Iikasha et al., Afr., J. Complement Altern Med. (2019) 16 (2): 15-23
https://doi.org/10.21010/ajtcam.v16i2.2

EVALUATION OF ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANT EXTRACTS AGAINST CLINICAL ISOLATES OF PATHOGENS FROM CHILDREN WITH ACUTE GASTROENTERITIS AT KATUTURA STATE HOSPITAL¸ WINDHOEK, NAMIBIA

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Article History

Received: Jan. 16th, 2019
Revised Received: Aug. 13th, 2019
Accepted: Oct. 2nd, 2019
Published Online: 9th, Jan. 2020

Abstract

Background: Diarrhea is a major health concern in Namibia with an estimate of 17000 cases in 2015 in Omusati and Kunene regions. The present study aimed at isolating and identifying gastrointestinal bacteria from stool samples of children admitted at Katutura hospital, Windhoek, and to determine the efficacy of selected medicinal plants used in treating gastroenteritis in Namibia.
Materials and Methods: Stool samples were collected for 6 months from children under age five admitted with acute diarrhea. Seeplex12 automated DNA extractor and PCR were used for isolation and identification of bacterial DNA from the samples. Serially diluted stool samples were cultured on selective media, sub-cultured in Nutrient broth and preserved in 80% glycerol. Parts of *Boscia albitrunca, Ziziphus mucronata, Combretum apiculatum, Solanum linnaeanum* and *Terminalia sericea* were collected, dried, ground and extracted using distilled water and ethanol. Disc diffusion method was used to determine the antibacterial activity of the plant extracts against isolated bacteria.
Results: A total of 12 out of the 18 stool samples collected had bacterial pathogens successfully identified by PCR. 33.3% were positive for *Salmonella*, 11.1% for *E. coli* 157:H7 VTEC and 22.2% for *E.coli* H7. *C. apiculatum* organic extracts exhibited potent antibacterial activity of (16± 0.57mm) at 1000 µg/ml against *Shigella* and *Salmonella* with a moderate minimum inhibitory concentration (MIC) of 250 µg/ml against *Salmonella. Salmonella* and *Shigella* showed resistance to 10 µg/ml of ampicillin.
Conclusion: Plant extracts showed *in vitro* antibacterial activity. However, toxicology and *in vivo* efficacy of these plant extracts should be determined before recommending their mainstream uses.

Key words: Acute Diarrhea, Medicinal Plants, Antibacterial, Resistance, Minimum Inhibitory Concentration.

List of abbreviations:- DNA: deoxyribonucleic acid, PCR: Polymerase chain reaction, SD: Standard Deviation, SE: Standard Error, MIC: Minimum Inhibitory Concentration, VTEC: Verotoxigenic *Escherichia coli*

Introduction

Gastroenteritis is defined as an inflammation of the mucus membranes of the gastrointestinal tract and is characterized by diarrhea or vomiting, increases in volume or fluidity of stools, changes in stool consistency, as well as an increase in frequency of defecation (Nonguera et al., 2014). Gastroenteritis and enteric ailments have become a greater burden worldwide with most cases reported in sub-Saharan Africa and Asia (Mostafa et al., 2017). Diarrheal diseases are preventable, yet account for 19% of the 10 million worldwide deaths of children under the age of five years worldwide (Bis-Johnson et al., 2017). In Africa, 800,000 children die each year from diarrhoea and dehydration which account for 25 to 75% of all childhood deaths (Centre for Disease Control and Prevention, 2012). In 2012, diarrhoea ranked fifth among the top ten causes of death in

Namibia with 5% mortality rate (Centre for Disease Control and Prevention, 2012). Moreover, there have been continuous outbreaks of diarrheal disease between 2014 and 2015 in northern Namibia, with as many as 17000 diarrheal cases reported in Omusati and Kunene regions (Media and Blog coverage, 2015). Bacteria such as *Shigella, Salmonella* spp., *Campylobacter, Vibrio* spp., and *E. coli* are responsible for many cases of bacterial gastroenteritis reported globally (Bisi-Johnson et al., 2017).

