IN VITRO ANTIMICROBIAL EVALUATION OF THE METHANOL EXTRACT AND ALKALOID FRACTION OF COTTON PLANT (*Gossypium hirsutum)* LEAVES AGAINST METHICILLIN RESISTANT *Staphylococcus aureus*

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Enwa Felix Oghenemaro[1], Ikpefan Emmanuel Oise[2], Umukoro Oke[1]
[1]Department of Pharmaceutical Microbiology, Delta State University, Abraka, Nigeria
[2]Department of Pharmacognosy & Traditional Medicine, Delta State University, Abraka, Nigeria

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Abstract: The study was conducted to determine the antimicrobial efficacy of the crude methanolic extract and alkaloid fraction of the leaf of Cotton plant (*Gossypium hirsutum*) against methicillin- resistant *Staphylococcus aureus.* Phytochemical screening was carried out to check for secondary metabolites following standard procedures and agar well diffusion technique was used to evaluate the sensitivity of the isolates to the crude extract and alkaloid fraction. New effective antimicrobials are needed; natural products can be a source of potential antimicrobial agents against *S. aureus*, especially MRS. Phytochemical screening on the methanolic crude extract of the Cotton plant (*Gossypium hirsutum*) leaves revealed the presence of alkaloids, steroids, reducing sugars, amino acids, tannins, terpenoids, saponins and general glycosides. The treatment with significant activity was the 400 mg/ml of the crude extract (antibacterial) with the highest zone of inhibition of 16 mm. The cloxacillin disc was used to check for the sensitivity of the bacteria, the bacteria showed 100% resistance to the cloxacillin sensitivity disc. The result of the antimicrobial effect of the crude extract showed the sensitivity of the bacteria to the crude extract in this order, Isolates 24>32>33>25 with mean zones of inhibition ranging from 4 to 16, 4 to14, 4 to 13, 4 to 8 mm, respectively. No antimicrobial activity was exerted on the bacteria strains by the alkaloid fraction. There was a significant difference between the activity of the methanol extract and the antibacterial disc against these bacteria at the average zones of inhibition. This study indicates that *Gossypium hirsutum* leaf might be a potential source of novel antimicrobial agent against MRSA and methanol was a good extracting solvent for the active phytochemicals from this plant.

Keywords: *Gossypium hirsutum*, MRSA, extract, antimicrobial

Introduction

Plants have widely been utilized for medicinal purposes long before recorded history (Stojanoski, 1999). Cotton plant (*Gossypium hirsutum*) a perennial shrub is used extensively in treating diarrhea, and as tonic for headache and migraine, treatment of burns, wound and scalds, in cough and asthma, and to induce abortion (Egbuta *et al*,, 2017). It is also used for the production of fabrics. The plant has been reported to possess antimicrobial, anticancer, anticonvulsant, antidepressant, hypotensive and diuretic activities (Aaron *et al*., 2017). Methicillin-resistant *Staphylococcus aureus* is a strain of *Staphylococcus aureus* that is resistant to synthetic penicillin - methicillin and other class of common antibiotics used in treatment (Chaturvedi *et al.,* 2010). Clinical manifestation of MRSA includes superbug infection, scaled skin syndrome, MRSA toxic shock syndrome and MRSA impetigo common in children etc.

Medicinal plants are a source for a wide variety of natural antioxidants and are used for treatment of diseases throughout the world (Rafiein-Kopaie *et al.,* 2013). Some of these properties are antimicrobial (Sharafati *et al.,* 2011), anticancer (Shirzad *et al.,* 2012), antidiabetic (Kazemi *et al.,* 2010), anti-atherosclerosis (Khosravi-Boroujeni *et al.,* 2012), immuno-modulatory (Shirzad *et al*, 2009) and impetigo common in children etc. Methicillin - resistant *Staphylococcus aureus* (MRSA) are strains of *Staphylococcus aureus* that have developed resistance to many common antibiotics, which includes the penicillin's, cephalosporins, aminoglycoside, quinolones, etc. (El-Gayer, 2014). These virulence characteristics of MRSA have resulted in prolonged hospital stay, poor treatment outcomes and increased health care costs amongst infected patients (Ali *et al.,* 2017). Hence, there is need to further investigate plants with established antimicrobial properties to ascertain they have antimicrobial activities against MRSA.

