CHARACTERIZATION AND SUSCEPTIBILITY OF *ESCHERICHIA COLI* ISOLATED FROM INFANTS DIARRHOEA TO *MOMORDICA CHARANTIA* AND *AESCHYNOMENE UNIFLORA* EXTRACTS

BY

AHMED Isma'ila

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(PhD/SCIEN/10682/2007-08)

A DESSERTATION SUBMITTED TO SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS OF THE AWARD OF DOCTOR OF PHILOSOPHY (PhD) DEGREE IN MICROBIOLOGY

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Title page

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BEING A DESSERTATION SUBMITTED TO SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARCIAL FULFILLMENT FOR THE REQUIREMENTS OF THE AWARD OF DOCTOR OF PHILOSOPHY (PhD) DEGREE IN MICROBIOLOGY

OCTOBER, 2014

Flyer leaf

DECLARATION

I hereby declare that this dissertation titled "Characterization and Susceptibility of *Escherichia coli* Isolated from Infants Diarrhoea to *Momordica charantia* and *Aeschynomene uniflora* Extracts" has been carried out and written by me and that it is exclusively the report of the investigation conducted. It has not been presented in any form for any other degree elsewhere. All quotations have been indicated and the sources of the information are specifically acknowledged by means of references.

Sing.: _____ _____

AHMED Isma'ila Date

(PhD/SCIEN/10682/2007-08)

CERTIFICATION

This is to certify that this dissertation was carried out by AHMED Isma'ila (PhD/SCIEN/10682/2007-08). The dissertation has been read and approved as having satisfied the requirements of the Department of Microbiology, Ahmadu Bello University, Zaria, under close supervision for the award of PhD degree in Microbiology.

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Prof. Joshua Adebayo Abiodun Dean, School of PG Studies	Date

DEDICATION

This dissertation is dedicated to my father **Alhaji Ahmed Inuwa Gaya** (Amadu Babban).

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October, 2014

ABSTRACT

A study was conducted to characterize and determine the susceptibility of *Escherichia coli* isolates obtained from infants' diarrhoea stool samples to extracts of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May in Kano state, Nigeria. Five hundred and eighty six (586) diarrhoeal samples of infants 0-5 years attending some hospitals in Kano state were examined for the presence of *Escherichia coli*. Standard methods of culturing, microscopy, Gram staining, biochemical tests and serotyping were used for the identification and characterization of the isolates. Samples were collected from some hospitals from the three senatorial zones. Some demographic data were also obtained from the infants' mothers/caregivers. Most common *E. coli* serotype isolates include enterotoxigenic *E. coli* O148:H28 and enterohaemorrhagic *E. coli* O157:H7 with number positive of 48 (23.53%) and 41 (20.10%) respectively and enteropathogenic *E. coli* O112 and enteroheamorrhagic *E. coli* O26:H11 with number positive of 35 (17.16%) each. Enteroinvasive *E. coli* strains O124 and O143 were the least common in the study with the least number positive of 0 (0.00%) and 7 (3.43%) respectively. Prevalence of 34.81% (n=204) of infants' diarrhoea was obtained from the study. Males infants' were more infected than females with number of males and females positive of 113 (19.28%) and 91 (15.53%) respectively. The prevalence is highest at age range of 24-35 month with total number infected of 54 (9.22%) while least infection was obtained in 0-11 month with number infected of 30 (5.12%). The results of susceptibility pattern of *E. coli* isolates to antibiotic showed that the *E. coli* are more susceptible to ciprofloxacin, ceftriaxone, augmentin and ofloxacin with numbers of susceptible isolates of 169 (83%), 159 (78%), 155 (76%) and 153 (75%) respectively. The isolates are however resistant to ampicillin, amoxycillin, sulphamethoxazole/trimethoprim and tetracycline with number resistance of 182 (89%), 170 (83%), 105 (51.47%) and 94 (46%) respectively. No significant difference between the means number resistant and susceptible organisms ($P = 0.1$). Resistance patterns of *E. coli* isolates showed that ampicillin and amoxycillin have the highest single antibiotics resistance with 11 (12.5%) and 10 (11.4%) number of isolates respectively. Multiple antibiotic resistance pattern shows high frequency of occurrence in AMP, SXT, AML with 6 (6.82%), AMP, SXT with 5 (5.68%) and SXT, AML with 4 (4.55%) number of isolate with the pattern. Highest number of antibiotics resistance pattern of 7 antibiotics in occurred in 1 (1.14%) isolate with resistance phenotype include; OFX, AMP, SXT, CN, AML, CIP, TE. Two local plants *Momordica charantia* Linn and *Aeschynomene uniflora* E. May extracts and fractions were tested for activity on the *E. coli* isolates. The leaves and the whole plant yielded the highest extract weight in both plants. Thus *Momordica charantia* yield 40.60g and 38.35g for MOL and MOW, while *Aeschynomene uniflora* yield 37.90g and 24.19g for AEL and AEW respectively. Aqueous and ethanol extracts have the highest weight of 48.25g and 36.79g in *Momordica charantia* and 36.33g and 36.38g in *Aeschynomene uniflora* respectively. The petroleum ether in both plants showed the least weight extract obtained with weight of 09.84g and 08.92g respectively. Preliminary phytochemical screening of the plants extracts include test for carbohydrates (Molish's test and Fehling's test), alkaloid test (Mayer's test, Wagner's test and Dragendoff's test), tannins (Fe_3Cl_2 test), glycosides (Fehlings test), cardiac glycosides (Kella-killiani test), steroids (Salkwoski test), saponins (frothing test and emulsion test), flavonoid (Shinoda test), resin (copper acetate test) and

anthraquinone (Borntrager's test). There was no significant difference between the mean weights of the albino mice before and after treatment (P=0.65 14 df) on pharmacological test. No death occurred of any mice after the 3 days experiment of haematological screening of albino mice blood after two weeks experiment on active MOL5 and AEW3 doses of methanol extract. The results of doses concentrations of 1000 mg/kg/day and 1600 mg/kg/day are not significantly different from the control, while 2900 mg/kg/day and 5000mg/kg/day results are significantly different from the control (P=0.05). Susceptibility pattern of *E. coli* isolates on extracts of *Momordica charantia* and *Aeschynomene uniflora* showed that ethanol and methanol proved to be effective for extraction of these plants having wider zones of inhibition of 18±2 mm and 18±1mm respectively. The isolates were more susceptible to leaves and whole plant extracts in both plants generally. Concentration has no significance effects in all the extracts tested (P=0.05). In *Momordica charantia* the isolates were more susceptible to leaves (methanol and ethanol) and whole plant (aqueous) with number susceptible of 52 (80%), 50(77%) and 48(74%) respectively, while more resistant to the root (methanol and petroleum ether extracts) with number of 53(82%) each. The organisms are more susceptible to whole plant and leave *Aeschynomene uniflora* methanol extracts with number susceptible of 49(75%) and 47(72%) respectively while resistant to the roots petroleum ether extract with number of 55(85%). All active extracts yielded the same MIC value of 62.5mg/ml except the methanolic extracts of MOL, MOS, MOW, AEL, AES and AEW with 31.2mg/ml. Low MBC of 31.2 mg/ml was obtained for MOL, AEL and AEW ethanolic extracts, MOL and AER for aqueous extracts, MOL, MOW, AEL and AEW for methanol extracts. Nine (9) TLC fractions were obtained from MOL methanol extract with R_f values ranging from 0.00 to 0.91 with solvent front of 7.9cm. Fraction number 5 (MOL5 deep purple) was the most active. MOL5 has R_f value of 0.51 and yielded 470mg (17.45%). Only three (3) fractions were obtained from the TLC of most active extract of *Aeschynomene uniflora* whole plant - AEW aqueous extract with solvent front of 7.8cm. Fraction number 3 (AEW3 green) showed more activity against the *E. coli* isolates with R_f value of 0.91 and yielded 370mg (46.84%). MOL5 fraction was more active than AEW3 fraction with zones of inhibition of 17±1 and 14±2 respectively. Zones of inhibitions did not differ significantly (P=0.05) from the values of *E. coli* (NTCT 10418) control. MOL5 showed MIC and MBC of 30µg/ml and 40µg/ml respectively, while AEW3 showed 40µg/ml in each case. Further phytochemicals screening revealed that each fraction contains 4 organic compounds. Steroids were found in MOL5 and more tannins than in AEW3. While AEW3 had more saponins compared to MOL5. This study revealed high prevalence of 34.81% infants' diarrhoea cases in Kano state. National policy on drugs disbursement, retail and usage should be observed strictly to avoid the antibiotics resistance shown by the *Escherichia coli* strains/isolates.

CHAPTER ONE

3.0 INTRODUCTION

Escherichia coli is a common member of the normal flora of the large intestine. As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals. Strains that acquire bacteriophage or plasmid Deoxyrhibonuecleic acid (DNA) encoding enterotoxins or invasion factors become virulent and can cause either plain, watery diarrhoea or inflammatory dysentery. These diseases are most familiar as traveler's diarrhoea, but they are also major health problems in endemic countries, particularly among infants in developing nations (Willey *et al*., 2010). *Escherichia coli,* a facultatively anaerobic Gram-negative bacillus, peritrichously flagellated is a major component of the normal intestinal flora and ubiquitous in the human environment. First described in 1885, *E. coli* has become recognized as both a harmless commensal and a versatile pathogen (Cheesbrough, 2006). In contrast to the essential and beneficial role of most *E. coli* isolates in the human intestine, pathogenic *E. coli* are responsible for broad spectrum of human diseases. *E. coli* has emerged as an important cause of diarrhoeal illness, with diverse phenotypes and pathogenic mechanisms. Hemolytic-uremic syndrome (HUS) is a potentially devastating consequence of enteric infection with specific *E. coli* strains (Brooks *et al*., 2007).

The Gastrointestinal Tract (GIT) of most warm-blooded animals is colonized by *E. coli* within a few hours or days after birth ingested in foods or water or directly from other individuals. The human bowel is usually colonized within 40 hours of birth. *E. coli* can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period (weeks to months), and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal flora. The basis for these shifts

and the ecology of *Escherichia coli* in the intestine of humans are poorly understood despite the vast amount of information on almost every other aspect of the organism's existence. In fact, the entire Deoxyribonucleic acid (DNA) base sequence of the *E. coli* genome is known (Brooks *et al*., 2007).

Diarrhoea is defined as an increased number of stools of liquid or semi-liquid consistently passed during a twenty-four-hour period after the age of three months (Martines *et al*., 1993). Diarrhoea infection occurs through water and food contamination with bacteria such as *Escherichia coli, Shigella* and other agents like *Vibrio cholerae, Compylobacter jejuni* and viruses such as Rotavirus; and protozoa such as *Giardia lamblia, Entamoeba histolytica* and *Cryptosporidium* (Okolocha and Umoh, 2002). It is acute when it lasts up to 21 days and chronic when it lasts beyond 21 days. Acute diarrhoeal diseases are one of the leading causes of childhood mortality and morbidity in the developing countries and a major contributor to malnutrition (Banerjee, 1988).

Plasmid-mediated antibiotic resistance is common in the treatment of *E. coli* diarrhoea, the use of antibiotics is in general only of minor important. Rehydration is always the most important measure taken to restore the lost of water and electrolyte (Cheesbrough, 2006). However, therapy with appropriate antibiotics can reduce the severity and duration of symptoms (Brooks *et al*., 2007). Various means have been proposed for the prevention of diarrhoea at times of epidemic, including daily ingestion of bismuth subsalicylate suspension and regular doses of tetracyclines or other antimicrobial drugs such as ciprofloxacin or trimethoprim-sulfamethoxazole for limited period prophylaxis (Brooks *et al*., 2007)

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1.1 *E. coli* Serotyping

Escherichia coli is routinely serotyped with specific antigen-antibody reactions involving flagella (H) antigens, capsular (K) antigens and lipopolysaccharide (O) antigens. Among *E. coli* there are over 167 different O antigens. Currently *E. coli* O55, O111 and O127 serotypes are most frequently associated with infantile diarrhoea, and *E. coli* O157:H7 is largely to blame for many life-threatening outbreaks. Thus the serotype of *E. coli* from stool sample is of diagnostic value and aids in the identifying the source of the infection (Willey *et al*., 2010). *Escherichia coli* serotypes are specific O-group/H-antigen combinations.

The H antigens are the flagella antigens, of which there are at least 56 types. *Escherichia coli* isolates may be nonmotile and nonflagellated and hence H negative (H). H typing is important for *E. coli* associated with diarrhoeal disease for two reasons. First, a strain causing an outbreak or epidemic can be differentiated from the normal stool flora by its unique O:H antigenic makeup. Second, most ETEC belong to specific serotypes; this relationship facilitates their identification even in isolated cases. The reason for the close association between specific serotypes and the production of plasmid-determined virulence factors remains a mystery. A plasmid-encoded but enterotoxin- and serotype-independent pilus (a long polar structure termed longus) has been reported recently in ETEC, like the CFAs, production of this pilus is restricted to *E. coli* isolated from human sources. Most *E. coli* isolates also produce heat-labile, surface-associated proteins antigenically unrelated to O and H (Blanco *et al*., 1992). These antigens can be seen in electron micrographs as filamentous structures called pili (fimbriae), which are much thinner and usually more rigid than flagella. Commensal *E. coli* strains usually produce so-called common pili, which are defined as a specific set of an antigen. When *E. coli* possessing common pili are mixed with erythrocytes (the standard test uses guinea pig erythrocytes), rapid

haemagglutination occurs. This haemagglutination is blocked and also reversed by millimolar concentrations of carbohydrate, mannose. ETEC possess specialized pili, antigenically unrelated to common pili, which act as ligands to bind the bacterial cells to specific complex carbohydrate receptors on the epithelial cell surfaces of the small intestine. Since this interaction results in colonization of the intestine by ETEC, with subsequent multiplication on the gut surface, these pili are termed colonization-factor antigens (CFAs). Most ETEC isolates produce either CFA/I, CFA/II or CFA/IV, whereas CFA/III and an undetermined number of other CFAs occur on other particular serotypes (Table 1.1). CFA-type pili play a major role in host specificity, for instance different CFAs (e.g., K88, K99, and 987P) are produced by *E. coli* that cause acute diarrhoea in domestic animals. A simple presumptive assay for CFAs on *E. coli* is a test for mannose-resistant (non-common pili) haemagglutination reaction with either human or bovine erythrocytes. However, identification must be confirmed by reaction of the bacteria with antibody directed against a specific CFA or polymerase chain reaction (PCR) assay for specific CFA genes. Genes coding for the production of CFAs reside on the ETEC virulence plasmids, usually on the same plasmids that carry the genes for one or both of the two types of *E. coli* enterotoxin, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST). Most cases of ETEC diarrhoea are caused by *E. coli* possessing a CFA and both LT and ST, fewer are caused by those possessing a CFA and only one toxin (usually LT) and the fewest are caused by *E. coli* that lack a CFA and possess only ST (Evans, 1990).

E coli are serogrouped/typed according to the presence or absence of specific heat-stable somatic antigens (O antigens) composed of polysaccharide chains linked to the core lipopolysaccharide (LPS) complex common to all Gram-negative bacteria. O specificity is determined by sugar or amino-sugar composition and by the sequence of these outer polysaccharide chains. More than

170 different O-specific antigens have been defined since Kauffmann began this method of typing *E. coli* in 1943. In normal smooth strains, which are typable, the core LPS is buried beneath the O antigen. Also occurring are untypable O-minus mutants in which the core LPS is exposed, these are called rough strains. There is considerable cross-reactivity among *E. coli* O antigens. Also many O groups of *E. coli* are cross-reactive or identical with specific O groups of *Shigella*, *Salmonella* or *Klebsiella* (Peter *et al*., 1998).

Escherichia coli strains that produce enterotoxins are called enterotoxigenic *E. coli* (ETEC). There are numerous types of enterotoxins. Some of these toxins are cytotoxic, damaging the mucosal cells, whereas others are merely cytotonic, inducing only the secretion of water and electrolytes (Table 1.1). A second group of *E. coli* strains have invasion factors and cause tissue destruction and inflammation resembling the effects of *Shigella* (EIEC). A third group of serotypes, called enteropathogenic *E. coli* (EPEC), are associated with outbreaks of diarrhoea in newborn nurseries, but produce no recognizable toxins or invasion factors (Donnenberg *et al*., 1993).

1.2 Forms and Types of Diarrhoea

Generally there are three forms of diarrhoea; acute watery, acute bloody and persistent diarrhoea. Types of diarrhoea however include; non-inflammatory - secretary, osmotic, motality-related and inflammatory diarrhoea (Banerjee, 1988).

Table 1.1 Examples of plasmid- and phage-encoded virulence determinants.

Organism	*Virulence Factor*	*Biologic Function*
1. Plasmid encoded		
Enterotoxigenic *Escherichia coli* (ETEC)	Heat-labile, heat-stable enterotoxins	Activation of adenylate/guanylate cyclase in the small bowel, which leads to diarrhoea.
	CFA/I and CFA/II	Adherence/colonization factors
Extraintestinal *E. coli*	Haemolysin	Cytotoxin
Shigella spp. and enteroinvasive *E. coli*	Gene products involved in invasion	Induces internalization by intestinal epithelial cells
Yersinia spp.	Adherence factors and gene products involved in invasion	Attachment/invasion
Bacillus anthracis	Edema factor, lethal factor, and protective antigen	Edema factor has adenylate cyclase activity; lethal factor is a metalloprotease that acts on host signaling molecules
Staphylococcus aureus	Exfoliative toxin	Causes toxic epidermal necrolysis.
Clostridium tetani	Tetanus neurotoxin	Blocks the release of inhibitory neurotransmitter, which leads to muscle spasms.
2. Phage encoded		
Corynebacterium diphtheriae	Diphtheria toxin	Inhibition of eukaryotic protein synthesis
Streptococcus pyogenes	Erythrogenic toxin	Rash of scarlet fever
Clostridium botulinum	Botulism neurotoxin	Blocks synaptic acetylcholine release, which leads to flaccid paralysis
Enterohemorrhagic *E. coli* (EHEC)	Shiga-like toxin	Inhibition of eukaryotic protein synthesis
Vibrio cholerae	Cholera toxin	Stimulates adenylate cyclase in host cells.

3. CFA, Colonization Factor Antigen.

Source: Elwell and Shipley (1980) and Cheetham and Katz (1995).

1.2.1 **Non Inflammatory Diarrhoea**

The diarrhoeal disease caused by ETEC is characterized by a rapid onset of watery, non-bloody diarrhoea of considerable volume, accompanied by fever or no fever. Other common symptoms are abdominal pain, malaise, nausea, and vomiting. Diarrhoea and other symptoms cease spontaneously after 24 to 72 hours. ETEC organisms are Gram-negative, short rods not visibly different from *E. coli* found in the normal flora of the human large intestine. Virulence-associated fimbriae are too small to be seen by light microscopy. All ETEC contain plasmids, but this is also not a distinguishing feature unless gene probe techniques are used to detect specific virulence-associated genes on these plasmids. *Escherichia coli* diarrhoeal disease is contracted orally by ingestion of food or water contaminated with a pathogenic strain shed by an infected person. ETEC diarrhoea occurs in all age groups, but mortality is most common in infants, particularly in the most undernourished or malnourished infants in developing nations (Blanco, 1996).

The pathogenesis of ETEC diarrhoea involves two steps: intestinal colonization, followed by elaboration of diarrhoeagenic enterotoxin(s). ST is actually a family of toxic peptides ranging from 18 to 50 amino acid residues in length. Those termed STa can stimulate intestinal guanylate cyclase, the enzyme that converts guanosine 5'-triphosphate (GTP) to cyclic guanosine 5'-monophosphate (cGMP). Increased intracellular cGMP inhibits intestinal fluid uptake, resulting in net fluid secretion. Those termed STb do not seem to cause diarrhoea by the same mechanism. One method for testing suspect *E. coli* isolates for ST production involves injection of culture supernatant fluids into the stomach of infant mice and seeing whether diarrhoea ensues. Specific DNA gene probes and PCR assays have been developed to test isolated colonies for the presence of genes encoding ST and LT (Sears and Kaper, 1996).

The *E. coli* LTs are antigenic proteins whose mechanism of action is similar to that of *Vibrio cholerae* enterotoxin. LT shares antigenic determinants with cholera toxin, and its primary amino acid sequence is similar. LT is composed of two types of subunits. One type of subunit (the B subunit) binds the toxin to the target cells via a specific receptor that has been identified as Gm1 ganglioside. The other type of subunit (the A subunit) is then activated by cleavage of a peptide bond and internalized. It then catalyzes the AD pribosylation (transfer of ADP-ribose from NAD; nicotinamide adenine dinucleotide) of a regulatory subunit of membrane-bound adenylate cyclase, the enzyme that converts ATP to cAMP. This activates the adenylate cyclase, which produces excess intracellular cAMP, which leads to hypersecretion of water and electrolytes into the bowel lumen. LT production is demonstrable by serologic methods, testing for diarrhoeagenic activity in ligated rabbit intestine, and by testing for specific cAMP-mediated morphological changes in cultured Y-1 adrenal tumor cells or Chinese hamster ovary (CHO) cells (Cheetham and Katz, 1995).

As in any faecal-orally transmitted disease, the first line of defense against ETEC diarrhoea is gastric acidity. Other non-specific defenses are small-intestinal motility and a large population of normal flora in the large intestine. Information about intestinal immunity against diarrhoeal disease is still somewhat superficial. However, intestinal secretary immunoglobulin (IgA) directed against surface antigens such as the CFAs and against LT appears to be the key to immunity from ETEC diarrhoea. Passive immune protection of infants by cholesterol antibody is important. Human breast milk also contains non immunoglobulin factors (receptor containing molecules) that can neutralize *E. coli* toxins and CFAs.

Escherichia coli diarrhoeal disease of all types is transmitted from person to person with no known important animal vectors. The prevalence of *E. coli* diarrhoea is clearly related to hygiene, food processing sophistication, general sanitation, and the opportunity for contact. The geographic frequency of ETEC diarrhoea is inversely proportional to the sanitation standards. Single-source outbreaks of ETEC diarrhoea involving contaminated water supplies or food have been found in adults in the United States and Japan. Adults traveling from temperate climates to more tropical areas typically experience traveler's diarrhoea caused by ETEC. This phenomenon is not readily explained, but contributing factors are low levels of immunity and an increased opportunity for infection. ETEC diarrhoea is characterized by copious watery diarrhoea with little or no fever. The diarrhoeal stool yields a virtually pure culture of *E coli*. Since the disease is self-limiting, virulence testing of isolates and serotyping is impractical except in an outbreak situation. Confirmation is achieved by serotyping, serologic identification of a specific CFA on isolates, demonstration of LT or ST production, and identification of genes encoding these virulence factors (Hasony and Muhammad, 1994).

Escherichia coli diarrhoeal disease is best controlled by preventing transmission and by stressing the importance of breast-feeding, especially where ETEC is endemic. The best treatment is oral fluid and electrolyte replacement (intravenous in severe cases). Antibiotics are not recommended because this practice leads to an increased burden of antibiotic-resistant pathogenic *E. coli* and of more life threatening enteropathogens (King *et al*., 2003).

1.2.2 Inflammatory Diarrhoea

Inflammatory diarrhoea caused by the enteroinvasive, cytotoxic, and enteropathogenic (EPEC) strains of *E. coli* ranges from very mild to severe. Illness is usually protracted and accompanied by fever. Infection with a few serogroups (O157, O26) is characterized by bloody diarrhoea

(hemorrhagic colitis). Infection with the Shigella-like serogroups presents as bacillary dysentery (i.e., abdominal pain and scanty stool containing blood and mucus). As in the case with ETEC, these strains of *E. coli* are not detectably different in structure from *E. coli* of the normal flora. The EPEC serogroups were the first *E. coli* groups to be recognized as causative agents of diarrhoea in infants. Their status as pathogens remained controversial for decades, mainly because the same O groups can be isolated from healthy contacts in outbreaks and from healthy adults. Recent work has proven that these *E. coli* serogroups possess an antigenic adherence factor (termed bundle-forming pilus, or BFP). The gene for BFP is carried by a plasmid termed EAE (enteroaggregative *Escherichia coli*) plasmid. BFP is responsible for the initial attachment of EPEC to intestinal target cells. A small but important group of EPEC includes serotypes O157:H7, O26: H11, and some O111 isolates. These cause epidemic haemorrhagic colitis. Serotype O157:H7 is often associated with food-borne outbreaks (Mackie and McCartny, 1993).

The Shigella-like enteroinvasive *E. coli* serotypes (i.e., those with somatic antigens reactive with specific anti-Shigella typing serum) like Shigella, are non-motile and therefore H negative. These serogroups usually do not harbor ETEC virulence plasmids and therefore are usually CFA negative (Blanco *et al*., 1992).

Escherichia coli strains belonging to the classic EPEC serogroups bind intimately to the epithelial surface of the intestine, usually the colon, via the adhesive BFP. The lesion caused by EPEC consists mainly of destruction of microvilli. There is no evidence of tissue invasion. Cell damage occurs in two steps, collectively termed attaching and effacing; first is intimate contact, sometimes characterized as pedestal formation; second is loss of microvilli which is the result of rearrangement of the host cell cytoskeleton. Loss of microvilli leads to malabsorption and

osmotic diarrhoea. Diarrhoea is persistent, often chronic, and accompanied by fever. EPEC are negative for ST and LT, but most strains produce relatively small amounts of a potent Shiga-like toxin that has both enterotoxin and cytotoxin activity.

The *E. coli* strains associated with haemorrhagic colitis (enterohemorrhagic *E. coli*, or EHEC) most notably O157:H7, produce relatively large amounts of the bacteriophage-mediated shiga-like toxin. This toxin is called vero toxin (VT), or Verocytotoxin after its cytotoxic effect on cultured Vero cells. Many strains of O157:H7 also produce a second cytotoxin (shiga-like toxin 2, or Vero toxin 2) which is similar in effect but antigenically different (Karch and Bielazewska, 2005). The Shigella-like *E. coli* strains are highly virulent, oral exposure to a very small number of these invasive bacteria causes severe illness (Riley *et al*., 1983). The site of the infection is the colon, where adherence is rapidly followed by invasion of the intestinal epithelial cells. An acute inflammatory response and tissue destruction produce diarrhoea with little fluid, much blood, and sheets of mucus containing polymorphonuclear cells. Invasive *E. coli*, like Shigella, causes a rapid keratoconjunctivitis when placed on the conjunctiva of the guinea pig eye (Sereny test). Virulent Sereny test-positive isolates carry a large (usually 140-megadalton) plasmid responsible for this property (Tesh and O'Brien, 1992).