The emergence of multidrug resistant enteric bacteria is a public health concern globally. Diarrheal pathogens, such as non-typhoidal *Salmonella spp., Campylobacter* and *E. coli* have been reported to have developed multiple antibiotic resistance to the 3rd and 4th generation beta-lactam antibiotic drugs, complicating antibacterial therapy worldwide (Mason et al., 2017; Bonyadian et al., 2014). Namibia has a standard treatment guideline. However, although antibiotics are only required in cases of severe diarrhoea, children, elderly people and immune-compromised patients, some doctors still use antibiotics as first-line treatment option - which is against the Namibia Standard Treatment Guidelines and the local and regional antimicrobial sensitivity data (Perekoa et al., 2015). This can contribute to the emergence and re-emergence of resistant pathogens. Moreover, despite the agency of microbial resistance and higher statistics of microbial morbidity and mortality cases, there is limited data on the microbial resistance profile in Namibia. The increase in microbial resistance calls for new alternative antibacterial agents to be used to combat gastroenteritis. Rural communities in Namibia rely on herbal medicine for primary healthcare for different ailments. In fact, out of 753 species of medicinal plants documented in Namibia, about 274 are used in the traditional setting to treat different ailments such as diarrhoea, cough, fever, gonorrhoea, infertility, chicken pox, male dysfunction, stomach pain and high blood pressure (Chinsembu et al., 2010; Cheikhyoussef et al., 2011). Plants, such as *Boscia albitrunca, Ziziphus mucronata, Combretum apiculatum, Solanum linnaeanum* and *Terminalia. Sericea*, are used as treatment options for diarrhoea and stomach pain (Chinsembu et al., 2010). Although, there is sufficient information on the ethno-medicinal uses of different plants in Namibia, there is limited scientific evidence on how effective they are and this limits their mainstream uses in treating gastroenteritis.

Methods and Materials

Study population and Stool sample collection

Between April and August 2015, 18 children under 5 years of age who were admitted into Katurura State Hospital Pediatric Ward with acute diarrhea were randomly enrolled in the study. Ethical clearance to conduct this study was obtained from the Ministry of Health and Social Services (Ethical Clearance Certificate Number: 17/3/3). Moreover, a written informed consent was obtained from guardians of the participants before collection of fecal samples from the children by a registered nurse. From each child, a stool sample and a rectal swab were collected, stored in Cary-Blair transport media and stored at -80 °C before use in code labeled containers until required for analysis.

Bacteria DNA extraction and amplification

The tip of the rectal swab specimens within the transport media were mixed by vortexing. The samples were further centrifuged for 10 minutes at 15,000 x g (13000 rpm). The supernatant was discarded and the pellets were re-suspended in 1000 µl of Phosphate Buffered Saline (PBS) and vortexed thoroughly to re-dissolve and disperse the sample. An aliquot of 240 µl of the supernatant was transferred into a micro-centrifuge tube containing 10 µl of proteinase K and mixed by flicking the tubes gently.1.5 ml elution tubes were placed on the automated Seeplex DNA extractor. The tubes containing the samples were then placed onto the automated Seeplex DNA extractor together with QIAampr DNA Mini-Kit reagents catalog number 51304, for automated extraction of total nucleic acids. The sample on the Seeplex DNA extractor volume was set at 250 µl and the elution volume at 60 µl. The extraction was run for 54 minutes.

Multiplex Polymerase chain reaction (PCR) was used for detection of *Salmonella* and *Shigella* species as well as *E. coli* from the extracted DNA under conditions shown in Table 1. Two kits were used for PCR detection namely "seeplex diarrhea-B1 ACE detector for extracting (*Salmonella* and *Shigella)* and seeplex diarrhea-B2 ACE detector (for extracting *E. coli*) each with specific primer mix. Primers used in this study were set as: Primer mixture containing specific primers of 200 µl for *Salmonella, Shigella* and *E. coli*, primer pair for internal control and template of internal control. A total volume of 20 µl of the PCR reaction was prepared using; 4 µL of the 3 diarrheal bacteria B1 and B2 primer mix containing positive and negative controls, 3 µL 8-Mop Solution to avoid contaminations, 10 µL 2X master multiple master mix and 3 µL of the sample's nucleic acid.

Table 1: Summary of PCR reactions used for pathogen detection

Segment	Number of cycles	Temperature (◦C)	Duration (minutes)
1	1	94	15
		94	0.5
2	40	60	1.5
		72	1.5
3	1	72	10

The PCR products were stained with ethidium bromide and analysed by using 2% agarose gel electrophoresis. Base pairs for identifying different pathogens were set at: 100 bp for an internal control, 395 bp for *Salmonella* spp, 330 bp for *Shigella* spp, 370 bp for *E. coli* H7 and *E. coli* 0157: H7, VTEC 476 bp

