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Comparative and combinatorial study of biogenic bismuth nanoparticles with silver nanoparticles and doxycycline against multidrug resistant *Staphylococcus aureus* BTCB02 and *Salmonella typhi* BTCB06

Estudio comparativo y combinatorio de nanopartículas de bismuto biogénico con nanopartículas de plata y doxiciclina contra *Staphylococcus aureus* resistente a múltiples fármacos BTCB02 y *Salmonella typhi* BTCB06

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Abstract

Currently, the world is facing a tremendous challenge to treat the infectious diseases caused by antibiotic resistant pathogens. Thus, there is a dire need to find alternative ways to treat the diseases caused by the resistant pathogens. In this context the current study was aimed at synthesizing biogenic bismuth nanoparticles (BiNPs) in combination with biogenic silver nanoparticles (AgNPs) and doxycycline hydrochloride against multidrug resistant pathogens. The MICs of BiNPs and biogenic AgNPs were 6 and 6 μg/L, respectively, against *Salmonella typhi* BTCB06, and 10 and 18 μg/L, respectively, against *Staphylococcus aureus* BTCB02. Whereas the combination of BiNPs and doxycycline resulted in MICs of 3 and 5 μg/L and the combination of BiNPs and AgNPs resulted in MICs of 8 and 9 μg/L against *S. typhi* and *S. aureus*, respectively. FICI value of BiNPs and Doxycycline combined was 0.75 against both *S. typhi* and *S. aureus*. However, the combination of AgNPs and BiNPs had FICI value of 1 against both the test strains. In conclusion, bismuth nanoparticles and doxycycline showed synergistic effect against both the pathogens, whereas BiNPs and AgNPs showed additive effects. Hence, biogenic BiNPs can be used as an excellent alternative alone or in combination to combat diseases caused by resistant pathogens.

Keywords: Bismuth, silver, nanoparticles, minimum inhibitory concentration, synergistic effect.

Resumen

Actualmente, el mundo se enfrenta a un tremendo desafío para tratar las enfermedades infecciosas causadas por patógenos resistentes a los antibióticos. Por tanto, existe una necesidad imperiosa de encontrar formas alternativas de tratar las enfermedades causadas por patógenos resistentes. En este contexto, el presente estudio tuvo como objetivo sintetizar nanopartículas de bismuto biogénico (BiNP) en combinación con nanopartículas de plata biogénicas (AgNP) y clorhidrato de doxiciclina contra patógenos resistentes a múltiples fármacos. Las CIM de BiNP y AgNP biogénicos fueron de 6 y 6 μg / L, respectivamente, contra *Salmonella typhi* BTCB06, y de 10 y 18 μg / L, respectivamente, contra *Staphylococcus aureus* BTCB02. Mientras que la combinación de BiNP y doxiciclina dio como resultado CMI de 3 y 5 μg / L y la combinación de BiNP y AgNP dio como resultado CMI de 8 y 9 μg / L contra *S. typhi* y *S. aureus*, respectivamente. El valor de FICI de BiNPs y doxiciclina combinados fue de 0,75 contra *S. typhi* y *S. aureus*. Sin embargo, la combinación de AgNP y BiNP tuvo un valor de FICI de 1 frente a ambas cepas de prueba. En conclusión, las nanopartículas de bismuto y la doxiciclina mostraron un efecto sinérgico contra ambos patógenos, mientras que los BiNP y los AgNP mostraron efectos aditivos. Por lo tanto, los BiNP biogénicos se pueden utilizar como una excelente alternativa, solos o en combinación, para combatir enfermedades causadas por patógenos resistentes.

Palabras clave: Bismuto, plata, nanopartículas, concentración inhibitoria mínima, efecto sinérgico.

