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Preliminary study on the anti-bacterial activity of 2 cultivars of *Acalypha wilkesiana* on bacterial isolates of clinical significance

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ABSTRACT

Background: Anti-microbial resistance has become a major problem in clinical practice making it necessary to search for newer molecules. *Acalypha wilkesiana*, is a herb commonly used traditionally in Nigeria to treat gastrointestinal disorders, wounds and skin infections like impetigo.

Objectives: To determine the *in-vitro* anti-bacterial activity of crude extracts of 2 cultivars of *Acalypha wilkesiana* (Macrophylla & Hoffmanii)

Methods: *In-vitro* antibacterial activity was investigated by agar diffusion and micro-broth dilution techniques. Bacterial isolates tested were *Escherichia coli*, Methicillin Resistant *Staphylococcus aureus*, *Staphylococcus aureus*, *Salmonella paratyphi*, *Pseudomonas aeruginosa and Klebsiella pneumoniae.*

Results: The ethanol extract of both cultivars were more active than the ethyl acetate and n-hexane extracts having diameter zones of inhibition against all the bacteria tested at a range of 12.00-22.00 mm. The lowest MIC of the crude ethanol extract was seen with *E. coli* (1000 µg/ml) and *S. aureus* (1000 µg/ml; 2000 µg/ml) while the most active fractions (R2, R4, G3 and G4) of the crude ethanol extract of both cultivars had greatest activity against MRSA and *P. aeruginosa* (156 µg/ml).

Conclusion: This study shows that *Acalypha wilkesiana* has good broad spectrum anti-bacterial activity with the potential for development as lead compounds to combat multi-drug resistant organisms like MRSA.

1. Introduction

Antimicrobial resistance has become a critical issue globally with significant impact on the prevention and treatment of infections[1]. Antibacterial resistance has been fuelled by inappropriate use of antibiotics in humans and animals. Over the years, many bacteria have become resistant to new antibiotics with the advent of multidrug resistant organisms. This has been worsened by a decline in the development of new drugs to combat resistance in the last few decade[2,3]. Some of these bacteria which have become resistant to several antibiotics to which they were previously susceptible include *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* that cause infections in the community and in healthcare facilities. Infection with resistant organisms has far reaching consequences like prolonged illness, increased morbidity and mortality, reduced quality of life associated with pain and discomfort, reduced man-hours and economic productivity and significant economic burden from increased health costs due to prolonged effect to the affected individual, the family, the community and the nation[6-8]. It has therefore become urgent to search for and develop new molecules to combat this rising scourge.

Plants have been the source of about a third of the drugs produced by industrialized nations. Some of these include Quinine, isolated from the bark of the cinchona tree, and Artemisinin isolated from the plant *Artemisia annua* L, used in Chinese traditional medicine; both of which are being used in the treatment of malaria[9,10]. About 60-90% of the developing world depend on traditional plants as primary health care modality[11]. The natural plants are more affordable, easier to access, more acceptable and are believed to have fewer side effects compared to conventional drugs[12]. In Nigeria and Africa, herbal preparations are culturally acceptable and are many times preferred to orthodox medicines. Bioactive molecules (phytochemicals) present in plants confer some of the antimicrobial activity seen. These molecules are also important because of lower tendency for antibacterial resistance to develop due to their mechanism of action[13].

