Original Research Article

In-Vitro Antimicrobial Susceptibility and Phytochemical Constituents of Methanol Leaf Extract of *Prosopis africana* against some Selected Microorganisms.

Abstract

The idea that certain plants had healing potential was known long before human beings discovered the existence of pathogens. The crude methanolic leaf extract of *Prosopis africana* was assayed for antimicrobial potency using Agar-well diffusion technique against *Salmonella typhi*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Candida albicans*. Qualitative phytochemical screening was also carried out. The result of the antimicrobial screening showed antimicrobial potency against the test isolates with various degrees of zone of inhibition which varied between 10mm – 22mm. The highest zone was noted against *Klebsiella pneumoniae* (22mm), followed by *Streptococcus pyogenes* and *Candida albicans* (21mm), *Salmonella typhi* (20mm), MRSA (19 mm) then *Pseudomonas aeruginosa* (18mm). Chloramphenicol and Fluconazole are used as reference standard and their zones of inhibitions ranged from 26mm–29mm. The Minimum Inhibitory Concentration (MIC) of the extract ranged between 12.50mg/mL – 50.00mg/mL whilst the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of the extract were at 50.00mg/mL. The result of phytochemical screening revealed the presence of carbohydrates, flavonoids, phenols/tannins, saponins, terpenes, steroids, cardiac glycosides and alkaloids as secondary metabolites. The result of this study showed that methanolic leaf extract of *P. africana* has proved its use in folklore as an alternative

antimicrobial agent and further research can lead to isolation of a new lead of medical importance.

Key worlds: Antimicrobial Susceptibility, *Prosopis africana*, Phytochemical Constituents, 96-well microplates, MIC, MBC and MFC.

1. Introduction

Infectious diseases are particularly a major challenge to public health, despite tremendous scientific discovery of medicines for their treatment [1]. This is due to increase in resistance to antibiotics by pathogenic microorganisms as a result of misuse and over prescription of antibiotics which has affected our ability to treat patients empirically [2][3]. So in recent years there has been continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because the incidence of new and re-emerging infectious diseases and development of resistance to the antibiotics in current clinical use [4]. However, nature is endowed with providing continuous new biomolecules with novel structures that are designed to interact with biological systems to provide defense against infectious diseases [5].

The presence of bioactive principles such as alkaloids, phenols, tannins, glycosides and essential oils amongst others is responsible for the medicinal properties of plants [6]. It is necessary to screen medicinal plants for the presence of these bioactive chemicals which may lead to a new active principle. Scientific studies available on medicinal plants indicate that promising phytochemical can be developed for many health problems [7]. The benefits of using plant derived medicines are that they are relatively cheaper and stable.

P. africana (Guill. & Perr.) Taub. belongs to kingdom plantae, division tracheophyta, class magnoliopsida, order fabales and family fabaceae. It is a renowned and versatile tree of immense economic value amid the rural communities in the Guinea savanna of Nigeria. It is mostly found in savanna countries of Africa like Senegal and Nigeria [8]. Literature gives an account of its uses as folk medicines for several ailment and virtually all its parts are of medicinal value. [9] reported that the stem bark is used as remedies for dysentery, gonorrhea, bronchitis and skin diseases. In Niger State of Nigeria, the twigs, leaves, bark, and secondary roots are used for treatment and relieve of typhoid fever, dental decay, malaria as well as stomach cramps while, [10] attested that the bark and root decoctions are utilized for the treatment of Trypanosomiasis in cattle and on lesions as a lotion.

It is in knowledge domain that antimicrobial resistance is a great challenge to holistic treatment of infectious diseases as a result of either the use of substandard antibiotics, misuse or over prescription. There is therefore, need for search to discovering new lead principles that will be effective, safe, readily available and cost effective. This study was aimed to determine the antimicrobial properties of *P. africana* towards the development of new antimicrobial agent.

2.0 Materials and Methods

2.1 Plant Sample Collection and Identification

The leaves of *P. africana* was collected from Bida in Niger State, Nigeria, voucher specimen was prepared, the plant was identified and voucher specimen was deposited in herbarium unit of the Department of Medicinal Plant Research and Traditional Medicine (MPR&TM), National Institute for Pharmaceutical Research and Development (NIPRD), Idu-Abuja, Nigeria.

