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Original Article

Bioassay-guided fractionation and antimicrobial and cytotoxic activities of *Cassia bakeriana* extracts

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ABSTRACT

The antimicrobial potential of extracts of bark and leaves of *Cassia bakeriana* Craib, Fabaceae, against aerobic and anaerobic oral bacteria was evaluated by the microdilution broth method. For crude ethanol extracts and organic fractions tested, the bark dichloromethane phase showed a significant antibacterial effect, with MIC values ranging from 12.5 to 100 μg/ml for most of the microorganisms tested. Thus, a bioassay-guided fractionation of this fraction was performed. This fractionation led to isolation of the 1,8-dihydroxy-anthraquinone-3-carboxylic acid, also known as cassic acid or rhein. It is the first time that this bioactive anthraquinone has been isolated from this plant. Rhein exhibited good selectivity and high activity against anaerobic microorganisms, with MIC values ranging between 3.12 μg/ml (11.0 μM) and 25 μg/ml (88.0 μM). These results were considered very promising since the most active samples and rhein showed greater selectivity against oral microorganisms than toxicity to Vero cells.

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Introduction

Cassia bakeriana Craib, Fabaceae, is a tree belonging to the genus *Cassia*. It is native in Thailand being also known as pink cassia (Lorenzi et al., 2003). A large variety of compounds has been isolated from species of the genus *Cassia*. The plants of this genus are considered an important source of anthraquinones, alkaloids, flavonoids and other phenolic bioactive compounds. These metabolites exhibit important biological activities such as anti-emetic (Ahmed et al., 2012), laxative, anti-diabetic, hepatoprotective (Dave and Lediwane, 2012), anti-inflammatory, antipyretic, antiviral, antioxidant, antibacterial, antifungal, and analgesic among others (Viegas Júnior et al., 2006; Mazumder et al., 2008).

Although the use of many *Cassia* species for the treatment of various diseases is well established, there is still a chemical and pharmacological potential to be explored in other species of this genus (Mazumder et al., 2008; Dave and Lediwane, 2012). The essential oils chemical composition from bark, wood and leaves of *C. bakeriana* have already been determined, and except for wood, the bark and leaves essential oils exhibited high antimicrobial activity against aerobic and anaerobic oral microorganisms, as well as low toxicity (Cunha et al., 2013).

The appearance of microorganisms that are resistant to various antibiotics and their side effects has led to interest in plants with antimicrobial properties (Namita and Mukesh, 2012). Several natural products, mainly obtained from plants, have been tested with the purpose of evaluating the antimicrobial activity on oral microorganisms (Cunha et al., 2007; Porto et al., 2009; Carvalho et al., 2011; Souza et al., 2011a,b; Waldner-Tomic et al., 2014; Bardaji et al., 2016).

In the oral cavity, as well as other parts of the human body, there is a characteristic microbiota in dynamic equilibrium with the host. If this equilibrium is broken, bacteria that were outnumbered may develop or allows the colonization of other more pathogenic microorganisms (Marsh and Devine, 2011). This condition can lead to the development of oral diseases as caries, endodontic lesions and periodontitis (Aas et al., 2005). In addition, oral microorganisms can trigger various systemic diseases, including cancer (Aas et al., 2005; Whitmore and Lamont, 2014). Some of the risk factors that promote disequilibrium between oral microbiota and host are unhealthy diet, tobacco use, harmful alcohol use, poor oral hygiene, and social determinants (WHO, 2012). When

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disequilibrium occurs, it is necessary to restore oral health. Then, in addition to the mechanical treatment and hygienisation, it becomes important to use antimicrobial agents to prevent and control the prevalence of oral pathogens (Teles and Teles, 2009).

Antimicrobial activity of crude extracts and isolated compounds from plants are often associated with toxicity tests using Vero cell line (Bagla et al., 2014), which is one of the most used cell line in the biology research (Ammerman et al., 2008). These tests are necessary to determine if the sample is selective, *i.e.*, if it exhibits antibacterial effect without showing significant toxicity to Vero cells (Bagla et al., 2014).

The aim of this study was to determine the antimicrobial activity of extracts of the leaves and bark of *C. bakeriana* against oral bacteria, performing concomitantly the phytochemical study of the most active extract. The toxicity of the most active samples was also tested.

Materials and methods

Plant material

Bark and leaves samples of *Cassia bakeriana* Craib., Fabaceae, were collected from specimens aged approximately eight years on March 2009 at the Federal University of Uberlândia, Minas Gerais, Brazil (18°55′8.95″ S; 48°15′34.01″ W). The plant was identified by specialists, and a voucher specimen was deposited in the Herbarium Uberlandenses of the Federal University of Uberlândia, under the number 63584 (Herbarium Code – HUFU).

General procedures

The NMR spectra were obtained on a Bruker DRX-400 spectrometer using tetramethylsilane as internal standard. The infrared analyses were performed on Shimadzu IR Prestige-21 in KBr. Sephadex LH-20® and silica gel 60 G (70–230 mesh) were used as the stationary phase in column chromatography. TLC was performed on silica gel 60 F 254 (5–40 μm) and silica gel 60 G (5–40 μm) plates and the spots were analyzed under UV light (254 and 366 nm) and the developing solutions used were methanolic solution of aluminium chloride 1% (w/v), ethanolic solution of potassium hydroxide 10% (w/v) (Bornträger reagent) and ammonia vapours (Waksmundzka-Hajnos et al., 2008).

Preparation of ethanol extracts and liquid–liquid partition

The leaves and bark of *C. bakeriana* were dried in an oven at 40 °C for ten days and pulverized in a ball mill. The dry powder of the leaves (1 kg) and bark (1 kg) was extracted three times with 2 l of ethanol 96% by maceration at room temperature for seven days. The mixture was filtered and the filtrate was concentrated on a rotary evaporator under reduced pressure at 40 °C, yielding 82 g and 68 g of extractives, respectively. The dry ethanolic extracts from leaves (EL) and bark (EB) with 70 and 60 g, respectively, were redissolved in 250 ml of a methanol/water solution (9:1, v/v). A successive partition of the EL extract yielding hexane (8.07 g), dichloromethane (34.35 g), ethyl acetate (10.8 g) and methanol (14.36 g) fractions and of the EB extract affording hexane (6.32 g), dichloromethane (9.84 g) and ethyl acetate (42.6 g) fractions. Subsequently, these fractions were subjected to tests of antimicrobial activity.

