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(RESEARCH ARTICLE)



Evaluation of activities of some plant leaf extract on typhoidal and non-typhoidal *Salmonella* isolate from selected hospitals in Bauchi, Nigeria

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Abstract

This study evaluated the antibacterial activity of aqueous and methanol extracts of *Cymbopogon citratus*, *Psidium guajava* and *Anacardium occidentale* on clinical isolates of typhoidal and non typhoidal *Salmonella*. The typhoidal *Salmonella* isolates were *S. typhi* and *S. paratyphi A*, while non typhoidal was *S. typhimurium*. Well diffusion method was used. The plant extracts were tested individually and in combinations for synergistic activity. Both methanol and aqueous extracts had significant ($p < 0.05$) in vitro activity. *Anacardium occidentale* was more active followed by *Psidium guajava* and the least active was *Cymbopogon citratus* with higher activity when plants extracts were combined for synergism. The Minimum Inhibitory Concentration (MIC) of extracts on the *salmonella* isolates ranged from 50 mg/ml to 200 mg/ml and the Minimum Bactericidal Concentration ranged from 25 mg/ml to 200 mg/ml. From the findings, it proved that plants possess some potentials as antibiotics and can be further studied to isolate the compound that is most active and formulated as drug against the disease.

Keywords: *Cymbopogon citratus*; *Psidium guajava*; *Anacardium occidentale*; Synergism; (MIC); (MBC)

1. Introduction

Salmonellosis is a disease caused by *salmonella* species which are members of the family enterobacteriaceae. They are gram negative facultative anaerobic rods. *Salmonella* species are classified into serovars (serotypes) based on the lipopolysaccharide (O) flagellar protein (H) and sometimes the capsular (VI) antigens. There are more than 2500 known serovars, within a serovar, there may be strains that differ in virulence [12]. *Salmonella* is generally divided into two categories; non typhoidal and typhoidal. The non typhoidal *salmonella* is the most common form and is carried by both humans and animals. Most serotypes of *salmonella* such as *Salmonella jariana* and *Salmonella enteritidis* cause non typhoidal salmonellosis. *Salmonella typhimurium* is involved in the invasive non-typhoidal. [12].

The serovars responsible for typhoid fever is restricted to human beings, which is transmitted through direct contact with the fecal matter of an infected person, that is to say it's transmitted through feaco-oral route. Typhoid fever is endemic in developing and under developed world where unsanitary conditions are more likely to prevail and which can affect as many as 21.5 million people, each year. Recorded cases of typhoid fever in the developed world are mostly related to recent travel to areas where, *salmonella* Typhoid is endemic.

Typhoid fever symptoms appear between 8-14 days after eating contaminated food and last anywhere from 3-60 days. They include fever, weakness, lethargy abdominal pain, coughing, nose bleeding and delirium and enlarged organs. Typhoid fever is a serious illness that can result in death [8].

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Herbal medicine is still the main stay of about 75-80 % of the world population, particularly, in developing countries for primary health care [11]. This is primarily because of the general belief that herbal drugs are without any side effects. Besides, they are cheap and locally available. Before scientists made impact into the research for drugs curing human infections, the traditional means of treating diseases were done by means of concoctions from plants either in single form or mixtures without knowing that the agents were used against some pathogenic micro-organism [19]. Limited knowledge about the practices of use of plant medication that is herbal medicine and lack of scientific studies of plants have led to the neglect of novel bioactive components that may bring about remarkable result in treatment of infectious diseases with little or no side effect [18].

In the last three decades, the search for natural bioactive compounds that can serve as antimicrobial agents had increased tremendously due to the increasing resistance possessed by microorganisms to synthetic antibiotic [15].

The scope of this study includes isolation of *Salmonella*, extraction of African lemon grass (*Cymbopogon citratus*) guava leaf (*Psidium guajava*) cashew leaf (*Anacardium Occidentale*) and the testing of the efficacy of these extract on the isolate.

2. Material and methods

2.1. Ethical clearance

The consent of the clinically suspected patients was sort, while the Ethics Committee of Abubakar Tafawa Balewa University teaching Hospital Bauchi gave approval for the study. Confidentiality of the subjects' identities was duly maintained.

