ANTIBACTERIAL AND PHYTOCHEMICAL ANALYSIS OF *Piper guineense, Ocimum gratissimum, Allium sativum, Zingiber officinale* AND *Cassytha filiformis*

ISSN: 2141 – 3290
www.wojast.com

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ABSTRACT

Medicinal plants remain important source to combat serious diseases in most developing countries. The ethanol, ethyl acetate, N-hexane and warm water extracts of the leaves of *Piper guineense*(Black pepper), *Ocimum gratissimum* (Scent plant),*Allium sativum* (Garlic), *Zingiber officinale* (Ginger) and the stem of *Cassytha filiformis* (Love vine) were examined for antibacterial activities and phytochemical constituents using standard methods. The antibacterial activity of the plants was assayed using agar well diffusion method. The minimum inhibitory concentration (MIC) values determined at concentrations of 200, 100, 50 and 25 mg/ml for all extracts against the test bacteria; Methicillin Resistant *Staphylococcus aureus* (MRSA), Methicillin Sensitive *Staphylococcus aureus* (MSSA), *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi*and *Bacillus subtilis*) ranged from 25 to 50 mg /ml. All the test bacteria but *E. coli* were susceptible to all the plants extracts assayed. The most effective extract was *Cassytha filiformis* and *Pseudomonas aeruginosa* was the most sensitive while *E. coli* was the least sensitive. Qualitative and quantitative assay of the extracts showed that their potency may be due to the presence of alkaloids, flavonoids,saponins, tannins, phlobatannins, steroids, glycosides and quinones. The presence and solubility of the bioactive compounds varied with the type of medicinal plant and solvent used. Alkaloid was detected only in *Cassytha filiformis* and recovered more with ethanol than ethyl acetate and N-hexane. The susceptibility test revealed that the ethanol, ethyl acetate, N-hexane and warm water extract of *Piper guineense, Ocimum gratissimum* and *Cassytha filiformis* were highly potent against MRSA (14.7±2.0 mm), MSSA (12.0±0.6 mm), *Escherichia coli* (19.0±2.5 mm), *Pseudomonas aeruginosa* (15.0±1.0 mm), *Klebsiella pneumonia* (16.0±1.0 mm),*Salmonella typhi* (16.6±1.5 mm) and *Bacillus subtilis* (14.5±0.5 mm) when compared with the values recorded for the control in MRSA (10.33±1.5) and *Pseudomonas aeruginosa* (9.7±2.1mm). The plants extracts showed effective potency against the putative bacterial pathogens and are recommended for use as antimicrobial agents for the treatment of ailments caused by pathogenic bacteria including those by Methicillin Resistant *Staphylococcus aureus* (MRSA).

INTRODUCTION

Plants play a key role in health care with about 80% of the world's populations relying on the use of medicinal plants for traditional medicine treatments (Obeidat *et al.*, 2012; Anyanwu and Nwosu, 2014). The medicinal value of these plants lies in some bioactive substances (alkaloids, tannin, flavonoid, saponins, phlobatannins, steroid, glycosides and quinones) which produce definite physiological action on the human body (Ameh, Eze and Omeje 2012) as well as on their antimicrobial potentialsv (Anyanwu and Nwosu, 2014). Of recent, bacterial infections have increased to a great extent and antibiotics resistance effects has become an increasing therapeutic problem (Manesh and Satish, 2008). Therefore, it is of great interest to ascertain the

potency of these plants in order to validate their use in folk medicine and to reveal the bioactive compound responsible for their efficacy.

In this study, the ethanol, ethyl acetate, N-hexane and warm water extracts of the leaves of *Piper guineense* (Black pepper), *Ocimumgratissimum* (Scent plant),*Allium sativum* (Garlic), *Zingiberofficinale* (Ginger)and the stem of *Cassythafiliformis* (Love vine) were examined for antibacterial activities and phytochemical constituents.

