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Phytochemistry and antibacterial activity of plants extracts compared with two commercial antibiotics against E coli responsible for avian colibacillosis in Benin

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Abstract

Despite its prominent place in development strategies, poultry breeding faces many constraints, including pathological ones. Among pathologies that affect poultry, colibacillosis is one of the most diseases that are communicable to humans and associated with heavy economic losses. To fight efficiently against avian colibacillosis, the work aimed to evaluate antimicrobial potential evaluation of non-volatile extracts of *Euphorbia hirta* and *Psidium guajava*, as well as the essential oils of *Clausena anisata* and *Aeollanthus pubescens* extracts and two commercial antibiotics namely tetracolivit and Oxytetracyclin against *Escherichia coli*. To achieve this goal, susceptibility tests were performed on a strain of *E. coli* using agar diffusion and microdilution methods. Results obtained showed that essential oils from *Clausena anisata* and non-volatile extracts tested were not active on *E coli*. However, the essential oils extracted from *Aeollanthus pubescens* were active on the *E. coli* with MIC of 0.44 ± 0.21 mg/ml and MBC of 0.87 ± 0.41mg / ml for the oil of the whole part, followed respectively by flower oil (MBC = 0.99 mg/ml) and leaf stem oil (MBC= 1.62 mg / ml). Comparing the activity of the essential oils of *Aeollanthus* with antibiotics tested, we noticed that tetracolivit was more active with MBC =0.15±0.07mg/ml on *E. coli* than these extracts which were more active than Oxytetracyclin (MBC=2.34±1.11mg/ml). In sum, the study showed that for a better management of avian collibacillosis in Benin, the tetracolivit is suitable as antibiotic which can be substituted by *Aeollanthus pubescens* essential oils.

Keywords: Avian colibacillosis; plant extracts; E coli; antibacterial activity

Introduction

Benin poultry farming has made great progress in recent decades. The number of poultry, all species combined, increased from 13 million in 2001 to more than 16 million in 2005 [1]. Today, the numbers have increased as a result of increased food needs and the growth of peri-urban poultry farming [2]. According to livestock management statistics, poultry is the second largest source of meat consumption after cattle (58% for cat-

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tle, 21% for poultry, 13% for sheep / goats and 7% for pork) in Benin [3]. However, the intensification of poultry rearing does not evolve without problems, because it is accompanied by many constraints, including pathological ones (viral, bacterial, parasitic, etc.) which seriously affect the budget of livestock farmers [4]. Among the main bacterial pathologies occurring in laying hen farms, we have avian colibacillosis [5], which, due to strains of *Escherichia coli*, causes significant economic losses (estimated about 6 million euros per year in England) due to stunting, septicemia, associated chronic respiratory conditions and conditions such as salpingitis, ovaritis, aerosacculitis, pericarditis and severe peri-hepatitis [6,7]. To control this avian pathology, the current treatment is based on the use of synthetic antibiotics including colistin, oxytetracyclin, second-generation fluoroquinolones, beta-lactams, etc. However, the misuse of these synthetic molecules, the high cost and inaccessibility of antibiotics, the non-knowledge of the disease by breeders and the lack of a quality control structure for pharmaceuticals have led to a worrying increase in the number of multi-resistant pathogenic strains. For example, strains of *E. coli* isolated from chickens showed high levels of resistance to oxytetracyclin (100%), amoxicillin (90.9%), trimethoprim + sulphamethoxazole (82.2%), enrofloxacin (75.9%) and florfenicol (61.5%) [8]. Regarding the increasing of resistance of *E coli* to conventional antibiotics, the emergence of new pathogens in both humans and animals, the increase in treatment costs, the decline in their effectiveness, antibiotics do not always guarantee a favorable response [9]. It is therefore clear that the search for alternative solutions to ensure a satisfactory succession of antibiotics in terms of zootechnical effects becomes an emergency. Fortunately, medicinal plants are a significant source of new drugs, especially since they have fewer side effects [10]. To remedy this problem of resistance and preserve animal and public health, the present work was undertaken to evaluate the antibacterial activities of extracts from medecinal plants whose traditional uses and therapeutic properties have been widely demonstrated in view of their uses to the detriment of antibiotics. Thus, the present work has objective to evaluate antibacterial activity of non-volatile extracts of the whole part of *Euphorbia hirta* and the leaves of *Psidium guajava*, as well as the essential oils of *Clausena anisata* and *Aeollanthus pubescens* on a strain of *Escherichia coli* isolated in poultry.

