

Original Article

## Use of *Vitex doniana* (black plum) and *Abutilon hirtum* (Florida keys) extracts as an integral part of phytomedicine in tackling multidrug-resistant *Salmonella*

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### Abstract

**Introduction:** The high prevalence and global spread of antibiotic resistance is driving the search for new antibacterial agents. Screening small molecules against specific bacterial targets has not yielded new compounds therefore functional assays and phenotypic screens are now being used. In Nigeria, drug resistance towards *Salmonella* is a major public health concern.

**Methodology:** Nine fully characterized clinical *Salmonella* isolates, from the Department of Medical Microbiology, Jos University Teaching Hospital, Plateau State, Nigeria, were screened by broth microdilution for susceptibility to fractionated ethanol extracts of *Vitex doniana* and *Abutilon hirtum*. This was compared to the control organism ATCC25922 and a range antibiotics: CH (chloramphenicol), SP (sparfloxacin), AM (amoxicillin), CN (gentamicin), S (streptomycin), PEF (pefloxacin).

**Results:** The most common resistance profile was AM,CN,S with most isolates susceptible to fluoroquinolones. Activity was detected from both plant extracts with MICs of extracted fractions ranging from 150 - 300 µg/mL. Interestingly both plants produced extracts with bactericidal activity from 300 - 600 µg/mL. *V. doniana* exhibited better activity against the resistant *Salmonella* strains in terms of greater inhibition zones, but *A. hirtum* extracts were more consistently active against all isolates. In comparison with the synthetic drugs, both plant extracts exhibited activity against more isolates – this activity was bactericidal.

**Conclusions:** Nigeria needs better anti-salmonella products and these results represent a starting point for antibiotic drug discovery.

**Key words:** Antibacterial activity; multi-drug resistant salmonellae; medicinal plants.

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### Introduction

The continued emergence and spread of multidrug-resistant (MDR) bacterial pathogens has reduced the potency of current antibiotics [1]. Infections caused by MDR bacteria have been reported to be responsible for increased mortality, longer in hospitals, and higher cost of treatment and care [2]. The ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species [2-4] are the major cause of MDR hospital infections but in case of community infections the lack of food chain surveillance is a major concern [5]. Food safety is a global problem and yet this issue is not adequately tackled in low to middle-income countries (LMICs) because of a lack of policy coherence among the different sectors. In sub-Saharan African countries contributing factors include inadequate food safety, inadequate financial investments, fragmented food

control systems, weak food-borne disease surveillance, obsolete food regulation, weak law enforcement, and the inability of small- and medium-scale producers to provide safe food [6]. Of further concern are outbreaks in both Uganda and Tanzania where 1,940 suspected cases of typhoid fever were registered at the beginning of the outbreak and *S. Typhi* was confirmed in 4 out of the 16 specimens screened [7,8]. In Nigeria, *Salmonella* strains in poultry that are resistant to antibiotics including third-generation antibiotics in some cases, are a major concern.

In West Africa, poultry imports mainly come from the European Union (EU), which has increased from 12,500 tons in 1996 to 86,000 tons in 2003. These exports mainly come to Benin, followed by Ghana, Nigeria, Senegal, Togo, and Ivory Coast [9]. Recently *Salmonella* Typhimurium ST313 isolates have been reported in imports from the UK containing virulence-resistance plasmids linked with invasive infections in

adults in Africa [10] and increasing resistance in the causal agent of typhoid fever, *Salmonella enteric serovar* Typhi (*S. Typhi*) [11-13]. It has been suggested that a further rise in the spread of multidrug-resistant *Salmonella* should be anticipated worldwide as a result of increasing international travel [14-16]. A ceftriaxone-resistant strain of *S. Typhi* has emerged and spread, in Pakistan [17].