MATERIALS AND METHODS

Collection and Identification of Plant Sample

The plants; *Piper guineense*, *Ocimum gratissimum, Allium sativum ,Zingiber officinale* , and *Cassytha filiformis* were collected from Ibesikpo Asutan, Local Government Area in Akwa Ibom State. The plants were identified with the help of a taxonomist in the Department of Botany and Ecological Studies, University of Uyo..

Preparation of Plant Extracts

The fresh leaves of *Piper guineense, Ocimum gratissimum, Allium sativum, Zingiber officinale* and stem of *Cassytha filiformis* were milled with grinding machine and dried for 7 to 23 days at room temperature. Then 300g of each of the dried plants were dissolved in 400ml of different solvents(ethanol, ethylacetate, N-hexane and warm water) in conical flasks and soaked for one week. They were filtered using whatman No.1 filter paper into beakers, the extracts were dried using oven at 40 ^{0}Cand dried (concentrated) extracts were stored in a refrigerator at 4^{0}C for further analysis

Phytochemical Screening of Plant Extracts

The phytochemical screening of the sample was carried out according to standard methods described by Harborne (1994), Sofowora (1999), Trease and Evans (1989) and Gul *et al*.(2017). Mayer's and Frothing tests were employed for the detection of alkaloids amd saponnins respectively. The tannins were determined by Bromine water test, anthraquinones by Borntrager's test, phlobatannins by formaldehyde, flavonoids by Shinoda's test, Cardiac Glycosides by Keller killiani test and Steroids by Leberman's test.

Test Bacteria

The test bacteria used for the antibacterial activity screening were Methicillin-resistant *Staphylococcus aureus* (MRSA), Methicillin-sensitive *Staphylococcus aureus* (MSSA), *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis and Salmonella typhi.* The isolates were obtained from the culture collection of the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Science, Nnamdi Azikiwe University,Agulu, Anambra State.

Antibacterial Screening

The antibacterial activity of the crude plant extracts were determined by the Agar Well Diffusion method (Dubey and Maheshwari, 2004). The different bacterial isolates were subcultured on nutrient broth to obtain young cultures. Mueller-Hinton Agar (MHA, Hi-media) was used for the assay. Using different solvents, the crude plant extracts were derived and serially diluted to obtain concentrations: of 200, 100, 50 and 25 mg/ml. The MHA plates were prepared and 0.1 ml of each of the organism inoculated on the medium with the aid of a sterile spreader.

The wells made with sterilized cork-borer (6 mm in diameter) were filled up with the extracts (0.2 ml) and plates were incubated at 37^{0}C for 24 hrs. In this assay, the halo diameters are taken as an index of the degree of antibacterial activity of the plant extracts or sensitivity of the test organisms to each of the extract. Ethanol (70 %) and Vancomycin (VA_{30}), Cefoxitin (Fox_{30}), Oxacillin ($0x_1$) Cloxacilline (OB_5) were used as the control. At the end of incubation, inhibition zones produced were measured with a transparent ruler in millimeters and the diameter of inhibition compared with control disc. All the experiments were carried out in triplicates.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the plants extracts was determined according to a modified method described by Dubey and Maheshwari(2004)and Akinjogunla (2016). The different extracts diluted to concentrations of 200,100, 50, and 25 mg/ml. Then 2ml of the different concentrations of the extracts were mixed with 13 ml of sterilized molten Mueller- Hinton Agar (MHA), poured into sterile petri dishes and allowed to solidify. Precisely 0.1 ml of each test organism (24 hrs old) was streaked on the medium. The inoculated plates were aerobically incubated at 37^0C for 24 hrs. The least concentration that prevented the growth of test organism was recorded as the MIC of the plant extract.

Statistical Analysis

The inhibitory zones of all crude extracts were expressed as the mean ± standard Deviation.

