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Characterization of bacteria isolates colonizing the throat of hospitalized patients at Sobi Specialist Hospital, Ilorin, Nigeria and *in vitro* antimicrobial effects of *Citrus aurantifolia* and Alum on the isolates

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Abstract:

Background: Antibiotic resistance in microorganisms implicated in nosocomial respiratory infections is a major reason for prolonged hospital stay and increased cost of therapeutic treatment of hospital acquired pneumonia (HAP). This study was designed to isolate bacterial pathogens colonizing the throat of hospitalized patients at the Sobi Specialist Hospital, Ilorin, and to evaluate antibacterial effects of extracts of *Citrus aurantifolia* peel and Alum against these bacterial isolates.
Methodology: This was a cross sectional study of 100 randomly recruited hospitalized patients at the Sobi Specialist Hospital, Ilorin, Nigeria. Throat samples collected from consenting participants were cultured on selective agar media (MacConkey, Eosin-Methylene blue and Mannitol salt) for isolation of bacteria. Identification of isolates from culture plates was done by Gram reaction and conventional biochemical tests while confirmation of the isolates was done by the polymerase chain reaction (PCR) assay. Antibiotic susceptibility test for each isolate to selected antibiotics (ampicillin, amoxicillin-clavulanate, cefuroxime, ceftazidime, gentamicin, nitrofuran, ofloxacin and ciprofloxacin) was done by the Kirby Buer disc diffusion method. Aqueous extract of Alum ($[KAl(SO_4).12H_2O]$) was done to produce concentrations of 10, 20, 30, 40 and 50% (w/v) at pH 3.6 and tested on the bacterial isolates using agar diffusion method. *Citrus aurantifolia* peel was extracted using methanol and hexane solvents to produce extract concentrations of 500mg/ml, 250mg/ml and 150mg/ml, and tested on the isolates by agar diffusion, and by the broth dilution method to obtain minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *C. aurantifolia*.
Results: A total of 14 bacterial isolates were recovered from throat samples of 100 hospitalized patients with *Staphylococcus aureus* (43%, n=6) being the most frequent while *Escherichia coli* (14.5%, n=2) was the least frequent. The isolates were generally resistant to penicillin, aminoglycoside and fluoroquinolone groups of antibiotics tested. The zone of inhibition for hexane and methanol extracts of *C. aurantifolia* and aqueous extract of alum on the bacterial isolates ranged from 11.5-19.2mm, 9.8-15.8mm, and 9.3-21.2mm respectively while those of selected antibiotics ranged from 7.0-25.0mm. The MICs of hexane and methanol extracts of *C. aurantifolia* against *S. aureus* were 10mg/ml and 25mg/ml, while the MBCs were 50 and 100mg/ml respectively.
Conclusion: Findings from this study showed the presence of resistant pathogenic bacteria colonizing the throat of hospitalized patients receiving care at the Sobi Specialist Hospital, Ilorin, Nigeria. The crude extracts of *C. aurantifolia* and Alum in this study showed inhibitory effects (albeit at higher concentrations) on the bacterial isolates comparable to the standard antibiotics. We posit that based on the inhibition capacity, further studies to characterize, purify and isolate the active anti-bacterial components in the extracts should be considered for novelty.

Keywords: Antibacterial; antibiotic resistance; *Citrus aurantifolia*; MBC; MIC.

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Caractérisation des isolats de bactéries colonisant la gorge des patients hospitalisés à l'hôpital spécialisé Sobi, Ilorin, Nigeria et effets antimicrobiens *in vitro* de *Citrus aurantifolia* et Alum sur les isolats

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Résumé:

Contexte: La résistance aux antibiotiques chez les micro-organismes impliqués dans les infections respiratoires nosocomiales est une cause majeure de séjour prolongé à l'hôpital et d'augmentation du coût du traitement thérapeutique de la pneumonie nosocomiale. Cette étude a été conçue pour isoler les agents pathogènes bactériens colonisant la gorge des patients hospitalisés à l'hôpital spécialisé Sobi, Ilorin, et pour évaluer les effets antibactériens des extraits de zeste de *Citrus aurantifolia* et d'alun contre ces isolats bactériens.
Méthodologie: Il s'agissait d'une étude transversale de 100 patients hospitalisés recrutés au hasard à l'hôpital spécialisé de Sobi, à Ilorin, au Nigeria. Des échantillons de gorge prélevés sur des participants consentants ont été cultivés sur des milieux gélosés sélectifs (MacConkey, éosine-bleu de méthylène et sel de mannitol) pour l'isolement des bactéries. L'identification des isolats à partir de plaques de culture a été effectuée par la réaction de Gram et des tests biochimiques conventionnels tandis que la confirmation des isolats a été effectuée par le test de réaction en chaîne par polymérase (PCR). Le test de sensibilité aux antibiotiques de chaque isolat aux antibiotiques sélectionnés (ampicilline, amoxicilline-acide clavulanique, céfuroxime, ceftazidime, gentamicine, nitrofurane, ofloxacine et ciprofloxacine) a été effectué par la méthode de diffusion sur disque de Kirby Buer. Un extrait aqueux d'alun ([KAl(SO4).12H2O]) a été réalisé pour produire des concentrations de 10, 20, 30, 40 et 50 % (p/v) à pH 3,6 et testé sur les isolats bactériens en utilisant la méthode de diffusion sur gélose. La peau de *C. aurantifolia* a été extraite à l'aide de solvants méthanol et hexane pour produire des concentrations d'extrait de 500mg/ml, 250mg/ml et 150mg/ml, et testée sur les isolats par diffusion sur gélose, et par la méthode de dilution en bouillon pour obtenir un minimum inhibiteur (MIC) et concentrations minimales bactéricides (CMB) de *C. aurantifolia*.
Résultats: Un total de 14 isolats bactériens ont été récupérés à partir d'échantillons de gorge de 100 patients hospitalisés, *Staphylococcus aureus* (43.0%, n=6) étant le plus fréquent tandis qu'*Escherichia coli* (14,5%, n=2) était le moins fréquent. Les isolats étaient généralement résistants aux groupes d'antibiotiques testés pénicilline, aminoglycoside et fluoroquinolone. La zone d'inhibition pour les extraits à l'hexane et au méthanol de *C. aurantifolia* et l'extrait aqueux d'alun sur les isolats bactériens variait de 11,5 à 19,2mm, 9,8 à 15,8mm et 9,3 à 21,2mm respectivement, tandis que celles des antibiotiques sélectionnés variaient de 7,0 à 25,0mm. Les CMI des extraits à l'hexane et au méthanol de *C. aurantifolia* contre *S. aureus* étaient de 10 mg/ml et 25mg/ml, tandis que les MBC étaient respectivement de 50 et 100mg/ml.
Conclusion: Les résultats de cette étude ont montré la présence de bactéries pathogènes résistantes colonisant la gorge des patients hospitalisés recevant des soins à l'hôpital spécialisé de Sobi, à Ilorin, au Nigeria. Les extraits bruts de *C. aurantifolia* et d'alun dans cette étude ont montré des effets inhibiteurs (bien qu'à des concentrations plus élevées) sur les isolats bactériens comparables aux antibiotiques standard. Nous postulons que sur la base de la capacité d'inhibition, d'autres études pour caractériser, purifier et isoler les composants antibactériens actifs dans les extraits devraient être envisagées pour la nouveauté.

Mots clés: Antibactérien; résistance aux antibiotiques; *Citrus aurantifolia*; CMB; MIC

Introduction:

Hospital acquired pneumonia (HAP) also known as nosocomial pneumonia accounts for the high mortality and morbidity reported amongst hospitalized patients. Generally, pneumonia accounts for about 15% of all children death according to World Health Organizations (1). Hospital acquired pneumonia (HAP) is a pulmonary infection developing during hospitalization, 48 hours or more after admission, and not present or incubating at the time of admission. Ventilator-associated pneumonia (VAP) is a type of HAP that arises more than 48–72hours after endotracheal intubation. The spectrum of pathogens involved in HAP and VAP is certainly different from that of community acquired pneumonia and is influenced by the severity of illness, presence of risk for specific pathogens and time of onset of the pneumonia (2). The pathogens that are most frequently involved in HAP are aerobic Gram-negative bacilli such as *Pseudo-*

monas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Acinetobacter sp. and *Staphylococcus aureus*. These bacteria can be considered the "core" pathogens in HAP. The role of a polymicrobial etiology of HAP has been proposed in 50% of cases (1,3).

According to World Health Organization, medicinal plants are used for therapeutic purpose and used as a pioneer in the synthesis of semi-synthetic and chemical drugs (4). About 80% of the world population use herbal medicine to treat ailment (5) because medicinal plants contain many chemical compounds such as alkaloids, flavonoids, glycosides, saponins, resins, oleoresins, sesquiterpene, phenolic compounds, fats and oils amongst others that possess therapeutic or ameliorating ability (6). The plants are easily available and cheaper than the conventional drugs and one of such plants is *Citrus aurantifolia* (lime fruit). Lime in its natural state is widely used in West Africa, particularly in the southwestern part of Nigeria.

The peel of lime fruit is commonly used in the treatment of pneumonia together with aluminum potassium sulfate (KAl (SO_4).$12H_2O$) commonly known as Alum. When added to sugar and palm oil or to honey, the juice has been found to be an excellent cough relieving mixture. The rind is burnt in some homes to act as insecticides against mosquitoes while the mesocarp is also used as a very good facial scrub that helps in prevention of pimples due to its cleansing action on the skin (7). Due to the increasing rate of antibiotic resistance, there is need to explore medicinal plants or agents with reported ameliorating effects for the provision of less costly, relatively available and effective alternatives to combat the burden of antibiotic resistance

Materials and method:

Study area

This research was carried out at Sobi Specialist hospital in Ilorin, Kwara State Nigeria. The hospital is located in the eastern part of Ilorin on N 8°31' 29.0784" and E 4°33' 13. 3236".https://www.findlatitudeandlongitude.com/l/Sobi+Road%2C+Ilorin%2C+Nigeria/2962723/)

Study design

This was a cross sectional study using simple random sampling method for recruitment of consenting participants. The study further employed an *invitro* study for the antibacterial efficacy testing of isolates against commercially available antibiotics, alum and *Citrus aurantifolia* (lime fruit).

