Prevention of artificial dental plaque formation in vitro by plant extracts

Joanne Smullen*, Michelle Finney, David M. Storey and Howard A Foster

Centre for Parasitology and Disease Research, School of Environment and Life Sciences, University of Salford, Manchester, M5 4WT, U.K.

* Corresponding author

Running title: Anti-plaque activity of plant extracts

Address for correspondence:
Professor H. A. Foster, Centre for Parasitology and Disease Research, School of Environment and Life Sciences, University of Salford, Manchester, M5 4WT, U.K.

Tel 0161 295 3832 Facsimile 0161 295 5015
E-mail h.a.foster@salford.ac.uk

Key words
Antimicrobial, Streptococcus mutans, plaque, plant extracts, polyphenols, Rosmarinus, Salvia

*Present address

Harrogate District Hospital, Lancaster Park Road, Harrogate, North Yorkshire HG2 7SX, UK.
Abstract

Aims A number of previous studies have shown that plant extracts can inhibit formation of dental plaque. The ability of extracts of *Rosmarinus officinalis* L., *Salvia officinalis* L., unfermented cocoa, red grape seed and green tea to inhibit plaque bacteria, glucosyltransferase activity, glucan and plaque formation in an *in vitro* model using bovine teeth was examined.

Methods and Results The antimicrobial activity of the plant extracts against oral bacteria was determined using a standard susceptibility agar dilution technique. Inhibition of growth and acid production from glucose and sucrose by *Streptococcus mutans* in liquid culture was investigated. Prevention of plaque formation on bovine teeth initiated by *S. mutans* was studied using an artificial mouth. The plant extracts inhibited the growth of oral bacteria and prevented acid production by *S. mutans*. Extracts inhibited glucosyltransferase activity and glucan production and inhibited adhesion to glass. Extracts of *R. officinalis* L. and *S. officinalis* L. at 0.25 mg ml⁻¹ reduced plaque growth by >80%. Green tea extract completely inhibited plaque formation but resulted in a greenish discolouration of the teeth which could not be removed by scrubbing.

Conclusions The plant extracts, particularly those from *R. officinalis* L. and *S. officinalis* L., inhibited glucosyl transferase activity, glucan production and plaque formation *in vitro*.

Significance and Impact of Study The results suggest that the extracts of *R. officinalis* L. and *S. officinalis* L. may be useful as antiplaque agents in foods and dental preparations. Bovine teeth can be used as an alternative to hydroxyapatite for studies of plaque formation but they need to be carefully sterilised before use.

INTRODUCTION

Dental caries are caused by demineralization of the enamel of the tooth by acid produced from dietary sugars by microorganisms growing as a biofilm or plaque. Plaque is composed of up to 500 different organisms (Paster et al., 2001; Socransky and Haffajee, 2005). *Streptococcus*
*S. mutans* is regarded as the most potent cariogenic organism in the oral microbiota and is implicated in all types of dental caries (Loesche, 1986). The use of an antibacterial agent in the mouth, for example as mouthwash or toothpaste, may inhibit the growth of this organism and prevent the development of dental caries (Baehni and Takeuchi, 2003). Plant products are of interest as a source of safer or more effective substitutes for synthetically-produced antimicrobial agents and, as such, could have an anticariogenic role in food products, oral products and medicines.

Previous studies have demonstrated the antibacterial effect of plant extracts (Ross *et al*., 1980; Deans and Richie, 1987; Mitscher *et al*., 1987; Scheie, 1989; Heisey and Gorham, 1992; Shapiro *et al*., 1994; Larsen *et al*., 1996; Tichy and Novak, 1998; Van der Weijden *et al*., 1998; Hammer *et al*., 1999; Morgan *et al*., 2001) against oral bacteria. Extracts of green tea inhibited the growth of *S. mutans in vitro* (Sakanaka *et al*., 1989; Yoshino *et al*., 1995) and prevented its attachment to tooth enamel by inhibiting glucosyltransferase activity (GTA; Sakanaka *et al*., 1989; Otake *et al*., 1991). These activities were probably due to the presence of catechins (Hamilton-Miller, 2001). Oolong tea extracts inhibited experimental dental caries in Specific Pathogen Free rats infected with mutans streptococci (Ooshima *et al*., 1993, 1998; Matsumoto, 1999) and reduced dental plaque formation in humans (Ooshima *et al*., 1994). Various Chinese medicines rich in tannins (Kakiuchi *et al*., 1986), extracts of cocoa, coffee (Kashket *et al*., 1985), hops (Yaegaki *et al*., 2008) and propanone extracts of bark (Mitsunaga and Abe, 1997) also inhibited GTA. Aqueous extracts of various African plants inhibited attachment of *S. mutans* to glass or hydroxyapatite beads (Wolinsky and Sote, 1984). Extracts of cocoa bean husk have been shown to be cariostatic (Ooshima *et al*., 2000). *Rosmarinus officianalis* L. and *Salvia officianalis* L. have been widely studied for their antimicrobial activity (Shelef *et al*., 1980; Pintore *et al*., 2002; Angioni *et al*., 2004; Mitac-Culafic *et al*., 2005; Bosnic *et al*., 2006; Moreno *et al*., 2006). *R. officianalis* L. extracts have been shown to inhibit growth and GTA production in *Streptococcus sobrinus* (Tsai *et al*., 2007).

