Antimycobacterial activities of selected medicinal plants from Zimbabwe against Mycobacterium aurum and Corynebacterium glutamicum

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Abstract. The spread of multi-drug resistant tuberculosis necessitates the discovery of new classes of antibacterials and compounds that inhibit macromolecules involved in these resistant mechanisms. Thirty ethanol extracts from nineteen selected plants from Zimbabwe were screened against Mycobacterium aurum and Corynebacterium glutamicum using the agar disk diffusion method. These two organisms were used as models for Mycobacterium tuberculosis. The amount of ciprofloxacin accumulated and effluxed by the test organism was used to determine whether the plant extracts could also act as drug efflux pump inhibitors. Vernonia adoensis and Mangifera indica extracts at 500 mg/disk had the highest growth inhibitory activity against M. aurum and C. glutamicum respectively. The extract from Parinari curatellifolia had an MIC of 8 µg/disk and an MBC of 63 µg/disk; an MIC of 125 µg/disk and an MBC of >500 µg/disk against M. aurum and C. glutamicum respectively. All the plant extracts were bacteriostatic and showed antagonistic effects when combined with rifampicin. The extract from P. curatellifolia made M. aurum and C. glutamicum accumulate the highest amount of ciprofloxacin. The accumulation of ciprofloxacin caused by P. curatellifolia extract was greater than that caused by the drug efflux inhibitor reserpine. This plant may serve as a source of lead compounds in the search of new antimycobacterials with new mechanisms of action.

INTRODUCTION

Tuberculosis (TB) was thought to have been eradicated in the early second half of the past century. However, this old disease has resurged at an alarming speed both in developing and industrialised countries (Bamuamba et al., 2008). TB remains an important public health problem, accounting for 8 million new cases per year. Despite improvements in chemotherapy, the treatment of TB is severely affected by the development of multi-drug resistance in Mycobacterium tuberculosis strains (Higuchi et al., 2008). The situation has been worsened by the association of TB and HIV especially in Sub-Saharan Africa and many developing countries (WHO Global report, 2009).

Zimbabwe is ranked 17th on the list of 22 high burden TB countries in the world. According to the World Health Organization’s (WHO’s) Global tuberculosis report (2009), Zimbabwe had an estimated 71 961 tuberculosis cases in 2007 with an estimated incidence rate of 539 cases per 100 000 populations. She has the second highest mortality TB rate in the world and TB-HIV/AIDS co-infection rate is high with nearly 69% of new adult TB patients testing positive (WHO, 2009). National data suggests the actual figure to be about 80% after an increasing HIV surveillance in HIV patients. The rapid spread of TB calls for
decisive action and the need for newer antitybacterial agents (Suresh et al., 2008).

Although drug regimens exist for treating tuberculosis, they are far from ideal. The current treatment requires that a person take at least three or four antitybacterial drugs. The leading drug combinations are isoniazid and rifampicin and may be supplemented with pyrazinamide and ethambutol. However, due to poor patient compliance, there has been an emergence of multi-drug resistant (MDR) strains of bacteria. The spread of multi-drug resistant strains of bacteria necessitates the discovery of new classes of antibacterials and compounds that inhibit macromolecules involved in these resistant mechanisms (Mann et al., 2008). 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 (Jachak et al., 2007). Plants have, thus, been screened for antitybacterial activity as some of them have been shown to have this property (McGaw et al., 2008a). Buddleja saligna, for example, is used traditionally for the treatment of diarrhea and gastric disorders (Bamuamba et al., 2008). Furthermore, some of the plants screened are used in the traditional treatment of tuberculosis and other respiratory related ailments (Chigora et al., 2007) for example Vernonia adoensis. Phytochemicals can 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., 2007). Traditions of collecting, processing and applying plants and plant-based medications have been handed down from generation to generation. Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy (Fennell et al., 2004). 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 et al., 2009).

The populations of developing countries worldwide continue to rely heavily on traditional medicine as their primary source of health care. Ethnobotanical studies carried out throughout Africa confirm that indigenous plants are the main constituents of traditional African medicines (Mann et al., 2008). 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 very enormous particularly for the respiratory diseases (Chigora et al., 2007).

The spread of multi-drug resistant (MDR) strains of bacteria necessitates the discovery of new classes of antibacterials and compounds that inhibit these macromolecules involved in these resistant mechanisms (Mann et al., 2008). The aim of this study was to investigate the in vitro antitybacterial activities of nineteen Zimbabwean medicinal plant extracts against two model non-pathogenic actinomycetales species; Mycobacterium aurum and Corynebacterium glutamicum. Corynebacterium glutamicum like, M. tuberculosis also contains mycolic acids as part of its outer membrane components (Arun et al., 2008). These plants were selected based on literature survey of their ethnomedicinal usages in the treatment of microbial infections and are listed in Table 1.

