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Efficacy of *Cymbopogon citratus* and *Carica papaya* Used in the Traditional Treatment of Enteric Fever against *Salmonella* in Bayelsa State, Nigeria

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Abstract

This study screened for *Salmonella* from 212 stool samples of patients attending a tertiary health facility in Yenagoa using conventional, serological molecular techniques (polymerase chain reaction, PCR). Six isolates of *Salmonella* were identified. The efficacy of ethanol extracts of the leaves of *Cymbopogon citrates* (lemon grass) and *Carica papaya* (pawpaw) was carried out against *Salmonella*, the causative agent of enteric fever. The zone of inhibition ranges from (21.17 ± 1.37 mm) to (22.33 ± 1.03 mm) and (16.83 ± 3.58 to 21.18 ± 0.88) for lemon grass and pawpaw respectively. Statistical analysis of variance showed that there were no significant differences ($P > 0.05$) among the various *Salmonella* species for each of the plants extracts. The plants extract *C. citrates* induced the least Minimal Inhibitory Concentration (MIC) at 50 mg/ml, for 1 isolate (2) and 20 mg/ml for 3 isolates (1, 5 and 6). While *C. papaya* where susceptible to 2 isolates (5 and 6) at 50 mg/ml and 1isolate (2) at 20 mg/ml. The other concentrations demonstrated varying degrees of turbidity and so where not subjected to Minimal Inhibitory Concentration (MBC) test. The MBC test for isolates 2 and 4 of *C. papaya* as well as 4, 5 and 6 of *C. citratus*, had similar bacteriocidal properties on *Salmonella* spp. This study confirms the efficacy of *C. papaya* and *C. citrates* against *Salmonella typhi, S. paratyphi* and *S. typhimurium*, therefore providing alternative treatment for enteric fever.

Keywords: *Alternative Medicine; Antibiotic Sensitivity Testing; Enteric Fever; Multidrug Resistance; Traditional Medicine; Typhoid Fever*

Introduction

Enteric fever is an ancient disease, which has afflicted mankind since human populations grew large enough to contaminate their water and food supplies. This disease is mainly associated with low socioeconomic status and poor hygiene. This disease is caused by *salmonella* belonging to the serovar enterica of which medically important ones include *S. typhi, S. paratyphi* A.B.C. Estimates for the year 2000 suggest that there are approximately 21.5 million infections and 200,000 deaths from typhoid fever globally each year [1,2]. In Africa, about 4.36 cases occur out of an estimated population of 427 million and it is often encountered in tropical countries including Nigeria where they constitute serious source of morbidities and mortalities [3]. The disease is a cause of concern in coastal area of Nigeria due to poor sanitary conditions and lack of potable water especially among rural dwellers [4]. There has been no detailed epidemiological investigation of the source and spread of *Salmonella enterica serotype typhi* in Nigeria [5]. This febrile disease is among the major widely spread disease affecting both young children and young adults in their reproductive years [5]. Nigeria like many other tropical and developing countries has been described as endemic zone for typhoid fever [6]. It is thus considered one of the most serious infectious disease threats to public health on a global scale, despite recent advancement in water and sanitation. Recently, there are concerns over the rapid and widespread emergence of resistance to multiple antibiotics among pathogenic microbes [7]. However, studies in Nigeria also shows that the incidence of *salmonella* infection is increasing [8]. Also of concern is the incidence of multidrug resistance among *Salmonella*. An-

ecdotal evidence indicated the need to investigate the susceptibility of available antibiotics as antibiotic failure in the treatment of typhoid fever is increasing [9]. Resistance to antibiotics is becoming prevalent among microbes worldwide, particularly in developing countries including Nigeria [10,11]. In traditional African system of medicine, a large number of drugs of either herbal or mineral origin have been used in the treatment of various diseases and other pathological conditions in humans. The patronage of herbal drugs is increasing in Nigeria. A number of plants has been screened for anti-salmonella activities. Medicinal plants are the back bone of traditional medicine. Studies show that *Emblica officinalis* and *Terminalia chebula* have been found to exhibit full protection against *S. typhimurium* [12,13]. Immunomodulatory activity of *Terminalia chebula* against *S. typhimurium* in mice was also reported [14]. Moreover, the same author reported the activity of the same extract against the oxidative stress induced by *S. typhimurium* in Swiss albino mice [15,16] and protective effect of *Emblica officinalis* against *S. typhimurium* through its antioxidant activity [13].