Many of the previous reports used essential oils containing mostly volatile components. However activity has been shown to be associated with polyphenols (Moreno *et al*., 2006). In a previous report we showed that plant extracts made with 70% aqueous propanone, specifically
to extract polyphenols, inhibited growth, acid production and adhesion of *Streptococcus mutans* (Smullen *et al.*, 2007). In particular those from red grape seed, unfermented cocoa and green tea all had antimicrobial activity and a suggested mechanism was via inhibition of GTA. In this study, the activity of these plant extracts, together with extracts of *R. officianalis* L. and *S. officianalis* L., on growth, GTA and glucan production in *S. mutans* and on plaque formation initiated by *S. mutans* in an *in vitro* model using bovine teeth were investigated.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**

Bacterial strains used were *S. mutans* Ingbritt (from the Dental School, University of Newcastle), *Actinomyces denticolens* NCTC 11490, *Lactobacillus acidophilus* NCTC 1723, *Streptococcus oralis* and *Veillonella* sp. GDH/94/1419 from The Dental School, University of Glasgow, *Actinomyces naeslundii* R12218 from the Public Health Laboratory, University of Wales; *Actinomyces odontolyticus* NCTC 9335 and *Actinomyces viscosus* WVU627 from the School of Dentistry, University of Leeds; *Candida albicans* JS, *C. utilis* JS1, *Enterococcus faecalis* JS2, *Escherichia coli* B. NCIMB10243, *Staphylococcus aureus* JS3 and *Streptococcus gordonii* JS from the Centre for Parasitology and Disease Research, University of Salford.

Bacteria were inoculated onto Pro-Lab Microbank™ beads (Pro-lab Diagnostics, U. K.) in accordance with the manufacturer’s instructions and stored at -20°C under glycerol. One bead was aseptically removed from the storage vial and inoculated into 10 cm³ of Brain heart Infusion Broth (BHI, Oxoid) and incubated for 16 h at 37°C (overnight culture). Fifty μl was inoculated onto pre-warmed blood agar (BHI plus 70 gl⁻¹ sterile horse blood) and grown at 37°C anaerobically for 48 h. The colonies were checked for purity by Gram staining before use.

**Plant extracts.**

Seventy percent propanone (P-70) extracts of unfermented cocoa, red grape seed and green tea were prepared as previously described (Smullen *et al.*, 2007) and extracts of leaves of *R.
officinalis L. (*Rosmarinus officinalis* L.) and *S. officinalis* L. (*Salvia officinalis*) were prepared by the same method.

**Determination of minimum Inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of extracts against oral bacteria.**

P 70 extract (1.28 g) was dissolved in 4.0 ml sterile distilled water with gentle heating where necessary. The MICs and MBCs were determined using a standard susceptibility broth dilution technique (National Committee of Clinical Laboratory Standards, 1993). Overnight cultures of oral bacteria were diluted to $10^5$ CFU ml$^{-1}$ and inoculated into Muller Hinton broth containing a doubling dilution series of extract from 64 to 0.25 mg ml$^{-1}$. The cultures were incubated overnight at 37°C and the MIC recorded as the lowest concentration inhibiting growth. A 10 μl loopful of each suspension was streaked over blood agar and the plates incubated for 48 h at 37°C. The MBC was recorded as the lowest concentration that completely inhibited growth.

