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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Anti-proliferative activity and possible mechanism of action of constituents identified in silkworm feces toward human lung carcinoma cell lines

잠분 유래 화합물의 인간 폐암세포에 대한 증식 억제 활성 및 작용 기구

By
JI YOUNG YOO

Major in WCU Biomodulation
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UNDER THE DIRECTION ADVISER YOUNG-JOON AHN
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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Anti-proliferative activity and possible mechanism of action of constituents identified in silkworm feces toward human lung carcinoma cell lines

WCU Biomodulation

Seoul National University

Ji Young Yoo

ABSTRACT

Lung cancer caused by diverse changes in cells resulted by exposure to carcinogens found in tobacco smoke, the environment, or sequential accumulation of genetic changes to the normal epithelial cells of the lung. Lung cancer is one of the most common malignancies. However, the fatality of lung cancer is highest than any other cancer in the whole world. Chemotherapy, radiotherapy, and targeted agents have been used for lung cancer treatment. Sometimes, there are serious side effects of these treatments occurred, such as bleeding, hair loss, vomiting, and difficulty getting and fatigue. Therefore, it needs to develop new impressive anticancer agents with effective target sites and low toxicity from natural products which have fewer side effects.

In this study, an assessment is made of the anti-proliferative activity of constituents isolated from silkworm feces against 10 human cancer cell lines, including A549 and H727 non-small cell lung cancer cell(NSCLC) lines, using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Results were compared with those of the commercially available anticancer agent with broad spectrum cisplatin. The air-dried silkworm feces (30.84 kg) yielded 660 g of a dark greenish ethanol extract. The ethanol extract of silkworm feces was proved to have anti-proliferative activity against all 10 species of human cancer cell lines including A549 and H727 lung, AGS stomach, PC-3 prostate, HeLa cervix, HT-29 colon, MCF-7 breast, SNU-213 pencreas, SK-Hep-1 liver, Hep-2 larynx and SK-OV-3 ovary cancer cell lines. The biologically active constituent was characterized as the Vomifoliol (Blumenol A) and Stigmasterol by spectroscopic analysis, including EI-MS and NMR. Vomifoliol (Blumenol A) and Stigmasterol were isolated from the silkworm feces as a new antiproliferative activity principle. Fifty percent inhibition concentration (IC_{50}) values of the constituent against A549 and NCI-H727 were conducted. The IC_{50} of vomifoliol was 57.24 and 55.05 μM toward A549 and NCI-H727 cell lines, respectively. The IC_{50} of stigmasterol was 46.91 and 44.90 μM toward A549 and NCI-H727, respectively. These compounds were less toxic than cisplatin (IC50, 16.53 μM for A549; IC50, 13.50 μM for NCI-H727). The mRNA gene expression of Akt1 and Bcl-2 gene was investigated by real-time RT-PCR. At the presence of vomifoliol and stigmasterol, the mRNA gene expression level of Akt1 which is famous for anti-apoptosis gene was reduced. Also, the mRNA gene expression of Bcl-2 which is important factor in anti-apoptosis mechanism was inhibited by these

two constituents, vomifoliol and stigmasterol identified in silkworm feces.

In conclusion, global efforts to reduce the level of antitcancer agents justify further studies on the silkworm feces-derived materials containing Vomifoliol (Blumenol A), Stigmasterol as potential anticancer products or a lead molecule for the prevention or eradication from human lung cancer.

Key words: Natural products anticancer agents, Lung cancer, Silkworm feces, Silkworm dropping, Vomifoliol, Stigmasterol

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INTRODUCTION

Lung cancer is occurs when some normal cells grow abnormally and out of control. Lung cancer cells break out of the lung and then have a possibility to invade to other parts of the body through the blood vessels (Lam et al., 2004). Lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer death in males. Among females, it is the fourth most commonly diagnosed cancer and the second leading cause of cancer death in the world (Jemal et al., 2011). Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined. In 2014, an estimated 159,260 deaths from lung cancer (86,930 in men and 72,330 among women in the United States), accounting for about 27% of all cancer deaths (American Cancer Society, 2014). The highest incidence rates of lung cancer are in Eastern and Southern Europe, North America, and Eastern Asia, while the lowest rates are low in sub-Saharan Africa (Lam et al., 2004). In the Republic of Korea (ROK), it has much lower incidence and mortality of lung cancer than those in the most Western countries in the past few decades. They have been increasing steadily over time because of risk factors such as active tobacco smoking, environment tobacco smoking, indoor air pollution, and fine dust. Since 1980, lung cancer has been the most rapidly increasing cause of cancer related deaths in the ROK (Lee et al., 1998). Recently, the incidence of lung cancer is gradually decreased by worldwide positive activity to stop tobacco smoking and restrain of second smoking, but because of its high fatality (the overall ratio of mortality to incidence is 0.87) and the relative lack of variability in survival in different world

regions, lung cancer caused a significant burden on world medicare system (IARC, 2012). For medicare patients with a diagnose of early stage lung cancer, it is estimated that medicare will pay an average of 70,000 USD over the remaining lifetime to these individuals. Lung cancer-related costs represented approximately one third of total medical care costs because of higher mortality and incidence of lung cancer (Brown et al., 2001).

Treatment of lung cancer has been achieved principally by the use of anticancer drugs, which continue to be effective chemicals (Kurup and Hanna, 2004). Prolonged treatment with anticancer agents has often resulted in the development of resistance (Semenas et al., 2012), which is a major global public health problem in both developed and developing countries. However, occasional side effects of anticancer agents have been reported that include hair loss, mouth sores, loss of appetite, nausea, vomiting, diarrhea or constipation, and chronic fatigue (Kurup and Hanna, 2004; Ihde et al., 1997). There is a pressing need for the development of selective anticancer agents with novel target sites and low toxicity.

Natural compounds extracted from natural products such as animals, plants, and minerals have been suggested as alternative sources for anticancer products. This approach is appealing, in part, because the natural products constitute a potential source of bioactive chemicals that have been perceived by the general public as relatively safe and often act at multiple and novel target sites, thereby reducing the potential for resistance (Raskin et al., 2002; Jassim and Naji, 2003; Dettner, 2011). Certain plant preparations and their constituents are regarded as potential sources for commercial

anticancer products for prevention or treatment of lung cancer. Previous studies have shown that an ethanol extract from the feces of silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae), possessed good anticancer activity against A549 and NCI-H727 non-small cell lung cancer lines. No work has been obtained concerning the potential use of silkworm feces to manage lung cancer, although historically silkworm feces is used as antiatopic dermatitis, antiinflammation, and antioxidant agents (Park et al., 2010; Costa-Neto, 2002).

In this study, an assessment was made of the antiproliferative activity of vomifoliol and stigmasterol identified in the silkworm feces against A549 and NCI-H727 non-small cell lung cancer lines using a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay. The antiproliferative activities of these materials were compared with those of cisplatin, a broad-spectrum anticancer agent currently used clinically to treat various cancers. The possible mechanisms of action of the test compounds also were elucidated using real-time RT-PCR with SYBR Green dye were applied.

Literature review

1. Lung cancer

Lung cancer is occurs when some normal cells grow abnormally and out of control. Lung cancer cells break out of the lung and then have a possibility to invade to other parts of the body through the blood vessels (Abratt and Morgan, 2002). Lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer in the world. Despite improvements in survival for many other types of cancer in recent years, 5-year survival for lung cancer has remained relatively poor, mainly because by the time a diagnosis is made, lung cancer is often well advanced and treatment options are limited (Spiroand Silvestri, 2005; Pirozynski, 2006; Schwarz et al., 2007). Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined. In 2014, an estimated 159,260 deaths from lung cancer (86,930 in men and 72,330 among women in the US), accounting for about 27% of all cancer deaths (American Cancer Society, 2014). The incidence rates of lung cancer in Asian countries are lower than those in Western countries. In recent few decades, mortality from lung cancer has increased rapidly in South East Asia countries including ROK, because of a high prevalence of smoking (Jee et al., 1998). Indeed, during the past 50 years, aggregate tobacco consumption in ROK has increased 8.6-fold, from a total of 12.4 thousand million cigarettes in 1945 to 106.3 thousand million cigarettes in 2007, while the ROK population has increased just 1.5-fold (Korea Tobacco and Ginseng Cooperation, 2007). Although there have been public health efforts to discourage cigarettes smoking and

positive activity of stop smoking, the other risk factors such as environmental air pollution, fine dust, and heavy metal dust threaten public Medicare of lung cancer. In ROK, lung cancer is the third most common incidence cancer, while the relative frequency cancer deaths are the first among the other cancers (Welfare Korea Central Cancer Registry, 2011). According to the WHO data published in April 2011 (WHO, 2011), lung cancer deaths in ROK reached 16,437 or 7.34 % of total deaths. Like other Asian countries, the incidence and mortality are low but the rates are rising sharply over the few years in ROK (Jee et al., 1998).

Lung cancer can be divided into two types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). These 2 types of lung cancer are treated very differently.

1.1 Small cell lung cancer

SCLC is also called oat cell cancer because it looks small and filled with the nucleus under the microscope. About 10–15 % of all lung cancers are SCLC. It often starts in the bronchi near the center of the chest. This type of lung cancer tends to grow and spread to hall bodies very quickly, and it has always spread to distant parts of the body before it is found. SCLC has two stages limited-stage disease and extensive-stage of disease (Simon and Wagner, 2003).

Patients with limited-stage disease of lung cancer are formed cancer in only one side of the chest. Unless tumors are widespread throughout the lung, limited-stage of SCLC can include one lung as well as the lymph nodes on the same side of the chest

(American Cancer Society, 2014). The important diagnosis criterion of limited-stage of SCLC is an area that is small enough to be treated with radiation therapy in one “port”. Unfortunately, only about 1 out of 3 people have limited-stage SCLC when it is first found (Murren et al., 2001). Approximately, 80–90% of patients with limited-stage of SCLC achieve a response to combination chemotherapy, with or without thoracic radiation (Hanna and Einhorn, 2002).

Extensive-stage of SCLC is defined as the presence of obvious metastatic disease (Simon and Wagner, 2003). Unlike limited-stage of SCLC, in this stage, the cancers have spread widely throughout the lung, to the other lung, to lymph nodes on the other side of the chest, or to distant organs including the bone marrow. About 2 out of 3 people with SCLC have extensive disease when their cancer is first found (American Cancer Society, 2014). Although there have been many studies in extensive-stage disease, there have not been many recent advances, and only a minimal improvement in median survival over the last 25 years (Chute et al., 1999). Approximately, 60–80% of extensive-stage SCLC patients will respond to chemotherapy, with median 1- and 2-year survival rates of 8–10 months, 35 and 10%, respectively (Hanna and Einhorn, 2004).

1.2 Non-small cell lung cancer (NSCLC)

Non-small cell lung cancer (NSCLC) is the most common form of lung cancer, accounting for approximately 85–90% of all cases (Barzi and Pennell, 2010). NSCLC grows and spreads more slowly than SCLC. Early stage disease is associated with few

specific symptoms; therefore approximately 70% of cases are not diagnosed until the disease is at an advanced stage when the chances for cure or significant patient benefit are limited (Schiller et al., 2002). NSCLC has four stages stage Ⅰ, Ⅱ, Ⅲ, and stageⅣ.

Stage Ⅰ

In stage Ⅰ, cancer has formed. Stage Ⅰ is divided into stage Ⅰ A and Ⅰ B. Stage Ⅰ A means that the tumor is in the lung only and is 3 centimeters or much smaller. Stage Ⅰ B means that cancer has not spread to the lymph nodes. In this stage Ⅰ B, the tumor is larger than 3 centimeters but not larger than 5 centimeters. Cancer has spread to the main bronchus and is at least 2 centimeters below where the trachea joins the bronchus. Cancer has spread to the innermost layer of the membrane that covers the lung. Part of the lung has collapsed or developed pneumonitis in the area where the trachea joins the bronchus (National Cancer Institute, 2014).

Stage Ⅱ

In this stage, normally, cancer has spread to the nearby lymph nodes or nearby tissues, such as the chest wall (WHO, 2008). Stage Ⅱ of NSCLC is a little larger than stage Ⅰ and it is divided into stage Ⅱ A and Ⅱ B. These two stages are classified depending on the size of tumor, where the tumor is founded, and whether there is cancer in the lymph nodes.

Stage Ⅱ A is that cancer has spread to certain lymph nodes on the same side of the

chest as the primary tumor. The size of tumor is not larger than 5 centimeters and/or has spread to the innermost layer of the lung lining. OR, cancer has not spread to lymph nodes; the cancer is larger than 5 cm but not larger than 7 cm and has spread to the innermost layer of the lung lining.

StageⅡB is that cancer has spread to nearby lymph nodes on the same side of the chest as the tumor, which is larger than 5 cm but not larger than 7 cm. In this stage, cancer has spread to the main bronchus, and/or has spread to the innermost layer of the lung lining. Cancer has not spread to lymph nodes and the tumor is larger than 7 cm. Also, cancer has spread to the membrane around the heart or lining the chest wall (National Cancer Institute, 2014).

StageⅢ

The tumor of stageⅢ has spread to lymph nodes beyond the same side of the chest, but does not appear to have spread to other organs outside the chest. Unfortunately, the tumor of stageⅢ often are unresectable because it spread to many other sites including lymph nodes in the center, chest wall, major structure of lung, heart or arteries and so on. Patients with this stageⅢ had poorest outcome, a cumulative survival rate of 9% at 5 years, compared to 13% for the group with stageⅡ (Mountain, 1997). StageⅢ of NSCLC is divided into two stages stageⅢA and ⅢB.

In stageⅢA, cancer has metastasized lymph nodes on the same side of the chest, chest wall, diaphragm, membrane around the lung or lining the chest wall and even

membrane around the heart. In more severe cases, cancers can metastasize heart, major blood vessels, chest bone, or backbone.

In stageⅢB, cancer has spread to the opposite side of the chest as the primary tumor. There may be one or more separate tumors in any of the lobes of the lung with cancer or in different lobes of the same lung (National Cancer Institute, 2014).

StageⅣ

In stageⅣ, the tumor may be any size and cancer may has spread to lymph nodes. There are one or more tumors in both lungs. Patients with this stage, cancer is found in fluid around the lungs or heart and has metastasized to other parts of body such as the brain, liver, kidney, or bone (National Cancer Institute, 2013).

2. Risk factors of lung cancer

Causes of lung cancer have not been clearly identified. Although cigarette smoking is considered to be the most important cause of lung cancer, smoking behavior cannot fully explain the epidemiological characteristics of lung cancer (Koet al., 1997). Risk factors of lung cancer also include associations with exposure to passive smoking, exposure to combustion products of heating and cooking fuels, and occupational exposures have been suggested (Wu et al., 1985). Recently, evidence has accumulated from observational studies that people eating more fruits and vegetables, with are rick in ß-carotene (a violet to yellow plant pigment that acts as an antioxidant and can be converted to vitamin A by enzyme in the intestinal wall and liver) and retinol (an alcohol

chemical form of vitamin A), and people having higher serum ß-carotene concentrations had lower rates of lung cancer (Omennet et al., 1996). Many people have at least one risk factor but will never develop lung cancer, while others with lung cancer may have had no known risk factors. Even if a person with lung cancer has a risk factor, it is usually hard to know how much that risk factor contributed to the development of their disease. While the causes of lung cancer are not fully understood, there are a number of factors associated with the risk of developing the disease. These factors are included.

2.1 Tobacco smoking

Smoking is the leading risk factor for lung cancer, causing the cells to grow abnormally. Tobacco smoke is a toxic mix of more than 7,000 chemicals, which of them are poisons. At least 70 are known to cause cancer in people or animals. In the early 20th century, lung cancer was much less common than some other types of cancer. But these changed once manufactured cigarettes became readily available and more people began smoking. At least 87% of lung cancer deaths are thought to result from smoking. The risk of lung cancer among smokers is many times higher than among non-smokers. The longer people smoke and the more packs a day people smoke, the greater people have risk of lung cancer (American Cancer Society, 2014). People who smoke are 15 to 30 times more likely to get lung cancer or die from lung cancer than people who do not smoke. Even smoking a few cigarettes a day or smoking occasionally increases the risk of lung cancer. The more years a person smokes and the more cigarettes smoked each day, the more risk goes up. People who quit smoking have a lower risk of lung cancer

than if they had continued to smoke, but their risk is higher than the risk for people who never smoked. Quitting smoking at any age can lower the risk of lung cancer (CDC, 2013). Smoking can cause not only lung cancer, but also other cancer anywhere in the body including cancer of the mouth, nose, throat, voice box (larynx), esophagus, bladder, kidney, pancreas, cervix, stomach, blood and bone marrow (acute myeloid leukemia). Most of the worldwide burden of lung cancer could be avoided by applying proven tobacco control interventions that include raising the price of cigarettes and other tobacco products, counter advertising, and treating tobacco dependence (US Department of Health and Human Services, 2007). To illustrate, a 10% increase in cigarette prices has been shown to reduce cigarette consumption by 3 to 5% (Shafey et al., 2009).

2.2 Passive smoking (Secondhand smoking)

Smoke from other people's cigarettes, pipes or cigars also cause lung cancer. When a person breathes in secondhand smoke, it is like he or she is smoking. In fact, a nonsmoker who lives with a smoker in the workplace or home is also more likely to get lung cancer diseases. Numerous studies have suggested an elevation in lung cancer risk for nonsmoking females who live with a smoker, with a summary excess risk of approximately 30% (Brownson et al., 1992). In the US, two out of five adults who don't smoke and half of children are exposed to secondhand smoke and about 3,000 people who never smoked die from lung cancer due to secondhand smoke every year (CDC, 2013). Aggregate exposure to ambient tobacco smoke is estimated to produce about 5,000 lung cancer deaths in America regions nonsmokers aged ≥ 35 year, with an

average loss of life expectancy of 17 ± 9 year per fatality. The estimated risk to the most-exposed passive smokers appears to be comparable to that from pipe and cigar smoking. Mortality from passive smoking is estimated to be about two orders of magnitude higher than that estimated for carcinogens currently regulated as hazardous air pollutants under the federal Clean Air Act (Repaceand and Lowrey, 1985). Nonsmokers exposed to secondhand smoke at home or at work increase their risk of developing lung cancer by 20% to 30%. Secondhand smoking also increases the risk of heart disease and other ailments (Reports of the Surgeon General, 2014). Therefore, to avoid secondhand smoking, government can ban smoking in public places.

