Anthelmintic and antimycobacterial activity of fractions and compounds isolated from *Cissampelos mucronata*

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Compounds studied: stigmasterol, hentriacontane, simiarenol, nonacosene, carbonic acid.

List of abbreviations: ABF, antibiofilm activity; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); ATCC, American Type Culture Collection; BuOH, butanol; CVS, crystal violet staining; DCM, dichloromethane; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EPS, extracellular polymeric substance; EtOAC, ethyl acetate; Hex, hexane; IC50, 50% inhibitory concentration; INT, *p*-iodonitrotetrazolium violet; LC_{50}, 50% lethal concentration; MBC, minimum bactericidal concentration; MeOH, methanol; MEM, minimal essential medium; MH, Mueller-Hinton; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; NMR, Nuclear Magnetic Resonance; OD, optical density; TLC, thin layer chromatography.

Highlights

- *Cissampelos mucronata* aerial part extracts were antimycobacterial (MIC as low as 0.015 mg/ml) and anthelmintic.
- Five compounds were isolated with varying anthelmintic and antimycobacterial activity.
- Isolated compounds nonacosene and hentriacontane inhibited mycobacterial biofilms with up to 100% inhibition.
- The study supports traditional use of *C. mucronata* in treating tuberculosis and parasites.

Abstract

Ethnopharmacological relevance: *Cissampelos mucronata* A.Rich., a perennial climber belonging to the family Menispermaceae, has been used traditionally to treat parasites and tuberculosis-related symptoms. Co-infection of helminth parasites and tuberculosis-causing pathogens heightens the risk of developing active tuberculosis.

Aim of the study: The aim was to isolate and characterize antimycobacterial compounds from *Cissampelos mucronata* and to investigate their antibiofilm and anthelmintic efficacy as well as cytotoxicity.

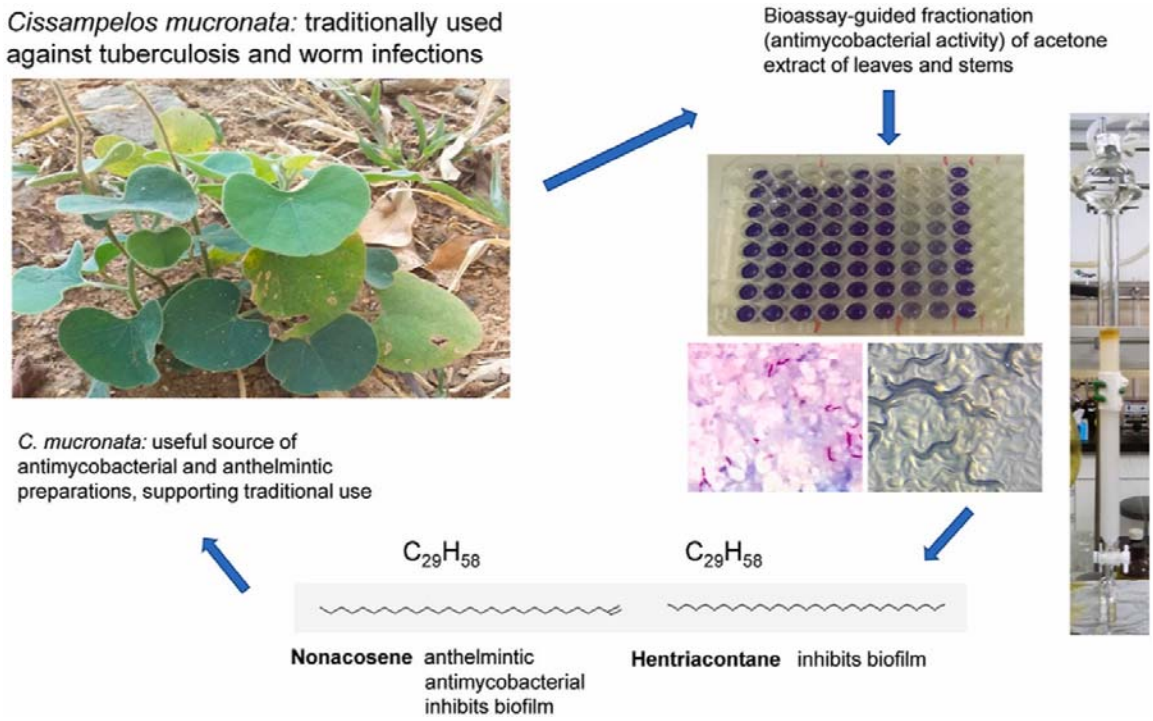
Materials and methods: The acetone extract of *C. mucronata* leaves and stems was fractionated by vacuum liquid chromatography using hexane, ethyl acetate, acetone and methanol:chloroform (3:7). Separation of the active ethyl acetate fraction by column and preparative thin layer chromatography led to the isolation and identification of five compounds using NMR and LC-MS, as well as GC-MS for non-polar compounds. The anthelmintic, antimycobacterial, antibiofilm, antioxidant and anti-inflammatory effects as well as cytotoxicity of the fractions and compounds were determined.

Results: The ethyl acetate fraction had the best antimycobacterial activity (MIC = 0.015 – 0.08 mg/ml). The fractions were relatively non-toxic to Vero cells (0.03 – 0.79 mg/ml) and had good anti-inflammatory and antibiofilm effects. Five compounds were identified as stigmasterol, hentriacontane, simiarenol, nonacosene and carbonic acid. Nonacosene had moderate anthelmintic effects but poor antimycobacterial activity (MIC = 0.375 mg/ml). Nonacosene and hentriacontane had good biofilm inhibitory effect (90 – 100%).

Conclusions: This study reveals that *C. mucronata* is a potential source of promising compounds with a range of useful bioactivities that support its use in traditional medicine. Development of plant-based remedies may assist in reducing the impact of co-infections with helminth parasites and tuberculosis-causing mycobacteria.

Keywords: *Cissampelos mucronata*, Menispermaceae, antimycobacterial, anthelmintic, anti-inflammatory, nonacosene, hentriacontane

Graphical abstract



List of abbreviations:

ABF	antibiofilm activity
ABTS 2	2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ATCC	American Type Culture Collection
BuOH	butanol
CVS	crystal violet staining
DCM	dichloromethane
DMSO	dimethyl sulfoxide
DPPH 2	2-diphenyl-1-picrylhydrazyl
EPS	extracellular polymeric substance
EtOAC	ethyl acetate
Hex	hexane
IC50	50% inhibitory concentration
INT	*p*-iodonitrotetrazolium violet
LC_{50}	50% lethal concentration
MBC	minimum bactericidal concentration
MeOH	methanol
MEM	minimal essential medium
MH	Mueller-Hinton
MIC	minimum inhibitory concentration
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear Magnetic Resonance
OD	optical density
TLC	thin layer chromatography

1. Introduction

Tuberculosis (TB) is a communicable disease of universal occurrence and a major cause of ill health, with the rate of mortality from TB higher than that of other bacterial infections (WHO, 2019, Grange and Zumla, 2002). Tuberculosis is mostly caused by *Mycobacterium tuberculosis* but other *Mycobacterium* species such as *M. bovis, M. africanum, M. canetti* and *M. microti* can cause TB in humans (Aro et al., 2015). India, China, Indonesia, Philippines, Pakistan, Nigeria, Bangladesh and South Africa constitute two-thirds of the global TB cases, and out of about half a million new cases of rifampicin resistant TB reported in 2018, 78% were multidrug resistant (WHO, 2019, WHO, 2018, Patel et al., 2020). The current TB regimen

has been a success with a slight reduction in annual average global incidence and death rate (WHO, 2019), but hepatotoxicity of current drugs combined with drug resistance requires extensive research for development of anti-tuberculosis drugs.

