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Phytochemical screening and antibacterial activity of the aqueous extract of the leaves of *Alchornea cordifolia* (Euphorbiaceae) on the *in-vitro* growth of tetracycline-resistant strains of avian *Escherichia coli*

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Abstract
Tetracycline is one of the most widely used antibiotics in poultry farming for the control of bacterial pathologies such as *Escherichia coli*, responsible for colibacillosis, causing significant economic losses in poultry farming. Unfortunately, high rates of resistance of these bacteria to tetracycline are increasingly observed. Thus, the search for new effective molecules is proving to be an alternative. It is in this context that this study is carried out with the objective of proposing a medicinal plant as an alternative to the fight against bacteria resistant to Tetracycline. Phytochemical analysis revealed the presence of phenols, flavonoids, catechic tannins, saponosides, sterols, polyterpenes, alkaloids and quinonics. The bacterial strains were sensitive to the aqueous extract with inhibition diameters ranging from 13.3± 0.3 and 18.3 ± 0.6 mm at 200 mg/ml. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract ranged from 3.12 to 50 mg/ml and 6.25 to 100 mg/ml respectively with MBC/MIC ratios ≤ 2 for all bacterial strains. Thus, the extract has bactericidal potential on all the strains after 24 hours and 48 hours of incubation. The antibacterial potential of this extract highlighted in this study could make this plant a candidate for further investigations that may lead to the discovery of new antibacterial molecules.

Keywords: A. *cordifolia*, antibacterial, *Escherichia coli*, avian, Resistant

Introduction
Tetracyclines are among the most widely used antibiotics in poultry production due to their broad spectrum of action (Chopra and Roberts, 2001) [1]. However, several studies have shown high rates of resistance of these bacteria to these antibiotics. Indeed, bacteria isolated from chickens by Brown and Alhassan (2014**)** [2] in Ghana, showed high resistance to tetracycline. Similarly the studies conducted by Hafed *et al*. (2016) [3], Messai *et al*. (2015) [4], showed resistance rates of 97.05% and 100%. Faced with this situation, the search for new antibiotic prototypes has become an absolute necessity. Thus the use of new molecules from natural substances is proving to be an alternative. In animal husbandry, a variety of medicinal plants are used to control pathologies (Koudandé *et al*. 2001) [5]. In addition, 25% of prescriptions worldwide are plant-based, with 60 to 70% of antibacterial and anticancer drugs made from natural substances (Diallo, 2005) [6]. Many plants are used in the African pharmacopoeia to treat several bacterial diseases. Among these plants is *Achlcornea cordifolia*, a plant used in traditional medicine for the treatment of diarrhea, tooth decay and wound healing (Adeleye *et al*., 2008; Idu *et al*., 2009) [7, 8]. So can *Alchornea cordifolia* treat bacterial infections in poultry?
The objective of this study is to evaluate the antibacterial activity of the aqueous extract of the leaves of *A. cordifolia* leaves on tetracycline-resistant strains of *Escherichia coli* of avian origin.

Materiels and Methods
Plant material
The plant material consists of the leaves of *Alchornea. cordifolia* (Euphorbiaceae). The leaves of this plant were chosen because, they are traditionally used in the treatment of diarrheal diseases (Alain *et al*., 2017; Saraka *et al*., 2018) [9, 10].

Bacterial material

The bacterial material (Table I) consisted of tetracycline-resistant Escherichia coli strains isolated from broiler droppings at the Antibiotics, Natural Substances and Surveillance of Anti-infectious Microorganisms Unit (ASSURMI) of the Bacteriology and Virology Department of the Institut Pasteur de Côte d'Ivoire (IPCI).

Table 1: Phenotypes of clinical strains of *Escherichia coli* of avian origin

Code	Clinical stains	Résistance phenotypes
1373CN/21CNRA	*Escherichia coli*	High level penicillinase Penicillinase + Cephalosporinase. Cross-resistance to fluoroquinolones Phenotype KTG
1355CN/21CNRA	*Escherichia coli*	Probable plasmid cephalosporinsase Cross-resistance to fluoroquinolone Phenotype G probable AAC (3-I production)
1395CN/21CNRA	*Escherichia coli*	High level penicinillase Pénicillinase + Cephalosporinase Quinolone wild type Phénotype sauvage aux Aminoglycosides wild type
1387CN/21CNRA	*Escherichia coli*	High level penicillinase Cephalosporinse medium expressed Cross-resistance to fluoroquinolone Phénotype K

CNRA: National Antibiotic Reference Center; K: Kanamycin, T: Tobramycin, G: Gentamycin

