Antimicrobial Compounds from *Coleonema album* (Rutaceae)

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Coleonema album, a member of the South African fynbos biome, was evaluated for its antimicrobial activity associated with its secondary metabolites. Ethanol- and acetone-based extracts obtained from plants from two different geographical areas were analyzed. A bioassay-guided fractionation methodology was followed for rapid and effective screening for the presence of bioactive compounds. The TLC-bioautographic method, used to screen the plant extracts for antimicrobial activity and localization of the active compounds, indicated the presence of a number of inhibitory compounds with activity against the microorganisms (*E. coli, B. subtilis, E. faecalis, P. aeruginosa, S. aureus, M. smegmatis, M. tuberculosis, C. albicans, C. cucumerinum*) tested. Evaluation of the inhibitory strength of each extract by the serial microdilution assay indicated that the *C. album* extracts inhibited effectively all the microorganisms, with the minimum inhibitory concentrations in the low mg ml^{-1} range. Identification and structural information of the bioactive components were obtained by a combination of preparative TLC and LC-MS. It revealed the presence of coumarin aglycones which were responsible for the observed antimicrobial activities. The results of this study indicate that *C. album* possesses strong antimicrobial activity against a wide range of microorganisms that warrants further investigation into the use of the extracts or their active constituents as a potential source for novel drugs.

Key words: Coleonema album, Antimicrobial, Coumarins

Introduction

The development of safe and effective antimicrobial drugs has revolutionized medicine in the last forty years, so that morbidity and mortality from microbial diseases have been drastically reduced by modern chemotherapy. However, the misuse, over-prescription and abuse of antibiotics has allowed resistant strains of bacteria to develop. Infectious diseases are still the world's leading cause of premature deaths, killing almost 50,000 people every day (Bax *et al.*, 2000; Carey, 2004). The current rates of resistance and cross-resistance development to all available classes of antibiotics agents has necessitated the search for new antimicrobial substances, with novel antimicrobial mechanisms.

Older antibiotic identification strategies have not identified additional useful classes of antimicrobials in recent years. As a result, new sources and ways to identify new bacterial targets for the discovery of novel inhibitors are of high priority (Barrett and Barrett, 2003). Various secondary metabolites have been reported to form part of the plant's chemical defense strategy against pathogen attack. From numerous studies it is evident that these antimicrobial agents from higher plants are plentiful and that there is a large amount of unexploited natural sources of antimicrobial compounds in higher plants (Mitscher *et al.*, 1987).

These defense chemicals not only have an obvious selective and survival value for the producing plants, but if detected and isolated, can also have a finite potential for use in the treatment of human and agricultural infections (Mitscher *et al.*, 1987). Because the toxicity of many phytoalexins appears to be rather unspecific, they are often toxic to a wide variety of microorganisms and can therefore also be potentially effective against human patho-

gens (Grayer and Kokubun, 2001). According to Eloff (1998), the possibility exists that these natural antimicrobial components in plants can inhibit the growth of bacteria by means of unknown mechanisms, different to that of known antibiotics. The ultimate solution to emerging resistance is thus to provide new agents, with novel mechanisms, unaffected by developing/emerging resistance mechanisms. It is therefore expected that extracts, showing target sites other than those used by antibiotics, might be active against multiple-drug resistant microbial pathogens. Bioassay-guided research could thus reveal new, renewable and less expensive compounds in these plants.

Antimicrobial properties of medicinal plants from Southern Africa are increasingly being reported. A wide variety of higher plants have been investigated for the presence of antibacterial (Rabe and Van Staden, 1997; Kelmanson *et al.*, 2000; Eloff, 2001; Kotze and Eloff, 2002), antifungal (Afolayan *et al.*, 2002; Motsei *et al.*, 2003) and antimycobacterial (Taylor, 2003; Seidel and Taylor, 2004) compounds. These studies have resulted in the isolation and characterization of a number of compounds with activity against a range of microbial pathogens, many of which may have a wide application area in the treatment of human and agricultural infections (Mitscher *et al.*, 1987).

