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Multi-Drug Resistant *Salmonella typhi* among Out-Patients in Hospitals within Ilorin, Nigeria and their Susceptibility to *Cymbopogon Citratus*

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ABSTRACT

The indiscriminate use of drugs, medication and non-adherence are one of the factors that has led to the emergence of multi-drug resistant *Salmonella typhi*. This study was conducted to assess the incidence of typhoid fever among out-patients in some selected Specialist Hospitals in Ilorin Metropolis and to determine the susceptibility of the causative agent to antibiotics and plant extract of *Cymbopogon citratus*. A cross-sectional survey was completed over a five-month surveillance period in these four locations namely: The Children Specialist Hospital Centre Igboro; Cottage Hospital; Adewole Specialist Hospital Alagbado; and Civil Service Hospital with a total of about 400 blood samples from out-patients across ages. Antibiogram profiling of the isolates to standard gram-negative antibiotics and antibacterial activity of *Cymbopogon citratus* extracts were determined using disc diffusion method. A total of Eight (8) *Salmonella typhi* isolates were recovered collectively from the patients. All the isolates were multi-drug resistant with a notable resistance to chloramphenicol, ampicillin and co-trimoxazole. Molecular confirmation of the isolates showed DNA size of 500 base pairs. The ethanolic extract of *Cymbopogon citratus* was more potent with MIC of 12.5mg/ml and MBC 25mg/ml while the phytochemical screening of the plants revealed the presence of alkaloids, flavonoids, phenols, tannins, saponins, steroids and glycosides. The promising outcome of the plant extract against *Salmonella typhi* posits its possible application in treatment against the infectious agent.

Keywords: Antibiotics, Antibacterial, Typhoid Fever, Resistance, Infection.

Introduction

Salmonella typhi, a motile, facultative anaerobe, is a typical *Salmonella* serovar that causes typhoid fever which pose a major threat to general health in developing countries (Qamar *et al.*, 2014). Typhoid fever is a systemic infection which may last for about three to four weeks and death rate

ranges between 12% and 30%. There has been a decrease in the worldwide spread of typhoid fever; however, the emergence of multi-drug resistant *S. typhi* (MDRST) is still a threat to public health. There exist about 107 strains of this organism with varying metabolic characteristics, virulence level,

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and multi-drug resistant genes that complicates treatment in prevalent areas (Parkhill *et al.*, 2001; Deng *et al.*, 2003).

Despite the emergence of newer antibacterial drugs, enteric fever has continued to be a major health problem (Zaki and Karande, 2011). Resistance of *S. typhi* to antibiotics like ampicillin, ceftriaxone, co-trimoxazole, and ciprofloxacin (Sehra *et al.*, 2013) further depicts the complications that could arise from the disease condition and thus poses greater challenge in its management.

***Cymbopogon citratus* (Lemongrass)**

Cymbopogon citratus of the Poaceae family is a tall, monocotyledonous aromatic perennial plant with slender sharp-edge green leaves and pointed apex. Lemongrass, *Cymbopogon citratus* is one of the important leaves among the species of grasses, and has various applications in traditional medicine (Ernst, 2008). In Nigeria, the Edos call it: *eti*, Efik: *Ikon eti*, Hausa call it: *tsauri*, Ibibio: *myobaka makara*, Igbo (Owerri) call it: *achara ehi* and Yoruba call it: *kooko oba* (Ahmad and Beg, 2001). Lemongrass ranked highest in inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA) infection, helpful in relieving colitis indigestion/gastro-enteritis ailments and relieves the symptoms of headache, body ache, nervous exhaustion and stress-related conditions amongst others (Joseph, 2005). The aim of the research is to molecularly detect the occurrence of multidrug resistant (MDR) *Salmonella typhi* among out-patients attending Major Health Institutions in Ilorin metropolis, Nigeria. The efficacy of commercially available antibiotics and different extracts of *Cymbopogon citratus* on the detected isolates was also evaluated.

