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Samples

Pine extract drink made of pine bark and phloem provided by the client.

Analyses

Analyses were performed as part of research contract funded by Technology Research Agency (Tekes) in Finland (Preclinic evaluation of health effects of plant derived raw materials; research funding # 400030/03, 2003-2004):

1. **Amount and characterisation of phenolic compounds** was performed using liquid chromatography (HPLC, UV-detection at 280 nm). The following Pine extract drink samples were analysed: best before 09.04.04, 24.04.04 ja 29.04.04. Analyses were made at the University of Turku, Department of Chemistry (prof. Kalevi Pihlaja).
2. **Antioxidative effect** was evaluated using an *ex vivo* LDL (low density lipoprotein) oxidation model system. Oxidation of LDL was monitored by following the formation of lipid oxidation product, hexanal, by head space gas chromatographic method. The sample analysed was Pine extract drink: best before 28.03.03. Analyses were made at the University of Helsinki, Department of Applied Chemistry and Microbiology (prof. Marina Heinonen).
3. **Anti-inflammatory effects** were investigated by measuring the effects of Pine extract drink on nitric oxide (NO) synthesis and COX-2 -mediated prostaglandin production in LPS-stimulated murine J774 macrophages *in vitro*. NO production was estimated by measuring its metabolite nitrite in the cell culture medium by Griess reaction. Expression of inducible nitric oxide synthase (iNOS) -enzyme was measured by Western blot method. Prostaglandin E_2 (PGE_2) production was measured by radioimmunoassay and the expression of inducible prostaglandin synthase (COX-2) -enzyme by Western blot method. Two concentrations of the Pine extract drink were used in the cell culture experiments: 1:100 (1 % of the Pine extraxt drink in cell culture medium) and 1:50 (2 % of the Pine extraxt drink in cell culture medium). The anti-inflammatory tests were carried out at University of Tampere Medical School (prof. Eeva Moilanen).
4. **Drug absorbtion** from small intestine and colon can be simulated with the use of Caco-2 cell line *in vitro* (originally obtained from colon tumour). In USA the FDA (Food and Drug Administration) has made a list of drugs and other compounds with known mechanisms of absorption. These compounds are

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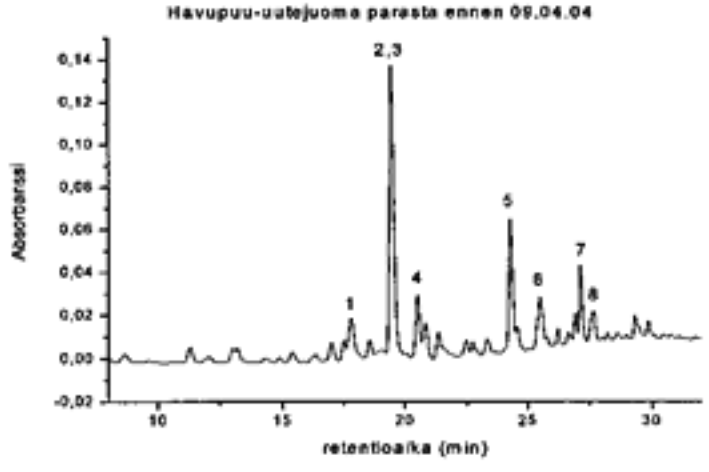
acceptable when absorption tests are validated. Also investigations on **enhancing and inhibitory effects** of absorption can be performed using these known compounds. These effects were investigated for freeze dried Pine bark extract at concentrations of 0.01, 0.1 and 1 mg/ml. The analyses were made at the University of Helsinki, Department of Pharmacology (prof. Heikki Vuorela). The drugs were selected on the basis of their different mechanisms of absorption (travelling) in epithelial cells:

- **Verapamil**: passive and fast absorption; absorption is slowed down by active efflux mechanism
- **Ketoprofen**: active and fast absorption
- **Metoprolol:** passive and fast absorption; both para- and transcellular
- **Paracetamol:** passive and fast absorption; only transcellular (not included in the FDA list, but well known)

