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Anti-*Salmonella* activity of plant species in the Benin republic: *Artemisia afra* and *Detarium senegalense* with promising *in vitro* and *in vivo* activities

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A B S T R A C T

Non-typhoidal invasive *Salmonella* (NTiS) diseases are one of the most important zoonoses in the world. This study explored the antipathogenic potential of twenty-four plants used in Benin folk medicine against NTiS diseases. The *in vitro* antibacterial and antibiofilm activities of ethanolic plant extracts were screened against clinical resistant isolates and ATCC reference strains of *Salmonella*. *Salmonella enterica* serovar Typhimurium-infected rat model was used to examine the *in vivo* antibacterial potential of plant extracts. Of the 24 plants, 18 plants exhibited antibacterial activity against *Salmonella enterica* strains with minimum inhibitory concentrations (MICs) ranging from 0.156 to 1.25 mg/mL. *Anacardium occidentale*, *Artemisia afra, Detarium microcarpum*, *Detarium senegalense*, and *Leucaena leucocephala* were the most active plant species. Extracts from *A. afra, D. microcarpum,* and *D. senegalense* showed biofilm inhibition greater than 50% against *Salmonella* clinical isolates. In the rat model of infection, *A. afra* and *D. senegalense* extracts were found to have an effective dose of less than 100 mg/kg and to stop the salmonellosis after 10 days of treatment. Additionally, these extracts did not produce any toxic effects in the treated animals. These results indicate clear evidence supporting the anti-*Salmonella* activity of *A. afra* and *D. senegalense*. Further studies are now needed to isolate bioactive compounds and to ensure the safety of these plant species.

1. Introduction

Zoonotic diseases represent about 60% of emerging infectious diseases worldwide and constitute a critical threat to global health security [1]. Human salmonellosis is one of the most common and important zoonoses, with about 93.8 million cases of gastroenteritis and 155,000 deaths occurring each year in the world [2]. Non-typhoidal *Salmonella* serovars, such as *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis are the principal cause of this zoonosis [3]. These serovars are carried by a wide spectrum of animals including poultry and cattle, and animal-based foods such as beef, pork, poultry, raw eggs, dairy products, as well as fruits and vegetables contaminated [4]. Besides diarrheal disorders, non-typhoidal salmonella infections can invade sterile sites and lead to focal infections (*e.g.*, bacteremia). These invasive non-typhoidal *Salmonella* (iNTS) infections are susceptible to cause mortality in vulnerable people such as children under 5 years old, immunocompromised individuals, and children with malaria, anemia, and malnutrition [5,6]. In 2017, there were about 535 000 cases of non-typhoidal *Salmonella* invasive diseases with 77 500 deaths reported. Sub-saharan Africa was the most impacted region of the world [7].

In Benin, the low level of sanitation and the excessive use of

Abbreviations: AB, Attached bacteria; ALT, Alanine transaminase; AST, Aspartate transaminase; ATCC, American Type Culture Collection; BF, Biofilm formed; BL, Bacterial load; CFU, Colony-forming unit; Crea, Creatinine; CW, Control well; ECDC, European Centre for Disease Prevention and Control; ED, Effective dose; EFSA, European Food Safety Authority; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; FI, Frequency index; Hb, Hemoglobin concentration; Hct, Hematocrit; LD, lethal dose; Ly, Lymphocytes; MCH, Mean corpuscular hemoglobin levels; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; MHA, Mueller Hinton Agar; MHB, Mueller Hinton Broth; MIC, Minimum Inhibitory Concentration; Mo, Monocytes; NTiS, Non-typhoidal invasive *Salmonella*; OD, Optical density; OECD, Organization for Economic Co-operation and Development.; SD, Standard Deviation; Rbc, Erythrocytes; Wbc, Leucocytes.

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antibiotics in farms by some breeders in remote areas, the lack of adequate conservation tools, and malnutrition are principal factors favoring the emergence of *Salmonella* diseases. The main strategies for controlling salmonellosis in humans have been based on the use of antibiotics, especially fluoroquinolones and third-generation cephalosporins [8]. This type of treatment is becoming less effective due to the emergence of antibiotic-resistant *Salmonella* serotypes, leading to an increase in the mortality rate of *Salmonella* infections, particularly in immunocompromised people [9]. Most importantly, developing countries are particularly affected by the rise of antibiotic resistance in nontyphoidal *Salmonella* serotypes and new therapeutic strategies are urgently needed to face this challenge [10-12].

Medicinal plants represent a great source of secondary metabolites with antimicrobial properties, and thus could be used to prevent or cure iNTS disease [13,14]. Recently, some medicinal plants traditionally used for treating typhoid fever have been documented in southern Benin [15]. This study highlights the high potential of the Beninese flora for its anti-*Salmonella* activity; however, the ethnobotanical survey deserves to be extended to other parts of Benin with great cultural and biological diversity. The Plateau department of Benin is made up of a predominantly rural community that depends mainly on plant resources for their health care. In this province, the dominant ethnic groups are the Nagot and Yoruba practicing agriculture and livestock as their main economic activities. So far, no studies have evaluated the traditional knowledge for tackling salmonellosis in this area. Thus, this study records information on the plants used by traditional practitioners from the Plateau department of Benin for treating salmonellosis and to provide pharmacological evidence for their activity, especially their *in vitro* antibacterial and antibiofilm activities, along with their *in vivo* effects on *Salmonella* infected-rats.

2. Materials and methods

2.1. Ethnobotanical survey

Ethnobotanical data (vernacular name, plant parts used, mode of preparation and administration) were obtained from local traditional healers in five communes (Ifangni, Sakété, Pobè, Adja-ouèrè and Kétou) of the Plateau department (Fig. 1). A structured questionnaire focusing on the plants used for treating affections related to salmonellosis (*i.e.*, diarrhoea, fever, nausea, vomiting and stomach cramps) was used. The communes lie approximately between 6° 40′ - 7° 21′ north and 2 ° 36′ - 2° 41′ east. In each commune, five traditional healers (25 in total) were interviewed in their local language (Nagot or Yoruba) after being informed about the objective of this study. Prior informed consent was obtained from each informant. Different fresh plant parts from all species commonly used by local traditional healers to treat *Salmonella*-related effects in the study area were collected from their natural habitats in Benin between October and December 2021. Voucher specimens in duplicate were deposited at the National Herbarium of Abomey

