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Original Research Article

Impact of *Vitex doniana* Extract in Elimination of *Salmonella* Infection-a Tropical Neglected Disease

Dawang D. Noel[1*], Opajobi O. Solomon[2] and Nvau B. John[3]

[1]Department of PRE-ND Science, Plateau State Polytechnic, B/Ladi, Nigeria

[2]Department of Medical Microbiology, University of Jos, Nigeria

[3]Department of Chemistry, Plateau State University, Bokkos, Nigeria

***Corresponding Author's Email: ademnaan@yahoo.com**
Tel.: +2348035864362

Abstract

The typhoidal and non-typhoidal *Salmonella* are the cause of typhoid fever, paratyphoid and food borne disease respectively and have not been given adequate attention in Africa. It is worrisome especially with the emergence of neglected invasive non typhoidal *Salmonella* (NTS). Thus, the aim of this research was to screen an indigenous plant (*Vitex doniana*) as an alternative agent in elimination of the disease. Fifteen serotyped clinical *Salmonella* isolates were obtained from Medical Microbiology Department of the University of Jos Teaching Hospital. Antibiotic pattern of the clinical isolates was determined by Kirby- Bauer's method. The isolates were also screened for their susceptibility to *V.doniana* crude extract by Agar Well Diffusion. Thirteen (86.6%) of the isolates were susceptible at the concentration of 200mg/ml while 9(60%) at 100mg/ml. The isolates were 100% susceptible to Pefloxacin, Ciprofloxacin and Chloramphenicol but exhibited high resistant against Amoxicillin+Clavulanic Acid, Amoxicillin and Ofloxacin. The Micro Well Dilution Method was used to determine the MICs of the plant fractions and the control drug (Pefloxacin). The both fractions, VF8 and VF3 had the same MICs within the range of 150µg/ml and 300µg/ml while that of Pefloxacin was between 9.37µg/ml and 600µg/ml. The MBC of both fractions, AF8, AF3 and Pefloxacin was between 300µg/ml and 600µg/ml and 150µg/ml-600µg/ml respectively. Nine (60%) were multidrug resistant while 6(40%) were susceptible suggesting high presence of MDR strains in the study area. The plant fractions killed (bactericidal) most of the isolates than Pefloxacin. Thus, it is likely that with further processing, this plant product could serve as therapeutic agent in eliminating this neglected tropical disease.

Keywords*:* Neglected tropical disease, *Vitex doniana* extract, multidrug resistant (MDR)

INTRODUCTION

The genus *Salmonella*, is medically classified as typhoidal and non-typhoidal. They are the causative agents of typhoid, paratyphoid and food borne disease (salmonellosis) respectively. Typhoid disease and salmonellosis have affected mankind since human population grew large enough to contaminate their water and food supplies (Smith *et al.,* 2010). Tropical countries are the most hit because of over crowdedness, poverty and poor sanitary condition and there has been no control centers and intervention from developed nations unlike HIV and malaria infections to curtail the spread. The situation is worrisome with the emergence of the invasive non-typhoidal *Salmonella* (iNTS) like Typhimurium ST 313 in the tropic. It has remained a vital cause of invasive disease especially in sub-Saharan African, likely secondary to high rate of coexisting

malnutrition, malaria and HIV infection (Curtis, 2013). It is an important etiology agent associated with malnourished children and adults infected with malaria and HIV (Feasey *et al*., 2012; Gilks *et al*., 2010; Arthur, 2001).A study carried out by Majowiscz *et al.* (2010) reported an estimate NTS burden to be 2.5 million cases and 410 deaths per year in Africa.

The emergence of typhoid and non typhoidal multi-drug resistant strains to primary antibiotics like ampicillin, amoxillin and sulphamethoxazole is in the increase. Again the resistance of the strains to even some fluoroquinolones has further complicated the health problems making treatment difficult and necessitating usage of expensive antimicrobial agents like pefloxacin and ceftriaxone. These third generation agents are not readily available and also not affordable in developing countries (Crump *et al*., 2011). The search for new drugs from plants to combat the problem of drug resistance nowadays has been receiving more attention (Coates *et al*., 2002; Henry, 2000).This is because plant products possess antimicrobial compounds that exhibit antimicrobial activities against various pathogens and have been used for centuries to inhibit microbial growth (Hamed *et al*., 2006; Abbey *et al*., 2005).Thus the aim of this study was to screen an indigenous plant (*Vitex doniana*) as an alternative agent in elimination of this neglected infection in the tropics.