Host defenses against EPEC are the same as those for ETEC. These defenses are frequently deficient or lacking in the infant and the elderly, which is consistent with the epidemiology of EPEC illness. An important example is the role of the immune system. Passive immune protection of infants by cholesterol antibody is important; breast-feeding is especially relevant where crowding and poor economic conditions prevail. Infection with these pathogens often excites an inflammatory cell response in the intestine, as is frequently reflected in the diarrhoeal symptoms.

The geographic distribution of all EPEC is generally the same as for the ETEC, with a more severe disease in infants and young children, and so EPEC are much less important in traveler's diarrhoea. Common-source community outbreaks are rare in geographic areas with satisfactory sanitation. However, sporadic cases are seen in the United States, Canada, and Europe, and outbreaks occur in these areas, but most commonly in close-contact institutions such as hospital nurseries, day-care centers, and nursing homes (Agbonlahor and Odugbemi, 1982). Diagnosis is usually based on the symptomatology described above. Enterohemorrhagic *E. coli*, such as serotype O157: H7 is suspected in the setting of copious bloody diarrhoea without fecal leukocytes or fever, especially when symptoms include hemolytic uremic syndrome, or HUS. *Escherichia coli* serotyping is useful in chronic cases and in outbreaks, because identification of the agent and its antibiotic sensitivity pattern are valuable in these situations. Testing for specific EPEC virulence factors is usually impractical because it can be done only in reference and specialized research laboratories. Prevention and control are generally the same as for ETEC. Intervention of the fecal-oral transmission cycle is most effective in institutional situations. Broad-spectrum antibiotics are recommended in chronic and/or life-threatening cases.

1.3 Traditional Medicine Practice; Problems and Prospects

The effectiveness and use of modern therapy are hampered by certain factors inherent in the drugs constituents, mode of administration, patient factors, storage condition etc. some of these limitations are outlined below.

The effectiveness of the chemical drugs is to a large extent defendant on their rates of absorption by the body or target organs. This is sometimes hampered by certain factors such as decrease blood flow and nature of the surface area (Anosike *et al*., 2008).

Some common antibiotics and other drugs have limitations associated with route of administration (Okogun, 2002). For instance, intravenous administration of drugs is not suitable for oily solutions or insoluble substances and there is increase risk of adverse reactions. Similarly, oral administration has risks of drugs been metabolized by gut enzymes in the mucosa, the intestinal flora or liver before gaining access to the blood circulation.

Also, drugs given in solution appeared to be more readily absorbed than those given in solid form and those introduced at administration sites at higher concentrations are absorbed more rapidly than those introduced in low concentrations (Sofowora, 1993). In some cases, antibiotics alone do not alter the clinical course of a disease, but only eradicate the carrier state (Brooks *et al*., 2007).

For pregnant mothers, the foetus is at least to some extent, exposed to essentially all the drugs taken by the mother and the potential transfer of drugs across the placenta may cause congenital abnormalities and or adverse effects on the neonate. Thus the view that the placenta is a barrier to the drugs is inaccurate (Patwari, 1999). Besides, the secretion of drugs in breast milk may cause unwanted pharmacological effects on the nursing infant.

It is common practice in modern therapy to generalize results and effects of drugs after clinical trials hoping that the drugs will have the same effect on most other people. This is not however the case, there is the need for individualization therapy through definitive diagnosis and accurate sensitivity testing for individual patients before administration of drugs (David, 1989).

Age is also another factor that affects therapeutic outcome; most drugs are developed and tested in the young and middle-aged adults. In real practice, moreover, individuals vary both in the way they handle drugs (pharmacokinetics) and their response to drugs (pharmacodynamics). Besides, in infants, antibiotics and other syrups containing sugars are not good for long term treatment for

they may lead to dental decay. There is also the problem of multiple-drug resistance as is experienced with some pathogens which calls for more and varied drugs use at a time. This increases toxicity risks. An example of this is the case of penicillin-resistant strains and *Pseudomonas aeruginosa* which is resistant to many antibiotics. Furthermore, all drugs no matter how trivial their therapeutic actions are have potential side effects. The anticipated benefit from any therapeutic decision must therefore be balanced by potential risks (David, 1989).

Lastly, the widespread use of antibiotics has led to the emergence of antibiotic-resistant pathogens. This observation has provided added impetus to the need and search for new drugs (WHO, 2009).

These limitations to the use of synthetic drugs make the search for new antimicrobial agents from plants sources inevitable, and provide a justification for researches such as this. The search for antimicrobial agents is an important aspect of human development and will remain relevant for as long as there are diseases and the need for new and better drugs for treatment. The potentials for finding remedies for various illnesses from plant sources or natural products are only limited by our imagination and capacity to screen. Sofowora (1993) suggested that only less than 10% of the world's flora have been screened for their medicinal value.

The search for antimicrobial agents began a long time ago. As far back as 1902, Paul Ehrlich had shown that methylene blue, a dye stuff of plant origin exhibited some activity against human malaria. Ehrlich pioneered methods that have since become the mainstay of the search for new drugs. One aspect of his approach is the use of the term 'screening' which is the application of a relatively simple test to a large number of compounds in order to obtain evidence of their biological activity (Becker *et al*., 2005).

Kano, like most other Nigerian states posses a vast wealth of traditional medicine that have potential of providing the very much needed succor to the large number of people from one ailment or the other. The full realization of this goal is hindered by number of problems associated with traditional medicine practice. Some of the problems include lack of scientific proof of the efficacy of herbal medicine which makes scientific research such as this imperative. There is also the problem of imprecise diagnosis, in that they tend to treat the symptoms rather than the disease. There is also a problem of the poor hygienic conditions under which traditional medicine practitioners operate. Another problem is the lack of precise dosage in the administration of the remedies. Also the occult practices and incantations associated with traditional medicine is a source of worry and suspicion to many (Sofowora, 1993).

The secrecy with which traditional medicine practitioners operate is also another point of criticism against the process. This hampers efforts towards verification of claims of the efficacy of herbal remedies. The task here for ethanobotanists and researchers is to encourage them to make known their recipe for verification, letting them realize that the information they are providing is strictly for research purpose only (Sofowora, 1993).

Despite the problem outlined above, traditional medicine practice has promising potentials in developing countries for the present future. For instance traditional medicine is a cheaper and a more readily accessible source of medication than orthodox medicine to most people in the developing countries. Besides, it has wider acceptability among people in such countries since it readily blends with the socio-cultural life of the people (Sofowora, 1993).

In developing countries such as Nigeria, traditional medical practitioners serve as additional source of manpower for the health care sector thereby increasing healthcare coverage. Perhaps the greatest importance of traditional medicine is that it is a potential source of new drugs as well

as a source of cheap starting products for synthesis of known drugs. It is even anticipated that the much needed breakthrough in cancer therapy may come from natural plant sources (Sofowora, 1993).

Another argument in favour of traditional medicine is the development of resistance which is experienced with orthodox drugs is not common with traditional medicine probably due to complex nature of the plant extracts (Sofowora, 1993).

There is an obvious relationship between traditional medicine practice and modern medicine. A number of useful drugs in use today were discovered after scientific examination of some plant extracts used as traditional remedies eg. quinine was isolated in 1820 from stem bark of Cinhona and remained the only treatment for malaria well into the 20^{th} century. Besides, the startling discovery of Penicillin in 1928 by Alexender Flemming was from a fungus extract and paved the way for the discovery of other synthetic antibiotics which revolutionalised modern therapy (Sofowora, 1993).

To say the least, there are great prospects for traditional medicine practice to play a complementary role and even compete with orthodox medicine, if scientific verification and standardization are improved. The best case scenario is the standardization and harmonization of traditional medicine practices with modern medicine as is currently being advocated (Sofowora, 1993).

Herbal medicine has been used since ancient times in many cultures either as dietary supplements or as therapeutic agents. Plants are known to synthesize a variety of chemical substances. These include phenolic compounds, terpenes, steroids, alkaloids, glycosides, fats and oils among others which are referred to as secondary metabolites. Thus plants are now sources of important drugs. For example quinine, from cinchona bark, reserpine from *Rauwolfia* sp roots,

digitoxin from digitalis leaf, morphine from opium capsule (Abdul, 1986). And recently artemisinin from "quinghaasu" a Chinese herbal medicinal plant (Okogun, 2002). All these show that medicinal plants still remain the primary source of important drugs used in orthodox medicine.

The use of natural products, especially plants for treatments of different aliments is as old as mankind. One of the earliest records of the use of plants for medicine was recorded in Pharmacopoeia of Emperor Shen Nung of China between 2730-3000 BC and also in the ancient Egyptian Tomb as far back as 1500 BC (Makhubu, 1998).

1.4 Anatomy of the Gastrointestinal Track

The Gastrointestinal Track (gut) has seven different regions consisting of the buccal cavity, oesopagus, duodenum, small intestine, large intestine; mainly concerned with the absorption of water from digested material regulated by hypothalamus and reabsorption of sodium as well as any nutrients that may have escaped primary digestion in the ileum and rectum (appendix vi). Although each region of the gut has its own special characteristics, all have a basic common structure. It consists of four distinct layers; the lumen, mucosa (glandular epithelium and muscularis mucosa) submucosa, muscularis externa and serosa (Roberts, 1986).

1.4.1 Lumen

This is a cavity where digested food passes through and from where nutrients are absorbed.

1.4.2 Mucosa

Mucosa is the innermost layer of the gut and has three layers, the epithelium, lamina propria and muscularis mucosa. It is the major absorbing and secreting layer. Along the whole length of the gut in the glandular epithelium are goblet cells. These secrete mucus which lubricate the passage of the food along and protect it from digestive enzymes. Villi are vaginations of the mucosa and

increase the overall surface area while also containing a lacteal, which is connected to the lymph system and aids in the removal of lipids and tissue fluid from the blood supply. Microvilli are present on the epithelium of a villus and further increase the surface area over which absorption can take place. The muscularis mucosa is a layer of smooth muscle that aids in the action of continued peristalsis and catastalsis along the gut.

1.4.3 Submucosa

This layer contains nerves endings (e.g. Meissner's plexus), blood and lymph vessels, and elastic fibre with collagen that stretches with increased capacity but maintains the shape of intestine.

1.4.4 Muscularis Externa

This layer composed of an inner circular and an outer longitudinal layer of smooth muscle. Coordinate movement of the two layers provide the wave-like peristaltic movement of the gut wall which force food along. Their movements also mix the food. Between the two layers is Auerbach's plexus (a mass of nerve tissue).

1.4.5 Serosa

It is the outermost coat of the gut wall. It is made up of loose fibrous connective tissue and coated in mucus so as to prevent friction damage from the intestine rubbing against other tissue. Holding all this in place are the mesenteries which suspend the intestine in the abdominal cavity and stop being disturbed when a person is physically active.

1.4.6 Normal flora

The gut hosts several kinds of bacteria that deal with molecules the human body is not able to break down itself. This is example of symbiosis. These bacteria also account for the production of gases inside our intestine released as flatulence through the anus.

1.5 Common GIT infections

Common gastrointestinal diseases and disorders include some of the followings; gastroenteritis, iIleus, ileitis, diarrhoea, constipation, colorectal cancer, hirschsprung's disease (aganglionosis), appendicitis, celiac disease, crohn's disease and ulcerative colitis, enteroviruses disease, irritable bowel syndrome (IBS), diverticular disease, endometriosis, bowel twist (bowel strangulation), angiodysplasia of the colon, chronic functional abdominal pain, colorectal cancer, intussusceptions, polyp (medicine), pseudomembranous colitis, ulcerative colitis, toxic megacolon etc (King *et al*., 2003).

1.6 Traditional Antidiarrhoeal Plants

The plants used in this study are *Mormordica charantia* Linn (Cucurbitaceae) and *Aeschynomene uniflora* E May (Leguminosae: Papilinodeae).

1.6.1 *Momordica charantia* L. (Cucurbitaceae)

Commonly refers to as "balsam apple", southern balsam pear or simply balsam pear, popularly known as "garahuni" in Hausa language is a twinner of cucumber family often growing over fences and huts, with yellow flowers and orange yellow tubercle fruits (Plate 1.1). When ripe, the fruits burst apart, revealing numerous seeds covered with a brilliant scarlet, extremely sticky coating. It is used medicinally in wounds and infants diarrhoea treatments and as well as in soup preparation (Hassan and Umar, 2007). It is also used in soap making, forming a viscid solution in water (Sofowora, 1993).

1.6.2 *Aeschynomene uniflora* E. May (Leguminosae: Papilinodeae)

It is an annual herb of 1 m high or more, grown on sandbanks on the edge of water of savanna countries in Senegal to Northern Nigeria, and widespread in tropical Africa and Madagascar. In Senegal the plant provides some forage. It may become a weed of rice-fields in Tangan. This is a

common weed popularly known in native Hausa language as “bagaruwar kasa” with yellow flowers and pinnate leaves (Sofowora, 1993). It also occurs in India, southern China and southward through Malaya to Australia. It is a low, diffuse shrub, 15 to 30 centimeters or more in height. The leaves are 7 to 10 centimeters long, with 40 to 60 pairs of narrow leaflets, and with a solitary, sessile gland on the rachis below the leaflets. The flowers grow one or two together in the axils and are shining, small, and yellow. The pods are strap-shaped, flat, and about 5 centimeters long, and contain rhomboid, dark-brown seeds (Plate 1.2). The leaves are used as cure for diarrhoea. The young stems and leaves are dried and used as a substitute for tea in Japan. The roots are used in tropical Africa to cure colic and for spasms of the stomach. It was also reported that a milk decoction is used in dysentery, and that the entire plant is used as a remedy for eruptions in the face (Sofowora, 1993).

Plate 1.1 *Momordica charantia*: a twinner plant

Plate 1.2 An Annual shrub, *Aeschynomene uniflora* plant

1.7 Research Problem

An analysis of the findings of 27 active surveillance studies conducted suggested that around 750 million children below 5 years of age in Asia, Africa and Latin America suffer from acute diarrhoea each year. It is also estimated that between three to six million in this age group die annually from acute diarrhoea. About 80% of these deaths occur in the first two years (Banerjee, 1988). Diarrheoal diseases account for the death of about 2.2 million children under 5 years annually (WHO, 2009) ie 250 deaths per hour. This figure may have increased because of the increasing level of poverty in many developing countries. Diarrhoea, related illnesses and complications are associated with dehydration, malnutrition, growth faltering or stuntedness and compromised immunity. During diarrhoea there is loss of water and electrolyte. Due to the anticipation of such problem it becomes a common practice for pregnant women keeping antidiarrhoeal traditional remedies such as plants materials for treatment. These remedies some in form of conqouations are normally taken by the nursing breast feeding mothers or given to the infants to arrest any diarrhoea and related illness. Diarrhoea is a catabolic disease during which there is increased breakdown of energy giving nutrients to maintain the body. This leads to increased nutrient demand which the body may not be able to meet readily coupled with decreased food intake due to lack of appetite.

In hospital based studies of acute diarrhoea, the rate of enteropathogenic *E. coli* (EPEC) ranged between 10% to 50% with an average of about 20% in children under five years of age. EPEC is also an important cause of sporadic infant diarrhoea in Brazil and south African infants accounting for 30% of cases (Hasony, 1996). Pathogenic *E. coli* was recovered from 4.8% of patients with gastroenteritis in Nigeria (Agbonlahor and Odugbemi, 1982). Frequencies of EPEC isolation rates ranging from 7.9% to 30% were reported throughout the world in infants.

Enteroinversive *E. coli* strains were detected in 9% of acute diarrhoea in infants in Columbia and Nigeria. An isolation rate of EIEC less than 2% was also reported.

Feeding practice plays a role in spreading the organism and breast feeding is an important factor in the prevention of infantile enteritis. Seasonal peak of *E. coli* diarrhoea is often observed during warmer season. Diarrhoeagenic *E. coli* have been reported to develop resistant to antibiotics. In South East Asia 72% of diarrhoeagenic *E. coli* isolates were found to be resistant to at least four drugs (Hasony and Mohammad, 1994).

In Nigeria 90,900 infants die every year from diarrheoa ie. 249 deaths per day and that is accounting for 11% of childhood mortality (PCN, 2013).

1.8 Research Justification

In situation of the dramatic infantile diarrhoea, there is urgent need to find new and cheap drugs and methods of treatment such as herbs and other much available plants materials as substitutes for the expensive and hard to get orthodox antibiotics. Thus, this research work would contribute significantly towards reducing these numbers of death due to infantile diarrhoea in Kano State in particular, and Nigeria, in general.

Despite the multifactorial nature of the aetiology of diarrhoeal diseases and link with malnutrition and heavy load of helminthes, they have some important common features (Okolocha and Umoh, 2002). Diarrhoea attack mostly infants and young children, and thus the expression “weaning diarrhoea” is often used. In fact the infant is protected from this in the early months of life by being fed with mother`s milk. As soon as a child starts to take a variety of different foods carrying various bacteria, the digestion is often upset by these bacteria and their toxins resulting in diarrhoea. As normal inhabitant of intestine, *Escherichia coli* often produces toxic substances that do not disturb adults but cause diarrhoea in infants when present in great

numbers (Martines *et al*., 1993). However, only in a small fraction of children with diarrhoea can recognized pathogenic organism be isolated.

The list of priority research questions focus on how to make the best use of interventions that are available today, in order to make the most difference, and ultimately save as many children's lives as possible. Nearly 2 million children die from diarrhoea every year. If childhood diarrhoea is not addressed urgently, the countries will fail to achieve the fourth Millennium Development Goal (MDG4) target of reducing child deaths by two-thirds by 2015. Despite the persistently high burden of disease, research into childhood diarrhoea has been steadily decreasing since the 1980s. Funds available for research into diarrhoea are less than those available for research in other diseases that cause fewer deaths. A lot is already known about effective treatments for diarrhoea, but critical knowledge is lacking on how to make sure the children who need it most get access to that treatment. World health organizations (WHO) has led a process to identify which types of research are most needed and would have greatest impact on mortality. The resulting top 20% of priority research questions are mainly targeted at better understanding the barriers to implementation, effectiveness and optimization of the use of available interventions and programmes such as oral rehydration salts (ORS) and zinc supplement, exclusive breastfeeding and the integrated management of childhood illness. However, very few donor agencies presently recognize the importance of these domains of health research. The life-saving treatment for diarrhoea is simple: ORS and zinc tablets. ORS is essentially a pinch of salt and a handful of sugar mixed with clean water. The cost of treating a child with ORS and zinc is about 30 US cents (PCN, 2013).

Children in poor countries get diarrhoea on average four times per year. Each of these episodes can be life-threatening. Administration of ORS and zinc reduce the risk of death down to almost zero. More than 50 million childrens' lives have been saved by ORS since its creation 25 years ago. The great challenge now is how to reach all children who are still suffering and dying from diarrhoea (Patwari, 1999).

Funding available for health research globally was US$ 126 billion in 2003, but that money has not always been targeted at diseases which affect the greatest number. Research on diarrhoea received less than US$ 10 per disability adjusted life years (DALYs) while conditions like diabetes type 2 received US$ 102 per DALYs. Similar exercises have been conducted by WHO in 2009 to identify priorities for research on other "neglected" childhood diseases, including acute respiratory infections, birth asphyxia, neonatal sepsis, and low birth weight.

The contribution of diarrhoea to the high morbidity and mortality in children has devastating emotional and psychological consequences. These suggest that diarrhoea is of great public significance and therefore increasing attention should be given to its study in children aged less than 5 years.

1.9 Aim and Objectives of the Study

This research is aimed to characterize and determine the susceptibility of *Escherichia coli* isolated from infants' diarrhoea to *Momordica charantia* L. (Curcurbitaceae), *Aeschynomene uniflora* E. May extracts.

This aim would be achieved through the following specific objectives;

1. **To isolate, identify and characterize *Escherichia coli* strains isolated from infantile diarrhoea samples obtained from children under 5 years of age attending some hospitals in Kano State, Nigeria.**
2. **To determine the *E. coli* isolates serotypes/serovars associated with infantile diarrhoea in Kano state, Nigeria.**
3. **To extract and fractionate the plant materials *Momordica charantia* L. (Curcurbitaceae), *Aeschynomene uniflora* E, May.**
4. **To screen the plant extracts and Thin Layer Chromatography (TLC) fractions for the presence of phytochemicals.**
5. **To test the pharmacological activity of the most active extracts *in-vivo* using laboratory mice.**
6. **To screen the *E. coli* isolates for susceptibility to antibiotics, extracts and the TLC fractions of *Momordica charantia* L. (Curcurbitaceae) and *Aeschynomene uniflora* E. May.**

1.10 Research Hypothesis

Null hypothesis, H_o = *Escherichia coli* is not associated with infantile diarrhoea, unsusceptible to antibiotics and plants extracts.

Alternate hypothesis, H_A = *Escherichia coli* is associated with infantile diarrhoea, susceptible to antibiotics and plants extracts.

CHAPTER TWO

4.0 LITERATURE REVIEW

Paul (2003) reported that *Escherichia coli* have had a central place in water microbiology for decades as an indicator of faecal pollution. It is only relatively recently that the role of *E. coli* as pathogen, rather than indicator, in drinking water has begun to be stressed. Interest in the role of *E. coli* as a cause of diarrhoeal disease has increased because of the emergence of *E. coli* O157:H7 and other enterohaemorrhagic *E. coli*, due to the severity of the related disease. There are enterotoxigenic, enteropathogenic, enterohaemorrhagic, enteroinvasive, enteroaggregative and diffusely adherent strains of *E. coli*. Each type of *E. coli* causes diarrhoeal disease through different mechanisms and each causes a different clinical presentation. Several of the types cause diarrhoea by the elaboration of one or more toxins, others by some other form of direct damage to epithelial cells.

Antibiotic resistance is a major global public health concern, particularly in settings where few treatment options are available. Limited research has been done on antibiotic resistance in *Escherichia coli* of Indian children at community level. Antibiotic resistance patterns in *E. coli* isolates from stool samples of children aged 3-14 years from Ujjain, Central India, to investigate associations of resistance with demographic variables was studied. Children, 3-14 years of age, were included from 30 randomly selected villages of Palwa demographic surveillance site, Ujjain, India. Parents were interviewed using a questionnaire and stool samples were collected from participating children. *E. coli* were isolated from stool samples (n= 529) and susceptibility testing to 18 different antibiotics was done using standard methods. The proportions of isolates resistant to various antibiotics were nalidixic acid, (45%), tetracycline (37%), ampicillin (37%), sulfamethoxazole/trimethoprim (29%) and amoxicillin/clavulanic acid (29%). No isolates were

resistant to imipenem. Overall, 72% of isolates were resistant to at least one antibiotic and 33% were multi-drug resistant. High rates of cross-resistance were seen for 15 (83%) of the antibiotics studied. *E. coli* isolates from children with illiterate mothers were more resistant to penicillins and fluoroquinolones. Extended Spectrum Beta Lactameses (ESBL) producers comprised 9% of the isolates. Antibiotic resistance and cross-resistance were common in *E. coli* from stools of children. Resistance rates were associated with maternal literacy (Shakya *et al*., 2013).

From a clinical perspective, it is important to know which serogroups, virulence genes and antibiotic resistance patterns are present in shiga toxin-producing *Escherichia coli* strains in paediatric patients suffering from diarrhoeic and non-diarrhoeic infections. This is the first study in Iran that has comprehensively investigated the shiga toxin-producing *Escherichia coli* -related infection characteristics in diarrhoeic and non-diarrhoeic pediatric patients of 0–60 months of age. Two hundred and twenty four diarrhoeic and 84 non-diarrhoeic stool specimens were collected from the Baqiyatallah hospital of Tehran, Iran. The stool samples were cultured immediately and those that were *E. coli*-positive were analyzed for the presence of antibiotic resistance genes and bacterial virulence factors using PCR. Antibacterial susceptibility testing was performed using disk diffusion method. One hundred and fifty four out of 224 (68.75%) diarrhoeic stools and 31 out of 84 (36.90%) non-diarrhoeic stools harbored *E. coli*. In addition, children in 13–24 month-old age group had the highest prevalence of infection with this bacterium (77.63%). A significant difference was found between the frequency of attaching and effacing *Escherichia coli* and Enterohaemorrhagic *Escherichia coli* (P =0.045). The genes encoding Shiga toxins and intimin were the most commonly detected virulence factors. Among all sero group studied, O26 (27.04%) and O111 (18.85%) had the highest prevalence in the diarrhoeic and non-diarrhoeic patients. The prevalence of genes encoding resistance against

sulfonamide (*sul*1), gentamicin (*aac*3IV), trimethoprim (*aad*A1), cephalothin (*bla*SHV) and tetracycline (*tet*A) were 82.78%, 68.03%, 60.65%, 56.55% and 51.63%, respectively. High resistance levels against penicillin (100%), tetracycline (86.88%), gentamicin (62.29%) and streptomycin (54.91%) were observed. Marked seasonality in the serogroup distributions was evident, while STEC infections were more common in summer (P=0.041). Findings raise awareness about antibiotic resistance in diarrhoeic pediatric patients in Iran (Momtaz *et al*., 2013).

2.1 Diarrhoea and Malnutrition

Khin *et al*. (1992) identify socioeconomic and behavioral risk factors for development of persistent diarrhoea and malnutrition in children in a case-control study carried out in Burma. Cases were 67 children 1–59 months old hospitalized for diarrhoea lasting >14 days and complicated by severe malnutrition; for each case, a healthy control child was selected who was age- and sex-matched from the same neighborhood. Homes of cases and controls were visited for interviews and for direct observation of household child-care practices. Risk factors were catalogued and calculations made for relative risk and etiologic fractions. Risk factors that were associated with persistent diarrhoea and malnutrition included low family income, low education of mothers, unhygienic latrines, flies in the house and on the child, dirty appearance of child and mother, mother not using soap and water when washing child's hands, defecation of child on floor, breastfeeding on demand, child eating food from floor, not feeding recommended weaning foods, and lack of knowledge by mother about causes of diarrhoea and about foods that prevent malnutrition. These results indicated that persistent diarrhoea and malnutrition in Burma is caused by a complex of several interrelated socioeconomic factors, unsanitary behaviour pertaining to personal

hygiene, the practice of demand breastfeeding and lack of certain weaning foods, and low education of mothers who showed less knowledge about causes of diarrhoea and prevention of malnutrition.

