Assessment of cytotoxicity exerted by leaf extracts from plants of the genus *Rhododendron* towards epidermal keratinocytes and intestine epithelial cells

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Abstract

**Background:** *Rhododendron* leaf extracts were previously found to exert antimicrobial activities against a range of Gram-positive bacteria. In this study, we investigated which of the extracts with these antimicrobial properties would be best suited for further exploitation. Specifically, the project aims to identify biologically active compounds that affect bacterial but not mammalian cells when applied in medical treatments such as lotions for ectopic application onto skin, or as orally administered drugs.

**Methods:** Different concentrations of DMSO-dissolved remnants of crude methanol *Rhododendron* leaf extracts were incubated for 24 h with cultured epidermal keratinocytes (human HaCaT cell line) and epithelial cells of the intestinal mucosa (rat IEC6 cell line) and tested for their cytotoxic potential. In particular, the cytotoxic potencies of the compounds contained in antimicrobial *Rhododendron* leaf extracts were assessed by quantifying their effects on (i) plasma membrane integrity, (ii) cell viability and proliferation rates, (iii) cellular metabolism, (iv) cytoskeletal architecture, and (v) determining initiation of cell death pathways by morphological and biochemical means.

**Results:** Extracts of almost all *Rhododendron* species, when applied at 500 µg/mL, were potent in negatively affecting both keratinocytes and intestine epithelial cells, except material from *R. hippophaeoides* var. *hippophaeoides*. Extracts of *R. minus* and *R. racemosum* were non-toxic towards both mammalian cell types when used at 50 µg/mL, which was equivalent to their minimal inhibitory concentration against bacteria. At this concentration, leaf extracts from three other highly potent antimicrobial *Rhododendron* species proved non-cytotoxic against one or the other mammalian cell type: Extracts of *R. ferrugineum* were non-toxic towards IEC6 cells, and extracts of *R. rubiginosum* as well as *R. concinnum* did not affect HaCaT cells. In general, keratinocytes proved more resistant than intestine epithelial cells against the treatment with compounds contained in *Rhododendron* leaf extracts.

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Conclusions: We conclude that leaf extracts from highly potent antimicrobial R. minus and R. racemosum are safe to use at 50 μg/mL in 24-h incubations with HaCaT keratinocytes and IEC6 intestine epithelial cells in monolayer cultures. Extracts from R. rubiginosum as well as R. concinnum or R. ferrugineum are applicable to either keratinocytes or intestinal epithelial cells, respectively. Beyond the scope of the current study, further experiments are required to identify the specific compounds contained in those Rhododendron leaf extracts that exert antimicrobial activity while being non-cytotoxic when applied onto human skin or gastrointestinal tract mucosa. Thus, this study supports the notion that detailed phytochemical profiling and compound identification is needed for characterization of the leaf extracts from specific Rhododendron species in order to exploit their components as supplementary agents in antimicrobial phyto-medical treatments.

Keywords: Rhododendron, Bio-active compounds, Cytotoxicity, Mitochondrial activity, Programmed cell death

Background
Plant extracts are commonly used in formulations of alternative and traditional medicine such as skin lotions, or when used as ingredients in dietary treatments and teas [1]. Plant-based medications are well-accepted by patients and are often preferred over chemically produced therapeutics because of their well-known health-benefitting bio-active ingredients [2–6]. Moreover, plant-extractable compounds have also gained a lot of attention in conventional medicine. For instance, plant-based drugs are now used for therapeutic treatment of diseases such as cancer and various inflammatory disorders [7, 8]. Therefore, knowing and assessing the potentials of plant-derived bio-active compounds is important for further drug development. This notion is deducible from the increasing interest of the pharmaceutical industry in gaining the rights to identify and exploit plant-borne compounds from species-rich rainforests in countries of tropical and subtropical regions [9–11]. While there is certainly a great potential in identifying plant-derived medication, the challenges associated with this venture must also be noted. Some of the current discussion revolving around this topic are: the protection of bio-diversity, acceptance of intellectual property rights, as well as biosafety of application [12, 13]. The aim of this study is to establish and provide an experimental, cell biological platform that allows for the identification of plant species that should be characterized and assessed in more detail.

So far, roughly 6 % of all higher plant species existing worldwide have been, or are currently being, assessed for their medicinal potential. In fact, only a minor proportion of these plant species have actually been subjected to detailed phytochemical profiling [14–16]. Bio-active compounds must first be purified before they can be assessed and eventually tested in clinical trials. Of course, the overall aim of the tests would be to ensure the efficacy of the biomolecules in particular therapeutic approaches. Simultaneously, drug safety and absence of undesirable side-effects are of the highest concern [17]. These considerations are important, regardless of whether pure compounds or crude extracts of an entire plant, or parts thereof, are used for the production of a pharmaceutically applicable plant ingredient [18].

The genus Rhododendron, comprising the species-richest group of wooden plants, belongs to the family Ericaceae and encompasses about one thousand species: the majority of which are indigenous to Asia [19]. In ethno-medicine, extracts of Rhododendron have been used traditionally in treating various disorders such as inflammatory conditions, common symptoms of cold, gastrointestinal disorders, skin diseases, or as pain killers [20]. Recent research highlighted that Rhododendron leaf extracts might be highly potent and beneficial to health due to properties they contain, such as anti-bacterial [21, 22], anti-allergic, and anti-inflammatory [23, 24] agents. The reported usefulness of crude extracts of R. ferrugineum and R. anthopogon [20, 25–27] is most likely due to the presence of terpenoids in high concentrations [25].

