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Antibacterial and Oxacillin Resistance Reversing Effect of Harmaline in Different Strains of *Staphylococcus Aureus*

Zaid O Ibraheem*
Department of Pharmacology, Faculty of Pharmacy, Ashur University, Baghdad, Iraq.
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ABSTRACT

Background: Harmaline is a naturally occurring indole alkaloid with many medicinal effects. Its antimicrobial effect against different pathogens has been reported previously.
Objectives: To explore the ability of harmaline to revers oxacillin resistance in the methicillin-resistant strain of *Staphylococcus aureus* (MRSA).
Materials and methods: Briefly, 124 isolates of MRSA were procured from different wards of the Medical Complex City, Baghdad, Iraq. The type of the bacteria and the strain had been checked using conventional methods to identify MRSA. Oxacillin resistance was checked using the conventional antibiotic sensitivity test. Moreover, the bacteriostatic and cidal effects of each harmaline and oxacillin were screened to determine their IC_{50} and IC_{90} for each effect. Eventually, the interaction between the two drugs for the bactericidal effect was screened using the well-known isobologram technique.
Results: The results showed that harmaline got a mild to moderate bactericidal effect against MRSA and produced a moderate synergistic effect when both drugs were combined at a ratio of 3:7 and 5:5 (harmaline/oxacillin). Besides, an additive effect or mild synergy was noticed in the other combinations.
Conclusion: Overall, harmaline can be suggested as a chemo-sensitizer or a pharmacophore to develop better synergistic drugs for oxacillin against MRSA but further safety and pharmacokinetic studies are indicated to optimize this effect.
Keywords: Oxacillin; Resistance; Harmaline; Staphylococcus aureus; Antibacterial.

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INTRODUCTION

Staphylococcus aureus has unique evolutionary adaptive mechanisms that confer for its high virulence and antibiotic resistance. For instance, its ability to express beta-lactamase enzyme turned it resistant to most of the penicillin derivatives. Only one group of the penicillin derivatives; known as methicillin derivatives, are insensitive to the beta-lactamase and retain activity against staphylococci. Oxacillin is a famous member of this group and is used widely against staphylococci. Long-term exposure to oxacillin or the other members of methicillin derivatives led to the emergence of methicillin-resistant strain of *Staphylococcus aureus* (MRSA) [1]. Like other penicillin derivatives, methicillin induces its antibacterial effect by binding to a type of cell wall protein called PBP (penicillin-binding protein) which is involved in the transpeptidation of the cell wall peptidoglycan matrix. MRSA expresses a type of penicillin binding protein (PBP) called PBP-2-α which is unresponsive to the inhibitory action of methicillin derivatives [1].

The issue of antibiotic resistance in Staphylococcus aureus perplexed the dedicated efforts to eradicate it and urged for exploring new alternatives or chemosensitizers that improve the action of the antibiotics. Implementation of phytochemicals for this purpose has been suggested [2]. Previous studies revealed that most of the phytochemicals have a prominent but weak to moderate antibacterial effect that does not entitle them to substitute the conventional antibiotics [3, 4]. Although some of them succeeded in producing a comparable effect but more clinical trials are still required to optimize them for human use [4]. Harmaline is a beta carboline indole alkaloid; which is obtained from a botanical herb called Peganum harmala. The plant has been extensively used in tra-

* Corresponding author: E-mail: zaid.osama@au.edu.iq

ditional medicine as an emmenagogue and antidepressant [2]. Recently its antimicrobial activity against bacteria [3], viruses [4] and parasites has been highlighted [5]. Accordingly, this study was designed to explore the activity of harmaline; as a chemo sensitizer for methicillin derivatives against *Staphylococcus aureus*.

MATERIALS AND METHODS

Study design

The experimental study was designed to evaluate the ability of harmaline to reverse oxacillin resistance in MRSA using bacterial samples procured from different wards in the Medical Complex City, Baghdad, Iraq. Most of the samples were collected from skin swabs (abscesses and boils, folliculitis, and impetigo). Few were blood samples obtained from diseased patients with septicaemia. The samples were obtained from patients used to visit the Dermatology Department or from hospitalized patients; suffering from burns infection, diabetic sores or other skin infections. The samples were taken along with the routine identification of the bacteria for the hospitalized patients and were used as representatives for MRSA. The other types of bacteria or the methicillin sensitive strains of *Staphylococcus aureus* were excluded from the study.