Study population and subject participants

The population of study included consenting participants/patients (age ≥ 10 years) from surgical, accident and emergency and maternity wards of Sobi specialist hospital. The population size was estimated to be <500 and thus, using the Conroy (8) table for sample size determination which required a minimum of 81 participants, a sample size of 100 was drawn over a period of three (3) months (Dec, 2019 to March, 2020).

A total of 100 hundred throat swab samples and structured questionnaires (closed ended) were utilized for the study. Inclusion criteria included patients with >48 hours on hospital admission, productive cough, breathing difficulty, chest pain and previous use of antibiotics. Approval for the study was obtained at the Kwara State Ministry of Health with approval number: MOH/KS/EU/777/341.

Data collection

The structured questionnaires was used to obtain information about the socio-demographic characteristics (age, gender) and socio-economic status (level of education, occupation) and other related factors such as duration of hospital stay, use of antibiotics and patients' exposure to risk factors (such as medical history, smoking, cough) associated with hospital acquired pneumonia.

Clinical sample collection

Throat swab samples were obtained from patients who meet the inclusion criteria through the laboratory technologist of the hospital. The swab stick was carefully labeled corresponding to the patients' identity number. The sealed container containing the swab sample was then transported to the Public Health and Infectious Laboratory, Department of Microbiology, University of Ilorin for analysis.

Citrus and Alum sample collection/preparation

Citrus aurantifolia (identification number; TSN852203) and Alum was purchased from Maraba Park in Ilorin, Kwara State, Nigeria. The peel of *Citrus aurantifolia* was extracted from the fruit, dried under the shade and blended to powdery form using an electric blender. The powdery form was weighed using electric weighing balance and Aluminum foil. The powdery form was divided into two different places, weighed and stored in separate conical flask for the respective solvents i. e. methanol and hexane.

Five (5), 2.5 and 1.5 gram of the powder samples were weighed into conical flask contain-

ing 100 ml of hexane and methanol separately to give 500mg/ml, 250mg/ml and 150mg/ml concentration respectively. The mixture in the conical flask was placed in flask shaker for 72 hours to ensure homogeneity of the sample in the solvent. Each of the solvent mixture was then filtered using a filter paper (Whatman No. 4) after 72 hours of incubation (9). Aqueous extraction of alum was done by dissolving crystals of alum [$KAl(SO_4).12H_2O$] completely in 100 ml hot distilled water at 92ºC to prepare concentrations of 10, 20, 30, 40 and 50 w/v % at pH 3.6 respectively.

Laboratory analysis of throat samples

Selective and general-purpose media was used and they include MacConkey, Eosin-Methylene blue agar, Mannitol salt agar, Mueller Hinton agar and Nutrient agar. The preparation of the respective media was with adherence to manufacturer protocol as well as use of laboratory manual of Fawole and Osho (10) and Anibijuwon et al., (9). The throat swab samples were cultured on the prepared selective media for isolation and screening of bacteria isolates (11). Identification was achieved through Gram staining technique and conventional biochemical tests while isolate confirmation was by polymerase chain reaction (PCR) assay.

DNA extraction and PCR procedure

The DNA of the isolates was extracted by suspending 4 to 5 pure colonies from culture media in 300 µl of 1 x TBE buffer in appropriately labelled Eppendorf tubes. The cells were boiled at 100ºC for 10 minutes and were cooled rapidly on ice for 30 minutes. Three (3) µl of proteinase K was added to the lysed cells and the mixture was incubated for 15 minutes at 60ºC. The enzyme was denatured by boiling at 100ºC for 10 minutes and was centrifuged at 13,400 rpm for 3 minutes. The supernatant containing the DNA was transferred into a fresh sterile Eppendorf tube and stored at -20ºC until required for PCR.

PCR was performed in a 20µl reaction containing 4µl of master mix, 0.5µl of forward primer, 0.5µl of reverse primer, 13µl of nuclease free water and 2µl of DNA lysate. Amplification conditions were initial denaturation at 95ºC for 5min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 58ºC, extension at 72ºC for 1 min, and final extension at 72ºC for 10min. Table 1 shows the details of the primers for the target genes of respective isolates.