MATERIALS AND METHODS

Mycobacteria and Reagents
Corynebacterium glutamicum (ATCC 13022) and M. aurum (A+) were used as model organisms for M. tuberculosis. Mycobacterium aurum was kind gift
from Prof. Smith of the Department of Pharmacology, University of Cape Town and Prof. Steenkamp, of the Department of Clinical Laboratory studies, University of Cape Town provided the \textit{C. glutamicum}. Luria Bertani media, glucose, Middlebrook 7H9, casein acid hydrolysate, Middlebrook 7H11, nutrient agar, sodium dihydrogen phosphate, disodium hydrogen phosphate, glycine, hydrochloric acid, reserpine, ciprofloxacin, dimethyl sulphoxide (DMSO) and sodium azide were obtained from Sigma Aldrich Company (Taufkirchen, Germany). All other chemicals used were of the highest grade and were obtained from various sources.

**Plant collection**
The plants used in this work were collected from three provincial localities of Zimbabwe, Norton (Mashonaland West), Centenary (Mashonaland Central) and University of Zimbabwe (Harare), Zimbabwe. The plants’ identity were authenticated and classified by Mr. Christopher Chapano, a taxonomist at the National Herbarium and Botanic Gardens, (Harare, Zimbabwe). Herbarium samples were kept at the Department of Biochemistry, University of Zimbabwe.

**Preparation of extracts**
Plant material were dried in an oven at 50°C and ground in an electric blender (Cole Parmer Instrument Company, Connecticut, USA) to a fine powder. A volume of 8 ml of ethanol was added to 2 g of powder and shaken for 5 minutes on a vortex and left to sit for 10 minutes. A syringe was prepared by inserting a piece of fine sieve. The plant suspension was then transferred into syringe and filtered into a small glass vial. The sterile suspension was filtered again using 0.45 µM Millipore® sterile filter (Sigma-Aldrich, Taufkirchen, Germany) into a labeled small glass vial. Ethanol was left to evaporate overnight in fume hood with air stream. A constant dry weight of each extract was obtained and the residues were stored at 4°C until when required.

**Culture of bacteria**
Middlebrook 7H9 medium was prepared by combining 4 g of Middlebrook 7H9 base and supplementing it with 1 g casein acid hydrolysate, and making up to 1 litre with distilled water. LB broth was prepared by combining 10 g sodium chloride, 5 g yeast extract, 10 g tryptone and 5 g glucose and distilled water was added to make a 1 litre solution. The media was sterilized by autoclaving. \textit{Mycobacterium aurum} was inoculated into 20 ml Middlebrook 7H9 solution supplemented with casein acid hydrolysate while \textit{C. glutamicum} was inoculated into 20 ml LB broth supplemented with glucose (LBG) from solid slants of the bacteria. The tubes were incubated at 37°C overnight and growth was checked for on the growth medium. Bacteria were enumerated and diluted to make up a concentration of 1 x 10^6 cfu/ml.

**Antimycobacterial susceptibility test**
Middlebrook 7H11 supplemented with casein acid hydrolysate and Luria bertani broth media supplemented with glucose were prepared and allowed to cool. A sample of 1 x 10^6 cfu/ml of \textit{M. aurum} and \textit{C. glutamicum} was inoculated into the media and mixed. The media now containing bacteria was allowed to solidify. The test material equivalent to 500 µg, dissolved in ethanol was applied on sterile paper disks (6 mm diameter, cartridge susceptibility disks, Mast Diagnostics, Mast Group Ltd, Merseyside, UK). The solvent was allowed to evaporate under a stream of air from filters deposited on 96-well plates at room temperature. The disks were then deposited on the surface of the inoculated agar plates. Plates were incubated at 4°C for 2 hours and then at 37°C in a Labcon incubator (Gallenkamp, England) for overnight. The inhibition zone which is the diameter of inhibition around each of the disc was measured and recorded in millimeters. Rifampicin, a standard antimycobacterial agent was used as the positive control whilst ethanol was used as the negative control.
Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)
The in-disk agar disk diffusion assay was carried out to determine the MIC and MBC according to Zaidan et al. (2005) with slight modifications. The disk diffusion test was carried out with twelve disks of 2-fold serially diluted concentrations of the plant extracts (500 µg/disk – 0 µg/disk). Exacts' concentrations were prepared by serial dilution. Rifampicin was used a positive control for both M. aurum and C. glutamicum. An aliquot of 1 x 10^6 cfu/ml of the bacteria was inoculated into the media, Middle Brook 7H11 and LB supplemented with glucose for M. aurum and C. glutamicum respectively. The agar was poured into 90 mm petri dishes (Concorde Plastics, Johannesburg, SA) and was allowed to cool. The filter disks impregnated with the plant extract were then placed onto the agar plates in duplicates and were incubated for 2 hours at 4ºC. The agar plates were then incubated overnight at 37ºC and 30ºC for M. aurum and C. glutamicum respectively. The lowest concentration that inhibited growth was taken as the minimum inhibitory concentration (MIC) value for each of the bacterial species. The MIC was then determined as the lowest concentration where there was a zone of inhibition for each plant extract. Minimum bactericidal concentration (MBC) assay were determined as the lowest concentration that completely kills bacteria. The MBC was determined from the MIC plates and the preceding plates by streaking MIC plates on the zone of inhibition and sub-culturing the organisms onto fresh Middle Brook 7H9 and LB agar for M. aurum and C. glutamicum respectively that had no extracts. The plates were incubated at 37ºC and 30ºC overnight for each particular for M. aurum and C. glutamicum respectively. The MBC was recorded, as the lowest concentration at which there was no growth of bacteria after the sub-culturing into an plant extract (or drug)-free medium. The assays were repeated four times.