METHODS

Bacterial Isolates

Fifteen clinical *Salmonella* isolates serotyped by polyvalent antiserum Poly O, 1-67 and Poly H-1+2(SIFIN-GERMANY) Subgroup and Monavalent sera (Carper Laboratories, London) were collected from Medical Microbiology Department of the University of Jos Teaching Hospital.

Antibiogram

Kirby-Bauer's method was adopted using Cotrimoxazole, Chloramphenicol, Streptomycin, Pefloxacin, Sparfloxacin, Pefloxacin, Gentamicin, Ofloxacin, Amoxicillin and Amoxicillin + Clavulanic Acid to obtain the antibiogram against the isolates.

Collection and Preparation of the Plant Sample

Vitex doniana was identified in the herbarium of the Federal College of Forestry, Jos, Plateau State after being collected from Doemak, Quaan-Pan of Plateau State. The procedure of Ndip *et al. (*2009) was used for the extraction of the plant material with slight modification by using thermostatic water Cabinet (model HH-W420, XMTD-204 and TT42D Multipurpose use. Techmel and Techmel, USA) at 1000C instead of rotor vapor to concentrate the plant extracts. Analytical grade 95.5% ethyl acetate was used for the cold extraction.

Column Chromatography

The extract of *V.doniana* was fractionated by column chromatography process using the following mobile phase; n-hexane: EtoAC as (10:0, 15:1,9:4,8:2,7: 3,6:4,5:5,4:6,3:7,2:8) and EtoAC: MeOH (10:0,9:1,8:2, 7:3,6:4,5:5) and the effluent was collected in a small fraction (150cm^3) in a beaker. Fractions from crude plant extract were pooled together based on similar profile on Thin Layer Chrmatography (TLC, Alugram Xtra SIL G/uv 254, MAC HERY-NAGEL GmbH and Co. Kg, Germany) to yield 11 *V.doniana* leaf fractions of VF1-VF11 due to variance in polarity and types of constituents extracted (Mouroge *et al*., 2013). However only 2 most effective fractions obtained after preliminary bioassay of the 11 fractions were tested against the isolates.

Phytochemical Screening

The phytochemical screening of ethyl acetate extract of *V.doniana* extract was carried out using standard qualitative procedure (Trease and Evans, 1989, Sofowara, 1993).

Sensitivity Test of the Crude Extract

Agar Well Diffusion Method was used to determine the sensitivity of the isolates to the plant crude extract. A Mueller-Hintor agar plate was inoculated with 0.7ml of suspended isolate of inoculum size equivalent to 1X10^{8}cfu/ml and the excess fluid at the edge of the petri dish was removed with sterile cotton wool to obtain confluent growth. The plates were then kept for few minutes to dry. Wells of 6ml in diameter were aseptically punched with a sterile cork borer. A stock concentration of the crude plant extract was obtained by dissolving 2g of crude plant extract in 10ml of DMSO and 100mg/ml of the crude extract was prepared. Two wells punched in a plate were filled with 200mg/ml and 100mg/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^{0}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 (Boyonova *et al*., 2005).

Table 1. The Susceptibility of isolates to Antibiotics

I.D No	Serogroup	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	S	MDR	SS
2	C	M	**S**	**S**	**S**	R	R	**S**	**S**	R	R	MDR	-
3	C	**S**	**S**	M	**S**	R	R	S	S	R	S	MDR	-
4	A	**S**	**S**	**S**	**S**	R	R	S	S	R	S	MDR	-
5	C	**S**	**S**	**S**	**S**	R	R	S	S	R	S	MDR	-
7	D	**S**	**S**	**S**	**S**	R	R	M	S	R	R	MDR	-
8	C	**S**	**S**	**S**	**S**	R	S	S	S	S	S	-	SS
9	D	**S**	**S**	**S**	**S**	M	R	S	S	S	R	-	SS
11	C	**S**	**S**	**S**	**S**	S	S	S	S	S	S	-	SS
12	D	**S**	**S**	**S**	**S**	R	R	S	S	R	S	MDR	-
13	E	**S**	**S**	**S**	**S**	S	S	S	S	S	S	-	SS
14		S	S	S	S	R	R	S	S	R	R	MDR	-
17	D	S	S	S	S	S	S	S	S	S	S	-	SS
20	Typhi	S	S	S	S	S	M	S	S	S	S	-	SS
26	D	M	S	S	S	R	R	S	S	S	R	MDR	-
27	D	M	S	S	S	R	R	M	S	S	R	MDR	-
ATCC 25922		S	S	S	S	M	R	M	S	M	M	-	-
Total												**9(60%)**	**6(40%)**

