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Polyphenols from *Myrothamnus flabellifolia* Welw. inhibit in vitro adhesion of *Porphyromonas gingivalis* and exert anti-inflammatory cytoprotective effects in KB cells

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Abstract

Aim: Identification of anti-adhesive plant extracts against cell surface binding of *Porphyromonas gingivalis* and underlying mechanisms; investigation of potential cytoprotective effects of anti-adhesive extract on KB cells.

Materials and Methods: Polyphenol-enriched extract, fully characterized concerning flavan-3-ols and oligomeric proanthocyanidins, from *Myrothamnus flabellifolia* (MF), traditionally used for periodontitis, was tested for inhibition of *P. gingivalis*-mediated adhesion to KB cells by flow cytometry, for influence on gingipain activity (protease assay), haemagglutination and by microarray analysis for effects on bacterial transcriptome. The influence of MF on *P. gingivalis*-induced cytokine gene expression was monitored by RT-PCR and IL-6 titres by ELISA.

Results: MF (100 μg/ml) reduced *P. gingivalis* adhesion/invasion about 50% by interacting with bacterial OMPs. As shown by RT-PCR, fimbrillin and Arg-gingipain encoding genes were up-regulated by MF. On the protein level, inhibition (70%) of Arg-gingipain activity was observed, while the corresponding Lys-gingipain was hardly influenced. MF also inhibited haemagglutination. While exposure to *P. gingivalis* resulted in an increased expression of inflammation-related genes in KB cells, pre-treatment of KB cells with MF evoked cytoprotective effects concerning IL-1β, IL-6, IL-8 and TNF-α gene expression as well as IL-6 release rates. Compounds from the plant extract belonging to the class of proanthocyanidins were shown to be responsible for the observed effects and were characterized for their respective structural features.

Conclusions: While being cytoprotective, MF exerts anti-adhesive effects against *P. gingivalis*. Thus, MF may be useful for the prevention of *P. gingivalis*-associated periodontal diseases.

Key words: adhesion; cytoprotection; gingipains; inflammation; *Myrothamnus flabellifolia*; outer membrane proteins; periodontal disease; *Phorphyromonas gingivalis*; polyphenols; proanthocyanidins

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Conflict of interest and sources of funding statement

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Although the majority of species colonizing the gingival sulcus at or below the gingival margin are compatible with periodontal health, a subset of species may cause or contribute to the progression of periodontitis. Among these, the gram-negative anaerobic bacterium *Porphyromonas gingivalis* is strongly associated with chronic forms of periodontal disease (Haffajee & Socransky 1994, Machtei et al. 1997, Darveau et al. 2000, Rescala et al. 2010). Furthermore, subgingival colonization with high rates of this bacterium has been demonstrated to increase the risk of disease progression significantly (Haffajee et al. 1991).

At present, periodontitis therapy generally relies on mechanical detachment of bacterial masses from the infected sites. Antibiotics are used to support the therapy, but often fail when exclusively applied because of several pharmacokinetic reasons: low concentrations in the gingival crevice fluids or wash out effects decrease antibiotic activity, and also biofilm organization of bacteria can prevent effective antibiotic concentration in an anaerobic environment with low bacterial multiplication rates. Therefore, blocking of very early bacterial adhesion instead of killing bacterial cells by antibiotics could overcome some obstacles, because the drug would act at an early stage of the disease process, and is independent from the atmospheric conditions and bacterial multiplication rates.

Development of anti-adhesive compounds against *P. gingivalis* therefore can be a promising new strategy. The multi-target adhesion of *P. gingivalis* to epithelial cells is very complex. Fimbriae mediate adherence to other oral bacterial species, to various host components, such as haemoglobin, collagen, fibronectin and to periodontal cell surface (Cutler et al. 1995, Amano 2003). Additionally, five different haemagglutinins, associated with lipopolysaccharides and lipids on the cell surface, and a secreted exo-haemagglutinin are responsible for effective binding to erythrocytes and to epithelial cells (Okuda et al. 1986).

The most potent adhesins and virulence factors are the gingipains, three cysteine proteases that bind and cleave a wide range of host proteins (Fitzpatrick et al. 2009). Gingipains are trypsin-like cysteine proteases and are classified into two groups based on substrate specificity. The arginine-specific cysteine protease (Arg-gingipain, Rgp) is encoded by genes *rgp*A and *rgp*B and the lysine-specific cysteine protease (Lys-gingipain, Kgp) is encoded by the *kgp* gene. The gingipains are located on the surface of *P. gingivalis* from where subfractions are secreted into the extracellular fluid (Chen et al. 2001). Because of their proteolytic activity, gingipains are capable of degrading host proteins, such as collagen, fibronectin, immunoglobulin G and TNF-α (Travis & Potempa 2000). Because of the adhesin function, the gingipains directly bind to extracellular matrix proteins (Lantz et al. 1991, Pike et al. 1996) or indirectly contribute to bacterial adhesion by processing the fimbrillin subunit (Nakayama et al. 1996).

Because of the complex adhesion strategy of *P. gingivalis*, a multi-target therapy using complex compound mixtures with affinity against the different bacterial adhesins has to be considered; use of a single compound with inhibitory activity against a single adhesion would probably not be sufficient for an effective inhibition of bacterial attachment to the host cells. During the search for potential anti-adhesive compounds with affinity to *P. gingivalis* fimbriae or OMPs, the use of plant-derived natural products, especially polyphenols and polysaccharides, is a new focus of attention (Niehues et al. 2010a). While polymeric carbohydrates interact with bacterial adhesins involved in protein–carbohydrate binding, polyphenols are capable of binding to adhesins, forming protein–protein bounds to the host membrane surface (M. Niehues, T. Stark, D. Keller, T. Hofmann, A. Hensel unpublished data). In case of *P. gingivalis*, the gingipain proteases are anticipated to be suitable targets for such tannin-like, polyphenolic compounds.

Polyphenols themselves form an inhomogenous group of natural products, with the general feature of multiple hydroxylation of complex aromatic systems. A special subgroup of polyphenols, the proanthocyanidins, were recently shown to influence bacterial entry into host cells (Lengsfeld et al. 2004a, b). Therefore, substances from this class could serve as lead structure for the development of typical entry blockers. On the other side and from an economic point of view, it is not possible for effective drug development to fractionate complex plant extracts until a purified active proanthocyanidin is obtained. Therefore, standardized proanthocyanidine-enriched plant extracts with a documented profile should be used for in vitro studies and clinical development.

For these reasons, a proanthocanydin-enriched extract from *Myrothamnus flabellifolia* Welw. (Myrothamnaceae) was used in this study to evaluate its anti-adhesive activity against the host cell adhesion of *P. gingivalis*. The aerial parts of this South African resurrection plant are traditionally used for treatment of gingivitis and periodontitis (Moore et al. 2007). This ethnopharmacological background and the very high content of phenolic compounds/proanthocyanidins (Engelhardt et al. 2007, Anke et al. 2008) indicate a sound scientific rationale for investigating the influence of standardized extract on the adhesion of *P. gingivalis*. Additionally, underlying mechanisms leading to the anti-adhesive effects had to be investigated concerning potential influence on bacterial adhesins, especially on gingipain protease activity and gene expression. Further on, it was to be investigated if a pre-treatment of KB cells with the anti-adhesive extract is able to initiate cytoprotective effects against *P. gingivalis*-induced inflammation parameters.

Materials and Methods

Extract from *M. flabellifolia* MF

Plant material (Myro AG, Greifensee, Switzerland, batch Myro1) (Anke et al. 2008) was powdered. Ten grams was extracted under stirring with EtOH:-water (1:1 v/v) for 30 min. at RT and the suspension was centrifuged (10,000 *g*, 10 min.). The supernatant was lyophilized to yield the extract named MF. Standardization of the extract and analytical quality control were performed by ultrahigh-pressure liquid chromatography (UPLC) by quantitation of the lead compounds of the extract (trigalloyl quinic acid, tetragalloyl quinic acid, tellimagrandin II) and of the proanthocyanidin content (Anke et al. 2008).