Bacteriological investigation by conventional methods

All collected stool specimens were tested for *Salmonella* spp., *Shigella* spp., and *E. coli* O157:H7 using the following methods: Stock solutions containing 500 mg stool samples in 5 ml (100mg/ml) were diluted using double fold serial dilution to form 50, 25, 12.5, 6.25 and 3.25 mg/ml concentrations. Ten microliters of each serially-diluted samples was cultured on *Salmonella Shigella* agar, Xylose Lysine Deoxycholate agar, and Sorbitol MacConkey agar. The plates were incubated for 24 hours at 37 °C. Briefly, 100 µl of 6.25 and 3.25 mg/ml concentrations were further inoculated into 20 ml of selenite cysteine broth and 20 ml Gram-negative broth and incubated for 24 hours at 37 °C. Sub culture was done to obtain pure colonies of *Salmonella* spp., *Shigella* spp., and *E. coli* O157:H7 on *Salmonella Shigella* agar and Sorbitol MacConkey agar respectively, and incubated at 37 °C for 18 hours. Plates with suspected *Salmonella*, *Shigella* and *E. coli* O157:H7 positive colonies were selected for further identification using biochemical tests: carbohydrate fermentation, urease test, indole test, lysine decarboxylase, as well as motility test as previously described (Elamreen et al., 2007). Pure colonies of isolated cultures were cultured in nutrient broth, preserved in 80% glycerol and stored at -80 °C till further uses.

Plant collection and extracts preparation

A plant collection permit (Report Number: 2016/388) was obtained from the Ministry of Environment and Tourism of Namibia (MET) for the collection of plant materials used in this study. Plant parts (bark, roots and leaves) of *B. albitrunca, Z. mucronata, C. apiculatum, S. linnaeanum* and *T. sericea* were collected from Kunene region in Namibia. Plants collected were authenticated by the National Herbarium at the National Herbarium of Namibia. The plants materials were air dried for 4 weeks at ambient temperature, and ground to a powder before preparation of extracts. Organic and aqueous plant extracts were prepared using 90% ethanol and distilled water respectively, by submerging 20 g of the powdered plant material in 200 ml of each respective solvent, and incubated at room temperature for 24 hours. Whatman No.1 filter papers were used for filtration of the extracts and the filtrates were then dried using rotary evaporation and freeze drying (Bayoub et al., 2010).

Inoculum preparation

Pure isolated colonies of *E. coli* 0157:H7, *Salmonella* spp. and *Shigella* spp. preserved in glycerol were further cultured in nutrient broth and used for antibacterial susceptibility of different plant extracts used this study. The turbidity of the actively growing broth culture was adjusted with sterile nutrient broth to obtain turbidity optimally comparable to that of 0.5 McFarland standards.

Antibacterial susceptibility testing of plant extracts and antibiotics

Antibiotic susceptibility of *Salmonella* spp., *Shigella* spp., and *E. coli* 0157:H7 isolates was determined using the disk diffusion method on Muller–Hinton agar as described by the Clinical and Laboratory Standards Institute. Antibiotics used to clinically treat bacterial infection (Ampicillin, 10µg/ml for *Salmonella* and *Shigella*, and tetracycline, 35µg/ml for *E. coli* 0157:H7) were tested against the isolated bacterial strains. Antibacterial activities of prepared aqueous and organic extracts were determined at three concentrations (250, 500 and 1000 µg/ml) (Khanam et al., 2014). The antibacterial activities of distilled water and 90% ethanol that were used in extracts preparation were also determined. All the plates were incubated at 37 °C for 24 hours (Coyle, 2005). Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition around plant extracts, and classified as strong (zone ≥15 mm), moderate (zone ≥ 7 mm) and inactive (zone ≤ 6 mm) (Dulger et al., 2004). The Minimum Inhibitory Concentration test was performed on extracts that showed antibacterial activity during the susceptibility test to determine the lowest concentration with no microbial growth observed with a naked eye. The MIC test was performed on Nutrient broth by broth dilution method at 9 different concentrations (1000 to 3.91µg/ml) (Coyle, 2005; Owuama, 2017). Minimum Bactericidal Activity assay was performed in order to confirm the MIC obtained. An inoculating loop was dipped into each test tube not showing growth and stroked on Nutrient agar plate. Inoculated agar plates were incubated for 24 hours at 37°C. Concentrations that showed no microbial growth after 24 hours incubation period at 37°C were considered to be the MIC. All experiments were repeated three times.

Data analysis

The results are expressed as Mean ± Standard Deviation (SD). Statistical analysis of the data were carried out using Student's t-test to determine whether there is a significant difference between antimicrobial activity of organic and aqueous extracts and the results were considered significant when $P<0.05$.