Carica papaya belongs to the family of *Caricaceae*, and several species of *Caricaceae* have been used as remedy against a variety of diseases [17]. *C. papaya* is commonly known for its food and nutritional values throughout the world and it is distributed throughout Nigeria. The medicinal properties of *C. papaya* leave and other parts of the plant are also known in their application in traditional medicine. Even though the active components are normally extracted from all parts of the plant, the concentration of these components varies.). *C. papaya* leaves are made into tea as a treatment for malaria. Antimalarial and antiplasmodial activity has been noted in some preparations of the plant, the leaves of the papaya plants contain chemical compounds of karpain, Substance which kills microorganisms that often interfere with the digestive function [18]. *C. papaya* leaf extracts have phenolic compounds, such as protocatechuic acid, p-coumaric acid, 5, 7- dimethoxycoumarin, caffeic acid, kaempferol, quercetin, chlorogenic acid [19]. Antimicrobials of plant origin effective in the treatment of infectious diseases and simultaneously mitigating many of the side effects often associated with synthetic antimicrobial agents have been discovered. Medical uses of plants range from the administration of the roots, barks, stems, leaves and seeds to the use of extracts and decoction from the plants [20].

Cymbopogon citrates is commonly known as lemon grass. It is used widely as an essential ingredient in Asian cuisines due to the sharp lemon savor. *C. citratus*, which belongs to the family of *Gramineae*, is commonly used in folk medicine for treatment of nervous and gastrointestinal disturbances [21]. It is also used as antispasmodic, analgesic, anti-inflammatory, anti-pyretic, diuretic and sedative [21-23]. Francisco., *et al*. [23] also mentioned that *C. citrates* leaves extract has potent antioxidant activity due to its polyphenolic content and is a potential source of new anti-inflammatory drug. It was reported that *C. citrates* to have antibacterial, antifungal, antitumoral, anticancer and insecticide activities [24]. The antimicrobial activity of *C. citrates* against a series of microorganisms is due to the abundance of citral and essential oil components i.e Geranial, Myrcene, 6-Methylhept-5-en-22-one [24,25]. This led to suggestion that *C. citrates* may have antimicrobial activities against *S. typhi, Bacillus cereus, Escherichia coli* O157:H7, *Klebsiella pneumoniae, Staphylococcus aureus* and *Candida albicans*. However, the objective of this study is to investigate the efficacy of two common plants traditionally used to manage feverish conditions locally in Bayelsa state.

Materials and Method

A total of 212 stool samples were collected from patients attending a tertiary hospital in Yenagoa, Bayelsa State, Nigeria. The samples were screened for *Salmonella* using cultural, serological and molecular techniques. The study identified six (6) pure isolates (Isolate 1-Isolate 6)using conventional laboratory techniques including gram stain, test for motility, oxidase, indole, urea and citrate test, followed by serotyping assay using the respective monovalent antisera (Salmonella sero-group kit from Statens Serum Institute Denmark, ranging from A-G and Vi antisera) to confirm the serotype belonging to *Salmonella enterica*. Amplification and detection of STR (fliC) STN (fliC) and the AroC genes present in *Salmonella* isolate were done with the following respective primer pairs. A regular multiplex Polymerase Chain Reaction (PCR) was carried out using the primer pairs STR (F) TATGCCGCTACATATGATGAG, STR(R) TTAACGCAGTAAAGA GAG, STN (F) ACTGCTAAAACC ACT ACT, STN (R) TGG AGA CTT CGG TCG CGTAG, aroCs for GGCACCAGTATTGGCCTGCT, aroCs rev CATATGCGCCACAATGTGTTG.

