AN *IN-VITRO* AND *IN-VIVO* ANTIBACTERIAL ACTIVITY OF *Vitexdoniana* CRUDE EXTRACTS ON *Salmonella typhi*

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ABSTRACT

Vitex doniana belongs to the family of Verbenaceae. It is the most abundant and widespread of the genus occurring in the savannah regions. This research was carried out to evaluate the antibacterial activity of *Vitex doniana* crude extracts on the clinical isolates of *Salmonella typhi*. The clinical isolates of *Salmonella typhi* were subjected to antimicrobial susceptibility test using agar diffusion technique. Phytochemistry of the *Vitex doniana* crude extracts revealed the presence of tannins, saponins, flavonoids, carbohydrates, glycosides, proteins, and steroids. Ethanolic and acetonic crude extract produced clear zones of inhibition at concentrations ranging from 50 to 150mg/ml. The Minimum inhibitory concentration was between 50 and 200mg/ml and the minimum bacteriocidal concentration was 100mg/ml. Five thousand milligrams per kilogram body weight of the crude extracts were administered to the mice orally and no case of death was recorded. *In vivo* antimicrobial assay revealed that mice treated with crude ethanolic and acetonic extracts survived after being infected with *Salmonella typhi*. Similarly, untreated mice (control) died after 48 hours of inoculation with *Salmonella typhi*. *Vitex doniana* crude extracts could be potential agents for the treatment of diseases associated with *Salmonella typhi.*

Key words: Antibacterial activity, *Salmonella typhi*, Crude extracts, *Vitex doniana* and Toxicity

INTRODUCTION

Vitex doniana belongs to the family of Verbenaceae. It is the most abundant and widespread of the genus occurring in the savannah regions (Agbede and Ibitoye, 2007; Nwachukwu and Uzoeto, 2010; Dauda *et al.*, 2011). It is a deciduous tree of coastal woodland, riverine and lowland forests. The height is about 8-18 m, with a heavy rounded crown and a clear bole up to 5 m (FAO, 1983). The flowers are small, blue or violet, 3-12 cm in diameter. The fruits are oblong, about 3 cm long, green when young, turning purplish-black on ripening and with a starchy black pulp. Each fruit contains 1 hard, conical seed, 1.5-2 cm long, and 1-1.2 cm wide. The fresh leaves are also used as vegetables (Agbede and Ibitoye, 2007; Dawang and Datup, 2012). *Vitex doniana*, according to traditional medical practitioners in Ebonyi State of Nigeria is known to be effective forthe treatment of fevers, particularly typhoid fever (Iroha *et al.*, 2010). Similarly, Survey of forest plants used for the treatment of typhoid fever was carried out by Faleyimu *et al.* (2010) and it was found that *Vitex doniana* was used frequently for the treatment of typhoid fever. Typhoid fever is an infectious disease caused by *Salmonella enteritica serovar typhi* (Willey *et al,* 2008, Sarkiyayi *et al.,* 2011). The organisms are Gram-negative, flagellated, non-encapsulated, non-sporulation and facultative anaerobic bacilli (Sarkiyayi *et al.,* 2011).

Typhoid fever is an endemic disease in the tropic and sub-tropic and has become a major public health problem in developing countries of the world,

particularly in Nigeria (Abdulkarim, 2012). Transmission is through fecal-oral route (Sarkiyayi *et al.,* 2011). The desire to investigate the use of medicinal plants as a remedy to typhoid fever is born from the fact that the disease has claimed many lives in Nigeria, coupled with continuous resistance to most orthodox drugs available (Ibekwe *et al.*, 2008). This study, therefore was an attempt to evaluate the antimicrobial potential of *Vitex doniana* crude extracts against *salmonella typhi* with a view to identifying the active part of the plant that have antimicrobial potentials of the plant.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The plant parts (leave, stem bark and root) were collected from Kuta in Shiroro Local Government Area of Niger State, Nigeria. Identification of the plant was carried out at Department of Biological Sciences (Plant Biology), Federal University of Technology, Minna, Niger State. The herbarium voucher No VD 4786 was deposited in the Plant Biology Department of the Federal University of Technology, Minna.

