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Antibacterial Activity of *Carica Papaya* (Leaf and Seed) Extracts on Methicillin Resistant *Staphylococcus Aureus* (MRSA) from Wound

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ABSTRACT

The aim of the study was to determine the antibacterial activity and phytochemical constituents of *Carica papaya* leaf and seeds extracts against *methicillin-resistant Staphylococcus aureus* (MRSA). Leaf and seed were collected and prepared using cold maceration technique. Phytochemicals screening were done**.** Three hundred of clinical wound samples were collected from Murtala Muhammad Specialist Hospital, Kano State. The isolation and identification of *Staphylococcus aureus* was done through culture on Nutrient agar and Mannitol salt agar, Gram staining, microscopic and standard biochemical tests such as (catalase, coagulase, oxidase and hemolysis test) were carried out. Cefoxitin disc diffusion test and molecular analysis was done for the detection of methicillin resistant strain of *S. aureus*. Evaluation of antibacterial activity of the extract against methicillin resistant strain was carried out. The phytochemical screening of leaf extract contains Saponin, Steroid, Terpenoid, Alkaloid, Flavonoid, Phenol and glycoside except Tannin and Anthraquinone in methanolic but in aqueous only anthraquinone and glycoside are not present, Seed also contains Saponin, Steroid, Terpenoid, Alkaloid and Phenol except Tannin, Flavonoid, Anthraquinone and Glycoside in both methanolic and aqueous extracts. A total of fifty six *Staphylococcus aureus* isolates were identified in the present study in which 8(14.3%) were MRSA and 48(85.7%) were negative MRSA. Polymerase chain reaction (PCR) analysis reveals the presence of *mec A* gene under 310base pairs nucleotide sequence in the positive MRSA. For the antibacterial activity leaf demonstrated antibacterial effects on 7 out of 8 tested MRSA isolates with higher activity and highest zone of inhibition of 19mm at 100mg/ml in methanolic extract. Seed evaluated antibacterial effects on 6 out 8 in methanolic extract with highest zone of inhibition of 15mm at 100mg/ml while no activity showed by aqueous extract. The calculated p value of leaf and seed is less than the table value (0.05) which is 0.00058 and 3.56E07 respectively. Based on statistical analysis there is significant.

Key words: Antibacterial, methicillin resistant, *Staphylococcus aureus*

INTRODUCTION

Antimicrobials of plant origin effective in the treatment of infectious diseases and simultaneously mitigating many of the side effects often associated with synthetic antimicrobial agents have been discovered. Medical uses of plants range from the administration of the roots, barks, stems, leaves and seeds to the use of extracts and decoction from the plants (Iwu *et al*., 1999). Herbal medicine is the most common form of traditional medicine that uses plants or other plant materials as active ingredients (WHO, 2008). Resistance to methicillin that indicated resistance to all beta-lactam agents was first reported in 1961, which marked the appearance of methicillin-resistant *Staphylococcus aureus* (MRSA) (Pantosti *et al*., 2007) (Molton *et al*., 2013) MRSA are most common in hospital and other institutional healthcare settings, thereby many researchers are aiming to scientifically prove the use of *Carica papaya* extract as an effective means of controlling antibiotics resistance (Que and Moreillon, 2009).

Aim

The main aim of the research work was the evaluation of antibacterial activity of *Carica papaya* seed and leaf extracts against isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) from burn wound.

Specific Objectives of the Study

1. To detect the phytochemical constituents and determine the active metabolites of *Carica papaya* seed and leaf extracts.
2. To isolate and identify *Staphylococcus aureus* from clinical specimens of burn wound and detect methicillin-resistant Strains using Cefoxitin disc diffusion and Molecular analysis.
3. To determine the antibacterial activity of *Carica papaya* extracts against the isolates of Methicillin Resistant *Staphylococcus aureus* (MRSA)

MATERIALS AND METHOD

Study area

The study was conducted in Murtala Muhammad Specialists Hospital (MMSH), Muhammad Abdullahi Wase Road, Kano municipal, Kano State. It was the largest state government hospital in northern Nigeria and has the highest patients' attendance in the region. The hospital was commissioned in 1926, presently with an official bed capacity of 826 and has total number of 30 wards and units and 9 operating theatre and 14 clinics. It was also has the staff strength of 1656 and it is an NHIS accredited hospital. An ethical approval was obtained from Murtala Muhammad Specialist Hospital (MMSH), Kano state based on the consent of the Hospital Ethical Committee, under the consideration of Ministry of Health Kano.

