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Antibacterial, antiviral, antiproliferative and apoptosis-inducing properties of *Brackenridgea zanguebarica* (Ochnaceae)

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Abstract

Brackenridgea zanguebarica is a small tree that is used in traditional African medicine as a type of cure-all for many diseases, including the treatment of wounds. The yellow bark of *B. zanguebarica* was used for the preparation of an ethanolic extract, which was tested in various concentrations against eleven bacteria, Herpes simplex virus type 1 (HSV-1) and different human tumour cell lines. The extract that contains different polyphenolic substances like calodenin B. Cell growth inhibition, assessed via MTT-assay, was found in all tested human cell lines with IC50 values (concentration of extract that reduced cell viability by 50%) between 33 μg dry extract/mL for HL-60 human myeloid leukaemia cells and 93 μg dry extract/mL for HaCaT human keratinocytes. Staining with Annexin-V-FLUOS and JC-1 followed by subsequent analysis via flow cytometry revealed significant apoptosis-inducing properties. Analysis of caspase activity using a fluorogenic caspase-3 substrate showed a significant caspase activity in Jurkat T-cells after incubation with the extract. The bark extract had a pronounced activity against free HSV-1 and a strong antibacterial activity against Gram-positive strains (MICs: 6–24 μg dry extract/mL), which are often involved in skin infections. Additionally, no irritating properties of the extract could be observed in hen-egg test chorioallantoic membrane (HET-CAM) assay. These findings give a rationale for the traditional use of *B. zanguebarica* and are a basis for further analysis of the plant's components, their biological activity, and its use in modern phytotherapy.

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Note: *Authors contributed equally to this work.

Introduction

Brackenridgea zanguebarica Oliv. (Ochnaceae) is a tree that can reach a height of up to 10 m. The species occurs in dry lowland forests and woodland of Tanzania and other countries in southern and eastern Africa. The bark consists of an outer grey and inner orange part and is frequently used as a yellow dye for mats. It has been reported that the powdered bark is used to treat wounds: "the yellow bark is powdered and sprinkled on" (Bally 1937). Yellow-coloured powdered roots are utilised in the same way (Kokwaro 1976). A bark decoction is applied as an eyewash in the treatment of conjunctivitis and the boiled bark is used in preparations of porridge against jaundice (Neuwinger 2000). Furthermore, in traditional medicine the plant was reported to be effective in the treatment of anaemia in East African people (Chhabara et al 1990). The root powder in combination with other herbal drugs is also well known for the treatment of swollen ankles, amenorrhoea, mental illness and against worms (Arnold & Gulumian 1984). Additionally, the powdered herb is inserted into scarification as an antidote against snake-bite (Bally 1937; Kokwaro 1976).

The secondary plant metabolites present in this plant have been analysed previously and various phenolic substances have been identified, such as brackenin (Drewes 1983), calodenin B (Drewes 1983, 1987), isochamaejasmin (Drewes 1987), a derivative of isoswertisin (Bombardelli et al 1974) and different flavonoids (Bombardelli et al 1974). The pharmacological properties of this plant have not been explored in detail, except for a report on antifungal activity of *B. zanguebarica* (Hostettmann et al 2000).

The aim of this study was to provide a rationale for the traditional use of the plant and to further analyse pharmacological properties of the extract. We have determined antimicrobial and antiviral activity of *B. zanguebarica* extracts and their ability to inhibit cell growth in different human cell lines and to induce apoptosis in Jurkat T-cells.

Materials and Methods

Plant material

The powdered bark of *Brackenridgea zanguebarica* was obtained from S. Kaltwasser, Heidelberg, Germany, a herbal therapist, who visited Tanzania in 2003. Plant material was identified by Dr M. Sawa, a botanist from the Amani Nature Reserve.

Preparation of the extract
Plant material (2 g) was extracted 5–10 times with 10–15 mL absolute ethanol until the coloured compounds were completely removed from the bark powder. The extract obtained was filtered, the solvent removed in vacuum and the dried extract resolved in 10 mL ethanol. Portions of the extract were dried completely in vacuum and the weight of the remaining dry extract was determined. Experiments were carried out with freshly prepared extracts.

