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Anti-bacterial Activity of Crude Flavonoid Fraction from *Bidens pilosa* Leaves against Selected Chronic Wound Bacterial Pathogens

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MKN, IN and AAA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JT, AB, DO, MO, BR, JNM, EE, EK and DCM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Bidens pilosa* is an extraordinary source of phytochemicals particularly flavonoids especially in leaves which have been attributed in various studies due to its antibacterial properties. The present study aimed at addressing bio-burden of chronic wound through proving a possible source of new antimicrobial product for wound healing.

Methodology: Solvent-solvent extraction method was used to isolate crude flavonoid fraction from leaves of *B. pilosa* using ether, chloroform, ethylacetate and methanol (1:1:1). Thin-layer chromatography was used to identify crude flavonoid fraction using methanol/n-hexane (1:2: v/v) as mobile phase solvents. Agar well diffusion method was used to determine anti-bacterial activity of crude flavonoid against bacterial pathogens: Susceptible *Pseudomonas aeruginosa* ATCC®27853™, resistant *Pseudomonas aeruginosa* susceptible *Staphylococcus aureus* ATCC®25923™, methicillin resistant *Staphylococcus aureus, Streptococcus pneumoniae* and methicillin resistant *Staphylococcus epidermidis*. Minimum inhibitory concentration (MIC) and Minimum bactericidal contrition (MBC) were also determined using broth dilution and culture methods.

Results: Thin-layer chromatographic profiling revealed an identity of three different spots with flavonoids from *B. pilosa* leaves showing three bands with Rf values 0.51, 0.60 and 0.63. The mean and standard deviation zone of inhibition of crude flavonoids ranged from 11.50±0.50 mm to 17.50±1.50 mm. The mean and standard deviation of positive controls (Ciproflaxacin, Co-Amoxiclay and Voncomycin) ranged from 0.00±0.00 to 22.50±0.50 mm. MIC and MBC of crude flavonoids ranged from 12.5-25.0 mg/mL and 50 to 200 mg/mL respectively. The flavonoid fraction was more effective against gram positive bacteria than on gram negative bacteria and it exhibited bactericidal effect on Methicillin resistant *Staphylococcus aureus*, resistant *P. aureginosa, s*ensitive *P.aureginosa* and *S. pneumonia.*

Conclusion: *B. pilosa* leaves could be a potential source for future drug development from flavonoid to address the issue of need for new antibiotics due to alarming burden of antimicrobial resistance in last resort antibiotics.

Keywords: Bidens pilosa leaves; flavonoids; chronic wounds; bacterial pathogens.

1. INTRODUCTION

Plants as natural sources of herbal drugs were used in traditional system and they represent a precious reservoir of innovative bioactive molecules. Among them; *Bidens pilosa* L. which is one of the important plants found in all tropical and subtropical regions of the world [1]. There are 230 to 240 known Bidens species. Among them, *B. pilosa* is a representative perennial herb, globally distributed across temperate and tropical regions. *B. pilosa* was first collected and named by Carl Linnaeus in 1753 taxonomically and assigned it to the Bidens genus [2]. It is an erect, perennial herb widely distributed across the temperate and tropical regions. It grows as either glabrous or hairy plant with green opposite leaves that are serrated, lobed, or dissected. It has white or yellow flowers and long narrow ribbed black achenes (seeds). It grows to an average height of 60 cm and maximum of 150 cm in favorable environments [1].

Previous phytochemical studies on *B. pilosa* have shown that it was affluent in alkaloids, saponins, tannins, aliphatics and aromatic, hydrocarbons, phenylheptatriyne, cytopiloyne, phytosterols, chalcones, aurones, centaurein, centauredin, caffeic acids, glycosides, polyacetylenes, terpenoids, porphrins, and nitrogen and sulphur containing compounds and many flavonoids derivatives [3-9].

According to Bartolome et al. [1], the whole plant of *B. pilosa* and all its parts including; aerial parts (Leaves, flowers, seeds), stems and roots are ingredients of folk medicine used locally. Additionally, either whole plant or different parts have been reported to be useful in the treatment of more than 40 disorders such as; chronic wounds, inflammation, immunological disorders, digestive disorders, infectious diseases, cancers, metabolic syndrome and many others.

Chronic wounds are injuries that heal poorly, failing to proceed through the normal healing stages, and generally taking over three months to close [10]. Pastar et al. [11] reported that several studies have shown that *Staphylococcus aureus* and *Pseudomonas aeruginosa* are among the most prevalent pathogens isolated

from both acute and chronic wounds non-healing wounds.

