Intracellular and membrane-damaging activities of methyl gallate isolated from *Terminalia chebula* against multidrug-resistant *Shigella* spp.

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*Shigella* spp. (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*) cause bacillary dysentery (shigellosis), which is characterized by bloody mucous diarrhoea. Although a variety of antibiotics have been effective for treatment of shigellosis, options are becoming limited due to globally emerging drug resistance. In the present study, *in vitro* antibacterial activity of methyl gallate (MG) isolated from *Terminalia chebula* was determined by performing MIC, minimal bactericidal concentration (MBC) and time-kill kinetic studies. Bacterial membrane-damaging activity of MG was determined by membrane perturbation and transmission electron microscopy (TEM). Cellular drug accumulation, cell infection and assessment of intracellular activities of MG and reference antibiotics were performed using HeLa cell cultures. The bactericidal activity of MG against multidrug-resistant (MDR) *Shigella* spp. in comparison with other commonly used drugs including fluoroquinolone was demonstrated here. TEM findings in the present study revealed that MG caused the total disintegration of inner and outer membranes, and leakage of the cytoplasmic contents of *S. dysenteriae*. The level of accumulation of MG and tetracycline in HeLa cells incubated for 24 h was relatively higher than that of ciprofloxacin and nalidixic acid (ratio of intracellular concentration/extracellular concentration of antibiotic for MG and tetracycline>ciprofloxacin and nalidixic acid). The viable number of intracellular *S. dysenteriae* was decreased in a time-dependent manner in the presence of MG (4 × MBC) and reduced to zero within 20 h. The significant intracellular activities of MG suggested that it could potentially be used as an effective antibacterial agent for the treatment of severe infections caused by MDR *Shigella* spp.

INTRODUCTION

*Shigella* spp. (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*) cause bacillary dysentery (shigellosis), an invasive disease of the human colon which reflects the capacity of these species to invade intestinal epithelial cells (Sansonetti, 2006). Invasion includes entry of the bacterium into epithelial cells, intracellular growth, intracellular movement and cell-to-cell spread, and eventually death of the host cell (Sansonetti, 2006). Shigellosis is a serious human health problem in both developing and industrialized countries. About 125 million cases of *Shigella* infection occur annually in Asia, of which ~14,000 are fatal (Bardhan et al., 2010; Ghosh et al., 2011). In the USA, there are up to 280,000 cases of shigellosis per year, most of which occur in preschool-age children with relatively mild disease (Bradley & Jackson, 2011). An increasing rate of ampicillin and trime-
thorprim/sulfamethoxazole (TMP/SMX) resistance has been reported, and multidrug-resistant (MDR) strains are becoming common. In an outbreak of MDR S. sonnei infection in the USA in 2005, 89 % of the strains were resistant to both ampicillin and TMP/SMX (CDC, 2006).

The distribution of serogroups of Shigella spp. differs from country to country: S. dysenteriae, S. flexneri, S. sonnei and S. boydii serogroups are predominant in developing countries, whilst S. sonnei is frequently reported in industrialized countries (Ghosh et al., 2011). Effective antimicrobial therapy for shigellosis shortens the duration of clinical symptoms, reduces the severity of the dysentery and the threat of potential lethal complications, and prevents the spread of disease (WHO, 2005a; Nagano et al., 2009). However, experimental treatment with first-line antimicrobial agents, including TMP/SMX, chloramphenicol, ampicillin, nalidixic acid and tetracycline, has become less effective due to the high prevalence of MDR clinical isolates amongst Shigella spp. (Dutta et al., 2003; Pazhani et al., 2005; Sivapalasingam et al., 2006; von Seidlein et al., 2006; Nagano et al., 2009). Fluoroquinolones, ceftriaxone and pivmecillinam are the drugs of choice at present recommended by the World Health Organization for the treatment of MDR Shigella infection in adults, children and immunocompromised hosts, as these drugs are effective at reducing mortality caused by Shigella spp. (Nagano et al., 2009; Traa et al., 2010; Ghosh et al., 2011). However, reduced susceptibility of Shigella spp., especially S. dysenteriae serotype 1, to fluoroquinolones has been reported (Sarkar et al., 2003). In addition, the use of antimicrobials for the treatment of shigellosis varies from country to country (Ghosh et al., 2011). Although pivmecillinam is being used in Bangladesh, it is not the drug of choice for the treatment of diarrhoea in many countries (Rahman et al., 2007). Ceftriaxone is an ideal drug for the treatment of shigellosis in regions where fluoroquinolone resistance is common. However, isolation of ceftriaxone-resistant Shigella spp. has also been reported (Rahman et al., 2007). The present antimicrobial resistance towards fluoroquinolones and ceftriaxone (Pazhani et al., 2005; Rahman et al., 2007; Wong et al., 2010; Ghosh et al., 2011) indicates that these drugs will no longer be useful, and emphasizes the need for using substitute drugs for the treatment of shigellosis.