Materials

Media and supplements for cell culture were obtained from Gibco (Invitrogen, Karlsruhe, Germany) or Oxoid (Wesel, Germany) and Becton Dickinson (Heidelberg, Germany); FBS was obtained from Biochrome (Berlin, Germany). MTT, propidium iodide (PI) and sodium dodecyl sulfate (SDS) were provided by Sigma-Aldrich (Taufkirchen, Germany). Annexin-V-FLUOS was from Roche Diagnostics (Mannheim, Germany) and JC-1 from Molecular Probes (MoBiTec, Göttingen, Germany). For the modified HET-CAM (hen-egg test chorioallantoic membrane) assay fertile chicken eggs (Lohmann's Selected White Leghorn) were obtained from LSL Rhein-Main (Schaafheim, Germany).

HPLC-grade amentoflavone was purchased from Fluka (Buchs, Switzerland). Methanol (p.a.) and HPLC-grade water were from VWR International (Fontenay sous Bois, France), acetonitrile (HPLC-grade) from Sigma Aldrich Chemie GmbH (Steinheim, Germany) and formic acid (p.a.) from Merck (Darmstadt, Germany).

Characterisation of the bark extract

An alcoholic extract of the bark was separated by semi-preparative reversed-phase HPLC (LiChrospher 100, RP-18 (5 μm); Merck, Darmstadt, Germany; acetonitrile, 0.1% formic acid). Different fractions were analysed via ESI-MS-MS.

Quantification of biflavonoids via LC-ESI-MS

The LC-ESI-MS system consisted of a solvent system from Latek Laborgeräte GmbH (Heidelberg, Germany) coupled with a Micromass VG Quattro II mass spectrometer (Waters, Manchester, UK). This was operated under MassLynx software (version 4.0, Waters). Nitrogen was used as nebulising and drying gas and was generated by a Parker nitrogen generator (Parker, Etten-Leur, The Netherlands). An electrospray interface was utilized for ionisation. LC-separation took part with a LiChrospher 100 RP-18 column (250×4 mm, 5 μm) from Merck (Darmstadt, Germany). Separation of the biflavonoids at ambient temperature was achieved using a binary gradient system consisting of water (A) and acetonitrile (B), both containing 0.1% formic acid. The pump program reached from 10 to 90% B within 35 min. A post-flow splitting of 1:5 was employed. Sample volumes of 20 μL were injected by a Rheodyne injection valve. Mass spectrometric detection of positively charged ions was performed over the range of m/z 50–1000 whereas the instrument was set to the following tune parameters: nebulising gas pressure of 13 L h^{-1} and drying gas pressure of 350 L h^{-1}, capillary voltage was set to 3.5 kV, HV lens to 0.5 kV, cone voltage to 90 V and the temperature of the heated transfer capillary was maintained at 120°C. Chromatograms were processed using MassLynx 4.0 software (Waters).

Quantification of the biflavonoids was performed using an external standard calibration curve with amentoflavone. Standard solutions of amentoflavone were prepared from stock solution (1 mg mL^{-1}) and stored at 4°C in the dark. Serial dilution was carried out with methanol to the following concentrations 6.25, 12.5, 25, 50 and 150 ng μL^{-1}.

Quantification of tannins

Tannins were quantified from the crude bark according to the method of the European Pharmacopoeia (5.2.08.14).

Bacteria, viruses and cell cultures

Bacterial strains were kindly provided by the Hygiene Institute, Dept. of Medical Microbiology, Heidelberg University. Strains were obtained originally from the DSMZ, Braunschweig, Germany; ATCC, USA; and NCTC, UK.

Herpes simplex virus type 1 strain KOS was obtained from PD Dr Schnitzler, Hygiene Institute, Dept. of Virology, University Hospital Heidelberg, and was cultivated in RC-37 cells (Rosen-Wolff et al 1988).

RC-37 cells (African green monkey kidney cells) were obtained from Hygiene Institute, Dept. of Virology, Heidelberg University, and were cultivated in DMEM supplemented with 5% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in 125 cm^2 culture flasks.

HL-60 and Jurkat cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany and maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U mL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin. Human HaCaT keratinocytes (kindly provided by Dr N. E. Fusenig, DKFZ, Heidelberg, Germany) were incubated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U mL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in 75 cm^2 culture flasks (Greiner bio-one, Frickenhausen, Germany).