RESULTS

The phytochemical and antibacterial activity of the medicinal plants analyzed are presented in Tables 1 and 2 respectively. The bioactive phytochemicals detected were alkaloids, saponins,flavonoids , anthraquinones, steroids, glycosides,and quinones. Their concentrations were found to vary with the different plants and sovents used for the extraction. The results of the antibacterial activity of the extracts against the pathogenic bacteria (MRSA,MSSA,*P. aeruginosa, B. subtilis, S. typhi, K. pnuemoniae and E. coli*) revealed varying degrees of growth inhibition. The mean inhibition zones is indicative of the antibacterial activity of the plant extracts. The positive control: Vancomycin antibiotics showed inhibition zone that ranged between 9.7 and 10, other antibiotics did not show any growth inhibition. The Minimum Inhibitory Concentration (MIC) of the crude extracts against the test bacteria are illustrated in Figures 1 to 5. Among the plants extracts tested, the water extracts of *Cassytha filiformis* showed the strongest activity against all of the test bacteria with the best MIC of 25 mg/ml. The highest sensitivity were exhibited by two organisms' *B. subtilis and P. aeruginosa*, while *E. coli* showed the least sensitivity of all the test isolates.

Table 1: Qualitative phytochemical screening of plant extracts

		Secondary Metabolites							
	Test	Alkaloids	Flavonoids	Saponins	Tannins	Phlobatannina	steriods	Glycosides	Quinones
Piper	E.E	-	+	+	++	+	++	+	+
guineense	E.A.E	-	++	-	+	-	++	+	+
	N.H.E	-	-	-	-	-	+	++	+
	W.W.E	-	+	++	++	++	+	+	++
Ocimum	E.E	-	+++	+	+	+	+	+	+
gratissimum	E.A.E	-	+	-	+	-	++	+	+
	N.H.E	-	+	-	+	-	-	-	-
	W.W.E	-	+	+	++	++	+	+	+
Allium	E.E	-	+	+	+	-	+	+	-
sativum	E.A.E	-	+	+	+	-	+	-	-
	N.H.E	-	+	+	++	-	+	-	-
	W.W.E	-	+	+	+	-	+	-	-
Zingiber	E.E	+++	+	+	+	-	+	+	-
officinale	E.A.E	+	+	+	+	-	+	+	-
	N.H.E	+++	++	+	+	-	+	-	-
	W.W.E	+	-	+++	+	-	+	-	-
Cassytha	E.E	++	++	+	+	+	-	+	+
filiformis	E.A.E	-	++	-	+	-	++	+	-
	N.H.E	-	+	-	-	-	-	+	-
	W.W.E	+	+	++	++	++	-	+	+

Key:-S/M= Secondary Metabolites, E.E = Ethanol extract, E.A.E = Ethyl acetate extract, N-H.E = N-Hexane extract, W.W.E = Warm water extract, (-) = Negative reaction, (+) = positive reaction

Table 2: Preliminary stage of the mean inhibition zone (mm)

