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Comparative study of antibacterial activity of some medicinal plants extracts against strains of *Salmonella* isolated from guinea fowl in Benin.

Boko KC[1]*, Kpodekon TM[1], Aguidissou ON[1], Sessou P[1], Sohounhloue D[2], and Farougou S[1].

[1]Laboratoire de Recherche en Biologie Appliquée/Ecole Polytechnique d'Abomey-Calavi, Université d'Abomey-Calavi, 01 BP 2009 Cotonou, Bénin
[2]Laboratoire d'Etude et de Recherche en Chimie Appliquée/ Ecole Polytechnique d'Abomey-Calavi, Université d'Abomey-Calavi, 01 BP 2009 Cotonou, Bénin

ABSTRACT

The present work has compared the in vitro effectiveness of ethanolic and semi-ethanolic extracts of *Annona muricta, Thalia geniculata* and the essential oils extracted from *Cymbopogon citratus, Syzygium aromaticum* and *Ocimum gratissimum* against strains of *Salmonella* Oakland, *Salmonella* Farakan, *Salmonella* Kingston and *Salmonella* Legon, one of causes of the keet deaths in farms recorded in Benin.. These effectivenesses had been assessed by the agar diffusion method and microdilution technic using 96 wells microplate. The results obtained from this test showed that non-volatile extracts of *Annona muricata* and *Thalia geniculata* have no antibacterial activity on the four strains tested. By contrast, the essential oils of *Cymbopogon citratus*, *Syzygium aromaticum* and *Ocimum gratissimum* have shown relative antibacterial effectiveness to these four strains with inhibitory diameters of strains varying between 10 to 11 mm for *Cymbopogon citratus* oil, 14 to 18 mm for *Syzygium aromaticum* and 20 to 24.5 mm for *Ocimum gratissimum*. The oil of *Ocimum gratissimum* has thus proven to be the most active on the strains followed by that of *Syzygium aromaticum*. Although the essential oil of *Ocimum gratissimum* presents the strongest activity among all the extracts, its activity remains low ($p < 0.05$) compared to that of ciprofloxacin.

Keywords: *Salmonella*, essential oils, extracts non-volatile, *Ocimum gratissimum*, *Syzygium aromaticum*, antibacterial activity.

Corresponding author

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INTRODUCTION

The livestock industry is one of the main activities carried out by man to satisfy its needs and to contribute to the fight against food insecurity. It is practiced by 33% of the Beninese population after agriculture [1]. Face the growing needs of African populations in proteins of animal origin, the developing countries will have to produce more than one hundred billion tones of meat by the year 2020 [2]. As well, in its policy of food self-sufficiency, Benin is interested in recent years in the livestock sector by the revival of the agricultural chain focused on the promotion of farming of short-cycle species especially poultry. In this pane poultry farms, the rearing of guinea fowl occupies a place of choice, taking account of its socio-economic importance and nutrition in the lives of people. Unfortunately, this livestock is confronted with many constraints which are at the origin of major economic losses recorded by the cattlemen. The number of these constraints, are infection related to *Salmonella*. The mortality rate of guinea fowl always remains high and varies from 60% to 65% [3]. Treatments against bacterial infections are therefore useful in the farms. But the high cost of antibiotics compared to the low purchasing power of peasants, the difficulty of supply by lack of adequate infrastructure for its routing to the remote areas as well as the antimicrobial resistance to synthetic veterinary products developed by the strains constitute a constraint to the development of the poultry industry in Benin [4]. In these conditions, other therapeutic alternatives deserve to be explored. Nowadays, the studies are oriented in the search for medicinal plants possessing interesting biological properties in the struggle against various animal pathologies. It is therefore necessary to explore the antimicrobial potential of some medicinal plants of the veterinary pharmacopoeia in view of their publicized in the fight against the avian salmonellosis.

MATERIAL AND METHODS

Plant Material

The plant material studied is composed of *Thalia geniculata* and *Annona muricata* leaves harvested respectively at Hevié in the commune of Abomey-Calavi and Atokoligbe in the township of Bantè. These plants were identified at National Herbarium of Benin.

