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To cite this article: D. E. M. Saravanakumar, P. I. Folb, B. W. Campbell & P. Smith (2008) Antimycobacterial Activity of the Red Alga *Polysiphonia virgata*., Pharmaceutical Biology, 46:4, 254-260, DOI: [10.1080/13880200701739413](https://doi.org/10.1080/13880200701739413)

To link to this article: <https://doi.org/10.1080/13880200701739413>



Published online: 07 Oct 2008.



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Antimycobacterial Activity of the Red Alga *Polysiphonia virgata*

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Abstract

The red marine alga (*Polysiphonia virgata* C. Agardh; Rhodomelaceae) was investigated for antimycobacterial activity. Bioassay-guided fractionation of the dichloromethane algal extract using direct bioautography resulted in isolation of a mixture of long-chain fatty acids, namely oleic acid, linoleic acid, lauric acid, and myristic acid as the major antimycobacterial compounds. Oleic acid showed the greatest inhibition of the growth of *Mycobacterium smegmatis* with a minimum inhibitory quantity (MIQ) of 0.8 µg; linoleic acid and lauric acid had MIQ values of 1.56 and 3.125 µg, respectively. Stearic acid, palmitic acid, and myristic acid did not inhibit the growth of *M. smegmatis*. Using the Bactec-460 radiometric method, oleic acid showed 100% inhibition of the growth of *Mycobacterium tuberculosis* at a minimum inhibitory concentrations (MIC) of 25 µg/mL; lauric acid, myristic acid, and linoleic acid all showed 100% inhibition at MIC values of 50 µg/mL. Myristic acid and lauric acid showed 90% and 76% inhibition at 50 µg/mL. Linoleic acid showed moderate inhibition of the growth of a clinical strain of multidrug-resistant *M. tuberculosis* 50 µg/mL.

Keywords: Antimycobacterial, marine algae, marine natural products, *Mycobacterium tuberculosis*, *Polysiphonia virgata*, seaweeds, tuberculosis.

Introduction

South Africa is among the 22 countries that bear 80% of the global tuberculosis (TB) case burden (Raviglione, 2002). The 2004 WHO estimates showed that the population of 44,759,187 had a TB case incidence of 558/100,000

population, with 60% of adult cases being co-infected with HIV (WHO Report, 2004). In the treatment of HIV infection, protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) form an integral part of the multi-drug regimens in AIDS patients (Fujiwara et al., 2000). However, these medications have serious consequences for the treatment of tuberculosis. They interact with rifamycins, the most important chemotherapeutic class of drugs in the treatment of tuberculosis, and particularly with rifampicin. Current tuberculosis drugs must be taken during at least 6 months and in combination to minimize the chances of multidrug-resistant tuberculosis (MDR-TB) developing (Kaufmann, 2000). The adverse reactions experienced by many patients lead to poor adherence and treatment failure (O'Brien, 2001). The drugs used to treat MDR-TB are expensive and more toxic than the first-line drugs (O'Brien, 2001). New TB-drug treatments are needed that would shorten the treatment period, be effective against MDR-TB, eliminate latent TB infection (O'Brien, 2001), and be affordable to all.

Marine natural products are increasingly receiving attention in the search for new and effective medicinal compounds. This is reflected in the many investigations into their bioactivities such as antiangiogenic (Koyanagi et al., 2003), antitumor (Hiroishi et al., 2001; Zhou et al., 2004), anticoagulant (Chevolot et al., 1999), immunomodulating (Shan et al., 1999), antioxidant (Ahn et al., 2004; Zhang et al., 2004), antiviral (Boyd et al., 1997; Carlucci et al., 1999; Huheihel et al., 2002; Ahn et al., 2002), anti-inflammatory (Jiang et al., 1999; Okai & Higashi-Okai, 1997), antimicrobial (Donia & Hamann, 2003; Haefner, 2003), hemagglutination (Kakita et al., 1999), and more.

Antimycobacterial compounds have been isolated from marine organisms such as sponges, gorgonia, and soft

Accepted: September 4, 2007.