Materials and Methods

Study Area and Population

This study was carried out in four selected tertiary hospitals within Ilorin, Kwara state, Nigeria, including: Children Specialist Hospital Centre Igboro (CSH); Cottage Hospital, Adewole (CHA);

Specialist Hospital Alagbado (SHA); and Civil Service Hospital, G.R.A (CSHG). These hospitals have facilities to attend to chronic health issues and they are accessible to patients seeking medical consultation. Consenting age groups (children, adults and aged) who were out-patients in the selected hospitals were sampled. A structured close-ended questionnaire was shared among consenting subjects to gather information about the socio-demographic characteristics such as age, gender, socioeconomic status such as level of education, occupation, the use of antibiotics and patients' exposures to the risk factors associated with typhoid fever. The survey/ sample collection was carried out in all the four locations from September, 2017 to June, 2018. A total of four hundred (400) subjects were recruited for this study. One hundred (100) blood samples was obtained from consenting subjects in each of the four (4) different hospitals.

Sample Collection

About 5ml of blood samples were aseptically obtained from subjects using a sterile needle and syringe, dispensed directly into sterile blood culture bottles containing thioglycolate broth, placed in ice packs and transported to the laboratory for analysis. Samples were labeled according to the serial number allotted to the questionnaire filled by the patients.

Identification and Phytochemical Screening of Plant Extracts

The leaf of *Cymbopogon citratus* (Lemon grass) was sourced from farms by herbal practitioners in Ilorin town, Nigeria. Identification and authentication of the plant was done at the Department of Plant Biology Herbarium, University of Ilorin, Ilorin, Nigeria, with voucher number UILH/001/1284. Phytochemical analysis was performed to screen the extracts for the presence of alkaloids, flavonoids, phenols, tannins, steroids, saponins and glycosides. All procedures

were as described by Geetha and Geetha, (2014) with slight modifications.

Bacteriological Analysis

Isolation and identification of *Salmonella typhi* from the samples was done by culturing on Salmonella-Shigella agar and incubated at 37°C for 24 hours. Biochemical test was carried out using Hi25™ Enterobacteriaceae identification kit (HiMedia laboratories, India). The *in vitro* sensitivity testing of the *S. typhi* isolates to different antibiotics including: Cefixime 5µg; Ofloxacin 5µg; Ciprofloxacin 5µg; Ampicillin 5µg; Chloramphenicol 30µg; Nalidixic acid 30µg; Co-trimoxazole 25µg; and Nitrofurantoin 30µg (Rapid labs, UK and Oxoid, UK) were carried out as recommended by the Clinical and Laboratory Standards Institute using the Kirby-Bauer agar disc diffusion method (Bauer *et al.*, 1966). The ethanolic and methanolic extracts were prepared according to the methods of Arya *et al.* (2014) and Al- Mansoub *et al.* (2013), to get the crude extract. The antibacterial activity of these extracts against these isolates was done using agar well diffusion method (Bauer *et al.*, 1966). The Minimum inhibitory and bactericidal concentrations of the extracts were determined using dilution susceptibility test against the seeded standardized isolates on Mueller Hinton broth.

Molecular Identification of Isolates

The Zymo Bacterial/Fungal DNA mini prep™ kit (Vesty *et al.*, 2017) was used for DNA extraction following the manufacturer's specifications and guidelines. Extracted DNA were subjected to polymerase chain reactions (PCR) using the primer of Haim *et al.*, 2008. Briefly, the PCR master mix was prepared containing 0.8µl deoxynucleoside triphosphate (dNTPs), 2.5µl PCR buffer, 0.2 µl thermos aquaticus (Taq) polymerase, 1.5 µl MgCl₂, 5µl DNA lysates, 11µl of PCR water and 2µl of primers. Twenty-five (25µl) total volume reaction was used containing 20µl of aliquot of master mix with 5µl of DNA lysate were put in eppendorf

tubes. Controls were set along with the samples and put in the PCR machine. Reactions consisted of an initial denaturation phase (95°C for 5 min), followed by 30 cycles of denaturation, annealing and elongation. The denaturation phase was performed at 95°C for 30 seconds. The annealing temperatures were varied according to the primers used and were also for 30 seconds. The elongation phase was also performed for 30 seconds at 72°C and a final elongation step of 10 min at 72°C. The PCR products with the DNA ladder were subjected to agarose gel electrophoresis at 100V for 25 minutes, after which the bands were visualized under ultra violet (UV) light on a Gene Genius' image analyzer (SYNGENE, Cambridge, UK).

