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A Novel Mathematical Model for Analysing Antimicrobial Susceptibility Using the Disc Diffusion Method

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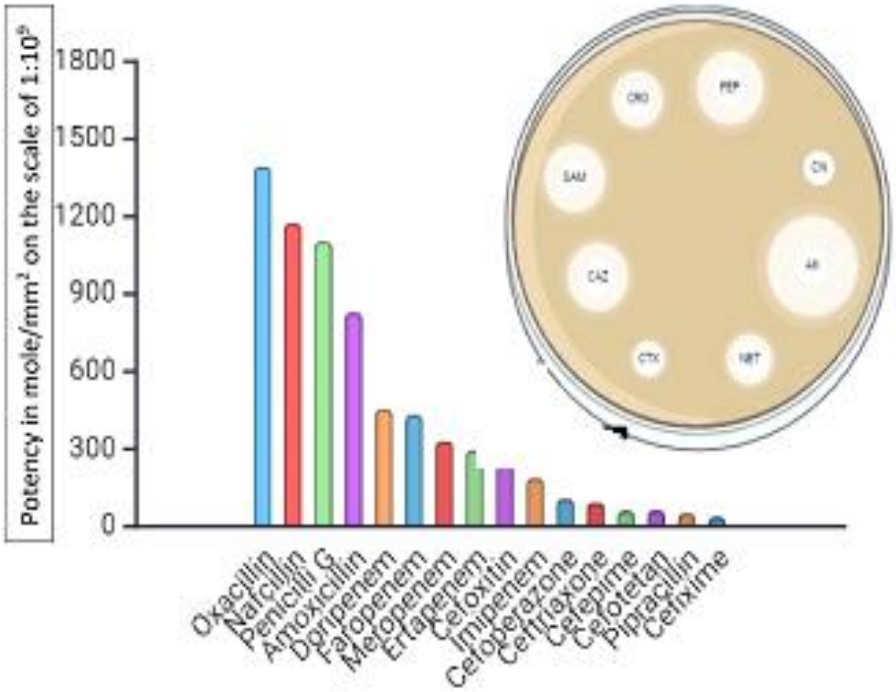
Graphical Abstract



Potency of β-lactam antibiotics based on mole/mm^2 of zone of inhibition

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ABSTRACT:

This study introduces a novel mathematical model designed to analyse the antimicrobial susceptibility of microorganisms using the disc diffusion method. The primary objective is to provide a more accurate and efficient interpretation of inhibition zone data, thereby improving the reliability of susceptibility testing. We developed the mathematical model based on a molar concentration of antibiotic and molar zone of inhibition concentration that account for key variables, including the model being validated using experimental data from disc diffusion tests diffusion coefficients, zone of inhibition, and antimicrobial agent potency. That was done on *Staphylococcus aureus* ATCC 25923 bacterial strains with 22 different antibiotics of beta-lactam group. Our results demonstrate that the proposed model offers significant improvements in predicting the comparative potency and enlisting the priority of antimicrobial agents based on Equivalent molar zone number and Zone of inhibition potency compared to traditional interpretation methods. Specifically, the model achieved a correlation coefficient of 0.9167 with experimental data, indicating high predictive accuracy. Additionally, it can adjust for factors of molar concentration, ZOI, and breakpoint, providing more nuanced insights into antimicrobial efficacy. These findings suggest that the novel mathematical model can be a valuable tool for microbiologists and clinicians, enabling more precise assessments of antimicrobial susceptibility and selecting effective treatment regimens.

Keywords: staphylococcus aureus, antimicrobial susceptibility test, Disk diffusion, Beta-lactam, zone of Inhibition.

1. Introduction

Antimicrobial resistance will lead to a major health problem worldwide shortly (Prestinaci, Pezzotti and Pantosti, 2015), superbugs are resistant to several antibiotics (Ghafur, 2013), and Nosocomial infections are challenging and threatening. It isn't easy to be treated with conventional antibiotics. Microbial resistance (Aedo and Tomasz, 2016) is not only a threat to

the community it also adds a burden on the economy, because advanced antibiotics are expensive and add a financial burden to patients (Alpert, 2016). The objective of antimicrobial susceptibility testing is to figure out possible drug resistance in the pathogen and to confirm susceptibility to the drug of choice for particular infections (Jorgensen and Ferraro, 2009). There are various methods including broth microdilution, disc diffusion method and gradient diffusion method current methods are competent enough to provide quantitative and qualitative results that accurately detect common antimicrobial susceptible intermediate or resistance (Jorgensen and Ferraro, 2009).

We analysed the beta-lactam group based on the zone of inhibition. The reliability of a single method of AST is not decisive (Khalili *et al.*, 2012). Assessment of antibiotics with the disc diffusion method is not new (Miftahussurur *et al.*, 2020) but arranging the priority list of antibiotic preferences according to molar concentration per unit square area of zoi is a novel approach to assess data under new parameters that may help the microbiologist and clinician to take a pinpoint decision (van Belkum *et al.*, 2019) on the bases of the priority list of antibiotics arranged according to the molar strength and area cleared about ZOI for a particular microorganism. It is a more accurate and precise method while selecting the antibiotic of choice based on molar concentration to ZOI and breakpoint (Asín *et al.*, 2012). MIC can be correlated with clinical effects and proper curing of bacterial infections (Hindler and Munro, 2022), (Kowalska-Krochmal and Dudek-Wicher, 2021)

