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In vitro killing rate of *Euphorbia heterophylla* and *Pterocarpus lucens* extracts minimum bactericidal concentration (MBC) on some clinical bacterial isolates

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In vitro **antibacterial activity of methanol and ethanol leaf extracts of** ***Euphorbia heterophylla*** **and** ***Pterocarpus lucens*** **were investigated against six bacterial clinical isolates using the tube dilution and agar diffusion methods.** ***Salmonella typhi*** **was the most susceptible to methanol leaf extracts of** ***E. heterophylla*** **with a zone of inhibition ranging from 16 to 24 mm for 12.5 to 100 mg/ml concentration. This was followed by** ***Streptococcus lactis, Escherichia coli, Staphylococcus aureus,*** **and** ***Shigella*** **species in that order with** ***Proteus vulgaris*** **not susceptible to the different test concentrations of both plant extracts.** ***E. heterophylla*** **had the least minimum inhibitory concentration (MIC) of 6.25 mg/ml against** ***E. coli*** **and** ***S. typhi*** **while** ***P. lucens*** **extract MIC of 25.00 mg/ml was the least against** ***S. typhi*****. Since there is an inverse relationship between MIC value and susceptibility of the clinical test isolates, the MIC values also shows that** ***E. heterophylla*** **methanol leaf extracts were more potent to the susceptible test organisms having lower MIC values than the corresponding ethanol leaf extract MIC value.** ***E. heterophylla*** **extract minimum bactericidal concentration (MBC) was 25.00 mg/ml for the sensitive isolates except for methanol extract with 12.50 mg/ml against** ***S. typhi*** **and ethanol extract with 12.50 mg/ml against** ***S. aureus. P. lucens*** **extract MBC was 100.00 mg/ml for the sensitive test isolates except for ethanol leaf extracts with 50.00 mg/ml against** ***S. typhi.*** **The killing rate of** ***E. heterophylla*** **methanol leaf extract MBC shows that** ***E. coli*** **was most rapidly killed at a rate of 4.53×10^6 CFU/min with** ***S. aureus*** **as the least killed at a rate of 0.62×10^6 CFU/min.** ***S. lactis*** **and** ***E. coli*** **were the most rapidly killed by** ***P. lucens*** **leaf extract MBC at a rate of 1.90×10^6 CFU/min. The killing rate of the extracts showed a positive support in the potential use of these plants in curing some infections as done by the traditional herbal healers in Anyigba, Kogi State, Nigeria.**

Key words: *In vitro*, antibacteria, extract, inhibitory.

INTRODUCTION

The primitive man lived at the mercy of nature in constant harassment of diseases from the earliest time. The search for agents to cure disease began long before people were aware of the existence of microbes (Larry and Judy, 1996). The use of medicinal plant in the treatment of disease is as old as the diseases themselves. Ijomah et al. (1997) noted that herbalism was the earliest form of medicine. It was the introduction of orthodox medicine that suppressed the growth and development of herbal medicine, the early indigenous health care system. Hence

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long before mankind observed the existence of microbes, the idea that plants contained healing potentials was accepted (Rios and Recio, 2005).

Records of early civilization in all parts of the world revealed that a considerable number of drugs that are used in modern medicine were in use even in the ancient times (Johnson, 2005). It was estimated that 25% of all prescribed medicines today are substances derived from plants (Belloin et al., 2005). In spite of the improved health system and longevity in the US and Europe, millions of people in this countries are turning back to herbal medicines in order to prevent or treat many illness (WHO, 2006) and to circumvent the resistance of many human pathogens to conventional drugs, some of which produced side effects like hypersensitivity and immuno-suppression (Beardsley, 1996). Medicinal plants are used in traditional societies all over the world for centuries to cure many infectious diseases. These early attempts used natural substances usually native plants or their extracts as remedies for the treatment of human diseases. This is largely based on experience handed down from one generation to another. The traditional herbal healing techniques are passed on as trade secrets in the families of certain communities, a practice protected by tradition. Indigenous people have demonstrated the therapeutic value and healing power of plants over the years (Edward and Ayansu, 1983) and over 60% of Nigeria rural population depends largely on traditional medicine for their health care needs (Ghani et al., 1986), while up to 80% of African population uses traditional medicine for their primary health care (WHO, 2006).