Epidemiological studies have demonstrated a marked negative relationship between diarrhoea and physical growth and development of a child. Each day of illness due to diarrhoea, produces a weight deficit of 20-40 g. Poor nutrition is associated with more serious prolonged diarrhoea. 'Catch-up growth' often does not occur in malnourished children. Malnutrition, particularly wasting, is a strong predictor of diarrhoeal duration and the prolonged illness could exacerbate nutritional faltering, thereby increasing the subsequent risk of death. Poor appetite, vomiting, deliberate withholding of food resulting in poor intake; malabsorption of macro and micronutrients; hastening of intestinal transit time; disturbance of metabolic and endocrine functions; and direct loss of protein and other nutrients in gastrointestinal tract are some of the known mechanisms which have an impact on the nutrition during an episode of diarrhoea. In addition diarrhoea of infectious origin causes cytokine induced malnutrition which results from the actions of proinflammatory cytokines like tumour necrosis factor and interleukin 1, 6 and 8. Preexisting malnutrition is associated with decreased turnover of epithelial cells resulting in delayed recovery which may prolong an episode of infectious diarrhoea by itself as well as by promoting tissue invasion by other enteropathogens. Malnutrition may also alter protective host factors and thereby favour intestinal colonization by the pathogenic microbes. Mucosal damage varying from moderately severe changes to flat lesions indistinguishable from those of celiac disease may occur in kwashiorkor. Diarrhoea malnutrition interaction represents a dangerous web which can be distangled by prevention of disease transmission by promoting exclusive breast feeding, hygienic weaning practices, safe drinking water and hand washing, improved host

defense by breast feeding, improved nutrition, measles vaccine and other vaccines against enteropathogens in the offing; and promotion of standard case management with special emphasis on nutritional support and rehabilitation (Patwari, 1999).

Using Demographic and Health Survey data from Ghana and Nigeria, a study examined whether the protective effects of breast (hyphen) feeding are greatest where the poorest sanitation conditions prevail. It was found that mixed hyphen fed infants aged between 0 and 11 months tend to have a higher risk of diarrhoea than fully breast fed children, while the risk of diarrhoea among weaned infants is twice that of mixed fed infants. The probit regression models employed in the analysis were used to predict the probability of diarrhoea associated with each breast feeding pattern for both 'poor' and 'good' sanitation areas. It was found that the risk of diarrhoea among mixed fed infants in the poor sanitation areas tends to be high while the same risk among fully breast fed infants tends to be minimal. In essence, the health risks of mixed feeding are real, particularly for infants aged less than 7 months, and are even worse for those weaned before 6 months of age (Ahiadeke *et al*., 2006).

2.2 *Escherichia coli* Serotypes

Rosa *et al*. (1998) studied faeces from urban children less than 2 years old with acute diarrhoeal illness and from non-diarrhoeal infants (controls). The diarrhoeal samples collected were examined for *Escherichia coli* and other enteropathogens. A total of 990 *E. coli* isolates from 100 patients and 50 controls were tested for enteropathogenic *E. coli* (EPEC) serotype (O:H), adherence to HEp-2 cells after incubation for 3 and 6 hours, fluorescent acting staining (FAS), DNA hybridisation with EAF, *eaeA*, STh, STp and EAggEC probes and production of heat-labile enterotoxin (LT) and verocytotoxin (VT) with Y1 and Vero cells. EPEC were the most prevalent enteropathogens in patients (32.7% and 14% in controls). Enteroinvasive *E. coli* (EIEC) and

Vero cytotoxin - producing *E. coli* (VTEC) were not detected. The rate of isolation of enterotoxigenic *E. coli* (ETEC) was identical in both groups. Among the EPEC isolates the prevalent serotypes were O111:H2, O55:NM and O119:H6. Localised adherence (LA) was found significantly more frequently in isolates, from patients (19.6%) than controls (2.1%). All LA-positive EPEC isolates were FAS+ and *eaeA*+, but only 75.2% of them hybridised with the EAF probe. Diffusely adhering *E. coli* (DAEC) and enteroaggregative *E. coli* (EAggEC) were found with equal frequency in patients and controls. Twenty-seven *E. coli* isolates were negative for EAF but positive for *eaeA* and FAS and produced LA in 6-h adherence tests. These EAF-/*eaeA*+ strains were the only putative enteropathogen identified in seven patients and were not found in controls. The ability of these strains to elicit ultrastructural cell alterations and cell-signalling events was evaluated in Caco-2 cells (human colon carcinoma cell line) by the gentamicin invasion assay and by transmission electron microscopy. The numbers of intracellular bacteria in cell invasion tests varied from 0.4% to 1.6% of the cell-associated bacteria after a 6-h incubation period. Tyrosine phosphorylation of host cell proteins was assessed in HEp-2 cells by immunofluorescence microscopy and all strains gave positive results. EAF-/*eaeA*+ *E. coli* strains express most of the virulence properties found among true EPEC strains and can be a relevant cause of infant diarrhoea in developing countries.

Enteropathogenic *E. coli* serotypes were searched in faecal specimen of 550 children with endemic diarrhoea and in 129 controls, in Sao Paulo, Brazil, in 1978 and 1979; serotypes O111ab:H-, O111ab:H2 and O1119:H6 were significantly associated with diarrhoea in children 0 to 5 months old and were the most frequent agents of diarrhoea in the age groups as compared with enterotoxigenic and enteroinvarsive *E. coli, Salmonella* sp., *Shigella* sp., and *Yersinia*

enterocolitica. It is concluded that various enteropathogenic *E. coli* serotypes may be of endemic infantile diarrhoea (Toledo *et al*., 1983).

Enteropathogenic *Escherichia coli* (0111:K58:H8) was isolated from the small bowel of two infants with chronic diarrhoea. Small bowel biopsy revealed focal adherence of the bacteria to the epithelium, accompanied by inflammation of the tissue. The isolates also adhered to tissue culture cells in densely packed aggregates. The pattern of adherence to tissue culture cells were found only among strains of serogroups 0111 and 0119 when 196 enteric *E. coli* isolates were tested. The pathogenic mechanism of enteropathogenic *E. coli* thus appears related to dense colonization of intestinal tissue. Adherent strains were detected by tissue culture assay (Clausen and Christie, 1982).

Three enteropathogenic *Escherichia coli* (EPEC) strains (0127:K63:H6, 0128:K67:H2, and 0142:K86:H6) isolated from outbreaks of infantile diarrhoea and one strain from the "normal" colonic flora (*E. coli* HS) of a healthy adult were fed in doses of 10^6, 10^8, and 10^{10} organisms in $NaHCO_3$ to adult volunteers. The strains, which had been stored for 7-9 years, gave negative results in sensitive tests for heat-labile (LT) enterotoxin (Y-1 adrenalcell test), heat-stable (ST) enterotoxin (infant mouse assay), invasiveness (guineapig eye test), and gross fluid accumulation (infant rabbit assay). Two strains (0142 and 0127) caused diarrhœa. LT or ST enterotoxins were not found in *E. coli* stool isolates from individuals with diarrhœa and no one had a rise in LT antitoxin titre; the findings suggest that LT and ST enterotoxins were not involved in pathogenesis of the diarrhœa. Non-invasive EPEC strains probably induce diarrhoea by a mechanism (presumably an enterotoxin) distinct from LT or ST enterotoxins (Clausen and Christie, 2004).

De Toni *et al*. (2009) conducted a Polymerase Chain Reaction (PCR) screening assay for *stx* genes used to examine a loopful of confluent colonies of 306 stool samples cultures. In *stx* (1.9%) of them DNA fragments of the expected size were observed, and the presence of six was confirmed by DNA sequencing. Then up to 100 single colonies from each of the six stool cultures were analyzed using the same PCR protocol. However, *stx*-positive colonies were found only in two of the cultures. The *E. coli* strains belonged to serotypes O69:H11 and O178:H19, and presented genotypes *stx*, *eae*, *ehx*A and *stx*1 respectively. Shiga toxin production was confirmed using the VTEC screen Seiken^{R}. Except ampicillin, they were susceptible to all the antimicrobials tested. These results showed that STEC may be an important cause of diarrhoea in children of Parana State, and that they are present in low numbers in stools. The strains belonged to serotypes not commonly found associated with STEC and probably present low virulence. The results also indicate that molecular methods are required in the diagnosis of STEC infections.

Iruka *et al*. (2003) analyzed stool specimens from 113 adult outpatients with diarrhoea in southwestern Nigeria and 63 controls were examined for bacterial and parasitic enteric pathogens. Enterohaemorrhagic *Escherichia coli* (EHEC) ($P < 0.02$), enteroaggregative *E. coli* (EAEC) ($P < 0.02$), and *Entamoeba histolytica* ($P < 0.0002$) were significantly associated with diarrhoea. *Salmonella, Shigella,* nontoxigenic *Vibrio cholerae*, other categories of diarrhoeagenic *E. coli*, as well as a variety of helminths were recovered more frequently from the stools of patients than from the stools of controls but did not show a significant association with disease. Multiple pathogens were recovered from 36.3% of specimens, and bloody diarrhoea was commonly associated with *E. histolytica* and diarrhoeagenic *E. coli* infections. The majority of EHEC isolates were non-O157 strains that carried the *stx*2 gene

of the 23 EHEC-infected patients, 12 (52.2%) presented during the 10th week of the study. EHEC strains isolated within this cluster were more likely to hybridize with the enterohemolysin gene probe to be nonmotile and sorbitol positive, and to fail to agglutinate O157 antisera. Pulsed-field gel electrophoresis demonstrated that the only strains with *xba*1 profile that occurred more than once were isolated during the 10th and 11th weeks of the study, suggesting an outbreak. The study has demonstrated that *E. histolytica*, EHEC, and EAEC are important diarrhoeal pathogens within the study area and that sporadic and epidemic EHEC infections occur in developing as well as developed countries. Routine surveillance for diarrhoeagenic *E. coli*, even only at the tertiary-care level, would be useful in identifying outbreaks and assist in identifying environmental reservoirs and transmission routes.

Momtaz *et al*. (2012) investigated the distribution of antibiotic-resistant genes in *Escherichia coli* isolates from slaughtered commercial chickens in Iran by PCR. The genes included *aad*A1, *tet*A, *tet*B, *dfr*A1, *qnr*A, *aac*3IV, *sul*1, *bla*SHV, *bla*CMY, *ere*A, *cat*A1 and *cml*A. According to biochemical experiments, 57 isolates from 360 chicken meat samples were recognized as *E. coli*. The distribution of antibiotic-resistance genes in the *E. coli* isolates included *tet*A and *tet*B (52.63%), *dfr*A1, *qnr*A, *cat*A1 and *cml*A (36.84%) and *sul*1 and *ere*A (47.36%), respectively. Nine strains (15.78%) were resistant to a single antimicrobial agent and 11 strains (19.29%) showed resistance to two antimicrobial agents. Multi-resistance which was defined as resistance to three or more tested agents was found in 64.91% of *E. coli* strains. The results indicate that all isolates harbour one or more of antibiotic resistance genes and that the PCR technique is a fast, practical and appropriate method for determining the presence of antibiotic-resistance genes.

2.3 Plant Extraction

Ahiadeke *et al*. (2006) studied the effectiveness of methods for extraction of traditional herbal medicine by comparing the antimicrobial and anti-inflammatory activities of the leaf extracts of *Piliostigma reticulatum* and *Piliostigma thonnigii*. The agar dilution method was employed to compare the activity of crude leaf extracts of the two species against sixty bacterial and fungal isolates selected from diverse genera. The antimicrobial spectra of the extracts from both species were very similar. In 44 (73.3%) of the test strains, MIC values for the two extracts were identical and in 6 (10.0%) the MIC values differed by only one dilution factor. Chlorocresol was used as standard. The crude extract and solvent fractions obtained from the leaf of *P. reticulatum* were evaluated for anti-inflammatory activity using the carrageenan-induced rat paw oedema model. The aqueous fraction at 100 mg/kg produced the highest inhibition of oedema (61.2%), while the crude extract at 100 mg/kg exerted a low anti-inflammatory effect (13.2%). Indomethacin at 5 mgkg-1 inhibited oedema by 86.2%. The study has established that *Piliostigma reticulatum* and *Piliostigma thonnigii* have similar antimicrobial spectra and anti-inflammatory activity. The traditional method is not effective in extracting the antimicrobial agents from the plants.

Abalaka *et al*. (2010) study crude extract of whole plant of *Momordica charantia* through fractionation processes using thin layer chromatography (TLC). The resultant fractions were tested for antibacterial activity against four pathogens. Chromatographic analysis yielded yellow fraction 690mg, dark green fraction 570mg and 740 mg of the blue black fraction. At the low concentration of about 40 µg/ml fraction 1 (yellow fraction) was active against all test organisms except *Salmonella typhi*, but at the same concentration fraction 2 (dark green fraction) was active against two of the four test organisms, *Staphylococcus aureus* and *Streptococcus pyogenes*, while the third faction (blue black fraction) was active

against only one organism *Streptococcus pyogenes.* Minimum Inhibitory Concentration (MIC) showed MIC between 40µg/ml and 60µg/ml for fractions 1 and 2 but 40µg/ml and 80µg/ml for fraction 3, while Minimum Bacteriocidal Concentration (MBC) studies revealed that MBC ranged from 40µg/ml-80µg/ml for fractions 1 and 2 but 60µg/ml-100µg/ml for fraction 3. The three fractions were active against the test organisms showing that a useful drug could be developed from this plant against some bacterial pathogens.

2.4 Lethality Testing

Isyaku *et al*. (2008) determined the antidiarrhoeal activity of the leaf extract of *Chrozophora senegalensis* (Euphorbiacea) used in the northern Nigeria Hausa ethnomedicine practices. The study was conducted on isolated rabbit jejunum and castor oil-induced diarrhoea in mice. The n-butanolic (NB) extract (0.4-3.2mg/ml) causes a dose defendant relaxation of isolated rabbit jejunum. The acute toxicity test for NB in mice was found to be 288.5mg/kg in castor oil induced diarrhoea. The antidiarrheoal activity was 50% compared to loperamide 5mg/kg (80% protection). The result revealed that the extracts have pharmacological activity against the diarrhoea causing organisms.

Anafi *et al*. (2010), study the *Combretum micrathum*, a small shrub that is widely distributed in African countries and readily available in Northern parts of Nigeria. The leaves of the plant have been reported to find the use in the treatment of diarrhoea. This study was carried out to investigate the antidiarrhoeal activity of the aqueous and methanolic leaf extracts of *C. micrathum* using castor oil induced diarrhoea in mice and isolated tissue preparation. The LD_{50} of the extract was found to be greater than 500 mg/kg. The aqueous methanolic extract was found to exhibit a concentration dependant inhibition of the spontaneous pendular movement of the rabbit jejenun and caused smooth muscle relaxation in guinea pig ileum, at high

concentration (0.8-8.0 mg/ml). In the castor oil induced diarrhoea, 60% protection was shown at highest doses of 1000 mg/kg and 2000mg/kg, compared to 80% protection showed by loperamide (5mg/kg). The results were analyzed using Chi-square and values considered significant at $p > 0.05$. The result obtained revealed that the aqueous methanol leaf extract of *C. micrathum* possesses pharmacological activity against diarrhoea.

Methanolic extract of the leaves of *Pullinia pinnata* Lin was evaluated for different behavioral effects such as diazepam-induced sleeping time, exploratory behavior and muscle relaxant activity in mice. The extract was found to cause significant ($P<0.001$) and dose dependent potentiation of the diazepam-induced sleeping time and a significant ($P<0.0005$) decrease in exploratory behavioral pattern by the head dip test. It also produces a significant motor coordination deficit (beem walking assay) in mice at 25 and 50mg/kg doses tested ($P<0.05$). The LD_{50} was found to be 288.2 mg/kg and >500mg/kg body weight intra prenially and orally in mice respectively. Preliminary phytochemical analysis of the methanoloic leaf extract of *Paulinia pinnata* revealed the presence of alkaloids, saponnins, tannins and flavonoids. The results suggested that the methanolic leaf extract of *P. pinnata* posses biologically active constituents that have sedative activity and lends pharmacological credence to the ethanomedical use of the plant in the management of some mental disorders (Aliyu *et al*., 2010).

The properties of 23 cell-detaching *Escherichia coli* strains were isolated from stool specimens in Nigeria in a work carried out by Iruka *et al*. (2002). Common properties of the strains included the presence of genes encoding-hemolysin (100%), pyelonephritis-associated pili (100%) and cytotoxic necrotizing factor 1 (70%) as well as lactose negativity (70%) and multiple antibiotic resistances (74%). Antibiotic resistance was shown in most cases to be transferable and associated with the presence of class 1 integrons. Phenotypic properties and pulsed-field gel

electrophoresis analysis demonstrated that the majority of the strains particularly multiple resistant, lactose negative O4:H40 strains were closely related. Multiple-resistant cell-detaching *E. coli* strains may represent an important reservoir for antibiotic resistance genes.

Christine and Valdemar (2004) assessed the importance of enterotoxigenic *Escherichia coli* (ETEC) as a diarrhoeal agent in developing countries. Odds ratios were calculated for incurring ETEC-associated diarrhoea based on data reported between 1970 and 1999. Carriage of ETEC was associated with diarrhoea in children aged less than five years, except for hospitalized infants aged 0-11 month(s) and children aged 1-4 year(s) at outpatient clinics. Two hundred and eighty million episodes of diarrhoea due to ETEC were seen yearly among those aged less than five years, and close to 50 million children of this age group were asymptomatic carriers of ETEC. Every 7th diarrhoeal episode in children aged less than one year and close to 25% of diarrhoeal cases in children aged 1-4 year(s) were due to ETEC. A child born in a developing country is likely to experience 0.5 diarrhoeal episodes per year caused by ETEC until the age of five years, after which the yearly prevalence drops to 0.1. ETEC remains an important diarrhoeal pathogen among children in the developing world.

MA6, an O157:H7-like strain did not react with most anti-O157 kits examined. However, it had the *rfbE* gene that is essential for O157 expression and carried O157:H7 virulence factors. Lipopolysaccharide analysis showed that MA6 is a rough strain that does not produce the O157 antigen, but genetically, it belongs in the O157:H7 clonal group (Peter *et al*., 1998).

Hasony (1996) found that diarrhoeagenic *E. coli* was detected in 148 (13.2%) of examined specimens. Among these 77 (52%) were enteropathogenic (EPEC), 96 (46.6%) were enterotoxigenic (ETEC) and 2 (1.4%) were enteroinversive *Escherichia coli* (EIEC). EPEC serotypes O119K69, O114K90 and O111K58 were the more frequent among twelve other

serotypes. Diarrhoeagenic *E. coli* showed high rates of resistance to antibiotics especially ampicillin (98%), tetracycline (93%) and chloramphenicol (86%).

Safety levels and chemotherapeutics of *Momordica charantia* were determined by evaluating the acute and subacute toxicity of the plant using thirty (30) wister rats. The rats were grouped into six (6) groups A, B, C, D, E and F with B-F administered with 100, 500, 800, 1200 and 1500 mg/kg of the body weight daily respectively for 2 weeks. The ones in group A serve as control. The animals in group F died after a couple of days indicating non tolerance of the extracts at that dose. The animals in group D showed 50% mortality at the end of the two weeks which indicates LD_{50} of the extract as 1200 mg/kg body weight. Microscopic examination of some visceral organs revealed changes in size, colouration as well as some phathological differences when compared with those of control. Acute treatment with 100, 500, 800 mg/kg per day did not produce any symptom of toxicity or mortality. The haemato-biochemical parameters show no significance differences ($P<0.05$). Administration of *Momordica charantia* extract up to 800mg/kg body weight is safe ($P>0.05$mg/kg) and tolerated by the body. *Momordica charantia* is therefore safe to use as ethanochemotherapeutic agent (Abalaka *et al*., 2009).

2.5 Susceptibility Test

Okeke *et al*. (2001) studied the activity of ethanolic and aqueous (cold and hot) extracts of *Landolphia owerrience* root parts (whole-root, root-bark and root-wood) against ten bacterial strains using agar-well diffusion and macro-broth dilution methods, respectively. The ethanolic extracts of the whole-root and root-wood were active against 100 and 80% of the test organisms, respectively. Ethanolic and aqueous extracts of the root-bark were moderately active while the aqueous (cold and hot) extracts of the root-wood exhibited little or no activity. Out of the nine

extracts prepared, 66.7% were active against *Staphylococcus aureous* ATCC 12600, 55.6% variously against each of *Pseudomonas aeruginosa* ATCC 10145 and local clinical isolates of *P. aeruginosa*, *S. aureus*, *Escherichia coli* and *Salmonella typhi*, 44.4% against Proteus sp., 33.3% against *Bacillus subtilis* ATCC 6051 and 22.2% against *E. coli* ATCC 11775. The agar-well-determined MIC values for the ethanolic whole-root extract (0.78-50 mg/ml) were higher (indicating lower activity) than the corresponding macro-broth-determined values (0.39-50 mg/ml) probably because of slow diffusion rates of the active constituents of the extract in agar. On the other hand, the differences could be due to the effects of DMSO used to dissolve the ethanolic extracts in the agar-well diffusion tests. Similar discrepancies in the MIC values detectable with the two test methods were apparent in the root-wood extract and the control drug, gentamicin, except that in the latter the agar-well-determined MIC values (0.125-8.0 microg/ml) were lower than the macro-broth-determined values (0.125-64 microg/ml). The strong activity of the ethanolic extracts against known etiologic agents of diseases traditionally treated with *L. owerrience* root of similar preparations provides scientific justification for the use of the herb in ethnomedical practice in Nigeria.

Vernonia amygdalina (bitter leaf), *Eucalyptus citriodora* (Eucalypt) and *Phyllanthus amarus* (Schum) were investigated for their antibacterial properties against pure cultures of clinical isolates of *E. coli*, *Klebsiella sp*., *Salmonella sp*. and *Shigella sp*. The isolates were obtained from the Department of Medical Microbiology and Parasitology of University of Ilorin Teaching Hospital. Water and ethanol were used in the crude extraction of the active constituents of the plants. Broth dilution and agar diffusion methods were used in determining the antimicrobial effects of the different plants extracts on the test organisms. The minimum inhibitory concentration (MIC) of the water extracts on the test organisms was 50mg/ml while the ethanolic

extract MIC was 6.25mg/ml - 50mg/ml. Similarly the diameters of zones of inhibition of the plant extracts at concentration of 100mg/ml ranged between 3.0-14.0mm and 3.0-18.0mm for the water and ethanolic extracts respectively on the test organisms. Water extracts of *Vernonia amygdalina (*Bitterleaf) and schum (*Phyllanthus amarus)* were not effective on majority of the test organisms. *Klebsiella sp.* was not inhibited by the water extracts at the test concentrations. The ethanolic extracts of *Eucalyptus citriodora (*Eucalypt) were most effective on all the test organisms. The least and the most susceptible organisms to the extracts were *Shigella sp.* and *E. coli* respectively. The results of this study suggest the possibility of using the ethanolic extracts of these plants in treating diseases caused by the test organisms (Sule and Agbabiaka, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

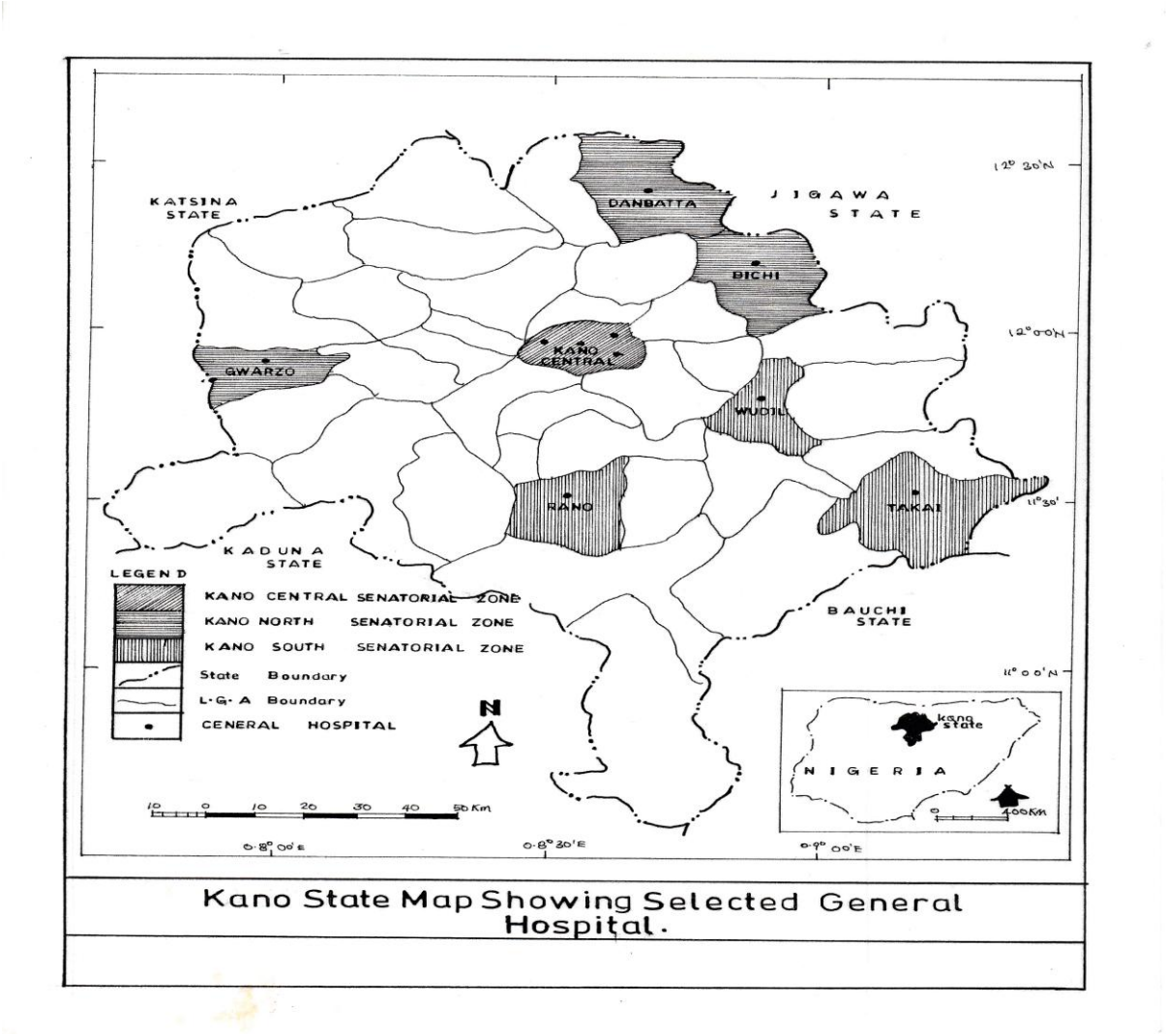
3.1 The Study Area

The study was carried out in Kano State, Nigeria (Fig. 3.1) located between latitudes and longitudes 12^{o}37'N, 9^{o}29'E and 9^{o}33'S, 7^{o}34'W respectively (Olofin *et al*., 2008). The state has a total of 20,680 square kilometers and population of 9,383,682, 2006 census (National Population Commission-NPC, 2006). The population is predominantly Hausa-Fulani and other tribes from different parts of Nigeria are also found in various parts of the state. The people of Kano are mostly livestock and crop farmers and traders, though other professions also exist. The study area has a large population density especially in the metropolis local government. The pattern of the settlement is nucleated in nature. It is common to find a market, motor park, schools etc. next to hospitals or residential areas. Sanitation is poor with inadequate waste management, open dumped and illegal roadside dumping from residential and commercial areas remains a problem. Most of the water drainages and town ditches have been turned into solid waste disposal places. However, government is putting some effort into sanitation and provision of healthcare facilities, yet diseases such as diarrheoa, cholera, malaria, measles and other illnesses occurring as epidemic in the study area.

