식물생명공학회지 제34권 제4호
J Plant Biotechnol
Vol. 34, No. 4, 313-322 (2007)

Biological Effect and Chemical Composition Variation During Self-Fermentation of Stored Needle Extracts from *Pinus densiflora* Siebold & Zucc.

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ABSTRACT Extract of Japanese red pine needles has been used in Asia pacific regions since long periods believing its valuable properties as tonic and ability of curing diseases of unidentified symptoms. Some selective compounds present in the extract and their effects were analyzed. Carbohydrates and vitamin c were identified using HPLC; terpenoid compounds by GC-MS; anti-bacterial analysis by paper discs, plates count and gastrointestinal motility by whole cell patch clamp. The extract is a mixture of compounds therefore its diverse effect was expected. Self-fermentation in extract proceeds after spontaneous appearance of yeast strains without inoculation. Effects and composition of the extract vary with varying period of self-fermentation. Extract inhibits the growth of bacteria dose dependently exhibiting its antibacterial properties however effectiveness increases with increase in fermentation period. The extract also can modulate gastrointestinal motility in murine small intestine by modulating pace maker currents in ICC mediated through ATP sensitive potassium channel.

Introduction

Pinus densiflora Siebold & Zucc. (Japanese red pine), a medium sized tree and a member of family Pinaceae, occurs either naturally or planted in the hills of Asia pacific region. The plant is believed contain highly valuable constituents therefore, its extract is in traditional use as a nourishing agent since long time in oriental systems (Cheong et al. 2005). 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). It is used as liquors like tea or some alcoholic beverages for tonic and the health-improving agent (Chung et al. 2002).

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 unknown period with considering 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 contains several different organic compounds including carbohydrates, proteins, lipids, terpenoids, alkaloids and several others. Among the compounds available, some of them play crucial role in human health. Plant not only supplies food but

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also provides tonic and medicinally important products, therefore being utilizing since unspecified periods. Not only useful but also some slow to highly toxic or poisonous constituents are also existed in the plant with varying concentrations.

Pine leaves have essential oils (0.3-1.3%) including α and β-pinene, camphene, borneol, phellandrene, etc. (Kim and Shin 2005) that are helpful in reducing cardiovascular diseases and possess anticancer properties (Kim et al. 2006). 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).Flavonoides and other plant phenolics such as phenolic acids, stilbenes and tannins are important for normal growth and defense against infection and injury (Jerez et al. 2007).

It was found that volatile compounds present in pine extract can completely inhibit the growth of bacterial strains (Kim and Shin 2005). The compounds isolated from various parts of Red pine are also found effective in inhibiting human intestinal bacteria (Dupont et al. 2006; Jeon et al. 2001; Koukos et al. 2000; Watanabe et al. 1991). In general, it is assumed that the plants products possess low side effects, nutritious and improve human health effectively therefore, they are consumed since long period of human history.

Traditional methods of processing foods and liquors are widely used since unspecified period. Self-fermentation of the pine products is one of such traditional food processing procedures practiced in asia-pacific regions. It is found that self-fermentation of the pine products improves its effectiveness (Park et al. 2006). Duration and storage conditionalso determines the major volatile compounds composition in the stored products (Perez-Prieto et al. 2003).

There is a myonteric movement of bowls that helps the downward movement of food in alimentarycanal where the continuous contracting and relaxing cells are occur (Thuneberg 1982; Sanders 1996). Such cells are called the Interstitial Cells of Cajal (ICC), small spindle-shaped or stellate cells having numerous mitochondria capable tomodulate the gastrointestinal movement through the alteration of the spontaneous inward currents generated throughinfluences of external agents (Thomsen et al. 1998; Koh et al. 1998; Tokutomi et al. 1995; Ward et al.1994). The current generated is called pacemaker current that enables the tissues producing continuous rhythmof contraction and relaxation in the smooth muscle tissues of bowl. These cells are affected by the chemicals and regulate the bowl motility.

