Antimycobacterial activity of diospyrin and its derivatives against *Mycobacterium aurum*

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Diospyrin, is a *bis*-naphthoquinone, which was isolated from *Diospyros montana* Roxb. The objective of this study was to determine the antimycobacterial activity of diospyrin (D1) and four of its derivatives (D2, D5, D7 and D17) against the non-pathogenic *Mycobacterium aurum*. The effect of these compounds was determined on growth parameters and drug efflux pumping activity. Diospyrin was shown to be the most active in inhibiting the growth of *M. aurum* whilst D2 was inactive. D17 was found to have the lowest MIC of < 0.1 µg/ml, while the MIC of other compounds were found to be as follows: D1= 0.1 µg/ml, D5= 0.39 µg/ml, D7= 0.78 µg/ml and D2 =3.13 µg/ml, in order of potency. The compounds were bacteriostatic rather than bactericidal as the MBCs were greater than 50 µg/ml. The compounds were potent efflux pump inhibitors as D5 enhanced ciprofloxacin accumulation by 160 %, D17 by 58 %, D7 by 41 %, D1 by 37 % when compared with the standard efflux pump inhibitor, reserpine, which enhanced accumulation by 51 %. D2 had no effect on drug efflux pumping activity. The modifications of diospyrin enhanced the activity of D17 and D5 by decreasing the MIC and enhancing accumulation of ciprofloxacin, respectively. In contrast, activity decreased significantly for D2 in the growth and accumulation assays. Diospyrin and its derivatives are potential antimycobacterial agents and drug efflux inhibitors and could be used to enhance the activity of known antimycobacterial agents that are actively effluxed from *M. tuberculosis*.

Keywords: Drug efflux, antimycobacterial activity, tuberculosis, ciprofloxacin, diospyrin

Tuberculosis is one of the most infectious diseases at the moment there are about 8 million infections a year worldwide, 3 million are clinical cases and result in 2 million deaths (Knechel, 2009). The other 5 million that are infected are asymptomatic, and able to infect other individuals. Infection is via droplets that can remain infective for several hours in the air before they are inhaled and suitable conditions for growth are provided (Palmer *et al.*, 2002). The World Health Organization (WHO) introduced the directly observed treatment system (DOTS) in order to ensure compliance to treatment and perhaps, lower the incidence of resistance. This treatment schedule consists of a combination of four anti-TB drugs, isoniazid, rifampicin, ethambutol and pyrazinamide that are taken over a six to eight month period (Scoy and Wilsoske, 1999). TB
treatment is available to the 3 million symptomatic individuals and sometimes these measures are unsuccessful leading to the development of resistance. Zimbabwe is ranked 22nd on the list of 22 high burden TB countries in the world and according to the World Health Organization’s (WHO Global report, 2011), Zimbabwe had an estimated 71,961 tuberculosis cases in 2007 with an estimated incidence rate of 539 cases per 100,000 populations. Zimbabwe has the second highest mortality TB rate in the world and TB-HIV/AIDS co-infection rate is high with nearly 79% of new adult TB patients testing positive (WHO country profile, 2011). HIV infection compromises the immune system leading to the activation of the replication of mycobacteria harbored by the macrophages. The rapid spread of TB calls for decisive action and the need for newer antimycobacterial agents (WHO Global TB report, 2011). There is need to better understand the mechanisms of resistance to the currently available drugs and to reduce the incidence of drug resistant cases. Drug efflux has been described as an important mechanism for intrinsic and acquired drug resistance in microbes (da Silva et al, 2011)

Plant-derived drugs have been used worldwide in clinical medicines for the treatment of various diseases. Plant species still serve as a rich source of many novel biologically active compounds, as very few plant species have been thoroughly investigated for their medicinal properties (Gautam et al., 2007). Several plants such as Buddleja saligna have been screened for their anti-mycobacterial activity (Bamuamba et al., 2008). Some plant extracts have been found to be active against M. tuberculosis and other model mycobacteria such as M. smegmatis and M. bovis (Gautam et al., 2007). Ethnobotanical information has also shown that plants such as the Euclaea species are used in folk medicine to treat diseases such as TB (McGaw et al., 2008). Many medicinal plants produce a variety of compounds of known therapeutic properties. Substances that can either inhibit the growth of pathogens or kill them and have little or no toxicity to host cells are considered good candidates for developing new antimicrobial drugs (Woods-Panzaru, 2009). With more than 80% of the population of developing countries relying on traditional medicines, the importance of the role of medicinal plants in the health care delivery is enormous, particularly for the respiratory diseases (Chigora et al., 2007).