Results

The present study was a part of a bigger study focusing on determining gut microbes of children with and without acute gastroenteritis in which a total of 164 samples were collected from children with acute gastroenteritis. However, only 18 samples were randomly selected out of the 164 stool samples that were collected from patients admitted into Katutura State

Hospital with acute diarrhea, to be used in this study. The diagnosis was done by an attending physician. Twelve samples showed positive results in PCR bacterial identification; on the detection of *Salmonella, E. coli* and *Shigella* species. *Salmonella* was detected in 33.3% of the samples (i.e., in sample numbers 14, 27, 87, 154, 52 and 64). *E. coli* 0157:H7 was detected in 11.1% of the samples (i.e., in sample numbers 6 and 64), while 22.2% of the samples showed the presence of *E. coli* H7 (i.e., sample numbers 6, 84, 44 and A157). Sample number 6 showed the presence of both *E. coli* 0157:H7 and *E. coli* H7, while sample 64 had both *E. coli* 157 and *Salmonella* spp. as depicted in Figure 1. *Shigella* spp. was not detected in any of the samples analyzed by PCR. Figure 1 depicts the amplified products of each sample on ethidium bromide stained 2.0% on agarose gel. All the samples were further analyzed using conventional methods of bacterial identification (culture techniques and biochemical tests) and the results were concordant with PCR. However, *Shigella* spp. was isolated and confirmed by biochemical tests in sample 158, although it was not detected by gel electrophoresis.

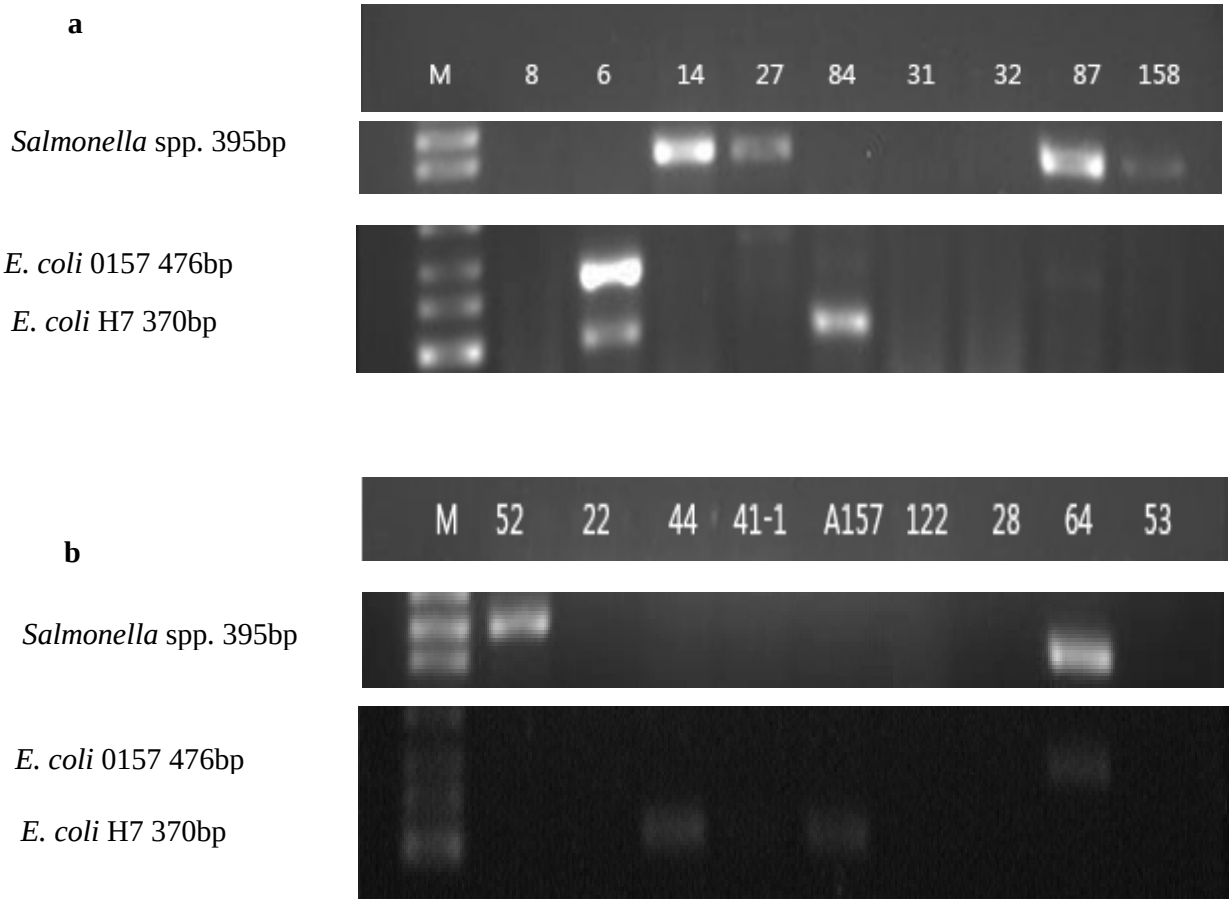

Figure 1: (a and b): Specific amplifications of *Salmonella* spp., *E. coli* 0157:H7 VTEC and *E. coli* H7 by PCR. M represents Ladder, while numbers 8, 6, 14, 27, 84, 31, 32, 87, 158, 52, 22, 44, 41-1, A157, 122, 64 and 53 are sample codes.