**Time-kill studies**

Time kill studies were as previously described (Smullen *et al.*, 2007). An overnight culture of *S. mutans* Ingbritt was centrifuged at 5,000 g for 10 min. The supernatant was discarded and the cells washed three times in 0.1 M potassium phosphate buffer (pH 7.0) by resuspension and centrifugation. Cells were resuspended in fresh buffer to 0.5 MacFarland standard and 0.5 ml added to 4.5 ml of fresh, pre-warmed buffer. At 0 h, a sample was removed and the viable count of the culture determined by dilution in saline and plating out on to CBA blood agar in duplicate. The MIC of P70 extract of unfermented cocoa, procyanidin polymer fraction, red grape seed or green tea was then added and the culture incubated in a shaking waterbath (150 strokes/min) at 37°C. The viable count was determined at 30 min intervals for 6 h. All plates were incubated for 48 h at 37°C. All plate counts were performed in duplicate.

An overnight culture (200 μl) of *S. mutans* Ingbritt was inoculated into 20 ml of chemically defined medium (CDM; van der Hoeven, 1985) supplemented with glucose or sucrose (10 g l$^{-1}$).
The culture was placed in a shaking waterbath as before. A sample was removed at time zero, at hourly intervals for 8 h and after 24 h. A viable count was performed as before and pH measured. Growth was monitored by measuring turbidity at 580 nm (M330 spectrophotometer; Camspec, Cambridge, UK). The MIC of the test extracts were added when the cultures were in exponential growth phase (4 h for CDM + glucose, 3 h for CDM + sucrose).

Preparation and assay of cell free glucosyltransferase

One-litre of pre-warmed Todd Hewitt Broth (Oxoid) plus 2 g l⁻¹ glucose was inoculated with 0.2 ml of an overnight culture of S. mutans Ingbrit in BHI and incubated at 37°C for 18 h. The culture was centrifuged at 10,000 g for 10 min at 4°C. The supernatant fluid was removed and protein precipitated with 291 gl⁻¹ (NH₄)₂SO₄ overnight at 4°C. The precipitate was collected by centrifugation at 10,000 g for 20 min. The supernatant was discarded and the precipitate dissolved in 0.1 M potassium phosphate buffer pH 6.5 (PB) and dialysed at 4°C with continuous gentle stirring against PB for 24 h. The mixture was centrifuged at 10,000 g for 10 min and the supernatant fluid (crude enzymes) stored at -20°C until required.

GTA was assayed indirectly by measuring the formation of fructose from sucrose with a fructose assay kit (kit no. F A-20, Sigma-Aldrich, UK) used as in the manufacturer’s instructions. Briefly the assay involved the conversion of fructose to glucose-6-phosphate using ATP, hexokinase and phosphoglucone isomerase. Glucose-6-phosphate was then oxidised by glucose-6-phosphate dehydrogenase with the formation of NADPH, which was measured by $E_{340}$ using a M330 UV-visible spectrophotometer (Spectronic Analytical Instruments UK). Crude enzyme preparation (0.3 ml), 0.1 ml of PB and 0.2 ml of 0.3 M sucrose were placed in a glass vial and incubated at 25°C for 20 min. Enzyme was replaced by buffer for sample blanks. Fructose assay reagent was added and incubated at room temperature for 15 min. The absorbance was read at 340 nm. The amount of fructose was calculated as described in the manufacturer’s instructions.
Determination of glucan production

Crude enzymes were diluted with PB to give an absorbance of 0.5 at 280 nm (approx. 0.5 mg ml$^{-1}$ protein). Samples (1.5 ml) were placed into sterile universal bottles, 0.5 ml PB and 1.0 ml 0.3 M sucrose added and the mixtures incubated overnight at 37°C. Water insoluble glucans were collected by centrifugation at 10,000 g for 10 min. The pellet was washed x 3 in PB by resuspension and centrifugation. The glucan content of the pellet was determined using the phenol sulphuric acid method (Dubois et al., 1956) and expressed as a % of the control.

Effects of plant extracts on glucanase activity

P-70 extracts were dissolved in PB with gentle heating where necessary and diluted to give final concentrations of 0.5, 1 and 2 MIC in the reaction mixture. Crude enzyme preparation (0.3 ml) was placed into a microcuvette together with 0.1 ml extract and pre-incubated at 25°C for 2 min. The cuvette was placed into a spectrophotometer and the instrument adjusted to read zero absorbance. The reaction was started by the addition of 0.2 ml 0.3 M sucrose. The E 550 nm was measured at 1 min intervals for 15 min. Controls contained PB alone.