2.2. Study area

The study area is Bauchi Local Government Area, where samples were collect from new General Hospital Bayara, Specialist Hospital and ATBU Teaching Hospital, Bauchi state is located on Lat. 10.6371 °N, long. 10.0807 °E the vegetation is that of savanna and Sahel according to Koppen's climate classification system. Bauchi LGA is located on Lat. 10.301 °N long. 9.8237 °E, it's area is 3,687 km² and has population of 493,810 according to 2006 population census.

3. Sample size/Sample population

Sample size is 210. Samples were collected from male, female and children. The sample size was determined using the Fisher formula for cross-sectional study [17].

3.1. Sample collection

3.1.1. Clinical samples

A total of 210 clinical samples were collected, blood and stool Samples were collected from patient with febrile or diarrheal illness attending new General Hospital Bayara, Specialist Hospital and ATBU Teaching Hospital, Bauchi on weekly basis from May to August 2016. Blood sample were collected using syringe and needle while stool samples were collected using sterile bottles, patient where served with sterile bottles to collect a small quantity of their stools.

3.1.2. Plants collection and identification

Plant samples collected or used are African lemon grass (*Cymbopogon citratus*), Cashew leaf (*Anacardium Occidentale*) and guava leaf (*Psidium guajava*). These were collected at Mbak area of Dass town a local government in Bauchi state where they are found to be growing naturally or planted and transported to ATBU herbarium for authentication with the following voucher numbers; *Psidium guajava* ATBUHB 2489, *Anacardium Occidentale* ATBUHB 2490 and *Cymbopogon citratus* ATBUHB 2491

4. Isolation, characterization and identification of Typhoidal and non-Typhoidal Salmonella

Stool sample were inoculated in a non-selective broth-selenite F enrichment broth and incubated at 37 °C for 24hrs, the pre cultured stool sample were sub cultured on deoxycholate citrate agar (DCA), *salmonella shigella* agar (SSA) agar and brilliant green agar (BGA) and incubated at 37 °C for 18-24 hrs where growth were detected based on their, colonial characteristic and morphological appearance on these media.

Blood samples were inoculated into Terathionate broth. A minimum of blood to broth ratio of 1 in 10 mls, was maintained, and this was incubated at 37 °C and checked for signs of bacterial growth daily for up to seven days. Bottles that show signs of growth were subculture on to Brilliant Green Agar (BGA) *Salmonella Shigela* Agar (SSA), and Mac conkey Agar.

Blood culture broth with no bacterial growth after seven days was sub-cultured before being reported as negative result. Typical colonies of *Salmonella* appear as pink colonies with or without black centers. Many cultures of *Salmonella* produce colonies with large, glossy black centers or may appear as almost completely black colonies. [22]

4.1. Biochemical screening and serology

Sugar fermentation tests

Nutrient broth cultures were prepared and used in Bijou bottles containing the basal medium and appropriately prepared. Carbohydrate (mannitol, maltose, dulcitol, sucrose and glucose) were inoculated with drop of the nutrient broth suspension of the test isolate and were loosely capped and incubated at 37 °C overnight. They were observed for change in colour from amber to red and for gas production (in the medium filled inverted Durham tube) [9, 13]

4.2. Serotyping of identified *Salmonella* species

Colonies considered to be of *Salmonella* spp. were further tested for somatic (O) and flagella (H) antigens with polyvalent antisera (OXOID), [9, 13].

4.3. Antibacterial sensitivity of commonly used drug for Typhoid treatment

The susceptibility testing was carried out by disc diffusion method using Mueller Hinton agar and it was tested in vitro for susceptibility to the following antibiotics (OXOID Ltd., UK) suggested by [23], Ciprofloxacin (CPX,10 µg), Amoxicillin (AMC,30 µg) and Chloramphenicol (CH,30 µg).

4.4. Plant sample preparation

The leaves and grass were washed, drained and air dried at room temperature and grinded to powder using mortar and pestle. All the powdered samples were labelled and stored in a dark polythene bags at room temperature prior utilization [21].

4.5. Extraction procedure of plants sample prepared

100 grams of each powder plant sample stored were weighed and extracted with methanol and aqueous (Water) for 3-6 hours using soxhlet extractor [7].