Phytochemicals could improve the activity of existing drugs by acting as efflux pump inhibitors and can also reduce the occurrence of drug resistant forms of bacteria (Stavri et al., 2008). Efflux pumps are able to remove antimicrobials from within the cell leaving sub-toxic concentrations (Fernades et al., 2003). Inhibition of efflux pumps makes them attractive drug targets. Instead of searching for new antimycobacterial agents, efflux pump inhibitors (EPI) can be co-administered with current antibiotics to reduce resistance by allowing the accumulation of the drug within the cell (Marquez et al., 2005). The spread of multi-drug resistant (MDR) strains of bacteria necessitates the discovery of new classes of antimycobacterials and compounds that could inhibit these resistant mechanisms (León-Díaz et al., 2010). Diospyrin, a bis-naphthoquinone (Fig 1) compound, occurring in some plants of Ebenaceae family, was first shown to possess strong inhibitory activity against murine tumor in vivo (Hazra et al., 1984). Thereafter, several of its semi-synthetic derivatives were found to induce significant apoptosis in human cancer cell lines (Chakrabarty et al., 2005), thereby, indicating a strong prospect of this ‘lead’ compound to be developed for novel chemotherapeutic applications (Das Sarma et al., 2008; Kumar et al., 2009). In fact, diospyrin and its analogues
have been observed to inhibit the growth of Mycobacterium tuberculosis in culture (Bansal et al., 2010). The present study was undertaken to determine the effect of synthetically modified diospyrin against Mycobacterium aurum, which is considered to be a good model for M. tuberculosis due to its non-pathogenicity potential and rapid growth rate. M. aurum has the same sensitivity profile as M. tuberculosis as their fatty acid chains are of similar length which plays an important role in susceptibility of mycobacteria (Chung et al., 1995). M. aurum, therefore, has been used frequently in drug screening where the actual pathogens cannot be grown without risk of pathogenesis in inappropriate laboratories (Shawar et al., 1997). This is the first study on diospyrin and its derivatives to explore their application as potential drug efflux inhibitors for the treatment of tuberculosis.

MATERIALS AND METHODS

Mycobacteria and Reagents

Rifampicin, ciprofloxacin, dimethyl sulfoxide (DMSO), reserpine, Middlebrook 7H9 base, agar and casein acid hydrolysate, glucose, ethanol, glycine, hydrochloric acid, sodium chloride, potassium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, iodonitrotetrazolium (INT) and 3-[4,5-dimethylthiazol-2-yl]-2,5-dipheny l tetrazolium bromide (MTT) were purchased from Fluka and Sigma-Aldrich (Darmstadt, Germany). M. aurum A+ was a kind gift from Professor P. Smith from the Department of Pharmacology, University of Cape Town, South Africa. The strain was obtained from Institute Pasteur, Paris, France. Diospyrin was isolated from the stem bark of Diospyros montana Roxb., which was collected from Bolangir district, Orissa, India, and the voucher specimen was authenticated at the Botanical Survey of India, Calcutta. The compound was isolated and purified as previously described by Hazra et al. (1984). Its structure has been established through routine spectroscopic methods, and it was reconfirmed to be 2,6’-bis(5-hydroxy-7-methyl-1,4-naphthoquinone) through total synthesis (Yoshida and Mori, 2000, Hazra 2004). The four derivatives of diospyrin were synthesised, purified and authenticated spectroscopically, as described before (Das Sarma et al., 2007).

Bacterial strains and growth conditions

Mycobacterium aurum A+ was grown in Middlebrook 7H9 media supplemented with casein acid hydrolysate (CAH) and in Middlebrook 7H11 solid media at 37°C under aerobic conditions.

Preparation of media

Middlebrook (MB) 7H9 broth or MB 7H11 agar were used for the growth of M. aurum A+. Middlebrook 7H9 and 7H11 media was made up with 5.2 g/L Middlebrook 7H9 base supplemented with 1 g/L casein acid hydrolysate with 15 g/L of agar for the solid media. The media components were dissolved in boiling water and autoclaved to sterilise the media. The five test compounds were all dissolved in DMSO to make stock solutions of 5 mg/ml. A 2.5 mg/ml stock solution of rifampicin was also prepared in DMSO. A 10 mM stock solution of reserpine was prepared in DMSO.

