Potential implications of the use of *Rapanea melanophloeos* (L.) Mez against mycobacteria

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Highlights:

- Antibiofilm activity of an ethanolic extract and hexane partition from *R. melanophloes.*
- Potential herb-drug interaction associated with the use of *R. melanophloeos* and associated harmful effect.
- Antagonistic effect of the extract on a fluoroquinolone drug, ciprofloxacin.
- Emphasises and highlights the importance of herb-drug interaction studies at an early stage of drug dicovery

ABSTRACT

Tuberculosis (TB) is a threat to a large population across the globe. *Rapanea melanophloeos* (L.) Mez, commonly known as the Cape beech, is a plant that is traditionally used in the treatment of tuberculosis and TB-related symptoms. The aim of this study, was to evaluate the pharmacological effects of a leaf ethanolic extract (1:10, w:v) of *R. melanophloeos,* as well as different polarity partitions, in conjunction with a fluoroquinolone antimycobacterial drug, Ciprofloxacin. The bioassays in this study included the antimycobacterial, antibiofilm and synergistic potential against *M. smegmatis*. Secondly the antiproliferative and hepatoprotective effect on hepatocytes (HepG2), and, lastly, a potential mechanistic investigation on mycothione, glutathione and thioredoxin reductases. The hexane fraction from the ethanolic plant extract showed relatively good antibiofilm activity with a mean IC_{50} value of 128.25±4.09 µg/mL. The drug-herb interaction activity of the extract was tested against a second-line anti-TB drug, ciprofloxacin, and it was found that the plant extract acted antagonistically towards the standard drug (FIC > 4). Antiproliferative and hepatoprotective tests were conducted on HepG2 hepatocytes to test the effects on cell viability. The plant was not toxic to the cells but showed no significant hepatoprotective activity against drug-induced hepatotoxic injury, in comparison to the positive control silymarin. Furthermore, the plant extract was tested against glutathione, mycothiol and thioredoxin reductase (human and bacterial analogs) for its inhibitory activity. *Rapanea melanophloeos* showed higher affinity for the glutathione and mycothiol reductase with relatively high inhibitory activity against the enzymes. The findings from this study indicate the potential adverse effects and drug-herb interactions, associated with the use of *R. melanophloeos* in the treatment of TB. In addition, this study emphasizes the importance of herb indications and drug interactions, and the term "natural" or "herbal" does not coincide with safe.

Keywords: antimycobacterial, biofilm inhibition, cytotoxicity, drug-herb interaction, enzyme inhibition, glutathione reductase, hepatoprotective, mycothione reductase, synergistic activity, thioredoxin reductase.

1. Introduction

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis*, has existed for centuries and affects a large part of the population across the globe. According to the World Health Organization (WHO), this disease affected an estimated 10 million people, with a mortality of 1.5 million in 2018 and remains one of the top ten causes of death. TB has been considered as the leading cause of death by a single infectious biological agent and has managed to rank above the human immunodeficiency virus, acquired immunodeficiency syndrome (HIV/AIDS). This is despite the diagnosis of the disease in a timely manner and having treatment for TB available. More challenges have risen due to the growing occurrence of resistant TB strains (World Health Organization, 2019). *Mycobacterium smegmatis* is often used as a model in tuberculosis studies due to its faster growth rate and experimental usage in lower biosafety level laboratories. As a nontuberculous mycobacterial species, its ability to assemble biofilms on various surfaces plays a role in their persistence and pathogenesis (Faria et al., 2015).

Biofilms are described as being microbially derived sessile communities characterized by cells that have attached to a substratum or each other. Microorganisms that grow biofilms are associated with chronic and reoccurring infections that are highly resistant to antimicrobial drug treatments (Hassan et al., 2011). Enzymes such as mycothiol and S-nitromycothiol reductase are important for biofilm formation in *M. smegmatis* (Vargas et al., 2016). Furthermore, biofilm enzymatic systems play a role in ensuring bacterial growth survival within the host, as well as the persistence of certain infections. To overcome oxidative stress by the host and ensure survival, many bacterial and mycobacterial species have systems that mitigate the effects of the host's defence mechanism. An enzyme that can be considered as a drug target of interest in this regard, is thioredoxin reductase. The thioredoxin system is often coupled with glutathione reductase in most bacterial species, however, mycobacteria lack the glutathione system and mycothiol reductase is the substitute (Kumar et al., 2011; Lin et al., 2016).

Plants have been used in several communities to treat various diseases and have been the test subject of many research investigations to determine their properties that are suitable for use as part of medicinal treatment. The use of traditional herbal medicine has expanded across the globe and continues, to remain a societal norm as a rich source of therapeutic agents. However, in modern society, the combination of both traditional herbal medicine and conventional Western medicine has become increasingly popular. With the use of whole plants or plant parts and their health-promoting benefits, the potential drug-herb interactions of the combinations are often not taken into consideration (Mehmood et al., 2018). Drug interactions can be defined as induced modifications by interactions with exogenous chemicals (from other drugs, food or herbs) which may result in a significant effect on pharmacodynamics and/or pharmacokinetic profiles of the drug. These effects may inherently influence the physiological responses within the body (Alissa, 2014; Cordier and Steenkamp, 2011). In the case

of TB treatment, the use of herbal medicines and the associated herb-drug interactions need to be assessed at an early stage of herbal drug discovery.