2. Material and methodology

Staphylococcus Aureus ATCC No-25923 (Sieuwerts *et al.*, 2008) was obtained from the Microbiology laboratory of Era Medical College under Era University Lucknow, India. The strains were maintained as stock strains in a Micro bank, cryovials were kept at 80^0 C until used. Nutrition Agar media (Sigma Aldrich) was obtained from the microbiology department. Antibiotic Disk of Cefixime 30µg, Cefepime 30µg, and Piperacillin 40µg purchased from Sigma Aldrich. And the rest standards of antibiotic average ZOI were considered from clinical and laboratory standard institutes (Treangen *et al.*, 2014).
Before Sensitivity testing *Staphylococcus aureus* ATCC No25923 was cultured in a nutrient agar plate and incubated for 24 hr at 37^o C. A single colony is then cultured in 5 ml liquid peptone media for 4 hr at 37^{o}C. 5 The density culture grown required for the test was adjusted to 0.5 Mc standards ($1.0x10^8$ CFU/ml) measured using a Turbidometer. LB-966 Moglix

Disc Diffusion Method
Antimicrobial susceptibility testing was carried out using the standard disc diffusion method to Zone of Inhibition (ZOI) *Staphylococcus aureus* Culture which has been maintained at 0.5 Mc Farland standard was used to lawn (Sieuwerts *et al.*, 2008). Nutrition agar plates evenly using a sterile swab, the plates were dried for 15 min before going to sensitivity testing. Antibiotics disc Cefixime 30µg, Cefepime30µg, and µg, Piperacillin40µgwere placed in a triplicate
The plates were then incubated at 37 ºC for 24 hr After incubation the plates were examined for Zone of Inhibition in triplicate (Hombach *et al.*, 2015)

S. No	Drug	Disc con in µg	Mol. Wt	limit in mm	ZOI in mm	Mole conc. of drugs	Area of zoi in mm2	MZOIC Mole per mm^2	Equivalent moler zone number	Potency In mm2 /mole	No. of Molecules in mole/mm^2

1	Oxacillin	1	401.43	18-24	21	2.49 x10^{-9}	346.5	7.19x10^{-12}	1	1.39x10^{11}	4.33x10^{12}
2	Nafcillin	1	414.47	16-22	19	2.41 x10^{-9}	283.64	8.51x10^{-12}	1.1	1.17 x10^{11}	5.12x10^{12}
3	Penicillin G	2	334.40	28-31	29	5.98 x10^{-9}	660.78	9.05x10^{-12}	1.25	1.10 x10^{11}	5.45x10^{12}
4	Amoxicillin	2	365.40	22-25	24	5.47 x10^{-9}	452.57	1.21x10^{-11}	1.68	8.26 x10^{10}	7.29x10^{12}
5	Penicillin G	1	334.40	12-18	15	2.99 x10^{-9}	176.78	1.69x10^{-11}	2.35	5.91 x10^{10}	1.02x10^{13}
6	Doripenem	10	420.50	33-42	37	2.38 x10^{-8}	1075.63	2.21x10^{-11}	3.07	4.52 x10^{10}	1.33x10^{13}
7	Faropenem	5	285.32	27-34	31	1.75 x10^{-8}	755.07	2.32x10^{-11}	3.22	4.30 x10^{10}	1.40x10^{13}
8	Meropenem	10	383.46	29-37	33	2.61 x10^{-8}	855.64	3.05x10^{-11}	4.23	3.28 x10^{10}	1.84x10^{13}
9	Ertapenem	10	475.51	24-31	28	2.1 x10^{-8}	616.00	3.41x10^{-11}	4.74	2.92 x10^{10}	2.05x10^{13}
10	Amoxicillin	10	365.40	28-33	31	2.74 x10^{-8}	755.07	3.62x10^{-11}	5.04	2.75 x10^{10}	2.18x10^{13}
11	Cefoxitin	10	427.454	24-29	27	2.34 x10^{-8}	572.78	4.08x10^{-11}	5.68	2.44 x10^{10}	2.46x10^{13}
12	Imipenem	10	299.34	24-31	28	3.34 x10^{-8}	616.00	5.42x10^{-11}	7.54	1.84 x10^{10}	3.26x10^{13}
13	Amoxicillin	25	365.40	29-35	32	6.84 x10^{-8}	804.57	8.50x10^{-11}	11.88	1.17 x10^{10}	5.12x10^{13}
14	Cefoperazone	30	645.67	22 - 28	25	4.65 x10^{-8}	491.07	9.46x10^{-11}	13.16	1.05 x10^{10}	5.70x10^{13}
15	Ceftriaxone	30	554.58	22-28	25	5.41x10^{-8}	491.07	1.10x10^{-10}	15.32	9.07 x10^{9}	6.62x10^{13}
16	Cefepime	30	480.56	23-29	26	6.24 x10^{-8}	531.14	1.18x10^{-10}	16.34	8.50 x10^{9}	7.11x10^{13}
17	Cefoxitin	30	427.45	23 - 29	26	7.02 x10^{-8}	531.14	1.32x10^{-10}	18.37	7.56 x10^{9}	7.95x10^{13}
18	Cefotetan	30	575.62	17 - 23	20	5.21 x10^{-8}	314.28	1.66x10^{-10}	23.06	6.03 x10^{9}	1.00x10^{14}
19	Cefoperazone	75	645.67	24-33	29	1.16 x10^{-7}	660.78	1.76x10^{-10}	24.45	5.68 x10^{9}	1.06x10^{14}
20	Pipracillin	40	516.54	20-24	22	7.74 x10^{-8}	380.28	2.04x10^{-10}	28.32	4.91 x10^{9}	1.23x10^{14}
21	Cefixime	30	453.45	16-19	17	6.62 x10^{-8}	227.07	2.91x10^{-10}	40.52	3.43 x10^{9}	1.75x10^{14}
22	Cefepime	30	480.56	15-18	16	6.24 x10^{-8}	201.14	3.10x10^{-10}	43.17	3.22 x10^{9}	1.87x10^{14}

