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Growth inhibiting activity of volatile oil from *Cistus creticus* L. against *Borrelia burgdorferi* s.s. *in vitro*

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Borreliosis patients from self-help groups reported considerable pain relief after ingestion of *Cistus creticus* leaf preparations. *C. creticus* leaf extracts of different polarities such as aqueous, ethyl acetate, hexane extracts as well as the volatile oil fraction obtained by steam distillation were tested for their antibacterial activity against *Borrelia burgdorferi sensu stricto* (*Bb*ss) *in vitro* using the antibiotic amoxicilline as standard and polysorbate 80 as solubilizer for lipophilic extracts. Comparison of the four plant preparations shows that the volatile oil exerts the strongest growth inhibitory effect. Even concentrations of 0.02% (w/v) volatile oil in cultivation media reduced the total number of bacteria to 2% in comparison to a growth control after an eight-day cultivation period. While the aqueous extract did not reduce bacterial growth, incubation with hexane and ethyl acetate extracts clearly inhibited microbial growth. The main volatile components of the three active extracts tested were analyzed by GC-MS. The number of different labdane-type diterpenes as well as the total relative amount of diterpenes in the samples tested was highest in the essential oil of *C. creticus*. Identification of ten different volatile labdane-type diterpenes was assigned to the essential oil of *C. creticus*. Among these, manoyl oxide, 13-epi-manoyl oxide, 3-acetoxy-manoyl oxide and the monoterpene carvacrol were determined to be major constituents, accompanied by minor amounts of 3-hydroxy-manoyl oxide, all of which are known to exert antimicrobial activity.

1. Introduction

Lyme disease is a multisystemic disorder caused by a group of spirochetes from the *Borrelia burgdorferi* complex (Burgdorfer et al. 1982; Richter et al. 2006; Stanek and Strle 2003). It is regarded as the most common tick-borne disease in Eurasia and North America (Hunfeld and Brade 2006). The outcome of Lyme borreliosis affected individuals is highly dependent on the still controversially discussed antibiotics-based pharmacotherapy (Baehr et al. 2008; Petric et al. 1998). At present, patients suffering from persistent borreliosis in Saxonian self-help groups have found *Cistus creticus* L. preparations such as tea infusions and nutraceuticals (aqueous dry extract in capsules) to improve their clinical conditions (for detailed information see Hückel and Rauwald 2009). Plants of the genus *Cistus* are indigenous to the Mediterranean area and have been traditionally used as a remedy for infections since early antiquity and the Greek-Roman period, e.g. for treatment of skin diseases, gastrointestinal disorders and wound healing (Zohary 1983; Lenz 1859; Madaus 1938). Recent investigations on *Cistus* species *in vitro* found evidence of antibacterial, antifungal, antiviral and antiinflammatory properties (Chinou et al. 1994; Yesilada et al. 1997a; Demetzos et al. 1997, 1999; Ehrhardt et al. 2007; Güvenc et al. 2005; Bouamama et al. 2006, 1999; Petereit et al. 1991), antiproliferative/cytotoxic (Dimas et al. 2001; Chinou et al. 1994; Angelopoulou et al. 2000; Lendeckel et al. 2002)