Therapeutic alternatives for these pathogens are not freely available in Nigeria and physicians are forced to use expensive or previously discarded drugs, such as colistin, which have significant side effects [1]. The spread of resistance has led to an analysis of the global herbal drug market which has been estimated at US \$62 billion with an expected increase of US \$5 trillion by the year 2050 [18]. This high demand has led to an increasing interest in plants as potential new sources for antibiotics. The World Health Organization has also suggested the inclusion of traditional medicine (TM) including phytomedicine, if they prove safe and effective, into national health care systems [19]. Previous research has confirmed that medicinal plants used in folk medicine are important in medical research [20] and the diversity seen in synthetic drugs can no longer supply effective new antibiotics against many emerging MDR infectious microorganisms; hence an alternative therapy is much needed [21]. Phytochemicals have played an integral part in maintaining human health and treating diseases all over the world [22] and the popularity of phytomedicine is increasing every day [23].

The actual number of medicinal plants on earth is not known but they have been recorded since the 18<sup>th</sup> century. Phytomedicine is on the increase as a more sustainable way to generate chemical diversity for medicine, particularly in the developing world [24]. In general, researchers are turning their attention to herbal products, looking for new pathways to develop better drugs against MDR microbe strains [25], and change of diet from manufactured products to pure plant products. The leaf extract of *Diocleareflexa* has been shown to exhibit anti-salmonellae potency [26]. It had been reported that the investigation of fractionated plant extracts and their bioactive constituents is set to play a vital role in controlling serious diseases as an important source of new medicines [27]. In many parts of Nigeria medicinal plants represent a resource that is readily available to the scientific community for local drug discovery [27]. Here we report the anti-salmonella activity of plant extracts against typical *Salmonella* isolated in our region.

## Methodology

### *Bacteria used in this study*

Bacteria isolated from the stools of patients were identified as *Salmonella* species (isolate 1,4,7,12,14,16,18,23,27, and 28) using biochemistry and antisera.

### *Collection and preparation of the plant samples*

*Vitex doniana*, collected from Doemak, Quaan-Pan of Plateau State, was identified using the herbarium of the Federal College of Forestry, Jos, Plateau State. *Abutilon hirtum* was obtained from Barkin Ladi and the identification was confirmed by a botanist. The procedure used for the extraction of the plant extracts was a slight modification of a published method [28] - using a water bath instead of rotor vapor to concentrate the plant extracts. The plant leaves were dried indoors at room temperature to prevent the ultraviolet rays from inactivating the chemical constituents. The dried leaves were then pulverized in a mortar using a pestle.

Analytical grade 95.5% ethyl acetate and 86% methanol were used for the cold extraction of *V. doniana* and *A. hirtum*, respectively. Pulverized *V. doniana* (527 g) and *A. hirtum* (420 g) were macerated in their respective solvents in extraction bottles with the level of the solvent above that of the plant materials and allowed to stay for 2-3 days. The liquid was then removed and filtered through Whatman No. 1 filter paper. The extraction solvents were added to the respective plant extracts again and the process was repeated. The filtrate from the mixture was then concentrated over a thermostatic water Cabinet (model HH-W420, XMTD-204, and TT42D Multipurpose use. Techmel & Techmel, USA) at 100 °C.

### *Column Chromatography*

The extracts of *V. doniana* and *A. hirtum* were fractionated by column chromatography process using the following mobile phases, n-hexane : Ethyl acetate (EtoAC) at 10:0, 15:1, 9:4, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, and 2:8) and EtoAC : MeOH at 10:0, 9:1, 8:2, 7:3, 6:4, and 5:5 for *V. doniana* while mobile phase solvent of n-hexane : EtoAC at 10:0, 9.8:0.2, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, and 2:8. EtoAC : MeOH at 10:0, 9:1, 8:2, 7:3, 5:5, 3:7, and 0:10 was adopted for *A. hirtum*. The effluent was collected in a 150 mL fraction in a beaker. Fractions from the crude plant extracts were then pooled together based on a similar profile on Thin Layer Chromatography (TLC, AlugramXtra SIL G/uv 254, MAC HERY-NAGEL GmbH & Co. Kg, Germany) to yield 11 *V. doniana* leaf fractions; VF1-VF11 and 11 *A. hirtum* leaf fractions of AF1-AF11 [29]. Only 1 fraction

for each plant (VF3, AF2) from the various fractions obtained were tested against the *Salmonella* isolates.

