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RESEARCH ARTICLE

In Vitro Inhibition of *Staphylococcus aureus subsp. aureus* (ATCC® 6538™) by Artemether-Lumefantrine Tablets: A Comparative Study of Three Dosage Strengths

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Abstract:

Purpose:

Antibiotics are progressively failing in the fight against infections due to *S. aureus* because the bacterium has an outstanding ability to acquire multi-antibiotic resistance and become resistant to most antibiotics. Multi-drug resistant *S. aureus* poses a major threat to the foundation upon which standard antibacterial chemotherapy stands, hence the need to consider non-antibiotic solutions to manage invasive bacterial infections. This study investigated the inhibitory activities of three dosage strengths of artemether-lumefantrine tablets against *Staphylococcus aureus subsp. aureus* (ATCC® 6538™) and determined the minimum concentrations of the tablets that are able to completely inhibit growth of the bacterium *in vitro*.

Methods:

The agar dilution and broth macrodilution techniques were used to determine the susceptibility of the *Staphylococcus aureus subsp. aureus* (ATCC® 6538™) strain to artemether-lumefantrine 20/120mg, 40/240mg and 80/480mg tablets.

Results:

The most active inhibitor was artemether-lumefantrine 80/480mg tablet with a minimum inhibitory concentration value of 2.5mg/mL while artemether-lumefantrine 20/120mg and 40/240mg tablets exhibited moderate but equal activities against the test strain.

Conclusions:

The study has revealed that artemether-lumefantrine, an antimalarial drug, also has anti-staphylococcal properties and inhibits *S. aureus in vitro*. This study presents the first report on the *in vitro* activity of artemether-lumefantrine tablet against *S. aureus* and suggests the need to consider it as an alternative in the treatment of staphylococcus infections.

Keywords: Minimum inhibitory concentrations, Multi-antibiotic resistance, Artemether-lumefantrine, Test strain, *S. aureus*, staphylococcus infections.

1. INTRODUCTION

Staphylococcus aureus is both a human pathogen and a commensal [1]. It colonizes about 30 percent of human population [2]. *S. aureus* is the most pathogenic species of the genus *Staphylococcus* and it is implicated in both

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community-acquired and nosocomial infections [3]. *S. aureus* has been reported as the causative agent of wide variety of diseases and infections such as boil, wound infection, pustule, subcutaneous and sub-mucosa abscesses, osteomyelitis, mastitis, impetigo, septicemia, meningitis, bronchopneumonia, food poisoning and urinary tract infections [4].

S. aureus infections are often exceptionally difficult to treat because of the large population heterogeneity, phenotypic switching, intra-strain diversity, hypermutability and the small colony variants [3]. The major factor for the success of *S. aureus* as a pathogen is its notable capacity to acquire antibiotic resistance [5]. There have been reports of emergence of *S. aureus* strains that are resistant to the following antibiotics: oxacillin, vancomycin, Mupirocin and Clindamycin [6].

In developed countries worldwide, *S. aureus* is the commonest cause of bacteremia and infective endocarditis and is associated with excess mortality relative to other pathogens [1]. *S. aureus* is a major pathogen in Africa and other developing countries [7]. In Sub-Saharan Africa and other tropical areas, it frequently causes invasive diseases [8]. Studies done in Ghana revealed that *S. aureus* is the third most frequently isolated microorganism from patients [9] and the second most prevalent bacterium among patients from teaching, regional and district hospitals and has a multi-drug resistant rate of 42.3% [10].

The emergence of Multi-Drug Resistant (MDR) microbial pathogens poses a major threat to the foundation upon which standard antibacterial chemotherapy stands hence the need to consider non-antibiotic solutions to manage invasive bacterial infections [11].

Antibiotics are progressively failing in the fight against infections due to *S. aureus* because the organism has an outstanding ability to acquire multi-antibiotic resistance [12] and become resistant to most antibiotics. This is a serious threat to global public health and requires stakeholders to come up with a harmonized set of approaches to fight antimicrobial resistance in a multifaceted manner [13]. As part of the efforts to develop approaches to fight antimicrobial resistance, this study sought to investigate the inhibitory effects of a non-antibiotic agent such as artemether-lumefantrine against *S. aureus*.