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1 Introduction

Recently, the emergence of diseases caused by multiple drug resistant pathogens has echoed serious concerns for the health professionals. In this context, scientists and health care providers are switching towards new ways of cure to solve the problem (Pornpattananangkul *et al*., 2013). With the advent of nanotechnology metal nanoparticles and nanocomposites are being tested as antimicrobial agents owing to their unique properties (Narkar *et al*., 2010). Metal nanoparticles possess potential applications in clinical diagnosis, catalysis, tissue engineering, drug delivery, biological tagging etc. Silver is already known to possess efficient antimicrobial, cytotoxic and insecticidal property with low toxicity with applications in both in vivo and in vitro settings (Elbeshehy *et al*., 2015; Hsueh *et al*., 2015; Mahmoud *et al*., 2016; Odeyemi, De La Mare *et al*., 2019). Among the various inorganic metal nanoparticles, silver (AgNPs) and bismuth (BiNPs) have received substantial consideration in the biomedical field (Sathishkumar *et al*., 2009; Vera Robles *et al*., 2016; Xia *et al*., 2014). Different chemicals as well as physical methods are being used to synthesize nanoparticles (Li, 2006; Yu *et al*., 2013). Chemical mode of synthesis is not only expensive but also increased the propensity of toxic compounds being adsorbed onto the nanoparticles formed via this process. Apart from that these methods are not ecofriendly as compared to biological methods. Microbes possess the potential to reduce metal ions in their vicinity to avoid metal toxicity, thereby generate nanoparticles (Xiong *et al*., *et al*., 2002). There is a constant need to develop a nontoxic, clean and environmental friendly procedures for the synthesis of nanoparticle (Hayes & Laws, 1991; G. H. Hwang *et al*., 2009; K. Wang *et al*., 2005). Plant extracts, fungal and bacterial metabolites have been used for the biological synthesis of nanoparticles (Alghuthaymi *et al*., 2015; Aritonang *et al*., 2019; Hsueh, *et al*., 2015; Odeyemi, *et al*., 2019). Though biological mode of synthesis is a slow process but it is an eco-friendly alternative (Dell'Agli *et al*., 1999; López-Salinas *et al*., 2010; Wu *et al*., 2010).

Among all the metals studied, silver and bismuth nanoparticles are reported to play a tremendous role in the field of medicine and biology. Like silver, bismuth nanoparticles can also find its place in techniques that involve the use of nano-vector such as nanomedicine; magnetic nanovector for delivering drugs, scanning and imaging like magnetic resonance imaging (MRI) (Bouchard *et al*., 2009; Cattaneo *et al*., 2010; Klostergaard & Seeney, 2012; Xia, *et al*., 2014). Bismuth ions exhibit very strong antimicrobial properties with no serious risk to human beings at certain concentrations that may lead to their extensive use as antimicrobial agent in soaps and detergents as well as in several biomedical applications (Dong *et al*., 2014; Garcia, Kao, & Strongin, 1972; Mégraud, 2012; Vera Robles, *et al*., 2016). Bi represents interesting photocatalytic and catalytic properties (Dong, *et al*., 2014; Vera Robles, *et al*., 2016). Among all the elements, semimetal bismuth possesses a wide range of applications, such as electronics due to its extremely anisotropic behavior and low effective mass (Fang *et al*., 2000; Sathishkumar, *et al*., 2009). Furthermore, compounds of bismuth such as bismuth sub citrate and bismuth subsalicylate have been extensively used to treat gastrointestinal disorders of 18th century (Dodge & Wackett, 2005).

Many reports have been published regarding bismuth NPs synthesis using a number of methods involving chemical, electron irradiation, radiolytic reducing technique and microwave treatment (Kharissova & Kharisov, 2008; Nazari *et al*., 2012; Vera Robles, *et al*., 2016; Xia, *et al*., 2014). However, the extracellular synthesis of BiNPs by microbes has not been investigated yet and should be understood due to its diverse applications. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Among many key areas to focus, one is to develop the new drugs.

Among many diseases, Typhoid fever, caused by *S. typhi*, is an emerging threat to the human population. According to World health organization, about 11-20 million people get sick because of typhoid and between 128,000 - 161,000 people die from it every year (WHO, 2018). The pathogen has shown resistance towards current known antibiotics and is becoming a treatment challenge for the health care professionals. On the other hand *S. aureus* that colonizes from 30 to 50% of healthy adults and is able to rapidly infect skin lesions, with a consequential inflammatory process (Karthik *et al*., 2019), has also shown resistance towards many antibiotics (Shakibaie *et al*., 2019). Therefore, the prominence of finding new effective and efficient antimicrobial agents cannot be overtly denied.

