Mine Closure 2009 — A.B. Fourie, M. Tibbett (eds)

https://papers.acg.uwa.edu.au/p/908_42_Regnier/

Chemical analysis and potential use of the tree *Combretum erythrophyllum* grown on gold and uranium mine tailings seepage

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Abstract

Combretum erythrophyllum *is a southern African riparian tree, which is widely used in traditional medicine to combat microbial infections. More than seven flavonoids with antimicrobial properties, including apigenin, genkwanin, rhamnocitrin and kaempferol, have been previously isolated from methanolic extracts of leaf material. The species occurs naturally in the Witwatersrand Basin gold fields, and is included in experimental trials of acid and salt-tolerant trees for seepage control purposes around gold mine tailings at AngloGold Ashanti Ltd (the Mine Woodlands Project). In this study,* C. erythrophyllum*, harvested from the mine woodland trials, was investigated as a source of secondary metabolites for the control of food-borne pathogens. The phenolic profiles of methanolic leaf extracts from trees growing on acid mine drainage and non-impacted surrounding soils were compared to those from a natural population, using high performance liquid chromatography. The concentrations of apigenin, genkwanin and kaempferol are currently being determined in dry leaf material. These extracts were evaluated for their ability to inhibit the growth of* Listeria monocytogenes *and* Candida albicans *biofilm formation. No significant differences between the phenolic profiles of samples from different substrata or regions could be discerned. Selected extracts of both mine and natural populations were able to significantly inhibit biofilm formation in the case of* C. albicans*. The activity against* L. monocytogenes *was substantially lower. Micrographs obtained using confocal laser scanning microscopy confirmed the inhibitory effects. These results demonstrate the potential of* C. erythrophyllum *as a good candidate for application in food safety. Such commercial attributes can add value to* C. erythrophyllum *and other species used for mine rehabilitation, and provide a basis for the development of secondary industries based on renewable natural resources alongside mine closure.*

1 Introduction

Mining has played an important role in the historical and social development of South Africa. Although the economic benefits of mining are numerous, mining operations have a severe impact on the environment. Most gold and uranium mine tailings in South Africa are acid-generating and metal-rich, and ions tend to leach from the tailings into the surrounding soil and water (McCarthy and Venter, 2006; Tutu et al., 2008), thereby rendering some lands risky for grazing and cultivation of edible crops (Sutton et al., 2006; Sutton and Weiersbye, 2007). Various processes worldwide, including chemical, physical and biological techniques such as soil amendment, soil replacement, solidification, washing strategies and phytotechnologies, are used to rehabilitate mine sites (Tordoff et al., 2000; Barceló and Poschenrieder, 2003). However, only phytotechnologies offer simultaneous decontamination and ecological rehabilitation of sites, and the costs are significantly lower than physical and chemical techniques (reviewed in Weiersbye, 2007). Plants used for restoration include a wide variety, some which are tolerant to high elemental availability and able to immobilise or conversely accumulate ions, as well as others that serve to stabilise the soil (Whiting et al., 2004). Mine closures due to depletion of the metal ore not only result in large-scale unemployment, but also increase crime levels and decrease the standard of social service delivery. In South Africa, where

