2008年 2 月

碩士學位論文

Characterization of secondary metabolites from fermented needle extract of *Pinus densiflora*

朝 鮮 大 學 校 大 學 院

生 命 工 學 科

朴 佳 永

Characterization of secondary metabolites from fermented needle extract of *Pinus densiflora*

솔잎 발효액으로부터 이차대사산물의 탐색 및 응용

2008年　2 月　日

朝 鮮 大 學 校 大 學 院

生 命 工 學 科

朴　佳　永

Characterization of secondary metabolites from fermented needle extract of *Pinus densiflora*

指導敎授　鄭　賢　淑

이 論文을 理學碩士學位 申請論文으로 제출함

2007年　12 月　　日

朝 鮮 大 學 校 大 學 院

生 命 工 學 科

朴　佳　永

朴 佳 永의 碩士學位論文을 認准함.

委員長 朝鮮大學校　　敎授　　印

委　員 朝鮮大學校　　敎授　　印

委　員 朝鮮大學校　　敎授　　印

2007年　11 月　28 日

朝 鮮 大 學 校 大 學 院

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AOAC..... Association of official analytical chemists

DMSO .....Dimethyl sulfoxide

DNP.....2,4-dinitrophenyl hydrazine

DPPH..... 1,1-diphenyl-2-picrylhydrazyl

GC-MS ..... Gas chromatrography – Mass spectrophotometric

HPLC..... High-performance liquid chromatography

ICC .....Interstitial cells of cajal

NBT.....Nitro blue tetrazolium

SFPE.....Self fermented pine needle extract

SMGM..... Smooth muscle growth medium

PE ..... Pine needle extract

ROS ..... Reactive oxygen species

ABSTRACT

Characterization of secondary metabolites from fermented needle extract of *Pinus densiflora*

Gayoung Park

Advisor: Prof. Hyeonsook Cheong, Ph. D.

Department of Biotechnology,

Graduate School of Chosun University

Pine needle (*Pinus densiflora* Sieb. *et* Zucc.) extract has been used to improve cardiovascular disorders, detoxification of nicotine, the infirmities of age and curing diseases of unidentified symptoms. In this study we have identified various useful components in extract including vitamin C, chlorophyll, amino acids, carbohydrate and fatty acids. GC-MS result reveals that self-fermentation leads terpenoid composition dynamics in the stored pine needle extract. The self-fermented pine extract (SFPE) inhibited the growth of some bacterial strains like *Salmonella typhimurium, Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis*. The SFPE (0.2 ㎕/ml-0.3 ㎕/ml) showed 90% NBT superoxide scavenging activities, which is similar for all tested samples of different ages.

Fibrinolytic activities of the extract indicated that activity depends on time and also with aging of the product. It was also found that the extract can lower the blood plasma cholesterol and triglyceride in cholesterol fed rat. When SFPE 7 (200 μg/ml) treated in ICC, under currents clamp mode decreased both the frequency and amplitude of pacemaker currents, and increased the resting currents in outward direction. Also, SFPE 7 inhibited the pacemaker currents in a dose-dependent manner. Glibenclamide, a blocker of potassium channel, reversed the effect developed by SFPE 7 indicating the SFPE 7 cause the opening of the potassium channels during modulation of pacemaker current. We have isolated and identified 8 different yeast strains that spontaneously grown and caused self-fermentation in the extract. Most abundantly grown yeast strain *Candida* sp. 2 possesses 99% sequence homologies with *Candida ernobii* disappeared within 4 years of storage. SFPEs also showed antioxidant, anti-bacterial properties, may improve the blood circulation and act against antherosclerosis, therefore it fermented pine needle extract could be a good source for functional food development.

I. INTRODUCTION

Pinus densiflora is an evergreen needle-leafed tree indigenous to Asia. Natural products have been used for millennia for the treatment of multiple ailments. It has been reported that pine needle extracts improved unidentified clinical syndrome such as fatigue, depression, anxiety, sleeping disturbance, etc. (Ichikawa *et al.*, 1998). Biological activity of pine needle is the essence for traditional medicine, which uses the pharmacological efficacy of natural compounds present in pine needle for treating human diseases. Furthermore, pine needles are used in preparation of teas, extracts, some alcoholic beverages for tonic and the health-improving agent (Chung *et al.*, 2002). In connection with this, evidence has supported the role that antioxidants, including several compounds, play in the prevention of anti-aging and several chronic diseases such as cardiovascular disease, cancer, diabetes, antihypertension (Cheong *et al.*, 2005; Yen *et al.*, 2007). Therefore, pine extract has been processed and used traditionally to treat multiple disorders.

In many areas, peoples adopt traditional methods in processing foods to increase its effectiveness or longevity during storage. These practices follow open storage systems to obtain alcoholic beverage, pickles and other products of desired tastes since unspecified period through critical consideration of storage conditions like temperature, humidity and light. Timing and micro-environmental conditions influence greatly in producing quality products. Microorganisms are expected to play a key role in changing composition and quality in targeted food, which is stored. To answer which organisms play the vital role in open system of fermentation is still a matter of search.

Pine extract contains several different organic compounds including carbohydrates, proteins, lipids, terpenoids, alkaloids and several others. Pine leaves have essential oils (0.3-1.3%)

including α-pinene, β-pinene, camphene, phellandrene, limonene, borneol (6.8%), and bornyl acetate (3.8%) (Kim and Shin, 2005; Shin, 2005) that are helpful in reducing cardiovascular diseases and possess anticancer properties (Kim *et al.*, 2006). Especially pine needle and bark are abundant in terpenoids. The essential oil of pine needles has found in wide commercial use (Lee *et al.*, 2005). Some terpenoid compounds like eucalyptol have been demonstrated to be capable of reducing inflammation and pain. It has also been found to be able to kill leukaemic cells (Moteki *et al.*, 2002). Plant terpenoids are aroma compounds that contribute smell of eucalyptus, the flavors of cinnamon, cloves and ginger and the color of yellow flowers. They may exist as vitamins, hormones, flavors and biopolymer. They play crucial roles in traditional herbal remedies and are under investigation for their antibacterial and other pharmaceutical effects. They are also employed for medical purposes. In addition, presumably large numbers of efficacious terpenoids are yet to be discovered. Antimalarial drug artemisinin and the anticancer drug paclitaxel are among such terpenoids with established medical applications. Artemisinin is a drug used to treat multi-drug resistant strains of *falcioarum* malaria. The compound (a sesquiterpene lactone) isolated from the shrub *Artemisia annua* is long been used in traditional Chinese medicine (http://en.wikipedia.org/wiki/Eucalyptol). And paclitaxel is a mitotic inhibitor used in cancer (used to treat patients with lung, ovarian, breast cancer, head and neck canner) chemotherapy (http://en.wikipedia.org/wiki/Paclitaxel.). Flavonoides and other plant phenolics such as phenolic acids, stilbenes and tannins are important for normal growth and defense against infection and injury (Jerez, 2007).

Bacteria cause several epidemic diseases like diarrhea, dysentery, tuberculosis, pneumonia, and several others. *Staphyloccus aures* and *Salmonella typhimurium* are food borne pathogenic bacteria (Dupont *et al.*, 2006) causing food poisoning (Lee *et al.*, 1996). Some plants products are being used traditionally in such complications as well. It was found that volatile compounds

present in pine extract have antibacterial properties that can completely inhibit the growth of bacterial strains like *Bacillus subtilis*, *Salmonella typhimurium* and *Staphylococcus aureus* (Kim and Shin, 2005). Volatile compounds isolated from various parts of Red pine are also found effective in inhibiting human intestinal bacteria (Jeon, 2001; Koukos *et al.*, 2000; Watanabe *et al.*, 1991). In general, plant products assumed have low side effects, nutritious, cure diseases and regularize normal physiological processes especially acting against oxidative stress.

Free radicals are constantly formed in the human body by regular metabolic action, and exert oxidative stress and cause various diseases including cancer, aging, diabetes and atherosclerosis. Reactive oxygen species (ROS) are originated ubiquitously in aerobic metabolism, and are generally removed by antioxidant such as a superoxide dismutase, glutathione peroxidase, metal-binding proteins, vitamin C, vitamin E, beta carotene, uric acid, bilirubin, albumin, DNA repair enzyme, methionine sulphoxide reductase repair (Seo *et al.*, 2004; Sung *et al.*, 2000). Recently, considerable attention has been paid on the use of natural antioxidants to scavenge free radicals or to prevent damage from free radicals. Various reports try showing the antioxidant effect in food. It is found that red wine and green tea contains high level of antioxidant (Maxwell *et al.*, 1994).

Fibrinolytic enzymes dissolve the blood clots, which are formed by the conversion of fibrinogen into fibrin via the proteolytic action of thrombin. When clots are not lysed, they accumulate in blood vessels and cause thrombosis leading to myocardial infarction and other cardiovascular diseases. Intravenous administration of urokinase and streptokinase, which are capable of degrading fibrin, has been widely used for this thrombosis therapy. However, these enzymes have a low specificity for fibrin and very expensive (Benerjee *et al.*, 2004; Jennings, 1996; Sumi, 1977). Therefore, it has been reported that there are some proteases of pine needle

showing fibrinolytic activity.

Hypercholesterolemia, resulting from cholesterol metabolic changes, is a major cause of cardiovascular disturbance, such as atherosclerosis and coronary heart disease (Kannel *et al*., 1971; Keys, 1970). Epidemiologic data showed that a high consumption of vegetables and fruits is consistently associated with a low risk of cancer and cardiovascular disease (Klerk *et al.*, 1998; MJ., 1999).

Alimentary canal of mammals is the main place for digestion of food materials taken. There is a myonteric movement of bowls that helps the downward movement of food in gut where the continuous contracting and relaxing cells are existed (Sanders, 1996; Thuneberg, 1982). Such cells are called the Interstitial Cells of Cajal (ICC), small spindle-shaped or stellate cells having numerous mitochondria capable to modulate the gastrointestinal movement through the alteration of the spontaneous inward currents generated through influences of external agents (Koh *et al.*, 1998; Thomsen *et al.*, 1998; Tokutomi *et al.*, 1995; Ward *et al.*, 1994). The current generated is called pacemaker current that enables the tissues producing continuous rhythm of contraction and relaxation in the smooth muscle tissues of bowl. Therefore, these cells play key role as basic regulators of gastrointestinal motility, many hormones, neurotransmitter, and various substances can modulate GI tract motility by influencing ICC. Abnormalities in these currents also cause gastrointestinal irregularities, which is also implicated in the use of certain drugs.

The aim of this study was the assessment of general compositions, and effects pattern of self fermented pine needle extract. The study also aimed to assess whether the extracts show multiples response with bacteria, fungi, free radical, cholesterol, triglycerides and intestinal motility. There is always lacking information in role of spontaneous fermentation in compound dynamics and functional efficacy of the pine needle extracts.

II. MATERIALS AND METHODS

II-1. Plant materials

Fresh pine needles were selected and harvested from Korean red pine trees (*Pinus densiflora* Sieb. *et* Zucc.) in Gokseong, Korea.

II-2. Preparation of self fermented pine needle extracts

Pine needles were cleaned 3, 4 times with tap water; dipped with charcaol water and dried and ground for 1 minute to homogenize. The preparation was allowed to put for 3 hours at 4℃ and the supernatant was recovered. This supernatant sample was stored at 4℃ for assays. PE was stored for years that favored emergence of microorganisms, which finally enabled spontaneous fermentation in extracts. The effects of the extract were examined after 3 and 7 years of spontaneous fermentation. The extract was freezing dried to obtain solid sample. Freeze dried sample was used only for electrophysiological studies whereas all other experiments were carried out from liquid extract.