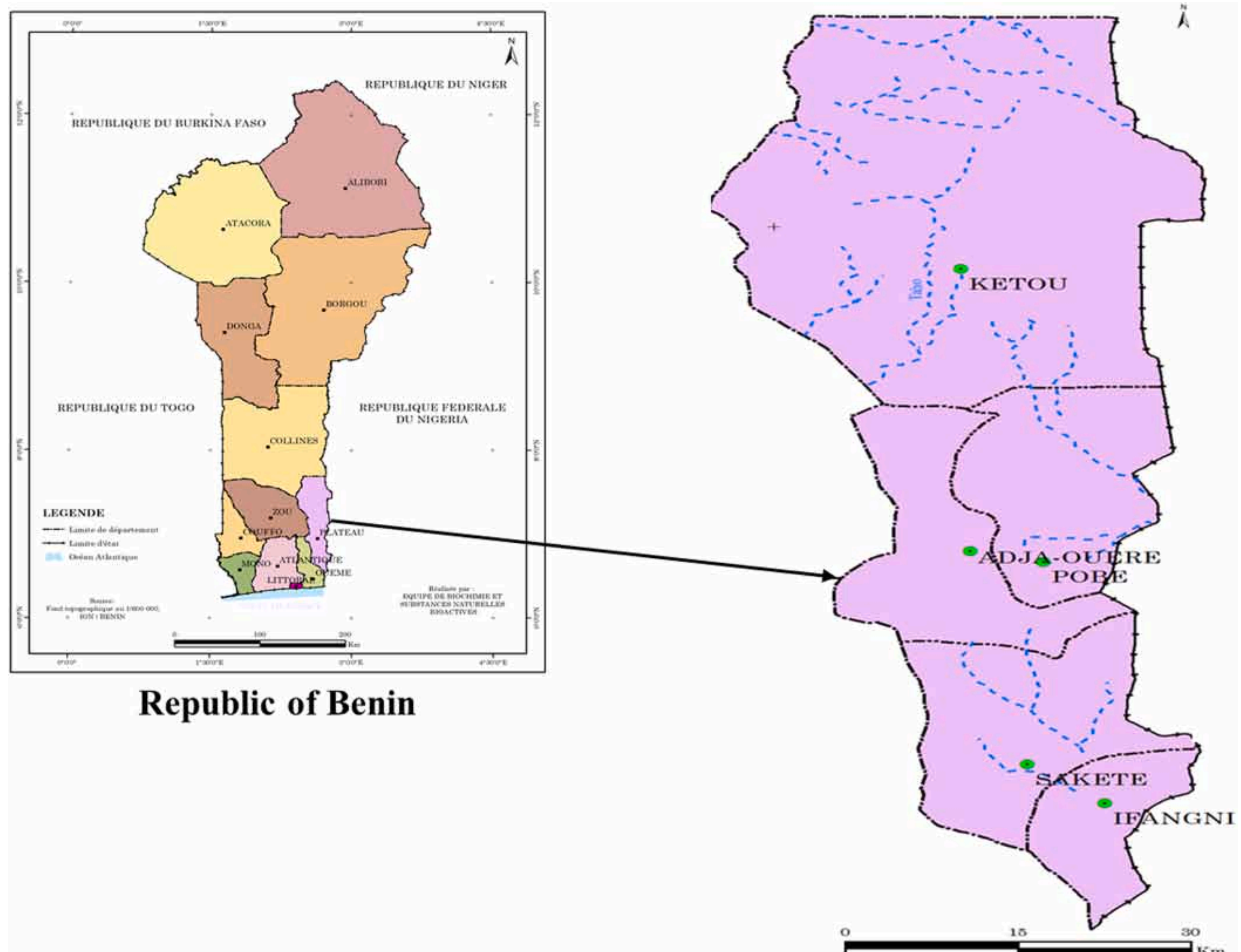

Fig. 1. Map showing the study area in the Plateau department of Benin

Calavi University (BENIN). Professor Hounankpon Yedomonhan, botanist and curator of this herbarium carried out the botanical identification. The frequency index (FI) was calculated using the following formula: FI = (n/N) x 100. Where n represent the number of traditional healers who mentioned the use of plant species, and N is the total number of traditional healers interviewed. Authorization to access plant samples and traditional knowledge was granted by the Abomey-Calavi University.

2.2. Preparation of plant extracts

The plant material collected was air-dried following laboratory conditions (22 °C ± 2) then reduced to a fine powder using an electric blender (Sokany SK-444) before being subjected to extraction. For each plant collected, the powdered material (500 g) was macerated with 5 L ethanol (70%) for 48 h. The plant residue was subjected twice to the same operation for 12 h. The extracts obtained were semi-evaporated under reduced pressure, below 40 °C (110 mbar) and then freeze-dried (see Supporting data file: Table S1 for extraction yield).

2.3. Experimental animals

Male and female Albinos rats aged of 6-weeks with body weights ranging from 180 to 200 g were obtained from the animal facility located in the Human Biology Laboratory, Faculty of Health Sciences, Abomey-Calavi University, Benin. All rat experiments were carried out with respect for the welfare of animals as recommended by WHO Ethical clearance for the animal. This study received ethical approval from the ethical committee of the Abomey-Calavi University (UAC/FAST/EDSV/LBSNB 10208507–2021). One week before beginning the experiment, rats were maintained in clean metabolic cages (1 rat/cage), acclimatized (22–25 °C on a light/dark cycle of 12 h) and had free access to food (rat pellets) and water *ad libitum*.

2.4. Salmonella strains and culture conditions

The *Salmonella* strains (Gram-negative bacteria) used in this study included the standard laboratory strains: *Salmonella enterica* subsp. *enterica* serovar Typhi (ATCC 19430), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), *Salmonella enterica* subsp. *enterica* serovar Enteritidis (ATCC 13076)] and three local isolates of *S.* Typhimurium (P70, L22, and R309) which were obtained from the Research Unit in Applied Microbiology, Abomey-Calavi University, Benin (see Supporting data file: Table S2). Salmonella–Shigella agar medium (SS agar) (Thermo Scientific™ Oxoid™) was used for purity analysis of all *Salmonella* strains, and the Mueller Hinton medium was employed for the plant extracts susceptibility tests. Bacterial strains were inoculated onto Mueller Hinton agar plates (MHA, Difco), then incubated at 37 °C to obtain fresh colonies of growing *Salmonella*. After 24 h of incubation, one or two colonies of the pre-cultures were inoculated into Mueller Hinton Broth (MHB, Difco), then incubated at 37 °C for 18 h. Bacterial turbidity was standardized to match the 0.5 McFarland standard ($\sim 10^8$ CFU/mL) according to Clinical and Laboratory Standard Institute guidelines [16]. The standardized bacterial solutions were subsequently diluted to 1:200 with fresh MHB medium to obtain the final testing inoculum (5×10^5 CFU/mL).

2.5. In vitro antibacterial activity

The minimum inhibitory concentrations (MIC) of plant extracts were determined using a modified version of the micro-dilution test using 96-well microtiter plates [17]. The crude extracts were dissolved in acetone 30%. Briefly, 25 μL of crude extracts (20 mg/mL) were added to 175 μL of sterile MHB previously contained in the first wells of a 96-well microplate. Then, two-fold serial dilution in sterile MHB was performed to obtain extract concentrations ranging from 2.5 to 0.019 mg/mL. The plate wells were finally inoculated with 100 μL of bacterial suspension (5×10^5 CFU/mL). Ciprofloxacin (Sigma-Aldrich) and acetone 4% were used as the positive and negative controls, respectively. Each experiment was performed in triplicate. Bacterial growth inhibition was determined using p-iodonitrotetrazolium chloride as a bacterial viability indicator [18], and was calculated by comparing bacterial growth in negative control wells and in treated wells.

2.6. Biofilm inhibition activity

Plant extracts showing the lowest MICs in the antibacterial assay (Section 2.5) were investigated for their anti-biofilm activity against local isolates of *S.* Typhimurium (P70, L22, and R309). The crystal violet assay was used to evaluate the anti-biofilm activity of plant extracts using a sterile flat-bottom polystyrene 96-well microtiter plate [19]. Briefly, 200 μL of *Salmonella* strains cultures with a standardized concentration (1.0×10^6 CFU/mL) were added to each well microtiter plate, and plant extracts reconstituted in acetone 4% were added to obtain concentrations of 2.5, 1.25, 0.625, 0.312, and 0.156 mg/mL. Wells containing (i) sterile bacteria-free MHB with acetone 4%, (ii) sterile MHB with inoculation and (iii) sterile MHB with inoculation and various concentrations of plant extracts were used as negative control, bacteria control and treatment, respectively. Plates were incubated at 37 °C during 24 h without shaking. After incubation, absorbance was measured at 600 nm using a microplate reader (Rayto rt-6500, China) to record the bacteria cell growth in MH broth. Then, the contents of each well were gently removed and microtiter plate wells were washed five times with sterile distilled water to remove the free-floating bacteria. The plates were air-dried for 45 min and the biofilm formed in each well was stained over 45 min at room temperature (22 °C) with 150 μL of crystal violet solution (1%). After staining, the excess of stain was gently removed and the plates were washed with sterile distilled water five times. Then, the microplates were air-dried for 15 min and 200 μL of ethanol (95%) was added to each well to elute the crystal violet bound to the biofilm. The plates covered were incubated for 15 min at room temperature and 100 μL from each well was transferred to a new microtiter plate where the absorbance was measured at 600 nm using a microplate ELISA reader (Rayto rt-6500, China). All assays were performed in triplicate and the mean optical density (OD) was considered. The bacteria biofilm formed in the presence of plant extracts was determinate using the following formula:

BF = (AB − CW), where BF is the biofilm formed, AB is the OD600 nm of the attached and stained bacteria (wells contaning sterile MHB with inoculation and plant extracts) and CW is the OD600 nm of the negative control wells (wells containing sterile bacteria-free MHB with acetone 4%). The antibiofilm activity of plant extracts was compared to bacteria control (average of OD600 nm of wells containing sterile MHB with inoculation).

