



Research Article

Inhibition of the Growth of Multidrug Resistant Avian *Salmonella* Strains by Aqueous and Ethanolic Extracts of *Mallotus oppositifolius* (Geisel.) Müll.-Arg (Euphorbiaceae)

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ABSTRACT

Acquired antibiotic resistance, observed in multi-antibiotic resistant strains of *Salmonella* in poultry, poses a risk to health consumer and a growing threat to public health. The objective of this work is to investigate the antimicrobial activity of plant extracts of *Mallotus oppositifolius* (Geisel.) Müll.-Arg (Euphorbiaceae) on strains of *Salmonella* spp isolated from raw chicken gizzards (*Salmonella* Bargny and Kentucky) and viscera of quail (*Salmonella*) serogroup O: 21), (multi) resistant to antibiotics. The diffusion method in agar medium, on Muller-Hinton® agar (BioRad, France), and the search for antibacterial parameters (Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (CMB)) made it possible to evaluate the effectiveness of plant extracts on the strains studied. The results obtained show that the aqueous and ethanolic extracts of *Mallotus oppositifolius* (Geisel.) Müll.-Arg (Euphorbiaceae) have antibacterial activity on the 03 avian *Salmonella* strains studied, with diameters (mm) varying from 15 ± 1^b to 19 ± 1^a for the Aqueous extract and from 12 ± 1^c to 27 ± 1^a for the Ethanolic extracts. Qualitative phytochemical screening of the active extracts shows the presence of Terpenes and sterols, Catechical tannins, Saponins, Anthraquinones and polyphenols. This work made that it possible to highlight the interest of a probable use of plant extracts from the Ivorian pharmacopoeia, to fight against salmonellosis of avian origin.

Key words: Antibacterial activity, *Mallotus oppositifolius*, multidrug resistant *Salmonella*, Poultry, Ivorian pharmacopoeia.

INTRODUCTION

During the last three decades, a number of antibiotics as well as certain synthetic molecules have been produced around the world to eradicate the microorganisms responsible for many diseases (Walsh, 2003). However, these antibiotics have caused the appearance and dissemination of mutations in the genetic makeup of these microorganisms, making them very resistant (Cohen, 1992). The spread of bacterial resistance to antibiotics is due to a number of factors. Several scientific studies have focused on the genetic exchanges that may exist between bacteria of the commensal flora, on the one hand, and between pathogenic bacteria and commensal bacteria, on the other hand. Indeed, the high concentration of bacteria present in the digestive tract and the cohabitation of commensal and pathogenic bacteria in the same environment, promotes interactions (Madec and Meunier,

2006). Likewise, the existence of transfers of plasmids carrying resistance genes within the commensal flora has already been proven in the digestive tract of rodents (Salyers *et al.*, 2004). More specifically, two studies (Salyers *et al.*, 2004; Salyers *et al.*, 2007) have shown that bacteria different from the commensal digestive flora of humans contain resistance genes with very strong homologies (more than 95 %). This suggests that these genes have the same origin and therefore, that they have been "transferred" from one bacterium to another. Consequently, these could favor a real public health problem, in the event of dissemination. To overcome the problem of the appearance and emergence of this type of microorganism, some scientific work is increasingly focusing on other antimicrobial agents, namely medicinal plants. Indeed, herbal remedies are recognized as rich sources of antimicrobial agents and are widely used in different countries for medicinal purposes. They are

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traditionally used for their therapeutic properties, also have antidiabetic, antioxidant, antibacterial, anti-inflammatory, antipyretic, gastro-protective, etc. potential. (Chopra *et al.*, 1956).