Gel electrophoresis

At the completion of the amplification, PCR products were resolved on l.5% agarose gel prepared by dissolving 1.5g of agarose powder in 100 ml of 1 x Tris-Borate-EDTA (TBE) buffer solution inside a clean conical flask and into which 0.5 µl of ethidium bromide has been added. The agarose plate was placed inside the electrophoresis tank containing 1 x TBE solution. Five (5) µl of the amplicon was mixed with 1µl of loading buffer and the mixture was loaded to the wells of the agarose gel. The power supply was adjusted to 100 volts for 25 minutes. For each run, a 100 base-pair molecular weight DNA standard (size marker) was used to determine the size of each PCR product. The DNA bands were then visualized with a short wave ultra-violet trans-illuminator and photographed using gene gel bio-imaging system.

Table 1: Primer sequences used for amplification confirmation of the bacteria isolates

Bacteria	Genes	Sequence	Base pair	Annealing temp	Reference
Acinetobacter baumannii	OXA-51-F OXA-51-R	TAA TGC TTT GAT CGG CCT TG TGG ATT GCA CTT CAT CTT GG	353bp	58ºC	Woodford et al. (27)
Staphylococcus aureus	Staph 756F Staph 756R	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	756bp	55ºC	Zhang et al. (28)
Escherichia coli	TEcol553 TEcol754	TGGGAAGCGAAAATCCTG CAGTACAGGTAGACTTCTG	258bp	58ºC	Maheux et al. (29)
Klebsiella pneumoniae	Pf Pr1	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130bp	58ºC	Einas et al. (30)

Antibacterial susceptibility of isolates against selected antibiotics

Antibiotic susceptibility testing of isolated organisms was carried out using the disc diffusion method. The isolates were spread on solidified Mueller Hinton agar and an antibiotic disc was aseptically placed on the surface of the media using sterile forceps. Plates was incubated at 37°C for 18-24 hours, after which plates was observed for zones of inhibition and characterized according to the most recent Clinical and Laboratory Standard Institutes (CLSI) guidelines (9,12,13,14). The following commercially available antibiotics were used against the isolates; ciprofloxacin (CPR), ofloxacin (OFL), augmentin (AUG), nitrofuran (NIT), ampicillin (AMP), ceftazidime (CAZ), cefuroxime (CRX), and gentamicin (GEN).

Antibacterial screening of *Citrus aurantifolia* peel against bacteria isolates

Suspension of the microorganisms were made in sterile normal saline and adjusted to 0.5 McFarland standards, which contains approximately 1 x 10^8 CFU/ml. A sterile swab stick was dipped into the peptone water containing the standardized organisms and then streaked on the solidified sterile Mueller-Hinton agar. Wells of 6 mm in diameter and about 2 cm apart were punched in the culture media with a sterile cork borer. The respective plant extract from the solvents were dropped into each well to fullness (approximately 100 µl) before incubating at 37°C for 24 hours. The zones of inhibition around the wells, measured in millimeters, were used as positive anti-bacterial activity.

Antibacterial screening of Alum against bacteria isolates

The agar well diffusion method was also used for the determination of antibacterial activity of aluminum potassium sulphate (Alum) aqueous extracts against the bacterial isolates. Loop-full growth from bacterial isolate was inoculated into peptone water, incubated at 37°C for 18 hours. The bacterial suspensions were diluted with normal saline, and the turbidity adjusted to 0.5 McFarland standards to yield a uniform suspension containing 1×10^8 CFU/ml. Muller Hinton agar was inoculated with bacterial inoculum, and a sterile cork borer was used to make wells of 6 mm diameter on the inoculated media. Then, 0.1ml of aqueous extracts of each alum concentration was added to the wells and incubated at 37°C for 24 hrs. The activity of alum aqueous extract was determined by measuring the diameter of inhibition zone in millimeter.

Determination of minimum inhibitory concentration (MIC) of *C. aurantifolia*

The MICs of the *Citrus* lime peel (methanol and hexane) extracts were determined for selected isolates using tube dilution susceptibility test. Two-fold serial dilutions of each extract i. e. 500 mg/ml was carried out in four tubes containing sterile 2 ml Mueller Hinton broth to give the following extract concentrations; 300, 200, 100 and 50 mg/ml. A loopful of the standardized test organisms was inoculated aseptically into the tubes containing the serially diluted extracts and incubated at 37°C for 24 hours. The concentration that showed no visible growth was taken as the MIC of the extract for each isolate (15).

Determination of minimum bactericidal concentration (MBC) of *C. aurantifolia*

Samples from the tubes used in MIC determination that showed no microbial growth were sub-cultured on Mueller Hinton agar and incubated at 37°C for 24 hours. The MIC tube with least concentration that showed no visible growth on the plate was taken as the MBC.

Data analysis

The data were analyzed to determine association and significance using the Statistical Package for the Social Sciences (IBM SPSS version 26.0). Informative and relevant data and result were presented in the best self-explanatory form either as figures, charts and/ or tables.

