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Effect of Latitude and Altitude on the Terpenoid and Soluble Phenolic Composition of Juniper (*Juniperus communis*) Needles and Evaluation of Their Antibacterial Activity in the Boreal Zone

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The demand for dry juniper (*Juniperus communis*) needles as a raw material for the food, pharmaceutical, and cosmetic industries has increased rapidly in recent years. Juniper needles are known to be rich in terpenoids and phenolics, but their chemical composition and antibacterial properties have not been well-characterized. In this study, we describe the soluble phenolic and terpenoid composition of juniper needles collected in Finland ($n = 125$) and demonstrate that the concentration of these compounds clearly increased with latitude and altitude with, however, a stronger latitudinal effect (a higher content of monoterpenoids, proanthocyanidins, and flavonols in northern latitudes). Analysis of methanolic extracts showed quite good activity against both antibiotic-sensitive and -resistant *Staphylococcus aureus* strains and suggested an important role of the soluble phenolic fraction. Finally, we demonstrate the relative lack of toxicity of juniper extracts on keratinocytes and fibroblastic cells, raising the possibility of their use in preventing bacterial skin infection.

KEYWORDS: *Juniperus communis*; terpenoid; phenolic; flavonoid; flavone; latitude; methanol extract; antibacterial; *Staphylococcus aureus*; minimum inhibitory concentration

INTRODUCTION

Genus *Juniperus* belongs to the family Cupressaceae, which comprises about 70 species distributed across the Northern Hemisphere. Among them, *Juniperus communis* L. (common juniper) is a familiar species with the characteristic flavor of distilled gin liquor. The ecological scale of occurrence of *J. communis* L. ssp. *communis* is very broad, but the best conditions for its development are found in coniferous forests. Juniper is known as a plant that is rich in secondary substances, particularly terpenoids and phenolics. Common juniper extract is an important natural product used widely in many pharmaceutical and technical preparations, in cosmetic products, and as a food additive (1, 2). Both berries and needles are collected as raw materials for the extraction of essential oils. Although the essential oil of juniper needles is not as commonly used as berry oils, the terpenoid composition of essential oils obtained by distillation of juniper needles from many countries in Europe has been previously described (3–10). However, very few data are available concerning the phenolic composition of juniper needles. Flavonoid glycosides (flavonols and flavones), phenol glycosides,

and recently neolignans have been isolated and characterized from *J. communis* (11–13). Interestingly, as far as is known, a combined analysis of both soluble phenolics and terpenoids in juniper needles has never been reported.

Although a very high variability in the data has generally been observed between individual juniper plants (9, 14), remarkable differences in the composition of essential oils of juniper needles have been observed, depending on the origin (3–10). Environmental conditions have an important effect on the secondary metabolisms (15), but correlations between the chemical composition and the geographical origin of juniper samples are scarce. A correlation between the altitude and the monoterpenoid composition of juniper needles was demonstrated in the Norwegian mountains, where α -pinene was dominant in lowlands, while sabinene was the major monoterpenoid in highlands (9). In Finland, a gradient was observed for different monoterpenoids and sesquiterpenoids in juniper berries from three maritime locations (14).

The demand for juniper needles as a raw material is rapidly increasing nowadays. In Finland, where young shoots are collected in the summer, the demand increased from 1 ton of fresh material in 2004 to 11 tons in 2006 and is expected to be 14 tons in 2010 (2). Latitudes in Finland range from 59°49' N to 70°04' N

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(about 400 km North of the Arctic Circle), ranging from southern boreal coniferous forests in the south of Finland to northern boreal and subarctic conditions in the northernmost parts of Finland. With an average of 143 m, altitudes of Finland are generally low. Increases in latitude coincide with increases in altitude, and in the northern latitudes, even a small shift in the altitude can have a great significance for the growing conditions. As the environmental growing conditions differ considerably from north to south in the country and might affect the secondary metabolisms, the quality and quantity of secondary compounds are very likely to differ among juniper shoots collected from different parts of the country. The effect of the origin of the sample on the composition of juniper shoots collected in the summer is thus a relevant factor to study since the composition of raw material is of great importance for its industrial use. To provide information on this topic, 125 juniper samples were collected in summer along a latitudinal gradient ranging from the south to the north of Finland and analyzed for their terpenoid and soluble phenolic composition.

The important increase in nosocomial infections caused by resistant or multiresistant bacteria is one of the most serious public health challenges of the 21st century, leading to an urgent need for new antibacterial compounds. In addition, food-borne diseases, predominantly involving *Staphylococcus aureus*, are of major concern worldwide, creating a need for the development of new food treatments (16). Numerous naturally occurring pure compounds as well as plant extracts have already shown inhibitory activity, especially on bacteria. Essential oils obtained from berries or needles of common juniper originating in Italy were reported to have no significant antimicrobial activity (4), and weak activity was detected against *Helicobacter pylori* when methanolic extract from American juniper berries was used (17). Although results from the disk diffusion method should be treated with care, due to the effect of the water solubility of the compounds on their antimicrobial activity (18, 19), some inhibitory activity was detected against several bacteria using juniper berry essential oil or oil fractions from Poland (20) and Serbia (7), respectively. In this study, the antimicrobial properties of methanolic extracts of juniper needles were evaluated against reference bacterial strains and antibiotic-resistant clinical isolates, using broth microdilution and agar dilution methods.

MATERIALS AND METHODS

Sampling. In the summer of 2006 (July 3 to August 30), 125 samples were collected from 125 sampling plots selected across Finland between the latitudes 59°58' N and 69°38' N (Figure 1a). The sampling period lasted 2 months mainly due to traveling reasons, as can be seen by the distribution of the sampling plot latitude as a function of the sampling date (Figure 1b). The whole sampling area was divided into five latitudinal zones A–E (Figure 1a,c). From each sampling plot, a minimum of 10 adult juniper (*J. communis* L.) individuals were selected. Several current-year juniper shoots were randomly collected from each individual and sent to the laboratory in paper bags, where the needles were detached, dried (1 day at 40 °C), finely milled, and stored in sealed plastic bags in the dark at 4 °C until extraction. A high number of shoots were collected from each site to minimize the possible individual variability that could result from, for example, plant height or the direction of branches and needles.