These compounds also act as efflux pump inhibitors (flavanoids, terpenoids, alkaloids), inhibitors of penicillin binding proteins (PLP), quinones, terpenoids, causing the permeability of bacterial membranes (terpenoids) and are also inhibitors of betalactamases (alkyl gallates). The active principles of medicinal plants being grouped into several chemical families, namely sulfur compounds (thiosulfonates, aldehydes, phenols, terpenes and tannins), act by blocking the multiplication of the microorganism or by destroying its wall. To this end, the World Health Organization considers them to be the richest source of multiple drugs and still favors the use of these traditional drugs in the treatment of microbial and non-microbial diseases (Anonymous, 1978).

In the present study, an attempt to use medicinal plants on enteropathogenic strains such as *Salmonella* sp. was made in order to research the antibacterial effect of aqueous and ethanolic extracts of plants of *Mallotus oppositifolius* (Geisel.) Müll.-Arg (Euphorbiaceae), on three strains of *Salmonella* (Derby, Kentucky, enterica serogroup O: 21) (multi) resistant to antibiotics, isolated from poultry, to control salmonellosis.

MATERIALS AND METHODS

Plant materials

The plant used in this study (*Mallotus oppositifolius* (Geisel.) Müll.-Arg (Euphorbiaceae)), was collected from traditional therapists, on the market for the sale of medicinal plants in the town of Bingerville. Once collected, the plant, known for its anti-diarrheal and / or anti-inflammatory property, was taken to the herbarium of the National Floristic Center of the Félix Houphouët-Boigny University in Abidjan (Côte d'Ivoire), for its identification.

Bacterial strains

The bacterial strains are composed of three (3) isolates of *Salmonella* sp. of known antibiotic profiles (Table 1). These strains come from the Microbiology Unit of Laboratory of Biotechnology, Agriculture and Development of Biological Resources, of the Biosciences Training and Research Unit of Félix Houphouët-Boigny University in Côte d'Ivoire.

Preparation of aqueous and ethanolic extracts

The leaves, washed and dried were finely ground using an electric grinder type Grinder GLG-450. The fine powder obtained was extracted according to the method of Zirihi *et al.*, 2003. Thus, one hundred grams (100) g of leaf powder were mixed in one liter (1L) of distilled water using a blender type mixer, three times for three minutes. The homogenate obtained is first of all wrung out in a square of fabric to remove the plant debris and then filtered twice successively through hydrophilic cotton to remove the debris which may have passed through the meshes of the fabric. Finally, a final filtration is done once on Wattman filter paper. The filtrate obtained is dried in an oven at 50°C. for five days. The powder thus obtained is the total aqueous extract of the powder of the leaves of *Mallotus*

oppositifolius noted, Mal A. The ethanolic extract was obtained according to the second variant of the method of Zirihi *et al.*, 2003. This extraction is carried out in a manner similar to that of the aqueous extract, with 70% ethanol as solvent. The ethanolic extract of the powder of the leaves of *Mallotus oppositifolius*, coded Mal E. The two extracts obtained were stored at 4°C. in the refrigerator.

Yield

The yield is determined by the ratio of the mass of the dry extract after evaporation to the mass of the powder of dry plant material used for the extraction. It is expressed as a percentage and is calculated according to the following formula:

$$Rd = (m \times 100) / M$$

Rd: extraction yield in percentage; m: the mass in grams of the dry extract; M: the mass in grams of the leaf powder.

Preparation of bacterial inocula

The strains of *Salmonella* sp. are revived in Muller-Hinton broth, then an aliquot of each bacterial suspension is seeded on Muller-Hinton agar and incubated at 37°C for 24 H, to obtain young colonies. From a few young colonies, the turbidity of each bacterial suspension is adjusted so that its opacity is equivalent to Mac Farland's 0.5 control, estimated at 10⁸ CFU / mL (Bauer *et al.*, 1966).

Subsequently, diffusion tests in agar medium are carried out as well as the determination of the Minimum Inhibitory Concentration (MIC) and of the Minimum Bactericidal Concentration (CMB), in order to assess the anti-bacterial power of each extract.