Sample size

The sample size was calculated using 28% prevalence of MRSA in Kano (Nwakwo *et al*., 2010). The following formula was used to calculate the sample size as described by Daniel (1999);

$N = Z^2 P (1-P) / d^2$

N=300

Inclusion Criteria

All consented patients admitted in the surgical ward with burns for at least 14 days were included in the study.

Collection of Carica papaya seed and leaf

Fresh green leaf was collected from Aminu Kano Teaching Hospital, (AKTH) garden while seed was collected from various fruit vendors along the hospital road, the plants material was carried to Herbarium in the Department of Plant Biology, Bayero University Kano, Nigeria where they were authenticated. The plant materials were washed thoroughly 5 times in sterile distilled water. Then air-dried under shade at room temperature for 14 days and pulverized to finely powdered form from using pestle and motor (Ali *et al*., 2017).

Preparation of plant extracts (Cold maceration)

According to Alabi *et al*. (2012) *Carica papaya* extract was prepared using cold maceration method, four Bama bottles containing the mixtures were allowed to stand at room temperature for a period of five days with frequent agitation. The extracts were filtered using Whatmann No.2 filter paper and the filtered mixtures was placed on water bath at 40^0C to obtained the various crude extracts, the extracts were kept in the refrigerator at 4^0C until used.

Phytochemical Screening

Test	Procedure
Saponins (emulsion test)	Zoopasharee *et al*, 2008
Tannins	Gul et al, 2017
Terpenoids	Gul et al, 2017
Steroids	Uma et al, 2017
Flavonoids	Audu et al, 2007
Alkaloids	Auwal et al, 2014
Glycoside	Raymund 2013
Anthraquinones	Emran et al, 2015
Phenol	Aka et al, 2009

Collection of Clinical Wound (burn) Samples

Samples (upper and lower limbs, surface area of stomach, neck and feet) were collected among the inpatients in Surgery Department (burn section) at the 14 days interval of collection in Murtala Muhammad Specialist Hospital (MMSH), Kano State using swab technique as described by Hilliard and Reddy (2018).

Isolation and Identification of Staphylococcus aureus

Five milliter of prepared nutrient broth was added to test tubes and sterilized by autoclaved at 121^0C for 15minutes then allowed to cool. Swabbed sample was inoculated into sterilized 5ml of nutrient broth and uninoculated test tube was served as control. The test tubes were then incubated at 37^0C for 24 hours. After the incubation, sterile swab stick was dipped into each test tube with reference turbidity and swabbed gently on to the prepared nutrient agar (NA) plate and incubated at 37^0 for 24 hours. After 24hours of incubation, the examined colonies on a nutrient agar (NA) plate was then picked and

inoculated on to the prepared mannitol salt agar (MSA) plate using sterile wire loop and incubated at 37^0 for 24-48hours. Typical *Staphylococcus* colonies were examined after two days of the incubation. (Cheesbrough, 2006; Forbes and Sahm, 2007).

Gram staining

Gram stain was achieved through the standard procedure as described by Colco, 2005

Biochemical Tests

Test	Procedure
Catalase (slide method)	Wheelis, 2008
Oxidase	Macfaddin, 2000
Hemolysis (blood agar test)	Kato et al, 2017
Coagulase (slide method)	Carson and Naber, 2014

Standardization of the bacterial inoculums

A loopful of bacterial colony of the test isolates was picked and inoculated into test tube containing 5ml of distilled water using sterile wire loop**,** turbidity was adjusted to equivalent 0.5 McFarland standards.

Detection of Methicillin-Resistant Staphylococcus aureus using Cefoxitin Disc Diffusion

All *Staphylococcus aureus* isolates were subjected to cefoxitin disc diffusion test using 30ug of cefoxitin (Oxoid CT0019B). Prepared Mueller-Hinton agar plates were swabbed with standardized bacterial inoculums. Thirty microgram of cefoxitin disc were placed on the centre of MHA plates using sterile forceps and incubated at 37 ^{0}C for 24hours. Positive methicillin resistant strain of *Staphylococcus aureus* and methicillin susceptible strain of *Staphylococcus aureus* were served as positive and negative plates control respectively obtained from Murtala Muhammad specialist hospital, Kano. Zone of diameter inhibition was measured (CLSI, 2011).