Helminth infections occur in about 1.5 billion people worldwide, with the bulk of those infected living in developing nations where TB is endemic (Bethony et al., 2006). Given the intersecting geographic distribution of helminths and *Mycobacterium tuberculosis* (Mtb) infections, the importance of investigating if a lung-dwelling helminth can potentially hinder the ability of the host to control pulmonary Mtb infection is clear. Co-infection with helminths can result in enhanced pathogenesis of TB (Abate et al., 2012) as well as TB chemotherapy and BCG vaccination failure (Resende Co et al., 2007).

A number of natural products, or secondary metabolites from plants and other sources have promising activity against different species of mycobacteria (McGaw et al., 2008). The *Cissampelos* L. genus falls under the Menispermaceae family. The genus has 21 species and diverse uses as sources of traditional medicines in tropical Africa (Semwal et al., 2014; Maroyi, 2020). *Cissampelos mucronata* A.Rich. is a shrub-like climber widely distributed in tropical Africa from Senegal east to Ethiopia and South Africa. The leaves and roots of *C. mucronata* are sold as traditional medicines in informal herbal medicine markets in Benin, Malawi and Mozambique, and it is commonly planted in home gardens as a medicinal plant in some countries in tropical Africa (Maroyi, 2020, Muzila, 2006). In South Africa, the roots are used to treat ailments such as fever, headache and backache (Van Wyk and Gericke, 2000) as well as stomach problems (De Wet and Van Wyk, 2008) and parasitic infections including bilharzia (Van Wyk and Gericke, 2000). The leaves are used to treat scrofula, a condition in which infection with tuberculosis bacteria causes symptoms outside the lungs, commonly associated with inflammation (Watt and Breyer-Brandwijk, 1962; Bryant, 1966). Leaves are also used to treat stomach problems (De Wet and Van Wyk, 2008). *C. mucronata* and related species are used in Nigerian traditional medicine to treat worm infections (Aska and Kubmarawa, 2016, Aska et al., 2019, Audu, 1995).

Several phytochemicals have been isolated from the plant (Semwal et al., 2014, Maroyi, 2020, Muzila, 2006). Some alkaloids were isolated from the leaves and rhizomes i.e., dicentrine, salutaridine, cycleanine, pronuciferine (De Wet, 2006). Triterpenes, anthraquinones, saponins, phenolics, glycosides and other phytochemicals have been identified in the leaves of *C. mucronata* (Maroyi, 2020). Antimalarial (Tshibangu et al., 2003), antiulcer (Nwafor and Okoye, 2005) and antibacterial (Tor-Anyiin et al., 2006) effects are among the biological activities noted.

This study reports on the isolation and structural elucidation of compounds identified from *C. mucronata* leaves. The antimycobacterial, antibiofilm and anthelmintic effects of the compounds were investigated to explore their effect on the helminth-tuberculosis link. Anti-inflammatory and antioxidant activities were also investigated as these are useful bioactivities in reducing the negative effects of mycobacterial and helminth infections (Muniandy et al., 2018).

2. Materials and methods

2.1 Collection of plant material, authentication, drying and storage

C. mucronata aerial parts (leaves and stems) were collected around the National Institute of Pharmaceutical Research and Development, Idu area, Abuja, Nigeria. The plant was identified and authenticated at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria. The voucher specimen number is PRU 0125290. The parts were air dried separately at room temperature. The dried plant was ground into powder using a Macsalab mill (Model 200 LAB, Eriez, South Africa] and stored in a brown paper bag in the dark at room temperature until needed.

2.2 Bulk extraction and fractionation of active extract

The dried pulverized material (*C. mucronata* leaves and stems) (777.43 g) was exhaustively extracted with acetone (15 L). Continuous extraction was performed whereby the plant material was macerated in acetone, agitated and filtered continuously until a pale colour of the extract (signifying exhaustive extraction) was observed. The filtrate was concentrated with a Buchi rotavapor [R-100, Labotec, Johannesburg, South Africa] under reduced pressure at 50 °C. The final weight of the crude extract obtained was 38.05 g.

Fractionation of the acetone crude extract was performed using vacuum liquid chromatography with hexane, ethyl acetate, acetone, methanol:chloroform (3:7). The acetone crude extract (36 g) was adsorbed with sufficient quantity of silica gel and filtered through a Büchner funnel twice with 500 ml hexane. The solvent was allowed to evaporate completely, and the residue was filtered with the same quantity of ethyl acetate. The procedure was repeated with acetone and methanol:chloroform (3:7). The fractions were concentrated and weighed. The mass of the resulting fractions was: hexane (16.9 g), ethyl acetate (4.36 g), acetone (3.78 g), methanol:chloroform (2.74 g).

2.3 Thin Layer Chromatography (TLC) of extract and fractions

Qualitative evaluation of *C. mucronata* extract and fractions (hexane, ethyl acetate, acetone, methanol:chloroform (3:7)) was done to separate the phytochemical constituents. Hexane:acetone (7:3) was used as eluent, and vanillin-sulphuric spray reagent was used to identify the compounds in the extracts and fractions.

2.4 Isolation of bioactive compounds

The ethyl acetate fraction (1.53 g) was subjected to further separation based on its superior antimycobacterial activity. Column chromatography using silica gel (Merck silica gel 60; 143 g), thin layer and preparative thin layer chromatography (TLC) (20 cm × 20 cm silica gel 60 TLC plates) (Merck, Germany) were used for the separation. The column was eluted with n-hexane (Hex) and acetone (Ace) (9:1), followed by an increasing polarity of solvent to acetone 50% (5:5). Volumes of 10 ml each (226 tubes) were collected. From TLC, using hexane:acetone (7:3) solvent system, the collected fractions were combined into 15 fractions based on the similarity of the chromatograms. A second column using the same quantity of the ethyl acetate fraction and silica gel was performed to obtain a better separation. The column was eluted with the n-hexane and acetone solvent mixture (9:1) with increasing polarity of solvent to acetone 40%. Tubes of 10 ml each (133) were collected.

Thin layer chromatography of the column fractionations with the same solvent system and spray reagent used initially (hex:ace, 7:3; vanillin) gave 8 fractions. Fractions from the first and second column were weighed and some fractions from the first and second column were combined due to the similarity in their TLC profile. Fractions 34, 41 (second column) and 50 (first column) (75.7 mg) were combined because they had a similar TLC profile to yield a powdery substance after evaporation of the solvent. Further purification using column chromatography, and thin layer chromatography with the above-mentioned solvent system and spray reagent yielded partially pure fractions (numbered 55, 124 and 107). Fraction 63 (24.6 mg), was further purified by extracting with acetone and refrigerating overnight at 4 °C before being filtered. Preparative thin layer chromatography of fractions 124 and 107 yielded three compounds each: 124 (230 mg) [124-3 (10 mg), 124-2(10 mg), 124-4 (10 mg)]. Fraction 107 (220 mg) also yielded three compounds [107-1 (10 mg), 107-2 (10 mg), 107-21 (10 mg)]. Fraction 55 (70 mg) was purified with column chromatography and was unstable, decomposing within a short period of time. The purified form of fraction 55 (16.9 mg) was obtained via preparative thin layer chromatography. The plates were viewed under UV light (254 nm) after separation and the prominent bands were scraped off the plate and extracted with a polar solvent. The procedure for fractionation and isolation of compounds is represented in Figure 1.