Overall assessment of the product is still lacking therefore the present study intends to express the many aspects of the experiments to verifywhether the Fresh Pine Needle extract (PE) and 3 and 7 years old Self-Fermented Pine Needle Extract (SFPE) have diversified and useful effects. Also, we intended to explore whether some toxic compounds present in PE disappears during the course of self-fermentation. Traditional users prefer old extract than fresh therefore, studies also aimed to explore the reason behind it.

Materials and Methods

Plant Materials

Preparation of pine needle extracts: Ten kilograms of fresh pine needles were selected and harvested from Japanese red pine tree (*Pinus densiflora* Siebold & Zucc.) in Changseung, Korea. The needles were washed with distilled water. The washed needles were squeezed with high pressure to obtain a juice extract. The juice obtained was then settled for 3 hours at 4℃ and the supernatants were isolated. This procedure was repeated three times and all the extracts collected were combined. The liquid extract was then centrifuged at 3000 RPM for 10 minutes and supernatant was obtained. The supernatant collected was then called Fresh pine needle extract (PE). PE was stored in room to allow self-fermentation for years in ambient condition without regulating heat, light and temperatures. The sample was further experimented after 3 years of self-fermentation (SFPE3) and 7 years of self-fermentation (SFPE7) taking at least 3 batches from each sample type for composition analysis and more than 10 batches analyzed for microorganism studies. 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 sample. Our next goal is optimizing environmental parameters in accelerating spontaneous fermentationand effectiveness of the pine product.

Chemicals Composition

Carbohydrates: Carbohydrate (soluble sugar) present in PE and SFPE 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 254 nm UV detector.

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.

Terpenoid compounds: Terpenoid compounds were analyzed by using GC-MS. The GC-MS analyses were performed using a HP-5973 MSD instrument. The data were obtained on a 5% phenylmethyl silicon fused silica capillary column, 30 m x 0.25 mm x 0.25 μm film thickness installed in a HP-6890 GC gas chromatograph. Other devices used were 25 ml frit sparger, gas-tight syringes, purge and trap concentrator (Tekmar 3000). Operation conditions were: carrier gas: He 1ml/min with a linear velocity of 36 cm/s; purge time 11 min; dry purge time 2 min; desorb preheat temperature 175°C desorb temperature 180°C (4 min); trap bake temperature 180°C (7 min); sample injection port temperature 230°C; ion conversion-EI, ionization voltage: 70 eV; ionization current: 60 μA; Detection mode: Scan (m/z 35-700).

Antibacterial Effect Assay

Bacterial specimens: 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*.

Antibacterial activity: Nutrient broth (bacto beef extract-3g, bacto-peptone 5g) agar culture of the test organisms was prepared as described (Ahmada et al.2005). Two different concentrations of the PE and SFPE sample (40 μl and 80 μl) to test the antibacterial activities were loaded onto each Whatman No.1 filter paper discs (φ, 6 mm) and placed on the previously inoculatednutrient 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.

Antibacterial effect of pine needles extract on *Bacillus subtilis* was also analyzed using total plate count. A colony of *Bacillus subtilis* was inoculatedinto 3 ml LB medium in a culture tube. The tube was incubated for 12 hours at 37°C in a shaking incubator. Three micro liters of the 12 hours cultured bacteria was diluted to 1 ml LB medium and incubated for 10 minutes at 37°C in a shaking incubator. 100 ul of bacterial culture was spread on to each LB-agar (2%) plates pre-added 10, 20, 30, 40 and 50 ul/ml of PE, SFPE3 and SFPE7. The plate was cultured at 37°C for 12 hours and the number of colony was counted taking LB agar plates as negative control.

Electrophysiological Study

Preparation of cells and tissues: Balb/C mice (8-13 days old) of either sex were anesthetized with ether and sacrificed by

cervical dislocation. Small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were removed by washing with Krebs-Ringer bicarbonate solution, tissues were pinned to the base of a Sylgard dish, and mucosae were removed by sharp dissection. Small tissue stripes of intestinal muscle (contained both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hanks solution for 30 min. The cells were then dispersed in an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) 1.3 mg/ml, bovine serum albumin (Sigma) 2 mg/ml, trypsin inhibitor (Sigma) 2 mg/ml and ATP 0.27 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD) in a 35 mm culture dish, and cultured at 37°C in a 95 % O_2-5 % CO_2 incubator in SMGM (smooth muscle growth medium, Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/ml, Sigma).

