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Isolation of Antibiotic Resistant Bacteria from Makelele River (Kinshasa, DR Congo) and Their Susceptibility Towards Plant-Derived Silver Nanoparticles

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Abstract

In Kinshasa city, Democratic Republic of the Congo (DRC) Rivers are highly affected by pollution mainly due to the discharge of garbage, domestic and industrial wastes without any prior treatment. The problem of waste management is a serious issue in this city. The main objective of the current research was to assess the antimicrobial activity of silver nanoparticles vis-à-vis certain bacteria indicative of faecal pollution from Makelele River. The preliminary characterization of silver nanoparticles was carried out using UV-visible spectrophotometer. Noble metals, such as silver nanoparticles, exhibit unique and adjustable optical properties due to their external plasmon resonance and the reduction of silver ions was monitored. The antibiotic susceptibility test results confirmed the inactivity of these antibiotics tested against the wild strain of *E. coli* and *Enterococcus sp.* The synthesized silver nanoparticles displayed a good antibacterial activity against *Enterococcus sp.* This synthesis is designed to bypass the situation of drug resistance and these results provide strong evidence that silver nanoparticles can be used to fight against antibiotic-resistant bacteria.

Keywords

Antibiotic Resistant Bacteria, Green Chemistry, Surface Water, Metallic Trace Elements, Pollution

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1. Introduction

It is well known that watercourses (streams, rivers) are closely bound to their environment and serve in many cases to eliminate waste from anthropogenic activities. Some authors described them as true "natural renal systems"; however, an excessive influx of wastes greatly disrupts the normal functioning of the receiving environments thru a modification of physico-chemical conditions. In addition, this situation can seriously threaten the public health through the development of water-borne diseases [1]. Currently, the insalubrity of the aquatic environment is very worrying in Kinshasa. The increasing population in the city and the intensification of urbanization do not respect any urbanistic rule influencing at first the waste evacuation conditions by waster courses. The situation is aggravated by the lack of ecological sensitivity of the population who is systematically using these rivers as an outlet for the disposal of waste from its activities. Up to now, there is no urban regulation laying down the procedures for waste disposal via watercourses as well as the nature and quantity of wastes carried by watercourses. The resulting degree of pollution is poorly understood and there are no standards for the ecological quality of natural watercourses [2, 3]. The problem of waste management is a serious issue in Kinshasa while almost all industries unload their liquid, solid and gaseous wastes without any slightest care of pre-treatment and around the whole city excreta disposal is not perfomed properly. Wastewater, excremental water and garbage are released into watercourses [4]. The major problem for Kinshasa city remains that relating to faecal pollution that results from the dumping of these wastes. In the middle of Kinshasa city, the dilapidated sanitation infrastructure, the proliferation of garbages, the state of widespread insalubrity remains the main cause of water pollution. This situation constitutes a great danger for the populations living in the surroundings of these rivers and to those who use these waters for various daily uses. These waters are also vulnerable to the proliferation of many pathogens and disease vectors. Henceforth, the treatment of these waters is more difficult and more expensive which leads to the contamination of fish and other wildlife components. The ecological imbalance generated by these pollutions adversely affects the biodiversity of these aquatic ecosystems [2].

The emergence of new infectious agents constitutes a potential risk associated with genetic engineering and culture in the field of genetically modified organisms (GMOs) and a new challenge in molecular epidemiology. In fact, it was proven that transgenic plants grown on the surface are likely to release their DNA and this genetic material can pass through different environmental compartments and end up in the groundwater which eventually can reach the gastrointestinal tract via consumption. During the plant transformation, it is well known that the gene of interest is fused with an antibiotic resistance gene in order to facilitate the selection of transgenic explants creating the uncertainty in the use of GMOs worldwide [5-7]. Regarding the case of transplatomic plants, dead leaves can release transgenic DNA into the soil by lysing the plant cells. In the ground, the transgenic DNA can be protected from nucleases by adsorption on clay particles. The high degree of homology between chloroplast DNA and the bacterial genome as well as the diversity of naturally occurring telluric bacteria are potential risks related to the environmental dissemination of recombinant DNA both in the biogeochemical cycles and in the contamination of the bacterium.

Bacteria have developed different mechanisms to render ineffective the antibiotics used against them. The genes encoding these defense mechanisms are located on the bacterial chromosome or on extrachromosomal plasmids, and are transmitted to the next generation (vertical gene transfer). Genetic elements, such as plasmids, can also be exchanged among bacteria of different taxonomic (horizontal gene transfer) [8]. The development of new resistant strains of bacteria to current antibiotics has become a serious public health concern worldwide; therefore, there is a strong incentive to develop new bactericides, henceforth the need of synthetizing nanoparticles from plants using the green chemistry [9].

The emergence of Nanotechnology as a rapid growing field of research with its application in science and technology has as a purpose the manufacturing of new materials at the nanoscale level. Lately, biosynthetic methods employing either biological microorganisms such as bacteria or plants extract have emerged as a simple and viable alternative to more complex chemical synthetic procedures to obtain nanomaterials. Silver nanoparticles have proved to be most effective as it has good antimicrobial efficacy against bacteria, viruses as well as other eukaryotic microorganisms and silver nanoparticles are playing a major role in the field of nanotechnology and nanomedicine [10].

They are among the most widely commercialized engineered nanomaterials, because of their antimicrobial properties. They are already commonly used in medical devices, household products and industry [11]. The use of nanoparticles for therapeutic purposes was envisaged some 20 years ago and continues to inspire active research in this field, particularly in the controlled release of drugs or the improvement medical imaging techniques [12, 13]. The biological syntheses of nanoparticles are being carried out by different macro–microscopic organisms such as plant, bacteria, fungi, seaweeds

and microalgae. The biosynthesized nanomaterials have been effectively controlling the various endemic diseases with less adverse effect. Non-biological methods were used in the synthesis of nanoparticles and they have a serious hazardous and high toxicity for living organisms while the biological synthesis of these nanoparticles is inexpensive, single step and eco-friendly methods. Plants are used successfully in the synthesis of various greener nanoparticles such as silver. In addition, plant mediated nanoparticles are potential remedy for various diseases such as malaria, cancer, HIV, hepatitis and other acute and chronic diseases [14]. The impact of the nanostructured materials can bring improvement on the quality of life and preservation of the environment, and also represents a promising field for generating new types of nanomaterials with biomedical and environmental applications [11].

