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Growth inhibiting activity of lipophilic extracts from *Dipsacus sylvestris* Huds. roots against *Borrelia burgdorferi* s. s. *in vitro*

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Fresh first year roots from *Dipsacus sylvestris* HUDS. were extracted with 70% ethanol, ethyl acetate as well as dichloromethane. Extracts were solubilized in water (lipophilic extracts with addition of polysorbate 80) and tested for their activity against *Borrelia burgdorferi* sensu stricto *in vitro* during an eight-day period using amoxicillin as standard. The hydroethanolic extract showed no growth inhibition whereas significant growth inhibiting activity could be shown in the two less polar fractions for the first time. Strongest inhibition was found in the ethyl acetate extract. The effect of polysorbate 80 on bacterial growth was examined and found to be negligible. As the nature of bioactive constituents has not been clarified yet, a micellar electrokinetic capillary chromatography fingerprint analysis for a methanolic extract was applied including loganin, chlorogenic acid, cantleyoside and caffeic acid as marker substances.

1. Introduction

Lyme borreliosis is a widespread tick-borne disease of the northern hemisphere (Stanek and Strle 2003). It is caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex (Burgdorfer et al. 1982; Barbour 1984). Affected individuals are treated with a controversially discussed antibiotic-based pharmacotherapy (von Baehr et al. 2008). Particularly with regard to chronic courses of borreliosis like arthritis (Steere et al. 1977), antibiotics are not always successful remedies (Clarissou et al. 2009). Novel ethnobotanical approaches are based on herbal medicines, such as treatment with hydroethanolic extracts of teasel, obtained from the roots of *Dipsacus sylvestris* HUDS. (Wood 1997; Storl 2007), Dipsacaceae, although antibacterial effects of the European teasel have not been described so far. In this context, solely grapefruit seed extract without any relation to therapeutical use (Brorson and Brorson 2007), and due to patient's reports *Cistus creticus* extracts by bioguided fractionation in our lab (Hutschenreuther et al. 2010) were tested against *Borrelia burgdorferi in vitro*.

The biennial *Dipsacus sylvestris,* introduced to Europe in antiquity, grows to a basal rosette with a strong tap root in its first year of cultivation, followed by the flowering period in the second year. Analogous to the use of the traditional Chinese medicine drug Xu Duan from D. asperoides against symptoms similar to borreliosis, roots from *Dipsacus sylvestris* are used by the ethnobotanists - only in the first-year stage due to philosophic thoughts. But this usage is heavily disputed: up to now there is no proof of any antibacterial effect of *Dipsacus sylvestris*.

2. Investigations and result

The present research work examined for the first time the growth inhibiting activity of defined plant extracts from *Dipsacus sylvestris* against high-passage *Borrelia burgdorferi* sensu stricto (*Bb*ss) *in vitro*. Over an eight-day period *Bb*ss in BSK-II-medium inoculated with different dry extracts was quantified by counting all visible individuals (mobile and immobile forms) in aliquots with the help of dark-field microscopy in a Petroff-Hausser counting chamber on days one to four and eight (Straubinger et al. 1995; Lakos et al. 1993). Those extracts were obtained out of fresh roots of *Dipsacus sylvestris* by ethyl acetate (drug-to-extract-ratio 117.8:1), dichloromethane (219.2:1) and ethanol 70% (V/V) (13.6:1). Each was repeatedly tested using polysorbate 80 as suitable solubilizer for the apolar extracts (Hutschenreuther et al. 2010). All calculated concentrations were correlated to a non-treated control group. Sensibility of *Bb*ss against outside influences was shown by amoxicillin. Extracts were investigated by several TLC methods to ensure reproducibility.

The growth inhibition rates achieved of growth by the apolar extracts (2 mg/ml) were comparable to those of amoxicillin (0.5 µg/ml) in contrast to the hydrophilic extract. The latter showed no inhibition, it even enhanced growth of *Bb*ss *in vitro*. The dichloromethane extract and the ethyl acetate extract caused decrease of individuals already on the first day (Fig. 1). Highest inhibition was found in the ethyl acetate fraction ($99.7 \pm 1.0\%$ on day 4). In this fraction all visible forms of *Bb*ss were immobile within the first day, their number did not increase within eight days. Polysorbate 80 did not significantly influence the growth in the applied concentration of 0.25% (inhibition $5.6 \pm 7.6\%$ on day 4). Thus growth inhibiting activity of lipophilic extracts has been shown *in vitro*, whereas by now there is no evidence for a therapeutic potential *in vivo*. Research work for structure-response relationship on individual substances is in progress.