Acalypha wilkesiana Muell Arg, (Euphorbiaceae -spurge family) is one of the medicinal plants which is commonly used in many parts of Nigeria. It is popularly known as "Red acalypha", "hibiscus" or "copper leaf". It is called "Jinwinini" among the Hausas of Northern Nigeria, and "aworoso" among the Yorubas of South Western part[14]. Most of the *Acalypha* species are used medicinally in Nigeria. Almost every part of the plant (leaves, stem and roots) is used but the leaf is the most widely used part to treat a wide range of ailments[15-17]. Some of these ailments include skin infections, neonatal jaundice, diabetes, dysentery and asthma[18]. Presence of secondary metabolites in the ethanol leaf extract include tannins, saponins, flavonoids, , reducing sugar glycosides and alkaloids, which are thought to be responsible for its anti-inflammatory and antimicrobial effects[19]. The expressed juice or decoction has been used for the treatment of gastrointestinal disorders[20]. Impetigo, a bacterial skin infection is one of the disorders that have been found to improve with the plant. Bacterial skin infections are common and can spread from the skin to the blood stream causing invasive disease. In the search for a potent anti-bacterial agent that will serve as a leading drug development, we investigated the *in-vitro* anti-bacterial activity of *Acalypha wilkesiana* against some gram-positive and gram-negative organisms that are of clinical significance.

2. Materials and methods

2.1 Collection and Processing of Plant materials

The fresh leaves of the two varieties, *Acalypha wilkesiana* Macrophylla (copperleaf plant) and *Acalypha wilkesiana* Hoffmanii (green leaf plant) were collected from the botanical garden of The National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria in July. Voucher specimens of plants were deposited in the NIPRD herbarium

with voucher numbers NIPRD/H/ 6788 for *Acalypha wilkesiana* Macrophylla and NIPRD/H/ 6789 for *Acalypha wilkesiana* Hoffmanii. The fresh leaves were separated, shade dried and pulverised into powder using mortar and pestle.

2.2 Extract Preparation

One kilogram of each variety of the powdered leaves was successively macerated in 10 L of n- hexane, ethyl acetate, ethanol and water for 48 hours and filtered. After each extraction, the extracts were concentrated using a rotary evaporator, dried in a water bath at 70^{o} C, weighed and stored in the refrigerator at temperature of 4^{o}C. The yield and percentage yield were calculated as follows

Yield = (weight of extract/weight of powdered leaves) 100.

2.3 Test Organisms

Typed bacterial isolates used in the study include

Escherichia coli ATCC 25952, Multi Resistant *Staphylococcus aureus* ATCC 33592, *Staphylococcus aureus* ATCC 25923, *Salmonella paratyphi* ATCC 9150, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC 13883 and *Bacillus subtilis* ATCC 21332. The bacterial isolates were stored in Nutrient agar (NA) slants and stored at 4 °C until needed.

2.4 Culture preparation

A loopful of 24 h surface growth of each of the culture bacteria was transferred to 0.9 % NaCl solution (with agitation to disperse spores) and homogenous suspension of it was used for inoculation. Turbidity was adjusted to match that of a 0.5 McFarland standard by visual observation[21].

2.5 Bioassay Guided Fractionation

Flash column chromatography was used to fractionate each of the ethanol extracts obtained from successive extraction . The wet method for packing of chromatographic columns was used. Adsorbent used was Silica mesh 60. The fractions were gradiently eluted in the presence of vacuum (reverse pressure) by addition of the solvent in decreasing order of polarity as follows: n-hexane (100 %), n-hexane – ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80), ethyl acetate (100 %), ethyl acetate – ethanol (80:20, 70:30, 60:40, 50:50, 40:60, 30:70) and ethanol (100 %). A total of 35 fractions were collected from the fractionation of each ethanol extract was collected in beakers and left to evaporate under a cool stream of air. Using different solvent systems, Thin layer chromatography was used to pool similar fractions. The pooled fractions were concentrated *in vacuo* and screened for anti-bacterial properties.

2.6 Antimicrobial screening of extracts

The extracts were screened for their antibacterial activity against the test organisms by disc diffusion method[23]. Muller Hinton agar plates were inoculated with the test isolate by spreading the standardized inoculum on the surface of the agar plate with sterile swab stick. Holes of diameter 6mm were bored in the inoculated agar plates and 100 µL of each of the extract solutions at 16 mg/mL, 8 mg/mL and 4 mg/mL at concentrations of 3.0, 2.0 and 1.0 % were introduced into the wells. Ciprofloxacin disc (5 µg) Oxoid, served as positive control whereas the disc containing 10% Dimethyl sulphoxide (DMSO) alone was used as a negative control. All the plates were incubated at 37 °C for 24 h. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition in millimetres from observation of the clear zones formed surrounding each well. The bioassay was performed in triplicate and the mean value calculated.

