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Antibacterial effects of the leaf ethyl acetate extract of *Diospyros mespiliformis* on methicillin resistant *Staphylococcus aureus* (MRSA)

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Abstract

Antimicrobial resistance (AMR) is currently one of the major public health issues in the world with Methicillin resistant *Staphylococcus aureus* (MRSA) being categorized among the highest in terms of global resistance by World Health Organization (WHO). It is one of the world's leading causes of bacterial infections with diseases caused ranging from mild skin infections to severe life-threatening septicemia. Hence the need to search for, novel therapeutic agents that may be of plant origin and effective against MRSA. These new approaches should, possibly be less expensive and more effective with minimal side effects. In the present study, antibacterial activity of leaf ethyl acetate extracts of *Diospyros mespiliformis* was evaluated against *mecA* positive isolates of MRSA strains obtained from backyard poultry. Well agar diffusion test was performed on the *mecA* positive isolates using leaf ethyl acetate extract of *D. mespiliformis* at 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL using 100% dimethyl sulfoxide as negative control.50 mg/mL had the highest activity of 22.4±0.90 mm followed by 25 mg/mL with 21.2±0.84 mm, 12.5 mg/mL with 19.6±1.14, 6.25 mg/mL with 13.2±4.87 while 100% DMSO had no antibacterial effect. Comparison between the different concentrations showed significant difference ($p < 0.05$) in all except in 50 mg/mL versus 25 mg/mL, 50 mg/mL versus 12.5 mg/mL and 25 mg/mL versus12.5 mg/mL. It was therefore concluded that leaf ethyl acetate extract of *D. mespiliformis* had antibacterial concentration dependent effect on MRSA.

Keywords: *Diospyros mespiliformis;* Methicillin resistant *staphylococcus aureus;* Antibacterial resistance; Multidrug resistance; Leaf extract

1 Introduction

Antimicrobial resistance (AMR) is currently one of the major public health issues in the world. It is a condition where microbial pathogens transform overtime, becoming no longer responsive to drugs and doses they were previously sensitive to [1]. This leads to difficulty in treating infection and increases the risk of disease spread, and even death. AMR is a global problem and till date, no known technique has been perfected to reverse the situation in microbial cells [2]. The invention of synthetic antibacterials and the discovery of antibiotics in the early 20th century revolutionized clinical therapy against bacteria [3]. The discovery of the ability of *Streptomyces* spp to produce antibiotics led to the golden age of antibiotic discovery and development. Different forms of these drugs were developed for commercial use [4]. Unfortunately, control over the use of these drugs was non-existent, and this contributed to the development and spread of antibiotic resistance by bacteria [1]. As it is now, our defense against bacteria is waning at an alarming rate as resistance to last line of defense antibiotics is gradually developing [5] and when complete resistance develops, we will return to the pre antibacterial and antibiotic era.

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Staphylococcus aureus was recently categorized among the highest in terms of global resistance by WHO [6]. It is one of the world's leading causes of bacterial infections. Diseases caused range from mild skin infections to severe life threatening septicemia [7]. There are records of humans been infected with animal strain of *S. aureus* and vice versa [8]. The practice of using antibiotics in livestock feed to promote growth has contributed a lot to the development of resistant genes by these microbes through selective pressure [9].

Few years after the introduction of penicillin into clinical use, resistance was developed by *S. aureus* to it. This was achieved by the production of a penicillinase enzyme that inactivates the penicillin. Shortly afterwards, methicillin was introduced in the 1940s and the organism countered it by the production of a gene later known as the *mecA* gene [10]. The gene encodes for the production of penicillin-binding protein 2a (PBP2a) which has low affinity for β-lactams. The strains of *S. aureus* with this feature are known as methicillin resistant *Staphylococcus aureus* (MRSA). In addition, resistance against other classes of antibiotics such as fluoroquinolone, aminoglycoside, tetracycline and vancomycin [11] were observed and these genes are acquired by the progeny either via horizontal or vertical transfer and by other bacteria via horizontal transfer [6]. The transfer of these genes acquired by the organism is aided by its ability to move between various hosts picking up required genes and discarding undesired ones and also transfer to its progeny by vertical transfer [2,4].