Material and Methods

Collect and identification of plant material

The studied plant material is consisted of four plants, including *Psidium guajava* leaves harvested in Lokossa municipality and the whole *Euphorbia hirta* plant harvested in Abomey-Calavi identified respectively as AA 6718 /HNB and AA 6719 / HNB at the National Herbarium of Benin (HNB). The leaves of *Clausena anisata* harvested by Adjalian et al. [11] and the organs (whole plant, flowers and leafy stem) of *Aeollanthus pubescens* harvested at Dassa (Benin) and previously studied by Alitonou et al. [12] and identified by the National Herbarium of Benin (HNB) during other projects are parts of our plant material.

Microorganism tested and antibiotics assayed

The *Escherichia coli* tested in the present was isolated from fresh diarrhea of poultry suffering for colibacillosis. The isolate was identified according to standard method using Mac Conkey agar medium and API 20E Kit. Two commercial antibiotics namely Tetracolivit and Oxytetracylin were purchased in veterinary pharmacy in Benin. They are in powder form.

MethodsExtraction of essential oils from plants

The essential oils were extracted from the leaves of *Clausena anisata* and flowers, leafy stem and whole part of *Aeollanthus pubescens*. Extraction of the essential oils was carried out by hydrodistillation using a Clevenger type apparatus according to protocol reported by Yovo et al. (2016). The essential oils obtained were collected in a sterile dark is kept in the refrigerator at 4°C and protected from light until use. Oils from the same plants were previously extracted and analyzed chemically respectively by Adjalian et al. [11] and Alitonou et al. [12].

Preparation of non-volatile extracts

The crude extracts were prepared from the whole part of *E. hirta* and *P. guajava* leaves. The technique used is that of maceration. 50 g of powder of each sample were macerated in 500 ml of extraction solvent, filtrated and evaporated for drying in an oven at 50 ° C according to protocol reported by Yovo et al. [13].

Phytochemical Screening

The identification of metabolites such as saponosides, tannins, flavonoids, alkaloids, coumarins, anthocyanins, leucoanthocyanins, reducing compounds, mucilages, and sterols and terpenes was made by staining and / or precipitation specific for most families of secondary metabolites according to the methods reported by Yovo et al. [13]. The identification of these secondary metabolites concerned only extracts of *E. hirta* and *P. guajava*.

Quantitative Analysis of Phenolic Compounds of *E. hirta* and *P. guajava* extracts

Total phenols content of extracts

The determination of total phenols was carried out with the Folin-Ciocalteu reagent according to the method described by Wong et al. [14]. This method consists of mixing 3 mL of distilled water with 50 μL of extract followed by 250 μL of Folin-ciocalteu reagent and 750 μL of 7% Na2CO3. After 8 min, 950 μL of distilled water was added to the mixture and the absorbance was measured at 765 nm after 2 hours against a blank without extract taken as reference. The quantification of the polyphenols is determined by the calibration line made with gallic acid at different concentrations under the same conditions as the sample. The results are expressed in mg of gallic acid per gram of dry material (mg GAE/g dry material).

Total flavonoids content of extracts

The aluminum trichloride ($AlCl_3$) method described by Enujiugha [15] reported by Yovo et al. [13] was used to quantify the flavonoids in the extracts. 2.5 mL of distilled water were mixed with 500 μL of extract, 300 μL of $NaNO_2$ and 300 μl of 10% $AlCl_3$ in ethanol. After 5 min, 1 mL of molar NaOH and 400 μL of distilled water were added and the absorbance was read with spectrophotometer at 510 nm against a blank without extract taken as reference. Quantification of total flavonoids was performed using the standard curve made with catechin at different concentrations. The results were expressed in mg equivalent of catechin per gram of dry matter (mgEC / gMS).

