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In vitro antisalmonellal activities of leaf extracts of *Adenia lobata* Jacq. (Passifloraceae) and mechanism of action of the most active extract on *Salmonella* Typhi ATCC6539

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Abstract
Typhoid fever is a major public health problem in developing countries, where it remains endemic because of the precariousness of lifestyle combined with the misuse and inappropriate use of antibiotics. As a result, the search for new sources of medicines to fight against salmonellosis is becoming a real challenge. Thanks to their richness in bioactive metabolites, medicinal plants are a good source for the production of new antityphoid substances accessible to all layers of the society. The *in vitro* antisalmonella activities of *Adenia lobata* leaf extracts were evaluated on *Salmonella* bacteria and the search for mechanisms by which the extract would act was evaluated on the strain of *Salmonella* Typhi (ATCC 6539).
In vitro antisalmonellal activity of *Adenia lobata* leaf extracts was assessed by liquid microdilution method on *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella* Typhimurium and *Salmonella* Typhi ATCC 6539 strains. Mechanisms of action of extracts that exhibited the best antimicrobial activity on the strain of *Salmonella* Typhi (ATCC 6539) were studied by following bacteriolysis, inhibition of biofilms and bacterial H^+/ATPase proton pumps. Phytochemical screening was performed to justify these activities.
70% ethanol extract of *Adenia lobata* leaves showed the best antisalmonellal activity with the Minimal Inhibitory Concentrations (MICs) values ranging from 8 to 64 µg/ml on the tested bacteria. This extract exhibited bacteriolysis after 4 hours at all concentrations (2MIC, MIC and MIC/2) and was a true inhibitor (IC_{50} = 3.76 µg/ml) of the formation of biofilms in *Salmonella*. H^+/ATPase proton pumps of strain S. Typhi ATCC6539 were inhibited at concentrations equivalent to 2MIC and MIC. Phytochemical analysis revealed the presence of steroids, terpenoids, tannins, phenols, anthraquinones, alkaloids and saponins in leaf extracts of *Adenia lobata.*
These results show that the 70% ethanol leaves' extract of *Adenia lobata* has an interesting antisalmonellal activity, and its bioactif compounds could act on many bacterial defense mechanims.

Keywords: antisalmonellal, *Adenia lobata*, phytochemical screening, mechanism of action

1. Introduction
Infectious diseases are a critical health problem and one of the leading causes of death worldwide, especially in developing countries in general and sub-Saharan Africa in particular [1]. Among these infections, typhoid fever (typhoid and paratyphoid) caused by bacteria of the genus *Salmonella* constitute a public health problem with an overall distribution of 21.6 million cases per year with a mortality rate of 10%, of which 90% in developing countries [2, 3]. Typhoid fever is characterized in the early stage by high fever, anorexia and continuous diarrhea. In advanced stages, prolonged fever is often observed, other symptoms may include intestinal bleeding, mild deafness [4]. The drugs used against these typhoid fevers have long been antibiotics such as penicillins and chloramphenicol. However, the resistance of the germs to these drugs has been observed [5]. One of these is the formation of biofilms, which protect bacteria against the host's immune system, dessication and biocides.
The exacerbation of these diseases in recent years could be explained by non-compliance with health standards, high prices of antibiotics, and the inappropriate use of antibiotics, which causes the phenomenon of resistance of germs to these drugs, the inaccessibility of medicines to some patients due to poverty, the growth of counterfeit medicines [6].
Plants may be an allternative for controlling these germs because they are a valuable and almost inexhaustible source of pharmacologically active molecules [7]. *Adenia lobata* is widely used in traditional medicine to fight against abscesses and wounds, palpitations, coughs, bronchitis, fever, rheumatic pains and abdominal pains. Some studies have shown that *Adenia lobata* has antihyperglycemic [8], antioxidants [9, 10] and anti-hemorrhoids [11] activities. The aim of this study was therefore to evaluate the antisalmonellal activities and the mechanisms of action of the most active extract from *A. lobata* on *Salmonella* Typhi ATCC6539 strain.

2. Materials and methods
2.1 Plant material
The leaves of *Adenia lobata* were harvested in Bandjoun (Koung-khi Division, West region of Cameroon) in September 2016. The plant was identified at the National Herbarium of Cameroon (Yaoundé by Dr. TCHIENGUE Basthelemy (Botanist)) in comparison with the reference sample kept under number 43292/HNC.