Bacteriological Material

The bacteria used in this study were isolated from herds of guinea fowl in the department of the Borgou in northern Benin. These bacteria have been typed in Belgium at the Veterinary and Agrochemical Study and Research Center (CERVA-CODA), Bacteriology section. These strains were then reduced and retained for the veterinary diagnostic laboratory of the Polytechnic School of Abomey-Calavi (EPAC).

Essential oils used

The essential oils of *Cymbopogon citratus*, *Ocimum gratissimum* and *Syzygium aromaticum* used in this study were previously extracted with hydrodistillation technic and analyzed using gas chromatography and gas chromatography coupled with mass spectrometry.by [5-7].

Methods

Preparation of ethanolic extracts and semi-ethanolic

The aerial parts of *Thalia geniculata* and *Annona muricata* have been dried in the open air at the shelter from the sun during a month, and then reduced to powder. For the ethanolic extract, 100g of powder from each plant were macerated in 500 ml of ethanol at 96 degrees. The mixture is left in maceration under manual shaking for 5 days and filter three times successively on absorbent cotton. Then the filtrate was evaporated to dryness at 40°C using a rotavapor and then in the oven at 50 °C. With regard to the semi-ethanolic extract, the same approach to preparation of the ethanolic extract was applied with ethanol at 50 degrees. The two extracts were kept at 4°C until use.

Phytochemical screening

The phytochemical screening of *Thalia geniculata* and *Annona muricata* was based on the coloring and/or precipitation reactions of the chemical compounds contained in the plants according to the standards methods described by [8]. The tannins have been identified by the test to the $FeCl_3$ and the reagent of Stiasny, flavonoids by reaction to the cyanidine ; The sterols and terpenes by the test of Liebermann-Burchard ; anthocyanins by reaction to H_2SO_4 and NH_4OH and finally the alkaloids by tests of Mayer and Dragendorf.

Evaluation of the antibacterial activity of the extracts studied

The evaluation of the effectiveness of antibacterial extracts on strains of *Salmonella* has consists of a part in the test of sensitivity of the strains to the extracted by agar diffusion and on the other hand to the determination of minimum inhibitory concentration, minimum bactericidal concentration and antibiotic power of extracts screws-to-screws of microbial strains tested by the microdilution method using 96 wells microplate.

Test of sensitivity of the strains to the extracts investigated

The assessment of the sensitivity of strains bolt-to-bolt of the extracts has been made by the agar diffusion method on Muller-Hinton agar medium according to National Committee for Clinical Laboratory Standards [9] reported by [10] for excerpts from non-volatile and [11] for the essential oils in order to identify the active extracts. The different tests were made in duplicate.

Test of sensitivity of strains bolt-to-bolt extracts non-volatile

The ethanolic ant semi-ethanolic extracts were prepared at a concentration of 100 mg/ml with a mixture of ethanol/water (4/6). The microbial suspensions in exponential phase of growth (0.5 on the scale of McFarland, approximately 1.5 10^6 cfu/ml) were sown on agar Muller Hinton agar (MHA) sterile medium. The disks impregnated with 25 µl of extract (2.5 mg), 25 µl of solvent for the negative control and the disk of antibiotic of reference, in this case the ciprofloxacin (10µg/disk) for the positive control, were deposited on the agar MHA previously inoculated. After a pre-broadcast from 30 min at ambient temperature, all the Petri dishes were incubated 18 to 24 hours at 37°C. After incubation only the extracts with a diameter of inhibition greater than or equal to 12 mm are qualified for extracts assets and will be the subject of determination of the CMI and CMB [10].

Test of sensitivity of strains bolt-to-bolt of essential oils

For this test, pure essential oils have been used. It has consisted to impregnate disc of 6mm of diameters with 5 µl of oil tested. Ciprofloxacin was used as positive control. These different discs impregnated and the antibiotic of reference have been deposited on the agar MHA previously inoculated with a microbial suspension of approximately 1.5 10^6 cfu/ml. All the Petri dishes were incubated 18 to 24 hours at 37°C. The extracts with a diameter of inhibition greater than or equal to 14 mm have been chosen for the determination of the MIC and MBC and antibiotical power [11].