Bioassay-guided fractionation and isolation of 1,8-dihydroxy-anthraquinone-3-carboxylic acid

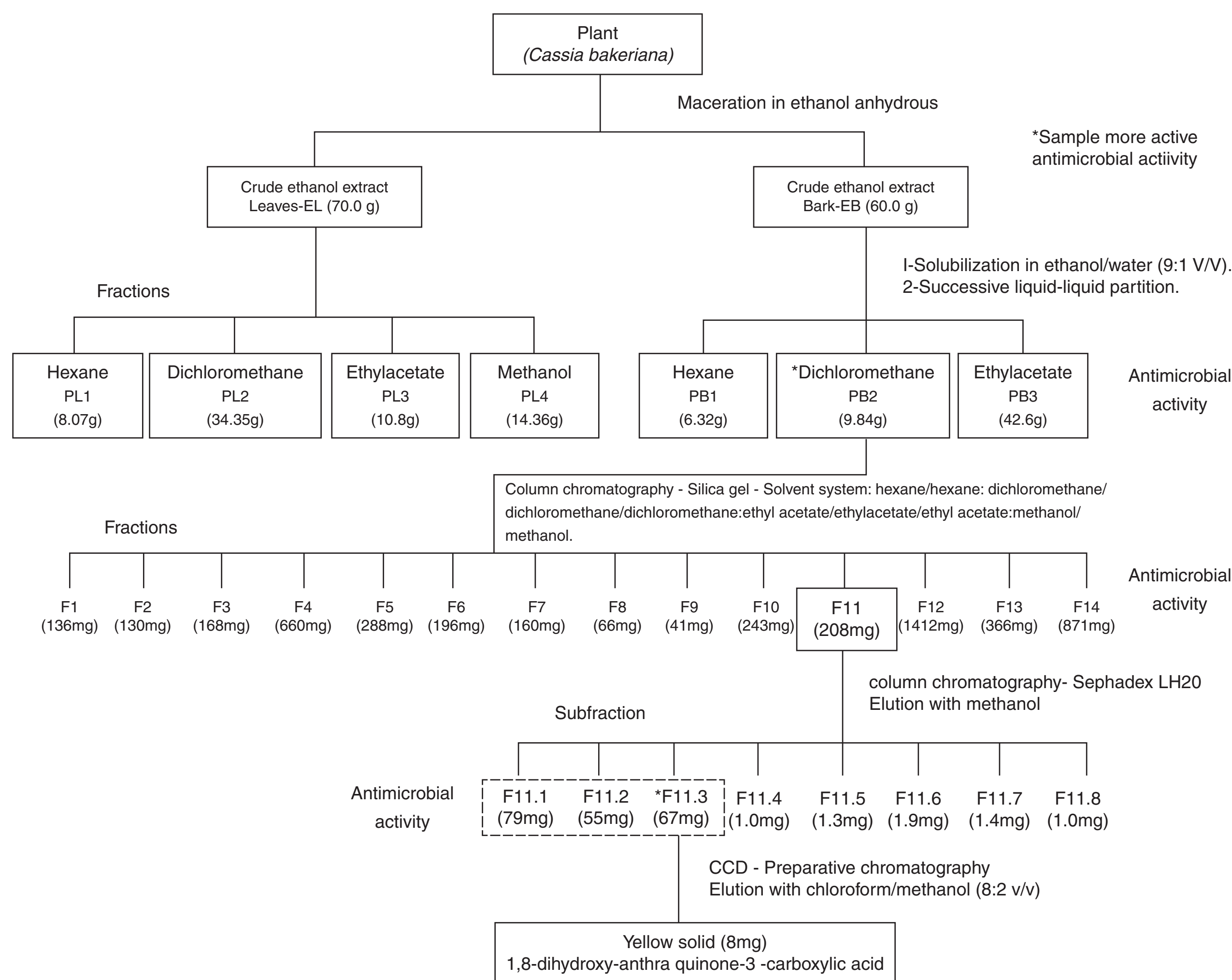
The bioassay-guided fractionation was only carried out with PB2 (Scheme 1) because it was the most active against the oral microorganisms evaluated. Seven grams of PB2 was fractionated using a glass column packed with silica gel 60H (Merck 70–230 mesh, 10 × 50 cm), eluted with hexane (500 ml), hexane/dichloromethane (1:1, 400 ml; 2:3, 400 ml), dichloromethane (400 ml), dichloromethane/ethyl acetate (3:2, 2100 ml; 1:1, 400 ml; 2:3, 300 ml; 1:4, 300 ml), ethyl acetate (2400 ml), ethyl(acetate/methanol) (1:1, 900 ml) and methanol (2000 ml) in order of increasing polarity. Twenty-four fractions were collected and grouped into fourteen fractions after monitoring by TLC (F1–F14) using hexane/ethyl acetate (1:1, 2:3) and chloroform/methanol (3:2, 1:1, 2:3) as mobile phase. The most active fraction, F-11 (208.0 mg), was resuspended in 5 ml of methanol and then fractionated with Sephadex LH-20 (Healthcare, 50 g, 5 × 50 cm) using methanol as mobile phase. Sixty fractions of approximately 10 ml were collected and monitored by TLC (mobile phase chloroform/methanol 3:2, 1:1, 2:3), generating eight subfractions F11.1 to F11.8. Only F11.1 (79.0 mg), F11.2 (55.0 mg) and F11.3 (67.0 mg) were evaluated against oral microorganisms as having presented sufficient quantity for antimicrobial tests. F11.3 showed the highest antimicrobial activity and, therefore, emphasis was placed on its analysis. The phytochemical prospection of F11.3 and its analysis were carried out by LC–ESI-MS/MS. F11.3 (65 mg) was submitted to preparative chromatography on glass plates with silica gel 60 G using chloroform/methanol (8:2) as mobile phase. The compound that presented R_f 0.32 was removed from the plates with silica and extracted with chloroform/methanol (1:1) to give a yellow solid (8 mg). The representation of continuous procedure of extraction and fractionation of *C. bakeriana* leaves and bark are shown in Scheme 1.

