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EFFECT OF METFORMIN ON THE EFFICACY OF ANTIBIOTICS (IN VITRO)

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Abstract:

The current work aims to determine the effect of metformin on some antibiotics' efficacy against some bacterial isolates; moreover, determination of fractional inhibitory concentration (FIC) index. Six bacterial isolates (*Staphylococcus aureus*, *Streptococcus viridans*, *Proteus mirabilis*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *E.coli*) diagnosed by VITEC-2-Compact. Several metformin concentrations (100 ug, 200 ug, 300 ug, 400 ug and 500 ug) were prepared and tested for the sensitivity of selected isolates to determine the best inhibitory concentration of bacteria. Used the disc diffusion method to determine the antimicrobial activity of metformin alone and when combined with antibiotics against all isolates after preparing MIC for each antibiotic. FIC index was measured by checkerboard assays of a combination of antibiotics with metformin to determine the types of reactions. Metformin showed the best antibacterial action against the bacterial strains at a 500 µg/ml concentration, it's increased the effectiveness of the antibiotic in inhibiting bacterial isolates by the desk diffusion method with a significant difference at ($P < 0.05$). About the FIC index, found that most of the interactions were partial synergies of 50%, metformin was recorded with amikacin as the highest reaction (partial synergy). Synergy interaction recorded in 11.11% of the tests, while additive and indifferent reactions was recorded similar rates at (16.16%), and only 5.55% of outcomes were indeterminate. Importantly, there were no antagonistic interactions between metformin and the used antibiotics. *E. coli* was the most bacteria that reported a synergistic reaction when metformin was combined with amikacin and gentamicin.

Key words: Metformin, partial synergy, *E.coli*, amikacin, Synergy interaction.

Introduction:

Metformin [dimethylbiguanide] is the first oral anti-diabetic drug used to treat diabetes type 2, especially in obese and overweight people and have normal renal function. It is restricted in gestational diabetes due to health concerns. Also, it's used to treat polycystic ovary syndrome and tested in other diseases where insulin resistance is essential. Metformin works by inhibition of the production of glucose by the liver (1). It Possesses a good safety profile, can control the side effects side reasonably and inexpensive (2). Can trace it back to *Galega officinalis*, known as a goat's rue or French lilac (3). In 1922, Dimethylbiguanidine was synthesized and observed to reduce glucose concentration in animal blood. In the Philippines, dimethylbiguanidine, called flu amine, was tested as an antimalarial and used to treat influenza. In 1957, Jean Stern used metformin as an anti-diabetic drug. In 1958, metformin was introduced to diabetes treatment in the UK and Europe. In 1994 Metformin was approved and in 1995 introduced in the USA in the treatment of DM. In 2002, metformin proved that it limits the development of pre diabetes to type 2 diabetes. Studies conducted in 2008 by UKPDS confirmed the benefits of metformin in reducing cardiovascular risks. In 2011, metformin was inserted in the WHO Essential Medicines List (4). Recently, interest in metformin as a new antibacterial agent for treating infections has increased due to its antimicrobial properties, some are difficult to treat (5). It was found as a promising antimicrobial in many bacterial and non bacterial infections (6). Metformin has a role as a limiting factor for the airway of bacterial load (2).

Laboratory discoveries showed the drug's effectiveness on several pathogenic microbes, Involving *Trichinella spiralis* (7), *Staphylococcus aureus* (8,9), *Pseudomonas aeruginosa*(10),

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hepatitis B virus (11), hepatitis C virus (12, 13, 14, 15), and human immunodeficiency virus (16). Development of different strains of multidrug by genetic mutations (17) or strains carrying the drug by bacterial stability make antibiotics ineffective (18). There is a need to develop or produce new antibiotics or to associate approved drugs with existing antibiotics as an efficient treatment strategy. Metformin proposed as a combined treatment with antibiotics on the front lines to attack antibiotic-resistant tuberculosis by inhibiting complex mitochondria-1, which is similar to the bacterial compound NDH-1 (19). Our study aimed to study the effect of metformin on the efficacy of some antibiotics and evaluate the antimicrobial effect of metformin on different pathogenic bacteria. The study measured the minimum inhibitory concentration (MIC) of the antibiotics and the fractional inhibitory concentration (FIC) for combining metformin with the antibiotics.