However according to Serra et al. [12], intensive use of antibiotics over many years is resulting in the emergence of antibiotic resistance which is a current global challenge. Furthermore, some bacteria are capable of transferring antibiotic resistance genes to other organism and over time this results in emergency of multi-drug resistant strains which are virtually impossible to treat, a prime superbug example being the methicillin-resistant *Staphylococcus aureus* (MRSA). *S. aureus* is a common opportunistic pathogen which is highly sensitive to several antibiotics (methicillin sensitive), even though lately pharmacological treatment is complicated by drug resistance by methicillin-resistant *S. aureus* strains. This poly-microbial bio-burden in wounds as mentioned by Pastar et al. [11] exists predominantly in the form of a biofilm resistant to antimicrobial treatments.

In Ugandan community *B. pilosa* is used in traditional medicine for treatment of wounds however, there is limited scientific data to enable their development into clinically useful products. Furthermore, in the study previously conducted to prove the efficacy of this plant on wound healing property didn't attribute the effects to a particular phytochemical rather the entire extract [13,14].

According to a study on flavonoid compounds by Cushnie et al. [15] reported their antimicrobial properties and this would be a strong attribute to *B. pilosa* wound healing effect. However, there is hardly any study conducted to scientifically prove the efficacy of flavonoid fraction of *B. pilosa* which this study seeks to address due to increased antimicrobial resistance. The results of this study if positive will aid in proving a source of new antimicrobial product for wound healing from *B. pilosa* which offers solution to drug development in the Uganda's health sector and the world at large. This study therefore seeks to address the above concerns through assessment of the in vitro antimicrobial effects of flavonoid [16] fraction extract from *B. pilosa* leaves against selected chronic wound invading pathogens such as; susceptible *Pseudomonas aeruginosa* ATCC®27853™, resistant *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, Methicillin resistant *Staphylococcus aureus* and Susceptible *Staphylococcus aureus* ATCC®25923™.

2. MATERIALS AND METHODS

2.1 Study Design and Study Area

An experimental laboratory design was used to determine the antibacterial susceptibility, MIC and MBC of crude flavonoid fraction from *B. pilosa* leaves crude extract. The flavonoid fraction was extracted using solvent-solvent extraction method and its characterization done using the thin layer chromatography at the pharmacology laboratory, Mbarara University of Science and Technology (MUST). Antibacterial susceptibility testing was conducted at Microbiology and Immunology laboratory, Kampala International University-Western Campus (KIU-WC).

2.2 Plant Collection and Identification

The fresh leaves of the plant were collected from the grounds behind KIU-WC Teaching Hospital 0º32'25.2"S 30º084'44.3"E. Plant was identified and authenticated by a taxonomist Dr. Eunice Olet, at Mbarara University of Science and Technology (MUST) and given a voucher number (#001). The voucher #001 specimen was archived in the herbarium at Mbarara University of Science and Technology Pharmacognosy unit.

2.3 Storage, Drying and Pulverization

The collected plant leaves were air dried in a dust free environment under shade at room temperature in the Pharmacognosy laboratory of Kampala International University-Western Campus. The dried samples were crushed using a mortar and pestle to get a fine powder according to method described by Sanghavi et al. [9].

2.4 Plant Extraction

Extraction was done using Soxhlet extraction method as described by Sanghavi et al. [9]. One hundred grams (100 g) of the coarse powder of *B. pilosa* leaves were packed in thimble of Soxhlet (Thomas Scientific) containing 250mL of 96% ether. The extraction was done at 60-70ºC. The powder with ether solvent was dried and placed back into the thimble and extracted with 250 mL chloroform at 60ºC. The powder with chloroform was dried and placed back into the thimble then further extracted with 250mL of methanol at 60ºC. The extract was then concentrated using a rotary evaporator at reduced pressure of 337 mbars and 40ºC. Sixteen (16 g) of methanol extract was dissolved

in 30 mL of distilled water and transferred into a 250 mL separating flask, 50 mL ethyl acetate was added and this was repeated three times. The extract was concentrated using a rotary evaporator at reduced pressure 337 m bars and 40ºC. The concentrated extract was considered as crude flavonoid fraction and was stored in a refrigerator at 4ºC till further study.

2.5 Identification of Crude Flavonoid Fraction

Characterization was done to confirm the flavonoid fraction extract of *B. pilosa* leaves according to a method by Sanghavi et al. [17] with slight modification. The identification was carried out without quercetine standard and also the ratio of mobile phase was changed to ethylacetate and methanol 1: 2.Thin Layer Chromatography (TLC): The pre-coated TLC plate (3.0×8.0 cm) was activated in hot air oven at 105ºC for 30 min and cooled to room temperature. About 1 g of crude flavonoids was dissolved in ethanol and then a drop of crude flavonoids was applied 1 cm above the edge of the plate. This plate was then developed in an air tight chromatography chamber containing about 8.5 mL of solvent mixture of ethyl acetate and methanol in the ratio of 1: 2 respectively. The developed plate was air dried and visualized under Ultra violet light at 254 nm using a UV hand lamp. The Rf (retention factor) values of the different spots were observed and calculated. The florescence of the compounds was observed too. The Rf was calculated using formulae:

$$\mathrm{Rf} = \frac{\text{Distance moved by the crude flavonoids}}{\text{Distance moved by the solvent front}}$$