Rapanea melanophloeos (L.) Mez, commonly known as Cape Beech, is an indigenous plant from the Myrsinaceae (Primulaceae) family. The plant has been used to treat various illnesses and conditions including respiratory problems, stomach and heart ailments, and it has been used as part of tuberculosis treatment (Xaba, 2005; Famewo et al., 2017). This plant is especially used in poly-herbal mixtures to treat Tuberculosis, and a study by Famewo et al., (2017), showed water preparations, from a mixture containing *R. melanophloeos*, having MIC values lower than 1.56 µg/mL. Contrary to their findings, a study testing the water and acetone extracts showed no activity from the water extract with an MIC value of 5 mg/mL for the acetone extract (Lall and Meyer, 1999). In a separate study, an acetone extract showed MIC values of 0.31 mg/mL (selective index (SI) of 0.87) and 0.16 mg/mL (SI of 1.74) on *M. smegmatis* and *M. tuberculosis*, respectively (Dzoyem et al., 2016). These findings indicate a broad range of reported activities as well as the potential toxicity linked to the acetone extract. Ethanol was selected as the extractant in this study to increase the extraction of medium and non-polar phytochemicals as well as to reduce the potential cellular toxicity.

This study aimed to evaluate the potential adverse effects of the use of *R. melanophloeos* ethanolic extract against planktonic and biofilm-forming *M. smegmatis,* as well as the antiproliferative vs hepatoprotective effect on the HepG2 liver cell line. Secondly, the combinatorial use of the plant extract together with the known drug, ciprofloxacin, was also assessed to evaluate any potential drug-herb interactions. Lastly, to determine a probable mechanism and further assess potential interactions, the extract was evaluated for its inhibitory activity against glutathione, mycothione and thioredoxin reductases.

2. Materials and methods

2.1. Chemicals and reagents

Solvents used for this study were purchased from Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa) and other reagents used were purchased from Sigma Aldrich (St. Louis, Missouri, United States).

2.2. Collection, identification, and preparation of plant material

Aerial parts (leaves and shoots) of *R. melanophloeos* were collected from the Manie van der Schijff Botanical Gardens located at the University of Pretoria. The identification and authentication were done at the H.G.W.J. Schweickerdt Herbarium at the University of Pretoria. Information regarding the sample name and herbarium voucher specimen numbers is provided in Table 1.

Table 1
Plant collection information

Plant name	Location	GPS co-ordinates	Specimen number
Rapanea melanophloeos (L.) Mez	Manie van der Schijff Botanical Gardens University of Pretoria	25°45'05.4"S 28°13'46.1"E	PRU [a]119005

[a] H. G. W. J. Schweickerdt Herbarium, University of Pretoria

Drying of the collected plant material was done at room temperature for 12 days and ground to a homogenous fine powder of 0.4 mm particle size using an IKA MF 10.1 mill (IKA®-Werke GmbH & Co. KG, Germany). A double exhaustive extraction of the ground plant material was done using absolute ethanol (96%) as the menstruum in a 1:10 (w:v) ratio and shaken for 48 hours at 170 rpm with the use of a Labcon 3086 U shaker. Ethanol was used as an extractant due to its broader spectrum of extraction of polar and non-polar phytochemicals as well as reduced toxicity due to residual solvent in the final extract. The filtrate was collected using a Büchner funnel and concentrated using a rotary evaporator to obtain a dry crude plant extract (Lall et al., 2016; Oosthuizen et al., 2018). The ethanolic extract was further separated into five partitions using liquid-liquid partitioning with the following solvents (polarity index): Hex (0), DCM (3.1), BuOH (4), EtOAc (4.4) and H_2O (9). The fractions were concentrated in vacuo and stored at 4 C until further testing.

2.3. *In vitro antimycobacterial activity*

The antimycobacterial activity of *R. melanophloeos* and its five fractions were tested using the microtitre Alamar blue assay method, as described by Collins & Franzblau (1998) with minor modifications as described by Lall et al., (2013). Briefly, 100 uL of 7H9 (supplemented with 0.4% glycerol, 0.5% Tween 80) broth was dispensed in each well of a sterile flat-bottom 96-well plate. Thereafter, 100 uL of the ethanolic plant extract, each fraction, and the positive control, ciprofloxacin, was added to the first row of wells followed by a 2-fold dilution resulting in a test concentration range of 15.6 to 1000 µg/mL for the plant sample and its fractions, and 0.08 to 5 µg/mL for ciprofloxacin. A negative, untreated bacterial and solvent control (DMSO 2.5%) were included in the assay. The *M. smegmatis* bacterial inoculum was prepared to a McFarland of 0.5 and diluted down to 1.5×10^6 cfu/mL. A hundred microliters of the inoculum was added to all the wells, except the negative control, to yield a final assay volume of 200 uL After a 24 hour incubation period at 37°C, 20 uL of Presto blue was added to the wells and incubated for 3-4 hours. The MIC values were defined as the lowest concentration where no colour change occurred from blue to pink by the conversion of resazurin (blue) to resorufin (pink). The absorbance was read using a BioTek PowerWave™ XS microplate reader and, quantitative analysis was conducted using KC Junior™ to determine the cell viability at a wavelength of 570 nm and a reference wavelength of 600 nm. The concentration where 50% of the bacterial growth was inhibited (IC_{50}) was determined using GraphPad Prism 4™.