Test Plant	Extract	Bact. Org. ± MRSA	MSSA	E. coli	P. a.	K. p.	S. t.	B. s.
Piper	E.E	11.71 ± 5	9.5± 1.0	8.7± 1.5	0.0± 0.0	0.0± 0.0	12.3± 2.5	0.0± 0.0
guineense	E.A.E	14.3± 2.5	0.0± 0.0	11.5± 0.5	0.0± 0.0	0.0± 0.0	10.5± 1.5	0.0± 0.0
	N.H.E	12.7± 2.5	14.0± 0.5	18.0± 1.0	0.0± 0.0	8.5± 2.5	13.5± 1.5	0.0± 0.0
	W.W.E	13.7± 2.5	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	17.7± 1.5	0.0± 0.0
Ocimum	E.E	0.0± 0.0	14.0± 2.0	17.5± 0.5	0.0± 0.0	10.5± 1.5	13.0± 5.9	9.5± 0.5
gratissimum	E.A.E	8.3± 2.1	10.0± 2.5	18.0± 2.0	0.0± 0.0	14.7± 1.5	11.5± 2.5	10.5± 1.5
	N.H.E	10.0± 3.0	9.0± 1.0	14.5± 2.0	16.5± 0.5	12.0± 1.0	0.0± 0.0	13.7± 1.5
	W.W.E	0.0± 0.0	7.0± 0.5	13.0± 0.5	0.0± 0.0	17.5± 0.5	0.0± 0.0	0.0± 0.0
Allium sativum	E.E	0.0± 0.0	8.7± 1.3	9.0± 2.0	5.1± 0.9	0.0± 0.0	6.1± 3.5	11.5± 1.7
	E.A.E	13.0± 41	9.3± 0.7	7.3± 1.2	0.0± 0.0	11.5± 0.5	7.7± 1.5	9.5± 2.3
	N.H.E	9.1± 0.0	4.2± 0.3	3.1± 0.5	7.5± 0.6	4.3± 0.7	11.3± 1.5	9.7± 1.5
	W.W.E	9.0± 60	0.0± 0.0	0.0± 0.0	0.0± 0.0	11.5± 0.5	0.0± 0.0	8.3± 0.5
Zingiber	E.E	11.0± 1.6	11.4± 1.3	5.0± 3.5	9.0± 1.3	0.0± 0.0	7.3± 1.9	8.1± 52.7
officinale	E.A.E	2.0± 1.3	7.1± 2.2	11.0± 5.0	5.1± 3.0	6.5± 5.1	0.0± 0.0	10.7± 0.5
	N.H.E	8.6± 2.3	6.5± 0.6	9.3± 1.0	8.0± 0.5	7.5± 1.5	8.1± 3.1	11.3± 1.7
	W.W.E	1.8± 1.6	0.0± 0.0	12.3± 55.0	0.0± 0.0	3.5± 0.7	0.0± 0.0	8.5± 1.3
Cassytha	E.E	12.7± 3.1	11.0± 3.5	9.0± 2.0	0.0± 0.0	14.5± 2.5	9.7± 2.5	10.5± 1.5
filiformis	E.A.E	11.0± 2.0	6.0± 2.0	7.5± 0.5	11.5± 0.5	0.0± 0.0	8.5± 2.5	0.0± 0.0
	N.H.E	9.7± 1.5	8.5± 0.5	0.0± 0.0	0.0± 0.0	9.3± 2.5	11.3± 1.0	7.5± 0.6
	W.W.E	1.5± 1.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
Susceptibility	**VA_{30}**	10.33±	10.33±	0.0± 0.0	9.7± 2.1	0.0± 0.0	0.0± 0.0	0.0± 0.0
Disc	**Fox_{30}**	1.5	1.5	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
	OX_1	0.0± 0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
	OB_5	0.0± 0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0
		0.0± 0	0.0± 0.0					

Key: E.E = Ethanol extract, E.A.E = Ethyl acetate extract, N-H.E= N-hexane extract, W.W.E = Warm water extract, VA_{30}= Vancomycin, Fox_{30} = Cefoxitin, OX_1= Oxacillin, OB_5= Cloxacilline, MM=Millimeter, MRSA =Methicillin Resistant *Staphylococcus aureus,* MSSA= Methicillin Sensitive *Staphylococcus aureus, E.coli= Escherichia coli, P.a.= Pseudomonas aeruginosa,* K.p.= *Klebsiella pneumoniae, S.t.= Salmonella typhi, B.s.=Bacillus subtilis*
Note: The results are the values of triplicate tests measured after 24hrs incubation at 37^0C.
Source: Researched Data , 2016

