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Bioassay-Guided Discovery of Antibacterial Agents: *In vitro* Susceptibility of Multi-drug Resistant *Staphylococcus aureus* to *Psidium guajava* Linn Extracts

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Authors' contributions

This work was carried out in collaboration between all authors. Author DB designed the study, wrote the protocol, performed the statistical analysis, managed the analyses of the study and wrote the first draft of the manuscript. Author GDF provided assistance in running the experiments and management of the analyses of the study. Author DAE provided suggestions and concept for the study. Author SK assisted in preparation of extracts and running the experiments. Author BE donated the S. aureus. Author HB supplied the Psidium guajava leaves. Author HBD assisted in extract concentration while Authors SA and MAA provided assistance in culturing the test organisms with AAA coordinating the research activities.

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ABSTRACT

Aims: Development of multi-drug resistance to antibiotics is a major health concern worldwide. This study assessed the antibacterial activities of leaves extracts from *Psidium guajava* against multi-drug resistant *Staphyllococcus aureus* strains.

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Study Design: Three (3) different extracts were prepared from the dried leaves of *P. guajava* and tested against the multi-drug resistant strains isolated from clinical samples.
Place and Duration of Study: Microbiology department, Centre for Plant Medicine Research. From October 2014 to March 2015.
Methodology: Absolute ethanol, 70% ethanol and aqueous extracts were prepared using maceration and concentrated by rotary evaporation techniques. The lyophilized materials were reconstituted in serial concentrations for antimicrobial assessments using the agar well diffusion method. The minimum inhibition concentration was determined by broth microdilutions method.
Results: Although, all the 14 strains exhibited various antibiotypes to 10 different commonly used antibiotics; none of them was resistant to the 3 extracts. There was 1/14 (KTHMDR-5) strain being highly susceptible to aqueous extract. In addition, the absolute ethanol extract was effective against 8/14 strains at 25 mg/ml and 4/14 strains at 12.5 mg/ml, whiles the aqueous extract was effective against 13/14 strains and 11/14 strains at 25 and 12.5 mg/ml respectively. The 70% ethanol extract showed much stronger activity against all the 14 strains than the aqueous and absolute ethanol extracts from 200 to 25 mg/ml with only 1/14 strain (KTHMDR-2) not being sensitive at a concentration of 12.5 mg/ml. The MIC values for all the strains were considerably low ranging from 0.78 to 6.25 mg/ml. There was a very strong significance difference ($P < 0.0001$) between the antimicrobial activities of 70% ethanol extract and the absolute ethanol.
Conclusion: The 14 multi-drug resistant *S. aureus* strains were susceptible to the 3 extracts with 70% ethanol extract exhibiting the most significant antimicrobial activity. Therefore, it is important to utilize this extract in drug formulations.

Keywords: Antibacterial; susceptibility; multi-drug; resistant; Staphylococcus aureus; Psidium guajava.

1. INTRODUCTION

Staphylococcus aureus is a Gram-positive, facultative anaerobe, non-motile, non-spore forming catalase and coagulase positive bacteria with diameter ranging from 0.5-1.5 µm. It belongs to the Staphylococcaceae family and considered to be a major pathogen of both humans and animals [1,2]. It is naturally found on the skin, mucous membranes and in the nasopharynx of the human body as normal micro-flora. Pathogenic *S. aureus* causes infections such as pustules, abscesses formation, septicemia, pneumonia, osteomyelitis, renal abscess, endocarditis, meningitis, gastroenteritis and toxic shock syndrome. In addition, it produces variety of extracellular enzymes and heat stable enterotoxins that cause food poisoning [3].

Multi-drug resistance (MDR) in *S. aureus* strains is a serious cause of public health concern. Resistance development in *S. aureus* strains is mediated by four main mechanisms: (1) Drug inactivation, (2) Alteration of target site, (3) alteration of metabolic pathway and (4) reduced drug accumulation [4]. The most frequent antibiotics resistance in *S. aureus* are methicillin and vancomycin.

Methicillin resistant *S. aureus* (MRSA) remains a global health concern as a result of resistance to the commonly used β-lactam antibiotics such as penicillin derivatives, cephalosporins, carbapenems and monobactams. Penicillin resistant strains of *S. aureus* typically produce *β*-lactamase enzyme that inactivates the β-lactam ring of the penicillin structure and thereby deactivates penicillin. It becomes difficult to successfully treat infections caused by such resistant strains. Consequently, treatment options for MRSA infected patients are limited, resulting in extended periods of ill health and in some cases high mortality rates. The MRSA is developed when methicillin-susceptible *S. aureus* (MSSA) acquires the methicillin-resistance gene mecA by horizontal gene transfer mediated by a mobile genetic element known as staphylococcal cassette chromosome (SCC). It is a site-specific transposon-like element mainly used among staphylococcal species [5].

Another important drug resistance situation is the vancomycin resistance in *S. aureus* (VISA). Some important antibiotic resistance phenotypes of MRSA are also acquired by spontaneous mutations. The most well known are rifampin resistance and fluoroquinolone resistance. Vancomycin resistance is also acquired by mutation. The current definition of VISA is *S. aureus* strain having vancomycin MIC of 4 or 8 mg/L whiles vancomycin-susceptible *S. aureus* (VSSA) should have MIC of 2 mg/L [5]. The VISA is developed by accumulation of several spontaneous mutations [6,7]. Nevertheless, vancomycin is still prescribed as the first-line antibiotic against MRSA. However, its clinical

effectiveness is compromised even against the strains whose vancomycin MICs are within the Clinical and Laboratory Standards Institute (CLSI) susceptible range of 2 mg/L [8,9]. There are other forms of vancomycin resistance that are acquired through adaptive mutations [5]. Considering the threats posed by MRSA and VISA as well as other antibiotics, it is critical to identify and develop a broad-spectrum drug for effective control.

Medicinal plants offer excellent source of therapeutic agents for many conditions and diseases due to the presence of important phytochemicals. One such plant is *Psidium guajava* Linn*.* It belongs to the Family Myrtaceae and considered a native to Mexico [10] and extends throughout the South America, Europe, Africa and Asia. The *Psidium guajava* Linn is commonly called guave (French), Guayave (German), banjiro (Japanese), guaiaba (Brazil), guayaba (Español) and guava (English) [11]. *Psidium guajava* is a small tree of about 10 m high with thin, smooth, patchy, peeling bark. Leaves are opposite, short-petiolate, the blade oval with prominent pinnate veins and about 5–15 cm long [12]. There has been a tremendous interest in this plant as evidenced by the voluminous work available [13].

Psidium guajava has been used in folk medicine to treat various diseases such as malaria, gastroenteritis, diarrhea, coughs, sore throat, dental plaque removal, antihyperglycaemic and a number of other conditions [14]. The part of the plant mostly used is the leaves, followed by the fruits, bark and the roots [15]. Though tremendous works have been conducted on *P. guajava*, few studies have demonstrated the antimicrobial effects of this medicinal plant on multiple drug *resistant S. aureus.* Recent study by Simone et al. [16] tested methanol and aqueous extracts of *P. guajava* stem bark against eight MRSA isolated from humans and animals. Both extracts were effective against the isolates with mean inhibition zones ranging from 5 to 20 mm. The minimum inhibitory concentration (MIC) of the stem bark aqueous extracts ranged from 125 to 500 µg/ml while that of the methanol extract from 62.5 to 250 µg/ml. The study also showed that *P. guajava* is an important source of carbohydrates, glycosides, tannins and proteins. Most recently, another study by Valle et al. [17] using ethanol leaf extract of *Psidium guajava*, showed significant antibacterial activity against four MRSA and one vancomycin-resistant *Enterococcus* isolated from humans with IZ ranging from 12–18 mm. Therefore, *P. guajava* extracts have potential to be explored for important drug formulation.