Results

1. Phenolic compounds

The Pine extract drink contains different phenolic compounds with higher amounts of resveratrol glucoside and catechin. The amount of identified phenolic compounds in the Pine extract drink is 489 µg/ml. In addition to the identified phenolic compounds, the Pine extract drink contains other phenolic compounds. According to the analysis performed using HPLC-chromatography the following main components are identified:



1	phenolic acid derivative	35 ± 2 µg/ml
2+3	resveratrol glucoside + catechin	249 ± 5 µg/ml
4	ferulic acid glucoside	35 ± 2 µg/ml
5	lignan glucoside	51 ± 1 µg/ml
6	lignan xyloside	32 ± 1 µg/ml
7	taxifolin glucoside	46 ± 2 µg/ml
8	taxifolin	41 ± 1 µg/ml

The amount of compounds 1,4,5 and 6 was quantitated as p-hydroxybenzoic acid, amount of compounds 2 and 3 as catechin and amount of compounds 7 and 8 as taxifolin.

2. Antioxidant activity

Oxidation of LDL is according to the current knowledge one of the biomarkers for the risk of cardiovascular disease. The more antioxidants the LDL particles contain the better the LDL particles can resist oxidative changes of its lipid constituents (fatty acids, cholesterol) and protein constituents. LDL oxidation model system is an *ex vivo* –model for simulating the LDL oxidation *in vivo*. The most efficient antioxidant inhibits LDL oxidation by 100 %. Pine extract drink is a very efficient antioxidant (LDL inhibition 95 %). Phenolic compounds in the pine extract drink contribute to the antioxidative effect.

3. Anti-inflammatory effects

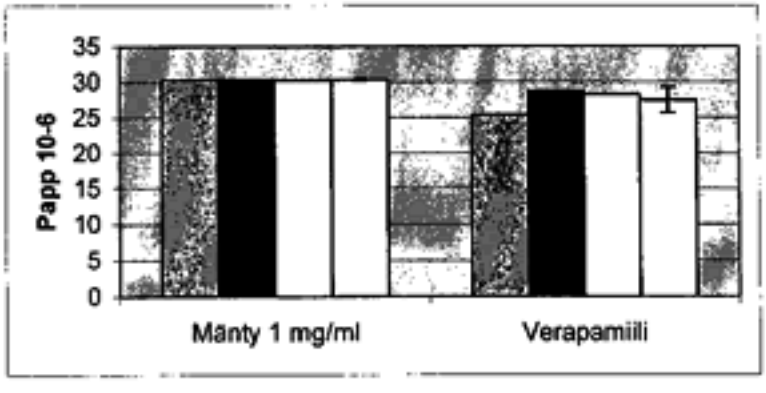
When macrophages are exposed to bacterial lipopolysaccharide, synthesis of inflammatory mediators like nitric oxide (NO) is induced. In these conditions high amounts of nitric oxide are produecd by enzyme inducible nitric oxide synthase (iNOS). Pine extract drink (2 % in cell culture medium) had a slight inhibitory effect on NO production and iNOS expression in these *in vitro* conditions .

Prostaglandin E_2 (PGE_2) is regarded as the most important prostanoid in inflammation. Pine extract drink (1-2 % in cell culture medium) inhibited PGE_2 production significantly, but no effect on COX-2 expression was found. These results suggest that the Pine extract drink contains compounds, that inhibit COX-2 enzyme activity and inhibit prostaglandin formation by that mechanism.

Effects on inflammatory mediators	Concentration of the Pine extract drink in cell culture medium	
	1 %	**2 %**
Inhibition of nitric oxide (NO) production (%)	10 %	17 %
Inhibition of the expression of inducible nitric oxide synthase (iNOS) (%)	No effect	24 %
Inhibition of prostaglandin (PGE_2) production (%)	54 %	60 %
Inhibition of the expression of inducible prostaglandin synthase (COX-2) (%)	No effect	No effect

4. Effects on drug absorption using Caco-2 cell line *in vitro*

Pine extract drink (freeze dried, conc. 1 mg/ml) did have an inhibitory effect on the absorption of metoprolol (see figures below). A dose-effect relation could also be established for inhibition of absorption. In addition, the Pine extract drink samples investigated were not cytotoxic measured in Caco-2 cells with the MTT test.