To characterize the drug and its possible antiinfectious compounds a methanolic extract of *Dipsaci sylvestris* radix was analysed by micellar electrokinetic capillary chromatogra-

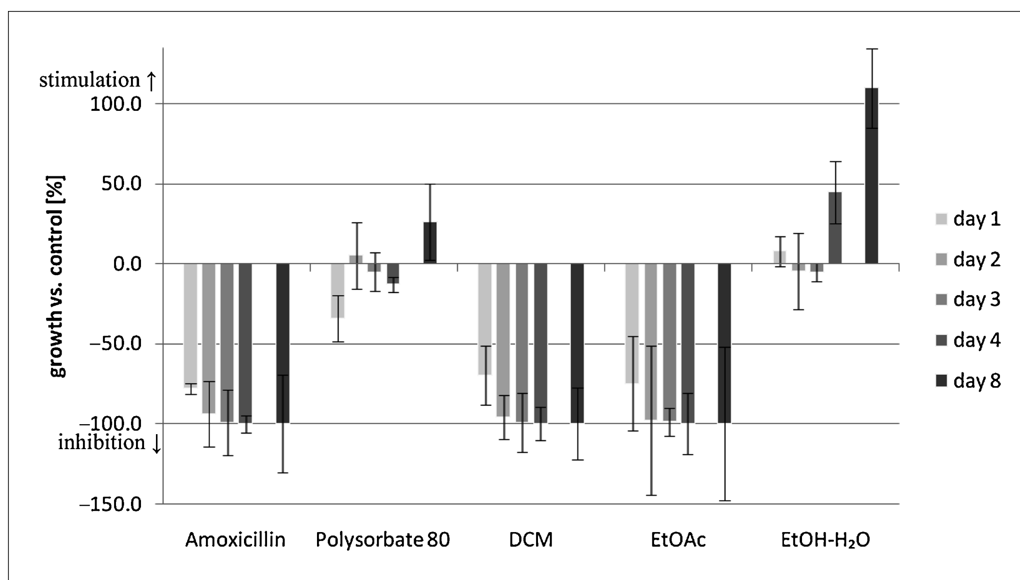

Fig. 1: Relative growth of *Borrelia burgdorferi* sensu stricto treated by extracts of *Dipsacus sylvestris* observed over 8 days vs. control (control = 0%)

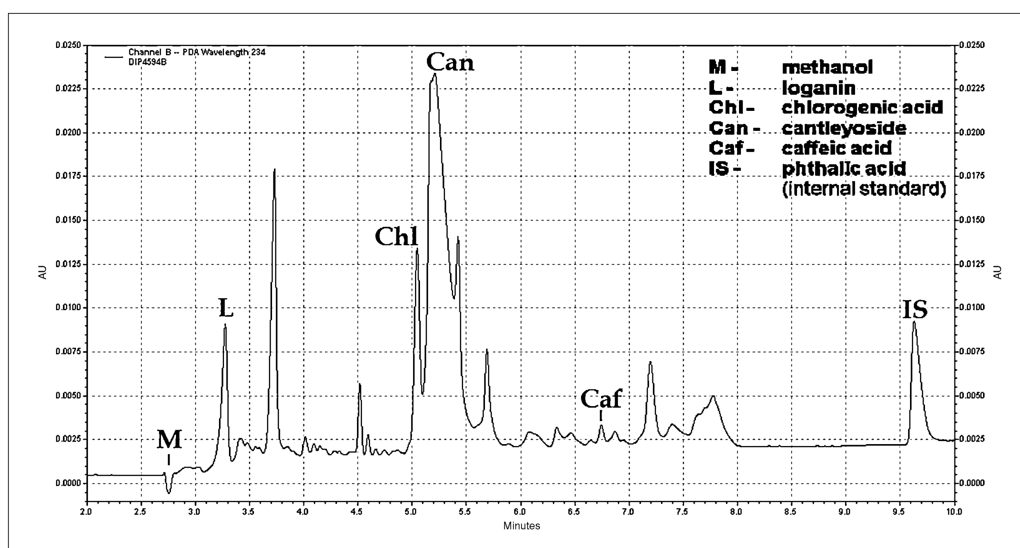

Fig. 2: Fingerprint MECC electropherogram of a methanolic extract of *Dipsacus sylvestris* with phthalic acid added as internal standard

phy (MECC). The MECC method yields typical fingerprint electropherograms mainly consisting of iridoids and phenolic acids useful for identification of *Dipsacus sylvestris.* Its marker substances loganin, cantleyoside, chlorogenic and caffeic acid were identified by their relative migration times using phthalic acid as internal standard (Fig. 2). With the help of the internal standard the concentration of loganin in the extract was quantified ($1.1 \pm 0.03\%$; corresponding to $0.267 \pm 0.007\%$ in the dried drug).

3. Experimental

3.1. Extraction method

The fractions for testing were obtained by repeated extraction of homogenized fresh roots of *Dipsacus sylvestris* (100 g each, Physiologica Naturprodukte, Ruppichteroth, Germany, gathered in October 2007 in the first year stadium), using ethanol 70% (V/V), ethyl acetate and dichloromethane (BASF, Ludwigshafen, Germany), respectively. All extracts were vacuum-dried and stored at −25 °C until testing (drug-to-extract-ratios in main text).