2.3 Radon

Radon is a naturally occurring radioactive gas that results from the breakdown of uranium in soil and rocks. It cannot be seen, tasted, or smelled. Radon is the second leading cause of lung cancer in US, and is the leading cause among nonsmokers (US EPA, 2014). In many countries exposure in the home to short lived radioactive disintegration products of the chemically inert gas radon-222 is responsible for about half of all non-medical exposure to ionizing radiation (Jay and William, 1984). Air pollution by radon is ubiquitous. Concentrations are low outdoors but can build up indoors, especially in homes, where most exposure of the general population occurs. The highest concentrations to which workers have been routinely exposed occur underground, particularly in uranium mines. Studies of exposed miners have consistently found associations between radon and lung cancer (National Research

Council, 1999; International Agency for Research on Cancer., 2001). Extrapolation from these studies suggests that in many countries residential radon, which involves lower exposure in much larger numbers of people, could cause a substantial minority of all lung cancers (Darby et al., 2004). In fact, outdoors, there is so little radon that it is not likely to be dangerous, but indoors, radon can be more concentrated. When it is breathed in, it enters the lungs, exposing them to small amount of radiation. This may increase a person's risk of lung cancer. The lung cancer risk from radon is much lower than that from tobacco smoke. However, the risk from radon is much higher in people who smoke than in those who don't. Studies from high level of radon soil regions have found that the risk of lung cancer is higher in those who have lived for many years in a radon-contaminated house (American Cancer Society, 2014). However, it is impossible to avoid radon gas completely, because radon is in the air both indoors and out. People can check radon levels and then be used to reduce radon levels by sealing cracks in floors and walls or increasing ventilation through using pipes and fans. By doing so, people can be reduce a bit of radon gas level which is risk factor of lung cancer.

2.4 Asbestos

Asbestos are hair-like crystals found in many types of rock and are often used as fireproof insulation in buildings. Asbestos fibers are found naturally in soil and rocks in many parts of the world. People who have been in prolonged or close contact with asbestos have a higher risk of developing lung cancer. Especially, when asbestos fibers are inhaled, they can irritate the lungs and can be develop lung cancer. Many people have in contact with asbestos during their working lives. Low-level exposure only

slightly increases the risk of lung cancer (compared to the risk from smoking), while heavy exposure may result in a much higher risk (International Agency for Research on Cancer., 2001). The relative risk of lung cancer from asbestos might be six times higher for nonsmokers than smokers (Berry et al., 1985). To avoid asbestos exposure, governments in worldwide countries can legislate strict law that protects workers from being exposed to asbestos which may help lower risk of developing lung cancer.

2.5 Family history

Lung cancer is frequently cited as an example of a malignancy solely attributable to environmental exposure. However, it has long been postulated that individuals may differ in their susceptibility to environmental risk factors (Matakidouet al., 2005).This is because although people are placed in a same environment of lung cancer risk factor, genetics may predispose certain people to lung cancer. A family history of lung cancer in a first-degree was associated with a 51% increased risk of lung cancer, independent of smoking and other relevant factors The effect is stronger when the affected relative is a sibling (82% risk increase) rather than a parent (25–37% risk increase) (Cote et al., 2012).

2.6 Air pollution

Air pollution has been looked at as a possible risk factor for lung cancer, because there is a significant difference between the incidence of lung cancer in urban and rural areas, with lung cancer being more prevalent in urban areas (Boffett, 2006). It is

uncertain to what degree air pollution contributes to what degree air pollution contributes to lung cancer in the US, but according to the largest study to date; more than 10% of lung cancers in Europe may be secondary to air pollution (Grant, 2009). Air pollution from traffic and the combustion of coal, diesel fuel, and wood, has a modest association with lung cancer risk. Large engines, including those used in many trucks, buses, trains, construction and farm equipment, generators, ships, and in some cars, run on coals and diesel fuel. The gas from those equipment include many toxic gases illustrating carbon dioxide, carbon monoxide, nitric oxide, nitrogen dioxide, sulfur oxides, and hydrocarbons, including polycyclic aromatic hydrocarbons, which can cause lung cancer. Also, air pollution affects the incidence of lung cancer in the worldwide. Despite their lower prevalence of smoking (less than 4% adult smokers), Chinese females have higher lung cancer rates (21.3 cases per 100,000 females) than those in certain European countries such as Germany (16.4) and Italy (11.4), with an adult smoking prevalence of about 20% (Mackay et al., 2006). The relatively high burden of lung cancer in women is thought to reflect indoor air pollution from unventilated coal-fueled stoves and from cooking fumes in China (Boffetta and Nyberg, 2003; Lam et al., 2004; Thun et al., 2008). Still, it has been uncertain whether air pollution is the culprit, or other factors that vary between people who live in urban versus rural areas.

2.7 Diet

Numerous retrospective and prospective studies have demonstrated that a reduced risk of lung cancer and other cancers is associated with an increased intake of provitamin A

carotenoids, the vitamin A precursors in vegetables and fruits. Elevated prediagnostic blood P-carotene levels have been consistently predictive of a reduced incidence of lung and other cancers (Ziegler, 1989; Fontham, 1990; Willett, 1990; Steinmetz and Potter, 1991; Ziegler, 1991; Byers and Perry, 1992). Placebo-controlled trial in Finland, male smokers took carotene daily for 5–8 years at a dose substantially higher than that linked to reduced risk in observational studies, and lung cancer incidence was increased 18% (95% confidence interval, 3–36%), contributing substantially to an 8% excess in total mortality (The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, 1994). Interim results from a second large, randomized trial conducted in the US with male and female smokers and men exposed to asbestos have corroborated that lung cancer incidence and total mortality are increased in subjects taking carotene supplements (Ziegler et al., 1996). The beta-Carotene and Retinol Efficacy Trial was initiated in 1983 to test the hypothesis that beta carotene and vitamin A, through complementary antioxidant and differentiation-promoting actions and possibly through immunologic protective effects, could reduce the incidence of lung cancer in high-risk populations (Omenn et al., 1996).

3. Therapeutic treatment of lung cancer

The therapy of lung cancer refers to the use of medical treatment such as surgery, radiation, chemotherapy, and palliative care alone or in combination in an attempt to cure or lessen the adverse impact of malignant neoplasm originating in lung tissue (Kelley and Meier, 2009; Preince-Paul, 2009; Temel et al., 2010). For the best clinical

and treatment purposes, patients with lung cancer be applied different treatments according to SCLC or NSCLC.

3.1 Therapy of small cell lung cancer

Depending on the stage of the lung cancer and other factors, the main treatment options for patients with SCLC. SCLC usually initially responds well to chemotherapy and/or radiation. However, SCLC has usually metastasized widely by the time it is discovered, making surgery ineffective because it is very fast to metastasis.

3.1.1 Radiation therapy

Radical radiotherapy may cure some nonresectable lung cancers and is more effective for small lesions (Deeley and Singh, 1967; Rissanen et al., 1968). Pre- or postoperative radiotherapy may improve these results theoretically by dealing with local cancer which is inaccessible to surgery, or by sterilizing micro metastases in mediastina lymph node (Houtte et al., 1980). Radiation therapy uses high-energy radiation to kill cancer cells. High-energy rays damage DNA in cells, causing them to die or stop dividing. Since lung cancer cells divide more frequently than normal cells, they are more susceptible to damage. The radiation can be delivered by a machine that directs the high-energy rays towards the cancer, or by a small radioactive pellet that gets implanted in or near the tumor (Canadian Lung Association, 2014). Mostly, patients with SCLC used to treatment called external beam radiation therapy. Before treatments start, radiation team will take careful measurements to find the correct angles for aiming the radiation beams

and the proper dose of radiation. Treatment is much like getting an x-ray, but the radiation is more intense. The procedure itself is painless. Each treatment lasts only a few minutes, although the setup time – getting you into place for treatment – usually takes longer (American Cancer Society, 2014). Most often, radiation treatments as part of the initial treatment for SCLC are given once or twice daily, 5 days a week, for 3 to 7 weeks. Radiation to relieve symptoms and prophylactic cranial radiation are given for shorter periods of time, typically less than 3 weeks. However, there are side effects of radiation therapy for lung cancer. Everyone responds to radiation therapy differently because of the location of lung cancer, general health of patients, and other treatments which patients are received. Radiation therapy is a local treatment, and therefore most symptoms arise in the area that is being treated (Abratt and Morgan, 2002). Short-term side effects often show up within the first few weeks of treatment, and resolve soon after treatment is completed. Long-term side effects can sometimes appear months or even years after treatment (Eldridge, 2014). Common possible side effects of radiation therapy can include skin irritation, hair loss, cough, fatigue, nausea, vomiting, loss of appetite, and weight loss. More seriously, radiation therapy can affect the blood-forming cells in bone marrow. This can lead to low blood cell counts, as a result, patients with radiation therapy can increase chance of infections from too few white blood cells. Also, it is easy to bruise or bleed from too few blood platelets and feel fatigue from too few red blood cells unlike normal people. In fact, fatigue is the most common symptom experienced by patients with radiation therapy (Chen, 1986). It substantially increases during the course of either radiation therapy, and persist higher than baseline rate,

sometimes for years, after the treatment is finished (Hickok et al., 1996). Especially, radiation therapy to the chest may damage normal lung cells, which might often cause a cough, problems breathing, and shortness of breath. However, most of these side effects go away after treatment, but sometimes can last a long time, or may even be permanent. When chemotherapy is given with radiation, the side effects are often worse (American Cancer Society, 2014).

3.1.2. Chemotherapy

Small cell lung cancer (SCLC), once considered one of the most rapidly lethal neoplasms of man, is the type of pulmonary carcinoma most responsive to chemotherapy. In the treatment of small cell lung cancer, chemotherapy has become a generally accepted and widely applied therapeutic modality. Since the majority of patients with this disease are not cured by surgery or radiotherapy and also many cases present with advanced stages of disease, chemotherapy is regarded as the most promising approach to the ultimate control of lung cancer (Papac, 1981). Chemotherapy medications work by killing rapidly dividing cells. Single agents, notably alkylating agents such as mustargen, leukeran, and alkeran, are effective in inducing tumor regression in about 25–30% of cases; although responses are generally brief (Green et al., 1969; Selawry, 1974). Different chemotherapy agents work at different stages of cell division. For this reason, two or more medications are given at the same time usually to kill as many cancer cells as possible (Eldridge, 2014). Chemotherapy agents for SCLC are generally given as a combination of two drugs at first. The combination most often

used to treat SCLC are; cisplatin etoposide, carboplatin etoposide, and irinotecan. If the cancer progresses get worse during treatment or returns after treatment is finished, other chemotherapy medications may be tried again. Also, if caner returns more than 6 months after treatment, it might respond again to the same chemotherapy medications that were given the first time, so these can be tried again. If the cancer comes back sooner, or if it keeps growing during treatment, further treatment with the same medications isn't likely to be helpful. If further chemotherapy is given, most doctors prefer treatment with a single, different drug at this point to help limit side effects (American Cancer Society, 2014). While chemotherapy targets cancer cells, it can also damage healthy cells and cause unpleasant side effects such as nausea, vomiting, hair loss, fatigue and mouth sores. The side effects of chemotherapy depend on the type and dose of drugs given and the length of time patient are taken. However, these side effects are usually short-term and go away after treatment is finished. Also, there are often ways to lessen these side effects. Therefore, people with cancer should report any side effects they notice while getting therapy so that they can be treated promptly.

3.2 Therapy of non-small cell lung cancer

Unlike SCLC, NSCLC is comparatively less sensitive to chemotherapy and/or radiation, so surgery is the treatment of choice in these NSCLC.

3.2.1 Radiation therapy

Preoperative radiation

Preoperative Radiation means that the treatments of radiation before surgery try to shrink a lung cancer size to make it easier to operate on. Controversy exists over the value of preoperative radiation for stage I and II non-small cell lung cancer. Indeed, pre-operative radiation in both prospective and retrospective studies has not shown significant benefit over the use of surgery alone in the treatment of early stage NSCLC (Komaki and Cox, 1994; Payne, 1994). On occasion, preoperative radiation may be indicated to increase operability of a lung tumor located adjacent to a vital organ, in hopes of minimizing surgical damage to that organ (Payne, 1994). Others still consider preoperative radiation to be worthwhile particularly for superior sulcus tumors (Pancoast's tumors), especially if the patient is considered curable with an acceptable complication rate (Emami and Perez, 1992). In a small number of patients with early stage NSCLC who are technically operable, but medically ineligible for surgery, radiation therapy becomes a definitive treatment modality. Radical radiation is an effective option for NSCLC patients who are medically inoperable, particularly if lesions are less than 3 cm in size. Doses of 65 Gray or higher should be used to achieve local control. For larger lesions, local failure after radical radiation is more common, and should be addressed by increasing the total radiation dose, adding chemotherapy, or employing special techniques such as endo-bronchial high dose-rate brachytherapy (Hilderley, 1996).

Postoperative radiation

Postoperative Radiation means that the therapy of radiation after surgery try to kill any small deposits of cancer that surgery may have missed. Patients undergoing surgical resection for stage II and III of NSCLC have shown poor overall survival, leading to the continuing search for adjuvant treatment (Hilderley, 1996). Postoperative radiation in patients with no demonstrable or mediastinal lymph node involvement has not improved survival. There may in fact be some negative effect on survival as well as increased morbidity due to radiation effect on surgically compromised pulmonary function. Because local recurrence is infrequent following complete resection (with no evidence of regional lymph node metastasis) radiation adds little to the overall outcome (Vanhoutte, 1991). However, there are many studies have shown that postoperative radiation combined with chemotherapy in the presence of residual disease improves median survival. The results of a trial comparing postoperative radiation therapy alone with chemotherapy plus radiation in patients with incompletely resected NSCLC. The radiation therapy alone group had a 13 month median survival, compared to 20 months in the group receiving post-operative combination chemotherapy and radiation (Abratt and Morgan, 2002). Moreover, patients treated by surgery followed by radiation therapy had a higher survival rate than those treated with definitive radiation alone. The study supports the contention of others that more favorable patients are selected for surgery than for treatment with definitive radiation therapy (Durci et al., 1991). It is recommended that a course of post-Operative radiation for all patients with NSCLC diseases affects positive surgical margins.

3.2.2 Chemotherapy

In past years, there has been considerable pessimism over the role of chemotherapy in non-small cell lung cancers. The pessimism was largely derived from the fact that alkylating agent-based therapies shortened survival and produced severe side effects This was especially important because the vast majority of patients (–85%) develop metastatic disease during their course. Randomized trials from the 1980s showed that cisplatin-based chemotherapy improved patient survival, improved quality of life as assessed by the patients, and relieved symptoms in the majority of symptomatic patients especially in stage IIIB and IV NSCLC patients. The activity of these new agents made it logical to combine them with other standard therapies, especially the platinum compounds. In all instances, the combination of one of these new agents with cisplatin or carboplatin led to higher response rates than those reported for either agent alone. In addition, median and 1-year survival rates were generally higher (Bunn and Kelly, 1998). There are positive effects of chemotherapy because patients improve the quality of life with advanced NSCLC. In a randomized study from the United Kingdom, cisplatin-based combination chemotherapy was shown to improve the quality of life as well as to prolong the survival of advanced-stage NSCLC patients (Billingham et al., 1997). In extensive-stage SCLC, chemotherapy improves median survival from 2 months to 9-10 months, but fewer than 20% are alive at 2 years, and 5-year survival rates are about 1%. In NSCLC, new agent-based chemotherapy improves survival from 4 months to about 10 months, 1-year survival from 10% to 40–50%, and 2-year survival to 20%. At the same time, these therapies improve quality of life, and their cost is similar to that of

other accepted medical therapies. Thus, it is reasonable to offer chemotherapy to advanced NSCLC patients (Bunn and Kelly, 1998).

3.2.3 Surgery

Surgery is the best chance to cure NSCLC. Lung cancer surgery is a complex operation that can have serious consequences, so it should be done by a surgeon who has a lot of experience operating on lung cancer. Surgery decided depending on the type, the stage, the location of lung cancer and general health of patients with NSCLC. Some evidence exists to suggest that, in Europe and the United States almost 30% of patients with NSCLC undergo surgery (Humphrey et al., 1990; Berrino et al., 1995). Lung cancer surgery involved 4 types: wedge resection, segmental resection, lobectomy, and pneumonectomy. More explaining, these 4 types of lung cancer surgery depend on removing a portion of the lung or the entire lung. An operation to remove the lung cancer and a small portion of healthy tissue is called a wedge resection. Removing a larger area of the lung is called segmental resection. Surgery to remove one of the lung's five lobes is called lobectomy. Removing an entire lung is called pneumonectomy. As with any surgery, cancer surgery does carry risks. In general, most lung cancer operations carry a risk of excess bleeding, wound infections and pneumonia. While it is rare, in some cases people may not survive the surgery, which is why it is very important that surgeons select patients carefully. Surgery for lung cancer is a major operation, and recovering from the operation typically takes weeks to months. The activity of patients who were taken lung surgery will be limited for at least a month or two. If the lungs are

in good condition (other than the presence of the cancer) patients can usually return to normal activities after some time if a lobe or even an entire lung has been removed. If patients also have noncancerous lung diseases such as emphysema or chronic bronchitis (which are common among heavy smokers), patients may become short of breathe with activity after surgery (American Cancer Society, 2014). Therefore, it is important to decide surgery treatment. In fact surgical resection, occasionally combined with chemo- and radiotherapy, still offers the best chances of survival in the early stages of the disease, although it is indicated in only 20%–25% of newly diagnosed cases of NSCLC (Reich, 2002). If cancer has spread to anywhere in body, then a major operation to remove cancer is usually not the right treatment. If there are cancer cells anywhere, patients with NSCLC will be suggested another type of treatment to cure cancer instead such as radiation therapy or/and chemotherapy. Therefore, it is important to catch in early stage NSCLC because it can do most curative lung surgery as soon as possible. In fact, the survival rates increase by lung cancer early stages. In Stage Ⅰ, the overall 5-year survival rates account for 60–80% and in stage Ⅱ lung cancer is 40–50%. Stage ⅢA lung cancer is 23%, but this varies widely among different cancers that are classified as stage. Stage ⅢB of the 5-year survival rate is only 10%. The median survival time with treatment is 13 months. Stage Ⅳ lung cancer is sadly less than 10% and the median survival time is about 8 months (Eldridge, 2014).