Patch clamp experiments: The whole-cell configuration patch-clamp technique was used to record cultured ICC membrane currents (voltage clamp) and potentials (current clamp, and Axopatch 1-D (Axon Instruments, Foster, CA, USA) amplified membrane currents and potentials. Command pulses were applied using 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, Valley View, OF, USA). The cells were bathed in a normal 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, K2ATP 2.7, Na_2GTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with tris. All experiments were performed at 30°C.

Results

Traditional methods of pine needle extracts processing involves an open system of storage in ambient temperature, humidity and other microclimatic conditions. Peoples believe that as long as the extract is stored, its effectiveness increases along with the taste. They also believe that the spontaneously fermented pine needles extract improves overall health and is effective over multiple ailments. Initial light-greenish color of the extract gradually turns into dark red with aging. Previous studies revealed *Saccharomyces cerevisiae*, several species of *Candia* and *Pichia* are the key yeast strains that play crucial role in self-fermentation process (Park et al. 2006). To explore the pattern of change effects and we tried to analyze the composition and their different biological effects.

Composition and Pattern of Compound Dynamism During Self-Fermentation

Carbohydrates: 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 here in PE and SFPE represents the sum of these three different simple carbohydrates. The concentration in SFPE7 was surprisingly increased to 10,648.56 (±158.13) from 7,253.63 (±138.112) ppm in PE (Figure 1). Carbohydrate shows increasing in aggregate with aging. Glucose and sucrose showed inconsistencies while

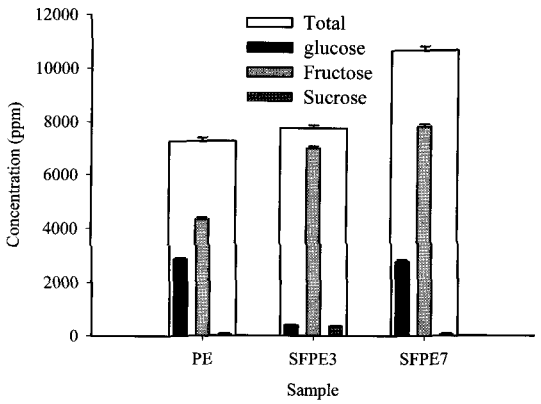

Figure 1. Trend of carbohydrate dynamics in PE and SFPE. Interchange in carbohydrates occurs and finally increases the total amount of carbohydrate due internal reactions during spontaneous fermentation. Glucose concentration decreased during 3 years old SFPE, which retained to its original level, but the fructose shows continuous increasing trend during spontaneous fermentation PE: Fresh Pine Needle extracts; SFPE3: Self-fermented pine extract 3 years old; SFPE7: Self-fermented pine extract 7 years old.

fructose showed gradual increase causing increase in aggregate. Yeasts might be the chief players in such concentration change in open fermentation system. Glucose concentration decreases from PE to SFPE3 (2,855 ± 56.72 to 395.2 ± 15.47 ppm) while increases in SFPE7 (2,765 ± 55.3 ppm) might indicate the microbialled degradation of other complex biomolecules however; it would need the further sudies.

Fatty acids/lipids: Fatty acids components in PE and SFPE were assessed. Saturated as well as unsaturated are present in all aged samples distributing among 9 known fatty acids. In terms of the total concentration percents, 9 known fatty acids possess 50.8 ± 4.56, 45.1 ± 2.77 and 37.3 ± 3.60% in PE, SFPE3 and SFPE7 respectively. Remaining unknown contributes to 40.9 ± 2.45, 51.2 ± 3.91 and 54.8 ± 4.78in PE, SFPE3 and SFPE7. Nearly 13% of the known fatty acid amount was changed to unknown fatty acid (Table 1) leading to disappear 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 SFPE (4.50 ± 0.35% in PE and 22.9 ± 2.54% in SFPE) (Table 1). 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.