3.2 Study Design

Three general hospitals each from the three senatorial zones of the state (Kano Central, Kano North and Kano South) and one additional hospital from Kano Central, making a

total of ten (10) hospitals were strategically selected at random for sampling stool of infants (0-5 months old) fortnightly for one year.



Source: **Ministry of Land Kano State Government (2008).**

Fig. 3.1 Map of Kano state showing selected general hospitals

3.3 Sample Size

The number of samples collected was calculated in accordance to Sarmukaddam and Garad (2006) formula thus:

$$n = \frac{Z^2 pq}{L^2}$$

Where;

n **is the number of samples to be collected.**

Z **is the standard normal deviation at 95% confidence limit = 1.96 from the Z Table.**

p **= 31 % (0.31) prevalence from past study (Isyaku *et al*., 2008).**

q **= (1- p) = 1 - 0.31 = 0.69**

L **= allowable error of 5% (0.05).**

$n = 1.96^2 \times 0.31 \times 0.69/0.05^2$

$= 3.8416 \times 0.31 \times 0.69/0.0025$

$= 0.8217/0.0025$

$= 328.68$

$= 329$ samples

Therefore, *n* = 329 as the threshold number of diarrhoea stool samples to be collected. However, from the nature of this study and the target population, a total of 586 diarrhoea stool samples were collected for analysis.

3.4 Sample Collection

Ethical committee clearance for collection of diarrhoeal sample from patients attending hospitals was obtained from the Kano State Health Services Management Board and the consent of the infants' care giver or parents was also solicited before collecting the diarrhoeal sample (appendix I, II and III). A structural questionnaire was designed and administered to the mother/care giver of the infants (appendix IV). Data obtained from the questionnaire include the followings: age, sex, main occupation of father, level of father's and/or mother's/care giver's formal education, main source of infants' drinking water, treatment of drinking water, type of toilet in use, habits of washing hands after toilet, causes and treatment of infants diarrhoea, use of Oral Rehydration Solution (ORS), effect on appetite, breast feeding, age at which food is introduced, symptoms of diarrhoea; vomiting, fever, dehydration and weight of infants. Stool samples were collected from infants and children aged less than five years attending the selected general hospitals in the study area. The diarrhoeal samples were collected in accordance with the procedure described by Cheesbrough (2006). The diarrhoeal stool samples of at least 1g or 10 ml was collected in a clean, sterilized wide open mouth plastic container with lid-spoon, labeled and transported to the laboratory within 3-6 hours after collection in a box containing ice blocks as described by Atata *et al.* (2003). The sample collection sites based on the three Senatorial districts of the state include the followings;

Kano Central.

1. **Hasiya Bayero Peadiatric Hospital, Kano.**
2. **Murtala Muhammed Specalist Hospital, Kano.**
3. **Muhammed Abdullahi Wase Specialist Hospital, Nassarawa.**
4. **Infectious Diseases Hospital (IDH), Kano.**

Kano South.

5. **Wudil General Hospital.**
6. **Rano General Hospital.**
7. **Takai NYSC Community Health Center.**

Kano North.

8. **Danbatta General Hospital.**
9. **Gwarzo General Hospital.**
10. **Bichi General Hospital.**

3.4.1 Collection and identification of plant materials

Table 3.1 shows reference number for identification of the plants used. *Momordica charantia* Linn has reference number of V/No 1139 and *Aeschynomene uniflora* E. May has V/No 2552 as obtained from Mal. Haruna Musa of herbarium section, Department of Biological sciences, Ahmadu Bello University Zaria.

3.5 Isolation and Identification of Isolates

3.5.1 Growth media preparation

Plates of Eosin Methylene Blue (EMB) agar (L:S Biotech), Mueller Hinton agar (Oxiod) and Nutrient Agar (Oxoid) were prepared according to the manufacture's guide. The media was sterilized in an autoclave at 121^{o}C for 15 minutes. The media was then allowed to cool, then poured in to sterile Petri-dishes and allowed to solidify. Excess moisture was removed in a dryer for 15 minutes.

3.5.2 Isolation and identification

An inoculum from the sample was streaked on Eosin Methylene Blue (EMB) agar plates and incubated overnight at 37^{o} C. Suspected *E. coli* colonies (bluish black with green metallic sheen colouration) from the EMB agar plate was streaked on Nutrient agar slant and refrigerated until further analysis. The suspected isolates were subjected to gram staining, microscopy and biochemical tests (indole, lysine decarboxylate (LDC), citrate utilization, oxidase, urease, Voges-Proskauer (VP), lactose, etc.) as described by Cheesbrough, (2006).

Table 3.1 Reference number for identification of the plants used obtained from Department of Biological sciences Ahmadu Bello University Zaria.

Family	Latin name	Reference number
Cucurbitaceae	*Momordica charantia* Linn	V/No 1139
Leguminosae: Papilinodeae	*Aeschynomene uniflora* E. May	V/No 2552

Key: V/No – Voucher Number

3.5.3 Gram staining

An inoculum from a well-isolated colony was transferred onto a glass slide with a drop of distilled water. A smear was prepared and heat-fixed. The smear was flooded with crystal violet stain for 60 seconds. The stain was then washed off with sterile distilled water. The smear was again flooded with Lugol's iodine (as mordant) for 60 seconds and then washed off with sterile distilled water. Decolourization of the smear was done by adding acetone-alcohol and then washed immediately with sterile distilled water. The smear was flooded with neutral red stain for 60 seconds then washed at once with sterile distilled water. The back of the glass slide was cleaned and the smear allowed to air-dried. The smear was examined microscopically with x 40 objective (thus to check the staining and the distribution of cells) and then with oil immersion objective. Gram positive bacteria appeared dark purple while Gram negative appeared pale to dark red (Cheesbrough, 2006).

3.5.4 Biochemical tests

a. Citrate utilization test

Citrate utilization test is based on the ability of an organism to use citrate as its only carbon source. The test was carried out by streaking and stabbing Simmon's citrate agar (Lab M) slant with an inoculum of the test organism. The culture was incubated for 48 hours at 35^{o} C. A positive test was indicated by the colour change from light green to blue (Cheesbrough, 2006).

b. Indole test

This detects the ability of bacteria to produce indole from tryphtophan. The test organism was inoculated into peptone water and incubated at 35^{o} C for 24 hours. A few drops of Kovac's reagent (*P*-dimethyl-aminobenzyldehyde in acidified amyl alcohol) was added, shaken gently and allowed to stand. The presence of indole was detected by the appearance of a ring that is deep red in colour at the top of the peptone culture medium (Cheesbrough, 2006).

c. Methyl red test

The methyl red test is based on the use of a pH indicator methyl red to determine the hydrogen iron concentration (pH) when organisms ferment glucose. All members of the Enterobacteriaceae are glucose fermenters. An inoculum of the test organism was inoculated into methyl red/Voges-proskauer (MR/VP) broth and incubated at 37 ^{0}C for 24 hours. About 2-3 drops of methyl red pH indicator was added. Red colouration indicates positive result and yellow colouration indicates negative result (Jean, 1976).

d. Voges-Proskauer test

Voges-proskauer test was carried out to determine the ability of some organisms to produce neutral end product, acetylmethylcarbinol (acetoin), from glucose fermentation. This differentiates between the genera; *Klebsiella* (positive) and Enterobacter (usually positive) from *Escherichia coli* (negative). Inoculum from a colony growth of the test organism was inoculated into MR/VP broth and incubated at 35^{0}C for 24 hours. Few drops of Barritts VP reagent was then added, pinkish-red colouration at the surface of the medium indicates VP positive result while yellow colouration (same colour of the reagent) or a copper-like colouration indicates a negative result (Jean, 1976).

e. Oxidase test

This test is used in the identification of cytochrome oxidase producing organisms. A piece of filter paper was soaked with a few drops of oxidase reagent. An inoculum of the test organism was then smeared on the filter paper. A deep purple colouration results after 10-30 seconds gives a positive result (Lamikanra, 1990).

f. Catalase test

An inoculum of the test organism was placed in a drop of hydrogen peroxide solution on a glass slide. Formation of air bubbles indicates a positive result (Cheesbrough, 2006).

g. Urease test

Urease production was detected by growing the test bacteria on Christensen's urea broth. After incubation at 37 ^{o} C for 12 hours, positive strains librated ammonia, which raised the pH and caused the medium to turn pink (Cheesbrough, 2000).

h. Lysine decarboxylase test

A dense suspension of the test organism was prepared in 0.25 normal saline, LDC/ indole tablet and three drops of paraffin oil was added and incubated at 37^{o}C overnight. Blue/violet colour gives positive result (Jaen, 1976) and red ring surface layer on addition of three drops of Kovac's reagent (Cheesbrough, 2000).

i. Kligler Iron Agar (KIA) test

KIA reactions are based on the fermentation of lactose and glucose (dextrose) and the production of hydrogen sulphide. Test tube containing Kligler iron agar (KIA) slant was inoculated by streaking the surface and stubbing the butt. This was incubated at 37 ^{o} C for overnight. The test was positive when the slope and the butt tuned yellow, cracked media and blackening along the

stab due to H_2S production (Jaen, 1976).

j. Motility test

A motility media NA (Oxoid) was inoculated with test culture using a straight wire loop as a single stab down the center of the medium and incubated at 37^{o} C and examined at interval of 12 hours. Diffuse hazy growth that spread through the medium rendering it slightly opaque was indicative of a positive result for motility. Growth that was confined to the stab line, having sharply defined margin, leaving the surrounding medium clearly transparent was indicative of negative result.

The results of biochemical tests were compared with the standard biochemical table provided by Cheesbrough (2006) for identification of bacteria (Appendix VII).

3.6 Preparation of Turbidity Standard

One per cent volume by volume (1% v/v) solution of hydrogen tetraoxosulphate vi acid (H_2SO_4) was prepared by adding 1 ml concentrated H_2SO_4 into 99ml of water. One per cent weight by volume (1% w/v) solution of barium chloride was also prepared by dissolving 0.5 g of dehydrated barium chloride in 50ml distilled water. Then 0.6ml of the barium chloride solution was combined with 99.4 ml H_2SO_4 solution to yield 1.0 % w/v barium sulphate suspension. The turbid solution (Macfarland standard scale No. 6) formed was transferred into a test tube for comparison with turbid standard bacterial inocula preparation (Cheesbruogh, 2006).

3.7 Standardization of Inoculum

The isolates were activated by subculture into nutrient broth (Oxoid) and incubated at 30^{o}C for 6 hours. To make the standard inocolum, some quantity of the activated culture

was transferred into a tube containing 2.0 ml normal saline (0.9% w/v) using sterile pipette, until the turbidity of the suspension matched the turbidity of the Macfarland scale number 1 standard (Cheesbruogh, 2006).

3.8 *E. coli* isolates Serotyping

Anti-sera for typing *E. coli* obtained from SEIKEN^{R} were used for the identification of the isolates serotypes in accordance with the manufacturers guide (Appendix X) and methods adopted by Guinee *et al*. (1999). A drop of MacFarland standard of bacterial suspension was mixed unto a drop of the antisera of each of the corresponding *E. coli* serotype/strains and mixed. Agglutination indicates positive result.

3.9 Susceptibility of *E. coli* Strains to Commercially Produced Antibiotics

One ml of the standard inoculum was evenly spread onto the surface of Muelar Hinton agar (Fluka BioChemica) plates in duplicates. This was incubated at 30^{o}C for 6 hours prior to the application of the antibiotic discs (Oxoid); ofloxacin, augmentin, sulphamethazole/trimethoprim, ciprofloxacin, tetracycline, ampicillin, amoxycillin, gentamicin and nitrofurantoin. The discs were placed firmly onto the surface of the solidified agar by means of sterile forceps (NCCLS, 1996). The plates were incubated at 37^{0} C for 24 hours. Diameters of the zones of inhibition were measured and recorded to the nearest mm in accordance with method adopted by Darwish *et al*. (2002).

3.10 Collection and Identification of the Plant Materials

The fresh plants: *Momordica charantia* and *Aeschynomene uniflora* were collected from the farm lands and bushes around Kano State (Gaya and Wudil local Government areas) with the help of local herbalists and methods adopted by Mukhtar and Tukur (2000). The plants

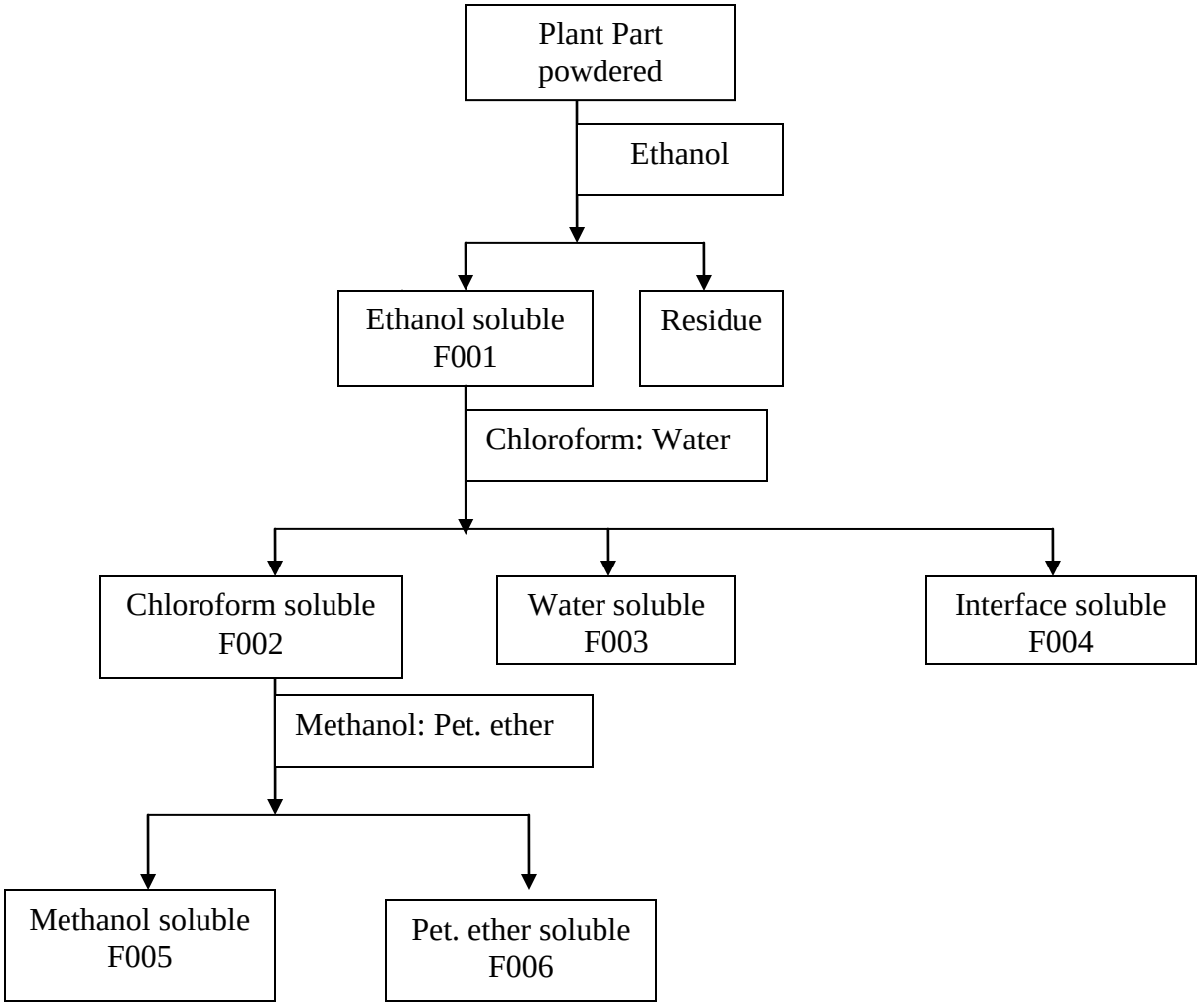
were identified and assigned reference numbers from the catalogue of herbarium section in the Department of Biological Sciences, Ahmadu Bello University, Zaria (Table 3.1).

3.11 Plants Extraction and Fractionation

The leaves, stems, roots and whole plants each plant specimens were separately air-dried under shed in a laboratory bench and grinded using pestle and motor. The powder plants were extracted to extraction in accordance with methods adapted by Fatope *et al*. (1993), Adoum *et al*. (1997) and Bukar *et al*. (2009). Two hundred grams of the powdered plant material was percolated in 1 litre of 97% ethanol for one week with constant shaking, after which a portion of the extract was filtered using Whatman number 1 filter paper (England). The percolate was concentrated using rotary evaporator (R110) at 40^{o} C. This extract (labeled ethanol extract) was weighed and kept in the refrigerator for further analysis. A fraction of the ethanol extract was partitioned between water and chloroform (H_2O/CH_3CL_3) mixture in the ratio of 1:1. This was shaken thoroughly for about an hour and allowed to settle for 24 hours in a separating funnel. The chloroform, water and interface fractions were separated in glass beakers and labeled F002, F003 and F004 respectively. The fractions were concentrated again, weighed and kept in the refrigerator for further analysis. Similarly, a fraction of chloroform soluble F002 was partitioned in a mixture of 97% methanol and petroleum ether of 1:1 ratio. These fractions were concentrated, weighed and labeled F005 and F006 respectively (Fig. 3.2). All these fractions were kept refrigerated at 4^{o}C for further use.

3.12 Phytochemical Screening of Plant Extracts

The extracts were screened for the presence of active components in according to methods adopted by Ibekwe *et al*. (2001). Tests conducted include carbohydrate (Molish's test and Fehling's test), alkaloid test (Mayer's test, Wagner's test and Dragendoff's test), tannins (Fe_3Cl_2 test), glycosides (Fehlings test), cardiac glycosides (Kella-killiani test), steroids (Salkwoski test), saponins (frothing test and emulsion test), flavonoid (Shinoda test), resin (copper acetate test) and anthraquinone (Borntrager's test).



Source: Salisu and Garba (2008).

Fig. 3.2 Schematic diagram of procedure steps for extraction and fractionation of plant materials.

3.12.1 Tests for carbohydrates

(a) Molisch test

A few drops of Molisch reagent were added to 2 ml of test extract in a test tube. One milliliter of concentrated tetraoxosulphate vi acid was allowed to run down the side of the inclined test tube so that the acid forms a layer beneath the aqueous solution without mixing it. The appearance of a reddish brown colour indicates a positive test.

(b) Fehling's test for free reducing sugar

Five milliliters of Fehling's solution A and B was added to two milliliters of test extract in a test tube. The resultant mixture was boiled for about two minutes. The appearance of a brick-red precipitate indicates a positive test.

3.12.2 Alkaloids

One cubic centimeter of 1% HCl was added to 3 ml portion of test fraction in a test tube. These solutions were treated separately with a few drops of Mayer, Wagner and Dragendroff reagents. A creamy white (Mayer), reddish-brown (Wagner) and an orange-brown (Dragendroff) precipitate indicates a positive observation for the test.

3.12.3 Tannins

Two drops of 5% $FeCl_3$ were added to 1 ml of test extract. A dirty-green precipitate is indicative of the presence of tannins in the extract.

3.12.4 Glycosides

A 10 ml of 30% H_2SO_4 were added to 1 ml of test extract in a test-tube. The mixture was heated to boiling for 15 minutes. A 10 ml aliquot of Fehling's solution were added and then

boiled for 15 minutes. A brick-red precipitate indicates the presence of glycosides in the extract.

3.12.5 Steroids (Salkowski test)

A 1 ml of concentrated H_2SO_4 was added to 1 ml of test extract. A red colour indicates the presence of steroids.

3.12.6 Saponins

(a) Frothing test

A 2 ml of test extract in a test tube were vigorously shaken for two minutes. Frothing in the test extract indicates the presence of saponins.

(b) Emulsion test

Five drops of olive oil were added to 3 ml of the test extract in a test tube and the mixture was vigorously shaken. A stable emulsion formed indicates the presence of saponins

3.12.7 Flavonoids

A little quantity of magnesium powder and a few drops of concentrated HCl were added to 3 ml of test extract. A red or intense red colouration indicates the presence of flavonoids.

3.12.8 Resin

A 5 ml of copper acetate solution were added to 5 ml of test extract. The resulting solution was shaken vigorously and allowed to separate. A green coloured solution is an evidence of presence of resin.

3.12.9 Test for anthraquinones derivatives

Borntrager's test (test for free anthraquinones)

A 2 ml portion of the extract was mixed with 10 ml benzene and filtered. Five ml of 10% ammonia solution was added to the filtered and stirred. The production of pink-red or violet colour indicates the presence of free anthraquinones.

3.12.10 Test for cardiac glycosides

Kella-Killiani test

The extract was dissolved in glacial acetic acid containing traces of iron (II) chloride. The test tube was held at an angle of 45 degree, 1 ml of concentrated H_2SO_4 was added down the side. Purple ring colour formation at the interface indicates cardiac glycosides (Trease and Evans, 1983).

3.13 Preparation of Extracts Concentrations for Sensitivity Test

This was carried out as described in Cheesbrough (2006). Stock solution of the plant extracts were prepared by adding 1g of each extract fraction in 1ml of appropriate diluents water for water soluble extract and dimethylsurphuroxide (DMSO) for non water soluble extracts to make 1000 mg/ml stock concentration. From the stock concentration, 500mg/ml, 250mg/ml, 125mg/ml and 62.5mg/ml were prepared by serial dilution techniques. These concentrations were kept in bijou bottles for further use.

3.14 Susceptibility of *E. coli* Isolates to Plant Extracts

Modified Kirby-Bauer agar diffusion method of sensitivity test as described by Cheesbrough (2006) was used to test the susceptibility of the *E. coli* strains isolated from the infants diarrhoea samples obtained. A 0.5 ml of the standard inoculum of the test organism was spread on to Mueller Hinton agar plates in duplicates and incubated at 30^{o}C for 6 hours prior to application of the extract. Using sterilized pipette for each, the various concentrations; 500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml of the plants extracts were poured into dug wells on the surface of the agar media at about 40mm apart. The plates were incubated at 37 ^{0} C for 24 hours. Diameters of the zones of inhibition in mililiteres (mm) were measured with a metric rule and the mean was recorded.

Standard strain of *Escherichia coli* (NCTC 10418) was sourced from National Institute for Pharmaceutical Research and Development (NIPRD) Abuja and Department of Pharmacognosy and Drugs Development ABU, Zaria for comparison with the test organism to serve as control.

3.15 Determination of Minimum Inhibitory Concentration (MIC)

Two-fold serial dilutions were made using nutrient broth in accordance with the method of Ibekwe *et al*. (2001) and the following concentrations were obtained: 250mg/ml, 125mg/ml, 62.5mg/ml, 31.25mg/ml and 15.625 mg/ml. The initial concentration was obtained by dissolving 0.25g of the extract in 1ml of nutrient broth. Three drops of the overnight broth cultures of the test organism (*E. coli* strains) were inoculated into the dilutions and incubated overnight at 37^{o}C for 24 hours. The lowest concentration of the extract, which inhibited the growth of the test organism, was recorded as Minimal Inhibitory Concentration (MIC). The growth of the inoculum in the broth was indicated by turbidity or cloudiness of the broth.

Negative controls were set up as follows: nutrient broth only, nutrient broth and sterile plant extract and finally positive control containing nutrient broth and a test organism.

3.16 Determination of Minimum Bactericidal Concentration (MBC)

Minimum Bactericidal Concentration (MBC) was detected from the MIC tubes showing no visible growth by subculturing an inoculum on nutrient agar (Oxoid) plate and incubated at 37^0C for 24 hours. The lowest concentration of the extract yielding no growth was recorded as the Minimal Bactericidal Concentration (MBC).

Negative controls were set up as follows: Nutrient agar only; Nutrient agar and sterile plant extract; and finally positive control containing nutrient agar and a test organism.

3.17 Plant Extracts Lethality Test

Swiss albino mice of both sexes weighing 113-264g obtained from Pharmacology Department ABU, Zaria were used for the study in accordance with the internationally accepted rules governing the use of laboratory animals (Garber *et al*., 2010). Acute toxicity test method LD_{50} was used to test the lethality of the various concentrations of the plants extracts using mice as described by Lorke (1983); Trease and Evans (1983). In the first phase, nine (9) mice were divided into three groups of three (3) mice each. The groups were treated with extract concentrations of 1000mg/kg, 100mg/kg and 10mg/kg oral doses respectively using gavage needle. The mice were observed for general signs and symptoms of toxicity including mortality over a period of 48 hours. Based on the results of phase one, four (4) fresh mice were divided into 4 groups of one mouse each in the second phase

experiment. The extract was administered at a dose of 5000 mg/kg, 2900 mg/kg, 1600 mg/kg and 1000 mg/kg orally. Signs and symptoms of toxicity such as restlessness, respiratory distress, coma and death were also monitored for 48 hours. The surviving animals were weighed again. Dead mice were autopsied and examined macroscopically for any pathological changes.

3.18 Haematological Screening of Albino Mice Blood

Haematological screening of albino mice blood after two weeks experiment on 1000 mg/kg/day, 1600 mg/kg/day, 2900 mg/kg/day, and 5000 mg/kg/day doses of methanol extract was carried out as described by (Abalaka *et al*., 2009). The mice blood were collected and analyzed for various blood cell count and distribution using automated blood counter (Beckman). Method of combination laser light scattering and impedance counting analysis was carried out according to the manufacturers guide.