The main hypotheses of this research were stated as follows: the accumulation of heavy metals such as Cd, Cu, Hg and Zn in tropical ecosystems (pollution) would favor the transformation of bacteria which would get the antibiotic resistance genes. The acquisition of this chemo-resistance may be a major public health problem; the dissemination of antibiotic resistance genes in the environment would be facilitated through hydro-dispersive transport of DNA via soil and/or groundwater [15, 16]. This DNA is biologically active and able to transform wild competent bacteria and it is from transgenic plants, or from the gastrointestinal tract (faecal pollution). Henceforth, the real need of conducting a molecular epidemiological survey in tropical regions is required.

The main objective of the current research was to assess the antimicrobial activity of silver nanoparticles towards certain bacteria indicative of faecal pollution from Makelele River. Specific objectives were: (1) to measure the following heavy metals: Cd, Cu, Hg, Zn, in water; (2) to synthesize silver nanoparticles (AgNPs) using green chemistry and their characterization and (3) to assess the antimicrobial activity of AgNPs by determining the Minimum Inhibitory Concentration (MIC). This research presents a double interest at the scientific level, it helps to provide data on the state of pollution of Makelele River while on the socio-cultural level, it is helpful for the sensitization of the population using water from this river on the importance of maintaining the quality of this river thru good practices of hygiene.

2. Material and Methods

2.1. Study Area

Makelele River is oriented from the South to the North of Kinshasa city, it drains part of the following districts: Bandalungwa, Kintambo, Ngaliema, Ngiri-Ngiri, Bumbu and Selembao. Its latitude South is 04 ° 19 '94' ', longitude East 15 ° 16' 26 " and has 270 m above sea level.

Figure 1. View of Makelele River.

Makelele River being entirely located in Kinshasa city is under AW_4 climate according to the Koppen classification. Makelele River has a hydrographic regime similar to that of an equatorial type river characterized by a sustained rate flow all year round with periods of floods between April, May, November and December. Both banks of Makelele River flooded during the flood period are often colonized by herbs. Several belts of vegetation are crossed as one move away from the open water. We can also find floating aquatic groups consisting of: *Salvinia nymphellula* L.; *Eichornia crassipes*; *Artemisia annua*; *Basella alba*; *Ricinus communis* and *Bambusa vulgaris*.

2.2. Material

In this study, leaves of *Aframomum alboviolaceum* (*A. alboviolaceum*) were used. Once dried under the required conditions of the traditional pharmacopoeia (ten days at 33°C in a dry atmosphere), the part of the plant is crushed and then sieved in order to obtain a fine powder. Two 24-hour-old bacterial strains isolated from the waters of Makelele River were tested and these strains were *Escherichia coli* (*E. coli*) and *Enterococcus sp.*

2.3. Methods

2.3.1. Collection and Sampling

In addition to sampling for physico-chemical analyzes using the multi-parametric probe, an observation was made on the anthropogenic activities that take place around this river. The landmark chosen was the exit of large liquid waste disposal channel from Kintambo hospital and at that point two samples were collected and marked as: $M_{0.1}$ and $M_{0.2}$; in the upstream, two samples were as well collected with a distance of 20 m respectively and marked as: $M_{1.1}$ and $M_{1.2}$; at the downstream two samples were also collected with a distance of 20 m and marked as: $M_{2.1}$ and $M_{2.2}$. These water samples were kept at 4°C in cool box.

2.3.2. Physico-chemical Parameters

The physico-chemical parameters of collected samples from Makelele River were the following: pH, conductivity, temperature, turbidity and dissolved oxygen. All these analyses were performed according to the standard methods as previously described by Ngbolua *et al.* [7].

2.3.3. Microbiological Analyses

We chose to isolate some bacteria indicators of water contamination notably *E. coli* and *Enterococcus sp*. *E. coli* is characterized by its rapidity to grow within the shortest generation time and it is fully sequenced having a well-known genome.

While for *Enterococcus sp.* the main reason is that this bacterium is indicative of a recent or old fecal contamination, and they are also responsible of various infections occurring mostly in hospitals (nosocomial infections) and *Enterococcus faecalis* as well *Enterococcus faecium* are considered as opportunist pathogens. The technique of series dilution was used along with appropriated culture media for the isolation of *E. coli* and *Enteroccoci.* Slanetz Bartley Agar (SBA) and Trypton Bile X-Glucuronide Agar (TBX) were used for isolating *Enterococcus sp.* and *E. coli* respectively. After preparing each medium, SBA was incubated for 48 hours at 44°C and TBX for 24 hours at 44°C. All analyses were performed in duplicate.

2.3.4. Antibiotic Susceptibility Test

The disc diffusion method is one of the oldest approaches used to determine the sensitivity of bacteria to antibiotics (antimicrobial susceptibility testing) and remains one of the most widely used methods up to date in routine Clinical microbiology laboratories. It is suitable to test the majority of pathogenic bacteria including slow growing bacteria and the more common fastidious bacteria which allows a variety in the choice of antibiotics and does not require any specific equipment [17].

2.3.5. Synthesis and Characterization of Silver Nanoparticles

In 100 mL of distilled water were macerated ten grams of *A. alboviolaceum* leaf powder for 48 hours and then filter using Whatmann's n°1 filter paper. Afterwards, 0.17 g of silver nitrate ($AgNO_3$) was added into 100 mL of distilled water, then five mL of *A. alboviolaceum* extract was collected and added into 95 mL of silver nitrate solution. The mixture was heated at 90°C for 10 min at 4°C. Having heated, this mixture was cooled for minutes then centrifuged at 10 000 rpm for 10 min followed by a washing of the obtained residue. At last, UV-visible spectrophotometer was used to read the results (wavelength between 200 and 700 nm).

The preliminary characterization of silver nanoparticles was carried out by UV-visible spectroscopy, using a spectrophotometer (HITACHI U-3900H brand). Noble metals, such as silver nanoparticles, exhibit unique and adjustable optical properties due to their external plasmon resonance, depending on the shape, size and distribution of nanoparticle sizes. The reduction of silver ions was monitored by measuring the UV-EIDENT spectra of the solutions after dilution of a small aliquot (0.2 mL) of the aqueous component.

2.3.6. Phytochemical Screening

The phytochemical screening is a chemical screening that includes a number of qualitative analysis that allows the identification of secondary metabolites present in a certain sample. The detection of these chemical groups is performed through color and precipitation reactions occurring with the addition of specific reagents [18-21]. This phytochemical screening was carried out according to the standard protocol as previously described by Ngbolua *et al.* [18] and it can be performed in aqueous as well as in organic phases [21].