SXT(Cotrimoxazole), CH(Chloramphenicol), S(Streptomycin), PEF(Pefloxacin), SP(Sparfloxacin), CPX(Pefloxacin), CN(Gentamicin), OFX(Ofloxacin), AM (Amoxicillin), AU(Amoxicillin + Clavulanic Acid, MDR (Multidrug Resistant Strain), SS(Suscepitble strain),M(moderate strain).

Determination of Minimum Inhibition Concentration (MIC) of Fractions of *A.hirtum*

The MIC of the leaf fractions (Ethyl acetate VF8 and Ethyl acetate VF3) against the various isolates was done by using the 96-well micro dilution method described by Nvau *et al.,* (2011), but with slight modification of using 8 wells instead of 12 wells. After about 24 hours sub-culturing of the isolates on nutrient agar and Xylose Lysine Deoxycholate (XLD), 4-5 colonies of the same appearance of each isolate was emulsified in sterile normal saline according to Ndip *et al.*(2007) documented by Nyenje and Ndip (2011) and adjusted to 0.5M_c Farland Scale ($1x10^8$cfu/ml). Fifty microlitres (50µl) of Brain Heart Infusion (BHI) broth was introduced into wells 2 to 8. One hundred microlitre of 0.006g of the extract dissolved in 10ml of DMSO was dispensed into well 1 and 50µl was then transferred from well 1 and delivered into well 2. After thorough mixing, 50µl was again transferred from well 2 to 3 and the same procedure was repeated through to well 8 and from well 8, 50µl was discarded. Thereafter, 50µl of inoculum was introduced to all the wells. The same amount of Pefloxacin (positive control) was processed alongside the 2 fractions of the plant. The wells were then covered with plastic tape, incubated for about 24 hours and observed for turbidity. The well before the one that showed turbidity (*growth*) was noted as Minimum Inhibitory Concentration (MIC). *Escherichia coli* (ATCC 25922), a standard reference strain was used as a quality control for disk diffusion and MIC (Beyene *et al*., 2011).

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 (Mourouge *et al.,* 2013).

RESULTS

Out of the 15 *Salmonella* isolates 9(60%) were multidrug resistant while 6(40%) were susceptible (Table 1). The isolates showed 100% susceptibility to ciprofloxacin, pefloxacin and chloramphenicol and 93.33% to sparfloxacin but 66.67% resistance to amoxicillin, amoxicillin + clavulanic acid and 46.67% to ofloxacin (Table 2). The sensitivity of isolates to the crude ethyl acetate extract of *Vitex doniana* ranged from 00mm – 20mm at 200mg/ml and from 100mm - 18mm at 100mg/ml. All the serogroups were sensitive to the plant extract except one of the serogroup C and D (Table 3). The Minimum Inhibitory Concentration (MIC) of *V.doniana* Fractions VF_8 and VF_3 varied from 150µg/ml to 300µg/ml while that of pefloxacin ranged from 9.37µg/ml to 600µg/ml. The phytochemical screening revealed the presence of alkaloids, flavonoids, cardiac glycosides, terpenes and steroids and resins.

Table 2. Percentage (%) Susceptibility and Resistance of Isolates to Antibiotics

Antibiotics	No. of Isolates Resistant	% Resistant	No.of Isolates Susceptible	% Susceptible	No.of Isolates Moderate	% Moderate
SXT	0	0	12	80	3	20
CH	0	0	15	100	0	0
SP	0	0	14	93.33	1	6.67
CPX	0	0	15	100	0	0
AM	10	66.67	4	26.67	1	6.67
AU	10	66.67	4	26.67	1	6.67
CN	0	0	13	86.67	2	0
PEF	0	0	15	100	0	0
OFX	7	46.67	8	53.33	0	0
S	6	40	9	60	0	0

SXT(Cotrimoxazole), CH(Chloramphenicol), S(Streptomycin), PEF(Pefloxacin), SP(Sparfloxacin), CPX(Pefloxacin), CN(Gentamicin), OFX(Ofloxacin), AM(Amoxicillin), AU(Amoxicillin + Clavulanic Acid).