Bacterial Strains. *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923 and ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains following the Clinical and Laboratory Standard Institute (formerly NCCLS) (21) and the CA-SFM (22) guidelines. Five clinical isolates, obtained from blood or rectal human specimens, were also tested in this study: methicillin-resistant *S. aureus* (MRSA) (*mecA* gene) (SaR1), vancomycin and teicoplanin-resistant *E. faecalis* (*vanA* gene) (EfR2), constitutive-MLSb *Streptococcus agalactiae* (StaS1),

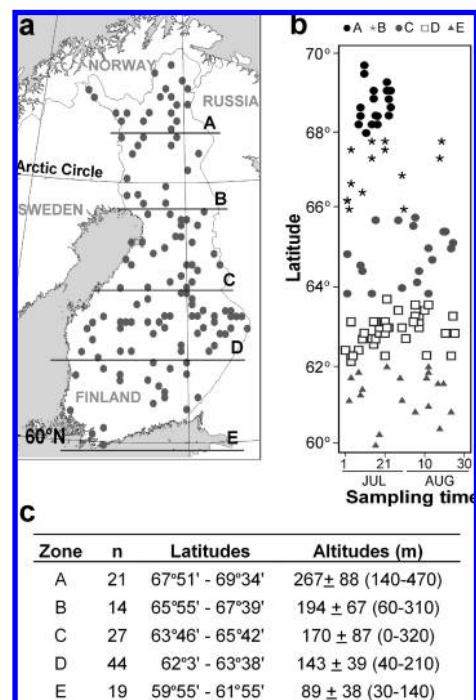


Figure 1. Location of juniper sampling plots in Finland (a), distribution of sampling during summer 2006 (b), and description of the latitudinal zones (c). The five latitudinal zones (A–E) covering the sampling area are indicated in panel a and are represented by different symbols in panel b.

Corynebacterium spp. (CspR1: β -Lactams^R, Macrolids^R, and Fluoroquinolons^R), and *P. aeruginosa* overexpressing efflux pumps (PaR2). Isolates were selected on the basis of their resistant antimicrobial susceptibility testing profile. Antimicrobial resistance was determined by the automated instrument VITEK2 (BioMérieux, France) or disk diffusion assay (23). Strains were grown on Mueller Hinton agar (MHA) (Difco, France) or Mueller Hinton broth (MHB) (Difco, France), complemented with 5% lysed sheep blood (MHS) for exigent bacteria (*S. agalactiae* and *Corynebacterium* spp.).

Extraction and Fractionation. Because of the large number of samples, a one-step extraction protocol was developed to extract terpenoids in hexane and soluble phenolics in aqueous methanol. Milled juniper needles (0.5 g) were extracted with 4 mL of methanol:H₂O (3:1, v/v) + 4 mL of *n*-hexane by shaking for 2 h in the dark at room temperature. Comparative extractions showed that a 2 h extraction time gave optimum yields when using finely milled needles; longer extraction times did not increase the yields and led to degradation of some specific compounds (monoterpenoids and flavones) (not shown). Isoborneol (200 μ g) was added before shaking as the internal standard for terpenoids. After centrifugation, the upper organic fraction was removed and slowly evaporated under a N₂ stream to a final volume of about 300 μ L, centrifuged, and stored at –20 °C for further analysis of terpenoid composition. The lower aqueous fraction was centrifuged and stored at –20 °C for further analysis of soluble phenolics. For microbiological analysis, methanolic extracts were obtained by overnight extraction under shaking in 100% methanol. After filtration, the extract was dried under vacuum, and the dry residue was used for dilution in growth media as described below. For minimum inhibitory concentration (MIC) and respiration measurements of terpenoid and phenolic fractions, 40 g of finely milled juniper needles was extracted in 200 mL of methanol:H₂O (3:1, v/v) + 200 mL of *n*-hexane by shaking for 2 h in the dark at room temperature. The organic and aqueous phases were separated, filtered, and dried under vacuum prior to MIC and respiration analysis.

Terpenoid Analysis. Terpenoids were analyzed from 1 μ L of the concentrated hexane extract by gas chromatography (GC) using an HP-5 (30 m \times 320 μ m \times 0.25 μ m) column (Agilent Technologies, Santa Clara, CA) under the following conditions: injector, 200 °C; detector, 280 °C;

helium as carrier gas (1 mL min⁻¹); and the following temperature program: 50–80 at 15 °C/min, 80–100 at 3 °C/min, 100–160 at 10 °C/min, 160–200 at 3 °C/min, and 200–250 at 15 °C/min. Alkane standard solution C₈–C₂₀ (Fluka, Switzerland) was used to measure the Kovat's index (KI) according to ref 24. Partitioning of isoborneol between the organic and the aqueous fractions during extraction was taken into account for quantification of terpenoids. Typical juniper hexane extracts were analyzed by GC/MS, as previously described (25), to confirm the identification of major terpenoids. Standard compounds were obtained from Fluka, Switzerland [β -pinene, myrcene, (+)-3-carene, α -terpinene, limonene, *p*-cymene, terpinolene, terpinen-4-ol, and bornyl acetate] and Aldrich, Germany (α -pinene, α -phellandrene, terpinyl acetate, α -terpineol, isoborneol, β -caryophyllene, and caryophyllene oxide). Quantification was done using the average response factor between α -pinene and limonene for all monoterpenoids and β -caryophyllene for all sesquiterpenoids.

Phenolic Analysis. Soluble phenolics were analyzed in 10 μ L of the aqueous extract by high-performance liquid chromatography (HPLC) (Waters, Milford, MA) with a Spherisorb ODS II column (4.6 mm \times 250 mm; particle size, 5 μ m) (Waters) using a binary solvent system (solvent A, 1% ammonium formate and 10% formic acid in water; solvent B, 1% ammonium formate and 10% formic acid in methanol). The elution program was as follows: 0–5 min, 0% B; 5–45 min, 0–100% B; 45–86 min, 100% B; 86–90 min, 100–0% B; and 90–120 min, 0% B, at 35 °C and at 1 mL min⁻¹. Detection and quantification were made at 280 nm using a UV/visible diode array detector (Waters PDA 996). The following compounds were used as references for quantification: catechin [for proanthocyanidins (PAs) and unknown compounds], rutin (for all flavonols), and apigetrin (for all flavones).

