South Asian Journal of Research in Microbiology

Volume 16, Issue 4, Page 1-13, 2023; Article no.SAJRM.106442
ISSN: 2582-1989

Antimicrobial Resistance Profile of Airborne *Aspergillus flavus* and Methicillin-Resistant *Staphylococcus aureus* in Public Toilets within Port Harcourt, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/SAJRM/2023/v16i4313

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/106442

Original Research Article

Received: 16/07/2023
Accepted: 20/09/2023
Published: 25/09/2023

ABSTRACT

MRSA is a major contributor to skin infections, bloodstream infections, toxic shock syndrome, and joint inflammation. *Aspergillus flavus* can result in a range of health issues these problems include allergic reactions, aspergilloma, both invasive and non-invasive. infections are a matter of importance for public health. *Aspergillus flavus* and Methicillin-resistant *Staphylococcus aureus* (MRSA) resistance to both antibiotics and antifungal medications put the public at risk.This study therefore was carried out to investigate the antimicrobial susceptibility pattern of *Aspergillus flavus* and Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from publicly used toilets in Port Harcourt, Rivers State Nigeria. Eighty (80) samples were collected for a period of two months from five public toilets using the sedimentation air sampling method. Samples were subjected to isolation

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and identification, antibiotics and antifungal susceptibility test using Kirby-Bauer disk diffusion method, plant extracts and molecular identification of bacterial isolates for analysis. The susceptibility profile showed that *MRSA* were resistant to Ofloxacin (61%), Ceftazidime (92.30%), Levoflaxcin (92.30%), Vancomycin (77 %), Gentamycin (61%), Azithromycin (46.2%) and cefotaxime (46.2%) and susceptible to Imipenem (100%), Meropenem (92.32%). Ketoconazole and nystatin both antifungals were both effective on the *A. flavus*. Methanol extract of *Ocimum grastissimum* was more effective followed by *Psidium guajava* and *moringa oleifera* on MRSA but also less effective in *A. flavus.* The MAR index ranged from 0.1 to 0.8 which showed that 60% of *MRSA* isolates had MAR index of 0.8, while 20% had MAR index of 0.4 and 0.5. The antimicrobial activity of the extracts is promising as the extracts could be used as a cheap antimicrobial for the treatment of infections cause by these test organisms. Conclusively, this study revealed both organisms present in toilet air, their vulnerability patterns was established, their resistance gene verified, and explore the potential use of natural plant compounds on them, would assist in mitigating public health.

Keywords: MRSA; Aspergillus flavus; susceptibility pattern; plant extract; public health; multi-drug resistance; resistance.

1. INTRODUCTION

1.1 *Aspergillus Flavus*

Aspergilli has continuously existed in the human environment. Micheli was the first person to identify the separate parts of fungi, such as the stalks and spore heads. Etymologia et al. [1]. However, it took until the mid-19th century for people to realize that these fungi were actually responsible for causing decay, diseases in humans and animals, and for producing useful metabolic products through fermentation. [2]. *A. flavus* is the second in rank, following only *A. fumigatus* is responsible for human invasive aspergillosis. Furthermore, *Aspergillus flavus* is the predominant fungal species contaminating foodstuffs and feeds and producing Afs worldwide. It is also the main contaminant during the food storage since its ability to produce Afs and its potential to persist as a pathogen and saprophyte in the food supply before and after harvest Lahouar et al. [3].

A. flavus fungus is responsible for a wide range of illnesses in people, ranging from allergic reactions to invasive infections that affect blood vessels Rudramurthy et al. [4]. After *A. fumigatus*, *A. flavus* can be the second most common reason for both invasive and non-invasive aspergillosis Denning [5]; Morgan et al. [6]. The main way the infection spreads is when someone breathes in the spores of the fungus. The larger dimensions of *A. flavus* have a larger diameter of 25 mm, while *A. flavus* has a smaller diameter of 23 mm. Nyongesa et al. [7].

1.2 Methicillin-Resistance *Staphylococcus aureus*

MRSA, also known as Methicillin-resistant *Staphylococcus aureus*, is a highly thriving and effective pathogen in contemporary times. The organism, which can live peacefully with another organism and is passed on in both healthcare and community environments, is also a primary source of bacteraemia, endocarditis, infections of the skin and soft tissues, infections of bones and joints, and infections acquired in hospitals Cogen et al. [8]. Even though the occurrence of MRSA has decreased in certain areas, it still presents a serious risk in the medical field, leading to consistently high rates of illness and death. Successfully treating a medical condition continues to be difficult and necessitates assessing new ways to combat infections as well as supplementary components of care, including seeking guidance from infectious disease specialists, conducting echocardiography, and implementing measures to control the source of the infection. In situations where the protection of the skin is compromised or the immune system is weakened, *Staphylococcus aureus* changes its behaviour from being a harmless resident to becoming the primary cause of skin infections. Zavadinack et al. [9]. This resistance is caused by beta-lactamases known as penicillinases, which are enzymes that break down the beta-lactam ring of the antibiotics found in the beta-lactam group, including penicillin, rendering them ineffective. Tavares [10]; Que, Moreillon et al. [11]. Swx2The first recorded instance of the Methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial strain in England occurred in 1961 when it was discovered in a patient who

was receiving medical care in a hospital. Ternover et al. [12] Shortly after, other European countries, as well as Japan, Australia, and the United States, began reporting similar cases, leading to the spread of this microorganism and making it the primary cause of infections acquired in hospitals. (Boyce, *et al*., 2005). MRSA, have developed resistance to all beta-lactams, except for cephalosporins that have anti-MRSA properties, as well as combinations of beta-lactamase inhibitors. Mimica et al. [13].