Material

Pure harmaline was purchased from Indofine Biochemical Company Inc. (Cat No.: A-006). Oxacillin vials (Hi-Midea -SD088-5VL) as well as the culture media for bacterial growth were obtained from Thermo-Fisher Scientific.

Ethical approval

The study was approved by the ethical committee for research at Ashur University (Reference number 177 on 18-12-2022). The samples were collected and disposed of as per the rules and regulations of the Ministry of Health. Besides, the patients were informed that their samples would be used for further research studies. The study did not involve any additional procedures or treatment interventions and it was done on samples already obtained from the patients for the routine investigation of the bacteria.

Sample collection

The collection was done during the period from April 2023 up to December 2023 and all the samples were exposed to the same conditions while performing the experiments.

Staphylococcus aureus identification and collection

The collected isolates were exposed to the routine procedure of bacterial identification using VITEK2 identification GP cards (BioMeeriex) as well as the conventional morphological and biochemical procedures for bacterial identification [6]. Only bacterial samples that got MRSA were used in the study. The strain was checked using the well-known latex agglutination technique for tracing of the penicillin binding protein 2-α. (PBP2a Latex Oxford-UK) [7].

Quantitative measurement of the antibacterial effect of the drugs

The quantitative measurement of the bacteriostatic and bactericidal effects of each of harmaline and oxacillin involved the determination of the IC_{50} and IC_{90} for each. They represent the concentrations required to inhibit the growth or kill 50% and 90% of the bacterial population (IC_{50i} and IC_{90i}) and (IC_{50k} and IC_{90k}) respectively. They were determined as per the protocols; recommended by the National Committee for Clinical Laboratory Standards (CLSI, 2014)[8].

Determination of the bacteriostatic effect (IC_{50i} and IC_{90i})

The bacteriostatic parameters were determined using the twofold micro-dilution method as previously described by (J. Nemeth *et al* 2014) [8]. Briefly, 180 μl of twofold serial dilution of each drug (1 nM -1 mM) were prepared from stocks using Mueller Hinton broth (Liofilchem -Italy) as a diluent. The dilutions were loaded in triplicates into wells of lidded 96 well microplate (Greiner-Germany). Then, 20 μL of a bacterial suspension; adjusted at 0.5 McFarland standard (1×10^8 CFU/mL), was added to each dilution. Triplicates of negative and positive control wells were allocated as well. The negative ones contained the maximum concentrations of each drug and were devoid of the bacteria while the positive ones contained bacteria and were unexposed to the antibiotics [8].

Eventually, the plates were incubated at 37°C for 24 hours using the Bio screen C MRB system (Särkiniementie 5 C 7, 00210 Helsinki, Finland). The optical density of each well was measured spectrophotometrically at λmax = 600 nm as an indicator for bacterial growth. Then, the percentage of the growth inhibition at each concentration was calculated and a plot of the log dose response curve (Percentage of growth inhibition versus log concentration curve) was plotted to determine (IC_{50i} and IC_{90i}) as shown in Figure 1 [8].

Determination of the bactericidal effect of each drug

At the end of incubation and after measuring the optical density to get the bacteriostatic parameters, 20 μl from each well were loaded onto new plates whose wells were prefilled with 180 μl of Mcfarland broth. Then, the plates were incubated using the same abovementioned incubation conditions for another 24 hours, and the bacterial growth was monitored

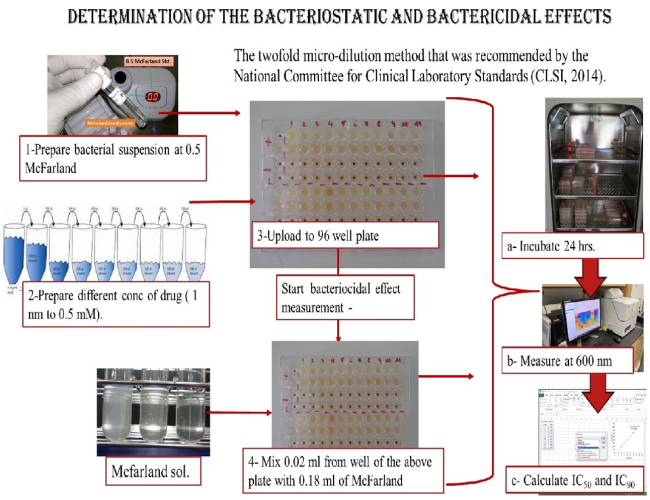

Figure 1. The sequence of steps followed to determine the bacteriostatic and bactericidal parameters of each drug.