Results:

Demographic characteristics and predisposing factors of subject participants

Of the 100 participants recruited for the study, 50% (n=50) had spent less than 48 hours on admission at the hospital while 50% (n=50) have spent beyond 48 hours on admission. There are 30% males represented while 70% are females (Table 2). Table 3 shows the predisposing factors and medical history amongst the participants. About 28% have a previous history of chronic cough, 10% had history of smoking, and 22% exhibited symptoms of chest pain. A total of 31% participants were admitted due to severe underlying medical conditions while 68% were on antibiotics treatment at the time of study.

Table 2: Demographic characteristics of hospitalized participants at Sobi Specialist Hospital, Ilorin, Nigeria

Demographic characteristics	No of participants (%)
Gender	
Male	30 (30.0)
Female	70 (70.0)
Age group (years)	
11-20	17 (17.0)
21-30	34 (34.0)
31-40	6 (6.0)
41-50	18 (18.0)
51-60	8 (8.0)
61-70	10 (10.0)
>70	7 (7.0)
Educational status	
Non-educated	12 (12.0)
Primary	18 (18.0)
Secondary	36 (36.0)
Tertiary	34 (34.0)
Religion	
Islam	26 (26.0)
Christianity	74 (74.0)
Traditional	0
Others	0
Marital status	
Single	19 (19.0)
Married	77 (77.0)
Divorced	0
Widowed	4 (4.0)
Occupation	
Student	19 (19.0)
Civil servant	8 (8.0)
Business	55 (55.0)
Artisan	10 (10.0)
Unemployed	19 (19.0)

Table 3: Clinical characteristics and medical history of hospitalized patients at Sobi Specialist Hospital, Ilorin

Factors	Frequency (%)
Underlying illnesses	
Severe	21 (21.0)
Non-severe	79 (79.0)
Smoking history	
Yes	10 (10.0)
No	90 (90.0)
Symptom of cough	
Yes	28 (28.0)
No	72 (72.0)
Chest pain	
Yes	22 (22.0)
No	78 (78.0)
Administration of antibiotics	
Yes	68 (68.0)
No	32 (32.0)

Table 4: Bacterial pathogens colonizing the throat of hospitalized patients at Sobi Specialist Hospital, Ilorin

Bacterial isolates	Number (%)
Staphylococcus aureus	6 (42.8)
Acinetobacter baumannii	4 (28.6)
Escherichia coli	2 (14.3)
Klebsiella pneumoniae	2 (14.3)
Total	**14 (100.0)**

Bacteria isolates

A total of 14 bacterial isolates were recovered from throat swab samples collected from the 100 patients (Table 4). The most frequent bacterium was *Staphylococcus aureus* 6 (43%), followed by *Acinetobacter baumannii* 4 (28%) while *Klebsiella pneumoniae* and *Escherichia coli* had 2 (14.5%) each.

PCR assay confirmed the identity of the bacterial isolates as shown in the plates. Plate 1 (lane 2 is positive control and lane 4 is test organism positive for *E. coli*), plate 2 (lane 1 and 7 are positive controls, lane 6 is test organism positive for *K. pneumoniae*), plate 3 (lane 1 is positive control and lane 5 and 6 are organisms positive for *A. baumannii*) and plate 4 is *S. aureus.* Ten bacterial isolates were recovered from the throat samples of female patients (33.3%, 10/70) while 4 were recovered from those of the male patients (13.3%, 4/30) (OR=0.01582, 95% CI=0.3111-3.773, *p*=1.00)

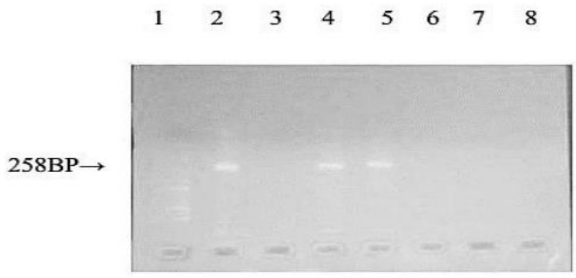

Plate 1: PCR product of *E. coli* on agarose gel (258bp)

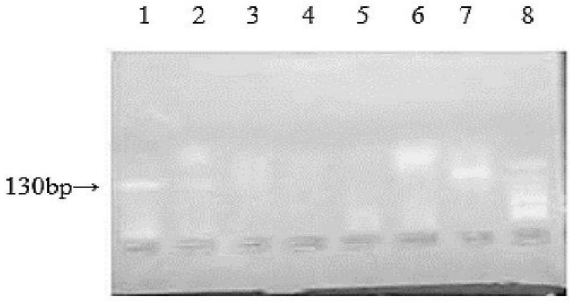

Plate 2: PCR product of *K. pneumoniae* on agarose gel (130bp)

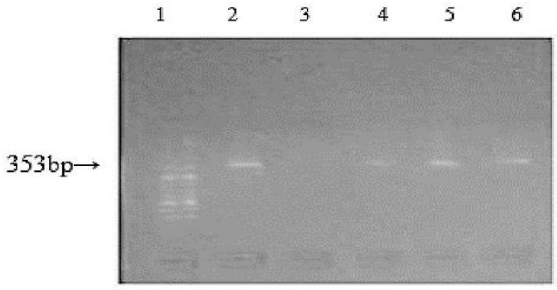

Plate 3: PCR product of *A. baumannii* on agarose gel (353bp)