Characterization of the isolated compound

The isolated compound was identified by spectroscopic analysis as FTIR, UV–vis, LC–ESI-MS/MS, H^1 NMR, COSY and HSQC and by melting point. The results were compared with spectroscopic data previously published (Danielsen et al., 1992; Wei et al., 2003; Ye et al., 2007; Dionex, 2009; Gavit and Laddha, 2010; Jiang et al., 2012).

HPLC-DAD-ESI-MS/MS conditions

F11.3 fraction at concentration 500 μg/ml was analyzed on a Shimadzu Prominence Liquid Chromatographic system equipped with quaternary high pressure pump (LC-20AD), automatic injector (autosampler) (SIL 20AC) and UV/vis photodiode array detector (DAD) model SPD-M20A. The chromatographic separation was performed on a reverse-phase Phenomenex C18 column (50 mm × 2.10 mm × 2.6 μm) maintained at 40 °C in an oven. The volume injected was 5 μl, in a flow of 0.13 ml/min using water acidified with formic acid (0.1%, v/v) as mobile phase A and methanol as mobile phase B in following program: 15–30% B (0–5 min); 30–50% B (5–10 min), 50–70% B (10–15 min); 70–100% B (15–30 min); 100% B (30–35 min), 100–15% B (35–40 min) and 15% B (40–43 min). The diode array UV/vis detector was set to 190–800 nm. The mass spectrometry detection was performed in a Shimadzu LC-IT-TOF with quadrupole ion trap (IT) and time of flight (TOF) sequential mass spectrometer, using N_2 as nebulizer gas at 1.5 l/min, temperature of the desorption curve line (DCL) at 200 °C, drying gas at 100 kPa, ESI ionization at +4.5 and −3.5 kV, and ion accumulation time of 10 ms. The TIC chromatograms were obtained in positive and negative mode with *m*/*z* 50–1000. The proposed molecular formula was selected according to the Formula Predictor® Software, real possibility of the existence of the molecule, equivalence of double bonds, nitrogen rule and error in ppm or mDa. Furthermore, the suggestions of possible structures took into account related data in



Scheme 1. Representation of continuous procedure of extraction and fractionation of *Cassia bakeriana*.

the literature, solvent system, retention times, ultraviolet spectrum (UV) and mass spectrum.

Microbial strains

The tested strains were purchased from the American Type Culture Collection (ATCC). The following microorganisms were used in the present work: aerobic *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556), *Streptococcus sobrinus* (ATCC 33478), *Enterococcus faecalis* (ATCC 4082) and *Agregatibacter actinomycetemcomitans* (ATCC 43717) and anaerobic *Fusobacterium nucleatum* (ATCC 25586), *Bacteroides fragilis* (ATCC 25285), *Actinomyces naeslundii* (ATCC 19039), *Prevotella nigrescens* (ATCC 33563) and *Porphyromonas gingivalis* (ATCC 48417).

Antimicrobial activity

The minimum inhibitory concentration (MIC) value is the lowest concentration of a compound, fraction or extract capable of inhibiting the growth of a microorganism. The antimicrobial activity of *C. bakeriana* was determined in triplicate using the microdilution broth method in 96-well microplates (CLSI, 2012a,b). The samples were dissolved in dimethyl sulphoxide (DMSO; Synth) at 8000 μg/ml, followed by dilution in tryptic soy broth (Difco) for aerobic and Schaedler broth (Difco) supplemented with hemin (5 μg/ml) and vitamin K1 (10.0 μg/ml) for anaerobic; concentrations tested ranged from 400 to 25 μg/ml and 25 to 0.39 μg/ml. The final DMSO content was 4% (v/v), and this solution was used as a negative control. The inoculum was adjusted for each organism to yield a cell concentration of 5×10^5 colony forming units (CFU) per ml. The microplates with the aerobic microorganisms were incubated aerobically at 37 °C for 24 h. The anaerobic microorganisms were incubated for 48–72 h in an anaerobic chamber (Don Whitley Scientific, Bradford, UK), in 5–10% H_2, 0% CO_2, 80–85% N_2 atmosphere at 37 °C. After that, resazurin (Acros Organics) (30 μl) in aqueous solution (0.01% w/v) was added to the microplates, to indicate microorganism viability (Carvalho et al., 2011). Chlorhexidine dihydrochloride (Aldrich) (CD) was used as a positive control, and the concentrations ranged from 0.0115 μg/ml to 5.9 μg/ml. Sterility tests were performed for the TSB and Schaedler broths, control culture (inoculum), positive control, extracts and DMSO.

Cytotoxic activity

Samples of the compound, fraction or extract were dissolved in methanol and diluted in DMEM (Dulbecco's modified Eagle's medium, Sigma–Aldrich) until form a stock solution with a concentration of 640 μg/ml. The cell viability test was done with Vero cells ATCC CCL 81. The cytotoxicity was evaluated using the microplate dilution method. A solution containing 1×10^6 cells in 10 ml supplemented DMEM was prepared and 100 μl of this solution was

Table 1
LC-DAD-ESI-MS/MS data of the major constituents of fraction F11.3.

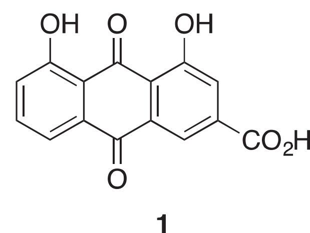
Compounds	T_R (min)	$[M-H]^-$ (Da)	Absorption UV/vis (nm)	Fragments (%)	Class of metabolite
1	5.99	299	228; 258; 288; 431	284 (100); 255 (41); 240 (21)	Anthraquinone
2	6.75	283	228; 258; 290; 433	257 (3); 255 (2); 239 (100); 211 (2); 183 (2)	Rhein
3	7.83	329	259; 277; 374	314 (100); 299 (14); 285 (20); 270 (14)	Flavonoid
4	8.53	313	224; 258; 270; 438	269 (100); 299 (11); 287 (5); 254 (19)	Anthraquinone
5	17.39	325	Not obtained	183 (100); 197 (2); 170 (3); 119 (4)	Not identified
6	27.83	283	Not obtained	Not obtained	Fatty acid