approximately 50 % of the mine workers are HIV positive (Churchyard et al., 2000), and people may also be subject to occupational health risks (Harington et al., 2004), the economic and social implications of mine closure is of great concern. The creation of alternative opportunities to retrenched mine workers to reduce unemployment in the mining regions of the world is one of the priorities of the mining industry (Limpitlaw et al., 2005). Due to the impaired immune status of so many mine workers, it is essential that such opportunities do not subject workers to increased health risks. Rehabilitation and revaluation of mines, both operating and abandoned, using plants can contribute towards the development of small businesses, such as community-based nurseries, planters, harvesters and secondary processes. The Ecological Engineering and Phytoremediation programme, initiated in 1995 by the University of the Witwatersrand, Johannesburg and AngloGold Ashanti Ltd (South Africa) (AGA), is developing safe technologies and employment opportunities to support the rehabilitation of mine tailings and polluted lands in the Witwatersrand Basin gold fields (Weiersbye and Witkowski, 2002), including the use of native trees for control of acid mine drainage (Dye et al., 2008). To date, approximately 500,000 trees, mostly native species, have been planted in trials on acid mine drainage (AMD) and tailings (AngloGold Ashanti Ltd, 2006, 2009), and numerous other plants on a high landfill site (Scaw Metals Group, 2009). In 2007, collaboration between the University of Witwatersrand (WITS) and the Department of Chemistry, Tshwane University of Technology (TUT), Pretoria, commenced in order to investigate the possibility of sourcing useful secondary metabolites (essential oils, antioxidants, anti-inflammatory and antimicrobial agents) from the wide variety of native plant species found to tolerate growth on mine tailings and polluted soils (Weiersbye et al., 2006). *Combretum erythrophyllum* (Burch.) Sond. is a native southern African tree of watercourses and floodplains. This species not only contributes to the control of seepage from AGA's gold mine tailings, but is known to produce an array of pharmacologically active secondary metabolites of phenolic nature (Eloff et al., 2001; Angeh et al., 2007). *C. erythrophyllum* is considered a safe candidate for seepage control and in situ stabilisation of some metals below-ground since the tree does not exhibit high metal uptake (Weiersbye and Cukrowksa, 2008).

The rapidly expanding number of food-borne pathogens, some of which have the ability to form biofilms, pose a serious risk to food safety. Biofilms are agglomerations of microorganisms encapsulated within a polysaccharide-based matrix, which lends protection to the microorganism, and is attached to a living or inert surface (Sandasi et al., 2008). *Listeria* and *Candida* species have been identified as pathogens with a high risk of compromising human health (Jin et al., 2003). In general, a high mortality rate results from meningitis and abortion related to *Listeria* infection while *Candida* is recognised as a public health problem, particularly for immune compromised individuals (Pereira-Cenci et al., 2008). Plant secondary metabolites are an important source of antimicrobial compounds, and hence good candidates to investigate as possible alternatives to synthetic drugs for the inhibition of biofilm formation by microorganisms.

The subject of this study is to determine whether methanolic leaf extracts of selected acid and salt-tolerant genotypes of *C. erythrophyllum* grown to control AMD, can be used as a sustainable source of secondary metabolites for application in the control of *Listeria monocytogenes* and *Candida albicans* biofilm growth and development.

2 Methodology

2.1 Plant extracts

Leaves of five year old *C. erythrophyllum* trees were collected from two different environments: a natural population was harvested in Pretoria (Gauteng Province, South Africa), whereas a mine woodland trial on gold tailings seepage was sampled at the West Wits operations of AGA near Carletonville (Gauteng Province). This mine woodland trial is planted on deep, well-draining red doleritic soils in receipt of moderately contaminated seepage (approximately 2000 mg/L sulphates) from gold mine tailings (slimes dams). The leaves were oven-dried at 35°C, and then milled to a fine powder using a coffee grinder (Russell Hobbs, Model No 9715), sieved (< 1 mm particle size) and stored in glass vials until required. Soluble phenolic compounds were extracted from 50 mg portions of the individual samples using 80 % aqueous methanol (v/v). The extracts were subsequently partitioned with chloroform to remove chlorophyll and leaf waxes. Extract solutions were adjusted to a concentration of 2.0 mg/mL with deionised water and stored at 4°C. The concentration of phenolic metabolites in each leaf extract was determined using the Folin-

Ciocalteau assay (Swain and Hillis, 1959; Singleton et al., 1999). A volume of 5 µL of each sample was added to all the wells in a 96-well Elisa plate, followed by 175 µL of deionised water. Then, 25 µL of Folin-Ciocalteau reagent was added to all the wells followed by 50 µL 20 % (m/v) aqueous sodium carbonate. The contents of each well were mixed and the plate was thereafter covered with aluminium foil and incubated at 40°C for 30 min to allow blue colour to develop. The absorbance of each solution was determined using a Universal Micro-plate Reader (Bio-Tek ELx800) at 750 nm and 25°C on quick read mode. A volume of 180 µL of deionised water was used as blank. All the analyses were performed using four replicates. The total phenolic content of the plant extract was expressed as milligram of gallic acid equivalents per gram of dry plant material. All solvents used were of analytical grade (Merck Chemical Company, Johannesburg, South Africa).

