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Effects of Pine Needle Extracts on Plasma Cholesterol, Fibrinolysis and Gastrointestinal Motility

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Abstract 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 folk medicine. To determine the facts behind the traditional belief, we tried to investigate the effects of fresh and self-fermented pine needle extracts of different aging. 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. Also, Self-Fermented Pine Needle Extracts 7 years old (SFPE 7) (200 μg/mL) reduce the frequency and amplitude of pacemaker currents in Interstitial Cells of Cajal (ICC) of murine small intestine by modulating ATP-sensitive potassium channels. Therefore, the investigation indicated that self-fermentation improves efficacy of the pine needle extracts reducing risk of cardio-vascular related disorders and would be an important source in nutraceutics. © KSBB

Keywords: pine needle extracts, self-fermentation, fibrinolytic activity, cholesterol and triglyceride, ICC

INTRODUCTION

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 liquors, 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 obtaining quality products. Microorganisms are expected to play a key role in changing composition and quality in targeted food, which is stored.

Pinus densiflora is an evergreen needle-leafed tree indigenous to Asia Pacific. Pine 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.* [1]. 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 [2]. 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, and antihypertension [3-6]. Therefore, pine extract has been processed and used traditionally to treat multiple disorders.

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%) [7] that are helpful in reducing cardiovascular diseases and possess anticancer properties [8]. Especially pine needle and bark are abundant in terpenoids. The essential oil of pine needles has found in wide commercial use [9]. Flavonoides and other plant phenolics such as phenolic acids, stilbenes, and tannins are important for normal growth and defense against infection and injury [10].

Fibrinolytic enzymes dissolve the blood clots, which are

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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 [11-15]. 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 [16,17]. Epidemiologic data showed that a high consumption of vegetables and fruits is consistently associated with a low risk of cancer and cardiovascular disease [18,19].

Pacemaker activities are ubiquitously existed in organs systems such as circulatory, vascular, digestive systems, *etc.* which are conducted by group of cells called pacemaker cells. The pacemaker cells have ability of modulating organ movements and the movements are easily modified from foods and drugs.

Alimentary canal 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 [20,21]. 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 [21-24]. 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 study also aimed to assess whether the extracts show multiples response with fibrinolysis, cholesterol, triglycerides, and intestinal motility viewing the points that the impairment of these components are vowed relate with cardio-vascular or circulatory disorders. There is always lacking information in role of self-fermentation in functional efficacy of the pine needle extracts. Therefore, present study would play an important role in fulfilling the existing gap.

MATERIALS AND METHODS

Plant Material

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

Preparation of PE and SFPEs

Harvested pine needles were cleaned with tap water, dipped with a charcoal in water to final wash, and dried, ground for 1 min to homogenize. The preparation was allowed to settle for 3 h at 4°C and the supernatant was recovered. This supernatant was used to sample and stored at 4°C for assays. PE was stored for years favor self-fermentation in the stored extracts. The effects of the extract were examined for fresh pine needle extract (PE) as well as after 3 and 7 years of self-fermentation designing as Self-Fermented Pine Needle Extracts 3 years old (SFPE 3) and Self-Fermented Pine Needle Extracts 7 years old (SFPE 7).

Fibrinolysis Assay

Fibrinolytic activity of self-fermented pine needle extract was measured on fibrin plate. The fibrin agarose plate was made to a 5 mm thickness. 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 μL of thrombin solution (0.1 U/μL). The solution was then poured into a Petri dish and allowed to stand for 1 h at room temperature to form a fibrin clot. Then 20 μL of self-fermented pine needle extract was carefully dropped onto the plate. The plate was incubated for 1 h at 37°C. 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.

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°C, 12 h light/dark cycle). Food (Mouse E.P. from Suprfeed Co. Ltd.) and tap water were supplied for animals. 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. Rats were grouped into control and the test groups where each group contained 3 individuals and the experiment was repeated for three times. Rats were separated into Cholesterol non-administered (−C), Cholesterol administered (+C), PE, and SFPEs as below. The animas were adjusted for two weeks before administration of cholesterol and/or pine needle extracts. Except the control, all rats were administered cholesterol (0.5 mL) continuously at once a day for 4 weeks (for first 2 weeks cholesterol only and remaining 2 weeks cholesterol along with pine needle extracts). The control group was fed DW (0.5 mL/day) during feeding periods. For one group of rats they were continuously administered cholesterol (0.5 mL) and SFPE 7 (0.5 mL) once a day throughout 4 weeks. Analysis was done using automatic biochemical analyzer (Hitachi 7600, Hitachi Tokyo, Japan).