3.2. Sample preparation

200.0 mg of each of the apolar extracts were resolved in ethyl acetate. To maintain a concentration of 40.0 mg/ml, 5.0 ml of a 5% aqueous solution of polysorbate 80 (Merck, Darmstadt, Germany) were added. Consecutively ethyl acetate and dichloromethane were evaporated slowly under vacuum. 200.0 mg of the hydroethanolic extract were soluted directly in 5.0 ml distilled water. All extract solutions were filtered through a 0.2 μm pore-size filter (Schubert-Laborfachhandel, Leipzig, Germany).

3.3. Growth inhibiting test

For the growth inhibiting test *Bb*ss (isolates N40, passage 52, Ch. A.H.20070305 from stocks of the institute of immunology, gained by extraction of ticks (Straubinger et al. 1995)) were grown for two days in BSK II medium to give a bacteria stock solution in logarithmic growth. 150 μl of each filtered fraction were mixed with 2.8 ml BSK II medium and 50 μl of bacteria stock solution, resulting in a final extract concentration of 2 mg/ml and a bacteria concentration of 10^6 to 10^7 *Bb*ss per ml. Additionally a pure growth control (50 μl bacteria and 2.95 ml BSK II), a polysorbate 80 growth control (50 μl bacteria, 2.8 ml BSK II and 150 μl of a sterile filtered 5% aqueous polysorbate 80 solution) and a sensibility control (1.5 μg amoxicillin in 3.0 ml BSK II) were made.

All samples were prepared in triplicate and incubated at 33 °C over 8 days in air tight vials. Immediately after preparation and on days one to four and day eight aliquots of all samples were counted after dilution with BSK II medium in a Petroff-Hauser counting chamber (1/400 mm^2; Hausser Scientific, Blue Bell, PA, USA) under dark field microscopy (Axiolab 2 plus, Carl Zeiss, Jena, Germany) with 400fold amplification. Concentrations were adjusted to 10^6 to 10^8 individuals per ml. The whole assay was repeated twice (extraction, preparation, testing) for evaluation.

3.4. MECC fingerprints

MECC fingerprints were received using a P/ACE^{TM} System MDQ (Beckman Instruments Inc., Fullerton, USA) with a multichannel UV-Vis diode array detector (Beckman Instruments Inc., Fullerton, USA), software Beckman P/ACE^{TM} System MDQ version 2.3, uncoated fused-silica capillary 75 μm i.d., 375 μm o.d. eCap^{TM} (Beckman Instruments Inc., Fullerton, USA) with an effective length of 30 cm to the detection window (40 cm total length), 100 x 800 μm aperture size. For MECC a 35 mM borate buffer pH 9.4 with an additive of 20 mM SDS as surfactant was used at 25 °C. A fingerprint chromatogram was received of a methanolic extract of *Dipsacus sylvestris* (drug-extract-ratio 4.15:1, concentration 23.90 mg/ml) at 12 kV using UV detection at $\lambda = 234$ nm. As marker substances loganin (Extrasynthese, Genay Cedex, France), chlorogenic acid, cantleyoside and caffeic acid (isolated in our laboratory) were employed. Phthalic acid (Merck, Darmstadt, Germany) was used as internal standard (0.1 mg/ml).

3.5. Quantification of loganin by MECC

With the same method as described under point 4 it was possible to quantify loganin in the range of 0.012 to 0.240 mg/ml. In the diluted methanolic extract (10.04 mg/ml) with an addition of 0.1 mg/ml phthalic acid the concentration of loganin was 0.111 ± 0.003 mg/ml (n = 5), giving 1.1% of the extract. Therefore the slope of the calibration curves (loganin: 40609, $R^2 = 0.9739$; phthalic acid: 39583, $R^2 = 0{,}9874$) and the corrected peak areas (area divided by migration time; loganin: 8381 ± 20; phthalic acid: 7369 ± 165) were used.

3.6. Thin layer chromatography

TLC investigations were carried out on silica gel 60 F254 (Merck KGaA, Darmstadt, Germany) using ethyl acetate – methyl ethyl ketone – formic acid – water (5: 3: 1: 1) (15 cm), dichloromethane – methanol (95: 5) (15 cm) and a combination of both (step-wise development 6/15 cm) with direct detection (UV_{254} and UV_{365}) and derivatisation with vanillyl-sulfuric acid (VIS and UV_{365}). As references chlorogenic acid, caffeic acid, loganin, oleanolic acid, β-sitosterol, fructose and saccharose were used (isolated in our laboratory; solvents from BASF, Ludwigshafen, Germany, distilled).

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