Natural products have been used for centuries as traditional remedies to combat a variety of ailments, including infectious diseases (Rios & Recio, 2005). Many plants conveniently available in India are used in traditional folklores medicine for the treatment of gastrointestinal disorders, such as cholera, diarrhoea and dysentery (Kala et al., 2004). Many modern and novel drugs have their origin in traditional medicine and ethnopharmacology. Terminalia chebula Retz. (Combretaceae), commonly known as haritaki, is used for the treatment of diarrhoea, indigestion, etc., in traditional Ayurvedic medicine (Kala et al., 2004). We have demonstrated previously that T. chebula shows promising broad-spectrum antibacterial properties, inhibiting the strains of enteropathogenic bacteria (Acharyya et al., 2009). Previously, in vitro activity of methyl gallate (MG) isolated from Galla rhosa alone and in combination with reference antibiotics such as ciprofloxacin against Salmonella, Escherichia, Clostridium and Enterobacter spp. was studied (Choi et al., 2009). However, the intracellular activity of MG against intracellular pathogens such as Shigella spp., the causative agents of shigellosis, has not yet been studied. In the present study, we demonstrated the intracellular and membrane-damaging activities of MG isolated from T. chebula against MDR Shigella spp.

**METHODS**

**Plant material.** MG was isolated, purified and identified from fruits of T. chebula as described previously by us (Acharyya et al., 2012).

**Bacterial strains.** The clinical strains of Shigella spp. used were S. flexneri 2a (B294), S. dysenteriae 1 (NT4907), S. boydii 4 (BC612) and S. sonnei (IDH00968SS) (strains were kindly provided by Dr Hemanta Koley, NICED, Kolkata, India). All the strains of Shigella spp. were positive for the Sereny test, and ipaH and virF genes. The strains were stored in 15 % glycerol stock at −70 °C.

**Antibiotic susceptibility test.** Antimicrobial susceptibility testing was performed by the disc diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010) with commercially available disks (BD) of ampicillin (10 μg), chloramphenicol (30 μg), co-trimoxazole (25 μg), ciprofloxacin (5 μg), furazolidone (100 μg), gentamicin (10 μg), neomycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), streptomycin (10 μg) and tetracycline (30 μg) on Mueller–Hinton agar (BD) using Escherichia coli ATCC 25922 for quality control.

**Antibacterial activity.** The MICs and minimal bactericidal concentrations (MBCs) of MG and reference antibiotics (streptomycin, ciprofloxacin, ampicillin, tetracycline and nalidixic acid) were determined using broth microdilution as described previously (Thakurta et al., 2007) following the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1997, 1999). A final inoculum of ~1 × 10^8 c.f.u. ml ^−1 was used and the concentrations of MG tested were 1–1000 μg ml ^−1.

**Selection of resistant mutants in vitro.** The strain S. dysenteriae 1 was grown and maintained in Mueller–Hinton agar broth (MHB) in the presence of sublethal doses of MG (32 and 64 μg ml ^−1) at 37 °C. Consecutive subcultures of S. dysenteriae 1 were exposed to sublethal doses of MG for 8 weeks and the bacteria were retested periodically (at 2 week intervals) to determine any increase in MIC following the method described above.

**Time-kill kinetic study.** Cultures of bacteria in MHB (~1 × 10^6 c.f.u. ml ^−1) were incubated separately in the absence (control) and presence of drug at the MBC for 24 h at 37 °C. Samples of the bacterial cultures were removed at 2, 4, 8, 12 and 24 h to record survival counts, expressed as c.f.u. ml ^−1. The surviving log_{10}c.f.u. ml ^−1 was plotted against time.