MIC Determination. MICs were determined by the broth microdilution (21) and agar dilution methods (18). The broth microdilution analysis was performed as has been previously described (26). Stock solutions of juniper extract were prepared by diluting the dry residue obtained after methanolic extraction at a final concentration of 1024 mg L⁻¹ in MHB for bacterial tests and in MHS for exigent bacterial tests. Starting from these stock solutions, 2-fold serial dilutions were prepared in appropriate media in 96-well plates (50 μ L/well) (Greiner, France). An equal volume of bacterial inoculum (1 day culture, 5×10^5 – 5×10^6 CFU/mL) was added to each well. After incubation for 24 h at 35 °C, the MICs were determined with an enzyme-linked immunosorbent assay (ELISA) reader ($A_{540\text{nm}}$ for MHB or $A_{690\text{nm}}$ for MHS, Multiskan EX, Thermo Electron Corp., France). The results are representative of three independent determinations. MICs were determined as well by the agar dilution method in accordance with ref 18 with slight modifications. The dry residue obtained after methanolic extraction of the juniper needles was dissolved in 500 mL of liquid MHA (Scharlau, Barcelona, Spain) supplemented with 1% (w/v) Tween-40 (MP Biomedicals, OH). The dry residue concentrations ranged from 5.0 to 0.5 g L⁻¹ (0.5 g L⁻¹ interval) or from 8.0 to 2.0 g L⁻¹ (1.0 g L⁻¹ interval) in test samples from zones A to D or E, respectively. The media/juniper extract mixtures were autoclaved at 121 °C for 15 min and poured into Petri dishes. *S. aureus* ATCC 25923 was grown overnight at 35 °C on MHA. Bacterial biomass was suspended in a 0.9% NaCl solution and adjusted to match the 0.5 McFarland turbidity standard ($A_{625} = 0.080$ – 0.100). Three aliquots of 1 μ L of the bacterial suspensions were delivered to the surface of three parallel MHA/juniper extract plates using a calibrated loop. The inoculated plates were incubated at 35 °C, and bacterial growth was visually observed after 24, 48, and 72 h of incubation.

Cell Lines and Cytotoxicity Tests. HaCaT [human keratinocytes (27)] were kindly provided by the Pierre Fabre Institute (Toulouse, France), and MRC-5 (human pulmonary embryonic fibroblasts) were obtained from BioMerieux (Lyon, France). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) for HaCaT and in modified Eagle's medium (MEM) (Invitrogen) for MRC-5 supplemented with 10% decomplemented fetal bovine serum (Invitrogen) without antibiotics at 37 °C, 5% CO₂, under humid conditions. HaCaT or MRC-5 cells were plated at 10⁴ cells/well in 96-well plates (Sarstedt, France). Forty-eight hours after plating, the growth medium was removed and replaced with diluted juniper extracts (100 μ L of dry juniper extract diluted in MEM for MRC-5 cells or in DMEM for HaCaT cells, both supplemented with 2% decomplemented fetal bovine serum).

After 24, 48, and 168 h of exposure, cytotoxicity tests were performed using the supravital dye Neutral Red [3-amino-7-(dimethylamino)-2-methylphenazine hydrochloride] (Sigma, France). The test was performed as described previously (23, 26). Eight wells per dose and time point were measured in three different experiments.

Respiration Assay. Nine milliliters of heart infusion broth (Becton Dickinson, Sparks, MD) was inoculated with 1 mL of an *S. aureus* ATCC 25923 suspension previously grown overnight at 35 °C in brain heart infusion broth. The suspension was incubated on a rotatory shaker at 35 °C for 5 h. One milliliter of this suspension was transferred to duplicate 100 mL vials containing 9 mL of brain heart infusion broth supplemented with 1% (w/v) Tween-40 and 1 g L⁻¹ of the dry juniper residue obtained from the terpenoid or phenolic fractions. After 20 h of incubation in headspace vials at 35 °C, the CO₂ produced was measured by headspace GC using a 10 ft Hayesep Q column (Agilent Technology, Santa Clara, CA) and a thermal conductivity detector (inlet temperature, 120 °C; oven temperature, 50 °C; and detector temperature, 200 °C). Microbial numbers were measured by the plate count method on brain heart infusion agar. CO₂ production was normalized to bacterial number.

Statistics. SPSS 15.0 (SPSS, Inc., Chicago, IL) for Windows was used for statistical analysis. After the normality of the variables was checked, linear regression analyses were done to measure the Pearson's correlation coefficient *R* and analysis of variance (ANOVA) *F* and *p* values between the variables and the latitude, altitude, and sampling date. To determine which factor (latitude or altitude) was most significant in predicting the variables, stepwise multiple linear regression analyses were conducted with both latitude and altitude included in the model.

RESULTS AND DISCUSSION

Phenolic and Terpenoid Composition. Because young juniper shoots are traditionally sold to companies as air-dried material, air drying was used as a general procedure before extraction. Different drying temperatures were used to study the evaporation of monoterpenoids in comparison with freeze drying. The yield of α -pinene, the major monoterpenoid (60% of the monoterpenoids, see below), was 33% lower after drying for 1 day at 40 °C and about 50% lower after drying for 1 day at 60 °C as compared to freeze-dried needles (100%). The tested drying temperature had no effect on the yield of sesquiterpenoids. In accordance with these results, a drying temperature of 40 °C was selected for the samples.

Phenolic Composition. Thirty-six peaks were identified by HPLC analysis of the methanolic extracts (Figure 2). According to their UV spectra and comparison with authentic standards, the peaks were gathered into seven groups. In order of decreasing abundance, we identified PAs (including catechin, group 1), flavones (mainly apigenin derivatives, group 2), flavonols (including quercetin derivatives, group 3), and unidentified compounds ("others", group 4). Although unidentified as well, a few compounds with similar spectra were detected and thus were not included in group 4: group 5 (two compounds with λ_{max} of 284 and 311 nm) and group 6 (two compounds with λ_{max} of 280, 296, and 305 or 277, 305, and 328 nm). In addition, two unidentified individual compounds, which were not included in group 4 because of their significant abundance, were detected as follows: U1 ($\lambda_{\text{max}} = 266$ nm and a shoulder at 300 nm, possibly a neolignan as previously described (13)) and U2 ($\lambda_{\text{max}} = 273$ nm). The phenolic composition observed over 125 samples is described in Table 1. An average total phenolic content of 65.1 ± 15.5 mg g⁻¹ dry weight (DW) was measured over all of the samples. High variability was observed in the phenolic content and composition. Flavonols, flavones, and flavan-3-ol derivatives have previously been described in juniper needles but not quantified (11–13).

Terpenoid Composition. Although several diterpenes were previously identified in *J. communis* ssp. *Hemisphaerica* (28), only monoterpenoids and sesquiterpenoids, which appeared as the