2.2 Preparation of extracts
The leaves of *Adenia lobata* were harvested, dried out of the

sun (about 25 °C) and crushed. The obtained powder was used for the preparation of hydroethanol (70% ethanol, 50% ethanol, 30% ethanol), ethanol 95°, and aqueous (infusion, decoction, maceration) extracts.

The preparation of these extracts was made by maceration of 100 g of powder for 48 hours in one liter (1 L) of each solvent (95° ethanol, 70% ethanol, 50% ethanol, 30% ethanol) while stirring twice each day. This mixture (solvent and extract) was subsequently filtered using Whatman N°1 paper. The filtrate obtained was left in an oven set at 45°C for two days for complete evaporation of the hydroethanolic solvents, and concentrated under vacuum at 45° C. Using a rotary evaporator (Büchi R200) for the ethanolic solvent 95°.

The preparation of the aqueous extracts (infusion and decoction) were made following the methods proposed by Duke [12].

- **Macerated extract:** It was obtained by maceration of 50 g of powder in 500 ml of distilled water for 48 hours, shaking three times a day. Subsequently, the mixture was filtered with Whatman N° 1 paper and finally dried in an oven (Memmert) set at 45 °C.
- **Infused extract:** A mass of 50 g of powder was mixed with 500 ml of distilled water previously boiled and the mixture was allowed to infuse for 15 minutes and then filtered with Whatman N°1 paper. The filtrate obtained was dried in an oven (Memmert) set at 45°C.
- **Decocted extract**: It was obtained by introducing 50 g of powder into 500 ml of distilled water and bringing the mixture to a boil for 15 minutes. After cooling, the mixture was filtered with Whatman N°1 paper and the filtrate was dried in a ventilator oven (Memmert) set at 40°C.

2.3 *In vitro* evaluation of the antisalmonellal activity of *Adenia lobata* leaf extracts

2.3.1 Preparation of stock solutions of *Adenia lobata* leaf extracts

The extract solutions were prepared at 4096 µg/ml in 5% DMSO and diluted so that the final concentration varied from 1024 µg/ml to 8 µg/ml. Stock solution of ciprofloxacin (positive control) was prepared at 256 µg/ml in 5% DMSO and diluted so that the final concentration ranged from 64 µg/ml to 0.5 µg/ml.

2.3.2 Preparation of bacterial inocula

The microorganisms used were bacteria of the genus *Salmonella*, consisting of a strain of *Salmonella* Typhi collection "American Type Culture Collection" (ATCC 6539) and isolates of *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella* Typhimurium, all from Bacteriology laboratory of the Pasteur Center of Yaounde (Cameroon). These isolates were stored in the Microbiology and Antimicrobial Substrate in a glycerol/Mueller-Hinton broth (MHB) mixture (1:1) at -4 °C and activation was performed with the streak technique on *Salmonella/Shigella* agar (SSA). This latter was then incubated at 37 °C for 18 hours for activation. The bacterial suspensions were therefore prepared by taking from this culture, colonies which were diluted in sterile physiological water until a turbidity identical to that of the point 0.5 on the scale of Mc Farland, corresponding to a concentration of 1.5×10^8 CFU/ml [13]. These suspensions were diluted with MHB until the desired bacteria concentration for the *in vitro* antibacterial test (1.5×10^6 CFU/ml) was obtained.

2.3.3 Determination of minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC)

The inhibitory potential of bacterial growth of *A. lobata* extracts was determined by the microdilution method as described by Mativandlela *et al.* [14]. Briefly, in each well of a 96-well microplate, 100 µl of culture broth (MHB) were introduced. Then, 100 µl of each extract were introduced respectively into the first 3 wells of the first line; subsequently, serial dilutions following a geometric progression of order 2 were performed. A volume of 100 µl of culture broth plus the bacterial inoculum was introduced into each well. Plates were incubated at 37° C for 18 hours. Wells containing the inoculum as well as those containing only culture media and DMSO were made and were negative controls. After this incubation time, 40 µl of an aqueous solution of 0.2% para-iodonitrotetrazolium chloride (INT) bromide was added to these wells. INT is a colorless reagent in its oxidized form. Bacteria during their growth release NADH into the medium from a bacterial enzyme such as threonine dehydrogenase (TDH), which catalyzes NAD-dependent threonine oxidation to give 2-amino-3-butobutyrate and NADH. The NADH reduces the INT which then becomes pink. The response to INT is based on electron transfer from NADH. Thus, wells that turn pink after addition of INT indicate bacterial growth [14]. All concentrations that prevented the appearance of pink color were taken as the inhibitory concentrations and the smallest was noted as MIC. For each extract, three columns were made and the revelation was made on two columns. The third was used to determine minimum bactericidal concentrations. This test was performed three times.

