

Full Length Research Paper

Extracts of *Eremophila longifolia* inhibit the cariogenic activities of *Streptococcus mutans* and *Streptococcus sobrinus*

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Accepted 4 April, 2011

Plants belonging to the *Eremophila* genus are an integral part of the traditional medicine of indigenous Australian populations. Extracts of a number of *Eremophila* species have demonstrated the presence of bioactivity, including inhibitory effects against Gram positive bacteria. The aim of the present study was to determine the activity of solvent and aqueous extracts of *Eremophila longifolia* stem and leaves against *Streptococcus mutans* and *Streptococcus sobrinus*. A stem ethanol extract (SEE) demonstrated growth inhibition of the two cariogenic bacteria with a minimum inhibitory concentration (MIC) of 0.5% (w/v). Time-kill experiments indicated bactericidal activity by SEE within a 2 h period. Furthermore, this study assessed the anticariogenic activity of SEE in terms of its effect on glycolytic pH drop, viability of cells within an artificial biofilm and cell attachment to a membrane. In all experiments, SEE demonstrated significant anticariogenic activity compared with controls ($p < 0.05$, Students t-test). SEE also compared favourably with commercial oral healthcare products. Preliminary phytochemical investigations suggested that the active components within SEE were phenolic compounds but unlikely to be flavonoids. Our study advocates SEE as a worthy candidate for further research into alternative chemotherapeutic approaches to dental caries.

Key words: *Eremophila longifolia*, dental caries, biofilm, antibacterial.

INTRODUCTION

The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals (Palombo, 2006). *Eremophila longifolia* F. Muell. (Myoporaceae), commonly known as “emu bush” is a large shrub that is found in the dry inland areas of all Australian mainland states (Latz, 1995). Ethnobotanic literature frequently cites the *Eremophila* genus as an integral part of the traditional medicine of indigenous Australian populations, and *E. longifolia* is often considered to be the most sacred and mystical plants

used within these cultures (Smith, 2007). Traditional therapeutic uses of *E. longifolia* include treatments for colds, headaches, sores (Lassak and McCarthy, 2001), skin ailments, eye conditions (Latz, 1995), boils and muscle ache (ANPSA, 2006). Recent studies investigating the medicinal properties of the genus have demonstrated the presence of bioactivity in a number of species. In particular, extracts of a number of *Eremophila* species have shown inhibitory effects against Gram positive bacteria (Palombo and Semple, 2001; Shah et al., 2004; Pennacchio et al., 2005; Owen and Palombo 2007; Ndi et al., 2007a, b), including antibiotic-resistant strains (Palombo and Semple, 2002; Ndi et al., 2007c). Previous studies have also revealed anti-mycobacterial activity (Meilak and Palombo, 2008), antiviral activity (Semple et al., 1998), cardioactive effects (Pennacchio et al., 1996) and *in vitro* inhibition of serotonin release and

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platelet aggregation (Rogers et al., 2000). However, there have been no studies specifically investigating the bioactivity of *Eremophila* species against the cariogenic bacteria, *Streptococcus mutans* and *Streptococcus sobrinus*.

Dental caries is an extremely prevalent infectious disease that has been shown to be associated with serious health problems. Although there has been a slight decline in the prevalence of dental caries in many developed countries, there is an increase in occurrence amongst people of lower socioeconomic status and those within indigenous populations (Jamieson et al., 2007; Arantes et al., 2009).

The disease is associated with the colonisation and biofilm development of the Gram positive bacteria *S. mutans* and *S. sobrinus* (Rasooli et al., 2008). These cariogenic pathogens utilise dietary sucrose and produce adhesive exopolysaccharides and acids which lead to plaque formation and carious lesions on susceptible tooth surfaces (Koo et al., 2004).

A range of chemical agents have been used in the prevention and treatment of dental caries, although concerns about the safety of some of these methods have increased consumer demand for alternative methods. Hence, there has been recent interest in testing natural products, including plant-derived compounds, for anti-cariogenic properties. The effectiveness of *E. longifolia* extracts as potential chemotherapeutic agents to prevent or treat dental caries was explored in the current study.

