Regular Article

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

Stanley Mukanganyama[1*], Elaine Chirisa[1] and Banasri Hazra[2]

[1]Biomolecular Interactions Analyses Group, Department of Biochemistry, University of Zimbabwe, P.O. Box MP 167, Mount Pleasant, Harare, Zimbabwe,
[2]Department of Pharmaceutical Technology, Jadavpur University, Calcutta, 700032, India
*Corresponding author e-mail: smukanganyama@medic.uz.ac.zw

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 Wilswoske, 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 plants 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 *Euclea* 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 sulphoxide (DMSO), reserpine, Middlebrook 7H9 base, agar and casein acid hydrosylate, 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-diphenyltetrazolium 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 hydrosylate (CAH) and in Middlebrook 7H11 solid media at 37^0C 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 hydrosylate 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 10⁶ 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 (Hermle 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 Comaprison 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

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

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

Compound	Effect	Percentage (%)
D5	Enhanced	301 ± 11 ***
D17	Enhanced	158 ± 7***
D7	Enhanced	141 ± 2***
D1	Enhanced	137 ± 11***
D2	No effect	100 ± 4
Reserpine	Enhanced	151 ± 2***
Control	No effect	100

D5- diacetyl, D17- dimethyl ether epoxide, D7- diethyl ether , D1- diospyrin, D2- dimethyl ether; Values are mean ± SD for n=4and 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 diospryin 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