DNA Extraction was carried out on the samples using Bacteria DNA Preparation Kit (Jena Bioscience, Germany). The purity and concentration of the extracted DNA was evaluated using a NANODROP (ND 1000) Spectrophotometer (Thermo Scientific, USA). All the samples showed a DNA yield between 65 and 120 ng, and the extracted DNA were optimally pure showing A260/A280 between 1.60 and 1.90 nm. PCR amplification and detection of STR, STN and the Aroc gene were carried out using the Solis Biodyne (Estonia) 5X FIREPol Master mix. PCR was performed in 20 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X PCR Master mix buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 20 pMol of each primer (Jena bioscience, Germany), 2 unit of FIREPol DNA polymerase (Solis Biodyne Estonia), 2 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf Vapo protected thermal cycler (Nexus Series, USA) for an initial denaturation of 95˚C for 5 minutes followed by 35 amplification cycles of 30 seconds at 95˚C; 30 seconds and 1 minute at 72˚C. This was followed by a final extension step of 10 minutes at 72˚C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100 bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker.

Collection of Plant Material

Fresh leaves of *Cymbopogon citrates* was bought from Swali Market-Yenagoa, Bayelsa state and *Carica papaya* leaves harvested within the Niger Delta University premises, Amassoma community Nigeria and identified at the Department of Biological Sciences, Niger Delta University, Bayelsa state. The plant materials were air dried at room temperature for seven days. They were weighed daily until a constant weight was attained. The dried leaves were homogenized into fine powder and stored in air tight containers.

Ethanol Extract Preparation of Leaves of *Cymbopogon citratus* and *Carica papaya*

Two hundred (200) grams of the powder of both medicinal plant was weighed separately using Satoric AG Gottingen Electronic weighing balance. The weighed samples were soaked in 400 mls of ethanol (99.9% concentration) contained in a conical flask. The mixture was swirled. After 72 hours with interval stirring, the mixture was filtered using Whatman no.1 filter paper [26] into a clean beaker and concentrated to dryness using a rotary evaporator at 70˚C. The extracts were stored in the refrigerator at 8˚C for use.

Antibacterial Activity Testing for Plant Extracts

Sensitivity of the pure culture of *Salmonella* isolates (*Salmonella typhi, Salmonella paratyphi, Salmonella typhimurium*) to ethanol extracts of *C. papaya* and *C. citratus* were determined using agar well diffusion method as reported by Kigigha and Atuzie [27], with slight modification. The well measuring 6mm were bored in agar. The six (6) test organisms were pre-adjusted to the 0.5 McFarland's turbidity standard in a different test tube, dipped with a sterile swab stick and used to seed on a solidified Mueller Hinton sensitivity test agar in an inoculating chamber already set aseptically. The wells in agar where filled, with pipetting 0.5 ml of the extract. The plates were incubated for 24 hours at 37˚C, after which the zone of inhibition was measured and recorded.

Minimal Inhibitory Concentration (MIC) and Minimal Bacteriocidal Concentration (MBc)

The Minimal Inhibitory Concentration (MIC) and Minimal Bacteriocidal Concentration (MBC) of the extracts were determined using the broth dilution technique [28]. The MIC helps to measure more exactly the concentration of an antibiotic necessary to inhibit growth of a standardized inoculum under defined conditions. Dilutions of the extract in Mueller Hinton broth were prepared in tubes. The concentration of inoculum was also standardized to 0.5 McFarland's turbidity, The Mueller Hinton broth in tubes containing the different concentration of plant extract, 50, 20, 10, 8.33, 6.67 and 5 mg/ml were then challenged with 0.5 ml of the standardized culture and inoculating it on the broth containing the extract solutions. The cultures were then incubated at 37˚C for 48 hours. The smallest concentration that inhibits the growth was taken as the MIC. Observation was made visually for turbidity of the test tubes. The determination of the value of MBC follows the determination of MIC by the broth dilution technique. The MBC is the lowest concentration of the antibacterial agent that kills at least 99.9% of the test organisms. To determine this value, about 0.5 ml of the sample was removed from the test tubes used

in the determination of MIC in which there was no desirable growth was spread over the surface of the oven dried nutrient agar plates. The lowest concentration of the agent that prevent the growth of less than 0.1% of the test organism on the recovery plate was taken to be the MBC value for the extract.