Evaluation of the antibacterial activities of the extracts of the studied plants

The antibacterial properties of the extracts were determined by two methods, namely the Mueller Hinton disk diffusion and well diffusion method, to preselect the extracts having effective antibacterial activity and the liquid microdilution method using the 96-well microplates to determine minimum inhibitory (MIC) and bactericidal (CMB) concentrations, as well as antibiotic potency [16].

Disc and well diffusion methods

The evaluation of the antibacterial activity of the various extracts was previously carried out by the disk diffusion method and wells on the Mueller-Hinton agar medium, in order to identify the active extracts on which subsequent studies will be conducted [16]. The test solutions were prepared at the concentration of 100 mg / mL with sterile distilled water for the aqueous extracts and ethanol / water (4/6) for the semi-ethanolic and ethanolic extracts. For essential oils, the pure extracts have been tested. Microbial suspensions in the exponential growth phase (0.5 on the Mc Farland scale, ie approximately 1.5×10^6 cells / mL) were inoculated on sterile MHA agar. Sterile disks impregnated with 1, 2, 3, 4, 5 and 10 μL of essential oil were deposited on the previously inoculated MHA agar. For the non-volatile extracts, 25 μL of extract were introduced into the 6 mm diameter wells in the center of the previously inoculated MHA agar. After pre-diffusion for 30 min at room temperature, the Petri dishes were incubated for 18-24 hours at 37 ° C. The test was done in duplicate. The diameters of the inhibition zones were measured and the averages were calculated. Non-volatile extracts with an inhibition diameter greater than or equal to 12 mm [17] and essential oils having a diameter greater than 14 mm at 10 μL [16], were selected for the determination of MIC, MBC and antibiotic potency. In addition, the sensitivity of the *E. coli* strain to two commercial antibiotics, namely Tetracolivit and Oxytetracyclin were treated under the same conditions as the non-volatile extracts.

Microdilution method in a liquid medium

MIC and MBC were determined using the liquid microdilution method using 96-well microplates as reported by Mamadou Sadou et al. [17] and Yovo et al. [13]. From a stock solution at 100 mg / mL for the non-volatile extracts and a suspension prepared from 40 μL of the pure essential oil diluted in 2000 μL of MHB, a successive dilution was performed well per well with Mueller-Hinton Broth and microbial suspension broth at 1.5 x 10^6 cells / mL. The extract solution diluted in MHB alone is taken as a negative control and the microbial suspension with MHB alone is considered as a positive control. The microplates thus seeded are coated with paraffin oil and incubated at 37 ° C for 24 hours. The MIC of the extract on the strain is the lowest concentration corresponding to the well for which turbidity is not observed. From the MIC, wells showing no visible microbial growth were re-isolated on Mueller-Hinton Agar and incubated at 37 ° C for 24 hours. The lowest concentration for which no microbial colonies (99.99% killed) is observed is the Minimal Bactericidal Concentration (MBC) of the extract on the strain tested [17].

Antibiotic potency

To better appreciate the antimicrobial properties of the tested samples, the ratio MBC/MIC was calculated for each extract was calculated to indicate a bactericidal (MBC/MIC $\leq$4) or bacteriostatic (MBC/MIC>4) activity [18].

Statistical analysis

The results obtained from two independent tests were processed using the Excel spreadsheet to calculate averages. The latter

were compared two by two by the student's t-test using the Statistica software. The level of significance retained is 5% ($p < 0.05$).

