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Evaluation of Potential Antimicrobial Activity of Stem Barks Extract of *Persea americana* (Mill) and its Solvent Fractions against Randomly Selected Pathogenic Bacteria and Fungi

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ABSTRACT: *Persea americana* stem barks are used locally in parts of Nigeria in treating bacterial infections. This study aims to determine the antimicrobial potential of the methanol extract and fractions against *S. aureus, B. substilis, E. coli, P. aeruginosa, C. albicans and Aspergilus species*. The plant material was extracted with 80% methanol and partitioned into chloroform and aqueous phases which were evaluated for antimicrobial activities using agar well diffusion method. The methanol extract inhibited the growth of the test organisms; *P. aeruginosa* showed the most activity at 100mg/mL. The $CHCl_3$ fraction of the plant extract was ineffective against gram negative bacteria (*E. coli* and *P. aeruginosa*) at low concentrations. The $CHCl_3$ fraction was most effective against *S. aureus* at 50mg/mL but ineffective against gram-negative organisms at lower concentrations. The extract's H_2O fraction had the most antimicrobial activity. The H_2O fraction inhibited *B. substilis* at 50mg/mL (8.50±1.08mm).Alkaloids, saponins, tannins, steroids, among others were observed in the extract and fractions. Owing to the higher antimicrobial activity in the H_2O fraction, the active antimicrobial compounds could be polar in nature. The higher inhibitory activity recorded by the H_2O fraction supports the folkloric use of *P. americana*

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Keywords: *Persea americana*; antimicrobial; microbes; phytochemicals; fungi; fractionation

Throughout history, natural remedies have been utilized to alleviate and combat illness for a long period of time, and many of the pharmaceuticals that have made modern medicine so successful were themselves derived from natural ingredients (Shabnam *et al*, 2016; Ukwubile *et al*, 2019). Because of their low cost and wide availability, these plants are commonly used in folk medicine (Boccolini and Boccolini, 2020). Many antimicrobial substances, both synthetic and natural, have been found to treat and prevent infectious diseases in the past (Ikpefan *et al*, 2021). The increasing prevalence of bacteria that are immune to antibiotics poses a serious threat to worldwide public health. This highlights the critical need for the invention of new antibiotics that can effectively combat microbial infections without breaking the bank (Li and Webster, 2018). The emergence of antibiotic insensitivity has been a major obstacle to their therapeutic use across a wide spectrum of microorganisms. Therefore, there is compelling demand for the discovery of new antimicrobials since this effectiveness of current drugs has been continuously declining (Enwa *et al*, 2021). The proliferation of microorganisms that are insensitive to antibiotics has boosted the discovery and development of new antibacterial medications (Saha and Sarkar, 2022). Multiple drug-resistant microbes are rising and it is attributable to the irresponsible use or mismanagement of antibiotics, which has resulted in many antibiotics losing their effectiveness against certain microorganisms (Llor and Bjerrum 2014). In recent time, there is a resurgent enthusiam in plants

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that have antimicrobial properties as a consequence of the current antimicrobial drug-related issues (Manandhar *et al*., 2019). As a result, the hunt for a new bioactive antibiotic is an essential component of contemporary medical practice, and it is necessary in order for modern medicine to overcome the socioeconomic and health repercussions of multidrug-resistant microbes (Roberto *et al*, 2019). The plant *Persea americana*, also known as avocado is known by its native names such as *"Igba/apoka", "Ube Oyibo" "Orhmwu" "Ebanmbakara"* in Yoruba, Ibo, Edo and Efik dialects in Nigeria (Nwauzoma *et al*, 2013), is a polymorphic tree crop that originated from a broad geographical area stretching from the eastern and central highlands of Mexico through Guatemala to the Pacific coast of Central America. It is also native to Puerto Rico, United States, Honduras and spans across Jamaica (Smith, 1966, 1969; Storey, *et al*., 1986; Dreher and Davenport, 2013). It inhabits the tropical parts of Africa, specifically the countries of Nigeria, Cameron, Benin republic, Togo, and a plethora of others. In Nigeria, the stem barks of *P. americana* are used ethnomedically to treat a wide range of illnesses induced by bacteria and fungi (Anyanwu and Okoye, 2017). Previous research conducted by our team on the three morphological components of the plant (leaves, stem bark, and root bark) demonstrated that the leaves extract exhibited higher levels of cytotoxic and growth inhibiting effects (Ayinde *et al*, 2011). The current research investigates potential antimicrobial activity of stem bark extract of *Persea americana* (Mill) and its solvent fractions against randomly selected pathogenic bacteria and fungi.