Determination of Minimum Inhibitory Concentration (MIC), Minimum bacteridal Concentration (MBC) and antibiotical power of extracts against microbial strains tested

The determination of the MIC, MBC and antibiotic power (p. a) of essential oils of *Ocimum gratissimum* and *Syzygium aromaticum* has been made following the technique of dilution in liquid medium on microplate 96 wells coupled to the plating on solid medium as described by [12] and reported by [4]. For this, a solution was obtained by mixing 40 µl of the essential oil to 2000 µl of the broth Mueller Hinton and a drop of Tween 80. 100 mL of the Mueller Hinton broth (MHB) have been distributed in the wells of a microplate. 100 mL of the solution of the extract (suspension prepared from 40 µl of pure essential Oil diluted in 2000 µl of MHB) have been added to each of the wells of the first column. The successive dilutions of reason 2 wells by wells until the last wells in each row were made and 100 µl from the last wells have been rejected. All the wells were seeded except those used to negative witness by 100 µl of the bacterial suspension at 10^6 germs/ml

(density equal to the scale 2 of MC Farland). The negative control consisted of the broth Mueller Hinton added with extract and the one used for positive control of the growth of germs was constituted of the inoculum and the broth Muller Hinton. The microplate was coated paper parafilm and incubated at 37°C for approximately 24 hours. For the reading, the wells corresponding to the smallest concentration of extract of essential oil for which we do not observe turbidity or visible growth to the naked eye is taken as the minimum inhibitory concentration (MIC) of the extract on the strain tested. The MBC which is the smallest concentration for which is not observed growth of the germ on Mueller Hinton Agar medium has been determined as a result of the MIC by a subculture of the dilutions whose concentration is greater than or equal to the MIC, on Mueller Hinton Agar poured in sterile Petri dishes and the antibiotical power (p. a) of the extract was calculated by the formula: p.a= MBC/CMI. When p.a is less than 4, it is said that the extract tested has an antibiotical power.

Statistical Analysis

The results of microbiological analyzes obtained have been treated through the Excel software for the calculation of averages and gap-types. The Stata software version 11 was used for the test of linear regression in order to compare the effectiveness of essential oils and the antibiotic of reference on strains of Salmonella from the diameters of inhibitions. The MIC and MBC of extracts against the strains were compared two by two by the student's t test.

RESULTS AND DISCUSSION

Phytochemical screening of plant species studied

The table 1 displays the large families of chemical compounds present in the leaves of *Thalia geniculata* and *Annona muricata*. It shows that these two plants contain cathechiques tannins, flavonoids, sterols and terpenes. Only *Thalia geniculata* contains alkaloids. These results are similar to those of [13] who had identified in the leaves and root of *Thalia geniculata* four compounds which were constitued of three steroids and a terpene. Concerning *Annona muricata*, the results obtained were similar to those obtained by [14] which in addition to its metabolites, have research and detected the presence of saponisides, reducing sugars, anthraquinones and glucosides.

Table 1: Secondary metabolites identified in the aerial parts

Secondary Metabolites	*Thalia geniculata*	*Annona muricata*
Alkaloids	+	-
Tannins fastness	-	-
Tannins catechiques	+	+
Flavonoides	+	+
Anthocyanins	-	-
Sterols	+	+
Terpenes	+	+

+ : Presence ; -: Absence

Sensitivity of the strains to the plants extracts

The study highlighted the antibacterial activity of some plants extracts on four strains of Salmonella at the origin of a high mortality of guinea fowl in Benin. The evaluation of the antimicrobial profile of ethanolic and semi-ethanolic extracts of *Thalia geniculata* and *Annona muricata* and essential oils of *Cymbopogon citratus*, *Ocimum gratissimum* and *Syzygium aromaticum* by agar diffusion method has shown that non-volatile extracts of plants studied have no antibacterial effect on the strains tested at the concentration tested of 100 mg/ml (25 µl/disc). In fact, the diameters of inhibition of ethanolic and semi-ethanolic extracts of two plants on the stumps were 6mm in diameter including the diameter of the disc paper (6mm). However, according to information reported by [10], an extract from non-volatile has a low antimicrobial activity on a strain when the diameter of inhibition of the strain is less than 12 mm in diameter. The ineffectiveness of these non-volatile extracts studied could probably be explained by the low content in active compounds. Moreover, this result may also be linked to the extraction solvent used (ethanol) because according to the work of [15], the methanolic extract of *Annona muricata* tested on different strains of

bacteria of references such as *Salmonella typhimurium* ATCC23564, *Klebsiella pneumoniae, Staphylococcus aureus, Proteus, Bacillus subtilis* ATCC12432 revealed an antibacterial activity on *Bacilus subtilis* and Staphylococcus *aureus* and *Klebsiella pneumoniae* . In addition, [16] and [17] have shown that this plant has many biological activities including anti-bacterial properties.