As part of the systematic investigation into biological activities associated with South Africa's indigenous plant biome, *Coleonema album*, a member of the Rutaceae and related to wild 'buchu' (Gray *et al.*, 1987), was evaluated for its antimicrobial activity. *C. album*, also known as the Cape-may or confetti bush, is a heath-like shrub covered with clusters of tiny white flowers and fine pleasant smelling aromatic leaves. The main population centre is the Cape peninsula, although it is also widely cultivated in gardens across South Africa (Goldblatt and Manning, 2002). Although *C. album* and *C. pulchellum* have previously been investigated for their antibacterial and antifungal activity, relatively few reports exist on the biological activities of these species (Brader *et al.*, 1997; Liz-Balchin *et al.*, 2001). The present study investigated the occurrence of natural antimicrobial compounds present in extracts of *Coleonema album* in order to identify the main active compounds present in the plant extracts and to clarify the structural elements conferring antimicrobial activity.

Material and Methods

Chemicals

Silica gel G60 F254 TLC plates and D-(+)-glucose monohydrate were from Merck (Germany), soy broth media and nutrient broth agar medium from Biolab (Merck, Germany), Bacto Middlebrook 7H9 broth and Middlebrook-Dubos 7H9 broth from Difco (USA), *p*-iodonitrotetrazolium violet (INT) and neomycin sulfate were from Sigma (Germany), fluconazole was from Pfizer (Sandton, South Africa) and PANTA from Becton Dickinson (USA).

Extract preparation

C. album plant material was collected from Kirstenbosh, Western Cape fynbos biome (plant group 1), and the Highveld region (plant group 2). Voucher specimens were deposited in the herbarium of the University of Johannesburg, Auckland Park, South Africa. Extracts were prepared according to the method described by Eloff (2000). Briefly, fresh plant material was homogenized (1:7 m/v) using an Ultraturrax homogenizer with 100% acetone (A-1 and A-2) or ethanol (E-1 and E-2) and clarified by centrifugation just before use.

Microorganisms

The following representative strains of Gram-negative bacteria, *Escherichia coli* (ATTC 1175) and *Pseudomonas aeruginosa* (ATTC 9027), Gram-positive bacteria, *Bacillus subtilis* (ATTC 6051) *Staphylococcus aureus* (ATTC) and *Enterococcus faecalis* (ATTC 29212), and fungi, *Candida albicans* (ATTC 10231) and *Cladosporum cucumerinum*, as well as *Mycobacterium smegmatis* were used as test organisms. All organisms were obtained from the Pharmacology Department, University of the Witwatersrand, Johannesburg, South Africa, except for *M. tuberculosis* cultures that were obtained from the Medical Research Council, Pretoria.

Culture of the microorganisms

All the Gram-positive and Gram-negative bacterial strains and *C. albicans,* were stored as frozen stocks in tryptone soy broth (TSB) medium or Bacto Middlebrook 7H9 broth in the case of *M. smegmatis*, supplemented with 50% glycerol (v/v) and kept at −20 °C. Working cultures were main-

tained by growing the microorganisms (all except for *M. tuberculosis* and *M. smegmatis*) on nutrient broth agar medium at 25 °C. *C. cucumerinum* was grown on potato dextrose agar plates.

Before the antibacterial screening, all the bacterial strains and *C. albicans* were incubated overnight in TSB medium, in an incubator at 37 °C equipped with a rotary shaker. The working bacterial inoculum for the TLC-bioautography method was prepared by diluting the organisms (1 ml) with 10 ml of TSB medium, and for the microwell dilution assay, by diluting the organisms (20 μl) with 50 ml TSB medium, just before the experiment was performed.

For preparation of the *Mycobacterium* strains, *M. smegmatis* was incubated overnight in Bacto Middlebrook 7H9 broth. The working bacterial inoculum for the TLC-bioautography method (Hamburger and Cordell, 1987) was prepared by diluting the overnight culture of *M. smegmatis* (2 ml) with 10 ml of Bacto Middlebrook 7H9 broth. For the radiometric method (Lall and Meyer, 1999), *M. tuberculosis* cultures were routinely tested for susceptibility to the primary drugs streptomycin, isoniazid, ethambutol and rifampicin. A sensitive strain of *M. tuberculosis*, H37Rv, was used in the screening procedure. Standard inoculums were prepared in Middlebrook-Dubos 7H9 broth containing 0.5% Tween 80 to obtain a concentration of 1 mg ml^{-1}. A sample of the bacteria was transferred to a sterile screw-capped tube containing 6–8 glass beads and 3–4 ml of Middlebrook-Dubos 7H9 broth. A homogenous suspension was prepared by placing the tube on a vortex mixer for 5 min. After the large particles have settled, the bacterial inoculum was adjusted to McFarland no. 1 turbidity standard by adding more media (Lall and Meyer, 1999).