Determination of the antibacterial activity of plant extracts by the agar medium diffusion method

Stock solutions concentrated at 200 mg / mL are prepared from each plant extract. Blotting paper discs (Bio-Rad), impregnated with each stock solution, were used for the diffusion tests in agar medium as described by Bauer *et al.*, 2003. From the bacterial inoculum equivalent to 0.5 Mc Farland (10⁸ CFU / mL), prepared beforehand, a dilution to 1/100 was carried out in sterile distilled water in order to obtain a bacterial suspension at 10⁶ CFU / mL. Each bacterial inoculum estimated at 10⁶ CFU / mL is inoculated by flooding onto Petri dishes containing Mueller-Hinton agar (Bio-Rad, France), previously poured. The antibacterial activity of the extracts is determined by measuring the diameter of the zone of inhibition around each disc (Doughari *et al.*, 2007).

Determination of the Minimum Inhibitory Concentration (MIC)

The determination of the antibacterial parameters (MIC) was carried out according to the method of dilution in liquid medium, as carried out by Kouadio *et al.*, 2015. Thus, in hemolysis tubes, one (1) milliliter with a concentration range of 100 to 3.12 mg / mL was prepared by the double dilution method. For each bacterial strain, a bacterial inoculum whose turbidity corresponds to the opacity of 0.5 Mc Farland is prepared in sterile distilled water, then diluted 1/100 in two times concentrated Mueller Hinton broth, for the obtaining a bacterial inoculum estimated at 10⁶ CFU / mL. Subsequently, a 1/2 dilution by adding one (1) mL of inoculated broth, at different

concentrations of extracts is carried out. The final concentration range was 50 to 1.56 mg / mL. After 24 h of incubation at 37°C, the turbidity of the medium reflects bacterial growth. The MIC of an extract for a given bacterial strain is the smallest concentration of extract, showing no visible growth of the bacteria.

Determination of the Minimum Bactericidal Concentration (MBC)

After inoculation of the concentration range, a count of viable colonies is carried out in order to determine the MBC. Using a 10 µL calibrated loop, a 5 cm and inoculation of the bacterial inoculum, having been used for the inoculation of the concentration range and its dilutions going from 10⁻¹ to 10⁻⁴ is carried out on Mueller-Hinton agar (BioRad, France), to constitute the bactericidal control. Subsequently, subcultures of the tubes without visible growth are carried out on Mueller-Hinton agar (BioRad, France). After 24 hours of incubation at 37°C, the observable colonies are compared to the bactericidal control. MBC is the smallest concentration whose subculture shows germ growth less than or equal to 0.01% of survivors.

Determination of the modality of action of different plant extracts

The antibacterial effect of the various plant extracts tested is judged to be bactericidal or bacteriostatic according to the MBC / MIC ratio (Fauchere and Avril, 2002), if the report:

- MBC / MIC ≤ 2, the substance is said to be bactericidal;
- MBC / MIC > 2, the substance is said to be bacteriostatic.

Phytochemical screening of aqueous and ethanolic extracts of plant extracts

This study was carried out according to the method described by Bagre *et al.*, (2007), for the demonstration of the presence of the different families of secondary metabolites which are: alkaloids, polyphenols, tannins, flavonoids, saponin, polyterpenes or sterols and anthraquinones.

✓ Identification of polyphenols

A drop of 2% alcoholic ferric chloride solution was added to 2 mL of extracts. The appearance of blackish-blue or more or less dark green color indicates a positive reaction.

✓ Demonstration of flavonoids

The aqueous and ethanolic extracts are taken up in 5 mL of hydrochloric alcohol (mixture of 10 mL of 96° ethanol, 10 mL of distilled water and 10 mL of concentrated hydrochloric acid). Subsequently, two to three shavings of magnesium were added to it. The appearance of a pink-orange or purple color after adding 3 drops of isoamyl alcohol indicates the presence of flavonoids.