2.7 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum inhibitory concentration (MIC) of extracts of the leaves of *A. Wilkesiana* were determined by broth microdilution method[24]. The 96-microtiter well was prepared by dispensing 95 µL of MHB and left for 15 minutes before adding 5 µL of the bacterial suspension into each well. One hundred µL from the stock solution of extracts was added into the first well followed by two-fold serial dilution down the remaining wells. The last row of wells did not contain the extract thus serving as organism viability control. Each plate was shaken for 20 seconds (with a shaker at low speed) and then incubated at 37 °C for 24 h. At the end of the incubation period, the plates were observed visually for the presence or absence of growth. MIC was calculated as the lowest concentration of the extracts showing no turbidity after incubation, where the turbidity was interpreted as visible growth of the micro-organisms. The test was performed in triplicate.

2.8 Statistical analysis

The diameter zones of inhibition and minimum inhibitory concentrations of the extracts, fractions and herbal formulation were presented as mean of three values standard deviation. A P value < 0.05 was considered as significant.

3. Results

3.1 Extraction of *Acalypha Wilkesiana* Leaves

The total yield and percentage yield of the different solvent extracts as shown in Table 1.0 revealed an increase in the extraction yield with increase in the polarity of the extraction solvents. As a result, the ethanol extract yielded the greatest quantities and least polar hexane extracted the least amount. (Table 1)

Table 1: Percentage yield of extracts from 100 g of *A. wilkesiana* leaves using various solvents.

Solvents	AWM Yield (g)	Percentage Yield`	AWH Yield (g)	Percentage Yield
Hexane	2.966	2.97	1.725	1.73
Ethyl acetate	1.437	1.44	2.237	2.24
Ethanol	3.700	3.70	3.880	3.88

AWH- *A. wilkesiana* green varietyAWM- *A. wilkesiana* red variety

3.3 Chromatographic Analysis of Ethanol Extracts of *A. wilkesiana* Leaves

A total of 35 fractions each were collected from the elution of the ethanol extracts of *A. wilkesiana* red variety (AWM) and *A. wilkesiana* green variety (AWH). Identical fractions were combined giving 8 and 7 fractions altogether for AWR and AWG respectively. (Table 2.0)

Table 2: Combination of fractions based on thin layer chromatography

AWM Fractions	Combined fractions	AWH Fractions	Combined fractions
R1	1-3	G1	1-4
R2	4-5	G2	5-7
R3	6	G3	8-10
R4	7-11	G4	11-14
R5	12-16	G5	15-21
R6	17-22	G6	22-31
R7	23-30	G7	32-35
R8	31-35		

AWH- *A. wilkesiana* green varietyAWM- *A. wilkesiana* red variety

3.4 Antibacterial activity of the leaves of *A. wilkesiana*

The antibacterial activity of extracts of *A. wilkesiana* shows that the solvent extracts have varying degrees of activity. Hexane extracts of the 2 varieties of plants produced lowest inhibitory effect. The ethanol extract was the most active as it inhibited all the test organisms with zones of inhibition ranging from 12.00-22.00 mm, activity that is comparable with that of standard drug. Table 3.0

