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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Anti-proliferative activity of constituent
identified in cicada slough on human prostate cancer

By
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Major in WCU Biomodulation
Department of Agricultural Biotechnology
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매미 껍질 유래 인간 전립선 암세포 증식 억제 물질의 분리 및 동정

UNDER THE DIRECTION ADVISER YOUNG JOON AHN
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Prostate cancer (PC) is the most common malignancy and the second leading cause of male death in the United States and many European countries. In the Republic of Korea, the prevalence of prostate cancer has increased rapidly and the incidence rate is highest in total forms of malignancy. Reasons for the increases in prostate cancer incidence and mortality are unclear but may be explained in part by the gradual westernization of lifestyle. Hormonal therapy, chemotherapy, and radiotherapy have been used for prostate cancer treatment. Sometimes, serious side effects of these treatments occur, such as bleeding, hair loss, vomiting, and difficulty getting and maintaining an erection and a lowered sex drive. There is, therefore, a critical need for the development of new improved anticancer agents with novel target sites and low toxicity.
In this study, an assessment is made of the anti-proliferative activity of cicada slough-derived materials against 10 human cancer cell lines, including PC-3 and DU145 prostate cancer cell 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 leaves (33 kg) of the cicada slough yielded 116.7 g of a dark greenish ethanol extract. The ethanol extract of Cryptotympana spp. slough was proved to have anti-proliferative activity against A549 lung, AGS stomach, PC-3 and DU145 prostate, Hela cervix, HT-29 colon, MCF-7 breast, and SK-Hep-1 liver cancer cell lines except for Hep-2 larynx and SK-OV-3 ovary cancer cell lines. The biologically active constituent was characterized as the nonprotein α-amino acid theanine [2-amino-4-(ethylcarbamoyl)butyric acid] by spectroscopic analysis, including EI-MS and NMR. Theanine was isolated from the cicada slough as a new cytotoxic principle. Fifty percent inhibition concentration (IC_{50}) values of the constituent against PC-3 was 6.52 µg/mL, respectively. The activity of theanine (IC_{50}, 6.52 µg/mL) did not differ significantly from that of the anticancer agent cisplatin (IC_{50}, 7.39 µg/mL) toward PC-3. In conclusion, global efforts to reduce the level of anticancer agents justify further studies on the cicada slough-derived materials containing theanine as potential anticancer products or a lead molecule for the prevention or eradication from human prostate cancer.
Key words: Prostate cancer, Natural anticancer agents, Cicada slough, Theanine

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INTRODUCTION

The prostate is an organ in the male reproductive system and is a doughnut shaped structure located below the bladder and in front of the bowel of males. Prostate cancer occurs when some cells in the prostate grow rapidly and out of control. Prostate cancer cells break out of the prostate and then invade to the other parts of the body through blood vessels (Prostate Cancer Fund, 2007). Prostate cancer is the leading cause of cancer deaths among men in the United States and many Western countries (Jemal et al., 2005). In 2013, an estimated 238,590 men in the United States were diagnosed with prostate cancer and 29,720 men were died of it, making it the second leading cause of cancer male death, behind only lung cancer (American Cancer Society, 2013). The incidence of prostate cancer varies worldwide, with the highest rates found in the United States, Canada, and Scandinavia, and the lowest rates found in China and other parts of Asia (Quinn and Babb, 2002; Gronberg, 2003). Currently, prostate cancer incidence and mortality rates are much lower in the Republic of Korea (ROK) than those in most Western countries, but they have been increasing steadily over time because of environmental elements, such as increased population age and Western dietary habits. The short period of time from 1996–1998 to 1999–2001, there were reports that the prostate cancer incidence had been rose by 28.2% (Hsing and Devesa, 2001; Sim and Cheng, 2005). Recently, the prevalence of prostate cancer has quadrupled from 2002 to 2008, and the incidence rate is highest among all forms of malignancy (Lee et al., 2013). Prostate cancer caused a significant burden on the U.S. Medicare system with average
per-patient life-time costs of approximately 34,000 USD. Prostate cancer-related costs represented approximately one third of total medical care costs (Stokes et al., 2011).

Therapeutic treatments for prostate cancer have been achieved principally by the use of conventional hormone therapy, radiation therapy, chemotherapy, and external surgery. Chemotherapy is the most effective tool. 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. Sometimes, serious side effects of anticancer agents occur, such as sexual dysfunction, thrombosis, and impotence (Galvao et al., 2006). There is, therefore, a high need for the development of selective anticancer agents with novel target sites and low toxicity.

The insects and other arthropods and the substances extracted from them have been used worldwide as medicinal resources to human (Dettner, 2011). Insects and other arthropods provide main constituents of traditional medicine for thousands of years in Asia, Africa and South America. Natural products are proven sources of drugs and almost 70 % of drugs are derived from natural compounds. These compounds have functions such as binding to specific target proteins or interacting with membranes. Also, there are some properties which might make toxic substances attractive as drug lead compounds including: 1) cytotoxicity, 2) neurotoxicity, and 3) efficacy against microbial pathogens. Numerous compounds from insects have all of these properties as well (Dossey, 2010). In addition, natural compounds extracted from insects or insect-derived materials have been suggested as alternative sources for anticancer agents (Ratcliffe et al., 2011).
Some effort has been focused on insect preparations and their constituents as potential sources of commercial anticancer products. In the screening of insect-derived materials for anti-proliferative activity against prostate cancer cell lines, an ethanol extract of the slough of *Cryptotympana* spp. was shown to have anti-proliferative activity against PC-3 prostate cancer cell line. However, no information has been obtained concerning the potential use of cicada slough-derived materials to manage prostate cancer, although cicada slough, the cast-off shell of the *Cryptotympana* spp. named ‘Seontoi’ in Korea and ‘Zentai’ in China, has been used for traditional Chinese medicine as an antifebrile, an antiphlogistic, and a spasmylytic (Okuda, 1986).