✓ Demonstration of sterols and polyterpenes

Liebermann's reagent was used for this demonstration. A mass of 0.1 g of dry extract was dissolved in 1 ml of acetic anhydride while hot and collected in a test tube. Then 0.5 mL of concentrated sulfuric acid (H₂SO₄) was added thereto. The appearance at the interphase of a purple or violet ring, turning blue then green, indicates the presence of polyterpenes and sterols.

✓ Identification of alkaloids

A mass of 1 g of dry extract was dissolved in 6 mL of 60° ethanol. A volume of 2 mL of the alcoholic solution thus

obtained was distributed in a test tube. 2 drops of DRAGENDORFF reagent (aqueous solution of potassium iodobismuth) are added to the tube. The appearance of a precipitate or an orange color indicates the presence of alkaloids.

✓ Highlighting tannins

- Catechetal tannins

A volume of 1 mL of hydrochloric alcohol (equivolumic mixture of alcohol, distilled water and hydrochloric acid) was added to 5 mL of dissolved extract. The mixture was brought to a boil for 15 min. the formation of a red precipitate soluble in isoamyl alcohol indicates the presence of catechetal tannins

✓ Demonstration of saponins

A mass of 0.1 g of dry extract was dissolved in 10 mL of distilled water. The resulting solution was stirred vigorously for 45 seconds. After stirring, the solution was allowed to stand for 15 minutes. The observation of a persistent foam, greater than 1 cm in height, indicates the presence of saponins.

✓ Demonstration of anthraquinones

To a few milliliters (3-5 mL) of extract, an equivalent volume of 10% aqueous potassium hydroxide (KOH) is added. After stirring, the presence of anthraquinones is confirmed by turning the aqueous phase to red.

Statistical analysis

The means of the diameters of inhibitions were calculated for each extract relative to each bacterial strain using the EXCEL 2013 software, at the end of the various tests. The STATISTICA 2019 software was used for the one-way analysis of variance (ANOVA 1). It was used to compare the means of the diameters of inhibition of the different extracts on each bacterial isolate, at the probability threshold p less than 5%. The results obtained were expressed as the mean ± Standard deviation.

RESULTS

The yield of the extracts varies depending on the drying conditions, the content of the plant species in metabolites and the nature of the solvent used in the types of extraction. Indeed, the leaf extraction results showed that for 200g of *Mallotus oppositifolius* powder the mass of dry extract powder after aqueous extraction is 21.37g, i.e. 10.68% and 14.67g after hydro-extraction. ethanolic or 7.33%.

The antibacterial activity of the aqueous and ethanolic extracts of *Mallotus oppositifolius*, on all three strains, in agar medium made it possible to obtain the results shown in Table 2.

The aqueous extract of *Mallotus oppositifolius* gives relatively average inhibition diameters of 15 ± 1^b mm for the strain of *Salmonella* Kentucky, and a large diameter of 19 ± 1^a mm for the strain of *Salmonella* enterica serogroup O: 21. However, none diameter showing a zone of inhibition was observed in the *Salmonella* Bargny strain. As for the ethanolic extract of *Mallotus oppositifolius*, these show antibacterial activity on all the strains studied with respective inhibition diameters of 12 ± 1^c mm for the strain of *Salmonella* Kentucky, of 16 ± 1^b mm for the strain. strain of *Salmonella* Bargny and 27 ± 1^a mm for the strain of *Salmonella* enterica serogroup O: 21.

Table 1: *Salmonella* strains used for the study

Strain code	Serogroup / Serotypes	Antibiotic profile	Original matrix	References
D 512 / A	Derby	A- AMC -Tic- Cf - G- SXT- Nal - Cip - Te	raw chicken gizzards	(Bonny <i>et al.</i> , 2014)
G121 / E	Kentucky	G- SXT- Nal- Cip -Te	quail viscera	(Bonny <i>et al.</i> , 2019)
S30H3C	O: 21	A-Tic-TE		

AM \mp : amoxicillin; AMC: amoxicillin / clavulanic acid; Tic: ticarcillin; Cf: cefalotin; CTX: cefotaxime; G: gentamicin; C: chloramphenicol; SXT: cotrimoxazole; Nal: nalidixic acid; Cip: ciprofloxacin; Te: tetracycline.