2.2 Preparation and Preservation of Plant Material

The leaves were properly washed under clean-running tap water to remove the dirt and air dried at room temperature for a week. The dried leaves were pulverized into powder with clean wooden pestle and mortar, and sieved. The pulverized sample was stored in a clean plastic container, properly labeled and tightly covered at 37°C prior for further analysis.

2.3 Extraction of Crude Extract

One hundred grams (100g) of the pulverized leave of *P. africana* was accurately weighed and subjected to cold maceration in 500mL of absolute methanol for 72 h at laboratory temperature. The macerated extract was filtered using Whatman No.1 filter paper. The extraction was repeated for the maximum extraction of the active ingredients and to also obtain reasonable yield (crude extract). The filtrate was dried using water bath at 45^{o}C until all the solvent evaporated out.

The percentage yield of the crude extract (PYCE) was calculated using the formula by [11].

$$PYCE = \frac{Mass\ of\ the\ crude\ extract\ obtained}{Mass\ of\ the\ pulverized\ plant\ sample} \times 100$$

2.4 Phytochemical Screening of the Extract

The qualitative phytochemical screening of the leaves of *P. africana* was carried out in Pharmacognosy unit, Department of Medicinal Plant Research and Traditional Medicine, NIPRID, Idu-Abuja. The standard methods illustrated by [12][13] [14] were adopted to test for the presence of carbohydrates, flavonoids, phenols/tannins, saponins, terpenes, steroids, alkaloids and cardiac glycosides.

3.4.1 Determination of carbohydrates: (Molisch's Reagent Test)

The fraction of the extract was mixed with 2mL of Molisch's reagent and the mixture was properly shaken. Thereafter, 2mL of concentrated H_2SO_4 was slowly and carefully added. The appearance of a purple ring/violet at the interphase signifies the presence of carbohydrates.

3.4.2 Determination of flavonoids

i. Alkaline Reagent Test

To the extract, 2mL of 2% solution of NaOH was mixed. An intense yellow colouration was formed which later turned colourless when few drops of diluted acid was added which signifies the presence of flavonoids.

ii. Shinoda Test

To the extract, few drops of Mg ribbon fragments were added followed by concentrated HCl. Pink scarlet colour appeared after few minutes which signifies the presence flavonoids.

3.4.3 Determination of phenols/tannins

The fraction of the extract was mixed with 2mL of 2% solution of $FeCl_3$. A blue-green or black colouration which appeared signifies the presence of phenols and tannins.

3.4.4 Determination of saponins: (Froth Test)

The fraction of the extract was mixed with 5mL of distilled H_2O in a test tube and was vigorously shaken for 30 seconds. The formation of the stable foam signifies the presence of saponins.

3.4.5 Determination of terpenes: (Libermann's Test)

The fraction of the extract was mixed with each of 2mL of chloroform and 2mL of acetic acid. The mixture was then cooled down. Thereafter, concentrated H_2SO_4 was slowly and carefully added and the colour changed from violet to blue which signifies the presence of terpenes.

3.4.6 Determination of steroids: (Salkoski's Test)

To the extract, about 2mL of the chloroform was mixed. Concentrated H_2SO_4 was slowly and carefully added and shaken gently. A reddish brown colour signifies the presence of steroids.

3.4.7 Determination of cardiac glycosides: (Keller-kilani Test)

The extract was mixed with 2mL of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2mL of concentrated H_2SO_4. A brown ring at the interphase signifies the presence of cardiac glycosides.

3.4.8 Determination of alkaloids

i. Dragendoff's Test

To 2mL of 1% HCl, the extract was added. Dragendoff's reagent was then added to the mixture. The resulting turbidity of the precipitate was considered as a proof for the presence of alkaloids.

ii. Hagner's Test

To another 2mL of 1% HCl, the extract was added. Hagner's reagent was then added to the mixture. The resulting turbidity of the precipitate was deemed as an evidence for the attendance of alkaloids.

iii. Wagner's Test

To another 2mL of 1% HCl again, the extract was added. Wagner's reagent was then added to the mixture. The resulting turbidity of the precipitate was believed to be a testimony for the existence of alkaloids.

