. L., Robin T., 1997. Development and validation of a genetic algorithm for flexible docking. *Journal of Molecular Biology*, Vol 267, P.727 – 748.
- Gewirtz DA., 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adrimycin and daunorubicin. *Biochemical Pharmacology*, Vol 57, Issue 7, P.727 – 741.
- Giang P. M., Son P. T., Matsunami K., Otsuka H., 2006. Anti-staphylococcal activity of *ent*-kaurane-type diterpenoids from *Croton tonkinensis*. *Journal of Natural Medicines*, Vol 60, P.93 – 95.
- Giang P. M., Jin H. Z., Son P. T., Lee J. H., Hong Y. S., Lee J. J., 2003. *Ent*-kaurane diterpenoids from *Croton tonkinensis* inhibit LPS-induced NF-kappaB activation and NO production. *Journal of Natural Products*, Vol 66, P.1217 – 1220.
- Giang P. M., Son P. T., Lee J. J., Otsuka H., 2004. Four *ent*-kaurane-type diterpenoids from *Croton tonkinensis* Gagnep. *Chemical and Pharmaceutical Bulletin (Tokyo)*, Vol 52, P.879 – 882.
- GLOBOCAN, 2012. *International Agency for Cancer Research – Liver cancer fact sheet*, <http://globocan.iarc.fr/> (accessed 25 September, 2015).
- Grover A., Priyandoko D., Gao R., Shandilya A., Widobo N., Bisaria V. S., Kaul S. C., Wadha R., Sundar D., 2012. Withanone binds to mortalin and abrogates mortalin-p53 complex: Computational and experimental evidence. *The International Journal of Biochemistry and Cell Biology*. Vol 44, P.496 – 504.
- Guéritte F., Fahy J., 2005. The Vinca Alkaloids. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. *Anticancer agents from natural products*. CRC Press, Taylor and Francis Group, Boca Raton, P.123 – 136.

- Hale K. J., Hummersone M. G., Manaviazar S., Frigerio M., 2002. The chemistry and biology of the bryostatin antitumor macrolides. *Natural Product Reports*, Vol 19, P.413 – 453.
- Harris C. C., 1976. The carcinogenicity of anticancer drugs: A hazard in man. *Cancer*, Vol 37, P.1014 – 1023.
- Harris SL, Levine AJ. 2005. The p53 pathway: positive and negative feedback loops. *Oncogene* Vol 24, P. 2899–2908
- Hattersley L., 2013. A second opinion – An insight into good health, disease and our relationship with them. *Lulu Press, Inc.* ISBN: 978-1291038941.
- He X. F., Yin S., Ji Y. C., Su Z. S., Geng M. Y., Yue J. M., 2010. Sesquiterpenes and dimeric sesquiterpenoids from *Sarcandra glabra*. *Journal of Natural Products*, Vol 73, P.45 – 50.
- Hecht S. M., 2005. Bleomycin group antitumor agents. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. Anticancer agents from natural products. *CRC Press, Taylor and Francis Grop, Boca Raton*, P.357 – 382.
- Henriquez R., Faircloth G., Cuevas C., 2005. Ecteinascidin 743 (Et743, Yondelis), aplidin and kahalalide F. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. Anticancer agents from natural products. *CRC Press, Taylor and Francis Grop, Boca Raton*, P.215 – 240.
- Hequet O., Le Q. H., Moullet I., Pauli E., Salles G., Espinoise D., Dumonlet C., Thieblemont C., Arnaud P., Antal D., Bouafia F., Coiffier B., 2004. Subclinical late cardiomyopathy after doxorubicin therapy for lymphoma in adults. *Journal of Clinical Oncology*, Vol 22, No. 10, P.1864 – 1871.
- Hocking G. M., 1997. A dictionary of natural products: Terms in the field of pharmacognosy relating to natural medicinal and pharmaceutical materials and the plants, animals and minerals from which they are derived. *Medford New Jersey, Plexus Publishing*, 994 pages.
- Hofle G., Reichenbach H., 2005. Epothilone, a myxobacterial metabolite with promising antitumor activity. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. Anticancer agents from natural products. *CRC Press, Taylor and Francis Grop, Boca Raton*, P.413 – 450.
- Hong Z., Chen W., Zhao J., Wu Z., Zhou J., Li T., Hu J., 2010. Hepatoprotective effects of *Rubus aleaefolius* Poir. and identification of its active constituents. *Journal of Ethnopharmacology*, Vol 129, P.267 – 272.

- Hou T., Xu X., 2004. Recent development and application of virtual screening in drug discovery: an overview. *Current Pharmaceutical Design*, Vol 10, No.9, P.1011 – 1033.
- Huong V. N., Sinh N. X., 2003. Isolation and identification of two triterpenoids from leaves of *Syzigium resinosum* Gagnep. 8th *Eurasia Conference on Chemical Science, Hanoi*, P.355 – 359.
- Iosefson O., Azem A., 2010. Reconstitution of the mitochondrial Hsp70 (mortalin)-p53 interaction using purified proteins – Identification of additional interaction regions. *FEBS Letters*, Vol 584, Issue 6, P.1080 – 1084.
- Irwin D. K., Jeffrey M. B., Stuart J. O., Robert L., Thomas E. F., 1982. A Geometric Approach to Macromolecule-Ligand Interactions. *Journal of Molecular Biology*, Vol 161, P.269 – 288.
- Itokawa H., Wang X., Lee K. H., 2005. Homoharringtonine and related compounds. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. *Anticancer agents from natural products*. CRC Press, Taylor and Francis Grop, Boca Raton, P.47 – 70.
- Ivanov A.A., Khuri F.R., and Haian F., 2013. Targeting protein-protein interactions as an anticancer strategy. *Trends in Pharmacological Sciences*, Vol 34, P.393-400.
- Jaki B. U., Franzblau S. G., Chadwick L. R., Lankin D. C., Zhang F., Wang Y., Pauli G. F., 2008. Purity – activity relationships of natural products: The case of anti-TB active ursolic acid. *Journal of Natural Products*, Vol 71, Issue 11, P.1742 – 1748.
- Jermal A., Bray F., Melissa M., Ferlay J., Ward E., Forman D., 2011. Global Cancer Statistics. *CA: A Cancer Journal for Clinicans*, Vol 61, No. 2, P.69 – 90.
- Kamei K., Matsuoka H., Furuhashi S. I., Fujisaki R. I., Kawakami T., Mogi S., Yoshihara H., Aoki N., Ishii A., 2000. Anti-malarial activity of leaf-extract of hydrangea macrophylla, a common Japanese plant. *Acta Medica Okayama*, Vol 54, No.5, P.227 – 232.
- Kaul S. C., Deocaris C. C., Wadhwa R., 2007. Three faces of mortalin: A housekeeper, guardian and killer. *Experimental Gerontology*, Vol 42, P.263 – 274.
- Kaul S. C., Aida S., Yaguchi T., Kaur K., Wadha R., 2005. Activation of wild type p53 function by its mortalin-binding cytoplasmically localizing carboxyl terminus peptides. *The Journal of Biological Chemistry*, Vol 280, No. 47, P.39373 – 39379.
- Kaul S. C., Yaguchi T., Taira K., Reddel R. R., Wadha R., 2003. Overexpressed mortalin (mot-2)/mthsp70/GRP75 and hTERT cooperate to extend the in vitro lifespan of human fibroblasts. *Experimental Cell Research*, Vol 286, Issue 1, P.96 – 101.

- Kaul S. C., Reddel R. R., Mitsui Y., Wadha R., 2001. An N-terminal region of Mot-2 binds to p53 in vitro. *Neoplasia*, Vol 3, No. 2, P.110 – 114.
- Kaur K., Widodo N., Nagpal A., Kaul S., Wadha R., 2007. Sensitization of human cancer cells to anti-cancer drugs by leaf extract of Ashwagandha (Lash). *Tissue culture research communications*, Vol 26, No. 4, P.193 – 199.
- Kennedy T., 1997. Managing the drug discovery/development interface. *Drug Discovery Today*, Vol 2, Issue 10, P.436 – 444.
- Khamis M. A., Gomaa W., Ahmed W. F., 2015. Machine learning in computational docking. *Artificial Intelligence in Medicine*, Vol 63, Issue 3, P.135 – 152.
- Kingston D. G. I., 2005. Taxol and its analogs. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. Anticancer agents from natural products. *CRC Press, Taylor and Francis Group, Boca Raton*, P.89 – 122.
- Komarov P. G., Komarova E. A., Kondratov R. V., Konstantin C., Coon J. S., Chernov M. V., Gudkov A. V., 1999. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science*, Vol 285, No.5434, P.1733 – 1737.
- Kuo K.W., Hsu S.H., Li Y.P., Lin W.L., Liu L.F., Chang L.C., Lin C.C., Lin C.N., Sheu H.M., 2000. Anticancer activity evaluation of the solanum glycoalkaloid solamargine. Triggering apoptosis in human hepatoma cells. *Biochem Pharmacology*, Vol 60, P.1865-1873.
- Kuo P. C., Shen Y. C., Yang M. L., Wang S. H., Thang T. D., Dung N. X., Chiang P. C., Lee K. H., Lee E. J., Wu T. S., 2007. Crotonkinins A and B and related diterpenoids from *Croton tonkinensis* as anti-inflammatory and antitumor agents. *Journal of Natural Products*, Vol 70, Issue 19, P.1906 – 1909.
- Kuo Y. H., Hsu Y. W., Liaw C. C., Lee J. K., Huang H. C., Kuo L. Y., 2005. Cytotoxic phenylpropanoid glycosides from the stems of *Smilax china*. *Journal of Natural Products*, Vol 68, Issue 10, P.1475 – 1478.
- Lavecchia A., Giovanni C. D., 2013. Virtual screening strategies in drug discovery: A critical review. *Current Medicinal Chemistry*, Vol 20, Issue 23, P.2839 – 2860.
- Lee K. H., Xiao Z., 2005. Podophyllotoxins and analogs. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. Anticancer agents from natural products. *CRC Press, Taylor and Francis Group, Boca Raton*, P.71 – 88.
- Lee K. H., Huang E. S., Piantadosi J. S., Geissman T. A., 1971. Cytotoxicity of sesquiterpene lactones. *Cancer Research*, Vol 31, P.1649 – 1654.