Table 2: Diameters of inhibition of plants extracts of *Mallotus oppositifolius*

Plants	Types of extracts	Bacterial strains		
		<i>Salmonella</i> Kentucky	<i>Salmonella</i> Bargny	<i>Salmonella</i> enterica serogroup O: 21
<i>Mallotus oppositifolius</i> (leaves)	Aqueous	15 \pm 1 ^b mm	0 \pm 0 ^c mm	19 \pm 1 ^a mm
	Ethanollic	12 \pm 1 ^c mm	16 \pm 1 ^b mm	27 \pm 1 ^a mm

Table 3: Antibacterial parameters of *Mallotus oppositifolius* extracts on the *Salmonella* strains studied

Strains studied	CMI (mg / mL)		CMB (mg / mL)		CMB / CMI		Power of the extract	
	Mal A	Mal E	Mal A	Mal E	Mal A	Mal E	Mal A	Mal E
<i>S. Derby</i>	12.5	12.5	25	25	2	2	Bactericidal	Bactericidal
<i>S. Kentucky</i>	6.25	6.25	25	25	4	4	Bacteriostatic	Bacteriostatic
<i>S. enterica</i> O: 21	25	50	50	50	2	1	Bactericidal	Bactericidal

CMI: Minimum inhibitory concentration; CMB: Minimum bactericidal concentration; Mal A: aqueous extract of *Mallotus oppositifolius*; Mal E: ethanolic extract of *Mallotus oppositifolius*.

The method of dilution in liquid medium used for the determination of the minimum inhibitory concentrations (MIC) on the one hand, and the minimum bactericidal concentrations (CMB) on the other hand made it possible to obtain the effect of extracts of *Mallotus oppositifolius* on the all the strains studied (Table 3).

For the strain of *Salmonella* Bargny, the minimum inhibitory concentration of the aqueous and ethanolic extracts of *Mallotus oppositifolius* is 12.5 mg / mL and the minimum bactericidal concentration is 25 mg / mL. With regard to the strain of *Salmonella* Kentucky, the minimum inhibitory concentration of the two extracts is also the same (6.25 mg / mL), with a minimum bactericidal concentration estimated at 25 mg / mL. However, at the level of the *Salmonella* enterica serogroup O: 21 strain, the minimum inhibitory concentrations of the two extracts differ. Indeed, the aqueous extract of *Mallotus oppositifolius* showed antibacterial activity at a minimum inhibitory concentration of 25 mg / mL and a minimum bactericidal concentration of 50 mg / mL,

The CMB / MIC ratios make it possible to determine the bactericidal and / or bacteriostatic power of the aqueous and ethanolic extracts of *Mallotus oppositifolius* on all the strains studied. Thus, the two extracts (aqueous and ethanolic) of *Mallotus oppositifolius* showed a bactericidal effect on the strains of *Salmonella* Bargny and of *Salmonella* enterica serogroup O: 21, and a bacteriostatic effect on the strain of *Salmonella* Kentucky (Table 3).

The phytochemical screening of *Mallotus Oppositifolius* plant extracts revealed the presence of certain secondary metabolites contained in the various plant extracts, thus justifying their antibacterial power. Thus, the phytochemical screening of the ethanolic extract of *Mallotus oppositifolius* revealed the presence of five (5) of the seven groups of the metabolites sought. These are terpenes and sterols, catechetal tannins, saponins, anthraquinones and polyphenols. The aqueous extract of *Mallotus oppositifolius*, on the other hand, contains only two (2) of the seven groups of secondary metabolites sought, namely catechetal tannins and polyphenols (Table 4).

