

The background of the cover features a teal surface with medical instruments. A silver stethoscope is positioned on the right side, and a white digital thermometer is at the top, showing a reading of 36.5°C. A red and white capsule is visible in the lower center, and a pink pill is partially seen on the right edge. A white rectangular box is present in the middle section of the cover.

African Journal of Pharmacy and Pharmacology

Volume 11 Number 28, 29 July, 2017
ISSN 1996-0816

MEDICAL



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Full Length Research Paper

Antibiotic sensitivity patterns of microbial isolates from fish ponds: A study in the Greater Accra Region of Ghana

John Antwi Apenteng*, Christina Osei-Asare, Esther Eshun Oppong, Ignatius Amihere and Mohammed Yahaya Hafiz

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Received 27 April, 2017; Accepted 14 June, 2017

Intensive use of antimicrobial agents in aquaculture provides a selective pressure creating reservoirs of drug-resistant bacteria and transferable resistance genes in fish pathogens and other bacteria in the aquatic environment. From these reservoirs, resistance genes may disseminate by horizontal gene transfer and reach human pathogens, or drug-resistant pathogens from the aquatic environment may reach humans directly. This study aims at identifying the antibiotic sensitivity patterns of *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli* isolates from fish ponds in two localities (Ashiaman and Dawhenya) in the Greater Accra region of Ghana. A total of 43 isolates were tested using the Kirby-Bauer agar disc diffusion method against Ciprofloxacin (5 µg), Erythromycin (15 µg), Cefuroxime (30 µg), Gentamicin (10 µg), and Tetracycline (30 µg). *P. aeruginosa* was the most isolated organism with 90% prevalence, followed by *E. coli* (75%) and *S. typhi* (50%). All the *P. aeruginosa* isolates were resistant to Erythromycin and Cefuroxime while 90% of the *S. typhi* isolates were resistant to Tetracycline and Erythromycin. *E. coli* isolates showed 100, 93.33 and 66.66% resistance to Erythromycin, Cefuroxime and Tetracycline respectively. Gentamicin, Tetracycline and Ciprofloxacin were sensitive to 66.66, 61.10 and 50% of *P. aeruginosa* isolates respectively. Ciprofloxacin was sensitive to 90% of the *S. typhi* isolates whiles Gentamicin was sensitive to 70% of the *S. typhi* isolates. Ciprofloxacin and Gentamicin were more sensitive to *E. coli* isolates (80 and 66.66% sensitivity respectively). Multi-drug resistant strains were obtained in 77.78% of *P. aeruginosa* and 70% of *S. typhi* isolates whiles 66.67% of *E. coli* isolates were also resistant to more than two classes of the antibiotics tested. High levels of microbial resistance were observed in the isolates with 72.09% of isolates being multidrug resistant strains.

Key words: Resistance patterns, multi-drug resistance, fish ponds, antibiotics.

INTRODUCTION

With the rise in public awareness about the loss of effectiveness of antibiotics due to over use; consumer

groups, public health experts and environmentalists have begun to challenge antibiotic usage in livestock, poultry

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and fish farming. Antibiotics in fish farming and other animal food production is widely believed to contribute to the dramatic increase in numbers of antibiotic-resistant bacterial strains now threatening human health (Benbrook, 2002). As a result of the non-hygienic and stressful conditions present in aquaculture facilities, the risk of bacterial infections among aquaculture fish is high, therefore, heavy amount of antimicrobials are used in fish feed for preventive and curative purposes in aquaculture facilities worldwide (Sapkota et al., 2008).

The heavy use of antimicrobial agents in aquaculture has resulted in increase of strains resistance to these agents. Potentially, these resistant strains can have impact on the therapy of fish and human diseases or the environment of the fish farms (Smith et al., 2003). It is therefore necessary to study the presence of clinically important bacteria in the environment and their antibiotic resistance pattern. This study shall focus on resistance strains found in fish farms and the specific isolates will be *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*.

P. aeruginosa is a clinically important gram negative bacterium, which is responsible for a variety of systemic infections like urinary tract infections, respiratory system infections, gastrointestinal infections, dermatitis, soft tissue infections, bone and joint infections (Favero et al., 1971). The overall prevalence of antibiotic resistant *P. aeruginosa* is increasing, with up to 10% of global isolates found to be multidrug resistance strains (Gales et al., 2001). It is recognized as the second leading cause of gram negative nosocomial infection and a major treatment challenge for *P. aeruginosa* infections (Carmeli et al., 1999).

Salmonella is a Gram-negative bacterium belonging to the family Enterobacteriaceae, and known as "enteric" bacteria. *Salmonella* are found in the intestinal tract of animals and humans. Some serotypes of *Salmonella*, such as *S. typhi* and *S. paratyphi* are only found in humans (Miller and Pegues, 2005). In humans, *Salmonella* are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a food borne infection or intoxication (Foley et al., 2006). *Salmonella* bacterium is one of the commonest causes of food poisoning worldwide.

E. coli is the most prevalent facultative anaerobic species in the gastrointestinal tract of human and animals, usually a harmless microbe, but it is also a medically important bacterium causing a number of significant illnesses. *E. coli* can be easily disseminated in different ecosystems through the food chain and water. One of the possible ways of entry of various microbes could be by the adoption of improper hygienic measures during handling and processing of harvested fishes. Antimicrobial resistance in *E. coli* has been reported worldwide.

The aim of this study is to isolate and assess the prevalence of *S. typhi*, *E. coli*, and *P. aeruginosa* in fish ponds and to determine their respective antibiotic resistance patterns against some clinically used antibiotics.

MATERIALS AND METHODS

Collection of samples

A total of 20 fresh water samples were collected from Ashaiman and Dawhenya Fish Ponds. The samples were collected into a sterile sample container at four different sites of the fish ponds. They were then transferred to the Microbiology Laboratory of Central University Pharmaceuticals department immediately and kept refrigerated for 24 h. A volume of 1.0 mL of the collected samples were then transferred into 10 mL peptone water and incubated at 37°C for 48 h.

Isolation of bacteria

The initial isolation of bacteria (*P. aeruginosa*, *S. typhi* and *E. coli*) was done using various selective, differential isolation media such as Cetrimide, MacConkey and Bismuth sulphite agar. Further isolation were performed on the initial isolates using biochemical methods using fermentation, gram staining and other microbe specific media such as brilliant coliform *E. coli* media, Methyl Red Vogues Proskauer (MRVP) tests and Citrate utilization tests. A total of 43 isolates comprising the organisms of interest were obtained. All microbial media used were purchased from Oxoid, UK.