Antibacterial activity

The antibacterial activity of an ethanolic extract of *B. zanguebarica* was evaluated in-vitro against 7 Gram-positive and 4 Gram-negative bacterial strains by determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) with a modified broth microdilution method according to the German DIN regulation 58940-8 (Weseler et al 2002). In a time-kill assay according to the NCCLS guidelines (1999) the time and concentration dependence of the bactericidal activity against *Staphylococcus aureus* was shown.

Before testing, a serial dilution of the extract was prepared in physiological saline solution in a 96-well-microtitre plate (Greiner bio-one) to obtain final extract concentrations of 385 to 6 μg dry extract/mL. The ethanol concentration in the test system did not exceed 3.5% (v/v). A bacterial inoculum adjusted to approximately 5×10^5 colony forming units (cfu) mL^{-1} was added to each well. After incubation of the plate at 37°C for 18–20 h the MIC was determined as the lowest concentration without bacterial growth (turbidity, precipitation) in the wells of the microtitre plate. The result was only recorded if the negative control (ethanol 3.5%) showed appropriate growth. For determination of MBC, 10 μL from the wells without visible growth were dropped onto agar plates and incubated overnight at 37°C. MBC was determined as the lowest concentration without bacterial colonies on the agar plate. Assays were performed in duplicate and repeated twice.

Antiviral activity

Cytotoxicity assay

To differentiate the antiviral activity from the cytotoxic effect on host cells, the extract was assayed for toxicity before testing in antiviral studies. The viability of cells was evaluated by neutral red uptake assay (Soderberg et al 1996). Plaque reduction assays were carried out with non-cytotoxic concentrations of the extract.

Direct plaque assay

The antiviral activity of the extract was evaluated via a plaque reduction assay as described elsewhere (Schnitzler et al 2001). Briefly, 2×10^2 plaque forming units (pfu) of Herpes simplex virus type 1 (HSV-1) strain KOS (Parris et al 1980) were treated with different concentrations of the extract for 1 h at room temperature and subsequently added onto confluent RC-37 monolayers to allow infection for 1 h. The residual inoculum was discarded and infected cells were overlaid with medium containing 0.5% carboxymethylcellulose. After 4 days of incubation (37°C and 5% CO_2), cell monolayers were fixed with 10% formalin and stained with 1% crystal violet. Plaques were counted and the concentration of extract that inhibited plaque formation by 50% (IC50) was determined with reference to the plaque number of RC-37 cells infected with untreated virus.

Mode of antiviral action

To elucidate the mode of antiviral action, the extract was added to virus and host cells at different stages during viral infection (Schnitzler et al 2001). Four separate assays were performed. Host cells were pre-treated with extract before viral infection or virus was incubated with extract before infection. In a third assay, cells and virus together were incubated with the extract. Finally, infected cells were incubated with overlay-medium containing the extract. The extract was used at non-cytotoxic concentrations (77 $\mu g\,mL^{-1}$; 7.7 $\mu g\,mL^{-1}$). In all experiments, cells infected with untreated virus were used as control. Each assay was performed in six replicates. The percent plaque reduction was calculated relative to the number of plaques in the control.

Anti-tumour activity (MTT-assay)

Cytotoxicity was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assays (Mosmann 1983). Exponentially growing cells were seeded in 96-well plates and treated with various concentrations of the extract (up to 0.5%, corresponding to 385 μg dry extract/mL). HaCaT cells were allowed to adhere to the cell culture plate for 48 h before treatment. Cells were incubated at 37°C for 48 h, MTT was added to each well (0.05 mg mL^{-1}) and incubation was continued for 2 or 4 h, respectively. MTT-formazan products were dissolved in DMSO with 10% SDS and 1% acetic acid or in 50% isopropanol containing 0.2 M HCl by mechanical destruction and absorbance was detected at 570 nm in an ELISA plate reader. Cell viability was calculated as relative to untreated cells. Assays were performed in 4–8 replicates and repeated at least 3 times.

Analysis of apoptosis

Jurkat T-cells were incubated with the extract for 24 h and apoptotic cells were quantified via flow cytometry using a FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Phosphatidylserine exposure was detected after staining with Annexin-V-FLUOS for 15 min on ice; distinction between early and late apoptotic cells was realised by co-staining with PI in annexin-binding buffer directly before analysis (Willingham 1999).