The aim of this study was to assess the antibacterial activities of *P. guajava* extracts against multi-drug resistant *S. aureus* (MDR-SA) strains obtained from different clinical samples. It was expected that the data generated from the effective anti-MDR-SA extract would be helpful for development of new therapeutic agents for better management of infections associated with MDR-SA.

2. EXPERIMENTAL DETAILS

2.1 Origin and Antibiotypes of Staphylococcal Strains

Staphylococcal strains were provided by the Bacteriology Department (Noguchi Memorial Institute for Medical Research), which carried out prospective cross-sectional study to identify and characterize 290 *Staphylococcus aureus* strains (between October 2010 – June 2012. The strains were obtained from clinical specimens (blood, wound swap and nasal swap) collected from inpatients seeking health care at 4 different facilities in Ghana: Sunyani Government Hospital (Central Zone), korle Konno Community hospital and Korle bu Teaching Hospital and University Hospital (Southern zone). The strains were identified as *S. aureus* by Gram staining, catalase, tube coagulase and slidex staphplus tests. Antimicrobial susceptibility testing was carried out to determine the resistance status of the strains. Briefly, disc diffusion technique was used in the susceptibility test according to the European Committee on Antimicrobial Susceptibility Testing guidelines. In all 21 antibiotics were screened. These were 1U penicillin (pen), 30 mg tetracycline (tet), 30 mg cefoxitin (cef), 2 mg clindamycin (cli), 15 mg erythromycin (ery), 10 mg norfloxacin (nor), 10 mg gentamicin (gen), 10 mg linezolid (lin), 5 mg rifampicin (rif), 1. 25 mg (+23.75 mg) trimethoprim/sulfamethoxazole (TMS), and 10 mg fusidic acid (fuc). The rest are kanamycin (kan), moxifloxacin (mox), 200 mg mupirocin (mup), oxacillin (oxa), teicoplanin (tei), tigecyline (tig), ceftaroline (ceft), ceftobiprole (cefto) daptomycin (dap) and vancomycin (van) [18]. In all, 14 multi-drug resistant *Staphylococcus aureus* strains were identified and characterized (Table 1).

Table 1. Origin and characteristics of multidrug resistant *Staphylococcus aureus* strains

MDR-SA code	Origin	Infection	Carrier/ place	Antibiotype
KTHMDR-1	blood	bacteremia	1, KTH	cef-pen-tet
KTHMDR-2	wound	wound infection	1, KTH	cef-pen-tet
KTHMDR-3	nasal swab	asyptomatic carriage	1, KTH	cef-pen-tet
KTHMDR-4	blood	bacteremia	1, KTH	cef-pen-tet
KTHMDR-5	blood	bacteremia	1, KTH	cef-cli-ery-fuc-gen-pen-tet
KTHMDR-6	wound	wound infection	1, KTH	cef-cli-ery-gen-nor-pen-tet
KTHMDR-7	wound	wound infection	1, KTH	cef-cli-ery-gen-nor-pen-tet
KTHMDR-8	wound	wound infection	1, KTH	cef-cli-ery-gen-kan-nor-mox-pen-tet
UHMDR-11	nasal swab	asyptomatic carriage	1, UH	cef-pen-tet
KGCHMDR-12	nasal swab	asyptomatic carriage	1, KGCH	cef-pen-tet
SGHMDR-16	-	-	1, SGH	cef-pen-tet
KTHMDR-22	nasal swab	asyptomatic carriage	1, KTH	cef-pen-tet
KTHMDR-23	nasal swab	asyptomatic carriage	1, KTH	cef-pen-tet
KTHMDR-24	nasal swab	asyptomatic carriage	1, KTH	cef-pen-tet

KTH: Korle bu Teaching Hospital, UH: University Hospital, KGCH: Korle Gonno Community Hospital, SGH: Sunyani Government Hospital. MDR: multi drug resistant, pen: penicillin, cef: cefoxitin, tet: tetracycline, gen: gentamicin, fuc: fucidic acid, cli: clindamycin, ery: erythromycin, nor: norfloxacin, kan: kanamycin, Mox: moxifloxacin

Multidrug resistance (MDR) was defined as resistance to at least three distinct antimicrobial classes including cefoxitin. Furthermore, inducible clindamycin resistance was detected by the D-test. The Brain Heart Infusion agar supplemented with teicoplanin (5 mg/L) was used to screen MRSA strains for glycopeptides resistance by a spot test; where 10 or more colonies survived on the plates, E-tests were performed for MIC with vancomycin and teicoplanin. Resistance status was further confirmed by standard molecular typing methods using multiplex PCR for the detection of *spa, lukS/F-pv* and *mecA* as well as DNA sequencing. There were 14 strains resistant to 10/21 commonly used antibiotics screened (penicillin, cefoxitin, tetracycline, gentamicin, fucidic acid, clindamycin, erythromycin, norfloxacin kanamycin and moxifloxacin). The strains were susceptible to only 11/21 antibiotics (ceftaroline, ceftobiprole, daptomycin, linezolid, teicoplanin, tigecyline, trimethoprim/sulfamethoxazole, *oxacillin, rifampicin, mupirocin* and vancomycin).

2.2 Preparation of Extracts

Fresh leaves of *Psidium guajava* were provided by the Plant Development Department at Centre for Plant Medicine Research (CPMR) where plant identification procedures were carried out by a taxonomist to ensure it authenticity. These were air-dried under strict hygienic conditions and pulverization using milling machine at CPMR. To prepare absolute or 70% ethanolic extracts, 150 g of the pulverized material was macerated in 1.5 L analytical grade absolute (not diluted) or 70% ethanol for 72 h and then filtered with Watmann paper 1 plugged in a funnel. Each filtrate was concentrated under vacuum using rotary evaporator (Eyela, N-1110, Tokyo Rikakikai Co. Ltd, Japan) at 50^{o}C and the residue freeze-dried (Eyela, FPU-1200, Tokyo Rikakikai Co. Ltd, Japan) to obtain lyophilized material. In the case of the aqueous extract, similar amount of the pulverized *P. guajava* leaves was added to a volume of 1.5 L sterile distilled water and then boiled in stainless steel container for 45 minutes with intermittent stirring for effective extraction. This was similarly filtered and freeze-dried at the CPMR. The lyophilized extracts obtained for each extract was stored at 4℃ until used.

2.3 Sub-culturing of MDR-SA and Standardization of Inoculum

To ensure that all the strains received (stored at -20) were viable, Mueller Hinton agar plates were

prepared for each isolate. About 50 µl of each strain pipetted from storage vial was spread on the respective plate followed by incubation at 37℃ for 18 – 24 h. The plates were store at 4 ^{o}C until used. In addition, a reference standard (ATCC 25923) obtained from the American Type Culture Collection was included. Each inoculum was standard as described by the Manual of Antimicrobial Susceptibility Testing of the American Society of Microbiology [19]. Briefly, 5 colonies of each MDR-SA was picked into 5 ml Nutrient Broth agar (Oxoid, Hampshire, England) and incubated at 37℃ for 18-24 h. The resulting turbidity was adjusted to the 0.5 McFarland's standard.

2.4 Antimicrobial Susceptibility Testing

Antibacterial activities of the aqueous, absolute and 70% ethanol extracts prepared from the *P. guajava* leaves were evaluated by the agar-well diffusion technique [20-22]. An inoculum of 0.5 McFarland turbidity standard [23] was spread on Mueller Hinton agar (Oxoid, Hampshire, England), plates using swap sticks followed by creation of 6 mm wells using a cork borer to ensure uniform wells on the plates. Respective wells were filled with 80 µl of reconstituted extracts in serial dilution pattern (200, 100, 50 25 and 12.5 mg/ml). Experiment for each strain was prepared in duplicate plates and incubate at 37℃ for 18 – 24 h. The zone of inhibition was measured in millimeter (mm) using a simple measuring rule. Ciprofloxacin (pure compound obtained from Ernest Chemist Ltd, Ghana), 15 µg/ml, a second-generation fluoroquinolone antibiotic [24,25], was used as positive control while either 5% dimethyl sulfoxide (DMSO) (Sigma, 472301) or deionized water was used as negative control.