2.2 High performance liquid chromatography (HPLC)

The prepared extracts were filtered through 0.45 µm nylon filters (millipore) prior to analysis by reversed phase-high performance liquid chromatography (RP-HPLC) (Hewlett Packard Agilent Series 1200) with diode array detection (DAD) using the modified method of Lin et al. (2007). A Zorbax C_{18} column (150 × 4 mm i.d.; 5 µm particle diameter: Agilent Technologies) at 40°C was used for separation and chromatograms were recorded at 280, 320 and 380 nm. A binary solvent gradient using acetonitrile and deionised water (pH 2.6 by addition of H_3PO_4) was applied as mobile phase at a flow rate of 1 mL/min. The initial mobile phase consisting of 5 % aqueous acetonitrile was linearly adjusted to 80 % aqueous acetonitrile over 30 minutes. All extracts were analysed in triplicate with an injection volume of 20 µL. Authentic standards of kaemferol, genkwanin, apigenin, ferulic acid, chlorogenic acid were purchased from Sigma Aldrich (Johannesburg, South Africa). The identification of phenolic compounds was carried out by comparing the retention times and UV apex spectra to those of purchased. Thin layer chromatography (Merck, silica gel coated plate; 20 × 20 cm × 0.25 mm with fluorescent indicator) using benzene/ethanol/ammonium hydroxide (90:10:1, v/v/v) as developing solvent was used to confirm the identification of the compounds. Phenolics were visualised by spraying the plate with 1 % methanolic diphenyl boric acid-2-aminoethyl ester, followed by 5 % ethanolic polyethylene glycol 4,000 and the plate was then viewed under ultraviolet light (360 nm).

2.3 Bioassay

Listeria monocytogenes (Tracking code ATCC 19111 and clinical isolate tracking code CI001) and *Candida albicans* (Tracking code ATCC 10231 and clinical isolate tracking code CI002) were revived from glycerol stock and sub-cultured onto tryptone soy agar as described by Sandasi et al. (2008). A crystal violet assay (CV) was performed as described by Djordjevic et al. (2002) to determine the amount of biomass formed. After each incubation stage, the 96-well flat-bottom microtitre plates were washed five times with sterile deionised water to remove planktonic bacteria and oven-dried for 45 minutes at 60°C. Then, 100 µL of a 1 % (m/v) ethanolic crystal violet was added to stain the wells, followed by incubation at room temperature for 15 minutes. The plates were subsequently washed five times with sterile deionised water to remove unabsorbed staining reagent. Excess water was removed and the well destained using 125 µL of ethanol. A volume of 100 µL of the destained solution was then transferred into a new plate and the absorbance was read at 590 nm by means of a Universal microplate reader ELx800.

The metabolic activities of the cells were quantitatively evaluated using tetrazolium salt {2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide} (XTT) as described by Pettit et al. (2005).

Amphotericin B, Ciprofloxacin and deionised sterile water were incorporated into the assay as standard antifungal agent, standard antibacterial agent and control, respectively. The experiment was performed in triplicate.