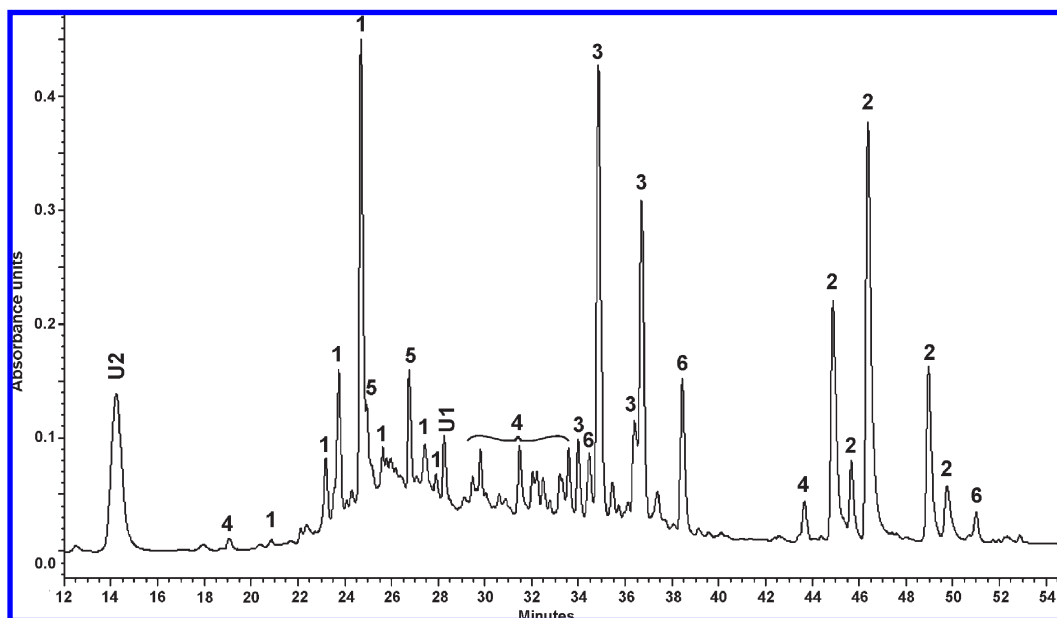


Figure 2. Typical HPLC chromatogram (280 nm) of soluble phenolics in a juniper methanolic extract. Peaks: 1, PAs; 2, flavones; 3, flavonols; 4, “others”; 5, group 5; and 6, group 6.

Table 1. Soluble Phenolic Content (mg g^{-1} DW) and Composition (Weight %) of Juniper Needles and Linear Correlation with the Sampling Time and the Geographical Characteristics (Latitude and Altitude) of the Sampling Plots ($n = 125$)^a

compound	mg g ⁻¹ DW		linear correlation				
			sampling time ^b		geographical factor		
	mean \pm SD	min max	R^c	F^d	factor	R^c	F^d
total phenolics	65.1 \pm 15.5	37.3 99.1	0.346	13.02 ^e	latitude	0.711	124.86 ^e
					latitude, altitude	0.748	76.90 ^e
	%						
PA (1)	29.9 \pm 3.0	21.6 36.6	0.010	0.01	latitude	0.461	32.88 ^e
others (4)	16.5 \pm 2.6	12.8 25.2	0.235	5.63			
flavonols (3)	15.7 \pm 2.4	9.9 22.2	-0.506	33.02 ^e	latitude	0.254	8.42
flavones (2)	11.1 \pm 2.1	6.6 16.7	-0.278	8.05	latitude	-0.613	73.62 ^e
group 5	8.7 \pm 1.2	4.9 12.7	0.147	2.13	latitude	-0.465	33.72 ^e
U1	7.7 \pm 2.5	3.5 13.9	0.413	19.72 ^e	latitude	0.368	19.16 ^e
U2	6.6 \pm 2.7	1.8 14.8	0.039	0.14	latitude	-0.534	48.59 ^e
group 6	3.8 \pm 0.8	1.9 7.1	-0.128	1.59	latitude	0.605	70.55 ^e

^a Only geographical factors that were significant in predicting juniper needle composition are indicated ($p < 0.05$). Content and composition values are mean values \pm SDs ($n = 125$). The group of compound is indicated in parentheses.

^b $n = 97$ because zone A was excluded due to the absence of sampling in August.

^c Pearson's standardized correlation coefficient. ^d Strength of the correlation: F value of ANOVA test. ^e $p < 0.001$.

major classes of terpenoids in juniper needles, were quantified in this study, and their sum is labeled “terpenoid content”. A minimum of 28 monoterpenoids and 22 sesquiterpenoids was detected in juniper needle extracts. Fractionation of hexane extract over a silica (60 Å) column and elution with hexane 100% (fraction I), hexane:diethyl-ether 95:5 (v/v) (fraction II), and hexane:diethyl-ether 90:10 (v/v) (fraction III) allowed fractionation of hydrocarbons in fraction I (most of the monoterpenoids and sesquiterpenoids, including germacrene D and B), and oxygenated compounds in fraction II (including terpinen-4-ol, bornyl acetate, terpinyl acetate, and germacrene D-ol). The average total content of terpenoids over all of the samples was $7.72 \pm 2.85 \text{ mg g}^{-1}$ DW, with monoterpenoids representing only $28.0 \pm 9.3\%$. High variability was measured in the content and

composition of terpenoids (Table 2). The most abundant monoterpenoid was α -pinene, followed by limonene, sabinene, and 3-carene. The lower proportion of monoterpenoids (28% of terpenoids as an average) reported in this study as compared to the composition of juniper essential oil as previously described in the literature is likely due to the drying process and the extraction method (29). High variability was observed between the samples, as has been described previously (9, 14), but the most variable compounds were monoterpenoids, particularly sabinene and 3-carene. Germacrene D-ol and germacrene D were the major sesquiterpenoids, representing together more than 40% of the terpenoids. Germacrene D has frequently been detected in juniper needles, but germacrene D-ol has rarely been described; it was the major compound in *J. sibirica* collected in Mongolia (2500 m) (3).

As far as is known, this is the first report of analysis of both soluble phenolics and terpenoids from juniper needles. Although many compounds remain unidentified, we report here that PAs (including catechin), flavonols, and flavones represent the majority of soluble phenolics in juniper needles with more than 60% of the total soluble phenolics, and germacrene derivatives are the most common terpenoids, with almost 45% of the terpenoids.

Effect of Sampling Time on the Chemical Composition. The period of sampling extended from early July to the end of August due to practical sampling reasons (Figure 1b). Before the effect of latitude and altitude was analyzed, the effect of the sampling date (needle maturation) on the composition of juniper needles was studied. Zone A was excluded from this analysis since no sampling was performed in August in this area. The soluble phenolic content increased significantly during the summer (Table 1), while no significant changes were measured in the terpenoid content (Table 2). Among the phenolics, the percentage of flavones decreased slightly, the proportion of flavonols decreased significantly during the summer, and U1 increased significantly (Table 1). The analysis of zone D alone, the most widely represented one with $n = 43$ samples, confirmed a clear decrease of flavonols during maturation ($R = -0.649$, $F = 30.58$, and $p < 0.001$). The composition of terpenoids remained rather stable during the summer with no change in the proportion