Table 3. *Invitro* Antibiacterial Activity of Ethyl acetate *V. doniana* Crude Extract against Isolates

Isolates i.d. No.	Serogroup	200mg/ml	100mg/ml
2	C	16	13
3	C	00	00
4	A	20	16
5	C	20	15
7	D	15	00
8	C	16	13
9	D	19	08
11	C	10	00
12	D	18	16
13	E	18	15
14	D	13	00
17	D	20	18
20	Typhi	13	10
26	D	17	11
27	D	20	15
ATCC 25922		18	14

Susceptibility at break points (≥ 11 mm), i.d No. (Identification Number)

Table 4. *Invitro* Antibiacterial Activity of Ethyl acetate *V. doniana* Fractions against Isolates

Isolates i.d. No.	VF_8	VF_3	Pefloxacin
2	300	300	300
3	300	150	150
4	300	150	300
5	300	300	600
7	300	300	150
8	300	300	300
9	150	300	34
11	300	300	159
12	150	300	150
13	300	300	75
14	150	150	150
17	150	150	150
20	150	300	9.37
26	300	300	75
27	300	300	37.5
ATCC 25922	75	150	150

i.d No. (Identification Number)

Table 5. Minimum Bactericidal Concentration (µg/m) of Ethyl acetate *Vitex doniana* fraction VF8, VF3 and Pefloxacin

Isolates i.d. No.	VF_8	VF_3	Pefloxacin
2	600	300	ND
3	600	600	ND
4	600	600	ND
5	600	600	ND
7	ND	300	600
8	600	300	ND
9	600	300	ND
11	600	300	600
12	600	300	600
13	600	ND	75
14	600	600	150
17	600	600	150
20	600	300	ND
26	600	300	600
27	600	600	300
ATCC 25922	300	300	300

i.d No. (Identification Number), +(Growth), -(No growth), ND(not determined).

DISCUSSION

The prevalence of typhoid infection and salmonellosis in tropical countries, Nigeria inclusive, is partly due to negligence. There is very limited scope of studies, lack of coordinated epidemiological surveillance system and lack of adequate laboratory facilities for correct diagnosis. Also, limited reporting of cases and the presence of other diseases considered to be of high priority could have over shadowed the problem of *Salmonella* infections (Ayala *et al*., 2015).

The high rate of multidrug resistance observed in the *isolates* screened from the patients attending the hospital is in conformity with the report of Mourouge *et al*. (2013) who reported that anti-biotic drug resistance is increasing worldwide in both hospitalized patients and outpatients. Again, in terms of geographical location relatedness, the high rate (60%) of MDR screened is in line with the earlier work of Ehwarieme (2011) who reported high presence of MDR in Warri, Nigeria. Also, this high rate is confirming the finding of Okonko *et al.* (2010) who reported 72.7% prevalence of MDR isolated from poultry feed in Calabar. Most of the isolates resisted amoxicillin+clavulanic acid, amoxicillin and co-trimoxazole. The resistance to these traditional first-line, inexpensive antibiotics have been reported from the same Jos, Nigeria (Opajobi *et al.,* 2014). The treatment of typhoid fever was initially successive with the use of first line antibiotics like chloramphenicol, amoxicillin and trimethoprim-sulfamethorazole (WHO, 2003). However, Jesudason *et al*. (1996), Kalu *et al.* (2008) and Threlfall *et al*.(1992) have reported progressive profile of prevalence of multi drug resistant (MDR) *S.*Typhi*,* most especially in developing nations like Nigeria. Thus the re-sistance exhibited by these e in this study to some of the antibiotics, confirm these earlier reports. Surprisingly, sensitivity to chloramphenicol which is also a primary antibiotic was high (100%).This observation is closely related to that of Prajapati *et al*. (2008) who documented high sensitivity of e to chloramphenicol. This finding also indicates that chloramphenicol which is no longer commonly used for treatment of typhoid in this research area has demonstrated to be active against these isolates even though they were resistant to usually prescribed antibiotics. This phenomenon is in line with the observation that an organism that was formerly resistant to a particular antibiotic may become susceptible if treatment with that antibiotic is suspended for a long time (Threlfall and Ward, 2001).However, the 100% susceptibility of the isolates to pefloxacin and ciprofloxacin is in line with the findings of Cajetan *et al*. (2013) from Abuja, Nigeria, where all the screened isolates were susceptible to ciprofloxacin. Nevertheless, several researchers have reported some serovars showing reduced susceptibility to ciprofloxacin which is at variance with the finding of this present study (Lunguya *et al.,*2012; Tabo *et a*l.,2013).Despite the newer antibiotics, continued selective antibiotic pressure and bacterial adaptation have compounded issue resulting in increase or prevalence of antibiotic resistant bacteria and emergence or multidrug resistance strains in many species that cause human disease (Mourouge *et al*., 2013). Again, the increase in multi-antimicrobial resistant strains isolated from humans has been reported to be linked with the widespread use of antimicrobial agents in food and animal production ((Raufu *et al*., 2014).