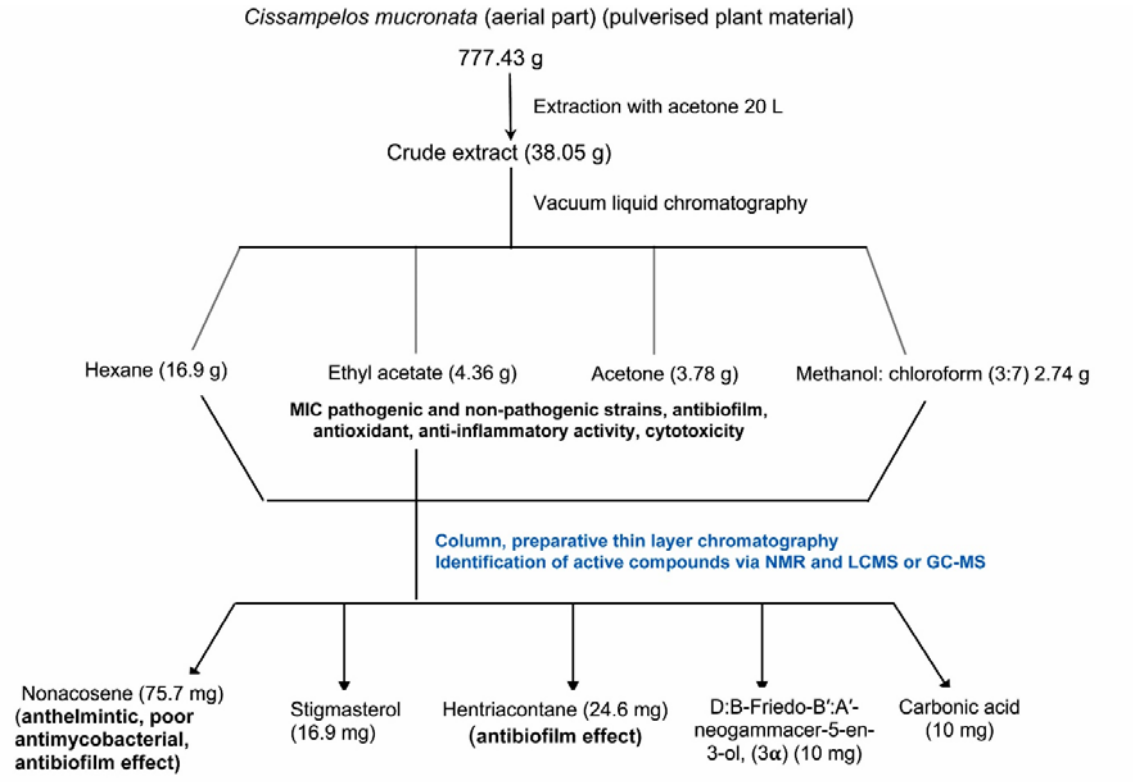


Figure 1. Flow chart for the isolation of bioactive compounds.

2.5 Structural elucidation of isolated compounds

The isolated compounds were characterized by analysis of proton (^{1}H) and carbon (^{13}C) Nuclear Magnetic Resonance (NMR) spectra (Bruker Avance III 400 MHz NMR Spectrometer, Bruker Avance III HD 500 MHz NMR Spectrometer with Prodigy Probe). The data obtained from the positive Electrospray Ionization-Mass Spectrometry (ESI-MS) (Waters Acquity Ultra Performance Liquid Chromatography (UPLC®) system hyphenated to a quadrupole-time-of-flight (QTOF) instrument) and Gas Chromatography-Mass spectrometry (Agilent GC-MSD) supported the structures. NMR data was processed with Mnova suite, Mestrelab.

2.6 Antimycobacterial effect of fractions and compounds

The antimycobacterial effect of the fractions was determined against non-pathogenic (*M. smegmatis* ATCC 1441, *M. fortuitum* ATCC 6841, *M. aurum* NCTC 10437) and pathogenic strains obtained from the American Type Culture Collection (*M. tuberculosis* ATCC 25177) and the Agricultural Research Council-Onderstepoort Veterinary Research (ARC-OVR) Tuberculosis Laboratory (*M. bovis* TB9350C and *M. tuberculosis* TB8993A). The minimum inhibitory concentration (MIC) of the fractions and compounds against non-pathogenic and biofilm-forming (*M. smegmatis* mc^2 155) strains was determined according to the method of

Tran et al. (2017) with slight modifications. The cultures were grown at 37°C for 24 h (*M. smegmatis*), 24-48 h (*M. fortuitum*) and 4-5 days (*M. aurum*). Single cell suspensions of the cultures were prepared. The cultures were diluted to OD_{600} 0.004 (1.12 x 10^6) in the 7H9 medium. A two-fold serial dilution was performed using 100 µl of extracts and 100 µl 7H9 medium. Streptomycin and rifampicin were used as positive controls at a concentration range of 25 – 0.20 µg/ml. DMSO (10%), bacterial culture and 7H9 medium were the negative controls. One hundred microliters of the mycobacterial culture were then added to all the wells. The plates were sealed in a plastic bag and incubated for 24 h for *M. smegmatis*, 48 h for *M. fortuitum* and 5 days for *M. aurum* at 37 °C.

M. tuberculosis and *M. bovis* field strains (TB8993A, TB9350C) and standard strain (ATCC25177) were grown on Lowenstein-Jensen (LJ) slants supplemented with glycerol, or pyruvate in the case of *M. bovis,* for 3 - 4 weeks. The colonies were sub-cultured in 7H9 medium for 14 - 15 days. The test inoculum was prepared in sterile water and adjusted to McFarland standard 1 (4×10^7 CFU/mL). The cultures were diluted 1:10 to a final density of 5×10^5 CFU/mL with freshly prepared supplemented Middlebrook 7H9 medium. Sterile water (200 µl) was added to the outer wells to reduce evaporation. Two-fold serial dilutions were performed using 100 µl of extracts and 100 µl 7H9 medium. Streptomycin and rifampicin were used as positive controls while 10% DMSO, bacterial culture and 7H9 medium served as negative controls. One hundred microliters of the mycobacterial culture were added to all the wells. The plates were sealed in a plastic bag and incubated for 7 days (*M. bovis* and *M. tuberculosis* strains) at 37 °C. MIC values were determined by adding 40 µl of 0.2 mg/ml of freshly prepared thiazolyl blue tetrazolium bromide (MTT, Inqaba Biotec, South Africa) as growth indicator (McGaw et al., 2008, Bhunu et al., 2017). The colour reaction occurred after incubation for 60-90 min for non-pathogenic strains and 6 h for pathogenic strains. The result was read as soon as a purple color became visible in the untreated control wells. MIC values are the concentrations with marked reduction in color formation corresponding to inhibition of mycobacterial growth. The assay was done in triplicate and the experiment was repeated twice.

2.7 Cytotoxicity of fractions and compounds

Cytotoxicity of the fractions and compounds was determined against Vero African Green monkey kidney cells (ATCC® CCL-81TM) using the 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (Mosmann, 1983; McGaw et al., 2007a). The Vero cells were maintained in minimal essential medium (MEM, PAN Biotech, Biocom Africa, South Africa) supplemented with 5% foetal calf serum and 0.1% gentamicin (Virbac). The cell suspensions were prepared from confluent monolayer cultures and plated at a density of 1×10^4 in 96-well microtitre plates. The plates were incubated for 24 h at 37°C

in a 5% CO_2 incubator prior to exposure. The fractions and compound were prepared in the appropriate solvent at 100 mg/ml and appropriate dilutions were prepared in MEM and added to the cells. The cells were exposed to various concentrations (0.0075 – 0.1 mg/ml) for 48 h. Doxorubicin (Pfizer Laboratories) was used as a positive control. After 48 h incubation, the wells were rinsed with 200 µl of phosphate buffered saline (PBS, Sigma) and 100 µl of fresh medium was dispensed into the wells. Thirty microliters of MTT (5 mg/mL) (Inqaba Biotec, South Africa) dissolved in PBS was added to each well and incubated for 4 h at 37ºC. The medium was removed and MTT formazan crystals were dissolved with 50 µl DMSO. Absorbance was measured on a microplate reader (BioTek Synergy) at a wavelength of 570 nm. Each concentration was tested in triplicate and the assay was repeated three times. The LC_{50} of the samples was calculated (LC_{50} is a measure of the concentration that kills 50% of the cells). The selectivity index (SI) was determined by dividing cytotoxicity (LC_{50}) by the MIC values (LC_{50}/MIC).