MATERIALS AND METHODS

Plant collection and extraction

Aerial parts of *E. longifolia* were supplied by Canopus Biopharma Inc. (Byrock, New South Wales, Australia). The identification of the plant was confirmed by Dr. Bob Chinnock (State Herbarium, Adelaide, South Australia) and the plant material was stored at -20°C until use. Stem and leaf material were separated, lyophilised, ground to a powder and extracted separately in ethanol, acetone and distilled water at room temperature. The six resulting extracts were filtered, evaporated and re-dissolved to a concentration of 100 mg/ml in their extraction solvent.

Bacterial media

Brain Heart Infusion Agar (BHIA) and Brain Heart Infusion Broth (BHIB) were supplied by Oxoid Pty Ltd. (Basingstoke, UK). All media were prepared in deionised water and autoclaved at 121°C for 15 min prior to use.

Bacterial strains

S. mutans (ACM 969) and *S. sobrinus* (6715-247) were provided by the Melbourne Dental School, University of Melbourne, and are part of a culture collection located at Swinburne University of Technology. Bacteria were sub-cultured on BHIA at 37°C under capnophilic conditions, prior to being grown in BHIB overnight.

Plate-hole and disk diffusion assays

For the plate-hole assays, 200 µl of overnight BHIB culture were added to 15 ml of molten BHIA and poured into a sterile Petri dish. Once set, a sterilised core-borer (6 mm diameter) was used to produce wells in the agar, and 10 µl of plant extract were dispensed into each well. Neat solvent and chlorhexidine (CHX: J & J Medical) (0.2%) were used as negative and positive controls, respectively. For disk diffusion assays, 10 µl of plant extract and controls were added to sterile paper disks (6 mm diameter, Oxoid) and allowed to dry. One hundred µl of each culture was spread on BHI agar and allowed to dry for 10 mins. Disks were transferred to the agar surface. All plates were incubated overnight at 37°C under capnophilic conditions and assays were performed in triplicate. Zones of inhibited bacterial growth with a diameter greater than 6 mm were considered as positive.

Determination of minimum inhibitory concentration (MIC)

The MIC of the active extract was determined by the plate-hole assay using two-fold dilutions of extract in ethanol (McRae et al., 2008) The MIC was considered to be the lowest concentration with a visible zone of inhibition. This assay was carried out in triplicate.

Time-kill assay

BHIB (0.5 ml) was inoculated with 0.5 ml of overnight *S. mutans* or *S. sobrinus* culture. Stem extract (100 µl) was added to each vial to give a final concentration of 1.0%. Vials were incubated at 37°C and 100 µl aliquots were taken immediately and after 1 and 2 h and decimally diluted in sterile BHIB. A viable count was performed after overnight incubation to determine the colony forming units (cfu)/ml. Controls were prepared following the same method without the addition of plant extract. Time-kill assays were performed in duplicate.

Inhibition of acid production by cariogenic bacteria

The effects of the active extract on acid production by both bacteria were measured by pH assays with dense cell suspensions as previously described by Duarte et al. (2006). Cells of *S. mutans* and *S. sobrinus* from suspension cultures were harvested by centrifugation, washed once with salt solution (50 mM KCl, 1 mM MgCl₂), and resuspended in 5 ml salt solution containing stem extract at a final concentration of 0.33%. The pH was adjusted to between 7.4 to 7.5 with 0.1 M KOH solution and sufficient glucose was added to give a concentration of 1% (w/v). The decrease in pH was measured every 5 min over a period of 30 min using a glass electrode. A solvent control was prepared by adding 100% ethanol to each bacterial system instead of stem extract and a 'no treatment' control involved measurement of pH drop without addition of extract or solvent.

Antibacterial activity against salivary bacteria

Non-stimulated saliva was collected from a healthy donor and 200 µl aliquots were transferred to four sterile microcentrifuge tubes. Stem extract was added to two tubes (0.5 and 1.0%, respectively) and CHX was added to another tube at a concentration of 2 mg/ml. Tubes were incubated for 1 h at 37°C and then 100 µl aliquots were taken and diluted 10-fold in sterile BHIB for viable counts.