The understanding of MRSA spread between animals and humans has been significantly influenced by the One Health approach. (Casey, *et al*. 2014). ST398 (CC398) has been extensively documented as a source of livestock-related community-acquired methicillin-resistant *Staphylococcus aureus* in Europe starting from 2005 Witte et al. [14]. ST398 has been identified as a source of livestock-connected MRSA in Asia, Australia, and the Americas. However, it is important to note that there are other strains present in livestock as well. Smith et al. [15]. Interspecies transmission of MRSA may create added evolutionary limitations, as certain genetic markers associated with evading the immune system, like *scn, chp,* and *sak,* seem to show divergent selection. These genetic markers are positively linked to human infection but negatively linked to colonization in livestock [16].

2. MATERIALS AND METHODS

2.1 Study Area

The study area was Port Harcourt Metropolis, Rivers State, Nigeria. Five major locations were chosen from the study area; Mile 3 Market, Nkpolu-Oroworukw Port Harcourt (4.8042° N, 6.9924° E) Rumuokoro, Motor Park (4.8670° N, 6.9944° E), Mile 1 Market Rumuwoji (4.7918° N, 6.9986° E) Model Girls Secondary, School Rumueme (4.83835,6.99588° E) and Rivers State University Nkpolu-Oroworukw (4.8522622, 6.9896428° E). These five (5) locations were selected because the high rate of human activities within the locations leading to spread of infections.

2.2 Toilet Air Sample Collection

The method of sampling used in this investigation was the direct sedimentation method of aerosol sampling which involves the aseptic exposure of growth media including Sabouraud' dextrose agar and Mannitol salt agar both produced by (Titan Biotech Limited, Bhiwadi, india) to the environment air. The agar plates used for the investigation were prepared in duplicates in the laboratory and transported to the point of sample collection aseptically. The agar plates containing adequate amounts of SDA was supplemented with Ampicillin and tetracycline and MSA were exposed in each of the toilet at various sampling stations and labelled adequately. Exposure period for MSA plates was for 10 Minutes and SDA plates for 1hour and users of the toilets were prevented for a period of 4 hours to prevent inaccurate sample collection as a result of flushing and agitating the toilet water thereby emitting more aerosols. Jung et al. [17]; Ohagim et al. [18].

2.3 Microbiological Analysis

2.3.1 Preparation of plant sample for extraction

This approach was carried out with accordance to Iheukwumere et al. [19]., the plants of *Psidii guajava*, *Morinda citrifolia* and *Ocimum gratissimum* was left to dry for a period of five days at normal room temperature before being transformed into a powdered state by crushing them. Afterward, 200g of the powder was put into their respective 500ml Erlenmeyer flasks. These flasks already had 200ml of both methanol (>95%) and sterile water. For a duration of three days, every sample was passed through Whatman No 1 filter paper into a 500ml vessel. Subsequently, the container was subjected to drying at a temperature of 400^0C using an oven drier. After the extracts were dried, 20ml of a solution containing DMSO was mixed with the extracts and placed in the refrigerator for future use. The size of the inoculum was determined to be one milliliter of suspended broth solution ($1.0x10^8$ml) of each resistant bacterial culture, which was then inoculated into Muller Hinton broth (MHB) and incubated for 24 hours at 37°C. To measure concentrations the agents, the culture was added to a set of test tubes containing, respectively, 100mg/ml, 50mg/ml, 25g/ml, and 12.5g/ml of plant extracts. The test tubes were then incubated at 37ºC for 24 hours. Colonies were chosen and inoculated into freshly made Muller Hinton agar plates after 0.1-millimeter (0.1ml) aliquots from each test tube were put into nutrient agar plate and incubated. Iheukwumere et al. [19]; Yogini et al. [20].

2.3.2 Characterisation and identification of bacterial and fungi isolates

Discrete colonies were picked based on their cultural, morphology, macroscopic and microscopic examinations and biochemical tests. The clumping factor, also known as bound coagulase, was examined using the coagulase test to see if it was present and bonded to the bacterial cell walls to differentiates strains of *Staphylococcus aureus* from *S. epidermidis* and other coagulase-negative species. The isolate was subculture on solid NA and SDA and subsequently on slants of the respective agar media and preserved at refrigeration temperature. Identification of the isolates as bacteria and fungi was carried out as described in Cheesbrough et al. [21].