After reading the different MICs, 150 µl of MHB were introduced into the wells of the new plates, then 50 µl of the contents of each well where there was inhibition of bacterial growth (absence of pink color) were collected using a micropipette, and introduced into the corresponding wells of the new plates. These plates were covered again with a sterile lid. Negative control wells containing only MHB and those containing the inoculum without extract or antibiotics were made. The new incubation was also done at 37°C for 48 hours. The revelation was made as that of the determination of MICs (40 µl of an aqueous solution of INT were added to each well). All extract concentrations for which the absence of bacterial growth was noted (nonappearance of pink color), were considered bactericidal concentrations and the smallest was noted as MBC. This test was repeated 3 times.

2.4 Phytochemical screening of *A. lobata* leaf extracts

Phytochemical tests were performed according to the standard methods as described by Harbone [15] in order to determine the different classes of potential bioactive compounds such as anthocyanins, anthraquinones, steroids, tannins, phenols, saponins, flavonoids, triterpenes, alkaloids present in extracts of *A. lobata.*

2.5 Determination of the mechanisms of action of the 70% ethanolic extract of *A. lobata* Leaf on the *Salmonella* Typhi Strain (ATCC 6539)

2.5.1 Evaluation of anti-biofilm activity of the 70% ethanol extract of *A. lobata*

The anti-biofilm potential of the 70% ethanol extract of *A. lobata* was determined according to the method used by Dong *et al.* [16] with some modifications. For this purpose, a volume of 100 µL of Mueller Hinton Broth was introduced into a 96-well microplate. Then, 100 µL of test substances were introduced into the upper wells and diluted in a geometric progression of order 2. Thereafter, 100 µL of a bacterial suspension equivalent to 1.5 10^7 CFU/ml was introduced into the wells. Final concentrations ranged from 1024 µg/ml to 8 µg/ml for the extract and from 64 to 0.5 µg/ml for ciprofloxacin. The medium consisting solely of the microorganism was used as a negative control and that without extract or antibiotic as a neutral control. Plates were incubated under constant shaking (130 rpm) for 24 hours at 37°C. At the end of incubation, the planktonic cells (those which did not form biofilms) were removed from the microplates by washing in 50 mM phosphate buffered saline (pH 7.4, 25 mM NaCl).

The biofilm formed by the adherent cells was fixed to 3-[4,5-dimethyl-2-thiazolyl] -2,5-diphenyl-2H-tetrazolium-bromide (MTT) for 30 minutes at laboratory temperature and then rinsed with sterile distilled water and dried at room temperature.. Subsequently, 100 µl of DMSO were introduced to solubilize the reduced formazan. The optical densities were measured using an ELISA microplate reader (Dialab, France) at 630 nm. The tests were repeated three times and the percentage inhibition of the biofilm was calculated according to the formula:% I = [(OD negative control – OD test) / OD negative control] × 100 (référence). The regression lines were used to determine the inhibitory concentration 50 (IC_{50}) of the extract as well as that of the reference antibiotic.

2.5.2 Measurement of the Bacteriolytic Activity of the Ethanol Extract 70% of *A. lobata*

The protocol established by Limsuwan and Voravuthikunchai [17], was used in this experimentation. Indeed, an unlysed bacterium absorbs light at 620 nm, so there will be bacteriolysis if the absorbance at 620 nm decreases over time [18]. Briefly, the bacterial colonies seeded for 18 h on agar medium were used for the preparation of standardized bacterial suspension at the scale of 0.5 Mc Farland in NaCl 0.9%. A volume of 500 µl of plant extract was introduced in triplet into different tubes containing this suspension so as to have concentrations of 2MIC, MIC and MIC/2 in the medium. The suspensions obtained were incubated at 37°C with stirring (130 rpm). At 0 h, 2 h, 4 h and 6 h, the absorbances were measured at 620 nm and those of the 0 h time were used to evaluate the relative absorbances at different times to plot the relative absorbance curve = f (t).

