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Study on the Subacute Toxicity of Complex of Pine Needle Oil and Korean Medicinal Herbs against Rats

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Abstract - Pine needle oil and Korean medicinal herbs (KMH) are known as effective therapeutic agents on various blood vessel disease. We have already reported the ameliorative effect of complex of pine needle oil and Korean medicinal herbs against hyperlipidemia. But safety and non-toxicity of pine needle oil and Korean medicinal herbs to normal animal cells have not been studied clearly. In this study, we investigated whether pine needle oil and Korean medicinal herbs show side effects on rat or not. These materials were administered to rats, and subacute toxicity was examined by measuring the hematological values, CBC differentiation, biochemical levels of blood (TP, total protein; albumin; ALP, alkaline phosphotase; AST, aspatate aminotransferase; ALT, alanine aminotransferase; T-Chol., total cholesterol; T-Bil., total bilirubin) and urine analysis, suggesting that the sample have no side effects and cytotoxicity. These results indicate that the complex of pine needle oil and Korean medicinal herbs may effective non-toxic, safety therapeutic agents on hepatocytes and hyperlipidemia.

Key words : pine needle oil, Korean medicinal herb, subacute toxicity, animal cells

INTRODUCTION

The steam-distilled essential oil from the balsam of *Pinus densiflora* is official in the oriental pharmacopeias. *Song-jie* (its Chinese name) was first mentioned in Chinese medical literature ca. 500 C.E. as an antiarthritic and analgesic drug. Today, it is used in the traditional medicines of Korea, China, and Japan, administered as a topical paint to treat rheumatism (But 1997). The approved modern therapeutic applications for pine needle oil are supportable based on its history of use in well established systems of traditional and conventional medicines, and on phytochemical investigations, and pharmacological studies. Pine needle oil is distilled from the finest pines using only the needles, differs from pine oil which may utilize all or any part of the tree when distilled (Koelling 1999). Pine needle oil was used by the ancient Romans and Greeks to treat respiratory problems and muscular aches. Distilled in Austria from the finest pines, pine needle can be diffused to help strengthen the respiratory tract and maintain sinus passages (Bown 1995). In the bath, pine needle improves the circulation and relieves muscular pains (Bown 1995; Krauze *et al.* 2002). Constituents include 50-97% monoterpene hydrocarbons, such as α-pinene, with lesser amounts of 3-carene, dipentene, β-pinen, D-limonene, α-terpinene, γ-terpinene, cis-β-ocimene, myrcene, camphene, sabinene, and terpinolene (Schulz *et al.* 1998). Other constituents include bornyl acetate, borneol, 1, 8-cineole, citral terpineol, T-cadinol, T-muurolol, α-cadinol, cayophyllene, chamazulen, butyric acid, valeric acid, caproic acid, and isocaproic acid. The active principles of some essential oils responsible for the antiviral and

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antibacterial activities are thought to be limonene, dipentene, and bornyl acetate (Leung and Foster 1996). Pine needle oil and other essential oils can cause a decongestant effect by stimulating reflex vasoconstriction (Schulz *et al.* 1998). Herbs are nutritional foundation nutrients and good alternative medicine to nourish the body's deepest and most basic elements. Medicinal herbs have been used safely and effectively since the time of recorded history for an endless list of reasons from health, healing, weight loss/gain/maintenance, to survival and more. Herbs can offer the body nutrients it does not always receive, either from a poor diet, or environmental deficiencies in the soil and air. They are great body balancers that help regulate body functions.

The benefits of herbs are many and varied. Even the once skeptical traditional medical community is starting to embrace alternative medicine practices using herbal remedies and healing philosophies and practices incorporating herbal medicine and medicinal herbs (Nadkarni 1976). Today, herbs are still the alternative medicine and primary source of health care for 80% of the world. Oriental herbal compositions are not mixed together by accident. These are sometimes quite complex mixtures of herbals. Combinations of herbs work better than they do singly, because specific combinations allow inclusion of herbs that can work at different aspects and stages of need, like short term energy, long term endurance, or weight control. Herbal combinations are not addictive or habit forming supplements, but are powerful nutritional agents that assist the body naturally (Taber 1962). Nevertheless, herbal drugs are frequently not sterile and may not be free of bacteria or fungi. Herbal products are not reviewed by any governmental agency for quality, dosage, safety or efficacy and having possible side effects. Therefore, in order to find an alternative to the traditional cure, studies have increasingly focused on the development of non-toxic therapeutic agents based on natural products and medicinal herbs.