Extraction of the Plant Material

The plant materials root, stem-bark and leaves were dried and grounded using mortar and pestle, pulverized to powdered form using blender. The method of Abalaka *et al.* (2011) was employed for the extraction. One hundred (100) grams of powdered sample of each plant part was mixed separately with 500ml of 99% acetone, 95% ethanol and distilled water at room (28 ± 2^0C) temperature with occasional shaking for a period of about 120 hours. The mixtures were filtered and the filtrates were evaporated in steam bath and further dried to constant weight at 60^0 C using hot air oven. The reconstitution of the extracts was done using distilled water and stored at 4^0C for further use (Trease and Evans, 1983).

Phytochemical Screening of the Plant Extracts

The crude extracts were subjected to phytochemical screening for possible detection of secondary metabolites such as alkaloid, tannin, saponin, flavonoid, glycoside, carbohydrate, protein, steroid anthroquinone and phenolic substances using standard procedures described by Harborne (1973), Trease and Evans (1983) as well as Sofowora (1983).

Source of the microorganisms

The test organisms (*Salmonella typhi)* were isolated from patients who were clinically diagnosed of typhoid fever at the General Hospital Minna, Niger state. The organisms were inoculated on Salmonella/Shigella agar and maintained at 37 ^{0}C for 48 hours. Finally, the organisms were kept in the refrigerator for further analysis (Chesebrough, 2010).

Identification of the Test Organisms

The clinical isolates (*Salmonella typhi*) were identified using Gram's stain procedures alongside Biochemical tests, which included triple sugar iron agar (TSI), citrate utilization, indole, urease, lactose, glucose fermentation and oxidase tests (Chesebrough, 2010).

In-vitro Antibacterial Assay:

The *Salmonella typhi* were subjected to anti-microbial susceptibility test using agar diffusion technique as described by Abalaka *et al.* (2011). The inoculum size of the isolate (*Salmonella typhi*) was standardized, inoculated into Mueller Hinton broth and incubated for 3 hours to obtain a suspension of 0.5 Macfarland turbidity standard (1.0×10^6cfu/ml). One milliliter of the standard suspension (1.0×10^6cfu/ml) was spread evenly on Mueller Hinton agar plate using sterile swab stick. Three holes (4mm) were bored on the surface of the agar medium equidistant from one another using 4mm cork borer. The bottom of each hole

was sealed with molten agar to avoid seepage. Concentrations of the crude extracts such (10mg/ml, 50mg/ml, 100 mg/ml and 150mg/ml) were introduced into 3 replicate wells. The plates were incubated at 37ºC for 24 hours. Observation for zones of inhibition was done, measurement taken in milliliter and the results recorded. Ciprofloxacin was used as positive control while one un-inoculated plate served as media-sterility control and one inoculated plate without the extracts, as organism-viability control (Abalaka *et al.,* 2011).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the crude extracts was determined using broth dilution method (Abalaka *et al.*, 2011). Each crude extract (0.0125g/ml, 0.025g/ml, 0.05g/ml, 0.1g/ml and 0.2g/ml) of *Vitex doniana* was introduced into 5 respective test tubes each containing 5ml of nutrient broth. Each test tube was labeled and 0.5ml of the bacterial suspension (1.0 x 10^6cfu/ml) was inoculated. The final volume in all the test tubes was adjusted by adding 10ml of distilled water to make concentrations of 12.5 mg/ml, 25.0 mg/ml, 50.0 mg/ml, 100.0 mg/ml and 200.0 mg/ml respectively. In each of the control tubes, the crude extract was not added. The un-inoculated test tubes were used to check the sterility of the medium as negative control while the positive control tube was used to check the suitability of the medium for growth of the *S. typhi* and the viability of the inoculums. All the test tubes were properly shaken and then incubated at 37^0C for 24 hours and the results were recorded. The MIC was determined by the lowest concentration of the crude extracts that prevented visible growth (turbidity).