2.8 Antioxidant activity of fractions

The antioxidant effect of the fractions was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assays. The DPPH assay was done according to Gyamfi et al. (1999) with slight modifications. The optical density (OD) of the DPPH solution was calibrated at 517 nm ranging from 0.9 - 1.00. The DPPH solution (160 µL) was added to 40 µL of different crude extracts and fractions at different concentrations (3.125 - 200 µg/mL). The mixture was incubated in the dark for 30 min, and the absorbance was measured at 517 nm using a microplate reader (Epoch, Biotek). Lower absorbance of the solution indicated higher free radical scavenging activity. The percentage of scavenging activity was calculated using the formula (1) below:

$$\% \text{ Scavenging activity} = [(A0 - As)/A0] \times 100$$

Where A0 is absorbance of control (DPPH solution without sample), As is absorbance of tested sample (DPPH plus sample).

The 50% inhibitory concentration (IC_{50}) values of the fractions were determined using a non-linear regression curve of percentage of scavenging activity against the logarithm of concentrations. Ascorbic acid and Trolox were used as positive controls. Each test was done in triplicate and results are presented as mean ± standard deviation (SD).

The antioxidant effect by ABTS was evaluated following the method of Re et al. (1999).

The stock solution of ABTS radical was prepared by mixing the ABTS (7 mM) with potassium persulfate (2.45 mM) at room temperature. The mixture was kept in the dark for 12-16 h. The working solution was prepared by calibrating the stock solution to obtain an optical density

(OD) of 0.70±0.02 at 734 nm. The ABTS working solution (160 µL) was mixed with the samples (40 µL) at different concentrations and incubated in the dark at room temperature. The absorbance was measured after 5 min at 734 nm using a microplate reader (Epoch, BioTek). The percentage of scavenging activity was calculated using the formula (1) above. The 50% inhibitory concentration (IC_{50}) values of the samples were determined using a non-linear regression curve of percentage of scavenging activity against the logarithm of concentrations. Ascorbic acid and trolox were used as positive controls. Each test was done in triplicate and results are presented as mean ± standard deviation (SD).

2.9 Anti-inflammatory activity of fractions

2.9.1 15-lipoxygenase assay

The activity of fractions against the 15-lipoxygenase enzyme was determined according to del Carmen Pinto et al. (2007) with slight modifications. This assay evaluated the inhibition of soybean 15-LOX activity by samples in the presence of linoleic acid based on the formation of the complex Fe3+/xylenol orange with absorption at 560 nm. The substrate linoleic acid (final concentration, 140 µM) was prepared in Tris-HCl buffer (50 mM pH 7.4). The crude extracts and fractions (10 mg/mL) with potent antioxidant activity were prepared in 100% DMSO and further diluted to 2 mg/mL in Tris-HCl buffer. Forty microliters of the enzyme (15-LOX), diluted in ice-cold Tris-HCl buffer (final concentration, 0.2 U/ml), was mixed with 20 µl of different concentrations (100 to 0.78 µg/ml) of test samples or quercetin (positive control) at 25 °C for 5 min. Linoleic acid (40 µl) was added to the mixture (except blank) and further incubated at 25 °C for 20 min in the dark. The assay was terminated by the addition of 100 µl of freshly prepared FOX reagent [sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulphate (100 µM) in methanol/water (9:1)]. The negative control was made of the enzyme 15-LOX solution, Tris-HCl buffer, substrate and FOX reagent while the blanks contained the enzyme 15-LOX and buffer, but the substrate was added after the FOX reagent. The lipoxygenase inhibitory activity was determined by calculating the percentage of inhibition of hydroperoxide production from the changes in absorbance values at 560 nm after 30 min at 25 °C as indicated in the formula below:

15−LOX inhibition (%) = Absorbance (control) − Absorbance (sample)/Absorbance (control) x 100.

The 50% inhibitory concentration (IC_{50}) was determined using the non-linear regression curve of the percentage (15-LOX) inhibition against the logarithm of concentrations tested.

2.9.2 Nitric oxide inhibition

Nitric oxide inhibition was investigated using RAW 264.7 macrophages as previously described (Adebayo et al. (2015).

2.9.2.1 Cell culture and treatment with samples

RAW 264.7 macrophages obtained from the American Type Culture Collection (ATCC TIB-71) (Rockville, MD, USA) were grown at 37 °C with 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L of glucose and 4 mM of L-glutamine (HycloneTM) supplemented with 10% fetal bovine serum (FBS) (Biocom Africa, South Africa) and 1% penicillin/streptomycin/fungizone (PSF) (Celtic Molecular Diagnostics). The cells were sub-cultured three times a week. One hundred microliters of a cell suspension (4×10^4 cells/ml) of RAW 264.7 cells at the logarithmic phase of growth (about 80% confluence) were seeded in wells of 96-well microtitre plates and incubated overnight at 37 °C with 5% CO_2 to allow attachment. The RAW 264.7 cells activated by incubation in medium containing 1 µg/ml of lipopolysaccharide (LPS) alone served as the negative control. The cells were treated simultaneously with different concentrations of the samples dissolved in 50% DMSO and further diluted at different concentrations in medium. Quercetin (Sigma-Aldrich, Johannesburg, South Africa), a known inhibitor of NO production in macrophages (Lawal et al., 2019) was the positive control.

2.9.2.2 Measurement of nitrites and determination of percentage of nitric oxide inhibition

The concentration of NO released from RAW 264.7 macrophages was assessed by determination of nitrite concentration in the supernatant from the above experiment using the Griess reagent (Adebayo et al., 2015). Briefly, 100 µl of cell supernatant from each well was transferred into a new 96-well microtitre plate after 24 h incubation. The same volume of Griess reagent was added. The plates were incubated in the dark for 15 min at room temperature and the absorbance was recorded at 550 nm (Epoch Biotek). The concentrations of the nitrites were calculated from the standard curve obtained from serial dilutions of sodium nitrite (0-50 µM). Percentage of NO inhibition was calculated based on the ability of extracts or fractions to inhibit nitric oxide formation by RAW 264.7 macrophages compared with the control (cells treated with LPS alone).

2.9.2.3 Determination of cell viability

The cytotoxicity of the fractions was determined against RAW 264.7 macrophage cells using the MTT assay as described above (Mosmann, 1983).

2.10 Antibiofilm activity of fractions and compounds

The biofilm inhibition and dispersion effect of the fractions was determined against the known biofilm-former *Mycobacterium smegmatis* mc^2 155 (Ishida et al., 2011; Cady et al., 2012). The biofilm inhibition effect of nonacosene and hentriacontane was also investigated.