Results and discussion

Main metabolites of non-volatile extracts studied

The secondary metabolites identified in the extracts of the plants studied are shown in Table 1. The analysis of this table shows that the extracts of the four plants all contain flavonoids, catechin tannins, sterols and terpenes. However, gallic tannins are present in the extracts of *E. hirta*. As for the saponosides and anthocyanins, they are contained in the extracts of *P. guajava*. Leucoanthocyanans are present in extracts of *P. guajava*; reducing compounds and mucilages are present in extracts of *E. hirta*. The extracts of *P. guajava* also contain mucilages. Coumarins are absent in all plants. Regarding these secondary metabolites identified in the extracts, the presence of flavonoids, tannins, terpenes and sterols in our semi-ethanolic extract of *E. hirta* sample is in agreement with the results obtained by Ayéna et al. [19] and Sadou Nassirou et al. [20] respectively with samples harvested in the Municipality of Aplahoué in southern Benin and with the aerial part of *E. hirta* harvested in Niger. However, we note the presence of alkaloids in the samples of the Municipality of Aplahoué and Niger, unlike our sample that does not. Regarding the leaf extracts of *P. guajava*, the secondary metabolites identified in our sample are also present in the hydroethanolic extract of *P. guajava* leaves studied in India by Gurpreet et al. [21]. These secondary metabolites are present in the leaves of *P. guajava* studied in Mali by Maïga [22], but the anthocyanins present in our sample, are absent in that of Mali and the alkaloids, coumarins and gallic tannins present in the sample from Mali, are absent in our case. This variation of secondary metabolites observed in our samples compared to previous work, could be related to various factors namely the geographical origin, the nature of the soil, the mode of extraction, the fresh or dry plant material, the organ of the plant used and the conditions of analysis [23, 24].

Table 1 Secondary Metabolites identified in the extracts of the plants studied

Secondary metabolites	*Euphorbia hirta*			*Psidium guajava*		
	EE	ESE	EA	EE	ESE	EA
Flavonoids	+	+	+	+	+	+
Catechic tanins	+	+	+	+	+	+
Gallic Tanins	+	+	+	-	-	-
Saponosides	-	-	-	+	+	+
Sterols et terpens	+	+	+	+	+	+
Leucoanthocyans	-	-	-	+	+	+
Anthocyans	-	-	-	+	+	+
Alkaloids	-	-	-	-	-	-
Reducing compounds	+	+	+	-	-	-
Mucilages	+	+	+	+	+	+
Coumarins	-	-	-	-	-	-

+: Presence; -: Absence; EE: Ethanolic extract;ESE: Semi ethanolic extract; EA: Aqueous extract

Levels of phenolic compounds in the extracts of the plants studied

Based on the absorbance values of the various extract solutions and referring to the calibration curves, the total phenol and flavonoid contents were calculated. The analysis in Table 2 reveals that all plants are richer in total phenols than in total flavonoids. The ethanolic extract of *P. guajava* leaves has the highest content of total phenols with an average of $3.39x10^5 \pm 126.26$ mg GAE /g of dry material and the aqueous extract of *E. hirta*, the lowest content that is equal at $5.5x10^4 \pm 757.61$ mg GAE/g of dry material. As for the total flavonoid contents, the highest content is noted in the ethanolic extract of *P. guajava* with a value of 400.84 ± 13.98 mg CE/g of dry material.

Table 2 Phenolic compound content of extracts

	Extracts	*E. hirta*	*P. guajava*
Total Phenols (mg GAE/g dry material)	EE	$1.67x10^5 \pm 252.53$bA	$3.39x10^5 \pm 126.26$aA
	ESE	$1.53x10^5 \pm 631.34$B	$2.83x10^5 \pm 126.26$aB
	EA	$5.51x10^4 \pm 757.61$bC	$2.15x10^5 \pm 126.26$aC
Total flavonoids (mg CE/g of dry material)	EE	146.94±7.77bB	400.84±13.98aA
	ESE	162.70 ±17.09bAB	373.74±27.97aA
	EA	165.52±8.54bA	275.98±18.65aB

EE: Ethanolic Extract, ESE: Semi-Ethanolic Extract, EA: Aqueous Extract;The average values of the same line followed by different letters in lower caseare significantly different at the 5% threshold. The average values of the samecolumn followed by different letters in capital letter are significantlydifferent at the 5% threshold