4. Anti-proliferative activity of natural products

Natural products have been a rich resource of agents of value to medicine. In fact, since ancient time, the role of natural products as a source for remedies has been recognized. Natural products is an attractive source of new therapeutic candidate compounds as a tremendous chemical diversity is found in millions of species of plants, animals, marine organisms, microorganisms, insects, and their metabolites. More than half of currently available drugs are natural compounds or are related to them, and in the case of cancer this proportion surpasses 60%. That means that an analysis of the number of chemotherapy agents and their sources indicates that over 60% of approved drugs are derived from natural compounds (Newman and Cragg, 2007). This area of research, which is continually expanding and is of enormous current interest, explores new natural products coming from different sources, among which the sea could be quoted as an almost infinite source of resources (Marris, 2006; Hamann et al., 2007), with a view to collecting more potent, more selective and less toxic compounds than today's drugs, and hence with better therapeutic indices (Gordaliza, 2007). Prevention of tumor metastasis is one of the goals for cancer patients and cytotoxic agents have been applied to tumor metastasis therapy. However, such therapy is recognized to have many serious side effects such as decreased white blood cells, nausea and stomatitis that could decrease the quality of life of cancer patients (Creemers et al., 1996). Therefore, non- or low cytotoxic agents have recently been demanded for tumor metastasis therapy from natural products. Anti-proliferative agents derived from natural products against cancer cell lines are listed in Table 1, along with their IC_{50} values. A number of anti-cancer

compounds-derived from natural product usually utilize late preclinical advancement and also use in early clinical trials.

Table 1. IC_{50} values of natural compounds for cancer cell proliferation

Natural compounds	IC_{50} (μg/ml)	Sources	Part	Reference
Alkaloids				
Evodiamine	25.1	*Evodia rutaecarpa* Bentham	Fruits	Yoshinori et al.
Berbamine	10.6	*Berberis koreana* Palib.	Plants	Qadir et al.
Cepharanthine	6.40	*Stephania cepharantha* Hayata	Plants	Nawawi et al.
Corydaline	48.7	*Corydalis yanhusuo* WT Wang	Plants	Masaru et al.
Cycleanine	23.1	*Synclisia scabrida* Miers	Plants	Ohiri et al.
Isotetrandine	19.1	*Berveris* sp.	Plants	Masaru et al.
Papaverine	49.6	*Papaver somniferum* L.	Plants	Fabricant and Farnsworth
Phenylporpanoids				
Honokiol	5.48	*Magnolia* sp.	Plants	Masaru et al.
Magnolol	23.8	*Magnolia* sp.	Plants	Masaru et al.
Terpenoids				
α-Pinene	19.7	*Quercus ilex* L.	Plants	Susanti et al.

Thymol	27.6	*Trachyspermum ammi* Sprague	Plants	Kaur and Arora
Betulin	3.41	*Betula platyphylla* Sukachev	Plants	Lee et al.
Saikosaponin a	3.13	*Bupleurum falcatnum* L.	Plants	Masaru et al.
Saikosaponin b_2	8.74	*B. falcatnum*		Masaru et al.
Saikosaponin d	3.11	*B. falcatnum*		Masaru et al.
Flavonoids				
Baicalein	2.57	*Scutellaria baicalensis* Georgi	Plants	Ye et al.
Wogonin	26.3	*S. baicalensis*	Plants	Scheck et al.
Kaemperol	>100	*Lysimachia clethroides* Duby	Plants	Liu et al.
Quercetin	15.9	*Euonymus alatus* (Thunb.) Siebold	Plants	Cha et al.
Steroids				
Bufalin	47.3	*Bufo vulgaris* L.	Venom	Yeh
Cinobufalin	61.3	*B. vulgaris*	Venom	Qadir et al.
The others				
Capsaicin	20.8	*Capsicum frutescens* L. and others	Fruits	Materska and Perucka
Capillarisin	76.7	*Artemisia capillaries* Thunb.	Plants	Chai et al.

4.1 Plant-derived agents

Plant species with a capacity to defend themselves from potential predators and to inhibit other plants competingfor space have been selected for natural selections (Mans et al., 2000). To survive in nature, plants have complicated mechanisms such as an elaborate chemical arsenal of toxic substances. For example, terpenes and alkaloids can inhibit the growth of other plants and make them unattractive to predators. Tannin is also useful to inhibit predators. Trees or plants release ethylene into the air. These signals to other trees or plants in the grove to increase leaf tannin production, making the poisonous and unpalatable to the predators like animal. When attacked by predators, some plants are also able to produce phenol and tannin, using similar signaling processes (Adriana et al., 2001).

In current pharmaceutical industry, several plant-derived compounds have been used patients to cure diseases. One of the most significant examples is the vinca alkaloid family isolated from the periwinkle *Catharanthus roseus* (L.) G.Don, which is found in the rain forests of Madagascar (Noble, 1990). Vincristine has been well known inhibiting microtubule assembly, inducing tubulin self-association into coiled spiral aggregates. Another example of a highly active agent derived from a natural product is etoposide, which has produced high cure rates in testicular cancer when used in combination chemotherapy (Williams et al., 1987). Etoposide is an epipodophyllotoxin, derived from the mandrake plant *Podophyllum peltatum* L. and thewild chervil *Podophyllum emodi* Wall. & Royle. It has also significant activity against SCLC (Chabner, 1991). This lead compound can have activity a topoisomerase II inhibitor,

stabilizing enzyme-DNA cleavable complexes leading to DNA breaks (Liu, 1989). In addition, the camptothecin derivatives, irinotecan and topotecan, have shown significant antitumor activity against colorectal and ovarian cancer, respectively (Creemers, 1996). These compounds were initially obtained from the bark and wood of *Camptotheca accuminata* Decne and act by inhibiting topoisomerase I (Liu, 2000). Flavopiridol is a synthetic flavone derived from the plant alkaloid rohitukine, which was isolated from the leaves and stems of *Amoora rohituka* Wall and from *Dysoxylum binectariferum* Blume. The mechanism of action of flavopiridol involves interfering with the phosphorylation of cyclin-dependent kinases, hampering their activation and blocking cell-cycle progression at growth phase 1 (G1) or G2 (Harmon et al., 1979; Cragg and Suffness, 1988). Currently, a number of additional plant-derived agents have been investigated (Table 2). These investigations will improve clinical chemotherapy to human carcinoma cells including ovary, colon, lung, prostate, and breast.

Table 2. Plant-derived anti-proliferative agents

Compound	Cancer use normally
Vincristine	Leukemia, lymphoma, breast, lung, pediatric solid cancers, and others cancers
Vinblastine	Breast, lymphoma, germ-cell, and renal cancer
Pacitaxel	Ovary, breast, lung, bladder, and head and neck cancer
Docetaxel	Breast and lung cancer
Topotecan	Ovarian, lung, and pediatric cancer
Irinotecan	Colorectal and lung cancer
Flavopiriol	Experimental
Acronyciline	Experimental
Bruceatin	Experimental
Thalicarpin	Experimental

4.2 Microorganisms-derived agents

Microorganisms have come into the spotlight to investigate anti-proliferative activity agents. In microorganisms include a number of compounds families to develop agents. Clinically, useful agents from these families are the daunomycin-related agents (e.g., daunomycinitself, doxorubicin, idarubicin, and epirubicin), the peptolides (e.g., dactinomycin), the mitosanes (e.g., mitomycin C) and the glycosylated anthracenonemithramycin (Adriana et al., 2001). For potential pharmaceutical agents in microorganisms, a number of researchers can progress experiences. For example, rapamycin, wortmannin and geldanamycin have been found to have anti-proliferative actions and may therefore find clinical use as novel chemotherapeutic agents (Patrick, 1997). The mode of action stages also indicates to promote using these agents.

Especially, rapamycin and its analogs are products of *Streptomyces hygroscopicus* subsp. and inhibit signaling pathways required for T-cell activation and proliferation (Adriana et al., 2001). Geldanamycin is a benzoquinone ansamycin natural fermentation product that was originally thought to be a direct protein tyrosinekinase inhibitor (Schulte and Neckers, 1998). Currently, there are many kinds of compounds from microorganisms to investigate anti-proliferative chemotherapy agents (Table 3). Therefore, microorganisms can have potential variety of physiological possibilities in human. These kinds of compounds are components of signal mechanism in human bodies, and then they have been considered attractive targets for anti-proliferative agents.

Table 3. Microorganism-derived anti-proliferative agents

Compound	Cancer use normally
Actinomycin	Sarcoma and germ-cell tumors
Bleomycin	Germ-cell, cervix, and head Phase III/IV and neck cancer
Daunomycin	Leukemia
Doxorubicin	Lymphoma, breast, ovary, lung, and sarcomas
Epirubicin	Breast cancer
Idarubicin	Breast cancer and leukemia
Mitomycin C	Gastric, colorectal, anal, and lung cancer
Streptozocin	Gastric and endocrine tumors
Wortmannin	Experimental
Rapamicin	Experimental
Geldanamycin	Experimental

4.3 Marine organisms-derived agents

Marine organisms are a rich source for natural products and many compounds that are derived from these organisms have generated interest both as challenging problems for structure elucidation and synthesis and for their cytotoxicities (Adriana et al., 2001). Since 20th centuries, in anticancer developments industries, marine organisms have been interested new resources to develop chemotherapy agents. Also many kinds of technologies allow the improvement of compounds to produce drug agents. Most of these compounds isolated from marine organisms have cytotoxicity against multiple tumor types. The first anticancer product derived from marine sources to enter clinical trials was didemnin B, a cyclic depsipeptide isolated from the tunicate *Tridemnum solidum*. Didemnin can inhibit protein synthesis and induce G1 cell cycle arrest. Aplidine is a related depsipeptide that appears to be more active than didemnin B in preclinical models, and so far does not seem to produce similar life-threatening neuromuscular toxicity (Rinehart, 2000). The other example of anti-proliferative agents identified in marine-organisms is dolastatins. The dolastatins are a class of peptides that were originally derived from a mollusk from the Indian Ocean, the seahare *Dolabella auricularia* Lightfoot. These peptides have cytotoxic activity and of the various compounds of this class, Dolastatin 10 and Dolastatin 15, have received the greatest clinical interest (Poncet, 1999). Its mechanism of action involves inhibition of microtubule assembly, which causes cell-cycle arrest in metaphase (Bai et al., 1990; Pathak et al., 1998). Apart from these compounds, there are existed a various compounds isolated from marine organisms which have anti-proliferative activities

against anticancer tumors and it have been found their mode of action *in vivo* (Table 4). To promote development of anti-proliferative agents in marine-organisms, progressing interests about these organisms are needed.

Table 4. Marineorganism-derived anti-proliferative agents

Compound	Cancer use normally	Mode of action
Citarabine	Leukemia, Lymphoma	Inhibition of DNA synthesis
Bryostatin 1	Experimental	Activation of PKC
Dolastatin 10	Experimental	Inhibition of microtubules and anti-apoptotic effects
Ecteinascidin 743	Experimental	Alkylation of DNA
Aplidine	Experimental	Inhibition of cell-cycle progression
Halicondrin B	Experimental	Interaction with tubulin
Discodermolide	Experimental	Stabilization of tubulin
Cryptophycin	Experimental	Hyperphosphorylation of Bcl-2

5. Pharmacological significance of Insects

Insects make up about 80–90% of the largest and diverse group of organisms on the Earth. Approximately 950,000 species of insects have been studied out of estimating total species 4,000,000 (Berenbaum and Eisner, 2008). Insects secrete a wide variety of chemical substances to ward off attacks and these substances are likely to produce a wealth of useful information with applications in the fields of ecology, biochemistry, and biotechnology. For these reasons, insects and their constituents become a valuable source as new medicinal compounds (Dossey, 2010). Since early times, insects, their

products and the substances have been used, directlyand indirectly, in the medical systems of different human cultures throughout the world (Costa-Neto, 2002). Indeed, insects have proven to be very important as sources of drugsfor modern medicine since they have immunological, analgesic, antibacterial, diuretic, anesthetic, and antirheumatic properties (Yamakawa, 1998). Also, insects and the substances extracted from them have been used as therapeutic resources in the medical systems of many cultures (Costa-Neto, 2005). Commonly considered to be disgusting and filthy animals, many insect species have been used live, cooked, ground, in infusions, in plasters, in salves, and as ointments, both in curative and preventive medicines, as well as in magic-religious rituals (Costa-Neto, 2002). The therapeutic use of insects and insect-derived products is known as entomotherapy. There are a number of articles describing the entomotherapy and the importance of insects-derived substances with medically relevant properties. Insects, their metabolites and the substances are potentially important sources for natural product drug discovery.

5.1 Anticancer activity of insect materials

Cytotoxicity (cell-killing) substances are often pursed as anticancer chemotherapeutics. This is a prime example of how chemical ecology can and does inform pursuits in pharmacognosy and drug discovery on which substances may be most promising to study (Dossey, 2010). There are a number of examples of cytotoxin from insects and their materials. One example of a cytotoxin from insects being explored for potential medical application is cantharidin from blister beetles (family Meloidae). The

blistering of the skin caused by cantharidin is due to the death of skin cells, which suggests potential efficacy against cancer (Moedet al., 2001). Indeed, cantharidin and its chemical derivatives have been explored due to its cytotoxic and apparent anticancer properties (Sakoffet al., 2002; Sagawa et al, 2008). Additionally, due to its cytotoxicity effects on skin, it is commonly used even in modern medicine for wart removal (Moedet al., 2001).

Even some common fatty acids from insects have been shown to possess anticancer properties. Several such compounds were isolated from the flower beetle *Protaetia brevitarsis* seulensis. In that study, the scarab larvae (or grubs) fraction was found to contain predominantly two fatty acids, palmitic acid, and oleic acid. An authentic standard of palmitic acid induced apoptosis in coloncancer cells. It was shown previously that high concentrations ofsome fatty acids cause cell death by apoptosis or necrosis (Andrade et al., 2005), and that palmitic acid can induce apoptosis in some cancer cell lines (Kong and Rabkin, 2002).

The solenopsins from red imported fire ants (*Solenopsis invicta* Buren) and related species have been pursued for a variety of medicinally relevant applications due to their ability to elicit necrosis in human tissue (Brand et al., 1973). They have been investigated for their ability to inhibit angiogenesis as inhibitors of nitric oxide production, as well as for their effect on the nervous system and on cardiosuppression in humans (Howell et al., 2005). In addition to venoms, other substances from insects can prove to be potent toxins that may be useful for fighting cancer.

Recently, new cytotoxic substances from ethanol extract of butterfly *Byasapolyeuctes termessa* Fruhstorfer, which were a part of their earlier arthropod anticancer survey work. Papilistatin which is a new cancer cell growth inhibitor has the activity on the mouse-derived leukemia model P388 (Pettit et al., 2010).

Often insects efficiently concentrate useful substances from their diet or other features of their environment. For example, the nests of wasps have been shown to contain anticancer substances. Many so-called ‘paper wasps’ (Family Vespidae) make their nests out of cellulosic plant materials collected from a diverse of sources. There are some compounds derived from the nest of the social wasp *Vespa simillima* Smith which include anticancer quinine compounds (Fujiwara et al., 2008).

In fact, promising anticancer drugs, such as isoxanthopterin and dichostatin, have been isolated from the wings of Asian sulfur butterflies (*Catopsilia crocale* Cr.) and the legs of Taiwanese stag beetles (*Allomyrina dichotomus* (L.)), respectively (Kunin and Lawton, 1996). About 4% of the extracts evaluated in the 1970s from 800 species of terrestrial arthropods (insects included) showed some anticancer activity (Oldfield, 1989). In addition, recent studies are proving that honey has good effects on human health. There have been discovered anticancer and anti-HIV activities in ethanolic extracts of propolis of *Apis mellifera* L., collected from different parts of Brazil (Part et al., 2000).

5.2 Antibacterial activity of insect materials

With the appearance of newly emerging pathogens and variousantibiotic-resistant microbial diseases, the search for new antibioticsis is a particularly important goal of modern drug discovery. Insects, as for all other organisms, are susceptible to infection by microorganisms. Various antimicrobial substances have been found in insects, many of which likely function as a defense against microbial attack and infection (Leem et al., 1996). Antibacterial proteins extracted from insects include cecropin A and B (from *Hyalophora cecropia* (L.), sarcotoxin IA, IB, and IC (*Sarcophaga peregrine* (Robineau-Desvoidy), sapecin (*S. peregrina*), defensin (*Drosophila melanogaster* Meigen), attacin (*B. mori*), diptericin (*D. melanogaster*), moricin (*B. mori*), and drosocin (*B. mori*) (Yamakawa, 1998). Cecropinsare is able to inhibit the growth of Gram-negative bacteria, while defensins are specific against Gram-positive bacteria (Montanõ-Pérez and Francisco, 2002). One of the most interesting applications of insects as therapeutic agents is maggot therapy, which is the treatment of superficial and deep wounds with the help of blowfly larvae. This medicinal use of live organisms, such as maggots, leeches, or fish, is known sometimes as biotherapy. Maggots, like those of *Phoenicia sericata* (Meigen), feed on dead tissue where gangrene-causing bacteria thrive. As they eat they secrete allantoin, a chemical that inhibits bacterial growth. They are primarily used to treat tumor-killed tissue and burns in people who would be endangered by surgery. Synthetically produced allantoin was commonly used as an antibacterial ointment until penicillin and other antibiotics became commercially available in the 1940s. Nowadays, maggot therapy is increasing around the world due to its efficacy, safety, and simplicity.

Medicinal maggots have three actions: they clean wounds by dissolving the dead (necrotic) infected tissue, they disinfect the wound by killing bacteria, and they stimulate wound healing (Shermanet al., 2000). Theoretical surveys recently carried out and have confirmed the benefits of this kind of therapy and state that it could be adopted widely in Brazil and overseas in the near future (Beraldo and von Zuben, 2004). Most of the known antibacterial activities of substances produced by insects are peptides, but in some cases antibacterial small-molecule secondary metabolites have been found (Ricciet al., 2005).

5.3 Insect neurotoxins

Most numerous insect venoms contain neurotoxins which are used to paralyze prey rather than for defense (Dossey, 2010). Acylpolyamines and peptides from a diverse spiders and their potential use in treating pain and central nervous system disease such as Huntington disease, Alzheimer's disease, and Parkinson's disease (Estrada et al., 2007). In fact, over 40 different polyamine neurotoxins have been isolated from the venoms of spider species (Nakanishi et al., 1994). Thus, even though spiders are an established source of toxins, they still represent a vast reservoir for discovery of useful substances such as potential pharmaceuticals. The venoms of many insects, particularly those of ants, bees, and wasps (order Hymenoptera) contain neurotoxins such as philanthotoxin which is originally discovered in the venom of the predatory wasp species *Philanthus triangulum* (Fab.) (Olsen et al., 2006). Philanthotoxin is a noncompetitive antagonist of both glutamate and nicotinic acetylcholine receptors

(Nakanishi et al., 1994). These receptors in humans have a side diverse of functions, including pre and postsynaptic neural transmission, memory formation, learning, and muscle contraction. They are, therefore, major drug targets for a lot of therapeutic applications, including treatment of neurodegenerative diseases (Dossey, 2010).