In vitro dental plaque model.

The in vitro dental plaque model used for testing the effect of food extracts on dental plaque was based on a design described by Bossmann (1979). The apparatus (Figure 1) comprised a 7 cm dia x 14 cm long cylindrical glass culture vessel with a Perspex lid with holes containing 3 glass tubes secured with silicone adhesive as supply inlets. These glass tubes were angled to guide drops of fluid onto the surface of a bovine tooth attached to the sample holder at an angle of about 25° using wax. A single waste tube was fitted to the base. The vessel was sterilized by autoclaving at 121°C for 30 min. The inlet tubes were connected to vessels containing artificial

© 2012 The Authors Journal of Applied Microbiology © 2012 The Society for Applied Microbiology
saliva, sucrose solution and the test extract with silicone tubing sterilized by pumping a 1.0% (w/v) sodium hypochlorite solution through for 30 min followed by flushing with sterile distilled water. Liquid flow to the culture vessel was regulated by peristaltic pumps (Model 2132, Microperpex® peristaltic pump, LKB) controlled by a timer switch (Model HR 5007, Philips).

Bovine teeth were obtained from the Manchester abattoir, Riverpark Road, Manchester, UK. The teeth were washed in 50 g l⁻¹ sodium hypochlorite for 10 h followed by thorough washing with sterile distilled water. The pellicle was removed by brushing with a sterile toothbrush, the teeth were washed in sterile distilled water and suspended in sterile distilled water. The teeth were then Tyndallised by heating to 80°C for 10 min on three successive d followed by a further wash in sterile distilled water. The teeth were attached to a sterile wax block and conditioned by immersion in freshly collected whole human saliva (centrifuged at 10,000 g for 10 min to remove solids) for 4 h at 28°C before use.

Several colonies of S. mutans Ingbritt from blood agar were inoculated into 100 ml pre-warmed BHI in a 250 ml conical flask. This was incubated at 37°C in a shaking waterbath (150 strokes/min) for 18 h. Conditioned teeth were inoculated by immersion in the culture for 4 h at 28°C followed by washing in sterile distilled water.

In vitro plaque assay

Teeth were attached to the sample holder using melted wax at an angle of approx 25°. Artificial saliva contained: Trypticase Peptone (Beckton Dickinson UK Ltd), 5 g l⁻¹; Proteose Peptone (Oxoid, Unipath Ltd, U.K), 10 g l⁻¹; Yeast Extract (Oxoid, Unipath Ltd, U.K), 5 g l⁻¹; KCl, 2.5 g l⁻¹; haemin 5 g l⁻¹; menadione, 1g l⁻¹; arginine , 0.174 g l⁻¹; urea, 0.6g l⁻¹ [Sissons et al., 1995]. Chemicals were obtained from Sigma-Aldrich, UK and were of Ultragrade unless otherwise stated. The mixture was sterlised by membrane filtration (0.22μm, Whatman International Ltd, England) and stored at 4°C. The saliva was dripped over the tooth continuously at 3.6 ml h⁻¹. Every 8 h, 50 g l⁻¹ aqueous sucrose, 1.5 ml delivered over 6 min, replaced the saliva. Two vessels were run side by side, a test vessel containing a tooth that had been incubated in saliva...
and then inoculated with *S. mutans* Ingbritt culture, and a control vessel containing a tooth that had not been exposed to either saliva or *S. mutans*.

Samples of plaque were removed and examined microscopically after Gram-staining and were cultured anaerobically on blood agar. Typical colonies were removed and identified using API 20Strep galleries (Biomerieux, UK).

**Effect of natural food ingredients on plaque development in vitro**

Extracts were dissolved in sterile distilled water with gentle heating where necessary to give the MIC of the extract. The extract was administered in the test vessel and distilled water in the control vessel concurrently with the sucrose solution i.e. 1.5 ml delivered over 6 min every 8 h. Each experiment was run for 5 d and each extract was tested three times. At the end of the experimental period the tooth was removed and placed in a bath containing disclosing tablets containing 12 mg erythrosine (The Boots Company PLC, UK) dissolved in 10 ml sterile distilled water. After five min the tooth was removed and rinsed in sterile distilled water to remove excess disclosing agent. The surface of the tooth was scanned (ScanJet 6100C, Hewlett-Packard) and the images stored as jpg files.