Materials and Methods:

Drugs

Metformin, Amikacin, Ciprofloxacin and Gentamicin were obtained in pure dry powder from SDI-Sammara -Iraq.

Bacterial isolates

The six bacterial isolates (*Staphylococcus aureus*, *Streptococcus viridans*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *E.coli*) diagnosed by VITEC-2-Compact, were obtained from Azadi Teaching Hospital - Laboratory Department.

Preparation of various concentrations of Metformin

Dissolved 50mg of metformin in 0.7ml of dimethyl sulphoxide (DMSO) and 50 ml of sterile water to obtain a 50µg/ml stock solution. From the solution, a 0.25ml, 0.3ml and 0.5ml were pipetted and diluted and supplemented volume to 10ml with distilled water, which gave the 250µg/ml, 300µg/ml and 500 µg/ml concentrations respectively . The solution of 10µl was dripped onto the disc such that each disc had 100 ug, 200 ug, 300 ug, 400 ug and 500 µg of test drug metformin.

Preparation of the bacterial suspensions

Specifically, each strain was cultured in Mueller–Hinton agar plate for 24h at 35 C0. Growth was collected using 5ml of sterile normal saline. Using a spectrophotometer set at 580 nm, tubes were diluted to get 1.5×10^8 CFU/ml of cells (20).

Preparation of Antimicrobial Agent Stock Solution

Made Stock solution by dissolving a specified amount of drug in a particular solvent by using the equation ($W = \frac{1000}{p} \times C \times V$)

Where P = manufacturer's potency (µg/mg) , V = required volume (ml) , C = the final solution's concentration (multiples on 1000) (mg/L), and W= antibiotic weight (mg) dissolved in a volume (ml) (21).

The drugs used for calculating MIC in this procedure were commonly used antibiotics (Ciprofloxacin, Amikacin and Gentamicin).

-Preparation of Stock Solution of Amikacin

Made it by adding 0.25gm of the drug in 10ml of distilled water .

-Preparation of Stock Solution of Ciprofloxacin

Made it by adding 0.1gm of the drug in 10ml of distilled water .

-Preparation of Stock Solution of Gentamicin

Made it by adding 0.5gm of the drug in 10ml of distilled water .

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Determination of the minimum inhibitory concentration (MIC) of antibiotics against bacterial isolates

The lowest inhibitory concentration was performed on bacteria that showed sensitivity to antibiotics. Broth dilution techniques carried out. The method's aim was to find the lowest concentration of antibiotics that inhibit the growth of bacteria. Briefly, the nutrient broth was prepared according to the manufacture and placed in a five serial dilution test tubes for each bacteria then sterilized at $121C^0$ for 15 min in an autoclave. McFarland Scale no. 0.5 was also prepared to measure the turbidity of suspensions. After these, bacteria were injected into 10% normal saline until the turbidity reached the McFarland scale using visual seeing. This coincidence was recorded as 1.5x108 cfa/mL, which was the factor of the serial dilution. Serial dilution of antibiotics in the nutrient broth was carried out for each bacterium to get solutions of (0, 2, 4, 8, 16, 32, and 64) µg /ml. Then used a sterile pipette of 20 mL volume to pipette 0.1mL of the bacterial suspension into each serially diluted tube, and incubated at 37 C^0 for 24 h. Finally, the tubes were observed for the presence of bacterial growth. The tubes with a clear solution and least antibiotic concentration were taken as the MIC (22).

Disk diffusion method

Used the method for the determination of the antibacterial activity of solutions. Briefly, using sterile clean paper discs (6 mm) in diameter, were saturated with (Mic of Cipro, Mic of Amk, Mic of Gent, Mic of Cipro + Met 500, Mic of Amk + Met 500, Mic of Gent + Met 500 (Each antibiotic was mixed with metformin in equal amounts) and Met 500 alone) and were placed on the inoculated previously prepared plates. After remaining at 4C° for 2 h, plates were incubated at 37C° for 24h. The inhibition zone's diameters of bacterial growth were measured in millimeters with a clear scale. All tests were carried out in triplicate (23).