We have previously reported the ameliorating effect of the complex of pine needle oil and KMH (composed as *Cornus officinalis* SIEB. *et* ZUCC., *Panax ginseng* C. A. Meyer, *Artemisia capillaris* Thunb. and *Astragalus membranaceus* Bunge) (Huh 1981) on liver damage and hyperlipidemia in alcohol fed rats (Park *et al.* 2003). In this study, we investigated whether complex of pine needle oil and Korean medicinal herbs have side effects or not. These materials were administered to rats, examined by measuring the hematological values and CBC differentiation, blood levels of the enzymes AST (aspartate aminotransferase) and ALT (alanine aminotransferase) and urine analysis. Serum levels of total cholesterol, important causes of hyperlipidemia and arteriosclerosis, were also measured.

MATERIALS AND METHODS

1. Animal models

Young adult male Sprague-Dawley (SD) rats, initial weight 200±10 g, were obtained from Daehan Biolink Co., Ltd. (Seoul, Korea). Animals were housed in individual cages under conditions of constant temperature (22±2°C) and humidity (55±5%). They were kept on a 12 h light dark^{-1} cycle and acclimatized to the housing situation for 10 days prior to the experiments. Rats were divided into two groups (n = 6) as follows : No. 1) normal control rats administered water, No. 2) rats administered diluted solution of KMH complex and pine needle oil (4 : 6,v/v)/water. Rats were treated with these various regimens for the same time period (Table 1).

2. Preparation and treatment of pine needle oil and Korean medicinal herbs

Pine needle oil is the steam-distilled essential oil extracted from the fresh needles, branch tips or from the combined fresh branches with needles and branch tips of *P. densiflora* or other essential oil-containing species of *Pinus*. Production of KMH was based on a recipe derived from Korean traditional medicine books

Table 1. Composition of groups

Group	No. of exam.	Treatment
No. 1 (Normal control)	6	food+water+distilled water
No. 2 (Test group1 : T1)	6	food+water+complex of KMH and pineneedle oil[1)]

Complex of KMH and pine needle oil[1)] : solution of KMH (Korean medicinal herbs composed with *Cornus officinalis* SIEB. *et.* ZUCC. *Panax ginseng* C. A. Meyer, *Artemisia capillaris* Thunb. and *Astragalus membranaceus* Bunge) and pine needle oil by the ratio of 4 : 6 (v/v).

and the recommendations of Korean traditional medical doctors. KMH are traditional Korean prescriptions containing a mixture of four herbs, *Panax ginseng* C. A. Meyer, *Cornus officinalis* SIEB. *et.* ZUCC., *Artemisia capillaris* Thunb., and *Astragalus membranaceus* Bunge. Boiling water extracts of KMH were prepared from the dried herbs. Each 25 g of mixed herbs was added to 1,000 mL of sterilized water and boiled for 150 min using a herbal and medicinal boiling pot (Daewoong Co., Ltd., Seoul, Korea). After centrifugation at 6,000 × g for 15 min, aqueous extracts from sample were filtered through 3 mm filter papers (Whatman, England), and the final volume was adjusted to around 400 mL in order to prepare an appropriate volume for administration (about 1.6 g kg^{-1} body weight day^{-1}). Pine needle oil and dilution complex were administered at a dose of about 0.023 g kg day^{-1} for 28 consecutive days (Park *et al.* 2003).

3. Observation of animal behavior and conditions

To conduct studies of medicinal herb safety, it is important to have a basic understanding on the behavior and general conditions of normal intact animals. We observed the state of skin, hair, conjunctiva, feces and pattern of movement, aggressive behavior in rat each other. In addition to above character, we measured the internal organ after dissection, mortality, body weight, amounts of ingested food and water during 28 days.

4. Urinary analysis

Urine was collected in sterilized cap tube before dissection and pH, protein, glucose, ketone, blood (hemoglobin) of urine were detected with indicating papers (Serotech Korea Co, LTd., Seoul).

5. Dissection and collecting blood

After fasting for 16h at last day of housing, rats were dissected under an anesthetic state and 5-6 mL of blood was collected using an EDTA free injector for biochemical analysis. Liver and kidney were removed and rinsed with cold 0.85% saline (Shinyo Pure Chemicals Co, Japan). Collected blood was allowed to clot for half an hour before separation of the serum by centrifugation at 3,000 × g for 15 m. Collected blood in another EDTA tube was used for hematological and CBC analysis.