Previously, we investigated leaf extracts of 120 different Rhododendron species for their efficacy as antimicrobials in killing a variety of Gram-positive and Gram-negative bacteria [25]. In the current study, extracts of 12 of the Rhododendron species with highest anti-bacterial potencies were applied in different concentrations to monolayer cultures of human HaCaT epidermal keratinocytes and rat intestine epithelial cell line IEC6. Intestinal epithelial cells and keratinocytes are considered to be among the first points of contact when drugs are administered orally or applied ectopically, respectively. In general, bio-active compounds are considered cytotoxic when they alter cellular morphology or metabolism, interfere with the cytoskeleton or cell adhesion, affect cell proliferation rates or cell differentiation processes, or initiate programmed cell death [28]. Different cell types might exhibit differential responses towards a specific compound or plant extract. Consequently, it is neither sufficient to use only one cell line nor to apply just a single cytotoxicity assay in any safety assessment study.
The aim of this study was to assess possible cytotoxic effects of antimicrobial *Rhododendron* leaf extracts on mammalian cells in order to identify a potential candidate species for further analysis of safe use. Thus, the study contributes to on-going investigations on the bioactivity potential of plant species such as the *Rhododendron*. Hence, the effects of *Rhododendron* leaf extracts on cell survival, metabolism, and growth as well as on different cellular structures were monitored *in vitro* by an array of cell biological assays employing differentiated cell lines.

**Methods**

**Collection of plant material and leaf extract preparation**

Fresh leaf material of reliably identified *Rhododendron* species was used in this study (Table 1). The material was collected from January 2012 to December 2013 from plants grown in the Rhododendron-Park Bremen (www.rhododendronparkbremen.de). Each plant species was sampled once without considering seasonal variations. The identities of the plant species used in this study (Table 1) have been verified by reference to the German Gene Bank Rhododendron Database provided by the Bundessortenamt (www.bundessortenamt.de/rhodo) [25]. Material from all plant species used is publicly and freely available from the Rhododendron-Park Bremen upon request.

Leaf material was frozen in liquid nitrogen and powdered using a KSW 3307 mill (Clatronic, Kempen, Germany). Crude extracts were prepared by soaking two grams of *Rhododendron* leaf powder in 10 mL of 80 % methanol for 24 h at 4 °C with constant shaking. Insoluble material was removed by centrifugation at 3,220 g for 30 min at 4 °C, and supernatants were stored at -20 °C for further use. Methanol was evaporated from the extracts using a Micro Modulyo lyophilizer (Edwards, Crawley, UK). Stock solutions were prepared by dissolving the residues in 100 % dimethyl sulfoxide (DMSO) (Carl Roth, Karlsruhe, Germany). Prior to the *in vitro* assays, the samples were mixed with the respective cell culture medium such that the final concentration of DMSO did not exceed 0.5 % (v/v), and 5, 50, or 500 μg lyophilized powder per mL culture medium were applied to confluent IEC6 and HaCaT cell monolayers.

**Cell culture**

The normal rat small intestine epithelial cell line IEC6 [29, 30] and the human keratinocyte cell line HaCaT [31, 32], purchased from the European Collection of Cell Cultures (Salisbury, UK), were used throughout this study. IEC6 cells were grown in Dulbecco's modified Eagle's Medium (DMEM High Glucose) (Lonza Group, Basel, Switzerland) supplemented with 10 % fetal calf serum (FCS) (Perbio Science, Bonn, Germany) and 10 μg/mL insulin (Sigma-Aldrich, Steinheim, Germany). IEC6 cells were incubated at 37 °C in a 5 % CO₂ atmosphere in an incubator (Heraeus, Osterode, Germany). HaCaT cells were cultured in DMEM containing 10 % FCS and incubated at 37 °C in an 8.4 % CO₂ atmosphere. Cell cultures were passaged once per week. All experiments were performed with cultures at approx. 70 % and 95 % confluence for IEC6 and HaCaT cells, respectively.

**Determination of cell viability and proliferative activity by MTT assays**

Effects of *Rhododendron* leaf extracts on the viability and proliferative activity of cultured IEC6 and HaCaT

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**Table 1** List of *Rhododendron* species from which leaves were collected and used to prepare extracts that were screened for exhibiting cytotoxicity towards intestine epithelial cell cultures and monolayers of keratinocytes

| Genebank-No. | Species Name | Section | Sub-section |
|--------------|--------------|---------|-------------|
| 100.345      | *R. ferrugineum* L. | Rhododendron | Rhododendron |
| 100.007      | *R. ambiguum* Hemsley | Rhododendron | Triflora |
| NA           | *R. anthopogon* Don ssp. *anthopogon* Betty Graham | Pogonanthum | - |
| NA           | *R. hirsutum* L. | Rhododendron | Rhododendron |
| 100.326      | *R. concinnum* Hemsley | Rhododendron | Triflora |
| 100.322      | *R. cinnabarum* Hooker | Rhododendron | Cinnabarina |
| NA           | *R. racemosum* Franchet | Rhododendron | Scabrifolia |
| 100.404      | *R. rubiginosum* Franchet | Rhododendron | Heliolepidia |
| 100.474      | *R. xanthostephanum* Merrill | Rhododendron | Tephropepla |
| 100.370      | *R. minus* Michaux | Rhododendron | Caroliniana |
| 100.392      | *R. polycladum* Franchet | Rhododendron | Lapponica |
| 100.353      | *R. hippophaeoides* var. *hippophaeoides* Hutchinson | Rhododendron | Lapponica |

*Gene bank numbers used in the collection of the Rhododendron-Park Bremen
NA Not a plant of the German Gene Bank Rhododendron but a verified plant of the Rhododendron-Park Bremen
cells were quantitated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Carl Roth). This test is indicative for mitochondrial NADH-dependent dehydrogenase activity, which is proportional to both cell viability and proliferation rates of treated cultures [33–35]. A total of 1 × 10^4 cells/well were seeded in single wells of 96-well plates (Greiner, Essen, Germany) and upon reaching the desired confluence, the cells were incubated with three different concentrations of Rhododendron leaf extracts (5, 50, and 500 µg/mL culture medium, not exceeding 0.5 % DMSO in content) for 24 h in complete medium and at standard culture conditions. Incubation of cells with culture medium containing DMSO at a final concentration of 0.5 % (v/v) was used as a negative control. Culture supernatants containing free-floating dead cells were removed at the end of the incubation period, replaced with fresh culture medium containing MTT at a final concentration of 0.5 % (w/v). The cell layers were then further incubated for another four hours. Subsequently, culture supernatants were removed, the cells adherent to the plate surface were collected in 100 % DMSO and incubated for 15 min at 37 °C to terminate the reaction and to dissolve formazan crystals. The absorbance of formazan formed by Rhododendron leaf extract-treated and non-treated control cultures was quantified at 595 nm in a microplate reader against the solvent (Tecan Group, Männedorf, Switzerland). Percentages of cell viability were calculated from triplicates using Eq. (1):

\[
\text{% of cell viability} = \left( \frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \right) \times 100
\]