In this study, an assessment is made of the anti-proliferative activity of the nonprotein α-amino acid theanine from the slough of *Cryptotympana* spp. against PC-3 and DU 145 prostate cancer cell lines using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay. The anti-proliferative activities of the constituent were compared with those of cisplatin, a currently used anticancer agent with broad spectrum, for use as future commercial anticancer products for prostate cancer.
Literature review

1. Prostate cancer

The prostate is an organ in the male reproductive system and is a doughnut shaped structure located below the bladder and in front of the bowel of males. Its main function is that helps produce semen, the fluid that carries sperm cells. The male sex hormone testosterone plays a key role in regulating the prostate. Prostate cancer occurs when some cells in the prostate grow rapidly and out of control. The cancer cells break out of the prostate and then invade to the other parts of the body through blood vessels (Prostate Cancer Fund, 2007). With the aging of the population, prostatic diseases have become an important health concern in men over the age of 50. Prostate cancer is the most common malignancy in men and the second leading cause of male death after lung cancer in many Western countries such as US, Europe, North America and some parts of Africa. Especially, it is predominantly a disease of elderly men and its incidence increases rapidly in the seventh decade of life (Quinn and Babb, 2002). Prostate cancer rates in Asian countries are lower than those in Western countries (Nakata et al., 1995; Hsing et al., 1998). Although the incidence and mortality rates of prostate cancer are low, the rates have risen rapidly and are still increasing steeply (Pu et al., 2004; Syed et al., 2007). In ROK, prostate cancer is the eighth most common cancer among men and is the fifth most common cancer among men over the age of 65. Total cancer cases and deaths among Korean men is 2.4% and 1.5% (Korea National Statistical Office, 2005; Shin et al., 2005). Like other Asian countries, the incidence and mortality are low but the rates
are rising sharply over the few years in ROK (Sim and Cheng, 2005).

2. Risk factors of prostate cancer

Even though there is little known about the causes of prostate cancer, the prostate cancer research have increased in the past years. Some risk factors have been revealed by continued interest in and funding for prostate cancer research (Gronberg, 2003).

Age, ethnicity, and family history

Age, ethnicity, and family history are the well-established risk factors of prostate cancer. The incidence of prostate cancer increases with age, more so than any other cancer. In the US, 60% of all cases of prostate cancer are diagnosed in men over 65 years and 97% of prostate cancer cases are occurred in men of 50 years and older in 2013 (American Cancer Society, 2013). When men get older, the biological function of prostate gradually decreases. For this reason, the prostatic diseases such as benign prostatic hyperplasia and prostate cancer are occurred because of unnecessarily enlarged prostate.

African American men and Jamaican men of African descent have the highest rates of prostate cancer incidence in the world. The incidence and death rates of African American (228.7 per 100,000) are much higher than Whites (141.0 per 100,000), Asian American or Pacific Islander (77.2 per 100,000), and American Indian or Alaska Native (98.8 per 100,000) in the US (American Cancer Society, 2013). In case of Black Africans, they have lower rates of prostate cancer incidence (Parkin et al, 1997). Asians
such as Japanese and Chinese have lower rates of prostate cancer relatively. However, the rates can be increased temporarily because of the migration from low-to high-risk regions (Giovannucci, 1995; Chan et al., 1998).

Men who have a first degree relative (father, brother, or son) affected by prostate cancer have a higher chance at getting the cancer. Furthermore, the men with family history are diagnosed at a 6–7 years earlier age than those without family history. For these reasons, family history could be an onset of prostate cancer of early age (Steinberg et al., 1990; Carter et al., 1992; Chan et al., 1998; Bratt, 2002). Especially, the growth factors of prostate cancer are autosomal dominant inherited; hence it may occur from maternal line also (Goldgar et al., 1996).

Hormones

The male hormones, androgens, play an important part in development of male reproductive system. The main male sex steroids, testosterone and dihydrotestosterone, play key roles in growth and development of prostate gland. The testosterone is converted to dihydrotestosterone by type II 5-reductase in the prostate (Culig et al., 2000). Prostate cancer is often male hormone dependent initially and can regress when testosterone stimulation is removed by surgical or medical castration. Several evidences supports that men castrated at an early age rarely get prostate cancer and continuous injection of high doses of testosterone can induce prostate cancer in vivo (Hovenanian and Deming, 1948; Noble, 1977; Pollard et al., 1982). Other hormones such as oestrogen and leptin have also been studied, but these have been not significantly related
to prostate cancer (Hsing et al., 2001; Stattin et al., 2001).

There are two main hormones (IGF-I and IGF-II) in the insulin growth factor (IGF). IGF-I is a peptide growth factor and plays an key roles in apoptosis, proliferation, differentiation, and metabolism of cancer cells. Some cohort studies reported that the people with high IGF-I concentrations had more incidence rates of prostate cancer than people with low concentrations (Cohen et al., 1994; Chan et al., 1998, Harman et al., 2000, Stattin et al., 2000). It has been suggested that IGF-I might be related to the changes in diet and lifestyle (Chan et al., 1998; Gronberg, 2003).

Diet

Ecological studies have reported the significant associations between prostate cancer and high intake of diet including fat, meat, and dairy products (Crawford, 2003). Two cohort studies of Seventh-Day Adventists were performed and the men with high intake of animal fat (especially from dairy products) had a greater incidence of fatal prostate cancer (Snowdon et al., 1984; Mills et al., 1989; Whittemore et al., 1995). In case of studies in animal, high intake of fatty acids were related to prostate cancer. These are because of the α-methyl-CoA remarcase (AMACR) gene. AMACR is expressed in prostate cancer but not in the normal prostate. AMACR plays a important role in peroxisomal oxidation of fatty acids and produces hydrogen peroxide which is a potential substance of carcinogenic oxidative damage. Because dairy products and meat are kind of dietary branched fatty acids, up-regulated AMACR may explain the relationship of dairy products and prostate cancer (Luo et al., 2002; Gronberg, 2003).
has been also suggested that consumption of red meat is linked with prostate cancer risk (Giovannucci et al., 1993; Veierod et al., 1997).