II-3. Composition and pattern of compound during self-fermentaion

II-3-1. General composition

General compositions present in SFPEs were determined using AOAC. Chlorophyll content of the extract was determined using spectrophotometric method. Powdered needles were dissolved in 85% acetone and ether was added in the mixture to dissolve fat-soluble substances. Acetone was removed using a separating funnel and the isolated colorful mixture was dehydrated using sodium acetate and the absorbance was recorded at OD_{660} and $OD_{642.5}$ to measure chlorophyll a, b and total chlorophyll. Vitamin C present in SFPEs were deterined using 2,4-dinitrophenyl hydrazine (DNP) method and HPLC. Vitamin C was isolated with 5% HPO_3, while nonpolar ingredient remained in the mixture was removed by hexane. Flow solution used was acetonitrile 30%, 0.05 M KH_2PO_4 and 70% isocratic in NH_2 column with the flow speed of 1 ml/min, using 254 nm UV detector. Amino acids content present in SFPEs was analyzed using amino acid analyzer and atomic absorption spectrophotometer.

II-3-2. Carbohydrate

Carbohydrate (soluble sugar) present in PE and SFPEs was determined using HPLC. A 10 ml sample was dissolved in 5% H_3PO_4 to make 50 ml. The mixture was filtered in 0.45 μm filter and examined in HPLC (SCL 10Avp Series, Shimadzu, Japan). Flow solution was acetonitrile 40%, 0.05M KH_2PO_4 and 60% isocratic with the flow speed of 1ml/min NH_2 column, using RI detector.

II-3-3. Fatty acids

The amount and type of fatty acids were determined by using total fat determination B-815/B-820 (Buchi, Switzerland). The 35 ml of sample was compressed to 10 ml and kept in the solvent vessels adding Potassium hydroxide and n-butanol to the solvent vessel accompanied with ascorbic acid when the determination of fatty acids is involved using tridecanoic acid C13 as standard. The extraction and simultaneous saponification were done in 30 minutes at boiling temperature. The potassium salts and fatty acids were converted to free fatty acids by addition of aqueous reagents. A two-phase system arises with organic phase containing fatty acids in the upper phase was separated. Operation conditions were: instrument: Fat determination system (B-820, Buchi, Switzerland), carrier gas: Hydrogen, injection temperature 220°C, detection (FID) temperature: 260°C, baking temperature 130°C increased by 6.5°C/min final steady temperature was 260°C for 4 min; hydrogen gas pressure 225 kPa, mixture gas pressure was 48 kPa used for obtaining data.

II-4. The effects and analytical assays of PE and SFPEs

II-4-1. Antibacterial activity

II-4-1-1. Bacterial strains

Bacterial culture was maintained in nutrient agar medium that were kept at 4°C. Six bacterial strains were used in this study. The bacteria were *Salmonella typhimurium* (KCTC1925), *Micrococcus luteus* (ATCC9341), *Staphylococcus aureus* (ATCC6538P), *Escherichia coli*

(KTCC1923), *Pseudomonas aeruginosa* (ATCC15692), and *Bacillus subtilis* (ATCC6633).

II-4-1-2. Antibacterial assay

Nutrient broth (bacto beef extract 3 g, bacto-peptone 5 g) agar culture of the test organisms was prepared as described (Ahmada *et al.*, 2005). Two different concentrations of the PE and SFPE sample (40 ㎕ and 80 ㎕) to test the antibacterial activities were loaded onto each Whatman No.1 filter paper discs (φ, 6 mm) and placed on the previously inoculated nutrient broth agar. The plates were inverted and incubated for 24 hours at 37°C. The clear inhibition zones around the discs indicated the presence of antimicrobial activities.

II-4-2. Antioxidant activity

The radical-scavenging activities of SFPEs were measured by using the NBT superoxide scavenging assay. The 100 ㎕ of SFPEs were treated in a solution: 20 ㎕ of 10 mM Na_2EDTA in buffer (50 mM KH_2PO_4 / KOH pH. 7.4), 50 ㎕ of 6 mM NBT in buffer, 30 ㎕ of 3 mM hypoxanthine in 50 mM KOH, 50 ㎕ of xanthine oxidase in buffer (1 unit in 10 ml buffer). The absorbance was read against a blank at 570 nm. The results were expressed as percentage

II-4-3. Fibrinolytic activity

Fibrinolytic activity of SFPEs was measured on fibrin plate. To prepare fibrin assay plates, 5

ml of 1% (w/v) fibrinogen solution in distilled water was mixed with 10 ml of 1.2% agarose solution and 20 ㎕ of thrombin solution (0.1 U/㎕). The solution was then poured into a Petri dish and allowed to stand for 1 hour at room temperature to form a fibrin clot. 5 mm wide holes were created with glass tubes on the fibrin plates. 20 ㎕ of SFPEs was carefully dropped onto the each hole in the plates. The plate was incubated for 1 hour at 37℃. And the diameter of the lytic circle was measured. In the fibrin plate method, a clear region is observed in which fibrin is hydrolyzed, and its diameter is directly proportional to the potency of the fibrinolytic activity.

II-4-4. Cholesterol and triglyceride

Male Sprague-Dawley rats (200 ± 20 g) approximately 12-weeks-old were used in all experiments. Animals were housed four per cage and maintained under control environmental conditions (22 ± 2 ℃, 12 hour light/dark cycle). Food and tap water were availed in libitum. All efforts were made to minimize animal suffering and to reduce the number of animals used. For study, 15% cholesterol and 1% sodium cholate were mixed with 84% corn oil to get cholesterol mixture. 12 weeks old male Sprague-Dawley rats were taken for the cholesterol and triglycerides examination. Rats were grouped into control and the test groups where each group contained 2 individuals. They were fed cholesterol continuously at once a day for 4 weeks. The control group was fed with 0.5 ml DW; and other test groups were fed with 0.5 ml cholesterol for 2 weeks and then with 0.5 ml pine extract with 0.5 ml cholesterol except the last group that was fed with 0.5 ml cholesterol and 0.5 ml SFPE 7 continuously for 4 weeks. Analysis was done using Automatic biochemical analyzer, Hitachi 7600, Hitachi Tokyo, Japan.

II-4-5. Electrophysiological assay

II-4-5-1. Materials

Glibenclamide and pinacidil were purchased from RBI (USA) and naproxen from Sigma (USA). All chemicals were dissolved in distilled water or dimethylsulfoxide for stock and stored at -20℃ until they were tested.

II-4-5-2. Preparation of cells and tissues

Balb/C mice (8-13 days old) of any sex were anesthetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed by washing with Krebs-Ringer bicarbonate solution, the tissues were pinned to the base of a Sylgard dish, and the mucosae were removed by sharp dissection. Small strips of intestinal muscle (containing both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hanks solution (containing in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9 and HEPES 11) for 30 minute. The cells were then dispersed with an enzyme solution containing collagenase (Worthington Bio-chemical Co., USA) 1.3 mg/ml, bovine serum albumin (Sigma) 2 mg/ml, trypsin inhibitor (Sigma, USA) 2 mg/ml and ATP 0.27 mg/ml. Thereafter they were plated onto sterile glass coverslips coated with murine collagen (2.5 ㎕/ml, Falcon/BD) in 35 minute culture dishes, and cultured at 37℃ in a 95% O_2-5%CO_2 incubator in SMGM (smooth muscle growth medium, Clonetics Crop., USA) supplemented with 2% antibiotics/antimycotics (Gibco, USA) and murine stem cell factor (SCF, 5 ng/ml,

Sigma).

II-4-5-3. Patch clamp experiments

The whole-cell configuration patch-clamp technique as used to record the membrane currents (voltage clamp) and potentials of the cultured ICC (current clamp, and Axopatch 1-D (Axon Instruments, USA). Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 7.2; Axon Instruments). Data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder (Gould 2200, Gould, USA). The cells were bathed in a solution containing (in Mm): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and HEPES 10, adjusted to pH 7.4 with Tris. The pipette solution contained (in mM) KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, disodium creatine phosphate 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with Tris. All experiments were performed at 30℃.

II-5. Identification of strains involved in self-fermentation

II-5-1. Culture media

GPYA (glucose 40 g/L, peptone 5 g/L, yeast extract 5 g/L, pH. 5.0~5.2), YM media (Yeast extract 3 g/L, Malt exteact 3 g/L, Peptone 5 g/L, Glucose 10 g/L), Bennett's media (beef extract 1 g/L, glucose 10 g/L, N-Z amine A 2 g/L, Yeast extract 1 g/L, pH. 7.3), Nutrient agar (peptone 5 g/L, beef extract 3 g/L), Nutrinet agar + SFPE 25% (peptone 5 g/L, beef extract 3 g/L, SFPEs

was centrifugated at 5,000 rpm at 4℃ for 30 min. The supernatant was autoclaved and mixed with media.)

II-5-2. Growth condition, assessment of suitable media and identification of yeast strains

To study the dynamic pattern of microbial involvement during the fermentation of PE, the SFPEs were spreaded on a GPYA media by diluting them 500 times. The SFPEs spread plates were incubated for 3 days at 28 ℃. Colonies obtained were then grown in GPYA, Yeast malt broth, Bennett's broth, Nutrient agar, Nutrinet agar supplied with autoclaved SFPE (25% v/v) to identify suitable media and growth condition. To determine suitable temperature for the growthyeast culture was spread on to nutrient agar medium plates supplied with 25 % autoclaved SFPEs and grown at 28 and 37℃. Temperature and media where the yeast strains were grown well was used for the further culture.

II-5-3. PCR and sequence analysis

Microorganisms present in SPFEs have been identified. The species present were identified with the help of rDNA isolated using Qiagen PCR purification kit and finally sequencing the purified product. A universal primer 5'- GCATATCAATAAGCGGAGGAAAAG-3', 5'-GGTCCGTGTTTCAAGACGG-3' have been used to detect the species existed in SFPEs.

II-6. Composition assessment and variation in PE and SFPEs

II-6-1. GC-MS analysis

Hewlett Packard HP-5973 MSD and HP-6890 GC for Gas chromatrography – Mass spectrophotometric device was used to detect compounds. Purge and trap method have been used to detect. Pretreatment of Sample was diluted 10 times with distilled water and analyzed with purge and trap methods. The 25 ml frit sparger, gas-tight syringes, purge and trap concentrator (Tekmar 3000). The conditions were Purge - 11 minutes, dry purge - 2 minutes, Desorb preheat - 175℃, desorb - 180℃, desorb time- 4 minutes Trap bake - 180℃, Trap bake time 7 minutes. HP-5 fusion silica capillary column having diameter of 0.25 mm, length 30 m and thickness 0.25 ㎛ were used. The carrier gas used was 1 ml helium in a minute with the speed of 36 cm/second.

II-6-2. The assay of some synthetic terpenoid compounds

II-6-2-1. Antibacterial activity

Antibacterial activities of synthetic terpenoids were tested against five bacterial strains. Camphor and 1,8-Cineole were purchashed from sigma-aldrich, and separately tested against bacterial strains including *Salmonella typhimurium* (KCTC1925), *Staphylococcus aureus* (ATCC9341), *Escherichia coli* (KTCC1923), *Pseudomonas aeruginosa* (ATCC15692), and *Bacillus subtilis* (ATCC6633). Bacteria were cultured overnight at 37℃ in nutrient media (bacto beef extract 3 g, bacto peptone 5 g). Antibacterial activities of the sample were

examined using paper disc method, 100 $\mu\ell$ of 16 hours grown bacterial suspension was spread on nutrient agar. Paper discs (6 mm diameter) were placed on the plate. 20 $\mu\ell$ sample was loaded on the center of the paper disk and incubated at 37℃ for 24 hours using DMSO as a negative control. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms. Concentrations of the 1,8-cineole used for the assay were 1, 10, 100, 500 and 1000 mM and for the camphor the concentration was 1000 mM.