Fig 1: showing the (a) fruit and leaf (b) surface stem bark of *P. americana* in its natural habitat

MATERIALS AND METHODS

Collection of Plant Materials: The stem barks of the plant was obtained in April 2022 at Abraka, Delta State. The identity of the plant was authenticated at Department of Plant Biology and Biotechnology, University of Benin, Benin City where a specimen number UBH-P408 was obtained.

Processing and Plant Material: The plant was gabbled for removal of contaminants and spread out to dry for at least 72 hours. Thereafter the stem barks was chopped into smaller sizes and was further dried in the oven maintained at 60°C. The plant was then grounded to coarse particle with the aid of a milling machine. The coarse plant material was then stored in an air tight container for future use. The plant material was extracted using the cold maceration technique.

Extraction: A total of 1.0kg of the powdered stem barks of *P. americana* was extracted adopting standard methods (Shabnam *et al*, 2016) carefully weighed and macerated in two (2) liters of 70% methanol in a stopper container and was left to stand for 72 hours with occasional agitation for proper extraction. Upon pressing the marc, the liquid was filtered through a cotton cloth as well as Whatman No. 1 filter paper. The filtrate was concentrated under vacuum using a rotary evaporator and was completed in a hot air oven. The extract's weight 1 (yield) was determined and recorded.

Partitioning of extract: The methanol extract having shown antimicrobial properties was subjected to liquid-liquid partitioning with the aid of separating funnel. 30g of the extract was first dissolved in equal amount of distilled water and methanol (1:1 of 200 mL). The mixture was then partitioned exhaustively with using chloroform (200 mL ×3). Both fractions were collected and dried in a rotary at 40°C and their yields calculated.

Phytochemical Screening: Phytochemical Screening was conducted according to standard specifications to determine the presence of phytochemicals in the extract and fractions of *P.americana* stem barks (Trease and Evans, 2002; Shaikh, *et al*., 2020).

Selected Organisms: Pure cultures of *S. aureus*, *B. substilis* (gram-positive bacteria); *P. aeruginosa*, *E. coli* (gram-negative bacteria); *Aspergilus spp* and *C. albicans* (fungi) were acquired from the pharmaceutical microbiology laboratory and biochemical tests were carried out to authenticate the identity of the organisms. These organisms were chosen because of their prevalence and clinical importance. A slant of pure culture of each test organism were prepared and stored in a refrigerator at 4°C. The culture of the bacteria and fungi were sub-cultured into a sterile nutrient broth and Saboraund dextrose broth respectively and were incubated for 24hours before the antimicrobial test was carried out.

Preparation of Culture Media: Muller Hinton agar and Saboraund dextrose agar was prepared according to the manufacturer's instructions and was autoclaved for 15minutes at 121°C. The media was then left to cool to 45°C. Thereafter, 20mL of the agar was measured out with a sterile measuring cylinder, poured aseptically into the petri dish, and left to set. The broth of the test organism was spread on the agar plate under aseptic conditions using a sterile swab stick. This procedure was repeated for all the organisms. A flame sterilized 6mm cork borer was used to bore seven (7) holes in each freshly prepared Muller Hinton agar plate containing the test organism. The hole was labeled using masking tape representing different concentrations and the positive control. Same process repeated for the Saboraund dextrose agar utilized for the fungi. The 6mm cork borer was sterilized before use and the process was carried out under aseptic conditions. The required concentration of the extracts was placed aseptically into the holes and left undisturbed for 20minutes before incubation at 37°C for 24hours

Measurement of Zones of Inhibition and MIC: A clear zone around each well indicates inhibition and each zone were measured with the aid of a meter rule in millimeters (mm). The diameter of the well was subtracted from the zone of inhibition and the results were recorded. The minimum inhibitory concentration was assessed and determined using the nutrient agar method. The Muller Hinton agar and Saboraund dextrose agar were prepared according to the manufacturer's instruction. A sterile wire loop was used to pick up the organism from the inoculums and streak the surface of the solid agar. The agar plate was incubated for 24hours at 37°C. This procedure was repeated for the other concentrations of the sample.

Statistical Analysis: The data obtained were evaluated using Graphpad Prism 7.0. One way analysis of variance (ANOVA) was used in the data analysis and was represented as mean ± Standard Error of Mean (SEM).