2.5.3 Evaluation of the effect of 70% ethanol extract of *A. lobata* on bacterial ATPases-H^+ proton pumps

The ability of 70% ethanolic extract of *A. lobata* to inhibit *Salmonella* Typhi ATPases-H^+ ATCC6539 was evaluated by monitoring the acidification of the external medium through pH measurement with the help of a pH meter (Hanna INSTRUMENTS: HI 2211, Woonsocket Rhode Island, USA), as described by Manavathu *et al.* [19], but with some modifications.

Briefly, a bacterial culture was previously prepared in a liquid medium. For this purpose, conserved bacterial cells (in a MHB-glycerol mixture) were streaked into 90 mm petri dishes, then the culture was incubated at 37°C for 18 hours at the end of which a bacterial colony was obtained and introduced into a volume of 20 ml of MHB contained in an conical flask. The mixture was then incubated at 37°C with stirring. After 18 hours of incubation, the obtained pre-culture of bacteria (OD_{600} = 1, corresponding to a bacterial load of approximately 3 × 10^8 CFU/mL) was used for bacterial culture for subsequent experiments. Aliquots of bacterial pre-culture were thus removed and introduced into conical flasks containing MHB at a rate of 1 ml of preculture for a final volume of 100 ml (1/100 v/v dilution). After 18 hours of incubation with stirring at 37°C, a volume of 100 ml of bacterial culture was centrifuged at 4000 rpm for 30 minutes under cold conditions. The obtained pellet was washed with distilled water, then 50 mM KCl and resuspended in 50 ml of KCl. The suspension was kept at 4°C for 18 hours. To a volume of 4 mL taken from this suspension, 0.5 ml of the sample solution (at MIC) dissolved in MHB was added. After 10 minutes of preincubation at 37° C, the acidification of the medium was started by adding 0.5 ml of a 20% (w/v) glucose solution.

Subsequently, the pH of the medium was measured every 15 minutes for 90 minutes. The negative control consisted of the suspension plus glucose solution 20% (w/v) and the positive control of ciprofloxacin. The test was repeated three times and the pH values noted allowed the plotting of the pH progression curves as a function of time [pH = f (time)].

The significant inhibition of the pH decrease in the presence of an antibacterial is attributed to an inhibitory effect of the operation of the ATPases-H^+ pumps. This inhibition may be detrimental to the survival of the bacterium in that; the proton pumps provide the bacteria with the energy necessary for the proper course of its metabolism and therefore its development (Kobayashi, 1985), this by extrusion of protons from bacterial cytoplasm.

2.6 Statistical analyses

The results of the various tests (mechanism of action) were subjected to analysis of the variance (ANOVA) and expressed in the form of means ± ESM. Differences between averages where it existed were assessed using the Waller-Duncan test at the 5% probability level. The SPSS software version 21 for Windows was used for this purpose.

3. Result and discussion

3.1 *In Vitro* Antisalmonellal Activities of leaf extracts of *Adenia lobata*

The various leaf extracts of *Adenia lobata* showed variable activity on the bacterial isolates and strain studied. The results of this test are presented in Table 1. It appears that the 95° and 70% ethanol extracts showed a high activity with MIC values of ≤ 64 µg/ml on less than 4/5 of the bacteria tested. In addition, the 70% ethanolic extract exhibited the best activity on the *Salmonella* Typhi ATCC6539 strain and the *Salmonella* Paratyphi B isolate with the MIC values of 64 and 8 µg/ml respectively. Among the aqueous extracts, the most active was the aqueous macerate on all the isolates and strain tested with MIC values between 64 and 128 µg/ml with the exception of the

Salmonella Paratyphi B isolate.

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of different leaf extracts of *A. lobata* on isolates and strains of *Salmonella*.