A systematic survey of biologically active substancesin solitary wasp venoms has identified novel peptide neurotoxins, pompilidotoxins (PMTXs), from the venom of the spider wasps *Anoplius samariensis* Pal. and *Pseudagenia maculifrons* Sm. in Japan (Konno et al., 1998). During this survey, a new mast cell degranulating peptide designated as eumeninemastoparan-AF (EMP-AF) was identified in the venom of the wasp *Anterhynchiumflavomarginatummicado* Kirsch, the most abundant eumenine wasp in Japan. These types of toxins may be useful not only for basicneuroscience research but also for the development of therapeutic agents for neurological disorders (Konnoet al., 2000).

5.4 Antivirals activity of insect materials

Some insect-derived substances, particularly peptides, have also been shown to be effective against virus infection and replication (Dossey, 2010). Anti-HIV activity has been reported for both melittin which is from the sting venom of honeybees and cecropins. Also, they have been shown to be effective against other viruses such as not only herpes simplex virus (Baghian et al., 1997) but also HIV-virus by inhibiting HIV-virus gene expression (Wachinger et al., 1998). Another group of insect-derived antiviral peptides, originally discovered in the hemolymph of experimentally infected blowflies

(*Calliphor avicina* Robineau-Desvoidy), are the alloferons (Chernysh et al., 2002). These peptides have been shown to be effective against influenza and herpes simplex virus (Ryu et al., 2008). A possible of mode of action is that alloferons against viruses could be activation of the NF-κB signaling pathway.

5.5 Other medicinally relevant properties of insect materials

Some insect-derived substances have been used to cure patients with diseases. Bee venom therapy is commonly used to treata variety of conditions such as arthritis, rheumatism, pain, and even cancer. It contains a variety of proteins and other substances with multiple pharmacologically relevant properties (Son et al., 2007). The use of bee venom and other natural products from bees is known as apitherapy. There is even an organization dedicated to promoting apitherapy, the American Apitherapy Society. Also, powdered silkworm larvae (silkworm powder) are often prescribed in Asian medicine and are commercially available. Silkworm powder has been tested and shown in modern bioassays to inhibit absorption of glucose in human intestinal epithelium cells (Han et al., 2007) and reduce vasopressin expression in the hypothalamus of diabetic mice (Kim et al., 2007). With potential medicinal value, the insect materials are valuable to study.

6. Silkworm feces

The mulberry tree, *Morusalba* L. (Moraceae), is distributed in Korea, China, and Japan (Choi et al., 1999), and its leaves are used to feed the silkworm, *Bombyx mori* (L.) (Lepidoptera: Bombycidae). When the silkworm ingests mulberry leaves, about 60% of the leaves were excreted without digestion (Leeand Lee, 1971), resulting in silkworm feces that are composed of not only intact mulberry leaf material but also various materials transformed by enzymes or microbes in the intestine of the silkworm (Park et al., 2011). Generally silkworm feces are collected for medicinal purpose during June to August, mainly in the second and third sleeps. As far as the quality concern, the autumn one is preferred to the spring one. Dried silkworm feces are cylinder-shaped particles, 2 to 5 mm in diameter. The surface is grey, rough, and with six obvious longitudinal ridge and 3 to 4 light horizontal lines. Both ends are slightly flat and with 6 edges in transverse section. The texture of silkworm feces is solid and crisp, and fragile in wet condition. It smells slightly like fresh grass. The preferred one is dry, black, solid, even, and inclusion free (Chinese Herbs Healing, 1988). Silkworm feces were reported in the ‘Dongeuibogam’ Korean medical text to be non-toxic, and can be used to strengthen the internal organs, to protect diplegia, and to treat diabetes (Kimet al., 1997). In the ‘Sinnongbonchogyung’ text, silkworm feces were used to cure hangovers, were thought to have antiaging effects, and were used to treat hemiplegia and urticaria (Lee, 1994). In China, silkworm feces have been used for traditional therapy. Indeed, it is widely used in ancient Chinese herbal remedies. It is sweet, acrid, and warm in nature and covers meridians of liver, spleen, and stomach. Main functions are to expel wind-damp and

harmonize stomach for transforming dampness. Main uses and indications are Bi syndrome (channel blockade), vomit, spasm, diarrhea, and rashes. Usually, usual dosage of silkworm feces is 5 to 15 g in decoction in Chines herbal remedies. These days, additionally, silkworm feces have been shown to lower blood pressure and cholesterol levels and to have anticancer and antidiabetes characteristics (Sugawaraet al., 1990; Lee et al., 1999). Even though the quantity of silkworm feces produced from one breeding cage of silkworm is about 104 kg, resulting in a yield of approximately 130 tons per a year in Korea, most of the droppings are disposed without any medicinal utilization (Sohn et al., 2007).

The constituents isolated from silkworm droppings include β-carotene (Tong and Xu, 2005), 1-deoxynojirimycin, fagomine, and 3-epifagomine (Zhouet al., 2007), and lipids such as corchorifatty acid E methyl ester, (8*E*)-10-hydroxydec-8-enoic acid methyl ester (Sohn et al., 2009).

In pharmacological study, the 80% aqueous methanolic extracts of the silkworm feces have antiinflammatory activity which is attributable to lignans derivatives including (+)-pinoresinol, (+)-lariciresinol, (+)-syringaresinol, syringaresinol-4-*O*-β-D-glucopyranoside, (+)-lariciresinol-4-*O*-β-D-glucopyranoside, and ehydrodiconiferylalcohol-9'-*O*-β-D-glucopyranoside (Park et al., 2010). Also, methanolic extracts make HO-1 which is considered to be an antioxidant enzyme to catabolize heme to carbon monoxide, free iron, and biliverdin. While SIRT1 is the mammalian homolog of the yeast silent information regulator involved in the suppression of inflammatory mediators or factors that may be used to improve atopic

dermatitis related symptoms to promote activity. These active compounds identified in silkworm feces are (3*S*,5*R*,8*R*)-3,5-dihydroxymegastigma-6,7-dien-9-one, (*S*)-dehydrovomifoliol, (6*R*,7*E*,9*R*)-9-hydroxy-4,7-megastigmadien-3-one, (3*S*,5*R*,6*S*,7*E*)-3,5,6-trihydroxy-7-megastigmen-9-one, (6*R*,9*R*)-9-hydroxy-4-megastigmen-3-one (Park et al., 2010). A new flavaneglucoside, 7,20-dihydroxy-8-hydroxyethyl-40-methoxyflavane-20-*O*-ß-D-glucopyranoside, along with three known flavonoids, 7,20-dihydroxy-8-prenyl-40-methoxyflavane, euchrenone a_7, and 7,20-dihydroxy-8-prenyl-40-methoxy-20-*O*-ß-D-glucopyranosylflavane have also promotion activity of HO-1 (Park et al., 2011).

7. Mode of action

In the past, natural products with cytotoxic activity often entered the clinic before any real understandings of their modes of activity was appreciated. Natural products' compound mode of action is still completely unknown, and it does show a considerable level of cytotoxicity (Mann, 2002). In this situation, many researchers would no longer allow such fundamental stages. They screen their natural products' extracts and then try to molecularly defined assays by distracting molecules. By doing so, they can increase their chances of identifying compounds with genuine anticancer activity. At this stage, numerous analogs are prepared for extensive structure–activity relationship studies, and a real effort can be made to elucidatethe mode of biological activity, before the choice of suitable preclinical candidates is made. In this way, the drug derived from natural

products that is finally selected for clinical trials is more likely to have beneficial effects with minimum of adverse side effects (Gordaliza, 2007).

7.1 Inhibitors of angiogenesis

Angiogenesis is the physiological process through which new blood vessels from preexisting vessels. Angiogenesis is a normal and vital process in growth and development, as well as in wound healing and in the formation of granulation tissue. However, it is also a fundamental step in the transition of tumors from a benign state to a malignant one, leading to the use of angiogenesis inhibitors in the treatment of cancer. Several natural products have shown effectively useful activity as inhibitors of angiogenesis, fumagillin from the fungus *Aspergillus fumigatus* Fresenius is probably the best studied (Liekens et al., 2001). More than ten years ago, its inhibitory activity was shown but its associatedtoxicity precluded clinical evaluation (Mann, 2002). However, its activity provided the basis for the preparation of a range of structural analogs. For instance, TNP-470 (chloroacetyl carbamoyl fumagillol) is effective against a wide variety of tumors *in vitro* (Folkmanet al., 1990). It has been treated the subject of Phase II and Phase III trials with solid tumors, and a Phase I trial against lymphomas and acute leukemia. Through these experiences, it is noticed that these compounds have little side effects compared with commercial anticancer drugs. Its mode of action is obscure, but there has been a recent demonstration that fumagillin inhibits expression of the ETS1 transcription factor, which regulates the expression of vascular endothelial growth factors (VEGFs) (Wernertet al., 1999). The other one of example is the

combretastatins from the Africanbush willow, *Combretum caffrum* (Eckl. & Zeyh.) Kuntze, provides a further example of native folk medicinesuggesting a lead compound (Pettitet al., 1989). This lead compound has shown selective cytotoxicity against proliferating endothelial cells in culture. The compound makes a hemorrhagic necrosis at doses that are no more than 10% maximum tolerated dose (Mann, 2002). In the United Kingdom (UK) and US, it used in phase I clinical trials to patients. This compound well known not only caused much necrosis but also failed to cause tumor regression. More recent results using combinations of combretastain A-4with cisplatin or 5-fluorouracil have provided better results (Grosios et al., 2000; Griggs et al., 2001). Therefore, like these examples, it needs to be put more experiences into mode of action practices to exploit practicable drugs in natural products industry. By doing so, the natural products industry will be increased giving benefits for patients with diseases.

7.2 Modifying cell signaling

For rational drug design, it is essential pathway to do experience cell signaling pathway. The signaling pathway is upgraded in cancer cells an obvious target. Most of the research in this area has been devoted to the identification of agents that will inhibit aspecific protein kinase of a signalling pathway, and the RAS–RAF–mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling pathway has received most attention (Weinstein-Oppenheimer, 2000). There are some natural products having useful inhibitory activity and therefore it is very effective starting points for the design of more potent molecules. For example, radicicol from the microparasite

Monocilliumnordinii acts as a RAF destabilizer by binding to heat-shock protein 90 (HSP90), which stabilizes RAF (Sano, 2001). Lavendustin from *Streptomyces griseolavendus* acts as a specific inhibitor of the epidermal growth factor receptor (EGFR) protein tyrosinekinase, and has been used as a template for the production of a range of analogues (Cushmanet al., 2001). However, these analogies biological evaluation indicates that their primary mode of action, which has cytotoxicity, was through inhibition of tubulin polymerization. In this situation, therefore, it needs to be developing more resources from natural products to upgrade mode of action to disease. Patients with diseases will be taken advantages of getting effective chemotherapies.

8. Perspectives

An analysis of a number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds (Newman and Cragg, 2007). Undoubtedly, in the future pharmaceutical industry will try to obtain drugs from natural products through the use of combinatorial chemistry and high-throughput screening technologies. To develop new drugs from natural products, it needs to be set vast libraries of compounds exactly. By contrast, the much more subtle use of natural-product templates combined with chemistry to produces elective analogs will have a much greater chance of success. In a sense, it will accept that nature has already carried out the combinatorial chemistry; all have to do is refined the structures (Mann, 2002). In addition, it needs to be broad portion natural products. Most of the natural products in clinical use today were discovered through a routine examination of just terrestrial

plants or microorganisms, so serendipity is still an important route of discovery such as insects or insects' metabolites. Several studies have to enhance inhibitory activity in a range of tumor not only *in vitro* but also *in vivo*. Natural products with interesting anti-proliferative activity which have the basic skeleton can be used as a template for the production of libraries of analogs with potentially greater activity. Through these libraries database, researchers can take advantage of natural gift from natural products. To develop natural products industries, synthetic chemistry will also grow steadily. This coupling of synthetic chemistry with the new technologies will take positive effects of nature's gifts without exploitation of the often delicate ecologies that support the parent organisms. And it will ensure that natural products make as many future contributions to cancer chemotherapy as they have in the past 35 years (Mann, 2002). Although, insects make up about 80–90% of the largest and diverse group of organisms on the Earth, there are a few researches to develop new drugs utilizing insects and insects' metabolites. Insect resource industry has many potential possibilities to grow up developing valuable sources of new medicinal compounds for human. The experimental agents isolated from insects and insects' metabolites will give good opportunities to assess not only new chemical compounds families of anticancer agents, but also relevant mode of action potentially.

MATERIALS AND METHODS

1. Instrumental analyses

^{1}H and ^{13}C NMR spectra were recorded in CD_3OD on a AVANCE 600 spectrometer (Bruker, Rheinstetten, Baden-Württemberg, Germany) at 600 and 150 MHz, respectively, using tetramethylsilane as an internal standard, and chemical shifts are given in δ parts per million (ppm). Distortionless enhancement by polarization transfer (DEPT) spectra was acquired using the Bruker software. Mass spectra were obtained on a GSX 400 spectrometer (Jeol, Tokyo, Japan). UV spectra were obtained in methanol with a V-550 spectrophotometer (Jasco, Tokyo, Japan). Silica gel 60 (0.063–0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Merck precoated silica gel plates (Kieselgel 60 $F_{254,}$ 0.20 mm) were used for analytical thin layer chromatography (TLC). An Isolera one medium-pressure liquid chromatograph (MPLC) (Biotage, Uppsala, Sweden) and an Agilent 1200 high-performance liquid chromatograph (HPLC) (Santa Clara, CA, USA) were used for isolation of active principles.

2. Materials

Vomifoliol and stigmasterol were purchased from BOC Science (NY, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Commercially available anticancer agent cisplatin was obtained from Sigma-Aldrich (St. Louis, MO, USA). [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) was purchased from

Sigma-Aldrich. Minimum essential medium (MEM), Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were supplied by Life Technologies (Grand Island, NY, USA). Phosphate-buffered saline **(**PBS) was purchased from Sigma-Aldrich. Antibiotic-antimycotic solution and 0.5% trypsin-ethylenediaminetetraacetic acid (EDTA) was purchased from Invitrogen (Grand Island, NY, USA). Maxima SYBR Green/ROX qPCR Master Mix was supplied by Thermo Scientific (Foster, CA, USA). All of the other chemicals and reagents used in this study were of analytical grade quality and available commercially.

3. Silkworm feces and sample preparation

Air-dried of silkworm feces were purchased from a local farm in Suncheon-si, Jeollanam-do, Republic Korea, and used for extraction. A voucher specimen (SF-01) was deposited in the Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University.

4. Extraction and isolation of active constituents from silkworm feces

The air-dried silkworm feces (30.84 kg) was pulverized, extracted with ethanol (155 L) three times at room temperature for 3 days, and filtered through Whatman no. 2 filter paper (Maidstone, Kent, UK). The combined filtrate was concentrated to dryness by rotary evaporation at 40°C to yield approximately ~662.0 g of a dark yellowish green tar (based on the weight of the silkworm feces). The extract (660 g) was sequentially partitioned into hexane- (418.44 g), chloroform- (51.48 g), ethyl acetate- (8.58 g),

butanol- (60.39 g), and water-soluble (121.11 g) portions for subsequent bioassay (Figure 1). The organic solvent fractions were concentrated under vacuum at 40°C, and the butanol and water fractions were concentrated at 50°C.

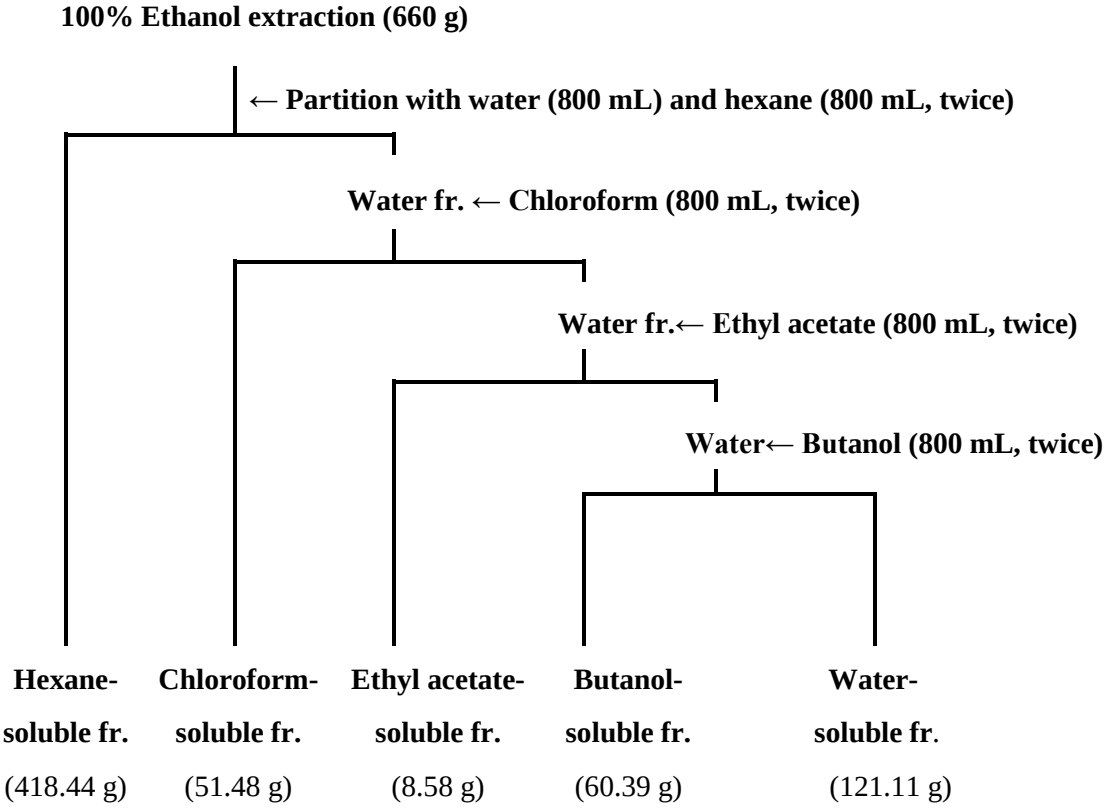

Figure 1. Solvent fractionation procedures of ethanol extract from silkworm feces

The chloroform-soluble fraction (51.48 g) was most active and open column chromatography was performed on a 5.5 cm i. d. × 70 cm silica gel (600 g) column by elution with chloroform and methanol [100:0 (1 L), 97:3 (1 L), 95:5 (2 L), 90:10 (1 L), 80:20 (1 L), 70:30 (1 L), and 0:100 (1 L) by volume] to provide 37 fractions (each about 180 mL). Column fractions were monitored by TLC on silica gel plates (Silica gel 60 F_{254}) developed with chloroform and methanol (95:5 by volume) mobile phase.

Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 5% sulfuric acid and then heating on a hot plate. Fractions 23 to 36 (C4, 14.86 g) was separated by MPLC using a Biotage Isolera apparatus equipped with a UV detector at 254 nm and 365 nm and a column cartridge SNAP (100 g silica gel) w ith column volume 132 mL. Separation was achieved with a gradient of chloroform and methanol [100:0 (847 mL), 98:2 (657 mL), 97:3 (382 mL), 95:5 (2740 mL), 92:8 (536 mL), 90:10 (281 mL), 86:14 (127 mL), 85:15 (369 mL), 80:20 (335 mL), and 0:100 (800 mL) by volume] at a flow rate 25 mL/min to provide 190 fractions (each about 22 mL). Column fractions were monitored by TLC on silica gel plates, as stated previously. Fractions 82 to 165 (C43, 5.40 g) was separated by MPLC with SNAP (100 g silica gel) column by elution of a gradient of chloroform and methanol [100:0 (924 mL), 99:1 (99 mL), 98:2 (46.2 mL), 95:5 (396 mL), 93:7 (567.6 mL), 90:10 (1379.4 mL), 85:15 (508.2 ml), 83:17 (481.8 ml), 80:20 (323.4 ml), and 0:100 (800 mL) by volume] at a flow rate 25 mL/min to provide 175 fractions (each about 22 mL). Column fractions were monitored by TLC on silica gel plates developed with chloroform and methanol (90:10 by volume). Fractions 17 to 40 (C432, 2.13 g) was also separated by MPLC with SNAP (100 g silica gel) column by elution with a gradient of chloroform and methanol [100:0 (699.6 mL), 97:3 (59.4 mL), 95:5 (1023 mL), 90:10 (910.8 mL), 85:15 (40.0 mL), 80:20 (699.6 ml), 70:30 (330 ml), and 0:100 (800 mL) by volume] at a flow rate 25 mL/min to provide 235 fractions (each about 22 mL). The TLC analysis was conducted with chloroform and methanol (90:10). Fractions 13 to 50 (C4322, 0.84 g) was also separated by MPLC with SNAP (100 g silica gel) column by elution with a

gradient of chloroform and methanol [100:0 (580.8 mL), 99:1 (165 mL), 98:2 (132 mL), 97:3 (270.6 ml), 96:4 (983.4 ml), 95:5 (534.6 ml), 94:6 (316.8 ml), 93:7 (415.8 ml), 92:8 (330 ml), 91:9 (297 ml), 90:10 (343.2 ml), and 0:100 (800 mL) by volume] at a flow rate 25 mL/min to provide 165 fractions (each about 22 mL). The TLC analysis was conducted with chloroform and methanol (90:10). A preparative HPLC was used for separation of the constituents from the active fractions 1 to 20 (C43221, 6.0 mg). The column was a 7.8 mm i.d. × 300 mm μBondapak C_{18} (Waters, Milford, MA, USA) using a mobile phase of methanol and water (50:50 by volume) at a flow rate of 1.0 mL/min. Chromatographic separations were monitored using a UV detector at 244 nm. Finally, a potent active principle **1** (C432212) was isolated at a retention time of 28.83 min (Figure. 3).

Fractions 35 to 82 (C43224, 0.76 g) was also separated by MPLC with SNAP (100 g silica gel) column by elution with a gradient of chloroform and methanol [100:0 (448.8 mL), 99:1 (59.4 mL), 98:2 (52.8 mL), 95:5 (19.8 ml), 92:8 (66 ml), 90:10 (257.4 ml), 89:11 (85.8 ml), 88:12 (158.4 ml), 85:15 (237.6 ml), 83:17 (237.6 ml), 80:20 (198 ml), and 0:100 (800 mL) by volume] at a flow rate 25 mL/min to provide 65 fractions (each about 22 mL). The TLC analysis was conducted with chloroform and methanol (90:10). Fractions 8 to 12 (C432242, 0.48 g) was also separated by reverse MPLC with SNAP KP-C18-HS (30 g) column by elution with a gradient of water and methanol [100:0 (462 mL), 65:35 (165 mL), 50:50 (719.4 mL), 40:60 (594 ml), 30:70 (640.2 ml), 20:80 (1260.6 ml), 10:90 (627 ml), 8:92 (488.4 ml), 5:95 (587.4 ml), and 0:100 (198 mL) by volume] at a flow rate 25 mL/min to provide 70 fractions (each about 22 mL). The TLC analysis on silica gel plates (silica gel RP-18 F_{254}, Merck) was conducted with methanol and water (80:20). A preparative HPLC was used for separation of the constituents from the active fractions 45 to 50 (C4322426, 9.5 mg). The column was a 7.8 mm i.d. × 300 mm Waters μBondapak C_{18} using a mobile phase of methanol and water (80:20 by volume) at a flow rate of 1.0 mL/min. Chromatographic separations were monitored using a UV detector at 214 nm. Finally, a potent active principle **2** (C4322426) was isolated at a retention time of 44.84 min (Figure. 4).

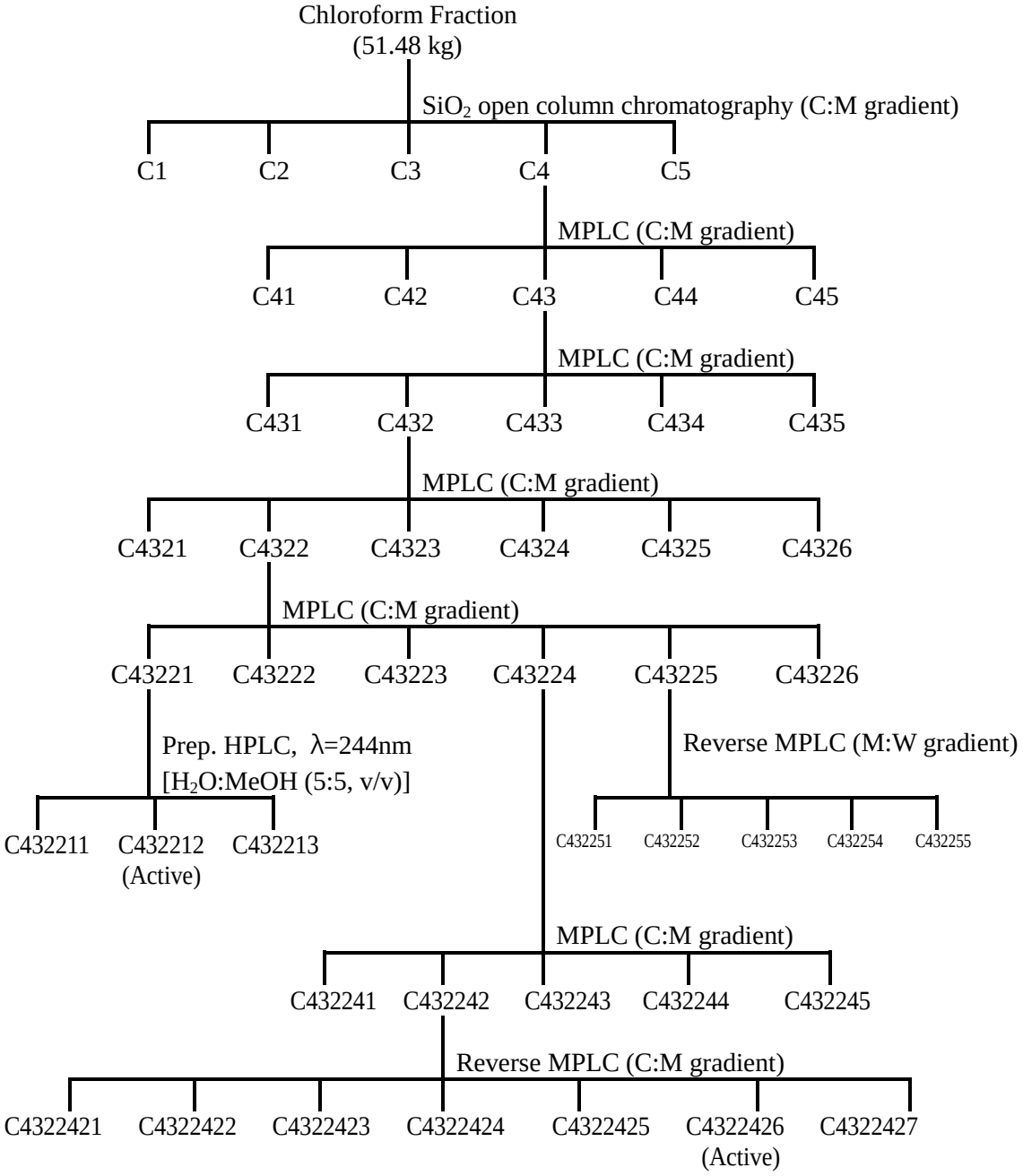

Figure 2. Isolation procedure of chloroform-soluble fraction from silkworm feces ethanol extract.

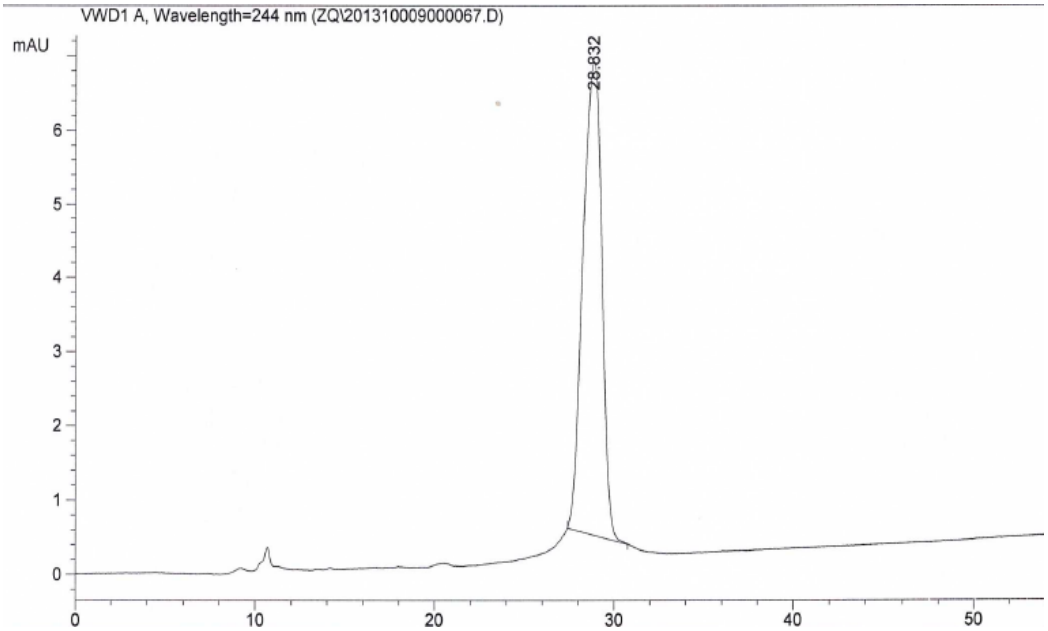

Figure 3. HPLC chromatogram of compound 1.

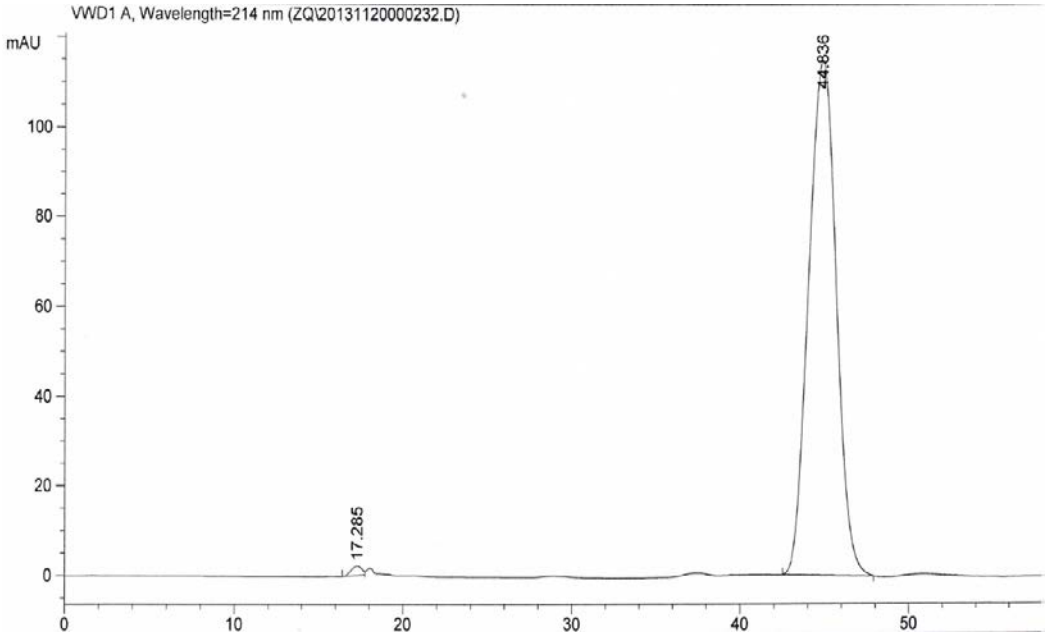

Figure 4. HPLC chromatogram of compound 2.

5. Cell lines and culture conditions

Ten human cancer cell lines used in this study were as follows: NCI-H727 (human lung carcinoma cell line), MRC-5 (human lung normal cell line), L-132 (human lung normal cell line), PC-3 (a human prostate adenocarcinoma cell line), HT-29 (human colon adenocarcinoma cell line), AGS (human stomach adenocarcinoma cell line), MCF-7 (human breast adenocarcinoma cell line), SK-OV-3 (human ovary adenocarcinoma cell line), Hep-2 (human larynx adenocarcinoma cell line), SNU-213 (human pancreas cancer), and SK-HEP-1 (human liver adenocarcinoma cell line) purchased from the Korean Cell Line Bank (KCLB) (Seoul, South Korea); HeLa (human cervix adenocarcinoma cell line) and A549 (human lung carcinoma cell line) purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

NCI-H727, PC-3, HT-29, AGS, MCF-7, and SK-OV-3 cell lines were cultured with RPMI 1640 containing 10% FBS and 1% antibiotic-antimycotic solution under 5% CO_2 and 95% air at 37°C. A549, HeLa, SK-HEP-1, and MRC-5 cell lines were cultured with MEM containing 10% FBS, 1% antibiotic-antimycotic solution, and 1% glutamine under 5% CO_2 and 95% air at 37°C. Hep-2, L-132 cell line was cultured with DMEM containing 10% FBS and 1% antibiotic-antimycotic solution under 5% CO_2 and 95% air at 37°C (Korean Cell Line Bank, 1982). Cells were grown in Corning Costar disposable Petri dishes (NY, USA).

6. Anti-proliferative assay

The anti-proliferative activity of the test materials to the human cancer cell lines was evaluated using a MTT assay described previously by Morgan (1998). A 10× stock solution of MTT (5 mg/mL) was prepared in PBS (pH 7.4). The stock solution was sterile-filtered and stored at −20°C. The cells were plated at 2×10^3 cells per well in 100 μL of complete culture medium containing several different concentrations of the test materials in 96-well microplates. The samples were dissolved in dimethyl sulfoxide (DMSO) Hybri-Max. The final concentration of DMSO Hybri-Max in all assays was 0.1% or less. The culture plates were incubated for 2 days in a 37°C incubator with a humidified atmosphere of 5% CO_2. The plates were then washed one time with 100 μL PBS. A volume of 100 μL medium containing 0.05% MTT was added to each well and then incubated for 4 h at the same condition. MTT solution was removed after 4 h of the incubation and 200 μL DMSO was added to each well. Finally, the plate was shaken for 10 min to dissolve the purple formazan crystals that had formed. Cisplatin served as positive controls and were similarly formulated. Negative controls only consisted of the DMSO solution only. The optical density (OD) values were recorded using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a 560 nm and a 670 nm reference. Blank values were subtracted from experimental values.

7. Quantitative real-time reverse transcription-PCR analysis

To evaluate the level of gene expression, real-time RT-PCR with SYBR Green dye was carried out. In brief, treated and nontreated cultures of A549 monolayers grown in

25 cm^2 cell culture flasks (Corning Costar, NY, USA) were treated with 12, 24, and 50 μg/mL of each test compound. After 2 days of treatments, the total RNA extraction from the nontreated and treated cultures was achieved with a RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The contaminated genomic DNA was removed using RQ1 RNeas-free DNAase (Promega, Madison, WI, USA). Complementary DNA (cDNA) was synthesized using 1 μg total RNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA). The cDNA products were stored in aliquots at –80°C until needed. Five log10-fold dilutions of cDNA for each RNA were performed to determine PCR efficiency (100 ng–10 pg per reaction). qRT-PCR was performed in 96-well plates using Applied Biosystem StepOne-plus real-time PCR system (Applied Biosystems, Foster, CA USA). Each reaction mixture consisted of 10 μL Maxima SYBR Green/ROX qPCR Master Mix (2×), 2 μL of forward and reverse primers (5 pmol of each), 1 μL cDNA (8 ng), and 7 μL diethylpyrocarbonate-treated water in a final volume of 20 μL. Oligonucleotide PCR primer pairs are listed in Table 1 and purchased from Bioneer (Daejeon, South Korea). The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 50 cycles of 95°C for 15 s and either 60°C (GAPDH, Akt1, or Bcl-2) for 30 s. mRNA expression level of target gene was normalized to mRNA level for the housekeeping gene GAPDH and analyzed by the $2^{-\Delta\Delta CT}$ method using StepOne Software v2.1 and DataAssist Software (Applied Biosystems).

Table 5. Primers used for real-time RT-PCR

Gene name	RefSeq ID	Forward primer and Reverse primer	cDNA amplicon size	Reference
GAPDH	M32599	TGCACCACCAACTGCTTAG GGATGCAGGGATGATGTTCTG	177	Uenoa et al., 2004
Akt1	NM_005163.2	CAAGCCCAAGCACCGC GGATCACCTTGCCGAAAGTG	80	Chin et al., 2014
Bcl-2	NM_000657	ATTGGGAAGTTTCAAATCAGC TGCATTCTTGGACGAGGG	301	Hu et al., 2007

8. Data analysis

Anti-proliferative activity was exposed as 50% inhibition concentration (IC_{50}) of the compound that reduced the viability of cells to 50% compared with the control wells. IC_{50} values of the test compounds were calculated using Prism 5 software program (GraphPad Software, La Jolla, CA, USA). The percent growth inhibition is calculated as % growth inhibition = $\frac{A}{B} \times 100$**,** where A and B are the OD values of treated cells and untreated cells, respectively. Results were expressed as mean ± SE of triplicate samples of three independent experiments. Statistical analyses were carried out using SAS 9.13 program (SAS Institute, Cary, NC). Significance between means was determined using one-way or two-way analysis of variance statistical test.