2.3.3 Susceptibility assay of the bacteria isolates

Antimicrobial sensitivity test was performed using Kirby-Bauer method to measure the ability of an antibiotic to inhibit bacteria growth in vitro by disc diffusion [22]. The pure cultures of bacteria isolated were aseptically inoculated into 5ml of sterile peptone water and incubated at 37^0C for 18-24hours. A turbid suspension of the isolate was made in distilled water using 0.5 McFarland Standard prepared as a comparator. A sterile swab was dipped into the bacteria suspension, pressed on the side of the test tubes to allow excess drip off and then evenly smeared on the entire surface of the Mueller Hinton agar. The antimicrobial agents used were Cefotaxime (30µg), Gentamicin (10µg), Oxacillin (1µg), Azithromycin (15µg), Imipenem (10µg), Ofloxacin (5µg), Cefuroxime (30µg), Vancomycin (30µg), Ceftazidime (10µg), Meropenem (10µg), Levofloxacin (5µg), Cefoxitin (30µg). Sterile forceps was used to position the commercial single antibiotic discs (Mast Group Ltd. Mast House Bootle, Merseyside, U.K). The plates were then incubated for 24 hours at 33^0C to 35^0C for and zone of inhibition were measured and interpreted as susceptible, intermediate or resistance in accordance with [22]. 250ml of Ketoconazole of the imidazole medication and Nystatin of the polyenes medication both antifungal drugs were used.

2.3.4 Antimicrobial susceptibility of extracts

The susceptibility of the extract to fight against bacteria and fungal was tested using the well method. The agar method, as explained by (Amadi *et al.,* 2016) involves a specific technique. In this technique, the bacterial and fungal isolates that were standardized using the McFarland standard were applied to labelled Mueller-Hinton agar plates with a 6mm swab and left to air dry. Then, using a sterile cork borer, wells with a diameter of 6mm were created on the agar plates at even intervals in the medium. These wells were labelled according to their concentrations. The concentrations of the extract were transferred into the wells in a sterile manner and then left to incubate. The diameter of the zone was determined by measuring the observed zones according to CLSI guidelines (2017). The tests were conducted twice.

2.3.5 Determination of Multiple Antibiotic Resistance (MAR) index

Multiple antibiotic resistance is the resistance of MRSA to three or more antibiotics. Multiple antibiotic resistance (MAR) index was determined for each of the MRSA isolate by using the formula MAR=a/b, where “a” represent the number of antibiotics to which the test isolate depicted resistance and “b” represent the total number of antibiotics to which the test isolate has been evaluated for susceptibility.

3. RESULTS

A total 16 isolates were obtained comprising of both *S. aureus* and *Aspergillus flavus* from the 5 locations with 8 isolates identified as methicillin susceptible *Staphylococcus aureus* (MSSA), 5 isolates as methicillin resistant *Staphylococcus aureus* (MRSA) after the use to Methicillin makers which are Oxacillin and Cefoxitin, while 3 isolates were identified as *Aspergillus flavus*.

Antibacterial susceptibility pattern of the bacterial and MAR index are presented in Table 1 and 2. The Analysis of the *S.aureus* susceptibility pattern, the majority of *S.aureus* isolates were sensitive to Imipenem (100%), followed by Meropenem (90.32%), Oxacillin (53.8%) and Cefoxitin (38.5%). Ofloxacin (100%), Ceftazidime (92.30%), Levoflaxacin (92.30%), Vancomycin (77%), Gentamycin (61.5%), Azithromycin (46.2%), Cefotaxime (46.2%) and were the drugs with the resistance. Therefore, in this study, it was found that 60.0% of the MRSA isolates had a MAR score above 0.8, as indicated by the Multiple Antibiotic resistance (MAR) index. 60% of the MRSA isolates examined in this study had a MAR index higher than 0.2, as indicated by the Multiple Antibiotic Resistance index. It is

important to note that areas where antibiotics are frequently used and sources of contamination typically have MAR index values greater than 0.2 Davis et al. [23]; Krumperman [24].