DNA extraction

Pure colonies of *Staphylococcus aureus* from those that were cefoxitin resistance were cultured in nutrient broth for 24hours at 37^{o}C with frequent agitation using micro centrifuge at 100rpm for 5minutes. Cell suspension were transferred into another test tube and centrifuged at 4500rpm for 5minutes. DNA was extracted using boiling lysis method, in which 1g of cell pellets were resuspended in 40ul of water and boiled at 100^{0}C in water bath for 10minutes. After it was cooled on ice, and then centrifuged at 15000 x g for 10seconds. Cold 95% of ethanol was added to the supernatant fluid and stored at -20^{0}C until polymerases chain reaction assay (PCR) (Asubel, 1999)**.**

Polymerase Chain Reaction (PCR) Assay

PCR mixture was prepared in thin walled 0.2ml tube, which contains 50ul of distilled water; Buffer 1x, $MgCl_2$ 0.5mM, Taq polymerase 0.05Units/ul, dNTP 200uM and mecA primer 0.5uM (forward) and 0.5uM (Reverse). 10ul of DNA sample was added to 50ul of PCR mixture, the mixture was placed inside the Bio-Rad thermal cycler for PCR amplification. Initial denaturation was carried out at 92^{0}C for 30minutes followed denaturation at 92^{0}C for 10minutes, annealing at 56^{0}C for 10minutes and extension at 72^{0}C for 10minutes. The final extension was performed at 72^{0}c for 30minutes; the amplified product was detected by 1% of agarose gel electrophoresis with (0.5ug/ml) ethidium bromide staining and was observed using Ultraviolet light (Abu-shady *et al.*, 2012).

Table1: Oligonucleotide sequence of PCR primers employed for the identification of *mecA* gene.

Name of primers	Sequence 5'-3'	Product Size bp
MecA-F	GTAGAAATGACTGAACGTCCGATTA	310bp
MecA-R	CGAATTCGACATTGTTTCCGTCTAA	310bp

Sources: Jonas *et al*. (2002).

Antibacterial activity of *Carica papaya* extracts against MRSA

Determination of antibacterial activity of *Carica papaya* leaf and seed extracts was done using agar well diffusion method as described by Aida *et al*, (2001). The antibacterial potential of test extract was determined on the basis of diameter zone of inhibition around the wells (CLSI, 2016).

Results

Extract yield of Carica papaya seed and leaf

Table 1 indicated that from 50g powdered of each dry samples of leaf and seed for methanolic and aqueous extracts, methanolic extracts has highest extract yield of 3.70g (7.4%) and 3.50 (7.0%) for the leaf and seed respectively.

Table 1: Extracts yield of *Carica papaya* seed and leaf

Extracts	Weight of dry sample (g)	Weight extract yield (g)	Percentages yield (%)
Methanolic Leaf	50.00	3.70	7.40
Aqueous Leaf	50.00	2.90	5.80
Methanolic Seed	50.00	3.50	7.00
Aqueous Seed	50.00	2.70	5.40

*Weight of extract yield (g) = weight of dry sample (g) + weight of empty bottle (g) - weight of empty (g)

*Percentages yield = weight of extract yield/weight of dry sample x 100

Phytochemicals constituents of Carica papaya extracts

Leaf extracts contains Saponin, Steroid, Terpenoid, Alkaloid, Flavonoid, Phenol and glycoside except Tannin and Anthraquinone in methanolic but in aqueous contains the listed phytochemical except anthraquinone and glycoside. Seed also contains Saponin, Steroid, Terpenoid, Alkaloid and Phenol except Tannin, Flavonoid, Anthraquinone and Glycoside in both methanolic and aqueous extracts as indicated in table 2.

Table 2: Phytochemical constituents of *Carica papaya* extracts

	Extracts			
Phytochemicals	**Methanolic Leaf**	**Aqueous Leaf**	**Methanolic Seed**	**Aqueous Seed**
Saponins	+	+	+	+
Tannins	-	+	-	-
Steroids	+	+	+	+
Terpenoids	+	+	+	+
Alkaloids	+	+	+	+
Flavonoids	+	+	-	-
Phenols	+	+	+	+
Anthraquinones	-	-	-	-
Glycosides	+	-	-	-

Percentage occurrences' of Methicillin-resistant *Staphylococcus aureus* (MRSA)

Percentage occurrence of MRSA was presented in table 3, in which out of the 56 total number of *Staphylococcus aureus* isolates, 8(14.3%) isolates were positive MRSA and 48(85.7%) were negative MRSA.