Several strategies are being considered, yet treatment by combining different drugs has achieved

maximum utility due to several reasons, such as increase in the spectrum of antimicrobial activity, prevention in the development of resistant strains, reduction in the toxicity and achieving the synergistic antimicrobial activity (Calzada *et al*., 2002; Martín-Arbella *et al*., 2011; Salata, 2004). Drug combination between antimicrobial agents and nanoparticles is a unique model and has been documented well in recent times (I.-s. Hwang *et al*., 2012; Lei *et al*., 2019; Pérez-Mezcua *et al*., 2014; Sharples & Collison, 2014; Zhao, Zhang, & Dang, 2004). In the current study, comparative and combinatorial effect of microbially synthesized bismuth and silver nanoparticles in comparison with the drug doxycycline is investigated.

2 Materials and methods

2.1 Nanoparticles

Bismuth nanoparticles prepared previously from *Bacillus cereus* BTCB 20 and silver nanoparticles from *Aspergillus fumigatus* BTCB 10 were obtained from Research Project Laboratory, Department of Biotechnology, Lahore College for Women University, Pakistan. AgNPs had size ranging 0.66-30 nm and negative zeta potential i.e. -13 mV. Whereas BiNPs had size ranging from 0.46-18 nm.

2.2 Preparation of test bacterial strains

Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) of AgNPs, BiNPs and doxycycline were determined for two multidrug resistant bacterial pathogens i.e. *Staphylococcus aureus* BTCB 02 and *Salmonella typhi* BTCB 06. For the preparation of microtitre plate, the inocula of the test organisms were prepared using colony suspension method (Irobi *et al*., 1994). Inoculum for each strain was prepared in nutrient broth and incubation for 24 h at 37 ºC in aerobic conditions. The optical density of the inoculum was maintained 1 OD at 600 nm.

2.3 Determination of Minimum Inhibitory Concentration MIC

MIC values for each strain against Bismuth, Silver nanoparticles and Doxycycline hydrochloride were determined using two fold serial micro dilution method according to Wiegand *et al*. (2008). About 50 μl of each dilution (2-20 μg/L) was placed in a 96 well microtitre plate. The 12th column, having 100 μL nutrient broth was considered as sterility control and 50 μl in the 11th column was used as growth control. The bacterial suspension was adjusted to 1×10^8 cfu/mL. Each well including the growth control except sterility control well was inoculated with 50 μL of the bacterial suspension. Microtiter plate was incubated at 37 ºC for 24h.

2.4 Determination of FIC and FIC (index) FICI

The combined effect of bismuth nanoparticles with silver nanoparticles and bismuth nanoparticles with doxycycline hydrochloride (FIC) was evaluated by micro dilution chequerboard method (Fratini *et al*., 2017a). Assays were performed on 96-well polypropylene microtiter plates on the basis of nanoparticle's MIC values obtained previously. Seven concentrations of both combinations i.e. BiNPs: AgNPs and BiNPs: Doxycycline were prepared (8 MIC, 4 MIC, 2 MIC, MIC, MIC/2, MIC/4 and MIC/8). For BiNPs: AgNPs about 190 μl of BiNPs was added along the x-axis across the chequerboard plate, while AgNPs along the y-axis. For BiNPs:Doxycycline each concentration of BiNPs was dispensed along the x-axis and Doxycycline along y-axis as illustrated in Fig 1c and 1d. Each well was inoculated with 10 μl of bacterial suspension standardized at 0.5 McFarland standard turbidity except the sterility column (negative control) that contained 10 μl of sterile nutrient broth. Microplates were incubated at 37 ºC for 24 h in a humid chamber. FIC determinations were performed in triplicate. For each replicate, the fractional inhibitory concentration (FIC) was thereafter derived from the least concentration of nanoparticles and antibiotic combination allowing no observable growth of the test bacteria on the plates (Kamatou *et al*., 2006). Each agent was estimated for their FIC value using the standard formula:

$FICI = FIC_{BiNps} + FIC_{dox}$
$FICI = FIC_{BiNps} + FIC_{AgNPs}$
Where,
$FIC_{BiNps} = MIC_{BiNps}$ in combination/MIC_{BiNPs} alone
$FIC_{AgNPs} = MIC_{AgNPs}$ in combination/MIC_{AgNPs} alone and
$FIC_{dox} = MIC_{dox}$ in combination/MIC_{dox} alone

The interfaces amongst the nanoparticles and the antibiotic were evaluated in expressions of the FIC guides calculated using the following formula:

FIC index = ∑FIC = FIC (Doxycycline) + FIC (BiNPs)
FIC index = ∑FIC = FIC (AgNPs) + FIC (BiNPs)

Combined action was categorized according to Odds (2003). A synergistic effect (SynO) is observed when FICI value ≤ 0.5, the effect was indifferent (IndO) when 0.5<FICI value ≤ 4 and the effect was antagonistic (AntO) when FICI value >4. Whereas according to EUCAST (2000) a Synergistic effect (SynE) is observed when FICI value ≤ 0.5; an Additive effect (AddE) when 0.5<FICI value ≤ 1; an Indifferent effect (IndE) when 1<FICI value <2 and an Antagonistic effect (AntE) when FICI value ≥ 2.