2.7. In vivo assay using Salmonella infected-rats

The animal experiments were conducted with strict respect to the guidelines of care and use of Laboratory Animals [20]. After being individually marked on the tail using different indelible ink markers, the rats were randomly distributed into thirteen experimental groups (G1, G2,…. G13) of eight rats each (4 males and 4 females). After the acclimatization period, animals were subjected to 16 h of starvation and only had water *ad libitum*. Salmonellosis was induced in animals by gavage method [21]. To facilitate infection in animals, all rats (except the control group: G1) were immunosuppressed with oral administration of cyclophosphamid (30 mg/kg body weight) two days before infection. After two days of immunosuppression, all animals were fasted overnight, then fed with 1 mL of *S.* Typhimurium suspension (1.5×10^8 CFU/mL) in 0.9% NaCl solution, except animals from groups 1 and 2. Rats were then daily treated for 10 days according to the following plan:

the groups G1, G2, G3, and G4 represented respectively the neutral control group (untreated and non-infected rats, which received sterile distilled water at 10 mL/kg body weight), immunosuppressive control group (uninfected rats, immunosuppressed for two days with cyclophosphamid and receiving sterile distilled water during the treatment period), *Salmonella* control group (immunosuppressed rats, *Salmonella* Typhimurium-infected, untreated and receiving sterile distilled water during the treatment period) and positive control group (immunosuppressed rats, *Salmonella* Typhimurium-infected and daily treated with ciprofloxacin at 8 mg/kg body weight). The nine remaining groups (G5 to G13) represented the test groups, which consisted of immunosuppressed rats, *Salmonella* Typhimurium-infected and respectively treated with three doses (100, 200 and 300 mg/kg) of aqueous extracts from *A. occidentale* (G5, G6, and G7), *D. senegalense* (G8, G9, and G10) and *A. afra* (G11, G12, and G13). The extract concentrations tested in the *in vivo* assay were chosen on the basis of the MIC results obtained against *S.* Typhimurium during the *in vitro* assay and the traditional healers indications (*i.e.* 120 mg/kg body weight). We also considered previous work on the oral lethal dose-50 (LD_{50}) of the selected plants (> 2000 mg/kg) to define the concentrations to be tested [22,23]. Feces samples were daily collected from each animal after *Salmonella* Typhimurium-infection and suspended (20 mg/mL) in saline-distilled water (0.9% NaCl). Serial dilutions of feces suspensions were performed and aliquots of 100 μL were inoculated on triplicate Salmonella-Shigella agar. Agar plates were subsequently incubated at 37 °C for 24 h. *In vivo* antibacterial activity of tested extracts was expressed in colonies forming units per gram of fecal matter (CFU/g) by counting viable colonies of *S.* Typhimurium on Salmonella-Shigella agar plates. At the end of 10 days of treatment, animals were overnight fasted on the 11th day and then anaesthetized *via* intraperitoneal injection of sodium thiopental (50 mg/kg). Blood samples were then collected through retro-orbital puncture and, respectively, dispensed into ethylenediaminetetraacetic acid (EDTA) and non-heparinized tubes for hematological and biochemical parameters analysis. The automata hematological (Sysmex, XP-300, Japan) and biochemical (Erba Chem 7, Germany) were used to analyze blood samples as previously described [24]. After euthanasia, the liver and kidneys of each animal were isolated and preserved in 10% buffered formalin for histological examination [24].

2.8. Statistical analysis

All experiments were carried out in triplicates and data were expressed as mean ± SD (Standard Deviation). The GraphPad Prism v.8.2 software was used to conduct statistical analysis. Analysis of variance (ANOVA) with a confidence level of 95% was used to determine the differences among the study parameters. Means were considered statistically different at $p < 0.05$.

3. Results

3.1. Ethnobotanical survey

A total of 25 traditional healers with an age ranging from 40 to 80 years were interviewed. A large proportion of the tradipractitioners were male (19.76%). Most of the informants were analphabetics and only 32% had a primary education level. The traditional healers mentioned 24 plant species belonging to 14 botanical families for treating salmonellosis. Table 1 summarizes the information (botanical family, scientific name, vernacular name, plant parts used, preparation and route of administration and relative frequency of citation) of the species collected during the ethnobotanical survey. Of the 14 botanical families, Fabaceae (25%) was the most represented family followed by Asteraceae (16.60%), Rubiaceae (8.30%) and Meliaceae (8.30%). Among the listed plants, *Anacardium occidentale* (FI=14.4%) were the most frequently cited plant species followed by *Acanthospermum hispidum* (11.30%), *Psidium guajava* (8.30%), *Kedrostis foetidissima* (7.60%), *Sesamum radiatum* (6.80%), *Petiveria alliacea* (6.80%), *Morinda citrifolia*

Table 1
Ethnobotanical information of plants used by traditional healers to treat *Salmonella*-retated effects in the Plateau department of Benin.

Botanical families	Scientific names [abbreviation]	Voucher specimens numbers	Vernacular names	Morphology	Plant parts used	Preparation (administration)	FI (%)
Amaranthaceae	*Pupalia lappacea* (L.) Juss. [PL]	YH 234/HNB	Eman agbo (Y)	Herb	L	D/M (Oral)	2.27
Anacardiaceae	*Anacardium occidentale* L. [AO]	YH 235/HNB	Egi yi cajou (N)	Tree	L, Sb	D (Oral)	14.39
Annonaceae	*Uvaria chamae* P.Beauv.[UC]	YH 241/HNB	Eruju (N,Y)	Shrub	L	D (Oral)	1.52
Asteraceae	*Acanthospermum hispidum* DC. [AH]	YH 252/HNB	Dagounro (N,Y)	Shrub	Wp	D/M (Oral)	11.36
	Artemisia afra Jacq. ex Willd [AAF]	YH 679/HNB	Assunwon funfun (N)	Shrub	Ap	M/I (Oral)	3.03
	Artemisia annua L. [AAN]	YH 680/HNB	Assunwon (N)	Shrub	Ap	M/I (Oral)	1.52
	Chromolaena odorata (L.) R.M.King & H.Rob. [CO]	YH 641/HNB	Akintola (N), Awolowo (Y)	Shrub	L	M (Oral)	2.27
Capparaceae	*Crateva adansonii* DC. [CA]	YH 260/HNB	Ewé égbona (N)	Tree	L	D/M (Oral)	3.03
Cucurbitaceae	*Kedrostis foetidissima* (Jacq.) Cogn. [Kf]	YH 670/HNB	Ewé imi (N)	Herb	Wp	M (Oral)	7.58
Fabaceae	*Baphia nitida* Lodd. [Bn]	YH 304/HNB	Irosun (N,Y)	Shrub	L	D/M (Oral)	1.52
	Caesalpinia pulcherrima (L.) Sw. [Cp]	YH 281/HNB	Egi ododo pupa (N)	Shrub	L	D (Oral)	1.52
	Detarium microcarpum Guill. & Perr. [Dm]	YH 668/HNB	Égi ogbobo (Y)	Tree	L, Sb	D (Oral)	4
	Detarium senegalense J.F.G [Ds]	YH 669/HNB	Égi ogbobo (Y)	Tree	L, Sb	D (Oral)	2.27
	Leucaena leucocephala (Lam.) de Wit [Ll]	YH 671/HNB	Awi fuufun (N)	Tree	L	D/M (Oral)	5.3
	Senna alata (L.) Roxb [Sa]	YH 673/HNB	Ewé asuwan (N,Y)	Shrub	L	D (Oral)	2.27
Lamiaceae	*Hyptis suaveolens* (L.) Poit. [Hs]	YH 502/HNB	Koutoubi (Y)	Shrub	L	D/M (Oral)	3.03
Lythraceae	*Lawsonia inermis* L. [Li]	YH 309/HNB	laali (N,Y)	Shrub	L	D (Oral)	5.3
Meliaceae	*Pseudocedrela kotschyi* Harms [Pk]	YH 674/HNB	Egi lilé (N)	Tree	L	D (Oral)	2.27
	Trichilia prieuriana A.Juss [Tp]	YH 675/HNB	Akorere (N,Y)	Shrub	L	D (Oral)	3.03
Myrtaceae	*Psidium guajava* L. [Pg]	YH 233/HNB	Arase (N,Y)	Tree	L	D (Oral)	8.33
Pedaliaceae	*Sesamum radiatum* Thonn. ex Hornem. [Sr]	YH 608/HNB	Ekuku gogoro (N)	Shrub	L	D/M (Oral)	6.82
Phytolaccaceae	*Petiveria alliacea* L. [Pa]	YH 323/HNB	Awogba (N)	Shrub	L	D/M (Oral)	6.82
Rubiaceae	*Morinda citrifolia* L. [Mc]	YH 672/HNB	Nori (N,Y)	Tree	L	D (Oral)	5.3
	Morinda lucida Benth. [Ml]	YH 333/HNB	Oruwo (N,Y)	Tree	L	D (Oral)	2.27

Ap: Aerial part; L: Leaf; Sb: Stem bark; D: Decoction; M: Maceration; I: Infusion; N: Nagot; Wp: Whole plant; Y: Yoruba.