Propidium iodide staining of nuclei in cells with ruptured plasma membranes

The two cell lines were grown on cover glasses in 24-well Bio-One Cellstar plates (Greiner) to reach the desired degree of confluence. Next, cells were incubated with three different concentrations of Rhododendron leaf extracts (i.e. 5, 50, or 500 µg/mL) for 24 h as described above. Subsequently, cells were washed three times with phosphate-buffered saline (PBS) before being incubated for 45 min in 2 mg/mL propidium iodide (PI) (Carl Roth) and 5 µM Draq5™ (Biostatus, Leicester, UK) in culture medium at 37 °C. After washing three times in PBS, cells were fixed in 4 % paraformaldehyde (PFA) (Carl Roth) in 200 mM HEPES (pH 7.4) at room temperature for 20 min. Cells on cover glasses were washed again in PBS and distilled water before mounting them in Mowiol for subsequent laser scanning microscopy as described previously [36]. PI is not capable of penetrating cells with intact plasma membranes, however, if plasma membrane integrity is lost, PI gains access to the nucleus and forms complexes with the DNA. In contrast, Draq5™ serves as a nuclear counter-stain that transverses the intact plasma membrane and can therefore be used to determine the total cell number. Special care had to be taken when analyzing total cell numbers, because some plant leaf extracts could have exhibited anti-adhesive effects such that total cell numbers were significantly diminished after washing steps. Therefore, total cell numbers were determined and reported herein as a measure for anti-adhesive properties of Rhododendron-derived compounds.

Phalloidin staining of the filamentous actin cytoskeleton

IEC6 and HaCaT cells were grown on cover glasses in 24-well plates to reach 70 % and 95 % confluence, respectively, and exposed to Rhododendron leaf extracts for 24 h as described above, while 0.5 % DMSO was used as a negative control. Cells were washed three times with PBS before fixation in 4 % PFA in 200 mM HEPES (pH 7.4) at room temperature for 20 min. After fixation, cells were washed with PBS before applying 0.2 % Triton X-100 in PBS for 5 min at room temperature, followed by several washing steps in PBS. Finally, cells were stained for 30 min at room temperature with a mixture of 3 µM FITC-labeled phalloidin (Sigma Aldrich) and 5 µM Draq5™ in PBS, the latter used as a counter-stain of nuclear DNA. Cover glasses were mounted in Mowiol for subsequent inspection by laser scanning microscopy (see below).

MitoTracker® Red CMXRos staining of the mitochondrial matrix

Cells were incubated and treated as described above, before washing twice in phenol red-free HEPES-buffered culture medium for 5 min. Subsequently, the cells were incubated with phenol red-free culture medium containing 20 mM HEPES and 500 nM MitoTracker® Red CMXRos (Molecular Probes, Oregon, USA) for 45 min at 37 °C followed by several washes. The fluorescent dye accumulates in the mitochondrion matrix only when an intact membrane potential, due to active cellular metabolism, is present across the inner mitochondrial membrane. Cells were fixed with 4 % PFA in 200 mM HEPES (pH 7.4) for 20 min at room temperature, rinsed, and mounted on microscope slides as described above for subsequent microscopic inspection.

Microscopy techniques

Stained cells were visualized with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) at excitation wavelengths of 488 nm, 543 nm and 633 nm for fluorophore excitation to visualize FITC-phalloidin, PI or
MitoTracker® Red CMXRos, and Draq5−, respectively. Scans at a resolution of 1024 x1024 pixels were taken in the line averaging mode and at a pinhole setting of one airy unit. Color coding and image analysis was performed by using the LSM 510 software, release 3.2 (Carl Zeiss).

Caspase-3 activity assay
For IEC6 cells, induction of apoptosis upon incubation with R. ferrugineum and R. cinnabarinum leaf extracts at the highest concentration, i.e. 500 μg/mL, was evaluated at different time intervals ranging from 1 to 24 h. The apoptosis assay was performed using the EnzChek Caspase-3 assay kit (Invitrogen, Karlsruhe, Germany) detecting activation of procaspase-3 and other Asp-Glu-Val-Asp (DEVD)-specific proteases. Lysates of treated IEC6 cells and non-treated controls were prepared according to the manufacturer’s protocol. Following clearance by centrifugation, the samples were incubated with 5 mM Z-DEVD-R110 substrate for 30 min at 4 °C. Lysates of IEC6 cells treated for 4 h at 37 °C with apoptosis-inducing staurosporin (10 mM) (Sigma-Aldrich) were used as positive controls, whereas no treatment or incubation with the solvent served as negative controls. Additionally, staurosporin-treated cells incubated with 1 mM of Ac-DEVD-CHO for 10 min served as a negative control since caspase-3 activity is blocked under these conditions. The extent of procaspase-3 activation was determined by fluorescence of liberated rhodamine upon excitation at 496 nm and reading the emission at 520 nm, using a microplate reader (Tecan Group, Männedorf, Switzerland). The values were normalized to equal amounts of DNA in the pellets after lysis, as determined by the Burton assay [37].

Determination of minimum inhibitory concentrations
The minimum inhibitory concentration (MIC) was defined as the lowest concentration of Rhododendron leaf extract that inhibits visible growth of microorganisms after overnight incubation. The MIC was determined by a two-fold dilution assay in Mueller-Hinton broth (MHB) (Becton Dickinson, Heidelberg, Germany). The Bacillus subtilis strain S168 was tested against 12 Rhododendron crude extracts (Table 1) [25]. All tests were performed in triplicates following the National Center for Clinical Laboratory Standards recommendations [38].