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corals (Donia & Hamann, 2003). König et al. (2000) screened 39 marine-derived natural products for antimycobacterial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium*. The compounds represented various structural classes including terpenes, aliphatics, aromatics, alkaloids, and sterols. Fifteen compounds were derived from red algae, one from a brown alga, three from cyanobacteria, 10 from sponges, one from a soft coral, three from a gorgonian, three from lichens, and three were semisynthetic. Seven of the sponge-derived compounds were the most active against *M. tuberculosis*, with minimum inhibitory concentrations (MIC) values less than 10 µg/mL. The compounds isolated from the red algae *Laurencia obtusa* Hudson (Rhodomelaceae), *Laurencia flexilis* Setchell (Rhodomelaceae), *L. rigida* J. Agardh (Rhodophyceae), and *Plocamium cartilagineum* Linnaeus (Plocamiaceae) had antimycobacterial activity ranging from MIC values of 16 to 64 µg/mL. South Africa is home to a diverse seaweed population (Silva et al., 1996), and it is estimated that there are about 800 species (Stegenga et al., 1997). Red seaweeds constitute most of the 800 species, and more red than brown and green seaweeds are potentially harvestable and/or economically important (Critchley et al., 1998). Considering the amount of seaweeds available along the South African coast, there has been little investigation into their pharmaceutical potential. Vlachos et al. (1997) screened 56 seaweeds for antimicrobial activity against 16 microorganisms. The brown alga, *Zonaria subarticulata* Lamouroux (Dictyotaceae), showed the greatest inhibition against the microorganisms tested. Stirk et al. (1996) tested crude algal extracts of 35 seaweed species for inhibitory activity to prostaglandin synthesis and found species from the red, brown, and green seaweeds with potential novel anti-inflammatory activity. Stirk and Van Staden (1997) screened 20 algal species for cytokinin-like activity and found it present in most of the extracts.

In this investigation, *Polysiphonia virgata* C. Agardh (Rhodomelaceae) was screened for antimycobacterial activity. This dark-red to blackish plant grows to 30 cm or more. It often exists within the tissues of other plants, but it is not necessarily parasitic; for example, it may be an epiphyte on the kelp (*Ecklonia maxima* Osbeck; Alariaceae) (Stegenga et al., 1997). *Polysiphonia virgata* C. Agardh is endemic to southern Africa and is found from the coast of Namibia to Brandfontein near Cape Agulhas. Other species of the genus *Polysiphonia* such as *Polysiphonia furcellata* C. Agardh (Rhodomelaceae) (Caccamese et al., 1985), *P. lanosa* Linnaeus (Rhodomelaceae), *P. elongata* Hudson (Rhodomelaceae), *P. nigra* Hudson (Rhodomelaceae), *P. nigrescens* Hudson (Rhodomelaceae), *P. urceolata* Lightfoot ex Dillwyn (Rhodomelaceae) (Hornsey & Hide, 1976), and *P. virgata* C. Agardh (Vlachos et al., 1997) contain antimicrobial compounds, and *P. hendryi* Gardner (Rhodomelaceae) has antiviral compounds (Kim et al., 1997).

Materials and Methods

Sample collection

A sample of *P. virgata* was collected at Sweet Water, Western Cape coast, on 4 July 2005. The alga was collected and verified by Mark Rothman and Chris Boothroyd from the Seaweed Research Unit of the Department of Environmental Affairs and Tourism: Marine and Coastal Management, which is based in the Botany Department at the University of Cape Town. A voucher specimen of the seaweed (BH 126936) was deposited at the Bolus Herbarium at the Botany Department of the University of Cape Town (UCT).

Extraction

The seaweed sample was placed in plastic bags and kept cool on ice. In the laboratory, the alga was washed with distilled water to remove surface salts, sand, and epiphytes. The seaweed was dabbed with paper towels to remove excess water, cut into small pieces, and placed in an oven at 50°C until completely dry (782.50 g). The dried material was milled using a blender and soaked in cold methanol for 2 h at 4°C. The methanol was filtered and the algal material steeped in 2 L dichloromethane (DCM) overnight at 4°C. The cold DCM was filtered and replaced with more DCM (5 × 2000 mL) at room temperature. The extracts were concentrated under vacuum using a Büchi Rotavapor R-205, (Switzerland) at 50°C.

Preparative fractionation using flash chromatography

Flash silica gel 60 chromatography of the resultant sample material (7.074 g) was done with hexane and increasing proportions of ethyl acetate (EtOAc) as eluant, resulting in 44 main fractions. Fraction 45 was obtained by washing the silica column with absolute ethanol until the eluant became colorless. The fractions were concentrated under vacuum using a Büchi Rotavapor R-205 at 50°C and dried completely under a stream of nitrogen.