(i) Preparation of the Aqueous and Organic Extracts

Ten g of the powder was weighed and placed in an Erlenmeyer where 100 mL of distilled water and methanol as well the mixture was incubated for two days then filtered using Whatmann's n°1 filter paper to obtain the aqueous and organic extracts respectively. The filtrate constitutes the basic product to be used for the detection of polyphenols including flavonoids, anthocyanins, leucoanthocyanins, tannins, bound quinones, alkaloids and saponins as well as for organic acids.

(ii) Search for Steroids and Triterpenoids

To one mL of anhydride acetic acid, five mL of dry evaporated organic extract and 0.5 mL of concentrated H_2SO_4 (Leibermann reagent) were added. The presence of triterpenoids and steroids is shown by a purple color while mixed. Separately, terpenes display a complex purple color while steroids display a green color.

2.3.7. Assessment of the Antibacterial Activity

The antibacterial activity was assessed Using the micro-dilution method in liquid medium [22]. The extract to be tested (20 mg) was dissolved in 250 µL of DMSO of which the final volume was adjusted to five mL using Mueller Hinton culture medium. The bacterial suspension was prepared in introducing into two mL of the saline solution, three isolated colonies of strains to be tested and incubated for 24 hours in order to obtain 0.5 McFarland (10^8 cells.mL^{-1}). Then, the bacterial

suspension was diluted to obtain 10^6 cells/mL.

The micro-dilution test was carried out in a sterile 96 well microplate. Briefly, 100 µL of culture medium was introduced in different wells (A_2 to A_8 and then in the 11th and 12th columns which served as controls). Using a micropipette, 200 µL of extract to be tested (1000 µg.mL^{-1}) was introduced in well A_1 (extract 1: AgNP), 100 µL of the stock solution of extract was then collected in order to perform serial dilutions of two by two up to the 8th column and the last 100 µL were thrown away. Aseptically five µL of the inoculum (10^8 CFU/mL) were collected using a micropipette and transferred to the wells of the microplate used except for the wells of the 11th column where the bacterial growth was observed (inoculum and culture medium). While the 12th column served as control for the sterility of culture medium. The microplate was incubated in an oven at 37°C for 24 hours. Having incubated, five µL of Reasazurin 1% dye (7-Hydroxy-3H-phenoxazin-3-one-10-oxide) was added to each well and the microplate was re-incubated for 5 hours. The minimum inhibitory concentration (first wells with no bacterial growth) was determined after 24, 48 and 72 hours respectively.

2.3.8. Chemical Characterization of Water Samples

The analysis of chemical parameters focused on the determination of copper (Cu^{2+}, Cu^{3+}), Cadmium (Cd^{2+}), Mercury (Hg^{2+}) and Zinc (Zn^{2+}) ions using a computer assisted ED-XRF Xepos III spectrometer.

The analyses of different chemical parameters were performed using X-ray fluorescence spectroscopy, X-ray dispersive energy version (ED-XRF), XEPOS III, a multi-elemental method. The samples were measured on X-ray fluorescence spectrometer, using the four secondary targets, namely Molybdenum (39.76KV voltage and 0.88 mA current), Aluminum oxide (49.15 KV voltage and 0.7mA current), cobalt (35.79KV current and 1mA current) and finally HOPG Crystal Bragg (17.4KV voltage and 1.99mA current) of the palladium anode respectively. Generally, the sample to be analyzed is placed under an X-ray beam. Under the effect of X-rays, the sample resonates and re-emits its own X rays - this is the fluorescence. Having a look at the energy spectrum of fluorescent X-rays, characteristic peaks of present elements can be observed so that it allows to know which elements are present and the height of the peaks determine the quantity of these elements.

3. Results and Discussion

3.1. Physico-chemical Parameters

Different physico-parameters of wastewater samples of Makelele River are presented table 1 below.

Table 1. Physico-chemical parameters of wastewater samples of Makelele river.

Parameters	WHO standards	Sites			Mean± SD
		M_0	M_1	M_2	
pH	6.5 to 8.5	6.89	7	6.87	6.92±0.07
Conductivity (µ.cm^{-1})	400 to 1200	568	748	555	623.6±107.87
Temperature (°C)	12 to 25	27	27	25.3	26.43±0.98
Turbidity (UNT)	≤ 5	238	231	262	243.6±16.25
Dissolved oxygen (mg.L^{-1})	5	2.61	3.92	2.33	2.95±0.84

Legend: M_0: Landmark, M_1: Upstream and M_2: Downstream

Out of the above table, it can be observed that the pH of water from Makelele river ranges between 6.89 and 7 having an average of 6.92 which is within the range as established by WHO (6.5 – 8.5). This pH value varies from one site to another such as at the landmark (M_0) the pH is neutral, upstream (M_1) the pH is acid as well as at downstream (M_2). The pH of natural waters is related to the nature of the lands crossed and generally this value is around 7 such as the case of Makelele river but also for most rivers located in Kinshasa [23, 24]. The conductivity at M_0 from the outflow of liquid waste coming from Kintambo hospital is higher than at M_1 and M_2. The findings of the current research show that the conductivity values ranges between 555 µS.cm^{-1} and 748 µS.cm^{-1} having an average of 623.6 ± 107.87. The standard established by WHO is between 400 and 1250 µS.cm^{-1} and our findings are in this range. Water conductivity is due to the degree of mineralization of this water and depends on the solubility of dissolved and dissociated compounds issued from ion mobility and the temperature of the medium. In this study, the temperature of Makelele river was between 25.3 and 27°C having an average of 26.4 ± 0.98 and these values are in the range established by WHO (25°C to 29°C). Seeing the conditions of this river, the variation of temperature might be due to the season and the time at which samples were collected.

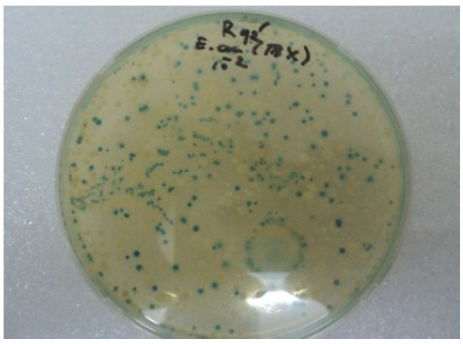
Concerning turbidity, its values increased from upstream passing through the landmark to downstream i.e. between 231 and 262 ppm (244 ± 16.25). The findings of the current research are not in the range as per the standards set by WHO because they are lower to the standards (500 to 1500 ppm).

This may be due in part to the dilution of ions in rainwater and their transport. This parameter being important to indicate the degree of pollution due to chemical fertilizers and other agricultural products that the farmers are using for their fields at the edges of the Makelele river. In fact, the turbidity of the water is partly linked to the presence of finely divided organic suspended matter.