as well as gastric antiulcer activities (Yesilada 1997b; Yesilada et al. 1999) of *Cistus* leaf extracts, of the oleoresin labdanum and of the volatile oil prepared from *Cistus* plants. Chemical studies, conducted on different species of the genus, revealed that their active constituents mainly consisted of 'polyphenols' with considerable qualitative and quantitative variability, such as flavonoids (flavonol, flavone, flavan-3-ol derivatives) as well as oligomeric and polymeric proanthocyanidins and ellagitannins, in addition to individual low molecular phenolics (Petereit et al. 1991; Danne et al. 1993). On the other hand, labdane- and clerodane-type diterpenes are the most characteristic constituents of the oleoresin labdanum along with mono-, sesquiterpenes, higher-chain fatty acid esters and alkanes (Chinou et al. 1994: Demetzos et al. 1994). In the essential oil of *C. creticus*, a high amount of labdane diterpenes was found (Demetzos et al. 1989), which exhibited strong antimicrobial activity (Yesilada et al. 1997b, 1999; Angelopoulou et al. 2000). Although various *Cistus* preparations have been tested against different microorganisms, to our knowledge an investigation of antispirochetal, especially of potential antiborrelia activity has never been published before. From the view of phytotherapy, solely grapefruit seed extract has been tested against *Borrelia burgdorferi* s.s. (*Bb*ss) *in vitro*, but without any relation to therapeutical use (Brorson and Brorson 2007). Here, the activity of *C. creticus* leaf preparations of different polarities against mobile forms of *Bb*ss *in vitro* is reported for the first time.
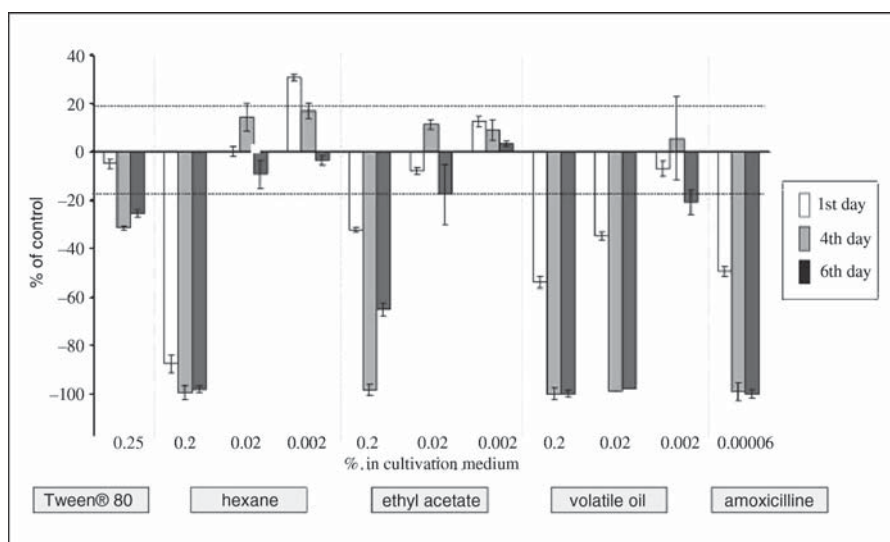
Fig. 1: Changes in *Borrelia* growth in comparison to the control after incubation with hexane and ethyl acetate extracts as well as essential oil from *C. creticus* in three different concentrations over an eight-day cultivation period. The growth of the negative control is set to zero. The impact of the different extract preparations of *C. creticus* on bacteria growth is expressed in percent growth reduction compared to the negative control. The dashed lines (- - -) represent $1 \times SE$ of the negative growth control