6. Biochemical analysis

Serum AST, ALT and alkaline phosphatase (ALP) activities were determined using the AST kit (Boehringer Mannheim, Germany), ALT kit (Boehringer Mannheim) and ALP kit (Boehringer Mannheim). The enzymatic colorimetric test for cholesterol content was performed using the Total Cholesterol kit (Boehringer Mannheim), albumin content was measured by using ALB kit (Boehringer Mannheim). Total bilirubin (T-Bil.) and total protein (TP) were determined using the Bil-T kit (Boehringer Mannheim) and TP kit (Boehringer Mannheim), respectively.

7. Measurement of hematological value and CBC differentiation

Blood in EDTA tube was homogenized by mixing gently, and WBC (white blood cell), RBC (red blood cell), HGB (hemoglobin), HCT (hematocrit) and platelet were measured using Coulter JT (Coulter Electronics Inc, USA, PN 4235846B). For microscopic study, blood was fixed and stained with Wright buffer or staining solution (Yong Dong Pharm, Co. Kyungki, Korea). The kinds of WBC were distingushed with manual WBC differentiation counter, and reticulocytes were counted after stain using 1% new methylene blue (Sigma).

8. Statistical analysis.

All results are shown as mean ± standard deviation. Statistical evaluation of data was performed at $p < 0.05$ by student's t-test to make comparisons between groups.

RESULTS AND DISCUSSION

1. Observation of animal behavior and conditions

Leung and Foster (1996) reported that intensified irritation may occur on skin and mucous membranes as the side effects of medicinal herb. Bronchospasms may be intensified. Also asian ginseng is a safe herb with low toxicity, but excessive amounts may cause headaches, insomnia, heart palpitations, or a rise in blood pressure in some people. These side effects may cause change the

color on skin, pattern of movement and having more aggressive behavior but there were no such symptoms in testing rat groups (No. 2) compared with normal control group (No. 1) in our study.

2. Observation of internal organ abnormality

After fasting for 16hs at last day of housing, rats were dissected under an anesthetic state and internal organs (liver, heart, lung, spleen, adrena, stomach, testis and kidney) were removed to comparing with control group. Their shape and size were normal, and the color of them were clear.

3. Amounts of ingested food and water, mortality

The daily intake of KMH complex and pine needle oil (No. 2) did not affect amounts of ingested food and water compared with normal control. There was no dead animals in all test group during the study.

4. Weight gain and ratio of liver, kidney weight to body weight

The ratio of body weight change and total body weight gains in treated young adult male Sprague-Dawley (SD) rats during four weeks were no difference with normal control group as shown in Table 2. That is, the daily intake of KMH complex and pine needle oil (No. 2) did not affect body weight gain during the study compared with normal control group ($p<0.05$). The groups administered KMH complex and pine needle oil (No. 2) also exhibited a same results in ratio (%) of liver, kidney weight to body weight compared with normal control. Pirola and Lieber (1975) reported that body weight gain decreased in alcohol-treated rats and body weight decreased by 50% alcohol ingestion instead of sugar in total energy source of man (Piola and Lieber 1972; Karsentry 1985; Kono *et al.* 2001). These results suggested that significant increase or decrease in body weight was due to side effect of medicinal herb like alcohol. But KMH and pine needle oil proved safe in our study.

Table 2. Total body weight gains and the weight ratio of liver and kidney

Group	Total body weight gains (g) Mean±S.D.	Liver (% of body weight) Mean±S.D.	Kidney (% of body weight) Mean±S.D.
No. 1 (Normal control)	120.17±7.84[1)]	2.94±0.077	0.711±0.043
No. 2 (Test group 1)	119.83±16.27	2.98±0.143	0.713±0.042

[1)]Each value represents the mean±S.D. of 6 rats.

5. Urinary analysis

Routine urinary analysis of collected urine from each group is shown at Table 3. Generally, biochemical characters of urine are not only reflected in symptom of kidney and urethral system but also in metabolic disorder, diabetes, liver disease and balance of electrolytes. There was no specific urine abnormality in all test group rats compared with normal control rats.

1) pH of urine

pH 7.5-8.5 of urine were measured in all control and tested groups, there was no specific difference.