Ethical Approval

Ethical approval was obtained from the University Ethical Review Committee, University of Ilorin, Ilorin, Nigeria (UERC/ASN/2018/1113) and Ministry of Health, Ilorin, Nigeria. Patients consent were sought and questionnaire administered before the collection of samples.

Results

The percentage age distribution of the subjects used in this study at each location is shown in Figure 1. Amongst all the subjects, only CHA had 4% occurrence of aged 70 years and above while CSH had 2% of aged 61-70. Others had varying percentage distribution across the age groups between 0 – 60 years. The percentage characteristics of the subjects as regard to gender, education, religion, marital status and profession is shown in Table 1. The evaluation of the medical history and predisposing factors associated with typhoid fever is shown in Table 2. In all four locations, the records of previous diagnosis of typhoid fever was over 45%. Poor personal hygiene and sanitation habit was zero (0) in all locations except SHA with 13%.

A total of Eight (8) blood samples were positive for *S. typhi* in all the sample location, except CSHG as

shown in Table 3. Isolates B7, B23, B47 and B57 were recovered from CSH; B28, B51, B76 from CHA; and B58 from SHA. Higher occurrence was observed in males compared to females.

Sensitivity pattern of isolates to commercial antibiotics

The isolates displayed varying sensitivities to the test antibiotics as shown in Table 4. All the isolates were resistant to Ampicillin, chloramphenicol, Nalidixic acid and cotrimoxazole. *S. typhi* B47 showed the highest multi resistant pattern to all the test antibiotics, while *S. typhi* B7 and B51 were the most sensitive.

Antibacterial activity of *Cymbopogon citratus* against the test isolates

The ethanolic and methanolic extracts of *Cymbopogon citratus* were effective on the isolates;

however, the ethanolic extract had better antibacterial activity with zone of inhibition ranging from 11.5mm – 16.9mm as shown in Figure 2. The MIC carried out on the isolates that were sensitive to the extracts recorded a concentration of at least 6.25mg/ml while the least MBC concentration was recorded at 12.5 mg/ml as shown in Table 5.

The phytochemicals present in the extracts of *Cymbopogon citratus* are shown in Table 6. Only alkaloids and saponins were absent in methanol extract compared to ethanol extract.

The molecular identification of *S. typhi* isolates revealed clear bands around 500bp in lane 1 and 2 as shown in Figure 3.

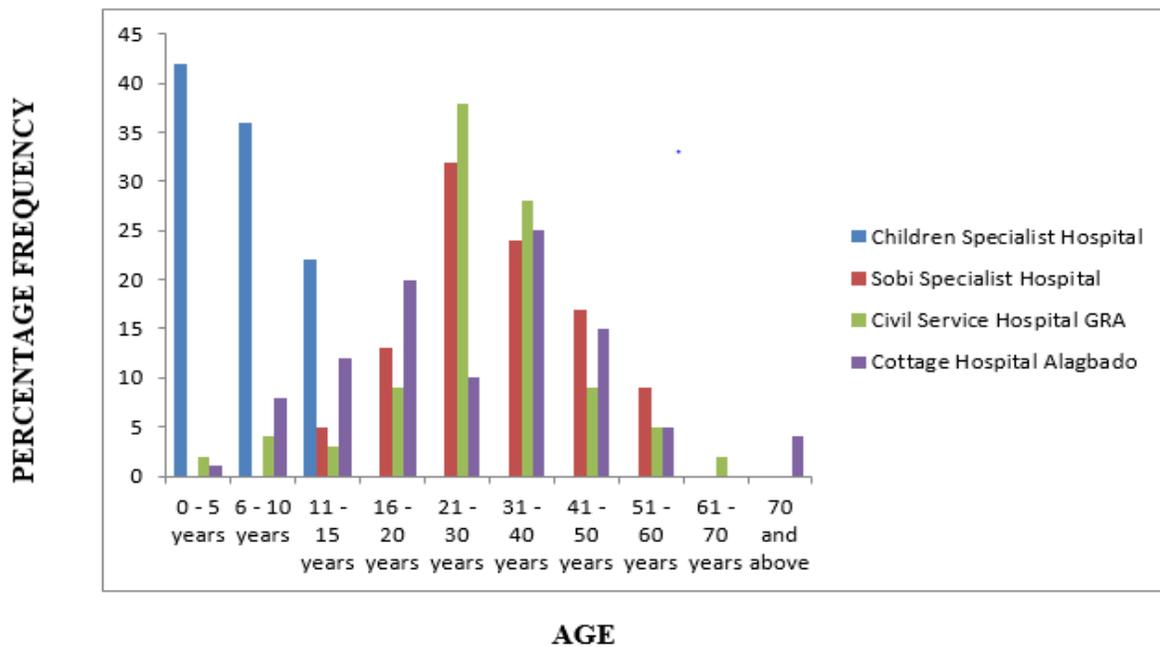


Figure 1: Age distribution amongst the respondents.