Centrifugal preparative layer chromatography

Centrifugal preparative layer chromatography was performed using a Chromatotron model 8924 from Harrison Research Inc. (Palo Alto, CA, USA). The sample was fractionated on the Chromatotron using silica gel 60 PF-254 coated plates (2 or 1 mm thickness) and hexane-DCM-MeOH (7:2:1) as solvent. The polarity of the solvent system was gradually increased to remove the more polar fractions until only MeOH was effective. The less polar fractions were further fractionated using hexane-DCM-MeOH (5:3:2) or DCM-MeOH (5:5), and the polar fractions resolved well with either toluene or hexane:DCM:MeOH (7:2:1). In some cases, starting with hexane and introducing more polar solvents enabled the bands to elute from

the silica plate with better separation. The fractions were observed under ultraviolet light at 254 nm and 366 nm.

Gas-liquid chromatography analysis of methyl esters

The sample was treated with methanol HCl, 3 M, to obtain the methyl esters of the lipids present, by refluxing 6 to 10 mg in 5 mL methanol HCl for 2 h. The reaction mixture was evaporated to dryness under vacuum. The fatty acid methyl esters were purified by silica gel column chromatography using a Pasteur pipette and eluting with hexane/ether (8:2 v/v). The solvent was evaporated to dryness and the methyl esters dissolved in hexane and analyzed by gas chromatography–mass spectrometry (GC-MS). The GC-MS analyses were recorded at 70 eV using a Carlo Erba GC 6000 Vega Series (Milan, Italy) equipped with a 40 m × 0.3 mm special performance capillary column (Lexus, PS089-OH) silanol-terminated (95%)-methyl-(5%)-phenylpolysiloxane copolymer stationary phase and He as the carrier gas. Analyses were performed under the following conditions: initial temperature, 130°C; rate of increase, 3°C/min; final temperature, 270°C, held for 10 min.

Direct bioautography

Each fraction (500 µg) was applied to an analytical thin-layer chromatography (TLC) 20 × 20 cm glass-backed plate with a fluorescent indicator and allowed to develop to the middle of the plate (about 9 cm) using hexane:chloroform:methanol (7:2:1) as the mobile phase. This permitted 18 fractions to be evaluated per plate. After development, the solvents were allowed to evaporate completely. Antimycobacterial activity was assessed using a Pasteur Institute strain of *Mycobacterium aurum* and a clinical strain of *Mycobacterium smegmatis*. These mycobacteria were cultured in Middlebrook-7H9 broth. The developed TLC plates were dabbed with the bacterial suspension using sterile cotton balls and incubated in a moist atmosphere at 37°C. Plates with *M. aurum* were incubated for 3 days and *M. smegmatis* for 2 days. At the end of the incubation period, the plates were sprayed with a *p*-iodonitrotetrazolium violet aqueous solution (0.4 mg/mL), which was reduced to a pink color by the mitochondrial enzymes of live organisms. The sprayed plates were incubated for 1 h at 37°C, after which the clear zones were recorded as inhibition zones. The R_f value was calculated as the inhibition zone distance from origin (midpoint) (mm)/solvent front distance from origin (mm). Purified fractions (500 µg) were applied to the plates, but not allowed to develop in a solvent system. Inhibition zones were observed around the bioactive samples, and the zone sizes were measured. The results of the pure compounds were presented in minimum inhibitory quantity (MIQ), which is the minimal quantity of compound applied to the TLC plate that showed inhibition of the microorganisms as done by Moulari et al. (2006). The MIQ

values were determined by applying sample sizes ranging from 500 µg to 0.4 µg to the TLC plates. Ciprofloxacin (12 µL of 1 mg/mL solution, i.e., 12 µg) was used as a drug control.

Radiometric method

The radiometric method used was described by S.H. Siddiqi in the Product and Procedure Manual of the Bactec 460 TB System (1995). The main component of the method is the 7H12 Middlebrook TB medium that contains ^{14}C -labeled substrate (palmitic acid) as a source carbon. As the bacterium grows, it consumes the palmitic acid, with release of $^{14}\text{CO}_2$ into the atmosphere of the sealed vial. The Bactec-460 instrument (Johnston Laboratories, Towson, MD, USA) records the amount of $^{14}\text{CO}_2$ and processes it as a growth index (GI) on the scale 0–999 (Lall & Meyer, 1999).