2.6 *In-vitro* Antibacterial Activity of Crude Flavonoid Fraction of *B. pilosa*

Origin of the bacteria strains: Standard bacterial strains were obtained from Microbiology and Immunology laboratory KIU-WC which included: *susceptible Staphylococcus aureus* ATCC®25923™*, susceptible Pseudomonas aeruginosa* ATCC®27853™ while the clinical isolates were obtained from KIU-Teaching hospital microbiology laboratory: *resistant Pseudomonas aeruginosa, methicillin resistant Staphylococcus epidermidis, Streptococcus pneumonia* and *methicillin resistant Staphylococcus aureus.*

The respective standard bacteria were tested to confirm their identity with following tests according to methods described as follows;

- Tube coagulase test by Tiwary et al. [18] was done for susceptible *S. aureus* in which the bacteria was cultured overnight in brain heart infusion broth, then 100µl of the bacteria suspension was added to EDTA anticoagulated commercial rabbit plasma contained in a tube. This was incubated in a water bath at 37ºC for 4 hours. The tube was then checked for any clot formation for a positive test. A clot was observed hence confirming a positive test for *S. aureus* both clinical and standard isolates.

Methicillin sensitivity test was done using Cefoxitin Disc diffusion method for methicillin resistant *S. aureus and* methicillin resistant *S. epidermidis* as described by Zurita et al. [19] where a stock solution of the standard bacterial inoculum was prepared using normal saline to make a bacterial suspension equivalent to McFarland standards of 1.5×10^8 CFU/mL in a test tube. Using a cotton swab a bacterial suspension was picked from the solution and streaked on the entire surface of Mueller Hinton agar (HiMedia-M173, India). Cefoxitin disc was placed firmly onto the surface of the agar media with a sterile forceps and the plates were incubated for 24 hours at 37ºC. No zone of inhibition was observed which confirmed methicillin resistance.

Tube Acylamidase test is as specific test for *P. aeruginosa* and resistant *P. aeruginosa* by Arai et al. [20] where basal medium containing 2.0 g of Potassium Hydrogen Phosphate, 5.0 g Sodium Chloride, 0.1 g hydrated Magnesium Sulphate and 100mL redistilled water at pH 6.8 was used.

To this basal solution 0.5% Acetamide was added and 1mL of the solution was dispensed into a test-tube and autoclaved at 121ºC for 15mins. Then a loop-full of the standardized bacterial inoculum with normal saline at $1.5 \times 10^{\wedge 8}$ cfu/mL were added to the 1 mL of the Acetamide solution and then incubated for 24 hours. One drop of Nessler's reagent was added to the culture. Reddish brown sediment was observed for positive results confirming *Pseudomonas aeruginosa*.

Streptococcus pneumonia was tested for catalase test in which it was catalase negative. A colony was placed on glass slide, then 0.1mL of 3% H_2O_2 was added to the slide and mixed with the bacteria and stored at 4ºC in a tightly closed bottle as it would slowly lose potency if left open.

No vigorous bubbles were observed in the suspension confirming a catalase negative *S. pneumonia* [21]. The bacterial isolates were then sub cultured prior to experimental use.

2.7 Preparation of Bacterial Standards

The standard bacterial inoculum were sub-cultured on pre-prepared Mueller Hinton agar and incubated at 37ºC for 24hrs to obtain visible bacterial inoculum**.** The viable bacterial strains were standardized in normal saline to form bacterial inoculum of 1.5 × $10^{\wedge 8}$ CFU/mL equivalent to 0.5 MacFarland standard solution.

2.8 Antibacterial Sensitivity Testing

The antibacterial sensitivity study for crude flavonoid fraction of *B. pilosa* was done by agar well diffusion method according to Alghazeer et al. [22] but with slight modification where the concentration of the extract of flavonoid fraction of this study was increased at a concentration of 500 mg/mL. The bacterial inoculum of 1.5 × $10^{\wedge 8}$ CFU/mL was surface spread on pre-prepared Mueller-Hinton agar (MHA) media using a sterile cotton swab. Four (4) wells (6 mm diameter and 4mm depth) were made into the agar media using a sterile cork borer. Fifty microliter (50 µl) of negative control (10% DMSO), 50 µl/mL positive controls: Ciprofloxacin (2.5 mg/mL) and Amoxicillin/ Clavulanic acid (2.5 mg/mL) and then 500 mg/mL flavonoid fraction extract were placed respectively in the wells. The plates were kept for 1hour at 4ºC to allow better diffusion of the extract into the agar. Subsequently, plates were incubated at 37ºC for 24 hours. Diameters of inhibition zones were measured in mm and the results recorded as the mean ± SEM of duplicate experiments.