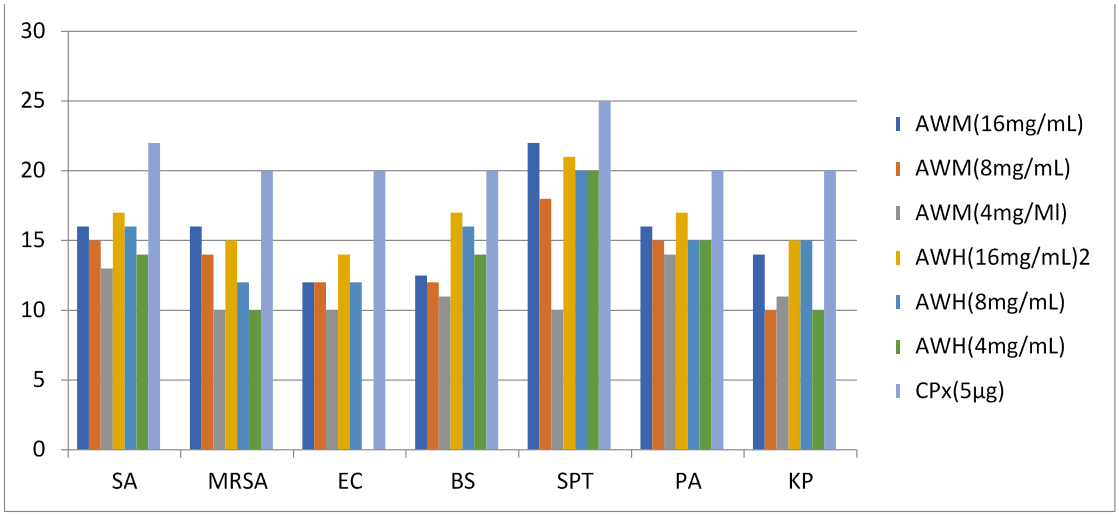
Table 3: Susceptibility of the bacterial isolates to different solvent extracts of *A. wilkesiana* leaves (Macrophylla and Hoffmanii) at 16 mg/mL

Organisms	**AWM (Zone of Inhibition ; mm)**			**AWH (Zone of Inhibition ; mm)**			**Ciprofloxacin**	**10 % DMSO**
	HEX	**ETA**	**ET**	**HEX**	**ETA**	**ET**	**(5 µg)**	
SA 95923	0.00±0.00	15.00±0.00	16.00±0.00	0.00±0.00	23.00±0.33	17.00±0.33	22.00±0.00	0.00±0.00
MRSA	0.00±0.00	0.00±0.00	16.00±0.00	0.00±0.00	0.00±0.00	17.00±0.00	20.00±0.00	0.00±0.00
EC	13.00±0.00	0.00±0.00	16.00±0.33	0.00±0.00	0.00±0.00	16.00±0.57	20.00±0.00	0.00±0.00
BS	12.00±0.00	14.00±0.57	12.50±0.57	12.0±0.00	14.00±0.57	17.00±0.00	20.00±0.00	0.00±0.00
SPT 9150	0.00±0.00	14.00±0.00	22.00±0.00	0.00±0.00	18.00±0.57	21.00±0.33	25.00±0.00	0.00±0.00
PA	0.00±0.00	16.00±0.57	16.00±0.0	0.00±0.0	0.00±0.0	17.00±0.0	20.00±0.00	0.00±0.00
KP	0.00±0.00	0.00±0.00	14.00±0.00	0.00±0.00	0.00±0.00	15.00±0.00	20.00±0.00	0.00±0.00

Key: HEX- hexane; ETA- ethyl acetate; ET- ethanol
SA; *Staph. aureus* ATCC 25952; MRSA: Methicillin Resistant *Staph. aureus*
EC- *E.* coli ATCC25952 BS- *Bacillus subtilis* ATCC 21332 *KP- Klebsiella. Pneumoniae*
SPT- *Salmonella paratyphi* ATCC 9150
AWM- *A. wilkesiana* Macrophylla AWH - *A. wilkesiana* Hoffmanii

3.5 Susceptibility of the test organisms to the ethanol extracts of *A. wilkesiana* leaves (Macrophylla and Hoffmanii).

Antibacterial activity of ethanol extract of *A. wilkesiana* leaves (AWM & AWH) show that the inhibitory effect is concentration dependent. At 16 mg/ml, all the test organisms were susceptible with zones of inhibition ranging from 12.00-22.00 mm for AWM and 14.00-21.00mm for AWH (Figure 1.0)