P. gingivalis strain and growth conditions

P. gingivalis (ATCC 33277) was cultured under anaerobic conditions (Anaerocult, Merck, Darmstadt, Germany) at 37°C on solid sheep blood agar. For quality control purposes (identity and purity), every third passage of the bacterium was subjected to quantitative *fimA* gene PCR analysis.

Bacteria used in assays were grown to late log phase, harvested and used to inoculate 20 ml of liquid media (the same as for solid medium without blood and agar) and grown overnight at 37°C. Growth was monitored by *OD* (660 nm) and bacteria in the exponential phase were used for further experiments.

Cell culture

KB cells (ATCC CCL-17, HeLa) were kindly provided by Dr. S. Eick (University of Jena, Germany). The cells were cultured in Earl's minimum essential medium (EMEM) (Lonza, Basel, Switzerland), supplemented with 8% (v/v) heat-inactivated FCS (PAA Laboratories, Cölbe, Germany) and 50 μg/ml gentamicin (MP Biomedicals, Irvine, CA, USA) at 5% CO_2/37°C. Passaging was performed twice a week to a maximum of 15 passages.

Adhesion assay with *P. gingivalis* and KB cells

KB cells (8×10^5 cells) were grown to 90% confluence in six-well plates. The supernatant was removed, the monolayer was washed with PBS and incubated in FCS- and antibiotic-free medium.

Solid-medium-grown *P. gingivalis* bacteria (3–5 days culture) were harvested and suspended in 0.5 M $NaHCO_3$, pH 8.0. After adjusting the OD_{660} to 4.0, fluorescein isothiocyanate FITC (50 μg/ml) (Sigma-Aldrich, Steinheim, Germany) was added for labelling of the bacteria followed by incubation for 30 min./37°C. Bacteria were sedimented (3600 *g*, 5 min.) and washed twice in PBS to remove unbound FITC. FITC-labelled bacteria were suspended in EMEM. Labelling efficiency was analysed by fluorescence microscopy.

To investigate the effect of MF on the adhesion of *P. gingivalis* on KB cells, various incubation procedures were applied.

i. For *pre-incubation of labelled bacteria*, bacteria were mixed with MF (1, 0.5, 0.1 and 0.01 mg/ml) and incubated for 30 min. at 37°C. After sedimentation and washing with PBS, bacteria were resuspended in EMEM and added to KB cells (BCR 100:1) for 90 min. at 37°C.

ii. For *pre-incubation of KB cells*, MF (0.1 mg/ml) was added to EMEM and the cells were incubated for 90 min. at 37°C. Subsequently, KB cells were washed vigorously three times with PBS and labelled *P. gingivalis* were added (BCR 100:1) and incubated for 90 min. at 37°C.

iii. For *co-incubation*, MF (0.1 mg/ml) and FITC-labelled *P. gingivalis* (BCR 100:1) were simultaneously added to KB cells and incubated for 90 min. at 37°C.

After each incubation variant, KB cells were washed three times with PBS, detached from the wells by incubation with trypsin–EDTA for 3 min. at 37°C and then suspended in EMEM, supplemented with FCS (8%). The adhesion intensity of FITC-labelled *P. gingivalis* on KB cells was immediately analysed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany). Instrument settings were as follows: FCS (Detector): E-1 (Voltage), 3.07 (Amp Gain), Lin (Mode); SSC: 332, 1.00, Lin; FL1: 360, 1.00, Log.

Gene expression analysis in KB cells

KB cells (8×10^5 cells) were grown to 90% confluence in six-well plates. Before incubation with *P. gingivalis*, the supernatant was removed, monolayers were washed with PBS and incubated in FCS- and antibiotic-free media (EMEM). MF (10 and 100 μg/ml) was added and cells were incubated for 2 h/37°C. Cells were isolated, washed three times with PBS and further incubated in EMEM.

Twenty-four hours before the experiment, a bacterial liquid culture was inoculated with solid-medium-grown *P. gingivalis* ($OD_{660} = 0.05$) and incubated into the exponential phase. Bacteria were pelleted (3600 *g*, 5 min.) and resuspended in EMEM. The OD_{660} of the bacterial suspension was adjusted to a BCR of 5:1 and added to the KB cells for an incubation period of 2 h at 37°C. Following this, the supernatants were removed, KB cells were washed three times with PBS and subsequently trypsinized. After collecting the KB cells by centrifugation (100 *g*, 5 min.), mRNA was isolated using the NucleoSpin® RNA XS Kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions.

The total RNA concentration in each sample was calculated from the OD_{260}. cDNA was synthesized from 1 μg of total RNA by using the High-Capacity cDNA Reverse Transcription Kit (ABI, Darmstadt, Germany) according to the manufacturer's instructions.

RT-PCR analysis was performed by 7300 RT-PCR (ABI). Two microlitres (100 ng) of cDNA was added to 1 μl of 20 × gene expression assay (ABI) (for assay IDs see Table 1), 10 μl of TaqMan Universal MasterMix (2 ×, w/o amperase, ABI) and 7 μl of RNase-free water in an optical 96-well plate. Enzyme activation for 10 min. at 96°C was followed by 45 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. mRNA levels were expressed as *RQ* values, related to the C_T value of the 18S rRNA and to the control (KB cells, not pre-treated with MF, only infected with *P. gingivalis*).

Gene expression analysis of *P. gingivalis* (RT-PCR, microarray analysis)

Solid-medium-grown *P. gingivalis* from a 3-day culture were harvested and used to inoculate liquid media ($OD_{660} = 0.05$). After 24 h, bacteria were transferred to 4 ml of liquid culture media ($OD_{660} = 0.1$). MF was added at different concentrations (10 and 100 μg/ml). Liquid culture with *P. gingivalis* served as a negative control. After incubation for 6 h at 37°C, bacteria were harvested by centrifugation (11,000 *g*, 5 min.) and washed with PBS. The pellet was resuspended in PBS (100 μl) and mixed with

Table 1. Assay IDs for primers and probes for gene expression analysis in KB cells, synthesized by ABI (Darmstadt, Germany)

Genes	Assay ID
IL-1β	Hs01555413_m1
IL-6	Hs99999032_m1
IL-8	Hs01567913_g1
COX-2	Hs01573472_g1
TNF-α	Hs00174128_m1
18S rRNA	Hs99999901_s1

TE buffer (500 μl), 1% SDS, phenol and lysing matrix B (0.4 g) (MP Biomedicals). Cells were lysed by a FastPrep®-24-System (MP Biomedicals) (2 × 50 s, 5.0 m/s^2) and RNA isolation was followed by phenol–chloroform extraction and ethanol precipitation. Removal of DNA was performed by NucleoSpin®rDNase (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. Total RNA concentration in each sample was calculated from OD_{260}. cDNA was synthesized from 1 μg of total RNA by using the QuantiTectReverse Transcription System (Qiagen, Hilden, Germany). RT-PCR was performed using 2 μl (100 ng) of cDNA, 10 μl of 2 × QuantiTect Probe PCR Master Mix (Qiagen), 1 μl of 20 × Primer Mix, 1 μl of 20 × QuantiProbe Solution (sequences listed in Table 2) and 6 μl of RNase-free water. The oligonucleotide primers and probes (Table 2) were synthesized by Qiagen.

PCR was performed on a 7300 Real Time PCR System (Applied Biosystems and ABI, Carlsbad, CA, USA) and 7300 System Sequence Detection Software (Version 1.2.3, ABI). A total of 45 cycles were performed after 15 min. at 95°C for enzyme activation. Elongation at 76°C/30 s, denaturation at 94°C/15 s and annealing at 56°C/30 s. mRNA levels were expressed as *RQ* values, related to the C_T value of the 16S rRNA and to the untreated control.