Table 3: Percentage occurrences' of Methicillin-resistant *Staphylococcus aureus* (MRSA)

Isolates	Number of occurrence	Percentages (%)
Positive MRSA	8	14.3
Negative MRSA	48	85.7
Total *S.aureus*	56	100

Antibacterial activity of leaf extracts against the isolates of Methicillin resistant Staphylococcus aureus (MRSA)

Table 4 indicates the antibacterial activity of methanolic and aqueous leaf extracts, in which 7 out of 8 isolates of MRSA has the activity against methanolic and aqueous extract; however methanolic extract has the highest zone of inhibition of 19mm at concentration of 100mg/ml evaluated on isolates number 5 while aqueous extract has the highest zone of inhibition of 17.00mm at also 100mg/ml concentration evaluated both in isolates number 1 and 7.

Table 4: Antibacterial activity of leaf extracts against the isolates of Methicillin resistant *Staphylococcus aureus* (MRSA)

Extracts	Conc. mg/ml	Isol.1	Isol.2	Zone Isol.3	of Isol.4	Inhibition Isol.5	(mm) Isol.6	Isol.7	Isol.8
Meth	100	16.5±0.5	12±00	10.5±0.5	14±00	19.5±0.5	00±00	18±00	14±00
	50	14.5±0.5	12±00	09±00	12±00	15.5±0.5	00±00	17±00	10±00
	25	13±00	09±00	8.5±0.5	09±00	13±00	00±00	15±00	10±00
	12.5	13±00	07±00	08±00	00±00	08.5±0.5	00±00	11±00	08±00
Aque	100	17.5±0.5	14±00	10±00	15±00	16.5±0.5	00±00	17±00	13±00
	50	13.5±0.5	14±00	08±00	10±00	10±00	00±00	17±00	11±00
	25	13±00	08±00	00±00	08±00	10.5±0.5	00±00	13±00	11±00
	12.5	12±00	00±00	00±00	08±00	08±00	00±00	11±00	00±00
Control (Cipro)	100	16.5±0.5	15±00	14±00	17±00	15.5±0.5	10±00	20±00	17±00

Antibacterial activity of seed extracts against the isolates of Methicillin resistant Staphylococcus aureus (MRSA)

Table 5 indicates the antibacterial activity of methanolic and aqueous seed extracts, in which 6 out 8 MRSA isolates has the activity against methanolic seed with highest zone of inhibition of 15.00mm at concentration of 100mg/ml evaluated on isolates number 5 and 7. No activity was shown at different concentration of aqueous seed extract against all tested MRSA isolates.

Table 6: Antibacterial activity of seed extracts against the isolates of Methicillin resistant *Staphylococcus aureus* (MRSA)

Extracts	Conc. mg/ml	Isol.1	Isol.2	Zone Isol.3	Of Isol.4	Inhibition Isol.5	(mm) Isol.6	Isol.7	Isol.8
Meth	100	10±01	00±00	09±01	10±01	15±01	00±00	15±00	13±01
	50	09±00	00±00	07±00	09±00	12±00	00±00	11±01	10±00
	25	09±00	00±00	00±00	09±00	12±00	00±00	11±00	09±00
	12.5	00±00	00±00	00±00	07±00	09±01	00±00	09±00	07±01
Aque	100	00±00	00±00	00±00	00±00	00±00	00±00	00±00	00±00
	50	00±00	00±00	00±00	00±00	00±00	00±00	00±00	00±00
	25	00±00	00±00	00±00	00±00	00±00	00±00	00±00	00±00
	12.5	00±00	00±00	00±00	00±00	00±00	00±00	00±00	00±00
Control (Cipro)	100	15.5±0.5	09±01	17±00	13±01	19±00	10±01	15±00	16±01

Key: Isol. = Isolates, Conc. = Concentration, Cipro = Ciprofloxacin, Meth = Methanol