Antibiogram study

A total of 43 isolates were tested for antimicrobial drug susceptibility against five commonly used antibiotics belonging to the following antibiotic classes; (Aminoglycosides, Fluoroquinolones, Macrolides, Cephalosporins and Tetracyclines) by Kirby-Bauer agar disc diffusion method (Bauer et al., 1966). The isolated bacteria (*P. aeruginosa*, *S. typhi* and *E. coli*) in the nutrient broth were spread on the surface of sterile nutrient agar. Antimicrobial discs (Oxoid, UK) were placed on the surfaces of inoculated nutrient agar plates using a multidisc dispenser (Oxoid, UK). The plates were kept on the bench for 30 min prior to incubation at 37°C for 24 h. After 24 h incubation, the plates were examined and the diameters of the inhibition zones were measured from the edge of the disc to the edge of the zone using a zone reader. Susceptibility and resistance were determined according to the interpretation criteria described in the Clinical and Laboratory Standard Institute (CLSI) Guidelines (2011) (Table 1).

Data analysis

All graphs were plotted and analysed using Microsoft Excel 2013.

RESULTS

Overall prevalence of isolates according to location

A total of 43 isolates were obtained out of which 18 were *P. aeruginosa*, 10 were *S. typhi* and 15 were *E. coli*. The

Table 1. CLSI inhibition zone diameter for *P. aeruginosa*, *S. typhi* and *E. coli*.

Antibiotic	Content	When testing	Susceptible (S)	Intermediately susceptible (I)	Resistant (R)
Cefuroxime	30 µg	Enterobacteriaceae <i>P. aeruginosa</i>	≥ 23	15-22	≤ 21
Ciprofloxacin	5 µg	Enterobacteriaceae <i>P. aeruginosa</i>	≥ 21 ≥ 21	16-20 16-20	≤ 15 ≤ 15
Erythromycin	15 µg	Enterobacteriaceae <i>P. aeruginosa</i>	≥ 23	14-22	≤ 13
Gentamicin	10 µg	Enterobacteriaceae <i>P. aeruginosa</i>	≥ 15 ≥ 15	13-14 13-14	≤ 12 ≤ 12
Tetracycline	30 µg	Enterobacteriaceae <i>P. aeruginosa</i>	≥ 15 ≥ 19	12-14 15-18	≤ 11 ≤ 14

Source: Clinical and Laboratory Standard Institute (CLSI) Guidelines (2011).

Table 2. Summary of prevalence of *P. aeruginosa*, *S. typhi* and *E. coli* according to location.

Location	Isolated bacteria (%)		
	<i>P. aeruginosa</i>	<i>S. typhi</i>	<i>E. coli</i>
Dawhenya fish pond	100	75	100
Ashaiman fish pond	87.5	43.75	68.75
Overall	90	50	75

overall prevalence (expressed as a percentage) of *P. aeruginosa*, *S. typhi* and *E. coli* in Dawhenya and Ashaiman Fish Ponds were recorded as shown in Table 2.

Antibiogram profile of isolated bacteria

Pseudomonas aeruginosa

Antibiotic sensitivity test of 18 isolates of *P. aeruginosa* showed different sensitivity and resistance patterns according different sources. Out of the five antibiotics tested, Gentamicin (CN) was sensitive to 66.66% of isolates. Tetracycline (TE) and Ciprofloxacin (CIP) were sensitive to 61.1 and 50% of isolates respectively. Ciprofloxacin (CIP) and Tetracycline (TE) were however intermediate resistant to 5.56% of isolates. Ciprofloxacin (CIP) was resistant to 44.44% of isolates. Erythromycin (E) and Cefuroxime (CXM) showed no antimicrobial activity against the isolates Figure 1.

Salmonella typhi

Antibiotic sensitivity test of 10 isolates of *S. typhi* showed different sensitivity and resistance patterns. Results from

the five antibiotics tested showed that, Ciprofloxacin (CIP), Gentamicin (CN) and Cefuroxime (CXM) were sensitive to 90, 70 and 30% of isolated *S. typhi* respectively. Tetracycline (TE) and Erythromycin (E) were sensitive to 10% of isolates. Gentamicin (CN) on the other hand was intermediately resistant to 10% of isolates. The highest resistance was found with Tetracycline (TE) and Erythromycin (E), showing resistance to 90% of isolates, followed by Cefuroxime (CXM), which showed resistance to 70% isolates. Gentamicin (CN) was resistant to 20% of isolates, whereas, Ciprofloxacin (CIP) to 10% of the isolated *S. typhi* shown in Figure 2.

Antibiogram profile of *Escherichia coli*

Out of the five antibiotics tested on the 15 isolates, Erythromycin (E) was resistant to 100% of isolates, followed by Cefuroxime (CXM), which was resistant to 93.33% of isolates. Ciprofloxacin (CIP), Gentamicin (CN), and Tetracycline (TE) showed resistant to 13.33%, 26.66%, and 66.66% of isolated *E. coli* respectively. Ciprofloxacin (CIP), Gentamicin (CN), and Cefuroxime (CXM) were intermediate resistant to 6.66% of isolates. Ciprofloxacin (CIP) on the other hand showed the highest sensitivity against 80% of isolates. Gentamicin (CN) and Tetracycline (TE), showed sensitivity to 66.66 and 33.33% of isolates respectively which is shown in Figure 3.

Multidrug resistant organisms

The presence of multidrug resistance strains were also studied among isolates. Of the isolates, 31 (72.09%) were resistance to more than two different classes of antibiotics out of which 14 (77.78%) isolates of