pipetted into each well; then, the plate was incubated for 6 h at 37 °C in a humidified atmosphere with 5% CO_2 to ensure cell adhesion to the well. Once attached, the culture medium was removed and sample solutions were added at concentrations of 512, 256, 128, 64, 32, 16, 8 and 4 μg/ml, starting from the stock solution. The final volume in each well was 100 μl and the quantity of cells present in each well was 1×10^4 cells. The final concentration of methanol in each well did not exceed 3%. Controls were prepared for growth (Cell viability 100%), positive (Cisplatin; Sigma–Aldrich), solvent (Methanol; Synth) and samples. The plates were incubated for 48 h at 37 °C in a humidified atmosphere with 5% CO_2. Next, 10 μl of developing solution of 3 mM resazurin in PBS was added to each well (Rolón et al., 2006) and the plate was incubated again for 24 h under the same conditions. Readings of absorbance at 594 nm were performed in a microplate spectrophotometer. The assays were done in triplicate and the results of the absorbances for each concentration were calculated according to the growth control. The CC_{50} (cytotoxic concentration at which 50% of the cells are viable) was calculated by a dose-response graph nonlinear regression (Pillay et al., 2007). The cytotoxic assays were tested with ANOVA with a significance level of 5%, using the Tukey method in GraphPad Prism 5. The results of cytotoxic activity were evaluated by comparing the values of cytotoxic concentrations (CC_{50}) to Vero cells with the values of minimal inhibitory concentrations obtained from tests for antibacterial activity using the selectivity index (SI). The SI was calculated by the logarithm of the ratio of cytotoxic concentration (CC_{50}) and the MIC value for microorganisms ($SI = \log [CC_{50}]/[MIC]$). A positive value represents higher selectivity against microorganisms than toxicity to Vero cells, and a negative value indicates a higher toxicity to Vero cells than to bacteria (Case et al., 2006).

Results and discussion

Identification of compound isolated from Cassia bakeriana

The compound isolated from the subfraction F11.3 presented proton NMR analysis the following chemical shifts: ^1H NMR (400 MHz, DMSO): δ ppm: 11.9 (3H, large, 3-COOH, 1-OH, 8-OH), 8.21 (1H, s, H-4), 7.36 (1H, d, J = 8.0 Hz, H-7), 7.82 (1H, t, J = 8.0 Hz, H-6), 7.76–7.74 (2H, m, H-2 and H-5). By HSQC spectrum, it was possible to assign the following signals ^{13}C δ ppm: 138.4 (C-6), 124.7 (C-7), 124.3 (C-2), 119.5 (C-5) and 119.1 (C-4). The signals of the H-5 and H-2 could only be identified by the HSQC spectrum (H-5, δ 7.74; H-2, δ 7.76). By COSY spectrum, it was possible to verify the couplings between H-6 and H-7 and between H-6 and H-5. The infrared analysis provided the following bands (KBr): O—H, 3630–3200 cm^{-1}; C—H aromatic, 3066 cm^{-1}; C=O (carboxyl), 1695 cm^{-1}; C=O (carbonyl), 1634 cm^{-1}; C—O, 1270 cm^{-1}; C=O, 1190 cm^{-1}; C—H aromatic, 751 cm^{-1}. Data from UV–vis: Band I (290 and 433 nm) and Band II (228 and 258 nm). Data obtained from LC–ESI-MS/MS m/z: 283 $[M-H]^-$, 257 $[M-H-C_2H_2]^-$, 255 $[M-H-CO]^-$, 239 $[M-H-CO_2]^-$, 211 $[M-H-CO_2-CO]^-$ and 183 $[M-H-CO_2-CO-CO]^-$; and molecular weight 284.0320 from Formula Predictor® Software. Fragmentation of rhein was recently reported (Zhu et al., 2014). The melting point was 320 °C. The above results are in accordance with data already determined in the literature for 1,8-dihydroxy-anthraquinone-3-carboxylic acid (**1**), also known as cassic acid or rhein (Danielsen et al., 1992; Dionex, 2009; Gavit and Laddha, 2010; Jiang et al., 2012; Wei et al., 2003; Ye et al., 2007).



Phytochemical prospection and LC–ESI-MS/MS analysis of the F11.3 active subfraction

The TLC of F11.3 active subfraction (SiO_2, chloroform/methanol 8:2 (v/v); methanolic solution of aluminium chloride 1%, w/v) suggested the presence of flavonoids, whereas TLC (SiO_2, chloroform/methanol 8:2 (v/v); ethanolic solution KOH 10%, w/v and ammonia vapours) suggested the presence of anthraquinones. These results with F11.3 were confirmed by LC–ESI-MS/MS. After LC–ESI-MS/MS, it was possible to determine that, in addition to rhein compound, other structures as anthraquinones, flavonoids and fatty acid are present in F11.3. The study of the major components of the subtraction F11.3 is indicated in Tables 1 and 2.

Antimicrobial and cytotoxic activities

The results of the antimicrobial activity of crude ethanol extracts and organic fractions of bark and leaves of *C. bakeriana* assessed

Table 2
LC–ESI-MS data of fraction F11.3 in negative mode.

Compounds	T_R (min)	Experimental mass $[M-H]^-$ (Da)	Theoretical mass $[M-H]^-$ (Da)	Suggested formula	IDH	Error (ppm)	Error (mDa)
1	5.99	2,990,536	2,990,555	$C_{16}H_{12}O_6$	11	−6.3	−1.9
2	6.75	2,830,214	2,830,242	$C_{15}H_8O_6$	12	−9.9	−2.8
3	7.83	3,290,651	3,290,661	$C_{17}H_{14}O_7$	11	−3.0	−1.0
4	8.53	3,130,336	3,130,348	$C_{16}H_{10}O_7$	12	−3.8	−1.2
5	17.39	3,251,790	–	–[a]	–	–	–
6	27.83	2,832,637	2,832,637	$C_{18}H_{36}O_2$	1	0	0

[a] Data were insufficient to determine the molecular formula.

Table 3
MIC for the ethanol extracts and fractions of bark and leaves of *Cassia bakeriana*.