Table 2. Terpenoid Content (mg g⁻¹ DW) and Composition (Weight %) of Juniper Needles and Linear Correlation with the Sampling Time and the Geographical Characteristics (Latitude and Altitude) of the Sampling Plots (*n* = 125)^a

compound	KI ^b	mg g ⁻¹ DW			linear correlation				
		mean ± SD	min	max	sampling time ^c		geographical factor		
					<i>R</i> ^d	<i>F</i> ^e	factor	<i>R</i> ^d	<i>F</i> ^e
terpenoids		7.72 ± 2.85	2.19	16.11	0.018	0.03	latitude	0.622	76.91 ^f
							latitude, altitude	0.662	47.15 ^f
		%							
monoterpenoids		28.0 ± 9.3	6.7	49.4	-0.100	0.97	latitude	0.447	30.41 ^f
sesquiterpenoids		72.0 ± 9.3	50.6	93.3	0.100	0.97	latitude	-0.447	30.41 ^f
monoterpenoids									
α-pinene	942	14.34 ± 6.14	1.65	31.66	0.021	0.04			
limonene	1034	3.05 ± 1.44	0.63	8.52	0.085	0.70	latitude	0.406	23.84 ^f
sabinene	979	2.65 ± 3.62	0.08	23.14	0.136	1.82	altitude	0.551	53.18 ^f
3-carene	1014	1.86 ± 1.53	0.24	9.86	-0.185	3.40	latitude	0.558	55.10 ^f
myrcene	993	1.05 ± 0.51	0.16	2.15	-0.188	3.53	latitude	0.575	60.41 ^f
terpinyl acetate	1355	0.92 ± 0.68	0.01	3.25	0.266	7.31			
β-pinene	982	0.76 ± 0.28	0.11	1.44	0.031	0.09	latitude	0.392	22.15 ^f
terpinolene	1091	0.68 ± 0.36	0.06	2.48	-0.097	0.91	altitude	0.383	21.03 ^f
							altitude, latitude	0.420	12.93 ^f
bornyl acetate	1290	0.62 ± 0.15	0.17	1.13	0.038	0.14	altitude	-0.370	19.32 ^f
sesquiterpenoids									
germacrene D-ol	1585	20.33 ± 6.76	5.45	46.49	-0.212	4.50	altitude	-0.228	6.69
germacrene D	1489	20.31 ± 5.76	1.57	36.10	-0.212	4.53			
α-humulene	1462	5.21 ± 1.68	2.16	11.85	-0.047	0.21	latitude	-0.225	6.52
β-caryophyllene	1427	5.18 ± 2.84	0.00	15.92	-0.048	0.23			
α-murolene	1503	4.13 ± 0.93	0.00	6.57	0.224	5.06	latitude	-0.343	16.31 ^f
β-elemene	1397	3.28 ± 0.96	0.26	6.65	-0.096	0.89			
germacrene B	1567	2.92 ± 1.83	0.35	10.97	0.142	1.97			
γ-cadinene	1522	1.49 ± 1.47	0.22	11.69	0.219	4.85	latitude	-0.254	7.77
δ-cadinene	1530	1.11 ± 0.41	0.39	3.18	0.195	3.81	altitude	-0.237	7.26

^a Only geographical factors that were significant in predicting juniper needle composition are indicated (*p* < 0.05), and only monoterpenoids more abundant than 0.5% and sesquiterpenoids more abundant than 1% are listed. Content and composition values are mean values ± SDs (*n* = 125). ^b KI calculated according to ref 24. ^c *n* = 97 since zone A was excluded due to the absence of sampling in August. ^d Pearson's standardized correlation coefficient. ^e Strength of the correlation: *F* value of ANOVA test. ^f *p* < 0.001.

of monoterpenoids or sesquiterpenoids or in the proportion of the major compounds (Table 2). Similarly, when zone D was considered alone, no significant changes were observed (not shown).

Effect of Latitude and Altitude on the Chemical Composition. Important changes in needle composition were measured with both changing latitude and altitude. Significant increases in the content of both soluble phenolics and terpenoids were measured with increasing latitude, with twice as much of each in northern locations as in southern locations (Figures 3a and 4a). However, the northern parts of Finland are characterized by higher altitudes (Figure 1c), and significant linear correlations can also be measured with increasing altitudes (not shown). Because latitudes and altitudes are closely correlated (*R* = 0.657, *F* = 92.47, and *p* < 0.001), stepwise multiple linear regression analyses were performed to determine whether latitude, altitude, or latitude and altitude is the best factor to explain changes in the composition of juniper needles. Such analyses showed that the content of total phenolics was best predicted by latitude alone, which explained 50.6% of the variation in the concentration of total phenolics (*R* = 0.711, *F* = 124.9, and *p* < 0.001) (Table 1). Latitude and altitude together explained 56.0% of the variation in the concentration of total phenolics (*R* = 0.748, *F* = 76.9, and *p* < 0.001). Similar analyses done for each type of compound showed that altitude was not significant in predicting the soluble phenolic composition (Table 1). In northern locations, the juniper extracts were enriched mainly in PAs but also in flavonols, group 6, and U1, while flavones decreased the most (Table 1). Compounds in

group 4 were unaffected by latitude and altitude. When expressed as mg per g of DW, even stronger positive correlations were calculated between the latitude and the PA (*R* = 0.780, *F* = 189.12, and *p* < 0.001) and flavonol content (*R* = 0.686, *F* = 108.40, and *p* < 0.001) due to the concomitant increase in the total amount of phenolics in needles as latitude increased (Figure 3). For the same reason, a significant positive relationship was found between the absolute amount of flavones and the latitude (*R* = 0.299, *F* = 11.99, and *p* = 0.001), even though the proportion of flavones decreased with increasing latitudes. A similar relationship was found in a parallel study with higher content of soluble phenolics in *Vaccinium myrtillus* leaves in higher latitudes as compared to lower latitudes (Martz, unpublished). In white birch leaves, an increase in flavonols and a decrease in flavones were also measured in higher latitudes in Finland (30).

The proportion of monoterpenoids significantly increased with increasing latitudes and altitudes due to significant changes in the proportion of limonene, sabinene, 3-carene, myrcene, β-pinene, and terpinolene, while the proportion of α-pinene was not affected by the geographical factors (Table 2). Stepwise linear regression analysis indicated that the concentration of total terpenes was correlated with latitude more strongly than with altitude. Latitude alone explained 38.7% of the variation in the concentration of total terpenes (*R* = 0.622, *F* = 76.9, and *p* < 0.001). Latitude and altitude together explained 43.8% of the variation in the concentration of total terpenes (*R* = 0.662,