RESULTS

1. Anti-proliferative activity of silkworm feces

The anti-proliferative activity of ethanol extract from silkworm feces was compared with that of the commercial anticancer agent cisplatin toward various human cancer cell lines using a MTT assay (Table 6). Responses varied according to cell line examined. Based on IC_{50} values, the silkworm feces ethanol extract was proved to have anti-proliferative activity against all test cancer cell lines (IC_{50}, 9.25–58.78 g/mL). Overall, the extract was 1.69–37.06 times less toxic than cisplatin toward all cancer cell lines. The cytotoxicity of the silkworm feces ethanol extract to two normal lung cell lines was likewise examined. The extract was not cytotoxic toward the normal lung cell lines L-132 and MRC-5 ($IC_{50,}$ >100 g/mL).

Table 6. A**nti-proliferative activity of ethanol extract from silkworm feces and anticancer agent cisplatin toward 10 cancer cell lines using a tetrazolium assay**

Cell line	IC_{50},[a] μg/mL (95% confidence limit)		RT[c]
	Ethanol extract	Cisplatin[b]	
A549	15.71 (11.27–22.54)	2.36 (1.19–4.01)	6.66
H727	9.25 (7.37–13.02)	5.48 (4.46–6.22)	1.69
SNU-213	58.78 (37.74–76.63)	22.40 (17.83–28.24)	2.62
PC-3	45.70 (44.16–49.68)	2.93 (1.69–4.86)	15.60
HT-29	30.76 (25.24–38.57)	16.26 (14.37–19.40)	1.89
HeLa	45.15 (32.16–63.38)	12.54 (5.05–30.30)	3.60
AGS	41.93 (35.13–50.05)	5.19 (4.05–6.63)	8.08
MCF-7	32.53 (26.57–36.89)	3.58 (1.59–8.90)	9.09
SK-HEP	53.37 (40.1–70.8)	1.44 (0.45–3.38)	37.06
SK-OV-3	50.33 (43.73–61.09)	1.98 (0.84–3.94)	25.42
HEP2	28.39 (20.30–35.39)	7.67 (3.38–12.53)	3.70

[a] The 50% anti-proliferative concentration for cell lines.

[b] The positive control.

[c] Relative toxicity, IC_{50} of slough ethanol extract/ IC_{50} of cisplatin.

2. Isolation and identification of active principles from silkworm feces

Fractions obtained from the solvent hydrolysable of the ethanol extract of the silkworm feces were likewise tested toward A549 lung cancer cell line (Table 7). Significant differences in toxicity in fractions of the extract were observed. As judged by IC_{50} values, the chloroform-soluble fraction showed the most pronounced anti-proliferative activity. The hexane- and ethyl acetate-soluble fractions exhibited moderate anti-proliferative activity. Weak and no activity were produced by the butanol- and water-soluble fractions, respectively. The chloroform-soluble fraction was used to identify peak activity fractions for the next step in the purification.

The anti-proliferative activities of each subfraction derived from the chloroform-soluble fraction are given in Table 8.

Table 7. Anti-proliferative activity of fractions obtained from the solvent hydrolysable of the ethanol extract of silkworm feces toward A549 lung cancer cell line using a tetrazolium assay

Fraction	IC_{50},[a] μg/mL (95% CL[b])	Slope ± SE	χ^{2c}	*P*-value
Ethanol fraction	15.71(13.53–18.23)	1.50 ± 0.92	4.00	0.9720
Hexane-soluble fr.	30.34(27.29–99.74)	1.08 ± 0.88	1.61	0.9964
Chloroform-soluble fr.	9.70(8.28–11.37)	1.06 ± 0.91	2.51	0.9662
Ethyl acetate-soluble fr.	34.63(27.27–43.98)	3.42 ± 0.86	7.82	0.9700
Butanol-soluble fr.	91.94(75.18–112.4)	1.06 ± 0.82	3.56	0.9874
Water-soluble fr.	>100			

[a] The 50% anti-proliferative concentration for cell lines.

[b] CL denotes confidence limit.

[c] Pearson χ^2, goodness-of-fit test.

Table 8. Anti-proliferative activity of each subfraction from chloroform-soluble fraction toward A549 lung cancer cell line

Fraction	IC_{50},[a] mg/mL (95% CL[b])	Slope ± SE	χ^{2c}	*P*-value
C1	>100			
C2	>100			
C3	35.19	0.9 ± 0.71	3.32	0.9845
C4	8.64	0.9 ± 0.73	2.82	0.9677
C5	50.95	1.5 ± 0.52	9.85	0.9399
C41	>100			
C42	>100			
C43	7.90	1.0 ± 0.71	1.84	0.9849
C44	9.14	0.9 ± 0.39	3.92	0.9463
C45	>100			
C431	10.21	1.6 ± 0.93	1.35	0.9929
C432	2.72	0.8 ± 0.34	2.02	0.9479
C433	3.40	0.8 ± 0.21	1.29	0.9639
C434	37.10	1.7 ± 0.96	5.40	0.9807
C435	>100			
C4321	23.84	1.6 ± 0.32	8.44	0.9311
C4322	1.12	0.5 ± 1.87	3.15	0.9244
C4323	2.87	0.8 ± 0.54	1.22	0.9760
C4324	>100			
C4325	>100			
C4326	>100			
C43221	2.01	0.5 ± 0.36	2.56	0.9119

Fraction	IC_{50},[a] mg/mL (95% CL[b])	Slope ± SE	χ^{2c}	*P*-value
C43222	3.52	0.5 ± 0.27	1.44	0.9781
C43223	4.65	0.6 ± 0.40	1.46	0.9827
C43224	1.63	0.5 ± 0.40	0.80	0.9898
C43225	1.84	0.5 ± 0.40	0.49	0.9964
C43226	54.46	1.0 ± 1.07	7.99	0.9337
C432211	-	-	-	-
C432212	-	-	-	-
C432213	-	-	-	-
C432241	8.80	0.9 ± 0.69	1.38	0.9921
C432242	1.41	0.5 ± 0.20	2.11	0.9321
C432243	61.01	1.2 ± 0.82	3.74	0.9862
C432244	51.14	1.5 ± 0.13	8.99	0.9354
C432245	>100	-	-	-
C432251	34.36	0.9 ± 0.46	5.69	0.9522
C432252	>100	-	-	-
C432253	5.75	0.7 ± 0.46	1.80	0.9789
C432254	2.41	0.7 ± 0.28	2.22	0.9393
C432255	1.73	0.4 ± 0.31	0.93	0.9867
C4322421	51.68	1.2 ± 0.93	2.70	0.9927
C4322422	>100	-	-	-
C4322423	26.80	1.4 ± 0.82	4.65	0.9782
C4322424	2.56	0.9 ± 0.42	1.06	0.9815
C4322425	2.23	0.9 ± 0.01	1.70	0.9387
C4322426	2.19	0.7 ± 0.55	0.82	0.9888

Fraction	IC_{50},[a] mg/mL (95% CL[b])	Slope ± SE	χ^{2c}	*P*-value
C4322427	26.63	1.2 ± 0.63	5.32	0.9639

[a] The 50% anti-proliferative concentration for cell lines.

[b] CL denotes confidence limit.

[c] Pearson χ^2, goodness-of-fit test.

MTT bioassay-guided fractionation of the ethanol extract from the silkworm feces afforded two active principles (compound **1 and 2**) identified by spectroscopic analysis, including EI-MS and NMR. Compound **1** was obtained as colorless powder. The mass spectrum of the isolate exhibited a molecular ion at *m/z* 124 $[M]^+$ (Figure 5) and its ^1H NMR spectra (Figure 6) showed 20 protons. Its ^{13}C NMR spectra (Figure 7) showed 13 carbons in the molecule comprising four methyl groups, two hydroxyl groups, and one ketone group carbons as indicated in DEPT (Figure 8), suggesting the molecular formula $C_{13}H_{20}O_3$. The interpretations of proton and carbon signals were largely consistent with those of Xiaoxi et al. (1999). This compound was characterized as vomifoliol (blumenol A) [CAS No. 23526-45-6, (4*S*)-4-hydroxy-4-[(*E*,3*R*)-3-hydroxybut-1-enyl]-3,5,5-trimethylcyclohex-2-en-1-one] (Figure 9). Vomifoliol was identified on the basis of the following evidence: colorless powder. EI-MS (70 eV), m/z (% relative intensity): 124 $[M]^+$ (100), 55 (10), 59 (10), 95 (10), 111 (15), 124 (100), 135 (11), 150 (11), 168 (13). ^1H NMR (MeOD, 600 MHz) and ^{13}C NMR (MeOD, 600 MHz): See Table 9.

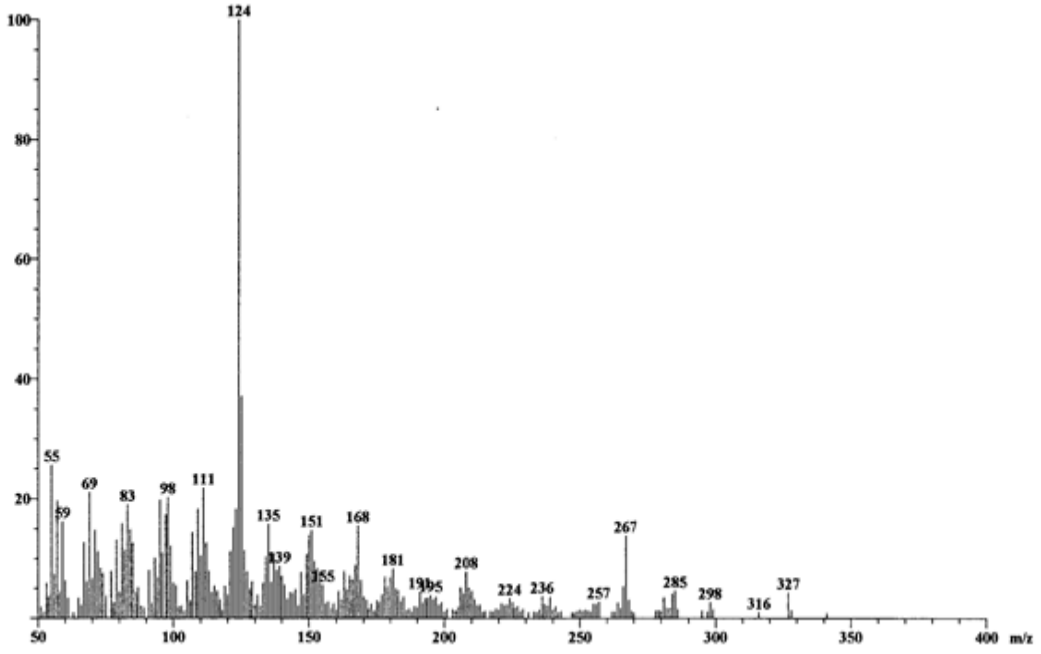

Figure 5. Mass spectrum of compound 1.

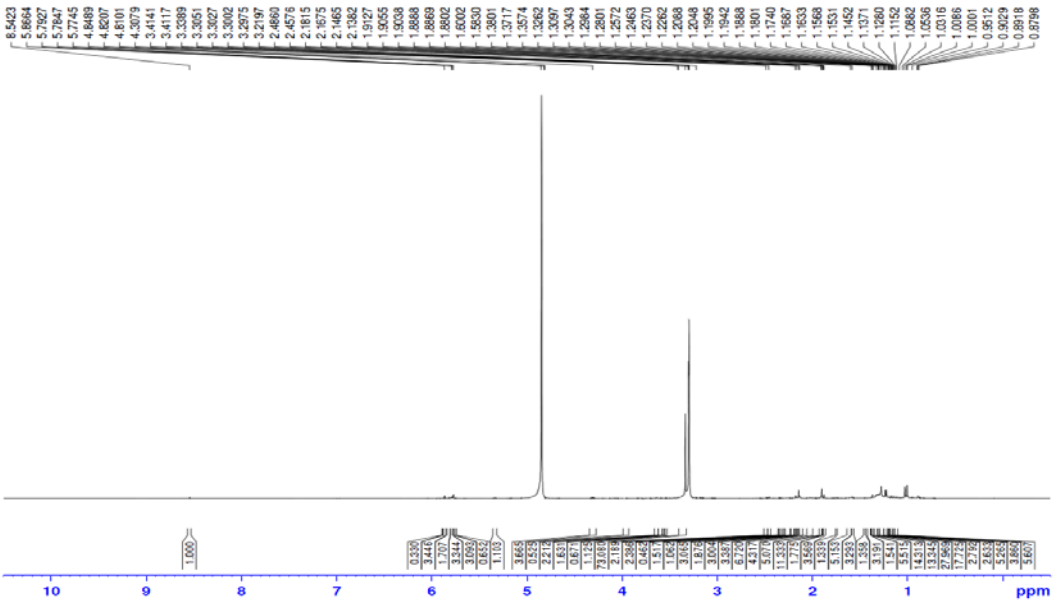

Figure 6. ^{1}H NMR spectrum of compound 1.

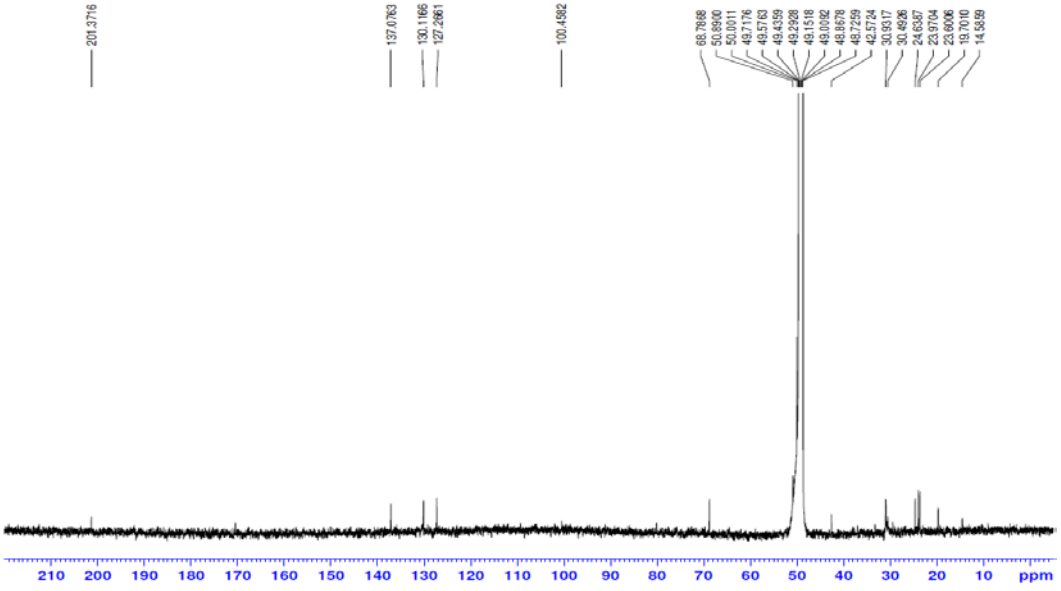

Figure 7. ^{13}C NMR spectrum of compound 1.

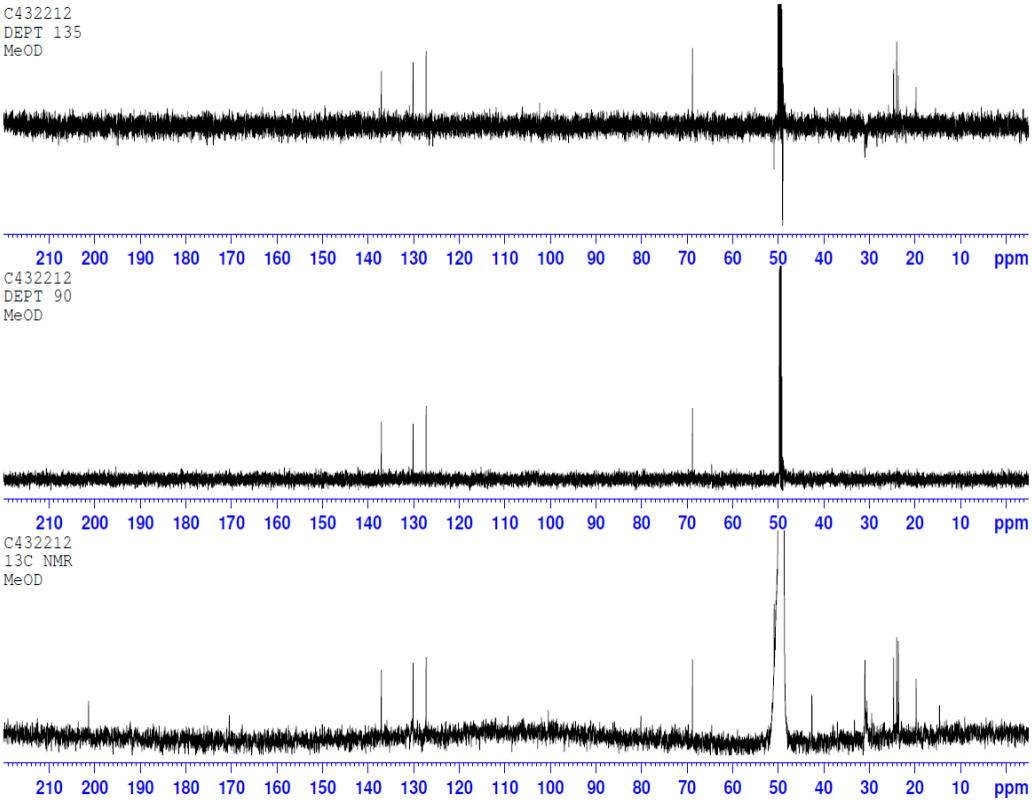

Figure 8. DEPT spectrum of compound 1.

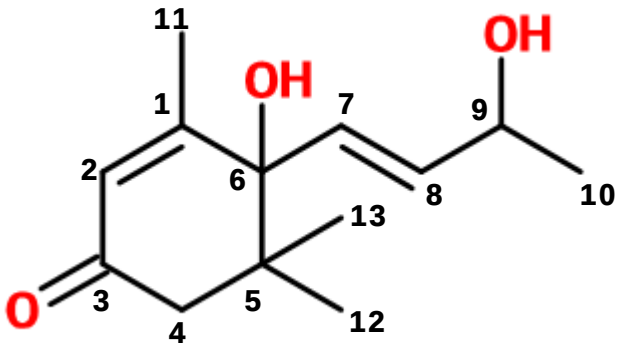

Figure 9. Structure of vomifoliol.