Bacteria	Minimum inhibitory concentrations (MIC) – µg/ml Ethanol extract crude and fractions – *C. bakeriana*									
	Bark				Leaves					[c]CD
	Ethanol extract EB	Hexane fraction PB1	Dichloromethane fraction PB2	Ethyl acetate fraction PB3	Ethanol extract EL	Hexane fraction PL1	Dichloromethane fraction PL2	Ethyl acetate fraction PL3	Methanol fraction PL4	
[a]*S. sanguinis*	400	>400	100	400	>400	>400	>400	>400	>400	1.84
[a]*S. mitis*	>400	300	200	200	>400	>400	>400	>400	>400	3.68
[a]*S. mutans*	>400	25	400	>400	>400	>400	12.5	>400	>400	0.92
[a]*S. sobrinus*	>400	25	100	>400	>400	>400	25	>400	>400	1.84
[a]*E. faecalis*	>400	>400	>400	>400	>400	>400	>400	>400	>400	7.37
[b]*B. Fragilis*	>400	200	20	>400	100	200	400	400	>400	1.84
[b]*P. nigrescens*	>400	>400	200	400	>400	>400	>400	>400	>400	1.84
[b]*A. naeslundii*	>400	>400	12.5	400	>400	>400	>400	>400	>400	1.84
[b]*F. Nucleatum*	>400	200	12.5	200	>400	>400	>400	>400	400	1.84
[b]*P. gingivalis*	>400	400	100	>400	>400	>400	200	400	>400	3.68

[a] Gram–positive bacteria.
[b] Gram–negative bacteria.
[c] CD, positive control (chlorhexidine dihydrochloride).
Solvent control (4% DMSO solution) did not affect the growth of microorganisms.

in vitro are shown in Table 3. The lowest inhibitory concentration was found to the bark fraction obtained from the phase in dichloromethane (PB2) with values of 12.5 µg/ml against the bacteria *A. naeslundii* and *F. nucleatum* and 20 µg/ml against *B. fragilis*. Except for *E. faecalis*, PB2 inhibited the growth of all microorganisms evaluated, showing a higher antibacterial effect against anaerobes. Crude extracts and compounds isolated from natural products with MIC values under 100 and 10 µg/ml, respectively, can be considered promising antimicrobial agents (Rios and Recio, 2005). Due to the fact that PB2 has shown antibacterial activity against most oral aerobic and anaerobic microorganisms evaluated and the MIC values are equal to or below 100 µg/ml, the fractionation of this sample was performed. The resulting F1–F14 fractions were subjected to antimicrobial activity tests and the results are presented in Table 4. Comparing the MIC values of fractions F1 to F14, lower MIC values were found for the F11 fraction. This fraction indicated higher antimicrobial activity to aerobic microorganisms when compared with PB2. F11 inhibited *A. actinomycetemcomitans* with MIC of 25 µg/ml, a very aggressive oral pathogen involved in cases of severe periodontitis in young and adult humans (Lorenzo, 2004). A very promising result was found for F11 against *P. gingivalis*, when its inhibitory concentration of 0.78 µg/ml was lower than that reported for the positive control. F11 showed higher MIC values than PB2 with respect to some anaerobes, although the activity of F11 remained relevant with concentrations of 100 µg/ml or below. The MIC results of F11 led us to work on its fractionation, therefore, the subfraction F11.1 to F11.3 were obtained. The results for MIC values are shown in Table 5.

The subfraction F11.3 showed the best results of antimicrobial activity, inhibiting all microorganisms studied. *E. faecalis* was resistant to all the extracts and fractions of bark and leaves of *C. bakeriana*, but had growth inhibition against F11.3 with an MIC of 200 µg/ml. The strong antibacterial activity and wide spectrum of action shown by the F11.3, against oral bacteria evaluated, may be related mainly to the presence of flavonoids and anthraquinones in its composition (Table 1). Metabolites of these classes of compounds has shown activity against various microorganisms, including oral (Dahija et al., 2014; Riihinen et al., 2014; Xiang et al., 2008). F11.3 has greater antibacterial effect against *S. mutans*, *F. nucleatum* and *P. gingivalis* that different propolis extracts, natural antibacterial product with recognized potential in the treatment of oral infections (Waldner-Tomic et al., 2014).

The preparative chromatography F11.3 led to the isolation of 1,8-dihydroxy-anthraquinone-3-carboxylic acid, and its antimicrobial and cytotoxic activities were tested. The antimicrobial activity found for the compound rhein, in comparison with PB2, F11 and F11.3 are shown in Table 6. Rhein was active on anaerobic bacteria with values ranging between 3.12 and 25 µg/ml, outstanding the strong antibacterial effect against *P. gingivalis* (MIC of 3.12 µg/ml). The MIC values for rhein show that it is a major contributor to

Table 4
Results of antimicrobial activity of fractions F1–F14.

Bacteria	Minimum inhibitory concentrations (MIC) – µg/ml Fractions														
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	[c]CD
[a]*S. sanguinis*	>400	400	400	100	400	400	–	>400	–	400	100	>400	>400	400	1.84
[a]*S. mitis*	200	400	25	100	200	100	–	400	–	200	100	400	400	200	3.68
[a]*S. mutans*	300	400	300	200	>400	200	–	400	–	>400	100	>400	400	>400	0.92
[b]*A. actinom*	200	400	200	200	400	200	–	400	–	200	25	400	400	200	7.37
[a]*E. faecalis*	>400	>400	>400	>400	>400	>400	–	>400	–	>400	200	>400	>400	>400	7.37
[b]*B. Fragilis*	>400	300	>400	>400	>400	>400	–	>400	–	>400	50	>400	>400	100	1.84
[b]*P. nigrescens*	>400	>400	>400	>400	>400	>400	–	>400	–	>400	100	>400	>400	>400	1.84
[b]*A. naeslundii*	>400	>400	>400	100	100	100	–	>400	–	100	100	>400	>400	200	1.84
[b]*F. Nucleatum*	>400	400	400	200	400	400	–	400	–	400	62.5	200	200	50	1.84
[b]*P. gingivalis*	300	200	25	25	100	100	–	200	–	100	0.78	6.25	6.25	3.12	3.68

[a] Gram–positive bacteria.
[b] Gram–negative bacteria.
[c] CD, positive control (chlorhexidine dihydrochloride); –, there was not enough mass for antimicrobial testing.

Table 5
Results of antimicrobial activity of F11.1, F11.2 and F11.3 subfractions.

Subfractions	Minimum inhibitory concentrations (MIC) – μg/ml Microorganisms									
	Anaerobic					Aerobic				
	[b]*F. nucleatum*	[b]*A. naeslundii*	[b]*P. gingivalis*	[b]*B. fragilis*	[b]*P. nigrescens*	[a]*S. sanguinis*	[a]*S. mitis*	[a]*S. mutans*	[a]*E. faecalis*	[b]*A. Actinom.*
F11.1	>400	>400	>400	>400	>400	>400	>400	>400	>400	>400
F11.2	>400	>400	>400	>400	>400	>400	>400	>400	>400	>400
F11.3	50	100	12.5	50	25	25	12.5	50	200	25
[c]CD	1.84	1.84	3.68	1.84	1.84	1.84	3.68	0.92	7.35	7.35

[a] Gram–positive bacteria.
[b] Gram–negative bacteria.
[c] CD, positive control (chlorhexidine dihydrochloride).