RESULTS AND DISCUSSION

Yield of the Plant: The 1.0kg powdered stem barks of *P.americana* yielded 62.3g of the extract. The 30g of the extract partitioned yielded 1.5 and 21.5g of the $CHCl_3$ and H_2O fractions respectively.

Result of Preliminary Phytochemical screening of extract and fractions of P.americana: The results of the preliminary phytochemical screening of the stem bark extract and fractions of *P.americana* showed the presence of secondary metabolites such as alkaloids, saponins, tannins, and terpenoids in varying intensities. (Table 1).

Table 1: Phytochemical screening results

Phytochemical group	Methanol extract	Aqueous Fraction	Chloroform Fraction
Alkaloids	+	++	-
Flavonoids	-	-	-
Saponins	+	+	-
Tannins	+	+	-
Terpenoids	+	+	+

Key: - = Absent. + = Present

Effects of the extract and fractions of P.americana on test organisms: The methanol extract and fractions of *P.americana* showed antimicrobial activity at varying degrees. The results showed that *P. aeruginosa* and *E.coli* were more susceptible to the extract and fractions. For example, at 12.50 mg/mL, the extract recorded 3.33 ±1.90 and 5.10 ±0.54mm against *P. aeruginosa* and *E.coli* with the other organisms showing little or no sign of inhibition. At the maximum concentration of 100 mg/mL, the zones of inhibitions increased to 5.80 ±0.47 and 8.33 ±0.54 mm for the same organisms. Although the extract showed some antimicrobial action, it was marginal compare to the control drugs (Table 2, Figure 2). Also, for the H_2O fraction, 5.50 ±0.47, 6.33 ±0.27 and 3.67 ±0.27 mm zones of inhibitions were recorded for *E.coli*, *P. aeruginosa* and *Aspergilus spp* at 25 mg/mL which later increased to 6.50 ±0.82, 9.00 ±0.47 and 4.67 ±0.27 mm at 100 mg/mL (Table 3, Figure 3). Similarly, the $CHCl_3$ fraction showed a pattern of activity against the same three organisms as the aqueous fraction, but at a significantly lower level of activity (Table 4, Figure 3).

Table 2: Antimicrobial activity of the methanol extract of *P. americana* stem barks

Organism	Concentrations (mg/mL)/Zones of inhibition (mm)						
	100	50	25	12.50	6.25	3.13	Control
S. aureus	4.00 ±0.82	3.67 ±2.13	2.33 ±1.36	-	-	-	24.00 ±0.00
B. substilis	3.67 ±1.66	3.67 ±0.27	1.35 ±0.01	-	-	-	25.33 ±0.27
E. coli	5.80 ±0.47	4.67 ±0.27	3.67 ±0.27	1.33 ±1.90	-	-	27.33 ±0.72
P. aeruginosa	5.33 ±0.54	5.00 ±0.47	4.67 ±0.47	2.10 ±0.54	-	-	30.67 ±0.72
Aspergillus spp	4.67 ±2.37	4.33 ±4.46	-	-	-	-	15.50 ±0.10
C.albican	1.00 ±0.47	-	-	-	-	-	5.00 ±1.11

Key: Control; Ciprofloxacin 200mg/mL was used for the bacteria while fluconazole 200 mg/mL was used for the fungi. Gram-positive bacteria = S. aureus and B. substilis; gram-negative bacteria = E. coli &P. aeruginosa; fungi = Aspergilus species and C. albicans Mean ± SEM, n=3

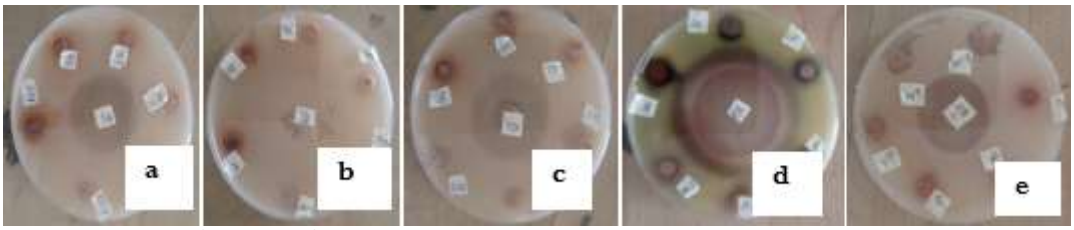

Fig 2: Showing the zone of inhibition of the crude extract against *S. aureus* (a), *B. substilis* (b), *Aspergilus spp* (c), *E. coli* (d), and *P. aeruginosa* (e)