Materials and Methods

Methanol, nutrient broth and agar, Mueller Hinton agar, mannitol salt agar, (Are all products of TM MEDIA, India), a pair of forceps, cotton plant (*Gossypium* spp) leaves, pressure cooker, Olympus microscope, marker pen, rotary evaporator, crystal violet, Lugol iodine, Safranin red, water bath shaker (Labtech A×F500,)

Collection of plant materials

The leaves of Cotton plant (*Gossypium hirsutum*) were collected from Campus 1, Delta State University Abraka and was identified by Dr. Ikpefan Emmanuel of the Department of Pharmacognosy, Faculty of Pharmacy, Delta State University Abraka

Preparation of plant materials for extraction

The leaves were washed with distilled water and air-dried. The dried leaves were grinded into coarse powdered form which was extracted using cold maceration method and 80% methanol with agitation every 6 hours intervals for 3 days after which it was filtered .The filtrate was concentrated using rotary evaporator at 40°C. The concentrated plant extract was placed in Petri dishes at a temperature of 4°C in the refrigerator until they are ready to be used. (De Silva *et al*., 2017).

Phytochemical screening of the extract

The Phytochemical screening of the extract was carried out using standard procedures (Sofowora, 2008; Khandewal, 2010).

Fractionation of methanol extract of cotton plant (Gossypium hirsutum) leaves

Forty gramms (40 g) of the extract was weighed and transferred into a beaker and 100 mL of 10% Acetic acid in methanol was added and the mixture was allowed to stand for four hours and was subsequently filtered using a filter paper (Whatman filter paper 90 mm). 100 ml of ammonia was added gradually, and the mixture was placed in a shaker (Amita and Shalini, 2014).

Fractionation of alkaloid from cotton plant (Gossypium hirsutum) leaves

The alkaloid fraction of the extract of was obtained following the method previously described by Abdullah *et al*. (1979). By this method, the powdered sample was extracted by Soxhlet using hexane and the hexane extract was washed with 1M sulfuric acid (3-50 mL) adjusted to pH 8 using solid $NaHCO_3$ and the free alkaloid fraction was extracted with CH_2Cl_2 (100 mL) and later with methanol in a Soxhlet for 48 h, after which it was evaporated under vacuum, and the residue was absorbed in 0.2 percent sulphuric acid. Furthermore, CH_2Cl_2 was used to extract the acidic solution to remove traces of fat. This was then followed by the addition of $NaHCO_3$ to the aqueous phase which raises the pH was to 8 with $NaHCO_3$ and extracted with CH_2Cl_2 (3 µ100 mL) to give a methanol fraction containing "free" alkaloids.

Isolation of S. aureus from nasal swab samples

Forty - one sterile swab sticks were used to swab the nostrils of forty - one different individuals. (Ethical approval was granted by Delta State Health Management Board with Ref. No. HMB/ETHICS 00238). Mannitol salt agar was prepared according to manufacturer's specification and the Mannitol agar was poured into forty-one (41) labelled petri dishes and allowed to solidify. After solidification, each swab stick was used to streak each petri dishes and the petri dishes were incubated at 37°C for 24 h. After incubation the organism grew on the mannitol salt agar, fermenting mannitol agar from pink to yellow confirming the organism to be *Staphylococcus aureus*. The mannitol agar test and the gram staining test served as confirmatory test for staphylococcus aureus. Subcultures were prepared and stored at 37 degrees centigrade**.**

Preparation of overnight broth culture

Nutrient broth was prepared according to manufacturer's specification and dispensed into bijou bottles, allowed to cool and the different strains of *Staphylococcus aureus* were inoculated into this broth using sterile wire loop and incubated at 37°C for 24 h.

Cloxacillin preparation

A spread of the overnight broth culture of the different strains of *Staphylococcus aureus* were made on already prepared Mueller Hinton agar plate. Then 500 mg of cloxacillin was diluted using ten-fold and two-fold to get 5 micrograms of cloxacillin. Sterile paper discs were soaked in 5 micrograms of cloxacillin solution and was allowed to dry before been placed on the surface of each culture plate and incubated at 37°C for 24 h. After incubation, zones of inhibitions were noted.