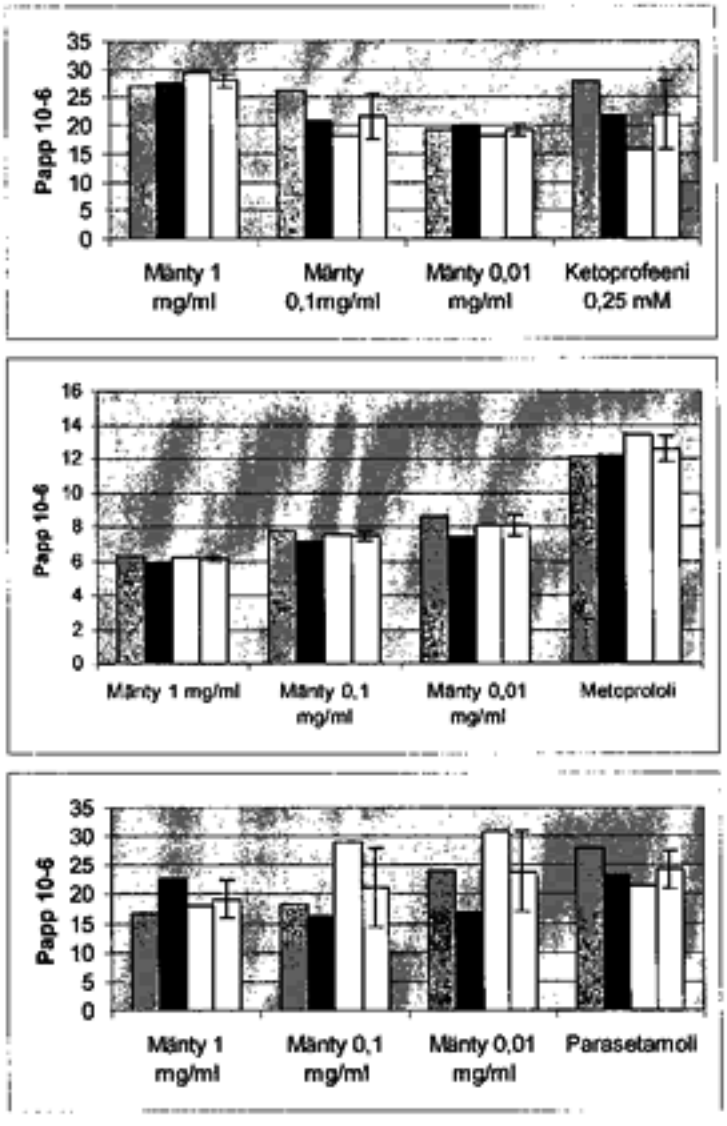


Fig. Effect of Pine extract drink (marked as "Mänty") on the absorption of model drugs across Caco-2 cells. P_{app}-value showns the rate of absorption per square area.

Summary

The Pine extract drink made of pine bark and phloem contains different phenolic compounds with higher amounts of resveratrol glucoside and catechin. The amount of identified phenolic compounds in the Pine extract drink is 489 µg/ml. Pine extract drink is a very efficient antioxidant inhibiting oxidation of human LDL *ex vivo* (95 % inhibition). Phenolic compounds in the Pine extract drink contribute to the antioxidative effect. Pine extract drink (1-2 % dilution) showed an inhibitory effect on prostaglandin production related to anti-inflammatory effects. However, more investigations are needed to verify the same effect *in vivo* after consumption of Pine extract drink. Pine extract drink (freeze dried) did have an effect also on absorption of certain drugs as the drink at concentration of 1 mg/ml inhibited absorption of metoprolol. The Pine extract drink (freeze dried) is not cytotoxic to Caco-2 cells.

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