**Determination of mammalian cell toxicity.** The haemolysis test was employed to determine cellular toxicity of MG (at concentrations of 1 × 10^4 M) as previously described (Situn & Bobek, 2000) using human red blood cells suspended in PBS (10 mM PBS, pH 7.4). The 100 % haemolytic control was the buffer containing 1 % Triton X-100 and the release of haemoglobin was determined at 540 nm. Toxicity of test compound was also assayed on HeLa cells as described previously (De La Fuente et al., 2006).
Membrane perturbation assay. The membrane perturbation assay was performed using propidium iodide (PI) fluorescence detection as described previously (Tsao et al., 2002). Bacteria (2 × 10^6 c.f.u. ml⁻¹) collected from the exponential phase were cultured with MG (1 × and 4 × MBC) at 37 °C. At various intervals, the bacteria were collected and stained with PI (10 µg ml⁻¹) for 10 min, and after washing the cells, dye penetration was measured by the presence of fluorescence (excitation at 535 nm and emission at 560 nm).

Transmission electron microscopy (TEM) examination. Cultures of bacteria in MHB (∼1 × 10^7 c.f.u. ml⁻¹) were incubated separately in the absence (control) and presence of drug at 1 × MBC (256 µg ml⁻¹) for a period of 12 h at 37 °C. Samples of the bacterial cultures were removed at different time intervals and harvested (5000 g, 10 min, 4 °C). Cells were washed with PBS, prefixed in 3 % glutaraldehyde overnight at 4 °C and post-fixed in 1 % osmium tetroxide for 1 h at room temperature. The cells were dehydrated in a graded series of ethanol, cleared in propylene oxide and embedded in epoxy resin. Ultrathin sections were cut in a Leica Ultracut UCT ultramicrotome. After staining the sections with uranyl acetate and lead citrate, thin sections were examined using a FEI Tecnai 12 Biotwin transmission electron microscope.

Determination of cellular antibiotic accumulation. HeLa cells were cultured in RPMI 1640 medium supplemented with 10 % FCS in an atmosphere of 95 % air/5 % CO₂ as described previously (Bag et al., 2008). Accumulation studies were performed as described previously (Carryn et al., 2002; Barcia-Macay et al., 2006). Cells were incubated with antibiotics for 24 h. Antibiotic concentration was measured by microbiological assay (by agar diffusion method) using Bacillus subtilis ATCC 6623 as the test organism.

Cell infection and assessment of intracellular activities of antibiotics. Invasion of HeLa cell monolayers by Shigella spp. has been used as a model which correlates with virulence in this organism, as penetration into epithelial cells is a requisite for virulence (Headley & Payne, 1990). In the present study, we used HeLa cells as a model to study Shigella infection and killing of intracellular bacteria. All experiments were performed with the HeLa cell line. The cells were maintained as described earlier. The cells (5 × 10⁵ cells ml⁻¹) were infected by using a fresh inoculum of S. dysenteriae (2.5 × 10⁶ c.f.u. ml⁻¹) that had been incubated for 1 h at 37 °C and washed extensively to remove non-firmly adherent bacteria (four successive sedimentations at 1300 r.p.m. followed by gentle resuspension in prewarmed sterile PBS), yielding a mean infection index of one bacterium per four HeLa cells (as determined by counting the c.f.u.) (Carryn et al., 2002; Barcia-Macay et al., 2006). The cells were then incubated in fresh medium (with or without antibiotic) for up to 24 h. To ensure the absence of extracellular bacteria, the culture medium that contained cells with firmly adherent S. dysenteriae and that had not been exposed to antibiotics after the washing procedure described here were incubated at 37 °C for 48 h; no bacterial growth was detected. After 24 h incubation, the cells were collected by centrifugation, washed with ice-cold sterile PBS and lysed in distilled water. The lysates were then plated on nutrient agar at appropriate dilutions for determination of the number of viable bacteria by counting of the colonies (determination of the number of c.f.u.) and used for total cell protein measurement (Carryn et al., 2002). All results were expressed as c.f.u. (mg HeLa cell protein)⁻¹.

RESULTS

The strains of Shigella spp. included in the present study were MDR clinical isolates. These were resistant/reduced-susceptible to chloramphenicol, furazolidone, norfloxacin, nalidixic acid, co-trimoxazole and tetracycline, and some of these isolates were resistant/reduced-susceptible to ciprofloxacin (fluoroquinolone), ampicillin and streptomycin (Table S1, available in the online Supplementary Material).

Mammalian cell toxicity

MG did not show cytotoxicity to human erythrocytes or HeLa cells (after 24 h of incubation) at concentrations of up to 4 × MBC (results not shown).