Changes in mitochondrial membrane potential were determined via staining with lipophilic cationic dye JC-1 for 20 min at room temperature in HBSS. Depolarisation of the mitochondrial membrane leads to a loss of JC-1 aggregates and an increase in green fluorescent JC-1 monomers (Cossarizza et al 1996). Cells with a shifted fluorescence were determined as apoptotic.

Activity of caspase-3

Activity was assessed with slight modifications as described elsewhere (Umansky et al 2001). In brief, Jurkat T-cells were treated with *Brackenridgea* extract for 6.5 h; for each sample 10^6 cells were harvested and resuspended in 50 μL ice-cold lysis buffer. After centrifugation 10 μL of the supernatant were incubated with 20 μL of the fluorogenic caspase-3 substrate Ac-DEVD-AMC (Bachem Biochemica, Heidelberg, Germany) in lysis buffer containing 10 mM dithiothreitol for 1 h at 37°C. Triplicates of each sample were analysed in a fluorometer (380 nm ex., 465 nm em.) and fluorescence units (FU) were detected. The caspase activity was calculated using the slope of an AMC calibration curve according to the following formula (ΔFU/h = the difference in FU between an un-induced control and an induced sample): Caspase activity = (ΔFU/h) × 1/curve slope.

Modified HET-CAM assay (detection of the irritating potential)

We evaluated the irritating potential of *B. zanguebarica* ethanol extract by determination of the irritation threshold in a modified HET-CAM (hen-egg test chorioallantoic membrane) assay, originally described by Luepke (1985). The irritation threshold is defined as the lowest concentration of the irritant leading to clearly visible haemorrhage within 5 min of application, comparable with the reaction of the CAM after exposure to 0.5% SDS solution.

White Leghorn Selected eggs were incubated at 37.8°C at 70% humidity. After 7 days of incubation the CAM was uncovered and rising concentrations of the *Brackenridgea* extract diluted in physiological saline solution were applied. The reaction of the CAM's vessel system was documented by digital photography for 5 min after application.

Statistical analysis

Statistical analysis of the effects of different extract concentrations on apoptosis induction was performed using SAS (version 8.02). The exact Kruskal–Wallis test was applied to compare the data of the three different concentrations in each assay. After a global test to significance level $\alpha = 0.05$, post-hoc pair-wise comparisons were performed using also the local significance level of $\alpha = 0.05$ according to the closed test procedure (closure method) (Hochberg & Tamhane 1987). In case of a rejection of the global null hypothesis, this procedure controls the global significance level of $\alpha = 0.05$. Statistical significance was assumed at a value of $P < 0.05$.

Results

Composition of the extract

Calodenin B (Figure 1B) could be identified unambiguously by comparing the MS-data (m/z 525, fragments m/z 137, 147, 295, 405) with those of the literature (Drewes 1987, Drewes et al 1984). Further MS data (e.g. molecular weights m/z 511, 527, 529, 543, 569, 585) indicate the presence of methoxy- and dihydro- derivatives and different biflavonoids (Bombardelli et al 1974; Drewes 1983, 1987).

Calodenin B was quantified to 1% in the bark, calculated as amentoflavone. One characteristic fragment is m/z 137, presenting a dihydroxybenzoate residue, which is also found as a fragment peak in other main components with a molecular weight higher than 500 (m/z 511, 513, 525, 527, 551, 513, 633) that we assume to be biflavonoids. Quantification of all these compounds lead to a total content of biflavonoids of 11% in the bark, corresponding to 29% in the dry extract.

The powdered bark contains 0.8% tannins.