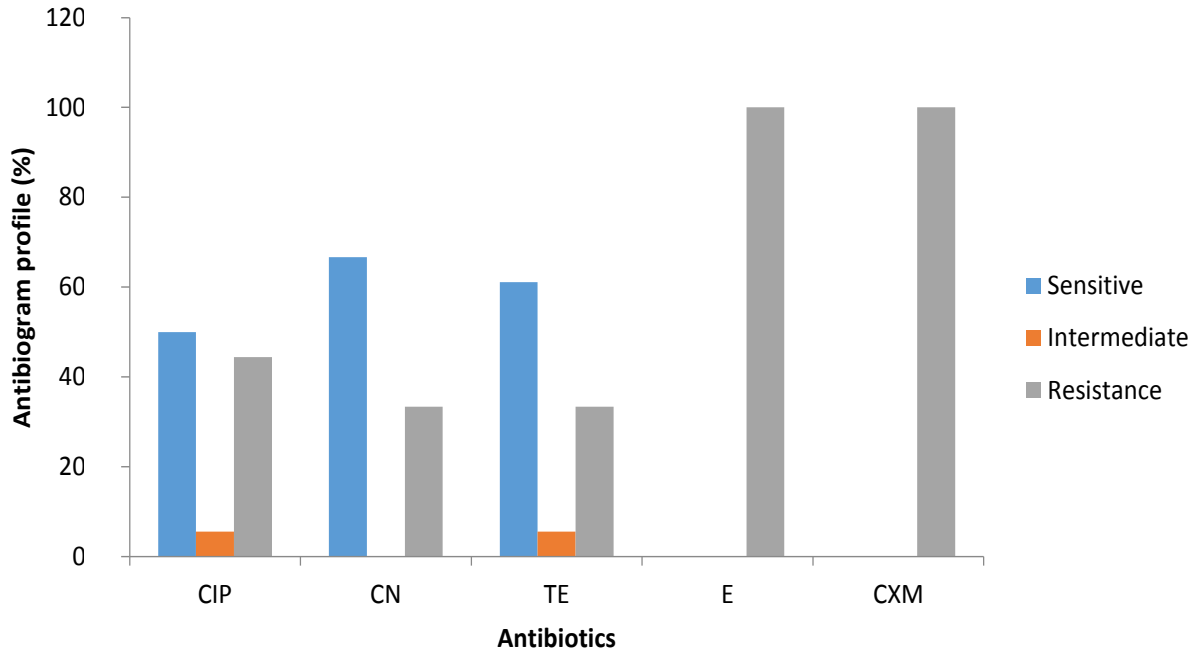


Figure 1. Antibiogram profile of *P. aeruginosa* isolates from Dawhenya and Ashaiman Fish Ponds. Ciprofloxacin (CIP), Gentamicin (CN), Tetracycline (TE), Erythromycin (E), and Cefuroxime (CXM).

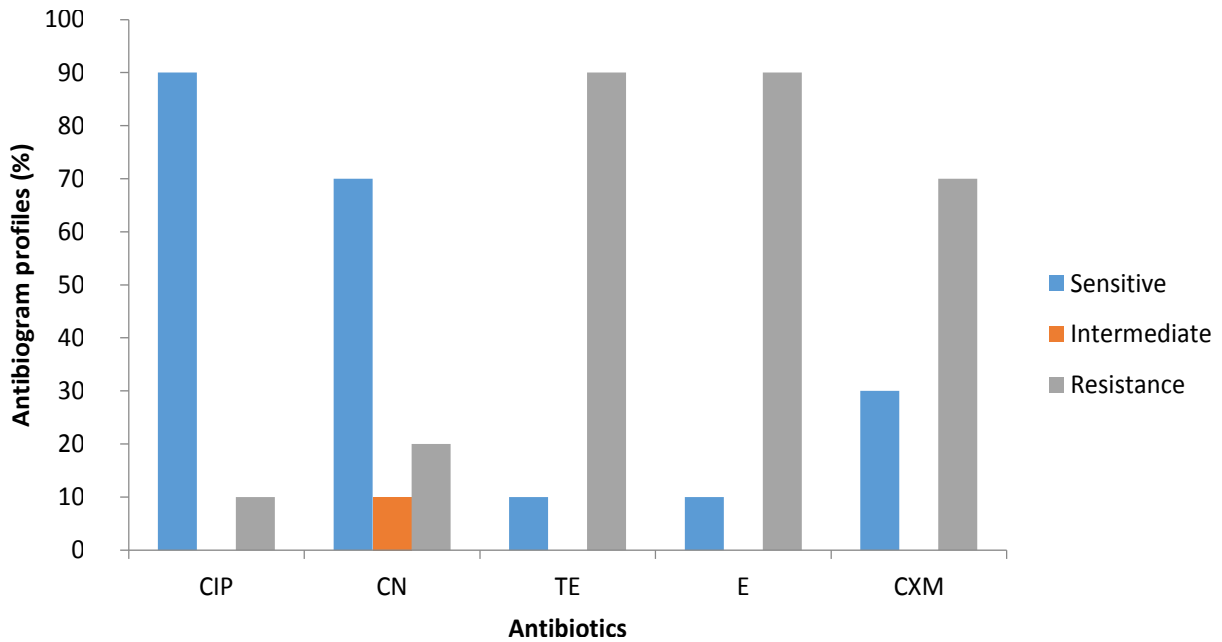


Figure 2. Antibiogram profile of *S. typhi* isolates from Dawhenya and Ashaiman Fish Ponds. Ciprofloxacin (CIP), Gentamicin (CN), Tetracycline (TE), Erythromycin (E), and Cefuroxime (CXM).

P. aeruginosa, 7 (70%) isolates of *S. typhi* and 10 (66.67) isolates of *E. coli* were all found to be multidrug resistant strains. *P. aeruginosa* had the highest number (14 out of 18 isolates) of multidrug resistant strains, followed by *S. typhi*.

DISCUSSION

Aquaculture is growing rapidly in many regions of the world, and aquaculture products constitute an important food supply with increasing economic importance. Use of

