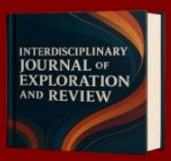

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SUSCEPTIBILITY STUDY OF ISOLATES FROM PHARMACEUTICAL EFFLUENTS

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ABSTRACT

Pharmaceutical effluents are wastes generated during the process of drug manufacturing by pharmaceutical industries. When these effluents are discharged directly into the environment without proper handling and treatment, they affect both human health and the environment. This study was undertaken to X-ray the molecular characteristics of isolates collected from five major pharmaceutical companies (P1, P2, P3, P4 and P5) in Anambra State. These samples were analyzed for nucleic acid content and Susceptibility patterns using instrumentation and gravimetric techniques. *Bacillus megaterium* strain QMB 1551 (BMQM), *Burkholderia cepacia* strain BC16 (BCBC), *Enterobacter cloacae* strain A117 (ECA1), *Klebsiella pneumoniae* strain 39427 (KP39), *Micrococcus luteus* strain SA211 (MLSA), *Pseudomonas fluorescens* strain Pt 14 (PFPt), *Aspergillus terreus* strain KAML04 (ATK04), *Aspergillus niger* strain CFL5 (ANC5), *Glomus mosseae* BEG119 (GMB1) and *Rhizophagus irregularis* strain C2 (RIC2) were the nutrient cycling microbes encountered from the experimented sites. UPEs showed pronounced inhibitory activities against the microbes, of which UPE P4 showed the highest activities against the microbes. Their risks to human health and the environment cannot be overemphasized. Increase in demand of pharmaceuticals in Nigeria has led to consequential increase in the amount of waste generated which most times contain recalcitrant substances that are either cytotoxic or genotoxic and sometimes both.

KEYWORDS: Waste, Microbes, Pharmaceuticals, Patterns

INTRODUCTION

Many pharmaceutical industries produce toxic effluents from their production operation. The waste water generated from these industries possess solids, biodegradable and non-biodegradable organic compounds etc. Pharmaceutical effluents propose basic information about the reliability of the aquatic habitat in rivers and streams into which they are discharged. The physicochemical analysis of the effluents should point toward that most of these industries obey the standard guidelines of Federal Environmental Protection Agency (Nwakoby and Ejimofor, 2023).

A significant pollution index of industrial wastewaters is the oxygen content in chemical oxygen demand and biological oxygen demand, where the nutrients status are measured in terms of amount of nitrogen and phosphorus in the wastewater. Besides this, other significant water quality parameters include pH, temperature and total suspended solids (Devesh, 2015).

Pharmaceutical waste is not one single waste stream, but many distinct waste streams that reflect the complexity and diversity of the chemicals that comprise pharmaceuticals. Pharmaceutical waste is potentially generated through a wide variety of activities in a healthcare facility, including but not limited to intravenous (IV) preparations, general compounding, spills/breakages, partially used vials, syringes and intravenous discontinued, unused preparations, unused unit dose repacks, patients personal medications and outdated pharmaceuticals. Treatment of pharmaceutical effluents can be carried out in the following ways:

Microwaving: Application of an electromagnetic field over the wastes provokes the liquid in the wastes to oscillate and heat up, destroying the infectious components by conduction. This technology is effective if the ultraviolet radiation reaches the waste material. Before microwaving, the waste require shredding to an acceptable size and humidification (Devesh, 2015).

Incineration: This is a disposal method in which solid organic wastes are subjected to ignition so as to alter them into residue and gaseous products. This method is constructive for disposal of residue of both solid waste management and solid residue from waste water management. This process reduces the volumes of solid waste to 20 – 30% of the original volume. Incineration and other high temperature waste treatment systems are sometimes described as "thermal treatment" (Devesh, 2015).

Deep Burial: The Biomedical waste rules require that human anatomical and animal wastes in cities with the population less than 500,000 and in rural areas be disposed of by deep burial. Accordingly, the deep burial site should be prepared by digging a pit or trench of about 2 meters deep in an area that is not prone to flooding or erosion, and where the soil is relatively impermeable, there are no inhabitants or shallow wells in the vicinity, and the risk to surface water contamination is remote (Devesh, 2015)

Autoclaving: Autoclaving uses saturated steam in direct contact with the waste in a pressure vessel at time lengths and

temperatures sufficient to kill the pathogens. The biomedical waste rules specify the minimum temperature, pressure and residence time for autoclaves for safe disinfection (Devesh, 2015).

Chemical Disinfection: Chemical disinfection is most suitable for treating liquid wastes such as blood, urine, stools, or health care facility sewage. Addition of strong oxidants like chlorine compounds, ammonium salts, aldehydes or phenol compounds kills or inactivates pathogens in the biomedical wastes (Devesh, 2015).