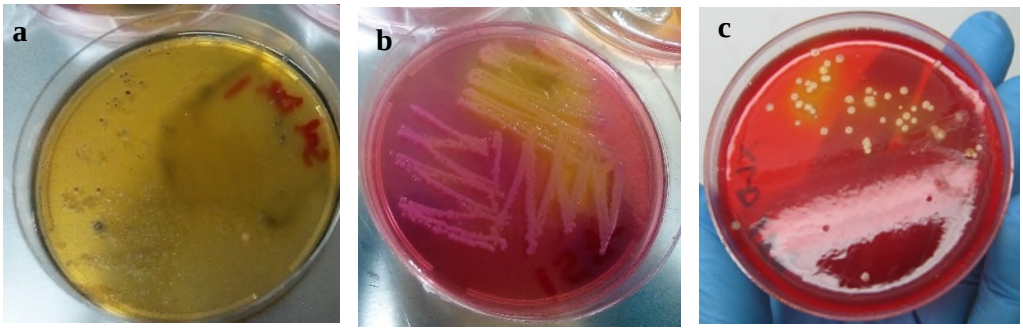

Figure 2: Conventional identification and isolation of (a) *Salmonella* spp. on *Salmonella Shigella* **agar, (b)** *Shigella* **on** *Salmonella Shigella* **agar and** *E. coli* **0157:H7 VTEC on MacConkey agar.**

Aqueous and organic crude extracts were prepared from different plant parts based on their ethno-medicinal uses to treat stomach pain, diarrhea, nauseous and abdominal pain in Kunene. The organic plant extracts for each plant showed the highest quantity of crude dry extract, except for aqueous plant extracts of *B. albitrunca* that showed higher percentage yield of 14%, double the yield of the organic extracts (Table 2). *C. apiculatum* and *S. linnaeanum* extracts showed the same percent yields for the aqueous and organic extraction.

Table 2: Effect of solvents on percentage yield of different plant extracts

Plant name	**Plant part used**	**% Yield**	
		Organic extracts	**Aqueous extracts**
B. albitrunca	Roots	7	14
Z. mucronata	Bark	7	4
C. apiculatum	Bark	8	8
S. linnaeanum	Fruit	6	6
T. sericea	Twig	11	5

Different plant extracts showed antibacterial activity against *Salmonella* spp., *Shigella* spp. and *E. coli* 0157:H7 in a concentration-dependent manner. *C. apiculatum* organic extract showed antibacterial activity against *Salmonella* spp with strong antibacterial activity (mean zone of inhibition, (16± 0.57) at 1000 µg/ml. *B. albitrunca Z. mucronata* and *S. linnaeanum* also showed antibacterial activity at this concentration. *T. sericea* organic extracts did not show antibacterial activity against *Salmonella* spp. (Figure 3).

T. sericea organic extracts showed concentration-dependent activity against *E. coli* 0157:H7, with a moderate average inhibitory zone of 11.3±0.57 at 1000 µg/ml. *B. albitrunca, Z. mucronata* and *C. apiculatum* also showed antibacterial activity against *E. coli* 0157:H7 at 1000 µg/ml. *S. linnaeanum* did not show antibacterial activity against *E. coli* 0157:H7 (Figure 4). Only *Z. mucronata* and *C. apiculatum* showed moderate to strong antibacterial activity against *Shigella* at 1000 µg/ml (average inhibition zones 9.3 and 16 ± 0.0.57 respectively) as depicted in Figure 5.

The isolated clinical strains of *Shigella* spp. and *Salmonella* spp. were resistant to 10 µg/ml of ampicillin. However, *E. coli* 0157:H7 was sensitive to 35 µg/ml of tetracycline with an average inhibition zone of 20± 0.58.