Artemether-lumefantrine is an artemisinin-based combination therapy approved for treatment of un-complicated malaria [14]. Artemisinin derivatives are endorsed internationally for treatment of malaria because of their high potency, rapid onset of action, broad malaria stage specificity and favourable safety profile [15]. Oral formulations of artemether-lumefantrine are available as tablet and dispersible formulations and have similar pharmacokinetic properties [16]. Studies have reported antimicrobial activities of artemisinin and its derivatives against a range of pathogenic bacteria including *Staphylococcus aureus* [17, 18].

This study examined the inhibitory activities of three dosage strengths of artemether-lumefantrine tablets against *Staphylococcus aureus subsp. aureus* (ATCC® 6538™) and determined their Minimum Inhibitory Concentrations (MICs) using the agar dilution and broth macrodilution methods.

2. METHODS

2.1. Bacterial Strain

The *Staphylococcus aureus subsp. aureus* (ATCC® 6538™) strain studied was obtained from Microbiologics Inc, St. Cloud, Minnesota USA. It was a lyophilized organism and first passage from reference strain. The strain was verified and confirmed in accordance with supplier's protocol and the certificate of analysis. The organism was maintained at 4°C on Mueller-Hinton agar slants (HiMedia Laboratories Private Limited, Mumbai, India) using seed-lot culture maintenance technique with three passages [19]. Prior to testing, the strain was subcultured onto Mueller-Hinton agar and incubated at 35°C for three days to ensure the viability and purity of the inoculum.

2.2. Culture Media

The Mueller-Hinton agar and Mueller-Hinton broth used for the study were obtained from HiMedia Laboratories Private Limited, Mumbai, India. Each batch of the Mueller-Hinton agar was confirmed for growth promotion and Minimum Inhibitory Concentration (MIC) performance according to Clinical and Laboratory Standards Institute (CLSI) procedures [20]. The growth promotion and minimum inhibitory concentration performance characteristics of each batch of the Mueller-Hinton broth were confirmed using standard set of quality control microorganisms recommended by CLSI [21].

2.3. Artemether-Lumefantrine Samples

The artemether-lumefantrine tablet samples used in this study were manufactured by Entrance Pharmaceuticals and Research Centre, a pharmaceutical manufacturing industry located in Accra, Ghana. The three artemether-lumefantrine tablet dosage strengths studied were 20/120mg, 40/240mg and 80/480mg. The excipients used in the tablet formulations were microcrystalline cellulose, aerosol, crosscarmellose sodium, polysorbate 80, talcum and magnesium stearate. These excipients were the same for all the different tablet strengths. The average masses of the tablets studied were 308mg, 582mg and 685mg for 20/120mg, 40/240mg and 80/480mg respectively. The ages of artemether-lumefantrine 20/120mg, 40/240mg and 80/480mg tablets at the time of the study were 8 months, 16 months and 8 months respectively. The tablets were aseptically ground into fine powder and prepared as stock solutions. Two-fold serial dilutions were then performed to obtain a concentration range of 0.04 mg/mL to 160 mg/mL (Tables **1** and **2**).

Table 1. Scheme for preparing dilutions of artemether-lumefantrine used in agar dilution susceptibility tests.

Artemether-lumefantrine Suspension					–		
Step	**Concentration of Powdered Tablet (mg/mL)**	**Source**	**Volume (mL)**	**Diluent (mL)**	**Intermediate Concentration (mg/mL)**	**Final Concentration at 1:10 in Agar (mg/mL)**	**Log2**
	1600	Stock	-	-	1600	160	7.32
1	1600	Stock	2	2	800	80	6.32
2	1600	Stock	1	3	400	40	5.32
3	1600	Stock	1	7	200	20	4.32
4	200	Step 3	2	2	100	10	3.32
5	200	Step 3	1	3	50	5	2.32
6	200	Step 3	1	7	25	2.5	1.32
7	25	Step 6	2	2	12.5	1.25	0.32
8	25	Step 6	1	3	6.25	0.63	-0.67
9	25	Step 6	1	7	3.1	0.32	-1.69
10	3.125	Step 9	2	2	1.6	0.16	-2.64
11	3.125	Step 9	1	3	0.8	0.08	-3.64
12	3.125	Step 9	1	7	0.4	0.04	-4.64

This table is modified from Clinical and Laboratory Standard Institute . (2016) Performance Standard for antimicrobial susceptibility : 26th ed. CLSI M100S. Clinical and Laboratory Standard Institute. Wayne, PA.