2.4. *In vitro antibiofilm activity*

A microtitre assay was conducted in a sterile flat bottomed 96 well plate for the determination of biofilm formation inhibitory activity. Firstly, 100 µL of 7H9 media, omitting the addition of Tween 80 to the media, was added to the plates. The subsequent addition of 100 µL of the plant extract, its five fractions, and the positive control, ciprofloxacin, was added to the first row of the wells followed by a 2-fold serial dilution yielding a test concentration range of 15.6 to 1000 µg/mL and 0.08 to 5 µg/mL for the plant samples and ciprofloxacin, respectively. For the *M. smegmatis* inoculum, a 0.5 McFarland was prepared (OD_{600} of 0.06) and diluted to 1.5 x 10^6 cfu/mL. A negative, untreated bacteria and solvent (DMSO 2.5%), control were included in the assay (Ishida et al., 2011; Oosthuizen et al., 2018). The plates were incubated for 3-4 days at 37°C. The minimum biofilm formation inhibitory concentration (MBFIC) was determined by observation of the minimum concentration at which no visible biofilm formation could be observed.

The biomass of the biofilm was quantified using the crystal violet staining method (O'Toole, 2011). Briefly, after the determination of the MBFIC's, the plates were dried at 45ºC for 48 hours. Two hundred microliters of sterile distilled water was used to wash away residual planktonic bacteria. The plates were left to air-dry for 10 minutes followed by fixation with, 200 µL of methanol. The methanol was aspirated and 200 µL of a 0.5% crystal violet solution was added to each well. The plates were left for 10 minutes for sufficient intercalation of the crystal violet into the biofilm structure. The plates were washed three times as described above. Finally, the bound crystal violet was recovered by the addition of 200 µL of 96% ethanol. The absorbance was read at 600 nm using a BioTek PowerWave™ XS microplate reader and the absorbance values were obtained from KC Junior™. The effective concentration where 50% of the biofilm formation was inhibited was calculated using a 4-parameter logistic equation.

2.5. *In vitro interaction studies*

The antibiotic, ciprofloxacin, was used in combination with the plant extract at varying ratios to analyze the interaction effect between the ethanolic plant extract and the antibiotic. Briefly, 100 µL of 7H9 Middlebrook media, was added to all wells of a sterile, clear, flat-bottomed microtitre 96 well plate. The plant sample was prepared at a stock concentration of 20 000 µg/mL, with 100% DMSO as the diluent, and ciprofloxacin prepared at a stock concentration of 20 µg/mL resulting in the highest test concentration at 1000 µg/mL and 5 µg/mL, respectively in the wells. The plant sample and ciprofloxacin were added at different ratios of 10:0, 9:1, 8:2, and 7:3 up to 0:10 into the first row of the wells with the final volume of each ratio mixture and media reaching 200 µL. Thereafter a 2-fold serial dilution was conducted. A negative control and untreated bacterial controls were added while ciprofloxacin (5 µg/mL) was used as the positive control. The *M. smegmatis* bacterial inoculum was

prepared at a McFarland of 0.5 and diluted down to 1.5×10^6 cfu/mL and 100 µL of the bacterial preparation was added to each well except the media control. The final assay volume was 200 µL. The plates were incubated at 37°C for 24 hours. The reagent Presto blue was added and after 3-4 hours, the absorbance was read at a wavelength of 570 nm (reference 600 nm) and analyzed using a BioTek PowerWave™ XS microplate reader and the KC Junior™ software.

2.6. *In vitro antiproliferative activity*

The antiproliferative activity of the ethanolic plant extract was conducted as described by Lall, 2016. Briefly, human liver cells, HepG2 (ATCC HB-8065), were cultured until 90% confluency at 37ºC and 5% CO_2. The cells were plated using 100 µL at a concentration of 2000 cells/mL in Dulbecco's Modified Eagle's Medium (DMEM), as the media. A negative control, containing 200 uL was also included to serve as the 0% cell viability control. The outer moats of the sterile well plates were filled with 2 mL of sterile distilled water to ensure that a humid environment is created during incubation and to compensate for evaporation. In a separate 24 well plate, a 2-fold sample dilution for both the plant extract and the positive control, actinomycin-D, were prepared separately and 100 µL of each dilution was added/transferred to the 96-well plates in triplicates. The final test concentrations ranged from 3.12 to 400 µg/mL and 0.05 to 3.90×10^{-4} µg/mL for the sample and positive control, respectively. A negative, solvent (2% DMSO) and an untreated cell control were included in the assay. The plates were incubated for 24 hours followed by the addition of 20 µL of Presto blue to all the wells. After 3 – 4 hours of incubation, the absorbance was read at a wavelength of 570 nm (reference 600 nm) and analyzed using a microtitre plate reader (Perkin Elmer, Victor Nivo). The concentration where 50% of the cell growth was inhibited was calculated using a 4-parameter logistic equation.