Table 2: Sensitivity of microbial strains tested to extracts

		Diameter of inhibition (mm)			
Strains	*C. citratus*	*S. aromaticum*	*O. gratissimum*	Ciprofloxa-cin	Significance test
S. Oakland	11±0.0d	17.5 ±0 ,71c	24.5 ±0.71b	39.5±0.71a	***
S. Farakan	10.5 ± 0.71d	18± 1.41c	24± 1.41 b	39.5 ± 0.71a	***
S. Kingston	12.5 ± 0.71d	16.5 ± 0.71c	23.5 ±2.12b	39.5 ± 0.71a	***
S. Legon	10±0.0d	14.5 ± 0.71c	20.5 ±0.71b	39.5 ± 0.71a	***

***P< 0.001; the averages in the same row followed by different letter, differ significantly on the threshold of 5%

Regarding the activity of essential oils, table 2 presents the diameters of inhibition of strains in contact with essential oils of *Cymbopogon citratus*, *Syzygium aromaticum* and *Ocimum gratissimum* compared to that of ciprofloxacin. From a reading of these results, we find that the four strains studied have shown a limited sensitivity to *Cymbopogon citratus* whereas they have shown an average sensitivity screws-to-screws of *Syzygium aromaticum* and a great sensitivity to *Ocimum gratissimum*. In fact, according to [11], the sensitivity of a germ is zero for a diameter less than or equal to 8 mm. It is limited to a diameter of between 8 and 14 mm, and average for a diameter between 14 and 20 mm. For a diameter greater than or equal to 20 mm, the germ is very sensitive. The analysis of these results shows that the three essential oils had an inhibitory effect on the four strains studied. However, the essential oil of *Ocimum gatissimum* showed the higher antibacterial activity among the oils tested followed by that of *Syzygium aromaticum*. These two oils were therefore chosen for the determination of the MIC and MBC. Overall, the study reveals that there is a significant difference ($p < 0.001$) between the antimicrobial efficacy of each of the three essential oils and the antibiotic of reference. The study of the sensitivity to the antibiotic of reference, shows that all strains were sensitive to ciprofloxacin with 39.5 mm in average of inhibition diameter.

Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) and antibiotical power of essential oils

The essential oils extracted from *Ocimum gratissimum* and *Syzygium aromaticum* are those who hold the most pronounced antimicrobial activities behind the antibiotic of reference which is ciprofloxacin. The minimum inhibitory concentration determined as well as the minimum bactericidal concentration to the two active oils have enabled us to assess their antibiotical power. The results of the MIC, MBC and the report MBC/MIC for determination of the antibiotical power of the essential oils of *Ocimum gratissimum* and *Syzygium aromaticum* are presented respectively in tables 3, 4 and 5. From a reading of these results, we can say that the lowest MIC were those of *Ocimum gratissimum* which vary from 0.20 mg/ml to 0.53 mg/ml on all the strains against those of *Syzygium aromaticum* which vary from 0.63 mg/ml to 1.26 mg/ml. The strain the more sensitive to the essential oil of *Ocimum gratissimum* is the strain of *Salmonella enterica* serotype Legon and the more resistant to this oil is the strain of *Salmonella enterica* serotype Farakan. With regard to the essential oil of *Syzygium aromaticum*, the most sensitive strains were *Salmonella enterica serotype* Oakland and *Salmonella enterica serotype* Farakan. Regarding the MBC, the same trends have been obtained where the essential oil of *Ocimum gratissimum* remains the most active with CMB varying between 0.26 and 1.05 mg/ml against 1.26 and 2.51 mg/ml for the essential oil of Syzygium *aromaticum*. The calculation of the report MBC/MICI reveals that these two active extracts possess an antibiotical power. In effect, according to [18] when the report MBC/MIC is less than or equal to 4, the extract is qualified of bactericide and when it is higher than 4, the extract is said bacteriostatic. These two essential oils could therefore validly be used in replacement of synthetic antibiotics.