Substances tests		Strain/isolates				
		STs	ST	SPA	SPB	STM
Ethanol 95°	MIC (µg/mL)	128	64	32	64	32
	MBC (µg/mL)	512	512	256	512	512
	MBC/MIC	4	8	8	8	16
Ethanol 70%	MIC (µg/mL)	64	64	32	8	32
	MBC (µg/mL)	256	256	256	128	512
	MBC/MIC	4	4	8	16	16
Ethanol 50%	MIC (µg/mL)	256	1024	512	-	1024
	MBC (µg/mL)	512	-	-	-	-
	MBC/MIC	2	-	-	-	-
Ethanol 30%	MIC (µg/mL)	128	64	256	128	128
	MBC (µg/mL)	512	256	512	512	512
	MBC/MIC	4	4	2	4	4
Infused extract	MIC (µg/mL)	512	256	256	512	256
	MBC (µg/mL)	-	1024	1024	-	1024
	MBC/MIC	-	4	4	-	4
Decocted extract	MIC (µg/mL)	256	512	128	128	256
	MBC (µg/mL)	1024	1024	1024	1024	-
	MBC/MIC	4	2	8	8	-
Macerated extract	MIC (µg/mL)	128	128	64	256	64
	MBC (µg/mL)	512	-	1024	-	1024
	MBC/MIC	4	-	16	-	16
Ciprofloxacin	MIC (µg/mL)	2	2	0,5	1	2
	MBC (µg/mL)	8	8	2	32	4
	MBC/MIC	4	4	4	32	2

STs: strain of *Salmonella* Typhi (ATCC 6539), ST: *Salmonella* Typhi, SPA: *Salmonella* Paratyphi A, SPB: *Salmonella* Paratyphi B, STM: *Salmonella* Typhimurium; MIC= Minimal Inhibitory concentration; MBC= Minimal Bactericidal Concentration; -: more than 1024 µg/ml.

Extracts of *A. lobata* for those that existed showed MBC values varying between 256 and 1024 µg/ml. The MBC/MIC ratios ≤ 4 were observed with the 30% ethanol extract and the aqueous infusion on the tested isolates and strain as well as all the *A. lobata* extracts on the *Salmonella* Typhi strain ATCC6539. However, the MBC/MIC> 4 ratios were observed with 95% ethanolic extract on all isolates.

3.2 Qualitative phytochemical composition of leaf extracts of *Adenia lobata*

Table 2 shows the phytochemical composition of leaf extracts of *Adenia lobata*. This table reveals the presence of different groups of secondary metabolites. Flavonoids, triterpenes, phenols, and steroids are present in all extracts while alkaloids were only absent in 50% ethanol extracts. The 70% and 95° ethanol extracts revealed the presence of all the tested groups of metabolites, with the exception of Anthraquinones, which were absent in the 95° ethanol extract and in the other extracts. The absence of anthocyanins, tannins, and saponins was observed in aqueous extracts with the exception of aqueous macerate which revealed the presence of saponins.

Table 2: Phytochemical composition of leaf extracts of *Adenia lobata*

Group of metabolite	Extracts						
	Ethanol 95°	Ethanol 70%	Ethanol 50%	Ethanol 30%	Aqueous infused	Aqueous Decocted	Aqueous Macerated
Anthocyanin	+	+	–	–	–	–	–
Anthraquinone	–	+	–	–	–	–	–
steroids	+	+	+	+	+	+	+
Tannins	+	+	+	+	–	–	–
Phénols	+	+	+	+	+	+	+
Saponins	+	+	+	+	–	–	+
Flavonoids	+	+	+	+	+	+	+
Triterpenes	+	+	+	+	+	+	+
Alkaloids	+	+	–	+	+	+	+

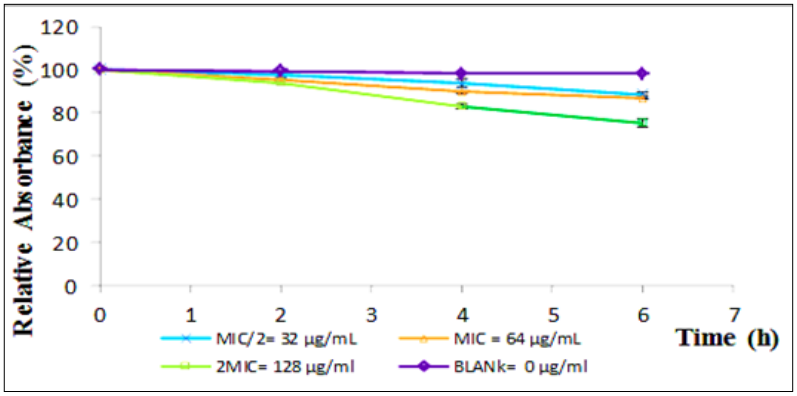
+ : Present; – : Absent.