2.5 Broth Microdilution

Minimum inhibition concentration (MIC) for the 70% *P. guajava* leaves extract was determined using the broth microdilution technique. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye [26]. The MIC was determined by broth microdilution method as described by Wiegand et al. [26]. Briefly, the extract was reconstituted in sterile bacteriological peptone (peptone water) to obtain 100 mg/ml. In addition, 15 µg/ml ciprofloxacin (cipro) was prepared with deionized water and used as positive control. To obtain very viable colonies, the strains were subcultured in Mueller Hinton agar plate over night (18-24 hrs) at 37℃. Inoculum for the MIC test was prepared by transferring 5 well-isolated colonies from each respective plate into culture tube containing 5 ml of Mueller Hinton broth. The broth culture of each strain was incubated (2 to 6h) at 37℃ until turbidity comparable to 0.5 McFarland standards (1.5×10^{8} CFU/ml) was achieved.

The broth microdilution was performed in a 96-well microtiter plate whereby the test wells were respectively filled with 100 µl of the serially diluted extract (100-0.05 mg/ml) followed by addition of 100 µl of the appropriate inoculum. Serially diluted ciprofloxacin antibiotic (15 - 0.007 µg/ml) was also accordingly inoculated. Blank wells contained inoculum with no extracts. All the procedures were performed aseptically and the plates cultured at 37^{o}C over night (18-24 h). After, 40 µl of 0.2 mg/ml *p*-Iodonitrotetrazolium violet (INT) prepared in deionized water was added to each well and further incubated for 30 minutes. To determine the MIC well, wells where bacterial growth occurred indicated the reduction of INT to red formazan precipitates. On the other hand, wells in which the strains did not survived showed no change in the INT purple colour. To read the MIC value for each strain, well which did not show INT colour change and next to a well having INT colour change was considered as the minimum concentration of the extract at which the growth of the strains could not be inhibited by the extract.

2.6 Determination of Percentage Inhibition

The percentage inhibition or relative inhibition ($\%C_r$) of the test extract with respect to positive control (ciprofloxacin) was computed to assess the relative strength of the concentrations used. This was performed by the following equation:

$$\%C_r = \frac{(x - y)\ x\ 100}{(z - y)}$$

Where, x = total area of inhibition zone of the test extract; y = total area of inhibition zone of the diluent; z = total area of inhibition of the ciprofloxacin. The total area of the inhibition zone was calculated by; area = πr^2; where, r = radius of zone of inhibition.

2.7 Statistical Analysis

The data generated were stored and analyzed in Microsoft Excel programme (version 2011). Values were presented as means±SD. Statistical differences among the extract were tested by Paired t test and ANOVA using Graphpad Prism 6. A difference in the mean values of $P = 0.05$ was considered as statistically significant.

3. RESULTS

3.1 Distribution of the MDR-SA

The *S. aureus* strains were obtained from patients seeking health care from 4 health care facilities (Korle bu Teaching Hospital, University Hospital, Korle Gonno Community Hospital and Sunyani Government Hospital) located in different municipalities of Ghana. There were 14 *S. aureus* strains (Fig. 1) that were isolated from blood, wounds, nose (6, 42.9%) and an unknown infection.

3.2 Antimicrobial Activities of the Extracts

The antibacterial activities of the aqueous, absolute and 70% ethanolic extracts of *P. guajava* leaves were evaluated by the agar-well diffusion technique against multi-drug resistance *S. aureus* (MDR-SA). The antimicrobial activities were evaluated to provide data on the effectiveness of the extracts against the strains, which could be formulated into a broad-spectrum anti-microbial product. The results obtained with regards to each extracts are represented in the following subsections.

3.2.1 Antimicrobial activity of the aqueous extract

The aqueous *P. guajava* leaves extract was screened against the 14 resistant strains at varying concentrations. As shown (Table 2), all the strains were susceptible to the extract at 200, 100 and 50 mg/ml. At 25 mg/ml, there was only 1/14 strain (SGHMDR-16) that was not susceptible and at 12.5 mg/ml 3/14 strains (KTHMDR-1, KTHMDR-3 and KTHMDR-16) were not susceptible since the extract concentration might be too weak to cause effect on these strains. However, the isolate KTHMDR-5 was highly susceptible showing average inhibition zones of 19±1.4, 17±1.4, 15.5±2.1, 14±2.1 and 11.5±2.1 mm at 200, 100, 50, 25 and 12.5 mg/ml respectively. The KTHMDR-8 appeared to be the next most susceptible strain with 18±0.0, 16±0.0, 15.5±0.7, 15±1.4 and 13±1.4 mm at the same concentrations respectively. The rest of the strains responded fairly well to the various concentrations. Though, all the strains were resistant to multiple antibiotics, none was found resistant to the aqueous.

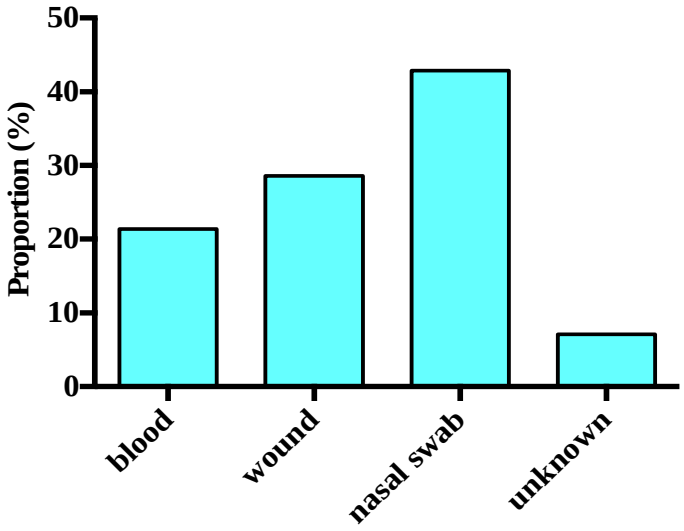

Fig. 1. The distribution of resistant *Staphylococcus aureus* that were tested for susceptibility to the *Psidium guajava* extracts
Origin of resistant Staphylococcus aureus

Table 2. Antimicrobial activities of aqueous *Psidua guajava* leaf extract on MDR-SA. The zones of inhibition values (mm) represent an average of duplicate experiments

MDR-SA code	Serial concentration of aqueous extract (mg/ml)					Cipro,15 µg/ml	H2O
	200	100	50	25	12.5		
KTHMDR-1	13.5±0.7	11.5±0.7	9±1.4	4±5.7	0±0.0	16±1.4	0±0.0
KTHMDR-2	15±0.0	13±0.0	10.5±0.7	9.5±0.7	4±5.7	20±0. 0	0±0.0
KTHMDR-3	14±1.4	11.5±2.1	9.5±2.1	4±5.7	0±0.0	18.5±2 .1	0±0.0
KTHMDR-4	15±1.4	14±1.4	12.5±0.7	10.5±0.7	9.5±0.7	19 ±0.0	0±0.0
KTHMDR-5	19±1.4	17±1.4	15.5±2.1	14±2.1	11.5±2.1	19± 1.4	0±0.0
KTHMDR-6	16.5±0.7	16.5±0.7	15.5±0.7	14±0.0	10.5±0.7	19.5±0.7	0±0.0
KTHMDR-7	13.5±0.7	12.5±0.7	11.5±0.7	10±0.0	8.5±0.7	15.5±2.1	0±0.0
KTHMDR-8	18±0.0	16±0.0	15.5±0.7	15±1.4	13±1.4	19.5± 0.7	0±0.0
UHMDR-11	17±0.0	16±0.0	15±1.4	14±0.0	10±0.0	18.5±0. 7	0±0.0
KGCHMDR-12	17.5±0.7	15±0.0	14±0.0	12.5±0.7	9±1.4	18 ±1.4	0±0.0
SGHMDR-16	13.5±2.1	11±1.4	4±5.7	0±0.0	0±0.0	15±0.0	0±0.0
KTHMDR-22	17.5±0.7	16±0.0	15±0.0	12.5±0.7	9±1.4	17. 5±0.7	0±0.0
KTHMDR-23	15±0.0	12.5±0.7	11.5±0.7	5±7.1	4±5.7	16.5 ±2.1	0±0.0
KTHMDR-24	15±0.0	13±1.4	11±0.0	9±1.4	4±5.7	14.5±0.7	0±0.0
ATCC 25923	15.5±0.7	14±0.0	12±1.4	10.5±0.7	8.5±0.7	18.5±0.7	0±0.0