Volatile terpenoid compounds in PE and SFPEs: There were 17 different volatile terpenoid compounds detected from PE and SFPEs. Out which 7 were detected only from PE, 7 from SFPE3 and SFPE7, only one was common between PE and SFPE3 and 2 were common in all (Table 2). The result indicated that the volatile compounds are sensitive and change rapidly within 3 years of fermentation with emerging new sets of compounds leading to change the odor of the extracts.

Antibacterial Properties of PE and SFPEs

The PE and SFPEs were tested for whether they have antibacterial properties. It was found that PE and SFPEs can retarded the growth of tested bacterial strains. The antibacterial test with *Bacillus subtilis*, *Salmonella typhimurium*, *Staphylococcus aureus*, using paper disk method revealed the strains are highly susceptible with SFPEs depending upon the dose. Similarly, *Escherichia coli*, *Micrococcus luteus*, Pseudomonas aeruginosa were also susceptible to SFPEs. *Bacillus subtilis* showed the highest growth inhibition (2.1 cm) to SFPE7 (80 μl) among the tested bacterial strains. This strains showed its growth inhibition even by 40 μl SFPE7 (Figure 2A).

Treatment of 40 μl on *Salmonella* showed growth retardation only with SFPE7 however, 80-μl treatments showed inhibition by SFPE3 also and then effect was high with SFPE7. Similar trend have been seen in *Staphylococcus*, *Microcystis*

Table 1. Lipids present in PE and SFPE

S. No.	Name of Lipid	No of carbon /Saturation	PE	SFPE3	SFPE7
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	31.8 ± 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

Table 2. Volatile terpenoid compounds and essential oils in PE and SFPEs

S. No.	Compound	Nature of compounds	Extracts (%)		
			PE	SFPE3	SFPE7
1.	α -pinene	Bicyclic terpene	+	-	-
2.	β -myrcene	Monoterpene	+	-	-
3.	β -phellandrene	Monoterpenes	+	-	-
4.	Camphene	Bicyclic monoterpene	+	-	-
5.	δ -3-carene	Terpene	+	-	-
6.	Styrene	Phenolic	+	-	-
7.	3-pentanone	Aromatic ketone	+	+	-
8.	Toluene	Phenolic	+	+	+
9.	1,8-cineole/Eucalyptol	Monoterpene	+	+	+
10.	2-methyl-1-butanol	Active amyl alcohol	-	+	+
11.	3-methyl-1-butanol	Alcohol	-	+	+
12.	5-isopyrenyl-2-2 methyl-2vinyltetrahydrofuran		-	+	+
13.	Bicyclo[2,2,1]heptan-2-ol	Terpene	-	+	+
14.	Camphor	Terpene	-	+	+
15.	Herboxide second isomer		-	+	+
16.	Hexanoic acid, ethyl ester	Fatty acid	-	+	+
17.	Isocineole	Terpene	-	+	+