For the antifungal screening, a fungal spore suspension of *C. cucumerinum* was prepared in a glucose-mineral salt medium containing 7 g KH_2PO_4, 3 g $Na_2HPO_4 \cdot 2H_2O$, 4 g KNO_3, 1 g $MgSO_4 \cdot 7H_2O$ and 1 g NaCl per liter distilled water. The solution was autoclaved at 120 °C for 20 min. Under sterile conditions, 10 ml of a 30% glucose solution were added (Homans and Fuchs, 1970).

Bioautographic antibacterial screening

A simple, direct bioautographic assay on TLC plates (Hamburger and Cordell, 1987) was used to screen for compounds with activity against the microorganisms. 100 μl extract were applied to the TLC plate and developed in a $CHCl_3$/MeOH (19:1 v/v) mobile phase. The developed chromatograms were dried for complete removal of the remaining solvents and the fluorescent compounds detected and marked. The prepared bacterial suspension (5 ml) was applied to the developed TLC plate (10 × 20 cm) with a roller device (glass rod) until just wet. This method allowed an even distribution of the organisms while minimizing airborne microorganisms. Plates were incubated in a clean chamber, lined with wet filter paper, for 8 h at 37 °C. Plates were then sprayed with a 2 mg ml^{-1} solution of INT and incubated overnight in a sealed container for colour development. Antibacterial compounds were identified as clear zones against a red coloured background that indicated bacterial growth. Comparison with a duplicate chromatogram developed under the same conditions and visualization of the separated compounds helped to localize the antibacterial compounds.

Antifungal screening

A bioautography method based on the direct spraying and growing of a fungal spore suspension on a developed TLC chromatogram (Homans and Fuchs, 1970) was used to screen for and identify compounds with antifungal activity present in the plant extracts. 100 μl plant extract were applied to the TLC plate. After development of the chromatogram in a $CHCl_3$/MeOH (19:1 v/v) mobile phase and localization of the UV-absorbing spots, the chromatogram was sprayed with a spore suspension of *C. cucumerinum*, in a glucose-mineral salt medium. The plates were incubated in a clean chamber, lined with wet filter paper, at 25 °C. The bioautogram was examined for fungal growth at regular intervals until the TLC plate was completely covered with a lawn of mycelial growth (after approx. 2 d). Inhibition was observed as reduced or lack of growth, visible as a clear zone against a darker background of mycelial growth.

Quantification of the antimicrobial activity

Quantification of the antibacterial activity of the plant extracts was carried out using the serial plate microdilution assay reported by Eloff (1998). The minimum inhibition concentration (MIC) value was determined for each plant extract that was active in the bioautography screening against the

most sensitive microorganisms. The MIC value was taken as the lowest concentration of the extract that inhibited any visible bacterial growth after 24 h of incubation. From an initial extract concentration of 100 mg ml^{-1} primary extract, doubling dilutions in sterile autoclaved water were prepared and tested at a concentration range from 2.5 mg ml^{-1} to 0.019 mg ml^{-1}. TSB medium (100 μl) was pipetted into each of the wells of the first column of the microtitre plate as a sterility control. The rest of the wells received 100 μl of the bacterial suspension. The antibiotics neomycin sulfate (for all the Gram-negative and Gram-positive bacteria) and fluconazole (for *C. albicans*), tested at a concentration range of 4 μg ml^{-1} to 500 μg ml^{-1}, were included as standards in each assay. Wells containing the organic solvents employed in sample preparation were also included in order to monitor sample sterility and to determine any antibacterial effects of the solvents. The microplates were incubated overnight at 37 °C. As an indicator of bacterial growth, 40 μl of INT, dissolved in sterile water, were added to the microplate wells and again incubated at 37 °C for at least 30 min to ensure adequate colour development. Since the colourless tetrazolium salt is reduced to a red-coloured product by biologically active organisms, the inhibition of growth could be visually assessed in the wells where the solution remained clear after incubation with INT. A clear solution or a definite decrease in colour reaction indicated inhibition of growth. All data represent at least three replicate experiments per microorganism. The resultant MIC values were determined as the mean of these replicate experiments.