KTHMDR: multi-drug resistant S. aureus isolated from Korle bu Teaching Hospital, UHMDR: multi-drug resistant S. aureus isolated from University Hospital, SGHMDR: multi-drug resistant S. aureus isolated from Sunyani Government Hospital, KGCH: multi-drug resistant S. aureus isolated from Korle Gonno Community Hospital, ATCC: reference standard from American Type Culture collection

3.2.2 Antimicrobial activity of the absolute ethanolic extract

The absolute ethanol *P. guajava* extract recorded quiet different level of efficacy yet similar to the aqueous extract (Table 3). There were activities at 100 up to 50 mg/ml against all the strains as observed for the aqueous extract. However, at 25 mg/ml concentration, 6/14 strains (KTHMDR-1, KTHMDR-2, KTHMDR-5, KTHMDR-7, UHMDR-11 and KTHMDR-23) were not susceptible while 4/14 strain (KTHMDR-4, KTHMDR-6, SGHMDR-16 and KTHMDR-24) were susceptible at 12.5 mg/ml with varying inhibition zones. As shown, the aqueous was able to kill 13/14 and 11/14 strains at 25 and 12.5 mg/ml concentrations respectively.

In comparison, the aqueous extract but not the absolute ethanol extract was effective at all concentrations against 4/14 strains that had at least 7 antibiotypes (KTHMDR-5, KTHMDR-6, KTHMDR-7 and KTHMDR-8) including cefoxitin. Among these 4 strains, 3 (KTHMDR-6, KTHMDR-7 and KTHMDR-8) were isolated from wound infections. Indicating how difficult it might be to successfully treat such patients.

3.2.3 Antimicrobial activities of 70% ethanolic extract

The 70% ethanol *P. guajava* extract was also tested against all the 14 and the reference standard strains for antimicrobial susceptibility. Interestingly, it showed much stronger activity against all the strains than the aqueous and absolute ethanol extracts as shown in Table 4. The 70% ethanol extract was active at concentrations from 200 to 25 mg/ml against all the strains. However, 1/14 strain (KTHMDR-2) was not susceptible at a concentration of 12.5 mg/ml and the same strain was also observed to be not susceptible at 25 and 12.5 mg/ml concentration of the absolute ethanol extract (Table 3). The KTHMDR-2 strain, which was isolated from wound infection and had antibiotype cef-pen-tet, might be very different from the strains with similar antibiotypes (Table 1).

3.3 Percentage Inhibition

The antimicrobial activities of the extracts were compared with the strength of the reference standard drug (ciprofloxacin) in terms of percentage inhibitions. The activity of ciprofloxacin was considered as 100% inhibitions (%C_r). As shown (Figs. 2 – 6), at 200 mg/ml, the 70% extract had 11/14 strains having %C_r greater than 100% (from 106.15 - 137.46%) with 1/11 (KTHMDR-8) having the highest value of 137.46%, that is, highly sensitive to the 70% extract than to the ciprofloxacin. The KTHMDR-2 was the least sensitive strain showing 66.02%. These observations were quiet different for the

aqueous and the absolute ethanol extracts, where only 4/14 MDR-SA (KTHMDR-24, SGHMDR-16, KTHMDR-22 and KTHMDR-5) and 2 MDR-SA strains (KTHMDR-5 and SGHMDR-16) had 100% similar to that of the cipro. However, with regards to the rest of the strains (Figs. 3-6), apart from the 100 mg/ml of the 70% extract where KTHMDR-5 showed greater (113.78%) than 100% inhibition, the various concentrations showed less than 100% value.

Table 3. Antimicrobial activities of absolute ethanol *Psidua guajava* leave extract on MDR-SA. The zones of inhibition values (mm) represent an average of duplicate experiments

MDR-SA code	Serial concentration of absolute ethanolic extract (mg/ml)					Cipro, 15 µg/ml	DMSO (5%)
	200	100	50	25	12.5		
KTHMDR-1	14.5±0.7	11.5±0.7	9.5±0.7	0±0.0	0±0.0	16.5 ±0.7	0±0.0
KTHMDR-2	13.5±0.7	11±1.4	8.5±0.7	0±0.0	0±0.0	15.5±0 .7	0±0.0
KTHMDR-3	13±0.0	11.5±0.7	10±0.0	8.5±0.7	0±0.0	17.5± 0.7	0±0.0
KTHMDR-4	17±0.0	16±0.0	11±0.0	10±0.0	9±0.0	17.5±0.7	0±0.0
KTHMDR-5	15±0.0	11±0.0	9±0.0	0±0.0	0±0.0	15±0.0	0±0 .0
KTHMDR-6	14.5±0.7	12±0.0	9.5±0.7	8.5±0.7	8±0.0	16±0 .0	0±0.0
KTHMDR-7	14±0.0	12±0.0	9.5±0.7	0±0.0	0±0.0	15±0.0	0 ±0.0
KTHMDR-8	13.5±0.7	11±0.0	10±0.0	8±0.0	0±0.0	14.5±0. 7	0±0.0
UHMDR-11	13.5±0.7	11.5±0.7	9.5±0.7	0±0.0	0±0.0	15±0 .7	0±0.0
KGCHMDR-12	14.5±0.7	12.5±0.7	10.5±0.7	9.5±0.7	0±0.0	16.5±0.7	0±0.0
SGHMDR-16	14±0.7	13±0.7	11±0.7	9±0.0	8±0.0	14±0.0	0 ±0.0
KTHMDR-22	13±0.0	12±0.0	10±0.0	8±0.0	0±0.0	17±0.7	0 ±0.0
KTHMDR-23	13.5±0.7	10.5±0.7	9±0.0	0±0.0	0±0.0	16±0.0	0±0.0
KTHMDR-24	13.5±0.7	12.5±0.7	10±0.0	9±0.0	8±0.0	17.5 ±0.7	0±0.0
ATCC 25923	18.5±0.7	16.5±0.7	13.5±2.1	12±1.4	10.5±0 .7	21.5±0.7	0±0.0

KTHMDR: multi-drug resistant S. aureus isolated from Korle bu Teaching Hospital, UHMDR: multi-drug resistant S. aureus isolated from University Hospital, SGHMDR: multi-drug resistant S. aureus isolated from Sunyani Government Hospital, KGCH: multi-drug resistant S. aureus isolated from Korle Gonno Community Hospital, ATCC: reference standard from American Type Culture collection

Table 4. Antimicrobial activities of 70% ethanol *Psidua guajava* leaves extract on MDR-SA. The zones of inhibition values (mm) represent an average of duplicate experiments