Table 1: Demographic characteristics of the subjects used in the study

FEATURES	Percentage Frequency of respondents			
	CSH	SHA	CSHG	CHA
SEX				
Female	58	63	68	60
Male	42	37	32	40
TYPE OF DUCATION				
Informal	52	73	46	63
Formal	48	27	54	37
HIGHEST LEVEL OF EDUCATION				
Primary	15	18	10	7
Post Primary	12	4	7	15
Secondary	11	2	10	5
Tertiary	10	3	27	10
RELIGION				
Christianity	33	30	46	43
Islam	67	70	54	57
Others	0	0	0	0
MARITAL STATUS				
Single	15	33	27	38
Married	82	64	71	60
Widowed	2	3	1	2
Divorced	1	0	1	0
PROFESSION				
Top Civil servant executive	8	5	26	10
Middle class/small scale business	20	22	40	29
Low class	40	31	11	26
Artisan	12	26	19	22
Others	20	16	4	13

Values represent the frequency and corresponding percentage (n=100)

CSH= Children Specialist Hospital; SHA= Specialist Hospital Adewole; CSHG= Civil Service Hospital, G.R.A CHA= Cottage Hospital, Adewole

Table 2: Medical history and predisposing factors estimated among subjects

FEATURES	Percentage frequency of respondents (%)			
	CSH	SHA	CSHG	CHA
PREVIOUS DIAGNOSIS OF TYPHOID				
Yes	46	63	53	60
No	54	37	47	40
MODE OF TREATMENT				
Self-medication	41	42	49	43
Pharmacy	21	35	31	37
Hospital	38	23	20	20
MEDICATION ADHERENCE				
Yes	57	29	40	25
No	43	71	60	75
PERSONAL HYGIENE AND SANITATION				
Poor	0	13	0	0
Fair	31	49	10	25
Good	59	25	31	56
Excellent	10	13	59	19
WATER SOURCE				
Tap/ Borehole	26	5	79	22
Well	74	87	21	76
Stream	0	8	0	2

Values represent the frequency and corresponding percentage (n=100)
 CSH= Children Specialist Hospital; SHA= Specialist Hospital Adewole; CSHG= Civil Service Hospital, G.R.A
 CHA= Cottage Hospital, Adewole

Table 3: Distribution of *S. typhi* recovered from blood samples of subjects

STUDY	AGE	MALE	FEMALE	TOTAL
LOCATION				
CSH	0 – 5	0	1	1
	6 – 10	3	0	3
SHA	51 – 60	0	1	1
CHA	16 – 20	3	0	3
TOTAL		6	2	8

Table 4: Susceptibility pattern of *S. typhi* isolates to commercial antibiotics

Isolates	Antibiotics							
	CXM	OFL	AMP	CPR	CPL	NAL	COT	NIT
<i>S. typhi</i> B7	S	S	R	S	R	R	R	S
<i>S. typhi</i> B23	R	S	R	R	R	R	R	S
<i>S. typhi</i> B28	S	S	R	S	R	R	R	R
<i>S. typhi</i> B47	S	R	R	R	R	R	R	R
<i>S. typhi</i> B51	S	S	R	S	R	R	R	S
<i>S. typhi</i> B57	S	R	R	S	R	R	R	S
<i>S. typhi</i> B58	S	S	R	S	R	R	R	R
<i>S. typhi</i> B76	S	S	R	S	R	R	R	R

CXM: Cefixime 5µg, OFL: Ofloxacin 5µg, CPR: Ciprofloxacin 5µg, AMP: Ampicillin 5µg, CPL: Chloramphenicol 30µg, NAL: Nalidixic acid 30µg, COT: Co-trimoxazole 25µg, NIT: Nitrofurantion 30µg, S: Sensitive, R: Resistant.