2) Albuminuria

Based on standard value of normal albuminuria is between negative (−) and trace (±), but positive albuminuria were detected all tested groups including normal control as shown Table 3. If the value of control is negative and also positive in tested group, it is thought to be diseased albuminuria due to administered sample. This result is suggested as physiological albuminuria by active movement not due to be KMH or pine needle oil because rats are nocturnal animal and active at night.

3) Glucosuria and hematuria

Glucosuria is symptom of diabetes, endocrine disorder,

Table 3. Urinary analysis

	pH	Protein ($U\,mL^{-1}$)	Glucose ($U\,mL^{-1}$)	Ketone ($U\,mL^{-1}$)	Blood ($U\,mL^{-1}$)
No. 1 (Normal control)	7.5	1	0	0	(±)
	8.5	(±)	0	1	0
	8.5	1	0	0	0
	8.5	2	0	1	0
	8.5	0	0	0	0
	8.5	1	0	0	0
No. 2 (Test Group 1)	8.5	1	0	1	0
	8.5	1	0	0	0
	8.5	0	0	0	0
	9.0	2	0	1	0
	8.5	1	0	0	0
	8.0	1	0	1	(±)

Table 4. Biochemical values

	No. 1 (Normal control)	No. 2 (Test group 1)
TP	6.27±0.23[1)]	6.38±0.23
Albumin	3.75±0.14	3.87±0.20
T-Bil.	0.1±0.00	0.1±0.00
ALP	95.00±5.77	102.50±6.98
AST	111.33±35.42	91.00±13.19
ALT	40.33±6.07	37.00±2.65
T-Chol.	75.67±4.82	74.17±6.87

[1)]Each value represents the mean±S.D. of 6 rats. TP, total protein (mg dL^{-1}); Albumin (g dL^{-1}); ALP, alkaline phosphotase (U L^{-1}); AST, aspartate aminotransferase (U L^{-1}); ALT, alanine aminotransferase (U L^{-1}); T-Chol., total cholesterol (g dL^{-1}); T-Bil., total bilirubin (mg dL^{-1}).

pancreatic disease, liver disease and brain tumor. Hematuria is reflected disease in urethral system. The daily intake of KMH and pine needle oil complex (No. 1) did not affect the level of glucosuria and hematuria during the study.

4) Ketone of urine

Urine ketone is a good indicator of fatty acid oxidation, that level is increased by deficiency of insulin. Therefore detecting much ketone in urine is symptom of severe diabetes or insufficiency of glucose in food. As shown in Table 3, positive results were exhibited in all tested group rats and normal control rats. Two possibilities can be suggested, diabetes due to KMH and pine needle oil, or insufficiency of glucose from ingested food. Former is of low possibility because of no detection of glucosuria, and also because of the composition of ingested food (millet protein 22.1%, millet fat 3.5%, millet fiber 5.0%).

6. Biochemical analysis

The results of biochemical analysis were shown at Table 4. The daily intake of KMH and pine needle oil complex (No. 2) did not exhibit significant differences ($p<0.05$) compared with normal control group (No. 1). Therefore, all administered materials in our study have no biochemical toxicity.

7. Measurement of hematological value and CBC differentiation

The results of hematological value and CBC differentiation were shown at Table 5. The daily intake of KMH and pine needle oil complex (No. 2) did not exhibit significant differences ($p<0.05$) compared with normal control group (No. 1). Therefore all administered materials in our study have no hematological toxicity.

Table 5. Hematological results

	No. 1 (Normal control)	No. 2 (Test group 1)
RBC	7.86±0.131)	8.06±0.15
WBC	9.97±1.57	9.60±1.62
HCT	47.80±3.19	48.20±2.14
HGB	15.28±0.17	15.62±0.43
PLT	1072±188.71	1093.67±146.56
Reticulocyte	3.26±0.25	3.07±0.49
Neutrophil Stab.	–	–
Neutrophil Seq.	8.60±3.38	8.00±2.94
Lymphocyte	88.20±5.56	89.00±3.90
Monocyte	3.20±2.48	3.83±1.67
Eosinophil	–	–
Basophil	–	–
Normoblast	–	–
Blast	–	–

[1)]Each value represents the mean±S.D. of 6 rats. RBC, red blood cell count (10^6 mm^{-3}); WBC, white blood cell count (10^3 mm^{-3}); HCT, hematocrit (%); HGB, hemoglobin (g dL^{-1}); PLT, and platelet (10^3 mm^{-3})

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