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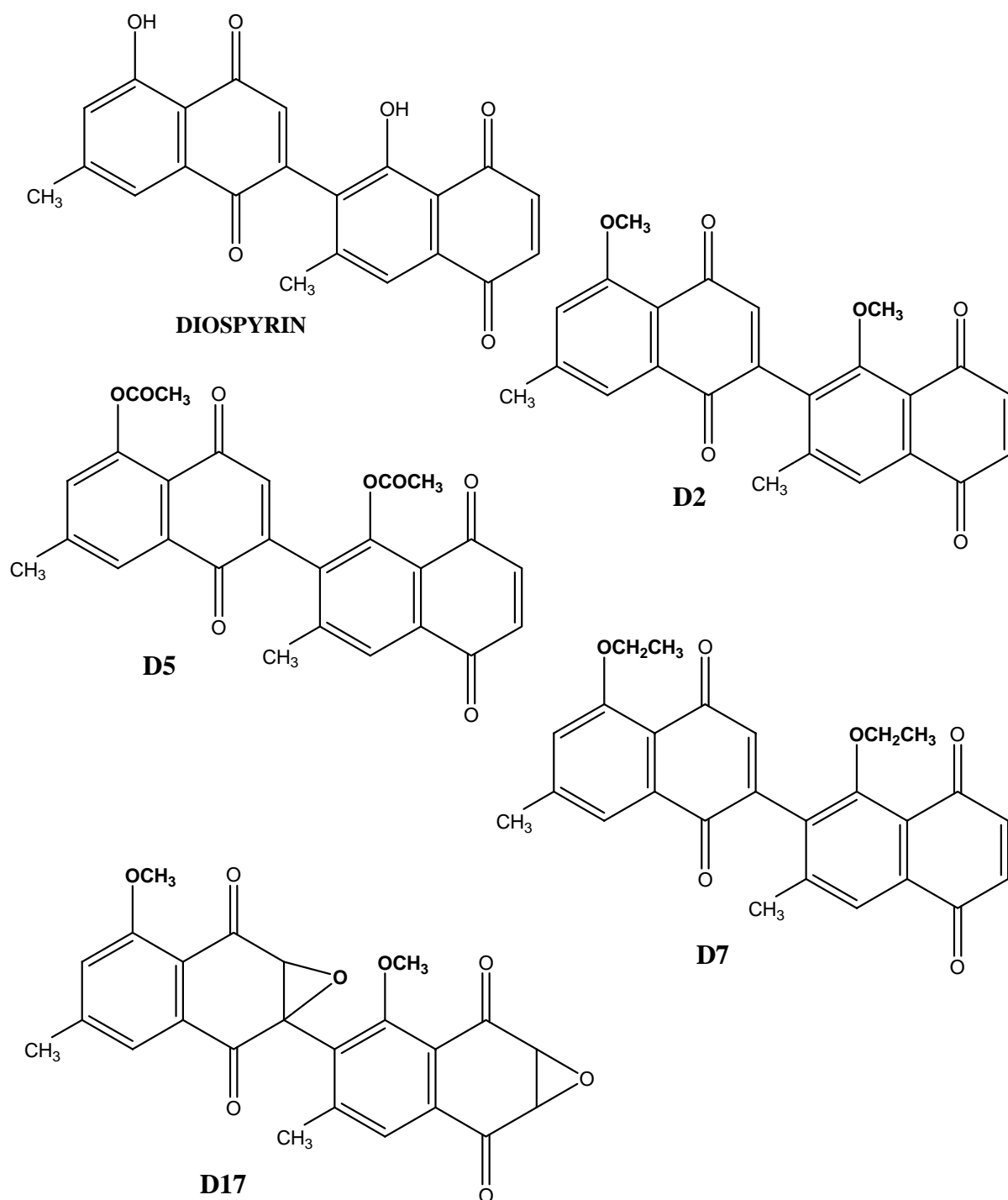
Figure 1: Structures of diospyrin and semi-synthetic derivatives. Diospyrin, D2- dimethyl ether derivative, D5- diacetyl derivative, D7- diethyl ether derivative, D17- diepoxide methyl ether derivative.
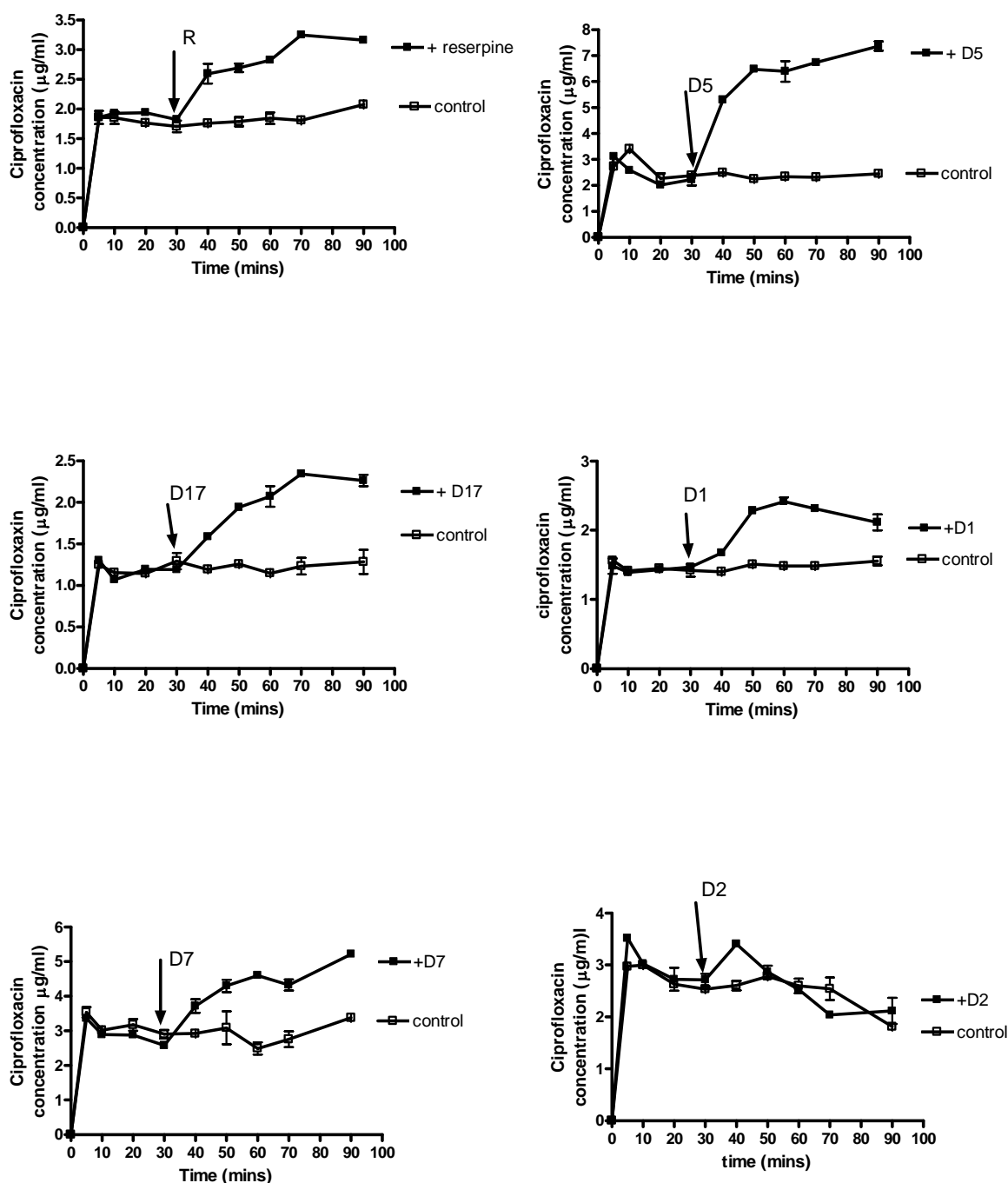
Figure 2: Ciprofloxacin accumulation in *M. aurum* cells after incubation with potential efflux pump inhibitors. The readings were taken at 10 minute intervals. The arrows indicate the time point at which the standard or potential inhibitor was added to the reaction. The values are mean ± SD for n =4.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