- Lee K. H., Hall I. H., Mar E. C., Starnes C. O., ElGebaly S. A., Waddell T. G., Hadgraft R. I., Ruffner C. G., Weidner I., 1977. Sesquiterpene antitumor agents: inhibitors of cellular metabolism. *Science*, Vol 196, P.533 – 536.
- Lee W. Y., Cheung C. C., Liu K. W., Fung K. P., Wong J., Lai P. B., Yeung J. H., 2010. Cytotoxic effects of tanshinones from *Salvia miltiorrhiza* on doxorubicin-resistant human liver cancer cells. *Journal of Natural Products*, Vol 73, Issue 5, P.854 – 859.
- Levine A.J., 1997. p53, the cellular gatekeeper for growth and division. *Cell*, Vol 88, Issue 3, P.323 – 331.
- Levrero M., 2006. Viral hepatitis and liver cancer: the case of hepatitis C. *Oncogene*, Vol 25, P.3834 – 3847.
- Liao Y. J., Bai H. Y., Li Z. H., Zou J., Chen J. W., Zheng F., Zhang J. X., Mai S. J., Zeng M. S., Sun H. D., Pu J. X., Xie D., 2014. Longikaurin A, a natural ent-kaurane, induces G2/M phase arrest via downregulation of Skp2 and apoptosis induction through ROS/JNK/c-Jun pathway in hepatocellular carcinoma cells. *Cell Death and Disease*, Vol 5, e1137, doi:10.1038/cddis2014.66.
- Li W. Y., Chiu L., Lam W. S., Wong W. Y., Chan Y. T., Ho Y. P., Wong E., Wong Y. S., Ooi V., 2007. Ethyl acetate extracts of Chinese medicinal herb *Sarcandra glabra* induces growth inhibition on human leukemic HL-60 cells, associated with cell cycle arrest and up-regulation of pro-apoptotic Bax/Bcl-2 ratio. *Oncology Reports*, Vol 17, P.425 – 431.
- Li X., Zhao Y., Wu W.K., Liu S., Cui M., Lou H., 2011. Solamargine induces apoptosis associated with p53 transcription-dependent and transcription-independent pathways in human osteosarcoma U2OS cells. *Life Sciences*, Vol 88, P.314-21.
- Li Y., Zhang D. M., Li J. B., Yu S. S., Li Y., Luo Y. M., 2006. Hepatoprotective sesquiterpene glycosides from *Sarcandra glabra*. *Journal of Natural Products*, Vol 69, P.616 – 620.
- Lin Z., Guo Y., Gao Y., Wang S., Wang X., Xie Z., Niu H., Chang W., Liu L., Yuan H., Lou H., 2015. Ent-Kaurane diterpenoids from Chinese liverworts and their antitumor activities through Michael addition as detected in situ by a fluorescence probe. *Journal of Medicinal Chemistry*, Vol 58, Issue 9, P.3944 – 3956.
- Liu C. X., Yin Q. Q., Zhou H. C., Wu Y. L., Pu J. X., Xia L., Liu W., Huang X., Jiang T., Wu M. X., He L. C., Zhao Y. X., Wang X. L., Xiao W. L., Chen H. Z., Zhao Q., Zhou A. W., Wang L. S., Sun H. D., Chen G. Q., 2012. Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. *Nature Chemical Biology*, Vol 8, Issue 5, P.486 – 493.

- Llovet J. M., 2005. Updated treatment approach to hepatocellular carcinoma. *Journal of Gastroenterology*, Vol 40, P.225 – 235.
- Long P. Q., Minh C. V., Dam N. T., 2009. Anticancer agents from Vietnam current status and future potential. *Institute of Natural Product Chemistry, Vietnam Academy of Science and Technology*. Les Ecoles de Do Son : Les Bio-ressources marines et leurs utilisations, 02-12 Mars, CNRS-VAST.
- Lu W-J., Lee N. P., Kaul S. C., Lan F., Poon R. TP., Wadhwa R., Luk J. M., 2011. Mortalin – p53 interaction in cancer cells is stress dependent and constitutes a selective target for cancer therapy. *Cell Death and Differentiation*, Vol 18, P.1046 – 1056.
- Lu W-J., Lee N. P., Kaul S. C., Lan F., Poon R. TP., Wadhwa R., Luk J. M., 2011. Induction of mutant p53-dependent apoptosis in human hepatocellular carcinoma by targeting stress protein mortalin. *International Journal of Cancer*, Vol 129, P.1806 – 1814.
- Meijer L., 2003. Le cycle de division cellulaire et sa regulation. *Oncologie*, Vol 5, P.311 – 326.
- Moi L. D., 2005. Plant resources in Vietnam: Plants contain compounds with biological activities. *Natural Science and Technology Publish House*, Hanoi, Vietnam.
- Mosmann T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity Assays. *Journal Immunological Methods*, Vol 65, P.55 - 63.
- Minh P. T., Ngoc P. H., Quang D. N., Hashimoto T., Takaoka S., Asakawa Y., 2003. A novel *ent*-kaurane diterpenoid from the *Croton tonkinensis* GAGNEP. *Chemical and Pharmaceutical Bulletin (Tokyo)*, Vol 56, P.590 – 591.
- Minotti G., Menna P., Salvatorelli E., Cairo G., Gianni L., 2004. Anthracyclines : molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacological reviews*, Vol 56, No. 2, P.185 – 229.
- Morris G. M., Goodsell D. S., Halliday R. S., Huey R., Hart W. E., Belew R. K., Olson A. J., 1999. Automated docking using a lamarckian genetic algorithm and an empirical binding free energy function. *Journal of Computational Chemistry*, Vol 19, P.1639 – 1662.
- Munari C. C., de Oliveira P. F., Campos J.C., Martins Sde P., Da Costa J.C., Bastos J.K., Tavares D.C. 2014. Antiproliferative activity of *Solanum lycocarpum* alkaloidic extract and their constituents, solamargine and solasonine, in tumor cell lines. *Journal of Natural Medicines*, Vol 68, P.236 - 41.

- Mutter R., Wills M., 2000. Chemistry and clinical biology of the brystatins. *Bioorganic & Medicinal Chemistry*, Vol 8, P.1841 – 1860.
- Nakagiri R., Hashizume E., Kayahashi S., Sakai Y., Kamiya T., 2003. Supression by hydrangeae Dulcis Folium of D-galactosamine-induced liver injury in vitro and in vivo. *Bioscience, Biotechnology, and Biochemistry*, Vol 67, Issue 12, P.2641 – 2643.
- Newman D. J., 2005. The bryostatins. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. *Anticancer agents from natural products*. CRC Press, Taylor and Francis Grop, Boca Raton, P.137 – 150.
- Newman D. J., Cragg G. M., 2016. Natural Products as sources of new drugs from 1981 to 2014. *Journal of Natural Products*, Vol 79, P.629 – 661.
- Nigam N., Grover A., Goyal S., Katiyar S. P., Bhargava P., Wang P., Sundar D., Kaul S. C., Wadha R., 2015. Targeting Mortalin by Embelin causes activation of tumor suppressor p53 and deactivation of metastatic signaling in human breast cancer cells. *PLOS ONE*, Vol 10, Issue 9:e0138192. Doi:10.1371/journal.pone.0138192.e Collection 2015.
- Nguyen V.T., Law M.G., Dore G.J., 2008 An enormous hepatitis B virus-related liver disease burden projected in Vietnam by 2025. *Liver International*, Vol 28, P.525 – 531.
- Oleksandr V. B., Anthony C. B., Kevan M. S., 2002. Modified autodock for accurate docking of protein kinase inhibitors. *Journal of Computer-aided Molecular Design*, Vol 16, Issue 2, P.113 – 127.
- Ooi L. S., Wong E. Y., Chiu L. C., Sun S. M., Ooi V. E., 2008. Antiviral and anti-proliferative glycoproteins from the rhizome of *Smilax glabra* Roxb (Liliaceae). *The American Journal of Chinese Medicine*, Vol 36, No. 1, P.185 – 195.
- Paraskevi A. F., Ronald A. D., 2006. Hepatocellular carcinoma pathogenesis : from genes to environment. *Nature Reviews Cancer*, Vol 6, P.674 – 687.
- Perez L. B., Still P. C., Naman C. B., Ren Y., Pan L., Chai H-B., Blanco E. J. C., Ninh T. N., Thanh B. V., Swanson S. M., Soejarto D. D., Kinghorn A. D., 2014. Investigation of Vietnamese plants for potential anticancer agents. *Phytochemistry Reviews*, Vol 13, No. 4, P.727 – 739.
- Phan M. G., Phan T. S., Hamada Y., Otsuka H., 2005. Cytotoxic diterpenoids from Vietnamese medicinal plant *Croton tonkinensis* GAGNEP. *Chemical and Pharmaceutical Bulletin (Tokyo)*, Vol 53, No. 3, P.296 – 300.
- Pinney K. G., Jelinek C., Edvardsen K., 2005. The discovery and development of the combretastatins. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. *Anticancer*