II-6-2-1. Antioxidant activity

The radical-scavenging activities of Camphor and 1,8-Cineole were evaluated by using the DPPH radical scavenging assay. 150 uM DPPH (3 mg) was dissolved in 50 ml of MeOH. Samples (camphor and 1,8-cineol) were prepared in MeOH. 100 $\mu\ell$ of each sample was added to 150 $\mu\ell$ of DPPH solution and incubated for 30 minutes at room temperature. The absorbance was read against a blank at 517 nm. The results were expressed as percentage of reduction of the initial DPPH adsorption by test samples.

III. RESULTS AND DISCUSSION

III-1. Composition and pattern differences during self-fermentaion

III-1-1. General composition

Crude lipid, crude proteins, moisture, crude ash and carbohydrate present in the pine needle extract were determined. The results of these general components are projected in the table 1. Chlrophyll content, vitamin C and amino acids content of pine needle extracts and powdered pine needle were determined. Chlorophyll a and chlorophyll b in pine needle extract was recorded as 40.8 mg/100 g while the pine needle powder contained 178.5 mg/100 g. Similarly, vitamin C content of the PE was recorded as 349.0 mg/100 g while the pine powder contained 569.8 mg/100 g (Table 1). The pine needle contain higher proportion essential amino acids such as aspartic acid, proline, cystein and lysine. The concentrations of these amino acids were found to be higher than 0.82 g out of 100 g powder (Table 2).

III-1-2. Carbohydrate

Carbohydrates are major components of any plants that occur in different forms. We have tested three different simple carbohydrates in PE and SFPEs *viz.* glucose, fructose and sucrose. A total carbohydrate presented in PE and SFPEs test results correspond to the sum of these three different simple carbohydrates. The concentration in SFPE 7 was surprisingly increased

to 10,648.56 (±158.13) from 7,253.63 (±138.112) ppm in PE (Fig. 1). Carbohydrate shows increasing in aggregate with increase the period of spontaneous fermentation. Glucose and sucrose showed inconsistencies in their concentration change; while fructose showed gradual increase causing increase in total carbohydrate concentration. Microorganisms might be the chief players in such concentration change in open fermentation system. Glucose concentration decreases from PE to SFPE 3 (2,855 ± 56.72 to 395.2 ± 15.47 ppm) while increases in SFPE 7 (2,765± 55.3 ppm) might indicate the microbial led degradation of other complex biomolecules however; it would need the further research.

III-1-3. Fatty acids

Fatty acids components in PE and SFPEs were assessed. Saturated as well as unsaturated fatty acids were present in all aged samples distributing among 9 known fatty acids. In terms of percent, the total fatty acids present in the extracts, 9 known possess 50.8 ± 4.56, 45.1 ± 2.77 and 37.3 ± 3.60 % in PE, SFPE 3 and SFPE 7 respectively. Remaining unknown contributes to 40.9 ± 2.45, 51.2 ± 3.91 and 54.8 ± 4.78 in PE, SFPE 3 and SFPE 7. Nearly 13% of the known fatty acid amount was changed to unknown fatty acid leading to disapear individual fatty acid or changed in its concentration. Palmitoleic acid, lauric acid, and myristic acid were common in all aged samples. Myristic acid, a rare component of dairy product and a component of the membrane lipid, was increased in SFPEs (4.50 ± 0.35% in PE and 22.9 ± 2.54 % in SFPEs). Linoleic acid, a two double bond fatty acid, found in PE was completely disappeared while a new mono-unsaturated Oleic acid was appeared after 7 years of spontaneous fermentation. Two acids viz. lauric acid and myristic acids had shown opposite but remarkable pattern in their concentrations change. Higher concentration of lauric acid in fresh extract as 31.8 ± 3.37

was reduced to 6.07 ± 0.13 in three years and finally to 3.82 ± 0.15 at 7 years. While myristic acid from 4.50 ± 0.35 in fresh was increased to 25.9 ± 1.41 in 3 years and finally reduced to 22.9 ± 2.54 in 7 years (Table 3).

Table 1. General composition, chrolopyhll and vitamin C contents of self fermented pine needle extracts. P.powder: pine needle extract powder

		pine needle extract (%)	P.powder (%)
Crude Lipid		2.49±0.126	6.91±0.073
Crude Protein		3.35±0.015	11.92±0.812
Moisture		68.88±0.416	3.70±0.102
Crude Ash		1.01±0.041	1.01±0.041
Carbohydrate		4.20±0.375	14.22±1.208
		pine needle extract (mg/100 g)	**P.powder (mg/100 g)**
Chlorophyll	Chlorophyll a	27.3±0.126	108.0±0.126
	Chlorophyll b	13.5±0.015	70.5±0.812
Vitamin C	oxidative vitamin c	146.0±0.416	180.4±0.416
	reductive vitamin c	203.0±0.416	389.4±0.416

Table 2. Amino acids analysis in self fermented pine needle extracts. P.powder: pine needle extract powder; PE: pine needle extract

Amino acids	Molecular Weight	P.powder (g/100 g)	PE (g/100 g)
Aspartic acid	133.10	0.82	0.30
Threonine	119.12	0.39	0.14
Serine	105.09	0.34	0.13
Glutamic acid	147.13	0.55	0.21
Proline	115.13	0.83	0.33
Glycine	75.02	0.49	0.18
Alanine	89.09	0.58	0.22
Valine	117.15	0.67	0.25
Cystein	240.30	1.36	0.51
Methionine	149.21	not detected	not detected
Isoleucine	131.17	0.58	0.22
Leucine	131.17	0.58	0.22
Phenylalanine	165.19	0.12	0.06
Tyrosine	181.19	0.13	0.07
Histidine	155.15	0.50	0.31
Lysine	146.19	0.84	0.12
Ammonium chloride	53.49	0.10	0.06
Arginine	174.20	0.49	not detected

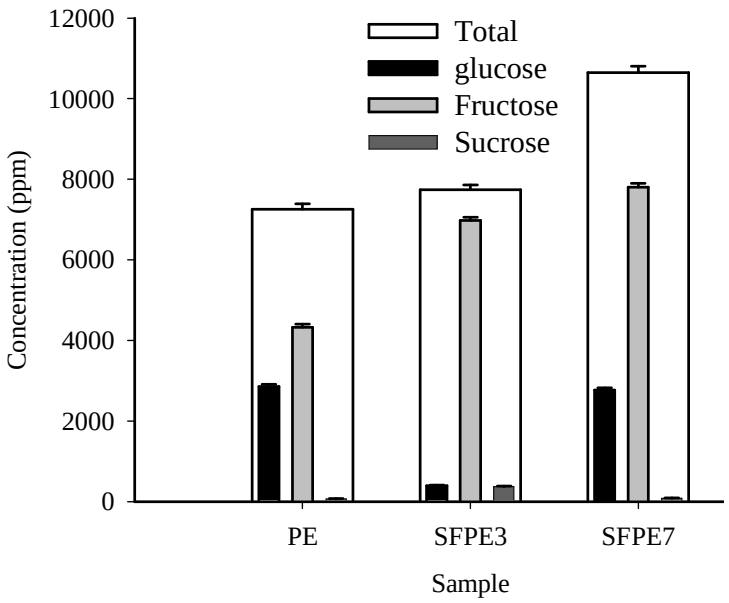

Figure 1. Trend of carbohydrate dynamics in pine needle extract and self fermented pine needle extracts. Interchange in carbohydrates occurs and finally increases the total amount of carbohydrate due to internal reactions during spontaneous fermentation. Glucose concentration decreased during 3 years old SFPE, which retained to its original level, but the fructose shows continuous increasing trend during spontaneous fermentation. PE: Fresh Pine Needle extracts; SFPE 3: Self-fermented pine extract 3 years old; SFPE 7: Self-fermented pine extract 7 years old.

Table 3. Lipids analysis in pine needle extract and self fermented pine needle extracts

S. No	Name of Lipid	No of carbon /Saturation	PE	SFPE 3	SFPE 7
1	Caproic acid	6:0	4.12 ± 0.02	1.47 ± 0.02	1.15 ± 0.05
2	Caprylic acid	8:0	2.05 ± 0.13	2.14 ± 0.02	0.70 ± 0.10
3	Capric acid	10:0	0.97 ± 0.03	1.37 ± 0.15	0.90 ± 0.01
4	Lauric acid	12:0	3.18 ± 3.37	6.07 ± 0.13	3.82 ± 0.15
5	Myristic acid	14:0	4.50 ± 0.35	25.9 ± 1.41	22.9 ± 2.54
6	Stearic acid	18:0	0	1.35 ± 0.52	0
7	Palmitoleic acid	16:1	6 ± 0. 2	6.81 ± 0.11	6.75 ± 0.15
8	Oleic acid	18:1 (ω-9)	0	0	1.10 ± 0.05
9	Linoleic acid	18:2 (ω-6)	1.38 ± 0.19	0	0
10	Unknown		40.9 ± 2.45	51.2 ± 3.91	54.8 ± 4.78
	Total Fatty acids		50.8 ± 4.56	45.1 ± 2.77	37.3 ± 3.60

III-2. The activities of self fermented pine needle extracts (SFPEs)

III-2-1. Antibacterial activity of SFPEs

The PE and SFPEs were tested for whether they have antibacterial properties. It was found that PE and SFPEs could retard the growth of tested bacterial strains. The antibacterial assay with *Bacillus subtilis, Salmonella typhimurium, Staphylococcus aureus, Micrococcus luteus, Escherichia coli* and *Pseudomonas aeruginosa* using paper disc method revealed the strains are highly susceptible with SFPEs and effect was dose dependent. Similarly, *Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa* were also susceptible to SFPEs. Effect of SFPE 7 (80 ㎕) on *Bacillus subtilis was* highest (21 mm) and 40 ㎕ also inhibited its growth in some extent (Fig. 2). Treatment of 40 ㎕ SFPE 7 only showd growth retardation on *Salmonella typhimurium* however, 80 ㎕ SFPE3 and 7 both inhibited its growth while inhibition effect of SFPE7 was higher than others. Similar trend have been seen in *Staphylococcus aureus, Micrococcus luteus* and *Escherichia coli.* In these strains PE and SFPE 3 showed no growth inhibitory effect by 40 ㎕, while the growth was inhibited by 40 ㎕ SFPE 7. *Bacillus subtilis* showed high level of susceptibility with SFPE 3 as well SFPE 7. Pine extract is effective in growth inhibition of some bacterial strains (Kim and Shin, 2005), thus reflects the PE and SFPEs might be safe from bacterial contamination. *S. typhimurium, S. aureus* that cause serious health hazards in human, also lost its growth in SFPE treatment.

III-2-2. Antioxidant activity of SFPEs

NBT superoxide scavenging activities of SFPEs was also assessed (Fig. 3). PE, SFPE 3 and SFPE 7 scavenged 90, 89 and 93 % radicals from 0.3 ㎕/ml concentrations respectively. Superoxide scavenging activities of SFPE was neary similar to all samples of different ages. Therefore, PE and SFPEs might be the good source of natural antioxidant in a functional food.

III-2-3. Fibrinolytic activity of SFPEs

Fibrinolytic activity of the PE, SFPEs was assayed using fibrin plates. 20 ㎕ of each sample was carefully placed on fibrin plate. The plate was incubated for 30 min, 1 hour and 2 hours at 37℃ and the diameter of the lytic circle was measured. After 30 min incubation, lytic circle formed by PE, SFPE 3, SFPE 7 on fibrin plate were 11, 12 and 14 mm respectively. At after 1 hour, the circles were 13, 14 and 18 mm respectively. In 2 hours of incubation, lytic circle of PE, SFPE 3, SFPE 7 were 17, 18 and 23 mm respectively (Fig. 4). It seems that fermentation facillatetes fibrinolytic activity in pine extract. And SFPEs can be developed and used as a funcrional food (health related food) for thrombosis prevention.