Table1.Priority list of antibiotics (Paterson *et al.*, 2016) according to Molar zone of inhibition concentration (MZOIC) (Smith and Kirby, 2019)

3. Result and discussion

Molar Zone of Inhibition Concentration (MZOIC)

Enlisting the antibiotics while taking a microbial species and strain constant, *Staphylococcus aureus* ATCC 25923 will give a unique signature effect. The priority list is arranged in descending order of potency, with the most potent antibacterial Oxacillin on top and the least potent Cefepime at the bottom.

Under standard conditions of antimicrobial susceptibility testing of disc diffusion method, the priority list is in ascending order of number of moles of antimicrobial agent required to clear the area of 1mm^2 per unit area ZOI termed as 'molar zone of inhibition concentration'*(*MZOIC) Tab1. The Depth of nutrient agar plate was not taken into consideration for the calculation (Flanagan and Steck, 2017).

By using the formula

$$\text{MZOIC} = \frac{4\,x Drug\ concentration}{Gram\ molecular\ weight x \Pi(ZOI)^2}$$

That indicates Oxacillin with molar zone of inhibition concentration7.19 x 10^{-12} mole/mm^2 on top and cefepime with 3.1x10^{-10} mole/mm^2 on the bottom

Equivalent Molar Zone Number (EMZN)

While taking Oxacillin with molar zone concentration 7.19 x 10^{-12} mole/mm^2as unit and dividing all the other molar zone of inhibition concentration values with it we get an equivalent molar Zone number**.** That means Oxacillin is 43.170 times more potent than cefepime under in vitro conditions of the standard disc diffusion method or Cefoperazone with a disc concentration of 30 µg is 13.16 times less potent than Oxacillin and Cefoperazone with a disc concentration of 75µg is 24.451 times less potent than Oxacillin. Penicillin G disc concentration 2µg is 1.258 and Penicillin G concentration 1µg is 2.35 times less potent than Oxacillin. Amoxicillin with disc concentrations 2µg,10µg, and 25µg are 1.68,5.04, and 11.828 times less potent than Oxacillin respectively (Tab1)

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Zone Of Inhibition Potency (ZOIP)

The reciprocal of the molar zone of inhibition concentration is the Zone of inhibition potency and its unit is mm^2/mole, represented by the formula.

$$ZOIP = \frac{Gram\ molecular\ weight x \Pi(ZOI)^2}{4\ x Drug\ concentration}$$

It can be postulated from the above formula ZOIP is directly proportional to the square of the Zone of inhibition or area of the zone of inhibition and inversely proportional to the molar concentration. The mole concentration required to clear a unit area of 1mm^2 of the nutrient agar plate. Less the gram mole required to clear the unit zone of inhibition more potent the antibiotic agent.

In Table 1 Oxacillin having a value of 1.391 x $10^{11}$$mm^2$ /mole is at the top of the priority list of potency and Cefoperazone 5.96 x 10^9 is at the fourth last position.

Cefepime scored 3.22 x $10^9$$mm^2$ /mole and was placed at the bottom, the list is arranged in descending order to ZOIP.

Number of Molecules Present in Mole/Mm^2

In Table 1. The number of molecules in mole/mm^2 is multiple of Avogadro number (6.022×10^{23})($Becker\ and\ Schiel,$ 2013) with 'molar zone of inhibition concentration' **(**MZOIC)

$$number\ of\ molecules = \frac{4\ x Drug\ concentration}{Gram\ molecular\ weight x \Pi(ZOI)^2} \times 6.022 \times 10^{23}$$

It represents the number of molecules of antibiotic present in the 1 mm^2 area of zoi. Corresponding to molar zone of inhibition concentration' **(**MZOIC) it indicates concentration in numbers of molecules.

The Correlation between a Zone of Inhibition Concentration and Mole Concentration of a Drug

As the increase in the mole concentration of various moieties of the drug, there is an increase in the molar zone of Inhibition concentration, the relation is linear and positive correlation with R^2 Value 0.9167 by considering only 18 reference points from Oxacillin to cefotetan (fig-1) while including last four drugs this value of R^2 depreciated to 0.802 if we add Cefoperazone, Piperacillin, Cefixime and Cefepime. The reference point 19, 20,21, and 22 respectively (fig-2).