- agents from natural products. *CRC Press, Taylor and Francis Group, Boca Raton*, P.23 – 46.
- Puri S. C., Verma V., Amna T., Qazi G. N., Spiteller M., 2005. An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. *Journal of Natural Products*, Vol 68, No. 12, P.1717 – 1719.
- Qiu G. H., Xie X., Xu F., Shi X., Wang Y., Deng L., 2015. Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B. *Cytotechnology*, Vol 67, P.1 – 12.
- Rahier N. J., Thomas C. J., Hecht S. M., 2005. Camptothecin and its analogs. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. *Anticancer agents from natural products. CRC Press, Taylor and Francis Group, Boca Raton*, P. 5 – 22.
- Rahman M. Z. A., Azam A. T. M., Gafur M. A., 2000. In vitro antibacterial principles of extracts and two flavonoids from *Clerodendrum indicum* Linn. *Pakistan Journal of Biological Sciences*, Vol 3, No. 10, P.1769 – 1771.
- Raihan S. Z., Biswas P., Monir M., Biswas S. K., Chowdhury A., Rahman A. K., 2012. Phytochemical investigation and in vitro antinociceptive activity of *Clerodendrum indicum* leaves. *Pakistan Journal of Biological Sciences*, Vol 15, No. 3, P.152 – 155.
- Reddy L., Odhav B., Bhoola K. D., 2003. Natural products for cancer prevention: a global perspective. *Pharmacology & Therapeutics*, Vol 99, P.1 – 13.
- Rehman A. U., Begum S., Saied S., Choudhadry M. I., Akhtar F., 1997. A steroidal glycoside from *Clerodendron inerme*. *Phytochemistry*, Vol 45, No. 8, P.1721 – 1722.
- Renart J., Reiser J., Stark G. R., 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: A method for studying antibody specificity and antigen structure. *Proceedings of the National Academy of Sciences of the United States of America*, Vol 76, No. 7, P.3116 – 3120.
- Renu W., Kazunari T., Sunil C. K., 2002. An Hsp70 family chaperone, mortalin/mthsp70/PBP74/Grp75: what, when, and where?. *Cell Stress & Chaperones*, Vol 7, Issue 3, P.309 – 316.
- Richard A. F., Jay L. B., Robert B. M., Thomas A. H., Jasna J. K., Daniel T. M., Matthew P. R., Eric H. K., Mee S., Jason K. P., David E. S., Perry F., Peter S. S., 2004. Glide: A new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *Journal of Medicinal Chemistry*, vol 47, P.1739 – 1749.
- Rushton L., Hutchings S. J., Fortunato L., Young C., Evans G., Brown T., Bevan R., Slack R., Holmes P., Bagga S., Cherrie J. W., Tongeren M. V., 2012. Occupational cancer burden in Great Britain. *British Journal of Cancer*, Vol 107, S3 – S7.

- Santagata S., Xu Y. M., Wijeratne E. M., Kontnik R., Rooney C., Perley C. C., Kwon H., Clardy J., Kesari S., Whitesell L., Lindquist S., Gunatilaka A. A., 2012. Using the heatshock response to discover anticancer compounds that target protein homeostasis. *ACS Chemical Biology*, Vol 7, Issue 2, P.340 – 349.
- Schreiber E., Harshman K., Kemler I., Malipiero U., Schaffner W., Fontana A., 1990. Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. *Nucleic Acids Research*, Vol 18, No. 18, P.5495 – 5503.
- Shimoyama T., Hamano T., Natsume T., Koizumi F., Kiura K., Tanimoto M., Nishio K., 2006. Reference profiling of the genomic response induced by an antimicrotubule agent, TZT-1027 (Soblidotin), in vitro. *The Pharmacogenomics Journal*, Vol 6, P.388 – 396.
- Shrivastava N., Patel T., 2007. *Clerodendrum* and Healthcare: An Overview. *Medicinal and Aromatic Plant Science and Biotechnology*, Vol 1, P.142 – 150.
- Singh R., Wang Y., Xiang Y., Tanaka K. E., Gaarde W. A., Czaja M. J., 2009. Differential effects of JNK1 and JNK2 inhibition on murine steahepatitis and insulin resistance. *Hepatology*, Vol 49, Issue 1, P.87 – 96.
- Soan F. A., Gelband H., 2007. Cancer control opportunities in low- and middle-income countries. *National Academies Press (USA)*. ISBN: 978-0-309-10384-8.
- Sohn D., Graupner V., Neise D., Essmann F., Schulze-Osthoff K., Jänicke R. U., 2009. Pifithrin- α protects against DNA damage-induced apoptosis downstream of mitochondria independent of p53. *Cell Death and Differentiation*, Vol 16, P.869 – 878.
- Song C. M., Lim S. J., Tong J. C., 2009. Recent advances in computer-aided drug design. *Briefings in Bioinformatics*, Vol 10, No 5, P.579 – 591.
- Strobel G., Daisy B., Castillo U., Harper J., 2004. Natural products from endophytic micro-organisms. *Journal of Natural Products*, Vol 67, No. 2, P.257 – 268.
- Sul Y. H., Lee M. S., Cha E. Y., Thuong P. T., Khoi N. M., Song I. S., 2013. An ent-kaurane diterpenoid from *Croton tonkinensis* induces apoptosis by regulating AMP-activated protein kinase in SK-HEP1 human hepatocellular carcinoma cells. *Biological and Pharmaceutical Bulletin*, Vol 36, P.158 – 164.
- Sunil C. K., Custer C. D., Renu W., 2007. Three faces of mortalin: A housekeeper, guardian and killer. *Experimental Gerontology*, Vol 42, Issue 4, P.263 – 274.
- Tam K., 2013. The roles of Doxorubicin in Hepatocellular carcinoma. *ADMET & DMPK*, Vol 1, No. 3, P.29 – 44.

- Thanh H. V., Luu H. V., Kinh C. D., My P. T. T., 2008. Structure identification of some compounds isolated from root of *Syzigium resinosum* Gagnep. *Vietnamese Journal of Chemistry*, Vol 46, No. 5A, P.260 – 264.
- Thomas A. H., Robert B. M., Richard A. F., Hege S. B., Leah L. F., Pollard W. T., Jay L. B., 2004. Glide: A new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. *Journal of Medicinal Chemistry*, vol 47, P.1750 – 1759.
- Thuong P. T., Khoi N. M., Ohta S., Shiota S., Kanta H., Takeuchi K., Ito F., 2014. *Ent-kaurane* diterpenoids from *Croton tonkinensis* induce apoptosis in colorectal cancer cells through the phosphorylation of JNK mediated by reactive oxygen species and dual-specificity JNK kinase MKK4. *Anticancer Agents in Medicinal Chemistry*, Vol 14, Issue 7, P.1051 – 1061.
- Tornesello M.L., Buonaguro L., Tatangelo F., Botti G., Izzo F., Buonaguro F.M., 2013. Mutations in TP53, CTNNB1 and PIK3CA genes in hepatocellular carcinoma associated with hepatitis B and hepatitis C virus infections. *Genomics*, Vol 102, P.74 - 83.
- Towbin H., Staehelin T., Gordon J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*, Vol 76, No. 9, P.4350 – 4354.
- Utomo D. H., Widodo, Rifa'i M., 2012. Identifications small molecules inhibitor of p53-mortalin complex for cancer drug using virtual screening. *Bioinformation*, Vol 8, Issue 9, P.426 – 429.
- Vaishnavi K., Saxena N., Shah N., Singh R., Manjunath K., Uthayakumar M., Kanaujia S. P., Kaul S. C., Sekar K., Wadhwa R., 2012. Differential activities of the two closely related Withanolides, Withaferin A and Withanone: Bioinformatics and experimental evidences. *PLoS One*, Vol 7, Issue 9.
- Venkatesh S., Lipper R. A., 2000. Role of the development scientist in compound lead selection and optimization. *Journal of Pharmaceutical Sciences*, Vol 89, No.2, P.145 – 154.
- Wang L., Li D., Wang C., Zhang Y., Xu J., 2011. Recent progress in the development of natural *ent-kaurane* diterpenoids with anti-tumor activity. *Mini-Reviews in Medicinal Chemistry*, Vol 11, Issue 10, P.910 – 919.
- Weiss R. B., 1992. The anthracyclines : will we ever find a better doxorubicin?. *Seminars in Oncology*, Vol 19, Issue 6, P.670 – 686.
- Wender P. A., De Brabander J., Harran P. G., Jimenez J-M., Koehler M. F. T., Lippa B., Park C-M., Shiozaki M., 1998a. Synthesis of the first members of a new class of

- biologically active bryostatin analogues. *Journal of the American Chemical Society*, Vol 120, P.4534 – 4535.
- Wender P. A., De Brabander J., Harran P. G., Jimenez J-M., Koehler M. F. T., Lippa B., Park C-M., Siedenbiedel C., Pettit G. R., 1998b. The design, computer modeling, solution structure and biological evaluation of synthetic analogs of brystatin 1. *Proceddings of the National Academy of Sciences of the United States of America*, Vol 95, P.6624 – 629.
- Wender P. A., Hinkle K. W., Koehler M. F. T., Lippa B., 1999. The rational design of potential chemotherapeutic agents: Synthesis of bryostatin analogues. *Medicinal Research Reviews*, Vol 19, P.388 – 407.
- Wender P. A., Baryza J. L., Bennett C. E., Bi F. C., Brenner S. E., Clarke M. O., Horan J. C., Kan C., Lacote E., Lippa B., Nell P. G., Turner T. M., 2002. The practical synthesis of a novel and highly potent analogue of bryostatin. *Journal of the American Chemical Society*, Vol 124, P.13648 – 13649.
- Wender P. A., Mayweg A. V. W., Vandeusen C. L., 2003. A consice, selective synthesis of the polyketide spacer domain of a potent bryostatin analogue. *Organic Letters*, Vol 5, P.277 – 279.
- World Cancer Research Fund, American Institute for Cancer Research, 2007. Food, Nutrition, Physical activity, and the prevention of cancer: A global perspective. Washington DC: AICR. ISBN: 978-0-9722522-2-5.
- Xia D., Fan Y., Zhang P., Fu Y., Ju M., Zhang X., 2013. Protective effects of the flavonoid-rich fraction from rhizomes of *Smilax glabra* Roxb. on tetrachloride-induced hepatotoxicity in rats. *The Journal of Membrane Biology*, Vol 246, Issue 6, P.479 – 485.
- Xie X., Zhu H., Yang H., Huang W., Wu Y., Wang Y., Luo Y., Wang D., Shao G., 2015. Solamargine triggers hepatoma cell death through apoptosis. *Oncology Letters*, Vol 10, P.168-174.
- Xin Y., John M. L., Nikki P. L., Jirun P., Xisheng L., Xin-Yuan G., Geogre K. L., Laura B., Sheung-Tat F., 2008. Association of Mortalin (HSPA9) with liver cancer metastatis and prediction for early tumor recurrence. *Molecular and Cellular Proteomics*, Vol 7, Issue 2, P.315 – 325.
- Xu W., Sun J., Zhang T. T., Ma B., Cui S. M., Chen D. W., He Z. G., 2006. Pharmacokinetic behaviors and oral bioavailability of oridonin in rat plasma. *Acta Pharmacologica Sinica*, Vol 27, P.1642 – 1646.
- Yang T. S., Wang C. H., Hsieh R. K., Chen J. S., Fung M. C., 2002. Gemcitabine and doxorubicin for the treatment of patients with advanced hepatocellular carcinoma: a phase I-II trial. *Annals of Oncology*, Vol 13, P.1771 – 1778.