2.7. *In vitro hepatoprotective activity*

Liver, HepG2, cells were cultured for the hepatoprotective assay (Lall et al., 2016). In a 24 well plate, the plant sample dilutions and the positive control silymarin, a known hepatoprotective agent, were prepared. The plant samples were tested at different concentration of 150, 50, 10, and 5 µg/mL. In the 96-well plate, 50 µL of each prepared dilution was added in 6 wells. Thereafter 50 µL of acetaminophen was added to yield a final assay concentration of 25 mM and final volume of 200 µL. A positive (silymarin at 50 µg/mL) control, media, cell control, and negative (acetaminophen (25 mM)) control was added to the assay. The plates were incubated for 24 hours and thereafter 20 µl of Presto blue was added to all the wells. Absorbance values were obtained by use of a microtitre plate reader (Perkin Elmer, Victor Nivo) at a wavelength of 570 nm with a reference wavelength at 600 nm.

2.8. *Mechanistic studies*

The enzymatic inhibitory activity of the ethanolic plant extract was tested on thioredoxin reductase (TXR) from *E. coli* and human TXR, mycothione (MTR) and glutathione reductase (GTR) using experimental procedures described by Hamilton et al., (2009) and modified by Oosthuizen et al., (2018). The enzymatic inhibition assays consisting of 50 mM HEPES and 1 mM EDTA buffer (pH 7.6), 140 μM NADPH, 600 μM DTNB, mycothiol or glutathiol substrate, and varying concentrations of the ethanolic plant extract was carried out in 96-well plates for each enzyme with a final assay volume of 200 μL. The plant sample, dissolved in DMSO, was tested at different concentrations ranging from 0.01 to 1000 μg/mL. The plate was left to incubate for 5 minutes at a temperature of 37°C. The reaction was initiated by the addition of the mycothiol (1 μM), glutathiol (60 μM), or DTNB (600 μM) as the substrate for MTR, GTR and TXR respectively. The relative rates of each enzyme were determined by measuring the change in absorbance at 412 nm.

2.8.1. *Enzyme kinetics*

The relative rates of each enzyme were tested using the same method described above but varying concentrations of substrate for GTR (60 – 1.88 μM), MTR (5 – 0.004 μM), and TXR (100 – 0.10 μM) The enzymes were tested both with and without the ethanolic plant extract (at the IC_{50}) at constant concentrations with varying substrate levels. The change in absorbance at 412 nm was recorded and the Michaelis Menten constant (Km) and maximum velocities (Vmax) were calculated according to the following equation:

$$y = Vmax[Sub]/(Km + [Sub])$$

2.9. *Statistical analysis*

All data were analysed using GraphPad Prism 4. Sigmoidal dose-response curves were analysed using a 4-parameter nonlinear regression (y = Top + (Bottom - Top / [1 + (x / IC_{50}) ^ hillslope]). The absolute IC_{50} values were calculated with constraints on the top and bottom parameters (Top = 100 and Bottom = 0). One-way analysis of variance (ANOVA) was performed with Tukey's multiple comparison tests as a post-hoc analysis. A *p*-value lower than 0.05 was considered to be statistically significant. All data are expressed as Mean ± SEM.

3. Results and discussion

The use of plant material to treat diseases and conditions plays an integral role in South Africa's local communities and across Africa. Intensive studies, though limited, have been conducted on *R. melanophloeos,* contributing to the validation of its use in traditional medicine and in particular, against tuberculosis (Lall and Meyer, 1999; McGaw et al., 2008; Lukhele, 2009; Famewo et al., 2017).

Various extracts from *R. melanophloeos*, have exhibited biological activity including antifungal, antimicrobial, antiviral activity and, in addition to this, has been considered safe for use according to toxicity studies (Ohtani et al.,1993; Steenkamp et al., 2007; Amenya et al., 2014; Mehrbod et al., 2018).

3.1. Antimycobacterial vs antibiofilm activity

The ethanolic extract of *R. melanophloeos*, its fractions and the positive control, ciprofloxacin, were tested for their *in vitro* antimycobacterial and antibiofilm inhibitory activity against *M. smegmatis.* The susceptibility tests conducted are presented in Table 2. The order of the fraction activities is as follows: Hex > EtOAc > BuOH > DCM = Water. The hexane fraction exhibited the highest antimycobacterial activity with an MIC of 1000 μg/mL and an IC_{50} value of 644 μg/mL. The reference drug, ciprofloxacin, exhibited an IC_{50} value of 0.417 μg/mL in the test against *M. smegmatis.* According to a study conducted by Lall & Meyer, 1999, the water extract of the bark of *R. melanophloeos* showed no activity while the acetone extract exhibited antimycobacterial activity with an MIC value of 5000 μg/mL. Dzoyem et al. (2016) found a slightly better activity with an MIC value of 312 g/mL from an acetone extract. In the present study, the hexane fraction from the ethanolic extract exhibited relatively better antimycobacterial activity, indicating that the antimycobacterial activity relates to the non-polar partition. This might also explain the slightly better activity reported for the acetone extract, as acetone has a slightly lower polarity index when compared to ethanol. It should be noted that the use of *M. smegmatis* as a model, despite its questionable efficacy has contributed to significant advances in anti-TB drug discovery such as the discovery of the DARQ R207910 mycobacterial agent with an MIC equal to or lower than reference drugs (Andries et al., 2005).