Methods

Preparation of the aqueous extract

One hundred grams of *A. cordifolia* leaf powders were macerated in 1000 ml of distilled water in blinder. The filtrate obtained was obtained according to the method described by Zirihi *et al*.**,** (2003**)** [11]**.**

Phytochemical analysis

Phytochemical analysis was performed according to the protocols described by Bekro *et al*. (2007**)** [12]

Antibacterial test of the aqueous extract of *A. cordifolia* leaves

Sterility test of the extract

Before testing, a sterility test was performed on the extract to verify that it was germ free. Thus in 10 ml of thioglycholate broth 0.1 g of the extract was incubated at 37 °C for 24 hours. After this time, the broth was plated on Muller Hinton (MH) and Sabouraud geloses and incubated under the same conditions as before. The aqueous extract is declared sterile if no colonies are visible on the different agar plates after the incubation period (Akers, 1984) [13].

Preparation of concentrations

A solution with an initial concentration of 200 mg/ml of the extract was prepared. From this stock solution, a series of dilutions according to a geometric progression of reason 2 was performed in order to obtain 7 concencentrations ranging from 200 to 3.12 mg/ml (Konan *et al*. 2014) [14].

Preparation of inoculum for solid-state testing

The inoculum was prepared from a young colony of 18 to 24 hours. It was homogenized in 2 ml of 0.9% Nacl suspension. Then the optical density was adjusted to 0.5 Mc Farland. A volume of 100 µl of this suspension was diluted in 10 ml of 0.9% Nacl, thus constituting the bacterial inoculum.

Sensitivity test by diffusion in solid medium

The test consisted in depositing 50 µl of the aqueous extract of 200, 100 and 50 mg/ml in wells dug on the surface of the agar previously inoculated with the bacterial strain. (Wiegand *et al*., 2007) [5]. Using sterile forceps, the tetracycline disk was also placed on this agar. Then the Petri dish was closed and allowed to diffuse at room temperature for 30 minutes and incubated at 37 °C for 24 hours. Tetracycline was used because of its wide use in poultry farming for the treatment of bacterial infections. Interpretation of the results was done by measuring the diameter of the inhibition zone around each cup using a caliper.

The results were expressed as the diameter of the inhibition zone of the strains towards the extract (Ponce *et al*. 2003) [16]:

Resistant: Diameter less than 8 mm;

Sensitive: Between 9 and 14 mm;

Very sensitive: Diameter between 15 and 19 mm

Extremely sensitive: diameter greater than 20 mm.

Preparation of the inoculum for the liquid tests

A colony isolated from an 18 hour old bacterial culture was collected and homogenized in 10 ml of Nacl and incubated for 3 hours at 37 °C. From this bacterial suspension, 0.1 ml was diluted in 10 ml of 0.9% Nacl. This bacterial suspension constituted the starting bacterial inoculum.

Preparation of the inoculum for the liquid media tests

A colony isolated from an 18 hour old bacterial culture was collected and homogenized in 10 ml of Nacl and incubated for 3 hours at 37 °C. From this bacterial suspension, 0.1 ml was diluted in 10 ml of 0.9% Nacl. This bacterial suspension constituted the starting bacterial inoculum.

Counting of the bacterial inoculum

To do this, the bacterial inoculum was homogenized and then diluted from 10 to 10 until the 10^{-4} dilution. We obtain 4 successive dilutions from 10^{-1} to 10^{-4}. The initial bacterial inoculum and the 4 successive dilutions were inoculated with a calibrated loop of 2 µl in MH agar plates, bearing streaks of 5 cm long. This preparation constitutes the plate (A) that will help determine the minimum bactericidal concentration (MBC).

Determination of antibacterial parameters (MIC and MBC)

These parameters were performed using the liquid dilution method (Dosso and Faye-Kette, 2000; Koné *et al*., 2004) [17] [18]. Final concentrations ranging from 200 to 3.12 mg/ml were performed in a series of 7 experimental tubes, one growth control tube and one sterility control tube. A one milliliter volume of known concentration range extract was added to the experimental tubes. The growth control tube received 1 milliliter of sterile distilled water while all experimental tubes received 1 milliliter of bacterial inoculum. The sterility control tube received 2 ml of Nacl (0.9%). The tubes were incubated for 24 hours at 37 °C.The MIC is the lowest concentration of extract for which no bacterial growth is observed. The contents of the tubes in which there was no visible growth were used to inoculate Muller-Hinton agar on 5 cm strips using a 2 µl calibrated loop. This Petri dish is named B. Analysis of the results after 24 hours of incubation allowed the calculation of the BMC which corresponds to the

lowest concentration that kills 99.99% of the bacteria in culture.