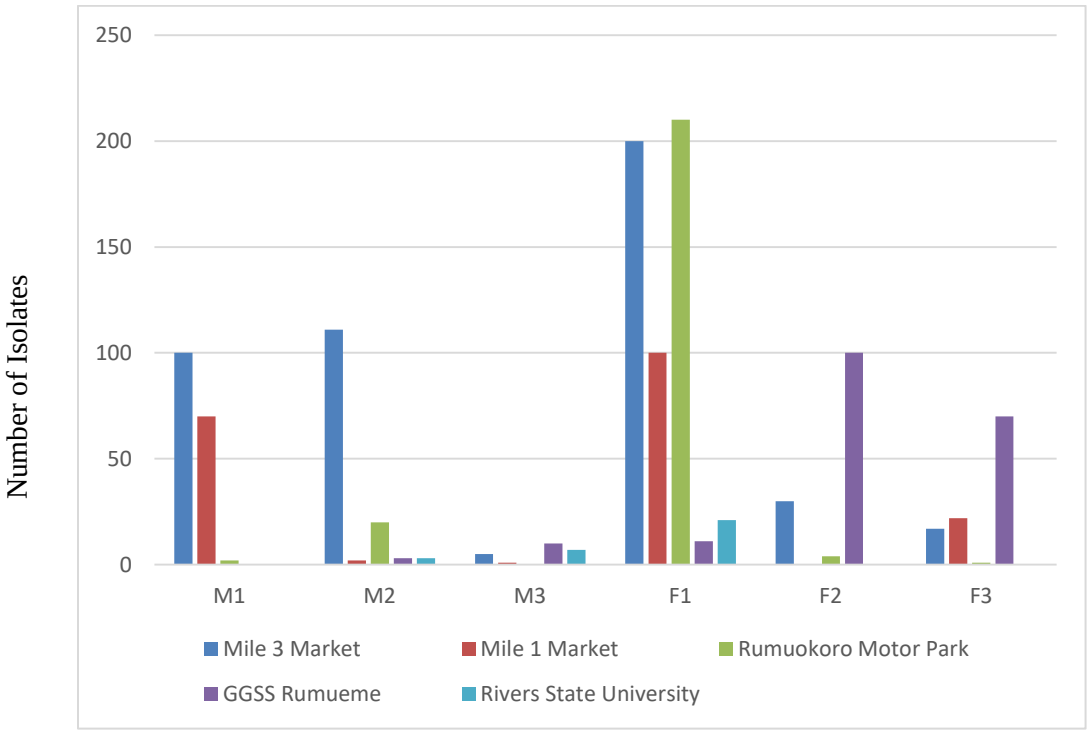

Fig. 1. Location distributions of *S. aureus*

Key: M1 = Male Toilet 1; M2 = Male Toilet 2; M3 = Male Toilet 3; F1 = Female Toilet 1;F2 = Female Toilet 2; F3 = Female Toilet 3

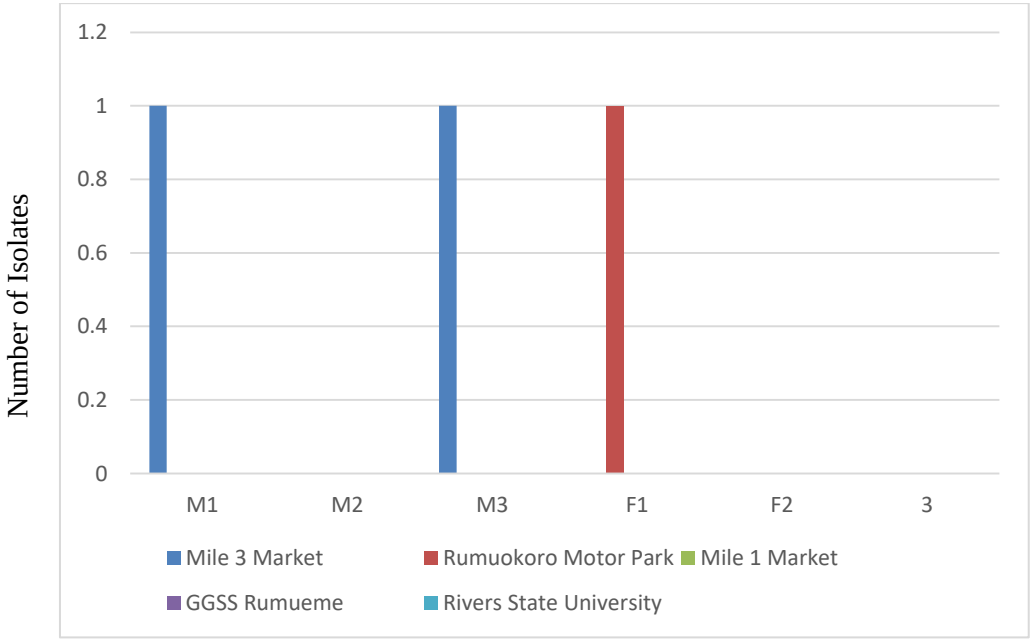

Fig. 2. Location distributions of *Aspergillus flavus*

Key: M1 = Male Toilet 1; M2 = Male Toilet 2; M3 = Male Toilet 3; F1 = Female Toilet 1; F2 = Female Toilet 2; F3 = Female Toilet 3