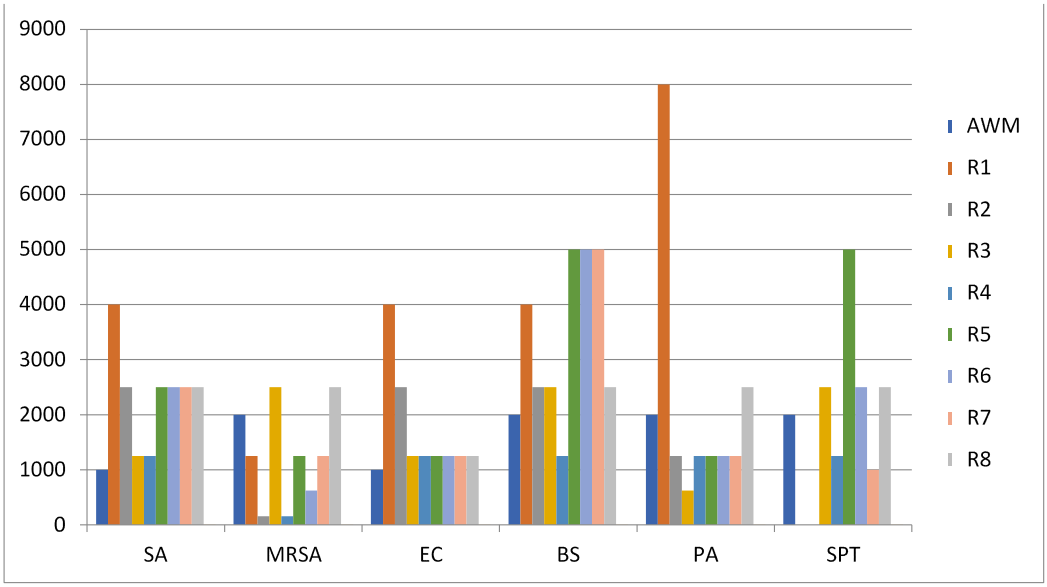


Key: SA; *Staph. aureus* ATCC 25952; MRSA: Methicillin Resistant *S. aureus* EC- *E. coli* ATCC 2595
BS- *Bacillus subtilis* ATCC 21332 *KP- Klebsiella. Pneumoniae* SPT- *Salmonella paratyphi* ATCC 9150 *Pseudomonas aeruginosa* ATCC 27853 AWM- *A. wilkesiana* Macrophylla AWH- *A. wilkesiana* Hoffmanii CPx- Ciprofloxacin

Figure 1: Susceptibility of the test organisms to the ethanol extracts of *A. wilkesiana* leaves (Macrophylla and Hoffmanii).

3.6 Minimum inhibitory concentration (MIC) of the ethanol extract and column fractions from ethanol extracts of *A. wilkesiana* Macrophylla (AWM) leaves.

The crude extract of AWM had the greatest inhibitory activity against *E. coli* and *S. aureus* at 1000 µg/ml. Fractionation of the ethanol extracts of AWM by column chromatography yielded 8 fractions (R1-R8). All the fractions had varying degree of activity ranging from 156-8000µg/ml. The best activity was produced by fractions R2, and R4 against MRSA (156µg/ml) and R3 against *P. aeruginosa* (625µg/ml), which is significant when compared to crude extract. (Figure 2.0)