The percentage area of tooth surface covered in plaque was calculated using Paint Shop Pro 5 (Jasc Software Incorporated). Each image was converted to monochrome in order to calculate the relative number of pixels forming individual background, tooth and plaque regions. Using the 'Freehand tool' on the 'Smart edge' setting, the irregular shape of the tooth was outlined and copied onto a white background as a layer. Areas of the tooth covered with plaque were selected and copied onto a second background image. The 'layer' of each new image was merged to its white background. A grid of 300 x 300 pixels, 28.35 pixel cm⁻¹ was placed over the images and the plaque areas determined as a percentage of the pixels of the plaque compared to the tooth area.
Statistical Analysis.

The means, standard deviations and standard error of the means of all experiments were calculated using Microsoft Excel (Microsoft Corporation, USA). Where appropriate T-tests were performed Excel and statistically significant differences recorded when P<0.05.

RESULTS

MIC

The MICs for 70% propanone-extracts of *R. officianalis* L. and *S. officianalis* L. against the organisms tested are shown in Table 1. *S. mutans* strains were most susceptible to the extracts followed by *A. denticolens*, *Lactobacillus* sp., *S. sobrinus*, *A. viscosus*, *Veillonella* sp., *S. salivarius*, *S. gordonii*, *S. oralis* and *S. aureus*. The MICs for *E. coli* were higher (8.0 mg ml⁻¹). *Candida* spp. were the least susceptible with MICs of ≥32.0 mg ml⁻¹. *Strep. mutans* was again most susceptible to the *S. officianalis* L. extract (MIC 0.25 mg ml⁻¹) followed by *A. odontolyticus*, *S. sobrinus*, *Veillonella* sp., *S. gordonii*, and *S. sanguis*.

Bacteriostatic vs Bactericidal activity

The effects of the MIC of P-70 extracts of red grape seed, green tea and unfermented cocoa on viability and growth of *S. mutans* have been reported previously (Smullen et al., 2007). Time-kill studies of *R. officianalis* L. and *S. officianalis* L. extracts on the viability of washed cells of *S. mutans* Ingbritt are shown in Figure 2. There was a decrease in viable cell numbers from approximately 10⁷ CFU.ml⁻¹ to 10⁶ CFU.ml⁻¹ over the 6 h incubation period in the control cultures. Addition of P70 extract of *R. officianalis* L. or *S. officianalis* L. resulted in a 10-fold reduction of cell viability within 1 h and viable cells had decreased to approximately 10⁴ CFU ml⁻¹ after 6 h, circa 100 fold less than the control.

The effects of adding the MIC of the extracts to *S. mutans* Ingbritt in CDM supplemented with 1.0% (w/v) sucrose are shown in Figure 3. There was an increase from 7.6 x 10⁶ cfu ml⁻¹ at 0 h to 1.3 x 10¹⁰ cfu ml⁻¹ after 8 h and 4.4 x 10⁹ cfu ml⁻¹ after 24 h in the control cultures. The initial
pH of control cultures was 6.99 which decreased to 5.90 (± 0.3) after 8 h and 4.03 (± 0.04) after 24 h. The addition of P-70 extract of *S. officianalis* L. (0.25 mg ml⁻¹) after 3 h caused a decrease in the growth rate but the cell concentration continued to increase for a further 3 h. Growth then ceased and the numbers began to decrease slowly. After 8 h, cell count was 2.8 x 10⁸ CFU ml⁻¹ decreasing to 1.3 x 10⁷ CFU ml⁻¹ after 24 h. The addition of P70 extract of *R. officianalis* L. (0.25 mg ml⁻¹) slowed growth during the first 2 h then cell numbers decreased to 1.3 x 10⁷ CFU l⁻¹ after 8 h. Only a small decrease in pH occurred after the addition of the extracts and the mean pH after 24 h for both extracts was above 6.5, significantly higher than the control (P<0.05). Similar effects were seen in medium supplemented with glucose rather than sucrose (data not shown).