Table 2
Antimycobacterial and antibiofilm activity of *R. melanophloeos* against *M. smegmatis*

Samples	**MIC[a] (μg/mL)**	**IC_{50}[b]±SD[c] (μg/mL)**	**MBFIC[d] (μg/mL)**	**IC_{50}[e]±SD (μg/mL)**	**SI[f]**
Rm ethanolic extract	>1000	>1000	500	462.56±46.75	2.2
Hex Fraction	1000	644±59.82	250	128.25±4.09	7.8
DCM Fraction	>1000	n.a	>1000	n.a	-
BuOH Fraction	>1000	> 1000	>1000	>1000	-
EtOAc Fraction	1000	> 1000	1000	>1000	-
Water Fraction	>1000	n.a	>1000	n.a	-
Ciprofloxacin	0.625	0.417±0.29	0.303	0.257±0.02	2.4

[a] Minimum inhibitory concentration, [b] Inhibitory concentration where 50% of the bacterial growth was inhibited, [c] Standard deviation [d] Minimum biofilm formation inhibitory concentration, [e] Inhibitory concentration where 50% of the bacterial biofilm growth was inhibited, [f] Selectivity index = ($^{MIC}/_{Biofilm}$ IC_{50}), [g] No activity

Mycobacteria have a tendency to form complex exopolymer structures called biofilms that aid in the restriction of the diffusion of substances and bind antimicrobials which substantially increases drug tolerance of several bacterial species (Al-refi, 2016; Oosthuizen et al., 2018). Biofilms have therefore

been considered an attractive drug target for various studies. The hexane fraction showed the best antibiofilm activity. The minimum biofilm formation inhibitory concentration was determined as the lowest concentration at which biofilm formation was completely inhibited. The hexane fraction exhibited antibiofilm activity with an MBFIC of 250 µg/mL (

Fig. 1) and an IC_{50} of 128.25±4.09 µg/mL. Ciprofloxacin exhibited an MBFIC of 0.30 µg/mL and an IC_{50} of 0.26 µg/mL (SI = 2.4). A microscopic view of the formation of biofilm is depicted in Fig. 2. This indicates that the fraction showed selectivity towards biofilm inhibition when compared to antimycobacterial activity (selective index (SI = MIC/IC_{50}) of 7.7). This finding indicates the potential adjuvant activity of *R. melanophloeos* more so than a direct mycobacterial inhibition. Many plant extracts have been reported to exhibit potent antibiofilm activity against several other bacterial species. At concentrations of 2 µg/mL, the *Capparis spinosa* (caper bush) significantly inhibited biofilm formation in bacterial species such as *E. coli., Pseudomonas aeruginosa, and Serratia marcescens* (Sadekuzzaman et al., 2015).

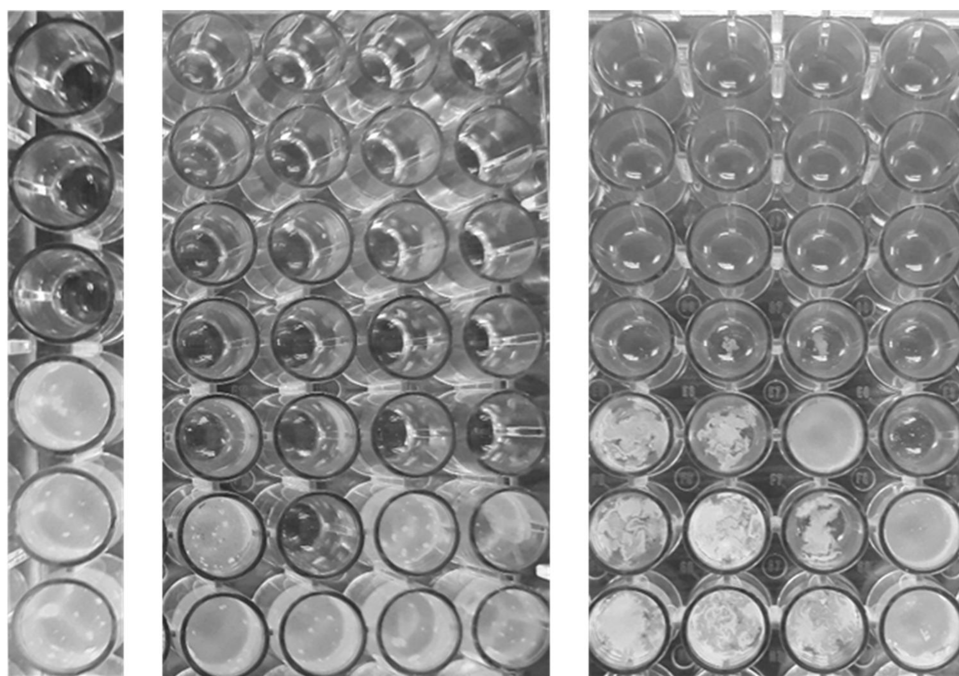

Fig. 1. A) Media and bacterial biofilm formation control. B) Ciprofloxacin positive control at a maximum test concentration of 5 µg/mL. C) Hexane fraction of the ethanolic *R. melanophloeos* at a maximum test concentration of 1000 µg/mL.