Table 6
Inhibitory effect of the bioactive fraction PB2, F11, F11.3 and rhein.

Bioactive samples	Minimum inhibitory concentrations (MIC) – μg/ml Microorganisms									
	Anaerobic					Aerobic				
	F. nucleatum	*A. naeslundii*	*P. gingivalis*	*B. fragilis*	*P. nigrescens*	*S. sanguinis*	*S. mitis*	*S. mutans*	*E. faecalis*	*A. Actinom.*
PB2	12.5	12.5	100	20	200	100	200	400	>400	–
F11	62.5	100	0.78	50	100	100	100	100	>400	25
F11.3	50	100	12.5	50	25	25	12.5	50	200	25
Rhein	[a]	20 (70.4 μM)	25 (88 μM)	[a]	3.12 (11 μM)	>200	>200	>200	[a]	[a]
CD	1.84	1.84	3.68	1.84	1.84	1.84	3.68	0.92	7.35	7.35

[a] Not determined (insufficient sample quantity for the assay).
–, was not performed; CD, positive control (chlorhexidine dihydrochloride).

the antibacterial effect of F11.3 on anaerobes. Due to the fact that rhein was not active on aerobic, others bioactive compounds are present in F11.3, justifying its strong activity against these bacteria. The structural formula of rhein has a carbonyl group and two β-hydroxyls at a linear position. This position has been suggested as favourable for antimicrobial activity of anthraquinones (Xiang et al., 2008).

There are other studies about antimicrobial effects seen with aerobic and anaerobic oral bacteria, involving compounds isolated from plants. For instance, the triterpenes ursolic and oleanoic acids and ursolic derivatives showed MIC between 40 μg/ml and 200 μg/ml against *S. mitis*, *S. sanguinis* and *S. mutans* (Cunha et al., 2007). For these bacteria, the sesquiterpene caryophyllene oxide and derivatives of diterpene copalic acid exhibited MIC ranging between 60 and 200 μg/ml. The (−)-copalic acid exhibited MIC between 3 and 6 μg/ml (Souza et al., 2011a) and pimarane-type diterpenes inhibited bacterial growth at concentrations ranging between 2.5 and 20 μg/ml (Porto et al., 2009). The sclareol and manool diterpenes were active against *A. naeslundii*, *P. gingivalis* and *P. nigrescens*, with MIC between 6.2 and 400 μg/ml, while (−)copalic acid showed MIC between 3.1 and 200 μg/ml and copalic acid derivatives between 25 and 200 μg/ml (Souza et al., 2011b). The diterpene kaurenoic acid and its derivatives showed MIC against *A. naeslundii*, *P. gingivalis* and *P. nigrescens* between 1.25 and 60 μg/ml (Carvalho et al., 2011). Although rhein not be active against aerobic bacteria, it exhibited very promising MIC value against *P. nigrescens* and also good results against *A. naeslundii* and *P. gingivalis*.

The fractions, the most active subfraction and rhein were tested for cytotoxicity. The relationship between cytotoxicity and antimicrobial activity was established through the selectivity index (SI) and is shown in Table 7.

PB2, F11, F11.3 and the pure compound showed lower toxicity to Vero cells compared to the positive control cisplatin. Regarding the cytotoxic activity, the values of CC_{50} decreased with fractionation of active samples indicating an increase in toxicity. However, when the selectivity index are considered, all bioactive fractions and rhein presented positive SI values at concentrations that exhibited strong antibacterial activity, indicating a great antibacterial effect and selectivity against oral microorganisms. In the cytotoxicity test, F11.3 and rhein showed no statistical difference at 5% by Tukey test. The positive values of selectivity index ranged from 0.21

Table 7
Cytotoxic activity and selectivity indexes of the bioactive samples.

Bioactive samples	Vero cells (ATCC CCL 81)	Selectivity index (SI)									
		Anaerobic					Aerobic				
	Cytotoxic activity CC_{50} – μg/ml	*F. nucleatum*	*A. naeslundii*	*P. gingivalis*	*B. fragilis*	*P. nigrescens*	*S. sanguinis*	*S. mitis*	*S. mutans*	*E. faecalis*	*A. Actinom.*
PB2	325 ± 24	1.41	1.41	0.51	1.21	0.21	0.51	0.21	−0.09	<−0.09	–
F11	263 ± 12	0.62	0.42	2.52	0.72	0.42	0.42	0.42	0.42	<−0.18	1.02
F11.3	196 ± 24	0.59	0.29	1.19	0.59	0.83	0.83	1.19	0.59	−0.07	0.83
Rhein	212 ± 16	[a]	1.02	0.92	[a]	1.83	<0.02	<0.02	<0.02	[a]	[a]
Cisplatin	7.01 ± 0.66	–	–	–	–	–	–	–	–	–	–

[a] Not determined (insufficient sample quantity for the assay).
–, was not performed. The SI was calculated by the logarithm of the ratio $[CC_{50}]/[MIC]$. Cisplatin (positive control).

to 2.52. The best SI values against aerobic were observed for F11.3. This fraction showed great toxicity to the Vero cells only against *E. faecalis*, which was notoriously the most resistant bacteria. The highest SI values observed for anaerobes occurred with *F. nucleatum*, *A. naeslundii* and *B. fragilis* (PB2), with *P. gingivalis* (F11) and with *P. nigrescens* (rhein).