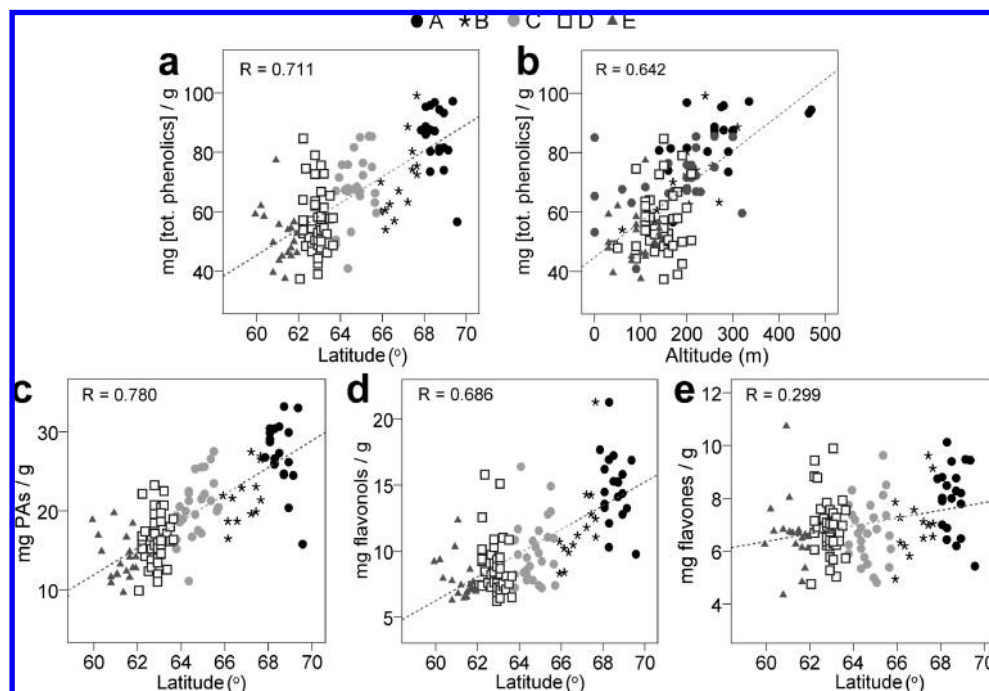


Figure 3. Linear correlation between the latitude, the altitude, and the content (mg g^{-1} DW) of total soluble phenolics (**a** and **b**) and between the latitude and the content in PAs (**c**), flavonols (**d**), and flavones (**e**) in juniper needles. The different symbols represent the sampling zones (A–E) described in **Figure 1**. The corresponding Pearson's coefficient R is indicated in each panel.

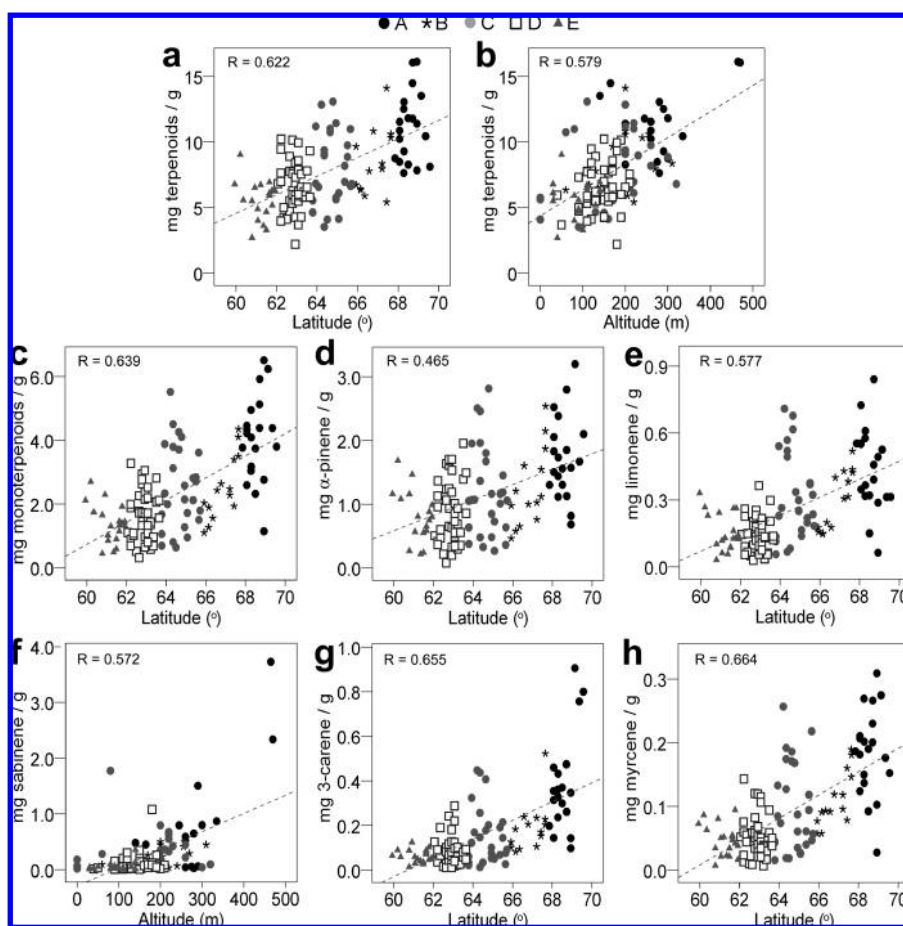


Figure 4. Linear correlation between the geographical factors (latitude and altitude) and the content (mg g^{-1} DW) of terpenoids (**a** and **b**), monoterpenoids (**c**), α -pinene (**d**), limonene (**e**), sabinene (**f**), 3-carene (**g**), and myrcene (**h**) in juniper needles. For each type of compound, only the most significant factor predicting the changes in the composition of juniper needles is represented. The different symbols represent the sampling zones (A–E) described in **Figure 1**. The corresponding Pearson's coefficient R is indicated in each panel.

$F = 47.1$, and $p < 0.001$). Similar analyses done for each terpenoid showed that altitude was significant in predicting the proportion of some specific mono- and sesquiterpenoids (sabinene, terpinolene, bornyl acetate, germacrene D-ol, and δ -cadinene) (Table 2). In general, geographical factors had less influence on the proportion of sesquiterpenoids than on the proportion of monoterpenoids. Interestingly, sabinene was almost completely absent from lower locations, but its content (expressed as mg g^{-1} DW) increased significantly in higher altitudes (Figure 4f). A similar trend was observed with the altitude and more extreme environmental conditions in the Norwegian mountains (9). Because of the high increase in the amount of terpenoids in northern latitudes, the absolute amount of α -pinene per g of DW also increased significantly in juniper needles, although its proportion was not significantly affected by latitude (Figure 4c). Similarly, needles from Scots pine of northern provenance were richer in monoterpenoids than ones of southern provenance (31).