III-2-4. The pattern of cholesterol and triglyceride contents

The levels of cholesterol in C-, C+ 4 weeks, C+ 2 weeks (Sample C+) 2 weeks, C+ SFPE 7 4 weeks after treatments are reported in Figure 5. The average cholesterol level in C+ 4 weeks (fed cholesterol continuously at once a day for 4 weeks) group was 50.5 ± 0.7 mg/dL. C+ 2 weeks (Sample C+) 2 weeks (0.5 ml cholesterol for 2 weeks and then with 0.5 ml SFPEs (PE,

SFPE 3, SFPE 7) with 0.5 ml cholesterol) group, the mean cholesterol level in this group was PE ; 34.5 ± 2.12 mg/dL, SFPE 3 ; 35 ± 0 mg/dL, SFPE 7 ; 23 ± 2.8 mg/dL. The level of cholesterol in C+ SFPE 7 4 weeks (fed with 0.5 ml cholesterol and 0.5 ml SFPE 7 continuously for four weeks) group was 29 ± 1.4 mg/dL. The plasma cholesterol was decreased by 0.5 ml cholesterol for 2 weeks and then with 0.5 ml PE ; 23.3 %, SFPE 3 ; 22.2 %, SFPE 7 ; 48.9 %, fed with 0.5 ml cholesterol and 0.5 ml SFPE 7 continuously for four weeks ; 35.6 %, respectively. And the average triglyceride level in C-, C+ 4 weeks, C+ 2 weeks (Sample C+) 2 weeks, C+ SFPE 7 4 weeks after treatments are reported in Figure 6. The average triglyceride level in C+ 4 weeks (fed cholesterol continuously at once a day for 4 weeks) group was 24 ± 4.3 mg/dL. C+ 2 weeks (Sample C+) 2 weeks (0.5 ml cholesterol for 2 weeks and then with 0.5 ml SFPEs (PE, SFPE 3, SFPE 7) with 0.5 ml cholesterol) group, the mean triglyceride level in this group was PE; 13.5 ± 4.9 mg/dL, SFPE 3; 15 ± 0 mg/dL, SFPE 7; 14.5 ± 7.8 mg/dL. The level of cholesterol in C+ SFPE 7 4 weeks (fed with 0.5 ml cholesterol and 0.5 ml SFPE 7 continuously for four weeks) group was 6 ± 5.7 mg/dL. The triglyceride level was decreased by 56 % to rats fed with 0.5 ml cholesterol and 0.5 ml SFPE 7 continuously for four weeks. We discovered that SFPEs decreased cholesterol and triglyceride levels in cholesterol-fed rats. Therefore, SFPE might be useful in improving blood circulation and could be a good source of functional food development.

III-2-5. Electrophysiological activity of SFPEs

ICCs cultured from the murine small intestine art cKit positive cells that have distinct morphology containing spindle shaped structures and form a network within smooth muscles. Recording from cultured ICC under current clamp mode (*I=0*) showed spontaneous pacemaker

potentials. The resting membrane potential was – 53 ± 3 mV and amplitude 23 ± 5 mV. In conversion of the ampliter to voltage clamp mode at a holding potential – 70 mV, ICC generated spontaneous inward currents called ‘pacemaker currents’. The average frequency of the currents was 14 ± 2 cycles/min and the amplitude averaged – 436 ± 62 pA. SFPE 7 has been tested for the analysis of the effects on alteration of pacemaker activities in the ICC. In whole cell patch clamp technique at 30℃, ICC generated spontaneous pacemaker potential under current clamp mode (*I=0*) and inward currents (pacemaker currents) under voltage clamp mode at a holding potential of -70 mV. When SFPE 7 (200 ㎍/ml) treated in ICC, under currents clamp mode decreased both the frequency and amplitude of pacemaker currents, and increased the resting currents in outward direction. Also, SFPE 7 inhibited the pacemaker currents in a dose-dependent manner. Glibenclamide, a blocker of potassium channel, reversed the effect developed by SFPE 7 indicating the SFPE 7 cause the opening of the potassium channels during modulation of pacemaker current (Fig. 7).

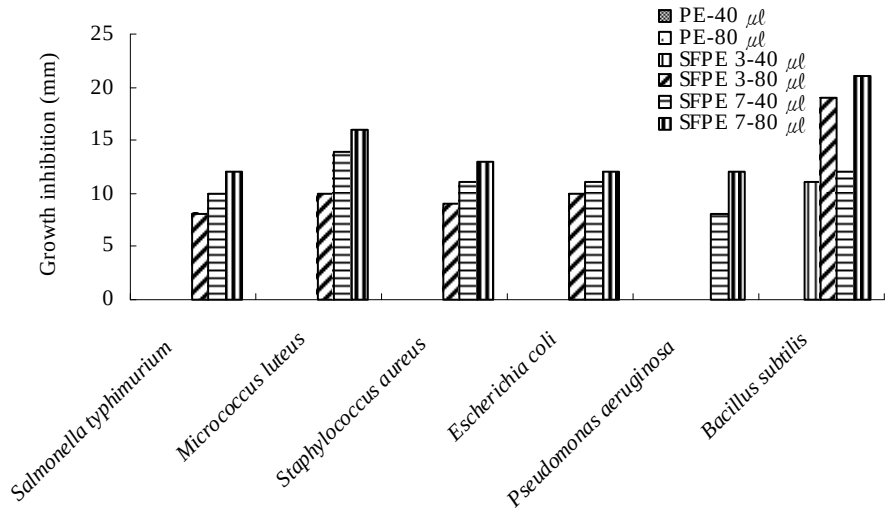

Figure 2. Antimicrobial property of pine needle extract and self fermented pine needle extracts. Figure shows the effect of PE and SFPE to different bacterial strains on paper disc. PE and SFPE suppressed the bacterial growth in dose dependent, self-fermentation aging dependent and strain specific. Effect was increased with increase in self-fermentation period. PE: Fresh Pine extract; SFPE 3: Self-fermented pine extract 3 years old; SFPE 7: Self-fermented pine extract 7 years old.

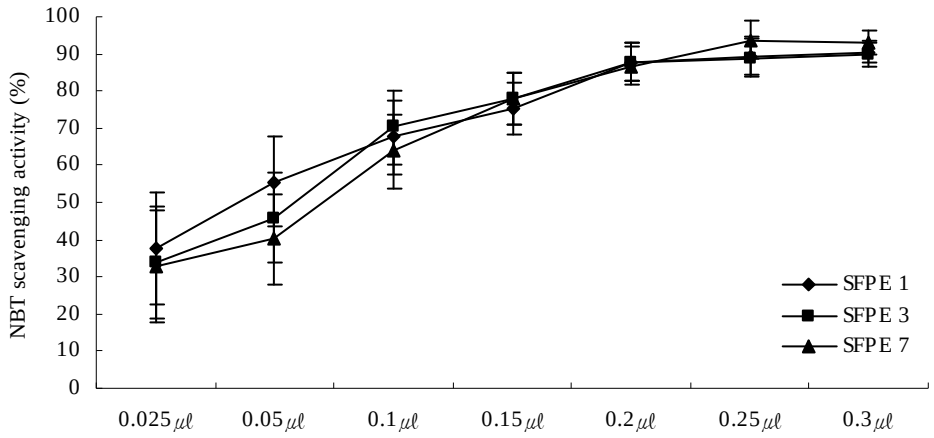

Figure 3. NBT superoxide scavenging activities of self fermented pine needle extracts 1, 3 and 7. NBT superoxide scavenging activities of SFPE 1, 3 and 7 (0.2 ㎕/ml-0.3 ㎕/ml) showed 90% superoxide scavenging activities which is similar to all samples of different ages.

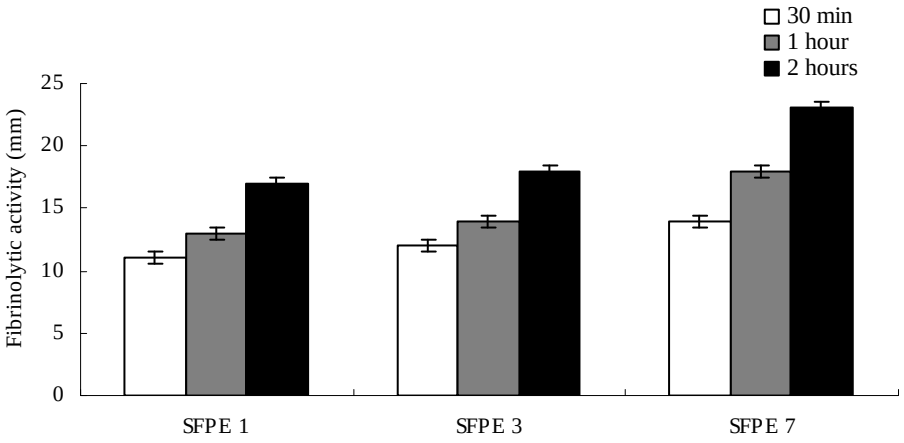

Figure 4. Fibrinolytic activity of self fermented pine needle extracts. Fibrinolytic activies of SFPEs increases with aging and it is the effect is time dependent.

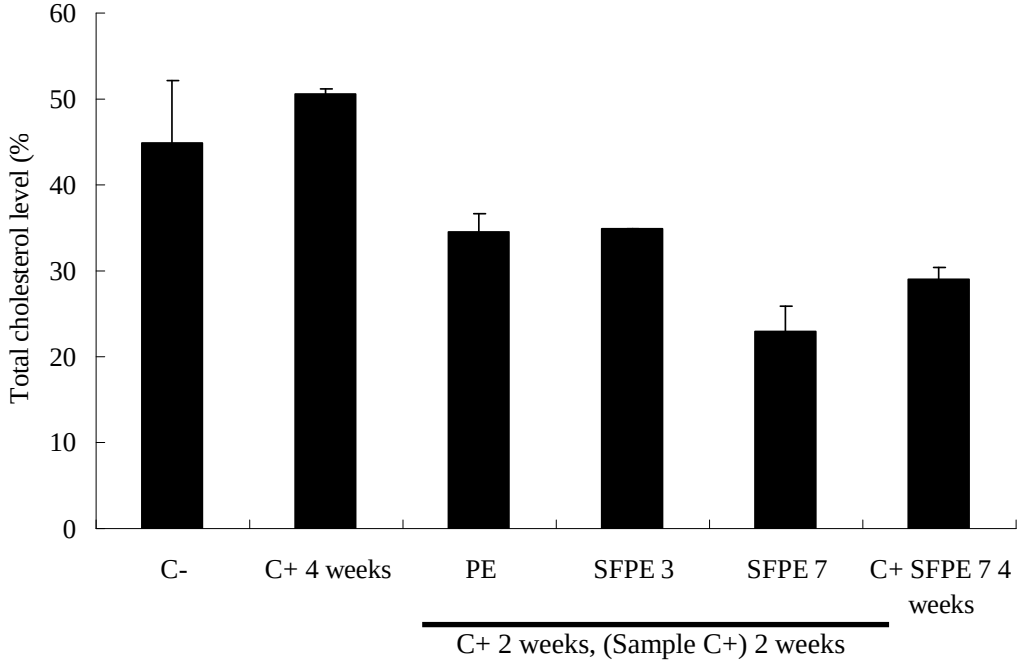

Figure 5. Assessment of total cholesterol in blood plasma of cholesterol fed rats. The level of cholesterol was found lowered after administration of PE and SFPEs. C+: Cholesterol administerd.