3 Results and discussion

3.1 *Nanoparticles*

In this study highly significant antimicrobial activity was observed at very low concentrations of AgNps (10 μg/l) and BiNPs (18μg/l) against *S. aureus* BTCB 02. Whereas against *S. typhi* BTCB 06, the effective concentration of AgNPs was 6 μg/L, and for BiNPs it was 16 μg/L in comparison to doxycycline (16 μg/L), Table 1 and Figure 1a. Antimicrobial activity of silver and bismuth salts has been reported previously and so their nanoparticles (Iftikhar *et al*., 2018; Iqtedar *et al*., 2019; Vega-Jiménez *et al*., 2017).

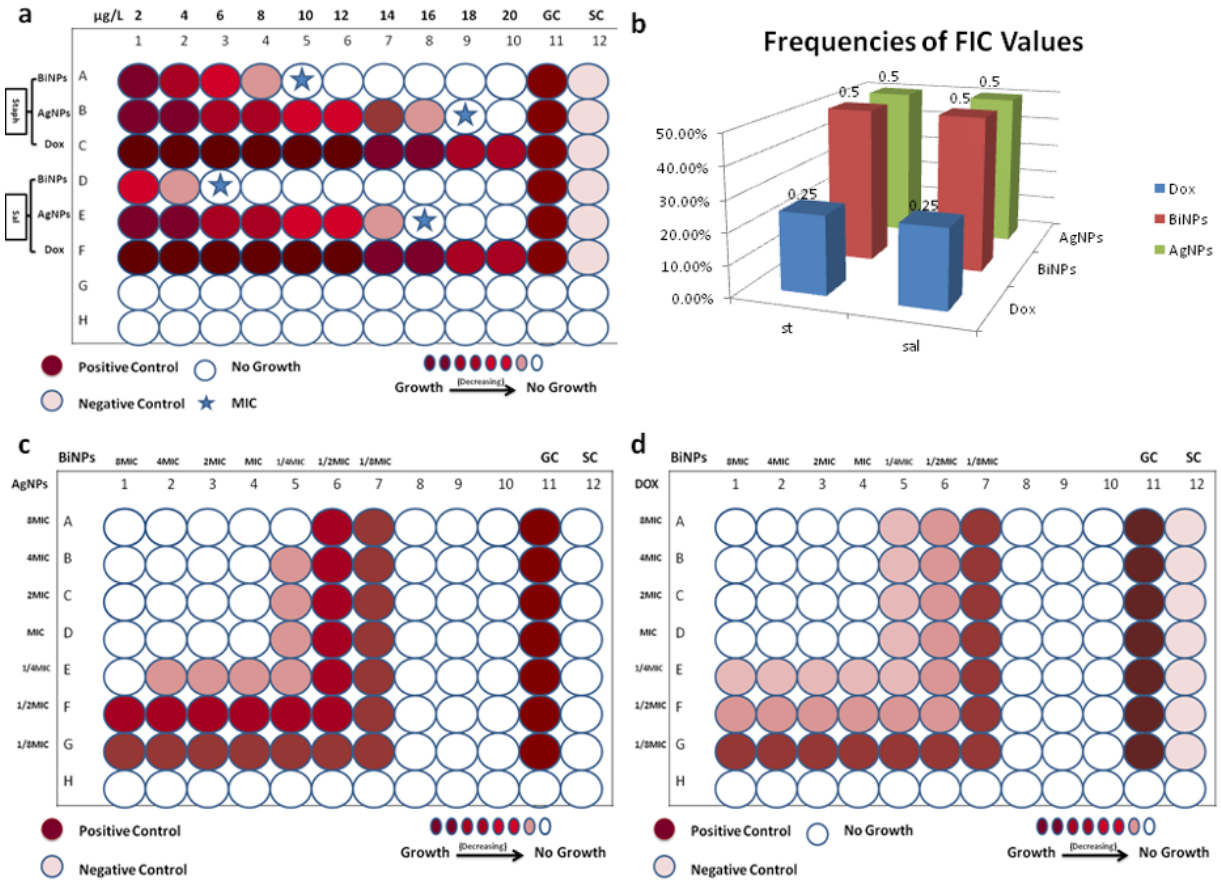

Fig. 1.) MIC determination of BiNPs, AgNPs and Doxycycline against *S. typhi* BTCB 06 and *S. aureus* BTCB 02 b) FIC percent values for the pathogens c) Determination of additive effect of BiNPs+AgNPs d) Determination of synergism of BiNPs+Dox.