As part of the unabated search for plants with antimicrobial activity, this study was carried out on *Euphorbia heterophylla* and *Pterocarpus lucens* to find out the antibacterial potential of these plants against some test clinical bacterial isolates and determine the killing rate of the extracts minimum bactericidal concentration (MBC). The choice of these plants was predicated on their use by the traditional herbal healers in Anyigba, Kogi State, Nigeria in the treatment of infections such as typhoid fever, gastrointestinal disorder, urinary tract infections, infected wounds and topical ulcers.

E. heterophylla Linn belongs to the family Euphorbiaceae with more than 1000 species included in this family. Members of the family are found in most parts of the world, but their diversity is greatest in the tropics (Peter et al., 1992). The plants ranged from prostrate herbs to tall trees. Several species of Euphorbia exhibit xerophytic adaptation that made them to resemble some members of the cactus family. The superficial similarity also found in some species of milkweed family is as a result of convergent evolution in unrelated plant species (Peter et al., 1992). Members of the Euphorbiaceae are of considerable importance by providing us with food; drugs, rubber and other products (Walter et al., 1999). They are also used as purgatives (Peter et al., 1992*). E. heterophylla* is an annual medicinal herb with common name 'spurge weeds' (Falodun et al., 2004). The plant extracts is used in ethno medicine for the treatment of constipation, bronchitis and asthma by traditional practitioners (Falodun et al., 2006). The herbal healers in Anyigba, Kogi State, Nigeria use *E. heterophylla* Linn commonly called 'Salime' in 'Igala' in the treatment of typhoid fever by drinking the tea made from cooking the leaves.

MATERIALS AND METHODS

Plant source and identification

The plant stem with leaves and reproductive structures were collected from different locations in Anyigba, Dekina Local Government Area, Kogi State, Nigeria. The plants were identified by Professor F. A. Oladele and Mr. S. A. Adebayo of the Herbarium, Department of Biological Sciences, University of Ilorin, Kwara State, Nigeria.

Plant treatment

The plant leaves were washed with distilled water and dried at room temperature in the Microbiology Laboratory, Kogi State University, Anyigba, for several days until the leaves became crispy and of constant weight. The dried leaves were ground separately using sterile pestle and mortar.

Preparation of crude extracts

Plant extracts were prepared using the modified method of Alade and Irobi (1993). 500 g of the powered dried leaves were soaked separately in 500 ml of 90% methanol and 98% ethanol for 72 h in the dark. It was then agitated at 200 rpm for 1 h on a mechanical shaker. The resulting suspension was filtered using sterile Whatman filter paper No. 1. The filtrate obtained was evaporated to dryness using a rotary evaporation (Falodun et al., 2006) and weighed on chemical balance.

Source of test clinical bacterial isolates

The test organisms were collected from the culture collections of the Federal Medical Centre, Owo and the Public Health Laboratory, Akure, both in Ondo State, Nigeria.

Culture media

The medium used for the activation and standardizations of inoculums of the test clinical bacterial isolates was nutrient broth, while the Mueller Hinton Agar was used for the antimicrobial sensitivity test. They were prepared according to the manufacturers' instruction.

Standardization of inoculums

The test clinical bacterial isolates were grown in a nutrient broth and incubated at 37°C for 24 h. Both cultures were standardized using the McFarland nephrometric method. McFarland standard No. 4 was chosen and prepared by adding 9.6 ml of 1.0% Na_2SO_4 solution to 0.4 ml of 1.0% BaCl which gives a corresponding

Table 1. Methanol and ethanol extracts filtrates.

Plant	Methanol extract filtrate (g)	Ethanol extract filtrate (g)
Euphorbia heterophylla	10.23	8.02
Pterocarpus lucens	10.01	7.3

approximate bacterial density of 1.2×10^7 CFU/ml of solution (Lenette et al., 1985; Bryant, 1981). The bacterial broth culture used was diluted to give the same turbidity with the solution prepared.

Preparation of extract concentrations

In preparing 100 mg/ml concentration, 1 g of the filtrate was reconstituted in 2 ml of the appropriate solvent (methanol or ethanol). 50 mg/ml concentration was prepared by adding equal volume of solvent and 100 mg/ml concentration, that is, double fold dilution. Double fold dilution was carried out on the 50 mg/ml concentration to give 25 mg/ml, while it was repeated for 25 mg/ml to get 12.5 mg/ml concentration.