3.19 Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out on the most active extracts in accordance with procedure adopted by Abalaka *et al*. (2010). A small amount of the extract was subjected to TLC over a glass coated with silica gel F254 using different gradient solvent system, chloroform-ethyl acetate in the ratio of 1:3 was the best mobile system used to obtain the best result. The extract was spotted on the silica coated TLC plate of thickness of about 0.2 mm. The plates were developed in saturated (with the solvent vapour) chromatography tank and spots detected in iodine tank following development and drying of the solvent. Bands of separated fractions were detected under ultraviolet light and in

iodine vapour. The retention fraction R_f of each fraction or band was calculated and recorded. Fractions with similar characteristic and R_f values were bulked parked and kept in clean sterilized bijou bottles for *E. coli* susceptibility test as described in 3.13 above.

The MIC and MBC of the different fractions were also determined as described in 3.14 and 3.15 respectively.

The susceptibility pattern of *E. coli* resistant to antibiotics and the plant extracts were tested against the TLC fractions. Preliminary phytochemical tests of most active TLC fractions were carried out as described by Bukar *et al*. (2009).

3.20 Statistical Analysis of the Results

Genstat statistical and Statistical Package for Social Sciences (SPSS) software was used in the analysis of most results obtained. Bioactivity of the different plant extracts were compared using t-test. The bioactivities of the plants extracts and that of the commercially orthodox antibiotics was compared by using ANOVA test (P = 0.05) in accordance to Norman (1995) and Sarmukaddam and Garad (2006). Values obtained from susceptibility of isolates to extracts and antibiotics were compared with the standard reference culture organisms.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic Data of Diarrhoeic Infants Care Giver Attending Some Hospitals in Kano State, Nigeria

Demographic data was obtained from the questionnaires filled by mothers/care givers of infants whose diarrhoeic stool were collected (Plate 4.1). Some results of the demographic data are presented (Figures 4.1-4.5 and Appendix V). Infection based on the infants' father occupation showed that traders and farmers infants have the highest prevalence of diarrhoea with number infected of 122 (60%) and 44 (22%) infants (Fig. 4.1). The results of infants parents formal education revealed that fathers have higher number of secondary and adult literacy with number of 62 (30.3%) and 61 (29.9%) respectively than primary, tertiary and none level of education with 17 (8.3%), 40 (19.6%) and 24 (11.8%) respectively (Fig. 4.2). Higher number was obtained from secondary and none in mothers/care givers with number of 51 (25%) and 55 (27%) respectively with lower count of 42 (21%) only in adult literacy (Fig. 4.3). Majority of the respondents (Fig. 4.4) obtained their drinking water meant for infants from pipe borne lines with number of 88 (43%). Most mothers/care givers do not treat the infant's drinking water. Only 16 (7.8%) and 5 (2.5%) mothers treated the infants' drinking water either by boiling or filtering respectively (Fig. 4.5).

Further findings revealed that, most of infant parents use pit latrine with 110 (53.9%) numbers. Majority of the population wash their hands after toilet use with soap and water with number infected infants of 87 (42.6%). Exactly 158 (77.5%) infants' mothers/care givers do not breast feed their infants at the time of this study, only 46 (22.5%) mothers are still breast feeding. Some 14 (6.9%) number of mothers diagnosed infants with diarrhoea in health facility before

treatment. Most mothers/care givers introduce food to the infants at the age of 5-6 months with number infected of 101 (49.5%).

4.2 Prevalence of Diarrhoea

The serotypes and associated O and H antigens of *E. coli* isolated from infants' diarrhoea attending some general hospital in Kano state, Nigeria was shown in Table 4.1. Amongst the different serotypes ETEC and EHEC are the most frequently occurring serovars with number positive of O148:H28 63 (30.88%) and O157:H7 55 (26.96%). There is no significant difference in the number positive among EPEC, ETEC and EHEC (P = 0.05) but EIEC is significantly difference (Appendix XIV) from EPEC, ETEC and EHEC (P = 0.01).

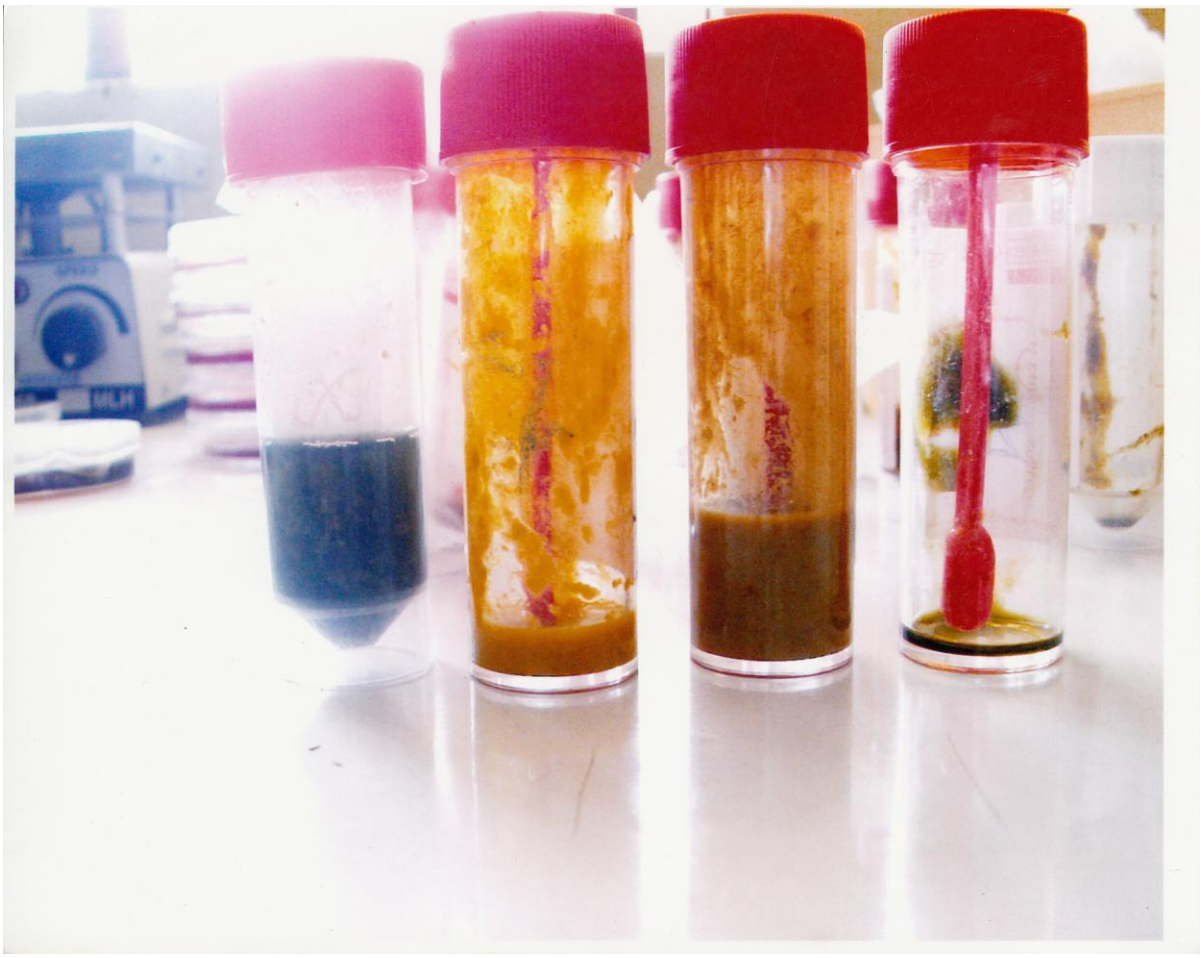


Plate 4.1 Samples of infants' diarrhoea collected from some general hospitals in Kano state, Nigeria showing deferent colours.

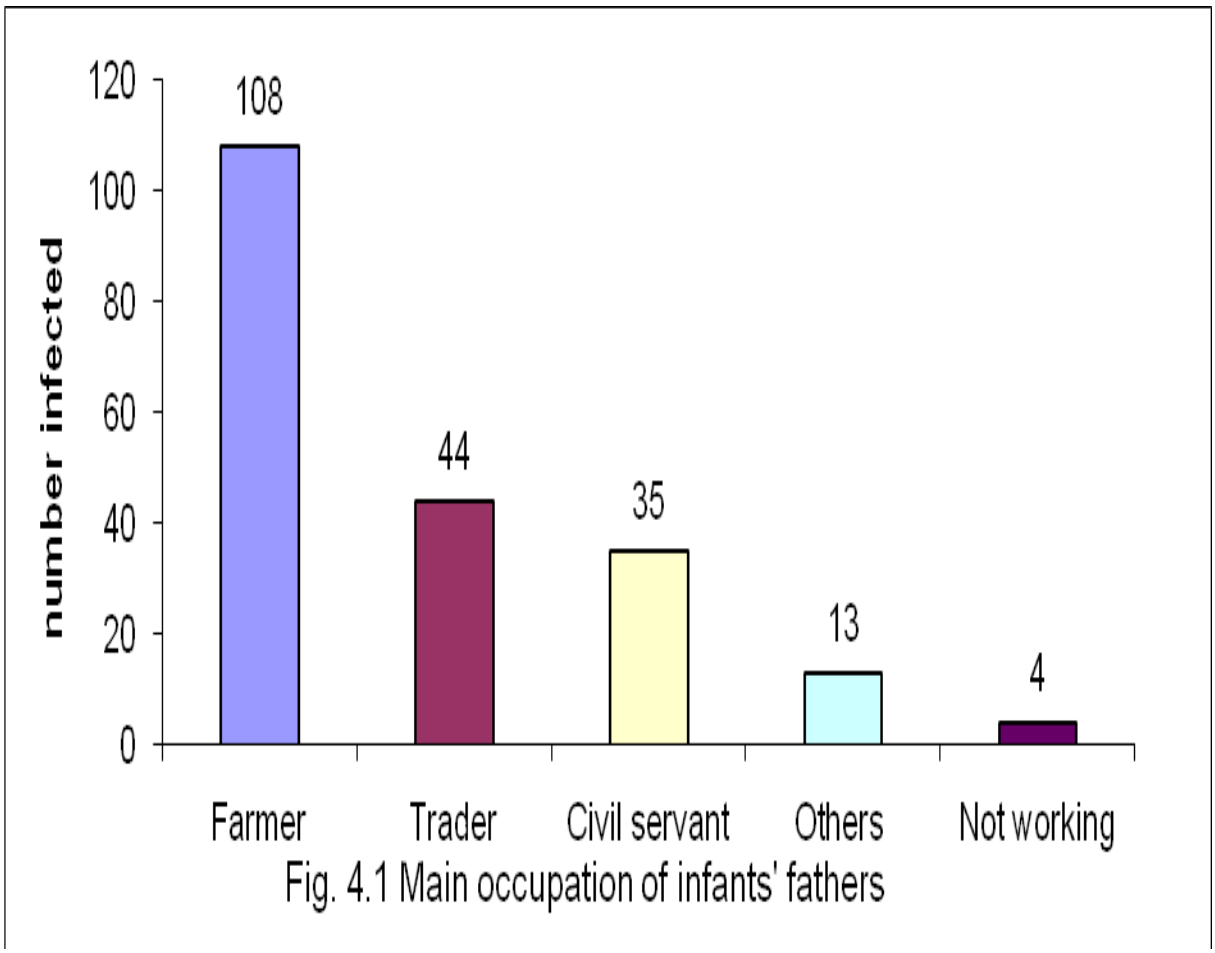


Fig. 4.1 Main occupation of infants' fathers

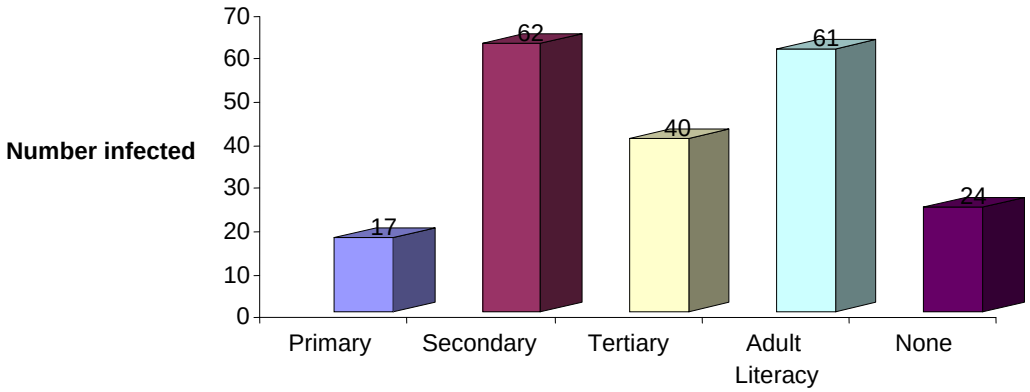


Fig.4.2 Level of infants with diarrhoea fathers formal education

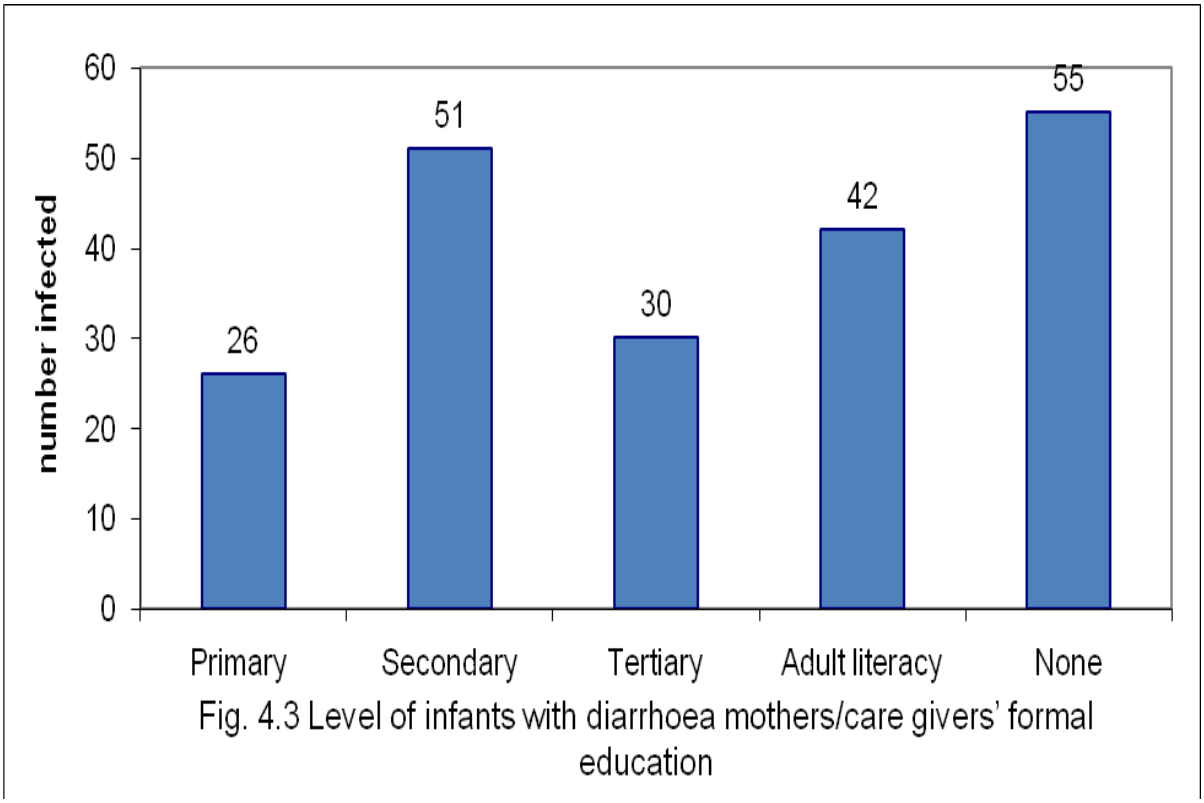


Fig. 4.3 Level of infants with diarrhoea mothers/care givers' formal education

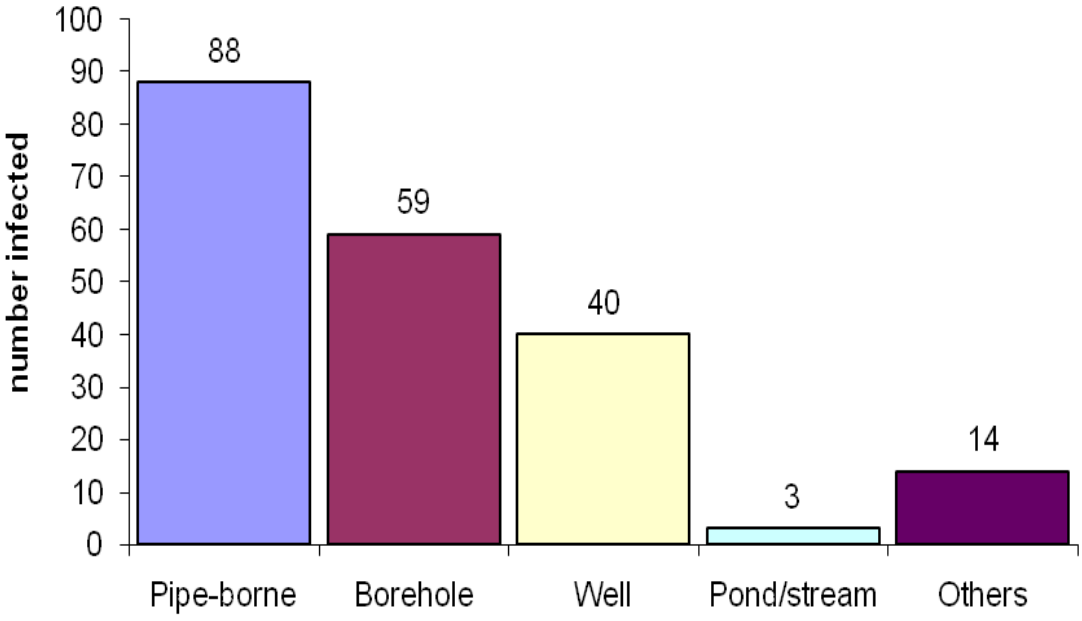


Fig. 4.4 Main source of drinking water for infants with diarrhoea cases attending some hospitals in Kano state, Nigeria.

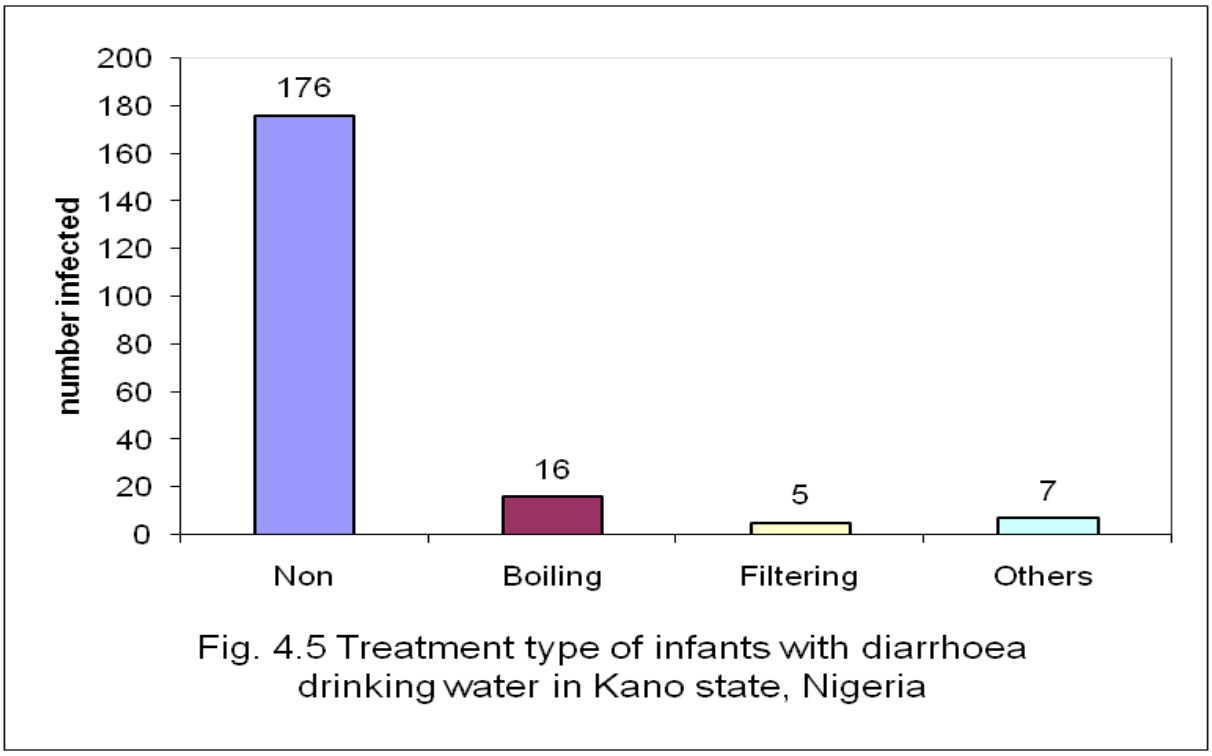


Fig. 4.5 Treatment type of infants with diarrhoea drinking water in Kano state, Nigeria

Table 4.1 Serotypes and associated O and H antigens of *E. coli* isolated from infants diarrhoea attending some general hospital in Kano state, Nigeria.

Serotype	Antisera	Number positive (%)
Enteropathogenic *E. coli* (EPEC)	O18	10(4.90)
	O112	35(17.16)
Enterotoxigenic *E. coli* (ETEC)	O148:H28	48(23.53)
	O29:H21	28(13.73)
Enterohaemorrhagic *E. coli* (EHEC/VTEC)	O157:H7	41(20.10)
	O26:H11	35(17.16)
Enteroinvasive *E. coli* (EIEC)	O124	00(0.00)
	O143	07(3.43)

Key: n- number of samples tested.

Table 4.2 shows the prevalence of diarrhoea caused by *E. coli* isolated from infants attending some hospitals in Kano state, Nigeria. A total of 204 (34.81%) infants were infected with *E. coli*. Murtala Muhammed Specialist Hospital, Hasiya Bayero Paediatrics hospital and Muhammed Abdullahi Wase special hospital have the highest prevalence of infants diarrhoea with 33 (16.18%), 29 (14.22%) and 25 (12.25%) tested positive respectively. While Takai NYSC community health center had the least prevalence of 5.39% (n= 11) number of infants infected. The results also showed that males are more infected than females with number infected of 113 (55%) and 91 (45%) male female respectively.

Table 4.3 shows the age range and gender distribution of diarrhoea cases of infants attending some hospitals in Kano state, Nigeria. Males have higher prevalence than females with number positive 113 (19.28%) and 91 (15.53%) respectively. In males the age group 24-35 months were more infected while the least prevalence occurred in the age group of 0-11 months with prevalence of 15.04% (n= 17). In females higher prevalence occurred in the age group of 24-35 and 36-47 months with prevalence of 25.27% (n=23) each, while the least prevalence was obtained in the age group of 0-11 month with occurrence of 13 (14.29%). The overall age range prevalence total showed that age range of 24-35 years has the highest prevalence of 54(9.22%) while age range 0-11 months has the least prevalence of 30 (5.12%). There is significant deference (Appendix XV) between the number of males positive and females (P=0.05).

Table 4.2 Prevalence of diarrhoea caused by *E. coli* isolated from infants attending some hospitals in Kano state, Nigeria.

Sampling site	Number examined (%)		Number Positive M	F	Total positive (%)		Total negative (%)	
Rano General Hospital	45	(7.68)	10.0	9.0	19.0	(9.31)	26	(6.81)
Takai NYSC Community Health Center	29	(4.95)	6.0	5.0	11.0	(5.39)	18	(4.71)
Wudil General Hospital	55	(9.39)	10.0	7.0	17.0	(8.33)	38	(9.95)
Bichi General Hospital	33	(5.63)	6.0	7.0	13.0	(6.37)	20	(5.24)
Danbatta General Hospital	52	(8.87)	10.0	7.0	17.0	(8.33)	35	(9.16)
Gwarzo General Hospital	61	(10.41)	10.0	8.0	18.0	(8.82)	43	(11.26)
Mohammed Abdullahi Wase Special Hospital Nassarawa	68	(11.60)	15.0	10.0	25.0	(12.25)	43	(11.26)
Murtala Muhammed Specialist Hospital	103	(17.58)	19.0	14.0	33.0	(16.18)	70	(18.32)
Hasiya Bayero Peadiatric Hospital	96	(16.38)	17.0	12.0	29.0	(14.22)	67	(17.54)
Infectious Disease Hospital (IDH), Kano	44	(7.51)	10.0	12.0	22.0	(10.78)	22	(5.76)
Total	**586**	**(100.0)**	**113.0**	**91.0**	**204**	**(34.81)**	**382**	**(65.19)**

Key: M-male, F-female.

Table 4.3 Age range and gender distribution of diarrhoea cases of infants attending some hospitals in Kano state, Nigeria.

Age range (month)	Number examined (%)		Number positive (%) M		F		Total +ve (%)	
0—11	101	(17.24)	17	(15.04)	13	(14.29)	30	(5.12)
12—23	154	(26.28)	20	(17.70)	18	(19.78)	38	(6.48)
24—35	103	(17.58)	31	(27.43)	23	(25.27)	54	(9.22)
36—47	126	(21.50)	28	(24.78)	23	(25.27)	51	(8.70)
48—59	102	(17.41)	17	(15.04)	14	(15.38)	31	(5.29)
Total	**586**	**(100.0)**	**113**	**(19.28)**	**91**	**(15.53)**	**204**	**(34.81)**

χ^2, P=0.1
Key: M-male, F-female, +ve-positive.

4.3 Antibiotic Resistance Pattern

The results of susceptibility pattern of *E. coli* isolated from infants' diarrhoea attending some hospitals in Kano on some commercial single disc antibiotics was showed (Plate 4.2 and table 4.4). The *E. coli* isolates were susceptible mostly to ciprofloxacin, ceftriaxone, augmentin and ofloxacin with numbers susceptible isolates with wider zones of inhibition of 169 (83%), 159 (78%), 155 (76%) and 153 (75%) respectively. The isolates are however resistant to ampicillin, amoxycillin, sulphamethoxazole/trimethoprim and tetracycline with resistant isolates of 182 (89%), 170 (83%), 105 (51.47%) and 94 (46%) respectively.

Table 4.5 shows resistance pattern of *E. coli* isolated from infants' diarrhoea attending some hospitals in Kano state, Nigeria. Ampicillin and amoxycillin have the highest single antibiotic resistance with 11 (12.5%) and 10 (11.4%) number of isolates resistant respectively. Multiple antibiotic resistance pattern of high frequency of occurrence were obtained in 3 combination of AMP, SXT, AML with 6 (6.82%), 2 combinations of AMP, SXT with 5 (5.68%) and SXT, AML with 4 (4.55%). Multiple resistance patterns of 4, 5, 6 and 7 number isolates with combination were also observed. Highest number of antibiotics resistant phenotypes with up to 7 number of antibiotics combination was obtained only with one isolate with 1 (1.14%) occurrence, resistance phenotype include; OFX, AMP, SXT, CN, AML, CIP, TE.