- Yang X., 2004. Preliminary study of a vincristine-producing endophytic fungus isolated from leaves of *Catharanthus roseus*. *Chinese Traditional and Herbal Drugs*, Vol 35, No. 1, P.79 – 81.
- Yoshikawa M., Matsuda H., Shimoda H., Shimada H., Harada E., Naitoh Y., Miki A., Yamahara J., Murakami N., 1996. Development of bioactive functions in *hydrangea dulcis* folium. V. on the antiallergic and antimicrobial principles of *hydrangea dulcis* folium. (2). thunberginols C, D, and E, thunberginol G 3'-O-glucoside, (-)-hydrangenol 4'-O-glucoside, and (+)-hydrangenol-4'-O-glucoside. *Chemical and Pharmaceutical Bulletin*, Vol 44, No. 8, P.1440 – 1447.
- Yu M. J., Kishi Y., Littlefield B. A., 2005. Discovery of E7389, a full synthetic macrocyclic ketone analog of halichondrin B. In: Cragg G. M., Kingston D. G. I., Newman D. J., editors. *Anticancer agents from natural products*. CRC Press, Taylor and Francis Group, Boca Raton, P.241 – 266.
- Zhang H., Matsuda H., Yamashita C., Nakamura S., Yoshikawa M., 2009. Hydrangenic acid from the processed leaves of *Hydrangea macrophylla* var. *thunbergii* as a new type of anti-diabetic compound. *European Journal of Pharmacology*, Vol 606, P.255 – 261.
- Zhao J., Liu L., Wan Y., Zhang Y., Zhuang Q., Zhong X., Hong Z., Peng J., 2015. Inhibition of hepatocellular carcinoma by total alkaloids of *Rubus alceifolius* Poir. involves suppression of hedgehog signaling. *Integrative Cancer Therapies*, Vol 14, No. 4, P.394 – 401.
- Zhao J., Lin W., Zhuang Q., Zhong X., Cao Z., Hong Z., Peng J., 2014. Total alkaloids of *Rubus alceifolius* Poir shows anti-angiogenic activity in vivo and in vitro. *Integrative Cancer Therapies*, Vol 13, No. 6, P.520 – 528.
- Zhao J., Zheng H., Liu Y., Lin J., Zhong X., Xu W., Hong Z., Peng J., 2013. Anti-inflammatory effects of total alkaloids from *Rubus alceifolius* Poir. on non-alcoholic fatty liver disease through regulation of the NF- κ B pathway. *International Journal of Molecular Medicine*, Vol 31, Issue 4, P.931 – 937.
- Zhao Z., Chen Y., 2014. Oridonin, a promising antitumor natural product in the chemotherapy of hematological malignancies. *Current Pharmaceutical Biotechnology*, Vol 15, Issue 11, P.1083 – 1092.
- Zou Q. F., Du J. K., Zhang H., Wang H. B., Hu Z. D., Chen S. P., Du Y., Li M. Z., Xie D., Zou J., Sun H. D., Pu J. X., Zeng M. S., 2013. Anti-tumor activity of longikaurin A(LK-A), a novel natural diterpenoid, in nasopharyngeal carcinoma. *Journal of Translational Medicine*, Vol 11:200; Doi:10.1186/1479-5876-11-200.

H - Annexes

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Cytotoxic, apoptotic, and sensitization properties of *ent*-kaurane-type diterpenoids from *Croton tonkinensis* Gagnep on human liver cancer HepG2 and Hep3b cell lines

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ABSTRACT

Human hepatocellular carcinoma (HCC) is the most common type of liver cancer, the second most common cause of death from cancer worldwide. A very poor prognosis and a lack of effective treatments make liver cancer a major public health problem, notably in less developed regions, particularly in eastern Asia. This fully justifies the search of new molecules and therapeutic strategies against HCC. *Ent*-kaurane diterpenoids are natural compounds displaying a broad spectrum of potential therapeutic effects including anticancer activity. In this study, we analyzed the pharmacological properties of a family of *ent*-kaurane diterpenoids from *Croton tonkinensis* Gagnep in human HepG2 and Hep3b cell lines, used as cellular reference models for in vitro evaluation of new molecules active on HCC. A structure-related cytotoxicity was observed against both HCC cell lines, enlighting the role of the 16-en-15-one skeleton of *ent*-kaurane diterpenoids. Cytotoxicity was closely correlated to apoptosis, evidenced by concentration-dependent subG1 cell accumulation, and increased annexin V expression. In addition, subtoxic concentration of *ent*-kaurane diterpenoid dramatically enhanced the sensitivity of HCC cells to doxorubicin. All together, our data bring strong support to the potential interest of *ent*-kaurane diterpenoids, alone or in combination with a cytotoxic agent, in cancer and more precisely against HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type (>80%) of liver cancer which has become the second most common cause of death from cancer worldwide, with estimated 746 000 deaths and 782 000 new cases in 2012, according to the most recent GLOBOCAN data from the International Agency for Research on Cancer (<http://www.globocan.iarc.fr/>) [1]. More than 80% of these deaths and new cases occur in the less

developed regions, particularly in eastern Asia, making liver cancer a major public health problem in these countries. The very poor prognosis for liver cancer (overall ratio mortality/incidence: 0.95) together with the lack of effective treatments makes the search of new molecules and/or new therapeutic strategies a challenging goal.

As a part of our ongoing program aimed at identifying Vietnamese natural compounds endowed with antiproliferative properties against HCC, we focused our

efforts on *ent*-kaurane diterpenoids from *Croton tonkinensis* Gagnep. *Ent*-kaurane diterpenoids isolated from various species display a wide variety of pharmacological effects such as anti-inflammatory, antibacterial, or antitumor activity, this latter one representing an attractive basis for the recent development of potential anticancer therapeutic agents [2,3]. The genus *Croton* L. (Euphorbiaceae) consists of about 800 species mainly distributed in tropical regions, among which 31 are cultivated or grow wild in northern Vietnam. *Croton tonkinensis* Gagnep, commonly named in Vietnamese as 'Kho sam Bac Bo', is a tropical shrub native to northern Vietnam [4]. It has been used commonly in traditional prescription to treat leprosy, psoriasis, malaria, and genital organ prolapse [4,5]. *Ent*-kaurane-type diterpenoids were isolated from the leaves of *Croton tonkinensis* Gagnep, and their structure determined from phytochemical investigations [6–8]. For some of them, the cytotoxic properties have been tested against breast MCF-7, lung A549 [6], liver SK-HEP1 [9], intestinal CaCo-2, and colon LS180 [10] cancer cell lines.

Regarding liver cancer, the well-characterized human cell lines HepG2 and Hep3b represent cellular reference models for in vitro pharmacological studies, proven to be well adapted for the study of new molecules active on HCC [11]. Of the various *ent*-kaurane diterpenoids from *Croton tonkinensis* described so far in the literature, only one has been tested against one HCC cell line (SK-HEP1) [9], and to our knowledge, none has been studied against the two HCC cell line pharmacological models. In this study, we selected a family of seven *ent*-kaurane diterpenoid compounds and tested their cytotoxic and apoptotic effects on both human liver HepG2 and Hep3b cell lines. Because it has been shown that natural products can enhance the sensitivity of human HCC cells to the cytotoxic effect of doxorubicin, an anthracycline used in HCC treatment [12,13], we further explored the ability of the *ent*-kaurane diterpenoids to enhance doxorubicin cytotoxicity in the HepG2 and Hep3b model cell lines.

MATERIALS AND METHODS

Plant material and chemical properties

The *ent*-kaurane diterpenoids were previously isolated from the leaves of the plant *Croton tonkinensis*. The purification and structure identification of the compounds were performed at the Institute of Natural Products Chemistry—Vietnam Academy of Science and

Technology. The purity of the compounds was determined as $\geq 97\%$ by HPLC. The list of the compounds 1 to 7 is presented in Table I and their structure shown in Figure 1. Doxorubicin (doxorubicin hydrochloride) was purchased from Sigma-Aldrich.

Cell lines and cell culture

Human liver cancer cell lines HepG2 (ATCC HB8065) and Hep3b (ATCC HB8064) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin at 37 °C in a humidified atmosphere with 5% CO₂ and 95% humidity.

Cell viability assay

Once reached about 80% confluency, cells were detached using 0.05% trypsin/EDTA and counted. Cells were then resuspended and added onto 96-well plate and allowed to attach for 24 h. Each tumor cell line was exposed to the *ent*-kaurane diterpenoid compounds 1 to 7 at various concentrations in triplicate for 24 or 48 h. Doxorubicin was used as positive control. Cell viability was assessed following the MTT assay protocol [14].

SubG1 analysis by flow cytometry

To investigate the pro-apoptotic effect of the *ent*-kaurane diterpenoids, HepG2 and Hep3b cell types were treated with compound 5 at various concentrations (0, 2, 4, 8, 16 μM) for 48 h at 37 °C. Cells were dissociated with 0.05% trypsin/EDTA, harvested, washed with the buffer solution containing sodium citrate, and then incubated with the DNA reagents according to the BD CycleTest™ Plus DNA Reagent kit protocol (BD Biosciences, Becton Dickinson France SA, Le Pont de Claix, France). The cellular DNA was stained with propidium iodide at a final concentration of at least 125 $\mu\text{g/mL}$ and analyzed. The flow cytometric analysis was performed using the LSRII flow cytometer (BD Biosciences).