(5.30%), *Hyptis suaveolens* (5.30%) and *Leucaena leucocephala* (5.30%). The others plant species had a frequency index of less than 5%. These plants are used alone or in combination (mixture of two or three plant species) by traditional healers. The plant parts used were principally the leaves (74.07%) followed by the stem bark (11.11%). The aerial part without roots (7.41%) and the whole plant (7.41%) were used in other cases for some plant species. Decoction (58.82%) and maceration (35.30%) were the most employed methods of preparation, and oral administration was the only route reported.

3.2. *In vitro* antibacterial bioassay

The antibacterial activity of the 27 extracts (from 24 plant species) is presented as a bar chart for each bacterial strain (Fig. 2). Plant extracts exhibited broad-spectrum activity against *Salmonella* strains tested with MIC values ranging from 0.156 to 2.5 mg/mL. Among all tested plant extracts, 41.67% (10 extracts out of 24) showed good antibacterial activity (MIC < 1 mg/mL) against isolates and reference strains of *Salmonella*, 33.33% (8/24) showed moderate activity (1 ≤ MIC < 1.5 mg/mL), while 25% (6/24) presented limited activity (1.5 ≤ MIC ≤ 2.5 mg/mL). *Salmonella* Typhimurium (ATCC 14028) was the most susceptible strain to crude extracts from all tested plants. *Anarcadium occidentale*,

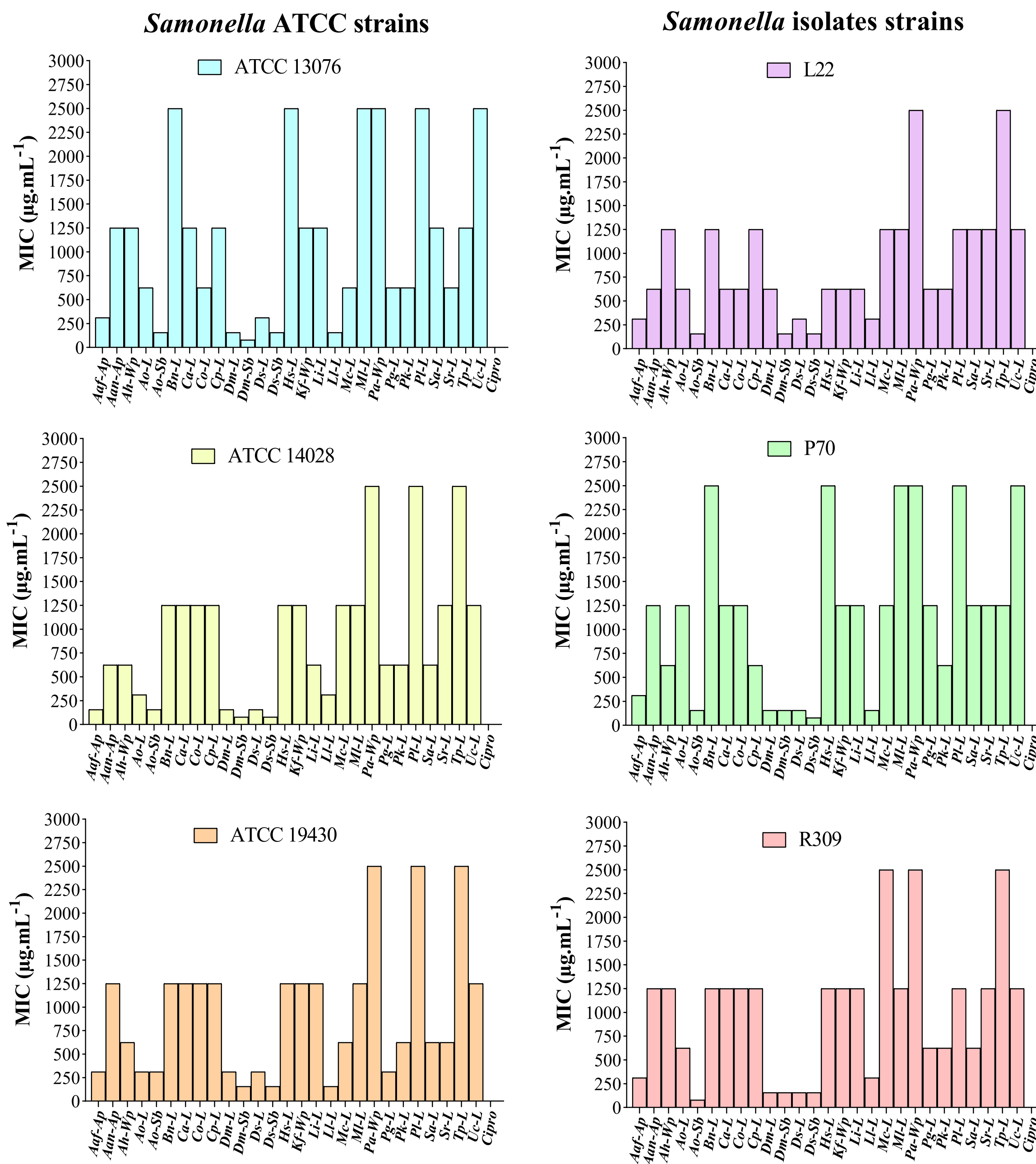

Fig. 2. Bar chart showing the MICs (µg/mL) of plant extracts against the ATCC strains (left) and isolates (right) of *Salmonella*. Aaf: *Artemisia afra*; Aan: *Artemisia annua*; Ah: *Acanthospermum hispidum*; Ao: *Anacardium occidentale*; Ap: Aerial part; Bn: *Baphia nitida;* Ca: *Crateva adansonii*; Cipro: ciprofloxacin Co: *Chromolaena odorata*; Cp: *Caesalpinia pulcherrima*; Dm: *Detarium microcarpum*; Ds: *Detarium senegalense*; Hs: *Hyptis suaveolens*; Kf: *Kedrostis foetidissima*; L: Leaves; Li: *Lawsonia inermis*; Mc: *Morinda citrifolia*; Ml: *Morinda lucida*; Pa: *Petiveria alliacea*; Pg: *Psidium guajava*; Pk: *Pseudocedrela kotschyi*; Pl: *Pupalia lappacea*; Sa: *Senna alata*; Sb: Stem bark; Sr: *Sesamum radiatum*; Tp: *Trichilia prieuriana*; Uc: *Uvaria chamae;* Wp: Whole plant. Positive control (ciprofloxacine) presented a MIC of 0.5–1 µg/mL. Further information are available in the supporting data file: Table S3.

Artemisia afra, *Detarium microcarpum*, *Detarium senegalense* and *Leucaena leucocephala,* showed the best antibacterial activities with MIC values less than 0.312 mg/mL against one or more strains of *Salmonella* tested. These plants were then selected to conduct antibiofilm bioassay.