4.6. Preparation of inoculum

McFarland standard; 0.5 McFarland standard was prepared by 0.5 ml of 1 % Barium chloride (BaCl₂) to 99.5 mls of 1 % Sulphuric acid (H₂SO₄). The turbidity of 0.5 McFarland standard was used for the estimation of the amount of *salmonella*. Colonies of *Salmonella* was suspended in sterilized 0.9 % sodium chloride solution (normal saline) which was compared with 0.5 McFarland solution the microbial suspension (1ml) in normal saline was added to 74 mls of sterile medium kept at 45 °C to give bacterial population density of 1.2×10⁷ Cells/ml [16].

4.7. Preparation of stock suspension of extract

Stock preparation of plant extract was prepared by dissolving 0.4 grams of extract in to 1 ml of dimethyl sulfoxide (DMSO) for methanol extract and 1 ml sterilized distilled water was used for aqueous extract to make 400 mg/ml and stored at room temperature pending usage.

4.8. Assays for antibacterial activity of plant extracts

The antibacterial activity was carried out by well diffusion method [10]. Preparation of inoculums was obtained using McFarland standard to have inoculums density of 1.2×10⁷ Cfu/mls, wells of 4 mm in diameter were bored on already inoculated Mueller Hilton agar plates using a sterile well borer and 60 µL of extracts was dispensed in the wells and stand for 40min. as pre-diffusion time, these were incubated at 37 °C for 18-24 hr. Diameters of zones of inhibition was determined by subtracting the diameter of the wells and recorded to the nearest millimeter.

Preliminary testing of this preparation was carried out using the stock concentration 400 mg/ml to test whether or not the plant is active. The antibacterial activity assay was done in duplicate at concentrations 400, 200, 100 50 and 25 mg/ml were the means were taken as the mean zones of inhibition (MZI). To investigate the synergistic activity of the extracts equal volume of 0.5 ml from the stock preparation of both solvents, this combination was made in twos and the three and diluted to make concentration of 400, 200, 100, 50 and 25 mg/ml.

4.9. Determination of minimum inhibitory concentration (MIC) of extract against the bacteria

This was determined by serial dilution method, where different concentration was obtained. 0.4 g of the Stock solution prepared from plant extract was dissolved in 1 ml of DMSO, making a solution of 400 mg/ml from where serial dilution of different concentrations was made 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml. Nutrient broth was used to produce overnight growth; 0.1 ml McFarland standard inoculums was inoculated in the tubes and incubated at 37 °C for 24 hr. The tubes least turbidity (bacterial growth) was the MIC. [14]

4.9.1. Determination of minimum bactericidal concentration (MBC)

The above tube that showed no bacterial growth was further subculture on to Mueller Hilton agar incubated at 37 °C for 18-24 hr. The least plate that showed no bacterial growth was the MBC. [14]

5. Results and discussion

5.1. Determination of activity of extract on bacterial isolates

The crude methanol and aqueous extract of *Anacardium occidentale*, *Psidium guajava* and *Cymbopogon citratus* was tested on *S. typhi*, these have MZI range as 0-19 mm Table 1, when tested on *S. paratyphi A*. it gave MZI range as 0-19.5 mm Table 2 and when it was tested on *S. typhimurium* the MZI ranged from 0-17 mm Table 3.

Table 1 Activity of individual plant extract on *Salmonella typhi* (Typhoidal)

Mean Zone of Inhibition in (mm)						
Concentration of plant extract in mg/ml	<i>Cymbopogon citratus</i>		<i>Psidium guajava</i>		<i>Anacardium occidentale</i>	
	ME	AE	ME	AE	ME	AE
400	9	11	17	16	19	13
200	5	10	17	15	15	10.5
100	2	7	14	13	11	10.5
50	2	4	11	9	8.5	6.5
25	0	0	4	4	4.5	5

Key: ME = Methanol Extract, AE = Aqueous Extract

Table 2 Activity of individual plant extracts on *Salmonella Paratyphi A*. (Typhoidal)

Mean Zone of Inhibition in (mm)						
Concentration of plant extract in mg/ml	<i>Cymbopogon citratus</i>		<i>Psidium guajava</i>		<i>Anacardium occidentale</i>	
	ME	AE	ME	AE	ME	AE
400	5	6.5	17	18.5	19.5	17
200	5.5	2	14.5	13	16	16
100	3	0	10	12	16.5	11
50	1	0	8	5	10	9
25	0	0	5	6	6	6