Susceptibility testing of cotton plant methanol extract against Staphylococcus aureus.

A spread of different strains of MRSA was made on Muller Hinton agar and a two-fold serial dilution of the plant extract was done (400, 200, 100, 50, 25, 12.5 mg). Six holes were punched on the agar using 6 mm cork borer and each hole was labelled according to these different concentrations (400, 200, 100, 50, 25, 12.5 mg). The methanolic Cotton plant (*Gossypium hirsutum*) extract was transferred into the culture plate according to their labelled concentrations and left for some time to diffuse. After that, it was incubated at 37°C for 24 h. After incubation, zones of inhibition for each concentration were measured and recorded.

Sensitivity test of the extract and alkaloidal fraction of Gossypium hirsutum

A spread of an overnight broth culture of MRSA was made on Muller Hinton agar and a two-fold serial dilution of the alkaloid fraction was done (400, 200, 100, 50, 25, 12.5 mg). Six holes were punched on the agar using 6 mm cork borer and each hole was labelled according to these different concentration (400, 200, 100, 50, 25, 12.5 mg). The alkaloid fraction was transferred into the culture plate according to their labelled concentrations and left for some time to diffuse. After that, it was incubated at 37°C for 24 h. After incubation, zones of inhibition for each concentration were measured and recorded.

Results and Discussion

Isolates of Staphylococcus aureus from nose swabs

The patient's samples 1 – 23 were negative for *Staph aureus* while patients samples 24, 25, 32 – 34, 39 – 41 were positive for *Staph aureus* on Mannitol salt agar.

Determination of methicillin resistant Staphylococcus aureus using cloxacillin sensitivity disc

Isolates 25 and 41 showed average Inhibition zone diameters (IZD) of 4+/-0.0 and 9.5 +/- 0.701, respectively; while the rest (24, 32, 33, 34, 39 and 40) are not susceptible. A zone of inhibition ≤ 10 mm indicates that the isolate of *Staphylococcus aureus* is methicillin- resistant. Hence all, the isolates of *Staphylococcus aureus* above are methicillin resistant (100% resistant to cloxacillin sensitivity disc).

Results of phytochemical screening

The results of the phytochemical screening of the extract showed the presence of phytochemical groups such as alkaloids, tannins, saponins, terpenes, etc. as shown in Table 1. The presence of alkaloid in the alkaloidal fraction was confirmed when reacted with Dragendorf's reagent.

Table1: Qualitative phytochemical screening of extract and alkaloidal fraction of cotton plant (*Gossypium hirsutum*) leaves

Phytochemicals	Extract	Alkaloidal fraction
Alkaloids	++	+++
Anthraquinones	–	–
Tannins (Phenolics)	+	–
Flavonoids	+	–
Saponins	+	–
Cardiac glycosides	+	–
Terpenes	+	–

+++ = appreciable amount; + = minute amounts; – = not detected

Sensitivity test of methanol extract and alkaloidal fraction of leaves of Gossypium hirsutum against MRSA

A concentration dependent sensitivity was recorded. The methanol extract of the cotton leaves recorded better zones of inhibition against the Isolates 24, 25, 32 and 33 at high concentrations with the exception of isolate 34 which was resistant at all concentration. The alkaloidal fraction recorded no zones of inhibition as the isolates were resistant to it (Table 2 and 3).

Table 2: Sensitivity test of methanol extract of leaves of *Gossypium hirsutum* against MRSA

S/N	Average Zones of inhibition (mm)/ Concentrations (mg/mL)					
	400	200	100	50	25	12.5
24	16.00±0.00	4.00±0.00	-	-	-	-
25	8.00±1.00	5.00±0.20	4.00±0.00	-	-	-
32	14.00±1.00	4.00±1.00	-	-	-	-
33	13.00±0.50	7.00±0.00	4.00±2.00	4.00±1.00	-	-
34	-	-	-	-	-	-

Table 3: Sensitivity test of alkaloid fraction of leaves of *Gossypium hirsutum* against MRSA

S/N	Average Zones of inhibition (mm)/Concentrations (mg/mL)					
	400	200	100	50	25	12.5
24	-	-	-	-	-	-
25	-	-	-	-	-	-
32	-	-	-	-	-	-
33	-	-	-	-	-	-
34	-	-	-	-	-	-

From Table 2, depicting the average zones of inhibition, it shows that the Methanol extract of the cotton leaves had a better zone of inhibition against the organisms (Isolates 24, 25, 32 and 33) at high level of concentration with the exception of isolate 34 which was resistant at all concentration. - Indicates no zone of inhibition.

