Oxidative Potential and woundhealing properties of extracts from wood bark of common temperate trees

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Abstract. Plant species have developed effective defense strategies to colonialize diverse habitats and protect themselves from numerous attacks from a wide range of organisms including insects, vertebrates, fungi and bacteria. Especially the bark of trees constitutes of a number of components that protect from unwanted intruders. The aim of this work was to identify beneficial properties of Austrian beech, birch, pine and oak bark extracts for their potential usage as additives or active ingredients in dermatological applications.
Bacterial agar diffusion assay was used to evaluate anti-bacterial activity. To gain more insight in cellular response to bark extracts, viability-, scratch-assays, qPCR and ELISAs were performed.
Birch and beech extracts showed strong antimicrobial activities against gram-positive bacteria, including *Staphylococcus aureus*, *Cutibacterium acnes, Staphylococcus epidermidis* and MRSA. Wound closure was enhanced with birch, beech, oak and pine extracts as compared to controls in the scratch-assays. Whereas beneficial properties of birch bark components have previously been described, the similar effects of beech, oak and pine extracts are novel. The combined positive effect in wound-healing and antimicrobial activity has great potential for treatment of various skin diseases, including acne in future dermal applications.
In addition, the sustainability aspect by utilizing the bark, which is considered a by-product in the forest industry, is addressed, as well as various extraction methods applied to retrieve extracts from bark.

Keywords: antimicrobial activity; oxidative stress; bark extract; birch; beech; oak; pine; wound-healing;

1 INTRODUCTION

Global resource consumption is steadily increasing. In the sense of the bioeconomy, renewable or hitherto unused raw materials will in future become more of a focus of interdisciplinary cooperation in the fields of biorefinery and life sciences. Phytochemical substances have been used for centuries to improve diseases in the course of natural medicine and are applied in the field of drug production. Not least due to the growing resistance to antibiotics, research on phytochemicals has gained momentum. In the course of this research work, the use of extracts from native woods as additives for dermatological applications will be investigated. The focus will be on certain skin diseases with characteristic manifestations such as misdirected inflammatory processes, wound healing disorders and bacterial infestation. Furthermore, attention will be paid to the circular economy, with a focus on the material utilization of forest biomass during various reprocessing processes.

Biocompatible extracts from bark, branches and seeds of native woody plants will be obtained by "green" extraction methods and tested for their properties in bacterial and human cell cultures. Expected results are the detailed characterization of extracts of native woods with regard to their chemical constituents, anti-oxidative spectrum of activity, wound-healing and immune-regulating properties.

2 METHODS

2.1 Wood bark extraction

Solvent extraction was performed by soxhlet extractions of about 5 g of biomass with 250 ml water and 18 h extraction time. The extractives solubilized were determined using 10 ml of the 250 ml extractive broth transferred to crystallisation dishes. The mass of the crystallisation dishes and those with extract after drying at 103 ± 2 °C were registered with a laboratory analytical balance from Sartorius AG (Goettingen, Germany) and the extractives solubilized were calculated as mg/ml.

2.2 Test microorganisms

To test the potential antimicrobial activities of wood compounds, four bacterial strains were investigated. All strains were purchased from the American Type Culture Collection (ATCC®; Dartford, England) and included *Cutibacterium acnes* (ATCC®11827, Gram-positive bacterium, anaerob), *Staphylococcus epidermidis* (ATCC®12228, Gram-positive bacterium, aerob), MRSA- *Staphylococcus aureus subsp. aureus Rosenbach* (ATCC®43300, Gram-positive bacterium, aerob) and *Escherichia coli* (ATCC®8739, Gram-negative bacterium, aerob).

2.3 Agar Diffusion assay

For the initial assessment of antimicrobial activity of certain wood extracts, Agar Diffusion assays were performed. Blank discs (Oxoid, Basingstoke, UK) were incubated in 100 µl of 10 mg/ml wood extracts overnight. The prepared inocula of different bacterial strains were evenly streaked with sterile cotton swabs on Mueller Hinton E agar (MHE) or for C. acnes on Mueller Hinton 2 agar, supplemented with 5% sheep blood (MHF) (Biomerieux, Marcy-l'Étoile, France). Discs, containing wood extracts were placed on striked agar plates with sterile tweezer. As positive controls, certain antibiotic discs (Oxoid, Basingstoke, UK) were used for aerob bacteria and Etest® (Biomerieux, Marcy-l'Étoile, France) was used for

C. acnes. The agar plates were incubated at 35°C for 20 h, C. acnes under anaerobic conditions (BD GasPak EZ Container System, Becton Dickinson, Sparks, USA) with extended incubation of 24 h - 28 h until visible growth could clearly be observed. Diameters of inhibition zones were measured with a ruler and pictures were taken.

2.4 Resazurin-based broth microdilution assay

For the broth microdilution assay, the prepared inoculum of McF 0.5 for all tested strains in NaCl was further diluted in Mueller Hinton Broth (MHB) (Becton Dickinson, Le Pont de Claix, France) or for *P. acnes* in Thioglycolate Broth (Merck KGaA, Darmstadt, Germany) to final concentrations of ~1 x 10^5, ~10 000 and ~1000 CFU/ml of bacteria. 100 µl of each concentration were seeded in triplicates in 96-well plates (CytoOne®, Starlab, Germany). Wood extracts were brought to room temperature and added immediately after seeding at 16.67 % (V/V). *E. coli*, MRSA and *S. epidermidis* were incubated over night at 35°C. *C. acnes* was cultured for 48 h at 35°C anaerob by using BD GasPak EZ Container System. To quantify the number of metabolically active bacteria, resazurin assay was used. Sterile filtered resazurin sodium salt (Alfa Aesar, Thermo Fisher, Germany) was diluted in PBS w/o Ca and Mg (pH 7.4) to a final concentration of 0,2 mg/ml. After incubation of the microdilution plates, 9.09 % (V/V) of the dye was added to each well and incubated for 45 – 60 min. at 35°C under aerobic conditions for all strains. Fluorescence signal of enzymatically converted resorufin was detected with Tecan Infinite 200.