Effect of combining plant extract and rifampicin
Extracts at concentration of MIC, double the MIC and ten times the MIC value of the plant extract with or without rifampicin was placed on filter disks (6 mm diameter, cartridge susceptibility discs, Mast Diagnostics, Mast Group Ltd, Merseyside, UK) and allowed to dry. The extract and the rifampicin maintained constant at 15 µg/ml were combined and placed on the disks. Rifampicin alone at concentrations of 15 µg/ml, 10 µg/ml and 5 µg/ml was also placed on the filter paper. Rifampicin at varying concentrations was combined with the test compound constant (up to ten times the MIC) and placed onto filter papers and allowed to dry. A quantity of 1 x 10^6 cfu/ml of the bacteria was inoculated into the media, Middle brook 7H11 and LB. The agar was poured onto petri dishes and was allowed to cool. The filter disks were then placed onto the agar plates in duplicates and were incubated for 2 hours at 4ºC and then overnight at 30ºC and 37ºC for C. glutamicum and M. aurum respectively. The zone of inhibition was then measured. All the experiments were performed in quadruplicate.

Flouroquinolone accumulation and active efflux
Accumulation and efflux of the flouroquinolone, ciprofloxacin was measured by the method of Mortimer & Piddock (1991) with some modifications. Bacteria were grown in Middlebrook 7H11 and LB media at 37ºC and 30ºC for M. aurum and C. glutamicum to an A 660 of 0.6-0.8 and harvested by centrifugation at 3000 rpm for 10 minutes. Bacteria were then washed twice with 50 mM sodium phosphate buffer (pH 7.0) at 4ºC. The cells were weighed and a volume needed to make up a volume of 40 mg/ml was made in 10 mM PBS containing sodium azide. The mixture was incubated at 37ºC for 15 minutes. The flouroquinolone ciprofloxacin was added to the mixture to a final concentration of 20 mg/ml. The reaction mixture was incubated at 37ºC with shaking at 120 rpm for 60 minutes. The sample was
divided into two aliquots, a 2/3 and 1/3 volume sample, which were then centrifuged for 5 minutes at 2800 x g. The supernatant was discarded and the pellet was weighed. PBS was added to the 1/3 sample to make up to a concentration of 40 mg/ml, this tube representing a sample without glucose. The 2/3 sample was further subdivided into 3 equal aliquots of 40 mg/ml containing reserpine, ethanol and plant extracts. Appropriate controls were set up to rule out the effects of the solvent. The samples were mixed and incubated at 37°C at 120 rpm for 30 minutes. The samples were centrifuged in a Rotofix® centrifuge (Zentrifugen, Germany) for 5 minutes at 2800 x g. The cells were washed in the chilled buffer and re-centrifuged for 5 minutes. Glycine hydrochloride 3.0 ml (0.1M, pH 3.0) was added to the pellet at 37°C and 30°C for M. aurum and C. glutamicum respectively with agitation to ensure exposure of the cells to the lysis buffer and the solutions were left overnight. The pellet was then centrifuged at 2800 x g for 10 minutes in a Rotofix centrifuge. The supernatant was centrifuged for another 5 minutes. The fluorescence of ciprofloxacin was determined at the excitation and emission wavelengths of 270 nm and 452 nm respectively using an RF-1501 Shimadzu spectrofluorimeter (Shimadzu Cooperation, Tokyo, Japan). Both supernatant samples from the intact cells (representing efflux samples) and the lysed cells (representing influx samples) were quantified for ciprofloxacin using a standard curve. To rule out interference from the reserpine and the plant extracts, their fluorescence was determined at the same excitation and emission wavelength as ciprofloxacin. The active efflux/influx of ciprofloxacin from the mycobacteria species was also examined in the presence of ethanolic extracts from the plants V. adoensis, Parinari curatellifolia, Mangifera indica for C. glutamicum and V. adoensis, P. curatellifolia and Faurea species for M. aurum.