The biofilm inhibition assay was performed as follows; one hundred and fifty microliters (2.06×10^3 CFU/ml) of *M. smegmatis* (mc^2 155) inoculum prepared in 7H9 media (7H9 broth supplemented with 2% glycerol, omitting Tween-80) was added to the wells of a 96-well microplate. The fractions and compounds (2 mg/ml; 150 µl) were added to yield a final assay volume of 300 µl. Rifampicin (200 µg/ml) was used as the positive control. The culture, samples, broth and solvent were the negative control. The plates were incubated at 37 °C for 4 days under stationary conditions which induce biofilm formation. The biofilm inhibition activity of the samples was quantitatively analyzed by using 0.01% crystal violet, to determine the percentage biofilm inhibition. The plates were dried in a desiccating oven to remove all the liquid media and washed thoroughly (thrice) with distilled water to remove planktonic cells and any broth residue. The plates were left to air dry. The biofilm was fixed to the surface of the plates by the addition of 250 µl of methanol for 15 minutes. Crystal violet solution (250 µl; 0.01%) was added to each well for 15 min as a biofilm biomass indicator followed by three wash steps as mentioned above. The bound crystal violet was solubilized using 96% ethanol (250 µl) and transferred (200 µl) to a new microtitre plate. The amount of crystal violet was quantified by measuring the absorbance at OD_{590} using a Biotek Synergy plate reader. The mean absorbance of the samples was determined, and percentage inhibition of biofilm was evaluated using the equation below (Famuyide et al., 2019):

Percentage (%) inhibition = OD Negative control - OD Experimental x 100 / OD Negative control

2.10.1 Biofilm dispersion

One hundred and fifty microliters (2.06×10^3 CFU/ml) of *M. smegmatis* (mc^2 155) inoculum prepared in 7H9 media (7H9 broth supplemented with 2% glycerol omitting Tween 80) was added into the wells of a 96-well microplate. The plates were incubated at 37 °C for 4 days under stationary conditions to allow biofilm formation. The fractions (2 mg/ml; 150 µl) were added to the preformed biofilms and incubated for 24 h. Rifampicin (200 µg/ml) was used as the positive control. The fractions, culture, broth and solvent were the negative control. The biofilm dispersion activity of the fractions was quantitatively analyzed using the same concentration of crystal violet and method as the biofilm inhibition assay above.

2.11 Anthelmintic effect of compound

The anthelmintic effect of nonacosene was determined in 24-well plates against *Caenorhabditis elegans* var. Bristol (N2) nematodes by the method of Rasoanaivo and Ratsimamanga-Urverg (1993) modified by McGaw et al. (2000). The nematodes were cultured on nematode growth (NG) agar seeded with *E. coli* (Brenner, 1974). Nematodes (7 to 10-day old cultures) were washed and diluted with M9 buffer until approximately 100 nematodes in 10 µl was obtained. Extracts were prepared from 100 mg/ml stock solutions to give concentrations of 0.5, 1 and 2 mg/ml which were incubated with nematodes in the dark for 2 h, 24 h and 48 h at 25 °C. Solvent control, standard (10 µg/ml levamisole) and blank (nematodes incubated with M9 buffer) were also included. Using an inverted microscope, the percentage of live (motile) worms was estimated, their movement was observed and compared to the controls. The experiment was performed in triplicate and repeated twice.

3 Results and Discussion

3.1 Antimycobacterial effect of fractions

Based on the promising antimycobacterial effect (MIC = 0.03 – 0.31 mg/ml) of the acetone extract of *C. mucronata* leaves and stems against pathogenic and non-pathogenic *Mycobacterium* strains (Akande, 2021), the combined leaves and stems were selected for isolation of active compounds. The yield of the crude extract was 38.05 g (4.89%). Fractionation of the acetone crude extract yielded four fractions i.e. hexane (16.9 g), ethyl acetate (4.36 g), acetone (3.78 g), and methanol:chloroform (3:7; 2.74 g). The ethyl acetate fraction was selected for further isolation of active compounds due to its promising antimycobacterial effect and *in vitro* safety. The ethyl acetate fraction was the most active against all the mycobacterial strains tested with MIC = 0.015-0.08 mg/ml followed by the hexane fraction (MIC = 0.03–0.120 mg/ml) (Table 1). Fractionation of the crude extract potentiated the antimycobacterial effect of the fractions as observed from the MIC of the extracts and fractions. Plant extracts with MIC values lower than 0.1 mg/mL are regarded as significantly active; those with MIC values >0.1 to 0.625 mg/mL are considered moderately active, and if the MIC is >0.625 mg/mL, the activity is weak (Kuete, 2010). The positive controls (rifampicin and streptomycin) had MIC of 0.03 – 0.47 mg/ml. The ethyl acetate and hexane fractions both had moderate inhibitory effect (MIC = 0.16 mg/ml) against the biofilm strain (*M. smegmatis* mc^2 155).

Table 1. Minimum inhibitory concentration of fractions against pathogenic and non-pathogenic strains (mg/ml).

Extract	Ms	Mf	Ma	Mtb (TB8993A)	Mtb (ATCC 25177)	M. bovis (TB9350C)
Hexane	0.04±0.00	0.08±0.00	0.03±0.01	0.03±0.01	0.12±0.057	0.08±0.00
Ethyl acetate	**0.015±0.007**	**0.08±0.00**	**0.03±0.014**	**0.03±0.014**	**0.02±0.00**	**0.04±0.00**
Methanol: chloroform (3:7)	0.31±0.00	1.25±0.00	0.08±0.00	2.5±0.00	2.5±0.00	>2.5±0.00
Acetone	2.5±0.00	0.94±0.44	0.08±0.00	0.31±0.00	0.63±0.00	0.63±0.00
Rifampicin	0.0031±0.00	0.0004±0.00	0.0002±0.00	0.0004±0.00	0.0008±0.00	0.0002±0.00
Streptomycin	0.0003±0.014	0.0008±0.00	0.0006±0.03	0.0063±0.00	0.0047±0.23	0.0016±0.00

Ms – *M. smegmatis*, Mf – *M. fortuitum*, Ma – *M. aurum*, Mb – *M. bovis*, Mtb – *M. tuberculosis*.

Figures in **bold** represent promising activity.

3.2 Structure elucidation of compounds

The isolated compounds were identified as stigmasterol, nonacosene, carbonic acid, hentriacontane and D:B-Friedo-B′:A′-neogammacer-5-en-3-ol, (3**α**). The molecular ion for stigmasterol is [M·⁺] *m/z* is 437.2291. Comparison of the NMR spectroscopic data with published literature confirmed the structure (Ee et al., 2006, Koay et al., 2013). GC-MS was used to support the NMR data of stigmasterol and hentriacontane. Nonacos-1-ene, carbonic acid, and D:B-Friedo-B′:A′-neogammacer-5-en-3-ol, (3**α**) are non-polar compounds and identification was based on GC-MS. Comparison of D:B-Friedo-B′:A′-neogammacer-5-en-3-ol, (3**α**) GC-MS data (using CAS number) with the Scifinder database revealed that D:B-Friedo-B′:A′-neogammacer-5-en-3-ol, (3**α**) is an epimer of D:B-Friedo-B′:A′-neogammacer-5-en-3-ol, (3β)- (ZCI), simiarenol. The molecular weight, molecular formula, similarity and percentage area of the compounds identified with GC-MS is reported in Table 2. The percentage areas of the compounds are 22.90, 28 and 27.2% for hentriacontane, D:B-Friedo-B′:A′-neogammacer-5-en-3-ol, (3**α**) and nonacosene respectively. Carbonic acid had the lowest percentage area of 6.7%. The similarity index of the compounds included 79.4%, 77.4% (D:B-Friedo-B′:A′-neogammacer-5-en-3-ol, (3**α**)), 93.3%, 93.8% (hentriacontane), 87.4% (nonacosene) and 84.9% (carbonic acid). None of these compounds have been previously identified in *C. mucronata*.