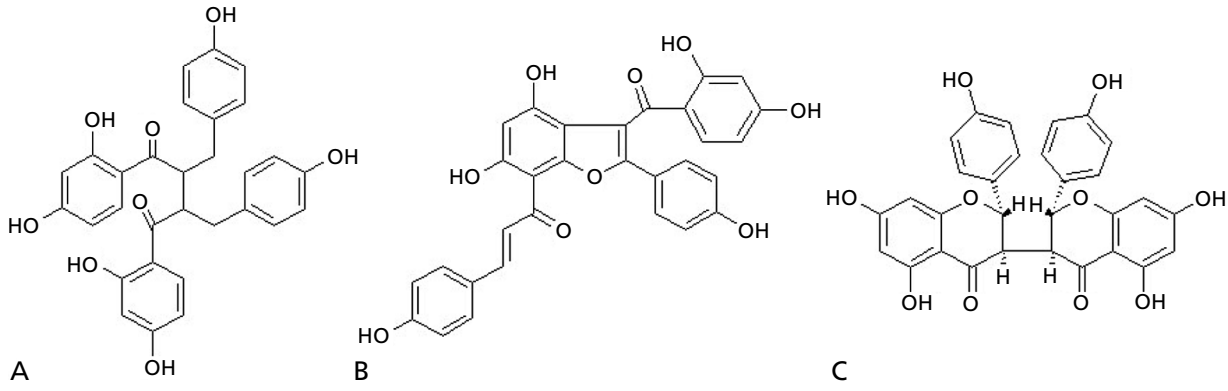

Figure 1 Phenolic compounds present in *Brackenridgea zanguebarica*: brackenin (A), calodenin B (B), isochamaejasmin (C).

Table 1 Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of ethanolic *Brackenridgea* extract

	MIC (μg dry extract/mL)	MBC (μg dry extract/mL)
Gram-positive bacteria		
Staphylococcus aureus ATCC 6538	12	48
Staphylococcus aureus, methicillin resistant (MRSA) NCTC 10442	24	48
Staphylococcus epidermidis ATCC 49134	24–12	48
Staphylococcus saprophyticus ATCC 15305	24	48
Enterococcus faecalis ATCC 19433	48	> 385
Enterococcus faecium ATCC 6057	48–24	> 385
Streptococcus pyogenes ATCC 12344	6	48
Gram-negative bacteria		
Escherichia coli ATCC 11229	> 385	> 385
Klebsiella pneumoniae ATCC 10031	> 385–385	> 385
Pseudomonas aeruginosa ATCC 15442	> 385	> 385
Proteus mirabilis ATCC 14153	385	385

Presented data are means of six replicates.

Activity against Gram-positive bacteria

Via a broth microdilution assay, the MIC and MBC of the ethanolic bark extract of *B. zanguebarica* were determined for 7 Gram-positive and 4 Gram-negative bacterial strains. The tested Gram-positive bacteria exhibited MIC values of 6–48 μg dry extract/mL (Table 1). The most sensitive strain was *Streptococcus pyogenes*. The tested Gram-negative bacteria were less susceptible; their MICs were above 385 μg dry extract/mL.

In a time-kill assay the extract exhibited a bacteriostatic effect on *Staphylococcus aureus* in concentrations of 48 μg dry extract/mL (4-fold MIC) and 24 μg dry extract/mL (2-fold MIC) (Figure 2). At higher concentrations, when the 8-fold MIC (96 μg dry extract/mL) was applied to the test system, a bactericidal effect resulted after 17 h (log-3-reduction time) of incubation. Figure 2 shows that the antibacterial activity of the extract was time and concentration dependent.

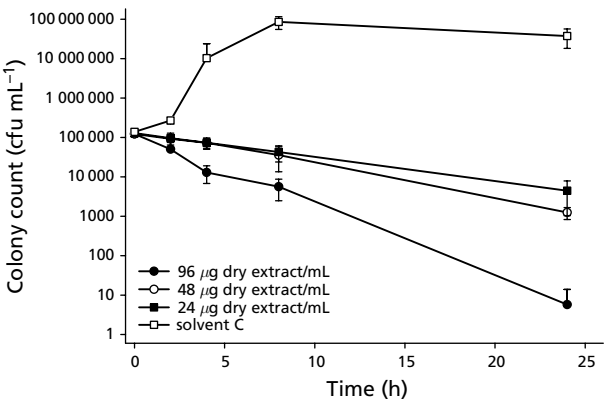

Figure 2 Time dependence of the antibacterial activity of *Brackenridgea* extract against S*taphylococcus aureus*. Bars represent standard deviations.

Antiviral activity of the extract is due to neutralisation of free viruses

The antiviral activity of the extract was assessed at four different stages during viral infection. Pre-treatment of cells 1 h before virus infection with the maximum non-cytotoxic concentration of 77 μg extract/mL caused a moderate plaque reduction of 27%, whereas a 10-fold lower concentration of 7.7 μg extract/mL exhibited no effect. When the extract was added during the adsorption period of the virus, a concentration of 77 μg dry extract/mL reduced plaque formation by > 99%; treatment with 7.7 μg dry extract/mL led to a reduction by > 62%. On the other hand, pre-treatment of viruses with the extract for 1 h before cell infection caused a significant plaque reduction of > 99% at both concentrations. In contrast, when the extract was added to the plaque assay after penetration of virus into host cells no significant plaque reduction could be observed.