Statistical evaluation
All assays were performed in triplicates and repeated at least three times in independent experiments unless stated otherwise. All data were expressed as means ± standard deviation (SD), as determined by using Origin software (MicroCal Software, Northampton, USA). The profile map shown in Fig. 9 was created using R (RStudio, Boston, USA). Levels of significance were calculated by One-Way ANOVA, and p < 0.05 was considered statistically significant. CellProfiler software [39] was used to determine total cell numbers (Draq5−-positive cells) versus numbers of dead cells (PI-positive cells). This software was also employed to quantify the MitoTracker® Red CMXRos and FITC-phalloidin fluorescence signal intensities as previously described by us [32].

Results
Classification of Rhododendron species based on antibacterial activities
In order to group the 12 selected Rhododendron species [25] according to their antibacterial activities, minimum inhibitory concentration (MIC) tests were conducted against B. subtilis. Accordingly, the plant species were classified into four major groups: six Rhododendron species formed the group with the highest antibacterial activity with an MIC of 50 μg/mL: R. minus, R. racemosum, R. ferrugineum, R. rubiginosum, R. anthopogon, and R. xanthostephanum. Another three species formed the group with moderately active extracts, with an MIC of 100 μg/mL: R. cinnabarinum, R. hirsutum, and R. ambiguum. The remaining Rhododendron species exhibited lower antibacterial activities with R. xanthostephanum and R. polycladum having an MIC of 150 μg/mL and R. hippophaeoides var. hippophaeoides requiring 300 μg/mL to efficiently produce an inhibition zone for B. subtilis.

Cell viability and proliferation rates as quantified by the MTT assay
The effects of leaf extracts prepared from 12 different Rhododendron species on cell viability and proliferation rates were initially estimated with the help of the MTT assay, as this test allows for a rapid screening of many samples. To this end, three different concentrations of Rhododendron leaf extract (5, 50, and 500 μg/mL) were applied to IEC6 and HaCaT cells for 24 h. As demonstrated in Fig. 1, incubation with extracts applied at lower concentrations (5 and 50 μg/mL) revealed no detectable change in MTT conversion rates of HaCaT cells, while the leaf extracts from R. polycladum, R. concinnum, and R. xanthostephanum affected IEC6 cellular metabolism negatively (arrows) in comparison to cells that were not treated at all, or treated with 0.5 % DMSO, suggesting that even 5 μg/mL of these extracts caused cytotoxic effects on intestine epithelial cells. Rhododendron leaf extracts used at the higher concentration of 500 μg/mL were more effective in reducing the MTT conversion ability of the treated cells (Fig. 1). Extracts of R. rubiginosum, R. cinnabarinum, and R. ferrugineum exerted cytotoxic effects as deduced from the significant decrease in the ability of both IEC6 and HaCaT cells to reduce MTT. In addition, samples from R. minus, R.
polycladum, R. concinnum, R. ambiguum, and R. hirsutum induced statistically significant reductions in the MTT conversion ability of IEC6 cells, but not HaCaT cells. Interestingly, treatments with leaf extracts of R. hippophaeoides var. hippophaeoides, R. anthopogon ssp. anthopogon, and R. racemosum did not exhibit any significant alterations in metabolic activities or cell viability highlighting their potential non-cytotoxicity (Fig. 1).

Analysis of plasma membrane integrity
In order to verify the initially observed cytotoxicity of the Rhododendron leaf extracts on IEC6 and HaCaT cell lines, changes in the integrity of the plasma membrane of the cells upon incubation with leaf extracts were tested. For this, PI acquisition was assayed, which occurs only in those cells that feature ruptured plasma membranes. Cell staining with Draq5™ allowed an estimation of the total cell number. The results summarized in
Table 2, Fig. 2, and Additional file 1: Figure S1 demonstrated that incubation of IEC6 cells with 5 and 50 μg/mL of most Rhododendron leaf extracts did not significantly reduce the total cell number nor affect plasma membrane integrity. However, incubation of IEC6 cells with 50 μg/mL leaf extracts of R. polycladum, R. concinnum, R. anthropogon ssp. anthropogon, and R. hirsutum resulted in a significant reduction in the total cell number as compared to controls (Fig. 2a). Application of the highest concentration of the majority of leaf extracts

| Treatments | Conc. μg/mL | IEC 6 Total cell numbers (Draq5™) | Dead cells (%) |
|------------|-------------|----------------------------------|----------------|
| R. hippophaeoides var. hippophaeoides | 500         | 99 ± 46                          | 81 ± 16        |
|           | 50          | 494 ± 188                        | 2 ± 0.6        |
|           | 5           | 297 ± 139                        | 1 ± 1          |
| R. minus | 500         | 74 ± 48                          | 79 ± 19        |
|           | 50          | 497 ± 106                        | 0.4 ± 0.4      |
|           | 5           | 683 ± 60                         | 0.8 ± 0.2      |
| R. rubiginosum | 500     | 288 ± 120                        | 97 ± 3         |
|           | 50          | 551 ± 147                        | 0.9 ± 1        |
|           | 5           | 672 ± 101                        | 0.5 ± 0.4      |
| R. cinnabarinum | 500 | 204 ± 40                         | 100 ± 0        |
|           | 50          | 475 ± 183                        | 0.8 ± 0.8      |
|           | 5           | 481 ± 128                        | 1 ± 1          |
| R. ferrugineum | 500     | 33 ± 11                          | 100 ± 0        |
|           | 50          | 522 ± 62                         | 1 ± 0.6        |
|           | 5           | 439 ± 85                         | 2 ± 2          |
| R. polycladum | 500   | 99 ± 25                          | 100 ± 0        |
|           | 50          | 274 ± 106                        | 1 ± 1          |
|           | 5           | 384 ± 104                        | 0.9 ± 0.5      |
| R. concinnum | 500    | 304 ± 109                        | 100 ± 0        |
|           | 50          | 252 ± 58                         | 45 ± 44        |
|           | 5           | 586 ± 160                        | 0.9 ± 0.8      |
| R. xanthostephanum | 500  | 211 ± 58                          | 12 ± 4         |
|           | 50          | 408 ± 68                         | 0 ± 0          |
|           | 5           | 460 ± 115                        | 0.7 ± 0.6      |
| R. anthropogon ssp. anthropogon | 500   | 164 ± 41                          | 100 ± 0        |
|           | 50          | 235 ± 72                         | 23 ± 9         |
|           | 5           | 578 ± 164                        | 2 ± 1          |
| R. ambiguum | 500  | 666 ± 220                         | 99 ± 2         |
|           | 50          | 439 ± 154                        | 1 ± 1          |
|           | 5           | 535 ± 119                        | 0.7 ± 0.7      |
| R. hirsutum | 500   | 189 ± 65                          | 99 ± 0.6       |
|           | 50          | 267 ± 64                         | 11 ± 15        |
|           | 5           | 271 ± 147                        | 1 ± 0.5        |
| R. racemosum | 500   | 224 ± 54                          | 81 ± 23        |
|           | 50          | 400 ± 262                        | 0.4 ± 0.6      |
|           | 5           | 471 ± 183                        | 1 ± 0.7        |