Antibacterial activity of extracts

Zone of inhibition

The modified agar well diffusion method of Perez et al. (1990) was used. 0.2 ml of the standardized bacterial broth culture of each test clinical bacterial isolate was mixed with 20 ml of molten Mueller Hinton Agar at 40°C. The seeded agar was poured aseptically into sterile Petri dishes and allowed to solidify. The solidified agar was punched with a 6 mm diameter sterile cork borer to create wells on the agar.

The wells were filled with 0.1 ml of each prepared extract concentration (one concentration per well). Sterile distilled water was used to fill one of the wells which served as the solvent control, while gentamycin sulphate (1 µg/ml) was used as the positive control. Tests were carried out in duplicates and plates incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the inhibition zone diameter (IZD) in millimeter (mm)

Determination of minimum inhibitory concentration (MIC)

Sterile nutrient broth was used to prepare the different extract concentrations. 4 ml of each extract concentration was introduced into sterile test tube. 1 ml of the standardized bacterial broth culture of test clinical bacterial isolate was added to each set of the extract concentrations.

The control test tube was inoculated with sterile distilled water. All the tubes were cotton plugged and incubated at 37°C for 24 h. The MIC was taken as the lowest inoculated extract concentration that did not permit any visible growth when compared with the turbidity of the test tube containing sterile nutrient broth and inoculated with water (control) (Rojas et al., 2006).

Determination of minimum bactericidal concentration (MBC)

The content of all the MIC tubes with no visible growth were plated out on sterile Mueller Hinton Agar and incubated at 37°C for 24 h. The MBC was taken to be the lowest inoculated extract concentration that did not produce bacterial colonies when platted out on sterile Mueller Hinton agar (Rojas et al., 2006).

Determination of the MBC killing rate

The modified method of Olowosulu et al. (2003) was used. 1 ml of the plant extract MCB was mixed with 3 ml of sterile nutrient broth and incubated at 37°C with 1 ml of standardized culture of the test isolate broth. 1 ml of standardized culture of the test isolate broth was added to 4 ml of normal saline to serve as control. The tubes were incubated at 37°C. 0.1 ml of the incubated tubes was removed at regular interval between 0 and 180 min. Each withdrawn sample was dispensed and serially diluted 10 fold to dilution of 10^{-5} with normal saline. 0.1 aliquot of the 10^{-5} dilution was plated out in duplicate on sterile Mueller Hinton Agar and incubated at 37°C for 24 h. The number of colony forming units (CFU) on each plate was counted. The total number of viable bacteria in the original sample per ml was computed from the number counted from the 0.1 ml aliquot of 10^{-5} dilution at time 0 min. The number of deaths of the bacteria was extrapolated from the number of bacterial survivors at each time withdrawn when compared with the number of bacteria counted at time 0 min.

RESULTS

Preparation of crude extracts

The leaf extract prepared showed that methanol gave a higher filtrate for *E. heterophylla* and *P. lucens* when compared with the ethanol extract filtrate for both plants as shown in Table 1.

Antibacterial activity of leaf extracts

Zone of inhibition

The methanol and ethanol leaf extracts of *E. heterophylla* were active against all the test clinical bacterial isolates except for *Proteus vulgaris* which was not sensitive to the extract concentrations as shown in Tables 2 and 3. *P. lucens* leaf extracts did not show antibacterial activity against *P. vulgaris*, *Staphylococcus aureus* and *Shigella* species as shown in Tables 2 and 3.

Minimum inhibitory concentration (MIC) of leaf extracts

The MIC of the methanol leaf extracts and ethanol leaf extracts were the same for both plants against all the test clinical bacterial isolates except for *Streptococcus lactis* which differs in both plants extracts as shown in Table 4. *E. heterophylla* extract concentration of 6.25 mg/ml was the least against *Escherichia coli* and *Salmonella typhi* as shown in Table 4.

Table 2. Antibacterial sensitivity of methanol leaf extract.