The microarrays were designed and synthesized by NimbleGen® (NimbleGen Systems GmbH, Waldkraiburg, Germany) investigating the expression of 1842 genes. Each open reading frame of the complete *P. gingivalis* W83 genome (strain 33277 sequences were not available at the time the work was undertaken) was represented by up to 13 probes per gene, if applicable. Each of these 60-mer oligonucleotides was presented in three replicates randomly located on the microarray to ensure utmost reproducibility of the hybridization process and read-out of the fluorescence signals. As negative controls served 3510 random probes spotted at various positions on the chips.

cDNA synthesis, labelling and hybridization

For gene array analysis, a 4 ml aliquot from a 3-day liquid culture was inoculated into fresh medium to render an OD_{660} of 0.05 and cultivated for 12 h. Using this culture, 4 ml with an OD_{660} value of 0.1 was incubated with 100 μg/ml MF or medium alone (control group) for 6 h. Bacteria were sedimented and washed. Total RNA was isolated and DNA was removed as described above. RNA was aliquoted to 10 μg samples. Two independent experiments were performed and RNA isolated from the experiments was pooled for the subsequent analysis.

cDNA synthesis was carried out utilizing the superscript II cDNA synthesis kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. An initial amount of 10 μg RNA was used for cDNA synthesis. The cDNA labelling, microarray hybridization and washing of the arrays were executed as recommended by the array user guide (Gene Expression Analysis v 2.0, NimbleGen®). This protocol determined labelling of cDNA with cy3. Dye swap experiments were not carried out as the cDNA of *P. gingivalis* with and without treatment of MF were hybridized on separate arrays and appropriate controls existed. The microarrays were stored dry and in the dark until scanning.

Scanning, quality control and interpretation of the microarrays

Scanning of microarrays and subsequent feature extraction were performed with the DNA Microarray Scanner (Agilent Technologies, Santa Clara, CA, USA) at a resolution of 5 μm and NimbleScan™ (NimbleGen Systems GmbH) version 2.4 software, respectively. First, all *Porphyromonas*-related data files obtained were corrected for background intensity by comparison with the average fluorescence intensity of the negative controls. Second, all *Porphyromonas*-related data files obtained were checked for inhomogeneous hybridization results among every set of probes. Replicate spots with more than a twofold aberration from the intensity of the other two replicates were excluded from analysis. In the case of more than twofold aberration between all three replicates of a single probe, all replicates of this probe set were excluded. Microarray data were then interpreted and analysed using the programs GeneSpring GX (GeneSpring GX 11.0, Agilent Technologies) and SAM (SAM version 2.23A: http://www-stat.stanford.edu/~tibs/SAM/). Genes with statistically significant differences were calculated using the *t*-test (unpaired, 100 permutations) with a *p*-value cut-off of 0.05 and an associated Benjamini and Hochberg False Discovery Rate (FDR) multiple testing correction (Benjamini & Hochberg 1995).

All fold changes were defined as relative to untreated *P. gingivalis* 33277.

The micorarray data are deposited at gene expression omnibus (http://www.ncbi.nlm.nih.gov/geo, accession number; GSE23939).

Table 2. Primers and probes used for gene expression analysis in *Porphyromonas gingivalis*

mRNA target		Oligonucleotides (5′ → 3′)
16S-rRNA	F	TAACAGTTTTCGCTGAGGA
	R	TGTAAGGGCCGTGTTGAATTGA
	P	GTGAGGAAGGTGTGGA
kpg	F	GTGTATGTTGGGCTATCT
	R	TCCTTACTGCCACCCCTTT
	P	TGGAGGGAAGAGGAGT
*rpg*A	F	GCAGGATGAGATGAACGAAA
	R	GTGACCACCGAAAGTA
	P	AAACACCCGAACAACA
*fim*A	F	ACGAATAACCCAGAGAAT
	R	CTGACCAACGAGAACCCA
	P	TCACAGAGTCTGCTCAC

F, forward primer; R, reverse primer; P, probe.

Haemagglutination assay with *P. gingivalis*

Colonies of solid-medium-grown *P. gingivalis* (3–5 days culture) were harvested and suspended in PBS, pH 7.4. One-hundred and fifty microlitres of the bacterial suspension (OD_{660} 2.0) was mixed with 150 μl of MF-containing solution and incubated at 37°C/30 min. Bacteria were washed (6000 *g*, 5 min.), resuspended and serially diluted in 96-well round bottom microtitre plates with PBS. A 50 μl aliquot of each dilution was mixed with 50 μl of human erythrocyte suspension (2% in PBS) and

incubated for 3 h at RT. Assays were carried out in duplicate and were repeated at least three times in independent experiments. Evaluation was performed by using an UV reader (Sunrise, Tecan Austria GmbH, Salzburg, Austria) and MicroWin® software.

Gingipain assay

Bacteria from liquid overnight culture were washed, resuspended in PBS and adjusted to OD_{660} of 1.0. One millilitre of this suspension was mixed with 10, 50 or 100 μg/ml of MF and incubated over 1, 10 or 30 min. at 37°C. Bacteria were washed, resuspended in PBS with 1 mM L-cysteine and adjusted to OD_{660} of 0.26. The fluorescent protease substrates α-*N*-benzoyl-L-arginine-7-amido-4-methylcoumarin and *t*-butyloxycarboyl-Val-Leu-Lys-7-amido-4-methylcoumarin (both from Sigma-Aldrich, Taufkirchen, Germany) were used to determine Arg-X- and Lys-X-specific activities, respectively. Arg-X activity was assayed in 100 μl of PBS containing 1 mM L-cysteine, 100 μM substrate and 5 μl of bacterial suspension. Lys-X activity was assayed in 100 μl of PBS containing 1 mM L-cysteine, 10 μM substrate and 50 μl of bacterial suspension. Released 7-amido-4-methylcoumarin was measured with a fluorimeter (Ascent FL, Thermoscientific, Waltham, MA, USA) using its kinetic measurement software program over a period of 10 min. (wave length settings: excitation 355 nm, emission 460 nm). Assays were carried out in duplicate and were repeated at least in three independent experiments. Protease activities were related to the untreated control.

Quantitation of IL-6 from KB cells

KB cells (8×10^5 cells, 70% confluence) were cultured in 96 flat-bottomed microtitre well plates. Seventeen hours before the experiment, the cells were washed with PBS and incubated in EMEM with 8% SerEx® (PAA Laboratories, Cölbe, Germany) and 50 μg/ml gentamicin. Treatment of KB cells started by washing them with 100 μl of PBS per well and adding MF (100 μg/ml in EMEM) for 2 h/37°C. Supernatants were collected in a sterile 96-well microtitre plate and the cell monolayers were washed with PBS. *P. gingivalis* grown to late exponential phase were resuspended in EMEM and added to KB cells at a BCR of 5:1 and 100:1, respectively. Cell culture plates were centrifuged (200 *g*, 5 min.) followed by incubation at 37°C/2 h. After incubation, the culture media were collected. Cells were washed three times with 150 μl of PBS per well and incubated for further 17 h at 37°C in KB cell culture medium. After this incubation period, the supernatants were again collected and stored at −80°C until analysed using Human ELISA Set IL-6 (ImmunoTools, Friesoythe, Germany) according to the manufacturer's protocol. Data are expressed as pg/ml as mean from three separate values from four independent experiments.

Analytical aspects for determination and characterization of proanthocyanidins

Isolation and structural characterization of polyphenols from MF were carried out as described by Anke et al. (2008).

For interaction studies of *P. gingivalis* with proanthocyanidins, 300 μl of bacteria suspension (*OD* 4.0, 1.0 and 0.1) was incubated with the test compounds and mixtures (1 mg/mL PBS) for 30 min. at 37°C. Control values were bacteria treated only with vehicle without test compounds, test compounds, dissolved in PBS but without bacteria. After incubation, the suspension was centrifuged (11,000 *g*, 20 min.). The clear supernatant was incubated for 30 min. at 56°C and lyophilized. The residue was dissolved in MeOH and used for HPLC on an Alliance 2690 system (Waters, Milford, MA, USA), stationary phase Silica Diol, pore size 6 μm, 120 Å, 250 mm × 4.6 mm (Interchim, Montlucon Cedex, France). The binary mobile phase consisted of (A) CH_3CN: HOAc (98:2, v/v) and (B) CH_3OH:H_2O:HOAc (95:3:2 v/v/v). Separations were effected by a linear gradient. Eluent was monitored by UV detection at 280 nm and by fluorescence detection with excitation at 280 nm and emission at 316 nm.

