Bacteriophage Lytic Enzymes as Antimicrobial Agent for Multi-Drug Resistant
Salmonella Typhi Enzybiotics for Multidrug Resistant Bacteria

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ABSTRACT

Bacteriophage lytic enzymes are peptidoglycan hydrolases responsible for lysis of bacteria and used as anti-microbial against multi-drug resistant bacteria. To evaluate the potentiality of bacteriophage's lytic enzymes against Salmonella typhi, both micro-organisms were isolated and identified according to WHO and ISO guidelines. Bacteria susceptibility was tested using the CLSI recommendations and bacteriophage assayed by double-layer agar technique. Lytic enzymes were extracted using ultracentrifugation and purified by dialysis in ammonium sulfate. Enzyme activity was determined measuring the reducing sugars released in the reaction culture. The phages and lytic enzyme lysis rates were measured in the reaction mixture spectrophotometrically at Optical Density (OD_{600nm}). Results showed S. typhi is multi-drug resistant and the allied phages multiplicity of infection (MOI) amplified virus particles in 1 ml of culture from n × 10^{-6} to n × 10^{-10} bt OD_{600nm}. The phage capability of bacteria lysing rates at serial titer 10^{-8}, 10^{-9} and 10^{-10} was calculated by \[ y = (3 \times 10^{-6}) e^{0.2908x}, y = (2 \times 10^{-10}) e^{0.0208x} \text{ and } y = (7 \times 10^{-11}) e^{0.0373x} \]. The initial amount of Salmonella = 1.0 \times 10^{-6} cell/ml absorbed at (OD_{600nm} < 1.0), after 4 h of incubation with the enzyme, the absorbance was decreased in all samples (1> OD_{600nm}). The lysing rate of enzyme from supernatant extraction \( y = (0.8712) e^{0.013x} \), and from the precipitated \( y = (0.8166) e^{0.036x} \). Since phages depend on their lytic cycle for destroying bacteria, their enzymes showed irretrievable relationship of host decay, they are safe and time saving when used in the treatment of antibiotics resistant S. Typhi.

Key words: Antibiotics/ bacteriophage/ enzybiotics/ lytic enzymes /Salmonella typhi.

INTRODUCTION

Multi-drug resistant (MDR) strains of Salmonella typhi have emerged worldwide in the last two decades (Mad et al., 2004). S. typhi has rapidly gained resistance to antibiotics like ampicillin, chloramphenicol and cotrimoxazole and to previously efficacious drugs like ciprofloxacin and many isolates with reduced sensitivity to fluoroquinolones (fourth generation of antibiotics) have now appeared globally (Jesudason et al., 1992; Butt et al., 2003). General hydrolysis of peptidoglycan by enzymes was observed in bacteria lysis and death. Some lysozymes can kill bacteria by stimulating autolysin activity upon interaction with the cell surface (Brüssow et al., 2005).

Bacteriophage lytic enzymes such as endolysins have been used as therapeutics (so-called enzybiotics); this word was coined to describe a treatment that uses purified phage-encoded enzymes as antibacterial agents. Phage encoded enzybiotics include two classes of peptidoglycan hydrolase enzymes namely: endolysins and virion associated hydrolase. Endolysins are better characterized as phage-encoded
proteins involved in the lytic cycle of phage replication (Nelson et al., 2001; Salazar and Asenjo, 2007). Therefore, phage therapy is a promising alternative drug to treat bacterial infections.

Phage therapy is the therapeutic use of bacteriophages to treat pathogenic bacterial infections. Lytic phages are preferred for the bio-control of pathogenic bacteria since they are highly specific and very effective in lysing targeted pathogenic bacteria. Furthermore, lytic phages do not contain integrase genes on their genomes, therefore, they are unable to coexist with the host and carry virulent genes from one host to another (Borris et al., 2001). Some investigations have attempted to circumvent problems associated with the clearance of phage by the immune system by utilizing lytic enzymes produced by bacteriophage (Iacono et al., 2010).