2.9 Determination of Minimum Inhibitory Concentration

The MIC is defined as the lowest concentration that completely inhibits the growth of test bacteria within 24 hours of incubation. The MIC for the crude flavonoid fraction extracts was determined by the macro-broth dilution method according to Alghazeer et al. [22] with modification of increasing in the initial concentration of the crude flavonoid fraction extract to 800 mg/mL. In this method, a two-fold serial dilution of the flavonoid extracts was prepared in test tubes containing 1 mL of sterile freshly prepared Mueller-Hinton broth used as diluents to achieve a decreasing concentration ranging from 800 mg/mL to 0.0244 mg/mL. Zero point one milliliter (0.1 mL) of each standardized inoculum 1.5 × $10^{\wedge 8}$ cfu/mL of bacteria was further diluted in 9.9 mL of normal saline to obtain 1.0x10^{6} cfu/mL concentration. Then 1 mL of this organism concentration was added into each of the extract dilutions contained in the tubes. All the test tubes were incubated 24 h at 37ºC. The tube with the lowest concentration of flavonoid fraction extract that inhibited bacterial growth was considered as the MIC.

2.10 Determination of Minimum Bactericidal Concentration

MBC is the minimum concentration that completely inhibits growth of all bacteria or 99.9% of the bacteria. The content of the preceding tubes from MIC were sub-cultured into the different freshly prepared MHA plates. All the inoculated culture plates were incubated at 37ºC for 24 h after which they were examined for presence of any visible bacterial colony. MBCs were recorded as the lowest concentration of flavonoid fraction extract that completely inhibited the growth of any bacterial colony on MHA [23].

2.11 Data Analysis

Data was entered in Microsoft excel and then exported to SPSS version 20 for analysis using One way Analysis of Variance (ANOVA).The inhibition zone diameters were obtained as mean ± SEM. Tukey's post hoc test was used to compare the antibacterial activity between flavonoid fraction extract and the positive controls against the different test bacteria that were used in the in-vitro study. The statistical significance of the comparisons was considered at 95% level of confidence ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1 Percentage Yield

The percentage yield of crude flavonoid fraction extract from leaves of *B. pilosa* was 3% (30 mg/mL) which was higher compared to 1.4% from Boldo (*Peumus boldus* Molina) leaves of which the same method of extraction (Soxhlet) and solvents were used [24]. The 3% which was 30mg/mL flavonoid fraction extract from *B. pilosa* leaves was slightly higher as compared to the 2.01% extract of flavonoid as reported by Barua et al. [25] and 19.623±0.1 mg/mL total flavonoid fraction extracted from *B. pilosa* L [26]. The

differences in percentage yields of flavonoid fractions from *B. pilosa* observed could be due to different areas of growth for plant species. This was in line with findings of Senjobi et al. [27]; Silivano et al. [28] who reported that, plants from different geographical area may have different phytochemical components and percentage yield compositions. The yield obtained suggests that plant could be a potential source for flavonoids for further studies and drug development.

The results of identification of crude flavonoid using Thin layer chromatography showed Retention factor values of 0.58, 0.60, 0.51 Rf confirming presence of flavonoids (Table 1). Martínez-Cano et al. [29] reported similar results in a TLC study done on *Citrus limetta* Risso crude flavonoids. In the same study the Rf values were included and recommended as values of reference under similar parameters which can be used for reference in identifying flavonoids. Previous study done on flavonoids identification reported Rf value within the range obtained in this study [30]. Flavonoid was also found present at Rf of 0.63 in *G. bicolar* in the report which is close to the Rf in this study of 0.60, 0.51. However, this discrepancy may be due to possibly the crude form of our extract and the different solvents used.

The flavones, flavonoles, chalcones and aurones fluorescence green, yellowish-green and brown colour were obtained (Table 1) after viewing under UV light [31]. Quenching was observed under UV light at 25 nm as reported to be observed at 254 nm UV light [32].