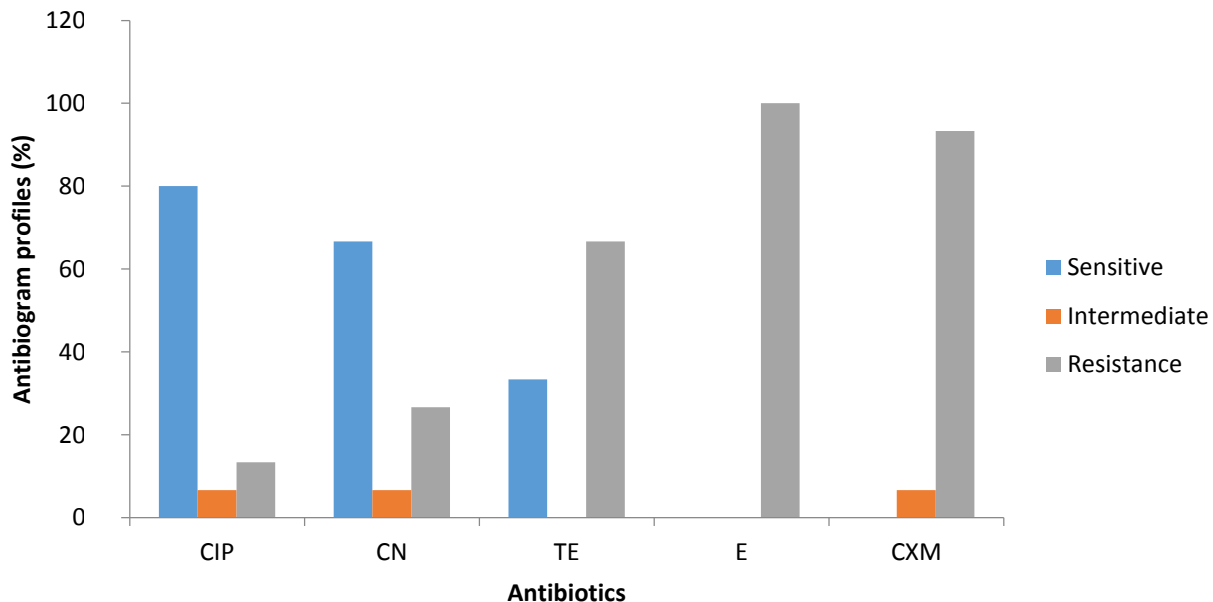


Figure 3. Antibiogram profile of *E. coli* isolates from Dawhenya and Ashaiman Fish Ponds. Ciprofloxacin (CIP), Gentamicin (CN), Tetracycline (TE), Erythromycin (E), and Cefuroxime (CXM).

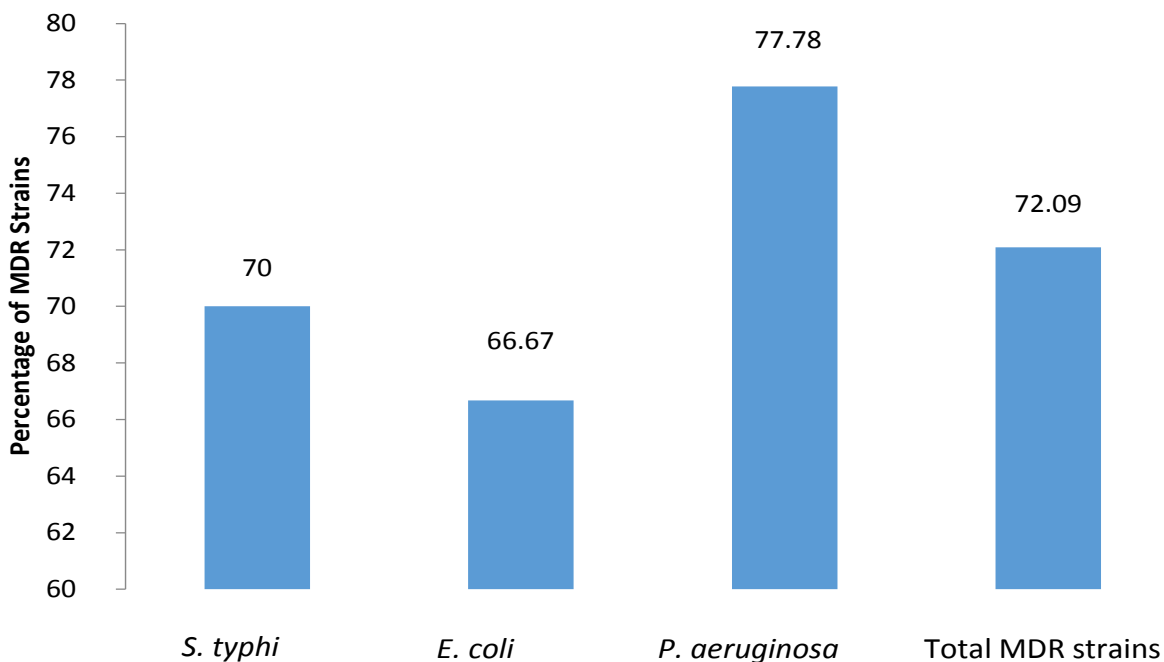


Figure 4. Multi-drug resistance (MDR) strains of isolates.

antimicrobial agents in aquaculture has resulted in the emergence of reservoirs of antimicrobial-resistant bacteria in fish and other aquatic animals, as well as in the aquatic environment (Akinbowale, 2006; Schmidt et al., 2000). The three bacteria studied in this project are of

public health importance (Bangtrakulnonth et al., 2004; Nadeem et al., 2009; Ministry of Health, 2002) and presence of resistance strains in fish pond water should be of much concern because, the people around and the environment are exposed to these microorganisms. A

total of 43 isolates were obtained in this study of which 18(41.36%) were *P. aeruginosa*, 15(34.88%) were *E. coli* and 10(23.26%) were *S. typhi*. Among the organisms isolated from Ashaiman and Dawhenya Fish Ponds, *P. aeruginosa* was the mostly found organism with 90% prevalence followed by *E. coli* (75%) and *S. typhi* (50%). Uğur et al. (2012) reported in their study in Turkey that, *Pseudomonas* species was the most frequently detected microorganism in fish ponds.

All the *P. aeruginosa* isolates obtained in this study were resistance to Erythromycin and Cefuroxime. This is similar to a study conducted by Sivanmaliappan and Sevanan (2011) in Coimbatore, India where 100% of *P. aeruginosa* isolates were resistant to Erythromycin. A study conducted in Egypt by Gad et al. (2008) also showed that 100% of *P. aeruginosa* isolates were resistant to Cefuroxime. Resistance to Cefuroxime is largely due to the production of extended spectrum β -lactamase (ESBL) enzymes by the bacteria. Resistance to Cefuroxime by *P. aeruginosa* could also be due to a combination of mechanisms such as the expression of chromosomal AmpCcephalosporinases and over expression of active efflux systems (McGowan, 2006). Resistance to Erythromycin may be due to the use of this agent in fish farming. Gentamicin, Tetracycline and Ciprofloxacin were potent against *P. aeruginosa* isolates with 66.66, 61.1% and 50% susceptibility, respectively. This is comparable to a study carried out in Dhaka, Bangladesh where both Gentamicin and Tetracycline were found to be sensitive to 93.70% of *P. aeruginosa* isolates (Nasreen et al., 2015). A similar observation has also been reported in Bangladesh where Ciprofloxacin was highly sensitive to *P. aeruginosa* isolates (Hossain et al., 2013). Gentamicin and Ciprofloxacin resistance to *P. aeruginosa* isolates is due to the ability of *P. aeruginosa* to acquire further resistance mechanisms to these agents (Streteva and Yordanov, 2009). This could be the reason why *P. aeruginosa* isolates were resistant (33.33%) to Gentamicin and (44.44%) to Ciprofloxacin, even though Gentamicin and Ciprofloxacin are hardly used in fish farming. *P. aeruginosa* isolates resistance to Tetracycline (33.33%) could be due to its frequent use in aquaculture facility.