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as above to plot the dose response curve and determine each of the IC_{50k} and IC_{90k} for each drug (Figure 1) [9, 10].

Measurement of the drugs interaction (Isobolgram plot for the combination effect)

Since the bactericidal effect was predominant for the two drugs against MRSA, the isobologram was designed to find the impact of their combination on the bactericidal parameters. The designs were done as previously described [11–13].

Briefly, as per the protocol, a working solution of each drug was prepared at 16 times the IC_{50k} such that the fourth dilution fell within its limit. Then, the prepared working solutions were mixed at different ratios (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 7:3, 8:2, 1:9 and 0:10 oxacillin/harmaline). Then, IC_{50k} & IC_{90k}, were determined for each drug within each combination as mentioned in the previous sections.

Later on, FIC (Fractional Inhibitory Concentration) values for each drug within each combination were determined by finding the quotient of IC_{50k} or IC_{90k} within each combination to that when the drug was incubated alone with the bacteria. After that, an isobologram table was established in which the FIC values of each drug were listed for each combination and the total FIC (sum of FIC for oxacillin with that of harmaline) was calculated. The interaction is considered as synergy if the total FIC is less than 0.5. Moreover, the interaction is considered mild synergy or additive if the value is between 0.5 and 1, indifference if the value is between 1–2, and antagonism if it is more than 2 [11–13].

In addition, the FIC values were used to build the isobologram by plotting the FIC for oxacillin on the Y-axis and harmaline on the X-axis. The line of additivity was plotted by connecting the FIC values when each drug was incubated alone. The interaction is considered additive if the plotted point falls on the line of additivity and is considered as antagonistic or synergistic if the points fell above or below the line of additivity respectively (Figure 2) [11–13].

Molinspiration (http://www.molinspiration.com) was used as a chemi-informatic software to determine the in silico physiochemical properties of harmaline and to get some predicted biological properties. It performs fragment-based virtual screening of some parameters, viz; cLOGP, PSA, nON and nrotb, which stand for the logarithm of octanol/water partition coefficient, molecular polar surface area, number of non-hydrogen atoms, number of hydrogens donating bonds, and number of rotatable bonds, respectively. For the biological properties, the software measures the predicted drug-likeness scores toward some biological intracellular targets, viz; GPCR (G protein coupled receptors), kinase, nuclear factors, ion channels, and protease enzyme [14].

Statistical analysis

Data were entered and analysed using Graphpad prism software version 5. The continuous variables were presented as a mean ± standard deviation. There are two parts in the study, the first was concerned about finding the growth inhibitory as well as the cidal parameters of each of oxacillin and harmaline against MRSA. The results obtained for oxacillin and harmaline were compared using one way ANOVA analysis. The growth and cidal parameters were determined using linear regression analysis and those for oxacillin were compared with their cohorts for harmaline. A statistically significant difference was considered at a P-value of less than 0.001.