Lytic enzymes are bacteriolytic agents that can cause bacterial lysis by cleaving bonds in the cell wall's peptidoglycan network responsible for cell rigidity and containment of the cytoplasmic membrane. They share the specificity found in the bacteriophage in which it was produced (Fischetti, 2011; Hermoso et al., 2007). Bacteriophages usually encode holins and lysins as part of their lytic system to achieve virus exit from the host bacterial cell (Young, 1992).

Holins are responsible for forming pores in the cytoplasmic membrane, following lysins accumulated in the cytoplasm responsible for degradation of the peptidoglycan layer. Damage to this layer results in rapid cell rupture and concomitant virus released through loss of osmotic integrity (Loeffler et al., 2001; Nelson et al., 2001). There are tremendous reviews that have dealt with the characteristics of lysins and their applications, both as alternative enzybiotics in medical-oriented in vitro and in vivo tests (Fischetti et al., 2006; Fenton et al., 2010; Knoll and Mylonakis, 2014). Using phage-encoded bacterial cell wall lytic enzymes to eliminate pathogenic bacteria has led to the designation of muraletic acids that include two classes of peptidoglycan hydrolase enzymes (virolysins) endolysins and virion associated hydrolase (Nelson et al., 2001).

Virolysins (phage lytic enzymes) are often composed of two structural domains, a C-terminal cell wall binding domain and one or two N-terminal catalytic domains (Fischetti, 2005; Yoon et al., 2008). The catalytic domain expresses at least one of the following five major types of lytic activity: N-acetyl muramidase, lytic transglycosylase, endo-b-N-acetyl glucosaminidase, and endopeptidase or N-acetyl muramoyl-L-alanine amidase. The first three classes include virolysins that act on sugar moieties of the peptidoglycan while the endopeptidases act on the peptide moiety and the amidases hydrolyze the amide bonds connecting the glycan strand and peptide moieties. Each C-terminal domain is specific to a substrate in the enzyme-sensitive bacteria cell wall. This binding domain is often but not always required for the action of virolysins (Borysowski et al., 2006). The enzyme acts by catalyzing the hydrolysis of 1,4-beta-linkages between N-acetyl muramic acid and N-acetyl-D-glucosamine residues in peptidoglycans and between the N-acetyl-D-glucosamine residues in chitodextrins. Although, lytic enzymes are more effective for the lysis of Gram-positive bacteria, they also facilitate the lysis of Gram-negative bacteria such as Salmonella and Shigella (Sekhon and Ahluwalia, 2012; Masschalck and Michiels, 2003).

However, virolysins are supposed to be the most promising enzybiotics for bacterial cell wall hydrolases, because they have the ability of rapidly killing bacteria, even if these bacteria are resistant to lysozymes. They have narrow spectra of sensitive bacteria, minimizing the disturbance to normal microflora and there is a tremendous diversity of lytic bacteriophages in the biosphere, which guarantees the availability of virolysins targeting almost any bacteria (Masschalck et al., 2003; Parisien et al., 2008). One possible problem of lysins may be their immunogenicity as protein molecules. However, studies thus far have illustrated that lysis-specific antibodies are non-neutralizing both in vitro and in vivo (Parisien et al., 2008; Rashel et al., 2007; Daniel et al., 2010) which means that lysins can be used repeatedly in the treatment of infections caused by susceptible pathogens.

To assess the safety and pharmacokinetic properties of lysins in humans, clinical trials are conducted or prepared (Yang et al., 2014). Therefore, isolation and purification phages enzymes was done for enhancing phages efficiency in lysing S. typhi to be used for antibiotics resistant bacterial infections.

MATERIALS AND METHODS

Isolation and identification of micro-organisms

S. typhi isolates were recovered from wastewater ponds located at Soba Stabilization Station and Omdurman Hospital Stabilization Stations according to the (ISO-6579, 2002) Standard, and preserved in nutrient agar slants until used. Each bacterium was tested for its susceptibility against seven-gram negative antibiotics using guidelines from the National Committee for Clinical and Laboratory Standards Institute (CLSI, 2005). The S. typhi phages were isolated from the same wastewater using the double-layer agar technique recommended by (ISO-10705-1, 2000).