Primary metabolism is an important source of precursors for the synthesis of secondary metabolites, which have a range of functions in metabolism, signaling, and defense against abiotic and biotic stresses (15). Flavonoids are synthesized via mixed-origin pathways from phenylalanine by a reaction sequence that leads to the recycling of ammonium and acetyl-malonyl units, and terpenoids are synthesized from acetyl CoA or glycolytic intermediates. This shows that biotic or abiotic factors affecting the general carbon metabolism, including the synthesis of substrates mentioned above, will have consequences on the secondary metabolite content. The northernmost samples in this study were collected from high latitudes corresponding to a subarctic environment, where plants are continuously subjected to several abiotic stresses, such as frequent frost spells, a great deal of light during the growing season, and nutrient-poor soils. Higher altitudes, especially in combination with northern latitudes, also contribute to more severe growing conditions. All of these factors are known to affect the secondary metabolism pathways (15, 32–34), which may explain the higher content of secondary metabolites found in juniper needles in the subarctic and north boreal sites, as compared with the south boreal sites. Given the differences in the concentrations of beneficial secondary metabolites, raw material collected from higher latitudes would constitute better quality material for the natural product industry.

Composition of Methanolic Extracts Used in Antimicrobial Assays. The juniper samples used in microbiology were prepared differently than the samples described above, with pure methanol without hexane used for needle extraction. The composition of such crude methanolic extracts used for the broth microdilution tests was analyzed using our regular extraction protocol (the crude methanolic extract was re-extracted with methanol and hexane). Two unique latitudinal samples were made by equally combining 14 randomly selected samples from both north (N extract, latitudes ranging from $67^{\circ} 42'$ to $69^{\circ} 38'$ N: zone A and the northern part of zone B) and south (S extract, latitudes ranging from $59^{\circ} 58'$ to $62^{\circ} 33'$ N: zone E and the southern part of zone D). The content of phenolics per g of DW was in the range described in Table 1, but the content of terpenoids was lower than when methanol + hexane were used for extraction (Table 3). An important difference was observed between the N and the S sample: The N sample contained 1.78 times more terpenoids and 1.67 times more soluble phenolics (both expressed as mg g^{-1} DW) as compared with the S sample, which is in agreement with our latitudinal results presented above (Figures 3 and 4). These values represent the content of soluble phenolics and terpenoids in the same volume of crude methanolic extracts, but because dry

Table 3. Terpenoid and Phenolic Content (mg g^{-1} DW) and Composition (Weight %) of the N and S Crude Methanolic Extracts of Juniper Needles

compounds	sample	
	S	N
global composition (mg g^{-1} DW)		
terpenoids	3.4	6.2
phenolics	45.0	75.1
TP composition (%)		
monoterpenoids	5.0	4.5
sesquiterpenoids	95.0	95.5
phenolic composition (%)		
PA	21.9	25.5
flavones	23.2	14.6
flavonols	13.1	19.8
"others"	17.2	14.3
group 5	11.1	9.3
U1	8.9	11.3
U2	3.4	2.7
group 6	1.2	2.5

Table 4. MIC (mg L^{-1}) Obtained for Six Reference Strains and Five Clinical Isolates Using the Broth Microdilution Method^a

bacterial strains	S sample	N sample
reference strains		
<i>S. aureus</i> ATCC 25923	256	256
<i>S. aureus</i> ATCC 29213	128	256
<i>S. epidermidis</i> ATCC 12228	256	256
<i>E. coli</i> ATCC 25922	≥ 256	≥ 256
<i>E. faecalis</i> ATCC 29212	≥ 256	≥ 256
<i>P. aeruginosa</i> ATCC 27853	≥ 256	≥ 256
antibiotic-resistant clinical isolates		
<i>S. aureus</i> SaR1	256	256
<i>E. faecalis</i> Efr2	≥ 256	≥ 256
<i>P. aeruginosa</i> PaR2	≥ 256	≥ 256
<i>S. agalactiae</i> StaS1	≥ 256	≥ 256
<i>Corynebacterium</i> spp. CspR1	≥ 256	≥ 256

^a MICs were measured turbidimetrically after 24 h of growth at 35°C in microplate wells ($n = 3$).

extracts obtained after methanol evaporation were used on a DW basis, only the relative composition of the extract is relevant here. Not surprisingly, the proportion of monoterpenoids was very low in both extracts. The terpenoid composition of each sample was similar (not shown), but some differences were measured in the phenolic composition: The N sample was richer in PAs and flavonols but contained fewer flavones than the S sample (Table 3). It is important to note that crude methanolic extracts contained not only soluble phenolics and terpenoids but also other compounds (sugars, lipids, etc.) not characterized in this study.

Antimicrobial Activity of Juniper Extracts. The antimicrobial activity of the juniper N and S extracts analyzed above for their composition was tested with the broth microdilution method against six antibiotic-sensitive reference bacterial strains and five antibiotic-resistant clinical isolates (23). Our study demonstrated a rather good antibacterial activity of the N and S juniper extracts on all *Staphylococcus* strains tested: *S. aureus* (ATCC 25923 and ATCC 29213) and *S. epidermidis* ATCC 12228 (Table 4). The activity is considered rather good when compared to MICs

Table 5. MICs (g L^{-1}) of Juniper Extracts Determined with the Agar Dilution Method against *S. aureus* ATCC 25923^a

extracts	24 h	48 h	72 h
crude methanolic extracts			
zone A	3.0	3.5	3.5
zone B	3.0	3.5	3.5
zone C	3.0	4.5	4.5
zone D	3.5	4.5	4.5
zone E	3.5	4.5	4.5
phenolic fraction			
zones A + B	5.0	5.0	5.0
terpenoid fraction			
zones A + B	6.0	>8.0	>8.0

^a MICs were determined visually after 24, 48, and 72 h of growth at 35 °C ($n = 3$).

measured with isolated compounds tested using identical experimental conditions (23, 26) or MICs found in other published data (4). No inhibitory activity was detected against the other reference strains. Interestingly, similar MIC values were obtained for the clinical isolates and particularly for the methicillin-resistant *S. aureus* (MRSA) strain. Except for *S. aureus* ATCC 29213, where one dilution higher was measured for the S sample than for the N sample, similar MICs were generally measured for the N and S samples.

In a second type of experiment, MIC determinations were performed using the agar dilution method against the reference strain *S. aureus* ATCC 25923 alone; five samples representing the five latitudinal zones were tested. Each plant sample was prepared by equally mixing randomly selected samples from each sampling zone ($n = 10$ from zones A–D and $n = 5$ from zone E). The plant methanolic extracts were mixed with the agar medium before sterilization by autoclaving as described in the section on methods. All five methanolic extracts showed similar antibacterial activity against *S. aureus* ATCC 25923 (Table 5), similar as well to spruce resin, a natural product of known antibacterial activity and tested using the same agar dilution method (35).