Key: ME = Methanol Extract, AE = Aqueous Extract

Table 3 Activity of individual plant extract on *Salmonella typhimurium* (Non-Typhoidal)

Concentration of plant extract in mg/ml	Mean Zone of Inhibition in (mm)					
	<i>Cymbopogon citratus</i>		<i>Psidium guajava</i>		<i>Anacadium occidentale</i>	
	ME	AE	ME	AE	ME	AE
400	14	11	11	14	17	17
200	9	2	10	11	15	11
100	3	1	5	6	13.5	6.5
50	1	0	3	3.5	13.5	5
25	0	0	1	3	10	3

Key: ME = Methanol Extract, AE = Aqueous Extract

5.2. Screening for synergistic activity of extracts on bacterial isolates

The result of the combination of extract in different combinations according to Solvents used varying ranges of MZI was observed. The activity of combination of two extracts on *S. typhi* have MZI range from 2-23 mm Table 4, on *S. Paratyphi A* have MZI range from 1-19 mm Table 5, when it was tested on *S. typhimurium* it gave MZI range from 1-15 mm Table 6. Table 7 is a result of when the three extracts where combined together depending on solvent and tested on the isolates, MZI result ranged from 2-17.5 mm.

Table 4 Activity of combined plant extracts for synergy on *Salmonella typhi* (Typhoidal)

Concentration of plant extract in mg/ml	Mean Zone of Inhibition in (mm)					
	<i>(Pg and Cc)</i>		<i>(Pg and Ao)</i>		<i>(Ao and Cc)</i>	
	ME	AE	ME	AE	ME	AE
400	19.5	17	23	16	17	19
200	17	15	20	11	11	11.5
100	15	6.5	13	9	10	4
50	3.5	3	12	8	10	2
25	3	2	9	4	7	1
Positive control streptomycin 1 g	22	20	20	20	22	22
Negative control DMSO	0	0	0	0	0	0

Key: ME = Methanol Extract, AE = Aqueous Extract, Cc = *Cymbopogon citratus*, Pg = *Psidium guajava*, Ao = *Anacadium occidentale*

Table 5 Activity of combined plant extracts for synergy on *salmonella Paratyphi A*. (Typhoidal)

Concentration of plant extract in mg/ml	Mean Zone of Inhibition in (mm)					
	<i>(Pg and Cc)</i>		<i>(Pg and Ao)</i>		<i>(Ao and Cc)</i>	
	ME	AE	ME	AE	ME	AE
400	18	15	19	14	16.5	14
200	12	12	17	10	14	11.5
100	10	9	15	9	11.5	10
50	8.5	5	8	6	10	8
25	8	1	8	3	9.5	5
Positive control streptomycin 1 g	24	22	22	20	22	20
Negative control DMSO	0	0	0	0	0	0

Key: Pg=*Psidium guajava*, Ao=*Anacadium occidentale*, Cc=*Cymbopogon citratus*
ME = Methanol Extract, AE = Aqueous Extract

Table 6 Activity of combined plant extracts for synergy on *Salmonella typhimurium* (Non-Typhoidal)

Mean Zone of Inhibition in (mm)						
Concentration of plant extract in mg/ml	<i>(Pg and Cc)</i>		<i>(Pg and Ao)</i>		<i>(Ao and Cc)</i>	
	ME	AE	ME	AE	ME	AE
400	20	17	18	16	17	15
200	13.5	9	18	15	17	12
100	12	7	14	12	15	6
50	12	4	10	5	12	6
25	11	1	8	3	9	4
Positive control streptomycin 1 g	22	20	20	20	22	22
Negative control DMSO	0	0	0	0	0	0

Key: Pg=*Psidium guajava*, Ao=*Anacardium occidentale*, Cc=*Cymbopogon citratus*
ME = Methanol Extract, AE = Aqueous Extract

Table 7 Activity of combination of the three plants extracts for synergy on the three *Salmonella* Soretotypes