2.4 Metal analysis

For metal analysis, five leaves harvested from each of the four aspects of five trees (n = 20 leaves per tree) per plot (n= 4) were pooled to constitute four representative samples. Each set of leaves was rinsed under running water to remove surface dust. The leaves were then washed three times with automatic stirring in

distilled deionised water before oven-drying at 40°C and milling in an agate pestle and mortar. Suprapure nitric acid (65% w/w; 6 mL; Merck, Darmstadt, Germany) was added to 0.25 g of the milled sample in a vent vessel and the leaf material was subsequently digested using a MARS CEM microwave digestion system (Mathews, NC, USA). The dissolutions were subsequently diluted to 50 mL using double deionised water. The samples were analysed for arsenic (As), chromium (Cr), uranium (U) and nickel (Ni) using a Perkin Elmer Elan DRC-e inductively-coupled plasma mass spectrometer (ICP-MS) (Perkin Elmer, Shelton, CT, USA) against inductively-coupled plasma (ICP) grade calibration standards (Fluka A.G., Switzerland). Standard curves ranging from 0–20 µg/L using five calibration standards were obtained ($r^2 = 0.999$). All digestions and ICP measurements were carried out in triplicate.

2.5 Statistical analysis

Differences between the parameters were subjected to an analysis of variance (ANOVA) using the general linear model (GLM). One way ANOVA (Pearson SPSS, version 14.0) was used to discriminate between the means.

3 Data

A typical HPLC profile of samples used in this study is illustrated in Figure 1.

The methanolic extracts of *C. erythrophyllum* represent two families of phenolic compounds. Peaks 1 and 2, with a maximum absorption at 280 nm, are flavonoids while peaks 3–8, with a maximum absorption at 320 nm, belong to the hydroxycinnamic family of compounds. Peaks 1 and 2 were tentatively identified as apigenin and kaemferol respectively, while Peaks 7 and 8 were preliminary identified as esters of ferulic acid. All compounds are currently in the process of being identified using a high performance liquid chromatography mass spectrometer (HPLC-MS). No significant differences in the phenolic profiles of methanolic leaf extracts from the trees grown on mine sites and naturally-occurring trees in the surrounding areas, or those of the natural population could be discerned using HPLC. The results from the Folin-Ciocalteau assay did also not reveal any significant differences in the amounts of total soluble phenolic compounds.

As illustrated in Figure 2a, the CV assay revealed that more than 68 % inhibition of the biofilm was achieved by all isolates when the *C. erythrophyllum* extracts were tested against *C. albicans*. There was no significant difference between the percentage of inhibition by extracts of the mine trees and those of the natural population trees. In contrast, the extract of the natural population of *C. erythrophyllum* caused greater inhibition of both clinical (Cl001) and laboratory (ATCC 19111) isolates of *L. monocytogenes* than that from the mine trees ($P < 0.05$) (Figure 2b).