Although water is the universal solvent used in preparing herbal medicine in many societies, the findings of this study are on the contrary with this theory since only *T. sericea* aqueous extract showed antibacterial activity in its aqueous form. Moreover, this activity was only observed against *Shigella* spp. with an average inhibition zone of 7.5± 0.47 at 1000 µg/ml (Figure 6). By comparing the level of significance of antibacterial activity of aqueous and organic extracts of *T. sericea* against clinical *Shigella*, a p-value of 0.787 was obtained. Hence, we accept the null hypothesis and conclude that there is no significant variation in the mean of inhibition zones of *T. sericea* aqueous and organic extracts against clinical *Shigella.*

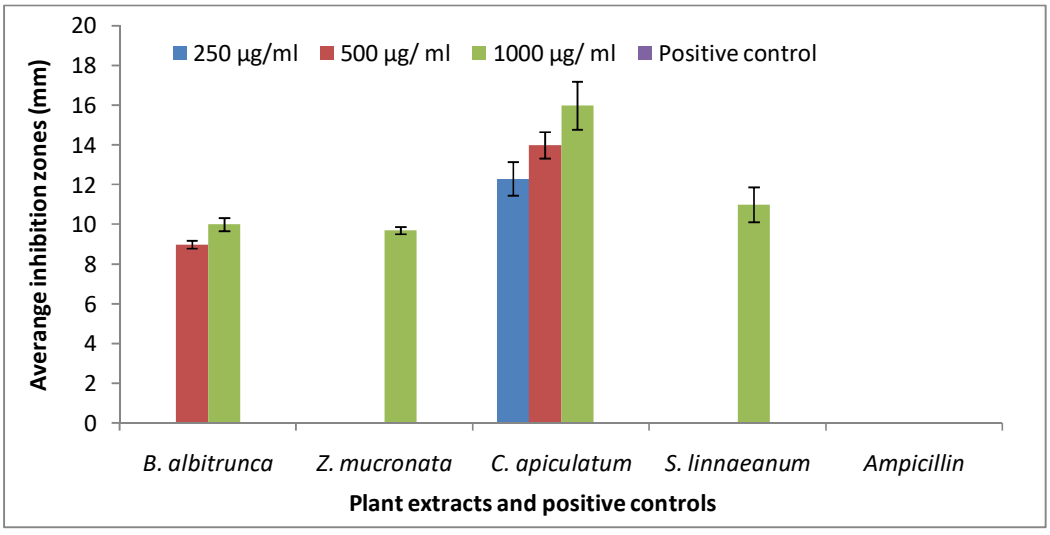

Figure 3: Antibacterial activity of organic extracts of *B. albitrunca* with Standard error (SE): 0.33, *Z. muronata SE; 0.19, C. apiculatum* SE: 1.20 and *S. linnaenum* SE: 0.88 at 1000 µg/ml and reference antibiotics against clinical *Salmonella* after 24 hours incubation.

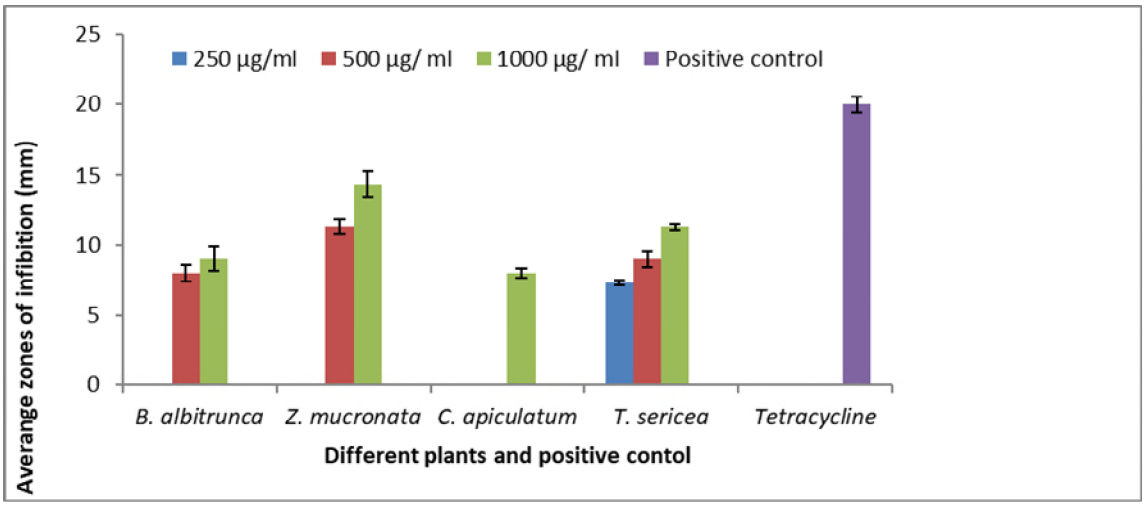

Figure 4: Antibacterial activity of organic extracts of *B. albitrunca* SE: 0.84, *Z. mucronata* SE: 0.96, *C. apiculatum* SE: 0.33 and *T. sericea* SE: 0.19 at 1000µg/ml against clinical *E. coli* after 24 hours incubation.