Anthocyanidin reaction in the washed (twice in PBS) bacteria pellet was performed after lyses with 1 mL of Triton® X-100 (10% in PBS) for 24 h at 4°C. Nine hundred and eighty microlitres of n-butanol/HCl (25%) (19:1 v/v) and 20 μL of 2% (m/v) solution $NH_4Fe(SO_4)$ were added to 500 μL of the centrifuged lysis solution. The mixture was incubated at 95°C for 15 min. The presence of proanthocyanidins was proven by the formation of an intensive red-orange colour.

Results

M. flabellifolia extract MF inhibits adhesion of *P. gingivalis* to KB cells

From the aerial parts of *Myrothamnus flabelifolia*, an ethanol/water extract MF was prepared with a phytochemical composition and fingerprinting as recently described (Engelhardt et al. 2007, Anke et al. 2008). The influence of MF on the adhesion of *P. gingivalis* to human epithelial mucosa cells (KB cell line) was analysed quantitatively by a flow cytometric assay. In principle, FITC-labelled bacteria were co-incubated with a monolayer of KB cells. Non-adhering bacteria were removed by washing. After trypsin treatment, suspended cells with adhering or invaded labelled bacteria were quantified by FACS. Measured values were related to the adhesion determined in the untreated control group. MF between 0.1 and 1 mg/ml evoked dose-dependent inhibition of bacterial epithelial cell attachment or invasion (Fig. 1).

In order to investigate whether MF influenced the bacterial cell surface and/or ligand properties on the eukaryotic cell membrane, three different incubation variants were performed, namely pre-treatment of bacteria, pre-treatment of KB cells and co-incubation of bacteria together with KB cells in the presence of MF (0.1 mg/ml). Data obtained indicated that pre-incubation of *P. gingivalis* as well as the pre-treatment of KB cells reduced bacterial binding to KB cells (Fig. 2). As expected, co-treatment also led to considerable effects. From these data, it is concluded that MF influences the bacterial and the eukaryotic cell surface.

MF (100 μg/ml) is not toxic for *P. gingivalis* and KB cells

For further evaluation of the mode of action, a potential direct cytotoxicity of MF (0.4 mg/ml) for *P. gingivalis* was tested by agar diffusion assay over 5 days (positive control amoxicillin). Bacterial growth was not influenced by MF (data not shown). In addition, the influence of MF (1–1000 μg) on *P. gingivalis* grown in liquid culture was investigated by determination of cellular viability (MTT assay) (Moosmann 1983). Exposure to MF at <150 μg/ml over 30 min. (the contact time used for the anti-adhesion assay) did not influence viability of *P. gingivalis*. EC_{50} values for a 24-h incubation were determined as

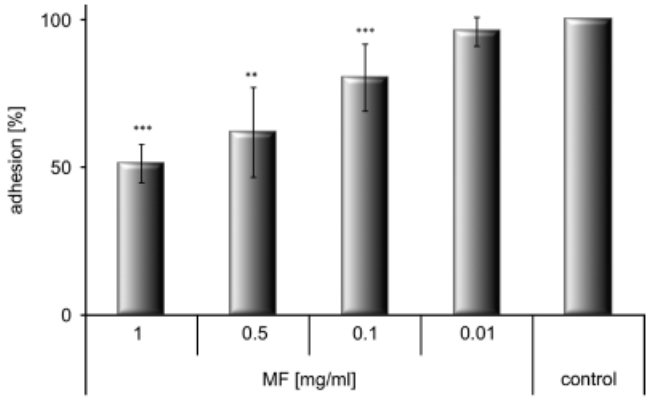

Fig. 1. Mean adhesion values of FITC-labelled MF-exposed *Porphyromonas gingivalis* to KB cells. The bacteria were pre-incubated for 30 min. at 37°C with different concentrations of MF. Data are related to the untreated control (= 100%). Data are mean values ± SD ($n \geqslant 3$); $^{**}p < 0.01$; $^{***}p < 0.005$.

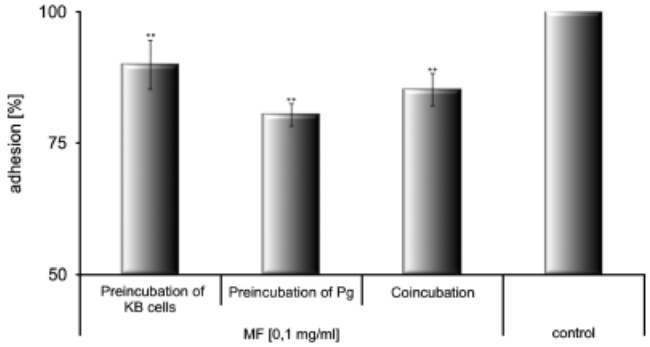

Fig. 2. Inhibitory effects of MF (0.1 mg/ml) on *P. gingivalis* adhesion to KB cells under different incubation variants: experiments were performed after pre-incubation of KB cells, pre-incubation of *P. gingivalis* and after co-incubation of KB cells together with *P. gingivalis* and MF. Data are mean ± SD ($n \geqslant 3$); $^{**}p < 0.01$ against control values; no significances between the different incubation groups.

114 μg/ml. From these data, cytotoxic effects of MF against the bacteria can be excluded under the assay conditions.

For evaluation of a potential cytotoxicity of MF against KB cells, cells were incubated for 2 and 6 h with different concentrations of MF (1, 10 and 100 μg/ml), followed by MTT test (Moosmann 1983). No signs of reduced mitochondrial activity were measured within these experiments. Prolonged incubation time for 24 h decreased cell viability at 100 μg/ml significantly, effects that are known for plant extracts containing higher amounts of galloylated proanthocyanidins (Actis-Goretta et al. 2008).

MF inhibits haemagglutination and gingipains

In order to investigate the influence of MF on the major adhesins, haemagglutinin, arginin- and lysine-gingipain activities after incubation with MF were monitored. An inhibiting activity of MF against bacterial haemagglutinin was confirmed by the haemagglutination assay. *P. gingivalis* were pre-treated for 30 min. with different concentrations of MF (1–1000 μg/ml) and incubated after a serial dilution together with human erythrocytes. MF-treated bacteria displayed a substantial inhibition of haemagglutination as shown by rise of the respective haemagglutination-inhibition titres. (MF 1000 μg/ml: shift 2.7, 500 μg/ml: shift 1.7, 100 μg/ml: shift 1.6, 50 μg/ml shift 1.4, 1 μg/ml shift 0.5).

For investigation of MF effects on the Arg- and Lys-gingipain activities, specific fluorescent-labelled peptide substrates were used for protease assays after incubating the bacteria with MF, namely α-BAAM (α-*N*-benzoyl-L-arginin-7-amino-4-methylcoumarin) for Arg-gingipain and t-boc-VLA (t-butyloxycarboyl-val-leu-lys-7-amino-4-methylcoumarin) for Lys-gingipain (Potempa & Nguyen 2007). The tripeptide leupeptide (100 μM), a specific inhibitor of Arg-gingipain, served as a positive control (Baba et al. 2001). MF inhibited Arg-gingipain in a concentration-dependent manner (Fig. 3). After 1 min. contact time, about 50% inhibition was observed at 1 μg/ml. Prolonged incubation time did not further increase the anti-protease effects. MF at 50 μg/ml reduced Arg-gingipain by 70–80%, at 100 μg/ml by about 80%. In contrast to this, Lys-gingipain activity was only slightly reduced by MF to about 50%. MF did also inhibit Lys-gingipain, but to a lesser extent. These findings indicate MF to be a potent gingipain inhibitor, predominantly because of its activity against Arg-gingipain.

Influence of MF on genome-wide gene expression by microarray analysis

For a thorough investigation on the influence of *MF* on *P. gingivalis* genes, a transcriptome microarray was performed. *P. gingivalis* were treated in liquid culture for 6 h with MF (100 μg/ml). Two independent experiments were performed, and pooled RNA was used for microarray. Levels of gene expression from treated and untreated bacterial cells were compared by the normalized fluorescence intensities (Table 3). In total, changes in transcript abundancies of 192 genes were monitored when using a threshold of twofold difference; judged by transcript amounts, 115 genes appeared as down-regulated and 77 genes as up-regulated. The calculated false discovery rate was 7.4% (Benjamini & Hochberg 1995). Generally, the majority of influenced genes showed three- to fourfold changes. Drastic differences in transcript abundancies with values of ⩾10-fold were found for five genes (Table 3).