Table 4: Phytochemical screening of aqueous and ethanolic extracts of *Mallotus oppositifolius*

Chemical compound	Extracts of <i>Mallotus oppositifolius</i>	
	Aqueous extract	Ethanolic extract
Terpenes and sterols	-	+
Catechetal tannins	+	+
Alkaloids	-	-
Flavonoids	-	-
Saponins	-	+
Anthraquinones	-	+
Polyphenols	+	+

DISCUSSION

The objective of this study was to determine the effect of aqueous and ethanolic extracts of *Mallotus oppositifolius* on the growth of enteropathogenic strains such as *Salmonella* of avian origin, (multi) resistant to antibiotics. All the extracts tested showed antibacterial activity on all the avian *Salmonella* strains tested, with inhibition diameters varying between 15 \pm 1^b and 19 \pm 1^a mm for the aqueous extracts and, 12 \pm 1^c and 27 \pm 1^a mm for ethanolic extracts from the same plant. The results of the inhibition diameters of the aqueous extracts obtained in this study are comparable to those obtained by Kouadio *et al.*, 2015, on strains of *E. coli* producing ESBL (extended spectrum beta-lactase), with diameters varying between 10.67 \pm 0.67 and 12.67 \pm 0.33 mm.

The antibacterial parameters (MBC / MIC), ranging from 2 to 4 for the aqueous extracts of *Mallotus oppositifolius*, give this extract a bactericidal power on two of the three strains, namely the strain of *Salmonella* Bargny and that of *Salmonella* enterica serogroup O : 21, and a static bacteriostatic power on the strain of *Salmonella* Kentucky. As for the ethanolic extract of *Mallotus oppositifolius*, produced to date only on phytogenic strains (Orsot *et al.*, 2016; Saraka *et al.*, 2019), nevertheless reveals the existence of an antibacterial power on the strains used in this study. In fact, the antibacterial parameters (MIC and CMB) of the ethanolic extract vary from 6.25 mg / L to 50 mg / mL for the minimum inhibitory concentrations, and from 25 mg / mL to 50 mg / mL for the

minimum concentrations. bactericides. The ratio of the antibacterial parameters (MBC / MIC), varying from 1 to 4 for the ethanolic extract of *Mallotus oppositifolius*, also gives this extract a bactericidal power on two of the three strains studied, namely the strain of *Salmonella* Bargny and that of *Salmonella* enterica serogroup O: 21; and bacteriostatic power on the strain of *Salmonella* Kentucky.

The phytochemical screening of the ethanolic extract of the leaves of *Mallotus oppositifolius* revealed the presence of five of the 7 groups of chemical compounds except the flavonoids and the alkaloids. On the other hand, two of the 7 groups (polyphenols and tannins) tested were demonstrated in the aqueous extracts. The chemical compounds being included in one or the other of the two extracts of the plant of *Mallotus oppositifolius*, would justify its use for the treatment of certain infections due to pathogenic microorganisms (Adeleye *et al.*, 2008; Mambé *et al.*, 2016; Orsot *et al.*, 2016; Owhe-Ureghe and Akpo, 2016). Indeed, tannins are renowned for their ability to inhibit the growth of many microorganisms including bacteria (Sepúlveda *et al.*, 2011); as for flavonoids, steroids, saponins,

In view of these preliminary results, the aqueous and ethanolic extracts of the leaves of *Mallotus oppositifolius* could be recommended in the treatment of food-borne infections involving the pathogen *Salmonella*, to guarantee the health of the consumer.

Conclusion

This study made it possible to show the antibacterial activity of the leaves of *Mallotus oppositifolius* on avian *Salmonella* strains (multi) resistant to antibiotics and to determine the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of the various extracts used. This activity is bactericidal in nature on two of the three strains studied. This preliminary work could open up other fields regarding the use of a set of plants from the Ivorian pharmacopoeia, for the resolution of the problem of the appearance and the dissemination of enteropathogenic strains (multi) resistant to antibiotics.

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