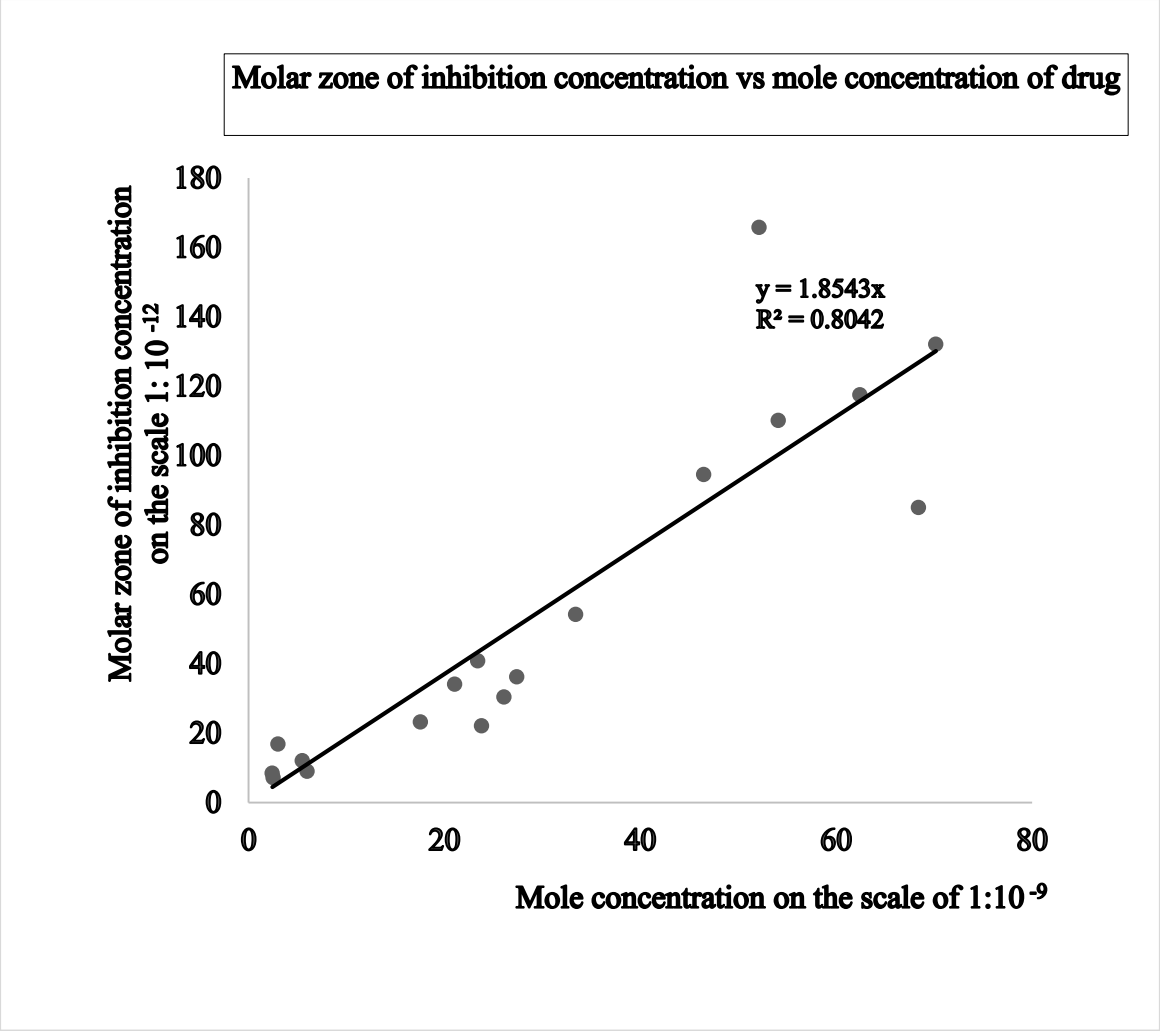

Figure 1. Molar zone of inhibition concentration vs mole concentration of the drug.

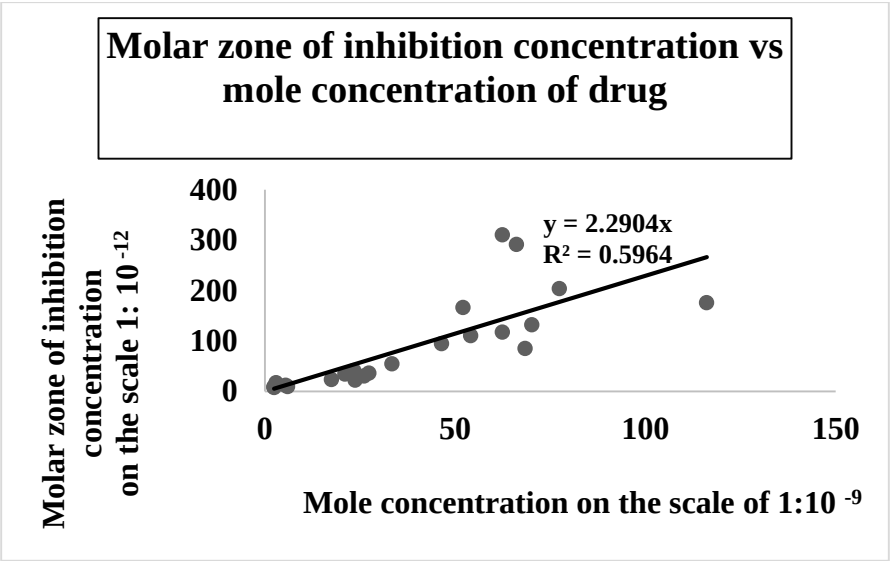

Figure 2. Molar zone of inhibition concentration vs mole concentration of the drug.