In contrast, there are many diets which reduce the risk of prostate cancer. The reason for the low incidence of prostate cancer in Asia may be consumption of dietary phyto-oestrogens. Soybean products that are rich in isoflavones, such as genistin and daidzin, have the highest amount of phyto-oestrogens and prophylactic effect on prostate cancer (Severson et al., 1989; Strom et al., 1999; Kolonel et al., 2000; Shirai et al., 2002; Stattin et al., 2002). Frequent consumption of tomato-based products can reduce the risk of prostate cancer. Tomatoes contain the carotenoid lycopene, and the constituent is well known for antioxidant. It has been reported that those who consumed high amounts of lycopene had a 16% lower risk than those who consumed small amounts of it among 2,481 men (Chen et al., 2001; Giovannucci et al., 2002).

3. Conventional therapy of prostate cancer

There are some types of standard treatment for prostate cancer. The therapies will be decided by considering patients age, co-morbidity, life expectancy, clinical stage, and tumor grade (Henry and O’Mahony, 1999).

Watchful waiting or active surveillance

Watchful waiting is monitoring prostate cancer that is not causing any problems or symptoms. Active surveillance is monitoring slow-growing prostate cancers which might never progress or cause any symptoms. These two treatments are used for older
men. The aim is to avoid unnecessary treatment which could cause side effects. Thus, the final goal is improving the quality of patient’s life (National Cancer Institute, 2013).

**Surgery**

Patients in good health whose tumor is in the prostate gland only can be treated with surgery. There are some kinds of surgery. Radical prostatectomy is a surgery that removes the prostate, surrounding tissue, and seminal vesicles. Radical prostatectomy rates have risen steeply over the past two decades. It can be conducted either using a retropubic or perineal approach (Sullivan et al., 2000). Pelvic lymphadenectomy is another surgery to remove the lymph nodes in the pelvis. It can be done in patients undergoing surgery by the perineal approach. There is a surgical procedure named transurethral resection of the prostate. This surgery uses a resectoscope (a thin, lighted tube with a cutting tool) and insert through the urethra and remove tissue from the prostate (National Cancer Institute, 2013).

**Radiation therapy**

Radiation therapy uses high-energy X-rays or other types of radiation to kill the cancer cells. The patients who diagnosed in the localized stage are treated with this therapy. There are two types of radiation therapy. One is external radiation therapy which uses a machine outside the body to transmit radiation toward the cancer and another one is internal radiation therapy which places directly near the cancer using needles with radioactive substance sealed (Stein et al., 2007).
**Hormone therapy**

Because male sex hormones can cause prostate cancer to grow, hormone therapy is a treatment that removes and reduces hormones and stops cancer cells from growing. Hormones are substances made by glands in the body and circulated in the bloodstream. Drugs, surgery, or other hormones are used to reduce the male hormones or block them.

Anti-androgens including flutamide, bicalutamide, nilutamide, and enzalutamide can block the action of androgens such as testosterone. Drugs such as ketoconazole and aminogluthethimide also can stop the adrenal glands from making androgens. The female sex hormone, estrogens, also can prevent the testicles from producing testosterone (National Cancer Institute, 2013). Orchiectomy is a surgery that removes one or both testicles to decrease the amount of hormone being made. For many years, orchiectomy has been mainly used as a standard treatment of advanced prostate cancer (Henry and O’Mahony, 1999).
Chemotherapy

Chemotherapy is a cancer treatment that uses drugs taken by mouth or injected into a vein or muscle to stop the growth of cancer cells. Several clinical studies have evaluated the role of both single agent and combination chemotherapy in the treatment of prostate cancer. Some have showed encouraging results in disease control, overall survival, and improvement in quality of life. The combination of docetaxel and prednisone is considered as the standard treatment in men with prostate cancer (Schrijvers, 2007). Unfortunately, this chemotherapy may cause some side effects even though there are merits on prostate cancer. Nausea and vomiting are the frequent side effects and thrombosis is also a severe side effect (Calabro and Sternberg, 2007).

Biologic therapy

Biologic therapy is a treatment that uses the immune system of patient. Compounds made from the body or made in a laboratory are used as natural defenses against cancer. Sipuleucel-T is an active cellular biologic therapy of prostate cancer, a type of therapeutic cancer vaccine, consisting of autologous peripheral-blood mononuclear cells (Kantoff et al., 2010). For patients with cancer, biological therapies may be used to treat the cancer itself or the side effects of other cancer treatments. Some forms of biological therapy have been approved by the U.S. Food and Drug Administration (FDA) but there still needs for experiments and clinical trials of biological therapy for prostate cancer (National Cancer Institute, 2013).
4. Medicinal use of insects

Insects make up about 80–90% of the largest and diverse group of organisms on 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). Ancient texts refer to medicinal use of insects in 16th century BC, and insects have been used medicinally for 3,000 years in China (Weiss, 1946; Zimian et al., 1997; Costa-Neto, 2005). There are number of articles describing the insect-derived substances with medically relevant properties. Insects are potentially valuable sources for natural product drug discovery.