Antimycobacterial susceptibility test by the disc diffusion assay

The agar disk diffusion method was to determine if the test compounds were able to inhibit the growth of M. aurum as described by Suresh et al., (2008) with modifications. The test material equivalent to 50 µg, dissolved in DMSO was applied on sterile paper discs (6 mm diameter, cartridge susceptibility discs, Mast Diagnostics, Mast Group Ltd, Merseyside, UK). The discs were sterilized and placed in microtitre plates and loaded with different volumes of the various
stock solutions of the five bis-naphthquinones. The compounds were dissolved in dimethyl sulfoxide (DMSO) to get final concentrations of 50, 25, 12.5 and 6.25 µg/ml. Rifampicin was used as the positive control at final concentrations of 50, 5, 0.5, 0.1 µg/ml and DMSO was used as the negative control. The filter papers with the loaded compounds were dried under an airstream to evaporate the solvent. A 1 x 10^6 colony forming units (cfu)/ml dilution of *M. aurum* was mixed with Middlebrook 7H11 at a ratio of 1: 20 ml respectively. The mixture was poured and spread on plates. The dried filter paper discs were placed on the surface of the solid agar and incubated at 4 °C in a refrigerator for two hours to allow diffusion of compounds into the agar. The plates were removed from the refrigerator and incubated at 37 °C overnight in an incubating shaker (Lab Companion, Woburn, Massachusetts, USA).

**Determination of MICs**

The method used was adopted from McGaw et al., (2008) with slight modifications. Diospyrin, the four derivatives and rifampicin were serially diluted two fold from 50 µg/ml with DMSO up to a final concentration of 0.1 µg/ml. Aliquots of 20 µL of each of the above compounds were taken and added to 80 µL of X 2 Middlebrook 7H9 supplemented with casein acid hydrosylate medium in wells of a 96 well microtitre plate in duplicate. A third row for each serially diluted compound was left with no inoculum so as to act as the control. *Mycobacterium aurum* was added to each of the wells in 100 µL aliquots with the exception of the sterility control wells where 200 µL of media was added. The microplate was covered and sealed with parafilm before being placed in a plastic container lined with absorbent paper saturated with sterile water. The container was wrapped in aluminium foil and incubated at 37 °C overnight in an incubating shaker (Lab Companion, Woburn, Massachusetts, USA). Aliquots of 40 µL of MTT were added to all the wells and the plate was incubated at 37 °C for 1 hour. The optical densities of the wells were read using Biokinetics Reader EL350 (Bio-Tek Instruments, Highland Park, Winooski, VT, USA) at 550 nm. The MBCs of the compounds were then determined on solid Middlebrook 7H11 media according to the method by Amsterdam, (1996) with modifications. Aliquots of 25 µL were taken from the wells that had no visible growth and spread on solid Middlebrook 7H11 using glass spreaders. The plates were then incubated overnight and checked for growth to determine the MBC of each compound.

**Determination of effects on drug efflux**

The accumulation of ciprofloxacin was measured according to the method by Piddock et al., (1998) with modifications. *M. aurum* was grown in Middlebrook 7H9 at 37 °C to OD_{600} of between 0.5 - 0.7 and harvested by centrifugation at 3000 rpm for 10 minutes. The bacteria were then re-suspended in 50 mM sodium phosphate buffer (pH 7.0) at 4 °C and made up to 40 mg/ml (dry weight of cells). The cells were placed in a water bath set at 37 °C for 15 minutes. Samples were split into sample A and B and maintained at the same temperature. Ciprofloxacin was added to both samples to a final concentration of 20 mg/L. Aliquots of 750 µL were taken at 0, 1, 5, 10, 15, 30, 45 and 60 minutes after the addition of ciprofloxacin from both sample A and B. Samples were diluted immediately into 750 µL chilled sodium phosphate buffer (pH 7.0) on ice and centrifuged in a micro centrifuge (Herme Labortechnik, Germany) at 8000 r/min, at 4 °C for 5 minutes. The cells were then washed with chilled phosphate buffer and centrifuged for 5 minutes. The cell pellet was
then suspended in 1.5 ml glycine hydrochloride (0.1 mol/L, pH 3.0) for 3 hours at 37 °C and centrifuged at 8000 rpm for 10 minutes. The supernatant was centrifuged for another 5 minutes. Fluorescence of ciprofloxacin in the supernatant was determined at an excitation wavelength of 270 nm and emission wavelength of 479 nm using a RF -1501 spectrofluorimeter (Shimadzu, Kyoto, Japan). The active efflux of ciprofloxacin was then examined with an efflux pump inhibitor, 50 mg/ml stock solution in DMSO, which was added to a final concentration of 100 µmol/ml but not sample B. Aliquots of 750 µL were removed at 5, 15 and 30 minutes after the addition of the efflux pump inhibitor reserpine. To rule out interference from the reserpine, the fluorescence of diospyrin and the four derivatives was also measured same the excitation and emission wavelength as ciprofloxacin. The compounds were diluted in 3 ml of 0.1 M glycine-HCl, pH 3, up to a concentration of 100 µM which was the concentration used in the ciprofloxacin accumulation assay.