Statistical Analysis

Statistical analysis was carried out using IBM SPSS version 20 (IBM SPSS Inc. Chicago, USA). Data were expressed as mean ± standard deviation. One-way analysis of variance was carried out at $\alpha = 0.05$ and Turkey HSD statistics was used for multiple comparison.

Results

Amplification and Detection of Test Isolates

Identification of *Salmonellae* using AroC gene sequence.

Results showed that the 2 isolates indicated an amplification product of 639 bps, which shows the specificity of primers for the identification of *Salmonella* strain (Figure 1).

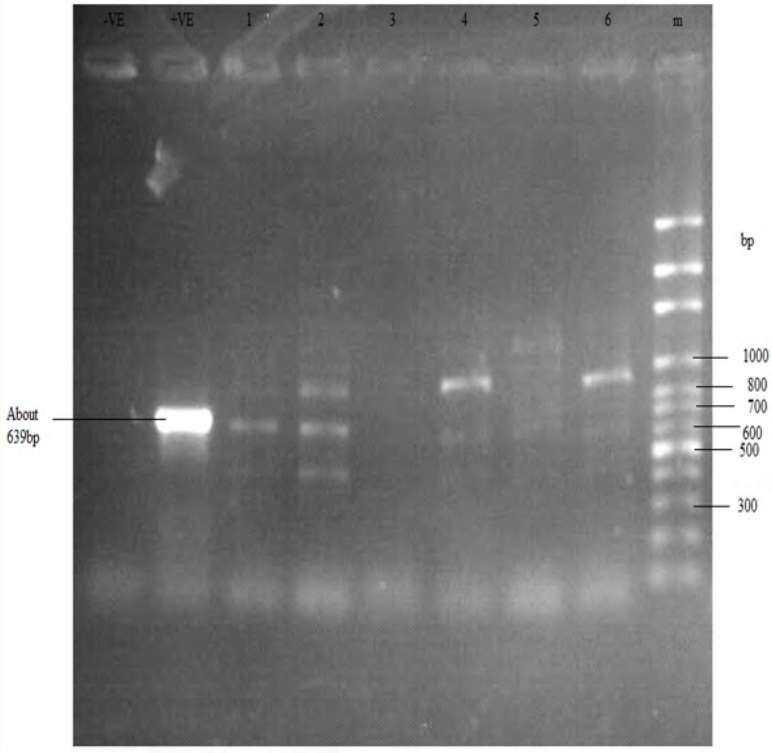

Figure 1: *Photograph of Salmonella genus by aroC gene amplification +ve control Salmonella Typhimurium. (639bp).*

Confirmation of *S. typhi* using the fliC gene sequence.

Out of the six tested isolates, result shows that only one isolate gave an amplification product of 495 bp. The primers sequence specific for the flagella unique to *S. typh*i reveals its specificity, despite difficulty in isolation and identification of *S. typhi* (Figure 2).

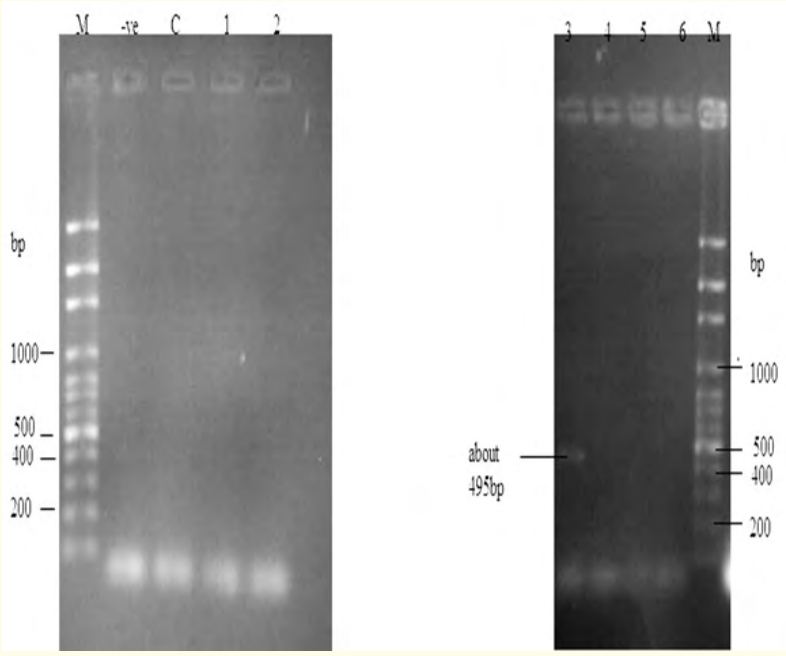

Figure 2: *Photograph showing Confirmation of Salmonellae enteric serovartyphi by amplification of fliC gene.*