2. Investigations, results and discussion

Extracts of different polarities were prepared, such as aqueous (A), ethyl acetate (B), hexane (C) extracts as well as the volatile oil fraction (D) obtained by steam distillation according to Ph.Eur. in order to investigate whether *C. creticus* leaf preparations do exert antispirochetal activity against *Borrelia burgdorferi* sensu stricto (*Bb*ss) *in vitro*. Data obtained from growth inhibition tests utilizing *Bb*ss organisms with 2 mg/mL of each extract, A, B, C and fraction D from *C. creticus,* demonstrated significant differences in the activities of these samples. While the aqueous extract (A) exhibited no growth-reducing activity in the concentrations applied over a cultivation period of 8 days (data not shown), growth reduction was obvious for the more lipophilic preparations B, C, D from the first day of incubation. The inhibitory effect lasted through the whole eight-day cultivation period. Fig. 1 shows susceptibility testing on bacterial growth of *Bb*ss for three different concentrations of the previously tested leaf preparations B, C and D. Incubation with B and C displayed a distinct growth inhibitory effect for 0.2% (w/v) concentration of the extracts in medium only. For this concentration, growth reduction occurred from the first day of incubation and lasted through the entire incubation period. While incubation with the hexane extract seemed to inhibit the regeneration of the *Borrelia* culture almost completely, cultures treated with ethyl acetate extract showed partly restored exponential growth after the fourth day of cultivation. Consequently, the ethyl acetate extract was less active in comparison to the hexane extract. However, most effective growth inhibition was achieved by incubating *Borrelia* organisms with the volatile *Cistus* oil (D). A concentration of 0.2% (w/v) volatile oil in the cultivation media led to a complete stagnation of bacteria growth, while a concentration of 0.02% (w/v) of D resulted in reduction of the total amount of bacteria to about 2% (w/v) after eight days in comparison to the negative control. Inhibitory effects were observed to occur at concentrations of 0.01% (w/v) volatile oil in cultivation medium and are strongest directly after incubation (Fig. 2B). Exponential growth of bacteria was clearly affected at a concentration of 0.015% (w/v) volatiles in cultivation media. At 0.015% (w/v), the amount of mobile spirochetes was reduced by approximately 85% compared to the control after the first day of incubation time. The number of bacteria not affected by treatment with 0.01% and 0.015% volatile oil could regenerate over the cultivation period to reach the amount
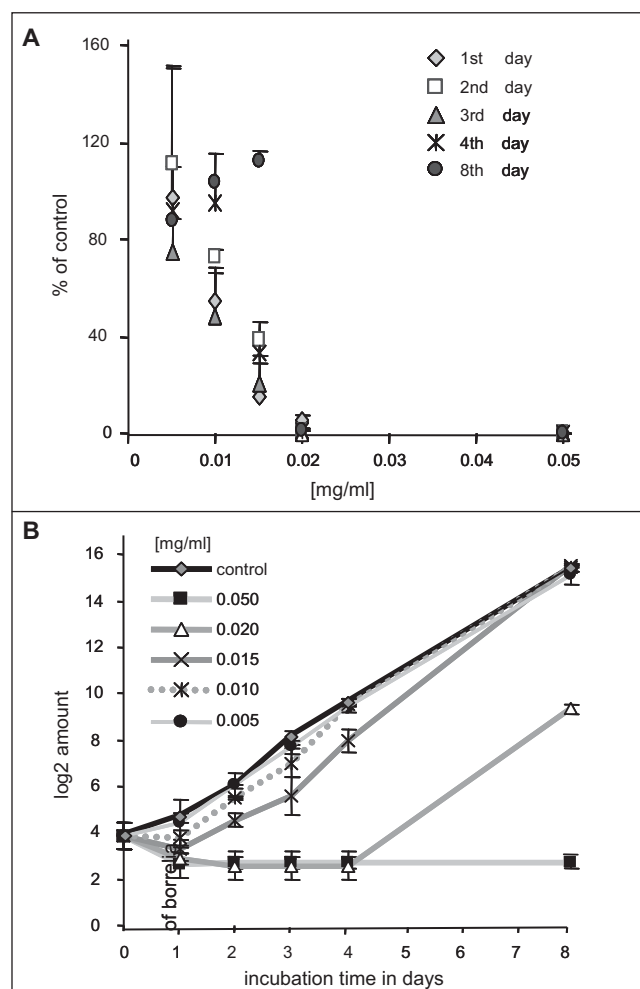