Test organism	*Euphorbia heterophylla* (mm) (Concentration in mg/ml)				*Pterocarpus lucens (mm)* (Concentration in mg/ml)				Gentamycim (1 µg/ml)
	100	50	25	12.5	100	50	25	12.5	
Staphylococcus aureus	18	15	13	9	6	6	6	6	12
Escherichia coli	20	16	12	8	10	7	6	6	12
Proteus vulgaris	6	6	6	6	6	6	6	6	25
Salmonella typhi	24	18	18	16	11	10	9	8	18
Streptococcus lactis	20	18	15	13	10	8	6	6	15
Shigella species	15	14	13	12	6	6	6	6	20

Table 3. Antibacterial sensitivity of ethanol leaf extract.

Test organism	*Euphorbia heterophylla* (mm) Concentration in mg/ml				*Pterocarpus lucens* (mm) Concentration in mg/ml				Gentamycin (1 µg/ml)
	100	50	25	12.5	100	50	25	12.5	
Staphylococcus aureu s	18	12	10	8	6	6	6	6	12
Escherichia coli	20	15	12	8	10	6	6	6	12
Proteus vulgaris	6	6	6	6	6	6	6	6	25
Salmonella typhi	20	17	15	11	13	12	11	10	18
Streptococcus lactis	18	17	15	14	10	8	8	6	15
Shigella species	15	14	13	12	6	6	6	6	20

Table 4. MIC of methanol and ethanol leaf extract of *E. heterophylla* and *P. lucens*.

Test organism	Methanol leaf extracts (mg/ml)		Ethanol leaf extracts (mg/ml)	
	E. heterophylla	*P. lucens*	*E. heterophylla*	*P. lucens*
Staphylococcus aureus	12.5	Nil	12.5	Nil
Escherichia coli	6.25	50	6.25	50
Proteus vulgaris	Nil	Nil	Nil	Nil
Salmonella typhi	6.25	25	6.25	25
Streptococcus lactis	12.5	50	6.25	25
Shigella species	12.5	Nil	12.5	Nil

Minimum bactericidal concentration (MBC) of leaf extracts

E. heterophylla methanol and ethanol leaf extract of 12.5 mg/ml was the least extract concentration bactericidal against *S. typhi* and *S. aureus*, respectively as shown in Table 5. *E. heterophylla* showed MBC of 25 mg/ml against all the other sensitive test clinical bacterial isolates, while *P. lucens* had MBC of 100 mg/ml against all the sensitive test clinical bacterial isolates except against *S. typhi* (Table 5).

Killing rate of plants extracts MBC

The killing rate of *E. heterophylla* methanol leaf extract MBC is as shown in Figure 1, while that of *P. lucens* is as shown in Figure 2. *E. coli* was the most rapidly killed by *E. heterophylla* methanol leaf extract MBC at the rate of 4.53×10^6 CFU/min (Figure 1). *E. coli* and *Streptococcus lactis* were the most rapidly killed by *P. lucens* methanol leaf extract MBC at the rate of 1.09×10^6 CFU/min (Figure 2).

DISCUSSION

Methanol was found to be a better extracting solvent for *E. heterophylla* and *P. lucens* than ethanol with respect to the extract filtrate yield, in which methanol yielded 10.23 and 10.01 g for *E. heterophylla* and *P. lucens*, respectively,

Table 5. MBC of methanol and ethanol leaf extract of *E. heterophylla* and *P. lucens*.

Test organism	Methanol leaf extract (mg/ml)		Ethanol leaf extract (mg/ml)	
	E. heterophylla	***P. lucens***	***E. heterophylla***	***P. lucens***
Staphylococcus aureus	25	Nil	12.5	Nil
Escherichia coli	25	100	25	100
Proteus vulgaris	Nil	Nil	Nil	Nil
Salmonella typhi	12.5	100	25	50
Streptococcus lactis	12.5	100	25	100
Shigella species	25	Nil	25	Nil