MATERIALS AND METHODS

Molecular characterization of the bacterial and fungal isolates

Extraction and purification of DNA: All strains were plated on Nutrient Agar (Biotech) and incubated at 37°C for 24 h for bacteria and Sabouraud Dextrose Agar (BIOTECH) and incubated at room temperature (30±2°C) for 5 days. By means of the procedures of Zymo Research (ZR) DNA miniprepTM kit, bacterial and fungal genomic DNA was then extracted and purified (Category No. D6005; Irvine, California, USA) as described in the study published by Iheukwumere *et al.* (2018).

Determination of the quality of extracted DNA: Using mass spectrophotometer (Nanodrop), One micro litre (1µL) was aseptically dropped into a fresh space in the chamber and the chamber was lightly closed which was then linked to a computer system which showed the window that discovered the value of the sample at 260/280nm as described in the study published by Iheukwumere *et al.* (2018).

Amplification of DNA and gel electrophoresis of PCR product: This was analysed using Master cycler Nexus Gradient (Eppendorf). A mixture of primer (20 µL), template DNA (20µL), water (72 µL) and master mix (108 µL), which comprises taq polymerase, dimethylsulfoxide (DMSO), magnesium chloride ($MgCl_2$) and nucleotides triphosphates (NdTPs), was made in 1.5 mL tube and homogenized using vortex mixer (Eppendorf). This was then positioned in the block chamber of the master cycler and then programmed. The PCR program for conditions were as follows: initial incubation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 secs, annealing at 55°C for 15 secs, elongation at 72°C for 21 secs and final extension period for 10 min at 72°C. The amplified products were electrophorezed in 1.0% agarose gel and a1kb DNA ladder was used as a size reference. After staining with 3µL of nucleic acid stain (GR green), the gel was documented with gel documentation apparatus as described in the study published by Iheukwumere *et al.* (2018).

DNA sequencing of 16S rRNA fragment : The 16S rRNA amplified PCR products generated from universal primer (16S), was used for the sequencing using ABI DNA sequencer (Applied Biosystem Inc) at International Institute of Tropical Agriculture (IITA), Ibadan using the method described in the study published by Iheukwumere *et al.* (2018).

Computational analysis: The chromatograms generated from the

sequences were cleaned to obtain regions with normal sequences. The cleaned nucleotides were aligned using pair wise alignment tool. The consensus sequences formed by the alignment of the forward and reverse sequences were used to perform the Basic Local Alignment Search Tool (BLAST) using National Centre for Biotechnology Information BLAST over the internet. The sequences of the isolates with 95% and above similarities were accepted. Also the maximum scores, total scores and accession numbers of the isolates were assessed.

RESULTS

The molecular study revealed that the nucleic acids had absorbance ratio of between 1.80 - 1.90 showing that they were all DNA. The organisms encountered were *Aspergillus terrus* Strain KAML04 (ATK04), *Aspergillus niger s*train CFL5 (ANC5), *Glomus mosseae* BEG119 (GMB1) and *Rhizophagus irregulavis s*train C2 (RIC2)

This was carried out by the modified method described in the study published by Iheukwumere and Umedum (2013). The test organisms were seeded in Muller Hinton Agar (MHA/BIOTECH) plates for bacterial isolates, whereas the fungal isolates were seeded in Sabouraud Dextrose Agar plates and this were placed in refrigerator at 4°C for 1 h. Each labeled plate was uniformly inoculated with the test organism using spread plate method. A sterile cork borer of 5 mm diameter was used to make the wells on the medium. One tenth millilitre of various pharmaceutical effluents were dropped into each labeled wells and then placed vertically in the Bacteriological incubator and incubated at 37±2°C for 24 h for bacterial isolates, whereas the fungal isolates were incubated at room temperature (30±2ºC) for 5 days. The susceptibility patterns were determined by measuring the diameter of the zones of inhibition (mm) produced after incubation

Susceptibility patterns of the isolates against the pharmaceutical effluents

The pharmaceutical effluents (P1, P2, P3, P4 and P5) showed significant ($P<0.05$) and pronounced activities against the bacterial isolates as shown in Table 20. Effluents P4 showed the highest activities against all the tested bacterial and fungal isolates (Table 3). P4 significantly ($P<0.05$) inhibited the test isolates more than P1, P3 and P5, and non-significantly ($P>0.05$) inhibited the test isolate more than P2. There was slight variations between the activities of P4 and Ciprofloxacin (CPX). Commercial antibacterial agent) and ketoconazole (KET) (Commercial antifungal agent). P2 significantly ($P<0.05$) inhibited the test isolates more than P1, P3 and P5, but non-significantly ($P>0.05$) inhibited the test isolates less than P4 and CPX. The highest activities of the effluents were against PFPt, and this deviated from the activities of CPX that inhibited ECA1 and KP39 (Enteric bacteria) most. Also, among the fungal isolates, the effluent's activities were mostly against ATK04, and this almost agreed with the activities of KET, but little deviation was seen against ANC5 as shown in Table 3.