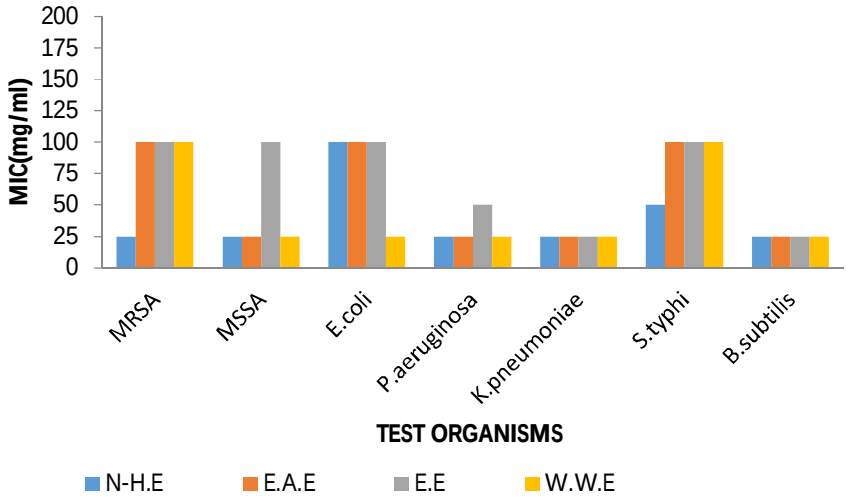

Figure 1 MIC of *Piper guineense* extract against tested pathogenic bacteria

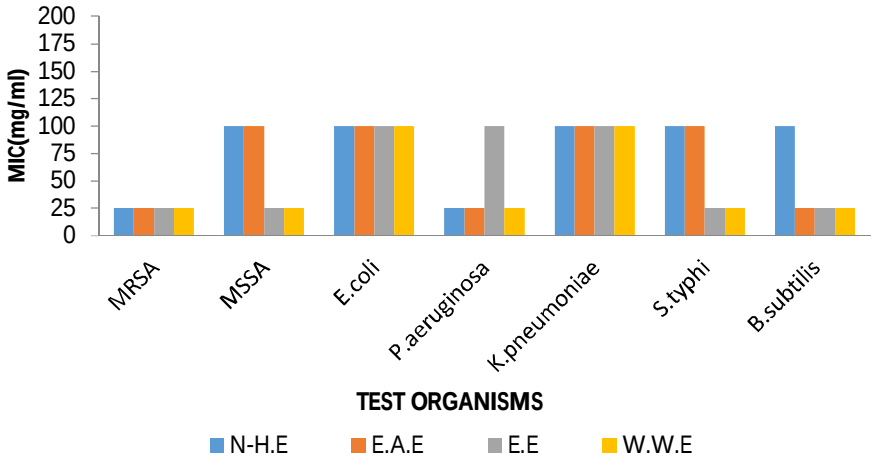

Figure 2 MIC of *Ocimumgratissimum* extracts against tested pathogenic bacteria

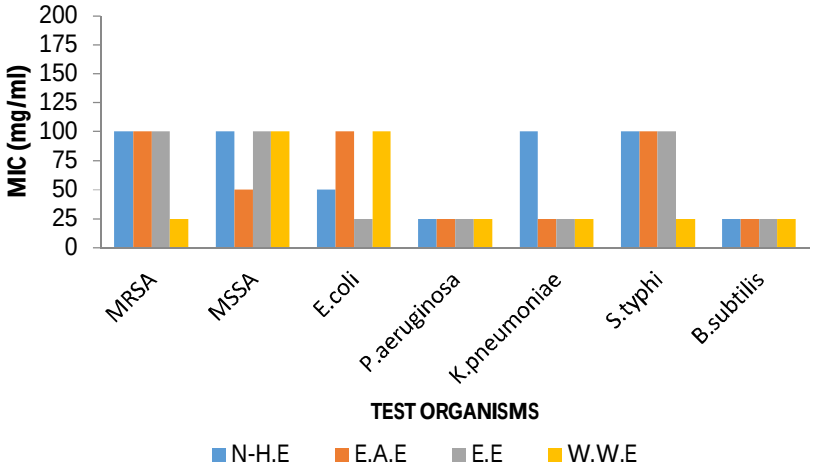

Figure 3 MIC of*Allium sativum*extractagainst tested pathogenic bacteria

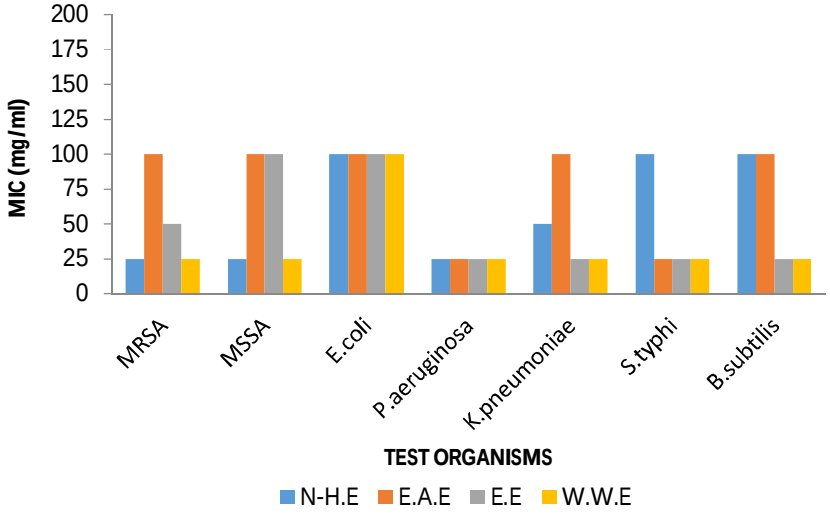

Figure 4 MIC of *Zingiber officinale* extract against tested pathogenic bacteria

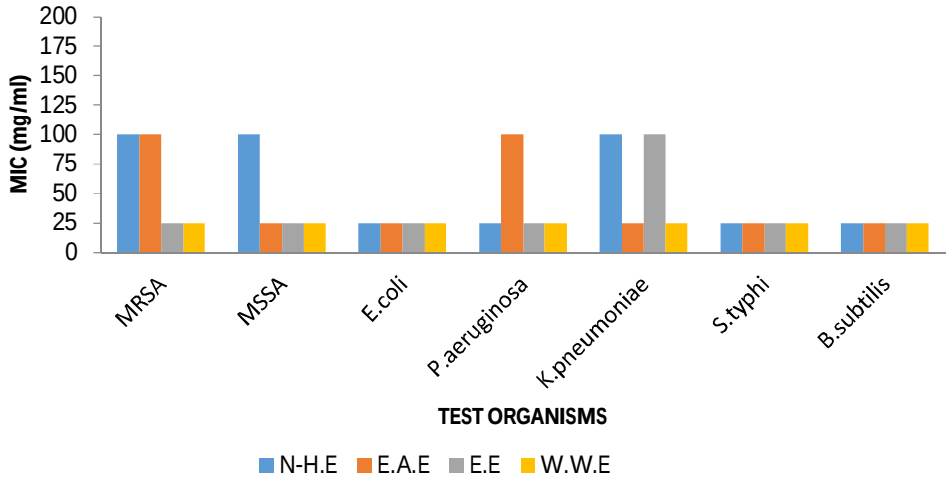

Figure 5 MIC of *Cassythafiliformis* extract against tested pathogenic bacteria

DISCUSSION

This study has shown that the test plants; *Piper guineense (black pepper)*, *Ocimum gratissimum* (scent leaf), *Allium sativum* (garlic), *Zingiber officinale* (ginger) and *Cassytha filiformis* (love vine) are good sources of antimicrobials, Their potency may be ascribed to the presence of bioactive compounds such as alkaloids, flavonoids ,saponins, tannins, phlobatannins, steroids, glycosides and quinones in appreciable quantities. The effective inhibitory potency observed against the tested pathogenic bacterial isolates with the plants parts; proved that the inhibitory compounds were extractable by the employed solvents. This is in agreement with previous observations by De and James (2002) and Gull *et al*. (2012) who emphasized that these compounds exhibit medicinal and physiological activities.