Antimycobacterial screening

The TLC-bioautography method was firstly used to screen the plant extracts for antimycobacterial activity against the fast growing, non-pathogenic *Mycobacterium smegmatis* species. The same basic procedure was followed as previously outlined. Before testing, *M. smegmatis* was incubated overnight in Bacto Middlebrook 7H9 broth. The working bacterial inoculum for the TLC-bioautography method was prepared by diluting the overnight culture of *M. smegmatis* (2 ml) with 10 ml of Bacto Middlebrook 7H9 broth.

The extracts that showed promising inhibitory activity against *M. smegmatis* were then screened for activity against a drug-sensitive strain of *Mycobacterium tuberculosis* (H37Rv) by employing a

rapid radiometric method described by Lall and Meyer (1999). Isoniazid and rifampicin were used as drug controls. Dried powdered extract was dissolved in dimethylsulfoxide at a stock concentration of 500 mg ml^{-1}. Plant extracts were analyzed for activity at the following concentrations: 1.0, 0.5 and 0.1 mg ml^{-1}. The inhibitory activity of the tested compounds on the growth of *M. tuberculosis* was expressed as a MIC value, which was defined as the lowest concentration of drug that inhibited more than 99% of the bacterial population (Lall and Meyer, 1999).

Partial characterization and fractionation of the extracted plant material by TLC

Silica gel G60 F$_{254}$ A1 sheet glass and aluminium plates were used for the chromatographic separation of the extracted plant material. The chromatograms were developed in a glass tank saturated with solvent vapour, using a mobile phase of CHCl$_3$/MeOH (19:1 or 16:4 v/v). Plates were viewed under UV (254 and 366 nm) light to locate the UV active compounds.

Isolation of the antimicrobial compounds

Preparative TLC was used for the initial fractionation of the crude extracts and isolation of the antimicrobial compounds. The developed chromatogram was subjected to the bioautographic procedure as described and the antibacterial and antifungal compounds were identified. Preparative, reference TLC plates, run under the same conditions, were used to locate the active spots. The bands containing the antibacterial and antifungal compounds were marked and the silica gel scraped off. The active compounds were repeatedly eluted from the silica gel by HPLC grade acetone. The samples were then centrifuged (13,000 × *g*, 15 min) to remove the silica gel and the fractions combined. The supernatants were dried under vacuum overnight and redissolved in a small volume of acetone and subjected to LC-MS for structure determination.

HPLC and LC-MS analysis

Chromatographic separation was performed on an HPLC system (Shimadzu) that consisted of a SCL 10AD VR Shimadzu system controller; LC-10AT VP Shimadzu binary gradient solvent delivery system; SIL 10AD VP Shimadzu auto-injector and a SPD-M10A VP photodiode array detector.

Data were interpreted by Shimadzu Class VP version 6 software. 10 μl of the fractions were injected directly onto the Xterra RP18 5 μm RP-HPLC column (3.9 × 150 mm; Waters). Chromatographic separation was obtained by gradient elution using a HPLC grade water and acetonitrile (Riedel de Haën) gradient as follows: 5% acetonitrile for 5 min, increased to 95% over a period of 35 min, kept there for 5 min and then decreased to 5% over a period of 5 min.

The active compounds isolated by preparative TLC were subjected to mass spectrometric analysis for structure determination. LC-MS analysis was carried out on a HPLC system (Waters 2690) equipped with both a 996 photodiode array detector and a tetra beam mass selector detector operated in the electron impact mode (maximum mass *m/z* 1000). The National Institute of Standards and Technology (NIST/EPA/NIH 2004; http://www.nist.gov) mass spectral database was used for identification.

Statistical analyses

Statistical analysis of results was performed on SPSS for Windows, version 11.0.11 (2001). ANOVA (analysis of variance) was used to determine the significant differences between the results obtained in each experiment. Each experiment, where possible, was performed at least three times, with each sample tested in triplicate. All results with $p \leq 0.05$ were considered significant.

Results and Discussion

The method currently available for quantifying antimicrobial susceptibility is the Kirby-Bauer broth dilution method in microdilution format. The microdilution method is more efficient, less labour intensive and ideal for screening large numbers of test samples. The colourless tetrazolium salt, used as an indicator of microbial growth, acts as an electron acceptor and is reduced to a coloured formazan-product by biologically active organisms. The relationship between the amount of reduced salt/colour change and bacterial concentration/number is well established (Tengerdy *et al.*, 1967; Gabrielson *et al.*, 2002). In an actively growing culture, the reducing activity is proportional to the number of bacteria in the culture; and antimicrobial activity was noted in the wells that showed a definite decrease in colour formation. Although chlorophyll was present in the plant extracts, it was generally easy to differentiate between the red-coloured formazan and the green colour of the plant extracts, especially when growth only took place at lower concentrations where much of the green colour was diluted out. The results suggested that several compounds were acting as antimicrobial agents in *C. album* extracts and supported further investigation into the antimicrobial potency of the crude extracts.