3.3. Biofilm inhibition activity

Extracts from the five most active plants showed varying levels of biofilm inhibition activity against the three clinical isolates of *S.* Typhimurium tested (P70, L22, and R309) (Fig. 3). Compared to *A. occidentale* and *L. leucocephala* extracts, *A. afra*, *D. microcarpum* and *D. senegalense* extracts showed a significant ($p < 0.05$) reduction of bacterial biofilm (see Supporting data file: Tables S4-S7 for details on all *p*-values found). Their antibiofilm activities were dose-dependent. At 0.156 mg/mL, these plant extracts showed the best activity with a biofilm inhibition greater than 50% against the clinical isolates P70 and R309. At 0.625 mg/mL, the extracts from these three plants inhibited more than 50% the biofilm formation of *S.* Typhimurium (L22). *Anarcadium occidentale* and *Leucaena leucocephala* showed moderate inhibition of biofilm formed by the three clinical isolates of *S.* Typhimurium.

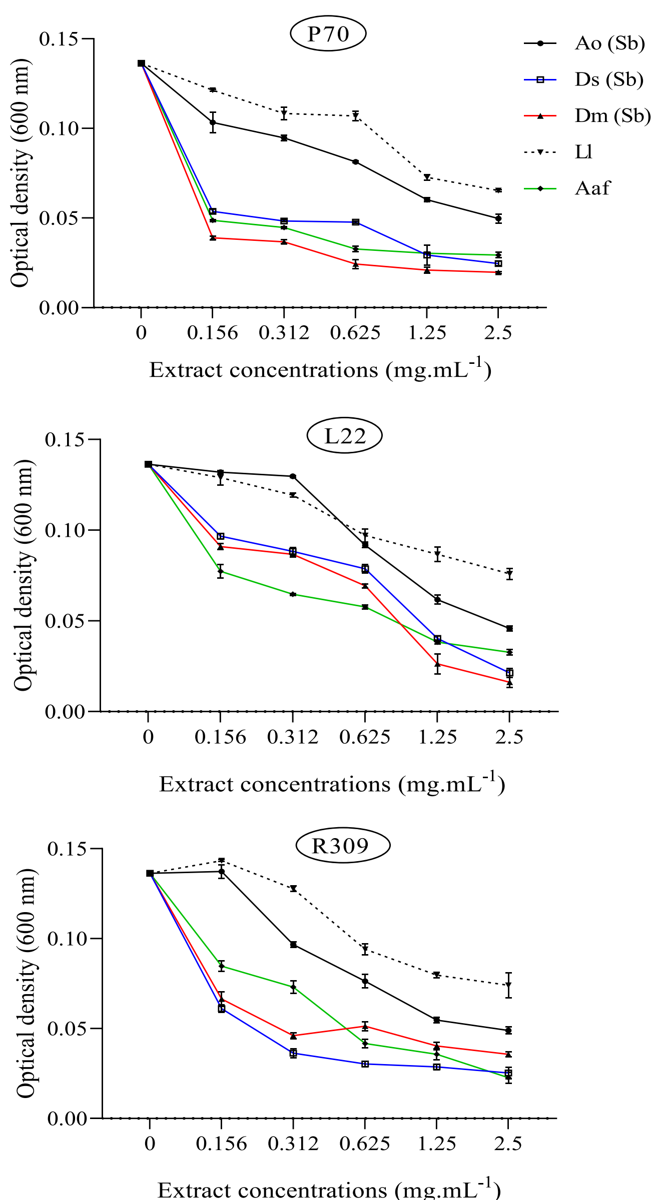

Fig. 3. Effect of different concentrations of plant extracts on the amount of biofilm formed (optical density-OD) by clinical isolates of *S.* Typhimurium. Aaf: *Artemisia afra*; Ao: *Anacardium occidentale*; Dm: *Detarium microcarpum*; Ds: *Detarium senegalense*; Ll: *Leucaena leucocephala*; Sb: Stem bark.

3.4. In vivo antibacterial activity of active plant extracts

The *in vivo* experiment was conducted with extracts from *A. afra, A. occidentale* and *D. senegalense*. Effects of plant extracts on the bacterial load (BL) of *S.* Typhimurium in animal feces is shown in Fig. 4. The administration of *Artemisia afra*, *Anacardium occidentale* and *Detarium senegalense* extracts induced a reduction of the BL in the feces of infected animals. This reduction of BL in feces of infected animals depends on the dose of the plant extracts administered and the treatment duration. At 100 mg/kg of body weight, none of the three plants tested significantly reduced BL in the feces of infected animals ($p > 0.05$). Considering both sexes, from the 4th to the 10th day of treatment, *A. afra* and *D. senegalense* extracts at 200 and 300 mg/kg/bw, significantly ($p < 0.05$) reduced the numbers of viable *S.* Typhimurium in feces of the treated rats with a BL less than 10 CFU/g at the end of treatment, while the *Salmonella* control group showed a BL more than 35 CFU/g and 70 CFU/g in male and female rats, respectively. The *in vivo* anti-*Salmonella* activity of plant extracts can be summarized as follows: *A. afra* > *D. senegalense* > *A. occidentale*. Although these extracts showed better anti-*Salmonella* activity *in vivo*, their activity at 100 mg/kg/bw remains however lower than that of ciprofloxacin at 8 mg/kg/bw, which showed a complete elimination of bacteria load from the eighth day after infection and until the end of treatment. However, there was no significant difference ($p > 0.05$) in the BL recorded in feces of animals treated with the extracts of *A. afra* and *D. senegalense* at 200 and 300 mg/kg/bw compared to the treatment with ciprofloxacin at 8 mg/kg/bw. The activity of *A. occidentale* at 300 mg/kg/bw was also comparable ($p = 0.0690$) to ciprofloxacin (8 mg/kg/bw).

3.5. Effects of plant extracts on the body weight of rats during in vivo studies

Considering the groups of animals infected and then treated with different doses (100, 200 and 300 mg/kg/bw) of *A. afra, A. occidentalis*, *D. senegalense* extracts or ciprofloxacin (8 mg/kg/bw), there were no significant changes ($p > 0.05$) in the body weight of rats compared to normal control (uninfected rats).

3.6. Effects of plant extracts on hematological, biochemical parameters and pathological changes in tissues and organs of rats during in vivo studies

Changes in the hematological and biochemical parameters in the experimental rats are summarized in Table 2. *D. senegalense* (200 mg/kg/bw) and *A. afra* extract at 200 and 300 mg/kg/bw did not induce changes in all parameters analyzed compared to normal control ($p > 0.05$). There were no significant changes ($p > 0.05$) in the mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin levels (MCH), alanine transaminase (ALT), aspartate transaminase (AST) and creatinine (Crea) in all groups of rats treated with *A. occidentale*, *D. senegalense* and *A. afra* extracts as well as ciprofloxacine (8 mg/kg/bw) compared with healthy normal control. Otherwise, significant increases ($p < 0.05$) in leucocytes (Wbc), lymphocytes (Ly), monocytes (Mo), creatinine (Crea), AST and ALT followed by significant decreases ($p < 0.05$) in hemoglobin concentration (Hb), hematocrit (Hct), erythrocytes (Rbc), MCV, MCH and MCHC were observed in the *Salmonella* control group. Alternatively, lymphocyte levels were significantly increased ($p < 0.05$) in groups treated with *A. occidentale* extracts (all tested concentrations) as well as infected-rats treated with *D. senegalense* extract at 100 mg/kg/bw, compared with normal control. The leucocyte (Wbc) and monocytes (Mo) levels were also significantly increased ($p < 0.05$) in animal groups