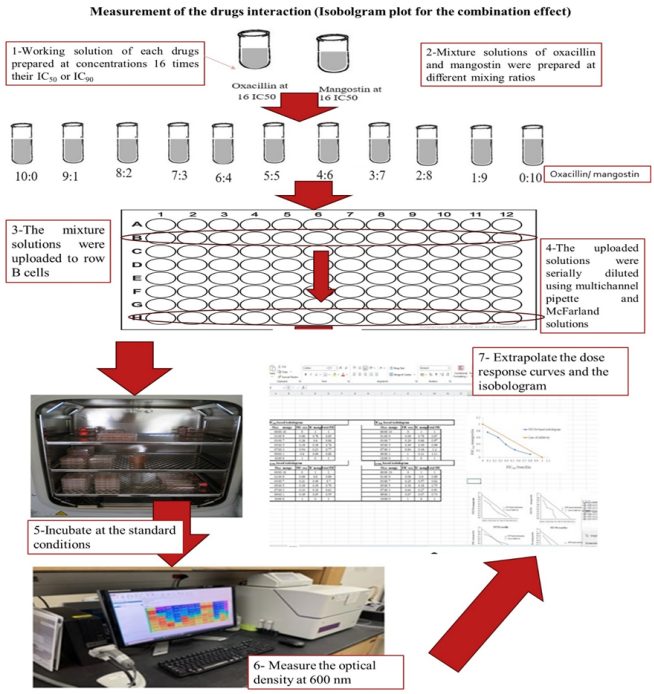

Figure 2. The sequence of steps of the isobologram technique mechanism elucidation (physiochemical properties calculation and bioactivity prediction).

RESULTS

Antibacterial effect of harmaline and oxacillin

Both oxacillin and harmaline showed a predominant bactericidal effect against MRSA, as the levels of the bactericidal parameters (IC_{50k} and IC_{90k}) were slightly higher or comparable to those of the bacteriostatic effect (IC_{50i} and IC_{90i}) (Table 1). The results showed the absence of any significance between IC_{50i} and IC_{50k} or between IC_{90i} and IC_{90k} for the two compounds (P-value < 0.05) (between IC_{50i} and IC_{50k} or between IC_{90i} and IC_{90k}) to find if there is a difference between the concentrations required to induce the bacteriostatic and bactericidal effects.

The second part was the isobologram analysis in which IC_{50} and IC_{90} values for each drug were calculated when the bacteria were exposed to the drug alone or within the combination. Their calculation was done using linear regression analysis, and then the values were used to calculate the FIC (Fractional inhibitory concentration) as mentioned above.

Isobologram technique for screening the pharmaceutical effect of different drugs combinations

A mild synergy to an additive effect was seen between harmaline and oxacillin when they were combined at all the ratios except at (7:3 and 5:5 oxacillin/ harmaline) wherein the synergy was stronger, and it was moderate to high with a total FIC value approaching to 0.5. (Table 2).

In-Silico molecular characterization

According to the *in Silico* physicochemical properties of harmaline, it does not violate the five Lipinski rules, making it a suitable candidate drug. Furthermore, the predictive tool

Table 1. The bacteriostatic and bactericidal parameters (IC_{50i}, IC_{90i}, IC_{50k} and IC_{90k}) for each of harmaline and oxacillin against MRSA. The results were expressed as mean ± Standard error of mean of the values obtained against each staphylococcus isolate.*

	Harmaline	Oxacillin
IC_{50i}	28.1.1 ± 1.85 μM^b	0.5 ± 0.2 μM^a
IC_{90i}	127.7 ± 2.12 μM^b	6.7 ± 0.035 μM^a
IC_{50k}	31.8 ± 2.32 μM^b	1.12 ± 0.0.09 μM^a
IC_{90k}	141.5 ± 3.12 μM^b	11.30.04 μM^a

* a,b represent a statistically significant value as compared to that of harmaline and oxacillin against MRSA respectively (P-value < 0.001). c represents a statistically significant difference (P-value < 0.001) between the cidal and growth inhibitory parameters for the same compound (between IC_{50i} and IC_{50k} or between IC_{90i} and IC_{90k})

Table 2. Results of the IC_{50i}, IC_{90i}, IC_{50k} and IC_{90k} based isobolograms for different combinations of oxacillin and harmaline at ratios of (10:0, 9:1, 7:3, 5:5, 7:3, 9:1 and 10:0) oxacillin/harmaline.*