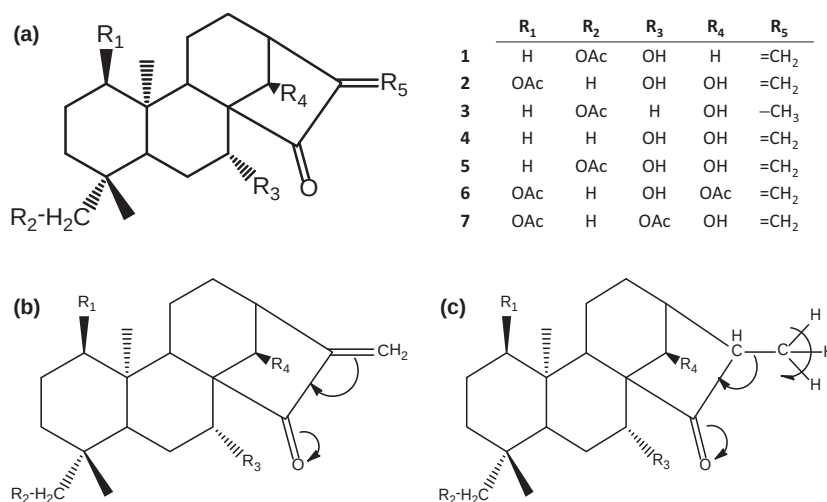
Apoptosis analysis by flow cytometry

The apoptosis process was evaluated using the BD Pharmigen™ Annexin V: FITC Apoptosis Detection Kit (BD Biosciences). Cells were seeded at a density of $4 \times 10^5/\text{mL}$ into 6-well plates for Hep3b cell type and $6 \times 10^5/\text{mL}$ into 24-well plates for HepG2 and then treated with varying concentrations (0, 2, 4, 8, 16 μM)

Table I Name (IUPAC) and chemical entities for R1 to R5 of the *ent*-kaurane-type diterpenoid compounds 1 to 7.

	Name (IUPAC)	R ₁	R ₂	R ₃	R ₄	R ₅
1	<i>ent</i> -18-acetoxy-7 β -hydroxykaur-16-en-15-one (C ₂₂ H ₃₂ O ₄)	H	OAc	OH	H	=CH ₂
2	<i>ent</i> -1 α -acetoxy-7 β ,14 α -dihydroxykaur-16-en-15-one (C ₂₂ H ₃₂ O ₅)	OAc	H	OH	OH	=CH ₂
3	<i>ent</i> -16(S)-18-acetoxy-7 β -hydroxykaur-15-one (C ₂₂ H ₃₄ O ₄)	H	OAc	H	OH	-CH ₃
4	<i>ent</i> -7 β ,14 α -dihydroxykaur-16-en-15-one (C ₂₀ H ₃₀ O ₃)	H	H	OH	OH	=CH ₂
5	<i>ent</i> -18 α -acetoxy-7 α ,14 β -dihydroxykaur-16-en-15-one (C ₂₂ H ₃₂ O ₅)	H	OAc	OH	OH	=CH ₂
6	<i>ent</i> -1 α ,14 α -diacetoxy-7 β -hydroxykaur-16-en-15-one (C ₂₄ H ₃₄ O ₆)	OAc	H	OH	OAc	=CH ₂
7	<i>ent</i> -1 α ,7 β -diacetoxy-14 α -hydroxykaur-16-en-15-one (C ₂₄ H ₃₄ O ₆)	OAc	H	OAc	OH	=CH ₂

Figure 1 Chemical structure of *ent*-kaurane-type diterpenoid compounds 1 to 7. (a) The structure common to all the seven compound is represented. For each compound (1 to 7), the chemical entities of group R1 to R5 are specified. (b) Structure with arrows illustrating the conjugation effect for compounds 1, 2, 4, 5, 6, and 7. (c) Structure with arrows illustrating the hyperconjugation effect for compound 3.



of compound 5. After incubation at 37 °C for 48 h, cells were dissociated with Gibco® Cell Dissociation Buffer, enzyme free, PBS (Life Technologies), harvested, washed in cold PBS, and then pelleted. According to the manufacturer's instructions, cells were stained in the dark for 15 min at room temperature and analyzed by flow cytometry within 1 hr of ending the staining procedure. Apoptosis was analyzed using the LSRII flow cytometer (BD Biosciences).

Time-course analysis of cell sensitization to doxorubicin cytotoxicity

HepG2 and Hep3b cells were incubated during 6, 12, 24, or 48 h in the absence or presence of doxorubicin (at 20 μ M or at increasing concentrations) alone or in combination with compound 5 at a subtoxic concentration (1 μ M). As control, cytotoxicity of compound 5 alone at 1 μ M was evaluated. Cell viability was measured using a MTT assay.

Statistical analysis

All experiments were performed at least three times. Values were expressed as mean $\pm$ SD. The half maxi-

mal inhibitory concentrations (IC₅₀) were determined using the GraphPad Prism software (GraphPad Software, Inc. San Diego, CA, USA). Differences were analyzed using the Student's *t*-test. Differences with *P* < 0.05 (*) or *P* < 0.001 (***) were considered statistically significant.

RESULTS

Cytotoxic effects of *ent*-kaurane diterpenoid compounds 1 to 7 on human HCC HepG2 and Hep3b cell lines

As shown in Table II, all the seven compounds elicited a cytotoxic activity against both HepG2 and Hep3b cell lines, mainly without marked difference between the two cell lines, for a given compound. The activity remained similar or weakly enhanced from 24 to 48 h of incubation, with most of the IC₅₀ values ranging from 3 to 10 μ M. Cytotoxicity varied, depending on the compound. Compound 5 was the most active, with IC₅₀ values around 3 μ M, followed by compound 6. Compound 1 and compound 4 and, to a lesser extent, compound 2 displayed similar

activities with IC₅₀ values below or close to 10 μM . Compound 7 exhibited a lower cytotoxicity with IC₅₀ values above 10 μM . Finally, compound 3 clearly displayed the weakest activity, with IC₅₀ values in the 100 μM range.

Induction of subG1 phase by *ent*-kaurane diterpenoid compound 5 on human HCC HepG2 and Hep3b cell lines

To further explore the cellular mechanisms underlying the observed cytotoxic effects, compound 5, for which the cytotoxic properties have not been tested in the literature, was used as the representative molecule of the series of compounds.

Compound 5 was tested in a range of concentrations framing its IC₅₀ values. Results are shown in Figure 2. Panel A illustrates the cell population observed in the absence or presence of increasing concentrations of compound 5 in representative experiments performed on HepG2 (upper panels) and Hep3b (lower panels) cell lines. Panel B shows that in the absence of compound 5, the subpopulation of both HepG2 and Hep3b cell lines in the subG1 phase was low, around 10% (11.2 ± 2.1 for HepG2 and 10.6 ± 6.9 for Hep3b). In the presence of compound 5, the % of cells in the subG1 phase increased in a concentration-dependent manner, from around 30% (35.0 ± 13.3 for HepG2 and 27.5 ± 3.3 for Hep3b) at 2 μM of compound 5 to around 80% (80.3 ± 15.0 for HepG2 and 73.7 ± 11.1 for Hep3b) at 16 μM of compound 5. At the concentration of 4 μM , about half of the cell popu-

lation was in the subG1 phase (56.0 ± 12.3 for HepG2 and 40.4 ± 9.7 for Hep3b).

Apoptotic properties of *ent*-kaurane diterpenoid compound 5 on human HCC HepG2 and Hep3b cell lines

Results are presented in Figure 3. Panel A illustrates the observed cell population in the absence or presence of increasing concentrations of compound 5 in a representative experiment performed on HepG2 cell line. A similar pattern was obtained with the Hep3b cell line (not shown). As shown in panels B (HepG2) and C (Hep3b), a high proportion (%) of cells was viable and nonapoptotic (73.8 ± 2.1 for HepG2 and 79.4 ± 8.3 for Hep3b) in the absence of compound 5. When treated with compound 5, the % of viable cells declined in a concentration-dependent manner. The population of viable cells at 4 μM was slightly below 50% (37.9 ± 4.1 for HepG2 and 43.5 ± 17.7 for Hep3b). At the highest concentration tested, viability of HepG2 cells was dramatically affected (4.1 ± 3.5) whereas that of Hep3b cells remained rather stable (19.3 ± 4.1). In parallel, there was an increase in early apoptotic cell populations which reached a plateau around 40% for both cell lines at the highest concentration of compound 5 (34.7 ± 7.2 for HepG2 and 38.9 ± 14.7 for Hep3b). The number of cells in the double positive cell population (which contains late apoptotic and/or necrotic cells) also increased, in a continuous manner for HepG2 (60.6 ± 3.5) but with a stabilization around 40% for Hep3b (41.4 ± 11.9) at 16 μM of compound 5.

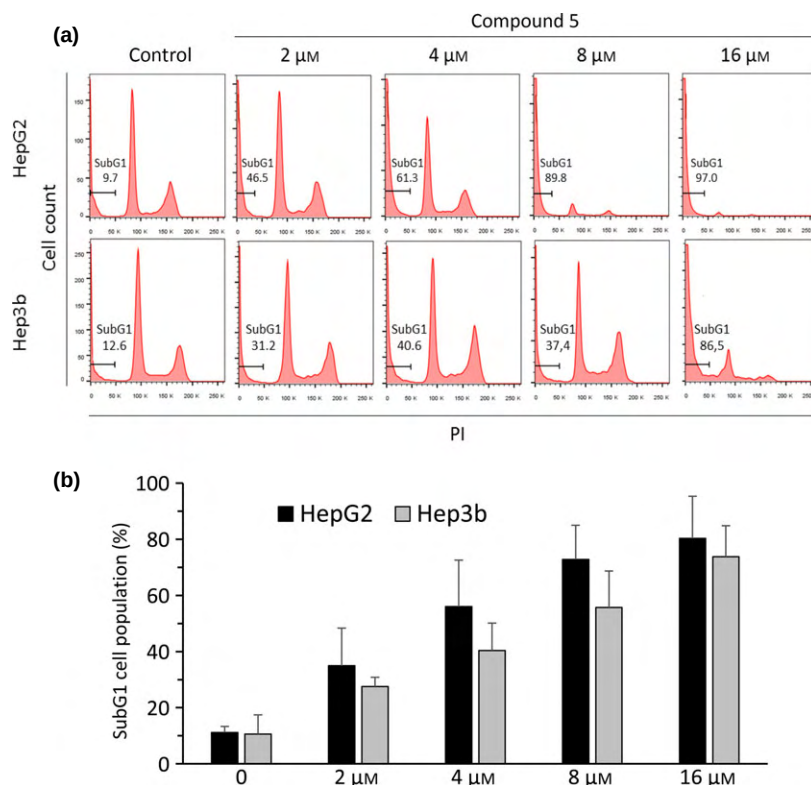
Table II Cytotoxic activity (IC₅₀, μM) of *ent*-kaurane diterpenoids (compounds 1 to 7) on human HCC HepG2 and Hep3b cell lines after 24 and 48 h of incubation. Doxorubicin (Dox) was used as positive control. Results are expressed as the mean $\pm$ SD values from three independent experiments.