Table 9. ^{1}H and ^{13}C NMR spectral data for compound 1

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Xiaoxi et al. (1999)
1	C	162.6		162.5	
2	CH	127.2	6.11, s	126.9	5.91, s
3	C	198.9		198.1	
4	CH_2	50.1	2.42, d (J = 18.0 Hz)	49.8	2.45, d (J = 17.0 Hz)
5	C	42.2		41.2	
6	C	79.8		79.0	
7	CH	135.7	5.88, d (J = 16.2 Hz)	135.7	5.79, d (J = 15.5 Hz)
8	CH	134.2	5.88, dd (J = 16.2, 6.2 Hz)	134.7	5.86, dd (J = 15.5 , 6.0 Hz)
9	CH	69.0	4.08, m	68.1	4.42, m
10	CH_3	23.6	1.27, d (J = 1.8 Hz)	22.9	1.30, d (J = 1.5 Hz)
11	CH_3	21.1	1.82, s	19.9	1.90, s
12	CH_3	24.1	0.99, s	23.7	1.02, s
13	CH_3	24.1	0.99, s	23.7	1.02, s

Compound **2** was obtained as colorless powder. The mass spectrum of the isolate exhibited a molecular ion at *m/z* 69 $[M]^+$ (Figure 10) and its ^1H NMR spectra (Figure 11) showed 20 protons. Its ^{13}C NMR spectra (Figure 12) showed 29 carbons in the molecule comprising a phytosterol which has a side-chain containing 10 carbon atoms attached at C-17 to the steroid skeleton as indicated in DEPT (Figure 13), suggesting the molecular formula $C_{29}H_{48}O$. The interpretations of proton and carbon signals were largely consistent with those of Peter and Katalin. (2004). This compound was characterized as stigmasterol ((3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol) (Figure 14). Stigmasterol was identified on the basis of the following evidence: white powder. EI-MS (70 eV), m/z (%relative intensity): 69 $[M]^+$ (100), 55 (71), 69 (100), 251 (65), 269 (40), 379 (73), 383 (50), 395 (67), 412 (88). ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 600 MHz): See Table 10.

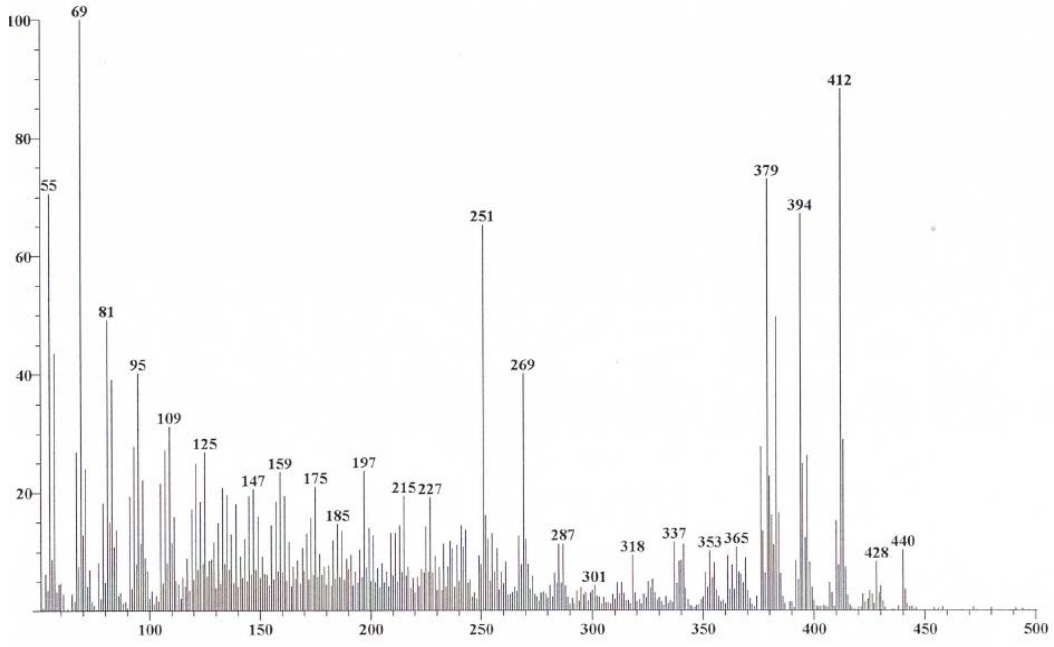

Figure 10. Mass spectrum of compound 2.

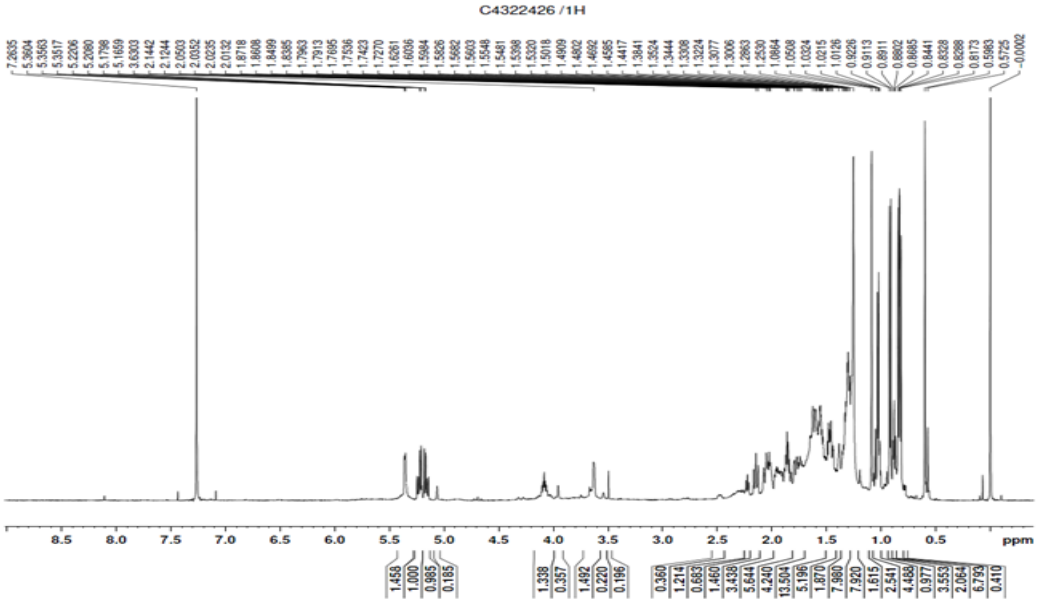

Figure 11. ^{1}H NMR spectrum of compound 2.

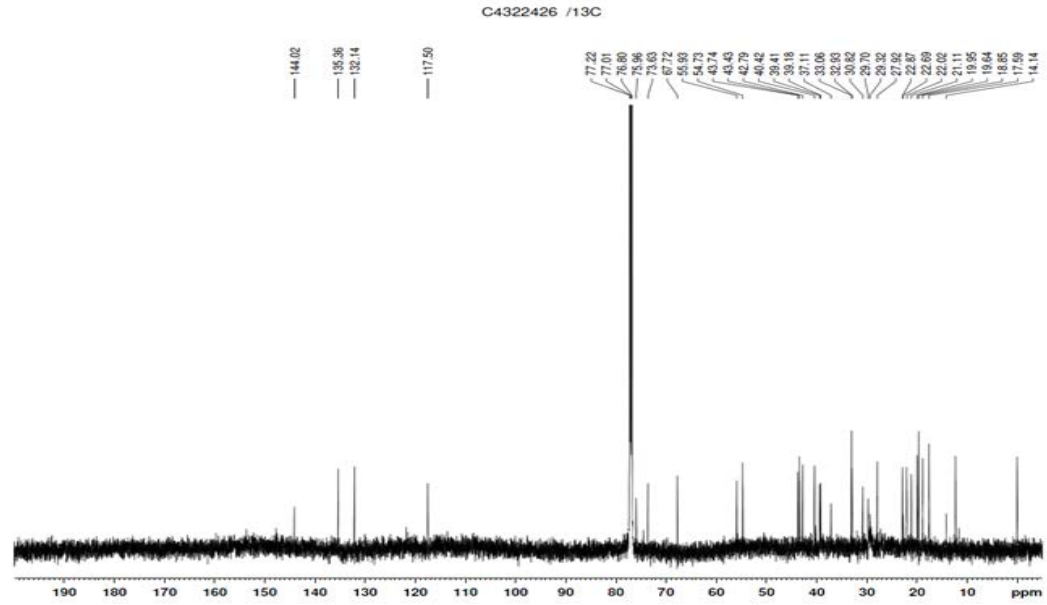

Figure 12. ^{13}C NMR spectrum of compound 2.

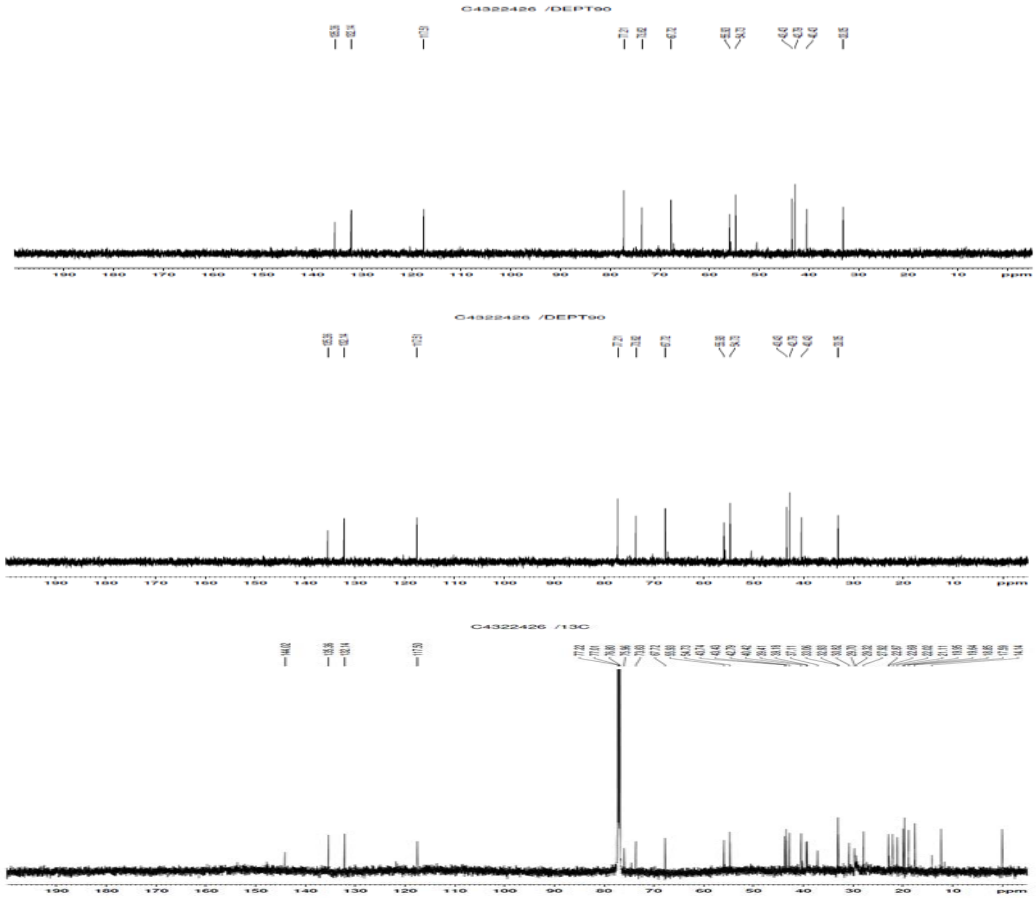

Figure 13. DEPT spectrum of compound 2.

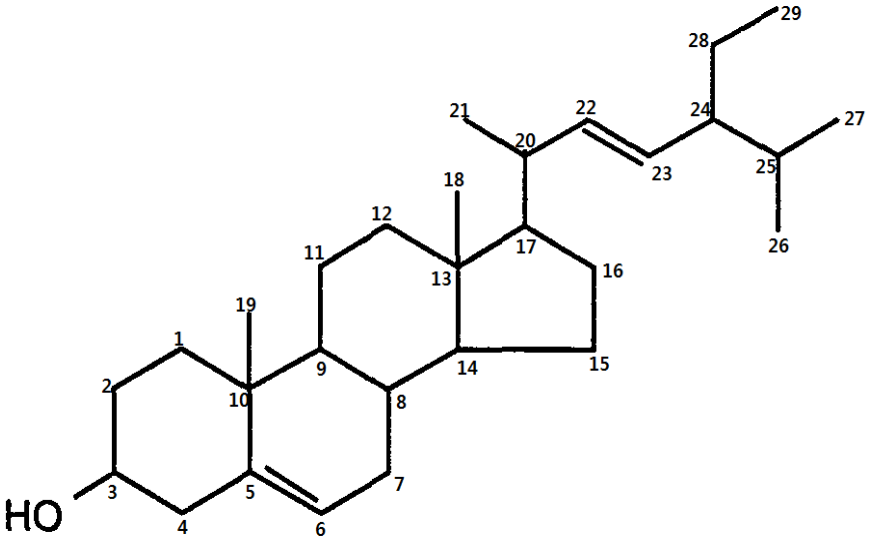

Figure 14. Structure of stigmasterol.

Table 10. ^{1}H and ^{13}C NMR spectral data for compound 2

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Peter and Katalin (2004)
1	CH_2	37.2	1.38;1.13, m	37.6	1.54;1.08, m
2	CH_2	31.7	1.56;1.31, m	31.9	1.51;1.31, m
3	CH	71.6	3.25, m	72.0	3.51, m
4	CH_2	41.8	2.23;1.98, m	42.5	2.23;2.30, m
5	C	140.8		140.8	
6	CH	121.8	5.37, m	121.8	5.34, m
7	CH_2	32.0	2.04;1.79, m	32.1	1.97;1.50, m
8	CH	31.8	1.45, m	32.2	1.46, m
9	CH	50.8	1.44, m	50.5	1.46, m
10	C	37.7		36.5	
11	CH_2	21.1	1.52;1.27, m	21.2	1.50;1.20, m
12	CH_2	39.9	1.56;1.31, m	40.0	1.51;1.18, m
13	C	43.0		42.2	
14	CH	56.6	1.40, m	57.1	1.20, m
15	CH_2	26.4	1.60;1.35, m	26.5	1.56;1.28, m
16	CH_2	28.6	1.60;1.35, m	28.9	1.56;1.28, m
17	CH	56.4	1.51, q	56.3	1.50, q
18	CH_3	12.3	1.04, s	12.2	1.06, s

Position	Partial structure	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm) Peter and Katalin (2004)
19	CH_3	19.3	1.30, s	19.5	1.24, s
20	CH	40.1	2.33, m	40.4	2.06, m
21	CH_3	20.2	1.11, d	21.4	1.06, d
22	CH	138.3	5.48, dd (J = 15.8 Hz)	138.3	5.18, dd (J = 15.2 Hz)
23	CH	129.5	5.48, dd (J = 9.2 Hz)	129.7	5.04, dd (J = 8.6 Hz)
24	CH	52.2	2.15, m	51.5	2.18, m
25	CH	31.9	1.86, m	32.0	1.55, m
26	CH_3	21.1	0.91, d	21.2	0.85, d
27	CH_3	21.1	0.91, d	21.2	0.85, d
28	CH_2	25.4	1.44, m	25.4	1.43, m
29	CH_3	12.3	0.90, m	12.2	0.81, m

3. Anti-proliferative activity of test compounds

The anti-proliferative activities of pure vomifoliol, pure stigmasterol, and anticancer agent cisplatin toward A549 lung cancer cell line were evaluated using a MTT assay (Table 11). Based on IC_{50} values, vomifoliol and stigmasterol were approximately 3 times less active than cisplatin toward A549 lung cancer cell line. The anti-proliferative activity of vomifoliol and stigmasterol did not differ significantly.

Table 11. Anti-proliferative activity of vomifoliol, stigmasterol, and anticancer agent cisplatin toward A549 lung cancer cell line using a MTT assay

Compound	IC_{50},[a] μM (95% CL[b])	Slope ± SE	χ^{2c}	*P*-value	RT[d]
Vomifoliol	51.66 (42.47–62.86)	0.03 ± 0.51	1.59	0.991	3.1
Stigmasterol	48.41 (42.43–55.25)	0.04 ± 0.71	1.62	0.995	2.9
Cisplatin	16.53 (14.20–19.26)	0.63 ± 0.55	0.52	0.998	1.0

[a] The 50% anti-proliferative concentration for cell lines.

[b] CL denotes confidence limit.

[c] Pearson χ^2, goodness-of-fit test.

[d] Relative toxicity, IC_{50} of vomifoliol or stigmasterol/IC_{50} of cisplatin.

The anti-proliferative activities of pure vomifoliol, pure stigmasterol, and anticancer agent cisplatin toward NCI-H727 lung cancer cell line were likewise compared (Table 12). As judged by IC_{50} values, vomifoliol and stigmasterol were approximately 5 and 3 times less effective than cisplatin toward NCI-H727 lung cancer cell line, respectively. The anti-proliferative activity of vomifoliol and stigmasterol did not differ significantly.

Table 12. Anti-proliferative activity of vomifoliol, stigmasterol, and anticancer agent cisplatin toward NCI- H727 lung cancer cell line using a MTT assay

Compound	IC_{50},[a] μM (95% CL[b])	Slope ± SE	χ^{2c}	*P*-value	RT[d]
Vomifoliol	51.84 (46.18–58.13)	0.01 ± 0.41	0.70	0.997	3.9
Stigmasterol	46.21 (38.75–55.13)	0.03 ± 0.45	1.60	0.991	3.4
Cisplatin	13.40 (9.87–18.16)	0.02 ± 0.34	0.70	0.996	1.0

[a] The 50% anti-proliferative concentration for cell lines.

[b] CL denotes confidence limit.

[c] Pearson χ^2, goodness-of-fit test.

[d] Relative toxicity, IC_{50} of vomifoliol or stigmasterol/IC_{50} of cisplatin.

4. Effect of vomifoliol and stigmasterol on the level of Akt1gene expression

mRNA gene expressions of Akt1 in A549 2 days after treatments with vomifoliol or stigmasterol were examined using real-time qRT-PCR (Figure 15). The Akt1 gene expression levels of A549 were significantly reduced. In the A549 cell cultures treated with 12.5, 25, and 50 μg/mL of vomifoliol, the RNA replication levels were reduced by 2.85, 3.33, and 6.25 fold, respectively, compared to the levels in the cell cultures without the compound. Similarly, the expression levels of the Akt1 in A549 cell culture treated with 12.5, 25, and 50 μg/mL of stigmasterol were also reduced by 1.22, 6.25, and 14.3 fold, respectively, compared with the untreated cultures.