A rifampicin, isoniazid, and ethambutol sensitive *M. tuberculosis* H37Rv strain was used in this investigation (supplied by the Microbiology Laboratory of the National Health Laboratory Services, based at Groote Schuur Hospital, Cape Town). The bacterium was grown on Löwenstein-Jensen slants or in Bacto Middlebrook 7H9 liquid medium. The same laboratory supplied a multidrug-resistant clinical isolate of *M. tuberculosis* that was resistant to rifampicin, isoniazid, and ethambutol. The sensitive *M. tuberculosis* H37Rv strain was inoculated into a Bactec vial containing 4 mL 7H12 Middlebrook TB medium. When the growth index of the bacterium vial reached values between 300 and 500, the broth culture was considered ready to be introduced into the test and control vials, each of which contained 4 mL 7H12 Middlebrook TB medium. The test vials contained 0.1 mL of the broth culture and fatty acids sample solution while the two positive control vials contained 0.1 mL of the broth culture and 0.1 µg/mL isoniazid and 2.0 µg/mL rifampicin, respectively. Two negative controls were used: one with 0.1 mL broth culture and the other was prepared by adding 0.1 mL of 1:100 dilution of the broth culture. The dilution was prepared in special diluting fluid. Test stock solutions were prepared in 12.5% ethanol and Bacto Middlebrook 7H9 broth. The test vials received 100 µL of each fatty acid stock solution resulting in a 40-fold dilution and final concentrations (µg/mL) of 50, 25, 12.5, 6.25, and 3.125. All the vials were incubated at 37°C and the growth index (GI) determined daily at the same time over a period of 4 to 12 days until the GI of the 1:100 control vial reached 30 or more. The sample vials with GI values below or equal to that of the 1:100 control vials on the final day of the assay were considered active against *M. tuberculosis*. The percent inhibition was calculated as (König et al., 2000):

$$1 - (\text{growth index of test sample} / \text{growth index of control}) \times 100 = \%$$

The MIC of each fatty acid solution was determined as the lowest concentration that yielded a growth index of less than that of the 1:100 control vial.

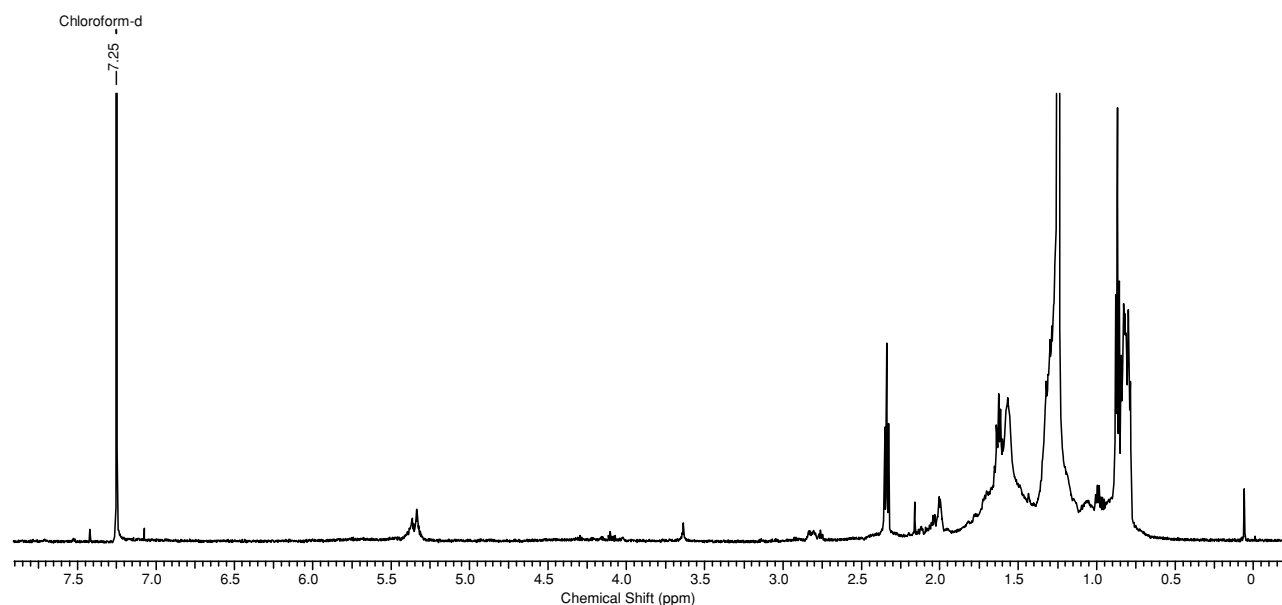


Figure 1. ^1H NMR spectrum of sample 6/9.