**Statistical analysis**

All values have been expressed as mean ± standard deviation and the comparison of the antibacterial activity of the samples with standard antibiotics was evaluated by applying One-way ANOVA with Dunnett’s Multiple Comparison Test using Graphpad Instat software® (Graphpad Prism Inc. San Diego, CA, USA, www. Graphpad.com). P values less than 0.05 were considered to be statistically significant difference.

**RESULTS**

**Screening by Disk diffusion assay**

The study was carried out to offer a better understanding of the ethnomedicinal and biological use of some of the plants widely distributed in Zimbabwe. A total of 30 ethanol extracts from nineteen different plants belonging to seventeen families were analysed. Plants were selected on the basis of their ethnomedicinal uses in the treatment of symptoms associated with TB in Zimbabwe (Table 1). Table 2 shows the growth inhibitory parameters of the ethanol plant extracts against M. aurum and C. glutamicum. V. adoensis was the most potent plant extract when using M. aurum and M. indica was the most potent plant extract when using C. glutamicum. Generally the antibacterial activity was low when using C. glutamicum as the test organism. The other plants showed mild to moderate antibacterial activities against the bacterial species.

**Determination of MIC and MBC**

Table 2 shows the antibacterial activities (MIC values in µg/disc) of five plant extracts which were found to have the most potent antibacterial activity by disc diffusion assay. The lowest MIC for C. glutamicum was that for the extract from Lippia javanica (32 µg/disk). The extract from Ziziphus mucronata had antibacterial activity at a high concentration of 500 µg/disk. These results suggest that the plant extracts had bacteriostatic activity when C. glutamicum was used as the test organism. For M. aurum, the extract from the stems of P. curatellifolia was the most potent with an
Table 1. Ethnobotanical information of investigated plants from Zimbabwe