			IC_{50k} based isobologram		
Oxa	:	Harm	FIC oxa	FIC Harm	Total FIC
0	:	1	0.00	1.00	1.00
1	:	9	0.09±0.001	0.75±0.065	$0.84±0.068^b$
3	:	7	0.29±0.019	0.52±0.041	$0.81±0.075^b$
5	:	5	0.32±0.024	0.31±0.024	$0.63±0.052^b$
7	:	3	0.49±0.031	0.18±0.009	$0.67±0.051^b$
9	:	1	0.61±0.052	0.28±0.017	$0.89±0.065^b$
1	:	0	1.00	0.00	1.00
			IC_{90k} based isobologram		
0	:	1	0.00	1.00	1.00
1	:	9	0.09±0.02	0.72±0.048	$0.81±0.065^b$
3	:	7	0.25±0.021	0.39±0.024	$0.64±0.055^{ab}$
5	:	5	0.29±0.022	0.23±0.018	$0.52±0.038^a$
7	:	3	0.28±0.024	0.31±0.021	$0.59±0.040^a$
9	:	1	0.67±0.051	0.21±0.014	$0.88±0.072^b$
1	:	0.00	1.00	0.00	1.00

* The results were expressed as mean ± Standard error of mean for all the bacterial samples (n = 124). The same letters indicate the absence of any statistically significant difference (P-value > 0.001). Meanwhile, the different ones indicate the presence of the difference (P-value < 0.001). The statistical analyses were done for the values of FIC_{total} for the different combinations.

for biological activity demonstrated that harmaline has a good enzyme inhibitory effect (Table 3).

Table 3. *In silico* molecular characters of harmaline.

Name of the parameter	Harmaline
ClogP	2.668
n violation	0
MWt	198.23
nNO	3
PSA	48.9
nOHNH	2
GPCR ligand	– 0.23
Ion channel modulator	– 0.11
Kinase inhibitor	– 0.67
Nuclear receptor ligand	– 0.61
Protease inhibitor	– 0.72
Enzyme inhibitor	0.03

DISCUSSION

This study is one of the trials to explore agents that reverse oxacillin resistance in MRSA among the sanctuary of phytochemicals. These events constitute a catastrophic challenge for the dedicated efforts to eradicate this pathogen. This phenomenon evolved due to the ability of the bacteria to express a type of penicillin binding protein called (PBP-2-α). PBP is a target of penicillin and is involved in the process of cell wall building. It is unresponsive to oxacillin and turns the bacteria resistant to it [15].

In the current study, harmaline was chosen as a chemo sensitizer based on previous literature about its antibacterial effect [16] and on the results of the *in silico* determination of physiochemical properties and biological effects on cells using the online free chemi-informatic software. The results revealed that harmaline can be accepted as a pharmaceutical agent as it does violate the Lipinski rules for pharmaceutical agents (Table 1). Furthermore, the software showed that harmaline had some biological effects against some cellular targets. Nevertheless, these results are just predictive tools and do not give a full confirmation about the drug's mechanism of action. The tool suggested a good potential for harmaline to inhibit different intracellular pathways.

The study showed that harmaline got a bactericidal effect s, as there has been a negligible discrepancy between the bacteriostatic and cidal parameters, although its antibacterial effect was moderate, as its IC_{50} was in the micro-molar range and was weaker than that of oxacillin against MRSA.

Previous studies have pointed out the antibacterial effect of harmaline and attributed it to its genotoxic effect that is characterized by its ability to induce DNA intercalation, chromosomal aberration, DNA damage, or suppression of DNA excision repair [17]. Furthermore, they can actively inhibit cellular growth through the inhibition of a bunch of enzymes related to DNA synthesis and cellular growth, such as; cyclin dependent kinase [18, 19]. Besides, harmala alkaloids may induce cell cycle arrest and induce apoptosis through regulation of the expression of the apoptosis genes. Although this effect was seen in cancer cell lines, further studies are recommended to look at this impact in bacteria [18–20]. Furthermore, previous investigations revealed an action of harmala alkaloids against metabolic enzymes such as kinases [21, 22], and by this, it has an anti-metabolic effect that might enhance its cytotoxic action. Besides, its genotoxic effect, previous studies revealed that incubation of beta carbolines with the bacteria may enhance the expression of some virulent genes and suppress other genes which are involved in the regulation of bacterial growth. For instance, they were found to enhance the expression of the intercellular adhesion gene (*icaA*) and the capsular polysaccharide synthesis gene (*CPS5*) in *Staphylococcus aureus*. On the other hand, they were found to sup-

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press some genes essential for the life of the bacteria like the flagellum gene (*flgK*), fimbriae protein gene (*pilA*), and fimbriae gene (*cupA1*) which are involved in cell motility and reproduction [16].