Table 2. Scheme for preparing dilutions of artemether-lumefantrine used in broth dilution susceptibility tests.

Artemether-lumefantrine suspension					–	–	–	–	
Step	**Concentration of Powdered Tablet (mg/mL)**	**Source**	**Volume (mL)**	**Diluent (mL)**	**Intermediate Concentration (mg/mL)**	**Volume (mL)**	**Inoculum Suspension with Broth (mL)**	**Final Concentration at 1:2 in Broth (mg/mL)**	**Log2**
1	320	Stock	-	-	320	0.5	0.5	160	7.32
2	320	Stock	0.5	0.5	160	0.5	0.5	80	6.32
3	320	Stock	0.5	1.5	80	0.5	0.5	40	5.32
4	320	Stock	0.5	3.5	40	0.5	0.5	20	4.32
5	40	Step 4	0.5	0.5	20	0.5	0.5	10	3.32
6	40	Step 4	0.5	1.5	10	0.5	0.5	5	2.32
7	40	Step 4	0.5	3.5	5	0.5	0.5	2.5	1.32
8	5	Step 7	0.5	0.5	2.5	0.5	0.5	1.25	0.32
9	5	Step 7	0.5	0.5	1.25	0.5	0.5	0.63	-0.67
10	5	Step 7	0.5	1.5	0.63	0.5	0.5	0.32	-1.69
11	0.63	Step 10	0.5	3.5	0.31	0.5	0.5	0.16	-2.64
12	0.63	Step 10	0.5	0.5	0.16	0.5	0.5	0.08	-3.64
13	0.63	Step 10	0.5	1.5	0.08	0.5	0.5	0.04	-4.64

This table is modified from Clinical and Laboratory Standard Institute . (2016) Performance Standard for antimicrobial susceptibility : 26th ed. CLSI M100S. Clinical and Laboratory Standard Institute. Wayne, PA.

2.4. Antimicrobial Susceptibility Testing

The agar dilution and broth macrodilution techniques described by CLSI (CLSI, 2015) were used to determine the

susceptibility of the *Staphylococcus aureus subsp. aureus* (ATCC® 6538™) strain to the artemether-lumefantrine samples.

2.5. Agar Dilution Method

2.5.1. Preparation of Agar Dilution Plates

Artemether-lumefantrine tablet suspensions were prepared by making successive 1:2, 1:4 and 1:8 dilutions to produce a concentration range of 0.4 mg/mL to 1600 mg/mL (Table **1**). One part (1.3mL) of each dilution was added to nine parts (11.7mL) of molten Mueller-Hinton agar that has been allowed to equilibrate in a water bath to 45°C. This produced a concentration range of 0.04 mg/mL to 160 mg/mL (Table **1**). Growth-control plates were prepared using sterilized distilled water in place of the artemether-lumefantrine suspensions. The tubes were thoroughly mixed and poured into 90mm diameter Petri plates to result in an agar depth of 4 mm. The agar plates were allowed to solidify at room temperature.

2.5.2. Inoculum Preparation

Inoculum was prepared by making saline suspension of colonies of *Staphylococcus aureussubsp. aureus* (ATCC® 6538™) selected from 24-hour Mueller-Hinton agar plate. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard which is equivalent to 1 x 10^8 CFU/mL [6]. The suspension was then diluted in sterile physiological saline to a concentration of 1 x 10^7 CFU/mL.

2.5.3. Inoculation and Incubation of Agar Dilution Plates

Each agar dilution plate was inoculated with thirty-six spots of the inoculum suspension using a thirty-six pin inoculum replicator. The replicator delivered 2µL of the inoculum suspension per spot to produce a final concentration of 10^4 CFU/spot. Growth-control plates were inoculated before and after the inoculation of the agar dilution plates to ensure that there was no contamination or significant antimicrobial carryover during the inoculation. The inoculated plates were allowed to stand for 20 minutes at room temperature for the moisture in the inoculum spots to be absorbed into the agar. The plates were inverted and incubated at 35°C for 20 hours.