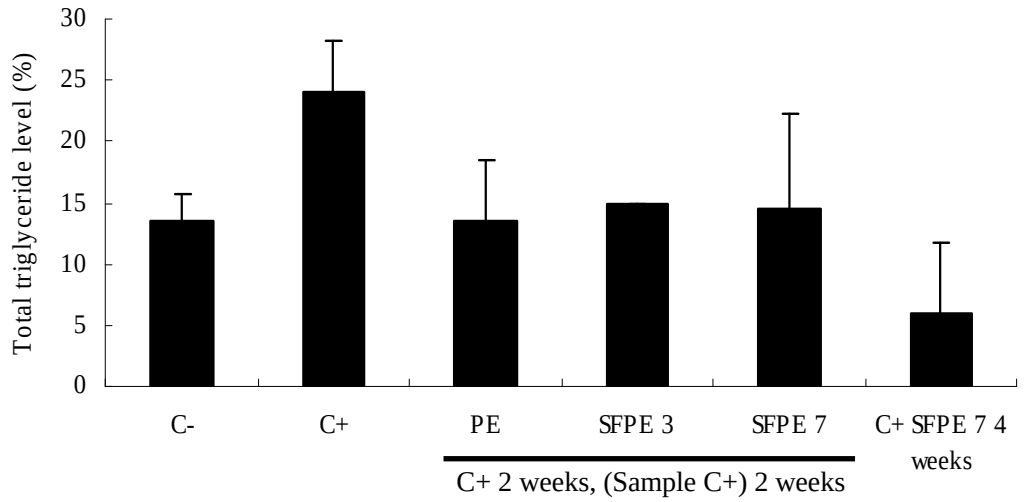

Figure 6. Level of triglyceride blood plasma of cholesterol fed rats. Blood plasma triglyceride decreases with administration of 0.5 ml PE and SFPEs. C+: Cholesterol administered.

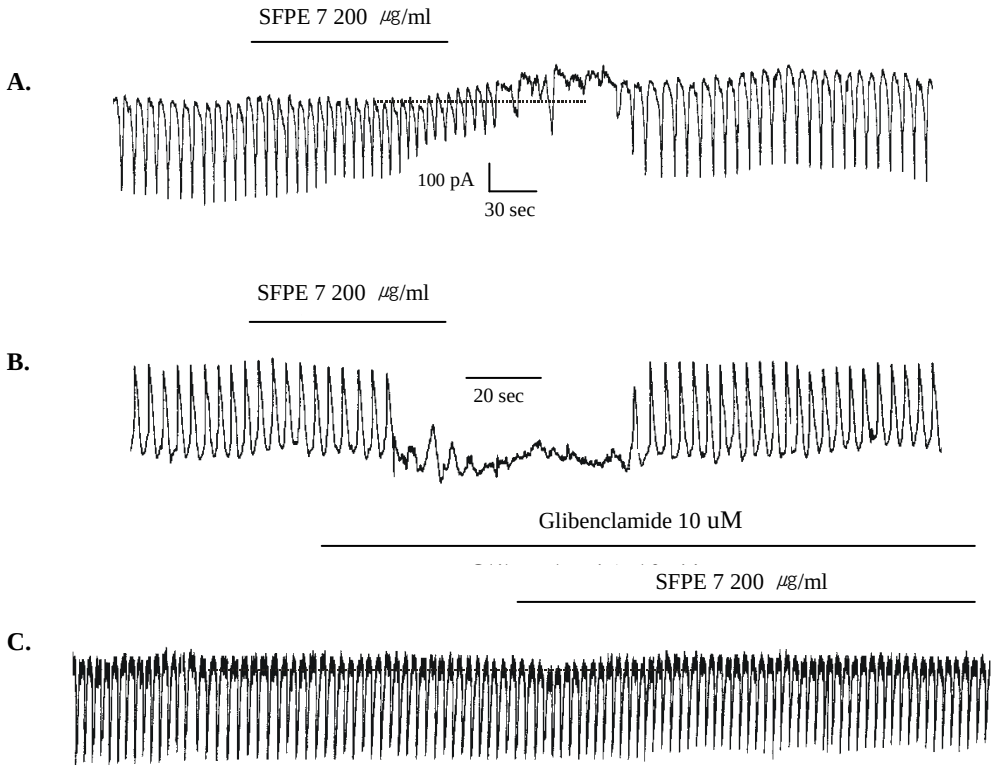

Figure 7. Effects of self fermented pine needle extracts 7 on pacemaker potentials and pacemaker currents recorded in cultured ICC from murine small intestine. (A) Shows the pacemaker currents of ICC recorded at a holding potential of –70 mV exposed to SFPE 7 (200 μg/ml). (B) Shows the pacemaker potentials of ICC exposed SFPE 7 (200 μg/ml) in the current clamping mode (*I*=0). The pine needle extracts induced membrane hyperpolarization. (C) Shows the effect of SFPE 7 on pacemaker currents after pre-treating cells with glibenclamide. The frequency and amplitude of pacemaker currents were decreased and the resting currents increased in the outward direction by SFPE 7. Dotted lines indicate zero current levels. (SFPE 7: Self-fermented pine extracts 7 years old).

III-3. Identification of strains involved in SFPEs

To identify and characterize yeast strains from SFPEs, we performed yeast strains screening. Several strains of yeasts were appeared in the stored extract without thier inoculation. There were altogether 8 different yeast strains isolated and identified (Fig. 8). Most of the yeast strains were appeared during first year of storage (SFPE 1). The strains number was reduced to 2 out of 8 at third year and all strains were disappeared at forth year of storage. The isolated strains were identified as *Pichia galeiformis*, *Candida boidinii*, *Pichia sp.*, *Candida sp.* 1, *Candida sp.* 2, *Candida ooidensis*, *Saccharomyces cerevisiae*, and *Candida Karawaiewii*. *Candida ooidensis* was appeared at the second year of storage. All species were seen in SFPE 2. Only *Candida sp.* 1, *Candida sp.* 2 were found in SFPE3 while all the species were disappeared after 4 years (Table 4A). We tried to determined the suitable media and growth condition for the isolated yeast strains. The result showed that GPYA was the best among the media tested for the growth (Table 4B). It also revealed that supply of 25% autoclaved SFPE facillated the growth of yeast strains at 28℃ however no colonies could be seen in nutrient agar without SFPE supplement. At 37℃, non of the strains could grown in both types of media (Table 4C). Therefore, the result reveals that the nutrient agar media supplement of 25% autoclaved SFPE provides the good substrate to facillate the growth of cultured yeast strains. Among species present, *Candida sp.* 2 was having 99.65% similarities with *Candida ernobii.* (Table 5, Fig. 9, Fig. 10).

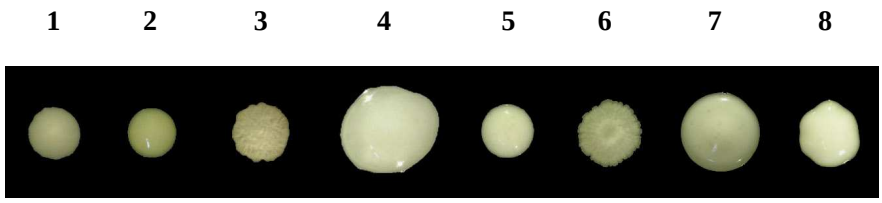

Figure 8. Structure of colonies of 8 different yeast strains isolated from self fermented pine needle extracts. They were identified as (from left to right). 1. *Pichia galeiformis*, 2. *Candida boidinii*, 3. *Pichia sp*., 4. *Candida sp.* 1, 5. *Candida sp.* 2, 6. *Candida ooidensis*, 7. *Saccharomyces cerevisiae*, 8. *Candida karawaiewii.*

Table 4. Growth condition for isolated yeast strains from self fermented pine needle extracts. A. Cell lines come out according to self-fermentation procedure of pine needle extract from 1 year to 4 years. B. Condition and isolated yeast strains from SFPE grown in different media. GPYA showed best growth than other media. No colony could be obtained from nutrient agar media, however, colony could be seen in nutrient agar media supplied with 25% SFPE. C. Screening medium and culture condition of microorganism for self-fermentation from pine needle extract.

A.

S. NO.	Species	SFPE 1	SFPE 2	SFPE 3	SFPE 4
1.	*Pichia galeiformis*	18	0	0	0
2.	*Candida boidinii*	5	3	0	0
3.	*Pichia sp.*	3	0	0	0
4.	*Candida sp.* 1	18	0	6	0
5.	*Candida sp.* 2	27	14	43	0
6.	*Candida ooidensis*	0	79	0	0
7.	*Saccharomyces cerevisiae*	13	16	0	0
8.	*Candida karawaiewii*	28	0	0	0

B.

Medium cell	GPYA	YM	BA	Nutrient agar	Nutrient agar + SFPE 25%
*Candida sp.*1,2	+++	+++	+++	-	+++
Candida ernobii (Control)	+++	++	++	-	++

C.

Source	Medium	Culture condition – 2 days	
		37℃	28℃
SFPE	Nutrient agar + Autoclaved SFPE (25%)	-	+
	Nutrient agar	-	-

Table 5. Nucleotide similarity analysis among the yeast strains isolated from self fermented pine needle extracts

Strain	Accession NO.	Similarity	nt differences /compared
Candida ernobii NRRL Y-17782T	U94921	99.65	2/570
Candida karawaiewii NRRL Y-17784T	U70241	99.65	2/570
Pichia holstii NRRL Y-2155T	U75722	99.30	4/568
Candida ishiwadae NRRL Y-17654T	U71067	98.07	11/569
Candida wickerhamii NRRL Y-2563T	U70243	97.89	12/568
Candida wyomingensis NRRL YB-2152	AF153673	97.36	15/568
Candida pomicola CBS4242	AF245400	97.01	17/568
Candida populi NRRL Y-17681T	U70249	96.82	18/566
Candida anatomiae NRRL Y-17641T	U70244	96.47	20/566
Candida peltata NRRL Y-6888T	U71066	95.78	24/569
Pichia xylosa NRRL Y-12939T	U75718	95.22	27/565
Picha toletana NRRL YB-4247T	U75720	95.04	28/565
Pachysolen tannophilus NRRL Y-2460T	U76346	93.63	36/567

5'-AAACCAACAGGGATTACCTTCGTAACGGCGAGTGAAGCGGTAAAAG

CTCAAATTTGAAATCTAGCACTTTCAGTGTTCGAGTTGTAATTTGAAGA

AGGTATCTTTGGAGTTGGCCCCCGTCTATGTTCCTTGGAACAGGACGTC

ACAGAGGGTGAGAATCCCGTGCGATGGGGAGACCAATTCTATGTAAAG

TTCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGG

TAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAG

TACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAG

TACGTGAAATTGTTGAAAGGGAAGGGCTTTTGATCAGACTTGGTGTTTA

GTACTCTCTACTCCTTGTGGGTGGAGCTCTTGCTTTATACTGGGCCAGCA

TCGGTTTGGATGGTAGGATAATAGCATTGGAATGTGGCTTCATTCGTGAA

GTGTTATAGCCCTTGTTGATACTACCTATCTAGACCGAGGACTGCGTCTTT

TGACTAGGATGCTGGCGTAATGATTAAAAGCCGC-3'

Figure 9. 26S rDNA D1/D2 region sequenced from *Candida sp.* 2

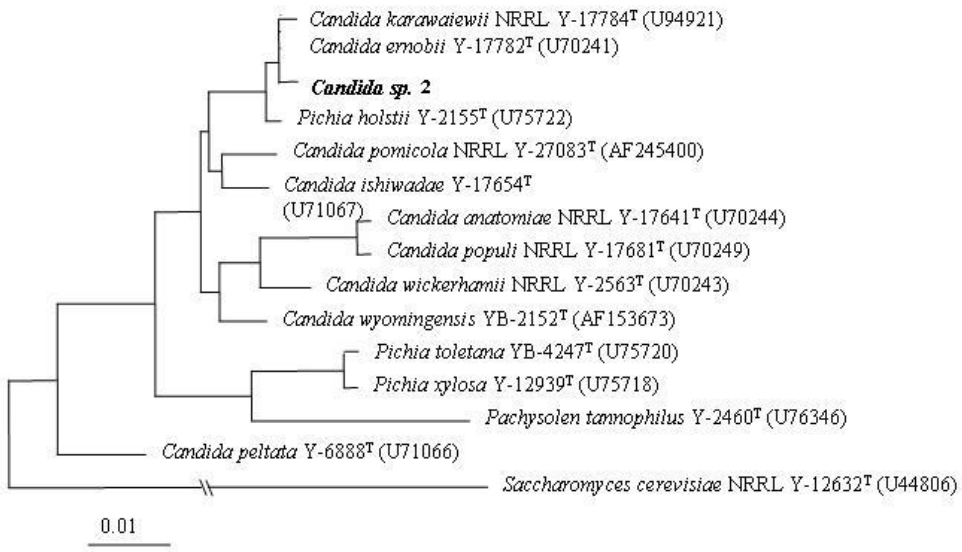

Figure 10. Phylogenetic tree based on partial 26S rDNA sequences. It shows the positions of strains 2 and some other related taxa, Gen Bank accession number. Scale bar represents 0.01 substitutions per nucleotide position.