Sensitivity of the E. coli strain to extracts and antibiotics

The table 3 shows the averages of the diameters of the inhibition zones of the various extracts on *E. coli* strain. Analysis of this table indicates that extracts of *E. hirta* and *P. guajava* are not active on *E. coli* strain at (100 mg/mL). As for the diameters of inhibition of essential oils and antibiotics presented in Table 3, we note that up to 10 μL, the essential oils of *Clausena anisata* showed no inhibitory activity on the two strain of *E. coli*. On the other hand, the essential oils of the organs of *A. pubescens*, as well as the antibiotics tested, showed a good inhibitory activity on the two strains. Indeed, on the *E. coli* strain, the largest inhibition diameter equal to 25.50 ± 2.1 mm, is noted with the essential oil of the whole plant of *A. pubescens*. Also, the inhibition

diameter is function of the quantity of oil tested, the more the quantity increases, the greater the diameter is important. With respect to the two antibiotics tested, Tetracolivit further inhibited (17 ± 0.0 mm) the strain of *E. coli* isolated from chickens than Oxytetracyclin (15.25 ± 1.7 mm).

Minimal inhibitory concentrations (MIC), bactericidal (MBC) and antibiotic potency of extracts and antibiotics

The results regarding Minimal Inhibitory Concentration, bactericidal (MBC) and antibiotic potency of the plant extracts and antibiotics are shown in Table 4. This reveals that on the strain *E. coli*, Tetracolivit has the lowest MIC equal to 0.07 ± 0.03 mg / mL and Oxytetracyclin, the highest MIC which is equal to 1.17 ± 0.55 mg / mL. The same trend was noted at the level of MBC with an average of 0.15 ± 0.07 mg / mL for Tetracolivit and 2.34 ± 1.11 mg / mL for Oxytetracyclin. This shows that Tetracolivit is more active on this strain than the essential oils of *A. pubescens* which are more active on this strain than Oxytetracyclin. Regarding antibiotic potency, *A. pubescens* essential oils and both antibiotics have a bactericidal effect on the *E. coli* tested. From the analysis of the results of the sensitivity of the *E. coli* strain to the extracts, it appears that the essential oils of the various organs of *A. pubescens* are active on the strain of *E. coli*. These same results were obtained by Yovo [24], which showed that these same oils have good antibacterial activity on the reference strain *E. coli* ATCC 25922 with MICs of 0.40 mg / ml for the whole part, 0. , 40 mg / mL for flowers and 0.27 mg / mL for leafy stem and MBC of 0.80 mg / mL, 0.80 mg / mL and 0.54 mg / mL respectively. We therefore note that these MICs and MBC are slightly lower than those found in our study, except for the oil of the stem with the MICs and MBC being three times lower than those obtained on the E. coli strain used in this study. our case (0.81 ± 0.38 mg / mL for MIC and 1.62 ± 0.76 mg / mL for MBC); which shows that the strain of E. coli studied by Yovo [24] is more sensitive to the essential oils of *A. pubescens* than the strain of E. coli used in our study. The antibacterial activity of these oils could be attributed to the action of the thymol they contain or the synergistic action of thymol and other major compounds such as thymyl acetate, p-cymene, γ-terpinene and carvacrol [12].

As for *E. hirta* extracts, no inhibitory activity was observed on the *E. coli* strain. However, Ayena et al. [19], have shown that the hydroethanolic extract of *E. hirta* has good antibacterial activity on an *E. coli* strain isolated from the offending foods during gastroenteritis, with a minimum inhibitory concentration of 1.25 mg / mL. Similarly, Nikunj and Kaushik [25] showed that the aqueous extract of *E. hirta* has moderate inhibitory activity on an isolated *E. coli* strain in patients with a MIC of 50 μg / μL. This result obtained in our case, could be related to the absence of the alkaloids in our sample contrary to that of Ayéna et al. [19]. Indeed, alkaloids are known to have antimicrobial properties. The mechanism of action of alkaloids is attributed to their ability to intercalate with bacterial DNA [26]. With regard to *P. guajava* leaves, the inactivity of the extracts of this plant observed on the *E. coli* strain in this study is in agreement with the results obtained by Biswas et al. [27], which showed that the ethanolic and aqueous extracts of the same plant are not active on the *E. coli* strain. However, these results contrast with those obtained by Suresh et al. [28], which showed that the aqueous leaf extract of *Psidium guajava* significantly inhibited *E. coli* strain.