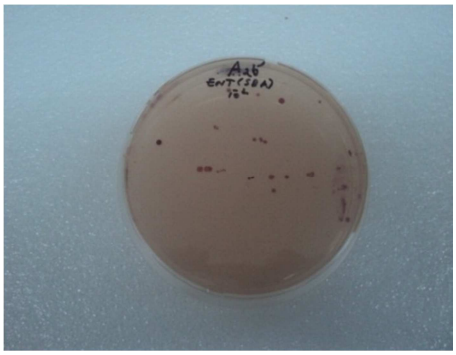
3.2. Antibacterial Activity

3.2.1. Isolation

Figure 2 displays the trend of colonies isolated from the wild strains of *E. coli* and *Enterococcus sp.*



(a) *E. coli* in TBX



(b) Enterococcus sp. in SBA

Figure 2. Isolation of wild bacterial strains from Makelele river wastewaters.

The above figure shows that Makelele wastewater samples contains several germs indicative of faecal pollution namely *E. coli* and this indicates a recent contamination. The culture of *Enterococci* on SBA of Makelele wastewater samples demonstrated the high level of pollution as well (figure 2b) and these media are specific for each strain isolated. This conclusion is raised from the specific characteristics and colors of different media as indicated by the manufacturer. Ngbolua *et al.* [7] reported similar findings with wild strains isolated from Kalamu river in Kinshasa. This shows how Kinshasa population is living in a very polluted environment.

3.2.2. Bacterial Load

The bacterial load of the wastewater samples of Makelele river is presented in table 2 below.

Table 2. Bacterial load of *E. coli* and *Enterococcus sp.* in wastewater samples of Makelele River.

Sites	Number of colonies (10^{-2})	Number of UFC.mL^{-1}	%
E. coli			
Landmark	86	8600	20.48
Upstream	1	100	0.24
Downstream	333	33300	79.28
Total	420	42000	100
Enterococcus sp.			
Landmark	2	200	5
Upstream	13	1300	32.5
Downstream	25	2500	62.5
Total	40	4000	100

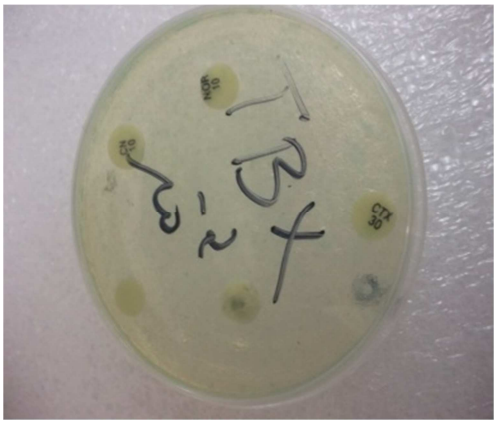
Regarding the findings on the above table, it is clearly observed that water is more polluted downstream than upstream along with the landmark. Although faecal pollution originates from a variety of human and non-human sources, faecal indicator bacteria namely *E. coli* and *Enterococcus sp.* contamination from animals and human faecal material is generally considered as a greater risk to human health as they are more likely to contain human enteric pathogens [25]. The presence of *E. coli* and *Enterococcus sp.* is due to the faecal pollution of which Makelele River is the main target because of the excretion of various wastes from Kintambo hospital and the surrounding populations. The ecological risk evaluation shows that this risk is higher while it is known that *E. coli* and *Enterococcus sp.* are human pathogens. The sources of contamination for humans are namely fish, vegetables and air. In brief, Makelele River contains several germs of faecal pollution. The presence of *Enterococcus sp.* in an aquatic ecosystem is indicating a recent and old pollution respectively, and it shows as well the presence of other pathogens which have the same characteristics than them [7] [26]. Most species of Enterococci do not grow in environmental waters. In this milieu, faecal enterococci are able to survive longer and are more resistant to drying and chlorination than *E. coli.* [27]. Meanwhile several authors reported that *E. coli* is a bacterial species indicating a recent pollution, and its presence in an aquatic ecosystem is indicating the presence of other pathogenic microorganisms [26]. Due to the presence of these pathogens, the entire array of life in water is affected due to pollution from the environment [28].

As Makelele River is close to Kintambo hospital, it receives the discharge of untreated wastewaters and excreta from its urban environment of this hospital which leads to the faecal contamination increasing the potential risks of human infections by direct uptake (drinking water). This phenomenon constitutes a possible source of bacterial contamination in raw vegetables or contamination during recreational activities [25]. In urban cities like Kinhsasa, rivers receive different urban

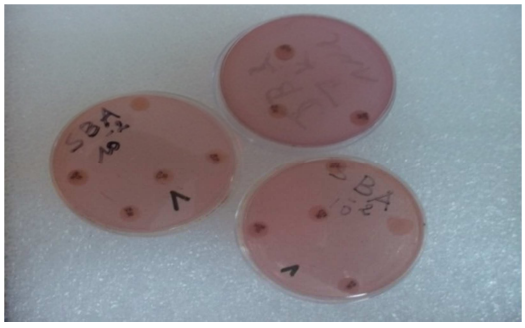
wastes and are considered as uncontrolled landfills exposed to other anthropogenic pollutions, including disposal of dead bodies, industrial and urban untreated effluent water discharge such as domestic waste, human open defecation, wild animal defecation, sewage wastes as well as runoff from the watershed. Because of the lack of public environmental awareness and education, these rivers have been receiving since many years not only wastewaters from diffuse sources but also the toilet wastes from the installation located on their banks. These situations are a major cause of ecological damage and pose serious health hazards. Similar situations have been reported in other countries such as Iran [25] [28-30]. Henceforth, appropriate eco-toxicological studies are needed in this country where cities are big bins and urban rivers are open dumping sites. What about other pollutants such as contraceptives that increase the feminization of fish which disturb the sex ratio? The GEC (Group of Consultants for the Environment) proposes to conduct an in-depth study on all issues related to urban and industrial wastes and their impact on aquatic biodiversity.

3.3. Antibiotic Susceptibility Test

The figures below illustrate the antibiotic discs on our different culture cells inoculated with the isolated bacterial strains.



(a) *E. coli* in TBX medium



(b) *Enterococcus sp.* in SBA medium

Figure 3. Antibiotic discs on different culture media inoculated with the isolated bacterial strains.

The above figures show that the insensitivity of *E. coli* and *Enterococcus sp.* to all antibiotics (resistant strains) available used in the current study. The inhibition average obtained at the concentration of 1000 μ g.mL^{-1} of the antibiotic discs on the wild isolates of *E. coli* and *Enterococcus sp.*, are presented in table 3 below.