Aque = Aqueous

Discussion

The present study was conducted to determine the antibacterial activity of *Carica papaya* leaf and seed extracts on methicilin resistant *Staphylococcus aureus* (MRSA) isolated from burn wound. The percentage yield for the methanolic leaf and seed gave the highest recovery yield of 3.7g (7.4%) and 3.50g (7.0%) respectively, the finding was corresponded to the study of Ayuba *et al*. (2017) on phytochemical screening, thin layer chromatography and antimicrobial activity of methanolic and aqueous *Citrus limon* who concluded the methanolic extract has the highest yield recovery while the result for the phytochemical screening showed that leaf extracts contains saponin, steroid, terpenoid, alkaloid, flavonoid, phenol and glycoside except tannin and anthraquinone presented in methanolic but in aqueous only anthraquinone and glycoside are not present, this finding can be certified to the work of Ikeyi *et al.* (2013) in his study of phytochemical analysis of paw-paw leaf extract who reported *Carica papaya* leaf extract contains listed secondary metabolites as an active chemical agent in the bacterial activity. Seed also contains saponin, steroid, terpenoid, alkaloid and phenol except tannin, flavonoid, anthraquinone and glycoside were not detected in both methanolic and aqueous extracts. Bais *et al.* (2002) and Marshall *et al*. (2015) in their studies reported that *Carica papaya* seed and root, contains phytochemical compounds such as alkaloids, saponins, phenol and terpenoid which were attributed to their antimicrobial effects. In addition, bioactive substances of *Carica papaya* have been reported to confer activity against bacteria, fungi and pests and therefore demonstrated the mode of activity by the compounds (Aravind *et al*, 2013).

Out of the total number of 56 isolates of *Staphylococcus aureus* obtained from 300 clinical samples of burn wound based on morphological and biochemical characterization, 8(14.3%) were methicillin resistant *Staphylococcus aureus* (MRSA) and 48(85.7%) isolates were negative MRSA. MRSA were determined by corresponded standard zone of inhibition given by Clinical Laboratory of Standards Institutes (CLSI), 2011. MRSA represented 14.3% which was evaluated in the present study from burn wound was below the findings of Mounir, 2014, in his study of phenotypic and genotypic characterization of nasocomial isolates of *S.aureus* with reference to methicillin resistant who reported out of total 98 isolates of *S.aureus* from burn wound 21(24.1%) were MRSA. The difference between 14.3% and 24.1% might be due the highest number of *Staphylococcus aureus* obtained by Mounir in his study. Polymerase chain reaction (PCR) analysis reveals the presence of *mec A* gene under 310base pairs nucleotide sequence in the positive MRSA, this present finding has similar to the previous findings by Garcia-alvarez *et al*. (2011) who reported a novel allele of *mec A* gene encoding an alternative penecillin binding protien that mediate methicillin resistance among bovine *S.aureus.* The *mec A* gene is highly conserved among Staphylococcal species that show resistance to methicillin and consequently the detection of this gene was by polymerase chain reaction machine (Maes *et al.*, 2002; Velesco *et al.*, 2004; Liu *et al.*, 2011).

From the results of antibacterial activity of the plant, leaf demonstrated antibacterial effects on 7 out of 8 tested MRSA isolates, with higher activity and highest zone of inhibition of 19mm at 100mg/ml in methanolic extract. Seed evaluated antibacterial effects on 6 out 8 in methanolic extract with highest zone of inhibition of 15mm at 100mg/ml while no activity showed by aqueous extract. The antibacterial activity of extracts used in this study shows that, leaf extract demonstrated higher antibacterial activity than the seed extract. It is in line with the finding of Baskaran *et al.* (2012) who studied the antibacterial activity of *Carica papaya* and reported the leaf to have significant antibacterial activity on *S.aureus* at various concentrations. Results of antibacterial activity also demonstrated that the methanolic extracts were more effective than aqueous extracts; this is in conformity with findings of Melo *et al.* (2008) who reported methanolic extract of *Carica papaya* has better antibacterial effect than aqueous extract. This may be due to the better solubility of the active components in the organic solvent. Oyoyede (2005) and Chandra *et al*. (2011) reported the extract of papaya leaf to have good antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Esherischia coli, Pseudomanas aeruginosa and Shigella flexneri*. There was evidence that *Carica papaya* accelerates wound healing (Mahmood *et al.*, 2005; Corral-Aguay *et al*., 2008; Otsuki *et al*., 2011.

The calculated p-value of leaf and seed extracts were 0.0005806 and 3.56E07 respectively Based on the findings of this research, statistical analysis of the antibacterial activity showed significant different on the susceptibility pattern of the isolates against the extracts used at $p<0.05$.

Conclusion

It was also concluded that there is antibacterial activity of *Carica papaya* against the MRSA, in which leaf possessed higher activity than seed extracts in both methanolic and aqueous extracts.

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