Incidence and Susceptibility of Beta-Lactamase Producing *Klebsiella pneumoniae* to Extract from *Phyllanthus niruri*

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Abstract- Antibacterial resistance to common antibiotic has increased overtime; there is thus the need to look for an alternative medicine to stem the trend. This study reported incidence of β-lactamase producing *Klebsiella pneumonia* from patients. The antibacterial activity of crude aqueous and ethanolic extract of *Phyllanthus niruri* was also investigated against some test bacteria. Antimicrobial activities were analyzed using agar diffusion method. Experiments were performed in triplicate and reported as mean ± standard error of mean and difference was analyzed using one way ANOVA at $p \leq 0.05$. Sixty five percent (65%) and 25% of urine and wound samples were positive to β-lactamase producing *K. pneumonia* respectively. The highest zone of inhibition (22.0mm) was observed in a concentration of 0.5g/mL from ethanolic extract against *K. pneumonia*. While the minimum inhibitory concentration (MIC) of aqueous and ethanolic extract of the plant was evidenced in 0.2g/mL and 0.1g/mL respectively. From the foregoing this study has shown that *P. niruri* extract have potential to be remedy for infectious disease caused by β-lactamase producing *K. pneumonia*.

Keywords- Antibacterial, β-lactamase, *Phyllanthus niruri*

I. INTRODUCTION

Plants have always been a major component of traditional system of healing in developing countries, which have also been an integral part of their history and culture. Medical plants offer alternative remedies with tremendous opportunities. Many traditional healing herbs and plant parts have been shown to have a medical value especially in the rural areas and that these can be used to prevent and cure several human diseases. Even today, majority of the world population depend on herbal health care practices (Kunle *et al*., 2012; Narendra *et al*., 2012).

Extracts of many plants are known to elicit certain reactions in human body when applied in a prescribed manner including *Phyllanthus niruri* L., (Syn.*P. franternus* Webster) belonging to the family Euphorbiaceae. It has been claimed to be an excellent remedy for jaundice and hepatitis (Abbasi *et al*., 2009). It also has antioxidant and hepatoprotective effect as reported by Makoshi *et al*. (2013) The plant is considered analgesic, aperitif, digestive, emmanagogue, laxative and stomachic tonic (Khanna *et al*., 2002) and is helpful in treating oedema, anorexia and diabetes (George and Pamplona-Roger, 2002). Its roots, leaves, fruits, milky juice as well as the whole plant are used as medicine with no adverse effect on the body organs (Ajibade and Famurewa, 2013). Recently, lignansniranthin, nirtetralin and phyltetralin have been isolated from the leaves (Tabasum *et al*., 2005). To buttress these points a recent study by Ajibade. (2014) reported saponin and alkaloid extracted from *P. niruri* posses antimicrobial properties thus may offer an alternative therapeutic agent against bacterial infections.

The strategic importance of reviving indigenous medical practice to provide safe and affordable primary health care to the people of the world is now recognized. During the last two decade or so WHO health assembly has passed a number of resolution in response to this resurgence of interest in the study and use of traditional medicine and in recognition of the importance of medical plant to health care of people in many developing countries (Kunle *et al*., 2012).

Klebsiella pneumoniae is ubiquitous as it is found in mammals and ecological environment. It has pathogenic effects worldwide. There is evidence of community-acquired and hospital-acquired infections in countries such as Taiwan and South Africa. Community-acquired *K. pneumoniae* has been found, in some places, to be associated with alcoholism. There are a large number of infections acquired when it affects different organs of the body. It can affect the liver, urinary tract and lungs (Vuotto *et al*., 2014).

The bacterium is an important cause of human infections. Infections or diseases are usually nosocomial or hospital-acquired. In 1998, *K. pneumoniae* and *K. oxytoca* accounted for 8% of nosocomial bacterial infections in the United States and in Europe. Diseases include urinary tract infections, pneumonia, septicemias, and soft tissue infections (Vuotto *et al*., 2014) The diseases caused by *K. pneumoniae* can result in death for patients who are immunodeficient. Bacteria from the genus Klebsiella causes numerous infections in human, which are often treated with β–lactam antibiotics. The indiscriminate use of antibiotics has revealed a considerable increase in outbreaks caused by microorganisms resistant to antimicrobial drugs, such *K. pneumoniae*. This research work is thus centered on investigating the potency of aqueous and alcoholic extract of *P. niruri* on Beta Lactam producing *K. pneumoniae,* thereby ascertaining the therapeutic importance of natural products.