#### Screening of Crude Extracts for activity

Agar well diffusion method was used to determine if the crude extracts of the 2 plants showed antibacterial activity. A Mueller-Hinton agar plate (Himedia, Pennsylvania, USA) was inoculated with a MacFarland 0.5 bacterial suspension (equivalent to  $1 \times 10^8$  cfu/mL *Salmonella*) to obtain semi-confluent growth. Excess fluid at the edge of the petri dish was removed with sterile cotton wool. A stock concentration of the crude plant extract was obtained by dissolving 2 g of crude plant extract in 10 mL of DMSO from which working solutions were prepared. Two wells (diameter 6.5 mm) were cut in each agar plate and filled with 200 mg/mL and 100 mg/mL of *V. doniana* separately and one well in the same plate was separately filled with DMSO (negative control). The plates were left again for some time for the extracts to diffuse into the agar, after which they were incubated at 37 °C for 24 hours. The zone of inhibition was measured to the nearest millimeter and mean zone of inhibition was calculated for each extract concentration [30]. The same procedure was repeated for *A. hirtum* extract.

#### Susceptibility testing of active extracts

To give an idea of the level of activity for the extracts, wells were cut into Muller Hinton agar plates which had been inoculated with *Salmonella* from a MacFarland 0.5 suspension and the resulting zone of inhibition was measured. Exactly 0.05 mL of the extract was added and the zone of inhibition was measured. Results were classified by comparison with the zone size for control organism ATCC 25922 using breakpoints from the CLSI guidelines 2019 as follows: for each extract susceptibility was reported if the zone around the extract was at least as great as the zone against the pan-susceptible control ATCC25922. This

organism was also used for quality assurance of reproducible antibiotic activity.

For selected extracts, *V. doniana* (Ethyl acetate VF3) and *A. hirtum* (Methanolic AF2), the MIC of leaf fraction against the various isolates was estimated using the 96-well microdilution method [31], but with a slight modification of using 8 instead of 12 wells. Dilutions of the test extract were prepared in DMSO to give a final concentration of extract of 200 or 400 mg/mL; 100 µL of the dilution was pipetted into each test well with an equal volume of bacterial suspension to give final concentrations of 100 and 200 mg/mL. All wells were incubated overnight at 37 °C in aerobic conditions. DMSO only was used as a control.

Four to five colonies of the same appearance of each isolate were emulsified in sterile normal saline according to Ndip *et al* [32] documented by Nyenje and Ndip [33] and adjusted to 0.5 McFarland Scale ( $1 \times 10^8$  cfu/mL) to obtain the required density of each isolate. *Escherichia coli* (ATCC 25922) was used as the control strain [34]

Determination of the Minimum Bactericidal Concentration (MBC) of Fractions of *V. doniana*. A sterile wire loop was dipped into the wells of minimum inhibitory concentration that showed no turbidity (no bacterial growth) and streaked on nutrient agar and incubated overnight. The MBC was obtained as the lowest concentration preventing the growth of bacteria [29].

## Results and Discussion

When tested against available antibiotics by disc diffusion all isolates were resistant to gentamicin (CN) and the most common resistance pattern exhibited by 8 of the *Salmonella* isolates showed resistance to aminoglycosides and ampicillin (Table 1). The isolates remained fully susceptible to chloramphenicol (CH) and sparfloracin (SP), but two isolates showed reduced susceptibility to pefloxacin. Overall, this shows that resistance is common but, at least for these isolates,

**Table 1.** Susceptible of *Salmonella* strains to selected antibiotics (zone size).

Isolate	CH (mm)	SP	AM	CN	S	PEF	R-pattern
1	S (22)	S (26)	R (00)	I (19)	R (00)	S (26)	AM,CN <sup>I</sup> ,S
4	S (26)	S (20)	R (00)	R (15)	R (00)	I (24)	AM,CN,S,PEF <sup>I</sup>
7	S (19)	S (22)	R (00)	R (18)	R (00)	S (26)	AM,CN,S
12	S (23)	S (23)	R (00)	I (23)	S (23)	I (23)	AM,CN <sup>I</sup> ,S, PEF <sup>I</sup>
14	S (21)	S (24)	R (00)	I (20)	R (00)	S (26)	AM,CN <sup>I</sup> ,S
18	S (26)	S (25)	R (00)	I (20)	R (00)	S (26)	AM,CN <sup>I</sup> ,S
23	S (21)	S (25)	S (22)	I (22)	S (23)	S (26)	CN <sup>I</sup>
27	S (20)	S (23)	R (00)	I (19)	R (00)	S (26)	AM,CN <sup>I</sup> ,S
28	S (26)	S (26)	R (00)	R (18)	R (00)	S (28)	AM,CN,S