Drug combination provide lots of benefits in the realm of bacterial chemotherapy, as they may expand the antibacterial spectrum, prevent the emergence of the multidrug resistance which is conferred by the P-glycoprotein system that expels xenobiotics outside the bacteria and help reduce the required dose of the antibiotic, and by this, it may reduce the chance of drug toxicity [23]. The results showed some mild synergy in the antibacterial effect of the combinations of harmaline and oxacillin. The synergy was more prominent for the FIC_{90} based isobolgram as compared to that of FIC_{50}. This synergy can be ascribed to the difference in the target of each of harmaline and oxacillin. Oxacillin interferes with the transpeptidation of the peptide glycan matrix [15] while harmaline acts by different mechanisms as mentioned previously. Besides, oxacillin action on the cell wall may provide a better opportunity for harmaline to penetrate the intracellular compartment and perform its action [15]. The synergy between harmaline and oxacillin may be ascribed to the abovementioned genotoxic effect or may be due to its cytotoxic effect on the cell membrane.

Previous studies revealed that incubation of the cells with harmala alkaloids results in the reduction of the steroid biosynthetic pathway and by this, the integrity of the cell membrane or the membranous structures of the cell wall is compromised [24]. Besides, the study showed that beta carbolines got a negative effect on the biosynthesis of SOD (superoxide dismutase) enzyme and CAT (Catalase) which are involved in the reduction of the flow of oxygen free radicals and by this they may help reduce the integrity of the cell membrane [24].

The highest synergy was obtained when both drugs were added at 5:5 and 7:3 oxacillin/ harmaline and this augmented the notion that oxacillin might have enhanced harmaline penetration. On the other hand, as per the *in Silico* study, harmaline may modulate the action of the ion channels, and this indeed affects the integrity of the cell membrane; an action that may cooperate with the cell wall lytic effect of oxacillin and lead to more cell hydrolysis. Previous investigations revealed that harmaline may enhance the release of reactive oxygen species near the cell membrane [22, 24].

This study highlights the significance of harmaline in reversing the resistance of MRSA toward anti-staphylococcus penicillin derivatives, and this may find its way to implementation as a chemo sensitizer for external use or consider it as a pharmacophore to develop drugs with higher safety and better chemo sensitizing and antibiotic resistance reversing effects. The study had some limitations it used MRSA; obtained from medical wards in Iraq as representative of these bacteria in Iraqi hospitals, and may be the sample size was insufficient to represent all the staphylococci. Besides, there may be a discrepancy in the responsiveness of MRSA in other places in comparison to those we procured in our study. Therefore, it is recommended to run the same experiment using MRSA from other parts of the world. It is worth noting that this study provides only information about the plausible synergistic effect of harmaline with oxacillin against MRSA. By this, we can suggest the phytochemical as a chemosensitizer or a pharmacophore to design new chemosensitizers for oxacillin. It is still not enough to screen its possible use *in vivo*. Further studies are recommended to check if it is possible to deliver it at the required concentration to the target site of action. Besides, further studies are required to check for the safety profile of harmaline at concentrations equivalent to the concentrations that provided the claimed pharmacological effect.

CONCLUSION

Harmaline has a moderate antibacterial effect, but it can be a good pharmacophore for developing drugs with a higher safety index and a strong chemo sensitizer effect. More structural modifications are recommended to get an efficient chemo sensitizer for oxacillin against MRSA. Besides, the implementation of harmaline as a chemo sensitizer for oxacillin or anti-staphylococcus penicillin derivatives needs more optimization and more safety study, especially for the external use of the anti-staphylococcus drugs.

ETHICAL DECLARATIONS

Acknowledgments

None.

Ethics Approval and Consent to Participate

The study was approved by the ethical committee for research and higher studies of the Iraqi Ministry of Health. Informed consent was obtained from each participant.

Consent for Publication

Not applicable (no individual personal data included).

Availability of Data and Material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests

The author declares that there is no conflict of interest.

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Authors' Contributions

Ibraheem ZO is responsible for the design of the study, analyzing the results, and writing the manuscript. Ibraheem ZO read and approved the final version to be published.

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