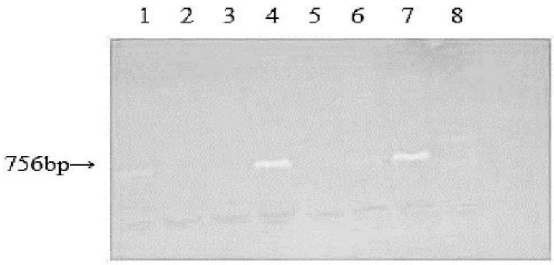

Plate 4: PCR product of *S. aureus* on agarose gel (756bp)

Antibacterial profile of isolates against selected antibiotics

Table 6 reveals the profile of the isolates in relation to the antibiotics. Of the 8 standard antibiotics tested, 3 isolates (*A. baumannii* TSS3*, K. pneumoniae* TSS6 and *A. baumannii* TSS37) were resistant to all while 3 others (*K. pneumoniae* TSS33*, S. aureus* TSS42 and *E. coli* TSS65) were resistant to at least five antibiotics. The inhibitory activity of hexane extract of *C. aurantifolia* compares favorably with the commercial antibiotics used in the study. The highest inhibition zone against *A. baumannii* TSS3 was 12 mm with nitrofuran (NIT) while hexane extract of *C. aurantifolia* gave highest inhibition zone of 19.2mm.

Although, the concentrations of the crude plant extracts were higher than the concentrations of the standard antibiotics tested, amoxicillin-clavulanate (augmentin AUG) was most active against *K. pneumoniae* TSS33 with zone diameter of inhibition of 24.5mm while the crude plant extract gave the highest inhibition zone diameter of 17.4mm. With exception of amoxicillin-clavulanate (AUG) for *K. pneumoniae* TSS 33, ciprofloxacin (CPR) and ofloxacin (OFL) for *S. aureus* TSS42 and ofloxacin for *E. coli* which had higher inhibitory effects, the plant crude extracts had comparative inhibitory effect or more active than the standard antibiotics, with diameters of inhibition zones ranging from 11.5-19.2mm (for hexane extract) and 9.8-15.8mm (for methanol extract).

Minimum inhibitory and bactericidal concentrations of *Citrus aurantifolia* peel

The MIC was carried out on isolates that were sensitive to the extract. Table 7 shows that the MIC of hexane extracts of *C. aurantifolia* for the isolates to range from 5-10 mg/ml while the MIC for methanol extracts range from 10-25 mg/ml. The MBC of hexane extracts for the isolates ranged from 25-50 mg/ml while the MBC of methanol extracts ranged from 50-100 mg/ml.

Table 5: Association of throat colonization by bacterial pathogens in relation to gender and age of hospitalized patients at Sobi Specialist Hospital, Ilorin, Nigeria

Characteristics	No of participants	No positive for enteropathogens (%)	X^2	OR (95% CI)	*p* value
Gender					
Male	30	4 (13.3)	0.016	1.083	1.000
Female	70	10 (33.3))		(0.3111-3.773)	
Total	100	14 (14.0)			
Age group (years)					
11-20	17	1	15.290	0.852	0.018
21-30	34	3		(0.737-0.967)	
31-40	6	1			
41-50	18	0			
51-60	8	3			
61-70	10	3			
>70	7	3			
Total	100	14 (14.0)			

X^2=Chi square; OR = Odd ratio; CI = Confidence interval

Table 6: Comparative inhibitory effects (inhibition zone diameters) of extracts of *Citrus aurantifolia* peel and standard antibiotics on bacterial isolates colonizing throats of hospitalized patients in Sobi Specialist Hospital, Ilorin, Nigeria

Bacterial isolates	**Antibiotic disc zone of inhibition (mm)**								**Hexane extract zone of inhibition (mm)**		**Methanol extract zone of inhibition (mm)**	
	CPR (5µg)	OFL (5µg)	AUG (30µg)	NIT (30µg)	AMP (30µg)	CAZ (30µg)	CRX (30µg)	GEN (10µg)	H (50mg/ml)	H (33mg/ml)	M (50mg/ml)	M (33mg/ml)
A. baumannii TSS3	6	11	8	12	9	7	10	9	19.2	16.20	13.3	10.0
K. pneumoniae TSS33	18	14	24.5	11.9	13	20	9	9	17.4	16.0	12.5	-
K. pneumoniae TSS6	9	10	8	6	7.7	8	9.2	8.3	13.7	11.5	15.8	-
A. baumannii TSS37	11	9.5	13	10	8	7	11	9.7	18.2	16.0	12.1	-
S. aureus TSS42	21	20	15	11	9	12	7	9	15.0	17.1	14.0	9.8
E. coli TSS65	14	25	8	9.3	13	12	15	17	15.1	13.7	10.1	-

CPR: Ciprofloxacin 5µg, OFL: Ofloxacin 5µg, AUG: Augmentin 30µg, NIT: Nitrofuran 300µg, AMP: Ampicillin 5µg, CAZ: Ceftazidime 30µg, CRX: Cefuroxime 30µg, GEN: Gentamicin 10µg. S: Sensitive, R: Resistant. H= Hexane and M= Methanol; - = no inhibition.