3.3 Mechanisms of action of 70% ethanol extract of *A. lobata*

3.3.1 Measurement of bacteriolytic activity

The results of the relative absorbances of the 70% ethanol extract of *A. lobata* are summarized in Figure 1. It appears that the relative absorbances of the different concentrations remained above 50% up to 6 hours. From a statistical point of view, the relative absorbances at 2 h remained insignificant ($p \geq 0.05$) compared to blanks (0 µg/ml). A significant decrease in absorbances ($p < 0.05$) concentration dependent was observed at 4 h. The lowest relative absorbance (75.49%) was obtained for 2MIC at 6 h while

that of the other concentrations (MIC and MIC/2) remained comparable at 6 h.



These values are the averages of three replicates of each extract concentration.

Fig 1: Relative Absorbance of Bacteria at Different Concentrations of 70% Ethanolic Extract

3.3.2 Effect of 70% ethanol extract of *A. lobata* on biofilm formation

Concentrations that inhibit 50% of biofilm formation were obtained by plotting regression lines of different percent inhibition of extract and ciprofloxacin (Figure 2). These results are summarized in (Figure 3). It appears that the extract inhibited the formation of biofilms with an Inhibitory Concentration 50 (IC_{50}) of 3.76 µg/ml. This value, however, remained very high compared to the reference molecule (ciprofloxacin).

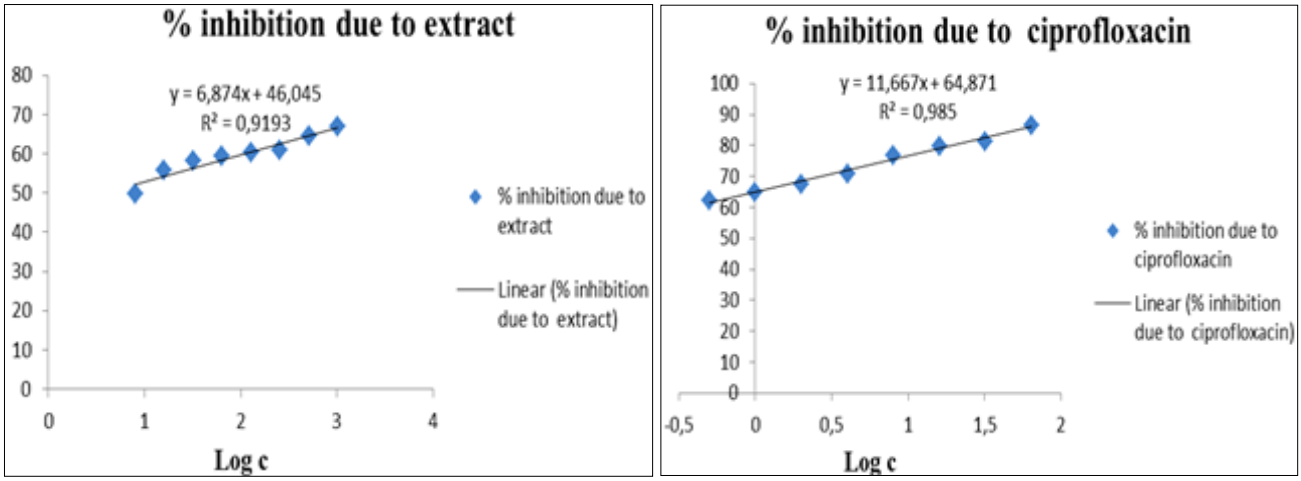


Fig 2: Line of regression inhibition percentages of the extract and ciprofloxacin

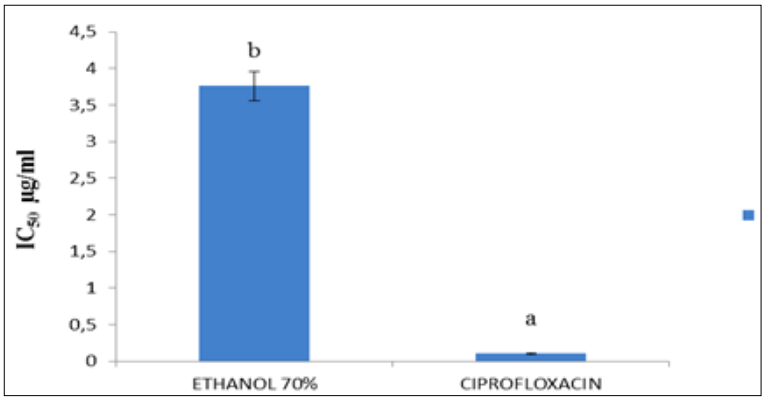


Fig 3: IC50 values of ethanol extract 70 % of *A. lobata* on inhibition of biofilms.