Amplification of bacteriophages (multiplicity of infection [MOI])

S. typhi isolates were cultured in nutrient broth and incubated overnight at 37°C to obtain a growth in concentration of approximately 10⁶ cell/ml at OD₆00 nm. Phage stocks were prepared by adding phages (multiplicity of infection [MOI] = 0.1–0.001) to S. typhi
culture that was in early exponential growth phase (OD_{600} < 0.3). Each isolate inoculated by its corresponding bacteriophage was added at the proportion of 1:10 and incubated at 37°C under shaking. To amplify and maximize the phages' concentrations in the sample, the bacteria were infected for the second time using serial dilutions 10^{-9}, 10^{-8} and 10^{-7} for releasing progeny phages using ciprofloxacin resistant *S. typhi* (I = 16 and R ≥32 µg/L) as a host bacterial strain and stored at 4°C (Pastaga et al., 2013). A standard *E. coli* ATCC25922 was used for quality control purposes. When the OD was below 0.1, the inocula were filtered through 0.45 µm Whatman filter paper; the filtrates were collected in screw cap bottles containing 50 ml SM buffer prepared by dissolving (% w/v) 2.0 gelatin, 2.0 MgSO_{4}·7H_{2}O, 5.8 NaCl in 200 ml tris HCl (1M, pH 7.5) and the demineralized water volume was completed to 1.0 L, (ISO-10705-1, 2000).

### Bacteriophage lysis rate

The lysis rate was determined by selection of serial dilution titer and the time bacteria needed to produce phages after infection. The value obtained from exponential growth of plotted trend lines that corresponds to the incubation period, which spans from the point of phage adsorption to the point at which host lysis occurs.

### Extraction of lytic enzymes

The bacteria free filtrates kept at 30°C without stirring for 2 days maintained the phages before extracting the lytic enzymes. Extraction of lytic enzymes was by centrifugation at 6,010 g for 30 min; ammonium sulfate (up to 40%) added to the supernatant was gently mixed for 4 h at 4°C. The filtrates were sealed in dialysis bags (molecular weight cut-off, 12,000 to 14,000), and dialyzed at 4°C for 2 days against deionized water to purify the enzyme according to the procedure of Fischetti et al. (1971).

### Detection of enzyme activity

Bacterial cultures were prepared and used to detect the bacteriophages lytic enzyme activity. Lytic enzymes were added to the culture (5 g/L) with a final proportion of 1:20 and incubated at 32°C for 4 h. The enzyme activity was determined by measuring the reducing sugars released in the reaction solution using Benedict's reagent (Nelson et al., 2001).

## Evaluation of enzyme lysis rate

*S. typhi* was added to the extracted enzyme to give an initial absorbance of 1.0 at OD_{600nm} for 1.0 × 10^8 cell/ml and incubated at 37°C for 4 h. The change in the mixture turbidity was recorded and the lysis rate expressed as a reduction in the turbidity.

## RESULTS

### Isolation of *Salmonella typhi*

Twelve isolates were recovered (9.0%) from 128 samples of wastewater and identified as *S. typhi*. The isolates were tested for their susceptibility to 7 different antibiotics, a total of 4(33.0%) namely (*S. Typhi*, *S7*, *Dr11* and *Sal C*) were found to be multi-drug resistant. The Minimum Inhibitory Concentrations (MICs) for these isolates were determined and 8 (67.0%) isolates resisted ciprofloxacin at 16 and 32 µg/ml concentrations as Minimum Bactericidal Concentration (MBCs) were selected for the bacteriophage susceptibility.

### Isolation and amplification of bacteriophages

The host range and the multiplicity of infection (MOI) for each phage was assessed on the eight (MBCs) isolates included as antibiotic resistant *S. typhi*. After growth was completed in broth media, bacteria were infected for the second time releasing progeny phages and amplified from n × 10^{-8} up to n × 10^{-10} phage/ ml. The lysis rate was determined by the time needed by bacteria to produce phages after infection as the reduction of optical density at 660 nm per min (Table 1).