MICs and MBCs of MG against Shigella spp

MG exhibited an MIC range of 128–256 µg ml⁻¹ against Shigella spp., whereas the MBC was found to be in the range 256–512 µg ml⁻¹ (Table 1). MICs and MBCs of streptomycin, tetracycline, ampicillin, nalidixic acid, ciprofloxacin and MG against Shigella spp. were determined in MHB and HeLa cell culture medium (RPMI 1640). Results are shown in Table 1.

Time-kill studies

The MBCs (S. flexneri 2a, 512 µg ml⁻¹; S. dysenteriae 1, 256 µg ml⁻¹; S. boydii 4, 256 µg ml⁻¹; and S. sonnei, 512

Table 1. MICs and MBCs (both µg ml⁻¹) of reference antibiotics and MG against Shigella spp.

<table>
<thead>
<tr>
<th>Test compound</th>
<th>S. dysenteriae 1 (NT4907)</th>
<th>S. flexneri 2a (B294)</th>
<th>S. boydii 4 (BCH612)</th>
<th>S. sonnei (IDH00968SS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHB</td>
<td>MIC</td>
<td>MBC</td>
<td>MHB</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>256</td>
<td>512</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>32</td>
<td>128</td>
<td>32</td>
<td>128</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>256</td>
<td>256</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>32</td>
<td>128</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>256</td>
<td>512</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td>MG</td>
<td>128</td>
<td>256</td>
<td>64</td>
<td>256</td>
</tr>
</tbody>
</table>

ND, Not determined.
μg ml⁻¹) of MG showed a ∼6 log reduction in viable cell number (c.f.u.) within 20 h, compared with the untreated control (Fig. 1). At the MBC, MG showed a 4.74, 4.75, 3.68 and 4.13 log reduction in c.f.u. for S. dysenteriae 1, S. boydii 4, S. flexneri 2a and S. sonnei within 12 h, respectively, whereas tetracycline showed a 5.15, 4.92, 4.96 and 5.35 log reduction in c.f.u. for S. dysenteriae 1, S. boydii 4, S. flexneri 2a and S. sonnei within 12 h, respectively.

**Selection of resistant mutants in vitro**

After consecutive subculturing of S. dysenteriae 1 in the presence of sublethal doses of MG (32 and 64 μg ml⁻¹) for a period of 8 weeks, the bacteria did not show any increase in MIC and the MIC remained constant (i.e. 128 μg ml⁻¹).

**TEM observations**

Untreated cells of S. dysenteriae in MHB medium after 12 h of incubation at 37 °C showed a normal cell shape with an undamaged structure of the inner membrane, and the outer membrane layer was smooth and intact with slight folds (Fig. 2a). The periplasmic space was thin with a uniform appearance. However, when cells were grown in MHB supplemented with MG (256 μg ml⁻¹; 1 × MBC), significant membrane damage and intracellular structure changes were seen (Fig. 2b, c); both the outer membrane structure and the intracellular structure were fatally damaged. The membranes of the bacterial cells were distorted and intracellular structures were disorganized. Many empty cells were found in bacterial samples treated with MG for 12 h (Fig. 2c), indicating that the intracellular contents had

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**Fig. 1.** Time-kill kinetic study of (a) S. dysenteriae 1, (b) S. flexneri 2a, (c) S. boydii 4 and (d) S. sonnei in the presence of MG at the MBC. Cells grown in the absence (control; ○), and in the presence of tetracycline (▲) and MG (▲) at the MBC.
**Fig. 2.** (a) TEM images of *S. dysenteriae* 1 cells grown in antibiotic-free MHB medium (control) for 12 h. (b, c) TEM images of *S. dysenteriae* 1 cells grown in MHB supplemented with MG at 256 μg ml⁻¹ (1 × MBC) after incubation for 8 (b) and 12 h (c). Arrows indicate the leakage of intracellular contents and disorganization of cell membranes.
leaked out of the cells due to damage to the cell membrane. In addition, in the membrane perturbation assay, it was found that PI uptake increased with time after treatment with MG in a dose-dependent manner (Fig. 3).

**Cellular antibiotic accumulation and assessment of intracellular activities of antibiotics**

Table 2 shows the levels of accumulation of ciprofloxacin, tetracycline, nalidixic acid and MG in HeLa cells incubated for 24 h in the presence of the drugs at 1 × MBC. The level of accumulation of MG and tetracycline was relatively higher than that of ciprofloxacin and nalidixic acid, as the ratio of intracellular concentration/extracellular concentration of antibiotic (C_i/C_e) was relatively higher for MG and tetracycline than that of ciprofloxacin and nalidixic acid.