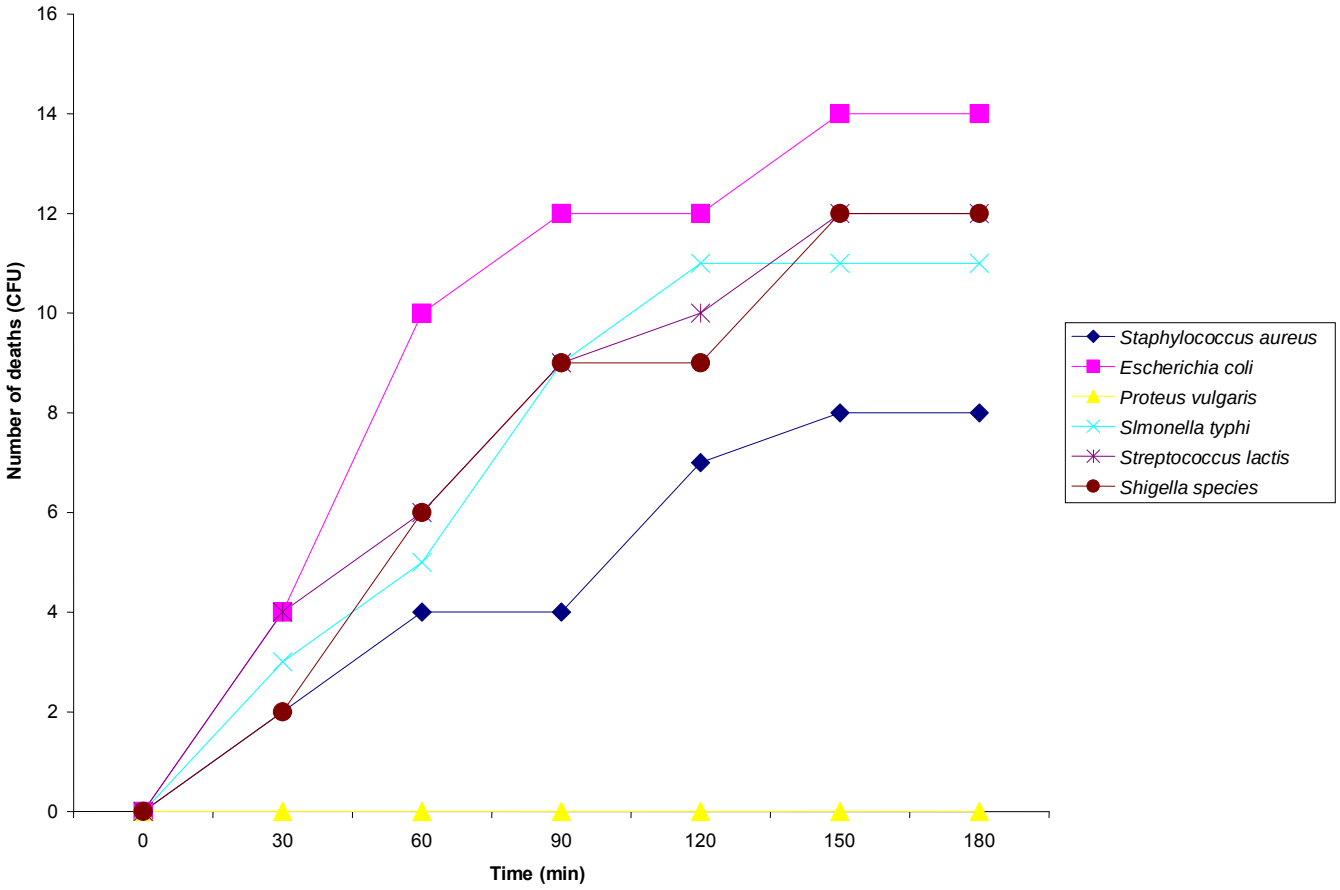


Figure 1. Killing Rate of *Euphorbia heterophylla* Methanol Leaf Extract MBC.

higher than ethanol extract filtrate yield of 8.02 and 7.34 g for the plants, respectively as shown in Table 1.

The sizes of the zone of inhibition are indicative of the level of the antimicrobial activities of the extracts. Therefore, it can be said that the methanol extracts of *E. heterophylla* and *P. lucens* had higher antibacterial activity against the susceptible test clinical bacterial isolates than the ethanol extracts as shown in Table 2. The diameters of the zones of inhibition were larger for *E. coli* methanol extract against *S. aureus*, *Salmonella typhi* and *S. lactis* than for *E. heterophylla* ethanol extracts (Table 2). Unlike in the case of *E. heterophylla*, ethanol extracts of *P. lucens* had a higher antibacterial activity than methanol extract against *S. typhi* as shown in Table 2.