<table>
<thead>
<tr>
<th>Family</th>
<th>Botanical name</th>
<th>Local name</th>
<th>Voucher</th>
<th>Plant part tested</th>
<th>Major traditional use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malvaceae</td>
<td>Abelmoschus esculentus</td>
<td>Derere</td>
<td>N15E10</td>
<td>fruit</td>
<td>Okra is a traditional food plant. It is used for the treatment of bronchitis, heart diseases and tuberculosis of the lungs (<a href="http://www.greenpatio">www.greenpatio</a>, 2010)</td>
</tr>
<tr>
<td>Aloaceae</td>
<td>Aloe vera barbadensis</td>
<td>Gavakava</td>
<td>N16E4</td>
<td>Leaves</td>
<td>Widely used for external treatment of minor wounds and inflammatory skin disorders. It is used for the treatment of pneumonia, tuberculosis and cough (Gelfand et al., 1985)</td>
</tr>
<tr>
<td>Rubiaceae</td>
<td>Catunaregam spinosa</td>
<td>Mutsvairchuru</td>
<td>C5E7</td>
<td>Leaves</td>
<td>Leaves are used for pulmonary infections, bark is an astrigent (Serada et al., 2002). Various respiratory ailments (Gelfand et al., 1985).</td>
</tr>
<tr>
<td>Leguminosae</td>
<td>Rhyphochisa insignis</td>
<td>Mukoyo</td>
<td>C15E7</td>
<td>Leaves</td>
<td>Treatment of diarrhea (Chinemapna et al., 1985).</td>
</tr>
<tr>
<td>Euphobiaceae</td>
<td>Croton gratissimus</td>
<td>Gunukira</td>
<td>UZ13E7</td>
<td>Leaves</td>
<td>Roots and bark infusions treat respiratory disorders (Roodt 1998).</td>
</tr>
<tr>
<td>Clusiaceae</td>
<td>Garcinia huillensis</td>
<td>Mutunduru</td>
<td>C10E7</td>
<td>Leaves</td>
<td>Treatment of cough, pneumonia and tuberculosis (Gelfand et al., 1985).</td>
</tr>
<tr>
<td>Celastraceae</td>
<td>Gymnosporia senegalensis</td>
<td>Chishuzhu</td>
<td>N3E7</td>
<td>Leaves</td>
<td>Treatment of coughs, pneumonia and TB (Gelfand et al., 1985).</td>
</tr>
<tr>
<td>Verbenaceae</td>
<td>Lippia javanica</td>
<td>Zimbani</td>
<td>C3E7</td>
<td>Leaves</td>
<td>Leave infusion used to treat coughs, colds and fever, influenza, measles, malaria and stomach ache (Van Wyk, 2009).</td>
</tr>
<tr>
<td>Anarcardiaceae</td>
<td>Mangifera indica</td>
<td>Mumango</td>
<td>N17E4</td>
<td>Stems</td>
<td>Treatment of tuberculosis (Kisangau et al., 2007).</td>
</tr>
<tr>
<td>Olacaceae</td>
<td>Olax obtusifolia</td>
<td>Gungwe</td>
<td>U28E7</td>
<td>Leaves</td>
<td>Treatment of abdominal pain (Chinemapna et al., 1985).</td>
</tr>
<tr>
<td>Chrysobalanaceae</td>
<td>Parmarini eucratifolia</td>
<td>Muhacha</td>
<td>C6E7</td>
<td>Leaves</td>
<td>Skin rashes, tuberculosis, Chronic diarrhea, herpes zoster, herpes simplex (Chigora et al., 2007).</td>
</tr>
<tr>
<td>Anarcardiaceae</td>
<td>Rhus longipes</td>
<td>Muchokochiana</td>
<td>C11E10</td>
<td>Flowers</td>
<td>Treatment of headache (Maliwichi, 2008).</td>
</tr>
<tr>
<td>Solanaceae</td>
<td>Solanum mauritianum</td>
<td>–</td>
<td>UZ10E7</td>
<td>Leaves</td>
<td>Treatment of menorrhagia (Lewu &amp; Afolayan, 2009).</td>
</tr>
<tr>
<td>Myrtaceae</td>
<td>Syzygium guineense</td>
<td>Muboo</td>
<td>C8E7</td>
<td>Leaves</td>
<td>Chest pain (De Boer et al., 2004).</td>
</tr>
<tr>
<td>Asteraceae</td>
<td>Vernonia adenosis</td>
<td>Musikavakadzi</td>
<td>C1E10</td>
<td>Flowers</td>
<td>The leaves are used for treatment of TB (Kisangau et al., 2007).</td>
</tr>
<tr>
<td>Leguminosae</td>
<td>Xeroderis stuhmannii</td>
<td>Murumanyama</td>
<td>C4E7</td>
<td>Leaves</td>
<td>Mastitis and backache (Ruffo, 1991).</td>
</tr>
<tr>
<td>Rhamnaceae</td>
<td>Ziziphus mucronata</td>
<td>Muchecheni</td>
<td>C7E4</td>
<td>Leaves</td>
<td>Paste of leaves treats boils, carbuncles and swollen glands, leaf infusion is taken against chest complaints (Roodt, 1998).</td>
</tr>
</tbody>
</table>
Table 2. Minimum inhibitory concentrations and minimum bactericidal concentrations of extracts towards the mycobacterial species

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Zone of inhibition (mm) at 500 µg/disc</th>
<th>Plant extract</th>
<th>Zone of inhibition (mm) at 500 µg/disc</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vernonia adoensis</em> leaves</td>
<td>28 ± 1</td>
<td><em>Mangifera Indica</em> (leaves)</td>
<td>17 ± 1</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td></td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>Faurea saligna</em> leaves</td>
<td>18 ± 1</td>
<td><em>Vernonia adoensis</em> leaves</td>
<td>15 ± 1</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>&gt;500</td>
<td></td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>Syzygium guineense</em> leaves</td>
<td>19 ± 2</td>
<td><em>Parinari curatellifolia</em> stems</td>
<td>13 ± 1</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td></td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td></td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>Xeroderris stuhlmannii</em> leaves</td>
<td>18 ± 1</td>
<td><em>Ziziphus Mucronata</em> stems</td>
<td>13 ± 1</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td></td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td></td>
<td>500</td>
</tr>
<tr>
<td><em>Parinari curatellifolia</em> stems</td>
<td>18 ± 1</td>
<td><em>Lippia javanica</em> roots</td>
<td>12 ± 1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td></td>
<td>&gt;500</td>
</tr>
<tr>
<td><em>Parinari curatellifolia</em> Leaves</td>
<td>18 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>29 ± 2</td>
<td>Rifampicin</td>
<td>40 ± 1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td></td>
<td>63</td>
</tr>
</tbody>
</table>

*aResults are the average (± SD) of two separate antibacterial susceptibility test (Each antibacterial susceptibility test was followed by a disk diffusion assay done in quadruplicate). The zone of inhibition being determined at a concentration of 500 µg/disk

MIC – Minimum inhibition concentration

MBC – Minimum bactericidal concentration

MIC of 8 µg/disk and an MBC of 63 µg/disk. Generally the plant extracts showed both bacteriostatic and bactericidal activities as compared to their effects on *C. glutamicum*. The values of the MBCs of the plant extracts in both species were less than that of the drug rifampicin.