To determine the 50% inhibitory concentration (IC50), viruses were exposed for 1 h to different extract concentrations in the range 0.0008–770 μg dry extract/mL, added on cell monolayers and incubated for 1 h at 37°C. The IC50 was determined to be 670 ng dry extract/mL (Figure 3). As the 50% cytotoxic concentration in RC-37 cells (TC50) was calculated to be 92 μg dry extract mL^{-1}, the selectivity index (TC50/IC50), which describes the relative distance between the cytotoxicity and the antiviral effect of *Brackenridgea* extract in-vitro, was determined at > 100.

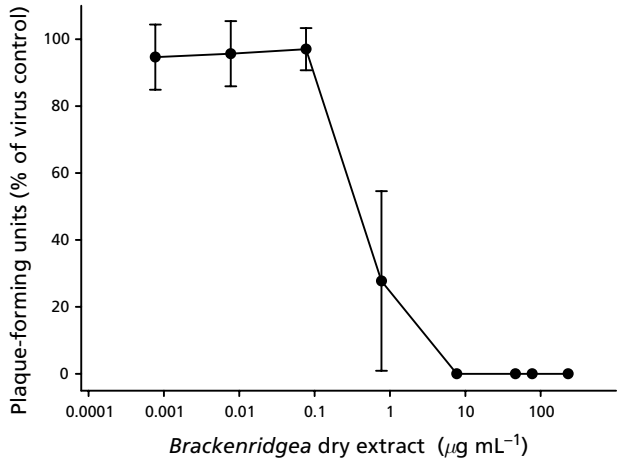

Figure 3 Dose-dependent plaque reduction after incubation of free HSV-1 for 1 h with *Brackenridgea* extract. Bars represent standard deviations. The assay was performed two times independently with six replicates each (n = 11–12).

Cell growth inhibition of HL-60, Jurkat and HaCaT cells

The extract was cytotoxic to tumour cells and keratinocytes in culture and inhibited cell proliferation. The cell viability was assessed by the MTT assay 48 h after incubation with the extract. Figure 4 shows the dose dependence of viability loss in HL-60 and HaCaT cells after incubation with *Brackenridgea* extract. For each cell line, the IC50 value was calculated using a four parameter logistic curve as a model. The IC50 for HL-60 cells was determined as 33 μg dry extract/mL and in Jurkat T-cells a very similar IC50 of 39 μg dry extract/mL was detected. For the benign HaCaT cell line the IC50 value was significantly higher at 67 μg dry extract/mL. When incubating confluent (10 days old) HaCaT cells for 48 h with *Brackenridgea* extract the IC50 was even higher, at 93 μg dry extract/mL.

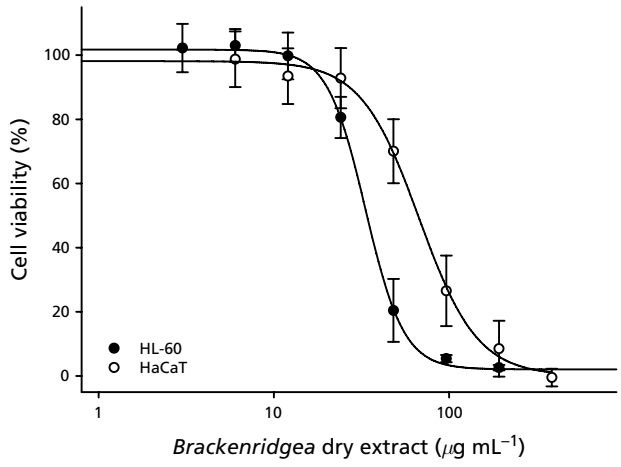

Figure 4 Viability (MTT-formazan production) of HL-60 and HaCaT cells after 48 h incubation with *Brackenridgea* extract. Bars represent standard deviations. Assays were performed three times independently with six replicates each (n = 18).