In search of new antimicrobial agents to help solve the problem of AMR, there is an increase in search of novel therapeutic agents that may be of plant origin, effective against antibiotic resistant bacteria. These new approaches should, possibly, be less expensive, more effective with minimal side effects as most of the current antibiotics/antibacterials are associated with one side effect or the other. However, some of the medicinal plants already established to have antibacterial properties are *Lawsonia inermis* [2], *Anthocleista djalonensis* [12] and *D. mespiliformis* [13]. It is therefore not out of place to study the antibacterial effects of any of them, in this case *D. mespiliformis,* for antibacterial effect against any multidrug resistant bacteria and this is what this research set out for against MRSA.

Several studies have been undertaken with regards to the plant's phytochemistry and a number of secondary metabolites have been isolated. Though variations exist in the composition of the compounds from one research to another, they include; saponins, tannins, alkaloids, flavonoids, steroids, terpenoides, glycosides, phlobatannins, anthraquinones, and carbohydrates [13–15]. Composition and concentration of these compounds are dependent on several factors such as geographical location, the plant part, stage of development of the plant part, age of the plant and nutrients, solvent and extraction method [16]. The leaf extracts of *D. mespiliformis* has been proven to possess antimicrobial activity against clinical isolates of *Salmonella typhi, Escherichia coli, Staphylococcus aureus, Streptococcus spp, Shigella sp* and *Klebsiella pneumonia* [17].

In this study, we demonstrated the multidrug resistance of methicillin resistant *S. aureus* against some antibiotics. Polymerase chain reaction was used in the detection of *mecA* gene. Although resistance was observed in 40% of the *S. aureus* isolates against antibiotics used for DAD, *mecA* gene expression was low. This led to the hypothesis of the presence of an intrinsic factor or *mecC* gene to be responsible for the resistance observed in the remaining ORSAB positive and *mecA* negative isolates. The leaf ethyl acetate extract of *D. mespiliformis* was found to have a dose dependent antibacterial effect on the MRSA isolates.

2 Materials and methods

2.1 Collection, identification and preparation of plant material

Diospyros mespiliformis leaves were plucked from the tree at Basawa Zaria, Kaduna State Nigeria. The leaves were identified at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria and voucher specimen number 938 was issued.

The leaves were washed with tap water and air dried. The dried leaves were broken into smaller pieces and pounded into powder using clean pestle and mortar. The powder was then dispensed into small plastic containers, labeled and stored at room temperature [18].

Two litres of ethyl acetate was poured into a container with 500g of powdered *Diospyros mespiliformis* leaf and mixed thoroughly. The mixture was filtered with Ocome® filter paper HO-103 (equivalent to whatmann size 2) with 8µm particle size range. The filtrate was poured into a container and kept open at room temperature under a stream of fast moving air to evaporate for 72 hours. Afterwards, the extract was scraped from the container and placed in plastic containers and labeled. The percentage yield for the leaf extract was calculated using the formula below.

$$\% \text{ Yield} = \frac{\text{weight of extract}}{\text{weight of puverized material}} \times 100$$

2.2 Test microorganism

One hundred samples were obtained by taking peri-cloacal swab from broiler chickens of backyard poultry. The swab samples were added to nutrient broth and later inoculated on nutrient agar. The primary cultures were sub-cultured on mannitol salt agar and identified by microscopic morphological and biochemical characteristics which include slide catalase and tube coagulase tests [19]. Cultures were grown on nutrient agar slants for further tests and stored at 4°C until further use.

2.3 Multidrug resistance test

Oxacillin resistance screening agar base (ORSAB) test was performed on the isolates that were positive for *S. aureus* according to [20].