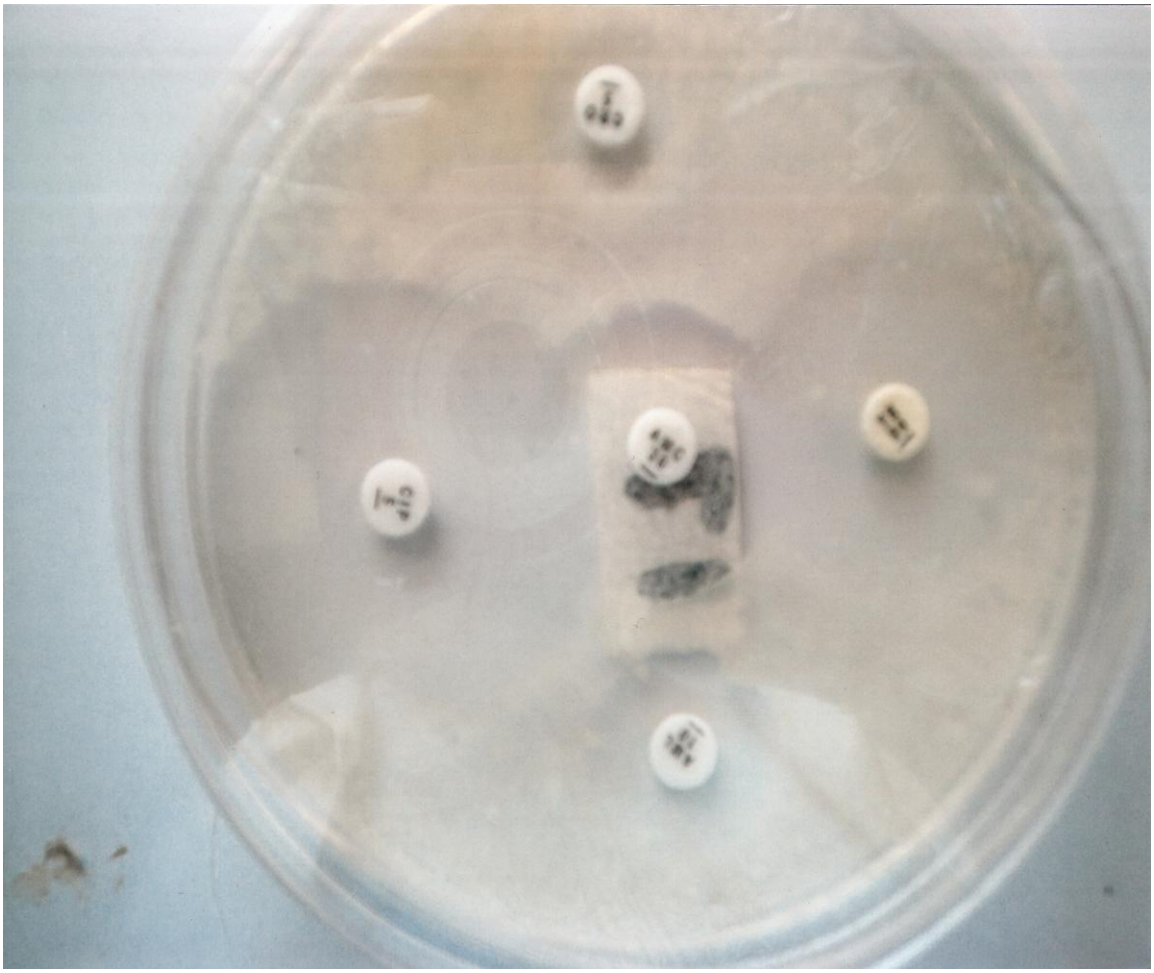


Plate 4.2 Zones of inhibition of some antibiotics discs sensitivity on *E. coli* isolates.

Table 4.4 Susceptibility pattern of *E. coli* isolated from infants diarrhoea attending some hospitals in Kano on some commercial antibiotics discs (n = 204).

Antibiotic	Abbreviation	Disc potency (µg)	Number (%) resistant organisms	Number (%) susceptible organisms
Ceftriaxone (β -lactam-3rd Generation Cephalosporin)	CRO	5	45(22)	159(78)
Ampicillin (β-lactam-amino-penicillin)	AMP	10	182(89)	22(11)
Amoxycillin-clavulanic acid (β -lactam-β-lactamase inhibitor)	AMC	30	35(17)	155(76)
Amoxycillin (Penicillin, β-lactamase)	AML	10	170(83)	34(17)
Tetracycline (Tetracyciles)	TE	30	94(46)	110(54)
Ciprofloxacin (Fluoroquinine, 2nd generation)	CIP	5	49(24)	169(83)
Ofloxacin (Fluoroquinine, 2nd generation)	OFX	5	51(25)	153(75)
Gentamicin (Aminoglycoside)	CN	30	80(39)	124(61)
Nitrofurantoin (β -lactam-3rd Generation Cephalosporin)	F	200	74(36)	130(64)
Sulphamethoxazole/ Trimethoprim (Sulphonamide)	SXT	25	105(51)	99(49)

χ^2, P=0.1 no significant difference
Key: n- number of samples tested.

Table 4.5 Resistance pattern of *E. coli* isolated from infants diarrhoea attending some hospitals in Kano, Nigeria (n = 88).

Single antibiotic resistance		Multiple antibiotic resistance		
Number of isolates (%)	Resistance phenotype	Number of antibiotic combination	Number of isolates (%)	Resistance phenotype with the pattern
11 (12.50)	AMP	2	1 (1.14)	F, SXT
10 (11.36)	AML		5 (5.68)	AMP, SXT
4 (4.55)	SXT		2 (2.27)	SXT AMC
2 (2.27)	TE		3 (3.41)	AMP, TE
1 (1.14)	F		4 (4.55)	SXT, AML
1 (1.14)	AME		1 (1.14)	AMP, CN
			3 (3.41)	AMP, AML
			2 (2.27)	TE, AMC
			1 (1.14)	AMP, AMC
			1 (1.14)	AML, TE
			1 (1.14)	CN, TE
		3	6 (6.82)	AMP, SXT, AML
			1 (1.14)	AMP, TE, AMC
			1 (1.14)	SXT, AML, CIP
			1 (1.14)	F, AML, CRO
			2 (2.27)	AMP, SXT, TE
			1 (1.14)	SXT, AML, AMC
			2 (2.27)	AMP, AML, TE
			2 (2.27)	AML, TE, AMC
			1 (1.14)	CN, AML, AMC
			1 (1.14)	AMP, AML, AMC
			1 (1.14)	OFX, SXT, CN
		4	1 (1.14)	AMP, CN, TE, AMC
			1 (1.14)	F, AMP, AML, CIP
			1 (1.14)	AMP, SXT, AML, CIP
			1 (1.14)	AMP, SXT, AML, AMC
			1 (1.14)	AMP, AML, TE, AMC
			2 (2.27)	AMP, SXT, AML, TE
			1 (1.14)	F, AMP, CN, AMC
		5	3 (3.41)	AMP, SXT, AML, TE, AMC
			1 (1.14)	F, SXT, AML, TE, AMC
			1 (1.14)	OFX, AMP, AML, TE, AMC
		6	1 (1.14)	OFX, SXT, CN, AML, CIP, TE
			1 (1.14)	OFX, AMP, SXT, CN, AML, TE
		7	1 (1.14)	OFX, AMP, SXT, CN, AML, CIP, TE

Key: OFX=ofloxacin, F=nitrofrantion, AMP=ampicillin, SXT= Sulphamethoxazole-trimethoprim (co-trimathoprim), CN= gentamicin, AML= amoxycilline, CIP=ciprofloxacine, TE= tetracycline, AMC= amoxicillin and clavulanic acid (augmentin), CRO= ceftriazone, n- number tested.

4.4 Extraction and Fractionation of Plant Materials

The weights of the different solvents extract of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May (Table 4.6). Among the different plant parts used, the leaves and the whole plant gave the highest extract weight in both plants used. Thus *Momordica charantia* yielding 40.60g (29.70%) and 38.35g (28.06%) extract for MOL and MOW, while in *Aeschynomene uniflora* E. may 37.90g (35.24%) and 24.19g (22.49%) for AEL and AEW respectively. From the solvents used aqueous and ethanol extracts have the highest weight of 48.25g (35.30%) and 36.79g 26.91(%) in *Momordica charantia* and 36.33g (33.78%) and 36.38g (33.83%) in *Aeschynomene uniflora* respectively. The petroleum ether in each plant shows the least weight extract obtained with of 9.84g (7.20%) and 8.92g (8.29%) only. There was extremely significant difference (Appendix XI) in the weights of plant parts (P□ 0.0001). Variation in solvent weights (Appendix XII) of extracts is significantly deference (P=0.0022).

4.5 Preliminary Phytochemical Screening of *Momordica charantia* Linn and *Aeschynomene uniflora*

Tables 4.7 to 4.11, Plates 4.3 and 4.4 shows some results of preliminary phytochemical screening of *Momordica charantia* Linn and *Aeschynomene uniflora* E. may ethanolic, aqueous, chloroform, methanol and pet ether extracts. There were more of the phytochemicals present in the leaves, whole plants, ethanolic, aqueous and methanol extracts then in stems, roots, chloroform and petroleum ether extracts.

4.6 Pharmacological and Haematological Study

Table 4.12 shows the pharmacological activity of *Momordica charantia* Lin and *Aeschynomene uniflora* E. May methanolic extract on experimentally infected albino mice. No significance different between the mean weights of the albino mice before and after treatment (P=0.65, 14 df). No death of any mice occurred during and 3 days after the experiment.

Table 4.6 Weight of different solvents extracts of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May.

Plant part	Solvent used, weight in grams (%) Ethanol	Chloroform	Aqueous	Methanol	Pet. ether	Total
MOL	15.45(11.30)	04.73(3.46)	10.19(7.45)	06.91(5.06)	03.32(2.43)	40.60(29.70)
MOS	08.34(6.10)	03.99(2.92)	14.13(10.34)	04.84(3.54)	02.12(1.55)	25.08(18.35)
MOR	09.12(6.67)	03.41(2.49)	12.59(9.21)	05.36(3.92)	02.18(1.59)	32.66(23.89)
MOW	12.22(8.94)	05.92(4.33)	11.34(8.30)	06.65(4.87)	02.22(1.62)	38.35(28.06)
Total	**36.79(26.91)**	**18.05(13.21)**	**48.25(35.30)**	**23.76(17.38)**	**09.84(7.20)**	**136.69(100)**
AEL	10.74(9.99)	03.51(3.26)	17.43(16.21)	03.87(3.60)	02.35(2.19)	37.90(35.24)
AES	07.67(7.13)	02.94(2.73)	06.00(5.58)	03.31(3.08)	02.14(1.99)	22.06(20.51)
AER	09.64(8.96)	02.83(2.63)	06.23(5.79)	02.50(2.32)	02.19(2.04)	23.39(21.75)
AEW	08.33(7.75)	02.84(2.64)	06.67(6.20)	04.11(3.82)	02.24(2.08)	24.19(22.49)
Total	**36.38(33.83)**	**12.12(11.27)**	**36.33(33.78)**	**13.79(12.82)**	**08.92(8.29)**	**107.54(100)**

MO, P=0.0001 extremely significant, AE, P=0.0022 very significant

Key: MOL – *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW -*Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant.

Table 4.7 Preliminary phytochemical screening of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May ethanolic extract.

Test	MOL	MOS	MOR	MOW	AEL	AES	AER	AEW
Carbohydrate	+	+	+	+	+	+	+	+
Alkaloid test	-	+	+	-	+	-	-	+
Tannins	+	+	-	+	+	+	+	+
Glycosides	-	-	+	-	+	+	+	+
Cardiac glycosides	-	+	+	-	+	+	+	+
Steroids	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+
Flavonoid	-	+	+	-	+	+	+	+
Resin	+	+	+	+	+	+	+	+
Anthraquinone	-	+	+	-	+	+	-	+

Key: + = positive, - = negative, MOL – *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW -*Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant.

Table 4.8 Preliminary phytochemical screening of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May aqueous extract.

Test	MOL	MOS	MOR	MOW	AEL	AES	AER	AEW
Carbohydrate	+	+	+	+	+	+	+	+
Alkaloid test	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+
Glycosides	-	-	+	-	+	+	+	+
Cardiac glycosides	-	+	+	+	+	+	-	-
Steroids	-	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+
Flavonoid	+	-	+	+	+	+	+	+
Resin	+	+	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-	+	-

Key: + = positive, - = negative, MOL – *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW -*Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant.

Table 4.9 Preliminary phytochemical screening of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May chloroform extract.

Test	MOL	MOS	MOR	MOW	AEL	AES	AER	AEW
Carbohydrate	+	+	+	+	+	+	+	+
Alkaloid test	+	+	-	+	+	+	-	+
Tannins	-	+	+	+	+	+	+	+
Glycosides	-	-	+	-	+	+	+	+
Cardiac glycosides	-	+	-	-	-	+	+	-
Steroids	-	+	-	-	-	+	+	+
Saponins	+	+	+	+	+	+	+	+
Flavonoid	-	+	-	-	-	+	+	-
Resin	+	+	-	+	-	+	+	+
Anthraquinone	+	-	-	-	-	-	-	-

Key: + = positive, - = negative, MOL – *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW -*Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant.

Table 4.10 Preliminary phytochemical screening of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May methanolic extract.

Test	MOL	MOS	MOR	MOW	AEL	AES	AER	AEW
Carbohydrate	+	+	+	+	+	+	+	+
Alkaloid test	+	-	-	-	+	-	-	+
Tannins	+	+	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+	+	+
Resin	+	+	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-	-	-

Key: + = positive, - = negative, MOL – *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW -*Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant.

Table 4.11 Preliminary phytochemical screening of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May petroleum ether extract.

Test	**MOL**	**MOS**	**MOR**	**MOW**	**AEL**	**AES**	**AER**	**AEW**
Carbohydrate	+	+	+	+	+	+	+	+
Alkaloid test	-	-	-	-	-	-	-	-
Tannins	+	+	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+	+	+
Resin	+	+	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-	-	-

Key: + = positive, - = negative, MOL – *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW -*Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant,

Plate 4.3 Frothing test for saponins from left *Momordica charantia* and right, *Aeschynomene uniflora*

Plate 4.4 Phytochemical tests: Above, left tube showing positive test for resin. Below, a purple ring colour at interface indicating the presence of cardiac glycosides

Table 4.12 Means and ranges weights of albino mice before and after treatment with *Momordica charantia* Lin and *Aeschynomene uniflora* E. May methanolic extracts for two weeks.

	weight of mice (grams)					
Plant part	before treatment mean	range	after treatment mean	range	% wg	death
MOL	145	113-188	151	120-191	4.14	Nil
MOS	138	120-177	145	128-182	5.07	Nil
MOR	156	124-233	157	130-196	0.64	Nil
MOW	141	113-204	149	120-209	5.67	Nil
AEL	145	105-215	149	113-205	2.76	Nil
AES	189	127-211	197	131-199	4.23	Nil
AER	168	125-210	172	136-181	2.38	Nil
AEW	158	125-200	163	120-177	3.16	Nil
Control	148	122-189	151	126-193	2.03	Nil

P=0.65, 14df not significant

Key: MOL - *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW - *Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant, % wg- percentage weight gain.

Table 4.13 shows the haematological profile of albino mice blood after two weeks experiment on active MOL doses of methanol extract. Concentrations of 1000 mg/kg/day and 1600 mg/kg/day doses were not significantly different from the control, while 2900 mg/kg/day and 5000mg/kg/day results were significantly different (P=0.05).

Table 4.14 shows the haematological profile of albino mice blood after two weeks experiment on active AEW doses of methanol extract. Results of 1000 mg/kg/day and 1600 mg/kg/day doses were not significantly different from the control, while 2900 mg/kg/day and 5000mg/kg/day results were significantly different from the control (P=0.05).

4.7 Susceptibility Pattern of *E. coli* Isolates to Plants Extracts

The susceptibility pattern of *E. coli* isolated from infants' diarrhoea attending some hospitals in Kano to extracts of *Momordica charantia* are shown in Table 4.15. Ethanol and methanol proved to be the most effective solvents for extraction of *Momordica charantia* in this study. Their extract shows wider zones of inhibition of 18±1.2 mm and 18±2.1 mm respectively. The organisms are more susceptible to leaves and the whole plant extracts. Concentration has no significance effect on zones of inhibition for ethanol, aqueous and methanol extracts (P=0.05).

Table 4.16 shows the susceptibility pattern of *E. coli* isolated from infants' diarrhoea attending some hospitals in Kano on extracts of *Aeschynomene uniflora*. Ethanol and methanol proved to be the most effective solvents for extraction of the plant in this study with extracts showing wider zones of inhibition of 17±2.1 mm each. The organisms were more susceptible to whole plant and leaves extracts. Concentration has no significance effect on zones of inhibition for ethanol, aqueous and methanol extracts (P=0.05).

Table 4.13 Haematological profile of albino mice blood after two weeks experiment on active MOL methanol extract doses.

Dose Conc. mg/kg/day	WBC (x10/µl)	RBC (x106/µl)	HGB (g/dl)	MCV (fi)	RDW (%)	PLT ($x10^3$/µl)
0 (control)	12.1±0.2	9.2±0.2	17.1±0.2	59.2±1.2	16.2±0.2	775±34.0
1000	11.1±0.1	9.1±0.2	17.2±0.2	61.2±0.4	16.2±02	703±72.0
1600	11.5±1.0	9.1±0.3	16.4±0.1	57.2±1.3	16.4±0.2	711±24.0
2900	12.3±0.2*	9.0±0.3*	16.5±0.1*	58.2±1.4*	16.4±0.2*	706±44.0*
5000	13.2±0.2*	8.6±0.3*	16.0±0.2*	50.1±1.2*	16.5±01*	826±46.0*

Results are mean ± S.D., n = 65, P=0.05, *- significant difference.
Key: WBC=white blood cells, RBC=red blood cells, HGB=haemoglobin, MCV=mean cell volume, RDW=red cell distribution width, PLT=platelets, MOL5- thin layer chromatography fraction number 5 of *Momordica charantia* leaves fraction.

Table 4.14 Haematological profile of albino mice blood after two weeks experiment on active AEW methanol extract doses.

Dose Conc. mg/kg/day	WBC (x10/µl)	RBC (x106/µl)	HGB (g/dl)	MCV (fi)	RDW (%)	PLT ($x10^3$/µl)
0 (control)	12.0±0.1	9.2±0.1	18.0±0.2	58.2±0.3	15.2±0.2	700±31.0
1000	11.8±0.1	9.1±0.1	17.6±0.2	60.2±0.4	15.0±01	705±22.0
1600	11.9±1.0	9.2±0.2	17.3±0.1	61.2±1.2	14.7±0.2	712±25.0
2900	12.4±0.2*	9.0±0.3*	16.3±0.1*	62.2±1.2*	14.0±0.2*	736±35.0*
5000	12.6±0.2*	8.2±0.2*	16.4±0.1*	62.1±1.2*	13.2±01*	721±24.0*

Results are mean ± S.D., n = 65, P=0.05, *- significant difference.

Key: WBC=white blood cells, RBC=red blood cells, HGB=haemoglobin, MCV=mean cell volume, RDW=red cell distribution width, PLT=platelets, AEW3- thin layer chromatography fraction number 3 of *Aeschynomene uniflora* whole plant fraction.

Table 4.15 Susceptibility pattern showing zones of inhibition of *E. coli* isolates obtained from infants diarrhoea attending some hospitals in Kano on extracts of *Momordica charantia*.

Extract (mg/ml) concentration	Plant part	Zones of inhibition (mm) Ethanol	Aqueous	Chloroform	Methanol	Pet.ether
500	MOL	18±1.2	16±1.4	12±1.0	18±2.1	12±0.5
	MOS	16±1.4	16±1.6	10±2.1	16±1.4	10±0.6
	MOR	07±1.3	09±1.8	08±1.1	09±1.6	08±1.1
	MOW	17±1.6	15±0.2	12±1.2	16±1.2	10±1.1
250	MOL	17±1.7	15±1.4	12±1.2	18±1.2	11±1.0
	MOS	14±1.2	14±1.5	10±1.3	15±1.3	10±1.2
	MOR	00±0.6	00±0.8	07±1.2	09±1.6	00±0.9
	MOW	15±1.1	12±1.2	10±1.0	15±1.7	10±1.3
125	MOL	14±1.3	12±1.7	10±1.2	16±1.7	10±1.2
	MOS	14±1.8	12±1.6	08±1.4	15±1.0	09±1.4
	MOR	00±0.4	00±1.1	07±0.8	08±1.2	00±0.7
	MOW	12±1.2	10±1.4	09±1.1	14±1.1	09±1.1
62.5	MOL	12±1.4	09±1.2	10±1.3	14±1.6	09±0.6
	MOS	10±1.2	09±1.0	08±1.8	12±1.4	00±0.3
	MOR	00±0.6	00±0.3	00±0.6	00±0.8	00±0.2
	MOW	10±1.8	09±1.2	00±0.2	12±1.1	00±0.3
E. coli (NCTC 10418)		21±1.2	20±1.3	18±1.2	20±1.4	16±1.0

Key: Results are mean±SD, n=65, MOL - *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW - *Momordica charantia* Whole plant, NCTC- National Culture Technology Commission.

Table 4.16 Susceptibility pattern showing zones of inhibition of *E. coli* isolates obtained from infants diarrhoea attending some hospitals in Kano on extracts of *Aeschynomene uniflora*.

Extract (mg/ml) Concentration	Plant part	Zones of inhibition (mm) Ethanol	Aqueous	Chloroform	Methanol	Petroleum ether
500	AEL	17±2.1	14±1.1	11±1.4	17±2.1	11±1.2
	AES	14±1.2	15±1.2	10±2.1	16±1.2	10±1.1
	AER	07±1.1	09±1.4	07±1.2	10±1.4	07±1.4
	AEW	18±0.6	16±1.6	11±1.2	18±1.3	09±1.2
250	AEL	17±1.3	14±1.3	11±1.1	16±1.6	10±1.3
	AES	14±1.5	13±1.7	09±1.2	14±1.2	09±1.5
	AER	07±0.9	07±0.9	00±1.5	09±1.3	00±0.3
	AEW	16±1.3	12±1.2	11±0.8	16±1.4	10±1.1
125	AEL	14±1.2	12±1.1	10±1.5	16±1.2	11±1.2
	AES	12±1.1	11±1.6	07±1.3	14±1.1	09±1.2
	AER	00±0.8	00±0.7	07±1.2	08±1.4	07±0.1
	AEW	14±1.5	11±0.5	09±1.1	15±1.2	10±1.0
62.5	AEL	11±1.3	09±1.3	10±1.8	14±1.1	09±0.4
	AES	10±1.4	08±1.2	08±1.3	12±1.4	07±0.7
	AER	00±0.5	00±0.6	00±0.4	07±0.8	00±0.2
	AEW	12±1.7	10±1.2	07±0.2	13±1.3	07±0.2
E. coli (NCTC 10418)		21±1.4	20±1.2	18±1.1	20±1.3	16±1.4

Key: Results are mean±SD, n=65, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant, NCTC- National Culture Technology Commission.

Table 4.17 shows the number of susceptible *Escherichia coli* isolated from infants diarrhoea attending some hospitals in Kano on extracts of *Momordica charantia.* The isolates were more susceptible to leaves methanol, ethanol and whole plant aqueous extracts with number susceptible of 52 (80%), 50(77%) and 48(74%) respectively. However, the isolates were resistant to the roots extract with number susceptible ranging from 05(08%) – 16(25%).

Number of susceptible *Escherichia coli* isolated from infants' diarrhoea attending some hospitals in Kano on extracts of *Aeschynomene uniflora* was shown (Table 4.18). The organisms were more susceptible to whole plant and leave methanol extracts with number susceptible of 49(75%) and 47(72%) respectively. However, the isolates are resistant to the roots extract with number susceptible ranging from 10(15%) – 23(35%).

Table 4.19 shows the Minimum Inhibitory Concentration of *Momordica charantia* and *Aeschynomene uniflora* extracts to *Escherichia coli* isolates. Low MIC of 31.2 mg/ml was obtained for; MOL,MOW, AEL and AEW ethanolic extracts, MOL and AER for aqueous extracts, MOL, MOS, MOW, AEL and AEW for methanol extracts.

The Minimum Bactericidal Concentration of *Momordica charantia* and *Aeschynomene uniflora* extracts to *Escherichia coli* isolates was shown (Table 4.20). All extracts have same MBC of 62.5mg/ml except the methanolic extract of MOL, MOS, MOW, AEL, AES and AEW having 31.2mg/ml.

Table 4.17 Number of susceptible *Escherichia coli* isolated from infants diarrhoea attending some hospitals in Kano on extracts of *Momordica charantia* (n = 65).

Extract concentration in mg/ml	Number of *E. coli* (%) leaves		stems		roots		whole plant	
	R	S	R	S	R	S	R	S
Ethanol	15(23)	50(77)	39(60)	26(40)	49(75)	16(25)	41(63)	24(37)
Aqueous	17(26)	48(74)	28(43)	37(57)	60(92)	05(08)	22(34)	43(66)
Chloroform	32(49)	33(51)	48(74)	17(26)	50(77)	15(23)	25(38)	40(62)
Methanol	13(20)	52(80)	25(38)	40(62)	53(82)	12(18)	31(48)	34(58)
Pet. ether	19(29)	46(71)	41(63)	24(37)	53(82)	12(18)	39(60)	26(40)

Key: S = susceptible, R = resistant, n = number tested.

Table 4.18 Number of susceptible *Escherichia coli* isolated from infants diarrhoea attending some hospitals in Kano on extracts of *Aeschynomene uniflora* (n = 65).

Extract concentration in mg/ml	Number of *E. coli* (%) leaves		stems		roots		whole plant	
	R	S	R	S	R	S	R	S
Ethanol	22(34)	43(66)	48(74)	17(26)	51(78)	14(22)	19(29)	46(71)
Aqueous	25(38)	40(62)	34(52)	31(48)	42(65)	23(35)	19(29)	46(71)
Chloroform	27(42)	38(58)	35(54)	30(46)	50(77)	15(23)	21(32)	44(68)
Methanol	18(28)	47(72)	36(55)	29(45)	43(66)	22(34)	16(25)	49(75)
Pet. ether	33(51)	32(49)	41(63)	24(37)	55(85)	10(15)	25(38)	40(62)

Key: S = susceptible, R = resistant, n = number tested.