Apoptosis induction in Jurkat T-cells

The apoptosis-inducing properties of secondary plant metabolites of *Brackenridgea* extract were analysed using a flow cytometric annexin-V assay. Detection of mitochondrial membrane depolarisation was carried out with JC-1, and caspase-3 activation was assessed.

Incubation with 39 μg dry extract/mL for 24 h resulted in phosphatidylserine exposure and mitochondrial membrane depolarisation for about 50% of the cells. A significant activation of caspase-3 was observed for this concentration after 6.5 h in Jurkat T-cells. Caspase-3 activity, annexin-V exposure and mitochondrial membrane depolarisation were shown to be clearly dose dependent (Table 2).

Brackenridgea extract is not irritating in the HET-CAM assay

Different concentrations of the ethanolic extract were applied to the CAM. The highest concentration tested was 7.7 mg mL^{-1}. Even at this concentration no haemorrhages could be detected on the CAM, indicating that the extract was not irritating (data not shown).

Discussion

The tested bark extract of *Brackenridgea zanguebarica* showed a high antibacterial activity against Gram-positive strains, especially when compared with the antibacterial activity of some other extracts of European medicinal plants that are traditionally used for prevention and treatment of skin lesions (Weseler et al 2002; Weseler 2004). Among the tested preparations, a tincture of Hamamelidis folium (*Hamamelis virginiana*), which is rich in tannins, is most effective against the chosen Gram-positive bacteria with MIC values of 120–480 μg dry extract/mL (Weseler 2004). In Germany this herbal drug is recommended by the Commission E for the treatment of minor skin injuries, local inflammation of skin and mucous membranes, haemorrhoids and for varicose veins. The corresponding MIC values of *Brackenridgea* extract are 5–20 times lower, emphasising its remarkably high activity against Gram-positive bacteria, especially against *S. pyogenes*. For *S. pyogenes* the MIC value of *Brackenridgea* extract (6 μg mL^{-1}) was only 10 times higher than the MIC value of the standard antibiotic levofloxacin (0.63 μg mL^{-1}); for *S. aureus* the corresponding MIC value (12 μg mL^{-1}) was about 100 times higher than the MIC of levofloxacin (0.16 μg mL^{-1}).

All tested bacteria play a role in infections of human skin and epithelia. Staphylococci belong to the resident skin flora, whereas the Gram-positive enterococci and Gram-negative enterobacteria occur physiologically in the intestine but can also be found temporarily on the

Table 2 Apoptosis induction in Jurkat T-cells

	Solvent C	*Brackenridgea extract*	
		39 μg mL^{-1}	77 μg mL^{-1}
Annexin-V positive cells (%)	8.67 ± 0.88	52.00 ± 5.03*	90.00 ± 3.00*
Cells with mitochondrial membrane depolarization (%)	8.00 ± 0.58	50.67 ± 3.28*	90.33 ± 2.6*
Caspase activity (relative to untreated control cells)	1.49 ± 0.04	8.04 ± 0.06*	22.96 ± 0.33*

Apoptotic cells were quantified after incubation with *Brackenridgea* extract for 24 h in Jurkat T-cells via annexin-V staining and detection of mitochondrial membrane depolarisation. Caspase activity was quantified after incubation for 6.5 h. Presented data are means ± s.e., n = 3. *$P < 0.05$ compared with control.

skin and even on epithelia of the urinary and respiratory tracts. When the skin is damaged or hurt, these bacteria are able to cause infections leading to complications in the treatment of the wound and slow down the healing process. *S. pyogenes* causes purulent infections of the skin and the upper respiratory tract (tonsillitis, sinusitis, otitis media), whereas the ubiquitously occurring *Pseudomonas aeruginosa* is especially dangerous for hospitalised and immune-suppressed patients. Because of the increasing number of multi-resistant bacterial strains the search for alternatives to the common antibiotics with a more non-specific mode of action is becoming more and more important. These substances or plant extracts might be used at least in the prevention but also in topical anti-infective therapy. The tested methicillin-resistant strain of *S. aureus* (MRSA) was nearly as susceptible to *Brackenridgea* extract (slightly higher MIC but same MBC value) as the methicillin-sensitive reference strain. Therefore, the extract is likely to act in a way that is different from the mode of action of the penicillins and will probably not lead to a selection of resistant strains. The pronounced antibacterial activity of the bark extract in-vitro is in agreement with the traditional use of the plant for the treatment of wounds. The time-kill assay implies that a long application time of the substance is necessary to obtain a significant decrease in the bacterial population. Therefore, it is required that the topically applied anti-infective preparation is not irritating to the skin at the effective concentration. The HET-CAM assay demonstrated that the extract is not irritant, even in concentrations of 7700 μg dry extract/mL, as no haemorrhages occurred within 5 min of application. This concentration is 80 times higher than the extract concentration of 96 μg dry extract/mL which is required to achieve a bactericidal threshold in-vitro.