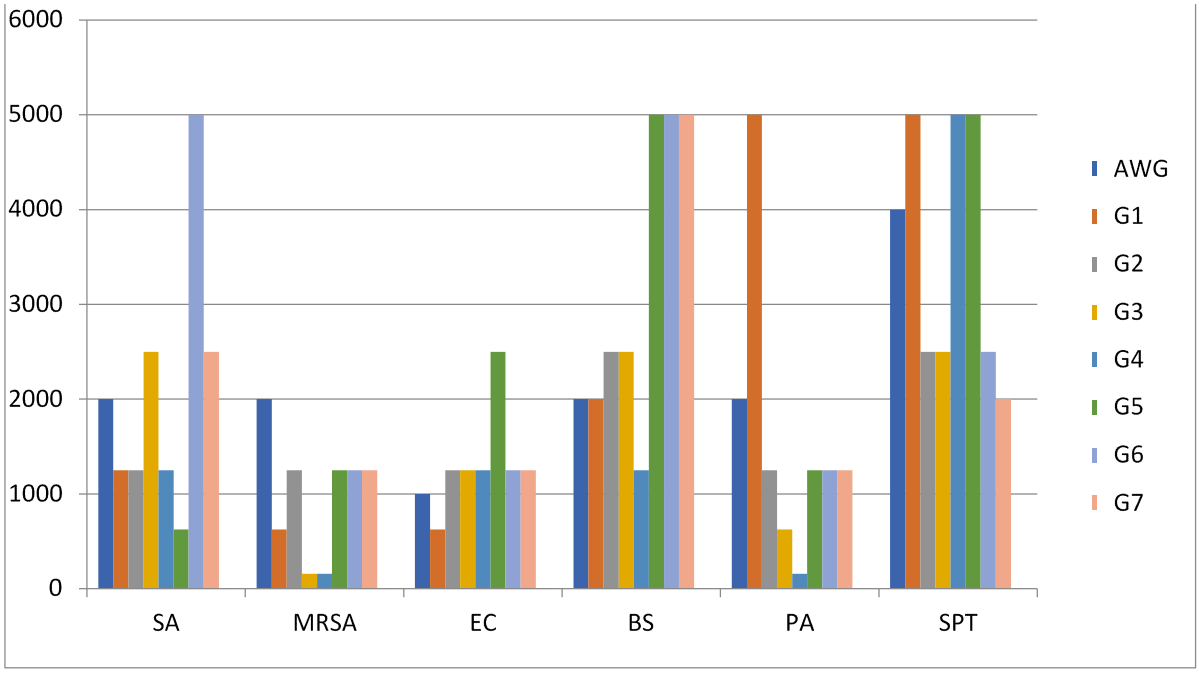


Key: SA; *Staph. aureus* ATCC 25952; MRSA: Methicillin Resistant *S. aureus* EC- *E. coli* ATCC 2595
BS- *Bacillus subtilis* ATCC 21332 *KP- Klebsiella. Pneumoniae* SPT- *Salmonella paratyphi* ATCC 9150 *Pseudomonas aeruginosa* ATCC 27853 AWM- *A. wilkesiana* Macrophylla
R1-R7- Column fractions of AWM

Figure 2: Minimum inhibitory concentration (MIC) of the ethanol extract and its column fractions of *A. wilkesiana* Macrophylla (AWM) leaves.

3.7 Minimum inhibitory concentration (MIC) of the ethanol extracts and column fractions from ethanol extract of *A. wilkesiana* Hoffmanii leaves (AWH).

The crude extract of AWH was most active against *E. coli* at 1000 µg/ml. Fractionation of the ethanol extracts of AWH yielded 7 fractions (G1-G7) with varying degrees of activity ranging from 156-5000µg/ml. The best activity was produced by fractions G1, G3 and G4 against MRSA, *E. coli* and *P. aeruginosa* at concentrations ranging from156-625 µg/ml (Figure 3.0)



Key: SA; *S. aureus* ATC 25952; MRSA: Methicillin Resistant *S. aureus;* EC- *E. coli* ATCC 25952 BS- *Bacillus subtilis* ATCC 21332 KP- *Klebsiella. pneumoniae* SPT- *Salmonella paratyphi* ATCC 9150 *Pseudomonas aeruginosa* ATCC 27853 AWH - *A. wilkesiana* Hoffmanii G1-G7- Column Fractions of AWH