Infection by *Salmonella* is a common cause of food poisoning in humans (Hobbs and Robert, 1993). In this study *S. typhi* was the least isolated organism compared to *P. aeruginosa* and *E. coli*. Erythromycin and Tetracycline exhibited the highest resistance to *S. typhi* (90%) isolates whiles Cefuroxime showed resistance against 70% *S. typhi* isolates. According to Chanda et al. (2011), most farms use tetracycline and erythromycin to eliminate pathogenic problems. Antibiotic resistance develops when microorganisms are exposed to effective doses of an antibiotic within a shorter period or when the microorganisms are exposed to smaller concentrations or residues of the antibiotic over a longer period of time (Todar, 2008). These theories may support

the high resistance observed for Tetracycline and Erythromycin as a result of prolong exposure to microorganisms. It was observed that the most sensitive antibiotics to *S. typhi* isolates were Ciprofloxacin and Gentamicin. A similar result was obtained in a study in Bangladesh where *S. typhi* was mostly sensitive against Ciprofloxacin and Gentamicin (Mannan et al., 2014). A total of 100, 93.33, and 66.66% of the *E. coli* isolates exhibited resistance to Erythromycin, Cefuroxime and Tetracycline respectively. A similar result was obtained by Kibret and Abera (2011), where Erythromycin and Tetracycline were resistance to 89.4 and 72.6% to *E. coli* isolates respectively. A study conducted by Ahmed et al. (2015) in Islamabad showed complete resistance of *E. coli* isolates to Cefuroxime. Ciprofloxacin and Gentamicin revealed the highest sensitivity against the *E. coli* isolates with 80 and 66.66% sensitivity, respectively. This in accordance with a study carried out in Nigeria where 78.9% of *E. coli* isolates showed sensitivity to Ciprofloxacin and same percentage was obtained for Gentamicin (Reuben and Owuna, 2013).

In general, the relatively higher proportion of susceptible responses of *E. coli*, *S. typhi*, and *P. aeruginosa* isolates to Ciprofloxacin and Gentamicin suggest the effectiveness of these agents in the treatment of infections caused by *E. coli*, *S. typhi* and *P. aeruginosa*. *P. aeruginosa* was the most isolated organism from the fish ponds. This is because *P. aeruginosa* thrives very well at habitats with adequate amount of moisture (Hardalo et al., 1997). Out of the 43 isolates, 31 (72.09%) were resistant to more than two different classes of antibiotics. *P. aeruginosa* had the highest percentage of multidrug resistance strains accounting for 77.78%. According to Gales et al. (2001), up to 10% of global *P. aeruginosa* isolates are found to be multidrug resistance. The high number of resistance of *E. coli*, *S. typhi*, and *P. aeruginosa* found in the two fish ponds serves as a reservoir which can be easily transferred to other pathogens and even humans.

Conclusion

The study has shown the presence of resistant strains in the fish ponds at Dawhenya and Ashiaman. All the *P. aeruginosa* isolates were resistant to Erythromycin and Cefuroxime whiles 90% of the *S. typhi* isolates were resistant to Tetracycline and Erythromycin. Cefuroxime, Erythromycin and Tetracycline respectively showed 100, 93.33 and 66.66% resistance to *E. coli* isolates. Ciprofloxacin and Gentamicin showed the highest sensitivity when tested on all the isolates. *P. aeruginosa* had the highest number of multi-drug resistant strains. This study has therefore proved the need for the monitoring of antibiotics usage in fish farming and the adoption of proper hygienic measures in aquaculture facilities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Authors acknowledge the immense contributions of Mr Harry Obliye Laryea, Mr Kwame Koomson and Mr Christopher Kyei Mensah of Central University School of Pharmacy for their technical support.

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Full Length Research Paper

Physicochemical characteristics and cytotoxic effect of the methanolic extract of *Croton heliotropiifolius* Kunth (Euphorbiaceae)

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Received 18 May, 2017; Accepted 27 June, 2017

Croton heliotropiifolius Kunth is an endemic specie in northeast of Brazil. It is renowned for its medicinal properties, larvicidal and insecticidal actions. This work investigated the chemical compounds present in the methanolic extract of *Croton heliotropiifolius* and the cytotoxic activity. Physical-chemical analyses were performed by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), Fourier transformation infrared spectroscopy (FT-IR), and ultraviolet-visible spectrophotometry (UV-Vis). Cytotoxic activity was evaluated in the following tumour cell lines; acute promyelocytic leukaemia (HL-60), human breast adenocarcinoma (MCF-7), human laryngeal carcinoma (Hep-2), human lung mucoepidermoid carcinoma (NCI-H292). Results revealed the presence of flavonoids and absence of alkaloids, coumarins, saponins and condensed tannins. HPLC identified Gallic acid with retention time of 1.80 min. The infrared spectrum identified the presence of esters group at 1661 cm^{-1} of absorbance. On the other hand, the UV-Vis spectrum revealed bands of light absorbance on 268, 316, 406 nm indicating the presence of phenols and flavonoids. Results showed inhibition of cell growth of 59.5% on HL-60 cells, 46.5% on NCI-H292, 26.7% on Hep-2, and 21.7% on MCF-7 cells. In conclusion, the presence of phenolic compounds in the extract was demonstrated and a low to medium percentage of cell growth inhibition on tumour cell lines tested.

Key words: *Croton*, *Croton heliotropiifolius*, flavonoids, gallic acid, cytotoxicity, tumour lines.

INTRODUCTION

The genre *Croton* comprises more than 1300 species of trees, bushes, and herbs distributes all over the world.

Such biodiversity is mainly found in India, Brazil and Madagascar where its ethnomedicinal value is

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recognized (Yankep et al., 2003; Ye et al., 2012). *Croton heliotropiifolius* Kunth is an endemic specie in the northeast of Brazil frequently found within “caatinga”, “brejo”, “resting” and “cerrado” vegetations. It is known by unofficial names such as “velame”, “velaminho” and “velame-de-cheiro” due to the presence of trichomes. Previous studies have described the presence of alkaloids, polyphenols and reducing agents in *C. heliotropiifolius*, including its medicinal use for relief of stomach pain, vomit, diarrhoea and as an antithermic (Randau et al., 2004). The essential oil has been described as larvicidal against *Aedes aegypti* (Dória et al., 2010) and the ethanolic extract demonstrated significant insecticidal activity against *Sitophilus zeamais* (Silva et al., 2012). The Human Toxicity Potential (HTP) is an important test to be performed early in the study of a medicinal herb. It is recommended that new herbs and those with unknown actions shall be analysed in a controlled cellular environment as free of complex interactions inherent an organism. The methodologies is aimed at analysing the cellular behaviour, display several advantages such as low costs, easy and quick execution and controlled cell environment (Freshney, 2000). Cancer is a progressive chronic disease responsible for approximately 13% of deaths during last year (WHO, 2016). Genre, race, age, genetic predisposition and environmental exposure to carcinogenic agents, are directly related to the distribution and incidence of tumours (Chu et al., 2004).