Table 7: Minimum inhibitory and minimum bactericidal concentrations of *Citrus aurantifolia* peel extracts against selected bacterial isolates colonizing the throats of hospitalized patients in Sobi Specialist Hospital, Ilorin, Nigeria

Isolates	**MIC (mg/ml)**										**MBC (mg/ml)**	
	Hexane extract					**Methanol extract**					**Hexane**	**Methanol**
	5	**10**	**25**	**50**	**100**	**5**	**10**	**25**	**50**	**100**		
A. baumannii TSS3	+	-	-	-	-	+	+	+	-	-	25	100
K. pneumoniae TSS33	+	+	-	-	-	-	+	-	-	-	50	50
K. pneumoniae TSS6	+	-	-	-	-	+	+	-	-	-	25	50
A. baumannii TSS37	+	-	-	-	-	+	+	-	-	-	25	50
S. aureus TSS 42	+	+	-	-	-	+	+	+	-	-	25	50
E. coli TSS65	+	-	-	-	-	+	+	-	-	-	25	50

'+' represents turbid (growth). '-' represents not turbid (no growth)

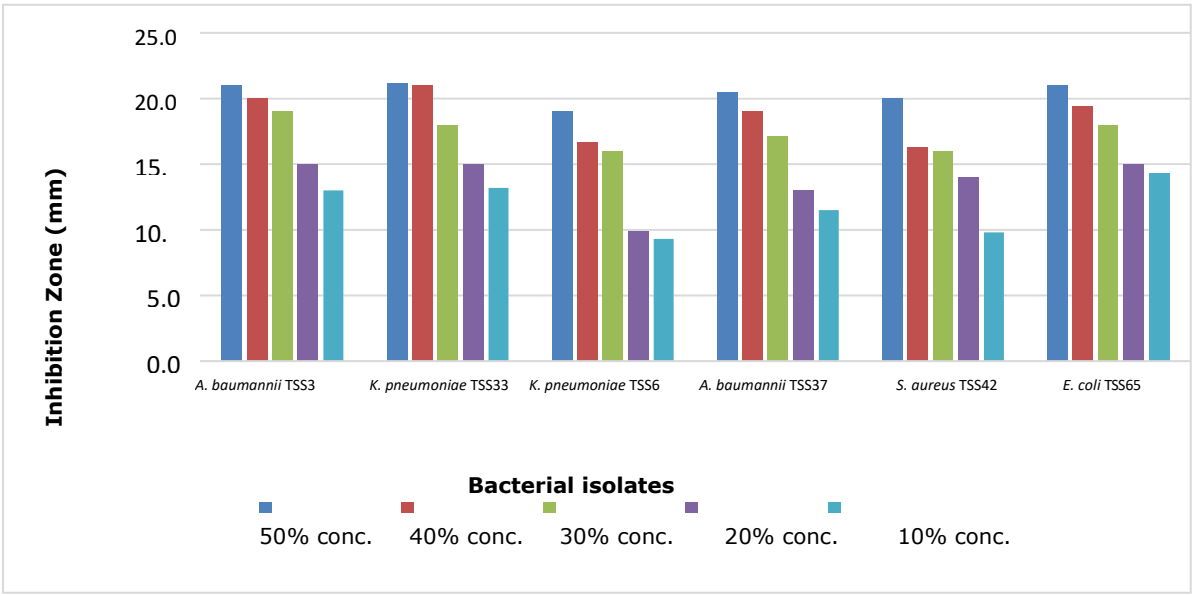

Fig 1: Antibacterial activity of aqueous extract of Alum on bacterial isolates colonizing the throats of hospitalized patients in Sobi Specialist Hospital, Ilorin, Nigeria

Antibacterial activity of Alum against isolates

Aqueous extracts of Alum [$KAl(SO_4).12H_2O$] at different concentrations (w/v%), produced zone diameters of inhibition for the isolates, ranging from 9.3 - 21.2 mm, which was concentration dependent, as shown in Fig 1.

Discussion:

The overall prevalence of bacteria pathogens colonizing the throat of hospitalized patients in this study was 14%. This is significant because presence of such pathogens can predispose to development of pneumonia as a result of immune compromise. The recorded prevalence is higher than 6.9% reported in Eastern Ethiopia in a nosocomial infection study (16) but lower than the reported 67% from patient in a referral hospital, Sikkim (17). The location as well as prevalence of predisposing factors such as underlying illness, hygiene and smoking history which could compromise the immunity of participants could account for the discrepancy of prevalence.