Artificial biofilm assay

Artificial biofilm assays were performed as described by Alviano et

Table 1. Antibacterial activity of leaf and stem extracts of *E. longifolia*^a

	Leaf extract (100 mg/ml)			Stem extract (100 mg/ml)			Chlorhexidine (2 mg/ml)
	Water	Ethanol	Acetone	Water	Ethanol	Acetone	
<i>S. mutans</i>	6.0 ± 0	6.4 ± 0.6	6.5 ± 0.7	6.4 ± 0.6	17.1 ± 0.7	17.9 ± 0.8	22.1 ± 0.6
<i>S. sobrinus</i>	6.0 ± 0	6.4 ± 0.8	6.7 ± 0.6	6.3 ± 0.5	15.9 ± 0.5	16.7 ± 0.7	20.5 ± 0.6

^a Values represent the mean diameter of the growth inhibition zone (mm) + SD, from three plate-hole assays and three disk diffusion assays.

al. (2008). Briefly, 20 µl of saliva or *S. mutans* were dispensed onto sterile 0.22 µm membrane disks (Millipore, 13 mm diameter) which were placed on the surface of BHIA. Plates were incubated for 48 h. After biofilm growth, the disks were removed and each disk was placed inside a bottle containing 3 ml of stem extract solution (0.5 or 1.0% in ethanol), 0.5% CHX, Listerine® (Johnson & Johnson Pacific Pty Ltd.), distilled water or ethanol for 1 h at 37°C with gently shaking. The disks were then briefly washed with distilled water to remove the plant extract and unbound bacterial cells, and the biofilm was removed by vortexing. Immediately, serial dilutions were performed and a viable count was performed after 48 h of incubation. Artificial biofilm assays were performed in duplicate.

Inhibition of bacterial cell attachment

This method was based on a beaker-wire test described by Kang et al. (2008). Stem extract (0.5 and 1.0%) and ethanol were added to three vials containing 3 ml of BHIB supplemented with 5% sucrose and 0.1 M of 2-(N-Morpholino) ethanesulfonic acid monohydrate (MES). The broths were inoculated with *S. mutans* and three nickel chromium wires attached to sterile 0.22 µm filter membranes were immersed in the system. The broths were incubated with slow agitation at 37°C for 24 h. The membranes were then removed, detached from the wire, and gently rinsed with distilled water and vortexed in 1 ml of BHIB. Serial dilutions and viable counts were then performed to determine the number of bacterial cells that were able to attach to the membrane over the 24 h time period.

Microscale column chromatography

A glass Pasteur pipette filled with silica gel (Labchem 100 to 200 mesh) was used to fractionate 150 µl of stem extract with a solvent gradient consisting of hexane and ethanol (10 to 100% ethanol). Fractions were collected according to colour change until the elution ran clear. All fractions were dried, diluted to 100 mg/ml in ethanol, and assessed for their antibacterial activity.

Thin-layer chromatography (TLC) and bioautography

Crude stem extract and active fractions were applied to a silica TLC plate with aluminium backing (Sigma). After drying, the plates were developed with toluene:ethanol 9:1. For bioautography, plates were allowed to dry, placed into sterile Petri dishes and covered with 15 ml of molten BHIA containing 200 µl of *S. mutans* or *S. sobrinus* culture. After overnight incubation, plates were sprayed with a 2% solution of methylthiazolyltetrazolium chloride (MTT) and inspected for zones of inhibited growth.

TLC spray reagents

Freshly prepared aluminium chloride (for detection of flavonoids),

Dragendorff reagent (alkaloids), Folin-Ciocalteu (phenolic compounds) and Liebermann-Burchard reagent (triterpenes, steroids and sterols) were sprayed onto developed TLC plates. For aluminium chloride, plates were observed under UV light (360 nm), whereas others were observed in visible light. The Liebermann-Burchard plates were heated at 100°C for 10 min prior to analysis (Krebs et al., 1969).

RESULTS

Assessment of antibacterial activity of extracts

Plate-hole and disk diffusion assays

The six *E. longifolia* extracts were screened for antibacterial activity against *S. mutans* and *S. sobrinus* using plate-hole and disk diffusion assays. Overall, the extracts of the stem material displayed greater antibacterial activity against both of the bacteria, with the acetone stem extract producing the largest zones of inhibition (Table 1). However, only the stem ethanol extract (SEE) was pursued for further investigation due to the fact that it achieved a much higher yield of extracted compounds (8.28% of dry stem mass, compared with 4.50% for acetone) whilst demonstrating comparable activity. This extract had an MIC of 0.5% against both *S. mutans* and *S. sobrinus*.

Time-kill assays

Time-kill assays were performed so that the killing kinetics of SEE could be observed. Within a two hour period, SEE completely eliminated all *S. sobrinus* cells and caused a gradual decline (approximately 3.0 log units) in the number of viable *S. mutans* cells (Figure 1).