Mean Zone of Inhibition in (mm)						
Concentration of plant extract in mg/ml	<i>S. Typhi</i>		<i>S. Paratyphi A.</i>		<i>S. Typhimurium</i>	
	ME	AE	ME	AE	ME	AE
400	17.5	15	17	15	18	16
200	14	14	13	13	15	14
100	13	12	10	11	14	12.5
50	10	5	9	7	12	11
25	5	4	6	2	9	6.5
Positive control streptomycin 1 g	20	22	22	20	20	20
Negative control DMSO	0	0	0	0	0	0

Key: Pg=*Psidium guajava*, Ao=*Anacardium occidentale*, Cc=*Cymbopogon citratus*
ME = Methanol Extract, AE = Aqueous Extract

5.3. Comparative antibacterial activity of different plant samples used

The inhibitory activity of different plant extract at concentration 400 ml/ml showed different inhibitory patterns, Figures 1, 2 and 3. *Anacardium occidentale* (Ao) of methanol extract have higher activity ranging from 17-19.5 mm MZI followed by *Psidium guajava* (Pg) 14-17 mm MZI then *Cymbopogon citratus* (Cc) having range from 5-14 mm MZI, aqueous extract had these range, (Ao) 13-17 mm MZI, (Pg) 14-18.5 mm MZI and (Cc) 6.5-11 mm MZI figure 1. On figure 2 Combination of two extracts according to solvents have MZI as- methanol extracts range of 18-20 mm MZI, on (Pg and Cc), combination (Pg and Ao) have MZI range from 18-23 mm and (Ao and Cc) combination have MZI range from 16.5 – 17 mm. aqueous extract have MZI ranges as these; (Pg and Cc) have 15 -1 mm MZI, (Pg and Ao) have 14-16 mm and (Ao and Cc) have 14-19 mm.

Figure 3 showed activities when extract was combined into three according to solvent, methanol extract had activity on *S. Typhi* with MZI 17.5 mm, *S. Paratyphi A*. 17 mm and *S. Typhimurium* 18 mm, aqueous extract had MZI on *S. typhi* 15 mm, *S. paratyphi A*. 15 mm and *S. typhimurium* 16 mm.

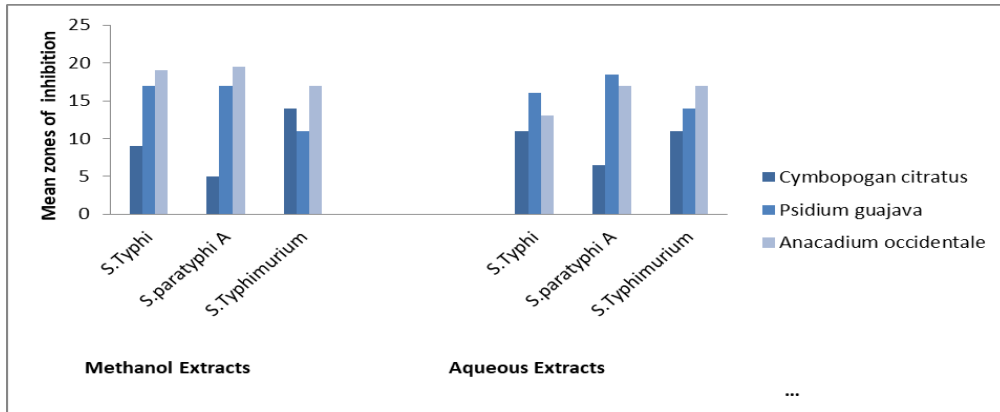


Figure 1 Comparative antibacterial activity of the individual plant extracts at (400 mg/ml) on clinical *Salmonella* isolates.