Cytotoxins and anticancer compounds

Blister beetles are beetles of the family Meloidae, so called for their defensive secretion of a blistering agent cantharidin (Greek, Kantharis, beetle), a cyclohexane monoterpenoid. The constituent is used medically to remove warts (Bhattacharjee and Brodell, 2003) and is collected for this purpose from Mylabris and Lytta species, especially the Spanish fly, Lytta vesicatoria L. Cantharidin is one example of a cytotoxin from insects. It is a poisonous chemical that causes blistering of the skin, which means it has a potential efficacy against cancer (Moed et al., 2001). Cantharidin and its derivatives have been studied that has apparent anticancer activities (Sakoff et al.,
It has been reported that two fatty acids which possess anticancer activity including the fatty acids palmitic acid (hexadecanoic acid) and oleic acid were isolated from the flower beetle *Protaetia brevitarsis* Lewis. Palmitic acid also induce apoptosis in colon cancer cells (Yoo et al., 2007). Recently, a new cancer cell growth inhibitor, the monoterpenoid papilistatin was isolated from ethanol extracts of the butterfly *Byasa polyeuctes termessa* Fruhstorfer. Papilistatin has the activity on the mouse-derived leukemia model P388 (Pettit et al., 2010). The polyketide derivative pederin is a compound discovered in the hemolymph of *Paderus fuscipes* (family Staphylinidae). Pederin’s toxic properties become an attractive candidate in anticancer studies (Pavan and Bo, 1953). The peptide derivative N-alamyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD) from the larvae of the flesh fly species *Sarcophaga preegrina* Robineau-Desvoidy. This compound has potencies in the range 0.5~20 M on human cancer cell lines such as melanoma and breast carcinoma (Leem et al., 1996; Akiyama and Natori, 2003). Often insects concentrate substances from their diet or other features of their environment. For example, paper wasps make their nests using cellulosic plant material collected from a various sources. It has been reported that the anticancer quinine, 7,8-seco-para-ferruginone, was isolated from nests of the social wasp *Vespa simillima* (Fujiwara et al., 2008).
Neurotoxins

Most spider venoms contain neurotoxins which are used to paralyze prey or enemy. Acylpolyamines and peptides from spiders have potential use in treating pain and central nervous system (CNS) diseases including Huntington’s, Alzheimer’s, and Parkinson’s diseases (Estrada et al., 2007). In addition, the venoms of insects including ants, bees, and wasps contain neurotoxins. Philanthotoxin is the venom of the European beewolf, Philanthus triangulum F. (Eldefrawi et al., 1988; Nakanishi et al., 1994; Olsen et al., 2006). Another example is a potent hymenopteran neurotoxin, the venom of the bullet ant (Paraponera clavata F.). This venom contains poneratoxin, a protein, and this protein blocks ion channels of insects. Also, poneratoxin has striking effect on humans so it strongly suggested its potential to contribute to the development of new drugs (Szolajskas et al., 2004).

Antibiotics

Insects are susceptible to infection by microorganisms so various antimicrobial substances have been found in insects because of their defenses against microbial attack and infection (Dossey, 2010).

Flies can be a valuable resource for antimicrobial agents. An antimicrobial lipid, 1-lysophosphatidylethanolamine, has been isolated from the common house fly, Musca domestica L. This compound inhibits growth of the Gram-positive bacterium Bacillus thuringiensis and the yeast Saccharomyces cerevisiae (Meylaers et al., 2004). p-Hydroxybenzoic acid, p-hydroxyphenylacetic acid, and octahydrodipyrolo [1,2-a;10,20-
d]pyrazine-5,10-dione (the cyclic dimer of the amino acid proline) have been identified from larval and hemolymph extracts of the common green bottle fly, *Lucilia sericata* Meigen. These compounds possess antibacterial activity against *Micrococcus luteus* and *Pseudomonas aeruginosa* (Huberman et al., 2007).

**Antivirals**

The substances from insect, particularly peptides, have effects against virus infection and replication. Melittin (derived from the sting venom of honeybees) and its analogs have anti-HIV activity and effects against other viruses such as herpes simplex virus and Junin virus (Matanic and Castilla, 2004). Another group of insect-derived antiviral peptides are the alloferons. Alloferons are discovered in the hemolymph of blowflies, *Calliphora vicina* Robineau-Desvoidy. These peptides have antiviral effects on influenza and herpes simplex virus (Chernysh et al., 2002).

**Other medicinally relevant properties**

Some insect-derived substances have been shown to possess biological activities. For example, bee venom therapy is commonly used to treat various diseases such as arthritis, rheumatism, pain, and cancer. This kind of therapy is known as apitherapy (Son et al., 2007). Powdered silkworm larvae have been used as a medicine in Asia. Silkworm powder inhibits absorption of glucose in human intestinal epithelium cells and reduces vasopressin expression in the hypothalamus of diabetic mice (Kim et al., 2007).
5. Cicada slough

Cicada is an insect of the order Homoptera, suborder Auchenorrhyncha, in the superfamily Cicadoidea. There are about 2,500 species around the world and still need to be classified (Sajomsang and Gonil, 2010). The cicada slough (Fig 1), the shell of the Cryptotympana spp. named ‘Seontoi’ in Korea and ‘Chan Tui’ in China, has been used for traditional medicine in Asia, especially in China (Okuda, 1986). The appearance is similar to cicada which has length and diameter of about 3.5 cm, 2 cm. The color of outer face is light brown and translucent and looks glossy. There is a pair of tactile organ in the head and the eyes are protruded aside. There are vestige of wings and legs in thoracodorsalis. The cicada slough has been used to defervesce, spasmolysis, and dephlogisticate (Okuda, 1986). It contains chitin, amino acid, four phenolic monomer including 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid, N-acetyldopamine, 2-oxo-N-acetyldopamine and the 1,4-benzodioxane derivatives such as (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-6-(N-acetyl-2′′-aminoethyl)-1,4-benzodioxane, and (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2′′-aminoethyl)-1,4-benzodioxane (Okuda, 1986; Naoki et al., 2000).