3.2 Antibacterial Susceptibility Study of Crude Flavonoid Extract

The activity of crude flavonoid fraction on *MRSA* with zone of inhibition 17.50±1.50 mm was significantly higher than on sensitive *P. aeruginosa* (P=0.04) and resistant *P. aeruginosa* (P=0.15) which had zones of inhibition 13.00±0.00 mm and 12.00±0.00 mm respectively. This was in line with the study by Singh et al. [30] where flavonoids had best activity against *S. aureus* and lower in *P. aeruginosa.* However, this was contrary to the findings of Nobakht et al. [33] who reported highest activity of flavonoids on *P. aerugionsa* and no activity on *S. aureus* with mean and standard deviation zone of inhibition of 24.7±2.9 mm and 0.0±0.0 respectively. Jaisinghani et al. [34] also reported higher antibacterial activity flavonoids in *S. aureus* than *P. aeruginosa* at 100-25 mg/mL concentration compared to 500 mg/mL of extract in this study. It was concluded by Singh et al. [30] that flavonoid studied had higher activity against gram positive bacteria than gram negative in which *P. aeruginosa* had zone of inhibition of 16.33 mm and 18.33 mm in *S. aureus*. The discrepancies could be explained by the various mechanisms of action of flavonoids on *S. aureus* species as reported in different studies including one in which radioactive precursors showed inhibition of RNA synthesis that was most affected in *S. aureus* [35], inhibition of energy metabolism in *S. aureus* by licochalcones flavonoids interfere in a similar way to respiratory-inhibiting antibiotics, since energy is required for active uptake of various metabolites and for biosynthesis of macromolecules as reported by Haraguchi et al. [36], and action on membrane integrity hence disrupting bacterial membrane according to a study done with catechin on *S. aureus* as reported by Stapleton et al.[37] where they formed pseudo multi-cellular aggregates formation. According to thestudy done on flavonoids of *Annona glabra* by Galvão et al. [38], the activity of the extract on *P. aeruginosa* was lower probably due to flavonoids ability to interact with the bacterial cell wall surface, resulting in an endothermic reaction with existence of a strong binding event between the flavonoids and *P. aeruginosa* for both sensitive and resistance species enabling it have some activity. Additionally, in the report by Kumar et al. [39] stated that flavonoids disrupted the interaction between acyl-homoserine lactones (AHLs; signal molecules used by Gram negative bacteria). The resistance due to increase in efflux pump and mutation phenotypes by the *P. aeruginosa* may have led to decreased activity shown in this study as in the report by Pang et al. [40].

Activity of crude flavonoid fraction on *MRSA* with zone of inhibition of 17.00±1.50 mm was significantly higher than on *S. pneumoniae* (P=0.006) which had a zone of inhibition of 11.50±0.50 mm. Extract activity was lower in *Streptococcus pneumonia* in this study with a smaller zone of inhibition which was in line with the report by Taleb-Contini et al. [41] whose study showed flavonoids activity lower also in *S. pneumonia* than in *S. aureus.* This was possibly because of some resistance mechanisms against the action of flavonoids due to alteration of protein binding proteins which may be used by flavonoid at less extent for attachment to alter its membrane fluidity as stated in the report by Cushnie et al. [15].

Activity of crude flavonoid fraction on *MRSE* with zone of inhibition of 15.50±0.50 mm was significantly higher than on *S. pneumoniae* (P=0.041) which had a zone of inhibition of 11.50±0.50 mm. The activity of the flavonoid fraction extract showed higher activity in MRSE compared to *S. pneumoniae* species as reported by Taleb-Contini et al. [41] where there was a greater activity for some flavonoids on MRSE as S. *aureus* species at concentration of 1000 mg/mL and lower on *Streptococcus* species. This may be due to the different mechanism of action exhibited through inhibition of nucleic acid synthesis by inhibition of DNA gyrase in MRSE as reported by Ohemeng et al. [42] and inhibition membrane fluidity of bacterial cells in *S. pneumoniae* as reported in the study by Sakagami et al. [43].

The activity of crude flavonoid fraction on MSSA with a zone of inhibition 17.00±0.00 mm was significantly higher than that on sensitive *P. aeruginosa* (P=0.04) and resistant *P. aeruginosa* (P= 0.015) with zones of inhibition 13.00±0.00 mm, 12.00±0.00 mm respectively. Activity of crude flavonoid fraction on MSSA with zone of inhibition of 17.00±1.50 mm was significantly higher than on *S. pneumoniae* (P=0.009) which had a zone of inhibition of 11.50±0.50 mm. In general gram positive bacteria (MSSA, MRSA) were the most susceptible organisms to the extract in this study followed by MRSE SPA, RPA with zones of inhibition as in Table 2 which supported the findings that these flavonoid fraction extracts are usually more active against gram-positive bacteria than gram-negative bacteria. Thus, this susceptibility difference between them might be due to differences in cell wall structures as in the study reported by Singhet al. [30] on antimicrobial screening of flavonoids from *Euphorbia hirta*.