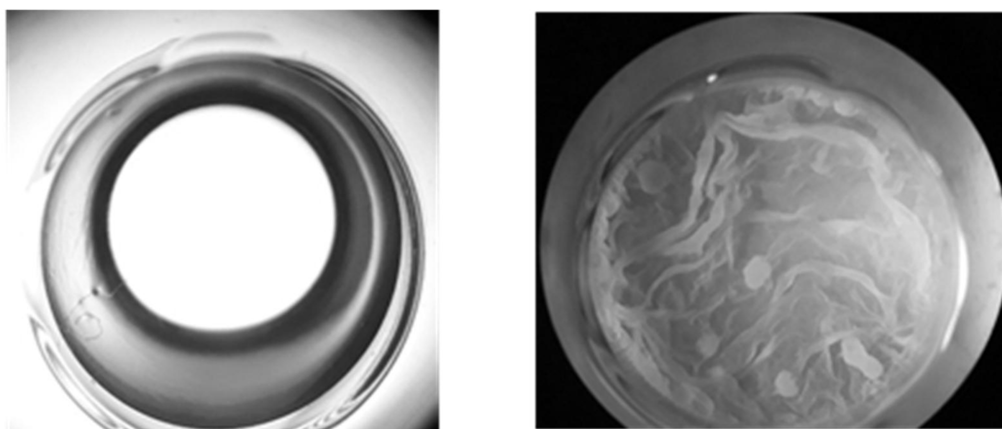

Fig. 2. No biofilm formation under light microscopy of bacterial control well. (Left). Biofilm matrix formation view under light microscopy (Right).

3.2. Drug-herb interaction

Combinational drug treatment with two or more drugs is a standard part of treatment for several diseases or infections. Strategies to produce the most effective combinations of drugs are necessary for the limitation of antimicrobial resistance development and illustrates the effectiveness of synergism between drug treatments (Xu et al., 2018). The dilution method was used to determine the interaction activity between the plant extract and the fluoroquinolone drug, ciprofloxacin. The fractional inhibitory concentration (FIC) was calculated as described by Jenkins & Schuetz, (2012). The FIC was calculated by comparing the MIC of the plant sample in combination with ciprofloxacin to the MIC of the plant sample alone. The ΣFIC was calculated as:

$$\sum FIC = FIC\ of\ agent\ A + FIC\ of\ agent\ B$$

Synergy is defined as having an $\Sigma FIC \leq 0.5$. Indifference between the plant samples is defined as $0.5 < \Sigma FIC \leq 4$. Antagonism is defined as having an $\Sigma FIC > 4$. The data (Table 3) indicate that at higher concentrations of the plant extract, the efficacy of ciprofloxacin was significantly decreased. This finding reveals that there is a potential of antagonism with other fluoroquinolone drugs in TB treatment. This would suggest caution in administering the plant with certain drug treatments. Synergistic activity with other plants may, however, counteract the negative effects of *R. melanophloeos*. Studies done by Famewo et al. (2017) show that *R. melanophloeos*, and a concoction of other plant species, have antimycobacterial activity against both drug-sensitive and drug-resistant *M. tuberculosis* strains hinting at the possible synergistic effect that may contribute to its high antimycobacterial activity. Further investigation for its synergistic or antagonistic effects on anti-TB drugs is however needed for future aspects.

Table 3
Summary of ΣFIC and synergistic activity between *R. melamophloeos (Rm)* and ciprofloxacin

Dilution Ratio (*Rm*/Cip)	**ΣFIC[a]**	**Effect**
9:1	9	Antagonistic
8:2	9	Antagonistic
7:3	9	Antagonistic
6:4	4.5	Antagonistic
5:5	4.5	Antagonistic
4:6	2.25	Indifference
3:7	2.25	Indifference
2:8	2.25	Indifference
1:9	2.25	Indifference

[a]Sum of the fractional inhibitory concentration

3.3. Antiproliferative activity

Rapanea melanophloeos showed low antiproliferative activity against HepG2 liver cells with an IC_{50} value of 319.5 ± 16.2 µg/mL (**Error! Reference source not found.**). This IC_{50} value was substantially higher than the positive control (IC_{50} 4 x10^{-2} ± 1x10^{-3} µg/mL). A value higher than 200 µg/mL was considered as having low to no toxicity (Kuete and Efferth, 2015). The HepG2 cells were therefore still viable in comparison to actinomycin D which exhibited a low IC_{50} value below the highest test concentration of 0.025 µg/mL. Induced toxicity, by the addition of a toxic inducing agent, acetaminophen, on the HepG2 cells were tested against the plant extract to determine the potential hepatoprotective effect. A concentration range lower than half the IC_{50} were selected to assess the protective effect. One-way ANOVA tests revealed that the various test concentrations were significantly different from acetaminophen and silymarin. All of the tested concentrations showed a protective effect with the exception of 150 µg/mL, which showed no added effect. Silymarin showed greater hepatoprotective activity at 54% compared to the various concentrations of the extract which varied between 6 – 9% (Fig. 4). Although some hepatoprotective effect has been observed at lower concentrations, the data suggest again that caution should be taken in the usage of this plant in combination with other drugs. Depending on the dose and pharmacodynamics properties of the extract and the associated absorption and metabolism profiles, serum concentrations can potentially lead to a concentration that could lead to an increase chance of drug-induced liver injury. This is especially true if the glutathione reductase inhibition by the extract is considered.