The clinical isolates used in this study were all members of the Staphylococcus family. The ability of the isolates to aerobically ferment mannitol salt agar, established their identity as *Staphylococcus aureus*, a further confirmatory test was carried out using gram staining. *S. aure*us appeared as golden colonies in the medium.

The result of the phytochemical screening of the methanol extract of Cotton plant (*Gossypium hirsutum*) leaves shows the presence of secondary metabolites as described in the work of Ayeni *et al*. (2015). Several research works have been done on the antimicrobial activity of Cotton plant (*Gossypium hirsutum*). However, the bacteriostatic and bactericidal activities of this plant are due to this bioactive components extracted being able to interfere with bacterial cell wall synthesis (Mades, 2014). Of all 41 isolates that were swabbed and cultured, only eight samples showed the growth of *Staphylococcus aureus* (isolates 24, 25, 32, 33, 34, 39, 40 and 41). Hence only 20% of the sample population showed growth of *S. aureus.* And all eight different samples of *Staphylococcus aureus* isolated were methicillin- resistant except for isolates 25 and 41 that was inhibited by Cloxacillin with an average zone of inhibition of 4 and 9 mm, respectively and this is less than 10 mm (zone of inhibition for organisms susceptible to Cloxacillin). From Table 3, the various concentrations of methanol extract of Cotton plant (*Gossypium hirsutum*) leaves did not inhibit the growth of isolates 34, 39, 40 and 41 which is in agreement with the work carried out by Marzieh *et al*, (2019). For isolate 33 of *Staphylococcus aureus*, the growth was inhibited by the various concentrations of methanol crude extract of the plant with an average zone of inhibition of 13, 7, 4 and 4 mm, respectively for the various concentrations, 25 and 12.5 mg/ml of the crude extract did not inhibit its growth. For isolate 32 of the Methicillin resistant *Staphylococcus aureus*, 400 and 200 mg/ml of the methanol extract inhibited its growth with an average zone of 14 and 4 mm, respectively while the other concentrations of the methanolic crude extract did not inhibit the growth of the bacteria. For isolate 25 of the MRSA, 400, 200 and 100 inhibited its growth with average zones of inhibition of 8, 5 and 4 mm, respectively; while the other concentrations of the crude extract did not inhibit the growth of the organisms. For isolate 24 of MRSA, 400 and 200 mg/ml of the methanol crude extract inhibited the growth of the MRSA with average zones of inhibition of 16 and 14 mm, respectively, while the other concentrations of the methanol crude extract did not inhibit the growth of the organisms.

Comparatively, the zones of inhibition of 5 microgram Cloxacillin disc tested against various strains of MRSA were considerably lower than the zones of inhibition of 400 mg concentration of the methanol extract of Cotton plant (*Gossypium hirsutum*).

All the different concentrations of the alkaloid fraction of the methanol extract of Cotton plant (*Gossypium hirsutum*) leaves did not inhibit the growth of all the strains of Methicillin resistant *Staphylococcus aureus.*

Conclusion

The study has shown that the methanol extract of Cotton plant (*Gossypium hirsutum*) leaves exhibits a moderate or mild degree of inhibition on different strains of MRSA (strains 24, 25, 32 and 33, respectively). Since the crude extracts (methanol extracts) of Cotton plant (*Gossypium hirsutum*) leaves was able to exert a greater antimicrobial effect on the MRSA than the control drug (Cloxacillin), it could be beneficial to use Cotton plant (*Gossypium hirsutum*) leaves as an adjunct or replacement therapy with antibiotics such as Penicillins, Amino glycosides, Cephalosporins and others to combat infections caused by MRSA. This may provide a synergistic effect in the treatment of MRSA related infection.

Conflict of Interest

Authors have declared that there is no conflict of interest reported in this work.

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