The microarray data indicated effects on the expression of adhesin genes in the presence/absence of MF. According to the microarray data, the fimbrillin message increased 2.5-fold, while the mRNA for the arginin-specific cysteine proteinase decreased fourfold. Also, the three haemagglutination genes were down-regulated (haemagglutinin-related protein: − 2.4, haemagglutinin protein HagE: − 2.8, haemagglutinin protein HagA: − 14.2).

Of note, mRNAs from gene clusters responsible for DNA transcription and

translation control reduced 3–10-fold, especially, messages from transposase genes and genes encoding translation-related enzymes with influence on ribosomal activity were considerably less concentrated in the presence of MF.

Influence of MF on the gene expression of major adhesins

For affirmation of gene array data, RT-PCR studies were performed on the influence of MF on the expression of *rgpA* for Arg-gingipain, *kgp* for Lys-gingipain and *fimA* for fimbrillin (Fig. 4). Expression rates of the respective genes were constant in *P. gingivalis* liquid cultures over incubation periods from 2 to 12 h, as monitored by the corresponding ΔC_T values. Treatment of the bacteria with MF (10 and 100 μg/ml) influenced the gene expression moderately; while *kgp* expression was unchanged, a significant, about three- to fourfold increase of *fimA* was observed, being in accordance with data obtained from the gene array. *RgpA* expression was significantly increased as monitored by RT-PCR. However, the increase of *rgpA* expression could not be substantiated by the microarray data. Together with the data from enzymatic gingipains assay (Fig. 3), it seems obvious that an effective blocking of Arg-gingipain by MF leads to an increased gene expression of rgpA in order to rebuild the restore protease activity. Moderate blocking of Lys-gingipain enables the enzyme to act enzymatically, but to a lower degree. A subsequent stimulation of gene expression seems not to be necessary.

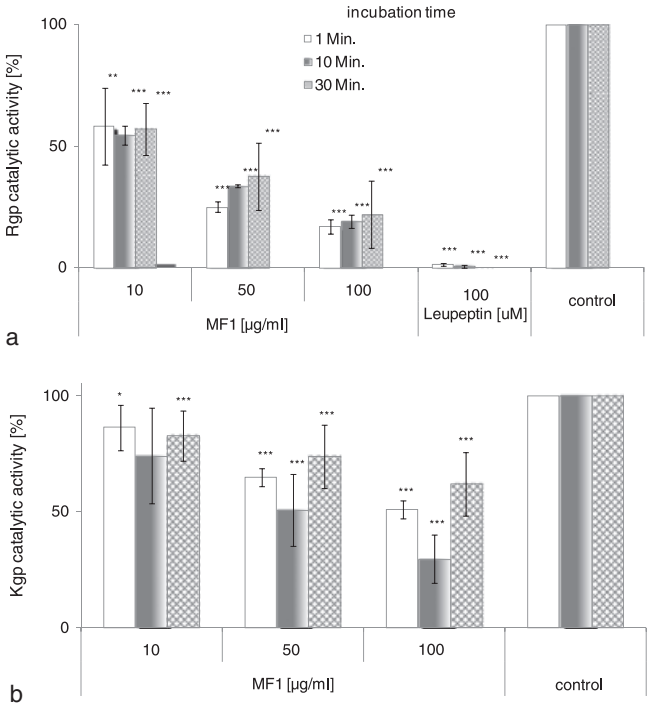

Fig. 3. Influence of MF (10, 50 and 100 μg/ml) on Arg-gingipain (a) and Lys-gingipain (b) activity of *P. gingivalis* within protease assay after incubation of bacterial over 1, 10 or 30 min. Positive control: leupeptin, negative control: untreated bacteria. Data are mean ± SD ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$.

Cytoprotective effects of MF against *P. gingivalis*-induced cytokine release

A prominent activity of *P. gingivalis* infection in KB cells is the induction of cellular inflammation response. Using gene expression studies for monitoring TNF-α, IL-1β, IL-6, IL-8 and COX-2 as typical inflammation markers, treatment of KB cells with bacteria (bacteria cell ratio BCR 5:1) for 6 h resulted in a significant, about fivefold increase of IL-1β, IL-6, IL-8 and TNF-α expression, while COX-2 was not influenced (Fig. 5a). When KB cells were pre-treated for 2 h with MF (10 and 100 μg/ml), followed by removal of the incubation medium, washing of the cells and treatment with *P. gingivalis* (BCR 5:1), this pre-treatment resulted in a clearly decreased gene expression for IL-1β, IL-8 and TNF-α, which were significantly lower than in control cells not exposed to MF (Fig. 5b).

Within the chronic interaction of *P. gingivalis* and the immunoactive host cells, IL-6 seems to play a major role. Owing to the fact that gingipains stimulate IL-6 secretion from KB cells (O'Brien-Simpson et al. 2009) and generalized aggressive periodontitis is dominantly associated with an increased IL-6 response to *P. gingivalis* (Borch et al. 2010), the influence of MF on the expression of IL-6 on protein level was investigated by pre-incubation of KB cells for 2 h with MF (100 μg/ml). The extract was removed and the cells were incubated with *P. gingivalis* (BCR 5:1 or 100:1 for 2 h). After removal of the bacteria from the cell culture, IL-6 amounts were quantified from the culture supernatant (2 h measurement). Cells were further incubated in bacteria-

Table 3. Changes in mRNA abundancies of *Porphyromonas gingivalis* ATCC 33277 genes after 6 h exposure to MF as assessed by NimbleChip™ microarray hybridization experiments

Gene ID	Corresponding protein	Classification	Fold change (− reduction)
PG_1765	Acyl carrier protein	Biosynthesis, lipid metabolism	− 15.1
PG_1320	ISPg1, transposase, internal deletion	DNA replication	− 7.5
PG_1827	RNA polymerase sigma-70, ECF subfamily	Transcription	− 8.03
PG_1914	Ribosomal protein S13	Ribosomal translation, ribosome structure	− 10.3
PG_0392	Ribosomal protein L10	Ribosomal translation, ribosome structure	− 15.4
PG_1837	Haemagglutinin protein HagA	Cell adhesion, virulence	− 14.2
PG_0103	Hypothetical protein	Unknown	− 10.5

Bacterial cells treated with MF (100 μg/ml) were compared with untreated cells. RNA from two independent experiments was combined for transcriptome analysis. Chosen/selected messages with expression changes > 7-fold are shown.

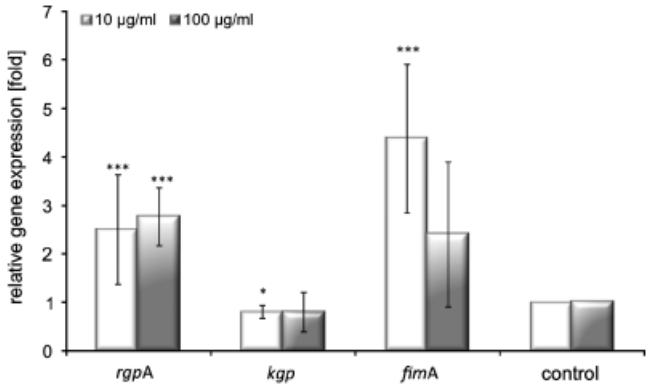

Fig. 4. Influence of MF on relative gene expression from *P. gingivalis* on *rgp*A, encoding for Arg-gingipain, *kgp*, encoding for Lys-gingipain and *fim*A, encoding for fimbrillin after 6 h incubation with 10 and 100 µg/ml MF. Data are mean ± SD ($n = 3$); $^{*}p < 0.05$; $^{***}p < 0.005$.