Determination of Minimum Bactericidal Concentration (MBC)

The Minimum bacteriocidal concentration was determined as follows: The test tubes that showed no turbidity were streaked on the surface of nutrient agar plates devoted of crude extracts and incubated at 37^0C for 24 hours. The lowest concentration of the crude extract that inhibited growth was detected on sub-culturing and was considered as the MBC (Abalaka *et al.*, 2011).

Animal study

Thirty five (35) mice of both sexes weighing between 18 – 30g were used. They were obtained from Department of Pharmaceutical science, Ahmadu Bello University, Zaria and Biochemistry Department, Ibrahim Badamasi Babangida University, Lapai. All the mice were kept in the Biochemistry Laboratory of Federal University of Technology, Minna to acclimatize for two weeks before the commencement of the study. The animals were handled according to Canadian Council on Animal Care (CCAC) guideline on animal use protocol review (1997).

Determination of LD_{50} of the V. doniana Crude Extracts

The oral acute toxicity (LD_{50}) value of the crude (leaf, stem-bark and root) acetonic, and ethanolic extracts of *Vitex doniana* was determined using procedures described by the Organization of Economic and Cooperative Development (OECD, 2008). In this procedure, the mice were grouped into 7 and each group had five mice. Six groups were administered orally with 5000 mg/kg body weight crude extracts of *Vitex doniana*. The seventh group served as the control. The mice were observed closely at 4, 24 and 72 hour intervals, up to 14 days for any delayed toxic signs of a general nature, such as: general activity, response to touch, grasping, tail twisting, and strength of grip, tremors, convulsions, stimulation and respiratory frequency. At the end of the observation period, the mice that survived were weighed and the results recorded (OECD, 2008).

In-vivo Antibacterial Assay of Crude Extracts of *Vitex doniana*

In-vivo antibacterial assay was carried out using the method described by Vakantesan *et al.* (2011).The *Salmonella typhi* was grown on Salmonella-Shigella Agar (SSA) and incubated at 37^0C for 24 hours. The fresh culture was transferred into nutrient broth and incubated for 12 hours at 37^0C. The culture suspension was diluted serially using sterile normal saline and from the dilution factor 10^{-7}, 0.5 ml of the inoculums was injected intraperitoneally into all the pre-labeled 7 groups of mice. The mice were observed for any sign of infection for 48 hours. The 300mg/body weight acetonic and ethanolic crude extracts of *Vitex doniana* was used to treat the mice daily after infection for a period of 7 days. The control (Group 7) was not treated. All the mice were under observation for 7days.

Thin Layer Chromatography (TLC) of Vitex doniana Crude Extracts

The crude extracts were subjected to thin layer chromatography and different fractions were identified and subjected to anti-microbial screening. The procedures for the TLC are as follows: one mg of each extracts was dissolved in 2 ml of water and 40 microliter (final concentration of 20 microgram/spot) was submitted to TLC on silica gel G60 F254 aluminum plates (Merck, 10cm×10cm). Effluent was ether/ethylacetate/formic acid (75:25:1), spot were detected by UV light at 254 365nm (Abalaka *et al.,* 2011).

STATISTICAL ANALYSIS

Data were analyzed using statistical package for social science (SPSS) version 16 and presented as means ± SEM. Comparisons between different groups was done using Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT). Values of $P<0.05$ were considered as statistically significant as described by Mahajan (1997).

RESULTS

The phytochemical analysis of the crude extracts of *Vitex doniana* revealed the presence of tannin, saponin, flavonoid, carbohydrate, glycoside, protein and steroid (Table I).