II. MATERIALS AND METHODS

A. Collection and Processing of Plant Material

K. Pnuemoniae were isolated from 40 samples each of urine, ear, sputum, pus and wound. Samples were immediately brought to the laboratory for analysis. The plant was collected from farmlands in the Federal Polytechnic Ado–Ekiti during the raining season between the months of September and December 2010. The plant was dried at room temperature ($28^oC \pm 1^oC$). The plant was milled using centrifugal milling machine (GallenKamp) and stored in air-tight plastic container. Identification and authentication of plant was done in the Department of Science Technology, Federal polytechnic Ado–Ekiti, Nigeria where a voucher specimen (No. MED. PLT 0-2008-10) was kept. 100grams of the milled samples was soaked with 400ml of distilled water and Alcohol respectively. It was allowed to stand for 24hours. Both solutions were filtered by using Whatman no. 4 filter paper and evaporated to dryness at 20^oC using a rotary evaporator. The residues were reconstituted to different concentrations using distilled water.

B. Isolation and identification of B-lactam producing K. pneumoniae

Clinical isolates of *K. pnuemoniae* were obtained from seven hospitals in four local government areas of Ekiti state, South-Western, Nigeria.

Identification was accomplished by colony characteristics and lactose fermentation in Macconkey agar (oxoid), biochemical reactions (gas from glucose, acid from lactose and acid from dulcitol and citrate utilization). Selection was based on non-outbreak isolates of *K. pneumonia* with resistance to *B- lactam* antibiotics.

Testing for antibacterial resistant of *K pneumoniae* was carried out by direct plating method (Clinical and Laboratory Standards Institute 2014).

Isolates were stored at -70^0C and were sub-cultured on trypticase soy agar plates containing 5% defribinated human blood. For each isolate, plates were inoculated by using cell suspension equivalent to a 0.5 Mcfarland standard which was spread on a Macconkey agar (oxoid) to yield uniform growth and multo-disk containing the B-lactam antibiotics (penicillin to cephalosporin) were asceptically placed on the agar surface. Diameter of zone of inhibition was calculated. Those with decreased zone of inhibition were indicative of *B-lactam* production and were isolated for further studies.

C. Susceptibility Testing

The agar diffusion method described in (Clinical and Laboratory Standards Institute 2014) was used. Reactivated culture from stock, diluted serially to 10^3 was used as inoculums. The inocula were spread uniformly on sensitivity agar (Oxoid) using a sterile glass spreader. Pasteur pipette was used to introduce different concentration of 0.1g/mL-0.5g/mL of the extract into the well bored (7.0mm) onto the surface of the culture. A control well containing extracting solvent was also incorporated. Standard antibiotic disc of Oflaxacin in concentrations of 20µg, 25µg and 30µg were used as positive control. The plates were incubated at 37^oC for 24hrs in an incubator. The diameter of zones of inhibition was measured in millimeter and recorded.

D. Statistical Analysis

Statistical analysis was performed by using SPSS software version 20.0 and Microsoft Excel. Data on antimicrobial activities were expressed as mean ± SEM and differences were statistically analyzed using one way ANOVA. P value was set at ≤ 0.05.

III. RESULTS AND DISCUSSION

A study by Podschun and Ullman (1998) have reported that *K. pneumoniae* is an enteric Gram-negative bacillus causing debilitating and dangerous infections in debilitated or immuno-compromised individuals. They accounted for up to 10% of all nosocomial bacterial infections (Spencer, 1996). Usually, these debilitating infections are treated with β-lactam antibiotics, which are usually hydrolyzed by β-lactamases produced by such microorganisms resulting in drug resistance (Bush *et al*., 1995). Therefore this study was designed to determine the efficacy of aqueous and methanolic extracts of *Phyllanthus niruri* against Extended spectrum β-lactamase producing *K. pneumoniae* to optimize empirical therapy in natural product which might lead to suggestion of a therapeutic regimen against it.

Forty *K. pneumoniae* strains were isolated from various sources (i.e. ear, sputum, wound, urine and pus) obtained from hospitalized patients. 32 (80%), 26(65%), 20(50%), 15(38%) and 10(10%) were isolated from sputum, urine, ear swab, pus and wound respectively (Table 1). This is not in congruent with results from Amin *et al*. (2009); where 52% and 7.5% were isolated from pus and urine respectively. A study among students in Nigeria reported that 55% of their sample yielded *K. pneumoniae* isolates (Chikwendu *et al*., 2010) this is not in tandem with our results. Prevalence of *K. pneumoniae* in urine sample was higher in females than in the male samples (Table 1). This level of prevalence agrees with other studies (Chikwendu *et al*., 2010; Anjun and Mir 2010) that also reported a similar trend with regards to sex. This increased prevalence in females could be attributed to the differences in physiology between the males and females, where the urethra is short, and the distance between the anal and vaginal opening is small. Bacteria therefore easily invade and colonize the urinary tracts (Chikwendu *et al*., 2010). But our result is not in congruent with a recent study by Akanbi *et al*. (2013) that reported non-significant relationship in occurrence of *K. pneumoniae* in male and female. As shown in Table 1; 66% of *K. pneumoniae* was isolated from male sputum, this is slightly higher than 53% recently reported by Al-Mussawi *et al*. (2015).