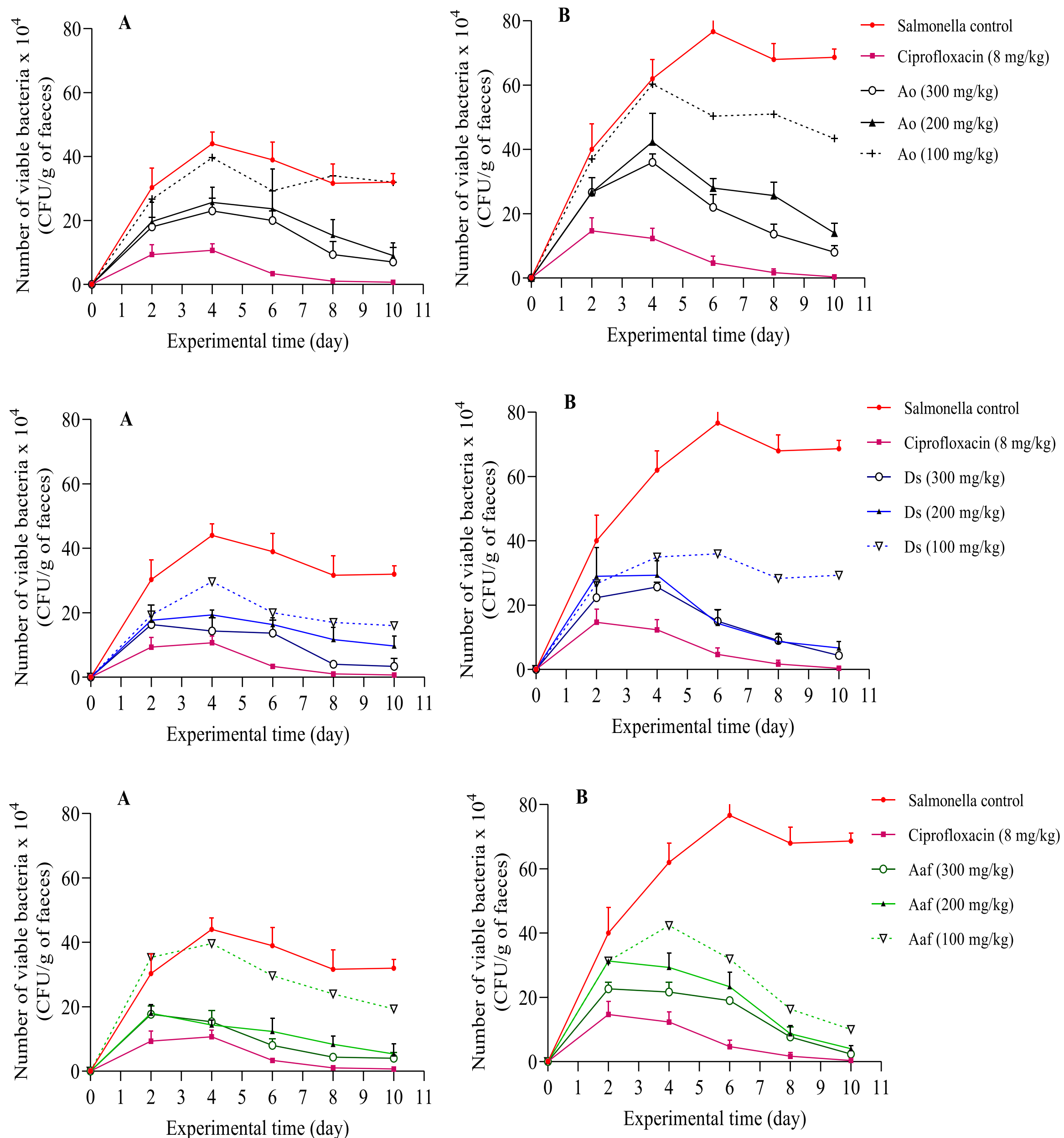

Fig. 4. Effects of plant extracts on bacterial load of *S.* typhimurium (CFU/g) in feces from male (A) and female (B) rats infected and treated with different concentrations of plant extracts comparing to untreated animals. Aaf: *Artemisia afra*; Ao: *Anacardium occidentale*; Ds: *Detarium senegalense*; Values are expressed as mean ± SEM. n = 4.

treated with these extract concentrations except *A. occidentale* at 300 mg/kg/bw ($p > 0.05$). As shown in Table 2, a significant decrease ($p < 0.05$) in the hemoglobin concentration (Hb) was observed in the group of animals treated with *A. afra* extract at 100 mg/kg/bw. However, the administration of *A. occidentale* and *D. senegalense* extracts at 300 mg/kg/bw increased significantly ($p < 0.05$) hemoglobin concentration (Hb) in the treated animals. Regarding histopathological analysis, there were no significant changes in the structural architecture of visceral organs (liver and kidney) animals treated with tested plants at 300 mg/kg/bw compared to normal control. However, significant alterations have been found in *Salmonella* control organs. This deterioration resulted in eosinophilia of the cell cytoplasm, a decrease in capillary space in the liver followed by restriction of cortical glomeruli in the kidneys of infected animals (Fig. 5).

4. Discussion

To the best of our knowledge, this is the first study to record and test the efficiency of plant species traditionally used in the Plateau department to treat salmonellosis. A similar survey has been performed in Southern Benin and focused on other provinces (Atlantique, Littoral, Ouémé) [25].

In our survey, a large proportion of traditional healers interviewed were men. This predominance of males among Beninese traditherapists has already been reported in various ethnobotanical surveys [26,27,28,29]. Although most traditional healers in Benin are males, herbal vendors in local markets are mainly females. This highlights the specificity of Beninese traditional medicine. Regarding botanical data, 24 plant species belonging to 14 botanical families were reported. Fabaceae was

Table 2
Hematological and biochemical parameters following the 10-day treatment of rats with ethanolic extracts of *A. occidentale*, *D. senegalense* and *A. afra*.

Parameters	Normal control	Immunosuppressed control	Salmonella control	Ciprofloxacin control	Ao100	Ao200	Ao300	Ds100	Ds200	Ds300	Afra100	Afra200	Afra300
Hb (g/dl)	13.17 ± 0.29	12.73 ± 0.15	9.93 ± 0.41 *	12.13 ± 0.65	12.13 ± 0.22	14.02 ± 0.60	24.97 ± 0.46	12.20 ± 0.76	14.07 ± 0.40	24.93 ± 0.7 *	10.23 ± 0.58 *	12.43 ± 0.49	12.90 ± 0.62
Hct (%)	46.67 ± 0.58	45.33 ± 1.15	30.50 ± 4.77	42.33 ± 1.15	43.67 ± 1.25	44.00 ± 1.00	68.67 ± 1.53	48.00 ± 2.00	46.67 ± 0.58	47.33 ± 3.06	43.17 ± 0.76	45.00 ± 1.73	46.67 ± 1.53
Rbc (x 10^6/ mm^3)	4.80 ± 0.10	4.37 ± 0.15	3.03 ± 0.32 *	3.90 ± 0.36	4.33 ± 0.26	4.17 ± 0.06	4.67 ± 0.32	4.70 ± 0.10	4.83 ± 0.12	4.47 ± 0.15	4.53 ± 0.31	4.30 ± 0.20	5.50 ± 0.20
MCV (fl)	84.67 ± 1.53	85.00 ± 2.65	52.33 ± 1.53 *	85.00 ± 2.00	85.33 ± 2.05	85.67 ± 1.15	82.75 ± 1.15	84.00 ± 1.73	85.33 ± 0.58	85.67 ± 1.53	86.69 ± 3.51	85.00 ± 1.73	85.00 ± 2.65
MCH (pg)	28.00 ± 2.00	29.00 ± 1.00	28.00 ± 2.65	28.00 ± 1.00	28.00 ± 1.63	29.33 ± 1.53	27.67 ± 1.53	28.00 ± 1.00	28.00 ± 1.00	29.00 ± 2.00	28.67 ± 1.53	28.00 ± 1.00	29.00 ± 2.00
MCHC (%)	33.00 ± 2.65	33.33 ± 1.53	18.67 ± 1.00 *	34.00 ± 1.73	33.00 ± 0.82	33.67 ± 1.15	34.33 ± 0.58	32.67 ± 0.58	32.67 ± 1.53	33.00 ± 4.00	34.33 ± 1.53	34.00 ± 2.00	33.00 ± 2.00
Wbc (x 10^3/ mm3)	10890 ± 17.32	10852 ± 34.51	21813 ± 15.94	10831 ± 12.12	14121 ± 9.23 *	13309 ± 9.46 *	10960 ± 10.46	12512 ± 8.84 *	10892 ± 9.41	10860 ± 9.50	10864 ± 14.01	10866 ± 15.04	10877 ± 12.64
Ly (cells/ mm^3)	2817.33 ± 8.08	2214.33 ± 13.28 *	8919 ± 10.15 *	2868.33 ± 2.89	6788 ± 4.11 *	5935 ± 8.66 *	2948 ± 8.66 *	4662 ± 10.79 *	2823 ± 9.07	2830 ± 9.70	2889 ± 6.73	2825 ± 2.31	2825 ± 5.69
Mo (cells/ mm^3)	469 ± 3.46	267.67 ± 4.93 *	2324 ± 14.74 *	489.67 ± 2.08	579.0 ± 4.24 *	500.7 ± 5.03 *	488.7 ± 5.03	577.0 ± 7.55 *	494.0 ± 7.2 *	478.7 ± 4.93	479.3 ± 3.06	488.3 ± 5.13	486.0 ± 3.61
Crea (mg/L)	8.33 ± 1.15	8.93 ± 0.58	16.37 ± 1.27 *	9.33 ± 1.53	10.00 ± 0.82	9.53 ± 0.92	8.67 ± 0.92	9.28 ± 1.73	8.70 ± 0.75	7.90 ± 0.58	11.20 ± 0.58 *	9.37 ± 0.58	8.73 ± 0.42
AST (UI/L)	24.00 ± 2.65	24.33 ± 1.15	51.33 ± 4.58 *	23.00 ± 2.00	24.33 ± 1.89	33.00 ± 4.36 *	28.67 ± 4.36 *	24.33 ± 3.51	23.67 ± 3.21	23.00 ± 1.73	25.33 ± 3.79	24.33 ± 4.16	24.10 ± 1.25
ALT (UI/L)	34.33 ± 3.06	34.67 ± 2.08	92.67 ± 5.29 *	35.00 ± 1.73	35.00 ± 1.63	34.67 ± 2.52	38.06 ± 2.52	35.67 ± 3.51	37.67 ± 2.08	34.33 ± 1.53	33.67 ± 2.08	36.00 ± 4.04	35.00 ± 2.00