Assessment of anti-cariogenic activity

Inhibition of acid production by cariogenic bacteria

The ability of sub-MIC concentrations of SEE to inhibit acid production by cariogenic bacteria was assessed by measuring changes in pH. In both the *S. mutans* and *S. sobrinus* assays, SEE resulted in significantly reduced acid production compared with both the ethanol control

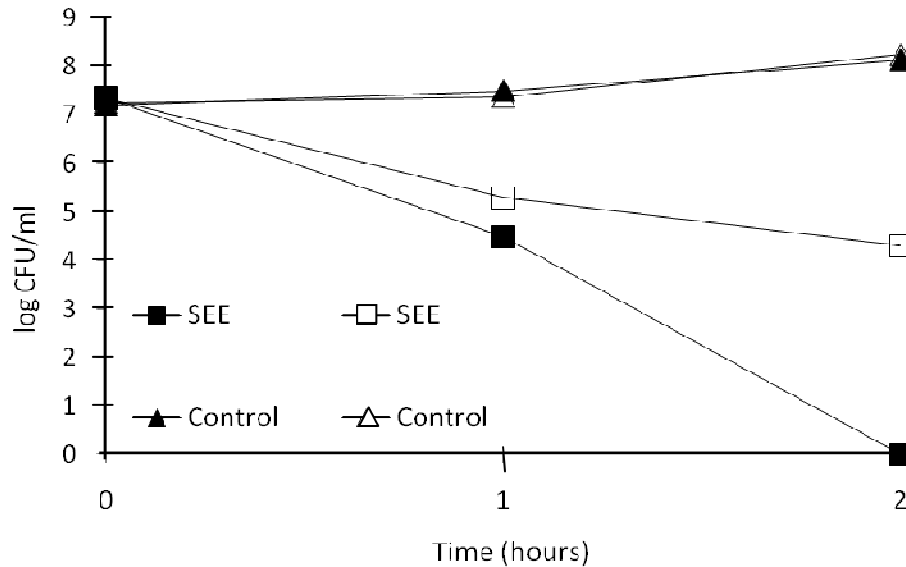


Figure 1. Time-kill assay for SEE (1.0%) against *S. mutans* (hollow marker) and *S. sobrinus* (filled marker). Viable cell counts at T= 0, 1 and 2 h represent the mean value of duplicate experiments. The extract exhibits a significant reduction in viable cells compared with the control after 1 h ($p < 0.01$, Student's t-test).

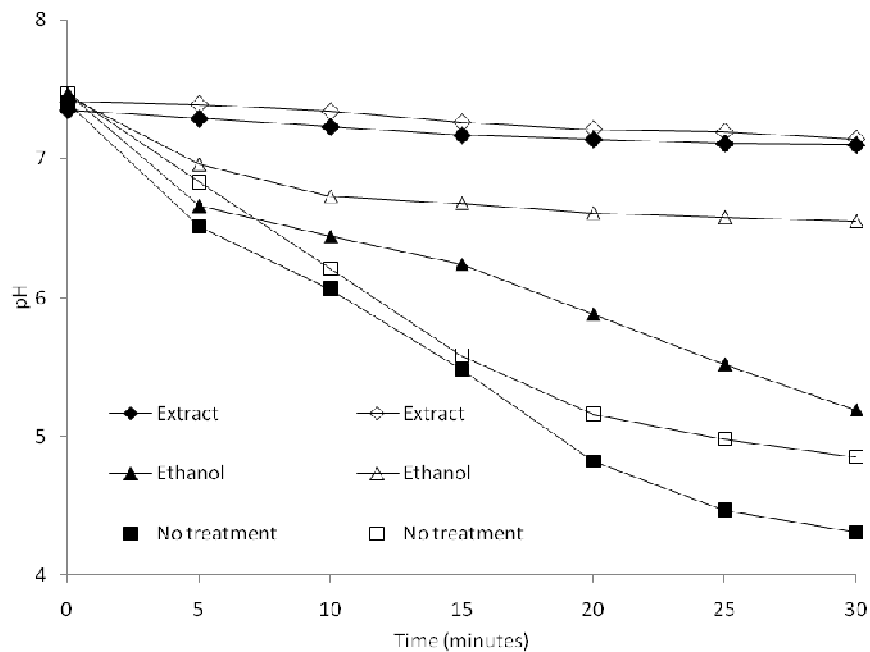


Figure 2. pH assay for SEE (0.33%) against *S. mutans* (hollow marker) and *S. sobrinus* (filled marker). pH values at 5 min intervals represent the mean value of duplicate experiments. The extract exhibits a significant reduction in pH drop compared with both the ethanol control and 'no treatment' control after 5 min ($p < 0.05$, Student's t-test).