MDR-SA code	Serial concentration of 70% ethanolic extract (mg/ml)					Cipro, 15 µg/ml	DMSO, 5%
	200	100	50	25	12.5		
KTHMDR-1	17.5±0.7	15±0.0	12.5±0.7	10±0.7	9.5±0.7	16 .5±0.7	0±0.0
KTHMDR-2	13±0.0	11.5±0.7	10.5±0.7	8.5±0.0	0±0.0	16±1.4	0±0.0
KTHMDR-3	16.5±0.7	14±1.4	12±1.4	10±1.4	4.5±0.7	17±1 .4	0±0.0
KTHMDR-4	17±0.0	16±0.7	12.5±0.7	10±0.0	9±0.0	16.5±2 .1	0±0.0
KTHMDR-5	16.5±0.7	15±1.4	11.5±0.7	9±1.4	4.5±0.7	15± 0.7	0±0.0
KTHMDR-6	16.5±0.7	14±0.0	11.5±0.7	10±0.7	9±0.7	16±0 .7	0±0.0
KTHMDR-7	16.5±0.0	14±0.7	10.5±0.7	9.5±0.7	8.5±0.7	1 5±0.7	0±0.0
KTHMDR-8	17±0.0	14.5±0.7	12.5±0.7	10.5±0.7	8.5±0.7	14.5±0.7	0±0.0
UHMDR-11	16±0.0	14.5±0.7	13±0.0	12±0.0	10.5±0.7	15. 5±0.7	0±0.0
KGCHMDR-12	16±0.0	13.5±0.7	12±0.0	11±0.0	10±0.0	14. 5±0.7	0±0.0
SGHMDR-16	16±0.0	14±0.0	12.5±0.7	12±0.0	10.5±0.7	15 .5±0.7	0±0.0
KTHMDR-22	17±0.7	14±0.0	12±0.7	10±0.0	10±0.0	16±0.7	0±0.0
KTHMDR-23	16.5±0.7	15±1.4	12.5±0.7	10.5±0.7	10±0.0	18.5±0.7	0±0.0
KTHMDR-24	16.5±0.7	14±1.4	12±0.0	11±0.0	9.5±0.7	17. 5±0.7	0±0.0
ATCC 25923	22±0.0	19.5±0.7	16.5±0.7	13±0.0	11±0.0	2 0.5±0.7	0±0.0

KTHMDR: multi-drug resistant S. aureus isolated from Korle bu Teaching Hospital, UHMDR: multi-drug resistant S. aureus isolated from University Hospital, SGHMDR: multi-drug resistant S. aureus isolated from Sunyani Government Hospital, KGCH: multi-drug resistant S. aureus isolated from Korle Gonno Community Hospital, ATCC: reference standard from American Type Culture collection

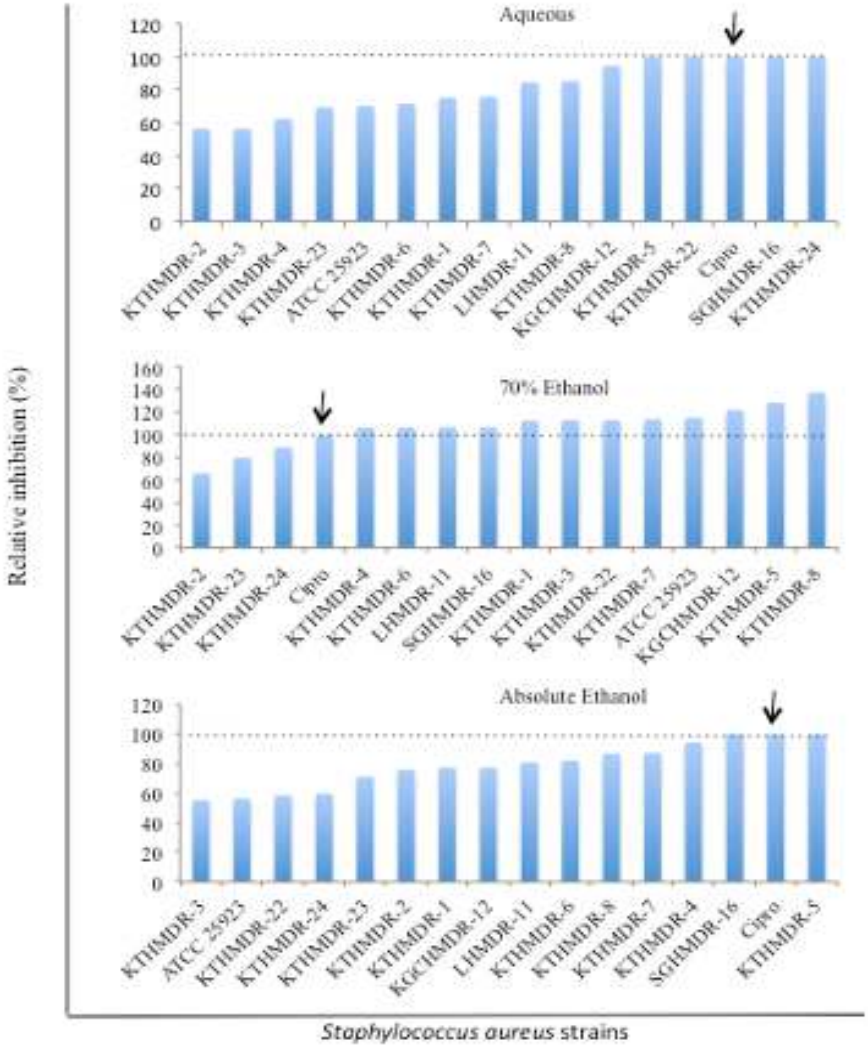

Fig. 2. Relative inhibitions of the 14 MDR-SA strains and reference standard strain at 200 mg/ml concentration of the absolute ethanol, 70% ethanol and aqueous extracts. The standard antibiotic control, ciprofloxacin is arrowed

3.4 Most Effective Extract

Three different solvents (aqueous, 70% ethanol and absolute ethanol) were used to extract the constituents in the *Psidium guajava* leaf. To assess which of the extracts is most effective and whether there were any significance differences in the antimicrobial activities, statistical analysis (P = .05) was carried out on the means of the inhibition zones obtained for each extract concentration, thus 200, 100, 50, 25 and 12.5 mg/ml. As shown (Fig. 7, A), there was a very strong significant difference (P < 0.0001) between the antimicrobial activities of the 70% ethanol extract (70%) verses that of the absolute ethanol (Abs). Again, the difference in microbial activity between the aqueous extract (Aq) verses absolute ethanol extract (Abs) was significantly different (P = 0.045) but not as strong as that of the 70% verses Abs extracts (70%/Abs). However, there was no significant difference between the 70% and Aq (P = 0.14). Nevertheless, there was an overall statistically significance difference (P = 0.003) when the three extracts were compared (Fig. 7, B – E). Therefore, the extract with most significant antimicrobial activity appears to be the 70% ethanol extract.