III-4. Terpenoids composition of SFPEs

Gas chromatography-mass spectrometry (GC-MS) was used to identify and quantify the volatile terpenoids in fresh and self-fermented pine needle extract (Byun *et al.*, 2007 ; Canini *et al.*, 2007). α – pinene, β – phellandrene, camphene, eucalyptol and camphor are some of the volatile terpenoids detected in the extracts. Camphor was not detected from the fresh extract, however it was found gradually increased with increase self-fermented duration. Similarly, no 1,8- Cineole detected in fresh was appeared and gradually increased from 1.816% to 2.003% in aging. Camphor and 1,8-cineole are volatile organic compounds that share a common carbon skeletal structure (Fig. 11A, B and C) and are biologically active. 5-isopyrenyl-2-2-methyl-2-vinyltetrahydrofuran (4.021 to 7.555%), herboxide second isomer (9.139 to 10.906%), isocineole (15.947 to 19.915%) are the characteristic changes occurred in the PE to SFPEs. The result showed that the terpenoids are changed rapidly during spontaneous fermentation process and also it makes the extract more effective and useful (Table 6). Volatile compounds of self-fermented pine needle extracts detected using GC-MS. Especially, peak a (11.03 min) corresponding to 1,8-cineole, peak b (13.02 min) corresponding to camphor. The time and mass spectra were compared with that of the standard compounds (Fig. 12). 1,8-cineole is natural organic compound pronounced variously as eucalyptol, eucalyptole, limonene oxide, cajeputol, 1,8-epoxy-p-menthane, 1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane, 1,8-oxido-p-menthane, cineol or cineole is basically a terpene and an important essential oil (http://en.wikipedia.org/wiki/Eucalyptol). It is a multipurpose compound widely used in pharmaceutical products such as nasal decongestant, anticough agent, aromatheraphy, dentistry and in cosmetics (Madyastha and Chadha, 1986; Soares *et al.*, 2005). Camphor is also terpenoid, white or lucid solid with a strong, aromatic scent. Camphor uses include as a moth

repellent, as an antimicrobial substance, in embalming, in medicine (http://en.wikipedia.org/wiki/Camphor.). Various experiment tries showing the possible trend of some compounds dynamics in fermented extracts. The aroma of wines is secondary products formed during the wine fermentation (fermentative aromas) and ageing (JH and Pretorius, 2005). The aroma complexity increases during alcoholic fermentation by the synthesis of important volatile compounds (JH *et al.*, 2005). Self-fermented pine needle extract tries to assess the functional perspective of the fermented product. Terpenoids of different sizes and composition are found in all classes of living things, and are the largest group of natural products, and are biologically active. Terpenoids and other nutrient compounds of plants have been recognizes as potential factors that can be useful to improve human health through their antioxidant activities (Chang *et al.*, 2008; Lee *et al.*, 2001; Pietri *et al.*, 1997).

III-5. Effect 1,8-cineole and Camphor

III-5-1. Antibacterial acitivity

1,8-cinoele and Camphor were tested for whether they have antibacterial activities. The experiment showed that 1,8-cinoele, Camphor can retard growth of bacteria. *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* showed that the strains are highly sensitive to 1,8-cineole and camphor. Treatment of 100 mM on 1,8-cineole *Escherichia coli* and *Bacillus subtilis* showed 6 mm and 7 mm growth inhibition. Treatment of 500 mM 1,8-cineole showed: *Salmonella typhimurium* (20 mm), *Staphylococcus aureus* (12 mm), *Escherichia coli* (20 mm), *Pseudomonas aeruginosa* (15

mm) and *Bacillus subtilis* (20 mm) inhibitions. Treatment of 1000 mM 1,8-cineole showed high level of inhibition activities to all bacterial strains. *Salmonella typhimurium* (25 mm), *Staphylococcus aureus* (16 mm), *Escherichia coli* (25 mm), *Pseudomonas aeruginosa* (20 mm), *Bacillus subtilis* (27 mm) have been inhibited by 1,8-cineole (Fig. 13). However, treatment of 1000 mM of camphor on *Salmonella typhimurium*, *Bacillus subtilis and Pseudomonas aeruginosa* showed equal (8 mm) inhibition to all the tested strains (Fig. 14). Tested bacteria were highly susceptible to 1,8-cineole and camphor. The food and cosmetic industry at present is facing an enormous problem from user for using chemical preservatives to prevent the growth of microorganisms (Deba *et al.*, 2008; Kim *et al.*, 2007). Antibacterial properties of the terpenoids (essential oils) and a variety of extracts from many plants have recently been of great interest in both study, the food and cosmetic industry, because their potential use as organic additives emerged from a increasing trend to replace synthetic preservatives with natural ones. A new approach to prevent the proliferation of microorganism might safeguard against biological invasion in various products, however needs detail assessments. These studies reveal that not only some compounds but combinations of the compounds involve in increasing antibacterial properties of the self-fermented pine needle extracts.

III-5-2. Antioxidant acitivity

DPPH radical scavenging activity of 1,8-cineole was also assessed (Fig. 15). 1,8-cineole scavenged 46, 82 and 84 % radicals from 10, 100, and 500 mM concentrations respectively and it is dose dependant. Damage by oxygen free radicals is known to be one of the mechanisms of chronic disorders and ageing. Oxygen free radicals are originated ubiquitously in aerobic metabolism, and are generally removed by antioxidant such as a superoxide dismutase,

glutathione peroxidase, metal-binding proteins, vitamin C, vitamin E, beta carotene, uric acid, bilirubin, albumin, DNA repair enzyme, methionine sulphoxide reductase repair (Seo *et al*, 2004; Sung *et al*, 2000). Various reports try showing the antioxidant effect in food. Red wine and green tea contains high level of antioxidant (S *et al*, 1994). Self-fermented pine needle extract contains 1,8-cineole and is good source for nutraceutical product.

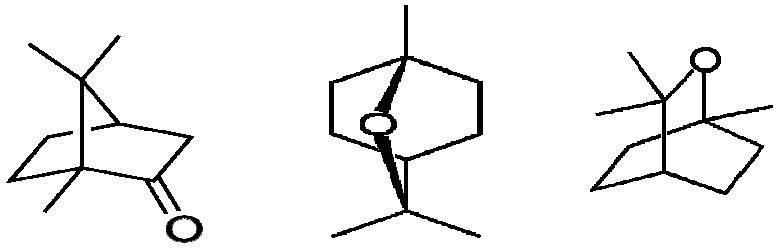

Figure 11. Structure of camphor and 1,8-cineole. Molecular structure of secondary metabolites analyzed. A. Structure of Camphor; B. Structure of 1,8-cineole; C. Structure of 1,8-cineole

Table 6. Volatile terpenoid compounds and essential oil contents in pine needle extract and self fermented pine needle extracts. Interchange in compounds occurs and finally increases the total amount of terpenoids (especially 1,8-cineole and camphor) due internal reactions during spontaneous fermentation. 1,8-cineole and camphor concentration increased during 3 years old self-fermented pine needle extract. Total concentration shows continuous increasing trend during spontaneous fermentation. PE: Fresh pine needle extracts; SFPE 3: Self-fermented pine extract 3 years old; SFPE 7: Self-fermented pine extract 7 years old.

Compounds	PE	SFPE 3	SFPE 7
Alpha-pinene	9.303		
Beta myrcene	0.536		
Beta phellandrene	1.370		
Camphene	2.230		
Delta 3-carene	0.630		
Eucalvptol	0.334		
Styrene	66.321		
3-Pentanone	11.707	7.439	
Tolune	7.586	7.175	7.843
1,8-Cineole/Eucalyptol		1.816	2.003
2-methyl-1-butanol		2.521	3.613
3-methyl-1-butanol		5.050	4.815

5-isopyrenyl-2-2-methyl-2-vinyltetrahydrofuran		4.021	7.555
Bicyclo[2,2,1]heptan-2-ol		38.645	17.962
Camphor		6.888	24.195
Herboxide second isomer		9.139	10.906
Hexanoic acid, ethyl ester		1.359	1.193
isocineole		15.947	19.915
Total terpenoic compounds (100%)	100	100	100

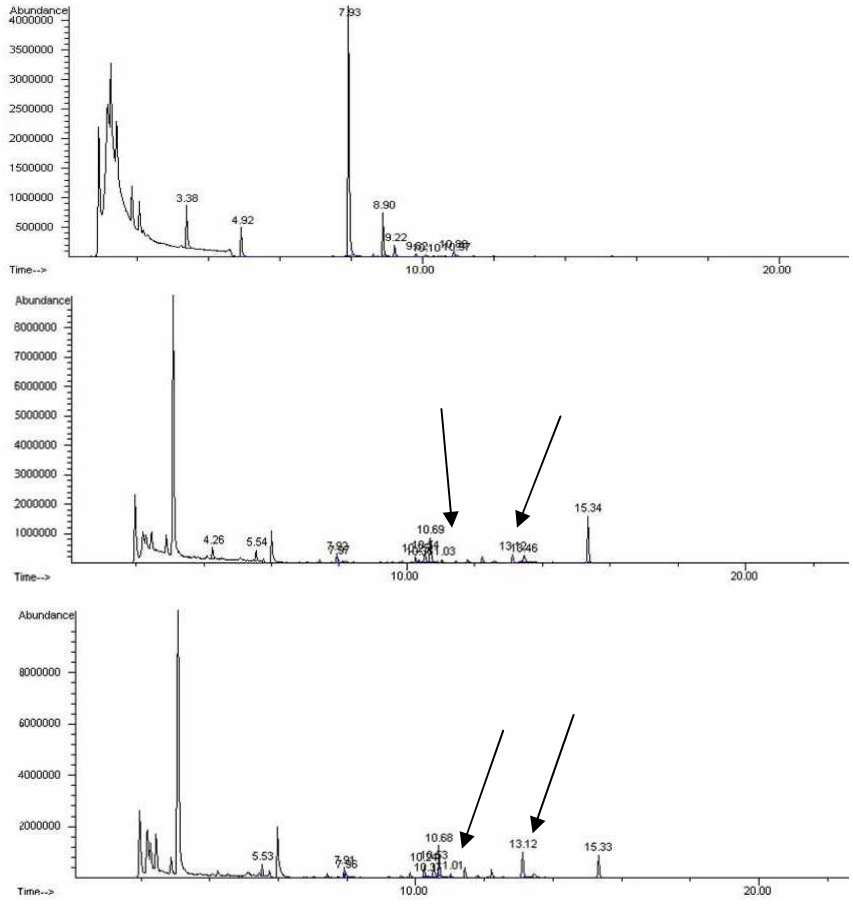

Figure. 12. Gas chromatography (GC-MS) analysis of self fermented pine needle extracts. Volatile compounds of self-fermented pine needle extract detected using GC-MS. Peak a (11.03 min) corresponding to 1,8-cineole, peak b (13.02 min) corresponding to camphor. The time and mass spectra were compared with that of the standard compounds. A. Fresh pine needle extract; B. Self-fermented pine needle extract 3 years old; C. Self-fermented pine needle extract 7 years old.