2.5 Cell culture

HaCaT cell line was purchased from Cell Lines Service in Germany. As recommended by the vendor, HaCaT keratinocytes were grown in DMEM high glucose (4.5 g/l) with stable L-glutamine (2 mM), supplemented with 10 % FBS and 1 % P/S (Penicillin G Sodium 107 Units/l Streptomycin Sulfate 10000 mg/l). This medium is referred to as growth medium. Untreated control samples (UT) contained sterile H_2O instead of bark extracts.

2.6 Scratch Assay

The wound healing assay was performed using 2 well silicone inserts with a 500 µm cell-free gap (Ibidi Germany) according to manufacturers instructions. Cells were imaged immediately, and after 16h, 23h, 38h and 44 h. Gaps were analyzed using IKOSA Platform (KML Vison GmbH).

2.7 Statistical Analysis

For statistical analysis two-way ANOVA using Dunnett´s multiple comparisons test (for analysis of scratch assay) and Tukey's multiple comparison test (for analysis of the inhibition zones, viability assay of bacteria and cell lines and ELISA) were performed. Statistics was performed using GraphPad Prism 8.3.0.

3 PRELIMINARY RESULTS

3.1 Anti-oxidative potential

The results of the different characterisation methods are listed in Table 1.

Table 1. Results extract concentration

sample [Nr.]	species [type]	TPC [B]* [µgGAE/mg]	DPPH [B]* [%inhibition]
1	Oak bark extract	263,2	76,2

2	Pine bark extract	289,65	37,13
4	Beech>500µm	283,71	47,05
5	Birch >500µm	328,64	65,05

3.2 Agar Diffusion Assay

The antimicrobial activity of bark extracts was tested via agar diffusion method and the inhibition zones of agar plates were measured. All tested gram-positive strains, including MRSA, *C. acnes*, *S. aureus* and *S. epidermidis* were susceptible to birch and beech extracts compared against negative control whereas gram-negative *E. coli* was resistant to all tested extracts. Pine and Oak extracts showed no inhibition against all tested bacteria.

3.3 Resazurin-based broth microdilution Assay

To further investigate the antimicrobial activity of the bark extracts, a resazurin-based broth microdilution assay was performed. The concentration-dependent reduction in bacterial growth at three seeding densities (100,000, 10,000 and 1000 CFU/mL) upon variable bark extract treatments was measured. The concentrations of bark extracts used in this assay differed depending on the sensitivity of each bacterial strain ranging from 25 to 100 ug/mL for C. acnes, 150 to 300 ug/mL for S. epidermidis and 200 to 600 ug/mL for MRSA. The birch and beech extracts led to a significant reduction in the viability of MRSA, C. acnes and S. epidermidis compared to the untreated control (UT). In line with the results from the agar diffusion test, Gram-negative E. coli showed no susceptibility to any of the extracts tested.

3.4 Scratch Assay

For testing the wound-healing capacity of the extracts, scratch assays were performed and the closure of the gap at 16, 23, 33 and 44h reported. All extracts accelerated the wound closure measured until 44 h.

3.5 Sustainability

3.5.1. Sustainability goals of the project

The project follows following Sustainability Development Goals:

-3: Ensure healthy lives and promote wellbeing

- 9: Industry, innovation and infrastructure (inclusive and sustainable industrialization)

- 12: Responsible consumption and production: sustainable production patterns

-15: Life on Land: Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss

Within the Green Deal, Europe aims to be climate neutral until 2050. Part of this goal is to develop a sustainable industry. Within this project, attention is paid to the use of natural, sustainable, renewable and unused resources, whereby the generation of valuable materials from forest biomass is a core topic of this project in view of the increasing scarcity of resources.

3.5.2 Sustainability in research

Research could gain in sustainability by increasing the transparence in the acquired data and sharing these between scientists. This way also negative results can be shared and experiments that do not provide any results do not need to be performed multiple times. Moreover, the data would be available for re-analysis in case of novel developments within a field. Another improvement in the sustainability would be to improve the exact protocols used for the laboratory based experiments, this would reduce the need for optimization in other laboratories, thereby reducing the costs and also the amount of material needed. In addition, research, the process, the tools and the results, should be transparent for the students within a certain field of studies. This way, they do not have to make the same mistakes as those that were made by the generation working on this subject before. This again saves a lot of time, but also reduces the frustration by these upcoming generations, hopefully leading to motivated and innovative generations of scientists for the years to come. In our research group, we already implemented many of these points. We make sure that our protocols are clear and can be well repeated by somebody else. Moreover, we include as many students as possible in our studies and teach them the complete research process, from acquiring the funding to disseminating the results.

4 CONCLUSIO

Our findings show a successful growth inhibition with birch and beech bark extracts of gram-positive bacteria, involved in skin diseases, like acne. Wound closing using an in-vitro scratch assay was shown to be highly accelerated with all extracts tested compared to controls. Whereas beneficial properties of birch components are already described in literature, the very similar effects of beech contents are new. Their combined positive effect in wound-healing and antimicrobial activity has great potential for treatment of various skin diseases. In addition, the use of biomolecules in form of an extract from bark, which is often considered as waste or by-product during a variety of industrial processes, enables a sustainable use of natural and renewable products.

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