2.5.4. Determination Agar Dilution End Points

The agar plates were placed on a dark nonreflecting surface and observed with a hand lens. The minimum inhibitory concentration was recorded as the lowest concentration of artemether-lumefantrine that completely inhibited growth, disregarding a single colony or a faint haze caused by the inoculum [6].

2.6. Broth Macrodilution Method

2.6.1. Inoculum Preparation

Inoculum was prepared by making saline suspension of colonies of *Staphylococcus aureussubsp. aureus* (ATCC® 6538™) selected from 24-hour Mueller-Hinton agar plate. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard which is equivalent to 1 x 10^8 CFU/mL [6]. The inoculum was diluted in Mueller-Hinton broth to a final concentration of 5 x 10^5 CFU/mL.

2.6.2. Preparation of Artemether-Lumefantrine Dilutions and Inoculation of Macrodilution Tubes

Stock solution of artemether-lumefantrine tablet of concentration 320 mg/mL was prepared. Intermediate solutions were prepared from the stock solution by making successive 1:2, 1:4, and 1:8 dilutions using the dilution format shown in Table **2**. Then, 0.5mL of the standardized inoculum was added to each macrodilution and growth control tubes to obtain a concentration range of 0.04 mg/mL to 160 mg/mL (Table **2**). Purity check of the inoculum suspension was performed by subculturing aliquots onto a Mueller-Hinton agar plate for simultaneous incubation [6].

2.6.3. Incubation of Macrodilution Tubes

The inoculated macrodilution tubes were incubated aerobically at 35°C for 20 hours.

2.6.4. Determination Broth Macrodilution End Points

The minimum inhibitory concentration was read as the lowest concentration of artemether-lumefantrine that completely inhibited the growth of *S. aureus* in the tubes detected by unaided eye [6]. A test was considered valid when definite turbidity occurred in growth-control tube. In instances where growth turbidity in the tubes were difficult to detect with unaided eye, due to higher concentrations of the powder particles, the macrodilution tube contents were cultured on Mueller-Hinton agar and observed for bacterial growth.

3. RESULTS

The three samples of artemether-lumefantrine tablets showed varying degrees of antibacterial activity against the assayed *S. aureus* strain and the inhibition was generally dependent upon tablet strength and concentration of tablet powder.

3.1. Agar Dilution Method

Out of 13 different concentrations of artemether-lumefantrine 80/480mg tablet tested against the *S. aureus* strain, 7 (53.8%) inhibited growth of *S. aureus* completely while 6 (46.2%) failed to inhibit *S. aureus* growth (Table **3**). The proportions of artemether-lumefantrine 40/240mg tablet concentrations that completely inhibited *S. aureus* growth was 15.4%. Artemether-lumefantrine 20/120mg tablet also produced two (15.4%) inhibitory concentrations. Artemether-lumefantrine 40/240mg and 20/120mg tablets had the same MIC value of 80 mg/mL. The most active inhibitor was artemether-lumefantrine 80/480mg tablet which had an MIC value of 2.5mg/mL (Table **3**).

Table 3. *In vitro* antimicrobial activity of artemether-lumefantrine tablets against *Staphylococcus aureus subsp. aureus* (ATCC® 6538™).

–	–	Concentration of Powdered Tablets (mg/mL)											
–	**Method**	**160**	**80**	**40**	**20**	**10**	**5**	**2.5**	**1.25**	**0.63**	**0.31**	**0.16**	**0.08**	**0.04**
Artemether-lumefantrine 80/480 mg tablet	Agar dilution	S	S	S	S	S	S	S	R	R	R	R	R	R
	Broth macrodilution	S	S	S	S	S	S	S	R	R	R	R	R	R
Artemether-lumefantrine 40/240 mg tablet	Agar dilution	S	S	R	R	R	R	R	R	R	R	R	R	R
	Broth macrodilution	S	R	R	R	R	R	R	R	R	R	R	R	R
Artemether-lumefantrine 20/120 mg tablet	Agar dilution	S	S	R	R	R	R	R	R	R	R	R	R	R
	Broth macrodilution	S	R	R	R	R	R	R	R	R	R	R	R	R

R = Incomplete growth inhibition / No growth inhibition, S = Complete growth inhibition.