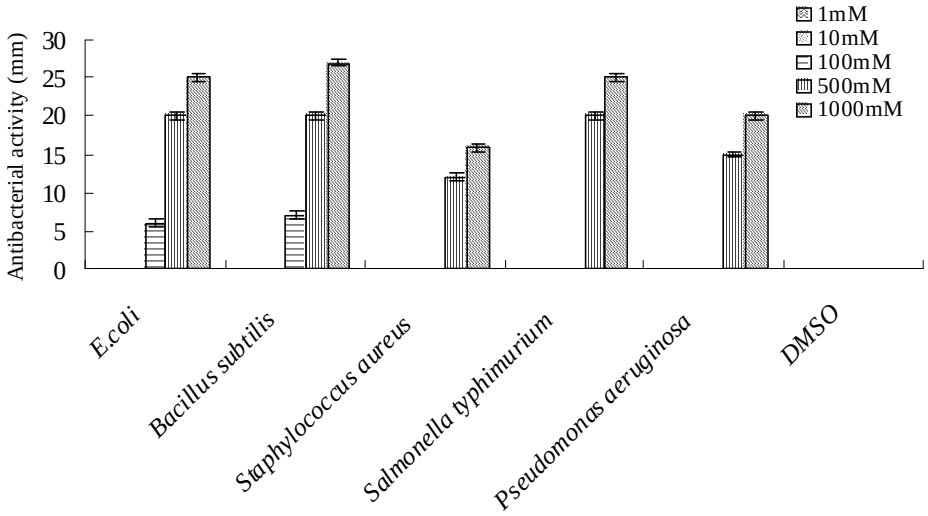

Figure 13. Antibacterial activity of 1,8-cineole. Antimicrobial property of 1,8-cineole was analyzed. Figure shows the effect of 1,8-cineole to different bacterial strains on paper disc. 1,8-cineole suppressed the bacterial growth in dose dependent manner. Effect was increased with increase according to concentration. DMSO: Negative control.

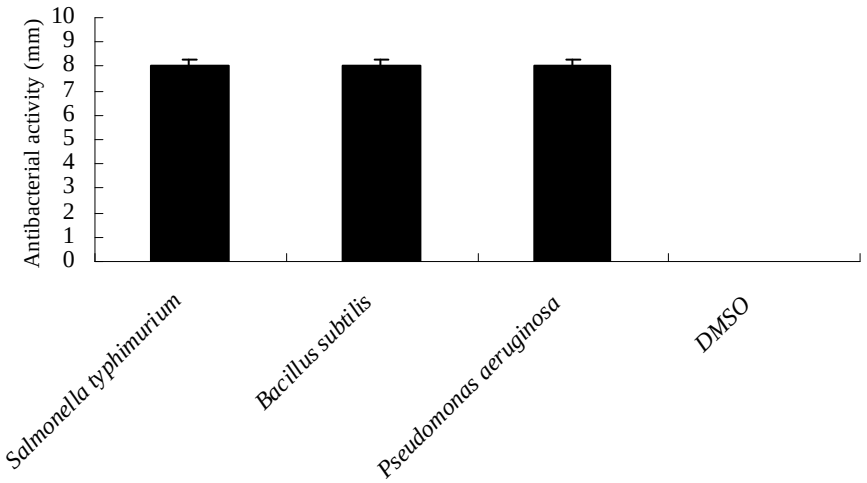

Figure 14. Antibacterial activity of camphor. Antimicrobial property of camphor was analyzed. Figure's shows the effect of camphor to different bacterial strains on paper disc. Camphor suppressed the bacterial growth. DMSO: Negative control.

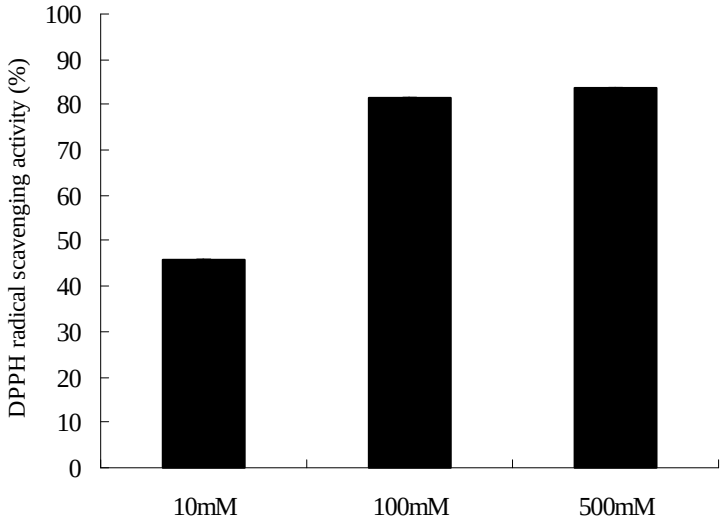

Figure 15. DPPH radical scavenging activity of 1,8-cineole. Free radical scavenging activities of the 1,8-cineole. DMSO: Negative control.

IV. Conclusion

Assessment of pine needle is the essence for traditional medicine using pharmacological efficacy of natural compounds present in pine needle for treating human diseases. In this study we have identified various useful components in extract including crude lipid, crude proteins, moisture, crude ash, carbohydrate, vitamin C, chlorophyll, amino acids, terpenoids and fatty acids. The self-fermented pine extract (SFPE) inhibited the growth of some bacterial strains like *Salmonella typhimurium*, *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. The SFPE showed 90% antioxidant activities, which is similar for all tested samples of different ages. Fibrinolytic activities of the extract indicated that activity depends on time and also with aging of the product. It was also found that the extract can lower the blood plasma cholesterol and triglyceride in cholesterol fed rat. When SFPE 7 (200 ㎍/ml) treated in ICC, under currents clamp mode decreased both the frequency and amplitude of pacemaker currents, and increased the resting currents in outward direction. Also, SFPE 7 inhibited the pacemaker currents in a dose-dependent manner. Glibenclamide, a blocker of potassium channel, reversed the effect developed by SFPE 7 indicating the SFPE 7 cause the opening of the potassium channels during modulation of pacemaker current. We have isolated and identified 8 different yeast strains that spontaneously grown and caused self-fermentation in the extract. Most abundantly grown yeast strain *Candida sp.* 2 possesses 99% sequence homologies with *Candida ernobii* was disappeared within 4 years of storage. SFPEs showed antioxidant, anti-bacterial properties, may improve the blood circulation and can act against antherosclerosis, therefore the fermented pine needle extract could be a good source for functional food development. GC-MS result reveals that

self-fermentation leads terpenoid composition dynamics in the stored pine needle extract. The study unveiled many aspects in the traditional methods of improving functional natural product processing that needs follow up studies in assessing them detail.

V. 적요

솔잎착즙액 (PE)은 엽록소 함유량 (40.8 mg/100 g)이 높고 비타민 C 도 다량함유 (149 mg/100 g)하고 있어 기능성 식품으로 개발가능성이 높으며, 솔잎 발효액은 향미가 좋아지고 기능성이 높아져서 고부가가치가 크므로 발효의 조건과 활성도를 조사 분석하여 고기능성 솔잎발효액 (SFPE)을 개발하고자 하였다. *Salmonella typhimurium, Micrococcus luteus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* 와 *Bacillus subtilis* 에 대해서 SFPE 를 농도별로 처리하였을 때 높은 항균활성을 확인하였다. SFPE 1, 3, 7 의 항산화 활성은 0.025 ㎕/ml 의 농도로 처리하였을 경우 SFPE 1, 3, 7 이 약 34.8%의 NBT scavenging 의 활성을 보여주었고, 0.2 ㎕/ml−0.3 ㎕/ml 의 농도에서는 SFPE 1, 3, 7 이 약 90%에 달하는 NBT scavenging 의 활성능력을 확인할 수 있었다. SFPEs 의 혈전분해의 경우 발효기간에 따라 분해 활성 또한 증가함을 확인하였다. 콜레스테롤을 섭취시킨 쥐에 SFPEs 를 처리하였을 경우 혈중 콜레스테롤과 중성지방의 수준이 감소함을 확인하였고, 전기생리학적 실험을 통하여 솔잎발효액이 쥐의 혈관수축 이완에 미치는 효과를 분석한 결과 Phenylephrine (PE)(10^{-6} M)으로 유발된 수축반응에 대해 SFPE 를 200 ㎕/m 를 첨가하였을 때 혈관이 다시 이완되었으며, ATP 민감성 K+ 채널 억제제인 glibenclamide (10^{-5} M)을 처리 하였을 때 SFPE 7 효과가 나타나지 않은 것으로 보아 SFPE 의 혈관 이완작용이 세포막 ATP−민감성 K^{+} 통로를 활성화 시켜 이루어짐을 확인하였다. 솔잎자체발효 (SFPE)에 관여하는 균을 발효단계에 따라 분리, 선발하고 동정하였다. GPYA 배지에서 발효균을 스크린하여

Pichia galeiformis, *Candida boidinii*, *Pichia species*, *Candida species 1*, *Candida species 2*, *Candida ooidensis*, *Saccharomyces cerevisiae*, *Candida karawaiewii* 를 선발하였으며, 발효가 진행됨에 따라 2 년차에는 *Candida boidinii*, *Candida species 2*, *Candida ooidensis*, *Saccharomyces cerevisiae* 가 선발되었고, 3 년차에는 *Candida species 1&2* 가 선발되어 *Candida species 2* 가 우점종으로 나타났다. 그러나 발효 4 년차에는 어떤 균도 선발되지 않았다. *Candida* species 2 는 *Candida ernobii* 와 99.65%의 homology 를 보였다. SFPEs 의 GC-MS 분석 결과 발효기간이 증가함에 따라 유용한 terpenoid 계열의 성분이 함유되어 있음을 확인하였고, 특히 camphor 와 1,8-cineole 의 경우 항균 및 항산화 활성이 있음을 확인하였다.

V. REFERENCES

A. K. 1970. Coronary heart disease in seven countries. *Circulation.* 41:1-211

Ahmada R, Alib A, Israf D, Ismaila N, Shaarid K, Lajis N. 2005. Antioxidant, radical scavenging, anti-inflammatory, cytotoxic and antibacterial activities of methanolic extracts of some Hedyotis species. *Life Sci.* 76:1953–64

Benerjee A, Chisti Y, Bannerjee UC. 2004. Streptokinase-a clinically useful thrombolytic agent. *Biotech. Adv.* 22:287-307

Byun H-G, Eom T-K, Jung W-K, Kim S-K. 2007. Lipase Catalyzes Production of Monoacylglycerols by the Esterification of Fish Oil Fatty Acids with Glycerol. *Biotechnology and Bioprocess Engineering.* 12:491-6

Canini A, Alesiani D, D'Arcangelo G, Tagliatesta P. 2007. Gas chromatography-mass spectrometry analysis of phenolic compounds from Carica papaya L. leaf. *Journal of Food Composition and Analysis.* 20:584-90

Chang S-F, Hsieh C-L, Yen G-C. 2008. The protective effect of Opuntia dillenii Haw fruit against low-density lipoprotein peroxidation and its active compounds. *Food Chemistry.* 106:569-75

Cheong H, Paudyal D, Jun J, Yeum C, Yoon P, *et al*. 2005. Effects of Pine Needle Extract on Pacemaker Currents in Interstitial Cells of Cajal from the Murine Small Intestine. *Mol. Cells.* 20:235-40

Chung Y, Bae M, Choung M, Lee J, S, Chung K. 2002. Cytotoxic effect of the distilled pine-needle extracts on several cancer cell lines in vitro. *J. Korean Soc. Food Sci. Nutr.* 31

Deba F, Xuan TD, Masaaki, Yasuda, Tawata S. 2008. Chemical composition and antioxidant, antibacterial and anti fungal activities of the essential oils from Bidens pilosa Linn. var. Radiata. *Food Control.* 19:346-52

Dupont S, Caffin N, Bhandari B, Dykes G. 2006. In vitro antibacterial activity of Australian native herb extracts against food-related bacteria. *Food Control.* 17:929–32

http://en.wikipedia.org/wiki/Camphor.

http://en.wikipedia.org/wiki/Eucalyptol.

http://en.wikipedia.org/wiki/Paclitaxel.