Determination of fractional inhibitory concentration (FIC) index

Checkerboard assays measured the fractional inhibitory concentrations index for the combination of antibiotics with metformin. Briefly, 100 µL of MHB was poured into each well of a 96 -well plate. Metformin was diluted along the ordinate while Antibacterial solutions were diluted along the abscissa. The overnight bacterial culture was standardized to 0.5 McFarland turbidity and diluted 1:100 in MHB broth. Then incubating for 18 hr at 37°C, the optical density of each well was determined by an Infinite Micro plate reader M200 at 600 nm (24).

The (FIC) index was calculated by equation:-

FIC= MIC drug A / MIC drug A + MIC drug B / MIC drug B…….

(in combination) (alone) (in combination) (alone)

Drugs combinations were classified into: - synergetic FIC $\leq$ 0.5, partial synergetic 0.5 < FIC< 1, additive FIC= 1, indifferent 1 < FIC < 4 and antagonism FIC $\geq$ 4 (25, 26).

Experimental design

Staphylococcus aureus and *Streptococcus viridans* as gram-positive bacteria, *Proteus mirabilis, Klebsiella pneumonia, Pseudomonas aeruginosa,* and *E.coli* as gram-negative bacteria were used in this study which was divided as follows:

A. Muller Hinton agar plates contain Mic of antibiotic for each bacteria disks.

B. Muller Hinton agar plates containing metformin 250 µg/ml and 500 µg/ml disks.

C. Muller Hinton agar plates containing (Metformin 500 µg/ml + Mic of Cipro disks, Metformin 500 µg/ml + Mic of Amk disks and Metformin 500 µg/ml + Mic of Gent disks) for each bacteria, and each antibiotic was mixed with metformin in equal quantities.

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Statistical Analysis

The statistical analysis was performed with the statistical package for Social Science (SPSS) version (18) and Microsoft Excel (2013) software. P <0.05 was regarded as statistically significant.

Results and Discussion:

In the present research, the zone diameter was used as a parameter to assess metformin's antibacterial effectiveness and the antibiotics. Metformin showed the best activity against the tested strains at a 500 µg/ml concentration, as shown in Table 1.

Table 1. Diameter zones of inhibition at different concentrations of metformin by the desk diffusion method.

Bacteria	Met 100 µg/ml	Met 200 µg/ml	Met 300 µg/ml	Met 400 µg/ml	Met 500 µg/ml
Staphylococcus aureus	–	–	–	0.5 mm	2 mm
Streptococcus viridans	–	–	–	–	3 mm
Pseudomonas aeruginosa	–	–	–	–	0.5 mm
Proteus mirabilis	–	–	–	–	1 mm
Klebsiella pneumoniae	–	–	–	–	3 mm
E. coli	–	–	–	1 mm	5 mm

Met: Metformin

Metformin was found to possess antibacterial activity for both gram +ve & gram –ve bacteria.

Non-antibiotics are assumed to exert their in-vitro antimicrobial action by impacts on the bacterial inner membrane, according to the suggested mechanism (27, 28).

Table 2 shows the combined effect between metformin and antibiotics; an increase in bacterial inhibition areas was observed when metformin was combined with antibiotics. It means that metformin increased the effectiveness of antibiotics when combined.

Table 2. The combined effect of metformin and antibiotics on inhibition zones of bacterial isolates.

Bacterial Strains	Metformin Concentration (500µg/ml)	Amikacin		Ciprofloxacin		Gentamicin	
		Amk	Amk + Met 500	Cip	Cip + Met 500	Gent	Gent + Met 500
Staphylococcus aureus	2 mm	17 mm	18 mm	16 mm	17 mm	28 mm	29 mm
Streptococcus viridans	3 mm	16 mm	18 mm	19 mm	20 mm	21 mm	21 mm
Pseudomonas aeruginosa	0 mm	13 mm	14 mm	15 mm	16 mm	26 mm	27 mm
Proteus mirabilis	1 mm	14 mm	15 mm	16 mm	18 mm	16 mm	18 mm
Klebsiella pneumoniae	3 mm	16 mm	18 mm	18 mm	19 mm	19 mm	21 mm
E. coli	5 mm	15 mm	19 mm	22 mm	24 mm	30 mm	31 mm

Met: Metformin; Amk: Amikacin; Cip: Ciprofloxacin; Gent: Gentamicin.