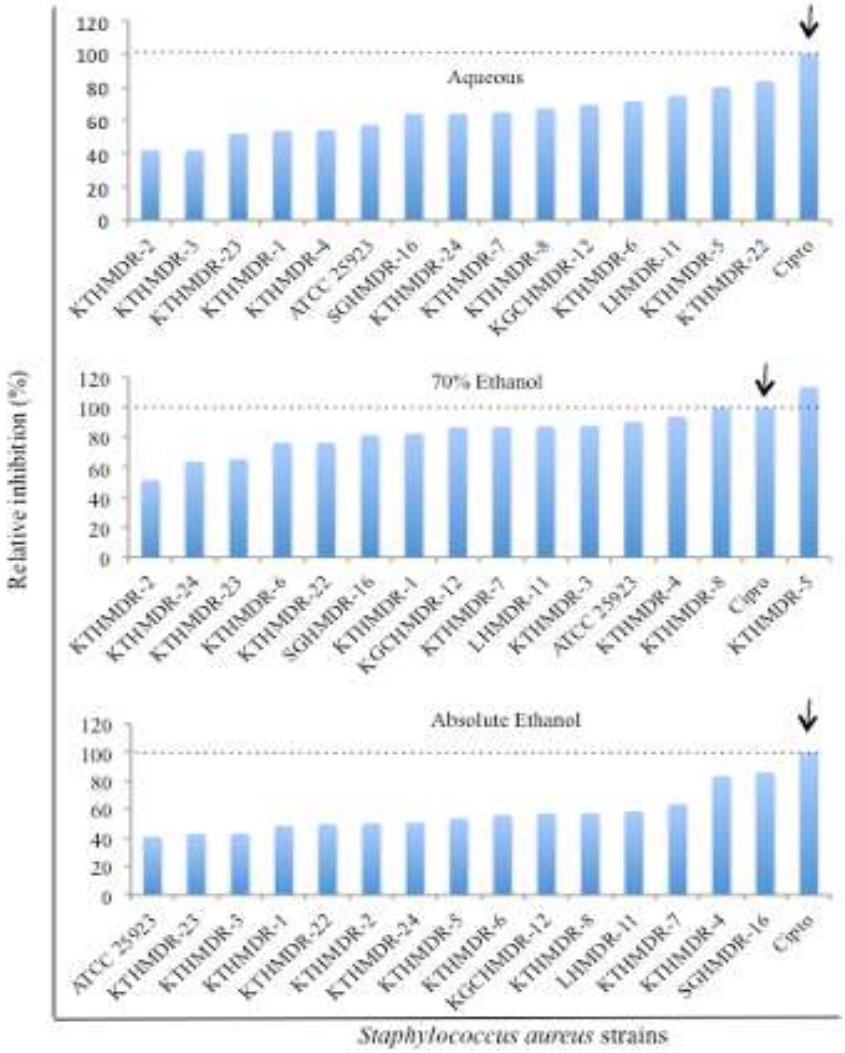

Fig. 3. Relative inhibitions of the 14 MDR-SA strains and reference standard strain at 100 mg/ml concentration of the absolute ethanol, 70% ethanol and aqueous extracts. The standard antibiotic control, ciprofloxacin is arrowed

3.5 Minimum Inhibition Concentration

The minimum inhibition concentration (MIC) values were determined for the 70% ethanol extract since it showed considerable much better antimicrobial activity against the strains. The MIC value is very essential for drug formulations and also necessary for assessing effectiveness of an antimicrobial agent. As shown (Fig. 8), the MIC values obtained for all the strains were considerably low, ranging from 0.78 mg/ml to 6.25 mg/ml. The least MIC value of 0.78 mg/ml was recorded for 1 strain (KTHMDR-23) followed by 1.58 mg/ml for KGCHMDR-12. Furthermore, 3 strains (KTHMDR-2, KTHMDR-5 and KTHMDR-8) had 6.25 mg/ml whiles the rest together with the reference standard (ATCC-25923) recorded 3.13 mg/ml.

4. DISCUSSION

Developments of multiple drug resistance of pathogenic bacterial strains are of great public health concern worldwide. *Staphylococcus aureus* has developed resistance to many antimicrobials through the acquisition of mobile drug resistance genes with the 2 most notable antibiotic resistance being methicillin and vancomycin. The methicillin resistance is acquired through interspecies transfer of mecA

gene from an ancestral *Staphylococcus* species through staphylococcal mobile genetic element, that is the staphylococcal cassette chromosome (SCC) [5,27], which is a site-specific transposon-like element used particularly among staphylococcal species [28]. The SCC elements carrying mecA (SCCmec) are integrated in the chromosomes of MRSA strains [29]. On the other hand, the vancomycin resistance is acquired from vancomycin-resistant *Enteriococcus* ancestor through horizontal transfer of plasmid containing vanA gene transposon. The ability of *Staphylococcus aureus* to evade host`s immune system facilitated by multi-drug resistance phenotype has made it one of the most perverse pathogenic bacteria in the history of antibiotic chemotherapy. Hence, there is continual search for effective antibiotics from both medicinal plants and conventional medicine.

Medicinal plants are useful source of therapeutic agents for many infections due to the inherit importance of pharmacologically active phytochemicals. Therefore, plants extracts play major role in drug-discovery systems. The study described here assessed the effectiveness of one such important medicinal plant (*Psidium guajava*) against multiple drug resistant *Staphylococcus aureus*. The *Psidium guajava* is used in folk medicine to treat various diseases including skin infections, gastroenteritis, diarrhea, coughs and sore throat [14]. Three different extracts (absolute ethanol, 70% ethanol and aqueous) were prepared from *P. guajava* leaves and screened against multi-drug resistant *S. aureus* (MDR-SA) strains obtained from clinical specimens.

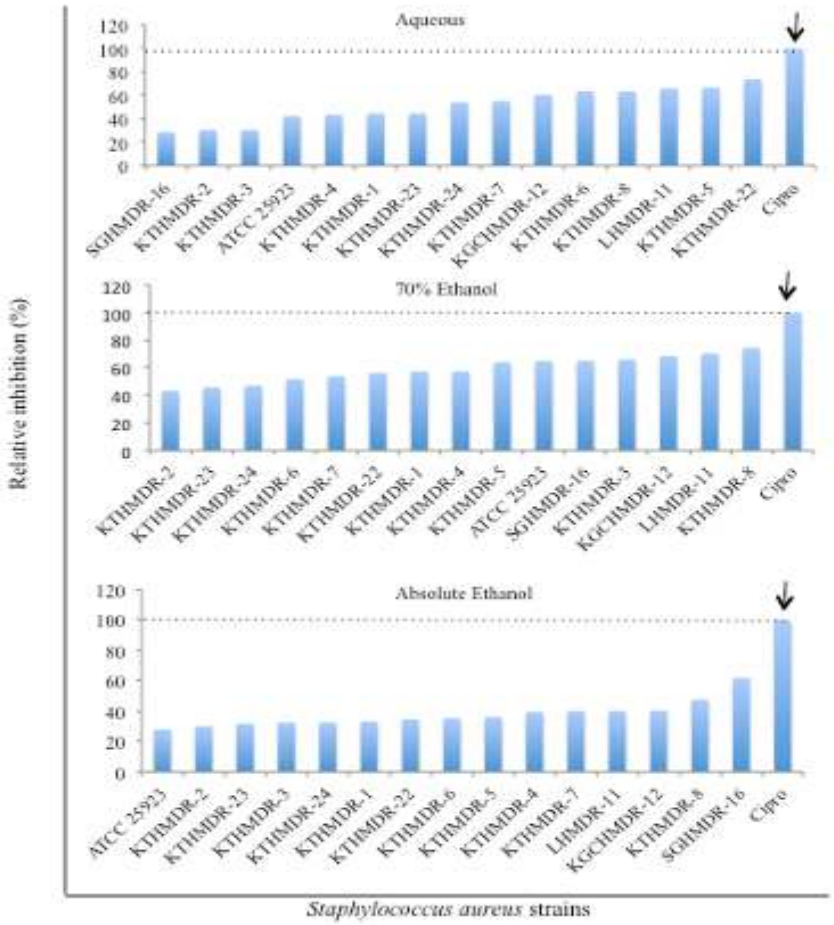

Fig. 4. Relative inhibitions of the 14 MDR-SA strains and reference standard strain at 50 mg/ml concentration of the absolute ethanol, 70% ethanol and aqueous extracts. The standard antibiotic control, ciprofloxacin is arrowed