All the extracts had significant antibacterial activity according to the criterion of 8.0 mg ml^{-1} chosen by Fabry *et al.* (1998), with all the MIC values in the 0.2–2.0 mg ml^{-1} range (Table I) and no significant difference between the two plant groups (1 and 2) tested (*p* value). The extracts displayed a varying degree of activity against the Gram-positive as well as the Gram-negative bacteria. The growth inhibition of test microorganisms ranged from 0.156 mg ml^{-1} to 2.083 mg ml^{-1} with

Table I. The minimum inhibitory concentrations (mg ml^{-1} ± SD), of the acetone and ethanol extracts from *C. album* with antibacterial activity against the bacterial and yeast isolates tested.

Extract/ test compound	*E. coli*	*P. aeruginosa*	*E. faecalis*	*S. aureus*	*C. albicans*	*M. tuber-culosis**
E-1	0.234 ± 0.039	0.556 ± 0.01	0.182 ± 0.022	0.729 ± 0.090	0.234 ± 0.039	
E-2	0.468 ± 0.078	0.182 ± 0.022	0.208 ± 0.045	0.729 ± 0.090	0.195 ± 0.051	
A-1	0.260 ± 0.022	0.182 ± 0.022	1.041 ± 0.152	2.083 ± 0.360	0.299 ± 0.088	5.0
A-2	0.312 ± 0.078	0.156 ± 0.001	1.145 ± 0.090	1.666 ± 0.360	0.338 ± 0.059	1.0
Neomycin	0.006 ± 0.001	0.026 ± 0.004	0.031 ± 0.001	0.010 ± 0.002		
Fluconazole					0.083 ± 0.018	
Isoniazid						$0.062 \cdot 10^{-3}$
Rifampicin						$0.125 \cdot 10^{-3}$

MIC values were taken as the lowest concentration of the extract that inhibited any visible bacterial growth after 24 h of incubation.
* MIC$_{99}$ defined as the lowest concentration of drug that inhibited more than 99% of the bacterial population.
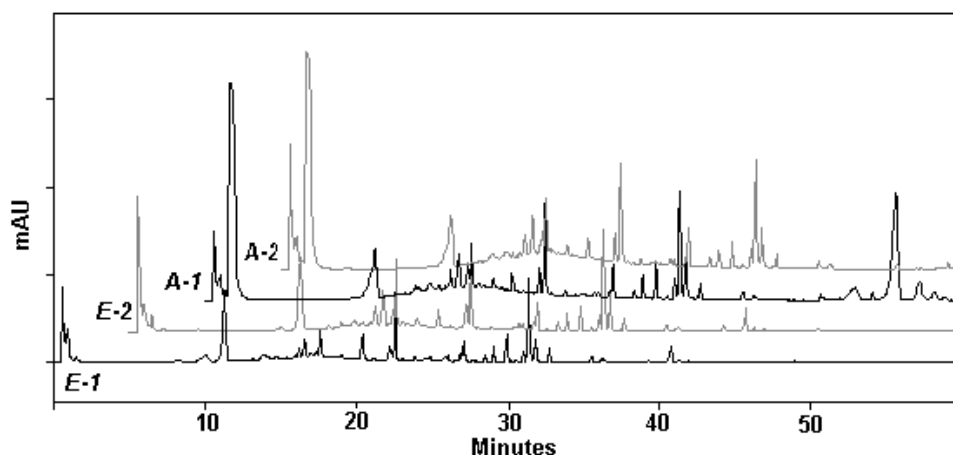
Fig. 1. RP-HPLC chromatograms indicating the different extracted constituents from the two *Coleonema album* plant groups (1 and 2) and solvent extracts (A and E). Separation of extracts was obtained by acetonitrile gradient elution (5%–95%) with a flow rate of 1 ml min^{-1} and monitoring of the elution by a diode array detector over the wavelength range 200–600 nm.

the lowest MIC values against the Gram-negative bacteria (*E. coli* and *P. aeruginosa*).