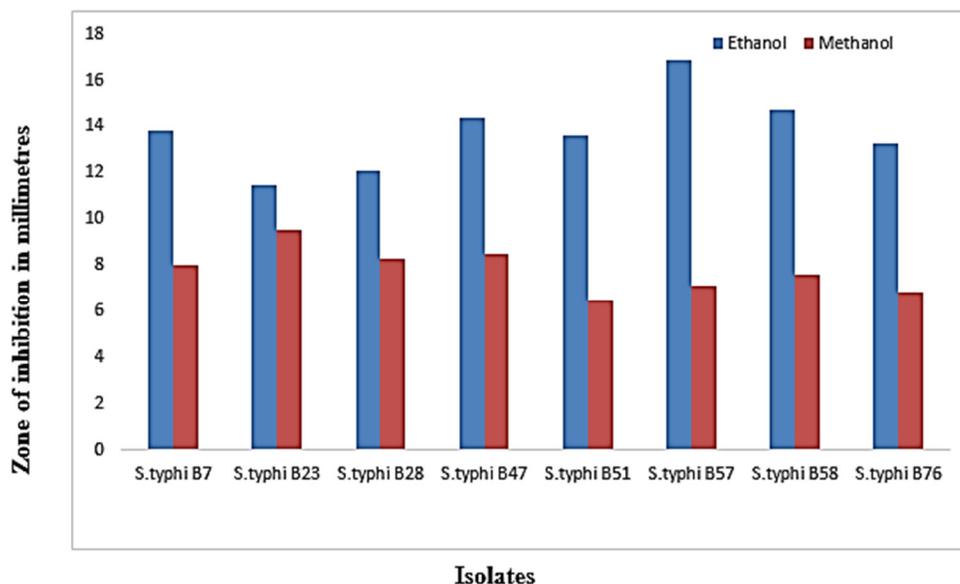


Figure 2: Antimicrobial activity of ethanol and methanol extracts of *Cymbopogon citratus* against the *S. typhi* isolates.

Table 5: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of ethanol and methanol extracts of *Cymbopogon citratus* against the *S. typhi* isolates.

Isolates	MIC (mg/ml)												MBC (mg/ml)	
	Ethanol						Methanol						Ethanol	Methanol
	3.125	6.25	12.5	25	50	100	3.125	6.25	12.5	25	50	100		
<i>S. typhi</i> B7	+	+	-	-	-	-	+	+	+	-	-	-	25	50
<i>S. typhi</i> B23	+	+	+	-	-	-	+	+	-	-	-	-	50	25
<i>S. typhi</i> B28	+	+	+	-	-	-	+	+	+	-	-	-	50	50
<i>S. typhi</i> B47	+	+	-	-	-	-	+	+	+	-	-	-	25	50
<i>S. typhi</i> B51	+	+	-	-	-	-	+	+	-	-	-	-	25	25
<i>S. typhi</i> B57	+	-	-	-	-	-	-	+	-	-	-	-	12.5	25
<i>S. typhi</i> B58	+	+	-	-	-	-	+	+	+	-	-	-	25	50
<i>S. typhi</i> B76	+	+	-	-	-	-	+	+	+	-	-	-	25	50

Key: + = bacterial growth, - = no bacterial growth

Table 2: Phytochemical screening of *Cymbopogon citratus* extracts.

Phytochemicals	Extracts	
	Ethanol	Methanol
Alkaloids	+	-
Flavonoids	+	+
Phenols	+	+
Tannins	+	+
Saponins	+	-
Steroids	+	+
Glycosides	+	+

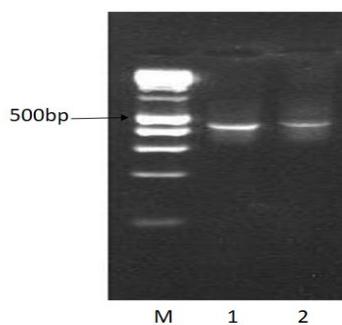


Figure 3: DNA bands of *S. typhi* isolates following agarose gel electrophoresis. (Key: M = DNA Marker, 1 and 2 = *S. typhi* isolates)