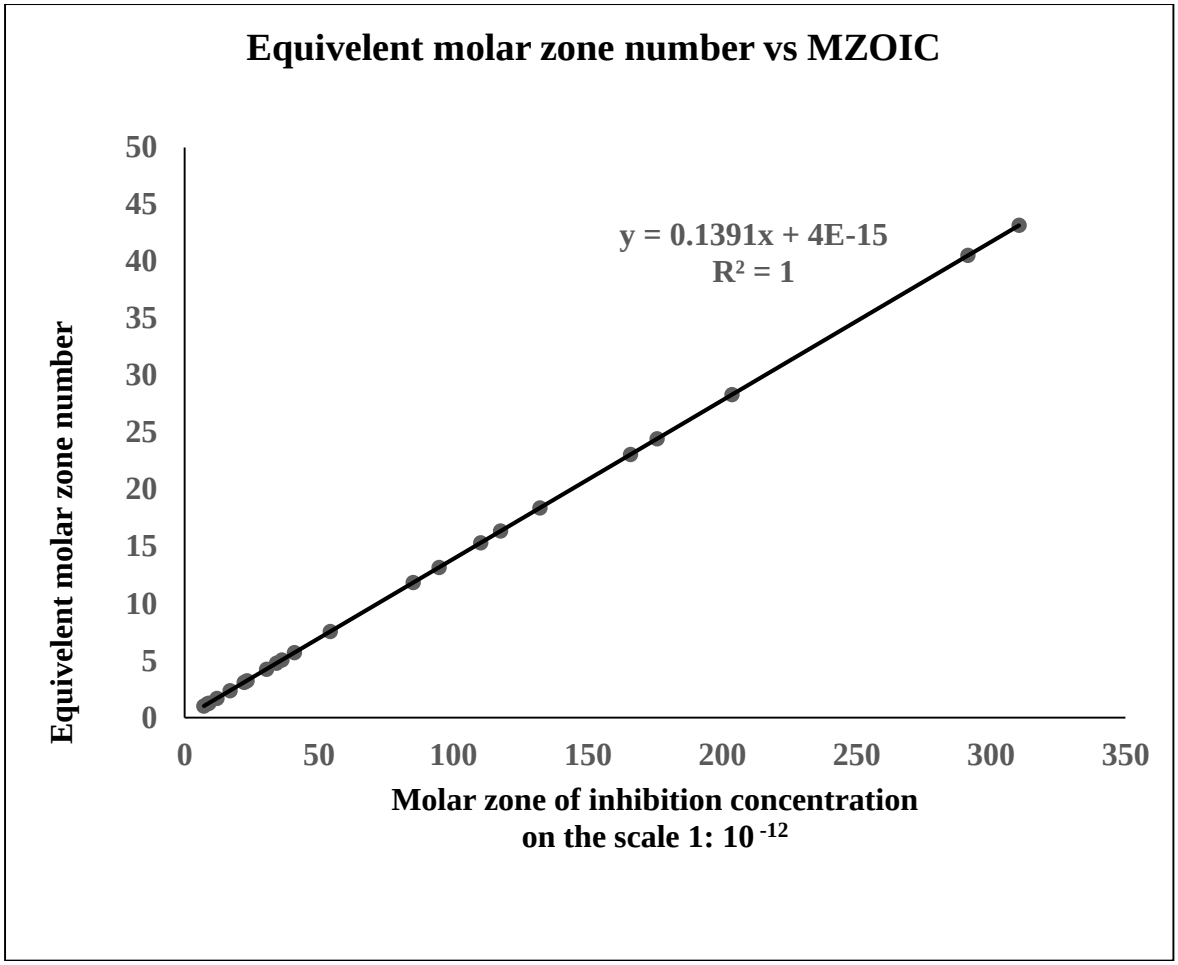

Figure 3. Equivalent molar zone number vs Molar zone of inhibition concentration (MZOIC)

Equivalent Molar Zone Number Vs Molar Zone of Inhibition Concentration (MZOIC)

The equivalent molar zone number is derived from the molar zone of inhibition concentration, it has a positive corelation with R^2 =1, less the molar zone of inhibition concentration low will be the equivalent zone number more potent will be the antibiotic in the priority list made according to the molar zone of inhibition concentration.

Equivalent Molar Zone Number (EMZN) Vs Molar Concentration

The mole concentration is an independent value that depends on the product of two variables 'molar zone of inhibition concentration'(MZOIC) and the area of the zone of inhibition of the corresponding antibiotic.

MZOIC= $\frac{4\,xDrug\ concentration}{Gram\ molecular\ weight x \Pi(ZOI)^2}$

$$\frac{Drug\ concentration}{Gram\ molecular weight} = \frac{molar\ zone\ of\ inhibition\ concentration\ x\ \Pi(ZOI)^2}{4}$$

$$Mole\ Concentration = \frac{molar\ zone\ of\ inhibition\ concentration\ x\ \Pi(ZOI)^2}{4}$$

The relationship between EMZN and molar concentration of antibiotic is polynomial with order 2 and R^2 = 0.8595 and equation y = -1E-10x^2 + 6E-09x - 2E-09 This Quadratic equation where mole concentration of antibiotic depends on equivalent molar zone number, where y represents the mole concentration and x represents the equivalent molar zone number (fig.4)

Quadratic Term$(-1 \times 10^{-10}x^2)$
The coefficient $(-1 \times 10^{-10}x^2)$ is very small and negative, meaning that as the equivalent molar zone number increases, the quadratic (Wiggins, Harding and et al, 2018) term decreases (mole concentration) the product of MZOIC and Area of ZOI but very slowly due to the small magnitude of the coefficient. This term indicates a downward curvature, suggesting a parabolic shape (fig 4).