There was significant difference of crude flavonoid fraction activity (P=0.00) and positive controls Vancomycin (P=0.00), Co-Amoxiclav (P=0.00) against all test bacteria from that showed by negative control DMSO with zone of inhibitions 0.00±0.00 mm in all test bacteria. The activity of crude flavonoid fraction in methicillin susceptible *S. aureus* with zone of inhibition 17.00±0.00 mm was lower than that of Co-Amoxiclav (P=0.00) with zone of inhibition 22.5±0.50 mm. The general activity of crude flavonoid fraction extract, Co-Amoxiclav, Ciprofloxacin and Vancomycin activities against the selected bacteria as reported in several previous studies could be attributed to the several antibacterial mechanisms of action [15,44,39] and no activity observed in 10% DMSO due to inactivity activities reported in previous studies hence recommended as a suitable solvent for standardization. Activity of crude flavonoid fraction on MRSA was significantly higher than that of Ciprofloxacin (P=0.00) and C0-Amoxiclav (P=0.00) which had zones of inhibitions 0.00±0.00 mm, 0.00±0.00 mm respectively.

Activity of crude flavonoid fraction on *MRSE* was significantly higher than that of Ciprofloxacin (P=0.00) and Co-Amoxiclav (P=0.00) which had zones of inhibition 0.00±0.00 mm each though a lower significant activity than that of Vancomycin (P=0.003) with Zone of Inhibition 18.25±0.25 mmwas obtained.The activity of extract was higher on *MRSE* than that of Ciprofloxacin and Co-Amoxiclav against MRSE due to a more stronger mechanism of action exhibited by crude flavonoids on the bacteria as reported by Cushine et al. [15] and Cushnie and Lamb [44] against bacterial nucleic acid synthesis but lower than that of Vancomycin in MRSE. This was due the more intense mechanism of action exhibited by Vancomycin through inhibiting bacterial cell wall synthesis by blocking glycopeptide polymerisation through binding tightly to D-Alanyl-D-alanine portion of cell wall precursor as stated by Akul [45].

Activity of crude flavonoid fraction on resistant *P. aeruginosa* with zone of inhibition 12.00±0.00 mm was significantly higher than that of Ciprofloxacin (P=0.03), Co-Amoxiclav (P=0.00) and Vancomycin (P=0.00) which had zones of inhibition 9.50±0.50 mm, 0.00±0.00 mm, 0.00±0.00 mm respectively.The activity of the

Table 1. Retention factor values and UV colour of crude flavonoid extract of *B. pilosa*

S/No.	DMCF	DM SF	Rf value	Fluorescence observed (254 nm)
1	3.0	5.6	0.53	Brown, green and yellowish green with quenching
2	3.3	5.6	0.58	
3	2.9	5.6	0.50	

Key: DMCF: distance moved by crude flavanoids, DM SF: distance moved by solvent front, Rf: Retention factor, nm: nanormiter

Table 2. Mean inhibition zone diameter of crude flavonoid extract against test bacteria

	Mean inhibition zone diameter ± Mean (SEM)					
Test bacteria	**MRSA**	**MSSA**	**RPA**	**SPA**	**MRSE**	**SP**
Flavonoid fraction	17.50±1.50^{a*}	17.00±0.00^{a*}	12.00±0.00^{b*}	13.00±0.00^{b*}	15.50±0.50^{a*}	11.50±0.50^{b*}
Ciprofloxacin	0.00±0.00**	15.50±0.50*	9.50±0.50**	19.00±1.00**	0.00±0.00**	11.00±0.00*
Co-Amoxiclav	0.00±0.00**	22.50±0.50**	0.00±0.00**	0.00±0.00**	0.00±0.00**	16.00±0.00**
Vancomycin	17.00±0.00*	-	-	-	18.25±0.25**	-
DMSO	0.00±0.00**	0.00±0.00**	0.00±0.00**	0.00±0.00**	0.00±0.00**	0.00±0.00**

Key: MRSE: Methicillin Resistant Staphylococcus epidermidis, SP: Streptococcus pneumoniae, RPA: Resistant Pseudomonas aeruginosa, MRSA: Methicillin Resistant Staphylococcus aureus, SPA: Susceptible Pseudomonas aeruginosaATCC®27853™, MSSA: Methicillin Susceptible Staphylococcus aureusATCC®25923™. Different superindexes of letters (a and b) in row for flavonoid fraction show a significant difference (p<0.05) between test bacteria. Different superindexes of asterisk (and **) in each column of bacteria show a significant difference (p<0.05) between flavonoid fraction and controls activity, - ; Not tested*