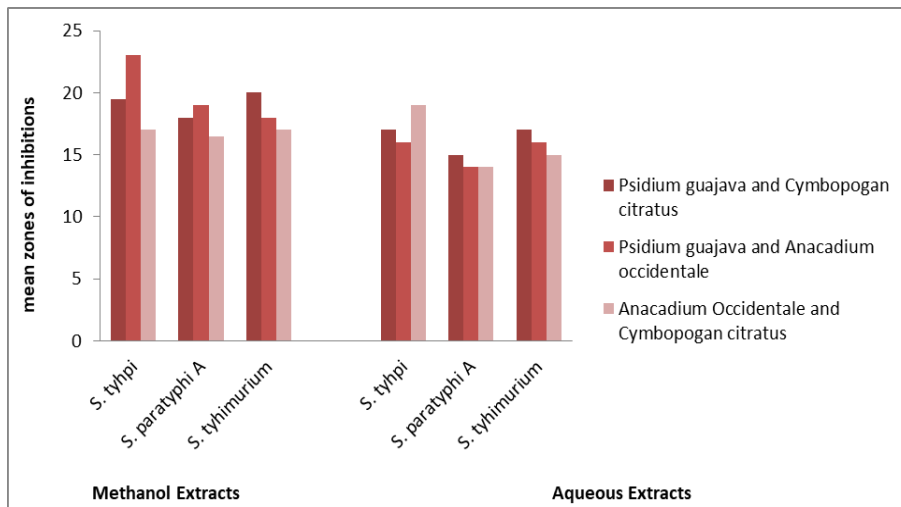


Figure 2 Comparative antibacterial activity of combinations of two plant extracts at (400 mg/ml) on clinical *Salmonella* isolates.

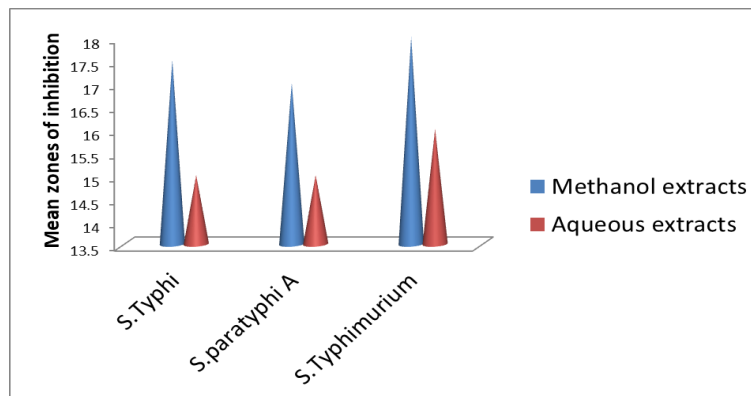


Figure 3 Comparative antibacterial activity of combination of the three plant extracts at (400 mg/ml) on clinical *Salmonella* isolates

5.4. Antibacterial activity of extract

The inhibitory activity of all the crude plant methanol and aqueous extract against the typhoidal and non-typhoidal *Salmonella* isolates with *A. occidentale*, and *P. guajava* being the most active Tables 1, 2 and 3 is in conformity with other reports by [6]. This justifies the use of these plants for Therapeutic purposes. The result clearly revealed pronounced activity with methanol extract on the organism than aqueous extracts,

This could be as a result of the antibacterial compounds present in the plant which may be more readily extracted with methanol than aqueous solvents. Data reports have shown that some antimicrobial agents are not extractible with aqueous solvents [5, 20]. The serotype that showed more susceptibility to the extracts is the non typhoidal, *S. typhimurium* followed by *S. Paratyphi A*. and *S. typhi* has the least susceptibility this could be due to its resistance and virulence as studied by so many researchers. [1, 2].

The high antimicrobial potential of many of these plants extracts against test organism is of obvious public health importance. Typhoid fever no doubt is frequently connected to resistance with a number of commercial antibiotics which result into complication as arthritis intestinal perforation and of course death.

The body on the other hand has been observed to accommodate plant extracts at relatively high dose, without visible side effects [3]. The stability of the natural drugs to antimicrobial resistance by the organisms is also an advantage.

5.5. Synergetic activity of extracts

The methanol and aqueous extracts where combined to observe for synergetic effect and the study revealed that the combination of the plant extract has higher antibacterial activity effects (Synergism) having a higher inhibitory effect than when used singly as shown on tables 4 ,5, 6 and 7. This clearly indicates that single plant extract could cause inhibitory effect ranging from 0 – 19 mm. However, when extract was combined the inhibitory surpassed that of single plant, 0 – 22 mm in combination of two tables 4, 5 and 6 and 2 – 19 mm, with combination of the three plants as on table 7. The synergetic effect may be due to formation of certain complexes which becomes more effective in the inhibition on the organism, similar conclusions were drawn by [10, 24].