Different MICs were measured for *S. aureus* ATCC 25923 using the broth and agar dilution methods. However, the values are not directly comparable due to methodological differences [exposure to plant extract in liquid/solid cultures, use of agar (36), and emulsifier (37)] and differences in microbial growth (analysis performed in different laboratories). Possible differences in MIC using these two methods were previously reported and appeared to be dependent on the plant extract, likely in relation to the extract composition (19). However, most importantly, the agar dilution method included a step in which the juniper extract/agar mixture was autoclaved. Autoclaving certainly affects the plant extract through the evaporation of volatile compounds (monoterpenoids) and possible destruction of active constituents by heat. This possibility was controlled by analyzing a crude methanolic extract of juniper needles obtained after filtration, the same dry extract resuspended in MHA before and after autoclaving, and the MHA alone. HPLC and GC analysis showed that (i) most of the monoterpenoids evaporated during the extract drying process (a small amount of monoterpenoids was present in the agar medium before autoclaving, as compared to the methanolic extract obtained after filtration, as analyzed in Table 3), (ii) autoclaving provoked the complete evaporation of the remaining monoterpenoids, and (iii) the HPLC and GC profiles of agar before and after autoclaving were qualitatively identical (same peaks), but the quantity of some compounds decreased due

to autoclaving (not shown). Lower amounts of some compounds might actually have contributed to higher MIC measurements when the agar dilution method was used than when the broth microdilution method was used. In addition, the other compounds present in the crude methanolic extract (sugars, lipids, etc.), which might play a role in the antibacterial activity, were not analyzed here. No matter what method of analysis was used, in comparison with published MICs of other plant crude extracts, the crude extracts of juniper needles in our study showed rather clear antibacterial activity against *S. aureus*, which suggests they may potentially be useful in meeting the challenges of public health and food quality.

When different latitudinal extracts were tested using the agar dilution method, slightly lower MICs were measured with northern juniper extracts (zones A and B) than with southern extracts (zones C–E) (Table 5). The difference between the samples became more obvious when the incubation time was longer. Because opposite results were obtained with the broth microdilution method (though against *S. aureus* ATCC 29213 and not 25923), the analysis performed in this study does not allow any clear conclusion about the effect of latitude on the antibacterial activity of juniper needle crude extracts. The opposite results are likely a consequence of the different methodologies used (see above) and highlight the importance of performing different types of microbiological tests when analyzing the activity of plant extracts (19).

Antimicrobial Activity of the Phenolic and Terpenoid Fractions.

Testing separated phenolic and terpenoid fractions of a juniper extract (composite sample made up of samples from zones A and B) with the agar dilution method showed that the phenolic fraction was more effective than the terpenoid fraction in inhibiting the growth of *S. aureus* ATCC 25923 (Table 5). Although the precise mechanism of the action of juniper extract is not known, our results suggest that compounds in the phenolic rather than terpenoid fraction are important for the antimicrobial activity of the juniper needle extract, as already proposed for other plant extracts (19). This hypothesis is also supported by the fact that juniper needle essential oil, which was mainly (90%) composed of monoterpenoids, did not show any significant antimicrobial activity (4, 17). Interestingly, MICs of isolated fractions were also higher than MICs obtained with nonfractionated methanolic extracts (Table 3), suggesting a synergistic antimicrobial effect when phenolic and terpenoid fractions were combined, as has been previously proposed (20).

The effect of the separated phenolic and terpenoid fractions was tested on cellular respiration of *S. aureus* ATCC 25923. The results showed that bacterial respiration was stimulated in the presence of the phenolic fraction as compared to the control (no juniper extract). The terpenoid fraction did not affect bacterial respiration (Figure 5). The stimulation of bacterial respiration in the presence of the phenolic fraction of juniper extract may indicate dissipation of membrane potential created by respiratory enzymes, as previously reported for *S. aureus* in contact with spruce resin (38). Bacterial cells as well as mitochondria may respond to this uncoupling activity of phenolic compounds by increasing their respiration to maintain the membrane potential needed for ATP synthesis (39, 40). Although the mechanisms explaining the antimicrobial activities of juniper oils and extracts are unclear, it can be speculated that due to the complex composition of juniper extracts, the identification of effective compounds or combinations of compounds would be very difficult.

Cell Cytotoxicity. Juniper extracts were tested for their cytotoxicity toward MRC-5 and HaCaT cell lines (CC₅₀, 50% inhibitory concentration). The cell lines used in this study were chosen as a model for the evaluation of antiseptics and/or

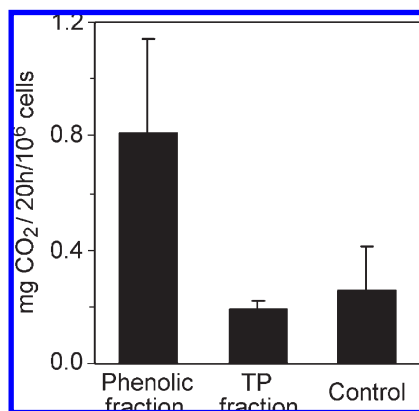


Figure 5. Respiration of *S. aureus* ATCC 25923 in the presence of juniper phenolic fraction (1 g L^{-1}), terpenoid fraction (1 g L^{-1}), and no juniper extract. The production of CO_2 was measured after 20 h of growth in headspace vials at 35°C ($n = 2$).

Table 6. Cytotoxicity (CC_{50} in mg L^{-1}) of Juniper Extracts toward Human Pulmonary Fibroblasts (MRC-5 Cells) and Human Keratinocytes (HaCaT Cells) Measured Using the Natural Red Assay ($n = 3$)

	exposure time (h)	N sample	S sample
MRC-5	24	128–256	≈ 128
	48	≈ 128	64–128
	168	≈ 64	≈ 64
	24	128–256	128
HaCaT	48	128–256	128–256
	168	128–256	128–256

antibiotics that could be used during acute skin infections, as previously proposed by some authors (41). The results using the Neutral Red assay showed that juniper extracts have a low and a very poor toxicity toward the MRC-5 and HaCaT cell lines, respectively (Table 6). A very slight difference, although not considered to be significant, considering the method of analysis used, was observed between the S and the N samples. Viability measurements using MTT [3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, France) assays were also performed as previously described (26). However, the results could not be considered because of technical difficulties with the MTT assay and juniper extracts (MTT was chemically reduced by juniper extracts in the absence of cells, Fontanay, personal communication), precluding evaluation of the selectivity index of the juniper needle extracts. Nevertheless, the poor cytotoxicity of juniper extracts toward human keratinocyte cells allows for the possible application of juniper extracts for the prevention of skin infection.

ABBREVIATIONS USED

CC_{50} , 50% cytotoxic concentration; DMEM, Dulbecco's modified Eagle's medium; DW, dry weight; KI, Kovat's index; MEM, modified Eagle's medium; MHA, Mueller Hinton agar; MHB, Mueller Hinton broth; MHS, Mueller Hinton broth complemented with 5% lysed sheep blood; MIC, minimum inhibitory concentration; PA, proanthocyanidin.

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