Hb: hemoglobin concentration; Hct: hematocrit; Rbc: erythrocytes; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin levels; MCHC: mean corpuscular hemoglobin concentration; Wbc: leucocytes; Ly: lymphocytes; Mo: monocytes; Crea: Creatinine; AST: aspartate transaminase; ALT: alanine aminotransferase; Ao: *Anarcadium occidentale*; Ds: *Detarium senegalense*; Afra: *Artemisia afra*. Values are expressed as mean ± SEM. n = 4. *p < 0.05: significant difference compared to normal control.

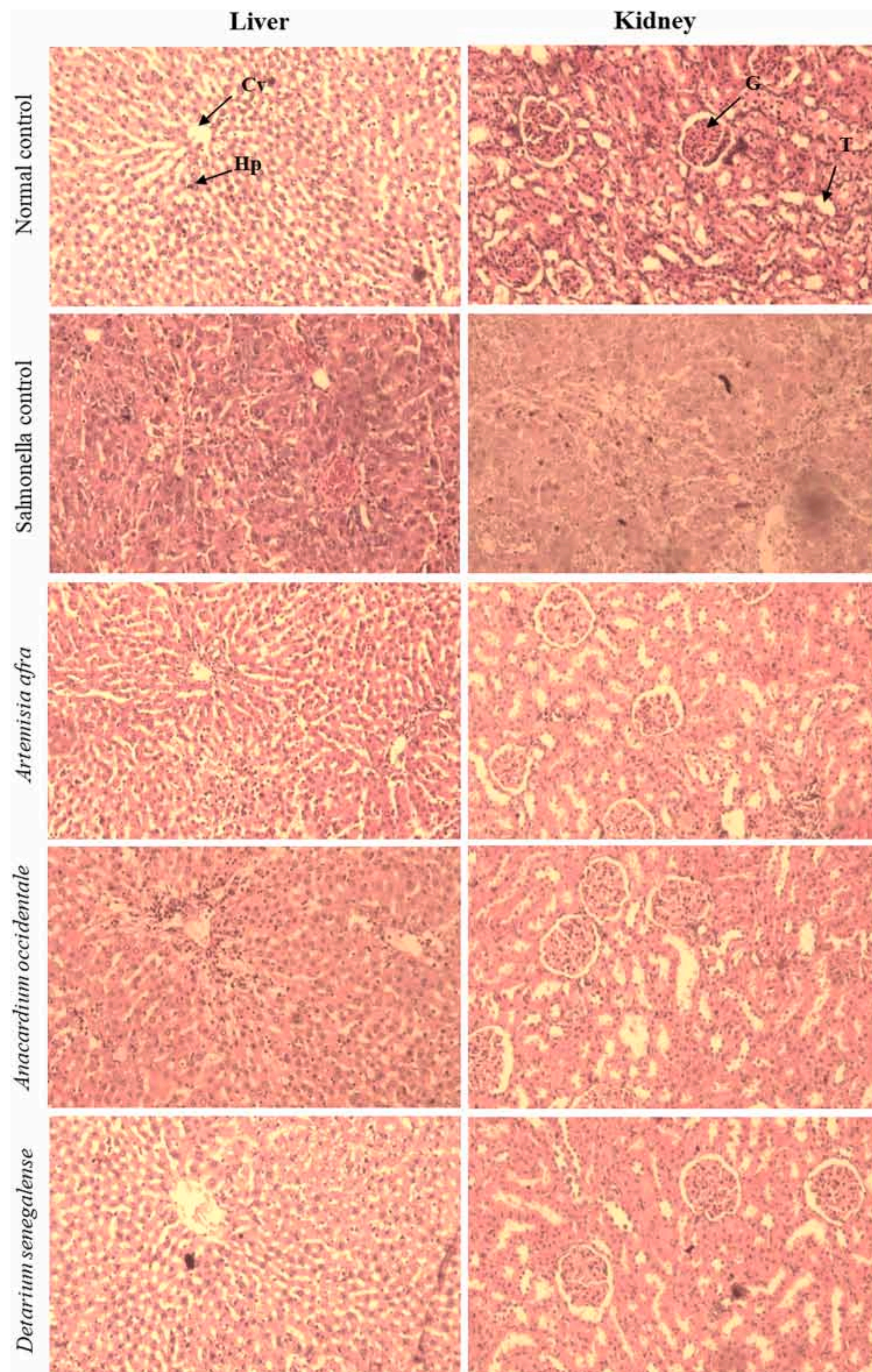

Fig. 5. Photomicrography (H and E, × 400) showing rats liver and kidney after 10-days exposure to plant ethanolic extracts (300 mg/kg/bw). Negative control is noted normal control, Positive control is noted *Salmonella* control. Cv: Centrolobular vein; G: Glomerulus; Hp: hepatocytes; T: tubules.

the most represented family, followed by Rubiaceae, Asteraceae and Meliaceae. Leaves were the most represented part of plant used and oral administration was the only route reported. In other surveys [30,31], the most frequently used medicinal plants for treating typhoid fever and/or gastrointestinal disorders were species from the Fabaceae and Asteraceae families. The preference for leaves among plant organs could indicate the importance of healers to the perpetuation of their activity through the conservation of plant species. Several surveys have reported leaves as the main plant organs used for treating typhoid fever and gastrointestinal disorders ([32,30,31]). Below, we provide an analysis of the safety and antibacterial efficacy of two plant species with the best *in vitro* and *in vivo* anti-*Salmonella* effect.

Artemisia afra (Asteraceae), also known as African wormwood, is a perennial woody shrub native to southern Africa and Ethiopia. It is also found in eastern Africa [33]. Based on information provided in the Prelude database (https://www.africamuseum.be/en/research/collections_libraries/biology/prelude), *A. afra* is mainly used in Africa for treating fever-related symptoms, malaria, cold, stomachache and as an antispasmodic. Of the 48 references reported in this database, only one mentioned the use of *A. afra* in diarrheal illnesses. This study was conducted in Uganda and used left ashes to treat diarrhea-associated health disorders. In our survey, aerial parts of *A. afra* were reported to be used as an infusion or decoction for treating salmonellosis, diarrhea and dysentery. Although many studies have explored the antibacterial potential of *A. afra* leaf extract and essential oil, only one study has focused on its anti-*Salmonella* effect. Mangena and Muyima [34] explored the antibacterial properties of *A. afra* leaf essential oils collected from South Africa and found inhibition zone diameters comprised between 10 and 14.5 mm on different *Salmonella* species [34]. In our study, the ethanolic extract of the aerial part of *A. afra* exhibited anti-*Salmonella* activity with a MIC ranging from 156 µg/mL to 312 µg/mL. At 156 µg/mL, the plant extract exhibited a biofilm inhibition greater than 50% against the clinically isolated strains of *S.* Typhimurium. Regarding other diarrhea-associated bacterial pathogens, McGaw et al. [35] did not find an MIC at the range of concentrations tested (0.38 µg/mL to 12.5 mg/mL) for the ethanolic extract of *A. afra* whole plant against *Escherichia coli*. However, van Vuuren and Muhlarhi [36] found an MIC ranging from 0.25 to 2 mg/mL against *Escherichia coli* for the organic extract of *A. afra*. In another study, More et al. [37] hypothesized that the chemical compounds responsible for the antibacterial activity of the crude extract of *A. afra* were betulinic acid, acacetin and scopoletin with an MIC ranging from 0.25 to 1 mg/mL. Besides *in vitro* studies, very few studies have focused on the *in vivo* effects of *A. afra*. To the best of our knowledge, there is no scientific report showing an *in vivo* antibacterial activity of this plant against *Salmonella* serovars or others diarrhea-associated bacterial pathogens. In our work, we demonstrated that the number of viable *S.* Typhimurium recovered from animal feces was significantly reduced at 4 days post-infection in the *A. afra* extracts treated groups compared with the non-treated group. Thus, our study provides important data to validate its use as an antidiarrheal.