Some of the tested bacterial isolates (MRSA, MSSA, *E.coli, P.aeruginosa S.typhi, K. pneumoniae and B. subtilis)* which are commonly associated with nosocomial infections were found to be susceptible to the test plants crude extracts. Results of the MIC of the extracts revealed that most of the isolates except *E. coli* were susceptible to all the extracts. *E. coli* was only resistant to extracts of *Ocimum gratissimum* and *Zingiber officinale*. This finding corraborates those of Gull et al. (2012) and Obeidat *et al*. (2012). All the extracts showed high antibacterial activity with inhibition zones range of of 25mg/ml to 50 mg/ml. This MIC range is consistent with the value of 32mg/ml observed by Obeidat *et al*. (2012) but higher than 10mg/ml reported for ethanol extract of ginger by Mostafa *et al*. (2018). Generally, ethanol extracts of all the test medicinal plants showed highest antibacterial activity followed by aqueous or warm water extracts. The ethanol extracts of *Cassytha filiformis* showed the strongest activity against all the test bacteria with the best MIC of 25 mg/ml. These findings are consistent with those of Anyanwu and Nwosu (2014) but contradicts the report of Mostafa *et al*. (2018) who reported that aqueous extract of *Piper guineense* had higher bactericidal potential than the ethanol extracts. Two organisms, *B subtilis* and *P. aeruginosa were more sensitive* while *E. coli* was the most resistant isolate.

Extraction is the main process by which bioactive compounds may be obtained from biomass materials. The objective of extraction process is to maximize the amount of target compounds and to obtain the highest biological activity of the plant extracts. This study has shown that high zones of inhibition were recorded for ethanol extracts of all the medicinal plants. This implies higher solubility of phyto-constituents in the ethanol compared to other solvents used. Though not quantitatively determined, the results showed that the used of solvents took an important role in the yield of extraction and the tested biological activity. The ethanolic extract was identified as the most effective solvent used in this study. Similar observation has previously been reported by Anyanwu and Nwosu (2014). However, Troung *et al*. (2019) had different

observations, in their study, distilled water showed high efficiency in the extraction of phenolic compound with 5.95 GAE/g DW of phenolics being extracted. However low levels of flavonoids, alkaloids and terpenoids were obtained in the water extracts (Ajanal *et al*., 2012 and Troung *et al*., 2019). Variations in the concentations of the phytochemical components of the studied plants, the inhibitory zones and the MIC concentrations obtained highlight the differences in the antimicrobial potency of the plants extracts and in the growth rate and resistance of the test isolates to antimicrobials. Similar assertion has been reported by Gail and Jon (1995) and Ajanal *et al.* (2012). This study has revealed the potency of the studied plants extracts on the selected pathogenic bacterial isolates than some highly rated antibiotics (reference drugs) in disease cure and prevention. More specifically the study has shown that some nosocomial infections could be prevented or alleviated with the use of most especially, the ethanol, ethyl acetate and warm water extracts of the test plants.

CONCLUSION AND RECOMMENDATION

The plants parts showed antibacterial effectiveness against the test bacterial isolates. The ethyl acetate and N-hexane extracts of the plants displayed extensively a competitive inhibitory potency with the ethanol extract which exhibited the highest level of efficiency against the test isolates including Methicillin-resistant *Staphylococcus aureus* (MRSA) which are commonly associated with many nosocomial infections. The results provide justification for the use of these plants in folk medicine to treat various infectious diseases. However, further phytochemical and antimicrobial potential screening to purify and characterize the active ingredients of the tested and other medicinal plants is necessary and is highly recommended.

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