**Effects of plant extracts on plaque formation in vitro**

No plaque developed when teeth were exposed to cultures of *S. mutans* alone. However, immersion of the teeth in *S. mutans* Ingbrit culture for 4 h at 28°C after exposure to natural saliva allowed development of a biofilm by *S. mutans* within 48 h and after 120 h the plaque covered nearly 80% of the teeth (Figure 4a, b and Table 2). Microscopic examination of the plaque revealed large numbers of Gram-positive cocci in chains, Gram-positive filaments and Gram-negative bacilli. More than 90% of the bacteria recovered from the teeth were identified as *S. mutans* but small numbers of *S. sanguis* and *S. salivarius* were also recovered. Other organisms were not identified further. Application of 0.25 mg ml⁻¹ P70 extracts of *R. officianalis* L. and *S. officianalis* L. reduced plaque growth by >80% whereas extracts of unfermented cocoa (4 mg ml⁻¹) or red grape seed (0.5 mg ml⁻¹) extracts only caused approx. 40% reduction (Figure 4 c-e and Table 2). Addition of green tea extract (2 mg ml⁻¹) completely inhibited plaque formation but resulted in a greenish discoloration of the teeth which could not be removed by scrubbing with a toothbrush (Figure 4f).

**Effects of plant extracts on glucosyl transferase activity and insoluble glucan formation**

The effects of P-70 extracts of *R. officianalis* L. and *S. officianalis* L. on adhesion of *S. mutans* to glass is shown in Table 3. Both extracts reduced adhesion by 85-90% at 0.5 MIC rising to
95% at 2 x MIC. The effects of P-70 *S. officianalis* L. extract on GTA measured by the formation of insoluble glucans is shown in Figure 5. Glucan production was reduced by 0.5 MIC of extract and almost completely inhibited by 2x MIC. The effects of all five extracts on the amount of glucan formed in the first 10 min is shown in Table 4. Red grape seed extract inhibited glucan formation by approx. 50% at 0.5 MIC and complete inhibition was seen at 1x and 2x MIC. *R. officianalis* L. and *S. officianalis* L. extracts reduced glucan formation by 30-40% at 0.5 MIC increasing to >90% at 2 MIC. Green tea extract was the least active and inhibited glucan formation by approx 60% at 0.5 MIC and 80% at 2 x MIC. All reductions were significant (P≤0.05).

**DISCUSSION**

The results show that the plant extracts previously shown to inhibit growth and adhesion of oral bacteria to glass, inhibited both GTA and glucan production by *S. mutans* and prevented plaque formation *in vitro*. The P70 extracts of *R. officianalis* L. and *S. officianalis* L. tested for the first time here were most active against *S. mutans* and had activity against other Gram positive oral bacteria. There was lower activity against *E. coli* and no activity against *C. albicans*. Similar results were previously obtained using a commercially available extract of *R. officianalis* L. (Del Campo et al., 2000). Both extracts were bacteriostatic when *S. mutans* was grown on glucose or sucrose as carbon source. Although growth was inhibited there was no rapid decrease in the number of viable organisms which would be expected with bactericidal activity. Although an extract of essential oil from *R. officianalis* L. had no activity against *S. mutans* (Bernardes et al., 2010b) another study by Shapiro et al. (1994) showed that *R. officianalis* L. and *S. officianalis* L. essential oils inhibited the growth of oral microorganisms including two species of *S. sobrinus*. Extracts of *R. officianalis* L. leaves had an MIC of 24 mg ml⁻¹ against *S. mutans* ATCC 25175 (Larsen et al., 1996). This was 100-fold higher than that determined in the present study (0.25 mg ml⁻¹), possibly because an aqueous infusion of *R. officianalis* L. leaves in buffer was used compared to the 70% propanone extract used here. The extracts were less active against Gram-negative bacteria and *Candida*. The inhibition of growth and acid production was similar to that seen by polyphenol-containing extracts of other plants in our previous study (Smullen et al., 2012).
The results show that the combined inhibition of growth and GTA inhibited plaque formation in vitro.

Previous studies have used hydroxyapatite discs for in vivo plaque culture (Guggenheim et al., 2001, Shapiro et al., 2002). We investigated the use of bovine teeth as a more natural substrate here but there were some problems. Preliminary experiments were contaminated and different methods of sterilization were tried. The final method used was laborious but subsequent runs were free from contamination. The plaque formed was composed of S. mutans from the inoculum plus small numbers of other organisms (<5%) that were probably derived from the saliva used for conditioning. The saliva used for conditioning of the teeth was centrifuged to remove particles but was not sterilized. It is possible that residual bacteria as well as salivary components such as glycoproteins facilitated subsequent biofilm formation by the S. mutans.