In pharmacological study, the water and ethanol extracts of cicada slough have been shown to possess sedative effect and antipyretic effect. Also, the study reported that the water extract of cicada slough have antiviral activity (Hsieh et al., 1991). Xu et al. (2006) reported the antioxidant and anti-inflammatory activities of (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2′′-aminoethyl)-1,4-benzodioxane and (2R,3S)-2-(3′,4′-dihydroxyphenyl)-3-acetylamino-7-(N-acetyl-2′′-aminoethylene)-1,4-
benzodioxane). In addition, N-acetyldopamine dimers were reported to have tyrosinase-inhibiting activity (Takahiro et al., 2002)

**Figure 1.** Slough of *Cryptotympana* spp.
MATERIALS AND METHODS

1. Instrumental analyses

$^1$H and $^{13}$C NMR spectra were recorded in MeOD on a Bruker AVANCE 600 spectrometer (Rheinstetten, Baden-Württemberg, Germany) at 600 and 150 MHz (TMS as an internal standard), 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. UV spectra were obtained in methanol with a JASCO V-550 spectrophotometer (Tokyo, Japan) and mass spectra on Jeol GSX 400 spectrometer (Tokyo, Japan). Merck silica gel (0.063–0.2 mm) (Darmstadt, Germany) was used for column chromatography. Merck pre-coated silica gel plates (Kieselgel 60 F$_{254}$, 0.20 mm) were used for analytical thin layer chromatography (TLC). An Isolera one Biotage® medium-pressure liquid chromatograph (MPLC) (Uppsala, Sweden) and an Agilent 1200 high-performance liquid chromatograph (HPLC) (Santa Clara, CA) were used for isolation of active principles.

2. Materials

Theanine was purchased from TCI (TOSHIMA, KITA-KU, TOKYO, JAPAN). 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. Roswell Park Memorial Institute (RPMI)
1640 Medium, Dulbecco’s modified Eagle’s medium (DMEM), minimum essential medium (MEM), 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). All of the other chemicals and reagents used in this study were of analytical grade quality and available commercially.

3. Cicada slough and sample preparation

Air-dried sloughs of Cryptotympana spp. were purchased from a local Chinese herb market in Shan Xi Province, P.R. China and used for extraction. A certified entomological taxonomist was used to identify the cicada. A voucher specimen (PS–01) was deposited in the Research Institute for Agriculture and Life Science, Seoul National University.

4. Extraction and isolation of active constituents

The cicada slough (33 kg) was finely powdered using a blender, extracted with ethanol (165 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 under vacuum at 40°C to yield approximately 117.0 g of an extract (based on the weight of the cicada slough). The extract (116.7 g) was sequentially partitioned into hexane- (7.00 g), chloroform- (8.17 g), ethyl acetate- (9.62 g), butanol- (23.34 g)
and water-soluble (68.57 g) portions for subsequent bioassay (Figure 2). The organic solvent fractions were concentrated to dryness by rotary evaporation at 40°C, and the butanol and water fractions were concentrated at 48°C.

![Diagram of solvent fractionation procedures](image)

**Figure 2.** Solvent fractionation procedures of ethanol extract from slough of *Cryptotympana* spp.

The chloroform-soluble fraction (8.17 g) was most active and MPLC was performed 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) with column volume 132 mL (Figure 2). Separation was achieved with a gradient of chloroform and methanol [100:0 (792 mL), 99:1 (396 mL), 98:2 (396 mL), 97:3 (396 mL), 95:5 (924 mL), 92:8
(528 mL), 90:10 (396 mL), 85:15 (396 mL), 70:30 (132 mL), and 0:100 (800 mL) by volume] at a flow rate 25 mL/min to provide 234 fractions (each about 22 mL). Column fractions were monitored by TLC on silica gel plates developed with chloroform and methanol (98:2 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 78 to 234 (C4, 2.70 g) was separated by MPLC with SNAP (100 g silica gel) column by elution of a gradient of chloroform and methanol [100:0 (910.8 mL), 95:5 (897.6 mL), 90:10 (633.6 mL), 85:15 (660 mL), 80:20 (396 mL), 70:30 (132 mL), and 0:100 (800 mL) by volume] at a flow rate 25 mL/min to provide 201 fractions (each about 22 mL). Column fractions were monitored by TLC on silica gel plates developed with chloroform and methanol (85:15 by volume). Fractions 1 to 22 (C41, 1.00 g) was also separated by MPLC with SNAP (100 g silica gel) column by elution with a gradient of chloroform and methanol [100:0 (1254 mL), 95:5 (1188 mL), 92:8 (488 mL), 90:10 (646.8 mL), 85:15 (990 mL), and 0:100 (600 mL) by volume] at a flow rate 25 mL/min to 235 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 17 to 33 (C412, 80 mg). The column was a 7.8 mm i.d. × 300 mm Waters µBondapak C18 (Milford, MA, USA) using a mobile phase of acetonitrile and water (20:80 by volume) at a flow rate of 1.0 mL/min. Chromatographic separations were monitored using a UV detector at 209 nm. Finally, a potent active principle 1 (C4121, 5.34 mg) were isolated at a retention time of 13.48 min (Figure 3).
Figure 3. Isolation procedure of slough of *Cryptotympana* spp. derived constituents.
5. **Cell lines and culture conditions**

Ten human cancer cell lines used in this study as follows: PC-3 (a human prostate adenocarcinoma cell line), DU 145 (human prostate carcinoma 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), 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
PC-3, DU 145, 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% CO₂ and 95% air at 37°C. Hela, A549, and SK-HEP-1 cell lines were cultured with MEM containing 10% FBS, 1% antibiotic-antimycotic solution, and 1% glutamine under 5% CO₂ and 95% air at 37°C. Hep-2 cell line was cultured with DMEM containing 10% FBS and 1% antibiotic-antimycotic solution under 5% CO₂ and 95% air at 37°C (Korean Cell Line Bank, 1982). Cells were grown in 2 × 10⁴ cm 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 5 × 10⁵ 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 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% CO₂. 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 and pure theanine served as positive controls and were similarly formulated. Negative controls consisted of the DMSO solution only. The optical density (OD) values were recorded using a Molecular Devices VersaMax microplate reader (Sunnyvale, CA, USA) at a 560 nm and a 670 nm reference. Blank values were subtracted from experimental values. All bioassays were replicated three times.
7. Data analysis

Anti-proliferative activity was exposed as 50% inhibition concentration (IC\textsubscript{50}) of the compound that reduced the viability of cells to 50% compared with the control wells. IC\textsubscript{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, B are the OD values of treated cells and untreated cells, respectively.
RESULTS