Table 3. Antibiotic susceptibility test of wastewater samples isolated from wastewaters of Makelele River.

Antibiotics	Critical charge of the disc	Diameter of inhibition (mm)	
		E. coli	*Enterococcus sp.*
Cefotaxim (CTX)	30 µg	1	0
Gentamicin (CN)	10 µg	2.1	1
Nalidixic Acid (NA)	30 µg	0	0
Norfloxacin (NOR)	10 µg	0	0
Amikacin (AK)	30 µg	3	1.5
Vancomycin (VA)	30 µg	0	0
Trimethoprime-sulfamethoxazole (SXT)	25 µg	1.2	0

From table 3, it is clearly shown that *E. coli* and *Enteroccocus sp*. are resistant to all tested antibiotics.

E. coli and *Enterococcus sp.* are common inhabitants of gastrointestinal tract of human and the majority of animals and are considered as practical "indicator bacteria" that could be used to track the evolution of antimicrobial resistance in different ecosystems [31]. Although *E. coli* and *Enterococci* are commensal bacteria that generally do not cause disease, they can transfer resistance genes to other bacteria. In short, the problem of antibiotic resistance is not just confined to resistant, but rather encompasses all resistance genes, in any type of bacteria. Moreover, many plasmids carry several resistance genes, leading to multi-resistant bacteria able to withstand simultaneously three, four, or even more different classes of antibiotics [31]. In human and Veterinary medicine, microbial resistance to antibiotics is a world-wide concern nowadays. It is generally accepted that the main risk factor for the increase in the antibiotic resistance is an extensive use of antibiotics. This has led to the emergence and dissemination of resistant bacteria and resistance genes in animals and humans [32]. Faeces can be a major source of resistance genes that can contamination environment and water sources [33]. Environmental contamination was blamed for the greater than expected prevalence of antimicrobial resistant bacteria from wild animals and humans. And it is noted that wild animals and birds are considered to be an important potential reservoir of bacteria [44].

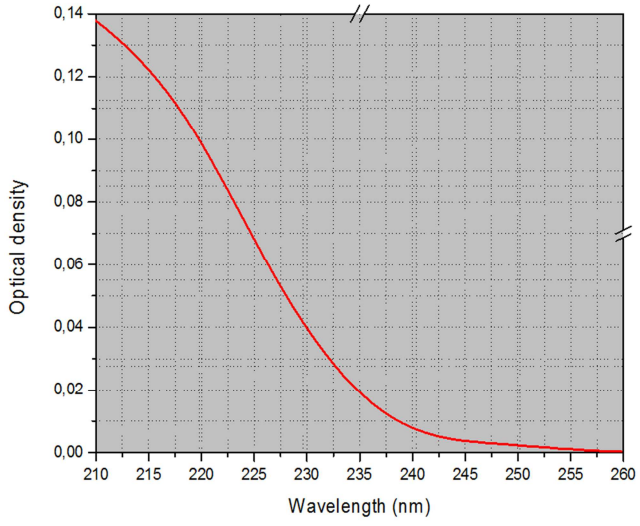
Multidrug-resistant and vancomycin-resistant enterococci are commonly isolated from humans, sewage, aquatic habitats, agricultural run-off and animal sources, which indicates their ability enter to human food chain. Vancomycin resistant

enterococci are emerging as a global threat to public health [32]. This situation is very crucial due to the fact that our environment contains these pathogens which are resistant to antibiotics of the third generation. Several authors reported the resistance of *E. coli* and Enterococcus sp. in foodstuffs, animals and in humans (precisely in children) as well as in water [31]. The persistence of resistance genes can occur incidentally as a result of co-selection with other genes that code for characteristics that enable the bacteria to survive exposure to environmental toxins such as heavy metals and disinfectants [33].

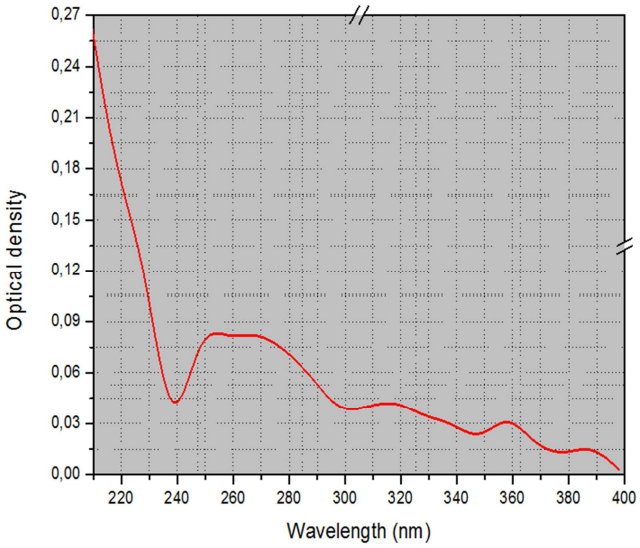
3.4. Characterization of Silver Nanoparticles

The following figures illustrate the pellets (residue) of AgNPs and various spectra of the aqueous extract of *A. alboviolaceum*, AgNPs as well as the compared and silver nanoparticles.

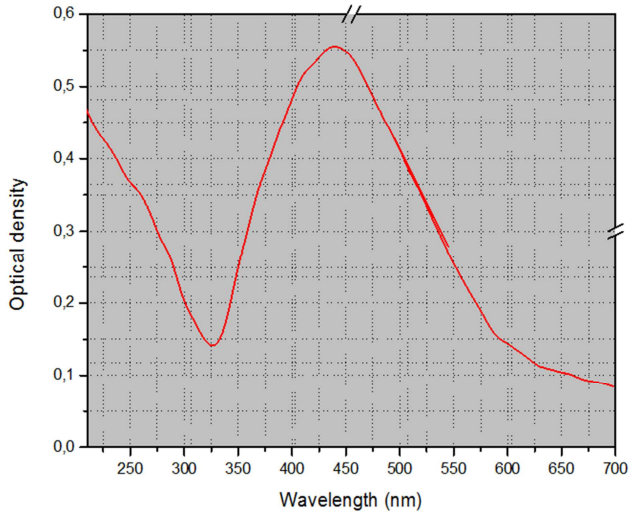
Figure 4. Residue of AgNPs.



(a). Spectrum of $AgNO_3$ aqueous solution (0.15 M)



(b). Aqueous extract of *A. alboviolaceum* (10%)



(c). Silver Nanoparticles (AgNPs)

Figure 5. UV-visible spectra of $AgNO_3$ solution, Plant extract and AgNPs.