Fig. 2: Dose-dependent impact of the volatile oil fraction of *C. creticus* on *Borrelia* growth. A: Growth curves of *Borrelia* cultures treated with different concentrations of the essential oil of *C. creticus* in cultivation medium. Logarithmic growth of bacteria cultures starts to be clearly inhibited at concentrations of 0,015% essential oils in cultivation media. B: Fraction of the *Borrelia* populations (in %) in comparison to control under the influence of different extract concentrations over the cultivation period. Bacteria cultures are able to regenerate under the influence of essential oils in cultivation media in concentrations below the treshold value of 0,02%
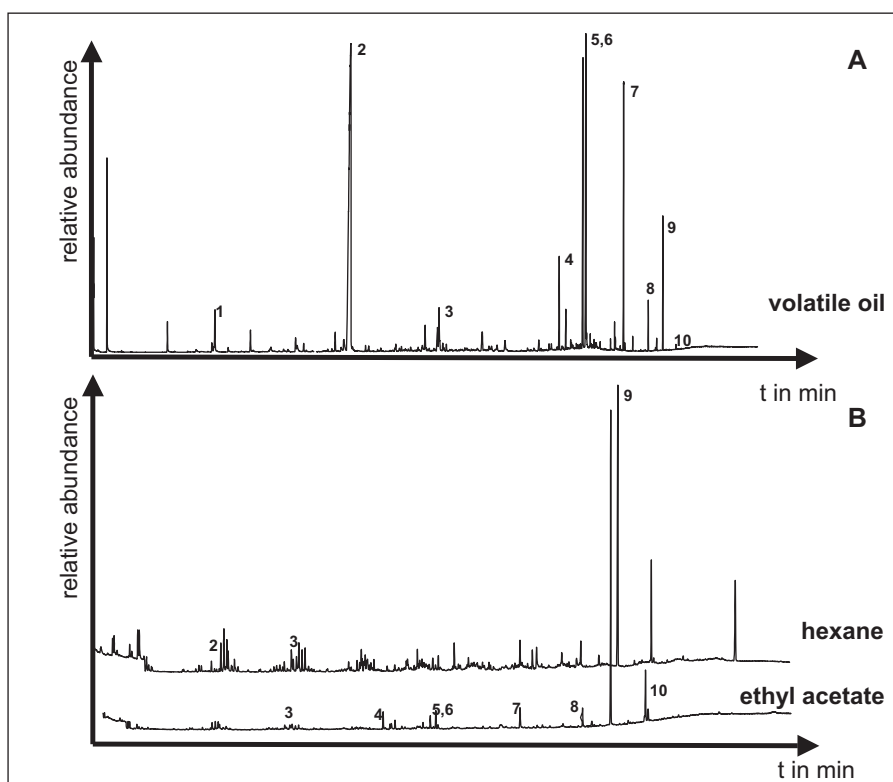
Fig. 3: Gas chromatographic comparison of the essential oil, the hexane and the ethyl acetate extracts of *C. creticus*, 1: cineole; 2: carvacrol; 3: calamanene; 4: hexahydrofarnesylacetone; 5: manoyl oxide; 6: epi-manoyl oxide; 7: 3-acetoxy-manoyl oxide; 8: heptacosane; 9: nonacosane; 10: hentriacontane

of bacteria in controls after eight days. Incubation with 0.02% (w/v) volatiles completely inhibited bacteria growth for the first four days. After eight days, a regeneration of the bacteria cultures was observed (Fig. 2A), even though the total amount of bacteria was only 2% (w/v) of the control. As there were no visible changes in the appearance of *Borrelia* examined by dark field microscopy (not shown), it is concluded, that the bacteriostatic and/or bactericide principle in *C. creticus* acts immediately. The observed effect appeared to be dose-dependent (Figs. 1 and 2). Complete growth inhibition started to occur at 0.05% of volatile oil (D) in cultivation media. With the effect of all active extracts declining over time and the strongest activity concentrated in the essential oil (D), the volatile components seem to be of major importance for the antispirochetal activity of *C. creticus*.

In all experiments, the standard antibiotic amoxicillin was used as positive control. At the applied concentration, it always caused a growth inhibition of 100% after four days (Fig. 1); in contrast, the also tested doxycycline did not have such an effect on bacteria growth at the applied concentration (data not shown), which is in agreement with Sicklinger (2003). In this context, we observed a strong inhibitory effect on *Borrelia* growth by means of DMSO as solubilizer. Even concentrations of 0.1% (v/v) DMSO in the cultivation medium reduced the amount of bacteria to about 60% compared to the control after eight-day cultivation periods. In contrast to DMSO, the effect of polysorbate 80 (Tween® 80) on the bacterial growth was found to be negligible (Fig. 1). Thus, the more apolar fractions were solubilized in water by addition of polysorbate 80.

With the strongest antibacterial activity concentrated in the volatile fractions, GC-MS was chosen for chemical characterization of the mentioned *Cistus* preparations. As expected, the composition of the active extracts B, C, D showed considerable differences between the gas chromatogram of the essential oil (D) and the chromatograms of the two less active extracts B and C (Fig. 3), particularly in the relative intensities of the main compounds. In the essential oil fraction, besides carvacrol

the labdane-type diterpenes manoyl oxide and epi-manoyl oxide are the most abundant constituents, followed by 3-acetoxy-manoyloxide and minor amounts of 3-hydroxy-manoyl oxide. Furthermore, the phenolic monoterpene carvacrol was determined as a major constituent of the essential oil, so that three of the five most prominent compounds belong to the group of labdanes (Table). In comparison, gas chromatograms of hexane and ethyl acetate extracts are dominated by nonacosane, followed by hentriacontane. Altogether, ten different labdane-type diterpenes were tentatively identified in the volatile oil of *C. creticus* using mass spectral libraries and automatic peak finding and matching (Table). Only seven different labdanes were found in the hexane (C) and six in the ethyl acetate (B) extracts. In direct GC-MS analysis of the aqueous extract (A), no labdane-type diterpenes were detected (data not shown). This might be due to too low concentrations, since TLC analysis of an enriched lipophilic fraction obtained by partition of the aqueous extract into dichloromethane indicated the presence of diterpenes in aqueous decocts such as A (data not shown).