Intracellular activity of MG and other reference antibiotics at 4 × MBC is shown in Fig. 4. At 1 h post-infection, reference antibiotic or MG was added to S. dysenteriae-infected HeLa cells and viability of intracellular bacteria was measured at different time points (0, 2, 5 and 24 h) of incubation. The viable number of intracellular bacteria was decreased in a time-dependent manner in the presence of the drugs. MG at 4 × MBC reduced the cell number [reduction of ~4.5 log_{10}c.f.u. (mg HeLa cells protein)^{-1}] of intracellular S. dysenteriae to zero (below the detection level) within 24 h at 37 °C. These observations confirmed the intracellular activity of MG.

**DISCUSSION**

*Shigella* is a highly infectious pathogenic micro-organism and as few as 10–100 bacterial cells can cause the disease in adults (Kweon, 2008). The choice of drugs for the treatment of shigellosis has become limited due to the emergence of MDR strains of *Shigella* spp. (Rahman et al., 2007; Ghosh et al., 2011). It was found in the present study that MG was significantly active against the MDR (including fluoroquinolone-resistant) clinical isolates of *Shigella* spp. MG at 1 × MBC exhibited a time-dependent killing effect against *S. flexneri*, *S. dysenteriae*, *S. boydii* and *S. sonnei*, and could reduce the number of viable cells (c.f.u. count) of these

**Table 2.** Cellular accumulation of antibiotics in HeLa cells after 24 h incubation

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Antibiotic added (µg ml^{-1})</th>
<th>C_i/C_e*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>64</td>
<td>39.63 ± 0.10</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>128</td>
<td>21.20 ± 2.12</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>512</td>
<td>11.21 ± 0.70</td>
</tr>
<tr>
<td>MG</td>
<td>256</td>
<td>32.74 ± 2.12</td>
</tr>
</tbody>
</table>

* C_i/C_e ratio (mean ± SD, n=5) was calculated from the drug contents per mg of cell protein by using a conversion factor of 5 µl (mg cell protein)^{-1}.
species below the detection limit within 20 h. Amongst four species and many subtypes of Shigella, S. dysenteriae 1 is the only Shigella that causes epidemics and pandemics, distinguished by high fever, cramps, seizures, bloody diarrhoea and dysentery, and haemolytic uraemic syndrome, and shows enormously high fatality rates amongst children and adults (Sur et al., 2004; Pozsgay et al., 2007; McKenzie et al., 2008; Barman et al., 2011). S. dysenteriae 1 causes recurrent disease in Asian countries such as India and Bangladesh (Dutta et al., 2003; von Seidlein et al., 2006; Emch et al., 2008). Several outbreaks of S. dysenteriae 1 infection were associated with fluoroquinolone-resistant bacteria (Sarkar et al., 2003; WHO, 2005b). In addition, amongst the strains of Shigella studied here, S. dysenteriae 1 showed the highest overall resistance in terms of MICs and MBCs against all of the reference drugs tested here, including quinolones and fluoroquinolones. Hence, S. dysenteriae 1 was chosen for further studies.

TEM findings in the present study revealed that exposure of S. dysenteriae 1 to MG at 1 × MBC resulted in disintegration of the bacterial cell membrane. Our TEM study demonstrated that MG caused damage to the outer membrane of Shigella, total disintegration of the inner and outer membranes, and leakage of the cytoplasmic contents. The increased uptake of PI in the MG-treated cells of S. dysenteriae 1 in our study further confirmed that MG altered the cell membrane structure, resulting in disruption of the permeability barrier of the microbial membrane structures. This antibacterial effect of MG observed in this study is consistent with the results reported recently (Sánchez et al., 2013) where the membrane-damaging activity of MG isolated from Acacia farnesiana against Vibrio cholerae was demonstrated. In addition, bactericidal activity of antibiotics and natural products such as paenibacterin, curcumin, etc., has been demonstrated previously, which was associated with disruption of the outer membrane of Gram-negative bacteria/damage of the cytoplasmic membrane of both Gram-negative and Gram-positive bacteria (Huang & Yousef, 2014; Tyagi et al., 2015). The bacterial cytoplasmic membrane is indispensable to bacterial life as it contains one-third of the proteins in a cell and is the location for many crucial processes (Hurdle et al., 2011; Huang & Yousef, 2014). Therefore, these antibacterial agents, including MG, may interfere with numerous cellular functions by damaging the cell membrane (Hurdle et al., 2011; Huang & Yousef, 2014).