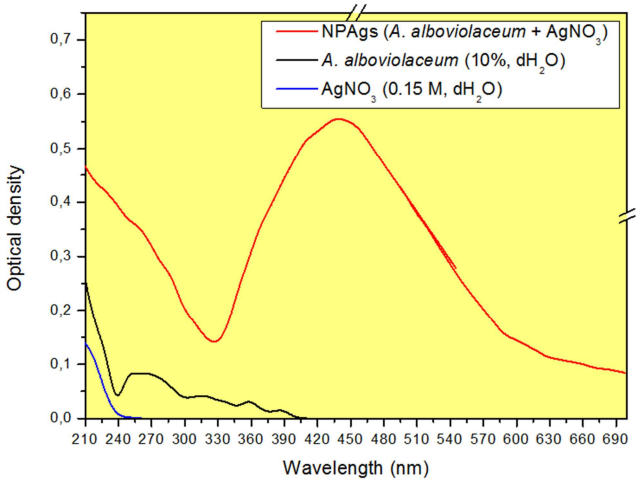


Figure 6. Comparative UV-visible spectra of aqueous solution of $AgNO_3$, aqueous extract of *A. alboviolaceum* and AgNPs.

Figures 5a, 5b, 5c and 6 describe the spectra of the $AgNO_3$ solution, aqueous extract, silver nanoparticles and the compared spectra of $AgNO_3$, aqueous extract and silver nanoparticles respectively. In view of the above, figure 6 provides sufficient evidence that silver nanoparticles are really present in our residue because this spectrum coincides with data in the literature, according to which the UV-visible spectrum shows a peak between 400 and 500 nm corresponding to the Plasmon absorbance of the AgNPs (surface plasmon resonance peak). This finding is similar to the one of the previous study carried out by our team in DRC [7] [34] [35].

3.5. Phytochemical Screening

The phytochemical screening carried out in aqueous and organic phases of *A. alboviolaceum* leaves extract is presented in table 4 below.

Table 4. Phytochemical screening of *A. alboviolaceum* leaves extract.

	Researched chemical groups	*A. alboviolaceum*
	Aqueous phase	
1.	Polyphenols	+
	Anthocyanins	+
	Leucoanthocyanins	+
	Bound quinones	+
	Tannins	+
	Flavonoids	+
2.	Alkaloids	-
3.	Saponins	-
	Organic phase	
1.	Triterpenoids	+
2.	Steroids	+
3.	Free quinones	-

Legend: + presence; -: absence

From the table, it is clearly showed that the leaves of *A. alboviolaceum* are enriched in secondary metabolites namely polyphenols precisely anthocyanins, leucoanthocyanins, bound quinones, tannins, and flavonoids as well as triterpenoids and steroids but devoided of alkaloids, saponins and free quinones. In fact, the presence of secondary metabolites such as flavonoids, anthocyanins and tannins could be justified by the physiological roles that they play in the plant namely protection against sunlight and predators as well they are responsible of plant color [18]. Moreover, the presence of quinones in the organic phase suggests that these metabolites are in their bound forms as heterosides [18]. All these secondary metabolites are endowed with the remarkable pharmacological properties that allow to partially justify their use in African traditional medicine against various infections.

3.6. Determination of the Minimum Inhibitory Concentration

The determination of the minimum inhibitory concentration of *A. alboviolaceum* extract + AgNPs is presented in the following table.

Table 5. Inhibitory action of silver nanoparticles.

	Concentration ($\mu g.mL^{-1}$)									MIC ($\mu g.mL^{-1}$)
	1000	**500**	**250**	**125**	**62.5**	**31.25**	**15.625**	**7.813**	**3.906**	
Enterococcus sp. and E. coli										
AgNPs	-	-	-	+	+	+	+	+	+	**250**

Legend: +: bacterial growth; -: growth inhibition, MIC: minimum inhibitory concentration, AgNPs: silver nanoparticles

From the above table, it is clearly shown that *Enterococcus sp.* and *E. coli* were sensitive to AgNPs (MIC = 250 $\mu g.mL^{-1}$). This antibacterial activity was due to the synthesized silver nanoparticles (AgNPs). The antibiotic susceptibility test results confirmed the inactivity of these antibiotics tested against both wild strains of *E. coli* and *Enterococcus sp.* i.e. these strains are resistant. The emergence of bacteria resistant to antibiotics is common in areas where antibiotics are used, but antibiotic-resistant bacteria also increasingly occur in aquatic environments [8]. These findings are different from the one as reported by Ngbolua et al. [7], he showed that *Enterococcus sp.* isolated from Kalamu River was sensitive to the action of AgNPs because this pathogen showed a resistance to antibiotics used but wild E. coli isolated was sensitive to antibiotics. The situation is crucial in Makelele River due to its environment especially as it receives as well wastes from Kintambo hospital while many cases of resistances have been reported in hospital facilities. Thus, the need of treating these wastes before being delivered to the environment and this destroys the aquatic ecosystem as well the surrounding population using this river for many purposes. Therefore, the synthesis of silver nanoparticles is designed precisely to alleviate this situation; and these results provide ample evidence that silver nanoparticles can be used to fight antibiotic-resistant bacteria [7].

Pareses *et al.* [37] reported that few new antibiotics have been introduced by the pharmaceutical industry, and none of them have improved activity against multi-resistant bacteria. However, AgNPs which are diverse compounds comprising silver, such as materials containing ionic silver (Ag+) or metallic silver (AgO), have been recently synthesized and demonstrated antimicrobial activity against Gram-negative bacteria such as *E. coli.* Consequently, AgNPs are arising as

new bacteriostatic agents, because they are comparable in efficacy and even more potent antimicrobial compounds than conventional antibiotics. Infectious diseases, caused by pathogenic and opportunistic bacteria, have instigated the development of both new pharmaceuticals and therapeutic targets [37]. Multiple mechanisms have been suggested to explain the antibacterial activity of AgNPs, such as release of silver ions from AgNPs, generation of reactive oxygen species, disruption of cellular morphology, inactivation of vital enzymes, DNA condensation and loss of DNA replication [38]. Yet, though multiple mechanism were suggested to explain the antibacterial activity of AgNPs, it should be noted that the major mechanism through which AgNPs manifested antibacterial properties was by anchoring to and penetrating the bacterial cell wall, and modulating cellular signalling by dephosphorylating putative key peptide substrates on tyrosine residues [9]. Besides their antimicrobial properties, AgNPs are also reported to exhibit anti-fungicidal, antiplasmodial, anti-cancer, antidiabetic, antiviral, antioxidant, anti-angiogenesis, anti-inflammatory, and anti-platelet activities. Hence, AgNPs have diverse medical applications. Therefore, silver nanoparticles (AgNPs) exhibit great potential as novel antimicrobial agents [14] [38].