Group	Week			
	1	2	3	4
–C	Distilled water (0.5 mL/day)			
+C	Cholesterol (0.5 mL/day)			
PE	Cholesterol (0.5 mL/day)		Cholesterol + PE (0.5 mL/day)	
SFPE 3	Cholesterol (0.5 mL/day)		Cholesterol + SFPE 3 (0.5 mL/day)	
SFPE 7	Cholesterol (0.5 mL/day)		Cholesterol + SFPE 7 (0.5 mL/day)	
SFPE 7-2	Cholesterol + SFPE 7 (0.5 mL/day)			

Electrophysiology

Materials

Glibenclamide and pinacidil were purchased from RBI (USA). For stock solutions, all chemicals were dissolved in distilled water or dimethylsulfoxide, and stored at −20°C until analyzed.

Preparation of Cells and Tissues

Balb/C mice (8~13 days old) of both sexes were anesthetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and washed 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 min. 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 cover slips coated with murine collagen (2.5 μL/mL, Falcon/BD) in 35 mm culture dishes, and cultured at 37°C 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).

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°C.

RESULTS AND DISCUSSION

Different parts of the red pine, such as the needles, cones, cortices, and pollen, have been used as folk medicine or as food. Particularly, pine needles are used in folk medicine to treat liver disease, gastrointestinal diseases, nervous system disease, circulatory diseases, and skin problems [25,26].

Pine needle extracts have been found to lower blood lipid levels, and to have antioxidative, antitumor, antimutagenic, and antibiotic effects [27-31]. In identifying the effects of pine needle extracts fresh or during and/or after self-fermentation we have tried to analyze in following aspects relating to vascular stimulation. Suspicion of hypercholesterolemia is relating to atherosclerosis that ultimately links with vascular related disease. Increasing concerns of people nowadays is linked with reducing cholesterol level which is hard to achieve. Our perspective was to assess whether the pine products lower the cholesterol level in rat blood and other blood related systems.

Role of PE and SFPEs in Fibrinolysis

Fibrinolysis is one of the important aspects in medicine linking to the blood clotting and its removal procedures. We tried to assess whether the pine needle extracts show fibrinolytic activities in fibrin plates experiments. Also we were intended in searching whether the fermentation has role in altering fibrinolytic properties. Twenty μL of each sample was carefully placed on fibrin plate. The plate was incubated for 30 min, 1 and 2 h at 37°C and the diameter of the lytic circle was measured. After 30 min incubation, lytic circle formed by PE, SFPE 3, and SFPE 7 on fibrin plate were 11, 12, and 14 mm, respectively. At after 1 h, the circles were 13, 14, and 18 mm, respectively. In 2 h of incubation, lytic circle of PE, SFPE 3, and SFPE 7 were 17, 18, and 23 mm, respectively (Fig. 1). Studies on extracts from bark or French pine was able to show the fibrinolytic behavior [32,33]. It seems that fermentation facilitates fibrinolytic activity in pine extract. And SFPEs might be an important ingredient and be potential source as a functional food (health related food) for thrombosis prevention.

Role of PE and SFPE in Total Plasma Cholesterol and Triglyceride Level

To determine whether the pine needle extracts involves in alteration of total cholesterol and triglyceride levels in blood plasma of rats, we checked the effects of PE and SFPEs. As the experimental animals were categorized into different test groups we administered them cholesterol and pine needle extracts. The average total plasma cholesterol level in cholesterol fed rats was 50.5 ± 0.7 mg/dL. The level was found

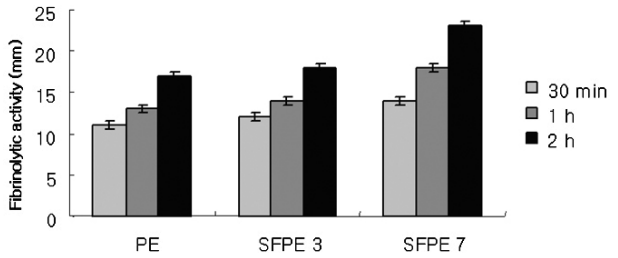

Fig. 1. Fibrinolytic activity of self-fermented pine needle extracts. Fibrinolytic activities of SFPEs increase with aging and it is the effect is time dependent.