Table 4.19 Minimum Inhibitory Concentration of *Momordica charantia* and *Aeschynomene uniflora* extracts to *Escherichia coli* isolates.

Extracts	Concentrations in mg/ml				
	Ethanol	Aqueous	Chloroform	Methanol	Petroleum ether
MOL	31.2	31.2	62.5	31.2	62.5
MOS	62.5	62.5	62.5	31.2	62.5
MOR	62.5	62.5	62.5	62.5	62.5
MOW	31.2	62.5	62.5	31.2	62.5
AEL	31.2	62.5	62.5	31.2	62.5
AES	62.5	62.5	62.5	62.5	62.5
AER	62.5	31.2	62.5	62.5	62.5
AEW	31.2	62.5	62.5	31.5	62.5

Key: MOL - *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW - *Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant.

Table 4.20 Minimum Bactericidal Concentration of *Momordica charantia* and *Aeschynomene uniflora* extracts to *Escherichia coli* isolates.

Extracts	Concentrations in mg/ml				
	Ethanol	Aqueous	Chloroform	Methanol	Petroleum ether
MOL	62.5	62.5	62.5	31.2	62.5
MOS	62.5	62.5	62.5	31.2	62.5
MOR	62.5	62.5	62.5	62.5	62.5
MOW	62.5	62.5	62.5	31.2	62.5
AEL	62.5	62.5	62.5	31.2	62.5
AES	62.5	62.5	62.5	31.2	62.5
AER	62.5	62.5	62.5	62.5	62.5
AEW	62.5	62.5	62.5	31.5	62.5

Key: MOL - *Momordica charantia* leaves, MOS - *Momordica charantia* Stem, MOR - *Momordica charantia* Root, MOW - *Momordica charantia* Whole plant, AEL - *Aeschynomene uniflora* Leaves, AES - *Aeschynomene uniflora* Stem, AER - *Aeschynomene uniflora* Root, AEW - *Aeschynomene uniflora* Whole plant.

4.8 Active thin Layer Chromatography Fractions

Nine 9 TLC fractions were obtained from MOL methanol extract based on their different colours, movement in the solvent system at different distances with R_f values ranging from the lowest 0.00 to the highest 0.91 with solvent front of 7.9cm (Table 4.21). Fraction MOL5 deep purple is the most active amongst the 9 fractions separated (Plate 4.5). MOL5 has R_f value of 0.51 and yielded 470mg (17.45%). The relation between the distant traveled by TCL fractions (Appendix XIII) and the R_f value is extremely significant (r=1.00).

Table 4.22 shows the thin layer chromatography fractions of AEW methanol and their R_f values. Only three (3) fractions were obtained from the TLC of the most active extract of *Aeschynomene uniflora* AEW aqueous extract with solvent front of 7.8cm. Fraction number AEW3 green showed activity against the *E. coli* isolates tested with R_f value of 0.91 and yielded 370mg (46.84%). The relation between the distant traveled by TCL fractions (Appendix XIII) and the R_f value is significant (r=1.00).

Further phytochemicals screening reveled that each fraction contains 4 organic compounds. Steroids are present and more tannins was found in MOL5 as compared to AEW3. While AEW3 have more saponins compared to MOL5 (Table 4.23).

MOL5 fraction is more active than AEW3 fraction having highest zones of inhibition of 17±1.2 and 14±2.2 respectively (Table 4.24). Zones of inhibition did not differ significantly (P=0.05) from the values of zones of inhibition of *E. coli* (NTCT 10418).

Table 4.25 shows the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of most active TLC fractions against *E. coli* isolates. MOL5 showed MIC and MBC of 30 μg/ml and 40 μg/ml respectively. While AEW3 fraction showed 40 μg/ml in each case.

Table 4.21 Thin Layer Chromatography (TLC) fractions of MOL methanol and their R_f values with solvent front of 7.9 cm.

Fraction	Colour description	Distant traveled (cm)	R_f value	Yield in mg (%)
MOL9	Pale brown	7.2	0.91	70 (2.60)
MOL8	Green	7.0	0.89	170 (6.32)
MOL7	Dark blue	5.5	0.70	80 (2.97)
MOL6	Light brown	4.6	0.58	390 (14.50)
MOL5	Deep purple	4.0 (active)	0.51	470 (17.45)
MOL4	Purple	2.9	0.37	1340 (49.81)
MOL3	Light brown	1.7	0.22	80 (2.97)
MOL2	Purple	0.9	0.11	90 (3.36)
MOL1	Dark Brown	0.0	0.00	00 (0.00)

Key: MOL-*Momordica charantia* Leaves, R_f – retardation factor.

Table 4.22 Thin Layer Chromatography (TLC) fractions AEW methanol and their R_f values with solvent front of 7.8 cm

Fraction	Colour description	Distant traveled (cm)	R_f value	Yield in mg (%)
AEW3	Green	7.1 (active)	0.91	370 (46.84)
AEW2	Purple	5.6	0.72	420 (53.16)
AEW1	Deep brown	0.0	0.00	0.00 (00.00)

Key: AEW-*Aeschynomene uniflora* whole plant, R_f – retardation factor.

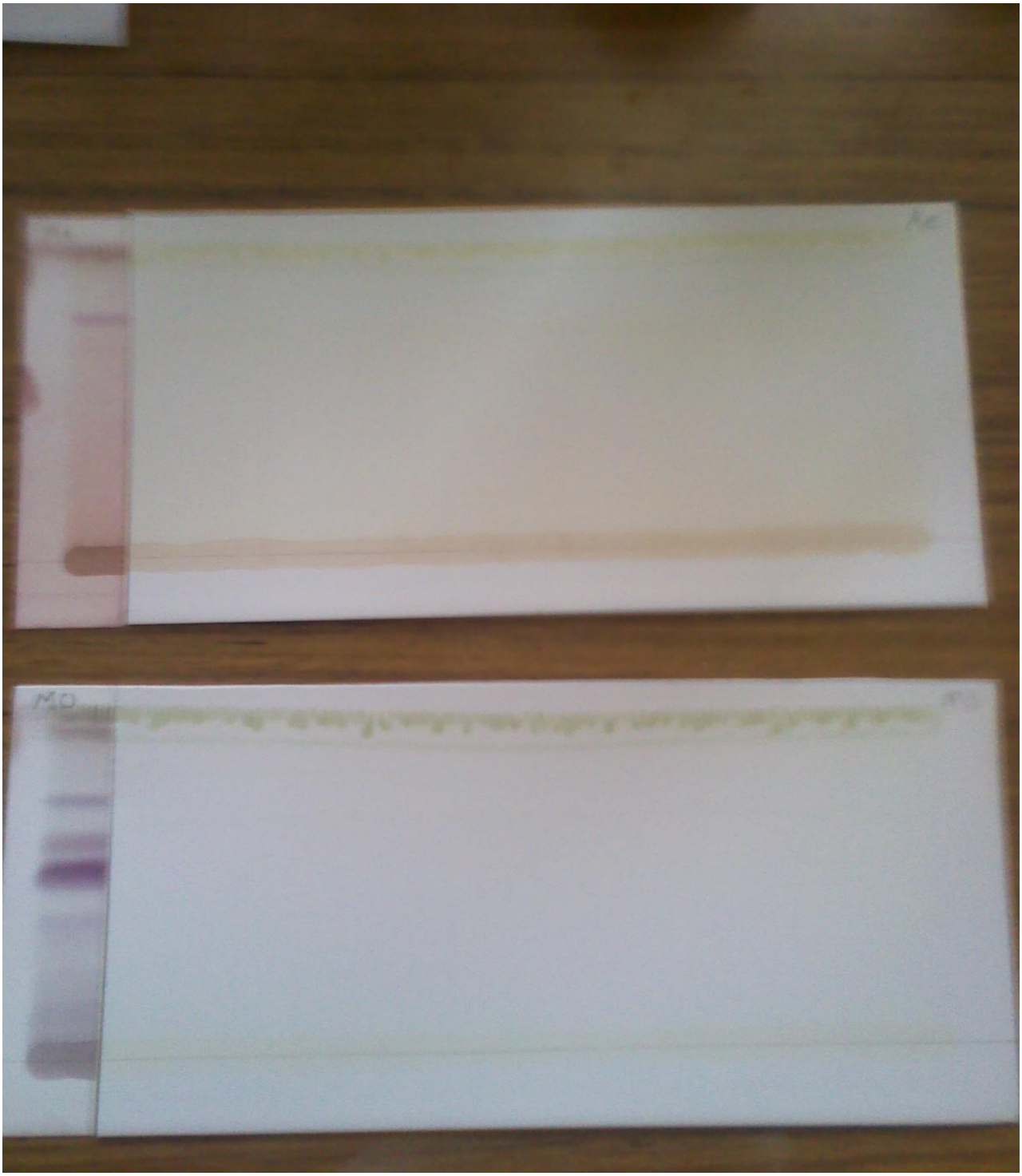


Plate 4.5 Showing Thin Layer Chromatography (TLC) bands for *Aeschynomene uniflora* above and for *Momordica charantia* below.

Table 4.23 Phytochemical screening of most active thin layer chromatography fractions for the presence of organic compounds.

Fraction	Colour	Phytochemical	result
MOL5	Deep purple	Tannins	++
		Alkaloids	++
		Cardiac glycoside	+
		Steroids	+
AEW3	Green	Tannins	+
		Alkaloids	++
		Cardiac glycoside	+
		Saponnings	+

Key: ++ indicate heavy presence of phytochemical substance, + indicate trace presence of phytochemical substance, MOL- *Momordica charantia* Leaves TLC fraction number 5, AEW- *Aeschynomene uniflora* TLC fraction number 3.

Table 4.24 Susceptibility of resistant of *E. coli* to most active thin layer chromatography fractions of *Momordica charantia* and *Aeschynomene uniflora*

Conc. of extract in µg/ml	Zones of inhibition (mm) MOL5	AEW3	*E. coli* NCTC 10418
30	0±0.2	0±0.1	7±1.2
40	9±1.1	8±1.4	8±1.6
50	10±2.4	10±2.2	8±1.4
60	12±1.2	11±2.1	12±2.2
70	13±2.1	12±1.4	14±1.2
80	17±1.2	14±2.2	18±1.3

P=0.05

Key: MOL- *Momordica charantia* Leaves TLC fraction number 5, AEW- *Aeschynomene uniflora* TLC fraction number 3, NCTC- National Culture Technology Commission.

Table 4.25 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of most active TLC fractions against *E. coli* isolates.

Fraction	Concentration in μg/ml	
	MIC	MBC
MOL5	30	40
AEW3	40	40

Key: MOL5- *Momordica charantia* Leaves TLC number 5 fraction, AEW- *Aeschynomene uniflora* TLC fraction number 3.

CHAPTER FIVE

5.0 DISCUSSION

5. 1 Demographic Data

Escherichia coli can cause a variety of infections in humans due to spread from the intestinal flora causing the infections as a result of direct or indirect faecal-oral from humans or animals (Paul, 2003). Therefore understanding the way we live is of great importance in the control of diarrhoea (Brussow *et al*., 1992). The demographic data obtained in this study did not differ much with that of Ibekwe *et al*. (2001). Findings from the demographic data revealed that infants' of farmers and traders have the highest prevalence of diarrhoea with number infected of 108 and 44 (Fig. 4.1). This could be as a result of higher number of the farmers and traders in the study area as compared to other occupations. It can also be as a result of their carelessness, lack of adequate education on the disease and its mode of transmission (Toledo *et al*., 1983).

Culture and tradition gave more emphasis on male than female formal education in the study area could be the factors responsible for high number of the secondary and adult literacy, with number of 62 and 61 diarrhoeic respectively than primary and secondary with 17 and 40 counts respectively (Fig. 4.2) in level of infants fathers formal education. As well as the same factors in the higher number obtained from secondary and none formal education in mothers/care givers with number of 51 and 55 respectively with lower count of 30 only in tertiary education (Fig. 4.3). Similar finfings were reported also by Hasony (1996).

Although majority of the respondents obtained their infants' drinking water from pipe borne lines (Fig. 4.4), there could be problem of storage and other utensils used that may bring contamination (Paul, 2003). Only 16 and 5 mothers treat the infants' drinking water by boiling or filtration respectively (Fig. 4.5). Most infants' parent uses pit latrine with a total of 110. Majority

of the population wash their hands after toilet with soap and water with number infected infants of 87. Only 14 mothers diagnosed infants with diarrhoea before giving treatment. Most mothers/care givers introduce food to the infants at the age of 5-6 months with a total number of 101 infected. Factors such as poverty, lack of basic amenities and ignorance have attributed with many diseases transmission including diarrhoea (Agbonlahor and Odugbemi, 1982).

5.2 *E. coli* Isolates

E. coli as Gram negative rod could easily be differentiated from other bacteria through Gram staining technique and biochemical reactions. Its vast specific biochemical reactions have serve as means of its identification (Kawo *et al*., 2009 and Abalaka *et al*., 2010). The results were compared with biochemical reactions of some *Enterobacteriae* and other organisms as described by Cheesbrough (2006).

5.3 *E. coli* Serotypes

Findings in this study revealed that ETEC and EHEC have the highest occurrence than EPEC and EIEC with serovars of O148:H28 and O157:H7 accounting for 48 (23.53%) and 41 (20.10%) respectively (Table 4.1). The infective dose and a range of transmission routes could be the factors accounting for the prevalence of these serovars (Kothary and Babu, 2001).

5.4 Prevalence of Diarrhoea

High prevalence of diarrhoea 34.81% (n=204) caused by *E. coli* isolated from infants attending some hospitals in Kano state, Nigeria obtained from the findings of this research work is alarming (Table 4.2). The high prevalence could be attributed to the cosmopolitan nature of the state in terms of human population as well as overcrowding of the buildings/homes (Christine and Valdemar, 2004). The results also shows that Murtala Muhammed Specialist Hospital, Hasiya Bayero Paediatrics and Muhammed Abdullahi Wase specialist hospitals all located in the

Kano city metropolitan has the highest prevalence of infants diarrhoea with 33 (16.18%), 29 (14.22%) and 25 (12.25%) tested positive respectively. Other factors may include the culture of rearing animals at home which could serve as source of the *E. coli* and other enteric bacteria, lack of good toilets and poor domestic wastes disposing system (Strachan *et al*., 2001). The least prevalence of 5.39% (n= 11) found at Takai NYSC community health center could be due to less population and vast free land of the area.

The results of the age range and gender distribution of diarrhoea cases of infants attending some hospitals in Kano state do not differ much from previous findings (Isyaku *et al*., 2008). Males have higher prevalence than females with number tested positive 113 (19.28%) and 91 (15.53%) respectively (Table 4.3). This may be due to the fact that male infants are more agile than females. This results in more chance of contacting the diarrhoea causing bacteria in males than females. High prevalence occurred in the age group of 24-35 for males with 31 (27.43%) and 24-35 and 36-47 months for females with prevalence of 25.27% (n = 23) each. Indeed the male agility and slight deference in the immunity could have been responsible. Amongst the age groups, 24-35 months infants are more infected with 54 (26.47%) than other age ranges. The weaning practice that is very common culture at this age range could be the reason to account for this. While the least prevalence occurred in the age group of 0-11 months with prevalence of 15.04% (n=17) could result despite their low immunity, due to close parental care. This low prevalence also revealed in positive male and female with number tested positive of 17 (15.04%) and 13 (14.29%). The drop in the prevalence total positive with 31 (15.02%) may indicate development of better health condition after stabilization from the weaning problems such as fever, vomiting, diarrhoea, dehydration, constipation and so on. Development of better immunity could be another factor (WHO, 2009). Number of infants that were tested positive in males and

females with age range of 48-59 months were 17 (15.04%) and 14 (15.38%) respectively. This might have been due to slight deference of the male immunity than females infants (Wenneras *et al*., 1992).

5.5 Antibiotic Resistance

Resistance of *E. coli* due to transfer of plasmid and other factors as well studied by (Donnenberg *et al*., 1993 and Cheetham and Katz, 1995) could be responsible for the results of susceptibility pattern of the *E. coli* isolated from infants' diarrhoea attending some hospitals in Kano on some commercial single discs antibiotics (Table 4.4). The result shows that the *E. coli* isolates were more susceptible to ciprofloxacin, ceftriaxone, augmentin and ofloxacin antibiotics with number of susceptible isolates of 169 (83%), 159 (78%), 155 (76%) and 153 (75%) respectively. The isolates are however resistant to ampicillin, amoxycillin, sulphamethoxazole/trimethoprim and tetracycline with number susceptible isolates of 182 (89%), 170 (83%), 105 (51.47%) and 94 (46%) respectively. No significant difference between the means for number resistance and susceptible organisms (P = 0.1302).

Ampicillin and amoxicillin have the highest single antibiotics disc resistance with 11 (12.5%) and 10 (11.4%) of the isolates respectively This could be as a result of many factors such as long term usage/misuse, production of TEM-type β-lactamases often produced by most *E. coli* to hydrolyse penicillin and cephalosporins (Abalaka *et al*., 2010). For the multiple antibiotic resistance pattern high frequency of occurrences were obtained in AMP, SXT, AML with 6 (2.94%), AMP, SXT with 5(2.45%) and SXT, AML with 4 (1.96%) number of isolates with the pattern. Highest number of antibiotics of 7 in only one isolate with 0.49% occurrence of resistance phenotype includes; OFX, AMP, SXT, CN, AML, CIP, TE (Table 4.5). This result indicates that *E. coli* resistance to antibiotics is increasing at alarming rate. However, the lower

percentage in multiple antibiotics resistance as compared to the single discs indicates multiple/combination drugs treatment is the best means of treatment (Sule and Agbabiaka, 2008).

5.6 Plant Extracts

The five solvents; ethanol, chloroform, water, methanol and petroleum ether used for the extraction of the different parts of the study plants yielded a total of 136.69g and 107.54g of *Momordica charantia* Linn and *Aeschynomene uniflora* E. may respectively (Table 4.6). This showed that *Momordica charantia* has more fibre materials and phytochemicals than *Aeschynomene uniflora*, thus equally MOL and AEL has more of these substances than other plants parts. In both plants water has more solubility than chloroform as well as methanol than petroleum ether. This could be as a result of water is more polar than chloroform and therefore will have much more dissolving power (Stephen *et al*., 1998) as "like dissolved like". Polar solvents have large dipole moments (partial charges) while nonpolar contain bonds between atoms with similar electronegativities. The constituents of the plant materials (phytochemical) may have effect on the yield obtained on specific solvent used. Thus having higher weights of yield of extracts both *Momordica charantia* Linn and *Aeschynomene uniflora* plants in water and methanol extracts than chloroform and petroleum ether cold be as a result of the above factors and many more. Solvents could also be either participatory; to dissolve the reactants or participatory to serve as an acid (proton donor), base (removing proton) or nucleophile (donating a lone pair of electron). Fractionation between water and chloroform yielding a total of 48.25g and 18.05g in *Momordica charantia* and 36.33g and 12.12g in *Aeschynomene uniflora* revealed that water is a better extractor solvent in water: chloroform fraction in both plants than chloroform. Same result pattern was obtained in methanol: petroleum ether fraction in both the two plants also revealed that methanol is better extractor than petroleum ether. These results of

high yield obtained from water and methanol conformed to common methods used by most traditional herbalist and many previous researchers (Abalaka *et al*., 2010 and Aliyu *et al*., 2010).

5.7 Preliminary Phytochemical Substances

Preliminary phytochemical screening of *Momordica charantia* Linn and *Aeschynomene uniflora* E. May plants parts extracts showed the presence of carbohydrates, tannins, saponnins, glycosides etc. (Tables 4.7 to 4.11). The presence of these plants metabolites conforms to the findings reported previously by various researchers as they try to identify a number of useful organo-chemical substances of plant origin (Abalaka *et al*,. 2010, Kawo *et al*., 2009 and Abdul, 1986). These compounds thus far identified, are now being widely used and considered to promote human health (Abalaka *et al*., 2009). The presence of these organic substances might have been responsible for the *Momordica charantia* and *Aeschynomene uniflora* plants' parts activity on the *E. coli* isolates used in this study. Alkaloid is one of the largest group of nitrogenous substances found naturally in plants, usually bitter containing pharmacologically active substances such as atropine, caffine, coniine, morphine, nicotine, quinine, strychnine, melonin and curare. Example of synthetic alkaloid is procaine. Tannins have been shown to form irreversible complex with praline-rich-protein which lead to inhibition of cell well protein synthesis, a property that may explain the mode of action of these extracts. Gibbons *et al*. (2003) reported that some phytochemicals have resistance modulating activities against stains of microorganisms which means that the extracts materials used in this study can act as antibiotic resistance inhibitors. Previous research investigations reveled that these phytochemicals are distributed in various plant parts in different concentrations (Okeke *et al*., 2001) and (Becker *et al*., 2005). This is in line with the findings of this study as the activity of the different plants parts extracts varies.

5.8 Pharmacology and Haematology

The extracts of *Momordica charantia* Lin and *Aeschynomene uniflora* E. may not be harmful to human consumers at lower therapeutic dose (Hassan and Umar, 2007). This is remarkably showed in (Table 4.12) that no significant difference between the means before and after treatment (P=0.65 14 df) and no death of any mouse occurs. Although there may be some slight effect on the consumers (Table 4.13 and 4.14) showing haematological screening of albino mices' blood after two weeks experiment on doses of active plants TLC fraction used in this study. Only results of higher doses of 2900 mg/kg/day and 5000mg/kg/day were significantly difference from the control (P=0.05). Therefore, even though there may be no effect on the consumers at lower doses, caution should be taken to avoid over dose as this could lead to liver problems and other body normal metabolic processes hindrances as also pointed by Ahiadeke *et al*. (2006); Okogun (2002). Generally, MOL extracts has more effect than AEW on the albino mice blood cells.

5.9 Susceptibility of *Escherichia coli* Isolates to Plant Extracts and TLC Fractions

According to NCCLS (1996) any plant material should be considered an effective therapeutic agent if it extracts produces zones of inhibition of between 15-22mm on the target pathogenic organism. Activity of plant extracts to test bacteria is normally expressed *in-vitro* as zones of inhibition in millimeter (greater than or equals to 7mm) around the substance on test (Gislene e*t al*., 2000) is a similar trend observed in this study. The activity of the crude extracts was compared with those of the standard antibiotics. An organism is considered sensitive only when the diameter of zone of inhibition is either equal to the control or wider than or not more than 3 mm smaller than the control (Becker *et al*., 2005). The results of the susceptibility pattern of *E. coli* isolated from infants diarrhoea to various solvents extracts of the test plants parts in this

study showed undoubtedly and clear that the ethanol, aqueous and methanol extracts are active while chloroform and petroleum ether extracts are not active against the isolates (Tables 4.15, 4.16, 4.17 and 4.18).

About 65/204 (32 %) and 29/65 (45%) resistant isolates obtained from antibiotic and plant extracts susceptibility tests in this study revealed that the isolates were more susceptible to the orthodox antibiotics than the plant extracts. The low activities of *E. coli* isolates observed on the plant extracts against the orthodox commercial antibiotics discs could be due to the following factors; organic extracts are in compound not in pure state like the synthetics antibiotics, therefore the secondary active metabolites could be present in low concentration or even be masked. (Sule and Agbabiaka, 2008) reiterated in their study were there was no activity against *E. coli* on *Ocimium gratissimum* leaf extract. Isyaku *et al*. (2008) explained further that Gram positive bacteria are often found to be more susceptible to plant extracts than the Gram negative, for Gram negative organisms has an additional outer phospholipid membrane and porines that make the cell wall impermeable to lipophilic and hydrophilic solutes. The test isolates used in this study are all Gram negative *E. coli* hence their low susceptibility to the plant extracts could be attributed to the impermeability of their cell wall.

Higher activities were obtained in the leaves and whole plants of both *M. charantia* and *A. uniflora* plants (Tables 4.15 and 4.16) respectively. Leaves are the center for photosynthesis, it then serves as a store for most of the plant manufactured organic molecules. It also contained many accumulated waste products of metabolism and periodically plants shed their leaves to get rid of these metabolites (Roberts, 1986). This could be the reason of the high susceptibility of the isolates to the leaves and whole plant in this study; number of isolates susceptible to the leaves methanol, ethanol and whole plant aqueous extracts of 52 (80%), 50(77%) and 48(74%)

respectively (Table 4.17) and also susceptible to whole plant and leave methanol extracts with 49(75%) and 47(72%) respectively (Table 4.18). The high organic molecules in leaves as a result of photosynthesis could also be the reason of higher mean weights of the MOL, MOW and AEL, AEW of 40.60g, 38.35g and 37.90g, 24.19g respectively (Table 4.6), as the leaves constitute most of the whole plants parts. These results supported the traditional use of these plants in the treatment of diarrhoea in the study area. The extraction capacity of these solvents has already been revealed by many previous researchers (Isyaku *et al*., 2008).

The 48(74%) and 43(66%) numbers susceptible (Table 4.17) for MOL and MOW aqueous extracts could serve as evidence of the local use of the plant in the treatment of diarrhoea for infants and mothers in the study community (Abalaka *et al*., 2010) as traditionally water preparation is normally made.

Momordica charantia leaves have more surface area than *Aeschynomene uniflora* leaves, therefore could store more of the synthesized organic molecule (Tables 4.21, 4.22 and 4.23) and might have more waste products as well for having more excretory glands (Anafi *et al*., 2010). This could have been the reason of MOL5 fraction more active generally than the AEW3 TLC fraction (Table 4.24).

5.9.1 MIC and MBC of Extracts and TLC Fractions

The general trend in this experiment is that lower concentrations inhibited the organisms while higher concentrations cause cidal effects on the organisms. According to NCCLS (1996) reported that for any plant material of clinical importance, it should be effective *invitro* at the concentration of 1.0 mg/ml. The lower the MIC and MBC of a given extract the higher the efficacy of such extract against the test organisms (Mukhtar and Tukur, 2000). The present work agrees with NCCLS position of *invitro* 1mg/ml and 15-22mm zones of inhibition on ethanol,

aqueous and methanol extracts of *Momordica charantia* and *Aeschynomene uniflora* and the TLC fractions respectively (Tables 4.19, 4.20 and 4.25). This finding suggests that the two plants could be considered to provide an effective therapeutic activity (Abalaka *et al*., 2010). The lower MIC and MBC values of 31.2mg/ml obtained in ethanol, aqueous and methanol extracts of *Momordica charantia* and *Aeschynomene uniflora* respectively (Tables 4.10 and 4.20) showed that these solvents are good extractors of the active components of *Momordica charantia* and *Aeschynomene uniflora*.