Surgical removal of tumours combined with chemotherapy have been efficient methods in the treatment of several cancers. However, the appearance of side effects is very characteristic. Those effects very often compromise the continuity of the treatment which may lead to advanced stages of malignity and morbidity. Therefore, continuing efforts are focused towards the discovery of new antitumor compounds with more secure and efficient characteristics. In this context, the study of natural products seems promising (Erharuyi et al., 2017).

Due to knowledge that chemical analysis and the human toxicity potential are important assays for the medical use of a plant, this work aimed to characterize the physical-chemical properties of *C. heliotropiifolius* leaves, and to evaluate its cytotoxic activity in several tumour cell lines.

MATERIALS AND METHODS

General experimental procedures

The methanolic extract of *C. heliotropiifolius* leaves was concentrated using a rotary evaporator with vacuum BUCHI Switzerland at 60°C. Thin-layer chromatography (TLC) was performed with TLC Silicagel 60G F254 (Merck®) on top of an aluminium base. Compounds were sprayed with visualization reagents, visualized under UV light (254 nm - 365 nm), and for flavonoids, was compared with the quercetin standard. The HPLC system used was a HPLC Waters (Self Cleaning System) attached

to an UV spectrophotometer with detector model Waters 2998 PDA containing photodiodes (Photodiode Array Detector). Values in the infrared region (FTIR) and UV- visible spectrum were registered respectively by an Agilent 630 and Shimadzu UV-vis 1800 spectrophotometers. All chemical products used in those procedures were for analytic use (Sigma® e Merck®). The Division of Antibiotics from the Federal University of Pernambuco (UFPE) supplied the cells, antibiotic solutions (penicillin and streptomycin), and the drug doxorubicin. Both culture media, Dulbecco MEM (DMEM) and Roswell Park Memorial Institute (RPMI), and the fetal bovine serum (FBS) were Gibco™ BRL. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was manufactured by Sigma®.

Plant material

C. heliotropiifolius (Euphorbiaceae) leaves were collected in July 2015 in the urban area of Garanhuns city, Pernambuco State, Brazil (Latitude: -8.89074, Longitude: -36.4966 8° 53' 27" Sul, 36° 29' 48" Oeste). The plant was identified by the Dárdano de Andrade Lima herbarium in the Agronomic Research Institute (IPA). It was registered in the referred herbarium under the catalog number 90440 in which an exsiccatae was deposited.

Extraction of compounds

The extract composed of plant leaves was prepared by soaking and maceration (Cechinel Filho and Yunes, 1998). About 150 g of powdered material was taken in a clean, flat bottomed glass container and soaked in 200 mL of methanol. The container with its contents was sealed and kept for a period of 10 days accompanying occasional shaking and stirring. Next, filtration was performed followed by evaporation of all solvent to obtain the crude extract. The presence of flavonoids, alkaloids and coumarins was tested by thin layer chromatography (TLC). The mobile phase used for flavonoids and alkaloids was ethyl acetate-formic acid-acetic acid-water (AcOEt- HCOOH-AcOH-H₂O 100:11:11:27v/v), for coumarins ether-toluene- acetic acid 10% (50:50:50v/v) was used. The visualization reagents used for flavonoids and alkaloids were respectively etilborilaminoester acid (*Neu*) and Dragendorff. For Coumarins, the visualization method used was UV light on 365 nm (Wagner et al., 1996). The presence of saponins was tested by mechanical shaking of the extract and visualization of foam (Simões et al., 2004). Formation of foam for 15 min was considered as positive for presence of saponins (Dewick, 2002). The presence of tannins was investigated by addition of iron chloride 0.5 M to the hydrated form of extract. In this method, the formation of blue precipitate indicates the presence of hydrolysed tannins awhile green precipitated the presence of condensed tannins (Matos, 1997). HPLC was conducted in model Shimadzu®HPLC LC-10, XBridge C18 column with 4.6 mm x 250 mm dimensions, 5 µm of particle size and 0.7 mL/min flow. The solvent system displayed; 94% of H₂O and 6% of acetonitrile on 0 min (A), 65% of H₂O and 35% of acetonitrile on 8 min (B), 94% of H₂O and 6% of acetonitrile on 9 min (C); with 40 µL of injection volume and sample concentration of 20 mg/mL in 50% MeOH. Identification was performed using the standard method and external integration of the peaks at 256 nm for gallic acid. The spectroscopic profile of the extract was obtained by Fourier transform infrared spectroscopy (FTIR). In the semisolid state, the extract was pressed against the diamond crystal of the equipment. Next, readings were recorded. The FTIR spectrum was obtained in less than 30 s and results compared against standards samples from the library. An aliquot (10 ml) of the extract (1 mg) diluted in MeOH (100%) was used to obtain the UV-visible spectrum, using the wavelength range of 200 to 700 nm.