Table 3: Antimicrobial activity index of the aqueous fraction of *Persea americana*

Organisms	**Concentrations (mg/mL)/Zones of inhibition (mm)**					
	50	25	12.5	6.25	3.13	Control
S. aureus	4.67 ±0.27	2.00 ±0.01	-	-	-	29.67 ±0.54
B. substilis	5.00 ±1.08	2.33 ±0.02	-	-	-	29.33 ±0.27
E. coli	6.50 ±0.82	5.50 ±0.47	1.00 ±0.47	-	-	29.33 ±0.27
P. aeruginosa	9.00 ±0.47	6.33 ±0.27	3.33 ±0.47	2.67 ±0.47	-	30.67 ±0.72
Aspergilus spp	4.67 ±0.27	3.67 ±0.27	-	-	-	14.00 ±0.47
C. albicans	2.15 ±0.27	-		-	-	13.33 ±0.27

Control; Ciprofloxacin 200mg/mL was used for the bacteria. Fluconazole 200 mg/mLwas used for the fungi. Mean ± SEM, n=5

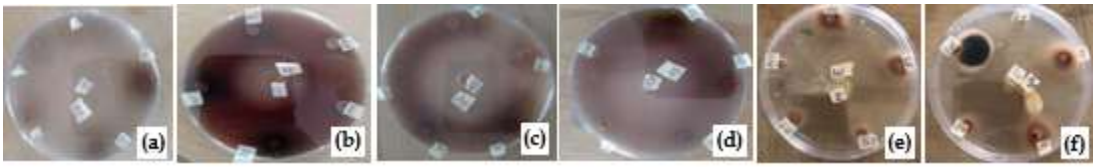

Fig 3: Showing the zone of inhibition of the aqueous extract against *S. aureus* (a), *E. coli* (b), *B. substilis* (c), *P. aeruginosa* (d), *Aspergilus spp* (e), and *C. albicans* (f)

Table 4: Antimicrobial activity index of the chloroform fraction of *P.americana* extract

Organism	**Concentrations (mg/mL)/Zones of inhibition (mm)**					
	50	25	12.5	6.25	3.13	Control
S. aureus	3.83 ±1.30	-	-	-	-	30.00 ±0.47
B. substilis	2.00 ±1.63	-	-	-	-	24.67 ±0.54
E. coli	5.67 ±0.27	2.00 ±1.63	-	-	-	24.67 ±0.54
P. aeruginosa	4.67 ±0.72	3.67 ±0.72	-	-	-	34.33 ±0.27
Aspergilus spp	2.33 ±0.02	-	-	-	-	13.33 ±0.27
C. albicans	3.27±0.01	-	-	-	-	16.67 ±0.54

Key: Control; Ciprofloxacin 200mg/ml was used for the bacteria while fluconazole 200 mg/mLwas used for the fungi. Mean ± SEM, n=5

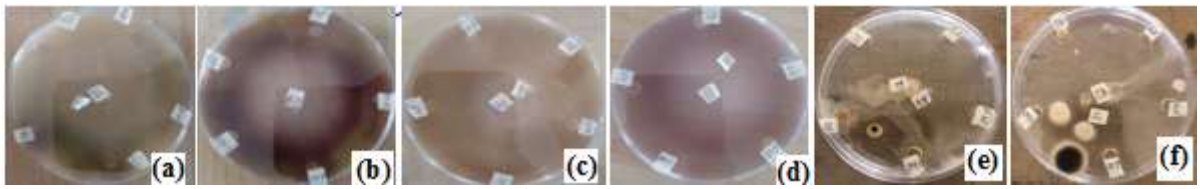

Fig 4: Showing the zone of inhibition of the chloroform fraction against *S. aureus* (a), *B .substilis* (b), *E. coli* (c), *P. aeruginosa* (d), Aspergilus spp (e), and *C. albicans* (f)

Results of Minimum inhibitory concentrations of extract and fractions of P. Americana: The MIC was the minimum concentration of extract, fraction, or chemical that inhibited growth for 24 h at 37°C. At a concentration of 1.56-50mg/mL of the extract, there was no growth inhibition in all test organisms. However at the maximum concentration (100 mg/mL), *S. aureus* , *E.coli* and *P. aeruginosa* had their growth inhibited by the methanol extract at a concentration of 100 mg/mL. This infers that the methanol extract had a good antimicrobial activity against the bacteria. Also, the MIC of the aqueous fraction indicated a substantive level of activity for the aqueous fraction of the extract. While the gram positive and negative bacteria both had MIC of 12.5mg/mL, the *Aspergilus* fungi spp recorded MIC at 25mg/mL (Table 6, Figure 6). The chloroform fraction only recorded MIC of 50 mg/mL against the gram negative bacteria (Table 7, Figure 7).