3.3.3 Effect of 70% ethanolic extract of *A. lobata* on bacterial H^+ proton pumps

Figure 4 below shows pH variation curves of culture media inoculated with the strain *Samonella* Typhi ATCC6539 as a function of time, and in the presence of the most active 70% ethanolic extract of *A. lobata*. It appears that the pH values of the medium decreased very slightly during the first 30 minutes at all concentrations. From 45 minutes, the pH decreased significantly ($p < 0.05$) to the concentration equivalent to MIC/2 compared to other concentrations but remained high compared to blank (0 µg/ml). However, at every minute the PH values remained comparable ($p \geq 0.05$) between the concentrations equivalent to 2MIC and MIC.

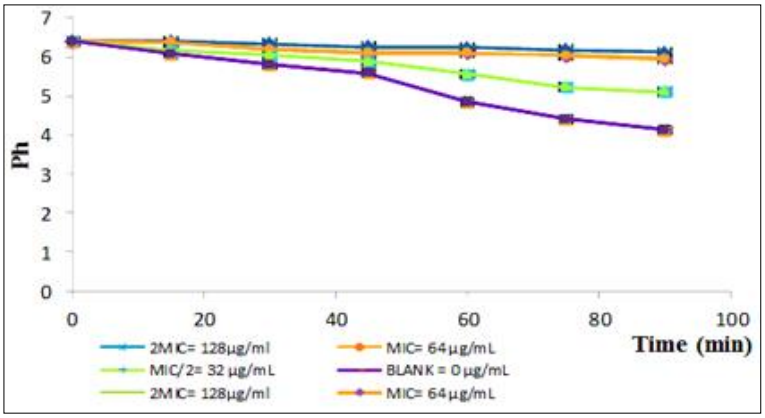


Fig 4: Effect of ethanol extract 70% of *A. lobata* on the variation of the pH of culture medium inoculated with the strain *S.* Typhi ATCC6539 (n = 3 repetitions)

4. Discussion

4.1 *In vitro* antibacterial activities

In Leaf extracts of *Adenia lobata* showed important antisalmonellal activities. This antibacterial activity varied according to the solvent and the extraction method. The 70% ethanol extract was the most active (MIC ≤ 100 µg / ml) against the strain and the four isolates tested. However, significant activities (MIC less than 100 µg/ml) were also found with the 95° ethanol extract on all isolates. In fact, the antibacterial activity of plant extracts is considered significant when the MIC <100 µg/ml, moderate when 100 µg/ml ≤ MIC <625 µg / ml and low when the MIC > 625 µg/ml [13]. The activity of leaf extracts of *Adenia lobata* also varied according to the extraction method (maceration, infusion, decoction). Macerate was the most active of the aqueous extracts. Indeed, The differences in inhibitory activity observed with the same extract against the isolates would be due to the difference in genetic and structural composition of the isolates and the tested strain. Whereas the differences observed for the same isolate or strain with the different extracts could be explained by the different mechanisms of action of the active principles present in the plant or by the quantitative variations of the active metabolites in each extract [20].

The presence of potential antimicrobial substances was identified by the phytochemical screening of *Adenia lobata* leaf extracts which revealed the presence of phenols, flavonoids, tannins, saponins, anthocyanins, triterpenes, steroids and alkaloids. This corroborates the work of Agoreyo [10] who had already demonstrated the presence of some of these compounds in the methanol leaf extract of *Adenia lobata*. These potential activities could be justified by the wealth of bioactive molecules in this extract. Indeed, several authors have already highlighted the antibacterial activity of terpenoids and steroids [21, 22]. The presence of tannins is also important in the antimicrobial activity of these extracts [23]. Anthraquinones have also been found in the ethanol extract and may also have antibacterial activity such as friedeline whose antibacterial activity has been demonstrated by Tamokou *et al.* [24]. These active ingredients may act at the level of the cell wall, at the level of the membrane constituents, or may enter the cytoplasm where they act at the level of ribosomes, or interfere with biochemical processes important for the life of the microorganism [25].

Depending on the mechanisms of action, these active ingredients could have a bacteriostatic or bactericidal effect on microorganisms. Many studies [26] indicate that the substances are considered bacteriostatic agents when the ratio MBC/MIC > 4 and as bactericidal agents when the MBC/MIC ratio ≤ 4. In this study, leaves' extracts from *A. lobata* were bactericidal on the *Salmonella* Typhi ATCC6539 strain and varied bacteriostatic activities on all isolates.