Rhein has been isolated in other species of *Cassia* (Dave and Lediwane, 2012) and others studies have proved its antimicrobial activity (Didry et al., 1994; Kavanagh, 1947; Hatano et al., 1999). Additionally to its antimicrobial potential, rhein is associated to antiviral (Barnard et al., 1992), antioxidant (Vargas et al., 2004), anti-angiogenic (He et al., 2011), anti-emetic (Ahmed et al., 2012), anticancer (Duraipandiyan et al., 2012) and antifibrotic (Tsang et al., 2013) activities. *C. bakeriana* is presented in this work as one more source of rhein, furthermore, this compound proved to be an important prototype for the development of an antimicrobial agent to target anaerobic oral microorganisms.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Author's contributions

LCSC (PhD student) conducted the work with the species *C. bakeriana*. SALM and FJTA were responsible for the research project with the species *C. bakeriana*. EAN, AO and RC contributed with mass spectrometry and nuclear magnetic resonance identifications. CHGM contributed to biological studies of antibacterial activity. CVS contributed to cytotoxicity assays. MMM (PhD student) contributed conducting the cytotoxicity assays. LCFS and TTB (undergraduate students) collaborated with the sample preparations, column chromatography and TLC analysis. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Aas, J.A., Paster, J.B., Stokes, N.L., Olsen, I., Dewhirst, E.F., 2005. Defining the normal bacterial flora of the oral cavity. J. Clin. Microbiol. 43, 5721–5732.

Ahmed, S., Zahid, A., Abidi, S., Meer, S., 2012. Anti-emetic activity of four species of genus *Cassia* in chicks. J. Pharm. 2, 380–384.

Ammerman, N.C., Beier-Sexton, M., Azad, A.F., 2008. Growth and maintenance of Vero cell lines. Curr. Protoc. Microbiol., 1–10, Appendix 4: Appendix-4E.

Bardaji, D.K.R., Silva, J.J.M., Bianchi, T.C., Eugênio, D.S., Oliveira, P.F., Leandro, L.F., Rogez, H.L.G., Venezianni, R.C.S., Ambrósio, S.R., Tavares, D.C., Bastos, J.K., Martins, C.H.G., 2016. *Copaifera reticulata* oleoresin: chemical characterization and antibacterial properties against oral pathogens. Anaerobe 40, 18–27.

Barnard, D.L., Huffman, J.H., Morris, J.L., Wood, S.G., Hughes, B.G., Sidwell, R.W., 1992. Evaluation of the antiviral activity of anthraquinones, anthrones and anthraquinone derivatives against human cytomegalovirus. Antiviral Res. 17, 63–77.

Bagla, V.P., McGaw, L.J., Elgorashi, E.E., Eloff, J.N., 2014. Antimicrobial activity, toxicity and selectivity index of two biflavonoids and a flavone isolated from *Podocarpus henkelii* (Podocarpaceae) leaves. BMC Complement. Altern. Med. 14, 383.

Carvalho, T.C., Simão, M.R., Ambrósio, S.R., Furtado, N.A.J.C., Veneziani, R.C.S., Heleno, V.C.G., Costa, F.B., Gomes, B.P.F.A., Souza, M.G.M., Reis, R.B., Martins, C.H.G., 2011. Antimicrobial activity of diterpenes from *Viguiera arenaria* against endodontic bacteria. Molecules 16, 543–555.

Case, R.J., Franzblau, S.G., Wang, Y., Cho, S.H., Soejarto, D.D., Pauli, G.F., 2006. Ethnopharmacological evaluation of the informant consensus model on anti-tuberculosis claims among the Manus. J. Ethnopharmacol. 106, 82–89.

CLSI, 2012a. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria. CLSI Document M11-A8, 8th ed. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

CLSI, 2012b. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. CLSI Document M7-A9, 9th ed. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Cunha, L.C.S., Morais, S.A.L., Martins, C.H.G., Martins, M.M., Chang, R., Aquino, F.J.T., Oliveira, A., Moraes, T.S., Machado, F.C., Silva, C.V., Nascimento, E.A., 2013. Chemical composition, cytotoxic and antimicrobial activity of essential oils from *Cassia bakeriana* Craib. against aerobic and anaerobic oral pathogens. Molecules 18, 4588–4598.

Cunha, L.C.S., Silva, M.L.A., Furtado, N.A.J.C., Vinhólis, A.H.C., Martins, C.H.G., Filho, A.A.S., Cunha, W.R., 2007. Antibacterial activity of triterpene acids and semi-synthetic derivatives against oral pathogens. Z. Naturforsch. 62, 668–672.

Dahija, S., Čakar, J., Vidic, D., Maksimović, M., Parić, A., 2014. Total phenolic and flavonoid contents, antioxidant and antimicrobial activities of *Alnus glutinosa* (L.) Gaertn., *Alnus incana* (L.) *Moench* and *Alnus viridis* (Chaix) DC. extracts. Nat. Prod. Res. 28, 2317–2320.

Danielsen, K., Aksnes, D.W., Francis, G.W., 1992. NMR study of some anthraquinones from *Rhubard*. Magn. Reson. Chem. 30, 359–363.

Dave, H., Lediwane, L., 2012. A review on anthraquinones isolated from *Cassia* species and their applications. Indian J. Nat. Prod. Resour. 3, 291–319.

Didry, N., Dubreuil, L., Pinkas, M., 1994. Activity of anthraquinonic and naphthoquinonic compounds on oral bacteria. Pharmazie 49, 681–683.

Dionex, 2009. Determination of Anthraquinones and Stilbenes in Giant Knotweed Rhizome by HPLC with UV detection. Application Note 232, Thermo Scientific, Available from: http://www.dionex.com/en-us/webdocs/77633-AN232-LC-Anthraquinones-Knotweed-21Aug2009-LPN2280-01.pdf (accessed October 2012).

Duraipandiyan, V., Baskar, A.A., Ignacimuthu, S., Muthukumar, C., Al-Harbi, N.A., 2012. Anticancer activity of rhein isolated from *Cassia fistula* L. flower. Asian Pac. J. Trop. Dis. 2, 517–523.

Gavit, R.S., Laddha, K.S., 2010. Synthesis of 4,5-dihydroxy-9,10-dioxoanthracene-2-benzyl carboxylate ester from rhein. Int. J. Pharm. Sci. Res. 1, 60–64.

Hatano, T., Uebayashi, H., Ito, H., Shiota, S., Tsuchiya, T., Yoshida, T., 1999. Phenolic constituents of *Cassia* seeds and antibacterial effect of some naphthalenes and anthraquinones on methicillin-resistant *Staphylococcus aureus*. Chem. Pharm. Bull. 47, 1121–1127.

He, Z.H., Zhou, R., He, M.F., Lau, C.B., Yue, G.G., Ge, W., But, P.P., 2011. Anti-angiogenic effect and mechanism of rhein from Rhizoma Rhei. Phytomedicine 18, 470–478.