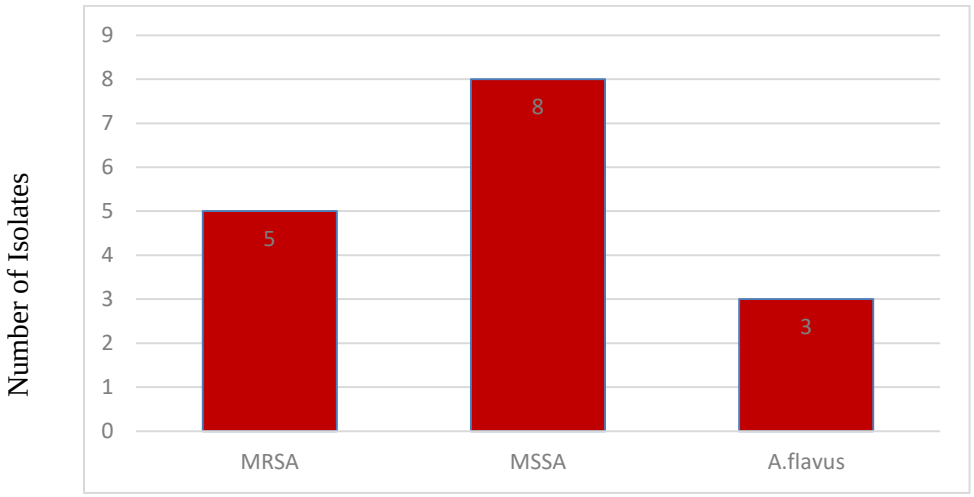

Fig. 3. Susceptibility pattern from different locations

Plate 1. Identified *Aspergillus flavus*

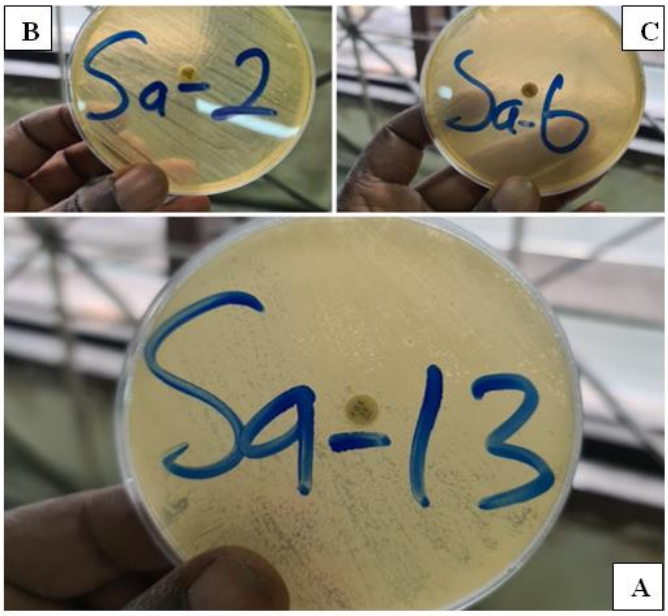

Plate 2. Confirmed *MRSA* after using the second maker (Cefoxitin)

Table 1. Susceptibility profile of *MRSA* to 12 antibiotics

Antibiotic Class/Conc. (µg)	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
Cephalosporin			
Cefuroxime (30) CXM	8(61.5)	5(38.5)	0(0.00)
Cefotaxime (30) CTX	6(46.2)	7(53.8)	0(0.00)
Ceftazidime (10) CAZ	12(92.30)	0(0.00)	1(7.7)
Aminoglycoside			
Gentamicin (10) GEN	8(61.5)	0(0.00)	5(38.5)
Macrolides			
Azithromycin. (10) AZM	6(46.2)	2(15.4)	5(38.4)
Carbopenem			
Imipenem (10) IMI	0(0.00)	0(0.00)	13(100)
Meropenem (10) MEM	0(0.00)	2(15.4)	11(84.6)
Fluoroquinolones			
Ofloxacin (5) OX	13(100)	0(0.00)	0(0.00)
Levofloxacin (5) LEV	12(92.30)	0(0.00)	1(7.7)
Glycopeptide			
Vancomycin (30) VAN	10(77)	0(0.00)	3(23)
Cephamycin			
Cefoxitin (30) OX	5(38.5)	0(0.00)	8(61.5)
Penicillin			
Oxacillin (10) FOX	6(46.2)	0(0.00)	7(53.8)

Table 2. MAR indices of methicillin-resistance S*taphylococcus aureus* (*MRSA*) isolated during the study

MAR Index	*MRSA* N=5
0.1	0(0.00)
0.2	0(0.00)
0.3	0(0.00)
0.4	1(20.0)
0.5	1(20.0)
0.6	0(0.00)
0.7	0(0.00)
0.8	3(60.0)

Key: Multiple Antibiotic Resistance (MAR)

4. DISCUSSION

The findings of the antibiotic sensitivity patterns in this study revealed that MRSA bacteria were sensitive to Imipenem (100%), Meropenem (90.32%), and Oxacillin (53.8%). Which corroborates previous report of other investigation sensitivity of these drugs to MRSA Fan et al. [25]; Kayser et al. [26]. The susceptibility pattern of MRSA showing resistance with Vancomycin at resistance rate of 77% due to its ability to hampers the peptidoglycan layer of bacterial cell walls but also hinders bacteria from effectively carrying out cell growth and division which aligns with Schultsz et al. in [27]. Gentamicin had a resistance rate of 61.5% with its ability to a permanent binding to the 30S ribosomal subunits, disrupting the creation of messenger RNA. R.R Culter et al. [28]. The organisms in this study have a strong resistance to the beta-lactam antibiotics (Cefuroxime 61.50%, Cefotaxime 46.2% and Ceftazidime 92.30%.) due to the widespread and unregulated use of these antibiotics, their low cost, and the acquisition of the blaCTX, blaSHV, and blaTEM genes. Nevertheless, the resistance to beta-lactam medications aligns with the findings of Bedasa et al. [29]. Ofloxacin had resistance rate of 100% and Levofloxacin with a resistance rate of 92.30%. were generally in agreement with report by other investigators Christof et al. [30]. Azithromycin at 46.2% that

interferes with the production of α-hemolysin and biofilm formation in *Staphylococcus aureus* Gui et al. [31].