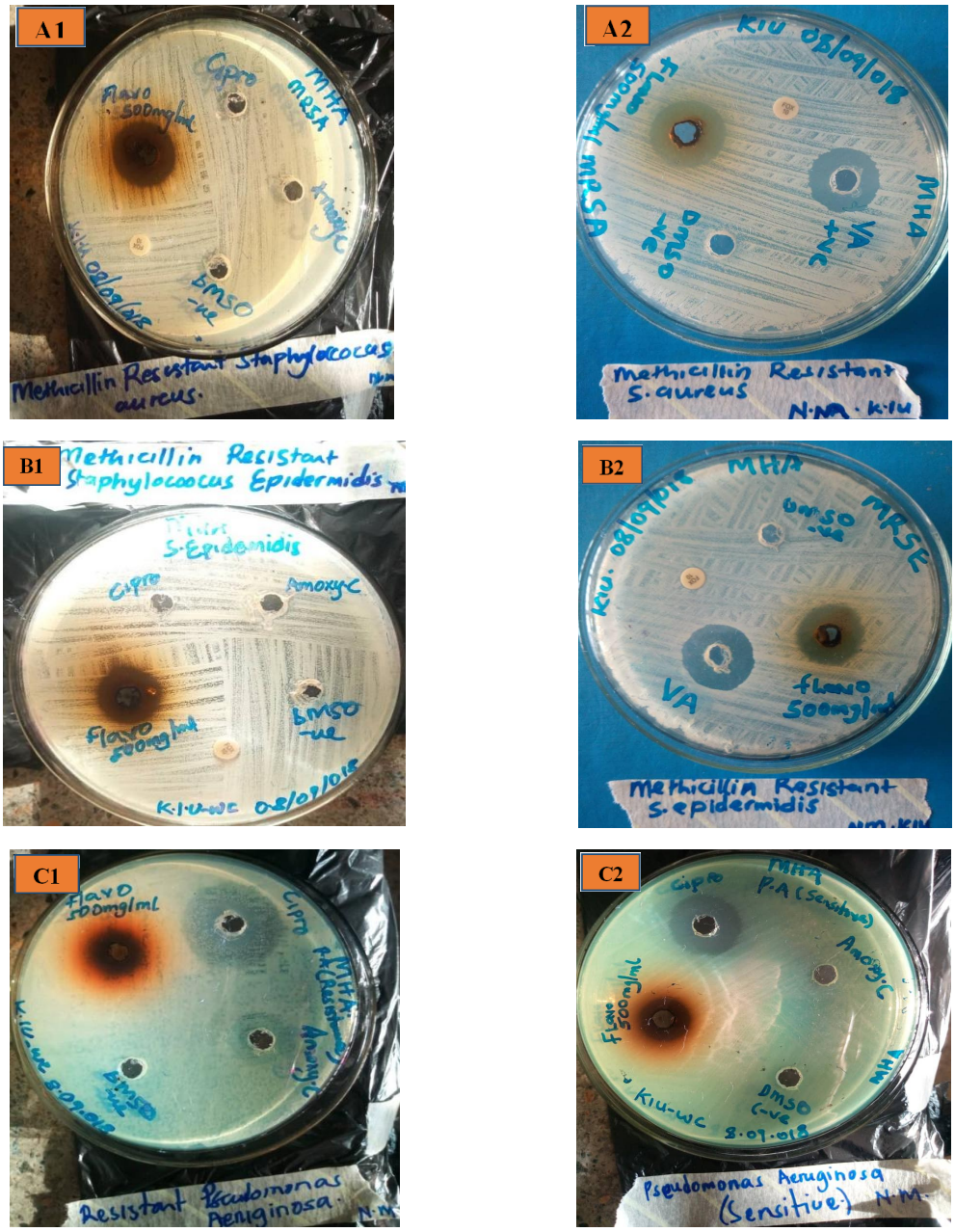

Fig. 1. Agar well diffusion assay to determine the antibacterial activity of crude flavonoid fraction of *B. pilosa* leaves and positive controls against drug resistant bacteria associated with chronic wound infections

A1; MRSA plate with flavonoid fraction and positive control antibiotics (Ciprofloxacin & Co-Amoxiclav plus Cefoxitin disc FOX, 30 mcg), A2; MRSA plate with flavonoid fraction and positive control antibiotics (Cefoxitin disc FOX, 30 mcg) and Vancomycin, 30 µg), B1; MRSE plate with flavonoid fraction and positive control antibiotics (Ciprofloxacin & Co-Amoxiclav plus Cefoxitin disc FOX (30 mcg), B2; MRSE plate with flavonoid fraction and positive control antibiotics (Cefoxitin disc FOX, 30 mcg and Vancomycin, 30 µg), C1 and C2; resistant Pseudomonas aureginosa and *Pseudomonas aureginosa ATCC®27853™ respectively, Flavo; Crude Flavonoid fraction*

extract was also higher in resistance *P. aeruginosa* than in Ciprofloxacin and Co-Amoxiclav due a possible more effective mechanism of action of cell wall interaction and

its strong hydrophobic interaction. This makes it less susceptible to efflux pump of the bacteria resistance mechanism that the standard antibiotics are susceptible to Galvão et al.[38] and also disrupting the interaction between acyl-homoserine lactones (AHLs; signal molecules used by Gram negative bacteria) Kumar et al.[39].