Jiang, J., Yang, M., Qian, W., Lin, H., Geng, Y., Zhou, Z., Xiao, D., 2012. Quantitative determination of rhein in human plasma by liquid chromatography–negative electrospray ionization tandem mass/mass spectrometry and the application in a pharmacokinetic study. J. Pharm. Biomed. Anal. 57, 19–25.

Kavanagh, F., 1947. Activities of twenty-two antibacterial substances against nine species of bacteria. J. Bacteriol. 54, 761–766.

Lorenzi, H., Souza, H.M., Torres, M.A.V., Bacher, L.B., 2003. Árvores exóticas no Brasil – madeireiras, ornamentais e exóticas. Instituto Plantarum, Nova Odessa.

Lorenzo, J.L., 2004. Microbiologia para o estudante de odontologia. Atheneu, São Paulo.

Marsh, P.D., Devine, D.A., 2011. How is the development of dental biofilms influenced by the host? J. Clin. Periodontol. 38, 28–35.

Mazumder, P.M., Percha, V., Farswan, M., Upaganlawar, A., 2008. Cassia: a wonder gift to medical sciences. Int. J. Clin. Pharm. 1, 16–38.

Namita, P., Mukesh, R., 2012. Medicinal plants used as antimicrobial agents: a review. Int. Res. J. Pharm. 3, 31–40.

Pillay, P., Vleggaar, R., Maharaj, V.J., Smith, P.J., Lategan, C.A., Chouteau, F., Chibale, K., 2007. Antiplasmodial hirsutinolides from *Vernonia staehelinoides* and their utilization towards a simplified pharmacophore. Phytochemistry 68, 1200–1205.

Porto, T.S., Rangel, R., Furtado, N.A.J.C., Carvalho, T.C., Martins, C.H.G., Veneziani, R.C.S., Costa, F.B., Vinhólis, A.H.C., Cunha, W.R., Heleno, V.C.G., Ambrosio, S.R., 2009. Pimarane-type diterpenes: antimicrobial activity against oral pathogens. Molecules 14, 191–199.

Riihinen, K.R., Ou, Z.M., Gödecke, T., Lankin, D.C., Pauli, G.F., Wu, C.D., 2014. The antibiofilm activity of lingonberry flavonoids against oral pathogens is a case connected to residual complexity. Fitoterapia 97, 78–86.

Rios, J.L., Recio, M.C., 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 100, 80–84.

Rolón, M., Vega, C., Escario, J.A., Gómez-Barrio, A., 2006. Development of resazurin microtiter assay for drug sensibility testing of *Trypanosoma cruzi* epimastigotes. Parasitol. Res. 99, 103–107.

Souza, A.B., Martins, C.H.G., Souza, M.G.M., Furtado, N.A.J.C., Heleno, V.C.G., Sousa, J.P.B., Rocha, E.M.P., Bastos, J.K., Cunha, W.R., Veneziani, R.C.S., Ambrósio, S.R., 2011a. Antimicrobial activity of terpenoids from *Copaifera langsdorffii* Desf. against cariogenic bacteria. Phytother. Res. 25, 215–220.

Souza, A.B., Souza, M.G.M., Moreira, M.A., Moreira, M.R., Furtado, N.A.J.C., Martins, C.H.G., Bastos, J.K., Santos, R.A.S., Heleno, V.C.G., Ambrosio, S.R., Veneziani, R.C.S., 2011b. Antimicrobial evaluation of diterpenes from *Copaifera langsdorffii* Oleoresin against periodontal anaerobic bacteria. Molecules 16, 9611–9619.

Teles, R.P., Teles, F.R.R., 2009. Antimicrobial agents used in the control of periodontal biofilms: effective adjuncts to mechanical plaque control? Braz. Oral Res. 23, 39–48.

Tsang, S.W., Zhang, H., Lin, C., Xiao, H., Wong, M., Shang, H., Yang, Z.J., Lu, A., Yung, K.K.L., Bian, Z., 2013. Rhein, a natural anthraquinone derivative, attenuates the activation of pancreatic stellate cells and ameliorates pancreatic fibrosis in mice with experimental chronic pancreatitis. Plos One 8, 1–15.

Vargas, F., Díaz, Y., Carbonell, K., 2004. Antioxidant and scavenging activity of emodin, aloe-emodin and rhein on free-radical and reactive oxygen species. Pharm. Biol. 42, 342–348.

Viegas Júnior, C., Rezende, A., Silva, D.H.S., Castro-Gambôa, I., Bolzani, V.S., 2006. Aspectos químicos, biológicos e etnofarmacológicos do gênero *Cassia*. Quim. Nova 29, 1279–1286.

Waksmundzka-Hajnos, M., Sherma, J., Kowalska, T., 2008. Thin Layer Chromatography in Phytochemistry. CRC Press, Boca Raton/London/New York.

Waldner-Tomic, N.M., Vanni, R., Belibasakis, G.N., Thurnheer, T., Attin, T., Schmidlin, P.R., 2014. The in vitro antimicrobial efficacy of propolis against four oral pathogens: a review. Dent. J. 2, 85–97.

Wei, Y., Zhang, T., Ito, Y., 2003. Preparative separation of rhein from Chinese traditional herb by repeated high-speed counter-current chromatography. J. Chromatogr. A 1017, 125–130.

Whitmore, S.E., Lamont, R.J., 2014. Oral bacteria and cancer. PLoS Pathog. 10, 1–3.

WHO, 2012. Oral Health. http://www.who.int/mediacentre/factsheets/fs318/en/ (accessed December 2015).

Xiang, W., Song, Q.S., Zhang, H.J., Guo, S.P., 2008. Antimicrobial anthraquinones from *Morinda angustifolia*. Fitoterapia 79, 501–504.

Ye, M., Han, J., Chen, H., Zheng, J., Guo, D., 2007. Analysis of phenolic compounds in rhubarbs using liquid chromatography coupled with electrospray ionization mass spectrometry. J. Am. Soc. Mass Spectrom. 18, 82–91.

Zhu, H., Yin, R., Han, F., Guan, J., Zhang, X., Mao, X., Zhao, L., Li, Q., Hou, X., Bi, K., 2014. Characterization of chemical constituents in Zhi-Zi-Da-Huang decoction by ultra high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. J. Sep. Sci. 37, 3489–3496.