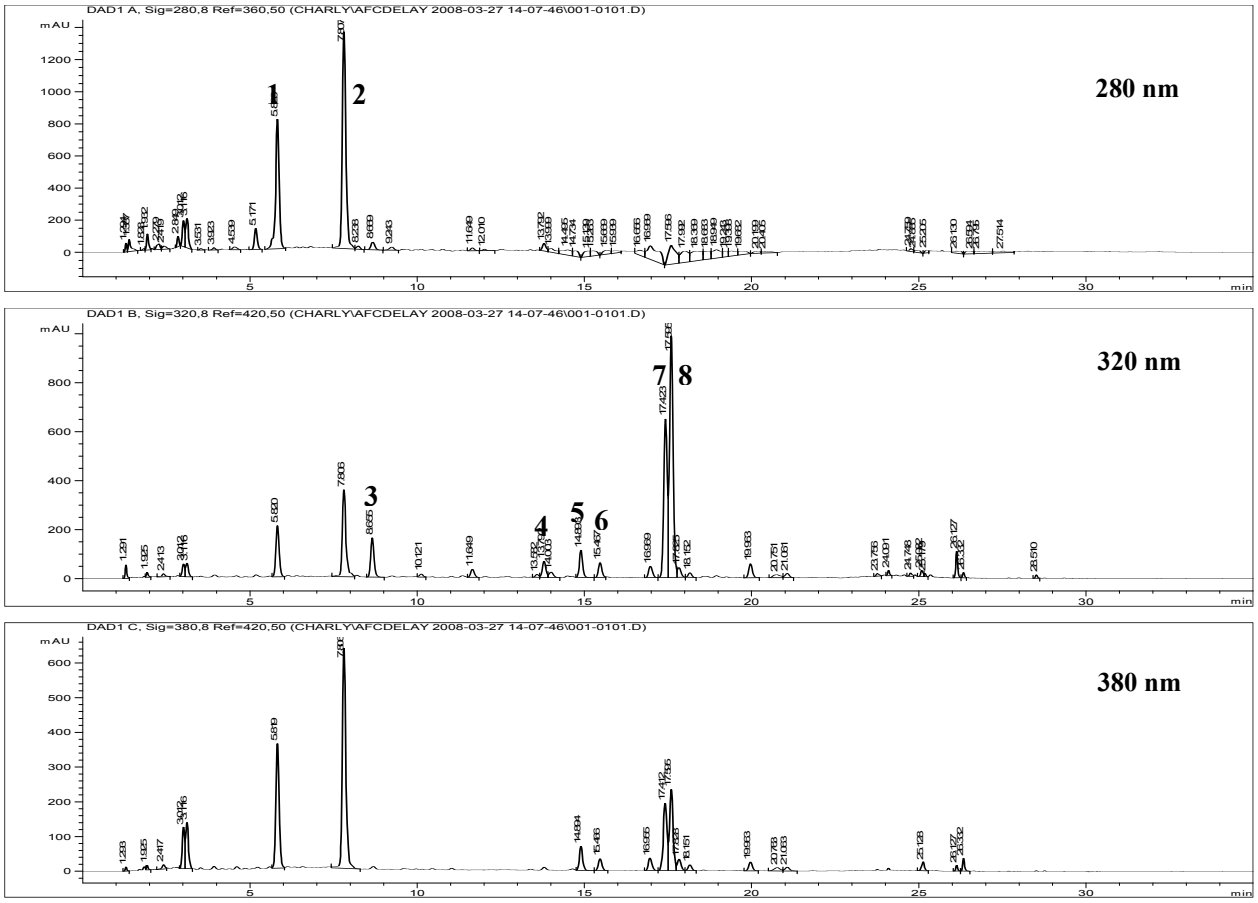
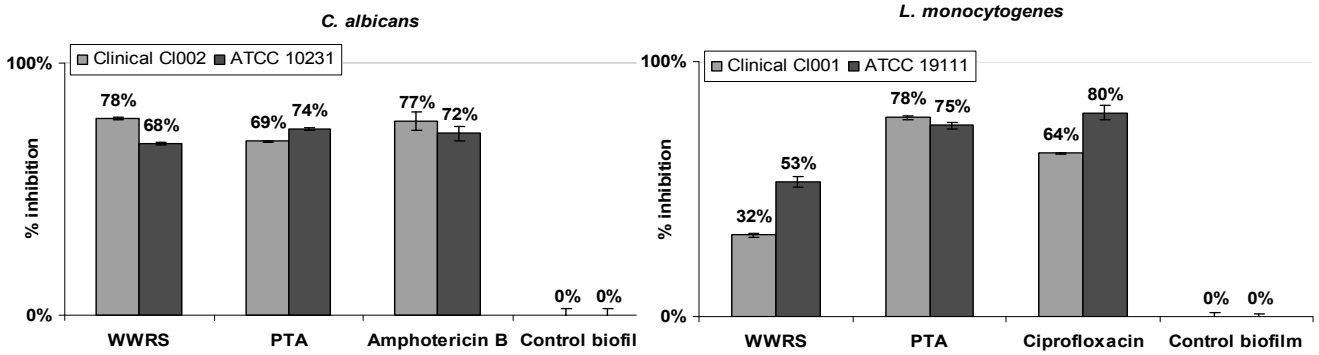