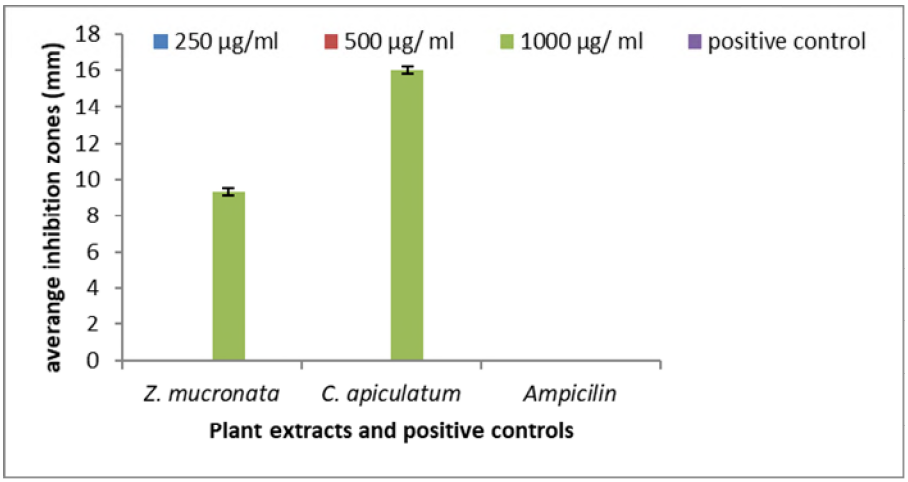

Figure 5: Antibacterial activity of organic extracts of Z. *muronata* SE: 0.19 and *C. apiculatum* SE: 0.19 and reference antibiotics against clinical *Shigella* after 24 hours incubation.

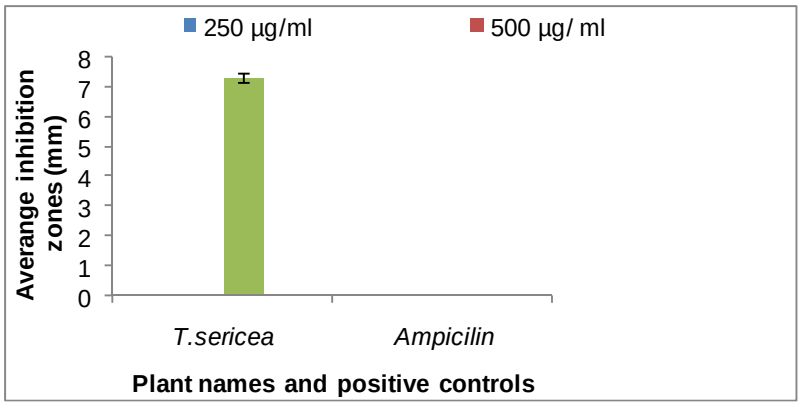

Figure 6: Antibacterial activity of aqueous extract of *T. sericea* SE: 0.15 and reference antibiotic against clinical *Shigella* after 24 hours incubation.

Among all the organic extracts that showed antibacterial activity, *T. sericea* and *C. apiculatum* showed moderate minimum inhibitory concentrations (at 250 µg/ml) against *E. coli* 0157:H7 and *Salmonella* spp respectively. *T. sericea* aqueous extract that showed antibacterial activity only showed activity at 1000 µg/ml against different pathogens as depicted in Table 3. None of the pathogens showed susceptibility to distilled water and ethanol.

Table 3: MIC of organic and aqueous plant extracts against clinical bacterial strains.

	MIC against test organisms (µg/ml)		
Organic plant extracts	***E. coli***	***Salmonella***	***Shigella***
B. albitrunca	500	500	>1000
Z. muronata	500	1000	1000
C. apiculatum	>1000	250	1000
T. sereciea	250	>1000	>1000
S. linnaenum	1000	1000	>1000
Aqueous plant extracts			
T. sereciea	>1000	>1000	1000

Discussion

Gastroenteritis has become a global burden in children under the age of 5 in developing countries. *Salmonella, Shigella, Campylobacter* and *E. coli* are among the top 10 causative agents of gastroenteritis globally (Fletcher et al., 2013). The findings of this study showed that *Salmonella* was responsible for most of the acute gastroenteritis cases in 18 samples collected from children under the age of 5 in Katutura State Hospital in 2016. However, a study by Langendorf et al. (2015) indicated that, among bacterial etiologies, typical entero-pathogenic *Escherichia coli* (EPEC) is associated with a higher risk of mortality in infants 0–11 months of age, while *Shigella* is the second cause of moderate-to-severe gastroenteritis among children 1 to 5 years old (Langendorf et al., 2015).