Ichikawa S, Takigawa H, Nara S. 1998. Effects of sho-ju-sen, an herbal medicine, on unidentified clinical syndrome. *J. Clin. Pharmacol. New Drugs.* 47,

Jennings K. 1996. Antibodies to streptokinase-one is enough. *BMI.* 321:393-4

Jeon H, Lee, KS, Ahn, YJ. 2001. Growth-inhibiting effects of constituents of Pinus densiflora leaves on human intestinal bacteria. *Food Sci. Biotech.* 10:403–7

Jerez M, Selga, A, Sineiro, J, Torrens, JL, Núñez, MJ. 2007. A comparison between bark extracts from Pinus pinaster and Pinus radiata: Antioxidant activity and procyanidin composition. *Food Chem.* 100

JH S, EJ B, PA H, IS P. 2005. Yeast and bacterial modulation of wine aroma and flavour. *Aust J Grape Wine Res.* 11:127-38

JH S, Pretorius. 2005. IS Yeast modulation of wine flavour. *Adv Appl Microbiol.* 57:131-75

Kim IH, Lee D-G, Lee SH, Ha J-M, Ha B-J, *et al*. 2007. Antibacterial Activity of Ulva lactuca against Methicillin-Resistant Staphylococcus aureus (MRSA). *Biotechnology and Bioprocess Engineering.* 12:579-82

Kim M, Kim Y, Park H, Chung J, Leem K, Kim H. 2006. Apoptotic effect of red wine polyphenols on human colon cancer SNU-C4 cells. *Food Chem. Toxicol.* 44

Kim Y, Shin D. 2005. Volatile components and antibacterial effects of pine needle (Pine densiflora S. and Z.) extracts. *Food microbiol.* 22

Koh S, Sanders K, Ward S. 1998. Spontaneous electrical rhythmicity in cultured interstitial

cells of Cajal from the murine small intestine. *J. Physiol.* 513:203-13

Koukos P, Papadopoulou K, Patiaka D, Papagiannopoulos A. 2000. Chemical composition of essential oils from needles and twigs of balkan pine (Pinus peuce Grisebach) grown in northern Greece. *J. Agric. Food Chem.* 48:1266–8

Lee J-G, Lee C-G, Kwag J-J, Buglass AJ, Lee G-H. 2005. Determination of optimum conditions for the analysis of volatile components in pine needles by double-shot pyrolysis0gas chromatography-mass spectrometry. *Journal of Chromatography A.* 1089:227-34.

Lee W, Sakai T, Lee M, Hamakawa M, Lee S, Lee I. 1996. An epidemiological study of food poisoning in Korea and Japan. *Food Microbiol.* 29:141-8

Lee YJ, Kim TY, Chung HW. 2001. Protective Effects of Ginkgo Biloba Leaf Extract (GBE) against 1,2,4-benzenetriol Induced Toxicity in vitro. *Kor. J. Env. Hlth. Soc.* 1:124-30

M K, MC J, P vtV, F. K. 1998. Fruits and vegetables in chronic disease prevention. *Wageningen: Wageningen Agricultural University*

Madyastha KM, Chadha A. 1986. Metabolism of 1,8-Cineole in Rat: Its Effects on Liver and Lung Microsomal Cytochrome P-450 Systems. *Bull. Environ. Contam. Toxicol.* 37:759-66

MJ. G. 1999. Food, nutrition, and the prevention of cancer: a global perspective. *American*

Institute of Cancer Research/World Cancer Research Fund. Nutrition. 15:523-6

Moteki H, Hibasami H, Yamada Y, Katsuzaki H, Imai K, Komiya T. 2002. Specific induction of apoptosis by 1,8-cineole in two human leukemia cell lines, but not in human stomach cancer cell line. *Oncology Reports* 9

Pietri S, Maurelli E, Drieu K, Culcasi M. 1997. Cardioprotective and Anti-oxidant Effects of the Terpenoid Constituents of Ginkgo biloba Extract (EGb 761). *J Mol Cell Cardiol.* 29:733-42

S. M., A. C., G. T. 1994. Red wine and antioxidant activity in serum. *Lancet* 344:193-4

Sanders K. 1996. A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology.* 111:492-515

Seo Y, Lee H-J, Park KE, Kim YA, Ahn JW, *et al*. 2004. Peroxynitrite-scavenging Constituents from the Brown Alga Sargassum thunbergii. *Biotechnology and Bioprocess Engineering.* 9:212-6

Shin Y-SKaD-H. 2005. Volatile components and antibacterial effects of pine needle (Pinus densiflora S. and Z.) extracts. *Food Microbiology* 22:37-45

Soares MCMS, Damiani CEN, Moreira CM, Stefanon I, Vassallo DV. 2005. Eucalyptol, an essential oil, reduces contractile activity in rat cardiac muscle. *Brazilian Journal of*

Medical and Biological Research 38:453-61

Sumi H. 1977. *Oral streptokinase*. Japan Tokyo

Sung H, Nah J, Chun S, Park H, Yang S, Min W. 2000. In vivo antioxidant effect of green tea. *European Journal of Clinical Nutrition.* 54:527-9

Thomsen L, Robinson T, Lee J, Farraway L, Hughes M, *et al.* 1998. Interstitial cells of Cajal generate a rhythmic pacemaker current. *Nat. Med.* 4:848-51

Thuneberg L. 1982. Interstitial cells of Cajal: intestinal pacemakers. *Adv. Anat. Embryol. Cell Biol.*:1-130

Tokutomi N, Maeda H, Tokutomi Y, Sato D, Sugita M, *et al.* 1995. Rhythmic Cl- currents and physiological roles of the intestinal c-kit-positive cells. *Pflugers Archiv.* 431:169-77

Ward S, Burns A, Torihashi S, Sanders K. 1994. Mutation of the proto-oncogene c-kit blocks development of interstitial cells and intestinal electrical rhythmicity in murine mutants. *J. Physiol.* 480:91-7

Watanabe T, Inaba K, Nakai A, Mitsunaga T, Ohnishi J, Koshijima T. 1991. Water-soluble polysaccharides from the root of Pinus densiflora. *Phytochem.* 30:1425–9

WB. K., WP. C., T. G., PM. M. 1971. Serum cholesterol, lipoproteins, and the risk of coronary

heart disease. . *Ann Intern Med* 74:1-12

Yen G-C., Duh P-D., Huang D-W., Hsu C-L., Fu TY-C. 2007. Protective effect of pine (Pinus morrisonicola Hay.) needle on LDL oxidation and its anti-inflammatory action by modulation of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. *Proceedings of Food and Chemical Toxicology.* Taiwan

감사의 글

대학원에 입학하고, 하루하루 반복되는 생활의 연속이지만 정신없이 달려왔더니 어느새 졸업이라고 합니다. 아직도 부족하고, 능숙하지 못하는 제가 석사학위를 받고 졸업한다고 하니 새삼 부끄러울 뿐입니다. 하지만 지금 이 자리까지 저를 이끌어 주시고 끊임없는 격려는 아끼지 않으셨던 모든 분들께 감사의 마음을 전하고 싶습니다.

제가 2년간의 석사과정을 잘 마칠 수 있도록 도와주시고, 지치고 힘들때마다 항상 이끌어주셨던 저의 인생의 멘토이신 정현숙 교수님께 진심으로 감사드리며, 김성준 교수님, 양영기 교수님, 박열 교수님, 이정섭 교수님, 전홍성 교수님, 박윤경 교수님 그리고 우은란 교수님께 진심으로 감사드립니다.

또한 저희실험실 식구들에게도 저의 마음을 전하고 싶습니다. 항상 티격태격 싸우고 지냈지만 그래도 옆에서 조언을 아끼지 않았던 황인덕 선생님, 영민오빠, 재영오빠 고맙습니다. 그리고 부족한 영어 실력 때문에 애를 많이 쓰셨던 dilli와 giri 고맙습니다. 특히 dilli 덕분에 졸업논문을 잘 쓸 수 있었습니다. 고맙습니다. 주영이도 항상 옆에서 파이팅 해줘서 고마워. 그리고 지금은 실험실에 있지 않지만 한때 오빠 겸 절친한 친구(?)로 지냈던 창수 오빠에게도 감사의 뜻을 전하고 싶습니다. 멀리 서울에 있는 이현주양에게도 감사의 뜻을 전하고 싶습니다. 현주야~ 광주오면 꼭 보자. 서울에서 너의 뜻을 모두 잘 이루길 바래.

분생방의 재성 선생님 지금은 미국에서 잘 지내고 계신가요? 선생님의 조언 덕

에 대학원 생활을 잘 마무리 할 수 있었던 것 같습니다. 또한 세은아 항상 고맙구나. 세은이 너가 있어서 대학원 생활이 더욱 힘났고, 좋았었어. 우리 평생 2년 간의 좋은 기억을 마음에 품고 가자꾸나. 그리고 지은이는 항상 재롱 피워줘서 언니가 웃을 수 있었다. 승선생님과 봉석 오빠도 항상 웃음으로 힘든 시기를 같이 지내줬고, 마지막으로 회창이 오빠에게는 대학원 생활을 잘 시작하라고 전해주고 싶습니다.

신경방의 홍석오빠, 효정이, 으뜸오빠 그 동안 놀러가면 잘 놀아줘서 고마웠구요, 상용오빠는 앞으로 같이 모임을 잘 이끌어 나가요. 정애도 대학원 생활 열심히 하고, 필요하거나 언니의 조언이 필요할 때 언제든지 전화주길 바래.

선영이 또한 항상 고민상담친구로써 역할을 잘 해주어서 고마워. 앞으로도 잘 지내자. 너의 해맑은 웃음이 그리울 거야.

그리고 나의 대학교 추억의 보물들 운혜, 현하, 선이, 선희... 정말 보고싶다. 지금은 다른 곳에서 각자 열심히 생활하고 있지만, 너희들의 격려가 너무 고마웠어. 운혜와 현하는 호주에서의 생활 잘 하길 바라고, 선이는 과기원에서 파이팅을, 선희는 수원에서 화이팅을!!! 그리고 학회실 생활을 같이 하였던 웅오빠, 형석오빠, 석준오빠도 고마워요. 그리고 성진오빠도 고마워요~

또한 수지, 아영, 미혜, 혜영. 나의 고등학교 친구들~ 그 동안의 끊임없는 격려 고마웠고, 앞으로도 우리의 우정 끝까지 가지고 가자. 수지는 취업 잘하고, 아영이도 원하는 일이 잘 이루어 졌으면 좋겠다. 미혜도 앞두고 있는 시험 잘 보고, 혜영이는 서울에서 너의 꿈을 펼치길 바래.

나의 짝궁 한군!! 항상 옆에서 지켜주고, 격려해주고, 용기잃지 않게 손 잡아줘서

고마워요. 앞으로도 잘 부탁드립니다.

마지막으로 항상 믿어주시고, 기도해주시고, 사랑으로 모든 것을 감싸주신 부모님께 정말정말 감사드리고, 나의 귀여운 동생들 지현이, 지성이에게도 감사의 마음을 전하고 싶습니다. 앞으로도 좋은 일만 있을거라고 약속드릴게요.

그 밖에 여기까지 이끌어 주신 많은 분들께 감사의 마음을 전합니다.

2007년 12월 04일

박가영 드림