Results

The fractionation of the crude extract using flash chromatography produced 45 fractions of which 38 were active against *M. aurum* and 27 against *M. smegmatis* in the bioautography method, with R_f values between 0.1 and 0.9. Samples with similar ^1H NMR spectra, thin-layer chromatography profiles, and inhibition zones were combined and further purified using the Chromatotron, such as fractions 2 and 3 and fractions 17 to 30. Some of the purified fractions inhibited the growth of *M. smegmatis* although the initial cruder fraction had shown no inhibition. The purified samples were screened for activity against *M. smegmatis* only using direct bioautography. Further bioassay-guided fractionation using centrifugal preparative layer chromatography and analytical thin-layer chromatography resulted in the isolation of a mixture of fatty acids and hydrocarbons as had been anticipated from the ^1H NMR spectra obtained (see Fig. 1).

The resultant sample (69.9 mg) was tested against *M. smegmatis* and the MIQ found to be $7.8\ \mu\text{g}$ (zone size = 4 mm), which compared well with ciprofloxacin, which

showed a 12-mm-diameter zone at an amount of $12.5\ \mu\text{g}$ on the thin-layer chromatography plate. The sample was methylated and submitted for GC-MS analysis (16.4 mg). Hydrocarbons and methyl esters were identified, confirming the presence of fatty acids. All the methyl esters in the mixture were identified by comparing the retention times of GC peaks with those of the standard compounds under the same chromatographic conditions using commercially available standards (Sigma Aldrich Co., Germany).

Of the fatty acids identified in the GC-MS analysis, only dodecanoic acid, stearic acid, palmitic acid, oleic acid, linoleic acid, and myristic acid could be obtained commercially for further analysis. Pentadecanoic acid had to be omitted. Antimycobacterial activity of the fatty acids was assessed against *M. smegmatis* using direct bioautography and *M. tuberculosis* H37Rv and a clinical multidrug-resistant (MDR) strain of *M. tuberculosis* in the Bactec-460 radiometric method. Dodecanoic acid, linoleic acid, and oleic acid inhibited the growth of *M. smegmatis* with MIQ values of 3.125, 1.56, and $0.8\ \mu\text{g}$, respectively (Table 1). Myristic acid, palmitic acid, and stearic acid showed no inhibition of *M. smegmatis*.

Table 1. Antimycobacterial activity of the bioactive fatty acids against *M. smegmatis*, *M. tuberculosis*, and MDR *M. tuberculosis*.

Fatty acid	<i>M. smegmatis</i>	<i>M. tuberculosis</i>	MDR <i>M. tuberculosis</i>
	Zone size in mm (MIQ) value in μg	% Inhibition (MIC) value in $\mu\text{g/mL}$	% Inhibition (MIC) value in $\mu\text{g/mL}$
Lauric acid	9 (3.125)	100 (50)	75 (50)
Linoleic acid	5 (1.56)	100 (50)	—86 (50)
Myristic acid	—	98 (50)	88 (50)
Oleic acid	5 (0.8)	100 (25)	—

In the Bactec-460 method, the fatty acid and hydrocarbon mixture showed moderate inhibition at 125 $\mu\text{g/mL}$ against *M. tuberculosis* H37Rv. Lauric acid and linoleic acid showed 100% inhibition at 50 $\mu\text{g/mL}$. Myristic acid showed 98% inhibition at 50 $\mu\text{g/mL}$, and oleic acid showed 100% inhibition at 25 $\mu\text{g/mL}$. Palmitic acid and stearic acid showed no significant inhibition. When tested against MDR *M. tuberculosis*, linoleic acid showed moderate inhibition at 50 $\mu\text{g/mL}$, whereas lauric acid and myristic acid showed 75% and 88% inhibition, respectively, at the same concentration (Table 1). None of the other fatty acids inhibited the growth of MDR *M. tuberculosis*.