Our data clearly indicate, that labdane-type diterpenes are an essential part of the active principle. The observed effect of the samples tested on bacteria growth was strongest directly after incubation, which could be due to a decreasing amount of volatile components over time. Furthermore, the effect on *Bb*ss growth was strongest in the essential oil fraction (D), which showed the highest relative abundance of the labdane-type diterpenes due to GC-MS analysis and the highest number of different labdane-diterpenes in total as well as significant amounts of carvacrol. Carvacrol, the major component of *Origanum* essential oils, has been described to exhibit considerable antibacterial activity (Sivropoulou et al. 1996; Dorman and Deans 2000). However, pronounced antimicrobial activity among *Cistus* species is discussed in the literature especially for the labdanes (Demetzos et al. 1997, 2001, 2002; Chinou et al. 1993; Anastasaki et al. 1999). In particular, diterpenes possessing an *ent*-13-epi-manoyl oxide skeleton, such as *ent*-13-*epi*-manoyl

Table: Proposed assignments of main components of the essential oil and their relative intensities normalised to the most abundant compound

Name	RT	RI	% Area[*]	Hexane	Ethyl acetate
α-Pinene	11.48	1000	9		
Cymene	15.89	1015	4		
1,8-Cineole	16.20	1022	13		
Limonene	16.09	1019	1		
γ-Terpinene	17.51	1050	2		
Linalool	19.74	1098	7		
α-Terpineole	24.22	1194	6		
Carvacrol (=p-cymen-2-ol)	29.46	1309	100	X	
α-Cububene	30.92	1342	2	X	
cis-Caryophyllene	33.92	1411	2		
β-Selinene	36.84	1481	7	X	X
Amorphene	38.07	1510	8		X
Calamanene	38.22	1514	12	X	X
cis-2-Cadina-1,4-diene	38.60	1522	2	X	X
α-Calacorene	38.94	1530	2		
Cubenol	42.52	1610	9		
α-Selin-11-en-4-ol	43.99	1643	2		
Ambroxide	48.14	1757	3		
Hexahydrofarnesylacetone	50.17	1845	20	X	X
Abietatrene	53.26	2058	2		
Tricosane	55.76	2300	2	X	X
Pentacosane	57.48	2497	2	X	X
Heptacosane	59.04	2690	8	X	X
Nonacosane	60.47	2869	22	X	X
Hentriacontane	61.79	3031	1	X	X
Labdane-type diterpenes					
Putative norlabdene	48.83	1781	0.06		
Sclareoloxide cis A/B	50.82	1881	11	X	X
Sclareol oxide cis B/C	51.88	1949	1		
Manoyl oxide	52.52	1993	100	X	X
13-epi-Manoyl oxide	52.80	2016	86	X	X
Manool	53.26	2058	1		
Putative labdienol	53.63	2092	2	X	
2-Keto-manoyl oxide	55.27	2248	3	X	X
3-Hydroxy-manoyl-oxid	55.67	2290	6	X	X
3-Acetoxy-manoyl oxide	56.58	2387	74	X	X

In the second part of the Table, labdane-type diterpenes are summarized. Manoyl oxide and carvacrol exhibited the same approximate absolute abundance. The presence of compounds in hexane or ethyl acetate extract is indicated in the two rightmost columns

[*] signal relative to most abundant signal; reference assignments mainly based on customer library, NIST05; Demetzos et al., Gonzales et al., Weyerstahl et al.

oxide or its derivatives hydroxylated in position C-3, *ent*-3β-hydroxy-13-*epi*-manoyl oxide (ribenol), also found in our GC analyses, have been proven to be powerful antimicrobial agents (Demetzos et al. 1994a, 2002; Kalpoutzakis et al. 1998) and have also been cited to possess interesting activity against *Leishmania donovani* (Garcia-Granados et al. 1997).