CH: Chloramphenicol; SP Sparfloracin; AM Amoxicillin; CN: Gentamicin; S: Streptomycin; PEF: Pefloxacin; Classification as Ssusceptible I intermediate or R Resistant according to CLSI guidelines.

**Table 2.** Efficacy of crude extracts against MDRSS measured as zone size in mm around a well containing 0.05 mL of extract.

Isolate	<i>Vitex doniana</i>			<i>Abutilon hirtum</i>		
	200 mg/mL	100 mg/mL	Susceptible	200 mg/mL	100 mg/mL	Susceptible
1	20	18	S	15	13	S
4	20	16	S	18	17	S
7	15	0	NS	16	0	NS
12	18	16	S	17	14	S
14	13	0	NS	17	10	S
18	20	12	NS	15	10	S
23	15	13	NS	17	5	NS
27	20	15	S	15	12	S
28	20	0	NS*	16	14	S
Control	18	14	S	12	6	S

Any isolates with a zone  $\geq$  the control (ATCC 25922) at both concentrations tested was considered susceptible, S (susceptible) where there was a discrepancy between the two concentrations the reading at 100 mg/mL was used. \*NS: not susceptible.

would not prevent treatment of invasive disease. Testing the crude extracts of *Vitex doniana* and *Abutilon hirtum* against the antibiotic-resistant *Salmonella* was carried out by comparing the activity of each extract against a susceptible control, *E. coli* ATCC 25922. Both plant extracts showed good activity when screened using disc diffusion; zones of inhibition were seen around all wells containing either extract at a concentration of 200 mg/mL (Table 2). For wells containing 100 mg/mL of the extract, the isolates were classified as susceptible if the activity against *Salmonella* was at least equivalent to that against the pan-susceptible control. In case of crude *A. hirtum* extract, 8/10 isolates were susceptible according to this method suggesting that the extract contained an active constituent. No further analysis was performed to identify the chemical composition; however, this is now warranted. For *V. doniana*, half of the *Salmonella* activity was equivalent to that against the control, suggesting that the extract was less active. However, activity against all the isolates when exposed to 200 mg/mL suggests that further work on the extraction protocols is needed (Table 2). We measured the bactericidal activity of the extracts by comparing MIC to MBC. Encouragingly the MBC for both plant fractions was always within 4-fold of the MIC and for *A. hirtum* the activity was within 2-fold for 9/10 of the

isolates. This also suggests that a more extensive drug discovery from the extracts this plant is worth exploring.

### Conclusions

Resistance to antibiotics has become a global health issue due to the danger of multiply resistant bacterial pathogens including *Salmonella*. The sensitivity of all the isolates to *Abutilon hirtum* and *Vitex doniana* extracts at a concentration of 200 mg/mL suggests that these plants have an inhibitory effect on *Salmonella*. In particular, the activity of *Vitex doniana* leaf extract is encouraging, especially as there is activity against *Salmonella typhi* [35] for which new antibiotics are needed.

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**Table 3.** Minimum Inhibitory (MIC) and Bactericidal (MBC) concentration of crude extracts against *Salmonella* isolates.

Isolate	<i>Vitex doniana</i>		<i>Abutilon hirtum</i>	
	MIC ( $\mu$ g/mL)	MBC ( $\mu$ g/mL)	MIC ( $\mu$ g/mL)	MBC ( $\mu$ g/mL)
1	300	300	300	600
4	150	600	300	300
7	300	300	300	300
12	300	300	300	600
14	150	600	150	300
18	150	600	150	300
23	75	300	150	300
27	300	600	150	600
28	300	600	300	300

Control result for ATCC 25922 was 300  $\mu$ g/mL for both extracts.

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