REFERENCES

Amin, M., Segatoleslami, S. and Hashemzadeh, M. (2009). Antimyco-bacterial activity of partial purified extract of *Allium ascalonicum*. *Jundishapur Journal of Microbiology*. **2**: 144-147.

Amsterdam, D. (1996). Susceptibility testing of antimicrobials in liquid media. Antibiotics in laboratory medicine. 4th edition. Williams and Wilkins. Baltimore. 52-111.

Bamuamba, K., Gammon, D.W., Meyers, P., Dijoux-Farca, M. and Scott, G. (2008). Antimycobacterial activity of five plant species used as traditional medicines in Western Cape Province (South Africa). *Journal of Ethnopharmacology*. **117**: 385-390.

Bansal, R., Bansal, P., Gupta, V., Kumar, S., Sharma, S. and Rao, M. M. (2010). Drug designing through anti-microbial potentials of Indian herbs. *Journal of Pharmacy Research*. **3**: 364-370.

Cannon, R. D., Lamping, E., Holmes, A. R., Niimi, K., Baret, P. V., Keniya, M. V., Tanabe, K., Niimi, M., Goffeau, A. and Monk, B. C. (2009). Efflux-mediated antifungal drug resistance. *Clinical Microbiology Reviews*. **22**: 291-321.

Chakrabarty, S., Roy, M., Hazra, B. and Bhattacharya, R.K. (2005). Induction of apoptosis in human cancer cell lines by diospyrin, a plant derived bis-naphthquinonoid and its synthetic derivatives. *Cancer Letters*. **188**: 85-93.

Chigora, P., Masocha, R. and Mutenheri, F. (2007). The role of indigenous medicinal knowledge (IMK) in the treatment of ailments in rural Zimbabwe: The case of Mutirikwi communal lands. *Journal sustainable development in Africa*. **9**: 26-43.

Chung GAC, Aktar Z, Jackson S, and Duncan K. (1995). High-Throughput Screen for Detecting Antimycobacterial Agents. *Antimicrobial agents and chemotherapy*,**39**: 2235–2238

Cockerill, F.R. (1998). Conventional and Genetic Laboratory tests used to guide Antimicrobial Therapy. *Mayo Clinical Protocol*, **73**: 1007-1021.

Das Sarma M., Ghosh R., Patra A., Hazra B. (2008). Synthesis of novel amino-quinonoid analogues of diospyrin and evaluation of their inhibitory activity against murine and human cancer cells, *European Journal of Medicinal Chemistry*, **43**: 1878- 1888.