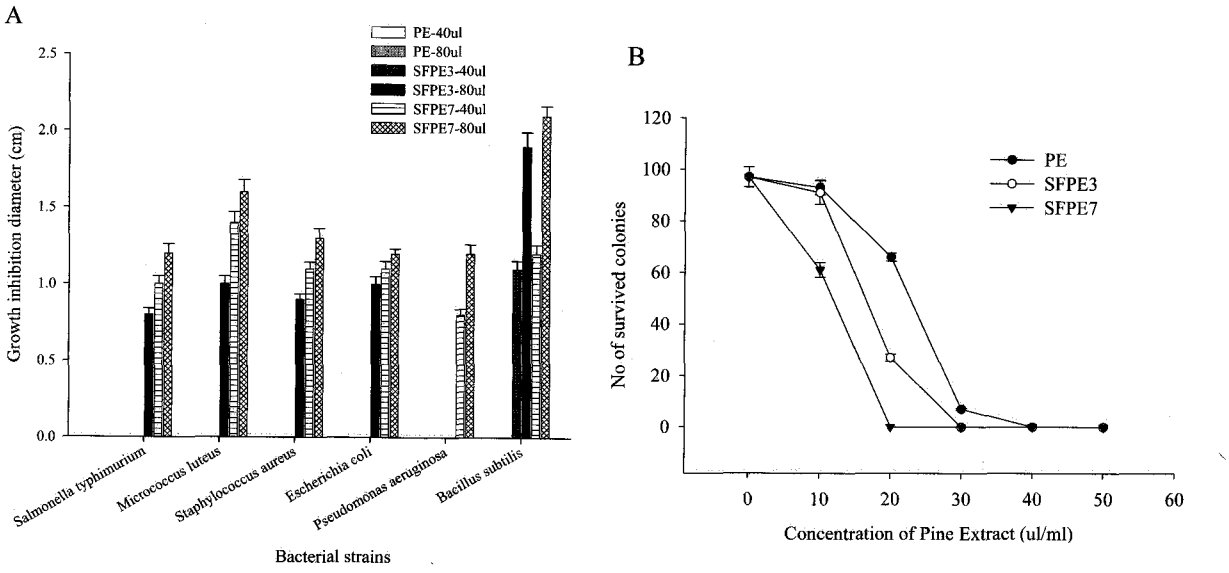

Figure 2. Antimicrobial property of PE and SFPEs were analyzed. A. shows the effect of PE and SFPE to different bacterial strains on paper disc. B. shows the effect of PE and SFPEs to *Bacillus subtilis* using total plate count method. PE and SFPE suppressed the bacterial growth in dose dependent manner, self-fermentation period dependent and strain specific. Effect was increased with increase in self-fermentation period. DW: Distilled water; PE: Fresh Pine extract; SFPE3: Self-fermented pine extract 3 years old; SFPE7: Self-fermented pine extract 7 years old.

and *E. coli*. In these strains PE and SFPE3 showed no growth inhibition by 40 μl, while the bacterial growth was inhibition by 40 μl SFPE7. Treatment of 80 μl extract showed inhibition of bacterial strains. *Bacillus subtilis* showed high level of susceptibility with SFPE3 as well SFPE7. In paper disk test, PE showed no inhibition to all bacterial strains however, PE as well as SFPEs showed inhibitory role to *Bacillus subtilis* in total plate count. Amongst, SFPE7 showed strong inhibition to the bacteria, which is followed by SFPE3 and finally low effect by PE. In the test in LB agar plates pretreated with 20 μl/ml and above concentrations of SFPE7 completely inhibited the growth of *Bacillus subtilis*. The SFPE3 showed complete

inhibition by 30 μl/ml and above concentrations. Similarly, PE showed complete inhibition by 40 μl/ml and above concentrations. The effect was shown in dose dependant manner (Figure 2B) revealing PE and SFPEs have antibacterial properties.

Effect of Liquid Extract in Murine Small Intestinal ICCs

ICCs cultured from the murine small intestine are 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 spontaneously pacemaker potentials. The resting membrane potential was -53 ± 3 mV and amplitude -23 ± 5 mV. In conversion of the amplifier 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.

SFPE7 has been tested for the analysis of the effects on alteration of pacemaker activities in the ICC. In whole cell patch technique at 30°C, 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 treated the SFPE7 (200 μg/ml) in ICC, under currents clamp mode it hyperpolarized the membrane potential and under voltage clamp mode decreased both the frequency and amplitude of pacemaker currents, and increased the resting currents in outward direction. Also, SFPE7 inhibited the pacemaker currents in a dose-dependent manner (data not shown).

Glibenclamide, a blocker of potassium channel, reversed the effect developed by SFPE7 indicating the SFPE7 cause the opening of the potassium channels during modulation of pacemaker current (Figure 3).