Table 1: Quality of nucleic acids from the fungal isolates

Isolate code	Conc (mg/l)	A_{280}	A_{260}	260/280
X1	71.40	1.701	3.113	1.83
X2	84.10	1.840	3.385	1.84
X3	88.60	1.846	3.415	1.85
X4	73.10	1.713	3.118	1.82

Table 2: Molecular characteristics of the fungal isolates

Isolate code	Max score	Total score	Query cover (%)	E-value	Percent identity (%)	Accession number	Description
X1	3153	3152	100	0.0	100	KC119206.1	*Aspergillus terrus* Strain KAML04 (ATK04)
X2	1035	1035	100	0.0	100	KF358715.1	*Aspergillus niger* Strain CFL5 (ANC5)
X3	1016	1016	100	0.0	100	AJ505614.1	*Glomus mosseae* BEG119 (GMB1)

X4	5635	8289	100	0.0	100	CP080904.1	*Rhizophagus irregulavis* Strain C2 (RIC2)

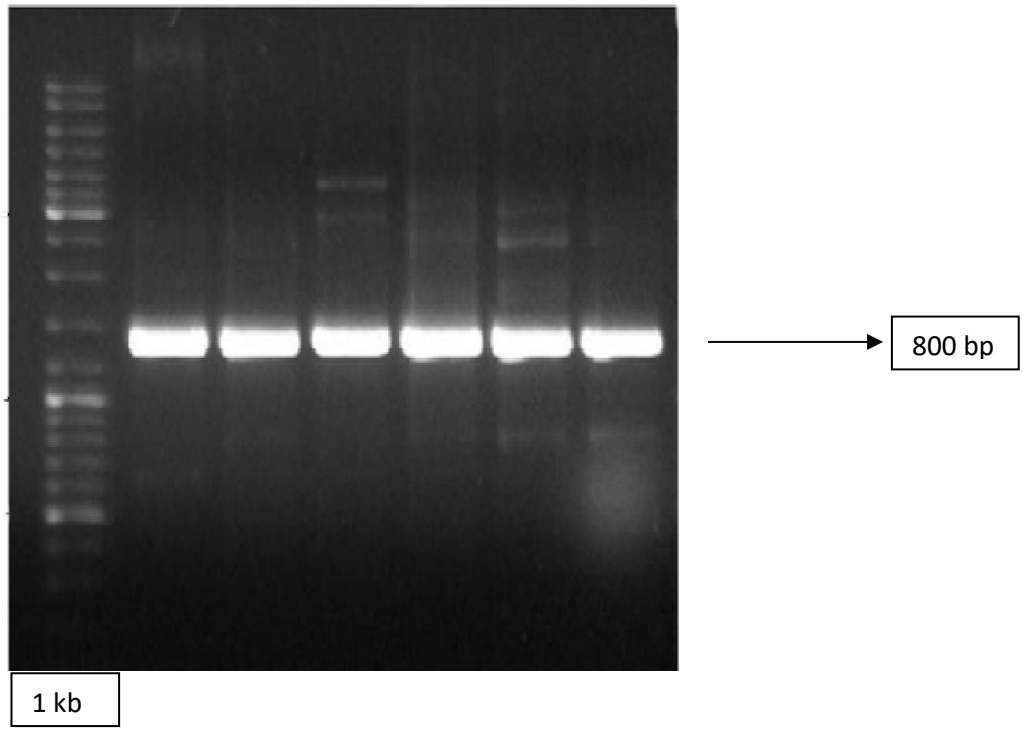

Plate 1: Gel representation of the amplicons (bacterial isolates)

Table 3: Diameter of zones of inhibition of the pharmaceutical effluents against the test isolates