2.4 Disc agar diffusion test

Antimicrobial susceptibility test was performed against MRSA using an antimicrobial susceptibility disc that contained the following antibiotics; amoxicillin clavulanate, cefotaxime, imipenem/cilastatin, ofloxacin, gentamicin, ceftriaxone, levofloxacin, cefexime, cefuroxime. Inoculum standardization was performed with a spectrophotometer at the wavelength of 630 nm. Values within the range of 0.08 to 0.1 were considered 0.5 McFarland's standard [19].

2.5 DNA extraction

Qiagen DNA easy extraction Kit was used for the extraction of the DNA templates. Twenty isolates that showed the most multi-drug resistance from the previous antibacterial susceptibility test were used [21].

2.6 Polymerase chain reaction

2.6.1 Amplification of mecA gene

S. aureus strains were subjected to PCR screening for the *mecA* gene. A 25 μL reaction was prepared which contained 12.5 μL 2X top taq master mix, 1 μL each of *mecA* forward (F: 5'-GTAGAAATGACTGAACGTCCGATGA-3') and reverse (R: 5'-CCAATTCCACATTGTTTCGGTCTAA-3') primers (10mM), 2.5 μL coral load, 5 μL of DNA template, and 3 μL of nuclease free water. A PCR program was conducted with initial denaturation at 94°C for 3 minutes followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds ended with a final extension at 72°C for 10 minutes [22].

2.6.2 Agar rose gel electrophoresis

100mL of 1X Tris Acetate EDTA (TAE) was added to 1g of agar gel for preparation. The amplicons where loaded alongside 100bp ladder (DSB China) in a Bio-Rad Horizontal electrophoresis tank containing 0.5 X TAE. The gel electrophoresis was conducted at 70v for one hour. Afterwards, the gel was taken into the trans-illuminator (gel doc) and observed for bands corresponding with the 100bp ladder at the expected amplicon size of 533bp [19].

2.6.3 Well agar diffusion test

The extracts were measured for the different concentrations to be used. 100% Dimethyl sulfoxide (DMSO) was used to dissolve the extract for 24 hours in a test tube. The concentrations were; 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL.

Eight isolates (including the *mecA* positive isolates) with the best resistance pattern were chosen. One plate was used as a positive control. Five holes were bored using size 4 cock borer (8µm) each for a leaf extract concentration to be used and negative control (DMSO 100%). Standardization of inoculum was done and the plates were inoculated with the MRSA isolates. The different concentrations and 100% DMSO were dispensed into the bored holes and incubated at 37˚C for 24 hours. The plates were observed and zone of inhibition was measured [13].

2.7 Statistical analysis

The data of average zones of inhibition produced by the isolates against leaf ethyl acetate extract of *D. mespiliformis* used were presented as the mean ± standard deviation and analyzed using one-way analysis of variance (ANOVA) with Microsoft excel and GraphPad Prism9. Significance level of the differences was set at $p<0.05$.

3 Results and discussion

3.1 Extract yield

At the end of the extraction process, 135 g of the crude leaf ethyl acetate extract of the *Diospyros mespiliformis* was obtained making the extract yield 27% (135/500 ×100).

3.1.1 Occurrence rate of bacteria isolated from the cloaca of broiler chicken

Of the hundred samples subjected to culture, isolation and identification, 88% (88/100) were observed to be *S. aureus*, followed by non-*Staphylococcus* spp with 5% (5/100) and non S. *Aureus* with 7% (7/100) of the isolated bacteria as shown in Table 1.

Table 1 Occurrence rate of isolated bacteria from broiler chicken cloaca

S. aureus	**Other *Staphylococcus* spp**	**Non-*Staphylococcus* bacteria**
88%	5%	7%

3.1.2 Percentage distribution of methicillin susceptible Staphylococcus aureus (MSSA) to methicillin resistant Staphylococcus aureus (MRSA)

The *Staphylococcus aureus* positive isolates were subjected to screening with chromogenic media, oxacillin resistance screening agar base (ORSAB). In table 2 below it showed that 41% were MRSA while 59% MSSA.