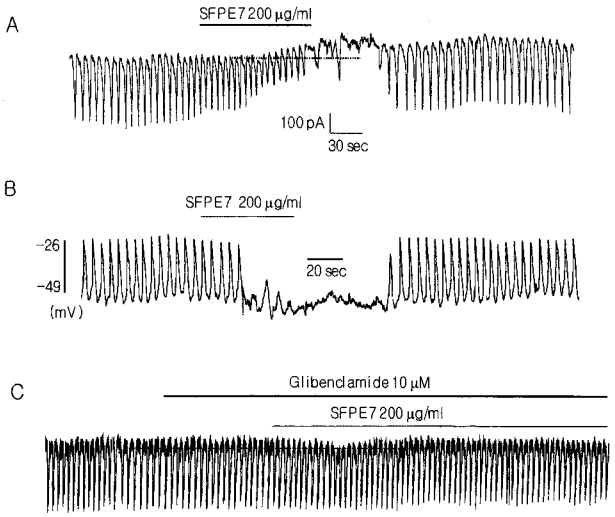

Figure 3. Effects of SFPE7 on pacemaker potentials and pacemaker currents recorded in cultured ICC from murine small intestine. (A) Shows the pacemaker currents of ICC recorded at a holding potential of -70 mV exposed to SFPE7 (200 μg/ml). (B) Shows the pacemaker potentials of ICC exposed SFPE7 (200 μg/ml) in the current clamping mode (I=0). The pine needle extracts induced membrane hyperpolarization. (C) Shows the effect of SFPE7 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 SFPE7. Dotted lines indicate zero current levels. [SFPE7: Self-fermented pine extracts 7 years old].

Discussion

Natural products are useful in various aspects therefore are being used since unspecified periods of human history. The Red pine, an evergreen needle-leaved tree, is considered as one of the natural resources contributing to human health in EastAsia (Chung et al. 1996). Natural products have been used for tonic as overall health improving agent, among them pine extract is one such products that have been used traditionally. Various parts of the pine tree, such as needles, cones, cortices, and pollen, have been widely used for promoting health as folk medicine or as food. Pine needles are used in folk medicine to treat liver disease, gastrointestinal diseases, nervous system disease, circulatory diseases, and skin problems (Yoon 1997, Hong 1999). Various studies have investigated the physiological functions of pine needles. Pine needle extracts have been found to lower blood lipid levels, to exert anti-oxidative, anti-tumor, anti-mutagenic and antibiotic effects (Moon et al. 1993, Kong et al. 1995, Choi et al. 1997, Kim et al. 1998).

Concentration of carbohydrates in the extract was found increased with the increase in period of self-fermentation (Figure 1). It is well known fact that carbohydrate is a chief food for many microorganisms, which they exploit and grow. In spite of the fact that the increased carbohydrate concentration with increased period of storage indicates certain other activities

existed during self-fermentation process. Possibly other complex form of carbohydrates might have degenerated by yielding a simpler one.

In analysis we found that PE and SFPEs contains terpenoids, lipids and carbohydrates in varied concentration. Existed terpenoids, essential oils and unsaturated fatty acids in pine needle extracts are useful compounds to play a vital role as antioxidant to anti-microbial function. Also such substances are regarded to reduce oxidative stress or damage in cells. Additionally, various experimental evidences showed that the dietary anti-oxidative substances are effective in reducing various coronary and cardiac problems (Tribble 1999, Esposito et al. 2003). From literatures it reveals that palmitoleic acid and linoleic acid are anticancerous fatty acids found in plants. The omega 6 and omega 9 fatty acids are designated as essential acids. Also mono or di-unsaturated fatty acids are considered good ingredients for functional food that are present in pine. The total amount of fatty acids decreases with increase in period of fermentation. Perez-Prieto 2003 also found that esters and fatty acids reduced with increase in the storage period of wine. Also fatty acids are taken as indicator for characterizing cider quality (Blanco-Gomis 2002). The decrease in concentration of lauric acid and increase of myristic acids (Table 1) may indicate the age of storage and quality of however needs further clarification.

Compounds like α-pinene, β-myrcene, β-phellandrene are volatile (Kim and Shin 2005; Petrakis et al. 2005) that may escape easily even at low temperature. Absence of these compounds from SFPE3 and SFPE7 (Table 2) indicated their possible escape or degradation or conversion to other form.

Results reveal that the compounds existed in fresh extract were under gone concentration and composition change (Table 1 and 2). Biological activities in spontaneous self-fermentation must have facilitated the conversion process. Emergence of several new terpenoid compounds, Linoleic acid, Stearic acid, Oleic acid, in SFPE3 or SFPE7 must be the resultant product of biologically mediated conversion. Complete disappearanceof toxic compound styrene in SFPE3 and SFPE7 are highly beneficial changes occurred during fermentation period.