34.5 ± 2.12, 35 ± 3.5, and 23 ± 2.8 mg/dL, respectively, in PE, SFPE 3, and SFPE 7. However, the level was remained 29 ± 1.4 mg/dL in rats which were administered cholesterol (0.5 mL) and SFPE 7 (0.5 mL) throughout for 4 weeks at once a day (Fig. 2A). It was revealed that the percentage decrease in total plasma cholesterol level in cholesterol and pine needle extracts fed rats was 23.3, 22.2, and 48.9%, by PE, SFPE 3, and SFPE 7, respectively. However, the level was 35.6%, in 4 weeks continuous SFPE 7 and cholesterol administered rats (Fig. 2B).

In cholesterol fed control, the level of plasma triglyceride was found as 24 ± 4.3 mg/dL. In PE, SFPE 3, and SFPE 7 (0.5 mL) administered rats for final two weeks were recorded as 13.5 ± 4.9, 15 ± 1.5, and 14.5 ± 7.8 mg/dL, respectively. However the level was sharply reduced to 6 ± 3.1 mg/dL in 4 week continuous administration of SFPE 7 with cholesterol (Fig. 3A). The plasma triglyceride level in cholesterol administered rats was effectively reduced by continuous feeding of SFPE 7 (0.5 mL) for all 4 weeks (Fig. 3B). Hypercholesterolemia, resulting from cholesterol metabolic changes, is a major cause of cardiovascular disturbance, such as atherosclerosis and coronary heart disease [16,17]. Epidemiologic data showed that a high consumption of vegetables and fruits is consistently associated with a low risk of cancer and cardiovascular disease [18,19]. Present study indicated that PE and SFPE are useful in lowering hypercholesteromic condition and which might improve blood circulation and could be a good source of functional food development.

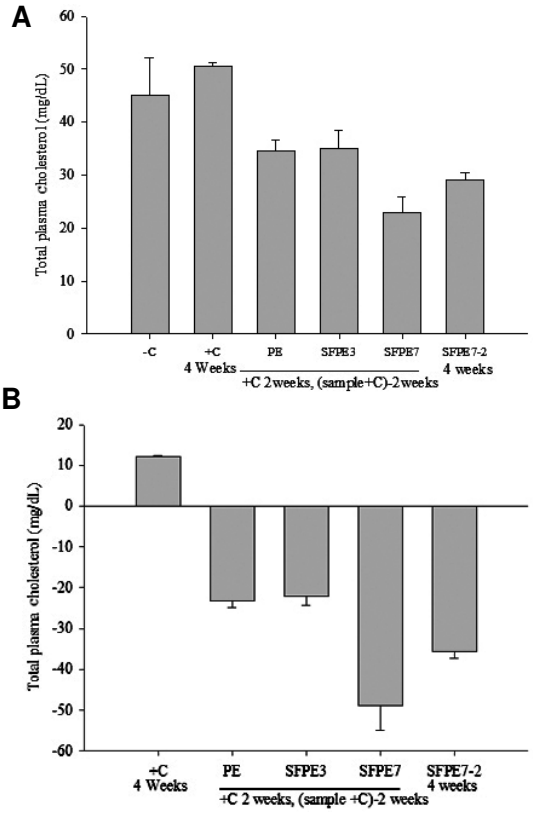

Fig. 2. Assessment of total cholesterol in blood plasma of cholesterol fed rats. The level of cholesterol was found lowered after administration of PE and SFPEs. (A) Shows total plasma cholesterol in cholesterol administered rats. (B) Shows the percentage change in total plasma cholesterol in cholesterol rats. +C, Cholesterol administered; PE, fresh pine needle extract; SFPE, self-fermented pine needle extracts.

Role of SFPE in Electrophysiological Activities in ICC of Murine Small Intestine

ICCs cultured from the murine small intestine are c-kit-positive 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 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°C, ICC generate 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 [21]. 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 inhibit the pacemaker currents in a dose-dependent manner [34]. 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 (Figs. 4A~4C). In identifying whether SFPE affects ATP-sensitive potassium channels, we had tested the effects of pinadicil, an ATP-sensitive potassium channel opener and glibenclamide, an ATP-sensitive potassium channel blocker on pacemaker current. The result demonstrated that the (Fig. 4A) pinacidil (10 μM) decreased the frequency and amplitude of pacemaker currents and increased the resting membrane potential