Table 2 MRSA and MSSA count and percentage to the nearest whole number

Isolates	**Count (%)**
MRSA (n=88)	36 (41)
MSSA (n=88)	52 (59)

3.2 Disc Agar Diffusion test

Table 3 Drug susceptibility pattern of the isolates

Antimicrobial Agent	**Disc content**	**Interpretive Categories and Zone Diameter Breakpoints (%)**	
		S	R
Amoxicillin clavulanate	30 µg	6	94
Cefotaxime	25 µg	6	94
Imipenem/cilastatin	10/10 µg	0	100
Ofloxacin	5 µg	39	61
Gentamicin	10 µg	22	78
Ceftriaxone	45 µg	8	92
Levofloxacin	5 µg	64	36
Cefexime	5 µg	6	94
Cefuroxime	30 µg	0	100

S- Susceptibility; R- Resistance

On antimicrobial susceptibility test on the MRSA isolates to further confirm the resistance pattern, the isolates were observed to have various degrees of antibiotic drug resistance to antibiotics outside of the β-lactam class. Susceptibility and resistance are presented in percentages in table 3. Highest resistance was recorded in cefuroxime and imipenem/cilastatin with 100% resistance. Resistance observed outside of the β-lactams was against gentamicin, ofloxacin and levofloxacin with 78%, 61% and 36% respectively.

3.3 Polymerase chain reaction

Two samples out of the thirty six ORSAB positive isolates were positive for *mec*A gene as shown in plate 1. 100 bp molecular weight ladder was used (first lane from the right) with lanes 3, 4 and 8 showing bands slightly above the 500 bp indicating the presence of the *mecA* gene.

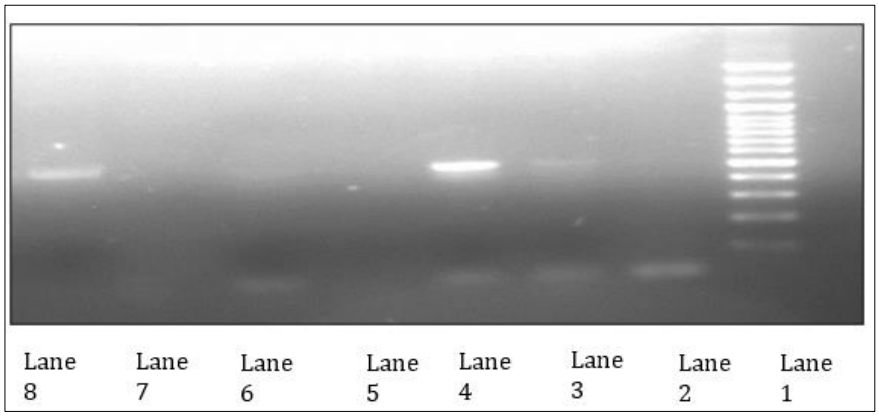

Figure 1 Amplicon of *mecA* gene; lane 1 (right): 100 bp molecular weight ladder, lane 4 positive control lanes 3 and 8 are test isolates with positively amplified *mecA* as indicated by 533 bp PCR amplicon; lanes 2,5,6 and 7 are *mecA* negative

3.4 Well agar diffusion test

The highest activity was seen in the 50 mg/mL concentration of the leaf extract followed by 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL, thus showing dose dependent activity.

Out of all the 8 isolates subjected to agar well assay of the leaf ethyl acetate extract of *Diospyros mespiliformis*, 6 were susceptible to the extract. However, resistance was observed in two of the isolates.

Table 4 Activity of leaf ethyl acetate extract of *D. mespiliformis* on MRSA

	50mg/ml(a)	**25mg/ml (b)**	**12.5mg/ml (c)**	**6.25mg/ml(d)**	**100% DMSO (e)**
MRSA Isolates	$22.4\pm0.90^{ad,\ ae}$	$21.2\pm0.84^{bd,\ be}$	$19.6\pm1.14^{cd,\ ce}$	13.2 ± 4.87^{cd}	8 ± 0^{de}

The results were expressed as mean ± SD. The significance of differences was determined by one-way analysis of variance using Graphpad Prism9. A $P < 0.05$ indicates that the difference is significant. $^{a\text{-}e}$Mean with superscript show significant difference.