Activity of crude flavonoid fraction on *S. pneumonia* with zone of inhibition 11.5.00±0.50 cm was significantly lower than that of Co-Amoxiclav (P=0.00) which had a zone of inhibition 16.00±0.00 mm.

Activity of crude flavonoid fraction on sensitive *P. aeruginosa* with zone of inhibition13.00±0.00mm was significantly higher than that exhibited by Co-Amoxiclav (P=0.000) and significantly lower than Ciprofloxacin (P=0.001) which had zones of inhibition 0.00±0.00 mm and 19.00±1.00 mmrespectively (Table 2 and Fig. 1).

3.3 Minimum Inhibitory Concentration

The lowest MIC was against MSSA with a value 12.5 mg/mL. The MIC was the same against MRSA, Resistant *P. aeruginosa,* sensitive *P. aeruginosa, S. pneumoniae* and MRSE with a value of 25 mg/mL as shown in Table 3. From this study all the MIC values obtained were lower than 100 mg/mL hence being in line with the report in the several studies to have more clinical relevance through inhibition of growth of bacteria with significant concentrations thus, showing potential bactericidal and bacteriostatic effects of the flavonoid fraction extract on all the test organisms. The potential for developing antimicrobials from higher plants like *B. pilosa* appears rewarding as it will lead to the development of phytomedicine from its flavonoid fraction to act against microbes [30]. The highest MIC was observed in MRSA, MRSE and lowest in MSSA with 25 mg/mL, 25 ml/mL and 12.5 mg/mL respectively which was in agreement with findings reported by Taleb-Contini et al. [41] with values lower than 500 mg/mL as compared to that in Bartmańska et al. [46] report in which the MIC was higher with values of 50 ug/mL and greater than 250 ug/mL against *S. aureus* species as reported by Johari et al. [47].

3.4 Minimum Bactericidal Concentration

The minimum bactericidal concentration was lowest in *S. pneumoniae* with value of 50 mg/mL followed by sensitive *P. aeruginosa*, resistant *P. aeruginosa*, MRSA and MSSA with same value of 100 mg/mL then finally followed by MRSE with highest value of 200 mg/mL. The MBC was the same in MSSA, MRSA, Resistant *P. aeruginosa* and sensitive *P. aeruginosa* with concentration of 100 mg/mL as shown in Table 4. The crude flavonoid fraction extract from *B. pilosa* leaves was found to be a potent bactericidalagentagainst MRSA, Sensitive *P. aeruginosa*, resistant *P. aeruginosa*and*S. pneumoniae* since their MBC values were less than four times the MIC values and bacteriostatic for MSSA and MRSE organisms since the MBC was more than four times the MIC as shown in Tables 4 and 3 respectively. The extract had a good bactericidal effect against MRSA, SPA, RPA, and *S. pneumoniae*.

Table 3. Minimum inhibitory concentration of crude flavonoid fraction extract against test bacteria

Test bacteria	Minimum inhibitory concentration (mg/mL)
MSSA	12.5
MRSA	25.0
RPA	25.0
MRSE	25.0
SPA	25.0
SP	25.0

Key: MRSE: Methicillin Resistant Staphylococcus epidermidis, SP: Streptococcus pneumoniae, RPA: Resistant Pseudomonas aeruginosa, MRSA: Methicillin Resistant Staphylococcus aureus, SPA: Susceptible Pseudomonas aeruginosaATCC®27853™, MSSA: methicillin susceptible Staphylococcus aureusATCC®25923™

Table 4. Minimum bactericidal concentrations of crude flavonoid fraction extract from *B. pilosa* leaves against test bacteria

Test bacteria	MBC (mg/mL)
MSSA	100
MRSA	100
RPA	100
MRSE	200
SPA	100
SP	50

Key: MBC: Minimum bactericidal concentrations, MRSE: Methicillin Resistant Staphylococcus epidermidis, SP: Streptococcus pneumoniae, RPA: Resistant Pseudomonas aeruginosa, MRSA: Methicillin Resistant Staphylococcus aureus. SPA: Susceptible Pseudomonas aeruginosaATCC®27853™, MSSA: Methicillin Susceptible Staphylococcus aureusATCC®25923™

4. CONCLUSION

From this study crude flavonoid fraction from *B. pilosa* showed activity against all the selected resistant bacteria found in biofilms of chronic wounds. Bio-activity was found to be more effective in gram positive bacteria than in gram negative bacteria. The extract had bactericidal effect on resistant isolates of MRSA and resistant *P. aeruginosa.*

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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