Statistical analysis

Statistical analysis was done using ANOVA-one way followed by Tukey's test for the comparison between the activity of the 200 mg/ml aqueous extract, that of sterile distilled water and the control. The results obtained were expressed as mean ± standard deviation. The different letters indicate significantly different activities (P<0.05).All results were analyzed using the statistical analysis software Graph Pad Prism 7.

Resultats

Phytochemical analysis

Phytochemical analysis revealed the presence of phenols, flavonoids, catechic tannins, saponosides, sterols, polyterpenes, alkaloids and quinonics.

Sterility test

Incubation at 37 °C of the aqueous extract of *A. cordifolia* leaves after 24 hours revealed no germ growth in the agar plates. The aqueous extract of *A. cordifolia* leaves therefore showed no signs of contamination.

Diameter of inhibition zones

At concentration of 200 mg/ml, the inhibition zone diameters of the aqueous extract of *A. cordifolia* leaves ranged from 13.33 ± 0.30 to 18.3 ± 0.6 mm on *E. coli* strains (Table II).The largest diameters of 18.3 ± 0.6 and 17.67 ± 0, 66 mm were obtained on *E. coli* strains 1395CN/21CNRA and 1373CN/21CNRA, respectively. In contrast, the smallest diameters of 13.33 ± 0.30 and 13.33 ± 0.33 mm were obtained on E.coli 1355CNR/21CNRA and *E.coli* 1387CNR/21CNRA. The tetracycline commonly used by farmers was resistant to these bacterial strains according to the Antibiogram Committee of the French Society of Microbiology (CASFM-Veterinaire 2021). The action of the aqueous extract of the leaves of *A. cordifolia* at the concentration of 200 mg/ml against the strains is better than that of the tetracycline tested even if the load of active principle of the raw aqueous extract is not known. At 200 mg/ml aqueous extract of *A. cordifolia* leaves, 50% of the tested strains are susceptible and 50% are very susceptible.

Antibacterial parameter

The minimum inhibitory concentrations (MIC) of the aqueous extract ranged from 3.12 to 50 mg/ml (Table III). The extract showed the highest MICs on *E. coli* strains 1387 CN/21CNRA and 1355 CN/21CNRA with the respective values of 50 mg/ml. The lowest MIC value of the aqueous extract of 3.12 mg/ml was obtained on *E.coli* 1373 CN/21CNRA.The minimum bactericidal concentrations (MBC) ranged from 6.25 to 100 mg/ml on the bacterial strains. The highest BMC of the extract was 100 mg/ml obtained on both *E. coli* 1387 CN/21CNRA and *E. coli* 1355 CN/21CNRA strains. In contrast, the smallest BMC values of the aqueous extract on the strains were 6.25 mg/ml on *E. coli* 1387 CN/21CNRA and *E.coli* 1355 CN/21CNRA respectively. The BMC/MIC ratios of the extract on the bacterial strains were all lower than 2. Therefore the extract showed bactericidal power on all tested strains.

Table 2: Diameter (mm) of the inhibition zones obtained with the aqueous extract of the leaves of *A. cordifolia* on the clinical strains of *E. coli*

Code	Strains	EAAC (200 mg/ml)	Control (0mg/ml)	TET (30µg)
1395CN/21CNRA	*E. coli*	$18,3 \pm 0,6^b$	$6 \pm 0,0^a$	$6 \pm 0,0^a$
1373CNR/21CNRA	*E. coli*	$17,67 \pm 0, 66^b$	$6 \pm 0,0^a$	$6 \pm 0,0^a$
1355CNR/21CNRA	*E. coli*	$13,33 \pm 0,33^b$	$6 \pm 0,0^a$	$6 \pm 0,0^a$
1387CNR/21CNRA	*E. coli*	$13,3 \pm 0,3^b$	$6 \pm 0,0^a$	$6 \pm 0,0^a$

Includes well diameter (6mm); Different letters indicate significantly different activities (P<0.05).CNRA: National Antibiotic Reference Center, EAAC: Aqueous extract of *A. cordifolia* leaves, TET: Tetracyclin

Table 3: Antibacterial parameter of the aqueous extract of *A. cordifolia* leaves on bacterial strains in liquid medium

E. coli	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	Antibacterial power
1387CN/21CNRA	50	100	2	Bactéricide
1373CN/21CNRA	3,12	6,25	2	Bactéricide
1395CN/21CNRA	6,25	6,25	1	Bactéricide
1355CN/21CNRA	50	100	2	Bactéricide