4 Discussion

Plants have been used for therapeutic purposes from time immemorial. This is because they contain bioactive compounds known as secondary metabolites [19]. The leaves of the test plant have been used by locals for the therapy of pain, leprosy, malaria and wound [20]. Methanol, water and ethyl acetate leaf extracts have been shown to possess anti mycobacterial [12] and antibacterial effects [13]. These findings made the basis for this research, which was to evaluate the anti-bacterial effect of leaf ethyl acetate extract of *D. mespiliformis* on MRSA. This is in pursuit of alternatives to conventional antibiotics in the fight against antibiotic drug resistance. 41% of the test isolates were positive for MRSA. This is in agreement with [22]. It is to be noted that this particular MRSA strain is resistant to antibiotics outside of the β-lactams. Resistance was observed against all the antibiotics used to a certain degree. The highest resistance was observed in imipenem/cilastatin and cefuroxime with 100% resistance to the isolates. Resistance was observed to be more than 50% in all the antibiotics except levofloxacin with 36% resistance.

Detection of the *mec*A gene is considered the "gold standard" for the identification of MRSA [21]. The discovery of a *mecA* gene homologue in the UK in cow milk sourced from different herds began to solve the mystery of absence of mec*A* gene in MRSA isolates identified via routine oxacillin and cefoxitin tests. The homologue was later named *mecC* gene which is 70% identical to *mecA* gene at DNA level [7] and the gene product is a penicillin-binding protein with 63% identity to PBP2a at amino acid level [10]. Since the discovery, the *mecC* gene has been found in other parts of the world. This can be a possible explanation to the low presence of the *mecA* (5.56%) gene observed in this study. It can therefore be extrapolated from this that another resistant gene or other intrinsic factors may be in play, possibly the *mecC* gene or β-lactamase hyper-production as observed by [25].

It was established that the leaf ethyl acetate extract of *D. mespiliformis* is effective against MRSA. This may be attributed to the presence of tannins, alkaloids, saponins and flavonoids. Tannins have the ability to react with protein on the cell wall of bacteria causing growth inhibition of the bacteria [14]. Alkaloids express antibacterial activity by inhibition of transcription and toxin production [26], it also inhibits bacterial growth by denaturing bacterial cell wall protein thereby increasing the permeability of the cell membrane [27]. Flavonoids inhibits bacterial enzymes and interfere in metabolism. Saponins exhibit antibacterial activity by forming complexes with bacterial cell wall cholesterol and altering its permeability [28]. Thus, the antibacterial activity observed in this study may be as a result of the pharmacological effect of one of the active compounds or a synergy between all the compounds. The 50 mg/mL concentration was the most effective against MRSA isolates used in the study. It had the highest zone of inhibition of 22.5 mm and was effective against six of the isolates. Next are the 25 mg/mL and 12.5 mg/mL concentrations with 21.2 mm and 19.6 mm as their mean ZOI respectively and effective against five isolates. The last and also with the least activity was the 6.25 mg/mL concentration, its highest ZOI was 16.67 mm and was effective against just three isolates. The negative control had no effect on any of the isolates. All except 6.25 mg/mL concentration were effective against the *mecA* positive isolates and the positive control. It was observed that the antibacterial effect decreased with decrease in the concentration of the leaf extract used. Also, two of the eight isolates were completely resistant to the leaf extract even at the highest concentration of 50 mg/mL.

5 Conclusion

- It can be concluded from this study that not all MRSA may possess *mecA* gene, as only 5.56% of the ORSAB positive isolates were positive for the gene.
- It can also be concluded from this study that the leaf ethyl acetate extract of *D. mespiliformis* has a dose dependent antibacterial effect on MRSA.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest.

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