Discussion

Fatty acids are important components of terrestrial plant oils (Evans, 1996), and the same is true for marine plants. Many investigations into marine animal and plant lipids have been published (Carballeira, 2002; Dembitsky & Srebnik, 2002; Huang & Wang, 2004; Sanina et al., 2004). The fatty acids identified in this investigation of *P. virgata* are common to red algae and specifically to the genus *Polysiphonia*. Johns et al. (1979) investigated the fatty acid composition of 10 seaweeds from Australian waters. *Polysiphonia pungens* Hollenberg (Rhodomelaceae) was one of the species investigated and showed high levels of polyunsaturated fatty acids, such as arachidonic acid. Palmitic acid was the main saturated fatty acid. *cis*-Vaccenic acid, *cis*-9-hexadecenoic or palmitoleic acid, and myristic acid were minor components in the fatty acid mixture. Pettitt et al. (1989) investigated the lipids of the algae *Chondrus crispus* Stackhouse (Gigartiniaceae) and *Polysiphonia lanosa* Linnaeus (Rhodomelaceae). The major fatty acids detected were palmitic, oleic, arachidonic, and eicosapentaenoic acids. A red alga with a lipophilic portion high in antioxidant activity, *Rhodomela confervoides* Hudson (Rhodomelaceae), had only 37.7% mg/g dry weight in lipid content but the most unsaturated fatty acids (46% of lipophilic content), such as 11-hexadecenoic acid and 5,8,11,14,17-eicosapentaenoic acid. Halogenated fatty acids are very common in the marine environment, and many have been isolated from algae and other marine organisms as shown in a review done by Dembitsky and Srebnik (2002).

Many publications are available about fatty acids with antibiotic activities. Findlay and Patil (1984) isolated the fatty acids (6Z,9Z,12Z,15Z)-hexadecatetraenoic acid, (6Z,9Z,12Z,15Z)-octadecatetraenoic acid, and (6Z,9Z,12Z)-hexadecatrienoic acid as the antibacterial compounds from the diatom (*Navicula delognei* f. *elliptica* Lobban). It inhibited the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Proteus vulgaris*. Benyagoub et al. (1996) isolated 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid as the compounds responsible for the antibiotic activity of the fungus *Sporothrix flocculosa*. Ohta et al. (1993) investigated the antibiotic activities of 10 fatty acids and their methyl esters and found

γ -linolenic acid (C18:3) to have the highest antibiotic activity. α -Linolenic acid, along with eicosapentaenoic acid, and docosahexaenoic acid also had strong antibiotic activity.

The antimycobacterial activities of long-chain fatty acids have been demonstrated by a few investigations. A potent antimycobacterial compound, 3-nitropropionic acid, was isolated from endophytic fungi found on Thai medicinal plants with a minimum inhibition concentration of 3.3 μM (Chomcheon et al., 2005). Antimycobacterial (MIC value of 25 $\mu\text{g/mL}$) and antiplasmodial activity (IC_{50} value of 7.2 $\mu\text{g/mL}$) was reported for scleropyric acid isolated from the twigs of *Scleropyrum wallichianum* Wight & Arn. (Santalaceae) (Suksamrarn et al., 2005). Stavri et al. (2004) investigated the hexane extract of hops, namely *Humulus lupulus* L. (Cannabaceae), for antimycobacterial compounds, and found a fatty acid mixture to be the bioactive principle. Among the fatty acids were palmitic, stearic, and oleic acid with small quantities of lignoceric, arachidic, behenic, and linoleic acids. All saturated fatty acids were inactive against *M. fortuitum*, whereas the unsaturated fatty acids, namely oleic and linoleic acids, showed MICs between 4 and 16 $\mu\text{g/mL}$. Kondo and Kanai (1972) looked at the lethal effect of long-chain fatty acids on mycobacteria and found that of 11 long-chain fatty acids, oleic, linoleic, and myristic acids showed the greatest activity against *M. tuberculosis* and *M. bovis*. Seidel and Taylor (2004) investigated the dried roots of *Pelargonium reniforme* Curtis (Geraniaceae) and *P. sidoides* D.C. (Geraniaceae) for antibacterial activity against *M. aurum* and *M. smegmatis*, and showed the active components to contain palmitic, oleic, and linoleic acids as the major bioactive components. Although the initial mixture of fatty acids and hydrocarbons was highly active against *M. smegmatis* in direct bioautograph but weakly active against *M. tuberculosis* in the radiometric method, the antimycobacterial activity of the individual fatty acids such as oleic acid, and linoleic acid are consistent with the above investigations.

Acknowledgments

The authors thank the National Research Foundation for financial assistance, Dr. Néstor M. Carballeira from the Department of Chemistry at the University of Puerto Rico for his invaluable guidance, Ms. Jean Mckenzie for the NMR spectra, and Dr. Stephan Louw, from the Central Analytical Facility, University of Stellenbosch, for the LC-MS and GC-MS analysis.

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