To obtain new leads in antimicrobial drug development, ten new hemisynthetic manoyl oxide derivates were prepared showing antimicrobial activity against all tested gram(+) and gram(−) bacteria (Kalpoutzakis et al. 2001). On the other hand, manoyl oxide derivatives were found as major constituents of the resin labdanum (ladano) obtained from aerial parts of *Cistus creticus*, e.g. ribenol constitutes 2% of the whole extract (Kalpoutzakis et al. 1998). Extracts from various Mediterranean *Cistus* species prepared according to different polarities, were found to exhibit some *in vitro* activity against individual bacterial strains for both polar, aqueous and methanolic extracts, in which polyphenols like proanthocyanidins are assumed to be the active principle, as well as for more lipophilic ethyl acetate, chloroform or hexane extracts, the essential oil or the resin labdanum (Bouamama et al. 2006; Güvenc et al. 2005). Our findings that lipophilic fractions containing labdane diterpenes display highest antimicrobial

activity against *Bb*ss correlate with literature data obtained with other microorganisms (Anastassaki et al. 1999; Chinou et al. 1994; Demetzos et al. 1997, 1999; Güvenc et al. 2005).

On this basis, further evaluations are necessary to elucidate the definite active principles of *C. creticus* against *Bb*ss growth. This will require isolation and structural elucidation, especially of labdane diterpenes, by stereochemical assignments by either enantioselective GC as shown by Pietsch (2000) for *ent*-13-epi-manoyl oxide from *Araucaria araucaria* and/or by conformational/configurational analysis using high field NMR, computational analysis or X-ray diffraction, as described for example by Kolocouris et al. (2006) and Weyerstahl et al. (1998), combined with antimicrobial assays for the isolated individual constituents.

In summary, our data show that lipophilic leaf extracts of *C. creticus* exert antimicrobial activity against *Bb*ss *in vitro*; the polar extract exhibited no anti-*Borrelia* activity. Among the tested lipophilic preparations, the ethyl acetate extract was less active than the hexane extract, while the essential oil exhibited the highest growth inhibitory activity. Considerable impact on exponential growth of the microorganisms started to occur at concentrations of 0.015% (w/v) essential oil in cultivation

media. GC-MS analysis of the active extracts showed that in the essential oil besides carvacrol three labdane-type diterpenes, manoyl oxide, epi-manoyl oxide and 3-acetoxy-manoyl oxide, displayed the highest relative abundance. In contrast, in ethyl acetate and hexane extracts nonacosane was the most prominent compound with a similar relative abundance as the manoyl oxides in the volatile oil. Altogether ten different labdane-type diterpenes could be identified in the essential oil, whereas only seven and six labdane-type diterpenes were found in the hexane and ethyl acetate extracts, respectively. Our data provides evidence that the group of labdane-type diterpenes, which possesses a wide spectrum of biological activities, and in particular antimicrobial activities, as also described for carvacrol, may clearly be regarded as an essential growth-reducing active principle of *C. creticus* on *Bb*ss. Nevertheless, further studies are necessary to elucidate the definite nature of the active principles.

3. Experimental

3.1. Plant material

Flowering aerial parts, mainly consisting of mature leaves from *Cistus creticus* L. were collected in the hills of Serik, province Antalya, Turkey. Specimens were identified by Ü. Karagöz, Antalya, according to Baytop (1994) and Demoly (2006). The dried and machine-cut plant material (Ch.-N. 1139610601) was obtained from LR Health and Beauty Company, Ahlen, Germany, where a voucher specimen is deposited.

3.2. Preparation of Cistus extracts of different polarities

Extraction of 150 g air-dried and grossly pulverized leaves was performed by repeated maceration with agitation at room temperature in hexane and ethyl acetate, respectively, filtered and evaporated to dryness to yield 1.2% (w/w) hexane (C) and 2.4% (w/w) ethyl acetate extract (B). The volatile oil fraction (D) was obtained by steam distillation according to Ph. Eur. for 3 h to yield 0.10% (w/w). The resulting yellow oil was stored at $-20\,^{\circ}$C until used. Analogously, 150 g *Cistus* leaves were extracted by boiling water for 30 min according traditional and commercial (aqueous dry extract in capsules) use. The extract was filtered, deep-frozen and lyophilized resulting in a final concentration of 23.6% (w/w) aqueous extract (A).