and 'no treatment' control after 5 min ($p < 0.05$, Figure 2, Student's t-test). The viability of the test bacteria was not affected as they were cultured on BHIA subsequent to testing.

Antibacterial activity of SEE against salivary bacteria

The inhibitory capabilities of SEE were tested in the presence of saliva. The 1.0% SEE caused a 5.0 log

Table 2. Activity of SEE against salivary and *S. mutans* biofilms^a

Treatment	Log ₁₀ reduction in viable cells compared with water control ^a	
	Salivary bacteria	<i>S. mutans</i>
CHX 0.5%	4.07	2.50
SEE 1.0%	3.22	2.00
Listerine	3.19	2.12
SEE 0.5%	2.97	1.69
EtOH control	0.45	0.05

^a Values are presented as a reduction in viable cells as compared with the water treatment. Values represent the mean value of duplicate experiments. The SEE treatments show a significant reduction in viable cells ($p < 0.01$, Student's t-test) compared with the ethanol and water controls and are not significantly different from the CHX and Listerine treatments ($p > 0.05$). The ethanol control did not cause a significant reduction compared with the water control ($p > 0.05$).

reduction in viable (aerobic) salivary bacteria and performed significantly better than 0.2% CHX, which only caused a 3.0 log reduction. At the MIC, SEE produced a significant (2.0 log) reduction in viable bacteria compared with the 'no treatment' control.

Artificial biofilm assays

SEE, at 0.5 and 1.0%, significantly reduced the viability of both the salivary and *S. mutans* biofilms compared to the ethanol and water controls ($p < 0.01$). The effects produced by both concentrations of SEE were not significantly different from those produced by Listerine[®] and CHX treatments (Table 2).

Inhibition of attachment

SEE, at 0.5 and 1.0%, was able to produce significant log reductions (2.3 and 1.8 respectively, $p < 0.05$) in the number of *S. mutans* cells that attached to a suspended membrane filter as compared to a 'no treatment control'. The ethanol control treatment did not cause a significant reduction in attached cells.

Preliminary phytochemical analysis

Microscale column chromatography

Chromatography was used to separate SEE into ten different visibly coloured fractions. These were collected, dried, diluted to 100 mg/ml in ethanol and tested against both cariogenic bacteria. Only three fractions were capable of inhibiting the growth of *S. mutans* and *S. sobrinus*, producing zones of inhibited growth with diameters between 7.5 and 11.5 mm.

Thin layer chromatography (TLC), bioautography and spray reagents

Crude SEE and the three active fractions were separated

by TLC and analysed by bioautography. All zones of inhibited growth were positioned on the lower half of the silica gel plate which indicated that the active compounds were relatively polar. Four different spray reagents were used on developed TLC plates of the three fractions to identify the compound class of the active component. Only the Folin-Ciocalteu reagent returned a positive result, indicating the presence of phenolic compounds.

DISCUSSION

In preliminary screening of solvent extracts of *E. longifolia* stems and leaves, it was found that ethanol and acetone extracts of stem material possessed inhibitory activity against the cariogenic bacteria, *S. mutans* and *S. sobrinus*. Given that ethanol extraction gave the greatest yield, further experiments were conducted only on the stem ethanol extract (SEE). Previous studies of *Eremophila* showed that antibacterial activity was restricted to leaf extracts. This is the first study to indicate that stems also contain antibacterial compounds. A standard broth dilution MIC assay relying on turbidity as the means of quantifying growth did not achieve satisfactory results due to the opacity of the extract. Instead, plate-hole assays were used to determine the MIC of the stem ethanolic extract against both bacteria. Given the semi-quantitative nature of plate-hole assays and their reliance on the diffusibility of active compounds through agar, the results of the assay were used as an estimate of the MIC. SEE had a minimum inhibitory concentration of 0.5% against both *S. mutans* and *S. sobrinus*. This is not excessively high considering that it is a crude extract resulting from a one-step extraction process.