5.10 Thin Layer Chromatography Fractions

Different solvent systems were studied, however only chloroform-ethyl acetate in the ratio of 1:3 was the best mobile system that could separate the fractions. Tables 4.21 and 4.22 show the TLC of MOL and AEW methanol fractions, their R_f value and solvent front. About 9 and 3 different numbers of fractions were obtained for the MOL and AEW TLCs. The results showed that MOL contained more organic compounds than AEW. Similar results were reported by Salisu and Garba (2008) that some plants may contain more phytochemicals then others and different plants may contain same or different phytochemicals substances. It is well known that in TLC the compounds are moving between two phases; the stationary phase - silica gel absorbed onto the glass plate and the mobile phase – substances are separated based on their molecular weights (other factors may include solvent used and the type or constituents of the compound in test) such that substances of higher molecular weight travel short distances compared to those of lower molecular weights which travel far in the solvent system thus producing different retention factor R_f values for the fractions. Abalaka *et al*. (2010) reported that the lighter the molecular weight of a substances the further it travels on TLC plate and the weaker the antibacterial activity. This could account of the low activity of AEW3 as compared to the MOL5 on the *E.*

coli isolates (Table 4.24) considering the distances travelled in the TLC plates by the AEW3 and MOL5 of 7.1cm and 4.0cm with R_f values of 0.9 and 0.51 respectively. Yield of the fractions MOL5, 70mg (2.60%) and AEW3, 370mg (46.84%) does not have any significant effect on the activity to the bacterial *E. coli* isolates (Tables 4.21 and 4.22). Fractions with small yield could be more active than fractions with large yield and vice versa (Cushnine and Lamb, 2005).

5.10.1 Further Phytochemical

The organic constituents of MOL TLC fraction exceeded that of the AEW by having more of tannins and added steroids (Table 4.23). This may be the reason for MOL TLC fraction being more active than AEW fraction to the *E. coli* isolates (Tables 4.24 and 4.25). The presence of these metabolites might have been responsible for the activity of these fractions to the bacterial isolates in this study. Pervious researchers such as Cushine and Lamb (2005) and Abalaka *et al*. (2010) reported the antimicrobial and resistance modulating potentials of some natural occurring compounds of plant origin. Tannins are known to form irreversible complex with proline-rich-protein which would lead to inhibition of cell wall protein synthesis (Zhu, 1990).

CHAPTER SIX

6.0 CONCLUSION, SUMMARY AND RECOMMENDATIONS

6.1 Conclusion

This study has shown that the prevalence of infants diarrhoea is 34.8 % (n=204) of the 586 sample examined. This signifies that the prevalence of infants' diarrhoea mostly caused by infectious agents such as *E. coli* is alarming in the study area. This study revealed the prevalence of infants' diarrhoea in Kano state of 34.81% to be on the higher side. The risk factors include age of infants, feeding practice, parental financial and educational status, awareness on the modes of transmission and taking measures, adequate sanitation and infants' drinking water. Both single and multiple resistances to orthodox antibiotics are also very common amongst the *Escherichia coli* isolates. Findings revealed that poor sanitation and personal hygiene, misuse and abuse of drugs could be some of the risk factors associated with the number of resistance and the pattern obtained.

The traditional plants used locally in this study to cure diarrhoea proved to contain some phytochemical substances such as carbohydrates, alkaloids, tannins, glycosides, steroids, saponins flavonoids, resins and anthraquinones especially in the leaves and the whole plants parts. Although aqueous and ethanol proved to yield more extracts than other solvents, however methanol extracts found to contain more of the phytochemicals gives more activity to the *E. coli* isolates. All the solvents extracts show some activity on the isolates except the petroleum ether extracts which showed least activity. The extracts do not have significant pharmacological and haematological effect on the laboratory mice tested. There was no death occurred of the mice after the experiment. The *E. coli* isolates are more susceptible to the leaves and whole plants extracts in both *Momordica charantia* and

Aeschynomene uniflora **plants. MIC and MBC of 31.2 mg/ml on the isolates was obtained from the methanol extracts. TLC of most active extracts gave 9 and 3 number of fractions for MOL and AEW respectively. The fractions proved further to contained phytochemical substances and shows activity on the isolates. The feasibility of integrating the use of these and related indigenous plants extracts into orthodox medicine form and the possibility of initiating local pharmaceutical industries that could utilize the plants as a raw materials could be one of the most useful success of this research. The susceptibilities of the various strains of** ***E. coli*** **to the extracts and the antibiotics will be of added knowledge for treatment of infantile diarrhoea in Kano state and Nigeria in general.**

6.2 Summary

6.2.1 Findings and contribution to knowledge from this study revealed the followings**;**

i. Infants diarrhoea is mostly caused by microbial infections including ***E. coli*** **not as thought by the traditional belief of such as teething, weaning etc.**

ii. ***Escherichia coli*** **known as opportunistic pathogens causing urinary tract infections is also responsible for infantile gastroenteritis as well as many different types of diarrhoea; prolong diarrhoea with fever and vomiting mainly in infants less than 2 years due to bacterial adhering to epithelial cells, watery (secretory) - travellers diarrhoea in infants and adults due to production of plasmid mediated toxins, life-threatening haemorrhagic diarrhoea (colitics) without pus cells and often without fever in all ages and lastly dysentery (similar to Shigellosis) with blood mucus and many pus due to bacterial invading and multiplying in epithelial cells.**

iii. The active plant extracts and fractions have revealed *invitro* activity against the test isolates.

iv. Toxicity study revealed that the extracts are safe at the dosages used in this study.

v. Ethanol, methanol and aqueous crude extracts in mg/ml and TLC fractions in µg/ml MOL5 and AEW3 of *M. charantia* and *A. uniflora* have shown activity against most of the isolates at different concentrations.

vi. *M. charantia* and *A. uniflora* extracts were safe for therapeutic consumption at concentration up to 1600mg/kg body weight.

vii. There is more concentration of the active substances in the leaves and whole plants in the two plants compared to other parts.

viii. Petroleum ether and chloroform are least active on the *E. coli* isolates and yield least weights of extracts of *M. charantia* and *A. uniflora*.

5.3 Recommendations

In view of the findings of this study, the following recommendations were made.

1. Effort should be made to further investigate the antibacterial as well as other antimicrobial activities of *M. charantia* and *A. uniflora* so as to fully harness the potentials of these plants. Further studies should be focused on the molecular weights of the phytochemicals in relation to their activity on test organisms. The use of the plants extracts in treatment of complicated diseases such as HIV should also be investigated.
2. The Government should provide a Ministry for traditional medicine at state and Federal levels. This is with a view to harnessing, ascertaining quality and safe traditional medication to the general public. The Government should also provide a research

institute on traditional medicine in every state in the Federation. This should serve as a center for research on traditional medicine. Modernization in the packaging of traditional medications; focus in the indication, the list of contents, dosage, manufacture date, expiry date and description for usage is important and should be put into practice.

3. Parents and caregivers should take extreme care on the sanity of food and drinking water for their infants. This could be achieved through exclusive breast feeding practice, use of bottled and/or boiled water for infants drinking purposes. Sanitary measures and precautions should be taken during the preparation of home-made infants' food formulations. This could be achieved through seminars on quality control and contamination in the infants' food preparation to be delivered in health centers during antenatal and post natal care visits of the pregnant/nursing women. Moreover, the feeding should also be introduced at the right time.

4. **A gene bank for the plants used and other related medicinal plants should be kept in the higher institutions of learning and research institutions. This will help prevent the plants from extinction and also provide very much easy access for future research.**

5. The states and the federal assemblies should pass a bill to compel all companies that produce products for infants to support researches on diarrhoea and related fields in tertiary institutions.

6. National policy on drugs disbursement, retail and usage should be observed strictly to avoid the antibiotics resistance shown by the *Escherichia coli* strains/isolates in this study.

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APPENDICES

Appendix I. Re-Request for ethical approval

Appendix II. Introduction letter

Appendix III. Patient consent form

Research Title: Characterization and Susceptibility of *Escherichia coli* Isolated from Infants Diarrhoea to Extracts of *Momordica charantia* and *Aeschynomene uniflora*.

Investigators: Mal. Isma'ila Ahmed, Prof. O. S. Olonitola, Prof. S. E. Yakubu and Prof. S. A. Ado of Department of Microbiology, Ahmadu Bello University, Zaria.

Description: As your infant/child been diagnosed by the physician at this hospital you are solicited to provide a stool sample of the child. This will be used for the research study mentioned above. We are to isolate and identify the diarrhoea causing bacteria from the samples collected and test if some plants extract (garahuni and bagaruwar kasa) and conventional antibiotics will have effect on the bacterial isolate so as to improve on the treatment of diarrhoea cases of our wards/children.

A questionnaire will be provided to get the following information; age, sex, history of therapy, clinical manifestation, occupation, etc. The findings of this study will be used for research purpose only. Sample collected will be assigned number instead of name.

I …….…… consent to my ward/child samples of diarrhoea to be collected by Mal. Isma'ila Ahmed a PhD student of Department of Microbiology, Ahmadu Bello University, Zaria and a staff of Kano University of Science and Technology, Wudil.

_____ _____

Signature of mother/care giver Date

_____ _____

Signature of person collecting consent Date

Appendix IV. Data collection questionnaire obtained from mothers/care givers of infants (0-59 months) attending some hospitals in Kano state, Nigeria.

Questionnaire No.: ……/…….

Title of the Study: Characterization and Susceptibility of *Escherichia coli* Isolated from Infants Diarrhoea to extracts of *Momordica charantia* and *Aeschynomene uniflora*.

Eligibility: Children > 0-5 years that have passed more than three lose/watery stool in the past 24 hours.

Name of child…… Sex: …… Age: ……..months
Name of care giver: …….. Sex: …… Age: ……..years
Name of Interviewer: …….. Signature and Date: ……..

Q1. Main occupation of father
(a) Farmer (b) Trader (c) civil servant (d) others (e) Not working

Q2. Level of father's formal education
(a) Primary (b) Secondary (c) Tertiary (d) Adult literacy (e) None

Q3. Level of mother's/care giver's formal education
(a) Primary (b) Secondary (c) Tertiary (d) Adult literacy (e) None

Q4. Main source of child drinking water
(a) Well (b) Pipe-borne (c) Borehole (d) Pond/stream (e) Others: ……

Q5. Treatment of drinking water
(a) Non (b) Boiling (c) Filtering (d) Others (specify): ……

Q6. Type of toilet
(a) pit latrine (b) Open- field (c) Water closet (d) Stream (e) Others:……

Q7. What did you used to wash your hands after cleaning your child diarrhoea?
(a) Water only (b) Soap and water (c) Detergent and water (d) Sand and water
(e) Others:……..……

Q8. How do you treat diarrhoea?
_____

Q9. What do you think is the cause of the diarrhoea? _____
_____

Q10. Have you used ORT/ORS/ SSS? _____

Q11. When your child has diarrhoea, how does it affect his/her appetite?
(a) Increase (b) Decrease (c) No effect

Q12. Are you still breast feeding the child? (a) Yes (b) No

Q13. At what age did you introduce foods other than breast milk to the child?
(a) Less than a month (b) 1-2 months (c) 3-4 months (d) 5-6 months (e) Above 6 months

Q14. Has there been vomiting? _____

Q15. Does the child have fever? _____

Q16. Degree of dehydration. (a) None (b) Moderate (c) Severe

Q17. Child weight (kg) _____

Thank you very much for your anticipated cooperation.

RESEARCH RESULT:

Presence of *E coli* organism (a) yes (b) no

Type of *E. coli* strain: ……

Most active plant extract: ……..

Most active antibiotics; …….

Appendix V. Demographic data obtained from diarrhoea infants care giver attending some hospitals in Kano state, Nigeria

1. Age (months)
   - 0-11 - 30 (5.12%), 12-23 - 38 (6.48%)
   - 24-35 - 54 (9.22%), 36-47 - 51 (8.70%)
   - 48-59 - 31 (5.29%)
2. Sex: M - 113, F - 91
3. Main occupation of father
   - (a) Farmer - 108 (b) Trader - 44
   - (c) Civil servant - 35 (d) Others - 13
   - (e) Not working - 04
4. Level of father's formal education
   - (a) Primary - 17 (b) Secondary - 62
   - (c) Tertiary - 40 (d) Adult literacy - 61
   - (e) None - 24
5. Level of mother's/care giver's formal education
   - (a) Primary - 26 (b) Secondary - 51
   - (c) Tertiary - 30 (d) Adult literacy - 42
   - (e) None - 55
6. Main source of child drinking water
   - (a) Well - 40 (b) Pipe-borne - 88
   - (c) Borehole - 59 (d) Pond/stream - 03
   - (e) Others: …… 14
7. Treatment of drinking water
   - (a) Non - 176 (b) Boiling - 16
   - (c) Filtering - 05 (d) Others (specify) - 07
8. Type of family toilet in use
   - (a) Pit latrine - 110 (b) Open- field - 22
   - (c) Water closet - 72 (d) Stream - 00
   - (e) Others - 00
9. What did you used to wash your hands after cleaning your child diarrhoea?
   - (a) Water only - 49 (b) Soap and water - 87
   - (c) Detergent and water - 33 (d) Sand and water - 12
   - (e) Others: ash, tissue - 23
10. Treatment of diarrhoea
    - Local treatments - 64 Hospital, clinic or chemist - 127
    - Lab. diagnosis - 14
11. Cause of the diarrhoea
    - Includes; teething, weaning, fever, file, worms, etc in this order
12. Use of ORT/ORS/ SSS
    - Yes - 98, No - 106
13. When your child has diarrhoea, how does it affect his/her appetite?
    - (a) Increase - 38 (b) Decrease - 106 (c) No effect - 64
14. Still breast feeding the child
    - (a) Yes - 46 (b) No - 158

Appendix V. Continued

15. Age did you introduce foods other than breast milk to the child?

(a) Less than a month	-	00	(b) 1-2 months	-	00
(c) 3-4 months	-	12	(d) 5-6 months	-	103
(e) Above 6 months	-	89			

16. Vomiting

Yes	-	32	No	-	172

17. Does the child have fever?

Yes - 51, No	-	119,	Sometime	-	34

18. Degree of dehydration.

(a) None	-	96	(b) Moderate	-	65
(c) Severe	-	43			

19. Child weight (kg)

<3.0kg	-	78,	3.1-3.9kg	-	92
>4kg	-	34			

…. end …

Thank you.

Appendix VI. Structure of Gastrointestinal Track (GIT).

Appendix VII. Biochemical reactions of some enterobacteria and other enteric organisms

												KIA Medium				
Species	**Urea**	**VP**	**ONPG**	**Lact**	**Man**	**Glu**	**Suc**	**Ox**	**Cit**	**Mot**	**Ind**	**LDC**	**Slope**	**Butt**	**H_2S**	**Gas**
Escherichia coli	-	-	+	+	+	+	*d*	-	-	+[5]	+[2]	+	Y[6]	Y	-	+[2]
Shigella species	-	-	-?	-	*d*	+	-1	-	-	-	*d*	-	R	Y	-	-3
Salmonella typhi	-	-	-	-	+	+	-	-	-	+	-	+	R	Y	+weak	-
Salmonella paratyphi A	-	-	-	-	+	+	-	-	-	+	-	-	R	Y	-	+
Most other Salmonellae	-	-	-	-	+	+	-	-	+	+	-	+	R	Y	+2	*d*
Cirobacter freundii	*d*	-	+	+ Late	+	+	*d*	-	+	+	-3	-	R or Y	Y	*d*	+
Klebsiella p. pneumonia	+	slow	+	+	+	+	+	-	+	-	-3	+	Y	Y	-	+
Enterobacter species	-	+	+	+	+	+	*d*	-	+2	+	-	*d*	Y	Y	-	+
Serratia marcescens	*d*	+	+	*d*	+	+	+	-	+	+	-	+	R or Y	Y	-	*d*
Proteus vulgaris	+	-	-	-	-	+	+	-	*d*	+	+	-	R	Y	+	*d*
Proteus mirabilis	+	*d*	-	-	-	+	*d*	-	+2	+	-	-	R	Y	+	+
Morganella morganii	+	-	-	-	-	+	-	-	-	+5	+	-	R	Y	-	*d*
Providencia species	*d*	-	-	-	*d*	+	*d*	-	+	+	+	-	R	Y	-	*d*
Yersinia enterocolitica4	+	slow	-	+	-	+	+	+	-	-	+	*d*	-	R	Y	-	-
Vibrio cholera	-	*d*	+	-24h	+	+	+	+	*d*	+	+	+	R	Y	-	-
Vibro parahaemolyticus	-3	-	+	-	+	+	-	+	*d*	+	+	+	R	Y	-	-

Source: Cheesbrough (2006).

Key: LDC = Lysine decarboxylase, **VP** = Voges-Proskauer, **ONPG** = β-galactosidase, **Lact** = Lactose, **Man** = Mannitol (mannite), **Glu** = Glucose, **Suc** = Sucrose, **Ox** = Oxidase test**, Cit** = Citrate test, **Mot** = Motility, **Ind** = Indole test, **Urea** = Urease, **H_2S** = Hydrogen sulphide (blackening), **R** = Red-pink (alkaline reaction), **Y** = Yellow (acid reaction), **d** = Different strains give different results.

Note:

1. *S. sonnei* ferments sucrose slowly.
2. A minority of strains give a negative result.
3. A minority of strains give a positive result.
4. Test should be incubated at 20-28^{o}C.
5. A few strains are non-motile.
6. A few strains give reactions similar to *Shigella* species.
7. *S. sonnei* is ONPG positive

Appendix VIII. Nomination as a Participant for STEP-B Diarrhoea Project

Appendix IX. Confirmation of Award for Local Study Fellowship

Appendix X. Determination of O antigen (SIENKEN^{R})

Preparation of O antigen suspension

- Inoculate the problem strain on tryptone soya agar (TSA) and incubate overnight at 37ºC.
- Suspend some of the growth from the TSA (approximately the equivalent to the confluence growth of 1 cm 2) in 2 ml of 0.85% NaCl saline solution (SS) and adjust the bacterial concentration comparing with the tube number 6 of the MacFarland Barium Sulfate Scale ($1.8x10^9$ bacteria per ml). Make this step in duplicate (in two tubes).
- Heat one tube in a boiling water (1 h at 100ºC) and the other in a autoclave (2.5 h at 121ºC) to inactivate K antigen. O antigen suspensions autoclaved at 121ºC for 2.5 h are only tested with O antisera O8, O9, O20 and O101, whereas O antigen suspensions heated at 100ºC are assayed with all O antisera.
- Allow suspensions to cool and add to each tube 2 ml of formalinized (0.5%,v/v) SS containing gentian violet (0.005%, w/v). Keep bacterial suspensions at 4ºC for no more than two months.

Presumptive serogrouping

- Polystyrene microtitre plates with 96 "V" wells are used instead of classical glass tubes. Thus, a high amount of antiserum is saved as we use microlitres. Dilute the pure antisera (1x) to 1/80 by using SS with sodium azide (1% NaN3, w/v). For 1/80 dilution, add 0.1 ml of serum to 7.9 ml of SS. Refrigerate diluted antisera at 4ºC. Never freeze antisera once diluted**.** It is recommended to prepare only 8 ml of diluted antiserum, because pure antiserum (1x) keeps better than diluted antiserum.
- Test O antigen suspension heated at 100ºC with all the antisera. If the result with all of them is negative, then use the bacterial suspension autoclaved and assay it with antisera O8, O9, O20 and O101. Add 50 µl of diluted O antiserum (Dilution 1/80). Add 50 µl of O antigen suspension. Cover the microtitre plate and incubate it at 37ºC overnight.
- Examine for agglutination. Negative reactions are indicated by a sharp point, whereas positive reactions by a carpet. The reactions can be read after 8 hours of incubation, but overnight incubation usually results in more clear-cut reactions. If the problem strain is positive with any of the antisera used, realize confirming test. If the strain is negative with all antisera, it is considered not-typeable (NT).

Confirmation of serogroup: O antigen titration

- Prepare serial dilutions of the O antiserum in the microtitre plate to give dilutions of 1/80 to 1/40,960. Add 100 µl of the diluted O antiserum (1/80) to the first well and 50 µl of SS to the remaining 11 wells. Transfer 50 µl from well 1 to well 2 and mix. Transfer 50 µl from well 2 to well 3, etc.., on through well 10; discard 50 µl of the mixture from well 10. Wells 11 and 12 serve as negative control.
- Place 50 µl of O antigen suspension into each of the 12 wells, from well 12 to well 1. Final dilution of the antiserum will be 1/160 in the first well and 1/81,920 in the last one.
- Incubate the microtitre plates to 37ºC overnight and examine for agglutination. Strains showing agglutination in dilutions of 1/160 or greater are considered to contain the same O antigens as the antiserum and thus constitutes final identification of the somatic antigen of the microorganism. However, when the O antiserum has a low titre, strains showing agglutination in dilutions of 1/80 can be considered positive.

Appendix XI. One-way Analysis of Variance (ANOVA) weight of extracts

The P value is < 0.0001, considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.367 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
Ethanol vs Chloroform	6.770	7.596 ***	P<0.001
Ethanol vs Aqueos	-0.7800	0.8752 ns	P>0.05
Ethanol vs Methanol	5.343	5.994 **	P<0.01
Ethanol vs Pet. ether	8.823	9.899 ***	P<0.001
Chloroform vs Aqueos	-7.550	8.471 ***	P<0.001
Chloroform vs Methanol	-1.428	1.602 ns	P>0.05
Chloroform vs Pet. ether	2.053	2.303 ns	P>0.05
Aqueos vs Methanol	6.123	6.869 **	P<0.01
Aqueos vs Pet. ether	9.603	10.774 ***	P<0.001
Methanol vs Pet. ether	3.480	3.905 ns	P>0.05

Difference	Mean Difference	95% Confidence Interval From	To
Ethanol - Chloroform	6.770	2.878	10.662
Ethanol - Aqueos	-0.7800	-4.672	3.112
Ethanol - Methanol	5.343	1.450	9.235
Ethanol - Pet. ether	8.823	4.930	12.715
Chloroform - Aqueos	-7.550	-11.442	-3.658
Chloroform - Methanol	-1.428	-5.320	2.465
Chloroform - Pet. ether	2.053	-1.840	5.945
Aqueos - Methanol	6.123	2.230	10.015
Aqueos - Pet. ether	9.603	5.710	13.495
Methanol - Pet. ether	3.480	-0.4122	7.372

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 8.806, The P value is 0.0661.

Bartlett's test suggests that the difference among the SDs is not quite significant.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

* * *

Appendix XII. One-way Analysis of Variance (ANOVA) on Weight of Extracts
The P value is 0.0022, considered very significant.
Variation among column means is significantly greater than expected by chance.
Student-Newman-Keuls Multiple Comparisons Test

Comparison	Mean Difference	q		P value
Pet. ether vs Ethanol	-6.865	5.301	*	P<0.05
Pet. ether vs Aqueous	-6.853	5.291	**	P<0.01
Pet. ether vs Methanol	-1.218	0.9401	ns	P>0.05
Pet. ether vs Chloroform	-0.8000	---	ns	P>0.05
Chloroform vs Ethanol	-6.065	4.683	*	P<0.05
Chloroform vs Aqueous	-6.053	4.674	*	P<0.05
Chloroform vs Methanol	-0.4175	---	ns	P>0.05
Methanol vs Ethanol	-5.648	4.361	*	P<0.05
Methanol vs Aqueous	-5.635	4.351	**	P<0.01
Aqueous vs Ethanol	-0.01250	0.009652	ns	P>0.05

With Student-Newman-Keuls test, it is impossible to calculate confidence intervals.
Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical
SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 34.941
The P value is < 0.0001.
Bartlett's test suggests that the difference among the SDs is extremely significant.
Since ANOVA assumes populations with equal SDs, you should consider transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?
ANOVA assumes that the data are sampled from populations that follow Gaussian distributions.
This assumption is tested using the method Kolmogorov and Smirnov:

* * *

Appendix XIII. TLC fractions Linear Correlation

Number of points = 9
Correlation coefficient (r) = 1.0000
95% confidence interval: 0.9998 to 1.0000
Coefficient of determination (r squared) = 0.9999
Test: Is r significantly different than zero?
The two-tailed P value is < 0.0001, considered extremely significant.

* * *

Linear Correlation
Number of points = 3
Correlation coefficient (r) = 1.0000
95% confidence interval: (Requires more than 3 points)
Coefficient of determination (r squared) = 1.0000

Test: is r significantly different than zero?
The two-tailed P value is 0.0016, considered very significant.

Appendix XIV. Unpaired t test between sero positive and sero negative *E. coli* isolates.

Do the means of Number positive and Number negative differ significantly?
P value. The one-tailed P value is < 0.0001, considered extremely significant.
t = 9.043 with 14 degrees of freedom. 95% confidence interval
Mean difference = 17.750 (Mean of Number negative minus mean of Number positive)
The 95% confidence interval of the difference: 13.540 to 21.960
Assumption test: Are the standard deviations equal? The t test assumes that the columns come from populations with equal SDs. The following calculations test that assumption.
F = 1.000. The P value is 0.5000.
This test suggests that the difference between the two SDs is not significant. Assumption test: Are the data sampled from Gaussian distributions? The t test assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
Number positive	0.1630	>0.10	Yes
Number negative	0.1630	>0.10	Yes

Summary of Data

Parameter:	Number positive	Number negative
Mean:	5.625	23.375
# of points:	8	8
Std deviation:	3.926	3.926
Std error:	1.388	1.388
Minimum:	0.000	18.000
Maximum:	11.000	29.000
Median:	6.500	22.500
Lower 95% CI:	2.343	20.093
Upper 95% CI:	8.907	26.657

* * *

Appendix XV. Linear regression on age range and gender distribution of diarrhoea cases of infants attending some hospitals in Kano state, Nigeria.

Number of points = 10, best-fit Standard 95% confidence interval

Parameter	Value	Error	from	to
Slope	0.5509	0.1251	0.2624	0.8393
Y intercept	2.875	1.504	-0.5939	6.345
X intercept	-5.220			

Correlation coefficient (r) = 0.8414. r squared = 0.7080, Standard deviation of residuals from line (Sy.x) = 1.631. Test: Is the slope significantly different from zero?

The P value is 0.0023, considered very significant. This result was obtained from the following ANOVA table.

Source of variation	Degrees of freedom	Sum of squares	Mean square
Linear regression (Model)	1	51.615	51.615
Deviations from linearity (Residual)	8	21.285	2.661
Total	9	72.900	

F = 19.399

Standard Curve Calculations

X Values	Y Values

* * *