Once set up the apparatus was completely self-sufficient and apart from the addition or removal of the teeth, no further manipulation was required. Estimation of the extent of plaque growth is difficult. The method used here, determination of the area covered by the plaque, was clearly an estimation but the results concurred with visual examination of the teeth. The method proved to be a simple and useful technique for determining anti-plaque activity.

Antimicrobial activity of plant extracts can be attributed to a variety of components including mono- and poly-hydric phenols (polyphenols; Cowan, 1999). Leaves of R. officianalis L. and S. officianalis L. contain a number of volatile phenolics including eugenol, isoeugenol and thymol and essential oils that have been shown to have antibacterial activity (Deans and Ritchie, 1987; Janssen et al., 1987; Shapiro et al., 1994; Hammer et al., 1999; Rasooli et al., 2008; Bernardes et al., 2010a). The precise composition depends upon a number of factors including country (Derwich et al., 2011), soil composition (Angioni et al., 2004) time of harvesting (Generalic et al., 2012). R. officianalis L. and S. officianalis L. also contain the diphenols carnosol, rosmanol, rosmariquinone and rosmaridiphenol (Nakatani, 1992) and components derived from aromatic amino acids such as caffeic acid, cinnamic acid and rosmarinic acid (Okuda et al., 1992).

Antimicrobial activity of methanol extracts was shown to correlate with polyphenol content (Moreno et al., 2006) and carnosic acid and carnosol were active components of ethanol/H₂O.
extracts (Bernardes et al., 2010b). Preliminary analysis of the extracts showed that both extracts had rosmarinic acid as a major component together with smaller amounts of cinnamic acid in the *R. officianalis* extract and flavanol glycosides in the *S. officianalis* extract. Neither contained catechins or procyanidins as was found in the other extracts (Smullen et al., 2007; data not shown). In a previous report we showed that polyphenol-containing plant extracts prevented adhesion of *S. mutans* to glass (Smullen et al., 2007). The results in the present study show that these extracts inhibited both GTA and plaque formation on bovine teeth. The results also show that extracts acted by inhibiting growth, acid production and plaque formation by *S. mutans*.

The quantities of extracts needed to inhibit *S. mutans* were much greater than the concentrations of commonly used antibacterial agents normally needed to produce similar antibacterial effects. Chlorhexidine and Triclosan were both more effective than herbal extracts in a previous study (Shapiro et al., 2002). However, the extracts used in this study are natural ingredients and may be acceptable for use in food products as well as dental preparations.

The initiation and progression of dental caries is related to a number of interrelated factors. Any preventive measure that interferes with a number of these respective factors would be a useful anticariogenic tool (Baehni and Takeuchi, 2003). The antiplaque activity of P70 extracts of green tea, unfermented cocoa, red grape seed, *R. officianalis* L., *S. officianalis* L. combined with reduction of acid production attacks two major stages in caries formation. The starting materials for these extracts were readily available all year round, were relatively inexpensive and preparation of the extracts was relatively easy. They may prove useful in prevention of caries. However the staining caused by the green tea extracts may prevent its use for cosmetic reasons despite its high anti-plaque activity.

**Acknowledgements**

The authors would like to thank Dr A. Lee of Reading Scientific Services Ltd for supplying the cocoa extracts and performing the analyses and Dr A. Oates for the diagram of the artificial mouth.
REFERENCES


© 2012The Authors Journal of Applied Microbiology © 2012 The Society for Applied Microbiology


Legends to Figures

**Figure 1** Artificial mouth used for studies on *in vitro* plaque formation.

**Figure 2** Time-kill studies of P-70 extracts *R. officianalis* L. and *S. officianalis* L. on the viability of *Streptococcus mutans* Ingbritt.


**Figure 3** Bacteriostatic activity of P-70 extracts of *R. officianalis* L. and *S. officianalis* L. on *Streptococcus mutans* Ingbritt.

The MIC of the extracts (0.25 mg ml\(^{-1}\)) were added to an overnight culture of *Streptococcus mutans* in CDM + 10 g l\(^{-1}\) glucose (arrow). Results are the means and SD of three experiments.

- ●, control; ○, *S. officianalis* L.; ▼, *R. officianalis* L.