Antibacterial Activity Testing For Plant Extracts

Antibacterial efficacy of *Cymbopogon citratus* and *Carica papaya* leaves against *S. typhimurium, S. typhi* and *Salmonella paratyphi*. For *S. typhimurium*, the zone of inhibition was higher for *Cymbopogon citrates* (21.17 ± 1.37 mm) than for *C. papaya* (16.83 ± 3.58 mm). For *S. typhi*, a zone of inhibition of 22.67 ± 0.88 mm and 18.67 ± 0.88 mm were observed for *C. citratus* and *C. papaya* respectively. The sensitivity of *S. paratyphi* were 22.33 ± 1.03 mm and 21.18 ± 0.88 mm for *C. citratus* and *C. papaya* respectively. There was no significant variation ($P > 0.05$) among both plant extract for the various *Salmonella* groups (Table 1).

Plant extract	*S. Typhimurium* (n = 3)	*S. Typhi* (3)	*Salmonella paratyphi* (n = 3)
Cymbopogon citratus	21.17 ± 1.37	22.67 ± 0.88	22.33 ± 1.03
Carica papaya	16.83 ± 3.58	18.67 ± 0.88	21.18 ± 0.88

Table 1: *Results of efficacy test of Cymbopogon citratus and Carica papaya against Salmonellae. Data is expressed as mean ± standard error; means across the row are not significantly different at $P > 0.05$ according to Turkey HSD statistics multiple comparison; N= number of isolates used for the sensitivity.*

Minimal Inhibitory Concentration (MIC)

The MIC for *C. citratus* shows that at 50 mg/ml five (5) out of the six isolates was not turbid, while at 20 mg/ml it was observed that three (3) isolate was not turbid; while four was slightly turbid at a concentration of 10 mg/ml, while others were moderately turbid as the concentrations decreases from 8.33 – 5 mg/ml, all tested isolates were either highly turbid or moderately turbid, indicating resistance to

plant extracts. The MIC thus for *C. citratus* against isolate 1, 5 and 6 was 20 mg/ml while isolate 2 was 50 mg/ml (Table 2).

No of isolates	Concentration (mg/ml)					
	50.00	20.00	10.00	8.33	6.67	5.00
Isolate 1	+	+	++	+++	+++	+++
Isolate 2	+	++	++	+++	+++	+++
Isolate 3	++	++	+++	+++	+++	++++
Isolate 4	+	++	++	+++	+++	+++
Isolate 5	+	+	+++	+++	+++	+++
Isolate 6	+	+	++	+++	+++	+++

Table 2: *Minimal inhibitory concentration (MIC) of C. citratus against Salmonellae.*

KEY: *+ = No turbidity, ++ = Slightly Turbid, +++ = Moderately Turbid, ++++ = Highly Turbid*

The MIC for *C. papaya*. Results showed that at 50 mg/ml only two (2) out of the six isolates were not turbid, while at 20 mg/ml it was observed that one (1) isolate were not turbid; while four were slightly turbid at a concentration of 10 mg/ml while others were moderately turbid as the concentrations decreases from 8.33 – 5 mg/ml, all tested isolates were either highly turbid or moderately turbid, indicating resistance to plant extracts. The MIC thus for *C. papaya* against the isolates, 5 and 6 was 50 mg/ml while isolate 2 was 20 mg/ml. (Table 3).