Statistical analysis
All values have been expressed as mean ± standard deviation was evaluated by applying Dunnett’s Multiple Comparison Test using Graphpad Prism 5 software® for Windows version 5.03(GraphPad Prism Inc. San Diego, CA, USA). P < 0.05 values or less were considered to indicate statistically significant difference.

RESULTS
Assessment of the antimycobacterial activity
Assessment of the antimycobacterial activity of the compounds was conducted using the agar disk diffusion method. Table 1 shows the zone of inhibition obtained from when 50 µg of a compound were loaded onto a filter paper disk. Rifampicin, the standard drug, at 5 µg/disc was the most active with an average diameter of 19.5 mm. Diospyrin was the most active of the test compounds with a diameter of 13.5 mm, followed by D17 with 11.5 mm. D5 and D7 were the next most active with average diameters of 10 and 8 mm respectively. D2 was inactive as it had a diameter of 6 mm, the diameter of the negative control. A rifampicin standard curve of the zone of inhibition against concentration was constructed and used to interpolate the concentrations of the test compounds that were equivalent to those of the standard drug, rifampicin (data not shown).

Table 1: Zones of growth inhibition, MIC and MBC values for diospyrin and derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>Zone of inhibition (mm)</th>
<th>Rifampicin equivalence (µg)</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
<th>MBC to MIC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17</td>
<td>12 ± 1**</td>
<td>2.5</td>
<td>&lt;0.10</td>
<td>&gt;50</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Diospyrin (D1)</td>
<td>14 ± 1**</td>
<td>3.4</td>
<td>0.10</td>
<td>&gt;50</td>
<td>&gt;500</td>
</tr>
<tr>
<td>D5</td>
<td>10 ± 0**</td>
<td>1.8</td>
<td>0.39</td>
<td>&gt;50</td>
<td>&gt;128</td>
</tr>
<tr>
<td>D7</td>
<td>8 ± 1*</td>
<td>0.8</td>
<td>0.78</td>
<td>&gt;50</td>
<td>&gt;64</td>
</tr>
<tr>
<td>D2</td>
<td>6 ± 0</td>
<td>-</td>
<td>3.13</td>
<td>&gt;50</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>17± 2**</td>
<td>-</td>
<td>0.20</td>
<td>&gt;50</td>
<td>&gt;250</td>
</tr>
<tr>
<td>DMSO</td>
<td>6± 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

D17- dimethyl ether epoxide, D1- diospyrin, D5- diacetyl, D7- diethyl ether, D2- dimethyl ether MIC- minimum inhibitory concentration, MBC- minimum bactericidal concentration. n= 2. The zone of inhibition were determined on 7H11 medium at 50, 25, 12.5 and 6.25 µg/ml. Rifampicin was used as the positive control at final concentrations of 50, 5, 0.5, 0.1 µg/ml and DMSO was used as the negative control. Values are mean ± SD for n = 4. * P < 0.05, ** P<0.001
Determination of MIC and MBC

In order to determine the effect of the test compounds on the growth parameters of *M. aurum*, the microbroth dilution and viable cell methods were used. Table 2 shows that D17 was the most active compound against *M. aurum* with an MIC of <0.1 µg/ml. This was followed by D1 with an MIC of 0.1 µg/ml. Rifampicin had an MIC of 0.2 µg/ml. The results showed that D17 and D1 were actually more potent against *M. aurum* than the standard drug. The MBC of all the test compounds and rifampicin were greater than 50 µg/ml, showing that these compounds were bacteriostatic rather than bactericidal.