Table 1: Incidence of B-lactam producing *Klebsiella pneumoniae* in 40 samples each

		Sex (n%)		Age (n%)	
Samples	**Positive sample (n%)**	**Male**	**Female**	**≤13 years**	**>13 years**
Urine	26(65)	6 (23)	20(77)	-	26(100)
Ear	20(50)	10 (50)	10(50)		20(100)
Sputum	32(80)	21(66)	12(34)	5(16)	27(84)
Pus	15(38)	9(60)	6(40)	8(53)	7(47)
Wound	10(25)	7(70)	3(30)	5(50)	5(50)

There is well documented evidence of the antibacterial activities of *P. niruri* which revealed that it contains bioactive compounds with significant potency (Ajibade and Famurewa, 2012; Oseni and Banini, 2014). This shows that this plant represents a potential source for the discovery of novel antimicrobial agent with little or no side effects (Ajibade and Famurewa, 2012; 2013; Ajibade and Ajenifuja, 2013; Ajibade, 2014).

The minimum inhibitory concentration (MIC) of aqueous and ethanolic extract of the plant was evidenced in 0.2g/mL and 0.1g/mL respectively (Table 2 and 3); this is in-tandem with a recent study in Ghana that reported MIC of 0.2gmL for ethanolic extract against *K. pneumoniae* (Oseni and Banini, 2014). The highest zone of inhibition (10.0mm) was observed in a concentration of 0.2g/mL from ethanolic extract against *K. pneumoniae*, this is in total agreement with 11.00mm reported recently by Oseni and Banini. (2014).

Table 2: Antibacterial effect of aqueous extracts on *K. pneumonia* at different concentrations

Samples	**Diameter of zones of inhibition (mm) at different concentration (g/mL)**				
	0.1	**0.2**	**0.3**	**0.4**	**0.5**
Ear	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Sputum	6.0 ± 1.2^{b}	10.0 ± 2.2^{d}	15.0 ± 3.2^{e}	9.0 ± 1.3^{g}	16.0 ± 3.2^{i}
Wound	6.0 ± 1.6^{b}	6.0 ± 1.8^{b}	7.0 ± 1.9^{f}	8.0 ± 2.1^{h}	12.0 ± 2.1^{j}
Urine	5.0 ± 1.1^{c}	6.0 ± 1.0^{b}	6.0 ± 1.2^{b}	7.0 ± 3.1^{f}	10.0 ± 1.2^{k}
Pus	5.0 ± 1.5^{c}	9.0 ± 2.2^{d}	6.0 ± 1.5^{b}	9.0 ± 1.2^{g}	12.0 ± 1.2^{j}

Data are expressed as mean ± SEM; data with different superscript are significantly different at $p \leq 0.05$
SEM: Standard error of mean.

Table 3: Antibacterial effect of ethanolic extracts on *K. pneumonia* at different concentrations

Samples	**Diameter of zones of inhibition (mm) at different concentration (g/mL)**				
	0.1	**0.2**	**0.3**	**0.4**	**0.5**
Ear	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Sputum	10.0 ± 1.9^{b}	10.0 ± 1.2^{b}	15.0 ± 3.2^{d}	19.0 ± 1.0^{e}	20.0 ± 3.2^{f}
Wound	10.0 ± 2.3^{b}	10.0 ± 2.2^{b}	11.0 ± 2.4^{b}	15.0 ± 2.4^{c}	15.0 ± 2.0^{c}
Urine	10.0 ± 1.5^{b}	16.0 ± 1.8^{c}	16.0 ± 1.2^{c}	16.0 ± 2.0^{c}	16.0 ± 1.2^{c}
Pus	15.0 ± 3.1^{c}	16.0 ± 3.9^{c}	16.0 ± 2.9^{c}	19.0 ± 3.5^{e}	22.0 ± 2.9^{g}

Data are expressed as mean ± SEM; data with different superscript are significantly different at $p \leq 0.05$
SEM: Standard error of mean.

In this study ethanolic extract of *P. niruri* showed better antibacterial activities when compared with aqueous extract, reason might be due to the fact that ethanol extract posses the ability to retain more active ingredient than water. The strong antibacterial activity of the P. niruri observed in this study has been strongly linked with the presence of bioactive compounds especially the flavonoids and alkaloids, this had been reported by other studies (Osuntokun and Ajayi, 2014; Oseni and Banini, 2014). One other strong bioactive compound that has been extracted from P. niruri and it is of high efficacy is saponin, this was reported by Ajibade and Famurewa, (2012).

IV. CONCLUSION

The extracts from this plant have been investigated to be active *in vitro* and *in vivo* when applied to infected rabbits that suffer from diarrhoae, dysentery and typhoid fever (Ajibade and Egbebi, 2011). This study has authenticated the phytotherapeutic position of *P. niruri* extracts and may be recommended as remedy for infectious disease caused by β-lactamase producing *K. pneumoniae*.

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