Linear Term$6 \times 10^{-9}x$
The coefficient $6 \times 10^{-9}x$ is also small and positive, suggesting that as mole concentration (MZOIC x Area of ZOI) of antibiotic increases as the equivalent molar zone number increases, but again very slowly. This term dominates the relationship for smaller values of equivalent molar zone numbers before the quadratic term becomes significant.

Constant Term$-2 \times 10^{-9}x$
This is the mole concentration intercept of the equation, meaning that when the Equivalent molar zone number = 0 than the value of mole concentration y= $-2 \times 10^{-9}x$is a hypothetical value as mole concentration can't be zero.

Shape of Curve (Fig 4)
The plot shows a parabolic curve that opens downwards, which is consistent with the negative coefficient of the $x^2(-1 \times 10^{-10}x^2)$
The curve initially rises to a peak and then starts to decline as x increases.

Behaviour of curve at Different Values of mole concentration
At lower values of x Equivalent molar zone number (close to 0), the mole concentration (y) is also close to zero the reason behind as more potent moieties requires less molar concentration to attain a susceptible zoi in standard in vitro conditions. As x increases, y increases due to the positive linear term $(6 \times 10^{-9}x)$The mole concentration reaches a maximum point, indicating the peak of the parabola. Beyond this peak, the mole concentration(MZOIC x Area of ZOI) decreases as "x" Equivalent molar zone number continues to increase, due to the dominant effect of the negative quadratic term$(-1 \times 10^{-10}x^2)$as the priority list was arranged in ascending order of number of moles of antimicrobial agent required to clear the area of $1mm^2$ per unit area ZOI termed as 'molar zone of inhibition concentration'*(*MZOIC) and ascending order of Equivalent molar zone number the least number allotted drug is most potent and larger the number assigned the potency decreases in vitro conditions of AST.

Peak Point:
The peak point represents the maximum mole concentration and is determined by the balance between the increasing linear term and the decreasing quadratic term.
Mathematically, the vertex of the parabola can be found using the vertex formula for a quadratic equation $ax^2 + bx + c$, which is $x = -\frac{b}{2a}$, substituting a=-1×10^{-10} and b = 6×10^{-9} we get

$$x = -\frac{6 \times 10^{-9}}{2 \times (-1 \times 10^{-10})} = 30$$

So, the peak is around 30 after that we got the down cure which means the molar concentration is lowered due to a decrease in the zone of inhibition to corresponding antibiotics Cefixime and Cefepime with zoi 17mm and 16 mm respectively [20].

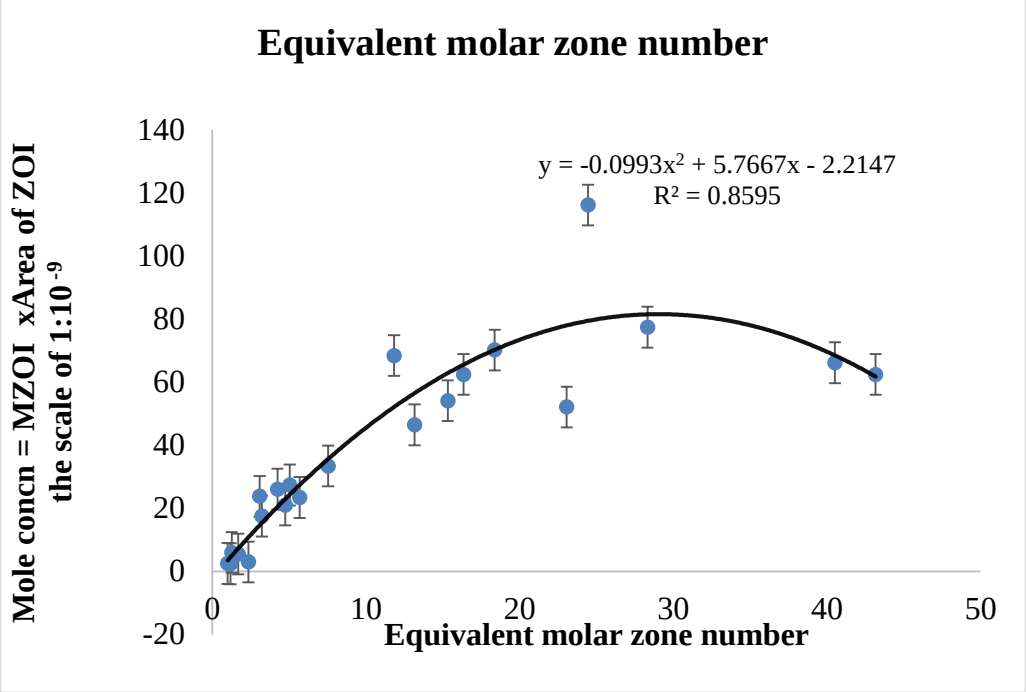

Figure 4. Equivalent molar zone number vs mole concentration at a confidence interval of 95%.

Data Distribution

The data points are scattered around the curve, and the fit is reasonably good, as indicated by the R^2 value (Hagquist and Stenbeck, 1998) of 0.8595. This means that 85.95% of the variation in the mole concentration (MZOIC x Area of ZOI) is explained by the equivalent molar zone number.

Classification of Zones on the Graph (Fig4)

The data points on the x-axis can be divided into three zones based on the polynomial equation:

Rising (Zone 1): x values from 0 to ~30, where mole concentration (MZOIC x Area of ZOI) increases with increasing x equivalent molar zone number Low y-values (0 to 6E-08): Represent the increasing part of the polynomial, where the linear term is dominant. This corresponds to x-values from 0 to around 25, data points from 1 to 19 Oxacillin to Cefoperazone lies in this zone (Tab.1)

Peak Point (Zone 2) Around x=30 where mole concentration is at its maximum(~1.2E-07). Represents the peak of the polynomial curve, around x = 30, where the contributions from both the linear and quadratic terms are balanced. Piperacillin lies in this zone.