The effect of combining rifampicin and plant extracts on growth inhibition

Plant extract and rifampicin were combined and the effects assessed on the growth of the bacteria. The concentration of the rifampicin was varied while that of the plant extract was maintained constant and vice-versa. Typical results are shown in Figure 1 for the combination of the extract from *X. stuhlmannii* and rifampicin. There was a gradual increase in rifampicin in the zone of inhibition with increase in concentration of rifampicin. When rifampicin, and the ethanol plant extract (maintained constant) were combined there was a decrease in the zone of inhibition as compared to rifampicin alone. The zone of inhibition obtained when
the ethanol extract of *X. stuhlmannii* and rifampicin (maintained constant) combined showed an increase in zone of inhibition but lower than when rifampicin was used on its own. *Xeroderris stuhlmannii* was, thus, decreasing the potency of rifampicin and, hence, the lower zone of inhibition. Generally these results showed an antagonistic activity between the plant extracts and rifampicin (Table 3).

Table 3. The effects of combining the plant extract and rifampicin on growth inhibition

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Test organism</th>
<th>Effects of combining extract + rifampicin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Parinari curatellifolia</em></td>
<td><em>M. aurum</em></td>
<td>Antagonism</td>
</tr>
<tr>
<td><em>Xeroderris stuhlmannii</em></td>
<td><em>M. aurum</em></td>
<td>Antagonism</td>
</tr>
<tr>
<td><em>Lippia javanica</em></td>
<td><em>C. glutamicum</em></td>
<td>No effect</td>
</tr>
<tr>
<td><em>Ziziphus mucronata</em></td>
<td><em>C. glutamicum</em></td>
<td>Antagonism</td>
</tr>
</tbody>
</table>

Antagonistic – A plant extract acting against rifampicin by decreasing its potency
No effect – A plant extract not showing a significant change in the activity of rifampicin

Figure 1. The effects of combining rifampicin and the extract from *X. stuhlmannii* on the growth of *M. aurum*. Rifampicin and the extract were kept constant (A/C) or varied (B/D). The combined effects of the drug and extract were assessed on the growth inhibition. Data are the mean + SD for n=4. ** Values differ significantly (P<0.01)
Ciprofloxacin accumulation assay
Overexpression of ATP binding cassette (ABC) transporters has been proposed as a major mechanism contributing to the innate drug resistance in *M. tuberculosis* and related *Mycobacterium* (Jiang et al., 2008). The aim of this part of the study was, therefore, to determine the effects of three most potent antimycobacterial extracts screened by disk diffusion assay, on the ATP mediated-drug efflux of the bacteria species. Bacterial cells were exposed to the plant extract, reserpine, glucose and buffer as shown in Figures 2 and 3. The fluorescence of the ciprofloxacin accumulated was then quantified. The extract from *Parinari curatellifolia* had the highest drug efflux pump inhibitory effect against both *C. glutamicum* and *M. aurum*. *Vernonia adoensis* and *Faurea saligna* leaf extracts also inhibited the action of the efflux pumps. The cells exposed to glucose showed a high amount of ciprofloxacin efflux compared to those without as glucose provides the ATP needed to drive the efflux pumps.

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 4. All the plant extracts at the same concentration as reserpine did not fluoresce at the excitation and emission wavelengths. The fluorescence by reserpine was not significant as compared to the fluorescence of ciprofloxacin as this compound was added at a concentration of 61 ng/ml whilst ciprofloxacin was at 20 mg/L.

DISCUSSION
Natural products continue to play a role in the drug discovery and development process, and plants are recognized as a very useful source of highly active antimycobacterial metabolites (McGaw et al., 2008b). Approximately 75% of the World’s population relies on medicinal plants as their primary source of medicine (Chigora et al., 2007; Gonzalez-Santiago et al., 2008).