Various antibiotics have been effective for the treatment of shigellosis, although options are becoming limited due to globally emerging drug resistance (Sack et al., 2001). Originally, both sulphonamides and tetracycline were effective, but Shigella strains rapidly developed resistance to these agents (Sack et al., 2001). Ampicillin and TMP/SMX were then used, and continue to be effective in many industrialized countries. Unfortunately, in many parts of the world, strains of all species of Shigella have become resistant to these low-cost agents and neither can now be confidently used as empiric therapy for shigellosis (Sack et al., 2001). One of the few remaining, relatively inexpensive and effective drugs for shigellosis is the quinolone, nalidixic acid. Unfortunately, resistance to nalidixic acid is also common; in regions where it was introduced to treat epidemic shigellosis due to shiga bacillus, resistance developed within 6 months (Sack et al., 2001). Although fluoroquinolones and ceftriaxone are able to penetrate mammalian cells and are effective against the MDR strains of Shigella spp., several reports on antimicrobial resistance/reduced susceptibility of Shigella spp., especially S. dysenteriae, towards fluoroquinolones and ceftriaxone indicate that these drugs will no longer be useful (Sarkar et al., 2003; Rahman et al., 2007; Nagano et al., 2009; Traa et al., 2010; Ghosh et al., 2011). Moreover, these drugs are expensive. Thus, there are very few antibiotic options. In our study, after consecutive subculturing of S. dysenteriae 1 in the presence of sublethal doses of MG for 8 weeks, the bacteria did not show any increase in MIC, indicating that exposure to MG for this long period of time did not produce any MG resistance. It has been suggested previously that unlike other conventional antibiotics, membrane-active agents are less likely to mediate antibiotic resistance in treated bacteria (Hurdle et al., 2011; Huang & Yousef, 2014). As MG clearly damages bacterial cytoplasmic membranes and S. dysenteriae treated with MG did not show any MG resistance, in terms of the challenges of antibacterial resistance, MG could be a potential candidate for the treatment of emerging infectious disease such as shigellosis. We have demonstrated here the killing effect of MG on extracellular and intracellular (in HeLa cells) growth of S. dysenteriae. Correlation of the killing effect of MG and tetracycline on S. dysenteriae between its extracellular and intracellular (in HeLa cells) growth has been compared by combining data for MG and tetracycline from Figs 1 and 4 (Fig. 5). It was interesting to note that there was no significant difference between the two sets of data (P ≥ 0.4659) for MG and tetracycline. The correlation coefficients between the effect on extracellular and intracellular growth of MG and tetracycline are 0.9999 and 0.9973, respectively. In addition, we have demonstrated here the significant penetration and accumulation of MG inside HeLa cells. The Cc/Ce ratios for ciprofloxacin, tetracycline and MG were 21.20, 39.63 and 32.74, respectively, i.e. ciprofloxacin, tetracycline and MG were accumulated up to 21.20-, 39.63- and 32.74-fold inside HeLa cells, respectively. Hence, it can be suggested following the analysis of Barcia-Macay et al. (2006) that ciprofloxacin, MG and tetracycline achieved high levels (21.20- to 39.63-fold) of accumulation. Penetration of antibiotics in effective concentrations inside cells is one of the important criteria to kill intracellular pathogens (Kadurugamuwa & Beveridge, 1998). The intracellular killing activity of MG was comparable with that of ciprofloxacin, and stronger than that of nalidixic acid and tetracycline.

The most striking features of MG were its activity against clinical isolates of Shigella, which are resistant/reduced-susceptible to the antimicrobial agents recommended by the World Health Organization, including TMP/SMX,
chloramphenicol, ampicillin, nalidixic acid, tetracycline and fluoroquinolone like ciprofloxacin, and its uniform bactericidal power against four species of MDR *Shigella*. In addition, exposure of *S. dysenteriae* to MG for a long period of time did not produce any MG resistance. Considering these results and the significant inhibitory, *in vitro* and intracellular bactericidal activities of MG demonstrated here, it is suggested that it could be considered as a potential effective antibacterial agent for the treatment of severe infections caused by MDR *Shigella* spp., particularly those resistant/reduced-susceptible to most of the recommended antibiotics. However, it is so far unclear whether the membrane damage was the sole bactericidal mode of MG action. It may have an effect on the cellular proteins and nucleic acid in addition to the membranes. The binding with the membrane and the transport of MG across the membrane and its effect on cell components could be studied in detail in the future.

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