Variation in the inhibitory activity against Gram-positive and Gram-negative bacterial can generally be attributed to the cell wall composition differences, with the Gram-negative bacteria being much more complex and less permeable to drugs (Atlas, 1997). The significant inhibition of the Gram-negative bacteria is therefore of great importance, due to their resistance to antibiotics and the fact that several Gram-negative bacteria cause serious infections in humans and are amongst the most difficult to treat by conventional antibiotics (Salie *et al.*, 1996).

C. album extracts that showed promising activity against *M. smegmatis* were subsequently analyzed for their activity against a drug-sensitive strain of *M. tuberculosis* by the radiometric method (Siddiqi *et al.*, 1981; Lall and Meyer, 1999). The ability of the extracts to inhibit the growth of *M. tuberculosis* bacilli was assessed by determining the lowest concentration of drug that inhibited more than 99% of the bacterial population (MIC$_{99}$). The A-1 extract exhibited moderate inhibition while A-2 had a stronger inhibitory activity, with a MIC value of 1 mg ml^{-1} (Table I) that is comparable to extracts with strong activity found in the literature (Lall and Meyer, 1999).

Due to the lack of knowledge about the specific chemical composition of the crude plant extracts, TLC and HPLC served as qualitative methods that effectively characterized and documented the multi-component plant constituents as a fingerprint. TLC separation of the extracted plant material revealed a highly complex crude extract, containing a large number of plant constituents with a wide range of polarities. RP-HPLC separation of the crude plant extracts adequately separated the extracted plant constituents and effectively characterized and documented the multi-component constituents as a metabolomic fingerprint. Upon comparison of the HPLC chromatographic profiles (Fig. 1) of the plant material obtained from the Cape and Highveld, it was observed that a similar complex secondary metabolite profile was present in both, with more metabolites present in the acetone preparations compared to the ethanol extracts. Due to the complexity of the chemical profiles it was difficult to interpret the results and only qualitative and quantitative differences were noted.

The direct bioautography assay, as a method to identify and localize compounds with antibacterial activity in crude plant extracts, has found widespread application in the search for new antimicrobials. This assay has the advantage of being quick, easy to perform and relatively cheap, requiring no sophisticated infrastructure, only requiring a small amount of the test compound, with high sample throughput and the results are easy to interpret. It is suitable for testing of all compounds, that can be separated by TLC, against or-

Table II. Antibacterial compounds exhibiting antibacterial activity were identified by LC-MS. The compounds were numbered according to the inhibition zone they were isolated from and by their R_f-values and retention times observed during TLC and RP-HPLC, respectively.

Compound	Retention time [min]	R_f	Chemical name
1	33.99	0.84	5,5′-(Tetrahydro-1H,3H-furo[3,4c]-furan-1,4-diyl)bis-[1S-(1α.,3a.α,4.β.,6a)]-1,3-benzodioxole, $C_{20}H_{18}O_6$ ($M_r = 354$)
2	25.60	0.78	6-(2,3-Dihydroxy-3-methylbutyl)-7-methoxy-2H-1-benzopyran-2-one (dihydroxydihydrosuberosin), $C_{15}H_{18}O_5$ ($M_r = 278$)
3	35.58	0.76	6-(3,3-Dimethyl-2-butenyl)-7-methoxy- 2H-1-benzopyran-2-one, $C_{15}H_{16}O_3$ ($M_r = 244$)
4	31.35	0.66	6-[(3,3-Dimethyloxiranyl)methyl]-7-methoxy-2H-1-benzopyran-2-one, $C_{15}H_{16}O_4$ ($M_r = 260$)
5	31.63	0.54	6,7-Dihydroxy-2H-1-benzopyran-2-one, $C_9H_6O_4$ ($M_r = 178$)
6	33.40	0.53	6,7-Dihydro-5-hydroxy-4,8,8-trimethyl-2H,8H-benzo[1,2-b:5,4-b′]dipyran-2-one, $C_{15}H_{16}O_4$ ($M_r = 260$)
7	27.3	0.51	2-(1-Hydroxy-1-methylethyl)-2,3-dihydrofuro[3,2-g]chromen-7-one, $C_{14}H_{14}O_4$ ($M_r = 246$)
8	30.51	0.50	6-(3-Methyl-2-oxobutyl)-7-methoxy-2H-1-benzopyran-2-one, $C_{15}H_{16}O_4$ ($M_r = 260$)
9	31.56	0.50	2,2-Dimethyl-pyrano(3,2-c)(1)benzopyran-5-one, $C_{14}H_{14}O_3$ ($M_r = 230$)
10	27.54	0.49	9,10-Dihydro-9-hydroxy-8,8-dimethyl-2H,8H-benzo[1,2-b:3,4-b′]dipyran-2-one, $C_{14}H_{14}O_4$ ($M_r = 246$)
11	42.60	0.48	n-Hexadecanoic acid, $C_{16}H_{32}O_2$ ($M_r = 256$)

ganisms that grow directly on the TLC plate's surface.