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration

Discussion

The phytochemical study of the aqueous extract of *A. cordifolia* leaves revealed the presence of several chemical constituents. These are phenols, flavonoids, catechic tannins, saponosides, sterols, polyterpenes, alkaloids and quinonics. On the other hand, gall tannins were absent. These same secondary metabolites as well as gall tannins were highlighted by Saraka *et al*. (2018) [10] and Mambé *et al*. (2016) [19] in an aqueous extract of *A. cordifolia* leaves. Among these compounds, several possess beneficial pharmacological activities for the functioning of the living organism. Flavonoids possess anti-allergic, anti-inflammatory, antithrombolytic and analgesic effects (Yamamoto and Gaynor, 2001;Sies *et al*., 2005) [20] [21]. As for the tannins, they are considered as good remedies in the treatment of respiratory diseases and against coughing and have an anti-diarrheal activity (Romani *et al*., 2002) [22]. They are also endowed with antibacterial, antifungal and antiviral activities (Kolodzie *et al*., 1999; Chen ***et al.***, 1999) [23] [24]. Tannins also possess antioxidant, immunostimulant (Feldman *et al*., 1999) [25], apoptotic (Yang *et al*., 2000;Wang *et al*., 2000) [26] [27], anti-inflammatory (Mota *et al*., 1985) [28], anti-hypertensive (Tachen *et al*., 1993) [29] and antitumor (Yoshida *et al*., 1995) [30] properties. Alkaloids exert positive effects on the cardiovascular system by reducing body fat, LDL-cholesterol levels and increasing HDL-cholesterol levels (Schmeda-hirschmann *et al*., 2000) [31]. At a dose of 200 mg/ml, the diameters of the inhibition zones of the aqueous extract of A. cordifolia leaves varied from 13.33 ± 0.30 to 18.3 ± 0.6 mm on *E. coli* strains. The aqueous extract of *A. cordifolia* leaves was reported to have antibacterial activity on *E. coli* strains. According to the solid-state diffusion method, an extract is considered effective when it induces a zone of inhibition greater than or equal to 10 mm (Biyiti, 2004). These results are in agreement with those of Owhe-Ureghe *et al*. (2016) [32]. Indeed, these authors showed that *A. cordifolia* exhibits antibacterial activities on *Kocuria varians, Streptococcus pyogenes, Klebsiella pneumoniae, Enterobacter aerogenes and Salmonella enterica*. These results would justify the

traditional use of *A. cordifolia* in Madagascar and Cote d'Ivoire in the respective treatment of diarrhea and ulcers (Nicolas**,** 2012; Alain *et al*., 2017 and Saraka *et al*. 2018) [33, 34] [10]. The antibacterial activity of the aqueous extract of A. cordifolia leaves could be explained by its chemical composition. Because, the aqueous extract of *A. cordifolia* leaves contains phenols, tannins, flavonoids, alkaloids, polyterpenes and quinone substances with antibacterial properties (Dosso *et al*., 2012) [35]. This antibacterial activity could also be explained by the lysis of bacterial membranes by these molecules. The diameters of the inhibition zones of the extract on the bacterial strains were significantly larger than that of tetracycline. These results indicate that the aqueous extract of *A. cordifolia* leaves would be more effective than tetracycline tested. The BMC/MIC ratio showed that the aqueous extract of A.cordifolia leaves has bactericidal actions (BMC/MIC ≤2) on all strains of *Escherichia coli*. This bactericidal power of the extract could be explained by the destruction of bacterial membranes. Indeed, flavonoids, alkaloids and even tannins could induce a leakage of potassium ions at the level of the membrane and consequently irreversible lesions at the level of this membrane. This permeability to potassium would be a precursor effect of the bactericidal power of this extract towards bacterial strains**.**

Conclusion

Phytochemical analysis of *A. cordifolia* leaves revealed the presence of several chemical constituents. These are phenols, flavonoids, catechic tannins, saponosides, sterols, polyterpenes, alkaloids and quinonics. The bacterial strains were sensitive to the aqueous extract of *A. cordifolia* leaves at the concentration of 200 mg/ml. The extract was bactericidal on all strains tested. The present results could justify some traditional uses of this plant in the treatment of certain pathologies such as diarrhea and wound healing. They show that this plant could be used in the treatment of bacterial infections in poultry. However, this work must be continued through *in-vivo* studies and evaluate the possible toxicity of the leaves of this plant on mammals.

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