**Figure 4** Inhibition of plaque formation on bovine teeth by plant extracts.

(a) Control 48 h, (b) control 120 h, (c) *R. officianalis* L. extract, (d) *S. officianalis* L. extract, (d) Unfermented cocoa extract (f) green tea extract.

**Figure 5** Effects of P-70 extract of *S. officianalis* L. on insoluble glucan formation by *Streptococcus mutans* Ingbritt.

Insoluble glucan was measured as absorbance at 550nm ●, control; ○, 0.5 MIC; ▼, 1 x MIC; ▼, 2 x MIC. MIC was 0.25 mg ml\(^{-1}\).
<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (mg ml⁻¹)</th>
<th>P70 R. officianalis</th>
<th>P70 S. officianalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomyces denticolens</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Actinomyces naeslundii</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Actinomyces odontolyticus</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Actinomyces viscosus</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>32</td>
<td>&gt;32</td>
<td></td>
</tr>
<tr>
<td>Candida utilis</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>4.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>8.0</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus acidophilus</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>8.0</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Streptococcus gordonii</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Streptococcus mutans Ingbritt</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Streptococcus oralis</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Veillonella sp.</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Minimal inhibitory and bactericidal concentrations of 70% propanone extracts of *S. officianalis* L. and *R. officianalis* L. against oral bacteria
<table>
<thead>
<tr>
<th>Food extract</th>
<th>Plaque area % (± SD)</th>
<th>Mean reduction in plaque area %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>76.9 (3.1)</td>
<td>0</td>
</tr>
<tr>
<td>Unfermented cocoa</td>
<td>49.1 (2.1)</td>
<td>36.2</td>
</tr>
<tr>
<td>Red grape seed</td>
<td>46.2 (3.1)</td>
<td>40.0</td>
</tr>
<tr>
<td><em>R. officianalis</em> L.</td>
<td>9.6 (2.6)</td>
<td>87.5</td>
</tr>
<tr>
<td><em>S. officianalis</em> L.</td>
<td>12.3 (4.0)</td>
<td>84.0</td>
</tr>
</tbody>
</table>

**Table 2** Inhibition of plaque formation on bovine teeth in an artificial mouth model by plant extracts.

<table>
<thead>
<tr>
<th>P70 Extract</th>
<th>Mean (± SD) turbidity at 480nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>Rosmarinus officianalis</em></td>
<td>1.54 (0.03)</td>
</tr>
<tr>
<td><em>Salvia officianalis</em></td>
<td>1.55 (0.05)</td>
</tr>
</tbody>
</table>

**Table 3** Inhibition of adhesion of *Streptococcus mutans* to glass by plant extracts.
<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration of extract</th>
<th>0.5 MIC</th>
<th>MIC</th>
<th>2 MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green tea</td>
<td></td>
<td>37.0 ± 3.8</td>
<td>29.0 ± 11.2</td>
<td>19.6 ± 7.8</td>
</tr>
<tr>
<td>Red grape seed</td>
<td></td>
<td>46.4 ± 10.7</td>
<td>0.0 ± 8.8</td>
<td>0.0 ± 2.8</td>
</tr>
<tr>
<td>Unfermented cocoa</td>
<td></td>
<td>14.5 ± 1.1</td>
<td>9.4 ± 3.6</td>
<td>0.7 ± 1.3</td>
</tr>
<tr>
<td>Procyanidin polymer</td>
<td></td>
<td>16.7 ± 4.5</td>
<td>6.5 ± 2.2</td>
<td>2.9 ± 1.3</td>
</tr>
<tr>
<td>R. officianalis L.</td>
<td></td>
<td>68.1 ± 5.5</td>
<td>30.0 ± 2.3</td>
<td>6.5 ± 2.2</td>
</tr>
<tr>
<td>S. officianalis L.</td>
<td></td>
<td>61.6 ± 10.3</td>
<td>27.5 ± 5.5</td>
<td>7.2 ± 1.3</td>
</tr>
</tbody>
</table>

Table 4 Inhibition of glucan production in *Streptococcus mutans* Ingbritt by plant extracts as a % of the control

MIC Minimum inhibitory concentration mg ml⁻¹. Red grape seed, 0.5; unfermented cocoa, 4; procyanidin polymer, 1; green tea, 2; *R. officianalis* 0.25; *S. officianalis* 0.25