Table 2: A summary of the effect of diospyrin and derivatives on the ciprofloxacin accumulation at the 90 minute point

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D5</td>
<td>Enhanced</td>
<td>301 ± 11 ***</td>
</tr>
<tr>
<td>D17</td>
<td>Enhanced</td>
<td>158 ± 7 ***</td>
</tr>
<tr>
<td>D7</td>
<td>Enhanced</td>
<td>141 ± 2 ***</td>
</tr>
<tr>
<td>D1</td>
<td>Enhanced</td>
<td>137 ± 11 ***</td>
</tr>
<tr>
<td>D2</td>
<td>No effect</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Reserpine</td>
<td>Enhanced</td>
<td>151 ± 2 ***</td>
</tr>
<tr>
<td>Control</td>
<td>No effect</td>
<td>100</td>
</tr>
</tbody>
</table>

D5- diacetyl, D17- dimethyl ether epoxide, D7- diethyl ether, D1- diospyrin, D2- dimethyl ether; Values are mean ± SD for n=4 and are expressed as the percentage of the control.

Effects of diospyrin and derivatives on drug efflux

Overexpression of ATP binding cassette (ABC) transporters has been proposed as a major mechanism contributing to the innate drug resistance in *Mycobacterium tuberculosis* and related mycobacteria (Alekshun *et al.*, 2007). The aim of this part of the study was, therefore, to determine the effects of diospyrin and its derivatives, on ATP mediated-drug efflux the bacteria species.

Figure 3 shows the amount of ciprofloxacin that accumulated inside *M. aurum* after exposure to the compounds. Reserpine or the test compounds was added at the 30 minute time point. At 35 minutes, there was an increase in the amount of CFX in the cells. Table 3 shows that the addition of the reserpine resulted in an increase of 51% of CFX. Figure 3 shows the effect of the derivative, D5, on CFX accumulation in *M. aurum* which resulted in an increase of 160%. Three compounds resulted in an increase in the amount of ciprofloxacin of 58%, 41% and 37% for D17, D7 and diospyrin itself. The derivative, D2, did not have a significant effect on the accumulation, with an increase of 1% on addition of the compound. In order to rule out interference due to the plant extracts, their fluorescence was determined separately at the same wavelengths and the results are shown in Figure 3. The fluorescence of the compounds was found to be negligible relative to that of ciprofloxacin at the concentrations that were used in the assay. Reserpine was added at an inhibitor concentration of 61 ng/ml whilst the concentration of ciprofloxacin added to the cells was 20 µg/ml.

Table 3: Summary of the nascent fluorescence of the compounds used in the ciprofloxacin accumulation assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fluorescence (f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>42.7760</td>
</tr>
<tr>
<td>Reserpine</td>
<td>2.8637</td>
</tr>
<tr>
<td>Diospyrin (D1)</td>
<td>-0.0809</td>
</tr>
<tr>
<td>D2</td>
<td>-0.0025</td>
</tr>
<tr>
<td>D5</td>
<td>0.0730</td>
</tr>
<tr>
<td>D7</td>
<td>-0.0098</td>
</tr>
<tr>
<td>D17</td>
<td>0.0716</td>
</tr>
</tbody>
</table>
Figure 1: Structures of diospyrin and semi-synthetic derivatives. Diospyrin, D2- dimethyl ether derivative, D5- diacetyl derivative, D7- diethyl ether derivative, D17- diepoxide methyl ether derivative.
Figure 2: Ciprofloxacin accumulation in M. aurum cells after incubation with potential efflux pump inhibitors. The readings were taken at 10 minute intervals. The arrows indicate the time point at which the standard or potential inhibitor was added to the reaction. The values are mean ± SD for n = 4. R- reserpine, D5- diacetyl, D17- dimethyl ether epoxide, D7- diethyl ether, D1- diospyrin, D2- dimethyl ether
DISCUSSION