Regarding its potential toxic effect, no significant changes were reported in our study in hematological and biochemical parameters in rats during 10 days of treatment with the ethanol extract of *A. afra* at 0.3 g/kg/bw. This is congruent with another work studying the pharmacotoxicological effects of the extract. In the Mukinda and Syce [22] study, the aqueous extract of *A. afra* was evaluated after acute and chronic administration in mice and rats. In this study, the authors showed that the LD50s in mice model were 2.45 and 8.96 g/kg after acute intraperitoneal and oral doses, respectively, while the LD50 is higher than 1 g/kg in rats given an oral dose. The authors also showed that repeated oral administration of *A. afra* extract at 100 or 1000 mg/kg/bw, during 3 months of dosing did not induce any morphological alteration of rat vital organs.

However, a sesquiterpene lactone called isoalantolactone and identified in the ethanolic extract of *A. afra* leaf was found to significantly contribute to the cytotoxic activity of *A. afra* [38]. This compound could be responsible for the allergic contact dermatitis reported in South Africa when manipulating *A. afra* [39].

Overall, *A. afra* extracts and isolated compounds exhibit weak to moderate antibacterial activity, thus highlighting additional mechanisms of action (*e.g.*, spasmolytic effect, antisecretory activity, beneficial effect on gut microbiota) susceptible to act on the antidiarrheal activity of *A. afra* extracts. Further studies are thus needed to identify the mechanisms of action of *A. afra* on salmonellosis and to provide additional clinical evidence for its safety. Because some studies have shown the phytochemical variation of *A. afra* depending on its geographical and growth conditions [40,41], quality control methods need to be developed to ensure its safety and efficacy.

Detarium senegalense (Fabaceae), also known as tallow tree, is a species native to West Tropical Africa and South Sudan. Regarding its traditional use in Benin, leaf, bark and root have already been mentioned for treating diarrheal illnesses as well as fever, dysentery, tiredness, and stomachache [42]. In Nigeria, the leaf and stem bark of *D. senegalense* is traditionally used for treating several diseases, including diarrhea and dysentery in both animals and humans [43,44]. In our study, the ethanolic extract of *D. senegalense* stem bark showed antibacterial activity with MICs ranging from 78 to 156 µg/mL against different *Salmonella* serovars. We also found that the plant extract had antibiofilm activity with biofilm inhibition greater than 50% against clinical *Salmonella* isolates. To the best of our knowledge, no previous studies have reported *in vitro* anti-*Salmonella* activity of *D. senegalense*. However, the effect of *D. senegalense* stem bark in castor oil-induced diarrhea in rats has already been studied [43]. In this study, the authors showed that the chloroformic, ethyl acetate, n-butanolic, methanolic, and residual aqueous fractions obtained from an aqueous extract of *D. senegalense* decreased the frequency of defecation in diarrheal-rats with percent protection ranging from 41.94% to 72.35%. Besides these few studies, there are few pharmacological and toxicological reports on *D. senegalense*. However, in the genus *Detarium*, only three species are present. From a phylogenetic view, they are all closely related [45]. Thus, similar compounds can be found in these three species, and so could be their pharmacological effects. For example, Hussain and Deeni [46] evaluated the antibacterial potential of *D. microcarpum* and found that the stem bark methanolic extract at a concentration of 2 mg/mL had an inhibition zone diameter of less than 8 mm on *Salmonella* spp. Also, Mbock et al. [47] tested an ethanolic extract along with the n-hexanic, dichloromethanic, ethyl acetate and n-butanolic fractions of *D. microcarpum* against three strains of *Salmonella* (*S.* Typhi, *S.* Typhimurium, *S.* Enteritidis) and found MICs ranging from 4.55 µg/mL to more than 500 µg/mL. The authors also showed that the ethanolic extract of *D. microcarpum* had anti-*Salmonella* activity in infected rats with an effective dose (ED50) of 75 mg/kg. In our study, we showed that the oral administration of *D. senegalense* ethanolic extract at tested doses, significantly reduced the bacterial load in infected-animals feces from 4th day post-infection. During the 10 days of treatment with the ethanol extract of *D. senegalense* at 0.3 g/kg/bw, no toxic side effects or changes in hematological and biochemical parameters were reported in the treated rats *vs.* uninfected-animals. These results are comparable to those reported for *D. microcarpum*. Indeed, Mbock et al. [47] found that the oral administration of ethanolic extract of *D. microcarpum* root bark had a lethal dose (LD50) greater than 5000 mg/kg in *an in vivo Salmonella* model of infection. They also found that the plant extract protected body weight loss and vital organs of treated rats.

Regarding phytochemical analysis, no previous rigorous studies have isolated the chemical compounds responsible for the biological activities of *D. senegalense* stem bark. However, Mbock et al. [47] isolated rhinocerotinoic acid and lupeol from the root bark of *D. microcarpum* and found that rhinocerotinoic had an MIC of 31.25 µg/mL against *S.* Typhimurium and *S.* Enteritidis, as well as an MIC of 62.50 µg/mL against *S.* typhi. In other studies, microcarposide, lupeol, betulinic acid, β-sitosterol glucoside, methyl gallate, luteolin, and epicatechin were isolated from the methanolic extract of *D. microcarpum* fruits and only microcarposide, an isovaleronitrile diglycoside, exhibited anti-*Salmonella* activity with MICs of 76.7–153.4 µM [48].

Overall, the results of our study represent the first scientific basis for demonstrating the anti-*Salmonella* potential of *D. senegalense*. Further studies, including phytochemical analysis, toxicity tests, and clinical studies, are needed to validate the safety and efficacy of *D. senegalense* for treating salmonellosis and diarrheal diseases.

5. Conclusion

In this study, we report for the first-time the traditional use of medicinal plants to treat salmonellosis in the Plateau department in Benin

republic. Our study demonstrated that five out of 24 selected plants (*Anacardium occidentale*, *Artemisia afra*, *Detarium microcarpum, Detarium senegalense,* and *Leucaena leucocephala*) showed an antibacterial effect *in vitro* against *Salmonella* serovars tested. In the antibacterial *in vivo* assays, *A. afra* and *D. senegalense* showed the best results. While *A. afra* is well known for its antimalarial activity, there are few studies focusing on its anti-*Salmonella* properties. Further studies are needed to elucidate the mechanism of action and to identify the chemical compounds responsible for the activity. In the case of *D. senegalense*, scientific studies on this species are scarce and various studies (*e.g.*, phytochemical analysis, toxicity studies) should be conducted to validate its use for treating salmonellosis. Experiments are ongoing in our laboratory to study their cytotoxicity and isolate phytochemical compounds.

CRediT authorship contribution statement

Abdou Madjid O. Amoussa: Investigation, Methodology, Writing – original draft, Writing – review & editing. **Latifou Lagnika:** Methodology, Supervision, Writing – review & editing. **Valérie Jullian:** Conceptualization, Project administration, Supervision, Writing – review & editing. **François Chassagne:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Conflict of interest statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.114119.

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