No of isolates	Concentration (Mg/ml)					
	50.00	20.00	10.00	8.33	6.67	5.00
Isolate 1	++	++	++	+++	+++	+++
Isolate 2	+	+	+++	+++	+++	+++
Isolate 3	++	++	+++	+++	+++	++++
Isolate 4	++	++	+++	++++	++++	++++
Isolate 5	+	++	++	++	+++	+++
Isolate 6	+	++	+++	+++	+++	+++

Table 3: *Minimal inhibitory concentration (MIC) of C. papaya against Salmonellae.*

KEY: *+ = No turbidity, ++ = Slightly Turbid, +++ = Moderately Turbid, ++++ = Highly Turbid*

Minimal Bacteriocidal Concentration (MBC)

The MBC for *C. citratus* shows that no growth was observed for isolate 4, 5 and 6. Growth was observed in isolate Is1 and Is2 thus bactericidal property of the *C. citratus* was observed in three (3) isolates at the concentrations of 20 mg/ml for isolate 5 and 6 while isolate 4 at 50 mg/ml. Table 4 presents the MBC for *C. papaya*. The result showed that no growth was observed for isolate 2. While growth was observed in isolate 5 and 6 thus bactericidal property of the *C. papaya* was observed in one (1) isolates at the concentrations of 20 mg/ml (Table 4).

No of Isolates	*C. citratus*		*C. papaya*	
	MIC (50 mg/ml) Inhibition	MBC Growth (48 hrs)	MIC (50 mg/ml) Inhibition	MBC Growth (48 hrs)
Isolate 1	Present	Growth	Absent	-
Isolate 2	Present	Growth	Present	No growth
Isolate 3	Absent	NA	Absent	NA
Isolate 4	Present	No growth	Absent	-
Isolate 5	Present	No growth	Present	Growth
Isolate 6	Present	No growth	Present	Growth

Table 4: *Minimal Bacteriocidal Concentration of C. Citratus and C. papaya against salmonellae.*

KEY: *MIC: Minimal Inhibitory Concentration. MBC: Minimal Bacteriocidal Concentration, NA: Not Applicable*

Discussion

This study showed that the ethanol extract of leaf of *C. citratus* and *C. papaya* possesses antibacterial properties. It was found that ethanol extract of the leaves of both plants were active against all the bacteria tested (Table 1). But on separation into various strain, the highest zone of inhibition was observed in *S. typhi* with a diameter of 22.67 ± 0.88 for *C. citratus*, while the least was observed for *S. typhimurium*, the activity against the test bacteria provides scientific bases for the local usage of these plants in the treatment of feverish conditions and in agreement with studies carried out for the justification of these plants having medicinal potentials [29]. Similar finding by Iroha., *et al*. (2010) in Ebonyi state Nigeria also proves the activity of *C. papaya* against *Salmonella* spp was found to be effective, while a study carried out by Evans., *et al*. [30] on a formulation containing *C. papay*a also reveals a promising potentials of this plant. Results also reported by Mohd., *et al*. [31] show the efficacy of *C. citratus* against some pathogenic organisms. In this study the concentrations observed to be the least among the different concentration used reveals that at 50 mg/ml there was still inhibition for some of the isolate (Tables 2 and 3). The other concentration shows varying degree of growth and so where not subjected to MBC test. The MBC test carried using *C. citratus*, isolate number 4, 5 and 6, reveals that *C. citratus* has a bactericidal property. Similarly, *C. papaya* reveals its bacteriocidal property on *Salmonellae* spp on isolates 2 and 4 (Table; 4). The result of the study also supports the traditional application of the plants and suggests that the plant extracts possesses antibacterial properties that can be used as antibacterial agents in novel drugs for the treatment of salmonellosis.

Conclusion/Recommendation

This study indicates that leaves of *C. papaya* and *C. citratus* has antityphodal and paratyphodal property with varying degrees of inhibition and are active against *Salmonellae*. Thus, it is suggested that *C. papaya* and *C. citrates* should be recommended as useful sources to prepare natural bioactive products from which to develop new antimicrobial drugs, which could be cost effective because the plants are available in Bayelsa state, Nigeria. These plants have also been proven locally in the management of feverish cases and thus the search for new pharmaceuticals, screening of this plants and identification of active agents must be considered as a fruitful approach. Further pharmacological evaluations, toxicological studies and possible isolation of the therapeutic antibacterial agents from this plant should be considered and used as an antibacterial agent in the formulation of novel drugs for the treatment of salmonellosis.

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