Table 1. Minimum inhibitory concentrations (MIC) of BiNPs and AgNPs against *Salmonella typhi* BTCB 06 and *Staphylococcus aureus* BTCB 02.

Bacterial pathogens	Minimum inhibitory concentrations MIC (μg/L)			
	BiNPs	AgNPs	BiNPs+Drug	BiNPs+AgNPs
S. typhi BTCB 06	6	16	3	8
S. aureus BTCB 02	10	18	5	9

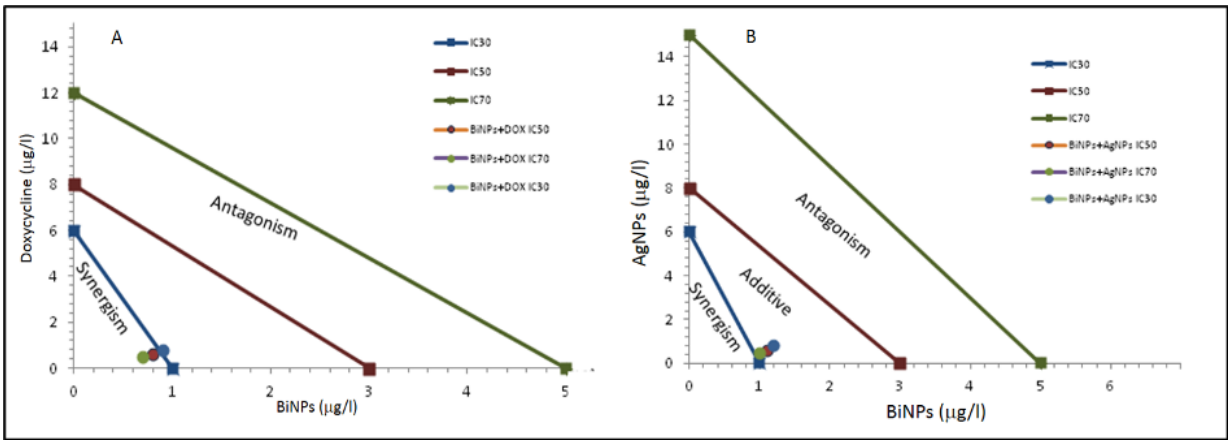

Fig. 2. Isobolograms showing the effect of different combinations against *S. typhi* BTCB 06 and *S. aureus* BTCB 02 a) The synergism for the combination BiNPs and Doxycycline b) The additive effect of AgNPs and BiNPs.

3.2 Determination of MIC values

Minimum inhibitory concentration was evaluated for microbially synthesized silver and bismuth nanoparticles (2-20 μg/l) against MDR pathogens *S. typhi* BTCB 06 and *S. aureus* BTCB 02. No growth was observed with silver nanoparticles after 24hrs of incubation at a concentration of 16 μg/L for *S. typhi* and 18 μg/L for *S. aureus* BTCB 02. The standard value for the doxycycline against these pathogens was reported to be 16 mcg/L (FDA, 2011). However, when the bismuth nanoparticles were used against the test strains, the results were very promising and significant. Bismuth nanoparticles at concentration of just 6 μg/L and 10 μg/L inhibited the growth of both *S. typhi* BTCB 06 and *S. aureus* BTCB 02, respectively. The results depict the efficacy of Bismuth nanoparticles over AgNPs and doxycycline against the test bacterial strains. Metal toxicity is an established phenomenon that causes bacterial growth inhibition by generating free radicals which ultimately disturbs the cell ionic balance and osmotic pressure, resulting into cell death (E. T. Hwang *et al*., 2008; Ishwarya *et al*., 2019; Lok *et al*., 2006). The MIC values of Bismuth and silver nanoparticles varied significantly when applied in combinations as shown in table 1.