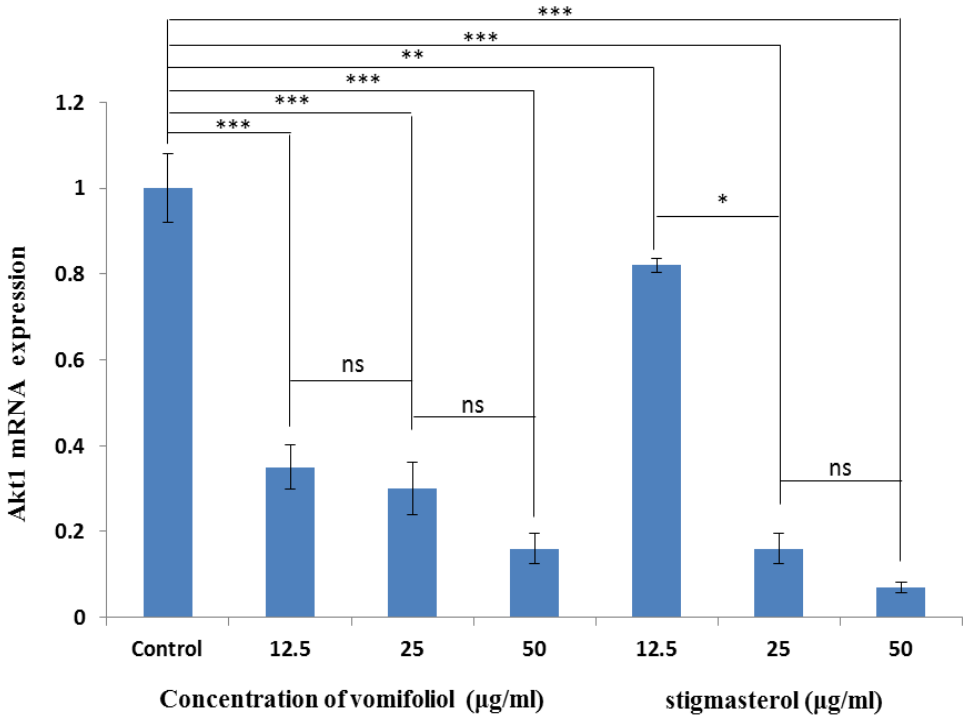

Figure 15. Effect on mRNA expressions of Akt1. The mRNA expressions of Akt1 were detected by real-time quantitative reverse transcription-polymerase chain reaction in A549 cells 2 days after treatments with 12.5, 25, and 50 μg/mL of vomifoliol or stigmasterol. Each bar represents the mean ± SE of duplicate samples of three independent experiments. (P = 0.05, using a Bonferroni multiple comparison post-test) (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns = no significant)

5. Effect of vomifoliol and stigmasterol on the level of Bcl-2 gene expression

mRNA gene expressions of Bcl-2 in A549 2 days after treatments with vomifoliol or stigmasterol were likewise compared (Figure 16). The Bcl-2 gene expression levels of A549 were significantly reduced. In the A549 cell cultures treated with 12.5, 25, and 50 μg/mL of vomifoliol, the RNA replication levels were reduced by 1.27, 6.67, and 12.5 fold, respectively, compared to the levels in the cell cultures without the compound. Similarly, the expression levels of the Bcl-2 in A549 cell culture treated with 12.5, 25, and 50 μg/mL of stigmasterol were also reduced by 14.3, 160, and 6666.7 fold, respectively, compared with the untreated cultures.

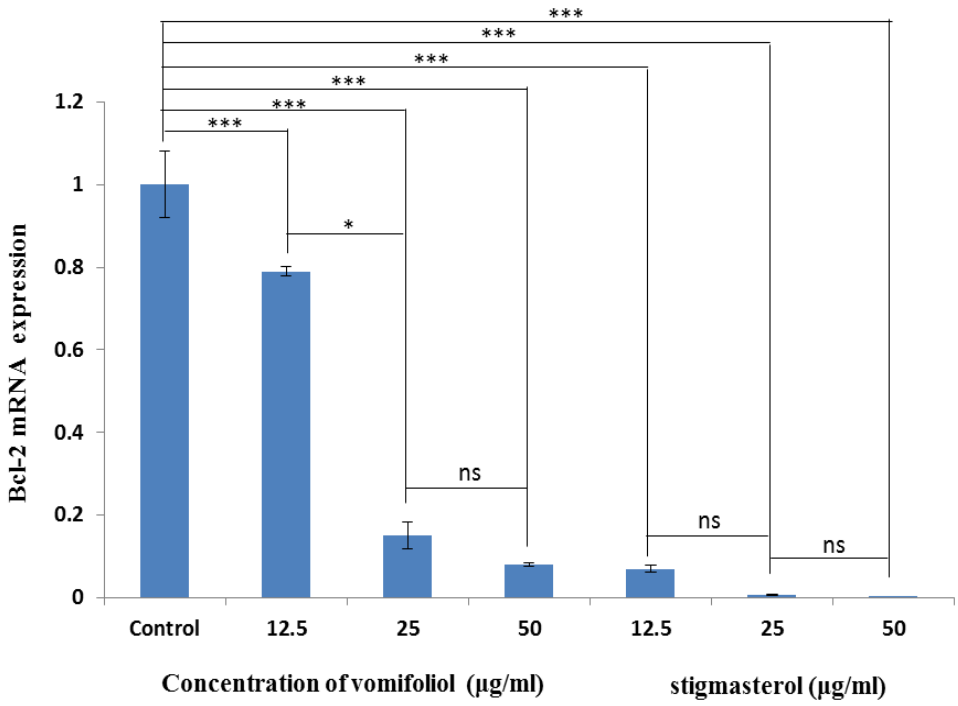

Figure 16. Effect on mRNA expressions of Bcl-2. The mRNA expressions of Bcl-2 were detected by real-time quantitative reverse transcription-polymerase chain reaction in A549 cells 2 days after treatments with 12.5, 25, and 50 μg/mL of vomifoliol. Each bar represents the mean ± SE of duplicate samples of three independent experiments. (*P* = 0.05, using a Bonferroni multiple comparison post-test) (***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns = no significant)

DISCUSSION

Lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer in the world. Despite improvements in survival for many other types of cancer in recent years, 5-year survival for lung cancer has remained relatively poor, mainly because by the time a diagnosis is made, lung cancer is often well advanced and treatment options are limited (Spiro and Silvestri, 2005; Pirozynski, 2006; Schwarz et al., 2007). Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined. The incidence rates of lung cancer in Asian countries are lower than those in Western countries. In recent few decades, mortality from lung cancer has increased rapidly in South East Asian countries including ROK because of a high prevalence of smoking (Jee et al., 1998). In ROK, lung cancer is the third most common incidence cancer, while the relative frequency cancer deaths are the first among the other cancers (Welfare Korea Central Cancer Registry, 2011). According to the WHO data published in April 2011 (WHO, 2011), lung cancer deaths in ROK reached 16,437 or 7.34 % of total deaths.

The therapy of cancer includes radiation therapy, chemotherapy, or surgical surgery. There is no effective cure for lung cancer of chemotherapy currently exists because of their adverse effects such as normal lung cell cytotoxicity, nausea, fatigue, vomiting, and hair loss (Evans and McLeod., 2003; Carelle et al., 2002). Moreover, in developing new anticancer drugs, chemical synthesis shows limitation because of cost, time, and low success rate (Katiya et al., 2012). In this situation, new treatment medical agents for

lung cancer have been developed from natural products.

Since early times, insects, their products, and their constituents have been used, directly and indirectly, in the medical systems of different human cultures throughout the world (Costa-Neto, 2002). Also, insects and the substances extracted from them have been used as therapeutic resources in the medical systems of many cultures including China, Brazil, India, Africa, and ROK (Costa-Neto, 2005). However, there are a few reports of anticancer agents from insects, which are the most diverse groups of organisms (Huang et al., 1997).

Silkworm feces were reported in the ‘Dongeuibogam’, Korean medical text, to be nontoxic, and can be used to strengthen the internal organs, to protect diplegia, and to treat diabetes (Kim et al., 1997). In the ‘Sinnongbonchogyung’ text, the feces were used to cure hangovers, were thought to have antiaging effects, and were used to treat hemiplegia and urticaria (Lee, 1994). In China, the feces have been used for traditional therapy to cure for antiproliferation and antiatopic dermatitis. In addition, they possess decrease in blood pressure and cholesterol levels and to have anticancer and antidiabetes characteristics (Sugawara et al., 1990; Lee et al., 1999). In pharmacological study, the 80% aqueous methanol extracts of silkworm feces have anti-inflammation activity due to lignans (Park et al., 2010). In addition, the aqueous extracts of silkworm feces make heme oxygenase 1 (HO-1), which is considered to be an antioxidant enzyme to increase. While extracts induce SIRT1, which is associated with the suppression of inflammatory mediators of factors that improve atopic dermatitis related symptoms to promote activity (Park et al., 2011).

Various compounds, including phenolics, terpenoids, and alkaloids, exist in natural products, and jointly or independently they contribute to anticancer efficacy (Dai and Mumper., 2010; Reddy et al., 2009; Kavallaris et al., 2002; Burres et al., 1989). Many natural products and their constituents manifest anticancer activity toward different cancer cell lines (Taraphdar et al., 2001; Molinski et al., 2009; Rajagopal et al., 2003; Sullivan et al., 2006) and have been proposed as alternatives to conventional anticancer drugs. Anticancer constituents derived from natural products include terpenoids [e.g., α-pinene, IC_{50} 144.85 μM; thymol, IC_{50} 184.0 μM; betulin, IC_{50} 7.71 μM; saikosaponin A, IC_{50} 4.00 μM (Damianaki et al., 2000; Seeram et al., 2006; Zhang et al., 2008)], phenylpropanoids [e.g., honokiol, IC_{50} 24.2 μM; magnolol, IC_{50} 89.4 μM (Thoppil and Bishayee., 2011)], flavonoids [e.g., baicalin, IC_{50} 9.51 μM; wogonin, IC_{50} 92.5 μM; kaempferol, IC_{50} 349.4 μM; quercetin, IC_{50} 52.6 μM (Ren et al., 2003)]; alkaloids [e.g., evodiamine, IC_{50} 82.8 μM; berbamine, IC_{50} 14.0 μM; corydaline, IC_{50} 131.8 μM; cycleanine, IC_{50} 37.1 μM; papaverine, IC_{50} 131.9 μM (Kruczynski and Hill, 2001)]; steroids [e.g., bufalin, IC_{50} 122.4μM; cinobufalin, IC_{50} 139.3 μM (Ogasawara et al., 2000)]; and the others [e.g., capsaicin, IC_{50} 68.1 μM; capillarisin, IC_{50} 240.5 μM (Ogasawara et al., 2000)].

In this study, the ethanol extract of silkworm feces was proved to have anti-proliferative activities toward 10 cancer cell lines, including lung, prostate, cervix, stomach, colon, breast, liver and pancreas, although the extract was less toxic than the

widely used anticancer agent cisplatin. Especially, the anti-proliferative activity of the feces was higher toward lung cancer cell lines A549 and NCI-H727 than toward the other cancer cell lines. The anti-proliferative principle was determined to be the megastigmane norterpenoid vomifoliol and the stigmastane steroid stigmasterol. The constituents exhibited potent anti-proliferative activity toward human lung carcinoma cell lines A549 and NCI-H727. Based on fifty percent inhibition concentration (IC_{50}), anti-proliferative activity of vomifoliol and stigmasterol did not differ significantly toward A549 cell line. The IC_{50} of vomifoliol and stigmasterol was between 44.90 and 57.24 μM, although IC_{50} of the natural compounds stated previously is between 4.00 and 349.4 μM. These compounds were less toxic than cisplatin and were nontoxic toward two normal cell lines. This original finding indicates that materials derived from silkworm faces can hold promise for the development of novel and effective naturally occurring anticancer agents for different cancer cell lines. In addition, vomifoliol was reported to possess antitumor (Ito et al., 2002), antituberculosis (Siddiqui et al., 2012), and antioxidant (Nile and Khobragade., 2010) activities. Stigmasterol was reported to possess antiprotozoal (Waechter et al., 1999), anti-hepatic cholesterol biosynthesis and to reduce plasma cholesterol level (Batta et al., 2005) activities.

Investigations on the modes of action natural anticancer agents may also contribute to the development of selective cancer therapeutic alternatives with novel target sites. Anti-proliferative activity of mechanism of action include inhibiting the polymerization of tubulin [e.g., podophyllotoxin (Buss and Waigh, 1995; Damayanthi and Lown, 1998;

Imbert, 1998)], arresting the cell cycle in the metaphase [cyclolignan (Stahelin and Von wartburg, 1989; Ayres and Loike, 1990; Srivastava et al., 2005)], blocking metastatic [e.g., topotecan (Malonne and Atassi, 1997; Dennis, 1997; Nelson, 2007; Shah et al., 2007)], inhibiting topoisomerase I, a cellular enzyme that maintains the topographic structure of DNA during translation, transcription and mitosis [e.g., camptothecin (Cersisimo, 1998; Erlichman and Sargent, 2004)], blocking the formation of microtubules and binds to the subunits of tubulin in the S phase of cell cycle [e.g., alkaloids, especially vincristine, vinblastine (Gordaliza, 2007)], disabling the mitotic apparatus by disturbing the normal function of microtubules [e.g., taxane (Sengupta, 2006; Bergstralh and Ting, 2006; Altmann and Gertsh, 2007)].

In this study, vomifoliol exhibited the reduction of the gene expression of Akt1 and Bcl-2 which are important anti-apoptosis gene in apoptosis mechanisms in cells. In particular, the constituent decreased sharply Akt1 gene expression than Bcl-2 gene expression. Stigmasterol also exhibited decrease in both the gene expressions of Akt1 and Bcl-2. The constituent showed different pattern, compared to vomifoliol. Stigmasterol significantly reduced in Bcl-2 gene expression as high concentration goes by. Results of this study indicate that the silkworm feces-derived preparations containing vomifoliol and stigmasterol could be useful as sources of anticancer products for prevention or eradication of lung cancer. The anti-proliferative action of vomifoliol and stigmasterol may be an indication of at least one of the pharmacological actions of silkworm feces. For the practical use of silkworm feces-derived preparations as novel

anticancer products to proceed, further research is needed to establish their human safety and whether this activity is exerted *in vivo* after consumption of silkworm feces-derived products by humans. Vomifoliol and stigmasterol have no acute oral toxicity on rat at 250 mg/kg (Al-Yahya, 2013) and 500 mg/kg (Turnbull, 1999), respectively. Lastly, detailed tests are needed to understand how to improve anti-proliferative potency and stability for eventual commercial development.

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잠분 유래 화합물의 인간 폐암세포에 대한

증식 억제 활성 및 작용 기구

서울대학교 대학원

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유 지 영

초 록

악성 폐 종양은 높은 사망률을 보이고 있는 5대 주요 빈발성 암이다. 최근, 전 세계적인 금연 운동으로 인해 폐 종양 발생 위험이 줄어들고는 있으나 선진국에서 현저한 고령화에 따른 폐 종양 환자 수는 지속적으로 증가하고 있다. 한국의 경우, 폐 종양은 발생 후, 5년 내에 사망할 확률이 가장 높은 암으로서 발생률 대비 사망률이 가장 높은 암으로 알려져 있다. 이와 같이 급증

하고 있는 폐 종양 발생률과 사망률에 관한 정확한 이유는 아직까지는 밝혀지지 않았지만 일차원적인 담배, 간접흡연, 가족력뿐만 아니라 최근 악화되고 있는 미세먼지와 환경적 요인으로 인해 증가하고 있다는 보고가 나오고 있다. 항암화학요법, 방사선치료, 외과적 제거술 등 폐 종양 치료법들이 행해지고 있지만 때로는 출혈, 탈모, 구토, 폐부종, 폐렴, 외과적 처치 후 수술 후유증 등 심각한 부작용들을 야기하기도 한다. 따라서 새로운 타겟 부위와 낮은 독성을 가지는, 보다 안전하고 경제적인 치료제 및 기술개발에 대한 필요가 시급한 실정이다.

본 연구에서는 폐종양 치료에 효과가 있을 것으로 예측되는 물질에 대한 효능검증을 실시하였다. 폐 암 세포주 A549 와 NCI-H727을 포함한 총 10개의 인간 암 세포주에 대한 잠분유래물질의 세포증식 억제활성 실험을 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 방법을 이용하여 진행하였다. 실험결과는 항암제로 널리 사용되고 있는 시스플라틴을 양성대조군으로 하여 비교하였다. 잠분 30.84 Kg에서 660 g의 에탄올 추출물을 얻게 되었다. 매미껍질 에탄올 추출물은 폐암 세포주 A549 와 NCI-727 뿐만

아니라 위암 세포주 AGS, 전립선 암 세포주 PC-3와 DU145, 자궁경부 암 세포주 Hela, 대장암 세포주 HT-29, 유방암 세포주 MCF-7, 간암 세포주 SK-Hep-1, 식도암 세포주 Hep-2와 난소암 세포주 SK-OV-3에서 상당히 높은 세포증식 억제활성을 나타내었다. 전자 이온화 질량 분석법과 핵자기 공명 분광법을 이용하여 매미껍질 에탄올 추출물에서 단일물질인 Vomifoliol (Blumenol A)와 Stigmasterol 의 물질을 분리하고 그 구조를 동정하였다. 분리한 물질 Vomifoliol은 폐암 세포주 A549 와 NCI-717 에 대해 세포증식 억제활성(IC_{50}, 51.66 μM, 48.41 μM)을 보였다. 반면 Stigmasterol은 폐암 세포주 A549 와 NCI-717 에 대해 세포증식 억제활성(IC_{50}, 51.84 μM, 46.21 μM)을 보였다. 분리한 물질들의 작용 기구는 안티 에이팝토시스 Akt1과 Bcl-2 유전자의 발현을 억제함으로써 폐 암세포의 세포자살을 촉진시키는 것을 알 수 있었다. 특히 Vomifoliol은 Akt1 유전자의 발현을 더 감소시켰고, Stigmasterol은 Bcl-2 유전자의 발현을 특이적으로 감소 시키는 것을 확인하였다.

이상의 결과를 바탕으로 본 논문의 연구는 잠분에 함유된 활성본체들을 분리동정 하였고, 활성 물질들의 작용기구를 연구했다는데 그 의의가 있고, 구

성 물질에 대한 생물검정을 통해 항암제로써의 가능성을 탐색하고 잠분의 새로운 생리활성을 밝혀내어 농업적, 산업적으로 그 활용 가능성이 높다고 판단되며 이에 더하여 추가적 연구가 요구된다.

검색어: 폐암, 천연 항암제, 잠분, Vomifoliol, Blumenol A, Stigmasterol,

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