The levels of the *Psidium guajava* extracts that were examined against *Staphylococcus aureus* resulted in different sizes of zones of inhibition around the discs soaked in the extracts. These ranges varied from 2.00 mm to 18.00 mm, showing that all the bacteria were responsive to at least one of the extracts. The variation in inhibition halo sizes ranged from 4mm to 17mm. The methanol extract was more effective at stopping bacterial growth compared to the aqueous solvents. The extracts and essential oil from the *Psidium guajava* plant are highly potent against MRSA, suggesting they could be valuable sources of new antimicrobial substances. This aligns with the findings reported by Gonçalves et al. [32]. The impact of guava leaf extracts was more significant on MRSA. The findings of the study indicated that *Moringa oleifera* leaf extract obtained with methanol demonstrated a wide range of effectiveness against various bacterial strains Maurya et al. [33]. The largest area of inhibition observed at a concentration of 100mg/ml was 21mm, but for the MRSA, the smallest area at a concentration of 12.5mg/ml was only 2.0mm. Comparatively, the largest zone of inhibition at a concentration of 100mg/ml was 15mm for the *A. flavus*, and the smallest zone at a concentration of 12.5mg/ml was 3.0mm. The methanol extract of flavus continues to significantly inhibit further growth. *Moringa oleifera* contains a range of beneficial plant compounds such as alkaloids, flavonoids, glycosides, saponins, and tannins. The current research study provided clear evidence of *Moringa oleifera's* ability to inhibit the growth of *A. flavus*. The study found that the methanol extracts of *Ocimum gratissimum* showed strong inhibitory effects on *Staphylococcus aureus* and *A. flavus* This suggests that *Ocimum gratissimum* has antimicrobial properties. The methanol extract showed greater inhibition in comparison to the aqueous extract. This can be determined by the fact that methanol has the capability to extract a larger amount of crucial oils and secondary compounds from plants, which are thought to have antibacterial properties on the test organisms. These compounds have demonstrated strong effectiveness in inhibiting the growth of both gram positive and gram-negative bacteria, as well as fungi. This aligns with the findings of the study conducted by Hamma et al. [34].

Ketaconazole is a type of imidazole medication that hinders the conversion of blastospores into the invasive mycelial form. This hindrance likely assists the function of immune cells and could be the main reason for eliminating the infection. Imidazoles are part of a category of antifungal drugs known as azole antifungals, which also include medications such as ketoconazole, miconazole, and clotrimazole. The triazoles, which consist of fluconazole, itraconazole, and voriconazole, are another group of azoles. Nystatin belongs to a group of antifungal drugs known as polyenes. Its function is to prevent the development of fungi that lead to infection.