Table 1: Phytochemical Properties of *Vitex doniana* Crude Extracts

Crude Extracts	Phytochemicals									
	Tannins	Saponins	Flavonoids	Glycosides	Carbohy.	Steroids	Proteins	Alkaloids	Anthroquinones	Phenols
L_{Aq}	-	+	-	-	-	-	-	-	-	-
B_{Aq}	-	-	+	-	-	-	-	-	-	-
R_{Aq}	+	-	-	-	-	-	-	-	-	-
L_{Eth}	+	+	+	+	+	-	+	-	-	-
B_{Eth}	+	-	+	+	+	+	+	-	-	-
R_{Eth}	+	+	+	+	+	+	+	-	-	-
L_{Ace}	+	+	-	+	+	-	+	-	-	-
B_{Ace}	+	+	+	+	+	+	+	-	-	-
R_{Ace}	+	+	-	+	+	+	+	-	-	-

Key Present = (+) L_{aq} = Aqueous leaf Leth = Ethanolic leaf L_{ace} = Acetone leafAbsent= (-) B_{aq} = Aqueous stem black B_{eth} = Ethanol stem black B_{ace}= Acetone stem back R_{aq}= Aqueous root R_{eth} = Acetone root R_{ace} = Acetone

The mean zone of inhibition by acetonic and ethanolic leave extracts at 50mg/ml was 2.66±0.33 and 3.33±0.33mm. At 100mg/ml, the mean zones of inhibition were 5.00±0.57 and 6.66±0.33 mm while 9.66±0.88 and 9.33±1.20 mm at 150 mg/ml was recorded respectively (Table 2).

The mean zone of inhibition for acetonic and ethanolic extracts of stem-bark had 3.66±0.33mm and 4.33±0.33mm at 50mg/ml. At 100mg/ml, it was 7.60±0.33 and 7.66±0.33mm while at 150mg/ml, it was 14.00±0.57 and 14.33±0.33mm (Table 3).

The mean zone of inhibition for acetonic and ethanolic extracts of the root produced 4.33±0.33 and 4.00±1.00mm at 50mg/ml. At 100mg/ml the extracts had 8.33±0.33mm and 7.00±0.57 mm while 14.66±0.33mm and 13.66±0.33mm was produced by acetonic and ethanolic root extracts of *Vitex doniana* at a concentration of 150mg/ml (Table 4).

Table 2: Antibacterial Activity of the Aqueous, Ethanolic and Acetone Leaf Extracts of *V. doniana*

Extract	Concentration (mg/ml)				Ciprofloxacin
	10	50	100	150	(5mg/ml)
Acetone	0.00±0.00^{a}	2.66±0.33^{b}	5.00±0.57^{c}	9.10±0.88^{d}	32.66±0.33^{e}
Ethanol	0.00±0.00^{a}	3.33±0.33^{b}	6.66±0.33^{c}	9.33±1.20^{d}	32.66±0.33^{e}
Aqueous	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	32.66±0.33^{e}

a,b,c,d,e value with the same superscript on are not significantly different ($p \leq 0.05$)

Table 3: Antibacterial Activity of the Aqueous, Ethanolic and Acetone stem-bark Extracts of *V. doniana*

Extract	Concentration (mg/ml)				Ciprofloxacin
	10	50	100	150	(5mg/ml)
Acetone	0.00±0.00^{a}	3.66±0.33^{b}	7.60±0.33^{c}	14.00±0.57^{d}	32.66±0.33^{e}
Ethanol	0.00±0.00^{a}	4.33±0.33^{b}	7.66±0.33^{c}	14.33±0.33^{d}	32.66±0.33^{e}
Aqueous	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	32.66±0.33^{e}

a,b,c,d,e value with the same superscript on are not significantly different (p≤ 0.05)

Table 4: Antibacterial Activity of the Aqueous, Ethanolic and Acetone Root Crude Extracts of *V. doniana*