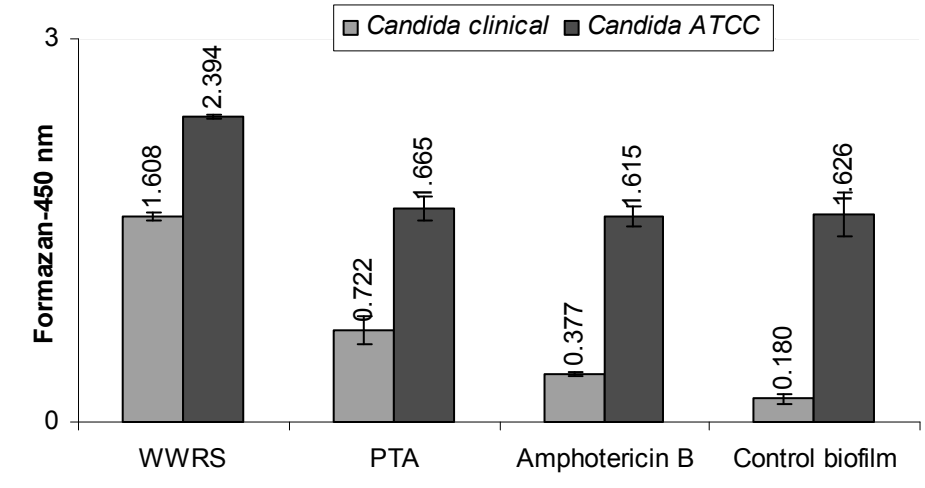
Figure 1 High performance liquid chromatograms (280, 320 and 280 nm) of a methanolic leaf extract of *Combretum erythrophyllum* grown on acid mine drainage



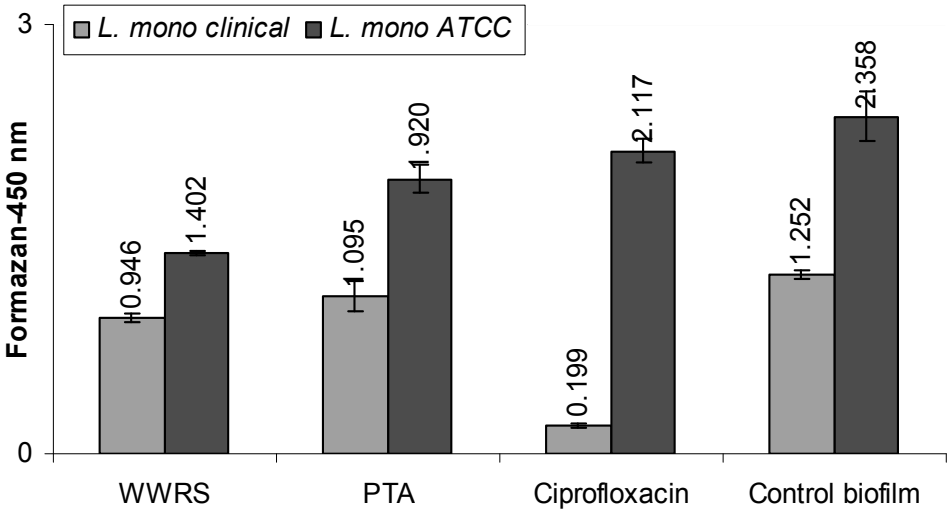
(a) (b)

Figure 2 Inhibition of the growth and development of *Candida* and *Listeria* biofilms by methanolic extracts of *C. erythrophyllum* (a) *C. albicans;* (b) *L. monocytogenes*, WWRS = West Wits Operations Red Soil (a mine woodlands trial area), PTA = Pretoria (natural population on uncontaminated soils)

The XTT results showed that the increase in percentage inhibition observed in the CV assay (Figure 3a) was associated with an increase in the metabolic activity of the biofilm (Figure 3b). Results obtained from the metal analyses indicated that the concentrations of As, U, Cr and Ni in the leaves of the mine samples and the natural populations were below the analytical detection limits of 1–10 ng/L.