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Table 3: Minimum inhibitory concentrations (MIC) of the extracts from against strains

Strains	MIC (mg/ml)		
	O. gratissimum	*S. aromaticum*	Significance Test
S. Oakland	0.26 ±0.00a	0.63 ±0.00 b	***
S. Farakan	0.53 ±0.00a	0.63 ±0.00 b	***
S. Kingston	0.26 ±0.00a	1.26 ±0.00 b	***
S. Legon	0.20 ±0.09a	1.26 ±0.00 b	***

***: P< 0.001; the averages in the same row followed by different letter, differ significantly on the threshold of 5%

Table 4: Minimum Bactericidal Concentration (MBC) extracts against strains

Strains	MBC (mg/ml)		
	O. *gratissimum*	*S. aromaticum*	Significance test
S. Oakland	0.53 ±0.00a	1.26 ±0.00 b	***
S. Farakan	1.05 ±0.00a	1.26 ±0.00b	***
S. Kingston	0.53 ±0.00a	2.51 ±0.00 b	***
S. Legon	0.26 ±0.00a	2.51 ±0.00 b	***

***: P< 0.001; the averages in the same row followed by different letter, differ significantly on the threshold of 5%

Table 5: Antibiotical power of essential oils against microbial strains tested

Strains	MBC /MIC		
	O. gratissimum	*S. aromaticum*	Antibiotical power
S. Oakland	2.04	2	Bactericide
S. Farakan	1.98	2	Bactericide
S. Kingston	2.04	1.99	Bactericide
S. Legon	1.30	1.99	Bactericide

In a comprehensive manner, we can remember that the two essential oils have antibacterial activity screw-to-screw strains of *Salmonella* studied. This activity varies significantly ($p < 0.001$) of an oil to another with an activity more pronounced of the essential oil of *Ocimum gratissimum* (table 3 and 4) whose effective antimicrobial has been reported by [4] on strains of *Salmonella* Oakland and *Salmonella* Legon. Comparing our results with those of [4], we observe that our MIC on strains of *Salmonella* Oakland and *Salmonella* legon are superior to those obtained (MIC for *Salmonell*a legon = 8.10^{-3} mg/ml against 16.10^{-3} mg/ml for *Salmonella* Oakland) by these authors. This low activity of our oil of essential of *Ocimum gratissimum* compared to that studied by [4] may be due to the lowest content (28% against 43 %) of our oil in thymol. In effect, the thymol has been reported in the literature as a powerful bioactive molecule possessing very interesting antibacterial properties [19]. With regard to the essential oil of *Syzygium aromaticum*, our results corroborate those of [20] where the essential oil held an antibiotical power on *Salmonella typhi* isolated from soft drinks with a MIC of 0.40 mg/ml and a MBC of 0.80 mg/ml. In sum, this work reveals the antimicrobial power of essential oils of *Ocimum gratissimum* and *Syzygium aromaticum* on *Salmonella* strains isolated from guinea fowl and opens the way to the fight against the avian *Salmonella* infections with natural substances extracted from plants and also the formulation of natural antimicrobials for this fact.

CONCLUSION

This study reveals that non-volatile extracts of *Thalia geniculata* and *Annona muricata* don't possess antibacterial activity against four strains of Salmonella isolated from guinea fowl in Benin contrary to essential oils of *Syzygium aromaticum* and *Ocimum gratissimum* which have shown antibacterial activity on strains studied with an activity more pronounced of the one of *Ocimum gratissimum*. In the light of the antimicrobial performance of these two plants, other researches such as in vivo test, isolation of active principles in these essential oils will be envisaged. In sum, in a general way the different results obtained constitute a first step which paves the way for the fight against the avian salmonella infections with natural substances extracted from plants and also the formulation of natural antimicrobials for this fact.

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