With regard to the essential oil of *Clausena anisata*, our results are contrary to those obtained by Osei-Safo et al. [29], who showed that the essential oil of with estragole as chemotype, has a good antibacterial activity on the strain of *E. coli*. This difference in results could be explained by the estragole content. Indeed, the essential oil of *C. anisata* used by Osei-Safo et al. [29] contains estragole at 98.76% and that used in this study [11] at 69.9%. The antibacterial activity of Tetracolivit consisting of oxytetracyclin, colistin and vitamins as active ingredients, would be more related to the action of colistin. Indeed, Rahmatallah et al. [8] showed that strains of *E. coli* isolated from broilers had low levels of resistance to colistin (2.94%), unlike oxytetracyclin, for which these strains showed 100% resistance.

Conclusion

Phytochemical analysis revealed that extracts of *E. hirta* and *P. guajava* are rich in flavonoids, catechin tannins, sterols and terpenes. With respect to the antibacterial activities of plant extracts and antibiotics, the study showed that *A. pubescens* HEs and the two commercial antibiotics tested had bactericidal activity on the E. coli isolated from poultry. These oils and antibiotics had bactericidal activity against the isolate investigated. Extracts of *E. hirta and P. guajava* and *C. anisata* essential oil showed no antibacterial activity on *E. coli* isolated from poultry.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

Table 3 Averages of the diameters of the inhibition zones of the various extracts on *E. coli*

Conc/qty	Diameters of inhibition zones of essential oils and antibiotics (mm)						
	100 mg/mL	1 µL	2 µL	3 µL	4 µL	5 µL	10 µL
HE *C. anisata*	-	6,0±0,0^{Ca}	6,0±0,0^{Ca}	6,0±0,0 aD	6,0±0,0 aC	6,0±0,0 aC	6,0±0,0 aC
HE APE	-	9,0±1,4^{BA}	10,5±3,5dB	18,0±0,0cB	19,0±1,4bcB	22,5±2,1abA	25,50±2,1Aa
HE APF	-	11,0±1,4^{Aa}	18,0±0,0dA	20,0±0,0cA	22±0,0bA	23,0±0,0aA	23,50±0,7aA
HE APTF	-	6,0±0,0^{Cb}	10,0±0,0dB	14,5±0,7cC	17,5±0,7bB	17,5±0,7bB	21,50±0,7aB
Oxytetracyclin	15,25±1,7^{A}	-	-	-	-	-	-
Tetracolivit	17±0,0^{B}	-	-	-	-	-	-

The average values of the sameline followed by different letters in lower case are significantly different atthe 5% threshold. The average values of the same column followed by differentletters in capital letter are significantly different at the 5% threshold;Conc/qty: concentration/quantity

Table 4 Minimum Inhibitory Concentration (MIC), Bactericides (MBC) and antibiotic potency of extracts and antibiotics

	Extracts/antibiotics	MIC (mg/mL)	MBC (mg/mL)	MBC/ MIC
E. coli	APE	0.44±0.21b	0.87±0.41b	2.00
	APF	0.49±0.00b	0.99±0.00b	2.02
	APTF	0.81±0.38ab	1.62±0.76ab	2.00
	Oxytetracyclin	1.17±0.55a	2.34±1.11a	2.00
	Tetracolivit	0.07±0.03c	0.15±0.07c	2.01

APE: Essential oil from wholepart of Aeollanthus pubescens; APF:Essential oil from flowers of Aeollanthuspubescens, APTF: Essential oil from leaf stem of Aeollanthus pubescens; The averagevalues of the same column followed by different letters in lower case aresignificantly different at the 5% threshold.

Authors' contributions

Philippe Sessou conceived the study and wrote the protocol; he performed antibacterial assay and wrote the manuscript. Yaovi and Yovo performed the plant collection and extracts prepartion and did together phytochemical analysis with the supervision of Alitonou and Professor Sohounhloue. Dossa has conducted on the supervision of Philippe Sessou and Boko Cyrille, the fecal sample collection and identification of E coli isolates. Oscar Aguidissou and Boko helped Philippe Sessou for antibacterial assay and results interpretation. Farougou had supervise the work. All authors had corrected and approved the final paper.

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