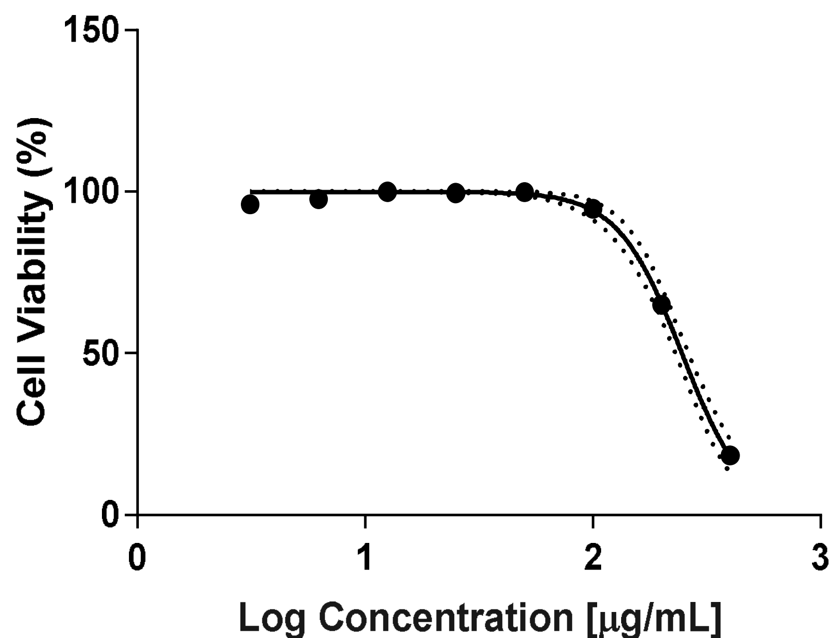

Fig. 3. Cytotoxic effect of *R. melanophloeos* on the cell viability of HepG2 cells tested at the highest test concentration of 400 µg/mL.

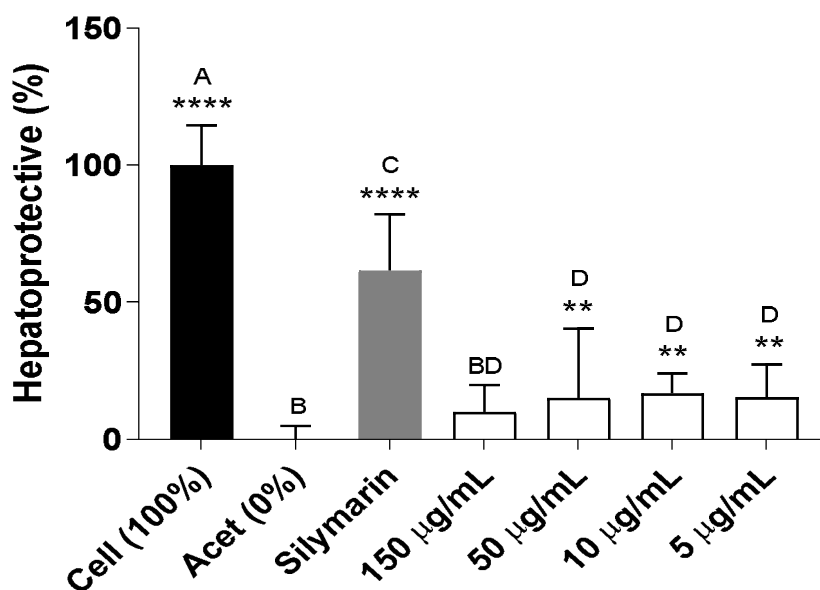

Fig. 4. The hepatoprotective activity of various concentrations of *R. melanophloeos* against acetaminophen (Acet at 25 mM) toxic induced HepG2 cells, with silymarin (50 µg/mL))as positive control. Data is represented as mean ± SEM. One-way ANOVA, with Tukey post-hoc analysis, p-value < 0.05. ** = $p < 0.01$, **** = $p < 0.0001$ when compared to the acetaminophen. Letters A-D indicate a statistical significance of p-value < 0.05.

3.4. Enzymatic inhibition

The extract was tested for its inhibitory activity against human and bacterial thioredoxin reductase (Txr), mycothiol disulfide reductase (Mtr) and glutathione reductase (Gtr). An inhibitory concentration at which 50% of the enzyme (IC_{50}) was inhibited was calculated for each enzyme (Table 4).

R. melanophloeos had a relatively higher affinity for Gtr and Mtr as oppose to Txr. From the Txr enzymes, the plant extract exhibited a slightly higher affinity for the human Txr in comparison with the bacterial isolated Txr.