3.3 FIC and FICI

FIC values for each nanoparticle and drug ranged from 0.25 to 0.5 for both the strains. Fig 1b depicts the FIC frequency values for both types of nanoparticles and antimicrobial drug doxycycline. Our results highlighted a notable effect of bismuth nanoparticles synthesized extracellularly for the first time by *Bacillus cereus* BTCB 20 having accession number MK118714 (Iftikhar, 2018). Antimicrobial activity is attributed to size, toxicity, semimetal nature and/or neutral charge potential of NPs as also reported various previous studies (Liu *et al*., 2019; R. Wang *et al*., 2019). FICI values were obtained that ranged from 0.75 - 1(Figure 2). FICI value was 0.75 for BiNPs +Doxycycline combination against both *Salmonella typhi* BTCB 06 and *Staphylococcus aureus* BTCB 02. However, AgNPs and BiNPs combination had FICI value of 1 against both the test strains (Fig 2).

Both bismuth and silver nanoparticles showed remarkable antibacterial activity as compared to doxycycline. The mechanism through which NPs showed antimicrobial activity on bacterial cell involves destruction of proton motive force and depletion of adenosine triphosphate which leaves the cell dead by depriving the cell of vital energy (Thormar, 2011). Many researchers have

investigated the antimicrobial properties of silver nanoparticles (Anwar *et al*., 2019; Bibi *et al*., 2019; Iftikhar, *et al*., 2018; Ishwarya, *et al*., 2019) but to date none of the report has documented the antibacterial activity of extracellularly synthesized biogenic bismuth nanoparticles. Studies have shown the synergistic effect of silver nanoparticles with antibiotics having FICI values of less than 1 (Barapatre *et al*., 2016; Bayroodi & Jalal, 2016; I.-s. Hwang, *et al*., 2012; Mala *et al*., 2012). This is in concurrence with our study where nanoparticles have shown synergism when employed with antibiotic. However, the studies mentioned above utilized higher concentrations of nanoparticles and drugs compared to our results (Figueiredo *et al*., 2019; Lee & Lee, 2019; Souza *et al*., 2019).

To the best of our knowledge, this is the first report on the antibacterial activity of extracellularly synthesized biogenic bismuth nanoparticles in combination with doxycycline and silver nanoparticles. Because of their inhibitory activity, these combinations could be used as a potential therapy against drug resistant pathogens as an alternative to conventional antibiotics.

3.4 FICI interpretations

As reported in fig 2, FICI values for BiNPs+Dox and BiNPs+AgNPs were 0.25 (synergistic) and 1(additive) against both the pathogens. According to Odds (2003) interpretation a Synergistic effect is present at 0.25 FICI value and indifferent effect at 1. According to interpretation of EUCAST (2000), synergistic effect (SynE) at 0.25 FICI and Additive effect at 1. According to both the interpretations' absence of antagonist effect (AntO and AntE) was observed. Whereas both the interpretations, Odds (2003) and EUCAST (2000), agreed in detecting a synergistic effect with FICI below or equal to 0.500. In the current study, MIC and the FIC values were obtained using microdilution method, that showed reproducibility and reduced uncertainty errors (Fratini *et al*., 2017b). The doxycycline at a lowest concentration showed antimicrobial activity when used in combination with BiNPs, shown in figure 1. If certain quantity of drug is replaced with BiNPs, the outcome will remain inhibitory, showing that both the compounds are in synergism. Thus the use of drug can be made possible at a very low dose to target pathogens which will lessen the burden on human body (Gustine *et al*., 2019; Mulder *et al*., 2019). The total dose of drug is reduced and a synergistic effect (SynA) would be attained at a lower quantity of both, the drug and the nanoparticles, compared to the single effective dose of a drug.

Conclusions

The findings of present study elucidate the potential of nanoparticles as antimicrobials that may replace the use of conventional antibiotics. Bismuth nanoparticles exhibited synergism when combined with doxycycline hydrochloride. Whereas both the nanoparticles (AgNPs+BiNPs) were found to display additive effect. These FICI interpretations showed that biogenically synthesized nanoparticles can effectively be used in combination against *S. typhi* and *S. aureus* for their synergistic activity. Presence of multidrug resistant microorganisms represents an increasingly widespread problem. Bismuth nanoparticles have the potential to be a clinically valid alternative to chemo-resistant therapies.

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