Compound	IC ₅₀ (μM)			
	24 h		48 h	
	HepG2	Hep3b	HepG2	Hep3b
1	8.3 ± 1.5	8.0 ± 0.6	3.6 ± 1.5	8.6 ± 0.4
2	10.2 ± 3.3	9.9 ± 1.2	9.6 ± 3.1	8.5 ± 0.7
3	98.6 ± 25.4	116.2 ± 41.6	85.2 ± 32.5	69.6 ± 13.9
4	8.4 ± 1.8	7.8 ± 2.3	3.9 ± 0.3	6.1 ± 0.5
5	3.1 ± 0.5	9.0 ± 0.4	3.5 ± 0.8	3.7 ± 0.1
6	5.7 ± 1.2	9.5 ± 0.5	5.0 ± 1.2	6.1 ± 0.5
7	12.1 ± 3.9	16.1 ± 5.8	13.7 ± 1.3	9.7 ± 0.1
Dox	50.8 ± 3.3	49.3 ± 17.3	26.5 ± 1.8	25.1 ± 2.1

Effect of *ent*-kaurane diterpenoid compound 5 on doxorubicin cytotoxicity in human HCC HepG2 and Hep3b cell lines

Results are shown in Figure 4 and Table III. Cytotoxicity of doxorubicin alone increased regularly with IC₅₀ values ranging from 109.3 ± 7.7 μM to 26.5 ± 1.8 μM for HepG2 and from 86.9 ± 7.3 μM to 25.1 ± 2.1 μM for Hep3b between 6 and 48 h of incubation (Table III). As shown in Figure 4, from 12 to 48 h, compound 5 dramatically enhanced the cytotoxicity of 20 μM doxorubicin, cell viability decreasing to less than 5% at 48 h (compared to 60% for doxorubicin alone). Accordingly, a significant—8- to 11-fold—decrease was measured for the IC₅₀ values (Table III). Conversely however at 6 h of incubation, compound 5 had only a weak to moderate effect on the IC₅₀ values (IC₅₀ ratio <2, Table III) and did not enhance the cytotoxic effect of 20 μM doxorubicin (Figure 4) in both cell lines.

Figure 2 Pro-apoptotic effect of *ent*-kaurane-type diterpenoid compound 5 on human HCC HepG2 and Hep3b cell lines. (a) Hep G2 (upper panels) and Hep3b (lower panels) cells were incubated with no (control) or increasing concentrations (2–16 μM) of compound 5 for 48 h followed by staining with propidium iodide (PI). DNA content was analyzed by flow cytometry. The number of cells (cell count) is represented as a function of DNA content (PI). The % of cells in subG1 is indicated. Data presented are from one representative experiment. (b) Histograms showing the % of cells in subG1 phase as a function of increasing concentrations of compound 5. Columns and bars are the mean and SD from 3 independent experiments.



DISCUSSION

Ent-kaurane diterpenoid compounds 1 to 7 display cytotoxicity in human HCC HepG2 and Hep3b cell lines

All the seven compounds elicited a concentration-dependent cytotoxic activity against both HepG2 and Hep3b cell lines without marked difference between the two cell lines. The fact that no significant difference was observed in the cytotoxic effect against HepG2 and Hep3b despite the distinct pharmacological properties of the two cell lines [11], suggested a cell line-independent mechanism of action of the compounds. Indeed, it is accepted that the cytotoxicity of *ent*-kaurane diterpenoids results from the formation of reactive oxygen species (ROS) through Michael reaction with regulatory thiol-containing enzymes or coenzymes [15–17], likely to be shared by the two HepG2 and Hep3b cell lines and others.

Cytotoxicity of compound 1 on HepG2 and Hep3b cell lines was in the same range as that measured on the SK-HEP1 cell line for the identical *ent*-kaurane diterpenoid named CrT1 [9]. Activity of compounds 1, 2, and 4 against HCC HepG2 and Hep3b cell lines was comparable to the activity of identical molecules

described against other tumor cell lines such as A549 (lung), MCF-7 (breast), or KB (epidermoid carcinoma) [6]. Compounds 1, 2, 6, and 7 were also found active in the brine shrimp lethality assay [18]. Interestingly, all the compounds except compound 3 share the $\text{O}=\text{C}-\text{CH}=\text{CH}_2$ system, described to be responsible of the cytotoxicity of sesquiterpene antitumor agents [19,20], and possess the 16-en-15-one basic skeleton shown to play an important role in the cytotoxic activity of *ent*-kaurane diterpenoids [6,10]. Conversely, the absence of an hydroxyl group at C7 position may contribute to a decreased activity [6]. Consistent with this observation, compound 7, bearing the 16-en-15-one structure but lacking the hydroxyl group at the C7 position, exhibited, to a lesser extent, a decreased activity with IC_{50} values above 10 μM . Further, our observation correlates with the weak cytotoxic activity measured for compound 7 in the brine shrimp lethality assay [18]. Compound 3 which lacks both the 16-en-15-one basic skeleton and an hydroxyl group at C7 position displayed the weakest activity, with IC_{50} values in the 100 μM range, that is, 10 to 30 times higher than those of compound 5. Interestingly, it must be noticed that the R5 methyl group, which results in a well-

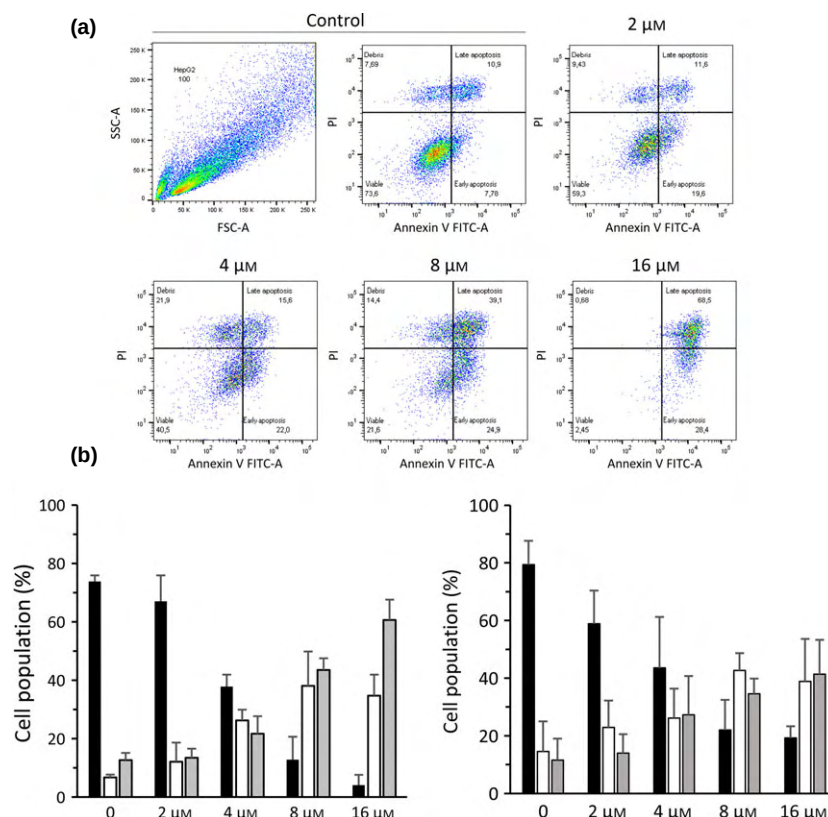


Figure 3 Induction of apoptosis of human HCC HepG2 and Hep3b cell lines by *ent*-kaurane-type diterpenoid compound 5. HepG2 and Hep3b cells were incubated with no (control) or increasing concentrations (2–16 μM) of compound 5 for 48 h followed by staining with Annexin V FITC and propidium iodide (PI). DNA content and Annexin V expression were analyzed by flow cytometry. (a) Data presented are from one representative experiment performed on HepG2 cell line. In the upper left panel, the light scattered properties of untreated cells (FSC-A, SSC-A) are shown. In each other panel, viable, early apoptotic, and late apoptotic cells are gated in lower left, lower right, and upper right quarters, respectively (debris are shown in upper left quarter). Comparable pattern was observed with Hep3b cell line (not shown). (b and c) Percentage (%) of viable, early apoptotic, and late apoptotic (black, white, and gray columns, respectively) HepG2 (b) and Hep3b (c) cells as a function of increasing concentrations of compound 5. Columns and bars are the mean and SD from 3 independent experiments.

known hyperconjugation effect (Figure 1), is only present in compound 3. Hyperconjugation leads to a more stable structure and, consequently, to a less reactive functionality, which may affect the biological properties of *ent*-kaurane diterpenoids. In accord with our observation, an *ent*-kaurane diterpenoid identical to compound 3 showed no cytotoxicity in the brine shrimp lethality assay [18] and was inactive in other biological assays such as inhibition of Silent information regulator two ortholog (SIRT1) [21] or antistaphylococcal activity [22]. Finally, compound 5, for which the cytotoxic properties have not been explored before, exhibited the more efficient activity and was therefore defined as the representative molecule of the series for further studies.