Table 2. Percentage composition of compounds identified by GCMS.

Name	Area %	Molecular mass	Molecular formula	Similarity	CAS number
D:B-Friedo-B':A′-neogammacer-5-en-3-ol, (3á)- (simiarenol)	9.994 18.019 (28.013)	426	$C_{30}H_{50}O$	79.4%	1615-94-7
Hentriacontane	14.031 8.895 (22.908)	436	$C_{31}H_{64}$	93.4%	630-04-6
Nonacos-1-ene	27.188	406	$C_{29}H_{58}$	87.4%	18835-35-3
Carbonic acid, methyl tetradecyl ester	6.730	272	$C_{16}H_{32}O_3$	84.9%	–

Stigmasterol is a triterpene that has been isolated from many plant species and some Menispermaceae species (Table 3). Terpenes are the most vast and varied group of naturally occurring compounds. They are grouped as mono-, di-, tri-, tetra- and sesquiterpenes based on the arrangement and number of the isoprene units they possess. They are the major constituents of essential oils in plants and possess a wide range of medicinal effects such as anti-plasmodial, antiviral, anticancer, antidiabetic, antioxidant and anti-inflammatory. Squalene and sterols are the animal sources of terpenes (Cox-Georgian et al., 2019). The reported biological activity of stigmasterol and other compounds are listed in Table 3. Simiarenol is a pentacyclic terpene with five isoprene units. It has been isolated from several plant species with numerous biological effects as listed in Table 3. Hentriacontane is also known as untriacontane or n-hentriacontane (https://pubchem.ncbi.nlm.nih.gov). It is a long chain alkane which has also been isolated from several plant species with some biological activity (Table 3). It is sometimes reported as a constituent of an active extract often detected by GC-MS (Table 3). Nonacos-1-ene or nonacosene is a dehydrogenated alkane with a double bond at position 1 and 2. It has been isolated from some plant species but it is mostly reported as a chemical constituent of an extract or essential oil (Table 3). Carbonic acid, also known as methyl tetradecyl ester, is mostly reported as a chemical constituent of an extract (Table 3).

Table 3. Reported biological activity of compounds isolated from *Cissampelos mucronata*.

Isolated Compound	Plant	Family	Biological effect	Reference
Stigmasterol	*Morinda citrifolia* L.	Rubiaceae	Antimycobacterial ($H_{37}Rv$) using BACTEC method	Saludes et al. (2002)
	Conyza canadensis (L.) Cronquist	Asteraceae	Antiproliferative	Csupor-Löffler et al. (2011)
	Garcinia bancana M_{lq}	Guttiferae	Antibacterial	Rukachaisirikul et al. (2005)
	Strobilanthes crispa L.	Acanthaceae	–	Koay et al. (2013)
	[a]*Legnephora moorei* (F.Muell.) Miers	Menispermaceae	–	Flor et al. (1974)
	Calophyllum inophyllum L.	Guttiferae	–	Ee et al. (2006)
	Tinospora crispa (L.) Hook.f. & Thomson	Menispermaceae	–	Lin (2009)
	[a]*Hyeronima alchorneoides* Allemão	Euphorbiaceae	–	Tinto et al. (1991)
	Prunella vulgaris L.	Labiatae	–	Kojima et al. (1990)
	[a]*Helicteres hirsute* Lour.	Sterculiaceae	–	Nguyen et al. (2019)
	[a]*Leptadenia reticulata*	Asclepiadaceae	–	Dhalani et al. (2019)
Simiarenol	*Hyeronima alchorneoides*	Euphorbiaceae	Analgesic	Kuroshima et al. (2005)

Allemáo			
Kayea stylosa Thwaites	Guttiferae	–	Gunasekera et al. (1975)
Erigeron annus (L.) Desf	Asteraceae	–	Yoo et al. (2008)
Ficus aurantiacea Griff.	Moraceae	–	Kardono et al. (2000)
Rhododendron brachycarpum fauriei (Franch.) D.F.Chamb.	Ericaceae	–	Youn and CHO (1991)
Dorstenia asaroides Gardn. in Hook.	Moraceae	–	Vilegas et al. (1997)
Rhododendron simiarum Hance	Ericaceae	–	Aplin et al. (1966)
Conyza canadensis (L.) Cronquist	Asteraceae	–	Csupor-Löffler et al. (2011)
Euphorbia neriifolia L.	Euphorbiaceae	–	Chang et al. (2012)
Artemisia stolonifera var. laciniata	Asteraceae	Weak anticancer	Kwon et al. (2001)
Helicteres hirsuta Lour.	Sterculiaceae	–	Nguyen et al. (2019)
Ficus glumosa Delile	Moraceae	–	Awolola et al. (2019)
Caraipa densifolia Mart.	Theaceae	–	Gunasekera et al. (1983)

	Euphorbia peplus L.	Euphorbiaceae	Antileishmanial	Amin et al. (2017)
	Euphorbia lathyris L.	Euphorbiaceae	–	Hemmers et al. (1989)
	Capparis decidua (Forssk) Edgew	Capparaceae	α-glucosidase inhibition	Anjum et al. (2020)
Hentriacontane	*Leucas nutans* (Roth) Spreng	Labiatae	–	Hasan et al. (1991)
	Terminalia arjuna (Roxb. ex DC.) Wight & Arn.	Combretaceae	–	Haque et al. (2008)
	Gymnosperma glutinosum (Spreng.) Less	Asteraceae	Weak cytotoxic	Quintanilla-Licea et al. (2012)
	Oldenlandia diffusa (Willd.) Roxb.	Rubiaceae	Improved the expression of inflammatory mediators (TNF-α, IL-6, PGE2, COX-2 and iNOS) and the activation of NF-κB and caspase-1 in LPS-stimulated peritoneal macrophages	Kim et al. (2011)
	Glycine max (L.) Merr. (Natto-fermented soybean)	Fabaceae	Anti-tumor promoting	Takahashi et al. (1995)
	Pure compound	Natural Product Chemistry Division, IIIM, Jammu	Suppresses inflammatory cytokines and other mediators. Regulatory effect on NF-kB.	Khajuria et al. (2017)
	Sarmienta repens Ruiz & Pav.	Gesneriaceae	–	Silva and Sammes (1971)

	Acanthopanax trifoliatus (L.) Voss.	Araliaceae	–	Chen et al. (1972)
	Leptadenia reticulata (Retz.) Wight & Arn.	Asclepiadaceae	–	Dhalani et al. (2019)
Nonacos-1-ene	[a]*Ganoderma lucidum*	Mushroom	Antiviral	Sangeetha et al. (2020)
	Balsamorhiza sagittate (Pursh) Nutt.	Heliantheae	–	Bohlmann et al. (1985)
	Syzygium dhaneshianum Ratheesh, Shareef & Nandakumar	Myrtaceae	–	Krishna et al. (2017)
	[a]*Colchicum autumnale* L.	Colchicaceae DC.	Antioxidant	Hailu et al. (2021)
	[a]*Hyssopus officinalis* L.	Lamiaceae	Chemical constituent of essential oil	Wesolowska and Jadczak (2018)
Carbonic acid	[a]*Halimeda* spp.	Macro-algae	Antibiofouling efficiency	Prasanth and Suresh Kumar (2020)
	[a]*Halimeda* spp.	Macro-algae	Skin cancer	Mahesh et al. (2021)

a Indicates that compounds were identified as a constituent of an active extract or as a mixture (stigmasterol).