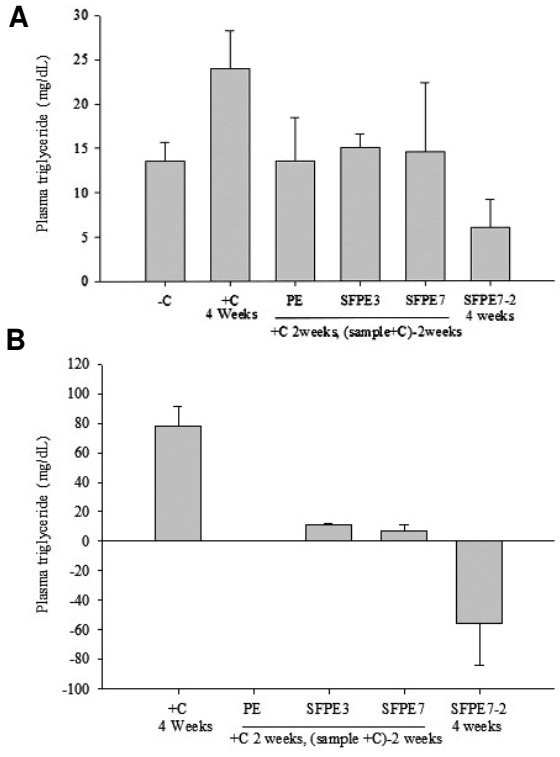

Fig. 3. Level of triglyceride blood plasma of cholesterol fed rats. Blood plasma triglyceride decreases with administration of 0.5 mL PE and SFPEs. (A) Shows plasma triglyceride in cholesterol administered rats. (B) Shows the percentage change in plasma triglycerides in cholesterol rats. +C, Cholesterol administered; PE, fresh pine needle extract; SFPE, self-fermented pine needle extract.

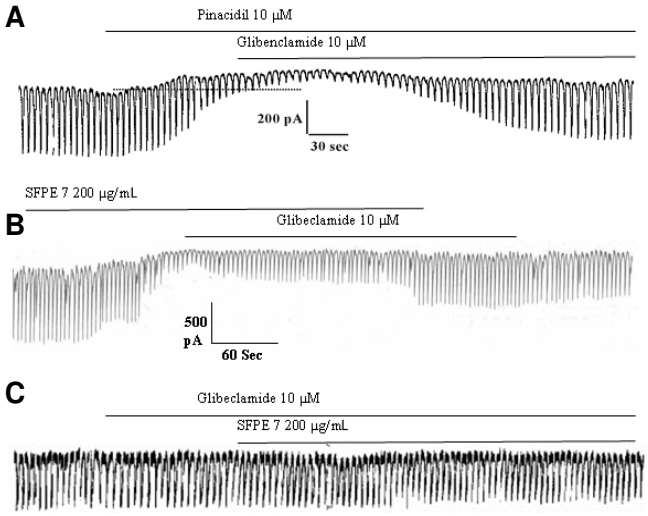

Fig. 4. Effects of self-fermented pine needle extracts on pacemaker currents recorded in cultured ICC from murine small intestine. Figure shows the effect of SFPE 7 on pacemaker currents pre and co-treating cells with glibenclamide. (A) Shows the effect of pinacidil, an ATP-sensitive potassium channel opener was reversed by application of glibenclamide. (B) SFPE7 (200 μg/mL) reduced the frequency and amplitude of pacemaker currents deviating resting current towards outward direction. (C) Pre- and co-treatment of glibenclamide stopped the effect of SFPE indicating the involvement of SFPE in modulating ATP-sensitive potassium channel. SFPE: self-fermented pine needle extract.

in outward direction which was reversed by glibenclamide (10 μM). The agonistical effect was observed in the test of SFPE 7 (200 μg/mL) with pinacidil which effect was also reversed by the application of glibenclamide. Further more, pretreatment of glibenclamide and co-treatment with SFPE 7 showed no alteration in pacemaker currents in ICC of murine small intestine indicated the role of SFPE in opening ATP-sensitive potassium channels (Figs. 4B and 4C).

CONCLUSION

Assessment of pine needle is the essence for traditional medicine using pharmacological efficacy of natural compounds present in pine needle for treating possible human diseases. Fibrinolytic activities of the extract indicated that activities depend on time and also with aging of the pine needle products. It was also found that the extract can lower the blood plasma cholesterol and triglyceride in cholesterol fed rat. SFPE 7 (200 μg/mL) inhibited the pacemaker current of ICC in murine small intestine. Therefore, the self-fermented pine needle products would be additive in lowering blood plasma cholesterol and also can reduce obesity and helpful in removing blood clots. The study unveils many potential aspects in the self-fermentation improve nutraceutical pine needle extracts product processing that further needs follow up studies in assessing them detail.

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