Previous studies showed that medicinal plants are rich in bioactive compounds which are easily isolated with organic solvents such as methanol, acetone and ethanol (Widyawati et al., 2014). The presence of polar compounds, such as: sugar, amino acid, glycosides, phenolic compounds, aglycon flavonoid, anthocyanin, terpenoid, saponin, tannin, xantoxilin, totarol, quacinoid, lacton, flavone, phenone, and polyphenol enable the plants to possess different biological activities. Organic solvents, such as ethanol used in this study, are good at extracting polar plant compounds (Widyawati et al., 2014). Whereas, water is effective in extracting glycoside compounds, amino acids, sugar and aglycon compounds. The presence of phytochemical compounds such as alkaloids, flavonoids, terpenoids, tannin and coumarins compounds in these plants is reported by Iikasha et al. (2017). The presence of different phytochemical compounds could explain the efficacy of the plant extracts against different diarrheal pathogens reported in this study. It is, however, interesting to note that although 25 to 50% of current pharmaceuticals are derived from plants, none are clinically proven for use as antidiarrheal agents (Cowan, 1999; Elisha et al., 2017). However, given the efficacy reported for numerous plant extracts, the long history on the ethno-medicinal uses of medicinal plants worldwide, their ready availability and lack of side-effects; plants could be good sources of next generation antimicrobial agents (Chandra, 2017).

The ampicillin resistance pattern reported in this study against *Shigella* spp. and *Salmonella* spp. are in line with the findings of Alam et al. (2013), who showed an increase in cases of enteric pathogens resistance to ampicillin and many other β-lactam antibiotics due their ability to produce enzyme β-Lactamases in India. The ampicillin resistance observed in this study by important gastric pathogens, *Shigella* spp. and *Salmonella* spp., highlights the need for new antibiotic treatments. The antibacterial activities of *C. apiculatum* and *T. sereciea* are significant because they exhibited measurable antibacterial activity against ampicillin resistant *Shigella* spp. and *Salmonella* as crude extracts with an MIC of 250µg/ml.

The antibacterial activity of *B. albitrunca* against *Salmonlella typhi* has been previously reported by Tshikalange et al. (2017). *B. albitrunca* extracts had activity against *Salmonlella typhi* in South Africa with an MIC 6300 µg/ml. This study reports an MIC that is 10-fold lower (500 µg/ml) for *B. albitrunca* growing in north western Namibia. This could be due to the difference of phytochemical compounds in Namibian *B. albitrunca* extracts which can be explained by their collection from different geographical locations with different environmental conditions. Kennedy et al. (2011) and Yang, et al. (2018) explained that accumulation of secondary metabolites in plants is strongly dependent on a variety of environmental factors, such as light, temperature, soil water, soil fertility and salinity. For most plants, a change in an individual factor may alter the content of secondary metabolites, even if other factors remain constant.

This study showed that organic solvents were superior in preparing extracts with potent antibacterial activity as compared to aqueous extracts, consistent with a previous report by Mudzengi et al. (2017). These findings do not necessarily contradict the effectiveness of aqueous herbal medicines reported in ethno-medicinal uses as compounds that are not effective

in vitro can still show efficacy *in vivo.* Some compounds can be activated once ingested and metabolized, or have activity under physiological conditions. Hence, studies should be conducted to study activity of extracts under *in vivo* conditions.

Conclusion

The extracts of *B. albitrunca, Z. mucronata, C. apiculatum, S. linnaeanum* and *T. sericea* showed potentials and should be considered in developing antibacterial agents as complementary or alternative treatments for gastro-intestinal ailments which are caused by *Salmonella, E. coli* and *Shigella*. However, the *in-vivo* efficacy and cytotoxicity of *B. albitrunca, Z. mucronata, C. apiculatum, S. linnaeanum* and *T. sericea* have to be evaluated before recommending their mainstream uses as alternative sources of next generation antibacterial agents.

Acknowledgements:-We would like to acknowledge the German Academic Exchange Service (DAAD) for funding this project. We would like to acknowledge the University of Namibia for providing the facility to conduct this study.

Conflict of interest:-We confirm that there are no known conflicts of interest associated with this publication.

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