Metabolites with antimicrobial activity were identified as clear inhibition zones on a red-coloured background that indicated bacterial growth. Two inhibition zones ($R_f = 0.45$, 0.6) resulting from compounds present in all the extracts were found to have inhibitory activity against both the Gram-negative bacteria (*P. aeruginosa* and *E. coli*) and Gram-positive bacteria (*S. aureus* and *E. faecalis*). A third zone ($R_f = 0.7$) of activity was noted against *P. aeruginosa* and *S. aureus*.

Initial screening against *Candida albicans* indicated two inhibition zones ($R_f = 0.60$ and 0.82) The effective inhibition of *C. albicans* is of significant importance due to the frequent manifestation of candidiasis diagnosed especially in immunocompromised hosts. In addition, *Candida* species are not very susceptible to antifungal agents and the presence of several resistance mechanisms, such as efflux pumps that take antifungals outside the cell and overexpression of the antifungal targets, have been reported in several *Candida* species (Eggiman *et al.*, 2003).

In an attempt to identify compounds with possible antimycobacterial activity, the plant extracts were screened for inhibitory activity against *M.*

smegmatis, a relatively fast growing, non-pathogenic bacterium. *M. smegmatis* has similar drug sensitivity profiles to *M. tuberculosis*, a slow growing parasitic organism that is difficult to manipulate and an accepted target for selection of antimycobacterial compounds (Seidel and Taylor, 2004). The initial screening indicated at least five compounds with inhibitory activity present in inhibition zones with $R_f = 0.28$, 0.40, 0.47, 0.58, 0.71, 0.82. These results support further investigation of the possible inhibitory activity of *C. album* plant extracts against *M. tuberculosis*. The inhibitory activity of *C. album* extracts against two mycobacteria strains is very significant, since mycobacteria species have been found to be highly resistant to a number of antibacterial drugs. Mycobacteria have a cell wall with high lipid content and an additional mycolic acid layer that forms a waxy outer protective barrier responsible for the high resistance observed against antibiotics. *C. album* may therefore represent a resource for new drugs that can be used in the treatment of tuberculosis, especially resistant forms of the disease.

Two inhibition zones ($R_f = 0.66$, 0.78) were identified from the bioautographic screen against *Cladosporum cucumerinum* using a spore suspension of the test fungus, indicating inhibition of spore germination and mycelial growth.
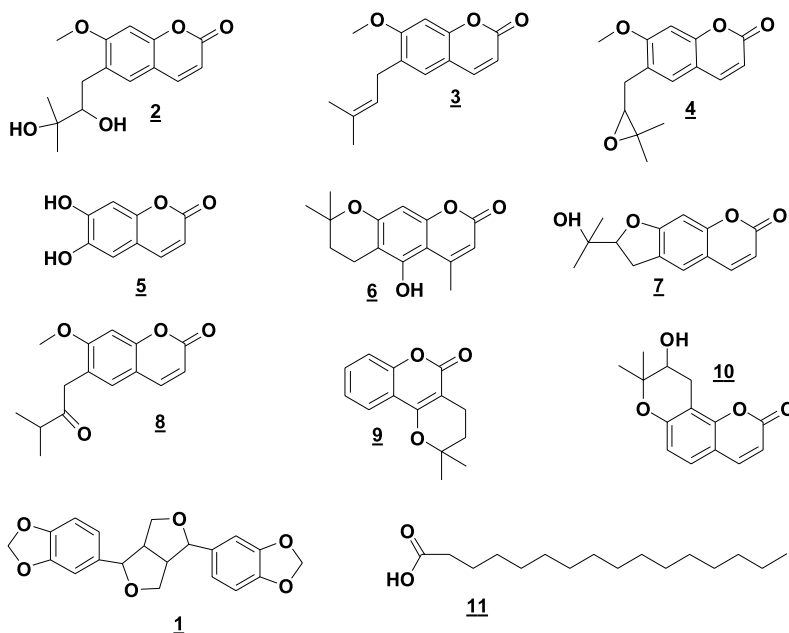
Scheme 1. Preliminary LC-MS identification of secondary metabolites present in the antibacterial zones from acetone extract (A-2) of C. album.