### Table 1. Bacteriophage isolation and amplification.

<table>
<thead>
<tr>
<th>Salmonella isolates</th>
<th>Salmonella isolates</th>
<th>Bacteriophage 1st infection</th>
<th>Bacteriophage 2nd infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD_{600nm} = 1.0</td>
<td>cell/ml</td>
<td>12 h OD_{600 nm} phage/ml</td>
<td>12 h OD_{600 nm} phage/ml</td>
</tr>
<tr>
<td>S. Typhi 16</td>
<td>1.6 × 10^{-6}</td>
<td>0.40</td>
<td>0.20</td>
</tr>
<tr>
<td>S. Typhi 32</td>
<td>2.8 × 10^{-6}</td>
<td>0.36</td>
<td>0.15</td>
</tr>
<tr>
<td>S. 7 16</td>
<td>1.7 × 10^{-6}</td>
<td>0.38</td>
<td>0.15</td>
</tr>
<tr>
<td>S. 7 32</td>
<td>1.1 × 10^{-6}</td>
<td>0.42</td>
<td>0.36</td>
</tr>
<tr>
<td>Dr11 16</td>
<td>0.9 × 10^{-6}</td>
<td>0.48</td>
<td>0.42</td>
</tr>
<tr>
<td>Dr11 32</td>
<td>1.3 × 10^{-6}</td>
<td>0.54</td>
<td>0.49</td>
</tr>
<tr>
<td>Sal C 16</td>
<td>1.5 × 10^{-6}</td>
<td>0.36</td>
<td>0.55</td>
</tr>
<tr>
<td>Sal C 32</td>
<td>1.6 × 10^{-6}</td>
<td>0.44</td>
<td>0.60</td>
</tr>
</tbody>
</table>

### Table 1. Bacteriophage isolation and amplification.
**Bacteriophage plaque assay**

The specific bacteriophage for each of the 8 MBCs was determined by the plaque assay. Clear plaques were observed when the *S. typhi* isolates were treated with the phage suspensions on solid medium (Figure 1). For confirming the phage specificity toward the specific bacteria, the numbers of plaques / phages were increased for each isolate by increasing the suspension dilution (Table 2).

The phages’ plaque assay was adjusted for bacteria first infection to produce plaques on solid medium and the most sensitive isolations are (Sal C 16, Sal C 32 and Dr11) at dilution $10^{-11}$. On the other hand, the isolate (S7 16) was the least sensitive to phage lysis at $10^{-8}$ concentration. Pure bacteriophages plaques were selected for the bacteria second infection or inoculation at high titers $10^{-9}$ and $10^{-10}$ (Table 2).

**Bacteriophage lysing rate**

The capacity of phage to lyse bacterial lawns and produce high numbers of pure plaques occurred when higher titers were selected in 12 h of incubation. To calculate the phages’ lysing rates of sample per serial dilution, the bacteria were tittered at $10^{-8}$, $10^{-9}$ and $10^{-10}$ using the formula $y = a \cdot 10^{e (bx)}$ where; $a$ = linear constant, $10^n =$ titer, $e =$ exponential number and $(x) =$ plaque formation / sample at certain point, (Figure 2).