Regarding the antiviral activity, pre-treatment of free HSV-1 with the extract 1 h before host cell infection led to the highest dose-dependent reduction of plaque formation. Using non-cytotoxic concentrations of the extract, virus plaque formation was reduced by more than 99%. Only a moderate plaque reduction (about 27%) was detected when host cells were pre-treated with the extract before virus infection. These data indicate that the extract preferably neutralizes the free virions, thus preventing adsorption of virus particles to host cells. When the extract was added during the adsorption period of the virus to host cells the maximum non-cytotoxic concentration reduced plaque formation by >99%, indicating that the extract interacts with free virus immediately after admixture. We suggest that the polyphenols of the extract interact with proteins of the virus envelope by forming multiple hydrogen and ionic bonds and therefore inhibit adsorption and entry into host cells. In contrast, when the extract was added to infected cells no significant plaque reduction could be observed. Therefore, we conclude that the extract and its components are ineffective in reducing intracellular virus replication.

In addition to the antibacterial and antiviral activity, a dose-dependent cytotoxic effect could be observed in the tested human cell lines. Among these the fast-dividing leukaemia cell lines (HL-60 and Jurkat cells) were more sensitive to the extract than the benign keratinocyte cell line (HaCaT). HaCaT cells were even more resistant to *Brackenridgea* extract when they had already reached a confluent state before treatment. Therefore, we conclude that the inhibition of proliferation is the most prominent effect. To analyse whether this effect is due to an apoptosis-inducing activity, two flow cytometric apoptosis assays were carried out documenting that *Brackenridgea* extract indeed led to induction of apoptosis in Jurkat T-cells. These findings were confirmed by detection of caspase-3 activity in Jurkat T-cells after 6.5 h of incubation with 39 μg dry extract/mL. The observed inhibitory effects might be due to some of the already known secondary plant metabolites, most likely polyphenols like the biflavonoid calodenin B, which is active against the growth of *Cladosporium cucumerinum* (Hostettmann et al 2000). Calodenin B, present in *Ochna macrocalyx*, has already been shown to be cytotoxic and antibacterial (Tang et al 2003). Since *Brackenridgea* has a wide biological activity against eukaryotic cells, bacteria and viruses, the mode of action is unspecific and is probably due to interaction of hydroxyl groups of the polyphenols with diverse proteins, which has already been

described for many other polyphenols. Plant phenolics were shown to neutralize herpes simplex viruses by blocking virus or host cell receptors (Vanden Berghe et al 1986; Jassim & Naji 2003). For bacteria, the toxicity of tannins and related compounds is well described (Kolodziej et al 1999) and in human cells apoptosis-inducing properties of many polyphenolic substances have been reported, (e.g. for resveratrol (Hsieh & Wu 1999; Surh et al 1999), epigallocatechin-3-gallate, genistein, quercetin and curcumin (Lambert et al 2005). For *Brackenridgea zanguebarica*, the described biochemical properties are apparently due to the high content of biflavonoids (29% in the dry extract). The concentration of tannins in the bark is lower than 1%, whereas typical tannin-containing herbs accumulate from 8% (*Hamamelis virginiana*) up to 50% (*Quercus infectoria*).

Conclusion

The bark extract of *Brackenridgea zanguebarica* is effective in-vitro in low dosages against Gram-positive cocci and shows a good tolerability. Since the MIC for *S. aureus* is more than 5 times, and the MIC for *S. pyogenes* more than 10 times, lower than the IC50 for the keratinocyte cell line HaCaT and since even at very high concentrations the extract did not show any irritating properties in the HET-CAM test, the application of a bark extract (or the bark powder itself) – as it is used for wound treatment (e.g. in Tanzania) – has a rational background and provides a promising perspective for its further use in modern phytotherapy of skin lesions and disorders.

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