The presence of phytochemical substances such as alkaloids, flavonoids and terpenes and steroids agree with the finding of Kumar et al., (2013) who reported the

presence of the same phytochemical substances in *Vitex negundo* and *Adhatoda vasica*. Several of phytochemicals have been reported to processes antimicrobial properties (Kumar *et al*., 2013). Alkaloids, flavonoids and sterols have been said to be active against some pathogenic bacteria including Typhi (Kennedy and Wightman, 2011; Choudhury *et al*., 2013) which were responsible for the anti- effect exhibited in this study. Flavonoids can function as bacteriostatic compounds by inhibiting the number of viable colonies and also as energy metabolism inhibitor (Konsam *et al*., 2015). Terpenoids present in the ethyl acetate extract might have added to the antibacterial properties since these compounds are bactericidal and fungicidal (Wiant, 2007).

The sensitivity of all the *Salmonella* isolates except two of them the plant extracts of *Vitex doniana* at concentration of 200mg/ml shows that this plant had inhibitory effects on *Typhi* and non-typhoidal e. This finding agrees with the various reports on medicinal plants exhibiting antimicrobial activity (Adegoke *et al*., 2010; Habtamu *et al*., 2010; Kamba and Hassan, 2010). The MICs of the different sub fractions (VF8, VF3) of *V.doniana* ranging from 150µg/ml to 300µg/ml confirms effectiveness of this plant against e even at low concentration (Doughari *et al.,* 2008).This also goes to show that the antibacterial properties were distributed into different fractions suggesting that the plant possesses different active antibacterial principles (Taiwo *et al*., 1999). This explains a recent move towards validating the activity of phytochemicals using fractionating guided protocol in realizing most antimicrobial uses from medicinal plants (Rufai *et al*., 2015).

The MBC assays showed that even at low concentration of 3000µg/ml, the plant fractions were bactericidal against some MDR but at higher concentration (600µg/ml) almost all the test organisms were sensitive. In term of effectiveness with respect to number of isolates being affected, pefloxacin was not bactericidal against 7 isolates, while VF3 and VF8 were not bactericidal only against 1 isolate each showing they had broad antibacterial activity than pefloxacin.

The activity of the fractions of these plants against even some of the isolates that showed resistance against many antibiotics indicates that these plant materials can be used in treatment of persistent typhoid fever and salmonellosis. The *V. doniana* extract had been earlier documented to be effective in diarrheal treatment (AbdulKarim *et al*., 2005) which is in line with this present finding. Dauda *et al*. (2011) also reported *V. doniana* leaf extract to exhibit a zone of inhibition of 20mm against *Typhi* and with the MIC at different concentrations inhibiting growth of some organisms, they considered the plant extracts as chemotherapeutic agents. The manner of the potency demonstrated by the plant extracts was concentration dependent which confirms the report of Adefuye *et al.* (2011) and Oboh and Abulu (1997).

CONCLUSION

It is evident from the result obtained in this present study that ethyl acetate extract of *Vitex doniana* was active as anti-typhoid and anti-salmonellosis agent against Typhi and Non Salmonella Typhi (NST) respectively. This is indeed a promising development, because with further processing of these bioactive substances, new chemical classes of medicines could be discovered and used against this infection which also exhibit MDR against orthodox medicines (antibiotics) thereby eliminating the neglected tropical disease.

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Conflict of Interest

All authors declare that there is no conflict of interest

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