3.2. Broth Macrodilution Method

Seven (53.8%) out of the 13 concentrations of artemether-lumefantrine 80/480mg tablet completely inhibited growth of *S. aureus* while 6 (46.2%) did not inhibit *S. aureus* growth completely (Table **3**). The proportion of artemether-lumefantrine 40/240mg tablet concentrations that completely inhibited *S. aureus* growth was 7.7%. Only one of the artemether-lumefantrine 20/120mg tablet concentrations inhibited growth of the test strain completely. Artemether-lumefantrine 40/240mg and 20/120mg tablets had the same MIC value of 160 mg/mL. Artemether-lumefantrine 80/480mg tablet was the most active inhibitor with an MIC of 2.5mg/mL (Table **3**).

4. DISCUSSION

In this study, all the three artemether-lumefantrine tablet samples exhibited antimicrobial activity against *S. aureus*. This is an indication of the presence of a common anti-staphylococcal agent in all three tablet samples. The ability of the artemether-lumefantrine tablets to inhibit *S. aureus* growth support the findings of some studies which reported that artemisinin derivatives have the potential to inhibit growth of *S. aureus* (Tajehmiri *et al*., 2014, Appalasamy *et al*, 2014). The antimicrobial activity of the tablets may also be due to the lumefantrine components or the excipients or synergistic effect of some or all the compounds in the formulation hence the need for further studies in order to pinpoint the *S. aureus* inhibitor(s) in artemether-lumefantrine tablet.

Artemether-lumefantrine 80/480mg tablets, having the highest strength, recorded the least MIC (2.5 mg/mL), making it the most efficacious among the three tablets studied in the *in vitro* inhibitions of *S. aureus*. The MIC, the degree of inhibition and trend of activity of artemether-lumefantrine 80/480mg tablet against the *S. aureus* strain did not differ in both agar dilution and broth macrodilution methods. For artemether-lumefantrine 40/240mg and 20/120mg tablets, MICs were higher using the broth macrodilution method when compared to the agar dilution method. This

finding is comparable to the one reported by Benning and Mathers in a similar study on veterinary antibiotics against *Clostridium perfringens* strains originating from porcine and avian sources [22].

Although artemether-lumefantrine 40/240mg tablet has twice the strength of artemether-lumefantrine 20/120mg tablet, interestingly, both tablets had same MIC value in the agar dilution method (80 mg/mL) and in the broth macrodilution method (160 mg/mL). This could be due to an intrinsic property of artemether-lumefantrine tablets that makes tablets with strengths of 40/240mg and below express antimicrobial activity that does not depend on tablet strength but rather on the concentration of the tablet suspension. This observation might also be as a result of the differences in the ages of the tablets. The artemether-lumefantrine 40/240mg tablet (16 months) were relatively older than the 20/120mg tablets (8 months). Since the Active Pharmaceutical Ingredients (APIs) of the drug combination has the ability to degrade over time [23], the artemether-lumefantrine 40/240mg tablets might have lost some API through aging and dropped to bactericidal threshold concentrations equivalent to that of the artemether-lumefantrine 20/120mg tablets. Further studies are necessary in order to understand this phenomenon fully.

This study presents the first report on anti-staphylococcal activities of artemether-lumefantrine tablets and has shown that the tablets exhibit fairly good antimicrobial activity against *Staphylococcus aureus subsp*. *aureus* (ATCC® 6538™) *in vitro*.

CONCLUSION

This study has shown that artemether-lumefantrine, an antimalarial drug, has anti-staphylococcal properties and inhibits *S. aureus in vitro*. Artemether-lumefantrine 80/480 mg tablet, which exhibited the highest inhibitory effects against the *S. aureus* strain, can be studied further and considered as an alternative in the treatment of staphylococcus infections. Further studies are invited to ascertain the molecular and genetic bases of this inhibition and to explain the mechanism of action of artemether-lumefantrine in inhibiting *Staphylococcus aureus*.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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