1. Anti-proliferative activity of slough of Cryptotympana spp.

The anti-proliferative activity of ethanol extract from the slough of Cryptotympana spp. was compared with that of the commercial cancer agent cisplatin against various human cancer cell lines using a MTT assay (Table 1). Potencies varied according to cell line tested. Based on IC$_{50}$ values, the slough ethanol extract was proved to have anti-proliferative activity against all test cancer cell lines (IC$_{50}$, 15.08–38.02 μg/mL) with the exception of Hep-2 and SK-OV-3 cell lines (>100 μg/mL). Overall, Cryptotympana spp. slough extract was 3.3–>40.5 times less toxic than cisplatin against the test cancer cell lines.
Table 1. Anti-proliferative activity of ethanol extract from slough of *Cryptotympana* spp. to 10 cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>EtOH extract (IC$_{50}$, $\mu$g/mL)</th>
<th>Cisplatin$^b$ (IC$_{50}$, $\mu$g/mL)</th>
<th>RT$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549 lung cancer</td>
<td>38.02</td>
<td>9.95</td>
<td>3.8</td>
</tr>
<tr>
<td>AGS stomach cancer</td>
<td>15.08</td>
<td>4.22</td>
<td>3.6</td>
</tr>
<tr>
<td>PC-3 prostate cancer</td>
<td>31.73</td>
<td>5.73</td>
<td>5.5</td>
</tr>
<tr>
<td>DU145 prostate cancer</td>
<td>36.15</td>
<td>5.51</td>
<td>6.6</td>
</tr>
<tr>
<td>Hela cervix cancer</td>
<td>35.32</td>
<td>4.99</td>
<td>7.1</td>
</tr>
<tr>
<td>Hep-2 larynx cancer</td>
<td>&gt;100</td>
<td>2.47</td>
<td>&gt;40.5</td>
</tr>
<tr>
<td>HT-29 colon cancer</td>
<td>32.65</td>
<td>9.89</td>
<td>3.3</td>
</tr>
<tr>
<td>MCF-7 breast cancer</td>
<td>25.11</td>
<td>3.28</td>
<td>7.7</td>
</tr>
<tr>
<td>SK-Hep-1 liver cancer</td>
<td>36.64</td>
<td>10.04</td>
<td>3.6</td>
</tr>
<tr>
<td>SK-OV-3 ovary cancer</td>
<td>&gt;100</td>
<td>8.41</td>
<td>11.9</td>
</tr>
</tbody>
</table>

$^a$ 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 slough of *Cryptotympana* spp.

Fractions obtained from the solvent hydrolysable of the ethanol extract of the slough of *Cryptotympana* spp. were likewise tested against PC-3 prostate cancer cell line (Table 2). As judged by IC$_{50}$ values, the hexane- and chloroform-soluble fractions showed the most pronounced anti-proliferative activity. Low activity was produced by the ethyl acetate-soluble fraction. The butanol- and water-soluble fractions were ineffective.
Therefore, the chloroform-soluble fraction was used to identify peak activity fractions for the next step in the purification.

The anti-proliferative activity and yield (%) of each subfraction derived from the chloroform-soluble fraction are given in table 3.

**Table 2. Anti-proliferative activity of fractions obtained from the solvent hydrolysable of the ethanol extract of slough of *Cryptotympana* spp. against PC-3 prostate cancer cell line using a MTT assay**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IC$_{50, a}$ µg/mL (95% CL$^b$)</th>
<th>Slope ± SE</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane-soluble fr.</td>
<td>17.78 (12.11–26.12)</td>
<td>0.5 ± 0.38</td>
<td>4.47</td>
<td>0.940</td>
</tr>
<tr>
<td>Chloroform-soluble fr.</td>
<td>9.53 (6.67–13.63)</td>
<td>0.3 ± 0.26</td>
<td>2.10</td>
<td>0.965</td>
</tr>
<tr>
<td>Ethyl acetate-soluble fr.</td>
<td>41.12 (32.31–52.33)</td>
<td>1.3 ± 0.95</td>
<td>6.14</td>
<td>0.972</td>
</tr>
<tr>
<td>Butanol-soluble fr.</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water-soluble fr.</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The 50% anti-proliferative concentration for cell lines.

$^b$ CL denotes confidence limit.

$^c$ Pearson $\chi^2$, goodness-of-fit test.
Table 3. Anti-proliferative activity of each subfraction from chloroform-soluble fraction against PC-3 prostate cancer cell line