Data are given as means ± standard deviation.
resulted in a significant decrease in the total cell number and a dramatic decrease in plasma membrane integrity (Table 2, Additional file 1: Figure S1). This suggested that the observed effects on IEC6 cell cultures were likely due to both, massive cell de-adhesion and cell death via plasma membrane rupturing of the majority of remaining cells, irrespective of the leaf extract used. Interestingly, the leaf extract of *R. ambiguum* had a remarkably divergent effect on IEC6 cells as opposed to all other extracts at 500 μg/mL: although almost all cells had lost their

![Fig. 2 Effects of *Rhododendron* leaf extracts on the cell numbers of IEC6 (a) and HaCaT (b) cells after 24 h incubation at 37 °C with three different concentrations (5, 50, and 500 μg/mL) of leaf extracts as indicated. The total number of cells as determined by Draq5™ staining reflects the effects of leaf extracts on cell viability and adhesion since only monolayer-associated cells were stained and counted in this assay. Values are given as mean ± standard deviations from three independent experiments, each performed in triplicates. Statistical evaluation was performed by one way ANOVA-analysis; levels of significance are indicated as *for p < 0.05.](image-url)
plasma membrane integrity, they remained adherent to the bottom of the incubation vessels (Additional file 1: Figure S1, Table 2), indicating that the extract of *R. ambiguum* potentially induces effects different from those of the other species.

HaCaT keratinocytes exposed to *Rhododendron* leaf extracts at any of the concentrations tested proved more tolerant than IEC6 cells under the same conditions. The total cell number was only significantly diminished upon incubation of HaCaT cells with 500 μg/mL leaf extracts from four *Rhododendron* species, i.e. *R. cinnabaratum*, *R. concinnum*, *R. xanthostephanum*, and *R. racemosum* (Fig. 2b, Additional file 2: Figure S2). Three out of those treatments followed the previously observed major trend: A combination of cell de-adhesion and plasma membrane disruption of HaCaT cells was observed when a high concentration of plant extract was applied (Table 2). Interestingly, the extract of *R. xanthostephanum* led to de-adhesion but not to disruption of plasma membrane integrity. Irrespective of the level of reduction in total cell number caused by 500 μg/mL of extract (Fig. 2b), five out of the 12 *Rhododendron* leaf extracts did not induce plasma membrane rupture in HaCaT cells (Table 2). This result indicated significant differences in the susceptibility of the two different cell types to the tested *Rhododendron* leaf extracts.

**Effects of *Rhododendron* leaf extracts on mitochondrial membrane potential**

Changes of the mitochondrial membrane potential of IEC6 and HaCaT cells, induced by *Rhododendron* leaf extracts were determined by MitoTracker® Red CMXRos. For this, fluorescence intensity of stained mitochondria was quantified by measuring the average intensity over arbitrarily chosen inspection areas (Fig. 3). The measured staining intensity is directly proportional to the extent of metabolically active mitochondria visualized by fluorescence. In addition, *Rhododendron* leaf extract-treated and MitoTracker® Red CMXRos-stained cells were inspected under a fluorescence microscope in accordance with morphological criteria that allow for the determination of the shape of mitochondria (Fig. 4, Additional file 3: Figure S3). Typically, mitochondria of metabolically active, well-adherent IEC6 cells with an intact cytoskeleton exhibited an elongated appearance (Fig. 4a, control), while the mitochondria of HaCaT keratinocytes appeared oval or doughnut-like in shape (Fig. 4b, control).

IEC6 cells incubated with leaf extracts from all *Rhododendron* species at the highest concentration were dramatically affected with regard to the mitochondrial membrane potential as deduced from the drastically reduced MitoTracker® Red CMXRos staining although effects were somewhat milder for leaf extracts from *R. hippophaeoides* var. *hippophaeoides*, *R. xanthostephanum*, *R. hirsutum*, and *R. racemosum* (Fig. 3a). Alterations in mitochondrial structure of IEC6 cells treated with leaf extracts were frequently observed at all three concentration (Additional file 3: Figure S3). However, IEC6 cells treated with 5 or 50 μg/mL extracts from *R. hippophaeoides* var. *hippophaeoides*, *R. xanthostephanum*, *R. hirsutum*, and *R. racemosum* did not show significant differences in the metabolic activity and mitochondrial structure when compared to controls (Figs. 3a and 4a).

Effects of *Rhododendron* leaf extracts on mitochondrial structure and metabolic activity, i.e. staining intensities, were much less pronounced in HaCaT keratinocytes (Figs. 3b and 4b, Additional file 3: Figure S3b). Exceptions were observed when HaCaT cell cultures were treated with high concentrations of leaf extracts prepared from *R. minus*, *R. cinnabaratum*, *R. ferrugineum*, *R. concinnum*, *R. anthropogon* ssp. *anthropogon*, and *R. ambiguum* (Fig. 3b) with mitochondria that no longer appeared elongated but were rounded up (Fig. 4b, Additional file 3: Figure S3b).