The time-kill assays provided a dynamic analysis of the decline in viable bacterial cells in the presence of SEE. The extract was particularly potent against *S. sobrinus* in a 2 h period, resulting in complete elimination of viable cells (Figure 2). Bactericidal activity was also demonstrated against *S. mutans* with an approximate 3.0

log unit reduction in viable cells. Although the extract did not cause complete elimination of cells, the reduction is still considerable when compared with similar time-kill assays reported in the literature. For example, Alviano et al. (2008) reported an approximate 1.8 to 1.5 log reduction in viable *S. mutans* cells over 2 h by aqueous *Cocos nucifera* and *Caesalpinia pyramidalis* extracts. These authors also tested an extract from *Ziziphus joazeiro* which, despite its use in commercial dentifrices, did not result in a reduction in the viable cell number.

Acid production by both *S. mutans* and *S. sobrinus* plays an important role in the pathology of dental caries. A pH assay showed that sub-MIC levels of SEE were capable of inhibiting acid production. In both the *S. mutans* and *S. sobrinus* assays, SEE significantly inhibited acid production compared with the ethanol and 'no treatment' controls after 5 min. As the extract concentration was sub-MIC, bacterial viability was unaffected (as confirmed by growth of recovered bacteria subsequent to assaying), which suggested that the reduction in acid production was caused by interference with the bacteria's metabolism of sugar. These results are very important as acid production has a direct influence on the aetiology of dental caries and advocates this extract as a candidate for the development of a natural product-based caries prevention product.

It is possible that components within saliva can interact with active compounds within a plant extract and render it inactive against its target bacteria. It is therefore important to assess the antibacterial activity of the plant extract in the presence of saliva. SEE at both 0.5 and 1.0% significantly reduced the number of viable salivary bacteria compared with the 'no treatment' control, indicating that the extract remained active in the presence of saliva, an essential prerequisite for any product to be utilised in the oral cavity.

The attachment of pathogenic bacteria to the tooth surface and the subsequent formation of a biofilm structure are key elements in the formation of dental caries. Furthermore, biofilm-associated bacteria are more capable of tolerating the presence of antimicrobial agents (Djordjevic et al., 2002). Therefore, a potential natural product capable of treating dental caries must be able to affect cariogenic bacteria within a biofilm. SEE reduced the viability of bacteria in artificial biofilms, suggesting that it was capable of detaching the cells from the biofilm and/or killing cells that remained attached. This first point is important as it may be preferential that an active agent is anti-adhesive rather than bactericidal in order to reduce the development of resistant strains (Duarte et al., 2006). Interestingly, the reduction in viability caused by SEE was not significantly different from the commercial mouthwash product Listerine® or the antiseptic agent chlorhexidine. SEE was also able to inhibit the attachment of *S. mutans*. While this is an important observation in the context of the anticariogenic activity of the extract, the results do not yet hold any implications for an *in vivo* application as the

sample was a monoculture and treatment was for 24 h. In addition, further testing is necessary to determine if SEE is as effective in the complex heterogeneous microbial environment of the oral cavity and *in-vivo* plaque biofilm.

Bioautography of the three active fractions of SEE revealed that each contained the antimicrobial components present in the crude extract, albeit at lower concentrations. Preliminary phytochemical analysis of the active fractions with spray reagents established that the active compounds were phenolics. The areas of inhibited growth that reacted with Folin-Ciocalteu reagent also exhibited dark spots under UV_{254nm} light. Phenolic compounds are able to quench fluorescence at this wavelength, resulting in dark spots. However, other structures are also known to cause this effect (Harbourne, 1973). Flavonoids are phenolic structures ubiquitous in plants; however, they were not present in any of the active fractions as none of the bands fluoresced yellow with the aluminium chloride spray as compared to a quercetin control. Therefore, a preliminary estimation of the active constituents of SEE was that they were phenolic compounds, but unlikely to be flavonoids.

In summary, the ethanolic stem extract of *E. longifolia* has demonstrated a number of anticariogenic characteristics that prove it to be a worthy candidate for further research into alternative chemotherapeutic approaches to dental caries.

ACKNOWLEDGMENTS

We are grateful to Canopus BioPharma Inc. for providing plant material and financial support, and to Mr Ngan Nguyen and Mr Chris Key for technical advice.

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