S. typhi was the most susceptible to *E. heterophylla* of all the test clinical bacterial isolates followed by *S. lactis, E. coli, S. aureus* and *Shigella* species with inhibition zone diameter range of 16 to 24 mm, 13 to 20 mm, 8 to 20 mm, 9 to 18 mm and 12 to 15 mm, respectively while

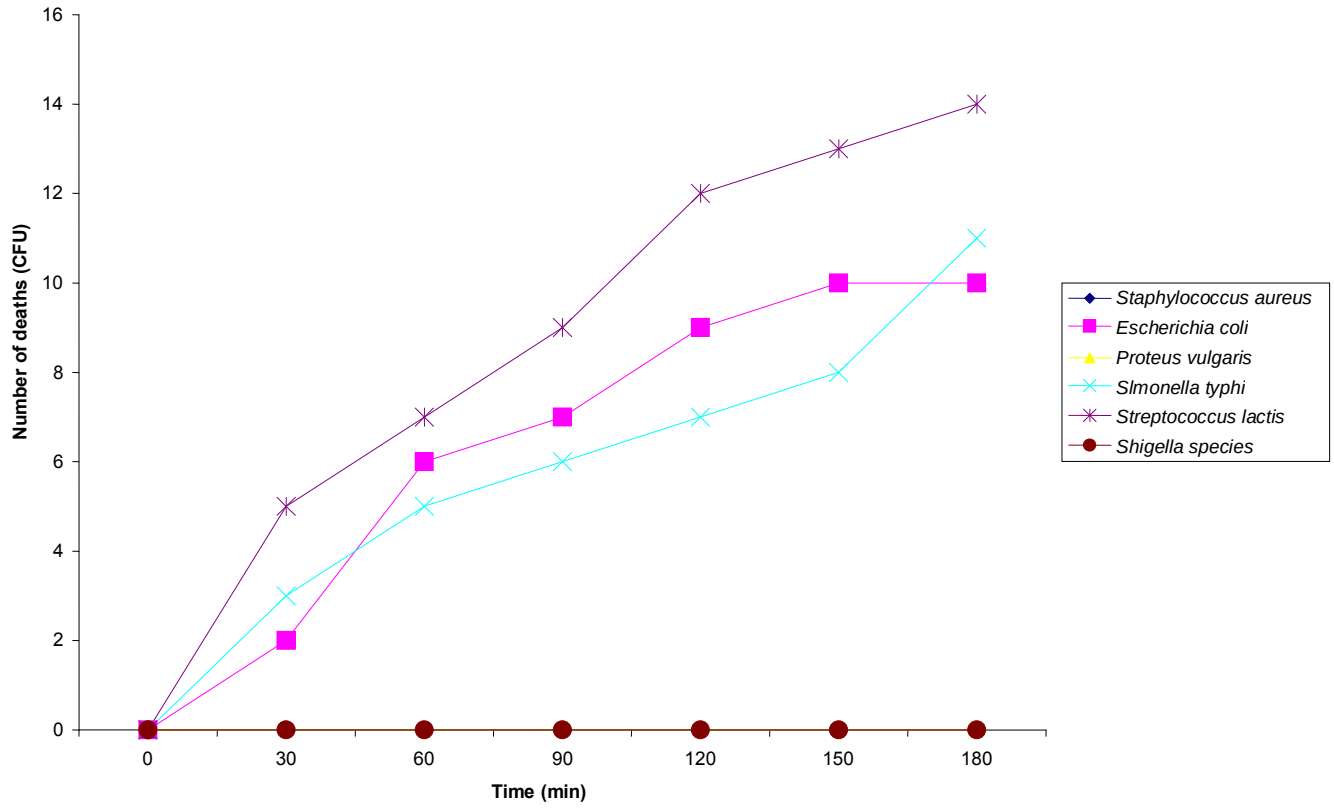


Figure 2. Killing rate of *Pterocarpus lucens* methanol leaf extract MBC.