Falling (Zone 3):x values greater than ~30, where mole concentration decreases with increasing x equivalent molar zone number. High y-values (1.2E-07 to 6E-08) Represent the decreasing part of the polynomial, where the quadratic term is dominant. This corresponds to x-values from 30 to 45. Cefixime and cefepime lie in this zone.

Equivalent Molar Zone Number vs. Zone of Inhibition Potency (ZOIP)

The x-axis represents the "Equivalent molar zone number." The y-axis represents the "Zone of Inhibition potency (ZOIP)." The power trendline equation $y = 1 \times 10^{11} x^{-1}$ describes the hyperbolic relationship between the Equivalent molar zone number and Zone of Inhibition potency (fig, 5).

In (fig6) both axes are on a logarithmic scale, which is appropriate for displaying data that span several orders of magnitude i.e. Zone of Inhibition Potency (ZOPI), the power trendline equation can be rewritten as $y = \frac{1\times10^{11}}{x}$

The R^2 =1 indicates that the fit is perfect (Hagquist and Stenbeck, 1998), meaning that the data points lie exactly on the trendline.

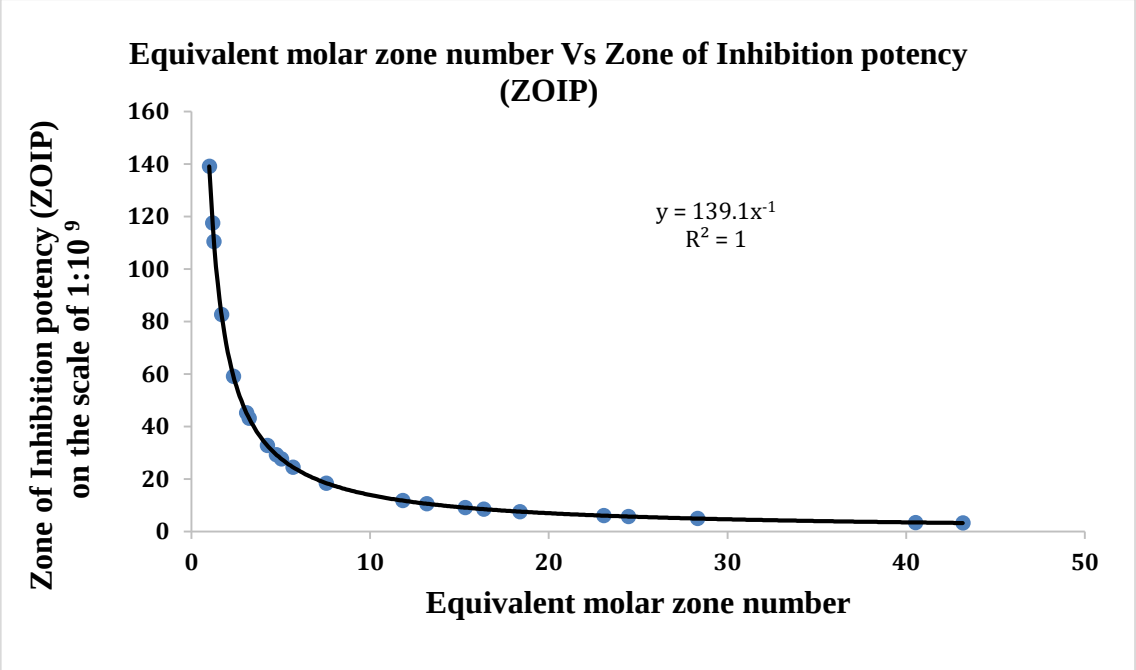

Figure 5. Equivalent molar zone number Vs zone of inhibition potency (ZOIP).