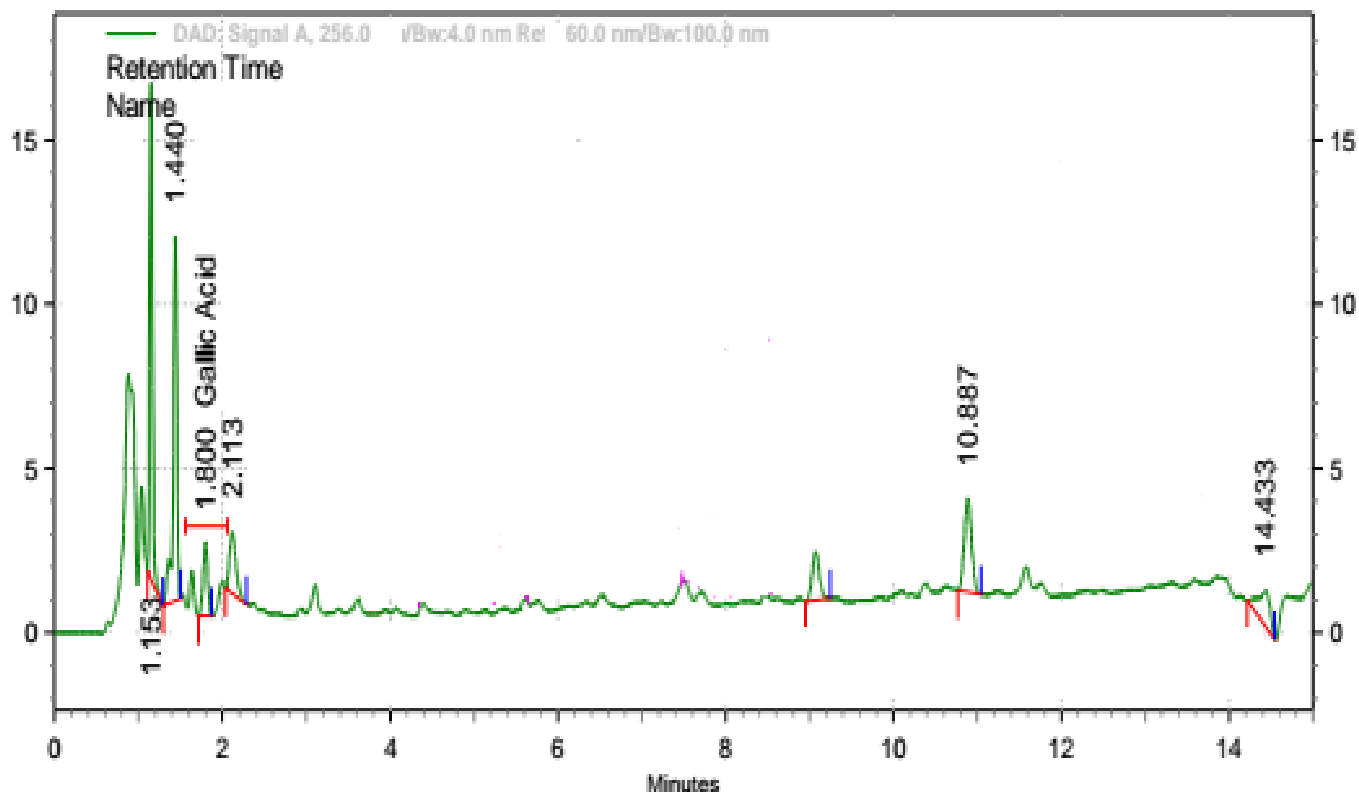


Figure 1. HPLC chromatogram shows the presence of gallic acid in the extract.

Cytotoxic activity

Cytotoxic activity was tested by MTT assay on tumour cell lines. DMEM culture medium was used for incubation of NCI-H292 (human lung mucoepidermoid carcinoma) MCF-7 (human breast adenocarcinoma), and Hep-2 (human laryngeal carcinoma) cells. HL-60 (acute promyelocytic leukaemia) cells were cultured in RPMI medium. In detail, all culture medium was supplemented with fetal bovine serum (10%) and antibiotic solution (1%) of penicillin and streptomycin. Cells were incubated on 37°C in rich humidity and 5% CO₂.

Cells were cultured on a 96 well plate and incubated for 24 h prior to addition of 50 µg/ml of the extract dissolved in 1% DMSO. NCI-H292, HT-29, Hep-2 cell lines were incubated with initial concentration of 10⁵ cell/mL and HL-60 containing 3 × 10⁵ cell/mL. After a total of 72 h of incubation, 25 µL of MTT (5 mg/mL) was added to the culture. After 3 h of incubation, the culture medium was removed by aspiration, and 100 µl of DMSO (1%) added to each well. Control samples received doxorubicin (50 µg/mL) for inhibition purposes. This method enables the visualization of enzymatic activity by formation of purpura colour. A plate reader was used to obtain absorbance values in the wavelength of 560 nm and cell viability values expressed in percentage in comparison with a negative control, considered as "100% inhibited". The experiment was performed with four sample replicates.

Statistical analysis

The percentage of growth inhibition was calculated through descriptive statistics methods on GraphPad Prism 5.0 software, for all cell lines.

RESULTS AND DISCUSSION

Results from this study showed the methanolic extract of *C. heliotropiifolius* leaves have a chemical profile similar to its genre and to the majority of Euphorbiaceae family members. Thin-layer chromatography (TLC) identified the presence of flavonoids and the absence of alkaloids and coumarins. In addition, an alkaloid was identified in the root skin (Schoefs, 2002). The test for presence of saponins revealed their absence. In fact, such result has been reported in another specie of the genre, *Croton linearifolius* (Cunha et al., 2014). Experiments showed the extract did not contain condensed tannins although they have been detected in such specie (Randau et al., 2004).

High-performance liquid chromatography (HPLC) identified gallic acid with retention time of 1.80 min (Figure 1). The hydrolysis of esters bonds on gallic acid have been reported as a natural defence mechanism (Matias et al., 2010).

The spectrum within the infrared region (Figure 2) revealed a band in the region of 3271 cm⁻¹ suggesting the presence of O-H in association to amides. The band in the region of 2958 cm⁻¹ suggests the presence of C-H aliphatic. The region of 1661 cm⁻¹ shows the presence of carbonyl, aliphatic ketones and esters. Those compounds may be related to esters from the gallic acid. Absorbance

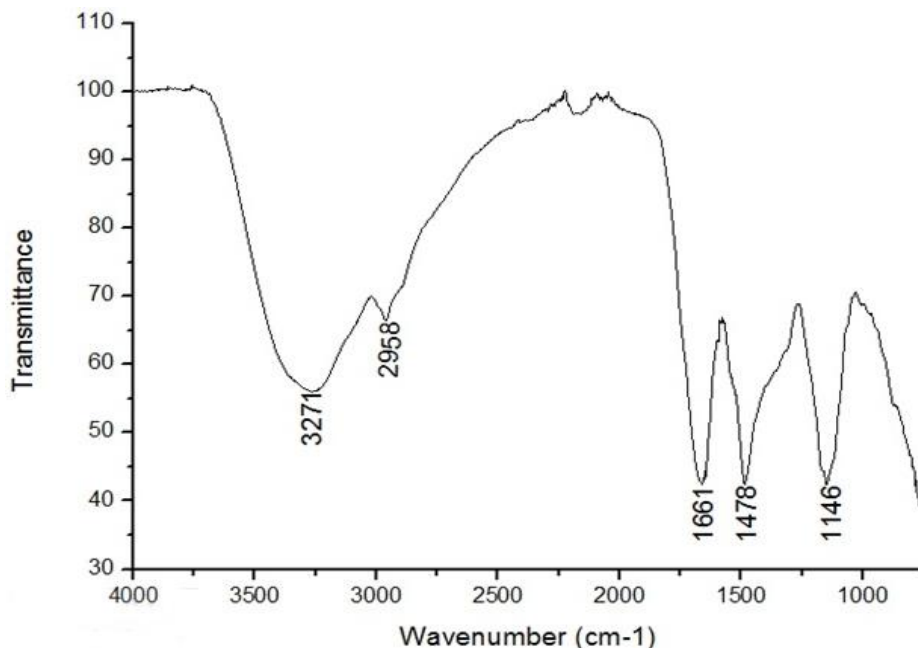


Figure 2. Infrared spectrum (FTIR) of the methanolic extract of *C. heliotropiifolius*.