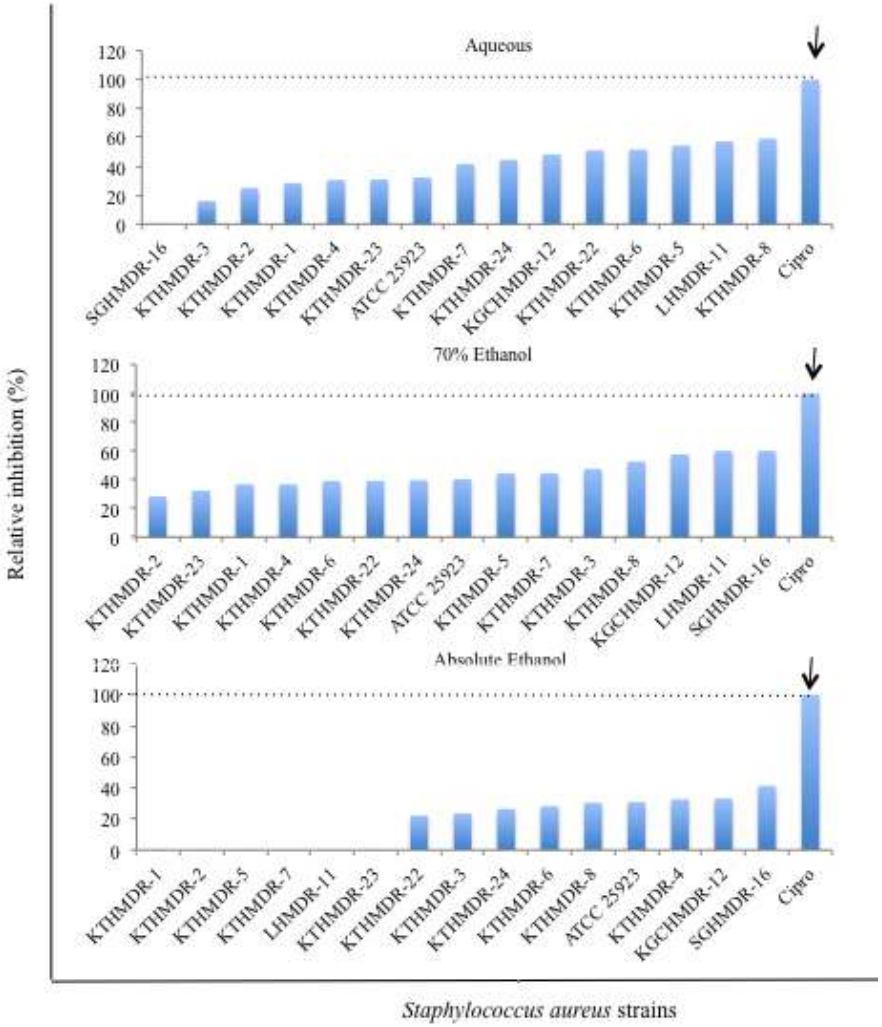

Fig. 5. Relative inhibitions of the 14 MDR-SA strains and reference standard strain at 25 mg/ml concentration of the absolute ethanol, 70% ethanol and aqueous extracts. The standard antibiotic control, ciprofloxacin is arrowed

The 3 extracts exhibited varying antimicrobial activities and were effective in inhibiting the growth of all strains screened at the concentrations (200 to 12.5 mg/ml). Similar observation was made by Chah et al. [30] when aqueous and alcoholic extracts of the *P. guajava* (root and leaves) were found to inhibit the growth of *Staphylococcus aureus, Streptococcus mutans, Pseudomonas aeruginosa, Salmonella enteritidis, Bacillus cereus, Proteus spp., Shigella spp*. and *Escherichia coli*, which are also causal agents of intestinal infections in humans. Ifeanyichukwu et al. [31], also reports of effective antimicrobial activities of methanol and ethanol leaf extracts against Gram negative and positive bacterial. Therefore, extracts from the leaves or roots could have broad-sprectrum antimicrobial activities.

The importance of antimicrobial properties of *P. guajava* becomes more intriguing when the aqueous extract of the leaves was found to be effective against more difficult bacteria such as *Clostridium perfringens* type A associated with food poisoning [32]. It inhibited the growth and spore formation and enterotoxin production. Wound healing properties of the plant was also observed when a methanolic leaf extract of the plant was studied using the excision wound model that recorded more than 90% wound

healing after 14 days post-surgery. This finding corroborate with the results obtained from the antimicrobial activities against the resistant strains isolated from wound infections (KTHMDR-2, KTHMDR-6, KTHMDR-7 and KTHMDR-8).

Interestingly, the 70% ethanol extract had significant activities against the strains making it more effective than the absolute and the aqueous extracts. Therefore, for production of a broad-spectrum antimicrobial agent from *P. guajava* leaves, this form of extraction should be considered. Moreover, it exhibited the highest percentage inhibition (137.46%) at 200 mg/ml concentration for 12/14 strains. Though the extract is in its crude form, it has proven very efficacious. The MIC is very important for monitoring development of antibiotic resistance [26] and drug formulations. The values determined for the 70% ethanolic extract was low and therefore very strong indicating that not large concentration of the extract is required to be effective.

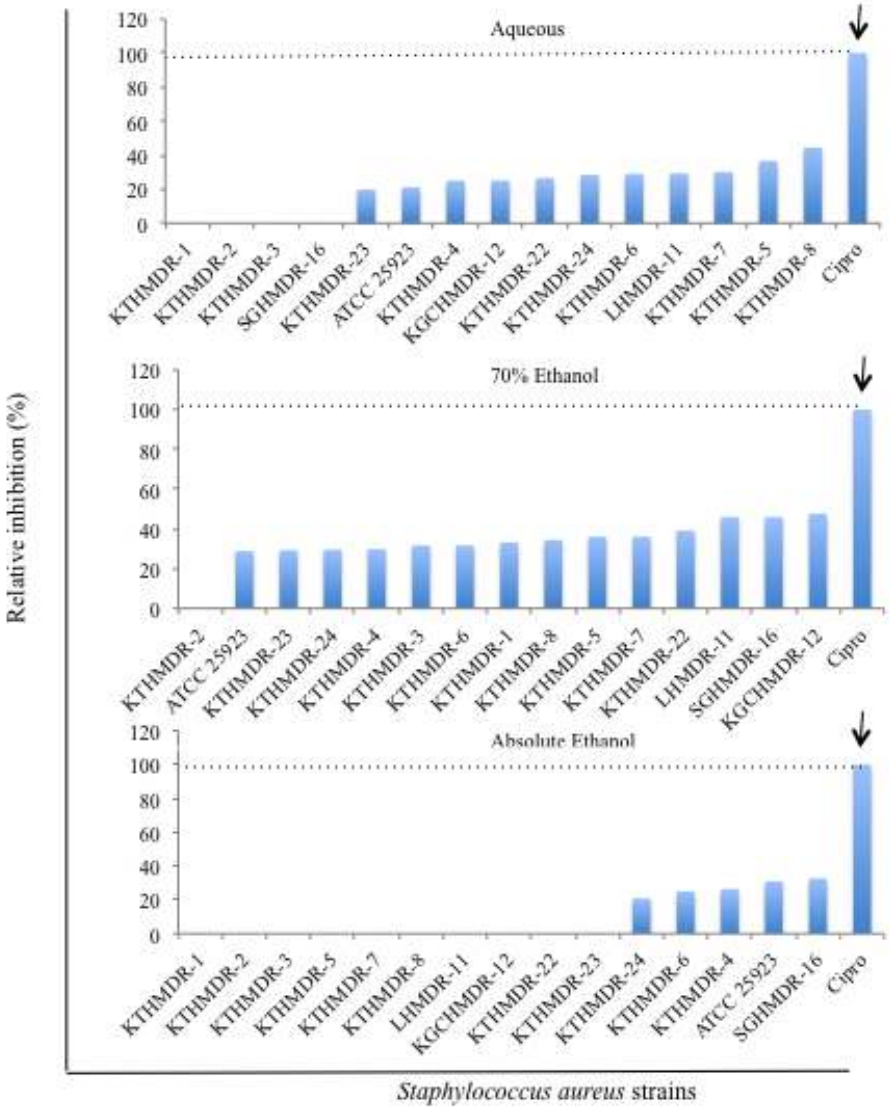

Fig. 6. Relative inhibitions of the 14 MDR-SA strains and reference standard strain at 12.5 mg/ml concentration of the absolute ethanol, 70% ethanol and aqueous extracts. The standard antibiotic control, ciprofloxacin is arrowed