3.3 Antimycobacterial effect of compounds

The isolated compounds had no inhibitory effect (MIC>0.500 mg/ml) against *M. smegmatis* at the highest concentration tested, except for nonacosene which had poor activity (MIC = 0.375 mg/ml) (Table 4). The antimicrobial activity of an isolated compound is significant if the MIC is 10 µg/ml or lower, moderate if MIC is between 10 and 100 µg/ml and low if MIC > 100 µg/ml (Kuete, 2010). The fractions were more active against *M. smegmatis* and the other mycobacterial strains (Table 1) with MIC values as low as 0.015 mg/ml. The MIC of the positive controls was 0.0063 and 0.0016 mg/ml (rifampicin and streptomycin, respectively). It is often reported that isolated compounds are generally less active than the crude extracts or fractions (Lewis and Ausubel, 2006). This implies that the high antimycobacterial effect observed with the extracts and fractions is most likely a result of synergy amongst the compounds of the extract and fractions (Madikizela and McGaw, 2018). The compounds could also utilize a different antimicrobial mechanism of action apart from bacteriostatic and bactericidal effect i.e. biofilm inhibition or quorum sensing (Lewis and Ausubel, 2006). Stigmasterol had no inhibitory effect on *M. smegmatis* but a previous report revealed that stigmasterol isolated from *Morinda citrifolia* had antimycobacterial effect against H_{37}Rv with the BACTEC method (Table 2).

Table 4. Minimum inhibitory concentration of compounds against *M. smegmatis*.

Compound	MIC (mg/ml)
107 (1)	>0.500
55 (stigmasterol)	>0.500
124 (4) (carbonic acid)	>0.500
124 (2)	>0.500
124 (3) (simiarenol)	0.250
107 (21)	>0.500
107 (2)	>0.500
63 (hentriacontane)	>0.500
50 (nonacosene)	0.375±0.18
Rifampicin	0.0063±0.00
Streptomycin	0.0016±0.00

3.4 Cytotoxic effect of fractions and compound

The cytotoxic effect of the fractions is presented in Table 5. A plant extract is considered highly cytotoxic when the LC_{50} is 0.020 mg/mL and below (Kuete et al., 2011). The LC_{50} of the ethyl acetate, methanol:chloroform and acetone fractions was >0.02 mg/ml (0.03 mg/ml) and hexane LC_{50} was 0.79 mg/ml. Doxorubicin, the positive control had an LC_{50} value of 0.010 mg/ml (Table 5). The selectivity index (SI) of each extract was calculated as the ratio of cytotoxicity (LC_{50}) to activity (MIC), and indicates the selective toxicity of the extract. The higher the SI value, the safer the extract (Bagla et al., 2014, Oosthuizen et al., 2019). Plant extracts with SI index less than 1 imply that the extracts are more toxic to the mammalian cells than to the bacteria (Eloff, 2000). The hexane fraction had the best selectivity index values, as high as 26.33 against the mycobacterial strains, followed by the ethyl acetate fraction with a selectivity index of 2 against *M. smegmatis*. This implies the observed biological activities of the hexane fraction is more selective toward the *Mycobacterium* strains than the mammalian cells. As a result of its safety to mammalian cells and promising activity against the strains, it can be considered for further studies. Nonacosene had LC_{50} of 0.13 mg/mL and selectivity index of 0.35. This implies that nonacosene is more active against the mammalian cells than the pathogens. To the best of our knowledge, this is the first report of the cytotoxic effect of nonacosene.

Table 5. Cytotoxicity (mg/ml) and selectivity index of fractions against Vero cells.

Fractions	LC_{50}	Selectivity index					
		Ms	Mf	Ma	Mb	Mtb TB8993A	Mtb ATCC25177
Hexane	0.79±0.17	**19.752**	**9.875**	**26.33**	**26.33**	**6.58**	**9.875**
Ethyl acetate	0.03±0.0068	0.1	0.375	1	1	1.5	0.75
Methanol: chloroform (3:7)	0.03±0.0088	0.097	0.024	0.375	0.012	0.012	0.012
Acetone	0.03±0.004	0.012	0.03	0.375	0.1	0.05	0.05
Nonacosene	0.13±0.00	0.35					
Doxorubicin (Positive control)	0.010±0.00						

Ms – *M. smegmatis*, Mf – *M. fortuitum*, Ma – *M. aurum*, Mb – *M. bovis*, Mtb – *M. tuberculosis*.

Figures in bold represent promising activity.

3.5 Antioxidant and anti-inflammatory effect of fractions

The antioxidant and anti-inflammatory effect of the fractions is presented in Table 6. Extracts with IC_{50}<50 µg/ml have significant antioxidant effect, while IC_{50}>50 ug/ml signifies a moderate effect (Omisore et al., 2005). Low IC_{50} values indicate potent antioxidant activity. The ethyl acetate and acetone fractions had potent antioxidant effect in the ABTS assay (IC_{50} = 27.94 and 22.46 µg/ml respectively). In the DPPH assay, the ethyl acetate and acetone fractions exhibited a moderate effect (IC_{50} = 64.22 and 56.32 µg/ml respectively). The positive controls ascorbic acid and Trolox had IC_{50} of 1.39 and 3.35 (ABTS), and 5.95 and 5.03 (DPPH), respectively. This implies that the acetone fraction had the most potent antioxidant effect followed by the ethyl acetate fraction.

Table 6. Antioxidant and anti-inflammatory effect (IC_{50} in µg/ml) of fractions, and cytotoxicity against RAW264.7 macrophages (LC_{50} in µg/ml).

Fractions	ABTS (IC_{50})	DPPH (IC_{50})	15-LOX (IC_{50})	Nitric oxide inhibition (IC_{50})	Cellular viability (LC_{50})
Ethyl acetate	27.94±0.86	64.22±0.20	16.62±3.55	64.60±3.90	270.00±70.00
Hexane	142.92±4.99	241.24±18.51		5.30±0.80	220.00±8.00
Methanol: Chloroform (7:3)	52.16±7.85	100.63±3.85		61.80±2.80	40.00±4.00
Acetone	22.46±0.23	56.32±1.04	18.33±2.02	58.50±0.40	200.00±20.00
Ascorbic acid	1.39±0.27	5.95±0.12			
Trolox	3.35±0.90	5.03±0.08			
Quercetin			12.40±0.70		
Indomethacin				19.30±4.00	370.00±11.00

The anti-inflammatory effect of the fractions was evaluated using 15-lipoxygenase and nitric oxide inhibitory assays. Ethyl acetate and acetone fractions had potent inhibitory effect on the 15-lipoxygenase enzyme (IC_{50} = 16.62 and 18.33 µg/ml respectively) compared to the positive control (quercetin, IC_{50} = 12.4 µg/ml). The fractions also had good NO inhibitory effect (Table 6). The NO inhibition of plant extracts >70% at a concentration of 250 µg/ml is viewed as being significant (Chinsamy et al., 2014). When converted to IC_{50} values, several of the fractions had IC_{50} below 100 µg/ml which is promising. The hexane fraction had the best activity with IC_{50} of 5.3 µg/ml, better than that of the positive control indomethacin. The LC_{50} of the RAW264.7 cells was 40.0 µg/ml when exposed to the methanol:chloroform fraction, so the NO

inhibitory effect of this fraction might be due to toxicity. LC_{50} values for the other extracts were 200 µg/ml and above, indicating low toxicity to the macrophages.