Das Sarma M., Ghosh R., Patra A., Hazra B. (2007). Synthesis and antiproliferative activity of some novel derivatives of diospyrin, a plant-derived naphtho-quinonoid. *Bioorganic Medicinal Chemistry*, **15**: 3672-3677.

da Silva PEA, Von Groll A, Martin A and Palomino JC (2011). Efflux as a mechanism of drug resistance in *Mycobacterium tuberculosis*. *FEMS Immunological and Medical Microbiology*, **63**:1-9.

Dewick, P.M. (2002). Medicinal natural products: Biosynthetic approach. 2nd Edition John Wiley and Sons Publishing, New York, USA 507 pages.

Fernandes, P., Ferreirra, B.S. and Cabral, J.M.S. (2003). Solvent tolerance in bacteria: role of efflux pumps and cross resistance with antibiotics. *International Journal of Antimicrobial agents*. **22**: 211-216.

Garvey, M.I. and Piddock, L.J.V. (2008). Efflux pump inhibitor reserpine selects multidrug resistant *Streptococcus pneumonia* that over express the ABC transporters PatA and PatB. *Journal of Antimicrobial Agents Chemotherapy*. **63**: 1102-1109.

Gautam, R., Saklani, A. and Jachak, S.M. (2007). Indian medicinal plants as a source of antimycobacterial agents. *Journal of Ethnopharmacology*. **110**: 200-225.

Global Tuberculosis Report (2010).

Hazra B., Das Sarma M. and Sanyal U. (2004) Separation methods of quinonoid constituents of plants used in Oriental traditional medicines. *Journal of Chromatography B*, **812:** 259-275.

Hazra, B., Sur, P., Sur, B., Banejree, A. and Roy, D.K. (1984). Studies of on the biological activity of diospyrin towards Elhrich ascisites carcinoma in Swiss A mice. *Planta Medica*, **58**: 295-298.

Ignacimuthu, S. and Shanmugam, N. (2010). Antimycobacterial activity of two natural alkaloids, vasicine acetate and 2-acetyl benzylamine, isolated from Indian shrub *Adhatoda vasica* Ness. leaves. *Journal of Biosciences*. **35:** 565–570

Knechel AN. Tuberculosis(2009): Pathophysiology, Clinical Features, and Diagnosis. *Critical Care Nurse ,*29:34-43 doi:10.4037/ccn2009968

Kumar, B., Kumar, A., Pandey, B.N., Mishra, K.P., Hazra, B. (2009) Role of mitochondrial oxidative stress in the apoptosis induced by diospyrin diethylether in human breast carcinoma (MCF-7) cells. *Molecular and Cellular Biochemistry*, **320**: 185-195.

Lall, N., Hazra, B., Das Sarma, M. and Meyer, J.JM. (2003). *In vitro* activity of derivatives of diospyrin against drug resistant and drug sensitive *Myco-bacterium tuberculosis. Journal of Anti-microbial Chemotherapy.* **51**: 435-438.

Lechner, D., Gibbons, S. and Bucar, F. (2008). Plant phenolic compounds as ethidium bromide efflux inhibitors in *Myco-bacterium smegmatis. Journal of Anti-microbial chemotherapy.* **62**: 345-348.

León-Díaz, R., Meckes, M., Said-Fernández, S., Molina-Salina, G. M., Vargas-Villarreal, J., Torres, J., Luna-Herrera, J. and Jiménez-Arellanes, A. (2010). Antimycobacterial neolignans isolated from *Aristolochia taliscana. Mem Inst Oswaldo Cruz.* **105**: 45-51.

Marquez, B., Neuville, L., Moreau, N.J., Genet, J., dos Santos, A.F., de Andrade, M.C. and Sant`Ana, A.E.G. (2005). Multidrug resistance reversal agent from *Jatropha elliptica. Phytochemistry.* **66**: 1804-1811.