The unique antimicrobial activities of the extracts observed in this study suggest possession of phytochemicals relevant for prevention of resistant *S. aureus* growth. Phytochemical studies of the leaves extract by several authors report of presence of secondary metabolites such as essential oil (e.g. limonene, α-pinene, menthol, terpenyl acetate, β-pinene, longicyclene and curcumene) [33], saponins and oleanolic acid [34]. In addition, the leaves contain flavonoids (avicularin and its derivatives 3-l-4-pyranoside) with strong antibacterial action [35]. Two triterpenoids; guavanoic acid (20β-acetoxy-2α,3β-dihydroxyurs-12-en-28-oic acid) and guavacoumaric acid (2α,3β-dihydroxy-24-*p*-*z*-coumaroyloxyurs-12-en-28-oic acid) have also been identified in the leaves [36]. Again, three antibacterial flavonoids were detected in the ethanolic and aqueous extracts, which were derivatives of quercetin [35,37]. Acylated flavonol glycoside guaijaverin from leaf and fruit showed antimicrobial activity against *Streptococcus mutans*, [37-40]. Acylated flavonol glycoside avicularin from leaf and fruit also showed antimicrobial activity against *Salmonella enteritidis and Bacillus cereus* [35]. Therefore, the effective antimicrobial activities observed in this study could be attributed to the phytochemicals found in the leaves especially the flavonoids.

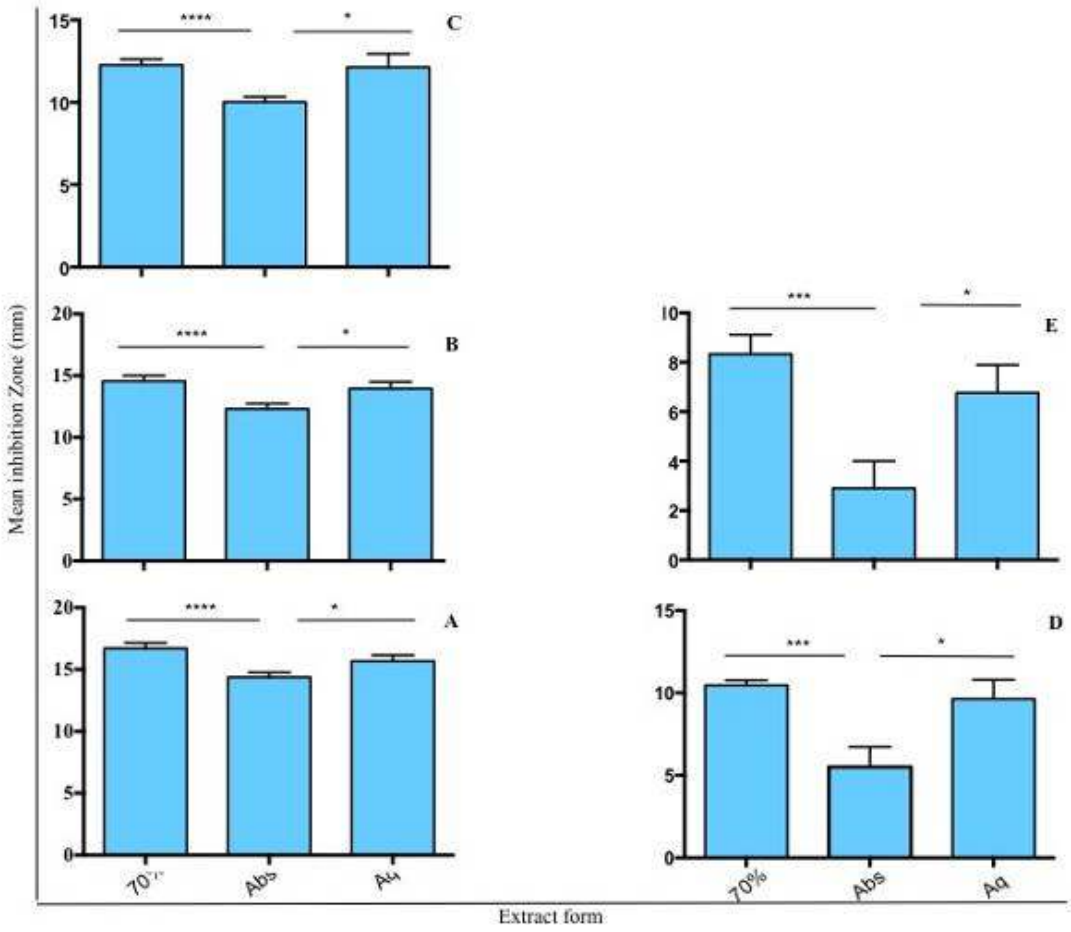

Fig. 7. Assessment of statistical differences among the three extracts in terms of antimicrobial activities. 70%: 70% ethanol extract, Abs: absolute ethanolic extract, Aq: aqueous extract, *: strength of significance differences

Extract concentrations tested were; A: 200 mg/ml, B: 100 mg/ml, C: 50 mg/ml, D: 25 mg/ml, E: 12.5 mg/ml. Statistical differences: A: *(70%/Abs, P = < 0.0001; Aq/Abs, P = 0.045; 70%/Aq, P = 0.14); B (70%/Abs, P = < 0.0001; Aq/Abs, P = 0.03; 70%/Aq, P = 0.37); C (70%/Abs, P = < 0.0001; Aq/Abs, P = 0.04; 70%/Aq, P = 0.88); D (70%/Abs, P = 0.0005; Aq/Abs, P = 0.03; 70%/Aq, P = 0.51); E (70%/Abs, P = 0.0002; Aq/Abs, P = 0.03; 70%/Aq, P = 0.24)*

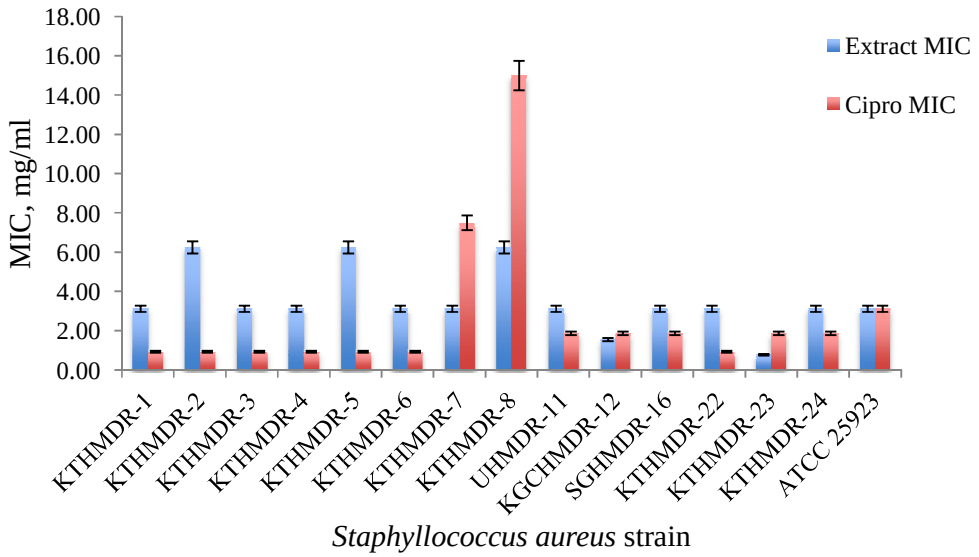

Fig. 8. The minimum inhibition concentrations of the 70% ethanol *P. guajava* extract against the resistance and reference standard strains as determined by broth microdilutions

4. CONCLUSION

All the 3 extracts tested did not only inhibit the bacterial growth (bacteriostatic activity) but effectively killed them (bactericidal activity) as well. The resulting 70% herbal extract with most effective anti-MDR-SA activity would be helpful for development of new therapeutic agent for better management of infections associated with multi-drug resistant *S. aureus* as well as other microbial infections.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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