2.5 The Test Microorganisms

Antimicrobial activity of methanol extract of leaves of *P. africana* was investigated against five bacterial isolates and one fungal isolate which were obtained from Vaccine Discovery and Research Laboratory, Centre for Genetic Engineering and Biotechnology, Federal University of Technology, Minna, Nigeria. The bacteria strains which were for the study include *Salmonella typhi, Klebsiella pneumoniae, Streptococcus pyogenes, Pseudomonas aeruginosa,* Methicillin-Resistant *Staphylococcus aureus* (MRSA) and the fungi used for the study was *Candida albicans*. The tested bacteria were maintained on Nutrient agar at 37°C for 24 h and *Candida albicans* on Potatoes Dextrose Agar at 30°C for 48-72 h.

2.6 Innoculum Preparation

A loopful of isolated colonies were inoculated into 4 mL sterile Mueller-Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi and incubated at 37°C for 2 h. The turbidity of actively growing microbial suspensions were adjusted with freshly prepared MHB and SDB using $BaSO_4$ turbidity standard to match turbidity standard of 0.5 McFarland. This turbidity was equivalent to approximately $1.5x10^8$ CFU/mL cells for bacteria, and $1.5x10^7$ spores/mL for fungi strain. The grown suspension was used for further testing.

2.7 Preparation of Crude Extract

For the preparation of the stock solution, 0.5g of the crude extract was accurately weighed using analytical weighing balance into a sterile tube containing 1mL of 2% Dimethylsulfoxide (DMSO). This was vortexed to allow the extract to completely dissolve and 9mL of sterile distilled water was added to give final extract concentration of 50mg/mL. Double fold dilutions was carried out to give extract concentrations of 25mg/mL, 12.5mg/mL and 06.25mg/mL using sterile distilled water respectively.

2.8 *In-vitro* Antimicrobial Susceptibility Assay of the Extract

Susceptibility test of the extract against the isolates were determined in the Microbiology Laboratory, Ibrahim Badamasi Babangida University Lapai in Niger State, Nigeria using Kirby-Bauer agar diffusion method according to NCCLS standards ([15][16]. The Mueller-Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) were used for the antimicrobial activity test. About 100 µL of MHB and SDB cultures containing 0.5 McFarland equivalent to approximately $1.5x10^8$ CFU/mL cells for bacteria, and $1.5x10^7$ spores/mL for fungi strain were dispensed into empty sterile petri dishes using micropipettes. Twenty three millilitres (23mL) of sterilized MHA and SDA maintained between 50 – 45°C was added to the appropriate petri dishes and rocked gently for even distribution of the organisms under aseptic condition and allowed to gel under safety hood for 1 h. On each of the plates containing bacteria isolates. Five wells of 8 mm in diameter were made on the agar plates using sterile metallic cork borer and labelled properly. The base of the wells was sealed with 30 µL of MHA and SDA. Thereafter, 200µL of different concentrations of the extract were carefully and aseptically added with the aid of micropipette into each well and left in the safety hood for 2 h for proper diffusion of the extracts into the agar and then incubated at 37°C for 24 h for bacteria. The same procedure was repeated for fungi strain and incubated at 25°C for 48 h for fungi. The experiment was set up in duplicates. The plates were observed for activity and zones of inhibitions were measured and recorded as mean zone of inhibition. The diameter of each zone was accurately measured with a spotless and translucent ruler in millimetre (mm) in vertical and horizontal manner and mean was determined.

Control experiments were set up by using standard antibiotics, Chloramphenicol (250mg) for bacteria strain and fluconazole (80mg) for fungi specie as reference standards for positive control. Sterile MHA and SDA plates were used as Media Sterility Control (MSC) and MHA and

SDA plates with the used organisms streaked as Organism Viability Control (OVC). All the controls were given the same treatment as the experiments.