***Ent*-kaurane diterpenoid compound 5 displayed pro-apoptotic properties in human HCC HepG2 and Hep3b cell lines**

Cytometry analysis of the cell population in the subG1 phase is commonly used to evaluate the pro-apoptotic properties of antitumor molecules. In the presence of compound 5, the number of cells in the subG1 phase increased in a concentration-dependent manner. This observation evidenced the pro-apoptotic effect of compound 5. Although the difference was not statistically different, the % of subG1 population in the presence of compound 5 was repeatedly and consistently higher for HepG2 than Hep3b at a given concentration of compound 5, suggesting a different sensitivity between the two cell lines. At

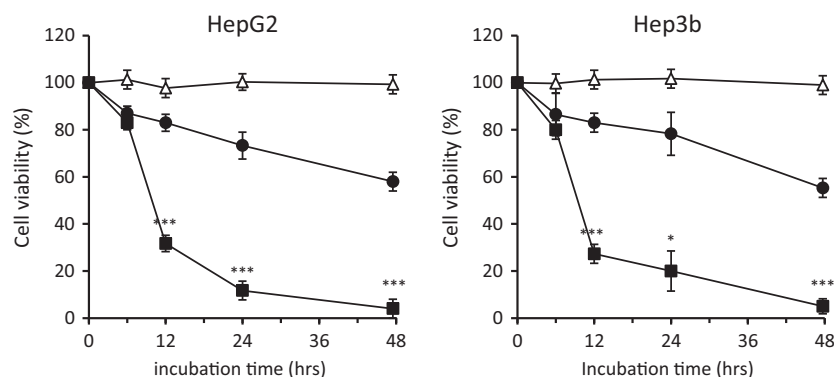


Figure 4 Sensitization of human HCC HepG2 and Hep3b cell lines to doxorubicin cytotoxicity by *ent*-kaurane-type diterpenoid compound 5. Cell viability was analyzed as a function of time and measured using a MTT assay. HepG2 (left panel) and Hep3b (right panel) cells were incubated during 6, 12, 24, or 48 h with 20 μM doxorubicin alone (full circle) or in combination (full square) to compound 5 at a subtoxic concentration (1 μM). As control, compound 5 alone at 1 μM (open triangle) exhibited no significant cytotoxicity. Values shown are the mean $\pm$ SD from three independent experiments. For a given time, *P* values <0.05 (*) or <0.001 (***) indicated statistically significant differences between measures performed with doxorubicin alone and in combination with compound 5.

Table III Cytotoxic activity (IC_{50} , μM) of doxorubicin (Dox) in the absence or in the presence of *ent*-kaurane diterpenoid compound 5 (cpd 5) on human HCC HepG2 and Hep3b cell lines after 6, 12, 24, and 48 h of incubation. Results are expressed as the mean $\pm$ SD values from three independent experiments. For a given time, the ratio of the IC_{50} values of doxorubicin in the absence/in the presence of compound 5 is shown in parentheses.

Incubation time (h)	IC_{50} (μM)			
	HepG2		Hep3b	
	Dox	Dox + cpd 5	Dox	Dox + cpd 5
6	109.3 $\pm$ 7.7	79.4 $\pm$ 6.0 (1.4)	86.9 $\pm$ 7.3	64.5 $\pm$ 3.8 (1.3)
12	66.6 $\pm$ 13.4	9.2 $\pm$ 0.9 (7.2)	56.2 $\pm$ 3.0	6.2 $\pm$ 0.5 (9.1)
24	50.8 $\pm$ 3.3	4.8 $\pm$ 0.6 (10.6)	49.3 $\pm$ 17.3	5.1 $\pm$ 0.3 (9.7)
48	26.5 $\pm$ 1.8	3.2 $\pm$ 0.6 (8.3)	25.1 $\pm$ 2.1	2.3 $\pm$ 0.3 (10.9)

a concentration of 4 μM , close to the IC_{50} values determined in the cytotoxicity assay at 48 h (see Table II), about half of the cell population was in the subG1 phase for HepG2 and for Hep3b. This is in favor of a correlation between the cytotoxic and pro-apoptotic properties of compound 5. Interestingly, the % of subG1 population of HepG2 and Hep3b cell lines induced by compound 5 in our study were quite comparable to those obtained with CrT1 (identical to compound 1) on the SK-HEP1 cell line [9]. These findings, observed on three different HCC cell lines, suggested that *ent*-kaurane diterpenoids might share common or closely related pro-apoptotic mechanisms of action leading to apoptosis in human HCC cell lines.

Concentration-dependent induction of apoptosis by *ent*-kaurane diterpenoid compound 5 in human HCC HepG2 and Hep3b cell lines

Concomitant analysis of annexin V and propidium iodide by flow cytometry allowed the identification of early and late apoptotic stages and therefore brought a more detailed insight on *ent*-kaurane diterpenoid-induced apoptosis. The number of viable cells treated with compound 5 declined in a concentration-dependent manner. In good agreement with the IC_{50} values and the observations made in subG1 experiments, the population of viable cells at 4 μM was slightly below 50%. This further supported the link, suggested above, between the cytotoxic and apoptotic properties of compound 5. At the highest concentration tested, the

important drop in cell viability of HepG2 cells contrasted with the rather stability of Hep3b cells, suggesting, as noted before, a different sensitivity of the two cell lines. In HepG2 cells, the high number of late apoptotic cells correlated with the low number of viable cells. In striking contrast with our observation, late apoptosis was barely detectable in HepG2 cells treated with longikaurin A, a natural *ent*-kaurane [23].

Two additional and complementary observations sustained the evidence of apoptosis in both HepG2 and Hep3b cell lines. First, under light microscope, we observed rounded and shrunk cells upon treatment of both cell lines. Second, in correlation with these morphological changes, treated cells showed altered flow cytometry light scatter properties, with a decrease in forward scatter (FSC-A, *x*-axis) and an increase in side scatter (SSC-A, *y*-axis), which resulted from cell shrinking and apoptotic vesicles formation, respectively (not shown). The cellular components involved in apoptosis of HepG2 and Hep3b cells lines induced by compound 5 remain to be identified. Along this line, two different, but not exclusive, apoptotic cellular pathways triggered by *ent*-kaurane diterpenoids in two different HCC cells have been reported recently: the regulation of AMP-activated protein kinase by CrT1, isolated from *Croton tonkinensis* in SK-Hep1 cells [9], and the activation of ROS/JNK/c-Jun pathway by longikaurin A, isolated from *Isodon ternifolius*, in SMMC-7721 cells [23].

Subtoxic concentration of *ent*-kaurane diterpenoid compound 5 strongly enhances the cytotoxicity of doxorubicin in human HCC HepG2 and Hep3b cell lines

Doxorubicin is one of the most commonly used chemotherapeutic agent against various cancers including HCC [12,13]. However, as a single agent, efficacy of doxorubicin against HCC remains limited, mainly due to chemoresistance and severe side effects [24]. Combined therapy has emerged as a strategy for improving the therapeutic effects of doxorubicin in HCC [25,26]. In this field, few natural molecules have been successfully used to enhance the cytotoxic effect of doxorubicin in ovarian [27] or liver [28–30] cancer cells. In our study, a low, subtoxic concentration of compound 5 efficiently sensitized both HepG2 and Hep3b cell lines to doxorubicin cytotoxicity in a time-dependent manner. However, this effect was not observed within the first 6 h. This 6 h-delay ruled out the hypothesis of an immediate, nonspecific action of

compound 5, such as disruption of the cellular membrane integrity, which would allow doxorubicin to better penetrate the cell. It indicated that the sensitization effect of compound 5 necessitated triggering of intracellular pathways, which remain to be characterized. Enhanced ROS production has been associated to the synergistic effects between doxorubicin and withaferin A in ovarian cancer [27] or selenocysteine in HCC [29]. Interestingly, as discussed above, *ent*-kaurane diterpenoid-induced formation of ROS also mediates cytotoxicity [17] and apoptosis [10]. The fact that ROS generation is also one of the mechanisms of action involved in doxorubicin antitumor activity [31,32] may be a possible explanation of the observed additive effects between compound 5 and doxorubicin.

All together, these data suggest that *ent*-kaurane diterpenoid-induced ROS formation may play a dual role in cancer cells: sensitization at low subtoxic concentrations on the one hand and apoptosis-mediated cytotoxicity at higher concentrations on the other.

As previously proposed for other natural products [27–30], interaction of *ent*-kaurane diterpenoids with doxorubicin may be viewed as a possible way to enhance the antitumor therapeutic effects of doxorubicin. Conversely, ROS accumulation is involved in the cardiotoxic side effects of doxorubicin [32,33] and cumulative ROS generation could then result in an unwished potentiation of cardiotoxicity.

CONCLUSION

Our study not only showed that *ent*-kaurane diterpenoids displayed a structure-related, concentration-dependent, apoptosis-induced cytotoxicity against two human HCC model cell lines but also revealed that they were active at subtoxic concentration, by sensitizing HCC cells to the anticancer agent doxorubicin. These data bring strong support to the potential interest of *ent*-kaurane diterpenoids in the cancer field and more precisely against HCC. Even though virtually no acute toxicity has been observed in vivo [34], the side effects of *ent*-kaurane diterpenoids remain poorly documented to date and their careful evaluation should be undertaken, in a perspective of future clinical applicability and development [2]. In parallel, one may expect that the intrinsic low aqueous solubility and weak bioavailability of these natural products [35] could be successfully overcome through, for example, *ent*-kaurane-based design of synthetic molecules [36].