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IC$_{50}$, a mg/mL (95% CL)$^b$</th>
<th>Slope ± SE</th>
<th>$\chi^2$$^c$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>17.06 (0.59–1.70)</td>
<td>1.1 ± 0.59</td>
<td>7.96</td>
<td>0.942</td>
</tr>
<tr>
<td>C5</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C41</td>
<td>23.35 (19.15–28.47)</td>
<td>0.7 ± 0.56</td>
<td>3.24</td>
<td>0.975</td>
</tr>
<tr>
<td>C42</td>
<td>50.14 (38.85–64.70)</td>
<td>0.8 ± 0.59</td>
<td>3.92</td>
<td>0.973</td>
</tr>
<tr>
<td>C43</td>
<td>27.24 (19.41–38.21)</td>
<td>0.4 ± 0.32</td>
<td>3.39</td>
<td>0.954</td>
</tr>
<tr>
<td>C44</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C45</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C46</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C411</td>
<td>36.30 (29.24–45.06)</td>
<td>1.0 ± 0.75</td>
<td>4.55</td>
<td>0.971</td>
</tr>
<tr>
<td>C412</td>
<td>13.93 (12.26–15.83)</td>
<td>1.2 ± 1.01</td>
<td>2.85</td>
<td>0.990</td>
</tr>
<tr>
<td>C413</td>
<td>88.69 (58.93–133.50)</td>
<td>0.6 ± 0.40</td>
<td>3.88</td>
<td>0.940</td>
</tr>
<tr>
<td>C414</td>
<td>72.92 (55.57–95.68)</td>
<td>1.9 ± 0.74</td>
<td>8.03</td>
<td>0.959</td>
</tr>
<tr>
<td>C415</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4121</td>
<td>6.52 (5.08–8.37)</td>
<td>1.3 ± 0.16</td>
<td>4.05</td>
<td>0.972</td>
</tr>
<tr>
<td>C4122</td>
<td>&gt;100</td>
<td></td>
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</tr>
</tbody>
</table>
MTT bioassay-guided fractionation of the ethanol extract of the slough of *Cryptotympana* spp. afforded an active principle identified by spectroscopic analysis, including EI-MS and NMR. The active principle was obtained as white powder. The mass spectrum of the isolate exhibited a molecular ion at *m/z* 174 [M]+ (Fig. 1) and its 1H NMR spectra (Fig. 2) showed 14 protons. Its 13C NMR spectra (Fig. 3) showed 7 carbons in the molecule comprising one methyl group, three ethyl groups, and two nonprotonated carbons as indicated in DEPT (Fig. 4), suggesting the molecular formula C7H14N2O3. The interpretations of proton and carbon signals were largely consistent with those of Yuan et al. (2013). This compound was characterized as theanine [2-amino-4-(ethylcarbamoyl) butyric acid] (Fig. 5). Theanine was identified on the basis of the following evidence: white powder. EI-MS (70 eV), m/z (relative intensity): 174 [M]+, 156 (3), 129 (61), 111 (30), 84 (100), 72 (59), 56 (39). 1H NMR (MeOD, 600 MHz) and 13C NMR (MeOD, 600 MHz): See Table 4.
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 theanine.
### Table 4. $^1$H and $^{13}$C NMR spectral data for compound 1

<table>
<thead>
<tr>
<th>Position</th>
<th>Partial structure</th>
<th>$\delta$C (ppm)</th>
<th>$\delta$H (ppm)</th>
<th>$\delta$C (ppm)</th>
<th>$\delta$H (ppm)</th>
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<tr>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>174.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CH</td>
<td>54.6</td>
<td>3.91, d</td>
<td>54.8</td>
<td>3.85, d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$(J = 7.08$ Hz)</td>
<td></td>
<td>$(J = 6.06$ Hz)</td>
</tr>
<tr>
<td>3</td>
<td>CH$_2$</td>
<td>29.3</td>
<td>2.35, m</td>
<td>32.8</td>
<td>2.45, m</td>
</tr>
<tr>
<td>4</td>
<td>CH$_2$</td>
<td>30.8</td>
<td>3.50, m</td>
<td>34.1</td>
<td>3.27, m</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>172.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-NH-</td>
<td>8.03, s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>CH$_2$</td>
<td>23.1</td>
<td>1.92, m</td>
<td>28.0</td>
<td>2.20, m</td>
</tr>
<tr>
<td>8</td>
<td>CH$_3$</td>
<td>15.8</td>
<td>1.36, d</td>
<td>15.0</td>
<td>1.12, d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$(J = 7.21$ Hz)</td>
<td></td>
<td>$(J = 7.26$ Hz)</td>
</tr>
</tbody>
</table>
3. Anti-proliferative activity of test compounds

The anti-proliferative activities of natural theanine, pure theanine, and anti-cancer agent cisplatin against PC-3 prostate cancer cell line were evaluated using a MTT assay (Table 5). Based on IC₅₀ values, the isolated theanine and commercial pure theanine did not differ significantly in the anti-proliferative activity against PC-3, indicating that the activity of the methanol-extracted theanine is purely due to theanine. Similarly, the anti-proliferative activity of the constituent and cisplatin did not differ significantly.

Table 5. Anti-proliferative activity of natural theanine, pure theanine, and anti-cancer agent cisplatin against PC-3 prostate cancer cell line using a MTT assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀, a μg/mL (95% CL b)</th>
<th>Slope ± SE</th>
<th>χ²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural theanine</td>
<td>6.52 (5.08–8.37)</td>
<td>1.3 ± 0.16</td>
<td>4.05</td>
<td>0.972</td>
</tr>
<tr>
<td>Pure theanine</td>
<td>9.13 (6.19–13.48)</td>
<td>0.9 ± 0.60</td>
<td>5.35</td>
<td>0.957</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>7.39 (6.28–8.70)</td>
<td>1.3 ± 1.00</td>
<td>3.32</td>
<td>0.949</td>
</tr>
</tbody>
</table>

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

b CL denotes confidence limit.

c Pearson χ², goodness-of-fit test.
DISCUSSION

Prostate cancer is the most commonly diagnosed invasive malignancy and has the second leading male death in Western countries. Especially, in 2013, an estimated 238,590 men in the United States were diagnosed with prostate cancer and 29,720 men were died of it (Parkin et al., 1997; Malik et al., 2005; American Cancer Society, 2013). In Asian countries, the rates of (20–30 %) prostate cancer have been increased for years (Nakata et al., 1995; Hsing et al., 1998). In ROK, the prostate cancer becomes a major cancer of male death (Park et al., 2006). Prostate cancer incidence and mortality rates in ROK had been rose 12.7 fold during the 20-year period from 1983 to 2002 (Park et al., 2006).

Hormone therapy, radiation therapy, surgical removal, and chemotherapy have been used for the various cancers therapies, but no effective cure for prostate cancer of chemotherapy currently exists because of their adverse effect such as nausea, vomiting and thrombosis (Calabro and Sternberg, 2007). Furthermore, in developing new anticancer drugs, chemical synthesis shows limitation because of cost, time, and low success rate (Katiyar et al., 2012). For these reasons, many researches have been studied the development of agents for prostate cancer from natural products.