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Metformin recorded significant differences at (P <0.05) when combined with antibiotics, as shown in Table 3.

Table 3. Statistical analysis of metformin + antibiotics in the Current Study.

Antibiotics	Mean ± SD of the antibiotic (alone)	Mean ± SD of the(antibiotic+ metformin)	T	DF	SIG
Amikacin	15.16 ± 1.34	17 ± 1.82	-3.84137	5	0.012 (S)
Ciprofloxacin	17.66 ± 2.35	19 ± 2.58	-5.71548	4	0.004 (S)
Gentamicin	23.33 ± 5.02	24.5 ± 4.75	-3.20713	4	0.032 (S)

SD: Standard deviation; T: T test; DF: Degree of freedom; SIG: Signification; S: Significant.

The interaction results of metformin combined with three antibiotics (FIC) against the six selected Gram-positive and Gram-negative bacteria isolates are summarized in Table 4.

Table 4. FIC index of metformin combinations with antibiotics against Bacterial Strains.

No. of tests	Synergy	Partial synergy	Additive	Indifferent	Antagonism	Indeterminate
Amikacin (6)	1	4	0	1	0	0
Ciprofloxacin (6)	0	3	1	1	0	1
Gentamicin (6)	1	2	2	1	0	0
Total (18)	(2)11.11%	(9)50%	(3)16.16%	(3)16.16%	0%	(1)5.55%

No: number.

The majority of the interaction results (50%) observed in partial synergy interactions, metformin was recorded with amikacin as the highest reaction. Synergy interaction was only present in 11.11% of the testing events, while additive and indifferent reactions recorded similar rates at (16.16%), and only 5.55% of results were discovered to be indeterminate. The most importantly, none of the examined antibiotics and metformin showed antagonistic interactions.

The combination of metformin and amikacin produced the most number of significant positive interactive outcoms (partial synergy), as the number of interacting samples was 4 out of a total of 6 (*Staphylococcus aureus*, *Streptococcus viridans*, *Proteus mirabilis* and *Klebsiella pneumoniae*), *E.coli* showed a synergy reaction and Pseudomonas aeruginosa showed indifferent interaction. Pseudomonas aeruginosa, Klebsiella pneumoniae, and E.coli showed a partial synergistic reaction when metformin was combined with amoxicillin. At the same time, *Streptococcus viridans* recorded an additive reaction, *staphylococcus aureus* was an indeterminate reaction and indifferent reaction of *Proteus mirabilis*. As for the combination of metformin with gentamycin, *E. coli* showed a synergistic reaction, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* showed partial synergy reaction. In contrast, *Proteus mirabilis* and *Streptococcus viridans* showed an additive reaction. Finally, the *Staphylococcus aureus* showed an Indifferent reaction. In Gram- negative and Gram- positive bacteria, Metformin reduces the cytoplasmic membrane's potential as loperamide (29). Metformin could first damage the outer membrane of Gram-negative bacteria, which is extremely impermeable and serves as a barrier to many potent antibacterial drugs (30).

We hypothesized that metformin would damage the integrity of the outer membrane by displacing the divalent cat ions like Mg2+, which stabilize the outer membrane of bacteria, based on the result that Mg2+ eliminated the potentiation of metformin (31). Additionally, metformin severely impairs the PMF driven efflux pump abilities in resistant bacteria.

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Conclusion and Recommendations:

In summary, the best bactericidal action was obtained by metformin at a concentration of 500µg/ml. Also, the areas of bacterial inhibition was increased when metformin was combined with 3 antibiotics. 50% of the interactions' outcomes recorded partial synergy, especially when combining metformin with amikacin. Importantly, there are no antagonistic interactions between metformin and the antibiotics detected against 6 bacteria isolates. *E.coli* showed a synergy reaction when metformin was combined with amikacin and gentamicin. This study corresponds to several studies that determined that metformin has antibacterial activity. However, more research is still needed to clarify metformin's mechanism against different bacteria types in vivo and in vitro.

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