Proteus vulgaris was not susceptible to both plant extracts as shown in Table 2. Resistance of Gram negative bacteria is well known (Irvin et al., 1981). All the test clinical bacterial isolates were sensitive to *E. heterophylla* except against *P. vulgaris*. Extract of plants from different parts of the world have been shown to possess antimicrobial activity (Ummulkaltum et al., 2002; Ogbulie et al., 2004; Beloin et al., 2005, Bello et al., 2005; Ariyo and Akande, 2005). In addition to *P. vulgaris* that was not sensitive to *P. lucens, S. aureus* and *Shigella* spp. were also not sensitive to *P. lucens* methanol and ethanol extracts, while *S. typhi* was also the most susceptible to *P. lucens* extracts followed by *S. lactis* and *E. coli* with inhibition zone diameter range of 8 to 11 mm, 8 to 10 mm and 7 to 10 mm, respectively. The different antibacterial activity of the plants may not be unconnected to the different phytochemicals present in the plants.

E. heterophylla had minimum inhibitory concentration (MIC) of 6.25 mg/ml against the most susceptible test clinical bacterial isolate and *E. coli*. The MIC values also indicated that *E. heterophylla* leaf extracts were more potent to the susceptible test clinical bacterial isolates having shown lower MIC values than the corresponding *P. lucens* leaf extract MIC as shown in Table 3. This is implied based on the fact that there is an inverse relationship between MIC and susceptibility of the test clinical bacterial isolates. Apart from the less antibacterial activity of *P. lucens* extracts, it did not show activity against 50% of the test isolates with 16.6% being very susceptible as shown in Table 3.

The minimum bactericidal concentration (MBC) had a similar pattern to the MIC as shown in Table 4. *E. heterophylla* methanol and ethanol extracts had MBC of 12.5 and 25 mg/ml, respectively against *S. typhi* while *P. lucens* methanol and ethanol extracts had MBC of 100 and 50 mg/ml, respectively (Table 4). The MBC result shows that the action of *E. heterophylla* ethanol extract on *S. aureus* was bactericidal having the same MIC and MBC of 12.5 mg/ml (Tables 3 and 4). Unlike the other extracts with different MIC and MBC values, an indication of a possible bacteriostatic and bactericidal mode of action by the extracts as shown in Tables 3 and 4.

The antibacterial activity of the *E. heterophylla* extracts was pronounced against *S. typhi* and *S. lactis. E. coli* and *S. aureus* were also very susceptible to the *E. heterophylla extracts* and *P. lucens* except for *S. aureus*. The implication of this result is that bacteria species that have been implicated in infections such as typhoid fever, as in the case of *S. typhi* (Julie, 2000); gastrointestinal disorder such as *S. typhi* and *E. coli* (Lederberg, 2000) and urinary tract infections as in the case of *E. coli* and *Shigella* spp (Ebie et al., 2001) were found to be

susceptible to these plant extracts. Therefore, it can be said that the effort of the herbal healers in Anyigba using water extracts of *E. heterophylla* and *P. lucens* in treating and curing infections like typhoid fever, gastrointestinal disorder and urinary tract infection is probably in the right direction since this work has shown the antibacterial potential of these plants extracts on the etiologic agents of these infections.

The *in vitro* killing rate of *E. heterophylla* methanol extract MBC shows that *E. coli* that has been implicated in gastrointestinal disorder (Lederberg, 2000) was the most rapidly eliminated at a rate of 4.53×10^6 CFU/min, an indication of the fact that the use water extracts of *E. heterophylla* by the Anyigba herbal healers in treating gastrointestinal disorder may not be out of place as supported by the result of this work as shown in Figure 1. In the same vein, the *in vitro* rapid killing rate of the *E. heterophylla* extract on *S. typhi*, an organism implicated as causative agent of typhoid fever, at a rate of 1.32×10^6 CFU/min also shows the antibacterial potential of the plant extracts against the causative agent of typhoid fever has been used by the herbal healer in Anyigba, Kogi State, Nigeria. The *P. lucens* methanol extract MBC killing rate of 190×10^6 CFU/min on *E. coli* and *S. typhi* also shows the antibacterial potential of the plant extracts against etiologic agents of gastrointestinal disorder and typhoid fever as shown in Figure 2. In conclusion, if these extracts did not undergo biotransformation in the body to chemicals of different potencies from their crude extracts, the *in vitro* killing rate of the extracts of *E. heterophylla* and *P. lucens* show the potential of these plants in eliminating some of the etiologic agents of the infections which the herbal healers use the plants to treat in Anyigba, Kogi State, Nigeria.

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