To obtain information on the active components, the structures of the compounds present in the inhibition zones and presumably responsible for the antibacterial and antifungal activities were determined by LC-MS following preparative TLC. For some of the peaks the acquired mass spectra showed sufficient agreement with one of the library entries and thus allowed the assignment of a predicted structure, within a certain percentage of probability. The structures for the compounds that showed an 80% or more agreement with the library entries are indicated in Scheme 1 and Table II.

Most of the compounds identified (**2 – 10**) had a coumarin core, with various substitutions and conjugations at different sites in the basic structure being responsible for the extreme variability observed in the obtained spectrum of compounds. Coumarins comprise a very large class of phenolic substances found in plants. About 1300 different coumarins have been identified form natural sources, principally as secondary metabolites in green plants. Coumarins are one of the classes of secondary metabolites which are thought to form part of a defense strategy against microbial attack. Investigation of the pharmacological properties of the coumarins have repeatedly indicated that they dis-

play potent and relevant pharmacological activities that are structure-dependent, while at the same time appearing to lack toxicity in mammalian systems (Lake, 1999). The antimicrobial activity of various coumarins and furanocoumarins has been investigated (Kayser and Kolodziej, 1999; Laurin *et al.*, 1999; Okunade *et al.*, 2004) and it is concluded that they possess distinct antimicrobial activity against not only Gram-positive and Gram-negative bacteria, but also several *Mycobacterium* species. The investigation of a series of coumarins and isocoumarins with different substituents revealed that the inhibitory activity was structure-dependent, with certain substitution patterns enhancing the antibacterial activity (Kayser and Kolodziej, 1999; Devienne *et al.*, 2004). Coumarins with a methoxy function at C-7, additional phenolic groups (as reflected by the highly oxygenated coumarins) and, if present, an OH group at either the C-6 or C-8 position were highly effective against the spectrum of Gram-positive and Gram-negative bacteria tested. They also reported that catechol functions (*ortho*-dihydroxy substitutions) have been shown to be toxic to microorganisms and that the number of hydroxy groups on the phenol group are thought to be related to their toxicity to microorganisms with evidence that in-

creasing hydroxylation results in an increase of antimicrobial activity (Devienne *et al.*, 2004).

The suggested mechanism responsible for the phenolic toxicity includes enzyme inhibition by the oxidized groups or by non-specific action with proteins (Kayser and Kolodziej, 1999). It has also been suggested that the simplicity of aromatic substitution and avoidance of bulky side-chains in coumarins aid in the penetration of the molecule through the bacterial cell wall. Therefore, the simple structure, in addition to the lipophilic character of the isolated antimicrobial compounds that aid in the passive diffusion of the compound over the bacterial cell wall, can be an explanation for the higher antimicrobial potency against Gram-negative bacteria exhibited by the *C. album* extracts.

In addition to the coumarins, one of the inhibitory compounds was identified as isosesamin (**1**), a simple lignan that has previously been isolated from *Acronychia mulleri* and reported to have antitubercular activity (DNP, CAS 133-03-9). This compound could therefore be one of the extract constituents responsible for the antimycobacterial activity observed. *n*-Hexadecanoic acid (**11**) has been identified as one of the compounds that possess bacteriostatic or fungistatic properties against mammalian skin pathogens or other microorganisms. It has previously been reported to possess antibacterial activity against *S. aureus* and *Tricho-* *phyton mentagrophytes*, a zoophilic skin fungus responsible for athlete's foot (Wood and Weldon, 2002).

Conclusion

The emergence of microbial resistance and the decrease in effectiveness of currently available antimicrobial agents have spurred an increased effort to search for new and alternative antimicrobial substances with novel inhibitory mechanisms. Many species of the Rutaceae have been used in ethnic medicine and it would seem certain that coumarins are implicated in many of their activities (Gray and Waterman, 1978). The *C. album* extracts and the compounds identified displayed potent and relevant pharmacological activities, with considerable antibacterial, antimycobacterial and antifungal activity, against several strains responsible for important infectious diseases. The results obtained show that the compounds from *C. album* could be of pharmaceutical interest for therapeutic application as complementary antibacterial and antifungal agents in infectious diseases.

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