Discussion

Enteric fever is a persistent public health problem especially in economically developing countries characterized with lower level of hygiene and poor sanitary conditions. In Nigeria specifically, enteric fever constitutes a great socio-medical problem being responsible for many cases of pyrexia of unknown origin (Akinyemi *et al.*, 2008). Assessment of the possible influence of the mode of treatment on typhoid fever prevalence in the study sites reveals that the greater percentage participants reported to have used non-prescribed antibiotics. The persistent and unmonitored use of antibiotics has led to the emergence of resistant strains; it often results in a condition where the body becomes more vulnerable to diseases and then therapeutic efforts becomes difficult as the organism develops immunity against the antibiotic drugs in circulation (Wright, 2014). It was observed that over 40% of subjects in all study sites did not adhere to medication. Adherence is a primary determinant of the effectiveness of treatment because poor adherence lessens maximum clinical benefit (Dunbar-Jacob, 2000). Non-adherence could lead to gene modification and replacement which consequently increases the resistance of organism to antibiotics. Over 70% of the subjects sourced their water from wells except in CSHG where 79% sourced their water from tap. Contamination of most well water could be due to the fact that most wells are dug close to septic tanks. This encourages the seepage of human waste which have been proven to be a transmission route of infectious agents into well water (EPA, 2017). Broken or improperly installed well covers could encourage pollution of the water source as this allows the entry of contaminant. From this survey, it is apparent that inadequate water supply is a serious socio-economic problem and has caused the subjects to resort to untreated well water for domestic water supplies. High risk of infection could be also traced to poor hygiene practices and

sanitation conditions below standards and this was observed in the study sites.

The occurrence of typhoid fever among age brackets 0-20years being more susceptible is similar to the findings of Tran *et al.* (2005), that children and young adults were more susceptible than the elderly; however, an occurrence of typhoid fever in age group 51-60 years could be as a result of the immune system of the elderly waning over time. The susceptibility of children to typhoid fever is due to the fact that the immune system of children is not fully developed.

The multi-drug resistance observed in chloramphenicol, cotrimoxazole and ampicillin is similar to the findings of Oyedum *et al.* (2016) who carried out a retrospective research on multidrug resistant *S. typhi* in Nigeria and reported high rate of resistance to chloramphenicol, cotrimoxazole and ampicillin. The ethanol extract recorded better antibacterial activity as compared to the methanol extract which is in accordance with the findings of Geetha and Geetha (2014). This might be due to the extracting ability of the solvents. It therefore implies that ethanol may be a better extraction solvent for the leaf of this plant than methanol solvent. Most of the MIC values were lower than the MBC indicating that the extracts have bactericidal activity. Low MIC values are also an indication of high efficacy. All phytochemicals screened in this study were detected except alkaloids and saponins which were absent in the methanolic extract of the plant. This result is in accordance with the findings of Geetha and Geetha (2014).

The detection of these phytochemicals depends on the solvent used for extraction; as this study also suggests that ethanol is stronger solvent than methanol for the extraction of the phytochemicals of *Cymbopogon citratus*. The presence of phytochemicals in plant extracts can influence antimicrobial activities due to their important

roles in bioactivity of medicinal plants (Oluduro and Omoboye, 2010). These classes of compounds (such as alkaloids, saponins flavonoids etc.) have been reported to have inhibitory activity against microorganisms and could therefore be a promising candidate for therapy. The confirmed isolates with polymerase chain reaction (PCR) at 500bp differs from report by Zhu *et al.* with a cloned primer that had 300bp. However, report by Haim *et al.*, 2008 that detected rare *Salmonella* serovar Typhi strains possessing flagella antigens (fliC-j) was in line with the result from this study and thus suggesting a rare strain of the bacteria from this location.

Conclusions

This study has been able to evaluate the incidence of antibiotic resistant *S. typhi* among out-patients in four (4) selected tertiary hospitals within Ilorin, Nigeria. The risk factors associated with enteric fever such as use of non-prescribed antibiotics, medication non-adherence, poor personal hygiene and consumption of contaminated water played a crucial role in the prevalence of typhoid fever among the subjects. Phytochemical assessment of the plants revealed the presence of seven (7) phytochemicals which might have contributed to the antibacterial activity of the plant extracts. The efficacy of *Cymbopogon citratus* leaves can serve as a promising candidate for therapeutic purposes.

Conflict of Interest:

The authors declare no conflicting interest in the publication.

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