**Analysis of the actin cytoskeleton of *Rhododendron* extract-treated cells**

Next, we inspected the filamentous actin cytoskeleton of *Rhododendron* leaf extract-treated IEC6 and HaCaT cells as a measure for the preservation of the overall cellular architecture. With regard to the intensity of FITC-phalloidin staining of the F-actin system of both IEC6 and HaCaT cells, the analyses revealed mostly mild effects of the *Rhododendron* leaf extracts when applied at concentrations of 5 or 50 μg/mL (Fig. 5). Likewise, when morphologically inspecting the cytoskeleton of either cell type, no visible changes to the cortical F-actin system were caused by the lower concentrations of leaf extracts. The corresponding structures remained detectable underneath the plasma membranes of most cells (Fig. 6). Conversely, IEC6 cells exposed to any of the concentrations of *R. ambiguum* leaf extracts showed a significant decrease in the intensity of phalloidin-staining (Fig. 5a). Five of the *Rhododendron* leaf extracts applied at the highest concentration, namely *R. cinnabaratum*, *R. ferrugineum*, *R. concinnum*, *R. xanthostephanum*, and *R. anthropogon* ssp. *anthropogon*, resulted in a significantly reduced staining intensity of the filamentous actin system in both cell lines (Fig. 6). In contrast, the extracts of *R. minus*, *R. rubiginosum*, and *R. polycladum* exerted negative effects on the staining of F-actin in IEC6 cells only (Fig. 6a). This suggested a rather heterogeneous spectrum of effects on the actin cytoskeleton by various *Rhododendron* extracts. No particular morphological phenotype could be observed in association with Rhododendron extract-treated HaCaT keratinocytes (Fig. 6b, Additional file 4: Figure S4).
Inspection of sub-cellular architecture of floating cells that detached from monolayers

The findings detailed above suggested that IEC6 and HaCaT cells remained either adherent within or to the monolayers, or that they detached upon incubation with specific *Rhododendron* leaf extracts. Such observations could be falsely interpreted as both cell types being able to tolerate exposure to cytotoxic agents only to some extent. Because the above assays were technically restricted to adherent cells in monolayers, we next analyzed the fraction of free-floating cells which detached during treatment with *Rhododendron* leaf extracts using the same staining methods as described above. Therefore, Drq5™ staining additionally served to examine the

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**Fig. 3** Effects of *Rhododendron* leaf extracts on the mitochondrial membrane potential of IEC6 (a) and HaCaT (b) after 24 h incubation at 37 °C with three different concentrations (5, 50, and 500 μg/mL) of leaf extract as indicated. The intensity of MitoTracker® Red CMXRos signal reflects the accumulation of the dye within the mitochondrial matrix, which depends on an intact inner mitochondrial membrane potential, and thus on the metabolic activity of the cells. Values are given as mean ± standard deviations from three independent experiments, each performed in triplicate. Statistical evaluation was performed by one way ANOVA-analysis; levels of significance are indicated as * for p < 0.05.
status of nuclear DNA and to identify morphological alterations of the nuclei, such as those that are typical for cells undergoing programmed cell death.

Treatment of IEC6 cells with 500 μg/mL leaf extracts from R. cinnabarinum and subsequent analysis of the detached cells revealed nuclear condensation, cell shrinkage, rounding-up, loss of contacts with adjacent cells, formation of typical membrane blebs and occurrence of apoptotic bodies (Fig. 7a). These results suggested that leaf extracts of this particular plant species may induce apoptosis.

However, IEC6 cells exposed to 500 μg/mL R. ferrugineum leaf extracts became pycnotic and the actin filaments formed a ring surrounding the nucleus. In addition, IEC6 cells treated with leaf extracts from R. minus, R. rubiginosum, and R. ambiguum showed different stages of chromatin condensation and shrinkage of the nuclei (Fig. 7). Thus, five Rhododendron leaf extracts, namely R. hippocastanoides var. hippocastanoides, R. cinnabarinum, R. ferrugineum, R. xanthostephanum, and R. racemosum induced signs closely related to the classical symptoms of programmed cell death – apoptosis – where the treated cells also exhibited typical phenotypes like formation of plasma membrane blebs.

HaCaT cells too showed cellular changes indicative of cell death upon exposure to 500 μg/mL Rhododendron leaf extracts. However, these were different from the phenotypes observed in treated IEC6 cell cultures. HaCaT cells treated with leaf extracts from either R. cinnabarinum or R. ferrugineum displayed signs of the final stages of cell death, reminiscent of cornification, because they exhibited intense PI staining throughout the nuclei and the cytoplasm, while some cells had lost their nuclei altogether (Fig. 7b, G and H).

Moreover, exposure of HaCaT cells to R. minus, R. concinnum, and R. anthropogon ssp. anthropogon induced
changes that featured shrinkage of nuclei and chromatin condensation (Fig. 7b, I).

Investigation of apoptotic cell death pathways through determination of procaspase-3 activation
In order to substantiate the interpretation of some of the observed morphological changes induced by specific Rhododendron leaf extracts, the cells grown in monolayers and treated with the extracts were subjected to an additional apoptosis-proving assay. Besides some of the above noted symptoms, one definitive characteristic of apoptosis is the activation of procaspase-3. Therefore, a caspase-3 activity assay was applied to all treatments of IEC6 cells. Incubation of the cells with 500 μg/mL leaf extracts from any of the 12 Rhododendron species indeed induced apoptosis as evidenced by a significant increase in the levels of caspase-3 activity (data not shown). There was no significant activation of procaspase-
3 when IEC6 cells were treated with 5 or 50 μg/mL of all leaf extracts prepared from *Rhododendron* species. Interestingly, cells treated with extracts from *R. cinnabarinum* and *R. ferrugineum* at concentrations of 500 μg/mL clearly exhibited phenotypic changes characteristic to apoptosis (Fig. 7). Thus, we selected the leaf extracts of these two *Rhododendron* species to analyze procaspase-3 activation in free-floating IEC6 cells in a time-dependent manner (Fig. 8). The results revealed a steady increase in caspase-3 activity levels until 12 h of treatment with extracts of *R. cinnabarinum*, and to a five-fold lesser extent upon treatment with *R. ferrugineum* extracts. However, caspase-3 activity decreased during the next 12 h. These results argue that components contained in *Rhododendron* leaf extracts induce apoptosis in IEC6 cells when applied in high enough concentrations.