Natural products continue to play a role in the drug discovery and development process, and plants are recognized as very useful source of highly active antimycobacterial metabolites (McGaw et al., 2008). Approximately three-quarters of the World’s population rely on medicinal plants as their primary source of medicine. Several plants with antimycobacterial activity have been screened, resulting in the isolation and characterization of several active compounds (Ignacimuthu and Shanmugam, 2010; Amin et al., 2009; León-Díaz et al., 2010). Presently, the potential of the natural plant product, diospyrin, and its semi-synthetic derivatives as antimycobacterial agents was assessed against the non-pathogenic model organism, *Mycobacterium aurum*. The basis for testing the antimycobacterial activity was to investigate how effective the compounds as potential antimycobacterials and as possible drug efflux inhibitors. The differences in the zones of inhibitions the ranking in order of potency was D1 > D17 > D5 > D7 > D2. Thus, diospyrin was the most potent test compound and was more active than all its derivatives. The dimethyl epoxide derivative, D17 is a slightly larger molecule and it has groups that could hinder its diffusion into the agar. Motility of molecules is influenced by their size and shape. However, the MICs of the compounds were in the order of D17 > D1 > D5 > D7 > D2. Naphthoquinones are phytochemicals that have been shown to be quite active within cells, as they have been found to have anti-bacterial, antiplasmodial and anticancer properties (Hazra et al., 2004, Lall et al., 2003). The activity of the compounds seems to be based on the nucleophilicity of the substituent groups of the compounds that are able to complex irreversibly with nucleophilic amino acids in proteins (Bansal et al., 2010). The methyl ether epoxide diospyrin derivative, D17 has the most nucleophilic substituent groups with two epoxide groups and a methyl ether group. Epoxide groups have been found to be highly reactive, especially when forming adducts with macromolecules such as DNA and proteins (Dewick, 2002). The ether group is also slightly reactive due to the slightly polar oxygen atom that can associate with macromolecules as well. Naphthoquinones have also been found to form Michael adducts with nucleophilic molecules (Van der Kooy, 2007, Mukanganyama et al., 2010).

MIC to MBC ratios that are greater than 32 are considered to indicate that the test is bacteriostatic rather than bactericidal (Cockerill, 1998). The same has been shown to be true for diospyrin against other mycobacterial strains such as *M. bovis* ATCC (McGaw et al., 2008) where the MIC to MBC ratio of 40 was observed. Studies of other naphthoquinones, such as lapachol and its derivatives, against *Staphylococcus aureus* also found the compounds to be bacteriostatic rather than bactericidal, as this ratio for drug sensitive strain of *S. aureus* was > 64 µg/ml (Perreira et al., 2006).

Ciprofloxacin (CFX) fluoresces when it interacts with its target enzyme, topoisomerase II and it is a substrate of mycobacterial efflux pumps (Lechner et al., 2008). These characteristics are what make CFX ideal to monitor the activity of efflux pumps. The activity of efflux pumping inhibition in *M. aurum* was in the order D5 > D17 > D7 > D1 > D2. The diacetyl derivative of diospyrin, D5, enhanced the accumulation of CFX the most, at ~200 % of the control. The enhancement in CFX accumulation could have been as a result of the diospyrin derivatives acting as pseudosubstrates with them being effluxed in place of ciprofloxacin. The pseudosubstrate mechanism in literature has been postulated as one of the possible
mechanisms used to efflux pumps in fungi (Cannon et al., 2009). Several compounds have been proposed as acting as pseudosubstrates of P-glycoprotein (Tsujimura et al., 2008). Thus, the concentration of CFX was able to increase inside the cell. This is a mechanism that has been proposed in the development of EPIs that inhibit the extrusion of fluoroquinolones from within bacterial cells (van Bambeke et al., 2006). The other mechanism that could account for the increase is that the compound was able to interact with the efflux pump and inhibit the action of the pumps. The diospyrin pharmacophore is the quinoid group that is involved in the formation of the Michael adducts (Van der Kooy, 2007). The standard efflux pump inhibitor in this study, reserpine, has been found to be involved in the uncoupling the attachment of ATP from its nucleotide binding site or domain. However, the exact mechanism of action of how the uncoupling takes place has not been elucidated (Garvey and Piddock, 2008).

To determine the SAR of the compounds used in this work, comparisons must be made based on the different functional groups that are found on diospyrin, the parent compound, and those found on the derivatives. The dimethyl ether epoxide derivative, D17 had greater activity than diospyrin to a significant extent and this could be due to the presence of the epoxide groups. The diacetyl derivative, D5, was also potent in the efflux pump inhibition assay. The diacetyl groups appear to confer better activity to the diospyrin parent compound against drug efflux activity. The modification of diospyrin to dimethyl ether (D2) seems to significantly decrease the antimycobacterial activity of diospyrin as D2 was the least active test compound in all the parameters tested.

In summary, our present study showed that diospyrin was able to inhibit mycobacterial growth and enhanced the accumulation of drugs inside the bacteria. The naphthoquinone, diospyrin and its semi-synthetic derivatives, therefore, have potential as antimycobacterial agents and further modifications could improve activity. The derivatives may have the most potential as efflux pump inhibitors.

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