Antioxidants function by removing free radicals such as reactive oxygen species, hydroxyl radicals and nitric oxide from the human body while anti-inflammatory mediators regulate the activities of pro-inflammatory enzymes and cytokines. Nitric oxide acts as a pro-inflammatory mediator and is produced by inducible nitric oxide synthase (iNOS) in response to pro-inflammatory agents such as lipopolysaccharide (LPS) and inflammatory conditions(Aro et al., 2016). NO accumulation is an important prerequisite in the pathogenesis of various inflammatory conditions, thus inhibiting its production is significant in inflammatory related ailments (Muniandy et al., 2018). Granuloma formation following exposure to *M. tuberculosis* is connected to strong inflammatory and protective responses (Jung et al., 2013). The expression of NOS2, NOS3 and nitrotyrosine (N-tyr) are all intensified in the granuloma-associated inflammatory cells and in the pneumonitis regions of human tuberculous lungs (Jung et al., 2013). Furthermore, granuloma-associated macrophages from untreated patients with pleuropulmonary and pulmonary TB demonstrate high levels of NOS2-mediated NO production and N-tyr (Schön et al., 2004). Therefore, inhibition of nitric oxide production in tuberculosis infection could represent an important step in tuberculosis chemotherapy. Many compounds from medicinal plants are known inhibitors of iNOS expression in LPS-activated macrophages (Son et al., 2000). Extracts with good nitric oxide inhibitory effect and low cytotoxicity are more useful as potential anti-inflammatory agents (Chinsamy et al., 2014). The fractions could be beneficial as potential sources or leads in treating inflammatory-related ailments such as tuberculosis coupled with the potent antimycobacterial effect demonstrated by the hexane and ethyl acetate fractions.

3.6 Antibiofilm effects of fractions and compounds

Most of the fractions and compounds had excellent biofilm inhibitory and dispersion effects. According to previous studies by Sandasi et al. (2011), inhibition values above 50% denote good activity, while activity is poor if it is below 50%. The percentage biofilm inhibition and dispersion effect of the fractions ranged between 80 – 100%, and 50 – 100%, respectively. The biofilm inhibition effect of the compounds ranged between 90 – 100% (Table 7). The inhibitory and dispersion effect of rifampicin, the positive control, was 81 and 80%, respectively Based on these criteria, the fractions and compounds have good antibiofilm effect. *M. tuberculosis* has the ability to persist in stressful situations such as avoiding the host immune response and chemotherapy by sequestering within the infected host macrophages at the early stages of tuberculosis infection to form multicellular communities called biofilms (Bhunu et al., 2017, Nguyen et al., 2019). Extracts and compounds with good biofilm inhibition or dispersion effects may be used in synergy with current tuberculosis chemotherapy to shorten

its generally long treatment duration (Nguyen et al., 2019). Therefore, the fractions and compounds can potentially be used in synergy with rifampicin or any of the other tuberculosis first line drugs to break their persistence, thereby reducing the duration of chemotherapy. The compounds can also serve as leads for treatments against opportunistic infections or rapidly growing mycobacterial strains. To the best of our knowledge, this is the first report on the biofilm inhibitory effect of the compounds. Previous reports on hentriacontane revealed that it had cytotoxic, anti-inflammatory and immune-modulatory effects (Table 2). Nonacosene is known to have antiviral and antioxidant activity (Table 2).

Table 7. Biofilm inhibition (IC), dispersion effects (EC) and minimum inhibitory concentration (mg/ml) of fractions; biofilm inhibition effect of compounds.

Fractions and compounds	IC (%)	EC (%)	MIC *M. smegmatis* (mc^2 155)
Hexane	96.86±0.36	92.77±3.54	0.16
Methanol: Chloroform (3:7)	79.85±6.14	100.0±1.30	>2.5
Acetone	100.13±5.85	100.0±6.90	0.625
Ethyl acetate	100.0±10.73	55.45±3.49	0.16
Nonacosene	99.33±2.30		
Hentriacontane	100.0±0		
Rifampicin	81.14±0.55	80.40±0.87	0.0047
Ciprofloxacin			0.00003

3.7 Anthelmintic effect of nonacosene

The anthelmintic effect of nonacosene against *Caenorhabditis elegans* is presented in Table 8. Nonacosene exhibited the highest mortality effect at 48 h (0.5, 0.3, 0.25; 50 – 80% mortality, moderately active), at 2 and 24 h, (all concentrations; 20 – 50% mortality, with reduced activity). Levamisole, the positive control (10 µg/ml) exhibited 50 – 80% mortality at 2, 24 and 48 h. Generally, nonacosene had a moderate effect on *C. elegans*. Nonacosene could serve as a lead that inhibits the pathogenesis of tuberculosis in view of its biofilm inhibitory and anthelmintic effect as well as the connection between the indication of helminth infection and pathogenesis of tuberculosis. The result supports the traditional use of the plant for helminth or parasitic infections. The investigation of the compound with an additional parasitic test organism such as *Haemonchus contortus* might be beneficial. Many commercially available anthelmintics have inhibitory effects on *C. elegans*, which reinforces its use as a model for anthelmintic drug screening (McGaw et al., 2007b). The difficulty in correlating *in vitro* results

with *in vivo* data as well as a lack of acknowledgment of the complexity of the infectious process is a major drawback (Aremu et al., 2012). To the best of our knowledge, the anthelmintic effect of nonacosene has not been reported.

Table 8. Anthelmintic effect of nonacosene.

Nonacosene (at different conc. in mg/ml)	2h			24h			48h		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
Nonacos-1-ene (0.5)	+-	+-	+-	+	+	+	++	++	+
0.3	+-	+-	+-	+	+	+	+	+	++
0.25	+-	+-	+-	+	+	+	+	++	+
0.125	+-	+-	+-	+	+-	+	+	+	+
Levamisole 10μg/ml	+	+	+	++	++	+	++	++	++
Solvent control	–	–	–	–	–	–	+-	–	–
Blank	–	–	–	–	–	–	+-	–	–

R1, R2, R3 – replicates. Scoring system: "-" - Nematodes (100%) are alive and active, "+-" - 80% alive and reduced activity, "+" - 50% alive and not active, "++" - 20% alive and not active, "+++" - 5–10% alive, motility is very slow, "++++" - all dead.

4. Conclusion

Bioassay guided fractionation of the acetone extract of the leaves and stems of *C. mucronata* yielded five compounds identified as nonacosene, hentriacontane, stigmasterol, simiarenol, and carbonic acid. The compounds had little to no direct bacteriostatic effect, but inhibited mycobacterial biofilms, signifying the potential ability of the compounds to inhibit the persistent nature of mycobacterial infections. Extracts or compounds with good antibiofilm effect can be used in synergy or as adjunct therapy with current tuberculosis drugs to reduce the usually lengthy tuberculosis chemotherapy. To the best of our knowledge, this is the first report on the dual antibiofilm and anthelmintic effect exhibited by the compound, nonacosene. This is significant in view of the connection between the incidence of intestinal helminths and pathogenesis of tuberculosis. The fractions exhibited promising antimycobacterial activity coupled with excellent anti-inflammatory and antioxidant effects, which implies that *C. mucronata* could be a good source of anti-inflammatory agents in inflammation related conditions and can potentiate the effect of antituberculosis drugs when used in synergy to

reduce the effect of oxidative stress in inflammation. *C. mucronata* is also a good source of antimycobacterial agents in view of the antimycobacterial and antibiofilm effect observed with the fractions and compounds.

Author Contributions

RA conducted the experiments with the help of GF (isolation of compounds), IMF (anti-biofilm assays), FNM (fnmakhubu@gmail.com; anthelmintic assays) and SMN (cytotoxicity assays). RA wrote the first draft of the manuscript. LJM, PNK and AOA supervised the research project and provided research facilities and funding. All authors read, edited, and approved the final manuscript.

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