Figure 2. The effects of the plant extracts on the accumulation and efflux of ciprofloxacin in *M. aurum*. Cells were loaded with ciprofloxacin and then incubated with the plant extract. The accumulation (A) was measured by determining the fluorescence of ciprofloxacin in the lysed cells. The efflux (B) was determined from the amount of ciprofloxacin in the supernatant of cells before lysis. Experiments were performed in duplicate and each measurement was done twice. Values differ significantly (**P<0.01; *P<0.05). All values are compared to the value of the sample with glucose.
Figure 3. The effects of the plant extracts on the accumulation and efflux of ciprofloxacin from *C. glutamicum*. Cells were loaded with ciprofloxacin and then incubated with the plant extract. The accumulation (A) was measured by determining the fluorescence of ciprofloxacin in the lysed cells. The efflux (B) was determined from the amount of ciprofloxacin in the supernatant of cells before lysis. Experiments were performed in duplicate and each measurement was done twice. Values differ significantly (**P<0.01; *P<0.05). All values are compared to the value of the sample with glucose.

Figure 4. Baseline fluorescence of the plant extracts. Fluorescence of each extract, at 61 ng/ml, the same concentration as that of the standard inhibitor reserpine, was determined at 254 nm emission wavelength 270 nm excitation wavelength.
Plants with antimycobacterial activity are being studied resulting in the isolation and characterization of several bioactive compounds. Plant species still serve as sources of many novel biologically active compounds, as very few plants have been thoroughly investigated for their medicinal properties (Jachak et al., 2007). There has been a renewed interest in plants as sources of novel therapeutics the past decade and, hence, many plants are being investigated for their pharmacological effect.

The present study shows that 11 ethanol plant extracts had antimycobacterial effects as determined from the effects of the positive control, rifampicin and, therefore, may have potential use as antitymocobacterial agents (Table 2). Corynebacterium glutamicum was less sensitive to plant extracts than M. aurum. This may be due to the differences in their structure mainly the length of the mycolic acids. Corynebacterium glutamicum has a highly ordered outer mycolic acid layer which has low fluidity and permeability which plays a crucial role in the flux of solutes (Eggeling et al., 2001). Alteration in this layer may lead to an increase in efflux rendering the plant extracts inactive. M. aurum is a species of saprophytic, rapidly growing, non-pathogenic mycobacteria. Mycobacterium aurum is used as a test organism in the initial primary screening process of plant extracts and has a similar drug sensitivity profile to M. tuberculosis (Newton et al., 2001). The extract from V. adoensis was a potent inhibitor of growth in M. aurum with inhibitory effects rifampicin equivalent to 80 µg of rifampicin (MIC-31 µg/disk). Similar effects were also observed in C. glutamicum. V. adoensis leaves are used in the treatment of symptoms associated with TB in Tanzania. The family Asteracea to which V. adoensis belongs stands out in medicinal plant inventories with large numbers of medicinal species (Tabuti et al., 2010). The popularity of Asteracea is thought to be due to the large diversity of bioactive components members of this family possess. Vernonia adoensis has been found to have high levels of sequiterpene lactones which correlate with a high anti-proliferative activity (Fouche et al., 2009). In a study by Kisangau et al. (2007) the crude extract of V. adoensis had the highest activity against Escherichia coli with an activity index (AI) of 1.76. The extract from the leaves and stems of P. curatellifolia showed a considerable amount of activity against C. glutamicum. Both the leaves and the stems of P. curatellifolia are used traditionally in the treatment of symptoms associated with TB (Chigora et al., 2007). This could be the basis for its ethnobotanical use in the treatment of symptoms associated with TB in Zimbabwe. Parinari curatellifolia is also used to treat malaria, wounds, typhoid, fever and has been reported to have antiplasmodic effects (Audu & Amupitan, 2006). Extracts from Parinari curatellifolia have been found to have moderate anti-proliferative activity against breast cancer cell lines. The high levels of ent-kaurene terpenoids that have been isolated from the leaves of P. curatellifolia may be responsible for this property (Rundle et al., 2001). Parinari curatellifolia was found to have antimicrobial effect against Staphylococcus aureus, Corynebacterium ulceran, E. coli, Salmonella typhi and Candida albicans (Audu & Amupitan, 2006).

Mangifera indica stems and twigs extracts were the most potent inhibitor of growth using C. glutamicum. Based on ethnopharmacological knowledge, a standard aqueous extract of M. indica stem was shown to have anti-oxidant, anti-inflammatory, and immunomodulatory properties (Wauthoz et al., 2007) and was proposed to improve the quality of life in AIDS, cancer and asthma patients. Gallotannins isolated from M. indica have been shown to have antimicrobial activity (Schierer et al., 2009). Leaf extracts of M. indica have been shown to have antibacterial activity against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa (Bbosa et al., 2007). The activity of M. indica against Clostridium
tetani has been investigated and it has been shown that ethanolic extracts contained alkaloids, tannins, triterpenoids and these compounds could have antimycobacterial activity in the case of our study.