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES

- 1 GLOBOCAN. 2012. *International Agency for Cancer Research - Liver cancer fact sheet*, <http://globocan.iarc.fr/> (accessed 25 September, 2015)
- 2 Wang L., Li D., Wang C., Zhang Y., Xu J. Recent progress in the development of natural *ent*-kaurane diterpenoids with anti-tumor activity. *Mini Rev. Med. Chem.* (2011) **11** 910–919.
- 3 Zhao Z., Chen Y. Oridonin, a promising antitumor natural product in the chemotherapy of hematological malignancies. *Curr. Pharm. Biotechnol.* (2014) **15** 1083–1092.
- 4 Giang P.M., Jin H.Z., Son P.T., Lee J.H., Hong Y.S., Lee J.J. *ent*-kaurane diterpenoids from *Croton tonkinensis* inhibit LPS-induced NF-kappaB activation and NO production. *J. Nat. Prod.* (2003) **66** 1217–1220.
- 5 Chi V.V. Dictionary of Vietnamese Medicinal Plants. Medicine Publishing House, Ho Chi Minh City, 1997, pp. 622–623.
- 6 Kuo P.C., Shen Y.C., Yang M.L. et al. Crotonkinins A and B and related diterpenoids from *Croton tonkinensis* as anti-inflammatory and antitumor agents. *J. Nat. Prod.* (2007) **70** 1906–1909.
- 7 Minh P.T., Ngoc P.H., Quang D.N., Hashimoto T., Takaoka S., Asakawa Y. A novel *ent*-kaurane diterpenoid from the *Croton tonkinensis* Gagnep. *Chem. Pharm. Bull.* (2003) **51** 590–591.
- 8 Giang P.M., Son P.T., Lee J.J., Otsuka H. Four *ent*-kaurane-type diterpenoids from *Croton tonkinensis* Gagnep. *Chem. Pharm. Bull.* (2004) **52** 879–882.
- 9 Sul Y.H., Lee M.S., Cha E.Y., Thuong P.T., Khoi N.M., Song I.S. An *ent*-kaurane diterpenoid from *Croton tonkinensis* induces apoptosis by regulating AMP-activated protein kinase in SK-HEP1 human hepatocellular carcinoma cells. *Biol. Pharm. Bull.* (2013) **36** 158–164.
- 10 Thuong P.T., Khoi N.M., Ohta S. et al. *Ent*-kaurane diterpenoids from *Croton tonkinensis* induce apoptosis in colorectal cancer cells through the phosphorylation of JNK mediated by reactive oxygen species and dual-specificity JNK kinase MKK4. *Anticancer Agents Med. Chem.* (2014) **14** 1051–1061.
- 11 Qiu G.H., Xie X., Xu F., Shi X., Wang Y., Deng L. Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B. *Cytotechnology* (2015) **67** 1–12.
- 12 Weiss R.B. The anthracyclines: will we ever find a better doxorubicin? *Semin. Oncol.* (1992) **19** 670–686.
- 13 Tam K. The roles of doxorubicin in hepatocellular carcinoma. *ADMET & DMPK* (2013) **1** 29–44.
- 14 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* (1983) **65** 55–63.
- 15 Liu C.X., Yin Q.Q., Zhou H.C. et al. Adenanthin targets peroxiredoxin I and II to induce differentiation of leukemic cells. *Nat. Chem. Biol.* (2012) **2012**(8) 486–493.
- 16 Santagata S., Xu Y.M., Wijeratne E.M. et al. Using the heat-shock response to discover anticancer compounds that target protein homeostasis. *ACS Chem. Biol.* (2012) **7** 340–349.
- 17 Lin Z., Guo Y., Gao Y. et al. Ent-Kaurane Diterpenoids from Chinese Liverworts and Their Antitumor Activities through Michael Addition As Detected in Situ by a Fluorescence Probe. *J. Med. Chem.* (2015) **58** 3944–3956.
- 18 Phan M.G., Phan T.S., Hamada Y., Otsuka H. Cytotoxic diterpenoids from Vietnamese medicinal plant *Croton tonkinensis* Gagnep. *Chem. Pharm. Bull.* (2005) **3** 296–300.
- 19 Lee K.H., Huang E.S., Piantadosi C., Pagano J.S., Geissman T.A. Cytotoxicity of sesquiterpene lactones. *Cancer Res.* (1971) **31** 1649–1654.
- 20 Lee K.H., Hall I.H., Mar E.C. et al. Sesquiterpene antitumor agents: inhibitors of cellular metabolism. *Science* (1977) **196** 533–536.
- 21 Dao T.T., Le T.V., Nguyen P.H. et al. SIRT1 inhibitory diterpenoids from the Vietnamese medicinal plant *Croton tonkinensis*. *Planta Med.* (2010) **76** 1011–1014.
- 22 Giang P.M., Son P.T., Matsunami K., Otsuka H. Anti-staphylococcal activity of *ent*-kaurane-type diterpenoids from *Croton tonkinensis*. *J. Nat. Med.* (2006) **60** 93–95.
- 23 Liao Y.J., Bai H.Y., Li Z.H. et al. Longikaurin A, a natural *ent*-kaurane, induces G2/M phase arrest via downregulation of Skp2 and apoptosis induction through ROS/JNK/c-Jun pathway in hepatocellular carcinoma cells. *Cell Death Dis.* (2014) **20** 5.
- 24 Carvalho C., Santos R.X., Cardoso S. et al. Doxorubicin: the good, the bad and the ugly effect. *Curr. Med. Chem.* (2009) **16** 3267–3285.
- 25 Cervello M., McCubrey J.A., Cusimano A., Lampiasi N., Azzolina A., Montalto G. Targeted therapy for hepatocellular carcinoma: novel agents on the horizon. *Oncotarget* (2012) **3** 236–260.
- 26 Finn R.S. Emerging targeted strategies in advanced hepatocellular carcinoma. *Semin. Liver Dis.* (2013) **33**(Suppl 1) S11–S19.
- 27 Fong M.Y., Jin S., Rane M., Singh R.K., Gupta R., Kakar S.S. Withaferin A synergizes the therapeutic effect of doxorubicin through ROS-mediated autophagy in ovarian cancer. *PLoS ONE* (2012) **7**(7) e42265.

- 28 Lee W.Y., Cheung C.C., Liu K.W. et al. Cytotoxic effects of tanshinones from *Salvia miltiorrhiza* on doxorubicin-resistant human liver cancer cells. *J. Nat. Prod.* (2010) **73** 854–859.
- 29 Fan C., Zheng W., Fu X., Li X., Wong Y.S., Chen T. Strategy to enhance the therapeutic effect of doxorubicin in human hepatocellular carcinoma by selenocystine, a synergistic agent that regulates the ROS-mediated signaling. *Oncotarget* (2014) **5** 2853–2863.
- 30 Gambari R., Hau D.K., Wong W.Y., Chui C.H. Sensitization of Hep3B hepatoma cells to cisplatin and doxorubicin by corilagin. *Phytother. Res.* (2014) **28** 781–783.
- 31 Gewirtz D.A. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem. Pharmacol.* (1999) **57** 727–741.
- 32 Minotti G., Menna P., Salvatorelli E., Cairo G., Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol. Rev.* (2004) **56** 185–229.
- 33 Hequet O., Le Q.H., Moullet I. et al. Subclinical late cardiomyopathy after doxorubicin therapy for lymphoma in adults. *J. Clin. Oncol.* (2004) **22** 1864–1871.
- 34 Zou Q.F., Du J.K., Zhang H. et al. Anti-tumour activity of longikaurin A (LK-A), a novel natural diterpenoid, in nasopharyngeal carcinoma. *J. Transl. Med.* (2013) **11** 200.
- 35 Xu W., Sun J., Zhang T.T. et al. Pharmacokinetic behaviors and oral bioavailability of oridonin in rat plasma. *Acta Pharmacol. Sin.* (2006) **27** 1642–1646.
- 36 Ding C., Wang L., Chen H. et al. ent-Kaurane-based regio- and stereoselective inverse electron demand hetero-Diels-Alder reactions: synthesis of dihydropyran-fused diterpenoids. *Org. Biomol. Chem.* (2014) **12** 8442–8452.

Title : Bio-pharmacological screening on liver-protective and anti-hepatocarcinoma activities of Vietnam natural products

Abstract :

Human hepatocellular carcinoma (HCC) is the most common type of liver cancer, the second most common cause of death from cancer worldwide. A very poor prognosis and a lack of effective treatments make liver cancer a major public health problem, notably in less developed regions, particularly in Eastern Asia. This fully justifies the search of new molecules and therapeutic strategies against HCC. The present work focused on finding bioactive compounds from Vietnamese plants against HCC. The first approach used classical screening of 33 natural compounds which resulted in the identification of 7 *ent*-kaurane diterpenoids isolated from *Croton kongensis* Gagnep. as potential agents. The second approach aimed at identifying molecules that could abrogate the interaction between Mortalin and p53 by in silico screening of a database of 354 natural compounds, which allowed the identification of Solasonine as a potent inhibitor of p53 – mortalin interactions.

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TITRE : Bio-pharmacological screening on liver-protective and anti-hepatocarcinoma activities of Vietnam natural products

DIRECTEUR DE THESE : Pr. Jean Edouard GAIRIN

LIEU ET DATE DE SOUTENANCE : Université Paul Sabatier, Toulouse, le 30 Mai 2016

RESUME

Le carcinome hépatocellulaire (HCC) est le cancer du foie le plus répandu et représente la seconde cause de décès par cancer dans le monde. Un mauvais pronostic et l'absence de traitement efficace en font un problème majeur de santé publique dans les pays en voie de développement, notamment en Asie du Sud-Est, justifiant pleinement la recherche de molécules ou d'approches thérapeutiques nouvelles contre l'HCC. Ce travail porte sur la recherche de molécules isolées de plantes vietnamiennes actives contre l'HCC. La première approche a consisté en un criblage pharmacologique de 33 substances naturelles qui a conduit à l'identification de 7 ent-kaurane diterpénoïdes isolés de *Croton kongensis* Gagnep. présentant des propriétés antiprolifératives originales. La seconde approche, par criblage in silico d'une banque de 354 substances naturelles, a permis d'identifier la solasonine comme inhibiteur de l'interaction mortalin – p53 induisant l'apoptose dans la lignée cellulaire humaine HepG2.

MOTS-CLES

Hépatocarcinome, substances naturelles, criblage pharmacologique, criblage virtuel

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