2.9 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) value of the extract of *P. africana* leaves extract was determined by microdilution broth method in 96-well microplates [17]. Chloramphenicol (Fidson, Nigeria) and Fluconazole (Pfizer, UK) were used as the standard drug for bacteria and fungi at stock concentration of 50µg/ml. Controls of sterility for the Mueller-Hinton nutrient broth, control culture (inoculum), Chloramphenicol, Fluconazole, crude extract and DMSO were carried out. The microwell plates were closed and incubated aerobically at 37°C for 24 h. Thereafter, 50µL of tetrazolium dye was applied into each well with further 2 h incubation at 37^{o}C and colour change was observed. Any well with reddish-pink colour signifies the microbial growth, which was noted and documented as positive (MIC). All assays were carried out in triplicate.

MIC was carried out on the extract against the isolates using the broth microdilution method (BMM) in 96 micro wells titre plates using NCCLS method [16] with little modification. A volume of 50µL of the extract was dispensed into first row and the same volume of the sterilized media (MHB and SDB) was dispensed into each well except the first row. A two-fold dilution was carried out from row 2 by taking 50µL of the extract to the next row, mixed well and the serial dilution continued to row 7 where 50µL from the wells was discarded away. Then, 50µL of 0.5 McFarland of 2 h culture was added to each well in row 1-7. The rows 8 and 9 were the OVC and MSC. The plates were incubated at 37^{O}C for 24 h. The test was carried out in duplicate and the values are express in mean.

2.10 Determination of Minimum Bactericidal and Fungicidal Concentration (MBC and MFC)

The MBC and MFC were determined from the Minimum Inhibitory Concentration (MIC) result by subculturing from the wells that shows no any sign of turbidity in the MIC test and streak on the freshly prepared MHA and SDA plates and incubate at 37^{o}C for 24 to 48 h and the plates were checked for the present or absent of the growth.

3.0 Results

3.1 Phytochemical Constituents

Qualitative biological active compounds of the methanolic crude extract disclosed the occurrence of carbohydrates, flavonoids, phenols/tannins, saponins, terpenes, steroids, cardiac glycosides and alkaloids respectively as demonstrated in table 1 below.

3.2 Antimicrobial Activity

The methanolic crude extract has antimicrobial efficacy against all the test isolates at 50 and 25 mg/mL of concentration, while *Salmonella typhi*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) showed activity even at 12.50mg/mL. The extract at 0.625mg/mL however had no activity against all the tested organisms. The antimicrobial controls were active against all tested organisms except *Pseudomonas aeruginosa* (figure 1).