**Summarizing integration of the results achieved with a variety of cell toxicity assays**

The partially complex data acquired herein with different cell toxicity analysis assays are summarized by grouping the 12 *Rhododendron* species according to their antibacterial effectiveness with respective MICs of 50, 100, 150, or 300 μg/ml, and qualitatively comparing their effects against both cell types (Fig. 9). In general, most *Rhododendron* leaf extracts exerted more pronounced effects on IEC6 intestine epithelial cells as compared to HaCaT keratinocytes, when applied at high concentrations (500 μg/mL). *R. hippophaeoides* var. *hippophaeoides*, which exhibited the lowest antibacterial effect, also proved to be least toxic towards both mammalian cell types. A total of five *Rhododendron* extracts with high antibacterial potential (MIC of 50 μg/mL) did not reveal cytotoxicity against the mammalian cell lines in any of the tested
Fig. 7 Plasma membrane integrity and cell death by apoptosis as induced by a 24 h-exposure of IEC6 cells and HaCaT keratinocytes to 500 μg/mL of specific Rhododendron leaf extracts. Merged micrographs taken with a confocal laser scanning microscope depict IEC6 (a, panels A-E) and HaCaT cells (b, panels F-J). Violet signals in merged images are due to overlapping red, PI-derived signals, in cells with ruptured plasma membranes, and blue, Draq5™ staining of nuclei in all cells. Pictures A and F are control cells treated with 0.5 % DMSO, while cells in all other panels were incubated with extracts from R. cinnabarinum (B and G), R. ferrugineum (C and H), R. minus (D and I) and R. hippocaineoides var. hippocaineoides (E and J). Bars represent 50 μm.

Fig. 8 Detection of caspase-3 activity in IEC6 cells. Cells were treated with 500 μg/mL of leaf extracts from R. cinnabarinum (a) and R. ferrugineum (b) for the indicated time intervals. Reactions were carried out at room temperature and fluorescence was measured in a fluorescence microplate reader using 496 nm for excitation and emission was detected at 520 nm. Non-treated cells and cells treated with DMSO (0.5 %) were used as negative controls, while staurosporine (10 μg/mL) treatment was used as a positive control (apoptosis inducer). Values are given as mean ± standard deviations from three independent experiments, each performed in triplicates. Statistical evaluation was performed by one way ANOVA-analysis; levels of significance are indicated as *for p < 0.05.
assays when applied at 50 μg/mL, indicating that these extracts are unlikely to harm mammalian cells while killing bacterial cells. Thus, these five extracts are the candidates to be further assessed for possibly containing bio-active compounds with antimicrobial potencies, while still proving safe to be applied onto epidermal or intestine mucosal cell monolayers. The corresponding plant species were *R. minus*, *R. racemosum*, *R. ferrugineum*, *R. rubiginosum*, and *R. concinnum*. Interestingly, only the extracts of *R. minus* and *R. racemosum* proved to be non-cytotoxic to both intestine epithelial cells and keratinocytes (Fig. 9), suggesting they are the most promising candidates for future investigations on the search for optimized antibiotics in bio-active plant extract and, therefore, to be used for the identification and purification of specific compounds derived from *Rhododendron*.

**Discussion**

To date, there are only few medicinal formulations on the market that contain compounds derived from *Rhododendron*. These comprise ‘Rhomitoxin’ used to treat hypertension, and ’Rhododendron cp paste’ used to relieve pain in arthritis [10]. In addition, only few *in vitro* and *in vivo* studies with specific *Rhododendron* extracts and compounds isolated thereof have been reported that validated plant extracts as being useful in traditional remedies [20]. Importantly, plants of the genus *Rhododendron* are more commonly used as alternative medicine in the geographic regions of their natural habitats, i.e., Nepal, Northeastern India, Western and Central China, or Indonesia [20]. This may be due to the fact that the precise chemical composition of medicinal formulations is often not very well defined [40, 41]. However, *Rhododendron* plants are known to synthesize a large number of chemical compounds, some of which exhibit attested pharmacological activities [42–45]. Several of these chemical compounds have been identified to belong to the pro-anthocyanidins, polyphenols, or terpenoids which are typically synthesized by plants reacting in defense to pathogenic infection or inflictions caused by herbivores [46, 47].

Not surprisingly, various plant-derived compounds exert severe cytotoxic or mutagenic effects when applied
to animal cells and tissues [48, 49]. Intoxication of domesticated or wild animals feeding on *Rhododendron* plants have been repeatedly reported and were linked to the presence of grayano-toxins [50–52]. Therefore, a comprehensive number of cytotoxicity studies involving mammalian cells or tissue cultures must be conducted before a given extract or a defined *Rhododendron*-derived compound can eventually be considered for testing on animal models, or even enter clinical trials [53, 54].

To the best of our knowledge, none of the previous studies had comprehensively analyzed the cytotoxicity of a group of pharmaceutically interesting *Rhododendron* species. Consequently, the current study introduces a multi-faceted approach, consisting of five different cytotoxicity assays, in order to investigate the effects of *Rhododendron* leaf extracts on cellular structure, metabolic activity, and viability of two different types of mammalian cells.

The results obtained herein show that treating IEC6 and HaCaT cells with low concentrations of leaf extracts prepared from any of the 12 *Rhododendron* species exhibited rather mild or no cytotoxic effects, whereas the use of high concentrations (500 μg/mL) resulted in a rather expected and remarkable cytotoxicity. A total of five *Rhododendron* species exhibited high antibacterial activities with MICs of 50 μg/mL and proved to be non-cytotoxic at this concentration. Interestingly, extracts of *R. minus* and *R. racemosum* were non-toxic to either cell lines, which makes them promising candidates for future studies. In contrast, incubation of either of the two cell lines with 500 μg/mL of the other *Rhododendron* leaf extracts resulted in severe structural and functional alterations often associated with signs of apoptosis. Our study thus confirmed that simultaneous analysis of several, albeit partially unlinked or only indirectly linked cellular parameters, is a convenient tool to separate potentially cytotoxic extracts from their ‘safe-to-use’ *Rhododendron* extracts counterparts, thus overcoming technical shortcomings of previous studies aiming at high-throughput screening.