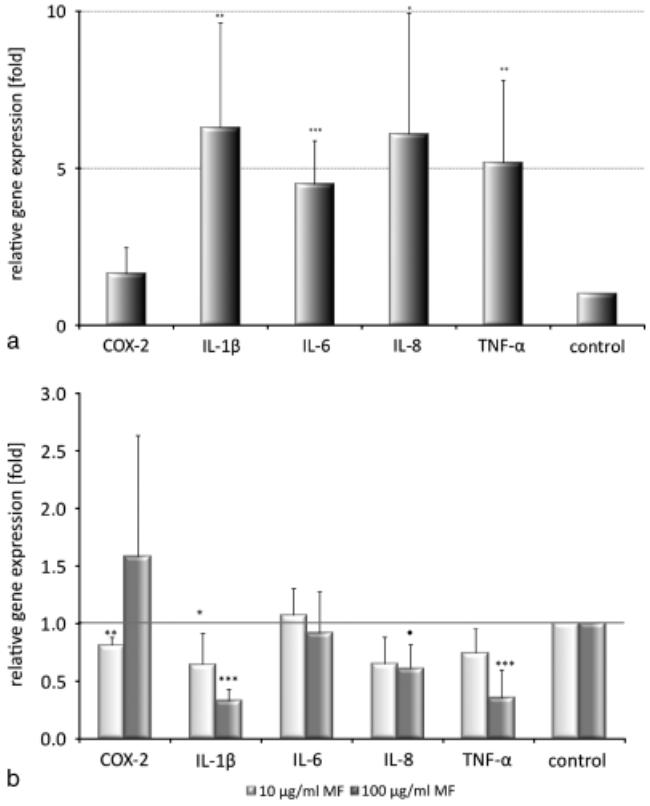

Fig. 5. Cytoprotective influence of MF on inflammation parameters of KB cells after exposure to *P. gingivalis*. (a) Influence of *P. gingivalis* exposure (BCR 5:1, 2 h) on the relative gene expression (RT-PCR) of COX-2, IL-1β, IL-6, IL-8 and TNF-α. (b) Influence of MF pre-treatment of KB cells (2 h; 10 and 100 µg/ml) before exposure to *P. gingivalis* (BCR 5:1, 2 h) on relative gene expression (RT-PCR) of COX-2, IL-1β, IL-6, IL-8 and TNF-α. Data are mean ± SD ($n = 4$ independent experiments) and related to untreated control groups, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$.

and MF-free media and IL-6 concentrations were additionally determined 17 h after infection. Differences were calculated against the respective values obtained from non-exposed cells. As shown in Fig. 6, the cytokine formation in untreated control groups was < 15 pg/ml after 2 h for cells infected with BCR 5:1 and 100:1. Similar low concentrations were measured for the MF-treated cells. This indicates that the IL-6 formation in KB cells is a rather slow process. Seventeen hours after treatment, IL-6 concentrations increased by about 10-fold within the untreated control groups. Pre-incubation of the KB cells with MF resulted in significant lower IL-6 amounts, indicating a cytoprotective effect of MF against the cells.

Proanthocyanidins as active ingredients

MF comprises a polyphenol-enriched extract, consisting mainly of proanthocyanidins and flavonoids (Engelhardt et al. 2007, Anke et al. 2008). Especially a variety of monomeric (epicatechin, gallocatechin epigallocatechin and their 3-*O*-galloylated homologues) and oligomeric flavan-3-ols (procyanidin B3 [catechin-(4$\alpha \rightarrow$ 8)-catechin], B4 [catechin-(4$\alpha \rightarrow$ 8)-epicatechin], B6 [catechin-(4$\alpha \rightarrow$ 6)-catechin] and catechin-(4$\alpha \rightarrow$ 8)-epigallocatechin along with the galloylated analogues B4-3′-*O*-gallate, procyanidin B2-3′-*O*-gallate [epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-3-*O*-gallate], B2-3,3′-di-*O*-gallate, procyanidin B5-3,3′-*O*-gallate [epicatechin-3-*O*-gallate-(4$\beta \rightarrow$ 6)-epicatechin-3-*O*-gallate], catechin-(4$\alpha \rightarrow$ 8)-epigallocatechin-3-*O*-gallate, the trimer procyanidin C1-3″-*O*-gallate [epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-(4$\beta \rightarrow$ 8)-epicatechin-3-*O*-gallate] and epicatechin-3-*O*-gallate-(4$\beta \rightarrow$ 6)-epicatechin-3-*O*-p-hydroxybenzoate) have been characterized as main constituents of the plant material (Fig. 7). Such proanthocyandins are know to have astringent properties against cell surfaces and proteins, leading to changed structural features and different enzymatic properties of the respective proteins (Sarni-Manchado et al. 2008).

Using the monomeric flavan-3-ols from MF gallocatechin and epigallocatechin as exemplary constituents (both at 3 mM) in the FACS assay significantly inhibited the bacterial adhesion/invasion of *P. gingivalis* to KB cells: epigallocatechin reduced the bacterial adhesion to 61.7 ± 2.4%, gallocatechin to 62.2 ± 9.4%.

Also, haemagglutination was inhibited in a dose-dependent manner: epicatechin-treated bacteria displayed a substantial inhibition of haemagglutination as shown by a rise of the respective haemagglutination-inhibition titres:

Epicatechin	300 µM: shift 2	150 µM: shift 2	30 µM: shift 1
Epigallocatechin	300 µM: shift 2	150 µM: shift 1.5	30 µM: no shift
Gallocatechin	300 µM: shift 2	150 µM: shift 2	30 µM: no shift

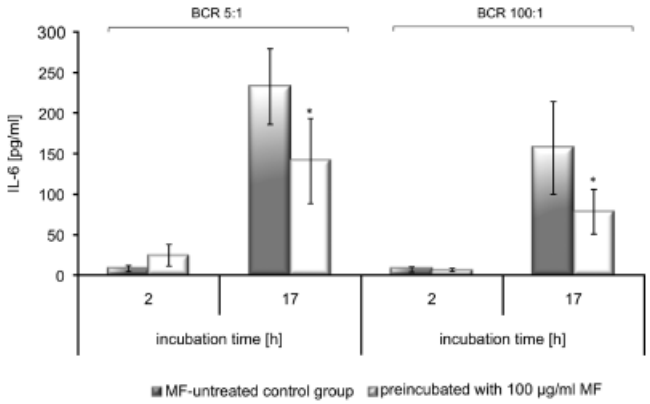

Fig. 6. IL-6 concentration in the culture supernatant of KB cells as determined by ELISA. KB cells were pre-incubated for 2 h with MF (10 and 100 μg/ml) before exposure to *P. gingivalis* (BCR 5:1 and 100:1). Co-incubation time with bacteria was 2 h, further incubation of the cells after removal of bacteria for 17 h in total. IL-6 was determined 2 and 17 h after the addition of the bacteria to the KB cells. Data are mean $\pm$ SD ($n = 4$) and related to the untreated control groups; $^{*}p < 0.05$.

In order to prove that proanthocyanidins interact directly with *P. ginigivalis*, a mixture of different oligomeric proanthocyanidins (1 mg/mL) was analysed by HPLC and the total proanthocyanidin peak areas in the chromatograms were set as 100%. Incubation (37°C, 30 min.) of the proanthocyanidin mixture together with *P. gingivalis* (1×10^{10} and 2.5×10^{9} CFU/mL) and subsequent HPLC analysis of the bacteria-free supernatant after centrifugation revealed a reduction of proanthocyanidin titres by about 40% (Fig. 8). This indicates interaction of the proanthocyanidins with the bacteria.

Additionally, proanthocyanidins were detected qualitatively by a specific colorimetric reaction in the bacteria pellet (anthocyanidin reaction with oxidative cleavage of the C–C inter-flavan linkage under acidic conditions, leading to the formation of red-coloured anthocyanidium cations) obtained after incubation with the proanthocyanidin mixture. The bacteria pellets were washed two times; the washing solution was proven by anthocyanidin reaction to contain no proanthocyanidins any more. The bacteria were lysed using Triton® X-100. In the cell lysates of MF-treated groups, proanthocyanidins were detected unambiguously by a strong anthocyanidin reaction, while untreated control group was anthocyanidin negative.

From these data, we assume that proanthocyanidins as the typical lead compounds from MF react with bacterial surface proteins, leading among others to an unspecific cross-linking or denaturing effects of adhesion proteins, which, for example, results in inhibition of Arg-gingipain and haemagglutinin activity.