3.3 Minimum Inhibitory and Bactericidal Concentrations of the Crude Extract against Sensitive Organisms

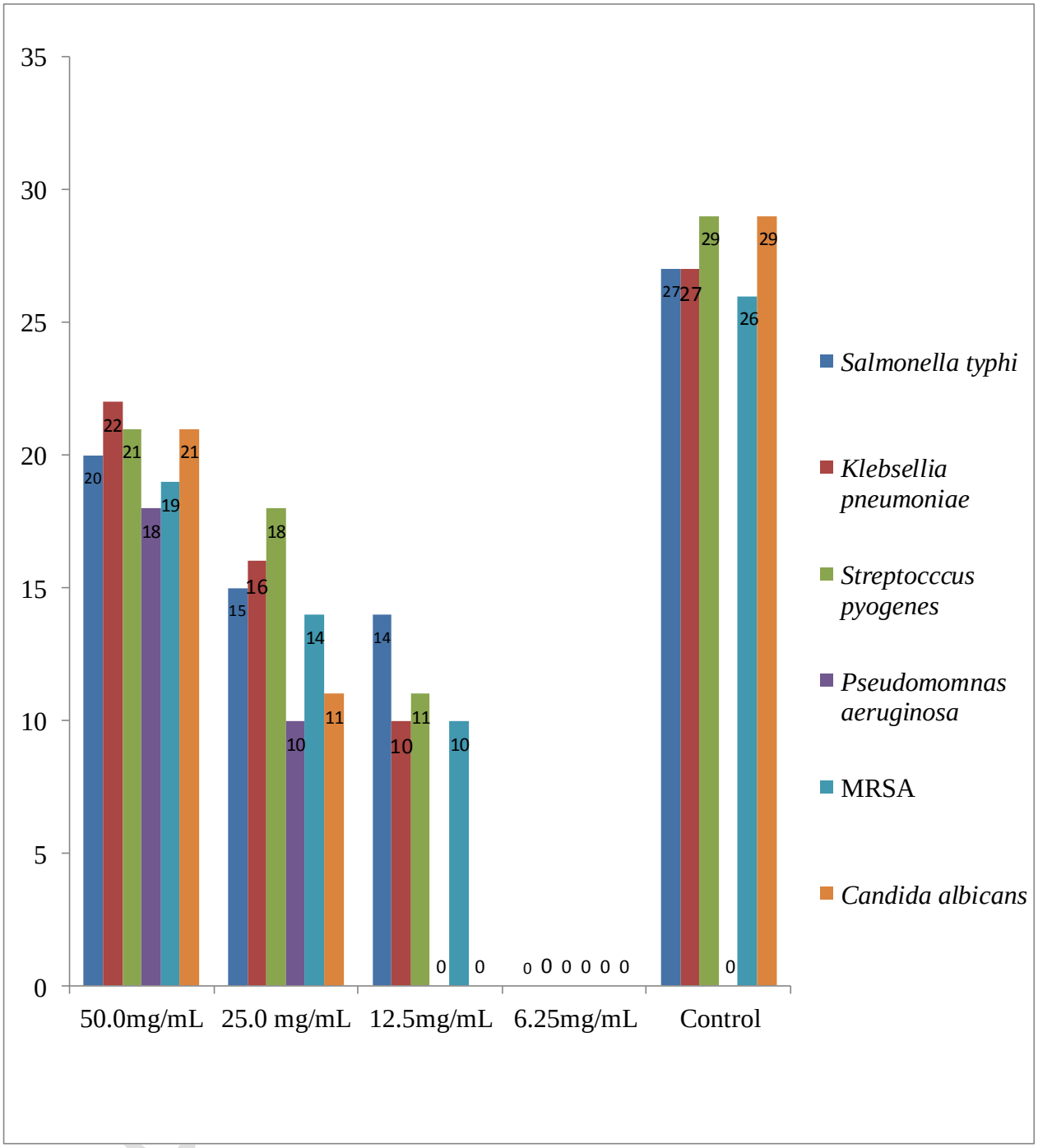
Minimum Inhibitory Concentration (MIC) was carried out on all the isolates where *Salmonella typh*i and *Streptococcus pyogenes* had MIC of 12.50mg/mL. MRSA, *Klebsiella pneumoniae* and

C. albicans had MIC of 25mg/mL while the MIC of *Pseudomonas aeruginosa* was at 50mg/mL (table 2). The MBC of *Salmonella typhi*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, MRSA were at 50mg/mL and *C. albicans* had the MFC of 50mg/mL (table 3).

Table1: Phytochemical constituents of methanolic leaves

Phytochemicals	**Tests**	**Inference**
Carbohydrates	Molisch	+
Flavonoids	Alkaline	+
Phenol/Tannins	Ferric Chloride	+
Saponins	Froth	+
Terpenes	Liebermann	+
Steroids	Salkowski	+
Alkaloids	Dragendoff's	+
	Hagner's	+
	Wagner's	+
Cardiac glycosides	Keller-Kilani	+

Key: + = Present - = Absent



234 Fig. 1: Mean zone of inhibition of methanolic extract of *P. africana* on the test organisms (mm).

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Table 2: Minimum Inhibitory Concentration of Methanol Extract of *P. africana* on the test Organisms

Organisms	Minimum Inhibitory Concentration (mg/mL)	Control (mg/mL)
Salmonella typhi	12.5	50
Streptococcus pyogenes	12.5	50
MRSA	25.0	50
Klebsiella pneumoniae	25.0	50
Pseudomonas aeruginosa	50.0	50
Candida albicans	25.0	50

Table 3: Minimum Bactericidal and Fungicidal Concentration of Methanol Extract of *P. africana* on the test Organisms

Organisms	MBC/MFC (mg/mL)
Salmonella typhi	50.0
Streptococcus pyogenes	50.0
MRSA	50.0
Klebsiella pneumoniae	50.0
Candida albicans	50.0

4.0 Discussion

Determining the phytochemical constituents to identify the possible therapeutic agents contained in this plant in order to establish the basis for their uses in folklore medical practice is important to discovery of new active principle.