Our results demonstrated that the incubation of cells with high concentrations of *Rhododendron* leaf extracts induced apoptosis specifically in intestine epithelial cells. Interestingly, only two extracts, namely those of *R. cinnabarinum* and *R. ferrugineum*, shared a similar pattern of cytotoxicity in all assays tested in this study. Leaf extracts of these two *Rhododendron* species were capable of inducing procaspase-3 activation prominently in IEC6 cells. The results of this study concur with other studies that have shown several secondary metabolic compounds from *Rhododendron* species to induce apoptosis in cultures of different mammalian cell lines [55, 57].

Overall, keratinocytes were more resistant to cytotoxicity exerted upon incubation with *Rhododendron* leaf extracts than IEC6 cells. Resistance of HaCaT cells against cytotoxic agents was observed by us previously when studying dust exposure [32]. This remarkable feature of keratinocytes might be due to the specific lipid composition of their membranes and their ability to build a stratified epithelium when exposed to air during cornification [32, 57, 58].

**Conclusion**

Using a comprehensive approach, the cytotoxicity of those *Rhododendron* species that had previously been shown to exhibit the highest antibacterial activities was determined. As such, we managed to continue our ongoing approach in identifying pharmaceutically feasible antibiotics or lead structures. Utilizing two test cell lines as relevant models for the envisioned ectopic or oral treatment and applying several different cell biological assays, proved to be a suitable combination of screening tools. Two out of the 12 *Rhododendron* species with antibacterial properties exhibit the desired traits: the extracts of *R. minus* and *R. racemosum* were both non-cytotoxic at a concentration at where they efficiently produced an inhibition zone for *B. subtilis*.

Furthermore, we could conclude that *Rhododendron* leaf extracts induced apoptosis, as evidenced by typical alterations of the cellular phenotypes (chromatin condensation and formation of plasma membrane blebs) as well as by the increasing levels of active caspase-3 when cells were exposed to higher extract concentrations. In the future, we will extend our current study in order to determine whether the specific apoptosis-inducing effects of *R. cinnabarinum* and *R. ferrugineum* can be used to selectively target cancer cells, such as colorectal carcinoma cells.

In our future research, we will focus on phyto-chemically identifying the actual active compounds present in the leaf extracts derived from different *Rhododendron* species. We plan to determine the IC 50 values and to study their potential cytotoxic effects through a repertoire of different methods similar to the cell biological screening tool box laid out in the current study.

**Additional files**

Additional file 1: Figure S1. Overview of plasma membrane integrity and apoptotic cell death induced by 24h exposure of IEC6 cells to three different concentrations (5, 50, and 500 μg/mL) of *Rhododendron* leaf extracts. Single channel fluorescence, phase contrast and merged micrographs taken with a confocal laser scanning microscope. Violet signals in merged pictures are due to overlapping red, PI-derived signals with blue Draq5™ staining of the nuclei. Cells treated with 0.5% DMSO served as controls: A) R. hippophaeoides var. hippophaeoides, B) R. minus, C) R. rubiginosum, D) R. cinnabarinum, E) R. ferrugineum, F) R. polycladum, G) R. concinnum, H) R. xanthostephanum, I) R. anthopogon spp. anthopogon, J) R. ambiguum, K) R. hirsutum, and L) R. racemosum. Bar represents 250 μm. (TIFF 6203 kb)
Additional file 2: Figure S2. Overview of plasma membrane integrity and apoptotic cell death induced by 24h exposure of HaCaT keratinocytes to three different concentrations (5, 50, and 500 µM) of Rhododendron leaf extracts. Single channel fluorescence, phase contrast and merged micrographs taken with a confocal laser scanning microscope. Violet signals in merged pictures are due to overlapping red, PI-derived signals with blue Draq5™ staining of the nuclei. Cells treated with 0.5 % DMSO served as controls, A) R. hypophoaeoides var. hypophoaeoides, B) R. minus, C) R. rubiginosum, D) R. cinnabarinum, E) R. ferrugineum, F) R. polycladum, G) R. concinnum, H) R. xanthostephanum, I) R. anthopogon ssp. anthopogon, J) R. ambiguum, K) R. hirsutum, and L) R. racemosum. Bars represent 250 µm. (TIFF 6203 kb)

Additional file 3: Figure S3. Overview of mitochondrial morphology in IECs and HaCaT cells after a 24h-exposure to three different concentrations (5, 50 and 500 µM) of Rhododendron leaf extracts. Confocal fluorescence images of IECs (a, left) and HaCaT (b, right) cells labeled with MitoTracker® Red CMXRos. Cells treated with 0.5 %, 5 % DMSO served as controls, A) R. hypophoaeoides var. hypophoaeoides, B) R. minus, C) R. rubiginosum, D) R. cinnabarinum, E) R. ferrugineum, F) R. polycladum, G) R. concinnum, H) R. xanthostephanum, I) R. anthopogon ssp. anthopogon, J) R. ambiguum, K) R. hirsutum, and L) R. racemosum. Bars represent 50 µm. (TIFF 11640 kb)

Additional file 4: Figure S4. Overview of the structure of the F-actin system in IECs and HaCaT cells after a 24h-exposure to three different concentrations (5, 50 and 500 µM) of Rhododendron leaf extracts. Confocal fluorescence images of IECs (a, left) and HaCaT (b, right) labeled with phallolidin (green) and Draq5™ (blue). Cells treated with 0.5 % DMSO served as controls, A) R. hypophoaeoides var. hypophoaeoides, B) R. minus, C) R. rubiginosum, D) R. cinnabarinum, E) R. ferrugineum, F) R. polycladum, G) R. concinnum, H) R. xanthostephanum, I) R. anthopogon ssp. anthopogon, J) R. ambiguum, K) R. hirsutum, and L) R. racemosum. Bars represent 50 µm. (TIFF 8731 kb)

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AR designed experiments, conducted the experimental work and the analysis, and contributed to manuscript writing; AH and WJ contributed to the experimental work presented in Figs. 4, 6 and 7; HS collected, identified, and prepared plant material. MU and KBr designed the study, supervised the experimental work presented in Figs. 4, 6 and 7; HS collected, identified, and contributed to manuscript writing; AH and WJ contributed to analysis, and contributed to manuscript writing. All authors read and approved the final manuscript.

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