In recent years, various methods notably chemical, physical or biological (plants or microorganisms) were employed to synthetize AgNPs. Currently, new strategies to obtained NPs are being tested using less aggressive compounds to the environment, henceforth the preference to biological synthesis over the chemical and physical syntheses and they are called green synthesis techniques [38-39]. Several authors reported that this method is safe, simple, dependable, environment friendly, cost effective, biocompatible, pollution-free and easily scaled up to large scale syntheses of nanoparticles and do not involve any toxic substrate or by-product. In addition, no need of using energy, high temperature, pressure and toxic chemicals [39-40]. Green synthesis is a key emerging branch of nanotechnology where the production of nanoparticles is carried out with the help of biological entities such microorganisms, plant extracts or plant biomass [41]. This approach uses microorganisms like bacteria (*E. coli*, *Lactobacillus* strains, *Pseudomonas aeruginosa*), fungi (*Fusarium oxysporum*) and plant extracts (*Allophylus cobbe*, *Artemisia princeps, Annona senegalensis*) as well as several biomolecules such as biopolymers, starch, fibrinolytic enzyme as well as amino acids and these materials used are always available [7] [40] [42]. The rich biodiversity and easy availability of plant entities have been highly explored for the nanomaterials synthesis [14]. Plant crude extract contains novel secondary metabolites such as phenolic acid, flavonoids, alkaloids and terpenoids in which these compounds are mainly responsible for the reduction of ionic into bulk metallic nanoparticles formation [14]. Therefore, nanomedicine makes a huge impact in healthcare sector in treating various chronic diseases. Hence, eco-friendly synthesis of nanoparticles is considered as building blocks of the forthcoming generations to control various diseases [14]. It should be noted that DRC is very rich in biodiversity, and with this huge biodiversity nanomedicine can explored deeply. Furthermore, the use of plant extracts reduces the cost of microorganisms and their culture media [42]. Therefore, the use of plants as the production assembly of silver nanoparticles has drawn attention, because of its rapid, ecofriendly, non-pathogenic, economical protocol and providing a single step technique for the biosynthetic processes. The reduction and stabilization of silver ions by combination of biomolecules such as proteins, amino acids, enzymes, polysaccharides, alkaloids, tannins, phenolics, saponins, terpinoids and vitamins which are already established in the plant extracts having medicinal values and are environmental benign, yet chemically complex structures [42].

3.7. Dosage of Heavy Metals

The following table presents selected heavy metals of wastewater samples collected from Makelele River.

Table 6. Concentration of selected heavy metals of wastewaters of Makelele River.

Samples	Al	Si	P	S	Cl	K	Ca	Cr	Mn
FSB1749	666	1514	159.2	37.0	<2.0	63.4	114.7	2.0	2.0
FSB1750	1200	2083	180.5	27.7	<2.0	51.3	129.3	2.6	4.5
FSB1751	2149	2412	177.2	47.7	<2.0	74.7	130.2	5.6	4.1
FSB1752	1445	2258	188.1	43.8	<2.0	63.3	185.6	2.7	5.1
FSB1753	2755	2974	202.2	38.7	<2.0	64.2	180.5	1.8	3.9
FSB1754	<20	752	127.7	19.2	<2.0	33.4	80.5	2.3	2.0
FSB1755	1148	2000	188.0	52.7	<2.0	76.3	216.4	2.8	5.6
FSB1756	1022	1772	518.2	38.7	<2.0	50.2	845.8	2.2	5.7
FSB1757	845	1694	170.6	24.7	<2.0	51.6	118.0	2.0	5.7

Samples	Fe	Co	Ni	Cu	Zn	As	Cd	Sn	Hg	Pb
FSB1749	117.1	<3.0	3.5	2.4	3.5	<0.5	0.2	<3.0	<1.0	0.8
FSB1750	235.1	<3.0	1.7	2.5	4.7	<0.5	<2.0	<3.0	<1.0	1.0
FSB1751	164.3	<3.0	3.3	3.5	3.1	<0.5	<2.0	<3.0	<1.0	<0. 3

Samples	Fe	Co	Ni	Cu	Zn	As	Cd	Sn	Hg	Pb
FSB1752	194.5	<3.0	2.3	2.2	3.2	<0.5	0.3	<3.0	<1.0	0.7
FSB1753	219.3	<3.0	3.2	2.7	3.3	<0.5	0.8	<3.0	<1.0	<0. 4
FSB1754	134.6	<3.0	3.1	<0.2	3.6	<0.5	<2.0	<3.0	<1.0	<0. 3
FSB1755	144.8	<3.0	2.7	1.4	3.1	<0.5	<2.0	<3.0	<1.0	0.8
FSB1756	193.7	<3.0	3.0	1.8	3.8	<0.5	1.5	<3.0	<1.0	0.8
FSB1757	176.5	<3.0	3.2	2.8	3.1	<0.5	<2.0	<3.0	<1.0	0.8

Legend: FSB1749: Upstream 1; FSB1750: Upstream: 2; FSB1751: Upstream 3; FSB1752: Landmark 1; FSB1753: Landmark 2; FSB1754: Landmark 3; FSB1755: Downstream 1; FSB1756: Downstream 2; FSB1757: Downstream 3.

Based on the hypothesis of the current study, only four chemical were reported to be heavy metals of environmental importance as involved in the resistance of microorganisms as previously described, and these elements are Cu, Cd, Zn and Cu [43]. It should be noted that the toxicity of heavy metals in the environment strongly depends on the environmental conditions because these conditions influence the valence of the metal ions and therefore their bioavailability [44]. In nature, heavy metals are persistent, accumulating in different components of the ecosystems. Although many antibiotics have relatively short half-lives, they are regarded as "pseudopersistent" due to their continuous introduction into the ecosystem. Such mixed contamination is causing considerable concerns, *i.e.*, whether their effects are combined with regard to selective ability of antibiotic resistance is unclear. This co-existence between heavy metals and antibiotics takes place in many kinds of environmental matrices [43]. In some natural environments with microbial communities, combined contaminations of heavy metals and antibiotics contribute to the occurrence and spread of microbial antibiotic resistance; and sometimes multidrug resistance evolves [43].