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 (Figure 2A). Also of the complete inhibition of growth of *Bacillus subtilis* by PE and SFPEs (30 μl/ml above) indicates their possible antibacterial properties (Figure 2B). It was found that α-pinene, δ-3-carene, eucalyptol containing essential oil from plants has anti-microbial activity to *Pseudomonas putida* (Oussalah et al. 2006).

Pine needle extract plays vital role in physiological process (Cheong et al. 2005). Electrophysiological studies imply that SFPE7 has role in modulating pacemaker current in ICC of murine small intestine. The extract caused reduction of frequency and amplitudes of pacemaker current and also it caused the hyperpolarization of membrane potential (Figure 3A and 3B). The test with glibenclamide (a closure of ATP sensitive potassium channel) reversed the effect of 200 μg/ml SFPE7 (Figure 3A and 3C) indicate that SFPE7 has function on opening ATP sensitive potassium channels and can modulate the gastrointestinal motility in murine small intestine.

Conclusion

Various aspects of analyses of PE and SFPEs reveal that they contain several useful compounds that are additive as nourishing agents. Monounsaturated fatty acids, essential oils and terpenoid compounds have been found in PE and SFPEs. The toxic substances like styrene in fresh extracts are also gettingremoved after self-fermentation. PE and SFPE possess antibacterial properties where effectiveness increases with increases in self-fermentation periods. Similarly, SFPE may modulate gastrointestinal motility through modulation of ICCs by activation of ATP dependant K^+ channel.

Acknowledgement

This work was supported by RCPM (grant #R11-2000-083-00000-0) from Chosun University and the Program for the Training of Graduate Students in Regional Innovation from the Ministry of Commerce, Industry and Energy of Korea.

References

Ahmada R, Alib AM, Israf DA, Ismaila NH, Shaarid K, Lajis NH (2005) Antioxidant, radical scavenging, anti- inflammatory, cytotoxic and antibacterial activities of methanolic extracts of some Hedyotis species. Life Sci. 76: 1953-1964

Blanco-Gomis D, MangasAlonso JJ, Cabrales IM, Abrodo PA (2002) Characterization of Cider Apples on the Basis of Their Fatty Acid Profiles. Agric. Food Chem 50: 1097-1100

Cheong HS, Paudyal DP, Jun JY, Yeum CH, Yoon PJ, Park CG, Kim MY, So I, Kim K, Choi S (2005) Effects of Pine Needle Extract on Pacemaker Currents in Interstitial Cells of Cajal from the Murine Small Intestine. Mol Cells 20: 235-240

Choi EJ, Lee E, Rhim TJ, Cha BC, Park HJ (1997) Antimicrobial activities of pine needle (*Pinus densiflora* Seib Zucc) extract. J Korean Soc Food Sci. Nutr 25: 293-297

Chung HJ, Hwang GH, Yoo MJ, Rhee SJ (1996) Chemical composition of pine sprouts and pine needles for the production of pine sprout tea. Koean J Dietary and Culture 11: 274-276

Chung YJ, Bae MW, Choung MI, Lee JS, Chung KS (2002) Cytotoxic effect of the distilled pine-needle extracts on several cancer cell lines in vitro. J Korean Soc Food Sci. Nutr 31: 691-695

Dupont S, Caffin N, Bhandari B, Dykes GA (2006) In vitro antibacterial activity of Australian native herb extracts against food-related bacteria. Food Control 17: 929-932

Esposito K, Nappo F, Giugliano F, Giugliano G, Marfella R, Giugliano D (2003) Effect of dietary antioxidants on postprandial endothelial dysfunction induced by a high-fat meal in healthy subjects. Am J Clin. Nutr 77: 139-143

Hong WS (1999) The ingredients and use of medicinal herb. Ilwonseogak Co, Seoul 42-144

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: 879-887

Jeon HJ, Lee KS, Ahn YJ (2001) Growth-inhibiting effects of constituents of Pinus densiflora leaves on human intestinal bacteria. Food Sci Biotech 10: 403-407