### Table 2. Bacteriophage plaque formation.

<table>
<thead>
<tr>
<th>Salmonella</th>
<th>Tested sample cell/ml</th>
<th>OD 600nm = 1</th>
<th>Dilution</th>
<th>Plaques no.</th>
<th>PFU/ml</th>
<th>Dilution</th>
<th>plaques no.</th>
<th>PFU/ml</th>
<th>Dilution</th>
<th>plaques no.</th>
<th>PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhi 16</td>
<td>1.6 x10^6</td>
<td>10^-8</td>
<td>62.0</td>
<td>6.2 x10^9</td>
<td>10^-9</td>
<td>42.0</td>
<td>4.2 x10^10</td>
<td>10^-10</td>
<td>52.0</td>
<td>5.2 x10^11</td>
<td></td>
</tr>
<tr>
<td>S. typhi 32</td>
<td>2.8 x10^6</td>
<td>10^-8</td>
<td>79.0</td>
<td>7.8 x10^9</td>
<td>10^-9</td>
<td>80.0</td>
<td>9.0 x10^9</td>
<td>10^-10</td>
<td>84.0</td>
<td>8.4 x10^11</td>
<td></td>
</tr>
<tr>
<td>S.7 16</td>
<td>1.7 x10^6</td>
<td>10^-8</td>
<td>46.0</td>
<td>4.6 x10^9</td>
<td>10^-9</td>
<td>66.0</td>
<td>6.6 x10^10</td>
<td>10^-10</td>
<td>94.0</td>
<td>9.4 x10^11</td>
<td></td>
</tr>
<tr>
<td>S.7 32</td>
<td>1.1 x10^6</td>
<td>10^-8</td>
<td>58.0</td>
<td>5.8 x10^9</td>
<td>10^-9</td>
<td>64.0</td>
<td>6.4 x10^10</td>
<td>10^-10</td>
<td>78.0</td>
<td>7.8 x10^11</td>
<td></td>
</tr>
<tr>
<td>Dr11 16</td>
<td>9.0 x10^5</td>
<td>10^-8</td>
<td>90.0</td>
<td>9.0 x10^9</td>
<td>10^-9</td>
<td>88.0</td>
<td>8.8 x10^10</td>
<td>10^-10</td>
<td>96.0</td>
<td>9.6 x10^11</td>
<td></td>
</tr>
<tr>
<td>Dr11 32</td>
<td>1.3 x10^6</td>
<td>10^-8</td>
<td>74.0</td>
<td>7.4 x10^10</td>
<td>10^-9</td>
<td>76.0</td>
<td>7.6 x10^10</td>
<td>10^-10</td>
<td>58.0</td>
<td>5.8 x10^11</td>
<td></td>
</tr>
<tr>
<td>Sal C 16</td>
<td>1.5 x10^6</td>
<td>10^-8</td>
<td>24.0</td>
<td>2.4 x10^10</td>
<td>10^-9</td>
<td>86.0</td>
<td>8.6 x10^10</td>
<td>10^-10</td>
<td>88.0</td>
<td>8.8 x10^11</td>
<td></td>
</tr>
<tr>
<td>Sal C 32</td>
<td>1.6 x10^6</td>
<td>10^-8</td>
<td>26.0</td>
<td>2.6 x10^10</td>
<td>10^-9</td>
<td>92.0</td>
<td>9.2 x10^10</td>
<td>10^-10</td>
<td>94.0</td>
<td>9.4 x10^11</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 1. Phage plaque formation (Pfu/ml 10^6).*
Bacteriophage lysing rate for titers was $10^{-8}$, $10^{-9}$ and $10^{-10}$: $y = (3 \times 10^{-9}) e^{0.2908x}$, $y = (2 \times 10^{-10}) e^{0.0208x}$, and $y = (7 \times 10^{-11}) e^{0.0373x}$ respectively, and their Spearman's rank correlation coefficient ($R^2$) = 0.5, 0.4 and 0.1. The exponent positive integer corresponds to the repeated multiplication of the base (bacteria) and $(e^{bx})$ is the product of phage multiplied in 12 h of incubation.

**Lytic enzyme**

The ability of the extracted phages’ enzymes to lyse the 8 *S. typhi* isolates, (each at 16 and 32 µg/ ml of ciprofloxacin) was determined by measuring its optical densities at 600 nm (Figure 3).

The ability of the extracted phages’ enzymes to lyse the 8 different *S. typhi* isolates was determined by measuring its optical density (Table 3).

The *S. typhi* lysis rate was determined as the reduction of OD$_{600nm}$ by the active lytic enzyme in the absence of bacteriophage particles and time saving from 12 to 4 h. The lysing rate of enzyme collected from the supernatant of phage extraction $y = 0.8712e^{-0.013x}$, and from the precipitated $y = 0.8166e^{-0.036x}$, $R^2$ was correlated and = 0.2 and 0.8. The negative exponent integer described the population decay and defines the exponential number $(e^{-bx})$ raised to a negative integer exponent as living bacteria decreased and in the absence of phages. No resistant bacteria occurred after enzyme lysing and the susceptibility was 100% after 4 h of incubation period.

**DISCUSSION**

Enteric fever or typhoid is still a significant public health issue all over the world. It is a dangerous disease because of its long course and associated complications unless early diagnosed and treatment. There are reports of...
changing clinical features in typhoid fever caused by drug resistant *S. typhi* leading to difficulty in clinical diagnosis (Robert et al., 2002). However, drug resistance in typhoid fever is considered as one of the important factors in the morbidity and mortality of the disease (Bhutta et al., 1991).