Isolate	Diameter of zones of inhibition (X±SD) mm					
	P1	P2	P3	P4	P5	CPX
BMQM	12.40±0.11	17.21±0.33	13.56±0.87	19.16±0.11	12.56±0.11	22.38±0.11
BCBC	16.34±0.33	20.08±0.17	17.34±0.12	22.08±0.07	16.76±0.11	22.76±0.51
ECA1	17.11±0.14	20.68±0.14	15.22±0.61	20.96±0.81	17.23±0.31	26.11±0.21
KP39	15.76±0.21	18.51±0.31	15.81±0.31	21.27±0.11	16.08±0.07	25.86±0.11
MLSA	14.38±0.11	18.07±0.41	16.21±0.51	21.86±0.12	14.68±0.42	22.44±0.22
PFPt	18.19±0.07	22.16±0.11	17.79±0.11	26.33±0.33	18.34±0.41	24.31±0.58
ATK04	13.42±0.21	18.01±0.07	15.46±0.11	21.18±0.07	14.08±0.07	0.00±0.00
ANC5	11.58±0.11	16.42±0.21	13.27±0.22	19.76±0.16	11.76±0.21	0.00±0.00
GMB1	12.52±0.11	16.86±0.42	14.08±0.31	20.81±0.62	13.03±0.17	0.00±0.00
RIC2	13.07±0.14	17.76±0.21	14.88±0.82	20.92±0.11	13.86±0.12	0.00±0.00

BMQM – *Bacillus megaterium* strain QMB1551; BCBC – *Burkholderia cepacia* strain BC16; ECA1 – *Enterobacter cloacae* strain A117; KP39 – *Klebsiella pneumoniae* strain 39427; KP39 – *Klebsiella pneumoniae* strain 39427; MLSA – *Micrococcus Luteus* strain SA211; PFPt – *Pseudomonas fluorescens* strain Pt14; ATK04 – *Aspergillus terrus* strain KAML04; ANC5 – *Aspergillus niger* strain CFL5; GMB1 – *Glomus mosseae* BEG119; RIC2 – *Rhizophagus irregularis* strain C2

DISCUSSION

The presence of coliforms, faecal coliforms and high total bacterial counts recorded in the studied UPE agreed with the findings of Ejimofor and Okigbo, (2023), but disagrees with the findings of Hutardo *et al*. (2017) who reported low counts of microbial loads from their studied effluents. The high level of microbial loads recorded in the present study could be attributed to faecal contamination, ability of the microorganisms to develop resistance to the antimicrobial substances in the effluents, and the ability of most of the microorganisms to metabolize some of the components of the pharmaceutical effluents as both energy and carbon sources. Several researchers (Anyaegbu and Okigbo, 2023) have reported the presence of pathogenic microorganisms in untreated pharmaceutical effluents but this report was not in line with the findings of Ejimofor and Adaugo (2024) who focused on the positive sides of UPE and the degradative potentials of some bacterial isolates without considering the negative impact to the environment and human health.

The susceptibility of the bacterial and fungal isolates to UPE supported the findings of many researchers (Xiaolei *et al*., 2020, Nwakoby *et al.,* 2024). The susceptibility of these microbes to UPE could be attributed to the presence of some antibiotics, acids, heavy metals that have been complexed with some metabolites in the effluents. Gomes *et al.* (2020) reported the inhibition of *Burkholderia cepacia* by pharmaceutical products. The susceptibility of these microbes to UPE is an indication of the loss of beneficial microbes and nutrients in agricultural soil polluted with UPE. The significant decrease in the growth performance of *Zea mays* (maize) planted in UPE-polluted soil in this study agreed with the findings of Sousa *et al*. (2018) but deviated from the reports of Shahmahdi *et al.* (2020) and Gworek *et al*. (2019) who argued that low concentration of pharmaceutical effluents contain nutrients that enhance the growth of root length without considering moderate or higher concentration of the effluents, even constant application of the effluents on the land. But in the present study, there were reduction in the percentage of germination, dimensions of the maize and cobs including the plant height, and these reductions were in line with the reports of many researchers (Baran *et al*., 2018; Barbara *et al.,* 2021) who also observed reductions in various plant parts in their respective studies.

CONCLUSION

There was significant inhibition of *Bacillus megaterium* strain QMB1551 (BMQM), *Burkholderia cepacia* strain BC 16 (BCBC), *Enterobacter cloacae* strain A117 (ECA1), *Klebsiella pneumoniae* strain 39427 (KP39), *Micrococcus lutteus* strain SA211(MLSA), *Pseudomonas fluorescens* strain Pt 14 (PFPt), *Aspergillus terreus* strain KAML04 (ATK04), *Aspergillus niger* strain CFL5 (ANC5), *Glomus mosseae* BEG 119 (GMB1) and *Rhizophagus irregulavis* strain C2 (RIC 2) by UPE. The migration of pollutants down into the soil profile depends on the intensity of their sorption on the

soil solid phase particles. The sorption intensity affects the bioavailability of active substances of medicines and their persistence in the soil environment. Therefore, the soil sorption capacity plays an important role in the exposure of living organisms to contamination

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