Discussion

The present study proves the concept that polyphenol-enriched plant extracts can strongly interfere with adhesion or invasion of *P. gingivalis* to KB cells under in vitro conditions. The adhesion assay by flow cytometry used in this study cannot differentiate clearly if MF inhibits only the bacterial attachment of *P. gingivalis* to the cells or if MF downregulates the invasion through the membrane. From the data, we have shown that MF interacts with surface proteins (e.g. gingipains, predominantly because of its activity against Arg-gingipain) and changes functionality and reactivity. Thus, we assume that MF mainly interacts with the initial adhesion process of the bacteria to the cell membrane. Additionally, cytoprotective effects of the extract against inflammation-related parameters of KB cells were shown.

Therefore, our hypothesis that the extract from *M. flabellifolia* can be used for prophylactic treatment against *P. gingivalis* infection was proven – at least under in vitro conditions. Despite the widespread and very frequent occurrence of periodontitis, this multi-factored and complex disease is still mainly treated by mechanical and adjunctive antibiotic therapies. *P. gingivalis* is one of the key players involved in the pathogenesis of periodontitis. Attachment of the bacteria to gingival epithelial cells is the initial step that leads to microinjuries, followed by a systematic destruction of infected tissue: once *P. gingivalis* has bound to its target cells, internalization into subepidermal tissue will help to mask the bacteria. After the initial binding, bacteria start degrading the host cells. Simultaneously, the local immune response is decreased during the first phases of infection. Later on, inflammation is induced, accompanied by tissue destruction. Eventually, at this phase, the bacteria can invade the systemic compartments.

Additionally, the low oxygen concentration at this anatomical site supports the vitality of the anaerobic bacteria and sustains its propensity to initiate acute and chronic disease.

Beside the wide range of virulence factors produced by *P. gingivalis*, the chronic interaction of the host immune system with the infecting bacteria is believed to be a main factor in the onset and progression of periodontitis (Taubman et al. 2005, Guentsch et al. 2009, Yilmaz 2008).

Patients who suffer from a chronic form of the disease have significantly increased amounts of proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α (Stashenko et al. 1987, Rescala et al. 2010). Moreover, prostaglandins, particularly PGE_2 as a strong stimulant of osteoclasts, are also believed to contribute to periodontal disease (Reinhardt et al. 1993). Therefore, reducing the attachment of the bacteria to the host cells and reducing the formation of cytokine mediators may help to improve the outcome of periodontal therapy. This approach has already been proved successful by Assuma et al. (1998), who observed a reduced destruction of tissue and alveolar bone loss in a non-human primate model by blocking IL-1β and TNF-α activity.

Also, results from our study clearly indicate reduced adhesion of *P. gingivalis* to host cells by MF, due to inhibition of Arg-gingipain and haemagglutinins. Blocking of the bacterial proteases (for review on gingipain functionality, see Fitzpatrick et al. 2009) has been described in some other cases (Curtis et al. 2002, Kadowaki et al. 2004) and also the use of antibodies directed against gingipains (Kuboniwa et al.

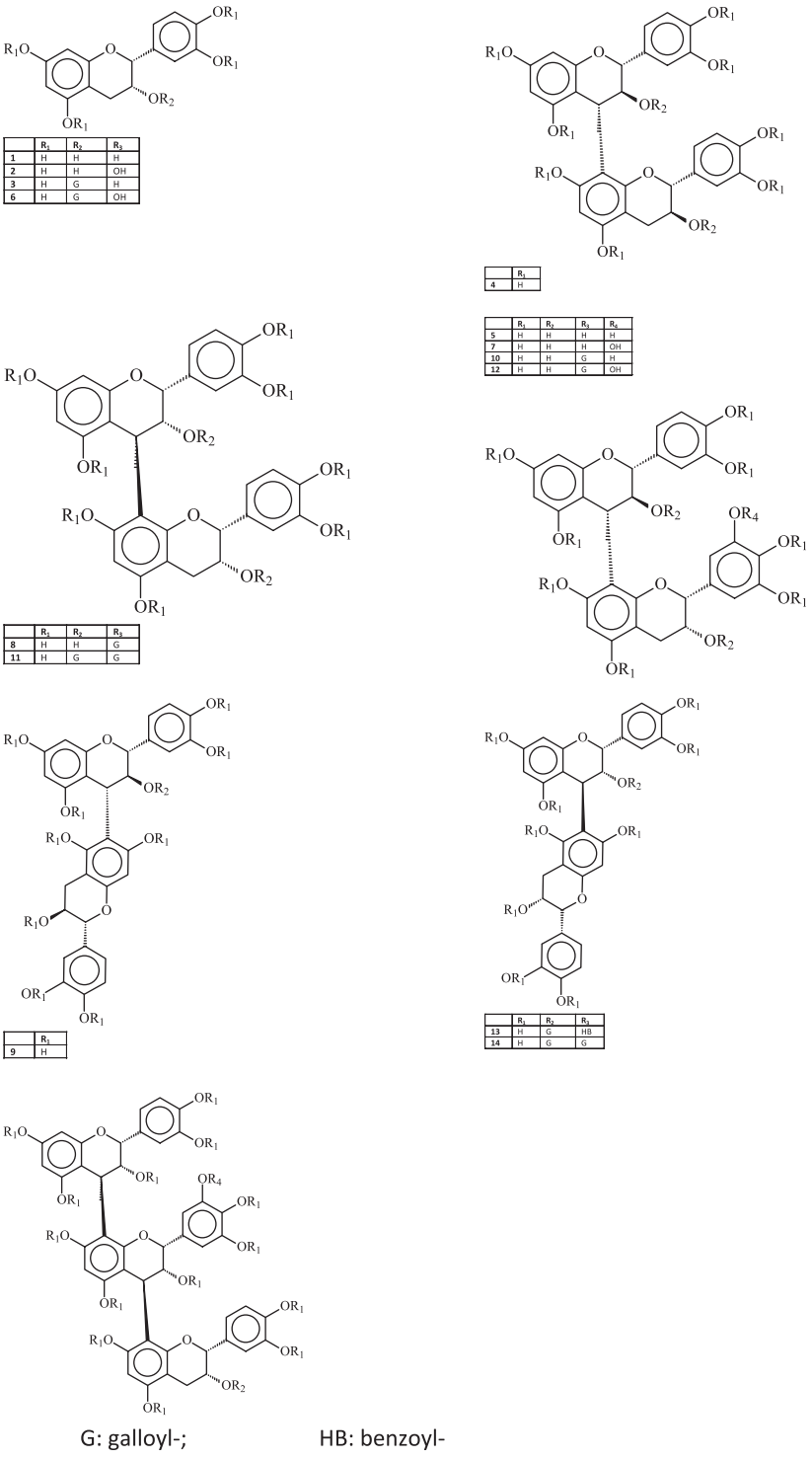

Fig. 7. Structural features of proanthocyanidins from MF.

2001, Yokoyama et al. 2007, Miyachi et al. 2007) led to reduced colonization and pathogenicity of *P. gingivalis* in animal infection models. It seems interesting that inhibition of the Arg-gingipain proteases in our in vitro study leads to a feed back mechanism of increased gene expression for *rgp*A, which indicates that the gingipain activity is controlled by bacterial cellular programmes. Recent studies on correlation of *Helicobacter pylori* adhesion with the gene expression for the respective OMP adhesins showed that the adhesion process for *H. pylori* is not related to changes in gene expression of the adhesins and virulence factors (Niehues et al. 2010a). In contrast, *P. gingvalis* seems to have a gene regulation system, which correlates gene expression with gingipain protease activity. This may also be due to the multivalent properties of gingipains, being on one side effective adhesion systems and on the other side strong virulence factors. A potential inhibition of gingipains, e.g. by the MF extract, would also influence cell-membrane-associated protein processing (Kadowaki et al. 2003).