5.6. Minimum inhibitory concentration (MIC) of extracts

A result of MIC for various species of *salmonella* subjected to the different plant extracts is presented on Table 8. The MIC of (*Ao*) on *S. typhi* with methanol extract was 50 mg/ml and aqueous extract 100 mg/ml, on *S. paratyphi A*, both extracts was 50 mg/ml, on *S. typhimurium* was 100 mg/ml with methanol extract and aqueous extract was 50 mg/ml.

The MIC of (*Pg*) with methanol extract on *S. typhi* was 50 mg/ml and aqueous extract 100 mg/ml, on *S. paratyphi A*, was 50 mg/ml for both solvents extract, on *S. typhimurium* methanol extract was 100 mg/ml and aqueous 200 mg/ml.

The MIC of (*Cc*) was 200 mg/ml for both solvent extract for the three *salmonella* isolates.

Table 8 Minimum inhibitory concentration of plants extracts on *Salmonella* Isolates

Organism	Minimum inhibitory concentration (MIC) in (mg/ml)					
	<i>A. occidentale</i>		<i>P. guajava</i>		<i>C. citratus</i>	
	ME	AE	ME	AE	ME	AE
<i>1.typhoidal</i>						
<i>S. typhi</i>	50	100	50	100	200	200
<i>S. paratyphi A</i>	50	50	50	50	200	200
<i>2.non-typhoidal</i>						
<i>S. typhimurium</i>	100	50	100	200	200	200

Key: - ME = Methanol extract AE = Aqueous extract, *S. typhi*=*Salmonella typhi*, *S. paratyphi A*= *Salmonella paratyphi A*, *S. typhimurium* = *Salmonella typhimurium*

5.7. Minimum bactericidal concentration (MBC)

Result of MBC is as shown on Table 9. The MBC of (*Ao*) of both solvent extracts on the isolates was 25 mg/ml except for *S. typhimurium* on aqueous extract which is 50 mg/ml. The MBC of (*Pg*) extract of both solvents on *S. typhi* and *S.*

typhimurium was 50 mg/ml and on *S. paratyphi A.* was 25 mg/ml. The MBC of (*Cc*) extract of both solvents on *S. typhi* was 50 mg/ml, on *S. paratyphi A.* was 100 mg/ml with methanol extract with and 200 mg/ml with aqueous extract while on *S. typhimurium* had MBC 100 mg/ml with methanol extract and 200 mg/ml with aqueous extract.

Table 9 Minimum bactericidal concentration of plants extract on *Salmonella* isolates

Minimum bacterial concentration (MBC) in (mg/ml)						
Organism	<i>A. occidentale</i>		<i>P. guajava</i>		<i>C. citratus</i>	
	ME	AE	ME	AE	ME	AE
<i>1.typhoidal</i>						
<i>S. typhi</i>	25	25	50	50	50	50
<i>S. paratyphi A</i>	25	25	25	25	100	200
<i>2.non-typhoidal</i>						
<i>S. typhimurium</i>	50	50	50	50	100	200

Key: - ME = Methanol Extract, AE = Aqueous Extract, *S. typhi*=*Salmonella typhi*, *S. paratyphi A*= *Salmonella paratyphi A*, *S. typhimurium* = *Salmonella typhimurium*.

6. Conclusion

Result obtained from this study indicates that the leaves of *Psidium guajava*, *Anacardium occidentale* and *Cymbopogon citratus* (grass) inhibited the medically important Bacteria-*Salmonella* species. This proves that the plant possesses some potential as alternative sources of antimicrobial substances.

Recommendation

Since herbal products are better in combinations as revealed by this studies, further work is required to carry out a further study into possible combinations of these extracts that would give an enhanced and exploitable activity by synergistic or additive reasons in a single therapeutic formulation. This will offer an advantage of using lower but effective therapeutic dosage of natural product as compared to the dose of synthetic substance that would be required to achieve similar therapeutic effect.

Compliance with ethical standards

In compliance with the ethical standard the consent of the clinically suspected patients was sort, while the Ethics Committee of Abubakar Tafawa Balewa University teaching Hospital Bauchi gave approval for the research. Confidentiality of the subjects' identities was duly maintained. Attached is a copy of the ethical approval and consent form.

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Disclosure of conflict of interest

The authors whose names are mentioned above declare that they have no conflict of interest as far as this publication is concerned, and should there be any conflict that may arise, it can be resolved amicably. The research is solely sponsored by the corresponding author.

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