*Lippia javanica* extract had the lowest MIC of 31.25 µg/disk when using *C. glutamicum*. Several plants that belong to the same genus have been found to possess antimycobacterial activity. These include *Lippia origanoides* and *Lippia alba* (Bueno-Sanchez et al., 2009). However, *V. adoensis* which was expected to show the lowest MIC, based on growth inhibition zones, ranked third with an MIC of 250 µg/disk and its MBC was greater than 500 µg/disk. This may be because its antimycobacterial activity exerts itself at high concentrations of the plant extract as the zone of inhibition was determined at a concentration of 500 µg/disk.

*Syzygium guineense* showed considerable activity in the agar disk diffusion assay. Djoukeng et al. (2005) found ten triterpenes in *Syzygium guineense* that had antibacterial activity against gram positive and gram negative bacteria and human pathogen bacteria. Triterpenes may also be responsible for the antimycobacterial activity observed in these studies.

The possibility of multiple plant secondary metabolites working together has to be taken into account when evaluating for biological activity of botanical and ethnomedical preparations (Wang et al., 2003). The mode of action of combination differs significantly than that of the same drugs acting individually; hence isolating a single component may make the plant extract lose its potency (Hemaiswarya et al., 2008). It has been shown that *Emblica officinalis* fractions prevent rifampicin, isoniazid and pyrazinamide induced toxicity (Tasduq et al., 2005). All the plant extracts examined in this study did not show any synergistic activity with the standard drug rifampicin. This may be because the plant extracts consists of different compounds which interact differently with rifampicin at the target site. Rifampicin inhibits bacterial RNA synthesis by binding to the β-subunit of bacterial DNA-dependent RNA polymerase. The compounds in the plant extract may have altered the drug target for rifampicin rendering it inactive or altered the transport of rifampicin to the target site.

Efflux pumps can cause multi-drug resistance and have recently received a lot of interest as promising new targets in antimicrobial therapy (Lechner et al., 2008). In *Mycobacterium smegmatis*, the isoflavone biochanin A, a phenolic compound, exhibited efflux pump inhibiting activity for ethidium bromide comparable to the effects of the standard efflux inhibitor verapamil. Ciprofloxacin is a fluoroquinolone that targets DNA gyrase (topoisomerase II) and, therefore, stabilizes the complex between DNA and DNA gyrase (Walker, 1999). It was used in this study to assess the effects of the plant extracts on the drug efflux pumping activity of ATP-binding cassette transporter proteins in the two mycobacterial species. The extract from *P. curatellifolia* made the bacteria accumulate a higher concentration of ciprofloxacin in comparison with the other plant extracts and that of reserpine, a known efflux pump inhibitor (Figure 3). These results suggest that inhibition of efflux pumps could be a mechanism for *P. curatellifolia* in its role as an antimycobacterial agent.

The effects observed in vitro may differ from those effects observed in vivo (Houghton et al., 2007). The lack of activity in vivo may be impracticable to extrapolate the dose from that activity in vitro, which will be required in equivalence for the size of a human adult. Metabolism and adsorption may lead to discrepancies as in vivo, there are enzymes which may bioconvert the active compound to an inactive compound that may have no effect on the target. Further tests need to be carried out of the effect of the plant extracts on *M. tuberculosis* to test the efficacy of the plant species with the pathogenic form of mycobacteria.

The results in this study provide leads for plant extracts that may be developed into antimycobacterial agents.
common solvent used to extract active compounds from plant sources. Dried plants can be ingested as teas or, rarely, tinctures, or inhaled via steam from boiling suspensions of the parts. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through ethanol and water extraction. The study tried to mimic traditional medicine by using absolute ethanol which has similar polarities to the traditional solvent water and, therefore, expected to extract similar compounds. Although, water could have been used in the study, ethanol was used instead as it is quicker to extract and this solvent reduces microbial contamination.

The plant extracts in this study are not only able to inhibit mycobacterial growth but are also able to enhance the accumulation of drugs inside the bacteria. This activity may contribute to the suppression of drug resistance to the existing antimycobacterials. Therefore, the antimycobacterial activity of these plants may be due to direct effects on growth as well as indirect effects due to inhibition of efflux of compounds from these cells. These results also indicate the potential of plants used by traditional healers in Zimbabwe as a source of possible bioactive compounds. Further investigations on isolation and characterization of antimycobacterial bioactive agents from these plants are in progress. In conclusion, the extracts from *P. curatellifolia* showed the greatest antibacterial activity through both inhibition of growth and inhibition of drug efflux in *M. aurum* and *C. glutamicum*. The active plant extracts were found to be bacteriostatic rather than bactericidal and showed antagonism with rifampicin. This plant may serve as a source of lead compounds in the search of new antimycobacterials.

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