For instance, the amendment of Cu in agricultural soils selects for Cu resistance and further co-selects for resistance to the following antibiotics namely ampicillin, chloramphenicol and tetracycline [45]. The co-exposure to Zn and antibiotics like oxytetracycline in activated sludge bioreactors appears to improve the resistance of the microbial community towards antibiotics. Both Ni and Cd increased the frequency of bacterial resistance in microcosms to chemically unrelated antibiotics including ampicillin or chloramphenicol [46-47]. We have to note that the environment acts both as a reservoir of resistance traits and a bioreactor containing chemical stressors and opportunities for genetic exchange. The potential for these traits to disseminate to clinically relevant pathogens becomes a consequence [15]. This could be explained as follows: the first possibility is that such improvement of antibiotic resistance is that the presence of heavy metals enhanced the enrichment and growth of indigenous bacteria in the microbial community, which are already bearing antibiotic resistance genes; and the second possibility is that the resistance in bacteria which is sensitive to antibiotics could be induced due to the co-existence of heavy metals and antibiotics in the environment. Some investigations have demonstrated the positive correlation between the abundance of antibiotic resistance genes and the elevated concentrations of antibiotic and heavy metals in environments [43]. The different concentrations of Cu, Cd, Zn and Hg found in wastewaters of Makelele river are presented in the table below.

Table 7. Different concentrations of the four heavy metals as per our hypothesis in wastewaters of Makelele river.

Elements	Concentration found ($mg.kg^{-1}$)	WHO standards ($mg.kg^{-1}$)	Mean ± SD
Cu	2.17	2	2.17 ± 0.95
Cd	175.5	0.3	175.5 ± 39.2
Zn	3.5	1.5 - 5	3.49 ± 0.52
Hg	1	0.006	1

Regarding these findings compared with WHO standards for drinking water quality, the above table shows that: Cu is in a very high concentration in the Makelele River, having an average of 2.17 ± 0.95 $mg.kg^{-1}$ compared to the WHO standards i.e. this river is polluted. This high concentration of Cu in Makelele River can be explained by the presence of metallurgical industries which are along this river and they evacuate their liquid wastes directly into it. This could be due not only to its use as an additive, but also to its use as raw material in the production of utensils from different production lines and effluent collectors. In addition, Cu acts as a cofactor for a wide range of metal-binding enzymes but its presence in excess amounts lead to its involvement in the generation of highly reactive oxidative species (such as hydroxyl radicals), well known for their devastating effects in cells, particularly DNA damage and oxidation of proteins and lipids [48].

The concentration of Cd ranges between 175 and 83.8 $mg.kg^{-1}$ having an average of 175.54 ± 39.24, from which we can state that Makelele river is very polluted with Cd compared to the threshold as per WHO standards (0.3 $mg.kg^{-1}$). This would be due to anthropogenic activities along this river and the discharge of rainwater. Cd is one of the most toxic element to which man can be exposed in the environment [49]. Cd is efficiently retained in the human body, in which it

accumulates throughout life once absorbed. It primarily attacks kidneys which are its main site of accumulation and can cause bone demineralization also cause bone demineralization, either through direct bone damage or indirectly as a result of renal dysfunction [49]. In the industry, excessive exposures to airborne Cd may impair lung function and increase the risk of lung cancer. These effects were described in populations with relatively high exposures to Cd living in heavily polluted environments [49].

The average recorded for Zn concentration is 3.49 ± 0.52, fortunately this value is within the range of WHO standards (1.5-5 $mg.kg^{-1}$); its presence could be justified by the leaching of water from the rain. Meanwhile Hg has a very high concentration in the Makelele River having an an average of 1 $mg.kg^{-1}$ which indicates that the river is highly polluted when compared to the WHO standards (0.006 $mg.kg^{-1}$), this high concentration may be due to the liquid waste evacuations coming from Kintambo Reference Hospital. Hg is toxic in its ionic form Hg^{2+} and it can cross the blood brain barrier in the organic forms like methyl-mercury and ethyl-mercury [50]. In nature, the majority of MeHg is contributed by the action of microorganisms in an aquatic ecosystem through biomethylation of inorganic mercury derived mainly from anthropogenic sources [48]. Possessing an enormous potential to undergo biomagnification, its accumulation in fishes renders communities highly vulnerable to its toxicity [48].

The risk of metal driven co-selection of antibiotic resistance in the environment was assessed based on heavy metal concentrations that potentially induce this co-selection process. However, the agricultural and aquacultural practice represent major sources of soil and water contamination with moderately to highly toxic metals such as mercury (Hg), cadmium (Cd), copper (Cu), and zinc (Zn) on the one hand and wastes from different other sources like the case of the current study on the other hand. If those metals reach the environment and accumulate to critical concentrations they can trigger co-selection of antibiotic resistance [44]. The threat of contamination and intoxication weighs on the life of the aquatic ecosystems of Kinshasa city. The harmful effects of heavy metals are not daunting because in the long run these metals are both toxic and bioaccumulative [7].

4. Conclusion

The main aim of the current research was to isolate some bacteria indicative of faecal pollution in Makelele River, test their sensitivity to some antibiotics of the third generation and synthesize AgNPs) using green chemistry and and assess the antimicrobial activity of these AgNPs as well measure some heavy metals of environmental relevance associated with antibiotic resistance such as: Cd, Cu, Hg, Zn, in water. The findings revealed that Makelele River is excessively charged with bacteria indicative of faecal pollution specifically *E. coli* and *Enterococci* and these bacteria are resistant to third generation of antibiotics. The leaves of *A. alboviolaceum* contain various secondary metabolites such as polyphenols, flavonoids, tannins, anthocyanins, alkaloids, leucoanthocyanins, triterpenoids, steroids, alkaloids and free quinones which serve as reluctant agents and from which AgNPs were synthetized. The antibacterial activity of AgNPs the nanoparticles gives a minimum inhibitory concentration (MIC = 250 $\mu g.mL^{-1}$) which shows that the synthesized AgNPs were active with respect to *Enterococci*. Makelele River is highly polluted with heavy metals. Furthermore, the environmental pollution by heavy metals not only triggers co-selection processes, but also increases the level of tolerance to antibiotics due to co-regulation of resistance genes. Heavy metal ions are known to co-regulate genes responsible for antibiotic resistance and decrease antibiotic susceptibility. Therefore, it should be noted that the release of liquid industrial effluents into various rivers of Kinshasa remains a serious problem to be solved for the environmental protection. In-depth studies are required where there would be a need of setting a water purification plant which would help to treat both hospital waste and liquid industrial effluent waste prior to any spill into a watercourse. Our suggestion to the policy makers of DRC is to establish standards concerning water management resources in order to overcome water pollution concern and protect in the same time riparian against waterborne diseases and other related epidemics water-related.

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