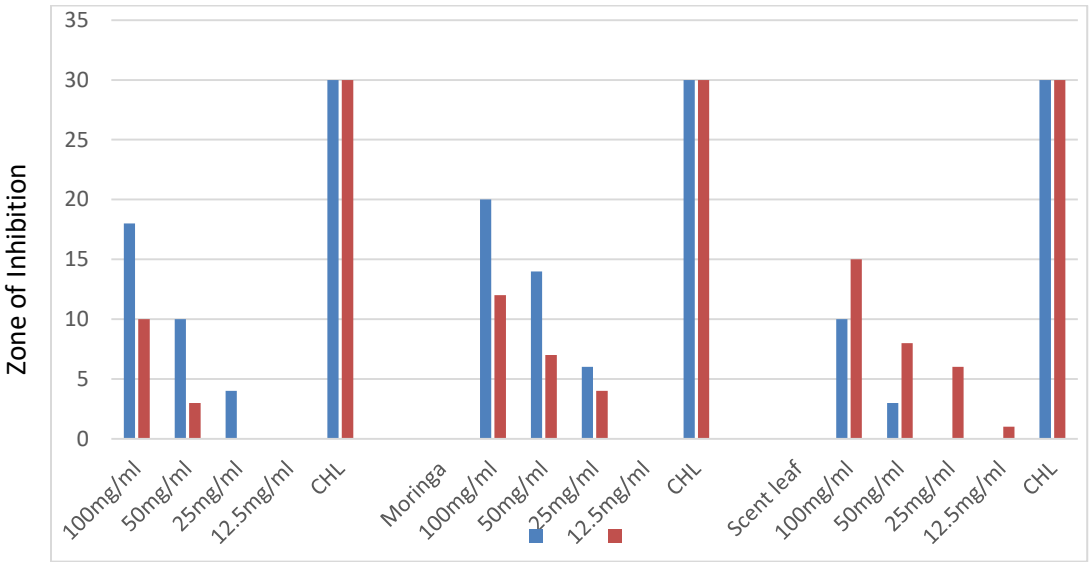

Fig. 4. Aqueous + plant extract for *MRSA*

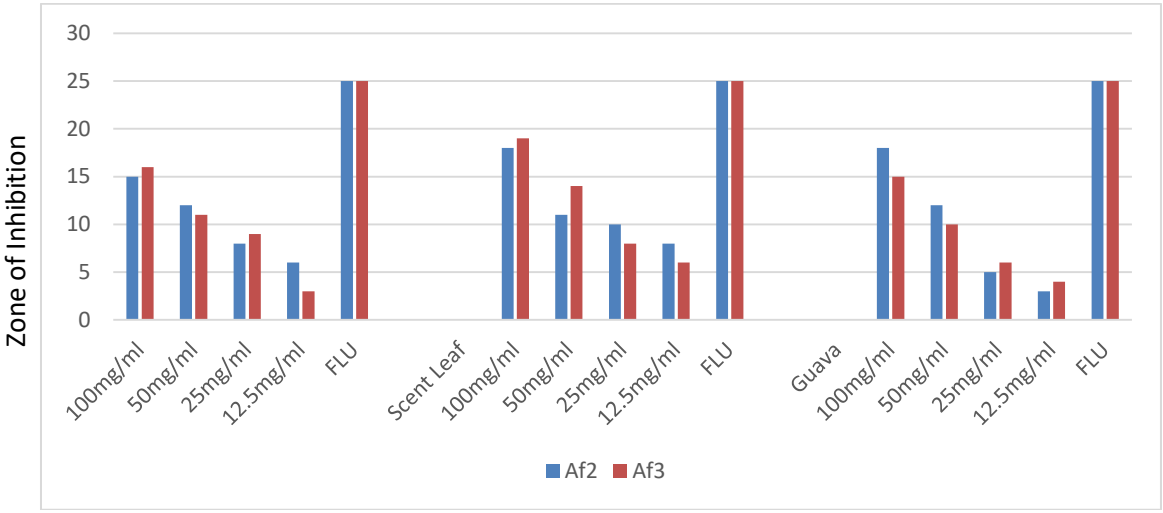

Fig 5. Aqueous + plant extract for *A. flavus*

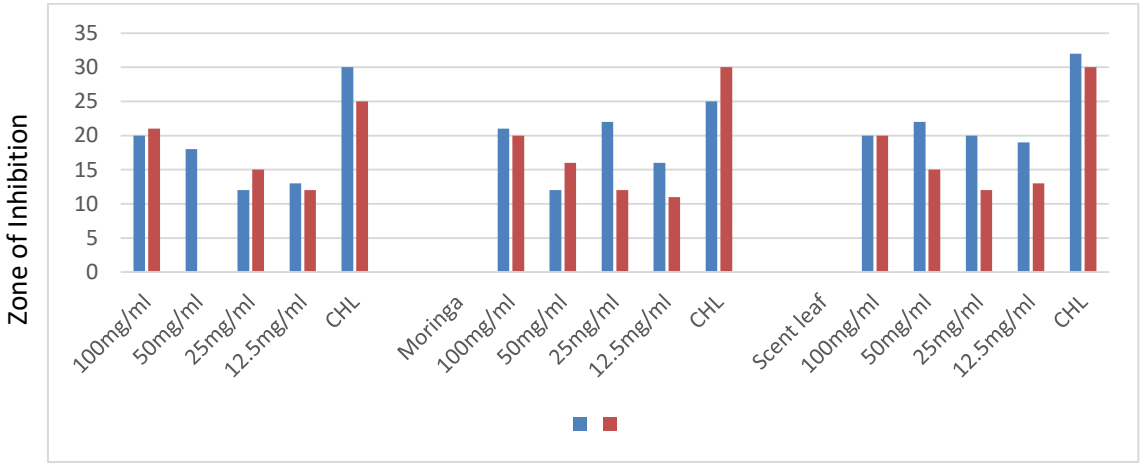

Fig. 6. Methanol + Plant extract for *MRSA*

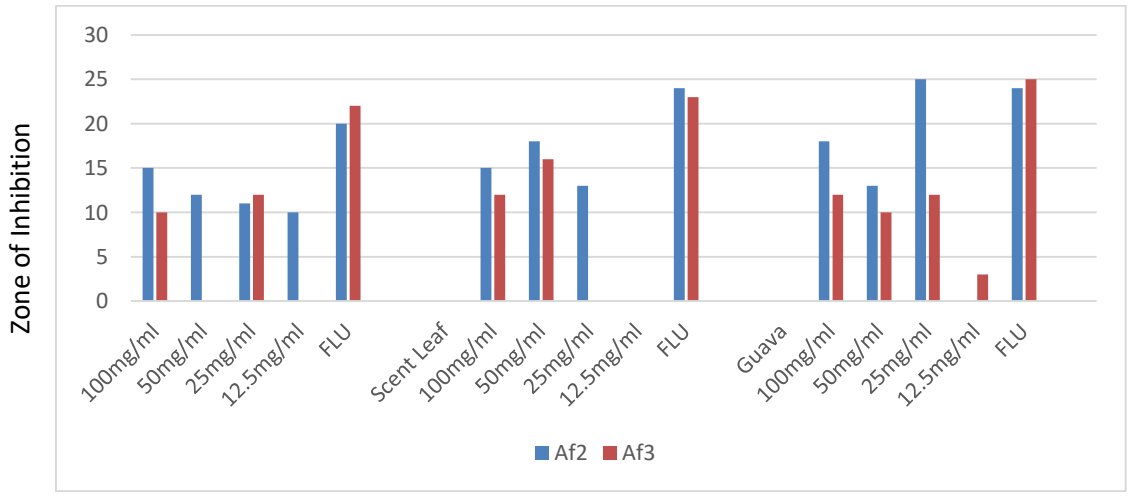

Fig. 7. Methanol + Plant extract for *A. flavus*

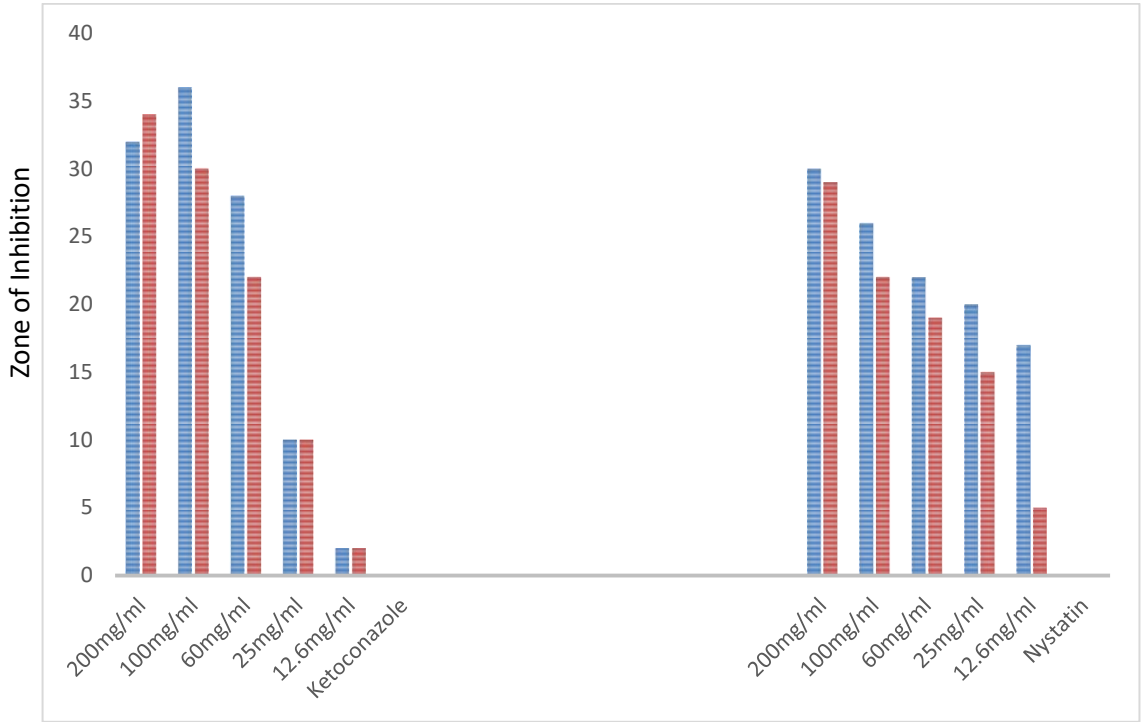

Fig. 8. Susceptibility pattern of antifungal drugs

A. flavus 3:

A. flavus 2:

Nystatin exhibited a wider range of inhibitory zone diameters, ranging from 29mm to 5mm, while ketoconazole displayed variability between 35mm and 8mm. Ketoconazole has been found to have a beneficial impact on reducing well-known *A. flavus* means yellow. This study is similar to Oji et al. [35] research, it was found that Ketoconazole has the ability to prevent and treat keratomycosis in rabbits. The antifungal effect of Nystatin is successful in combating fungus type *A. Flavus* is typically slower than many other types of *Aspergillus*. This aligns with a study conducted by Valerie et al. [36-38].

5. CONCLUSION

The result showed that *Staphylococcus aureus* (MRSA) was the highest prevalent organism. This report revealed that *Ocimum gratissimum* has more antimicrobial effect against both MRSA and *Aspergillus flavus*, compared to other plant extract. It also showed that MRSA isolates are resistant to Ofloxacin, Ceftazidime, Levoflaxacin, Vancomycin, Gentamicin, Azithromycin, Cefotaxime. The drugs that had no effect on the isolates may have no therapeutic value for infections caused by these isolates. This can pose a serious public health problem as the mentioned bacterial species can cause life-threatening infections.

The MRSA bacteria were vulnerable to Imipenem, Meropenem and had limited susceptibility to Oxacillin. This suggests that these medications could potentially be useful in treatment, as the organisms that were tested have no ability to resist them. As a result, they have the potential to be utilized in the treatment of infections that are caused by the bacterial species previously referenced. Ketoconazole and nystatin both showed influences on the *A. flavus* Ketoconazole is capable of preventing and treating certain conditions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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