The use of insects as a folk remedy has been common in China (Feng et al., 2009) and Brazil (Costa-Neto, 2002) and is introduced to many other countries such as India, Africa, Mexico, and ROK (Costa-Neto, 2005a and 2005b; Dossey, 2010). Pharmaceutical insects have been used in traditional Chinese medicine for over 3,000
years and an estimated 300 insect species including cicada are used to produce 1,700 traditional Chinese medicines (Huang et al., 1997; Costa-Neto, 2002; Feng et al., 2009). For example, the chitooligosaccharide, amino acid, glutamic acid, and the tridecapeptidesalloferons 1 and 2 from cicada slough were reported to possess antibacterial, antiviral, antitumor, anti-inflammatory, and antioxidant activities (Chernysh et al., 2002; Wu et al., 2013). However, there are a few reports of anticancer agents from insects, which are the most diverse groups of organisms (Huang et al., 1997). The water fraction extracted from the cicada slough showed quite strong antiproliferative activity against the mouse lymphocytic leukemia L1210 (IC$_{50}$, 1.51, μg/mL), Menogaril-resistant mouse leukaemia P388 (IC$_{50}$, 1.26 μg/mL) and human stomach SNU-1 (IC$_{50}$, 1.45 μg/mL) cancer cell lines (Huang et al., 1997). It has been also reported that the extract of the cicada slough had anti-proliferative activity on the mouse melanoma cell line B16 (IC$_{50}$, 23 μg/mL) and human skin malignant melanoma cell line G361 (IC$_{50}$, 29 μg/mL) (Takatsuki et al., 1996).

In this study, the ethanol extract of Cryptotympanaspp. slough was proved to have anti-proliferative activity against lung, stomach, prostate, cervix, colon, breast, and liver cancer cell lines except for Hep-2 larynx and SK-OV-3 ovary cancer cell lines, although the extract was less toxic than cisplatin. The anti-proliferative principle was determined to be the nonproteino-α-amino acid theanine. The constituent exhibited potent antiproliferative activity toward human prostate cancer lines PC-3 and DU 145. IC$_{50}$ of theanine was between 6 and 10μg/mL toward two human prostate cancer cell lines, although IC$_{50}$ of the natural compounds stated previously is between 10 and 100 μg/mL.
The activity of theanine did not differ significantly from that of the anticancer agent cisplatin. This original finding indicates that materials derived from the slough of Cryptotympanaspp. can hold promise for the development of novel and effective naturally occurring antiprostate agents. Theanine has some mechanisms of actions on human. Theanine increases alpha-brain wave activity, a sign of induced relaxation (Ito K et al., 1998). The antioxidant activity of theanine has been studied about its effect on the oxidation of LDL cholesterol (Yokozawa T and Dong E, 1997).

In conclusion, Cryptotympana spp. slough-derived materials containing theanine could be useful as an anticancer agent in the prevention or eradication of prostate cancer. The anti-proliferative action of theanine may be an indication of at least one of the pharmacological actions of slough of Cryptotympana spp. For the practical use of Cryptotympana spp. slough-derived materials as novel anticancer products to proceed, further research is needed to establish their human safety, exact mode of action, and whether this activity is exerted in vivo after consumption of Cryptotympanaspp. slough-derived products by humans. Lastly, detailed tests are needed to understand how to improve anti-proliferative potency and stability for eventual commercial development.


매미 겔질 유래 인간 전립선 암세포 증식 억제 물질의

분리 및 동정

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초 록

전립선 암은 가장 흔히 발생하는 악성종양으로 미국과 많은 유럽국가 등에서 두 번째로 높은 남성 사망률을 보이고 있다. 한국의 경우, 전립선 암의 발생빈도가 빠르게 증가하고 있는 추세이며 모든 암 중에서 가장 높은 발병률을 가지고 있다. 이와 같이 급증하고 있는 전립선 암 발생률과 사망률에 관한 정확한 이유는 아직까지는 밝혀지지 않았지만 최근 서구화된 식습관 등으로 인해 증가하고 있다는 보고가 나오고 있다. 호르몬치료, 항암화학요법, 방사선치료 등 전립선 암 치료법들이 행해지고 있지만 때로는 출혈, 탈모, 구토,
발기부전, 성욕감퇴 등 심각한 부작용들을 야기하기도 한다. 따라서 새로운 타겟 부위와 낮은 독성을 가지는 새로운 항암제의 개발에 대한 필요가 시급하다.

본 연구에서는 전립선 암 세포주 PC-3와 DU145를 포함한 총 10개의 인간 암 세포주에 대한 매미껍질유래물질의 세포증식 억제활성 실험을 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 방법을 이용하여 진행하였다. 실험결과는 항암제로 널리 사용되고 있는 시스플라틴을 양성 대조군으로 하여 비교하였다. 건조된 매미껍질 33 Kg에서 116.7 g의 에탄올 추출물을 얻게 되었다. 매미껍질 에탄올 추출물을 얻은 세포주로 페암 세포주 A549, 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-1에서 상당히 높은 세포증식 억제활동을 나타내었다. 하지만 식도암 세포주 Hep-2와 난소암 세포주 SK-OV-3에 대한 세포증식 억제활동은 보이지 않았다. 전자 이온화 질량 분석법과 핵자기 공명 분광법을 이용하여 매미껍질 에탄올 추출물에서 단일물질인 theanine [2-amino-4-(ethylcarbamoyl)butyric acid]의 물질을
분리하고 그 구조를 동정하였다. 분리한 물질은 전립선 암 세포주 PC-3에 대해 높은 세포증식 억제활성(IC₅₀, 6.52 μg/mL)을 보였다. 또한 시판되는 항암제인 시스플라틴의 세포증식 억제활성(IC₅₀, 7.39 μg/mL for PC-3)과 큰 차이를 보이지 않았다.

이상의 결과를 바탕으로 본 논문의 연구는 매미껍질에 함유된 활성본체를 밝혀냈다는데 그 의미가 있고, 구성 물질에 대한 생물검정을 통해 항암제로 써의 가능성을 탐색하고 매미껍질의 새로운 생리활성을 밝혀내어 농업적, 산업적으로 그 활용 가능성이 높다고 판단되며 추가적 연구가 요구된다.

검색어: 전립선암, 천연 항암제, 매미껍질, Theanine

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