Variety of different natural chemical compounds such as saponins, tannins, alkaloids, terpenoids, cyanoglycosides, oleic, flavonoids and stearic acids have been found to confer antimicrobial properties to plants and plant products [18] [19] [20]. [21] stated that flavonoids are polyphenolic phytocompounds which are found in plants and possess antifungal, antibacterial, anticancer, anti-inflammatory as well as antioxidant properties. In accordance with the study conducted by [22] the seed and pod of *Prosopis africana* shows the presence of saponin, alkaloids, steroids, flavonoids, phlabotanin and tannin, the result of this study also showed the presence of saponin, alkaloids, steroids, flavonoids, carbohydrates, terpenes, cardiac glycosides and tannin which is in agreement with findings by [6] but different from the findings by [23], where tannins, saponins and terpenes were absent in methanol leaf extract of *Prosopis africana*. The study by [24], showed the absence of tannins in *Prosopis lappacea*. The occurrence of these bioactive compounds in reasonable amount in the leaves of *P. africana* could have been responsible for its characteristic antimicrobial properties. These are recognized to have antibacterial agent and may be utilized traditionally for the treatment of infectious diseases [25]. [21] stated that flavonoids are polyphenolic phytocompounds which are found in plants and possess antifungal, antibacterial, anticancer, anti-inflammatory as well as antioxidant properties.

Antimicrobial analysis of methanol crude leaf extract of *P. africana* exhibited some level of antimicrobial properties against microorganisms which include *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, Methicillin-Resistant *Staphylococcus aureus* (MRSA) *Salmonella typhi, Klebsiella pneumoniae*, and the fungi used for the study was *Candida albicans* at various concentrations from 50mg/mL to 6.25mg/mL with varied diameters zones of inhibition ranging from 1mm for 29mm. [22] tested the aqueous and methanol extract of seed and pod of *P. africana* against fifteen bacteria at a fix concentration of 25mg/mL and recorded zone of

inhibition ranging from 5mm to 17mm. [26] also reported the antimicrobial activities of *P. africana* ethanol and aqueous stem and root extract against clinical isolates of oral pathogens namely *Streptococcus mutans*, *Staphylococcus saprophyticus* and *C. albicans* with significant action. The study by [25], revealed activity against *Pseudomonas aeruginosa, Staphylococcus aureus, and Bacillus subtilis* at concentrations comparable to the findings of this study. However, the study by [27], showed no activity against *K. pneumoniae.* The result of this study showed no activity against tested organisms at concentration of 6.25mg/mL. Interestingly, the extract had activity against MRSA at 12.5mg/mL concentration with MIC of 25.0mg/mL. This is very promising because of the facts that further purification may exhibit better activity which could lead to discovery of a new lead against antibiotics resistant *Staphylococcus aureus.* It is also worthy of notice that the extract is also active against *Pseudomonas aeruginosa* at crude MIC of 50.0mg/mL.

5. Conclusion

The methanolic leaf extract of *P. africana* has displayed varied activity against pathogenic microorganisms and could represent candidate of antimicrobial agent against some human pathogenic microbes. Furthermore, the bioactive ingredients indicated that the plant part have proved its usage in the folkloric medicine for the management of different ailments and could be the basis of alternative anti-infective therapy. Therefore, these findings shall broaden and enhance global data base of the antimicrobial property of the active ingredients present.

6. Limitation of the study

The limitation of this study includes lack of funding to permit us to buy solvents for fractionations which will enable us to obtain a pure compound.

Conflict of Interest

The authors declared no conflict of interest.

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