The outcome of antibiotic activity testing showed that isolates were multi-drug resistant bacteria with the isolates resistant to three or more classes of antibiotics, including aminoglycosides, fluoroquinolones, penicillin and cephalosporins. In Africa, higher rates of antibiotics resistant bacteria (10-100%) are reported among isolates from hospital-acquired infections (18), and multidrug resistant pathogens have continued to create critical health challenging issues especially in our environment with little or non-existent functional health infrastructure to cater for increasing population of the citizens.

In this study, both strains of *A. baumannii* and a strain of *K. pneumoniae* were completely resistant to antibiotics while others show 75% resistance to antibiotics and this agreed with the work of Levin et al. (19) but not in compliant with the work of Lim et al., (2) that reported HAP that were highly susceptible to antibiotics which could have resulted from geographical locations and practices such as controlled antibiotic usage and administration that could affect the emergence and distribution of resistant strains. However, it may possibly be thought that some of the reasons for the geometric rise of multi drug resistance cases might be connected to poor prescriptions expertise of antibiotic regimen and non-adherence to drug intake pattern recommendation by the subjects as suggested by the physician, even as it seems to be a verification of lack of health awareness education on the subject matter among the subjects, in our remote communities.

Increase in sensitivity of the test organisms to *C. aurantifolia* (lime) and alum solution was noticed to improve as the concentration increases and this corresponds to a previous report (20). Antibiotic susceptibility of test organisms was found to be relatively low compared to their susceptibility to lime peel extract and alum. This could have resulted from the difference in measurement where the crude extract had higher concentration compared to the standard antibiotics that was maintained at the standard quantity. The pattern of antibiotic activity could also be attributed to the general increase in antibiotic resistivity of bacteria isolates that is gradually on rise. Alum was however, most effective against test organisms having highest inhibition zone at 50% concentration. Out of the four organisms isolated from throat swab samples in this research, *S. aureus* was the only Gram-positive organism and it was observed to show the least sensitivity to the test agent. This finding is in accordance to the work of Nwankwo et al., (20) which posits that due to the nature of their cell wall, Gram-negative bacteria are more sensitive to antimicrobial agents.

Some compounds in citrus fruit can provide additional protection for the body against chronic disease and basic nutrition. Citrus fruits contain lots of phytochemicals, including essential oils, alkaloids, flavonoids, ouramin, psoralens, and carotenoids. Previous pharmacological studies revealed that citrus fruits have antimicrobial, anti-helminthic, insect repellent, antioxidant, anticancer, cardiovascular, anti-inflammatory, analgesic, anti- diabetic, reproductive, gastrointestinal, immunological, respiratory and many other pharmacological effects (21). Flavonoid compounds have properties that are effective in inhibiting the growth of bacteria, fungi, and viruses because these compounds include groups of phenol that can denaturize bacterial cell proteins and damage bacterial cell membranes (22).

Doughari et al., (23) stated that the anti-microbial effect of the plant could be due to the bioactive compounds such as the phytochemicals constituent present in the plant. The finding of this study showed that the efficacy of *C. aurantifolia* leaves extracts on the test isolates had different hierarchy of susceptibility among the organisms. The findings of this study indicated that higher concentration of the extract shows a larger zone of inhibition. This result agrees with that of Bisno and Stevens (24) which reported that the higher the concentration of antibacterial substance, the higher the zone of inhibition. However, this is most significant

for crude substances where higher quantity may be required to capture the active components. Thus, it is expected that with purified extract of concentrated bioactive agents, a lower quantity would yield required antibiotic activity.

The result of the antibacterial activity of *C. aurantifolia* leaves extracts in the present study was in conformity with studies that reported its antibacterial potentials (25,26). The molecular report of the target genes in the respective bacteria was confirmatory because they conform to the expected band size as reported by Woodford et al., (27), Zhang et al., (28) Maheux et al., (29) and Einas et al., (30).

Conclusion:

In conclusion, findings from this study showed the presence of resistant pathogenic bacteria colonizing the throat of hospitalized patients receiving care at the Sobi Specialist hospital, Ilorin. The isolates were resistant to aminoglycoside, fluoroquinolone and penicillin group of antimicrobials which are among the commercially available antibiotics. Although, the extract utilized were crude and of higher concentration, it can be concluded based on the recorded inhibition capacity that further studies to characterize, purify and isolate the active antibacterial components in the extracts should be considered. This can be harnessed as novel compounds for the production of antimicrobial drugs. There is need for proper health education to enlighten the public on the increasing trend of multi-drug resistant pathogen, transmission, prevention and control, which is not limited to eradication of indiscriminate use of antibiotics and self-medication.

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Authors contributions:

OAO, OMK, and MMS were involved in result computation, interpretation and manuscript preparation; OMK and OOA were involved in the study design while OAO, IBB, and OOA implemented the field work. All authors read the manuscript.

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