Table 5: Minimum Inhibitory Concentration of the methanol extract of *P.americana*

Organisms	Concentrations (mg/mL)						
	100	50	25	12.5	6.25	3.13	1.56
S. aureus	-	+	+	+	+	+	+
B. substilis	+	+	+	+	+	+	+
E. coli	-	+	+	+	+	+	+
P. aeruginosa	-	+	+	+	+	+	+
Aspergilus spp	+	+	+	+	+	+	+
C. albicans	+	+	+	+	+	+	+

Key: - = No Growth + = Growth

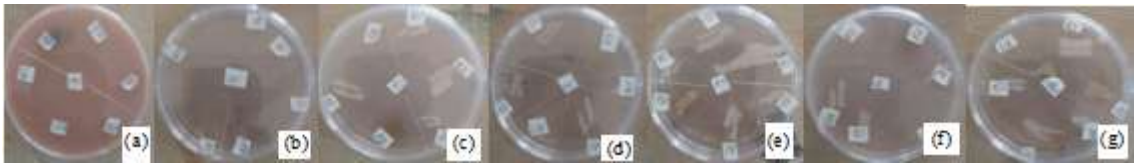

Fig 5: Plates showing the minimum inhibitory concentration of the crude extract against *S. aureus*, *B. substilis*, *Aspergilus spp*, *E. coli*, and *P. aeruginosa* at 100mg/mL (a), 50mg/mL (b), 25mg/mL (c), 12.5mg/mL (d), 6.25mg/mL (e), 3.13mg/mL (f), and 1.56mg/mL (g).

Table 6: Minimum Inhibitory Concentration of the aqueous fraction of *P. americana*

Organisms	Concentrations (mg/mL)					
	50	25	12.5	6.25	3.13	1.56
S. aureus	-	-	-	+	+	+
B. substilis	-	-	-	+	+	+
E. coli	-	-	-	+	+	+
P. aeruginosa	-	-	-	+	+	+
Aspergilus spp	-	-	+	+	+	+
C.albican	-	+	+	+	+	+

Key: - = No growth of organism. + = Growth of organism.

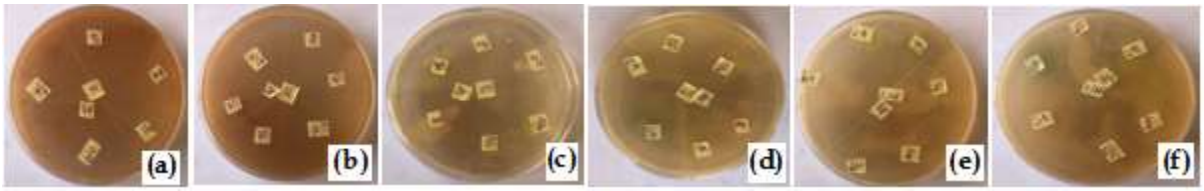

Fig 6: Showing the minimum inhibitory concentration of the crude extract against *S. aureus*, *B. substilis*, *Aspergilusspp*, *C. albicans*, *E. coli*, and *P. aeruginosa* at 50mg/mL (a), 25mg/mL (b), 12.5mg/mL (c), 6.25mg/mL (d), 3.13mg/mL (e), and 1.56mg/mL

Table 7: Minimum Inhibitory Concentration of the Chloroform fraction

Organisms	**Concentrations (mg/mL)**					
	50	25	12.5	6.25	3.13	1.56
S. aureus	+	+	+	+	+	+
B. substilis	+	+	+	+	+	+
E. coli	-	+	+	+	+	+
P. aeruginosa	-	+	+	+	+	+
Aspergilus spp	+	+	+	+	+	+
C. albicans	+	+	+	+	+	+

Key: — = No growth of organism. + = Growth of organism.