3.3. Sample preparation for antibacterial testing

For antispirochetal assay 40 mg/mL solutions from each extract were prepared as follows. The aqueous extract (A) was diluted in distilled water, the ethyl acetate (B), hexane (C) and volatile oil fractions were dissolved in aqueous solutions using 5% (w/v) polysorbate 80 (Tween® 80) or DMSO, respectively. These solutions were further diluted (1:10, 1:100) and sterile filtered through a 0.2 μm filter. Final concentrations in BSK II cultivation media (Straubinger et al. 1995) were 0.2% (aqueous extract); 0.2%, 0.02% and 0.002% (w/v) for hexane and ethyl acetate extracts. Essential oil was applied additionally in final concentrations of 0.005%, 0.01%, 0.015% and 0.05% (w/v).

3.4. Bbss cultivation and determination of antispirochetal activity

High-passage organisms of *Bb*ss N40 isolates (passages 52 to 60) were grown in BSK II medium in air-tight vials at 37 °C. Susceptibility testing of mobile spirochetes to the different extract types was performed in BSK II medium. Sterile filtered *C. creticus* extract 150 μL was added in concentrations as described above; 50 μL of bacterial suspensions in logarithmic growth were added making a final concentration of 10^5 or 10^6 bacteria/mL and a final volume of 3 mL. Negative controls were prepared with bacteria in medium only, with bacteria in medium and addition of distilled water (150 μL), or of distilled water containing 5% Tween® 80 or DMSO, each in concentrations of 5%, 2.5%, 0.5%, 0.1%, 0.05% (w/v), respectively. Since DMSO had an inhibiting impact on *Borrelia* growth even in the lowest concentration tested, it was therefore not used further. Positive controls were spirochete cultures with standard antibiotics: doxycycline at a final concentration of 16 μg/mL or amoxicillin at a final concentration of 0.5 μg/mL. Bacteria growth was monitored by dark-field microscopy (400×) at least on days 1, 4, and 8 of incubation using a Petroff-Hausser counting chamber. The minimal counting days were determined precedingly according to the growth curve of *Bb*ss. Each assay was performed in triplicates and repeated independently.

3.5. GC-MS analyses

The extracts prepared as described above (1 μL), were subjected to GC-MS-analysis on a GC-Quadrupol HP-MSD 5973 (Agilent, Waldbronn, Germany) using splitless injection. GC was equipped with a DB-5MS column, 30 m length, 0.25 mm id, 0.25 μm film and a 5 m guard column, run with helium as carrier gas at a flow rate of 1 mL/min. For GC analysis of volatile oils, injector temperature was set to 180 °C. After an initial period of 2 min at 35 °C, oven temperature was ramped at 3 °C/min to 150 °C, held for 5 min, and ramped further at 10 °C/min to 330 °C. For GC-MS analysis of hexane and ethyl acetate extracts, injection temperature was set to 250 °C. After an initial period of 2 min at 70 °C oven temperature was ramped with 9 °C/min to 330 °C and kept for 30 min. Kovacs indices of the compounds were estimated with respect to a parallel run of an alkane mixture (Van den Dool and Kratz 1963).

3.6. Evaluation of GC-MS files

GC-MS raw files were subjected to automated peak finding and matching using AMDIS-Software 2.65 (National Institute of Standards Technology, Gaithersburg, USA) against a customer library of volatile organic compounds and NIST05 mass spectral library (National Institute of Standards Technology, Gaithersburg, USA). The obtained report list of library hits was filtered for a maximum retention time index window of ±20, a minimum reverse match of 90, and a signal to noise ratio of minimum 200. After that, the selected list was validated manually by spectra comparison to reduce every peak down to a single hit for peak identification. In addition, labdane-type and other diterpenes known from the literature (Weyerstahl et al. 1998; Demetzos et al. 1999; Gonzalez et al. 1973) were searched for in the GC-MS-chromatograms using theoretical m/z values of their M+, [M-15]+, and reported typical fragments. Potential candidates were matched with reported spectra and after successful comparison incorporated into the list of analytes.

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