Table 4
Results summary of the inhibition and kinetic properties of *R. melanophloeos* on the bacterial and human analogs of thioredoxin reductase as well as the mycobacterial mycothione and human glutathione reductases.

	***E. coli* TXR**[f]	**Human TXR**	***M. tuberculosis* MTR**[g]	**Human GTR**[h]
		Inhibition		
IC_{50}[a]±SE[b] (µg/mL)	262.8 ± 48.4	> 1000	11.60 ± 1.67	1.57 ± 0.21**[j]
		Kinetics		
Substrate K_m[c] ± SE (µM)		-	13.26 ± 2.71	23.45 ± 2.24
Inhibitor K_m ± SE (µM) @ IC_{50}[d]	0.38 ± 0.1974	-	14.82 ± 4.51	32.84 ± 2.82
Substrate Vmax[e] ± SE (µOD/min)		-	34.66 ± 5.28	121.3 ± 3.37
Inhibitor Vmax ± SE (µOD/min) @ IC_{50}	190.20 ± 15.66	-	19.68 ± 4.59*[i]	83.39 ± 2.30***[i]

[a]Inhibitory concentration where 50% of the enzyme was inhibited. [b]Standard error. [c]Michaelis constant. [d]Inhibtor tested at the calculated IC_{50} value. [e]Maximum velocity. [f]Thioredoxin reductase. [g]Mycothiol reductase. [h]Glutathione reductase. [i]Student T-test, significant difference observed when compared to natural substrate, * *p*-value < 0.05, *** *p*-value < 0.001. [j]Student T-test, significant difference observed when compared to MTR inhibition, ** *p*-value < 0.01.

An inevitable challenge that invading pathogens have to endure within a host is the endogenous and exogenous oxidative stress imposed by the host. Production of antimicrobial oxidants is a host defence mechanism against *M. tuberculosis*. Several bacterial species have enzyme systems that ensure the growth, DNA repair and replication under oxidative stress conditions and thioredoxin reductase, in particular, is extensively investigated as a drug target for antibiotics (Lu et al., 2013). Many bacteria possess both thioredoxin and glutathione systems for regulation. *M. tuberculosis,* unlike many gram-negative bacteria, does not possess the glutathione system. Mycothiol is the substitute for glutathione (Lin et al., 2016). Mycothiol reductase is functionally equivalent to Gtr in mycobacteria (Sareen et al., 2003). Similar to Gtr, Mtr is involved in the detoxification and removal of selected antibiotics through the production of mycothiol-S-conjugates cleaved by the essential mycothiol-S-conjugate amidase enzyme (Newton et al., 2011). In addition, according to previous studies, Mtr and S-nitrosomycothiol reductase have been essential in the biofilm formation in *M. smegmatis* (Vargas et al., 2016).

In the present study, the ethanolic extract of *R. melanophloeos* had a high affinity for the Gtr and Mtr enzymes, exhibiting IC_{50} values of 1.57 µg/mL and 10.09 µg/mL respectively. This ten-fold increase in activity associated with the human analogue is concerning, suggesting that the extract could potentially have a bigger effect on the host as opposed to the bacteria. The Michaelis constant, Km (the substrate concentration at half the maximum velocity (½ Vmax)) of Gtr and Mtr was exhibited at 23.45 µM and 13.26 µM. When tested against the bacterial and human Txr enzymes, the plant extract had an IC_{50} of 262.8 µg/mL and the inhibitory activity was not significantly high enough in the human

reductase. In a study where natural product inhibitors that were largely derived from marine plants, were tested against *M. smegmatis* and *M. tuberculosis,* high inhibition was revealed by one the marine plant species at even lower IC_{50} values of 0.5 µM and 10 µM. Although the ethanolic extract of *R. melanophloeos* showed good inhibitory activity against Mtr and relatively good inhibitory activity against *E. coli* Txr, the inhibitory effect exhibited on human Txr and Gtr is where the cautionary warning of its use in the treatment of tuberculosis along with the antagonistic effect of fluoroquinolones becomes evident. The glutathione system acts as an intracellular redox buffer within cells and plays a role in the detoxification of H_2O_2 and organic hydroperoxides, therefore making its stimulation ideal for protection of the cells and aid in creating oxidative stress when a cell is invaded by a threat (Schafer and Buettner, 2001; Simons et al., 2009). Inhibition of Gtr and human Txr would subsequently render the oxidative defence response less effective, giving the pathogen a possible opportunity for extended periods of survival and intracellular replication.

4. Conclusion

Rapanea melanophloeos has had its many attributes in the community for its use for treatment against various disease ailments, including tuberculosis. Although promising adjuvant antibiofilm activity was exhibited, use of the plant alone with administered anti-TB drugs is advised to be taken with caution as it is evident that there is a potential of effects that may induce a favourable environment for the survival and replication of mycobacteria. This needs to be evaluated in a clinical setting and the phytochemicals reasonable needs to be identified not only in this extract but also in other plant extracts currently used in combination with current drugs.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors are thankful to the Medical Research Council (PTA) of South Africa as well as the National Research Foundation. The authors would also like to acknowledge Mothong Trust, and TDr Mabena for their support. This work was partly supported by the Department of Science and Technology, African Traditional Medicine flagship project [DST/CON0164/2010].

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