No investigations have been performed in this study on the functionality of fimbriae activity. Fimbrillin, the major subunit of the fimbriae, is encoded by the *fim*A gene and displays a molecular mass of approximately 45 kDa (Amano et al. 2004). Inactivation of the fimA gene results in a decreased attachment to epithelial cells compared with wild-type *P. gingivalis* (Weinberg et al. 1997). *Fim*A induces the release of proinflammatory cytokines in macrophages and is also involved in bacteria-induced inflammatory bone desorption (Hanazawa et al. 1995, Saito et al. 1997, Kesavalu et al. 2002). In our studies, we have proof for a slightly increased gene expression of *fim*A, which could be due to an influence of MF to the fimbriae. We assume that up-regulation of *fimA* gene expression after 6 h of incubation of the bacteria with MF is a reaction for replacement by MF disfunctionalized fimbrillin on the cell surface. What we clearly see is the reduced activation of inflammatory cytokines in infected KB cells. This may be due to the inactivation or inhibition of fimbriae by MF.

Conclusive preventive strategies for early inhibition of periodontitis are still not feasible. Oral hygiene products with anti-bacterial activity could serve that purpose, but due to the low specificity of those products, both the physiology of the oral microflora can change over the time and host tissues can be harmed by long-term use of such biocide products. Anti-adhesive compounds with a higher specificity could be an alternative strategy for early prevention of bacterial adhesion to the epithelium of the gingival sulci. Until now, only few in vitro studies have been performed on this subject, but the published data are promising concerning usage of complex plant extracts enriched with mixtures of polysaccharides and polyphenols as

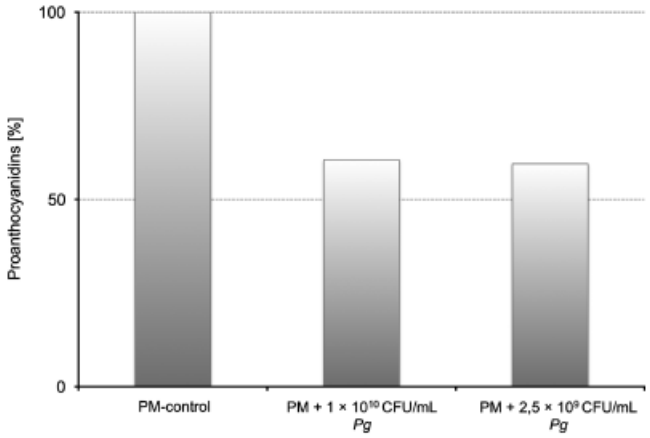

Fig. 8. Relative proanthocyanidin amounts from a complex mixture of different proanthocyanidins (PM, 1 mg/mL) before and after incubation (30 min., 37°C) of the test solution with *P. gingivalis* at 1×10^{10} and 2.5×10^{9} CFU/mL as determined by HPLC analysis.

an effective instrument against *P. gingivalis* (Sakanaka et al. 1996, Labrecques et al. 2006, Lee et al. 2006, Wittschier et al. 2007, Wittschier et al. 2009).

Owing to the fact that *P. gingivalis* cell adhesion predominantly relies on the functions of gingipain proteases, the use of anti-adhesive compounds influencing protein–protein interactions should be favoured. In contrast, carbohydrate–protein interactions, as known for other adhesive gram-negative bacteria (Niehues et al. 2010a), are less important for *P. gingivalis* binding to eukaryotic cells (M. Niehues, T. Stark, D. Keller, T. Hofmann, A. Hensel unpublished data). Manipulation of the *P. gingivalis* OMP functions can be achieved either by soluble, exogenous modified substrates of the gingipains, e.g. peptides or proteins, or by astringent polyphenols that influence protein structures via H–H-bound interaction (short-time contact) or by covalent binding and protein aggregation (longer contact time, oxidative conditions) (Canon et al. 2009). The use of polyphenol mixtures should be favoured due to two reasons: (i) adstringent affinity of proanthocyanidins to proteins is dependent on structural features, and (ii) high molecular compounds, especially longer oligomeric or polymeric proanthocyanidins, can exert strong interaction, but have a low solubility. In contrast, low molecular derivatives are completely soluble under aqueous conditions, acting very fast against proteins, but have a low astringency and do not adhere over longer time intervals to proteins. Complex mixtures of polyphenols with different clusters of polymerization and different substructures also have the advantage of influencing their own solubility, probably by formation of charge-transfer complexes between the different molecules. Therefore, standardized proanthocyanidin mixtures are anticipated to be more effective than the isolated and purified compounds, also leading to a multifaceted effect against various surface proteins of the target cells (Petrelli & Valabrega 2009). A clinical correlation between the intake of polyphenol mixtures from aqueous extracts from green tea and the incidence and severity of periodontitis has recently been documented (Kushiyama et al. 2009) by pinpointing a modest inverse association between the intake of polyphenol-enriched green tea (known to contain high amounts of galloylated proanthocyanidins) with periodontal disease.

A reliable and efficient source for polyphenol-enriched extracts is *M. flabellifolia*, traditionally used by African people for oral mouth hygiene against gingivitis. According to the results of our study, the polyphenol-enriched MF extract exhibits a clear inhibition of *P. gingivalis* adhesion to epithelial cells, with a strong influence against Arg-gingipain and less inhibition of the respective lysine protease, indicating a certain degree of selectivity of the polyphenols. Also, the haemagglutinin function is inhibited to a minor extent. The main target of the extract appears to be the bacterial surface, and also pre-incubation of KB cells with the extract led to a slightly reduced *P. gingivalis* attachment. This could be due to a binding of the multivalent polyphenols to surface proteins of KB cells, changing the structure of potential ligands for the bacterial binding.

A major problem of the *M. flabellifolia* extract for clinical use is the safety issue: at higher doses, the extract can exert a significant decrease of KB cell vitality and toxicity against *P. gingivalis*. However, our results demonstrate that concentrations below 150 μg/ml only influence bacterial cell surface structures without exerting negative effects on host cell physiology. Therefore, potential clinical use of such extracts should be restricted to multiple low-dose applications.

For the first time, we could show that polyphenol extracts do not only inhibit the host cell adhesion of *P. gingivalis* but also exert cytoprotective effects on KB cells. Pre-incubation of the KB cells with extract and subsequent exposure of the cells to *P. gingivalis* even in the absence of the extract substantially reduced the expression of inflammation-associated cytokines, either by decreasing the bacterial load or by down-regulation of the cellular response. Also from these aspects, the prophylactic use of *M. flabellifolia* extract could provide promising options.

An important issue is the effect of such anti-adhesive compounds on already adhering bacteria. For *H. pylori*, it was shown that the anti-adhesive 3-sialyllactose was able to efficiently detach bacteria already bound to epithelial cells (Simon et al. 1997). Such properties need to be investigated for MF. Also, potential anti-adhesive effects on other periodontal pathogens, e.g. *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythensis*, *Prevotella nigrecens*, *Prevotella intermedia*, *Treponema denticola*, *Campylobacter rectus*, *Fusobacterium* sp., *Micromonas micros* or *Streptococcus intermedius*, should be investigated in the future, paralleled by studies on the potential effects on biofilm formation.

Many questions about the properties and functions of *M. flabellifolia* extracts remain to be answered. However, the clear economical advantages of such polyphenol-enriched extracts, which can be easily and reliably produced from the plants by a simple process, seem to justify the efforts that have to be taken until clinical usage. Therefore and because of the interesting bioactivity, further developments towards toxicological and clinical testing are promising and should have a high potential.

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Clinical Relevance

Scientific rationale for the study: *P. gingivalis* has a strong tendency for adhesion to epithelial cells. Inhibition of this attachment should prevent subsequent inflammation and cell damage. Polyphenol-enriched extracts from *M. flabellifolia* are traditionally known to be used against periodontitis and are to be investigated on potential anti-adhesive effects against *P. gingivalis*.
Principal findings: MF inhibited the adhesion of *P. gingivalis* to epithelial cells by interaction with bacterial gingipains; inflammatory responses of the cells was decreased. Also, pre-incubation of epithelial cells with MF, followed by subsequent infection, proved to exhibit cytoprotective effects.
Practical implications: The anti-adhesive principle for in vitro prevention and cytoprotection was proven. MF should be developed further for the prevention of *P. gingivalis*-associated periodontal diseases.