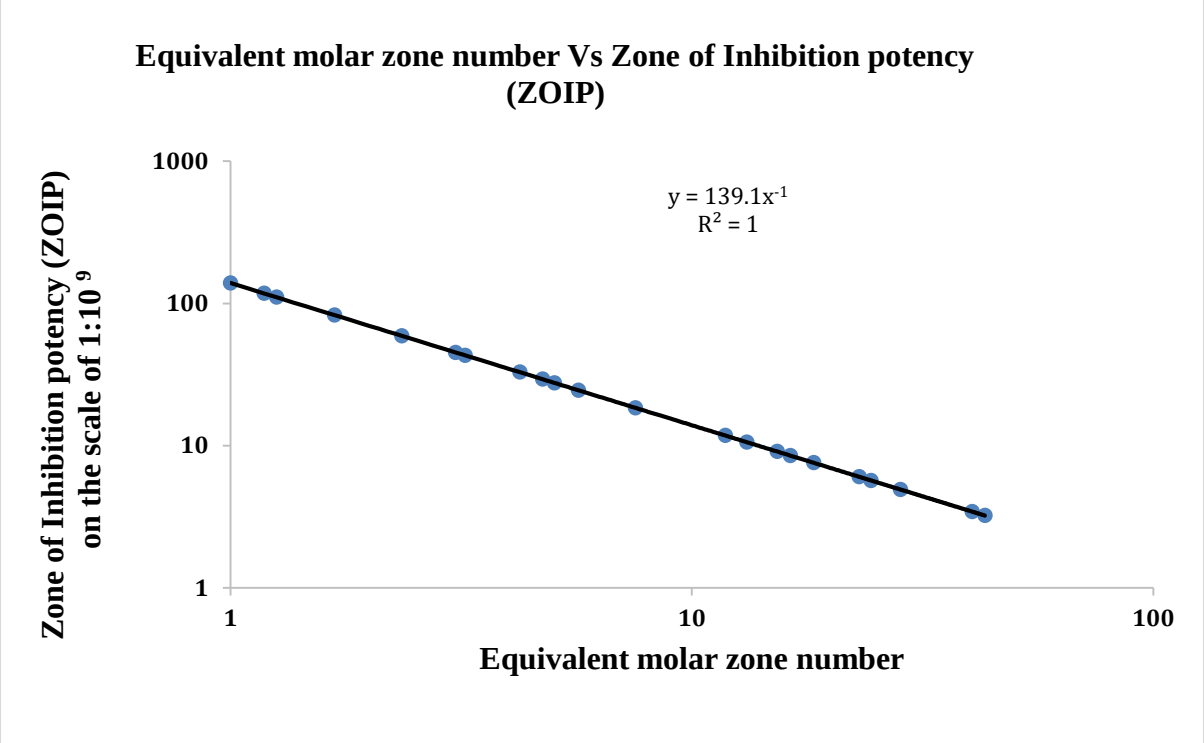

Figure 6. Equivalent molar zone number Vs zone of inhibition potency (ZOIP) log-log plot.

Interpretation of the Relationship Equivalent molar zone number (EMZN) Vs zone of inhibition potency (ZOIP)

Inverse Proportionality:
As the equivalent molar zone number (x) increases, the zone of inhibition potency (ZOIP, y) decreases. This inverse relationship means that higher molar zone numbers correspond to lower inhibition potency. Conversely, as the equivalent molar zone number (x) decreases, the zone of inhibition potency (ZOIP, y) increases significantly.

Magnitude:
The constant 1×10^{11} indicates that for small values of x, the value of y will be very large, emphasizing the strong inhibitory potency at low molar concentrations.

Logarithmic Scale:
The use of a logarithmic scale on both axes allows us to visualize the data across a wide range of values. It straightens out the hyperbolic relationship into a linear form on the log-log plot, making it easier to interpret the data. (fig6)

4. Discussion

The study includes *Staphylococcus aureus* ATCC no 25923. And will vary for species (Palmer *et al.*, 1995). Listing of the antibiotic according to the molar zone of inhibition concentration in ascending order of moles per unit square area in mm^2under standard conditions of AST, less the required moles of antibiotic to clear off the area of 1 mm^2, the more potent the antibiotic is... it will list at the top and more the mole required to clear unit square area in $mm^2$the least potent the antibiotic is and placed at the bottom of the table, a clearer term Equivalent molar zone number is appropriate for the nomenclature of novel terminology. Oxacillin 7.19 x 10^{-12} mole/mm^2 as standard to define the comparative priority list while taking and dividing all the other molar zone of inhibition concentration values with it we will obtain the Equivalent molar zone number. These are arranged in descending order to their potency, more potent at the top and least at the bottom. The reciprocal of the molar zone of inhibition concentration is the ZOIP arranged in descending order, the greater value of potency1.391 x 10^{11} of Oxacillin at the top and the lower value 3.22 x10^9 of cefepime at the bottom. In the priority list penicillin disc concentration with 2µg has Equivalent molar zone number 1.259 and ZOPI 1.105x10^{11}. Penicillin with 1 µg has Equivalent molar zone number 2.353 and ZOPI 5.912x10^{10} that showed the 2µgdisc concentration performs better than 1 µg whereas in the case of amoxicillin and Cefoperazone, as the disc concentration increases the EMZN and ZOPI decreases respectively, the possible reason may be 1 µg is very low dose for disc diffusion method or is a subject matter for further investigation which is beyond the scope of this journal article. There is a linear relationship between the Molar zone of inhibition unit mole/mm^2 and Mole concentration of antibiotics with an R^2 Value of 0.9167 while considering 19 data points, Piperacillin cefixime, and cefepime conventionally not considered as standards for assay of AST by disc diffusion method. Hence positioned last respectively in the priority table. While including the 22 points in the correlation table the R^2 lowers to 0.802

5. Conclusion

Using the molar zone of inhibition concentration (MZIC), equivalent molar zone number (EMZN), and zone of inhibition potency (ZOPI), the study effectively assesses and ranks the potency of beta-lactam antibiotics against *Staphylococcus aureus* ATCC 25923. The results

highlight the variability in potency with different concentrations and provide a clear ranking of antibiotics based on their effectiveness in vitro conditions. The system for ranking antibiotic potency is supported by the linear relationship between MZIC and mole concentration. As demonstrated by their ranking in the priority list, antibiotics, such as Piperacillin, Cefixime, and Cefepime, are less effective as standards in the disc diffusion method than Oxacillin, Nafcillin, and Penicillin. established by where they are on the list of priorities. Since we are in the era of antibiotic resistance, it is essential to take into account precise and accurate measurements based on mole concentration, zone of inhibition, and antibiotic breakpoints for better in-vitro results when deciding how to strike a wise balance between antibiotic concentration, resistance, and break points so that antibiotics can be administered safely and more effectively.

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Conflict of Interest
There is no potential conflict of interest.

6. References

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