4.2 Study of the mechanisms of action of 70% ethanol extract of *A. lobata, the* most active on *S.* Typhi ATCC6539

4.2.1 Effect of 70% ethanol extracts on the bacterial plasma membrane

The bacterial membrane is a barrier for protecting and maintaining cellular homeostasis since it controls the entry and exit of several substances. Its alteration, due to the presence of antibacterial substances can lead to complete lysis and could therefore be harmful to the bacteria. The measurement of the capacity of the 70% ethanol extract (the most active extract on *S.* Typhi ATCC6539) to cause the bacterial lysis was made by following the optical density at 620 nm. This lysis was marked by a decrease in the absorbance of the solution over time [18]. The significant decrease in the relative absorbance observed at all concentrations of the 70% ethanol extract after 4 h indicates the good bacteriolytic activity of this extract. This obtained bacteriolytic activity could be attributed to the presence of compounds belonging to the class of terpenes and flavonoids. Indeed, flavonoids by their lipophilic nature can disrupt the balance of the bacterial membrane resulting in membrane expansion, an increase in membrane fluidity and permeability, a disruption of membrane proteins, an inhibition of respiration and an alteration of the membrane ion transport process [27]. The study conducted by Lopez-Romero *et al.* [28], on the mechanisms of action of some constituents of essential oils such as citronellol and citronellal which are compounds belonging to the class of terpenes have a strong activity in the lysis of the bacterial membrane, especially in Gram negative bacteria (*E. coli*).

4.2.2 Effects of 70% ethanol extract of *A. lobata* on the operation of ATPases/H^+ proton pumps

The ability of the 70% ethanol extract of *A. lobata* to inhibit ATPase/H^+ proton pumps was evaluated against *Salmonella* strain Typhi ATCC6539. In fact, the bacterial cell imports from the culture medium, nutrients necessary for its growth. This transport involves a solute-H + osmo-osmotic coupling

performed by a symport and an ATP/H^+ chemo-osmotic coupling carried out by an H^+/ATPase pump [29]. The transport of nutrients is therefore largely dependent on the maintenance of an electrochemical proton gradient. For this reason, H^+/ATPase proton pumps are present in large quantities and can also regulate intracellular pH, which increases exponentially during the metabolism of sugars (production of organic acids) [29]. The inhibition of these pumps will therefore be deleterious for the bacterium because it will prevent the excretion of protons in the external medium, thus making the medium less acidic, compromising the survival of the bacterium. The 70% ethanolic extract exhibited complete inhibition of H^+/ATPase proton pumps of strain *S.* Typhi ATCC6539 at concentrations equivalent to MIC and 2MIC compared to control. This inhibitory potential could be justified by the varied presence of bacteriocidal compounds in this extract.

4.2.3 Effect of 70% ethanol extract of *A. lobata* on inhibition of biofilm formation in *S.* Typhi ATCC6539

Biofilms are structured clusters of bacterial cells coated with a polymeric matrix composed of exopolysaccharides, proteins and nucleic acids attached to a biotic or abiotic surface [30]. These biofilms protect bacteria against host immune system, desiccation and biocides. In addition, several resistance mechanisms are used by biofilms against antimicrobial agents and are responsible for much of the tolerance associated with biofilm [31, 32]. The capacity of the 70% ethanol extract to inhibit 50% of the formation of biofilms in *Salmonella* showed an important inhibitory effect (IC_{50} = 3.76 µg/ml) although *salmonella* of a biofilm are known to have a higher high resistance than planktonic *salmonella* [33]. This ability to inhibit the formation of biofilms resides in the rather rich and diverse phytochemical composition of the extract. In fact, these compounds can influence biofilm formation by damaging microbial membrane structures [34], inhibiting peptidoglycan synthesis and/or modulating quorum sensing detection [35]. Moreover, the existence in this extract of phenolic compounds which has already shown its ability to inhibit the formation of biofilms [36] would support this result.

5. Conclusion

The results presented in this work show that the hydroethanol and ethanolic leaves' extracts of *Adenia lobata* have interesting antisalmonella activities, and the bioactive compounds found in these extracts could act on bacterial defense mechanisms. However, further studies should be conducted to investigate the antimicrobial properties *in vivo* and the reduction of oxidative stress caused by this infection.

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7. References

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