(a)



(b)

Figure 3 Metabolic activities of (a) *C. albicans*; and (b) *L. monocytogenes* biofilm in the presence of leaf extracts of *C. erythrophyllum.* WWRS = West Wits Red Soil (mine area), PTA = Pretoria (natural environment)

4 Discussion

Plants are recognised as unique sources of compounds with useful properties. Amongst the thousands of secondary metabolites identified in plants, few are known to pose a risk to consumer health when consumed in moderation. Flavonoids are valuable plant metabolites that afford protection against cancer and heart disease (Lattanzio et al., 1994). Our results show that the flavonoids apigenin, kaempferol and genkwanin are present in the leaves of *C. erythrophyllum* established on AMD and surrounding areas. These findings are consistent with earlier reports for natural populations of *C. erythrophyllum* (Eloff et al., 2001; Angeh et al., 2007). Quantification of extracts from trees sampled during different seasons, and growing on a range of mine sites, is being conducted. It is well known that plants exposed to stress are stimulated to produce higher levels of secondary metabolites (Morant et al., 2008). However, the mine trial sampled was on a site that is considered to have relatively good growing conditions for *C. erythrophyllum* trees (i.e. deep, well drained soils and adequate water with only moderate sulphate concentrations), and no significant differences in the phenolic profiles were observed when compared to natural populations.

Despite the large number of reports on food safety, in practice, effective prevention of contamination of foods and feeds by *L. monocytogenes* and *C. albicans* is lacking (Sandasi et al., 2008). This is the first study where extracts of *C. erythrophyllum* have been tested for their potential value as antimicrobial agents for the control of *Listeria* and *Candida* biofilms. Application of leaf-extracts against two food-borne pathogens resulted in a general trend of increasing the percentage inhibition, while simultaneously enhancing the metabolic activity of the biofilm. This is an indication that under such conditions, the pathogens are not able to establish themselves and are therefore easier to control. Reports by Jin et al. (2004) with other microbial biofilms showed similar results. According to Matheron (2001), active compounds present in the plant extracts are able to disrupt the integrity of the microbial cell wall, leading to growth inhibition of the microbial population. As, Cr and Ni are known to have significant antimicrobial properties and have been used in pesticides for centuries, whereas As, Ni, Cr and U (amongst other metals and radionuclide) are known to be present in Witwatersrand gold and uranium tailings and/or seepage (McCarthy and Venter, 2006; Tutu et al., 2008). The presence of uranium has resulted in public and NGO concern regarding the safety of drinking water and edible crops in the vicinity of these mines in South Africa (M. Liefferlink, Friends Foundation NGO, pers. comm., 2008). The four elements assessed with below detection limits (1–10 ng/L) in the leaves of *C. erythrophyllum* in this study. A more comprehensive study is being performed to determine metal and radiological safety of *C. erythrophyllum* and other species at different phenological stages and under different site conditions (Weiersbye and Cukrowska, 2008), and better understand the mechanisms of biofilm inhibition involved. The importance of conducting studies on the non-target effects of such an extract will be compulsory if any product is to be registered.

5 Conclusions

This is the first report focusing on the biochemical and inhibitory effects of extracts of *C. erythrophyllum* on the growth and development of *L. monocytogenes* and *C. albicans* biofilms. The phenolic profiles of samples from mine tailings and surrounding areas did not differ significantly from those of natural populations, indicating that the antimicrobial properties of the tree extracts could be ascribed to the involvement of ubiquitous secondary metabolites, and not to the presence of site-specific contaminants in leaves or their effects on plant metabolic compounds. All extracts of both mine and natural populations were able to significantly inhibit biofilm formation in the case of *C. albicans*. This suggests that leaf extracts of *C. erythrophyllum* may be considered as potential candidates to protect foods and feeds from pathogens. If this study confirms that leaves and extracts from *C. erythrophyllum* trees grown on mine sites are radiologically and toxicologically safe then, woodlands of this species would be suitable for use, thereby creating job opportunities for local communities.

Acknowledgements

The authors would like to thank AngloGold Ashanti Ltd South Africa Region, the National Research Foundation of South Africa and the Department of Trade and Industry of South Africa (THRIP) and the University of the Witwatersrand, Johannesburg for funding, Ben Oageng and Jacob Mahlangu for field assistance, and Allan Hall for assistance with the microscopy work.

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