Figure 3: Minimum inhibitory concentration of the ethanol extracts and its column fractions of *A. wilkesiana* Hoffmanii leaves (AWH)

4. Discussion

This study has shown that *A. wilkesiana* has some antibacterial potentials as demonstrated by the crude extracts and the different column chromatography fractions although with varying degrees of activities. Ethanolic extraction produced better activity than ethyl acetate and hexane; and this agrees with reports of Alade *et al* (1993)[25] and Othman *et al* (2011)[26]. Akinyemi *et al* (2005)[27] and Adetutu *et al* (2011)[28] who reported some activities against MRSA, *S. aureus*, *E. coli*, *B. subtilis* and *P. aeruginosa*, found better activity with ethanol than the aqueous extract. Ethanol which was the most favourable solvent in this study is commonly used in traditional medicine. As depicted in results, extraction yield with polar solvents was higher in comparison to non-polar solvents. These variations may be due to the difference in their polarity as well as dielectric constant, which play vital role in the solubility of phytochemical compounds in respective solvents. Therefore, this result confirms the effect of solvent system

on the extract yield that consequently confirms the richness of this plant in polar substances[14,15].

Hence, its extract closely represents what is used traditionally. Although aqueous (water) extraction is also used indigenously, it has been shown that plant extracts obtained using organic solvents like alcohol give more potent and consistent antimicrobial activity than aqueous extracts[29-30]. Most antimicrobial components identified so far are not water soluble and thus extracts obtained with organic solvents have been found to be more potent[31]. The polarity of the solvent may play a role in the solubility of the phytochemicals present in the extract translating to the level of anti-microbial activity seen. It is possible that higher concentrations of the plant and longer duration of treatment are needed to achieve cure with the aqueous extracts as opposed to ethanol extracts when used indigenously.

Antimicrobial activity of the ethanol extract of the plant against the bacteria increased with increasing concentration of the crude extract. However, the fractions produced better activity at lower concentrations. Oladunmoye[32] demonstrated an exponential increase in killing rate of micro-organisms with increased contact time and higher concentrations of this plant. This suggests that rate of recovery from infection and hence clinical improvement will be faster with higher concentrations of the extract.

The findings in this study show good antibacterial activity of both variants of the plant against *Staphylococcus aureus* and *Methicillin Resistant Staphylococcus aureu*s (MRSA) which are gram-positive organisms. MRSA is a more virulent, usually multi-drug resistant organism, which gives rise to infections that are difficult to treat with commonly used antibiotics. Superficial infection with MRSA which may even be asymptomatic has been documented[33]. In a study of health workers in the critical care units in a tertiary hospital in Nigeria, over 50% of them were carriers of MRSA either in the nose or on the hands[33]. Hence, this plant has the potential of being developed as a topical agent that can be used to treat asymptomatic MRSA carriers.

The R2 and R4 fractions of the crude extracts of AWM were about 25 times more active against MRSA with much lower MIC than the crude extract itself. The G3 and G4 fractions of the AWH had a similarly better activity against MRSA than the crude extract with much lower MIC values. Similar findings were demonstrated by Santiago *et al*[34], in Malaysia, where a 4-fold increase in anti-MRSA activity of a semi-pure fraction compared to the crude extract of the *A. wilkesiana* was seen. They also reported synergistic (a 32-fold increase) activity of this fraction when added to ampicillin to inhibit MRSA, based on the MIC values. It was further demonstrated that overcoming of the resistance of the MRSA with the synergism of the fraction and ampicillin, was due to inhibition of penicillin-binding protein 2a (PBP2a) production. The PBP2a has low affinity for beta-lactam, conferring resistance to the organism thus making its treatment difficult by conventional antibiotics. This therefore suggests that the fractions in this study that were significantly active against MRSA need to be purified further by standard techniques to produce potent molecules.