Extract	Concentration (mg/ml)				Ciprofloxacin
	10	50	100	150	(control)(5mg/ml)
Acetone	0.00±0.00^{a}	4.33±0.33^{b}	8.33±0.33^{c}	14.66±0.33^{d}	32.66±0.33^{e}
Ethanol	0.00±0.00^{a}	4.00±1.00^{b}	7.00±0.57^{c}	13.66±0.33^{d}	32.66±0.33^{e}
Aqueous	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.66±0.33^{a}	32.66±0.33^{e}

a,b,c,d,e value with the same superscript on are not significantly different (p≤ 0.05)

The minimum inhibitory concentration (MIC) was between 50.0 - 200.0mg/ml and the minimum bactericidal concentration (MBC) was 100 mg/ml (Tables 5 and 6).

Table 5: Minimum Inhibitory Concentration (MIC) of the Crude Extracts on the Test Organism

Extracts	Concentration (mg/ml) 50			Control drug(mg/ml)	
	200.0	100.0		25.0	12.5.0
Acetone leaf	-	-	-	+	+
Ethanolic leaf	-	-	-	+	+
Acetone stem-bark	-	-	-	+	+
Ethanolicstem-bark	-	-	-	+	+
Acetone root	-	-	-	+	+
Ethanolic root	-	-	-	+	+

Key: (+) = Presence of turbidity (-) = Absence of turbidity

Table 6: Minimum Bactericidal Concentration (MBC) of the Crude Extracts on the Test Organism

Extracts	Concentration (mg/ml)			
	200	100	50	12.50
Acetone leaf	-	-	+	-
Ethanolic leaf	-	-	+	-
Acetone stem-bark	-	-	+	-
Ethanolic stem-bark	-	-	+	-
Acetone root	-	-	+	-
Ethanolic root	-	-	+	-

Key: (+) = Presence of turbidity (-) = Absence of turbidity

Table 7 shows the oral acute toxicity (LD_{50}) of the crude acetonic and ethanolic extracts of leaf, stem-bark and root of *Vitex doniana* at an exceptional dose of 5000 mg/kg/bw. There was no death recorded in all the groups after the observation period. The result shows that the LD_{50} is greater than 5000 mg/kg/bw.

Table 7: Oral acute toxicity (LD50) of the crude extracts of *V. doniana*

Extracts	Number of mice/extracts	Conc. of Extracts	Number Death
Acetone leaf	5	5000mg	0/5
Ethanolic leaf	5	5000mg	0/5
Acetone stem-bark	5	5000mg	0/5
Ethanolicstem-bark	5	5000mg	0/5
Acetone root	5	5000mg	0/5
Ethanolic root	5	5000mg	0/5
Control	5	5000mg	0/5

Table 8 shows the *in-vivo* antibacterial assay of *Vitex doniana* extracts on *Salmonella typhi*. The results shows that death was not recorded in all the groups of mice treated with the extract after inoculation with *Salmonella typhi* while in control (untreated) group, all the mice died within 7 days.

Table 8: *In-vivo* Antibacterial Activity of *Vitex doniana* Crude Extracts on *Salmonella tyhpi*

Extracts	No. of mice/ extract	Dilution factor	Volume of inoculum	No. of live	No. of Death	% of Survival	% of Death
Acetone leaf	5	10^7	0.5ml	5	0	5(100)	5(0)
Ethanolic leaf	5	10^7	0.5ml	5	0	5(100)	5(0)
Acetone stem-bark	5	10^7	0.5ml	5	0	5(100)	5(0)
Ethanol stem-bark	5	10^7	0.5ml	5	0	5(100)	5(0)
Acetone root	5	10^7	0.5ml	5	0	5(100)	5(0)
Ethanolic root	5	10^7	0.5ml	5	0	5(100)	5(0)
Control (untreated)	5	10^7	0.5ml	0	5	5(0)	5(100)

Table 9 shows the anti-bacterial activity of TLC fractions of *V. doniana* crude extracts. The fraction of acetone stem-bark and root extracts and fraction of ethanolic root exhibited weak activity against the test organism. The organism was resistant to all other fractions of the extracts and as well as the remaining fractions of acetone stem-bark and root and that of ethanol root.