McGaw, L. J., Lall, N., Hlokwe, T. M., Michel, A. L., Meyer, J. J. M. and Eloff, J. N. (2008). Purified compounds and extracts from *Euclea* species with antimycobacterial activity against *Myco-bacterium bovis* and fast-growing mycobacteria. *Biological Pharmacology Bulletin,***31**: 1429-1433.

Mukanganyama S, Bezabih M, Robert M , Ngadjui BT, Kapche GFW, Ngandeu F and Abegaz B. (2010) The evaluation of novel natural products as inhibitors of human glutathione transferase P1-1. *Journal of Enzyme Inhibition and Medicinal Chemistry.* DOI:10.3109/14756366.2010.526769.

Palmer MV, Ray Waters W and Whipple DL (2002). Aerosol delivery of virulent *Mycobacterium bovis* to cattle. *Tuberculosis*, **82**, 275−282.

Perreira, E. M., de Barros Machado, T., Leal, I.C.R., Jesus, D. M., de Almeida Damaso, C. R., Pinto, A. V., de Marval, M.G., Kuster, R. M. and des Santos, K. R. N. (2006). *Tabebuia avellanedae* naphthoquinones: against methicillin resistant staphylococcal strains, cytotoxicity activity and *in vivo* dermal irritability analysis. *Annals of Clinical Microbiology and Antimicrobials.* **5**: 5.

Piddock, L. J. V., Chung, G. A. C. and Williams, K. J. (1998). Accumulation of Norfloxacin by *Mycobacterium aurum* and *Mycobacterium smegmatis. Journal of Antimicrobial Agents Chemotherapy*, **42**: 795-800.

Scoy, R.E. and Wilkowske, C.J. 1997. Antimycobacterial therapy. *Mayo Clinical Protocols*, **74**: 1038-1048.

Shawar, R. M., Humble, D. J., Van Dalfsen, J. M., Stover, C. K., Hickey, M. J., Steele, S., Mitchier, L. A. and Baker, W. (1997). Rapid screening of natural products for antimycobacterial activity by using luciferase-expressing strains of *Mycobacterium bovis BCG* and *Myco-bacterium intracellulare. Journal of Antimicrobial Agents and Chemotherapy*, **41**: 570-574.

Stavri, M., Piddock, L. J. V. and Gibbons, S. (2007). Bacterial efflux pumps inhibitors

from natural sources. *Journal of Antimicrobial Chemotherapy*, **59**: 1247-1260.

Suresh, K., Babu, S.S. and Harisaranraj, R. (2008). Studies on in vitro antimicrobial activity of ethanol extract of *Rauvolfia tetraphylla*. *Ethnobotanical Leaflet*, **12:** 586-590.

Tsujimura, S., Saito, K., Nawata, M., Nakayamada, S. and Tanaka, Y. (2008). Overcoming drug resistance induced by P-glycoprotein on lymphocytes in patients with refractory rheumatoid arthritis. *Annual Rheum Dis*. **67**: 380–388.

Van Bambeke, F., Pages, J and Lee, V. J. (2006). Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. *Recent Patents on Anti-Infective Drug Discovery*, **1**: 157-175.

Van der Kooy, F. (2007). Naphthoquinones isolated from *Euclea natalensis*. University of Pretoria. 99-105. Ph.D. Thesis.

WHO tuberculosis country profile. Available at http://www.who.int/tb/country/data/ profiles/en/index.html

WHO Global tuberculosis control 2011 available at http://www.who.int/tb/country/en/index.html

WHO global report 2011. Available at http://www.who.int/tb/publications/global_report/2011/gtbr11

Woods-Panzaru, S., Nelson, D., McCollum, G., Ballard, L.M., Millar, B.C., Maeda, Y., Goldsmith, C.E., Rooney, P.J., Loughrey, A., Rao, J.R. and Moore, J.E. (2009). An examination of anti-bacterial and antifungal properties of constituents described in traditional Ulster cures and remedies. *Ulster Medical Journal*, **78**: 13-15.

Yoshida, M. and Mori, K. (2000). Synthesis of diospyrin, a potential agent against leishmaniasis and related parasitic protozoan diseases. *European Journal of Organic Chemistry*, 1313-1317.