The diameters of zones of inhibition 16.0 mm were seen against *E. coli,* which was also the most susceptible bacterium to the crude extracts of the 2 cultivars based on the MIC (1000 µg/ml). *Staphylococcus aureus* was also susceptible to the AWM cultivar (1000 µg/ml). Othman *et al*[25], demonstrated a lower susceptibility of *B. subtilis* while *E. coli* was just as susceptible as *S. aureus* to the ethanolic extract of AW. *S. paratyphi* appeared to be the least susceptible to the crude extracts (2000-4000 µg/ml), though the R7 and G7 fractions were more active against it (1000 -2000 µg/ml). The G4 and R3 and fractions of the crude extracts were much more active (156;625µg/ml) than the crude extract against *P. aeruginosa* (2000 µg/ml). *Pseudomonas aeruginosa* is one of the leading Gram-negative pathogens responsible for healthcare-associated infections worldwide, including pneumonia, urinary tract and surgical site infections[35]. Fractions that are particularly susceptible can be developed to combat this infection. The findings above demonstrate good activity of the crude extracts and some fractions against the gram-negative organisms.

Overall, the R4 fraction of the AWM and the G4 fraction of the AWH had the best broad-spectrum antibacterial activity with MIC that are mostly lower than the crude extract against most of the bacterial organisms making it more effective. However, the antibiotic (Ciprofloxacin) used as control had much lower MICs (2 µg/ml) against the organisms than the extracts and its fractions (156-5000µg/ml). This is not surprising as the antibiotic has been developed from an isolated lead compound with significant antimicrobial activity, unlike the crude which contains varied compounds and leafy parts of the plant. The

antimicrobial activity has been related to the presence of the polyphenol derivatives gallic acid, corilagin and geraniin, isolated from the leaves of this plant[36].

The demonstrated broad-spectrum antibacterial activity of this plant found in this study was corroborated by Awe *et al*[37] and Oladunmoye[38]. However, Awe *et al* who studied only the *Acalypha wilkesiana var* Macrophylla (AWM), documented similar activity against *S. aureus, Shigella spp, K. pneumoniae and E. coli* with much higher concentrations of the extract (200mg/mL) than used in this study. The *A. wilkesiana* plant in that study was collected in Abuja which is in the same vicinity as the location of the plant used in this study. Othman *et al*[25] in Malaysia documented similar broad-spectrum antibacterial activity as seen in this research. Oladunmoye[32] on the other hand, studied the 2 cultivars used in this study and documented lower MIC values suggesting better antibacterial activity of their extracts. This could be related to the difference in geographic region, extraction methods and even strains of organisms tested. A study conducted by Ubani-Ukoma et al.[39] revealed that organogels prepared from the methanolic extracts of both varieties of *A. wilkesiana* (Macrophylla and Hoffmanii) showed antibacterial activity against *B. subtilis*, *P. aeruginosa*, *S. aureus* and *S. albus*.

For most of the organisms, the spectrum of activity for both cultivars of the plant were similar with exception of. *S. paratyphi* and *S. aureus* which appeared to be more susceptible to crude extract of AWM than AWH.

The limitations of this study include we could not test for bacterial pathogens apart from those.

5. Conclusion

Our study showed that the Macrophylla and Hoffmanii varieties of *Acalypha wilkesiana* have good and comparable antibacterial activity against the pathogens tested (Gram negative and Gram positive) with the ethanolic extract producing the strongest activity against all organisms. against *E. coli* and *S. aureus* where most susceptible to the crude ethanol extracts while *MRSA and P. aeruginosa* were particularly susceptible to some fractions of both cultivars of this plant. The column fractions R4 and G4 from ethanol extract of *A. wilkesiana* produced the strongest anti-bacterial activity. This suggests that further preclinical and clinical studies on the efficacy of this plant in the treatment of skin and systemic infections should be carried out. More research is needed on the use of this plant in treating multidrug resistant infections.

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