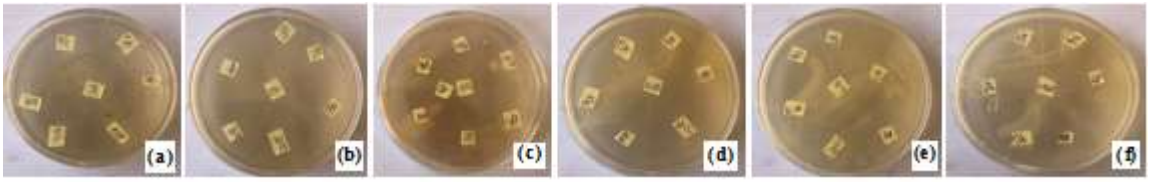

Fig 7: Photograph showing the minimum inhibitory concentration of the Chloroform fraction against *S. aureus*, *B. substilis*, *Aspergilusspp*, *E. coli*, *C. albicans*and *P. aeruginosa* at 50mg/mL (a), 25mg/mL (b), 12.5mg/mL (c), 6.25mg/mL (d), 3.13mg/mL (e), and 1.56mg/mL (f)

Drug-resistant organisms are on the rise, posing a severe threat to the treatment of many diseases (*Zhang et al*, 2022). The last ten years have seen an upsurge in microbial hazards to human health, many of which have been worsened by the spread of antibiotic-resistant genes (ARGs). The stem barks of *P. americana* is traditionally used to treat various bacterial infections ethno medically. On evaluation of the crude drug against some bacteria and fungi; the result confirmed its ethno medical use in the treatment and management of different bacteria and fungi infections. The antimicrobial studies revealed that the methanol extract of *P. americana* stem barks possessed growth inhibitory effect on both bacteria and fungi. However upon fractionation of the methanol extract using chloroform and distilled water, the antimicrobial activity was observed to be enhanced as recorded in by chloroform and aqueous fractions respectively. The aqueous fraction of the crude extract had the greatest activity against the test organisms (bacteria and fungi).When compared to the control the plant had marginal activity against the organisms. The difference in the efficiency of the extracts can be attributed to the difference in the polarity of the solvents. The solvents influence the solubility of the active compound which results in differences in chemical composition of the different extracts (Idris, *et al*., 2009). From the studies it can be deduced that the active compound responsible for the antimicrobial activity of *P.americana* can be extracted using polar solvents. The chloroform extract had no antimicrobial activity against gram negative bacteria at low concentrations this result correlates with observations made by Idris, *et al*., (2009) and Boadi, *et al*., (2015). Idris, *et al*., (2009) evaluated the antimicrobial activity of the seed of *Persea americana* and reported that the chloroform extract had no activity against *Escherichia coli* and *Salmonella typhi* (gram negative bacteria). This report correlates with the results of this study. Similarly Boadi, *et al*., (2015) reported that the petroleum ether and chloroform extract of *P. americana* leaves showed no antimicrobial activity against *E. coli*, a gram negative bacterium at low concentration. However the methanol extract of *P. americana* stem bark was found to be effective against gram positive bacteria, gram negative bacteria and fungi in contrast to observations made by Idris, *et al*., (2009) on the antimicrobial activity of *P. americana* seed's methanol extract. Several factors have been enumerated that influence the antimicrobial sensitivity of plant extracts against test organisms. These factors include; the volume and concentration of the extract in the hole prepared within the agar plate, thickness and composition of the medium, duration and diffusion phase before incubation (Hewitt and Marston, 1989). The low antimicrobial activity of the chloroform fraction could be due to the poor diffusibility of the constituents extracted by the chloroform. However this challenge can be surmounted through the use of high concentrations of the extract. This argument is supported by the data obtained from the studies (Table 6). There was an increase in antimicrobial activity of the chloroform fraction of the *P. americana* extract at a concentration of 50mg/mL. In order to have a reproducible and comparable result all agar plates used for the studies was ensured to have had the same level of thickness, volume and was subjected to the same temperature during incubation. In addition the antimicrobial studies was carried out in replicates. The aqueous fraction had greater antimicrobial activity than the extract and chloroform fraction. From the results obtained, gram negative bacteria (*E. coli* and *P. aeruginosa*) were resistant to the chloroform fraction of the extract.

Conclusion: This study indicate that the molecule responsible for the antimicrobial activity of *P. americana* stem barks may be polar in nature, and that maximum antimicrobial activity can be obtained by using polar solvents in the extraction procedure. However, more research are needed to confirm this as well as to isolate and identify the active component responsible for the antimicrobial activity of *P. americana* stem barks.

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