Therefore, bacteriophage therapy is one of the best alternative anti-microbial against resistant *S. typhi* due to their different mechanisms of action in lysing bacteria (Butta et al., 1996). The bacteriophages were specific, generally lysing only the hosts on which they were isolated. Consequently, it is required to isolate and characterize the infectious bacteria and matching it with an effective lytic phage before proceeding with treatment using the multiplicity of infection [MOI]. This is in agreement with the study of Oliveira et al. (2012) and Chan and Abedon (2012) who stated that the infection cycle utilized by a phage is an important consideration when choosing a phage for antibacterial application. Another innovative approach of phage therapy is the use of phages’ lytic enzymes instead of the whole virus generated through targeted bacterium by screening through phages-host interaction to increase lysis rates with a functional lytic enzyme; phages can infect and kill bacterial cells causing bacteriolysis, resulting in death but intact bacterial cells (Figure 2). Because the bacteria’s peptidoglycan was lysed to its primary amino sugars, the enzyme activity was visually detected in color’s variations using Benedict’s reagent, which changes the color in response to the enzyme activity and observes the progress of the reaction qualitatively and quantitatively.

One unit of lysing activity is defined as the amount of lytic enzyme necessary to decrease the optical density at 600 nm by 0.01/min (Keary et al., 2013). When lytic enzymes are mixed with bacterial cells in the solution, they adsorb to cell surfaces and cleave bonds, eventually causing lysis. The lysis rate is constant meaning an exponential decrease in OD<sub>600</sub> as shown in (Figure 4).

The bacteriophage lytic enzyme against *Salmonella typhi*.

<table>
<thead>
<tr>
<th>Tested sample</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt; = 1.0</th>
<th>Supernatant enzymes</th>
<th>Precipitated enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhi 16</td>
<td>0.75</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>S. typhi 32</td>
<td>0.98</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>S.7 16</td>
<td>0.80</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>S.7 32</td>
<td>0.80</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Dr11 16</td>
<td>0.92</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Dr11 32</td>
<td>0.81</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Sal C 16</td>
<td>0.85</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Sal C 32</td>
<td>0.69</td>
<td>0.57</td>
<td></td>
</tr>
</tbody>
</table>

*Lysing of *Salmonella typhi* using lytic enzymes showed irreversible interaction in 4 h, the optical density of tested samples was decreased (OD<sub>600</sub> < 1.0) as shown in (Figure 4).*
calculated to identify the area of each curve; the maximal determination coefficient ($R^2$) was calculated for an increasing sample size (n= number of measurements in time). This maximized $R^2$-value ensures the most reliable fit and hence, the most reliable linear regression. The slope of the curves were used to calculate the decrease in OD$_{590}$ per unit of titer dilution in certain time, the activity of which can be calculated based on the amount of lytic enzymes added in a volume of 1 ml of cell suspension (Cheng et al., 1994).

Time factor is very essential for lysing bacteria, either by bacteriophage for multiplicity of infection (MOI), or by lytic enzymes for bacteriolysis by reducing the incubation period from 12 h for the bacteriophage therapy to 4 h for enzybiotics, and after these incubation periods no resistant bacteria occurred with lysing efficiency 100% (Tables 1 and 2). Bacteria develop resistance against phage infection as with antibiotics. A significant point in relation to phage resistance is that it has been associated with reduced bacterial virulence (Smith et al., 1983; Laanto et al., 2012) and using enzybiotics is a great benefit to prevent phage resistance and safe manipulation.

**Conclusion**

For the elimination of a wide-range of infectious bacteria including antibiotic resistant strains, bacteriophage therapy and phage enzybiotics as effective agents has been approved. While bacteriophage therapy showed lytic multiplication in their bacterial host, the lytic enzymes showed irreversible relationship of host decay in the absence of phage. Lytic enzymes are safe and time saving in the *in vitro* treatment of antibiotics resistant strains of *S. typhi*.

**REFERENCES**


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