Jerez M, Selga A, Sineiro J, Torrens JL, Núñez MJ (2007) A comparisonbetween bark extracts from *Pinus pinaster* and *Pinus radiata*: Antioxidant activity and procyanidin composition. Food Chem 100: 439-444

Kim EJ, Jung SW, Choi KP, Ham SS (1998) Cytotoxic effect of the pine needle extracts. Korean J Food Sci Technol 30: 213-217

Kim MJ, Kim YJ, Park HJ, Chung JH, Leem KH, Kim HK (2006) Apoptotic effect of red wine polyphenols on human colon cancer SNU-C4 cells. Food Chem Toxicol 44: 898-902

Kim, YS, Shin, DH (2005) Volatile components and antibacterial effects of pine needle (*Pine densiflora* S. and Z.) extracts. Food microbiol 22: 37-45

Koh SD, Sanders KM, Ward SM (1998) Spontaneous electrical rhythmicity in cultured interstitial cells of Cajal from the murine small intestine. J Physiol 513: 203-213

Kong Z, Liu Z, Ding B, (1995) Study on antimutagenic effect of pine needle extract. Mutat. Res 347: 101-104

Koukos PK, Papadopoulou KI, Patiaka DT, Papagiannopoulos AD (2000) Chemical composition of essential oils from needles and twigs of balkan pine (*Pinus peuce* Grisebach) grown in northern Greece. JAgric Food Chem 48: 1266-1268

Moon JJ, Han YB, Kim JS (1993) Studies on antitumor effects of pine needles, *Pinus densiflora* Sieb et Zucc. Korean Vet Res 33: 701-710

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: 757-760

Oussalah M, Caillet S, Saucier L, Lacroix M (2006) Antimicrobial effects of selected plant essential oils on the growth of a *Pseudomonas putida* strain isolated from meat. Meat Sci. 73: 236-244

Park G, Li H, Hwang I, Cheong H (2006) The functional effects of fermented pine needle extracts. Korean JBioeng 21: 363-370

Perez-Prieto LJ, Lopez-Roca JM Gomez-Plaza E (2003) Difference in major volatile compounds of red wine according to storage length and storage conditions. J Food Composition Analysis 16: 697-705

Petrakis PV, Roussis V, Papadimitriou D, Vagias C, Tsitsimpikou C(2005) The effect of terpenoid extracts from 15 pine species on the feeding behavioural sequence of the late instars of the pine processionary caterpillar Thaumetopoea pityocampa. Behavioural Process 69: 303-322

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

Thomsen L, Robinson TL, Lee JC, Farraway LA, Hughes MJ, Andrews DW, Huizinga JD (1998) Interstitial cells of Cajal generate a rhythmic pacemaker current. Nat Med 4: 848-851

Thuneberg L (1982) Interstitial cells of Cajal: intestinal pacemakers. Adv. Anat. Embryol. Cell Biol 71: 1-130

Tokutomi N, Maeda H, Tokutomi Y, Sato D, Sugita M,

Nishikawa S, Nishikawa S, Nakao J, Imamura T, Nishi K (1995) Rhythmic Cl^- currents and physiological roles of the intestinal c-*kit*-positive cells. Pflugers Archiv 431: 169-177

Tribble DL (1999) Antioxidant Consumption and Risk of Coronary Heart Disease: Emphasis on Vitamin C, Vitamin E, and β-Carotene. Circulation 99: 591-595

Ward SM, Burns AJ, Torihashi S, Sanders KM (1994) Mutation of the proto-oncogene c-kit blocks development of interstitial cells andintestinal electrical rhythmicity in murine mutants. J Physiol 480: 91-97

Watanabe T, Inaba K, Nakai A, Mitsunaga T, Ohnishi J, Koshijima T (1991) Water-soluble polysaccharides from the root of Pinus densiflora Phytochem 30: 1425-1429

Yoon SU (1997) Pine trees and natural remedy. Academy Press Inc Seoul 40-43

(Received October 15, 2007; Accepted November 13, 2007)