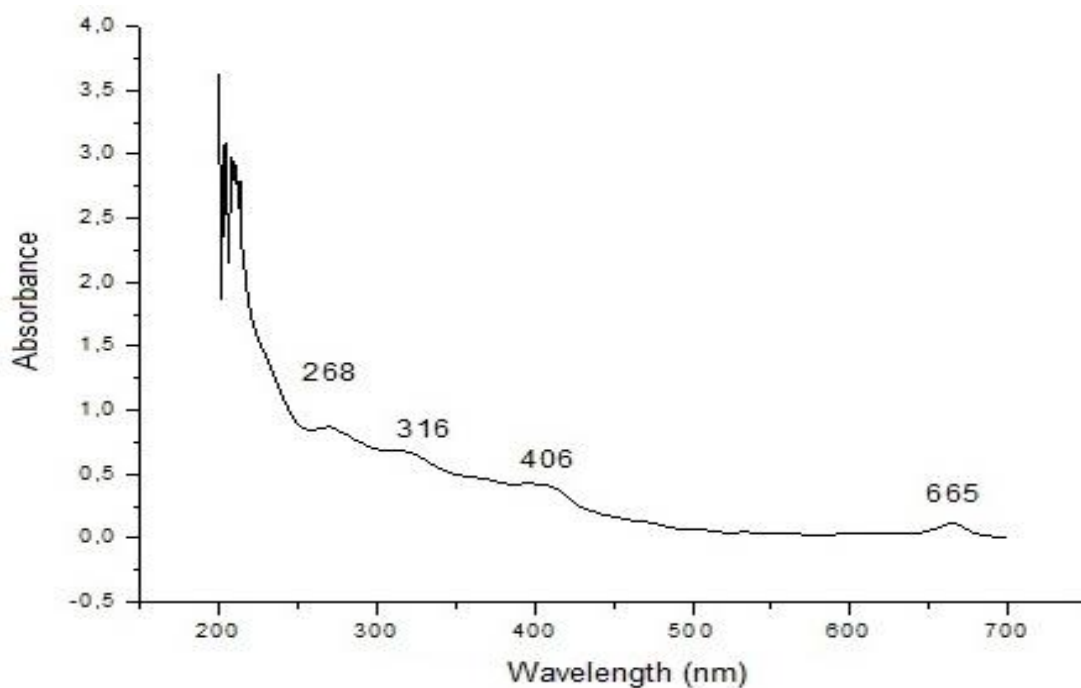


Figure 3. Ultraviolet-visible spectrum of the methanolic extract of *C. heliotropiifolius*

within the region of 1478 cm^{-1} refers to C=C. The range between $1140\text{--}1200\text{ cm}^{-1}$ indicates the presence of sulphones.

The UV spectrum (Figure 3) showed four absorbance bands with maximum values of 268, 316, 406 and 665

nm. Absorbance bands within 266–295 nm indicated the presence of simple phenols. The spectrum typically displays two maximum absorbance values for flavonoids; in 240–285 nm and 300–550 nm (Silva, 2015). Our results come in accordance to these values as demonstrated by

Table 1. Percentage of inhibitory activity of *C. heliotropiifolius* extract on HL-60, MCF-7, Hep-2 e NCI-H292 cells.

Cell line	<i>C. heliotropiifolius</i> extract (%)	Doxorubicin (%)
HL-60	59.5 (±2.9)	92.9 (±0.6)
MCF-7	21.7 (±3.7)	79.4(±2.6)
Hep-2	26.7 (±7.1)	79.4(±2.6)
NCI-H292	46.5 (±2.6)	94.1(±1.9)

chromatography and infrared spectrum results.

The toxicity of several secondary metabolites, present in vegetal, have been described (Silva et al., 2012). Flavonoids have their cytotoxic activity established in different tumour cells (Costa-Lotufo et al., 2003).

Experiments demonstrated cellular growth inhibition of 59.5, 21.7, 26.7 and 46.5% on the respective cell lines HL-60, MCF-7, Hep-2 e NCI-H292 (Table 1). Percentage values of 1-20% were classified as “without inhibitory activity”, between 20-50% as “low inhibitory activity”, for values between 50-70% as “moderate inhibitory activity”, and as “high inhibitory activity” for 70-100% (Andrade et al., 2015).

The extract here tested demonstrated to be more effective on acute promyelocytic leukaemia (HL-60) cells, with moderate growth inhibition. In a similar investigation, essential oil of *Croton zehntneri*, containing its major compound estragole, was tested on NCI-H292, MCF-7 and Hep-2 cell lines. Results revealed numbers lower than 20% of growth inhibition (Andrade et al., 2015). This study demonstrates that the physical-chemical profile of the extract is composed of phenolic compounds, flavonoids, and hydrolysed tannins. On the other hand, it devoid alkaloids and coumarins. The cytotoxic evaluation, within the conditions here tested, showed low inhibition rates on NCI-H292, MCF-7, Hep-2 cells but moderate on HL-60 cells which is potential for further investigations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations: **TLC**, Thin-layer Chromatography; **HPLC**, High-Performance Liquid Chromatography; **FT-IR**, Fourier Transformation Infrared spectroscopy; **UV-Vis**, Ultraviolet-visible spectrophotometry; **MTT**, brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio; **HL-60**, acute promyelocytic leukaemia; **MCF-7**, human breast adenocarcinoma; **hep-2**, human laryngeal carcinoma; **NCI-H292**, human lung mucoepidermoid carcinoma.

ACKNOWLEDGEMENTS

The authors wish to thank the Nacional Semi-arid

Institute (INSA), the Biophysics and Chemistry Laboratory and the Antibiotics Department of the Federal University of Pernambuco (UFPE). This work was funded by the Coordination for the Improvement of Higher Education Personnel (CAPES).

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The background of the entire page is a medical-themed collage. It features a teal surface with a white digital thermometer showing 36.5°C, a silver stethoscope, a red and white pill, a white pill, and a piece of brown adhesive tape. The text is overlaid on this background.

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