Table 9: Anti-bacterial Activity of the TLC Fractions of *V. doniana* Extracts

Extracts	Fractions (mg/ml) C			
	A	B		D
Acetone leaf	-	-	-	-
Ethanolic leaf	-	-	-	-
Acetone stem-bark	1	-	-	-
Ethanolicstem-bark	-	-	-	-
Acetone root	2	-	-	-
Ethanolic root	-	1	-	-

Key (-) = No activity A, B and C = Fractions

DISCUSSION

The presence (tannin, saponin, flavonoid, carbohydrate, glycoside, protein and steroid) of these metabolites in the ethanolic and acetonic extracts could have contributed substantially in the antibacterial activities recorded. The findings in this study agree with

the previous reports by Tijjani *et al.* (2012) and Katie *et al.* (2006). The results of the antibacterial susceptibility test shown that the extracts of *Vitex doniana* (leaf, stem-bark and root) were active against the test organisms at concentration ranging from 50 - 150mg/ml. The two crude extracts (ethanolic and acetonic) showed clear zones of inhibition in increasing order of concentration against *Salmonella typhi*. Although the inhibitory action exhibited by the crude extracts cannot be compared to that of the standard drugs used as the control, the fact that it inhibited the growth of the organisms in crude fashion makes the plant a potential source of drug for treatment of ailments associated with *salmonella typhi*. Statistical analysis showed that there is significant difference (p=0.05) between the zones of inhibition produced by the standard antibiotics and the crude extracts of the plant (*Vitex doniana*). The outcome in this report is in agreement with the report by Iroha *et al.* (2010).

The minimum inhibitory concentrations of both ethanolic and acetone crude extracts of all the plant parts were higher compared to the standard drug (control). The minimum bactericidal concentration of the ethanolic and acetonic crude extracts against the test organism was 100mg/ml. Although the MIC and MBC were higher compared to that of the standard drugs, this does not invalidate the antibacterial activity of the crude extracts. The values of MIC and MBC obtained from this sturdy are in agreement with the report by Faleyimu *et al.* (2010). The activity recorded by the components of this plant against *S. typhi* validates its use locally as an anti-typhoid agent. It also agrees with the findings of Iroha *et al.* (2010). The ineffective nature of the aqueous crude extract against the test organism could be attributed to the absence of phytochemical components alkaloids, anthroquinone and phenol in the aqueous extract (Table1).

The LD_{50} of all the extracts used in this study was 5000 mg/kg bwt and there was no death recorded. This lends credence to Sifuma (2011) who reported that extracts of *V. doniana* are safe for use orally in mice. Therefore, this report corroborates the finding of Sifuma (2011). The *in-vivo* antibacterial assay also revealed that the acetone and ethanolic crude extracts inhibited the growth of *S. typhi*. Furthermore, clinical signs of infection were rarely observed in the extract-treated mice after the period of treatment, whereas all the groups of untreated mice died within 7 days. The fact that treated mice survived, suggests that the extracts had activity against the *Salmonella typhi*. The various fractions identified after the TLC had no activity against *Salmonella typhi*. This is in agreement with the report by Harborne (1973) who asserted that the activity of plant extracts can sometimes change after TLC separation and the separated compound may eventually not possess the activity of the original crude extract.

CONCLUSION

From this study, it can be concluded that there is a pharmacological rationale for the use of the plant (*V. doniana*) in traditional medicine. The *in-vitro* and *in-vivo* antibacterial potential demonstrated by the crude extracts is a clear revelation that the plant could serve as an excellent candidate for the development of new drugs for the treatment of diseases associated with *Salmonella typhi*. Further investigation should be conducted to identify the chemical compounds in the plant extracts and their mechanism of action.

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