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Plant extracts and their constituents as alternatives to antibiotic treatment of diarrhoea in tropical regions

Bachelor Thesis

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Declaration

I declare that I have worked on this thesis independently, using only the sources listed in bibliography.

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Abstract

In vitro antimicrobial activity of 12 plant-derived compounds and four ethanol extracts obtained from various plant species that were both selected based on the previous studies showing their potential antimicrobial effect was determined by the broth microdilution method. The antimicrobial activity of both, plant-derived compound as well as ethanol extracts against two Gram-positive (*Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644) and two Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella enteritca* ATCC 13076) diarrhoea-causing pathogens was determined. The minimum inhibitory concentrations were obtained by measurement of bacterial growth. Six of all 12 compounds showed some antimicrobial activity at least against one of four bacteria. Zinc pyrithion showed the absolutely strongest antimicrobial activity with MICs 4 μg/mL for all tested bacteria. Against closely following sanguinarine with MIC values ranging from 16 to 32 μg/mL, Gram-positive bacteria have been shown to be more sensitive. With exception of ethanol extract from hardwood of Santalum album, which produced moderate antibacterial effect against both Gram-negative bacteria with MICs range (256-512 μg/mL), none of the plants tested exhibited significant growth-inhibitory effect against diarrhoea causing microorganisms in this study. These results call for further investigations, especially to perform more detailed toxicological studies for two compounds (zinc pyrithion and sanguinarine) showing the strongest antimicrobial activity to examine their safety for internal use, and thus to consider the potential possibility of their administration as the alternative antimicrobial drugs.

Keywords: antimicrobial, diarrhea, plant extracts, plant-derived compounds, tropical

Abstrakt

V rámci této práce byla zkoumána antimikrobiální aktivita dvanácti rostlinných látek a čtyřech ethanolových extraktů získaných z různých rostlinnýhch druhů. Všechny tyto rostlinné komponenty byly vybrány na základě získaných informací z předešlých studií prokazujících jejich animikrobiální aktivitu. *In vitro* antimikrobiální aktivita byla stanovena pomocí bujónové mikroduliční metody. Jak u rostlinných látek tak i u ethanolových extraktů byla antimikrobiální aktivita testována proti vybraným bakteriím obecně způsobujícím průjmová onemocnění. Dvě z nich byli zástupci Gram positivních druhů (*Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644) a zbývající dvě zástupci Gram negativních (*Escherichia coli* ATCC 25922, *Salmonella enteritca* ATCC 13076). Minimální inhibiční koncentrace byly získány na základě spektrofotometrického stanovení růstu testovaných mikroorganismů. Ze všech dvanácti testovaných látek, šest látek prokázalo antimikrobiální aktivitu přinejmenším na jednom ze čtyř bakterií. Absolutně nejvyšší antimikrobiální aktivitu prokázal zinc pyrithion s MIC hodnotamy 4 µg/mL u všech čtyřech bakterií. Sanguinarine prokázal také vysokou antimikrobiální aktivitu s MIC hodnotamy v rozsahu 16 až 32 µg/mL, sensitivita byla prokázana převážně u Gram positivních bakterií. Všechny ethanolové extrakty s vyjímkou extraktu ze santalového dřeva prokazující pouze mírnou antimikrobiální aktivitu (256-512 µg/mL) neprokázaly výrazný inhibiční efekt ani u jedné ze čtyř bakterií způsobujích průjem. Výsledky vyžadují další výzkum, zejména provest podrobnější toxikologické studie u dvou látek (zinc pyrithionu a sanguinarinu) prokazujících nejvyšší antimikrobiální aktivitu. Tyto studie by měly především prověřit zdravotní nezávanost těchto látek při jejich vnitřním použití a tím dále přispět k rozvahám zabývajícími se jejich použití při alternativní antimikrobiální léčbě.

Klíčová slova : antimikrobiální, průjem, rostlinné extrakty, rostlinné látky, tropický

Contents

1. Literature review

Diarrhoea generally represents a specific clinical symptom or health condition that can commonly occur in a broad range of several human diseases or health disorders of a wide variety of aetiology. Nevertheless, such diseases are always somehow associated with the however disrupted function of gastrointestinal tract, which usually results in diarrhoeal symptoms. Clinically, diarrhoea is defined as the condition of having a loose of unformed liquid or watery stools at least three times per day. Frequent passing of formed stools has not been accepted as diarrhoea, nor the passing of loose, "pasty" stools by breastfed babies. It is therefore more the stool consistency than the number of looses that principally characterizes diarrhoea (WHO, 2005). In general terms the condition usually results from the altered intestinal motility or imbalanced absorption and secretion of fluids in the gut lumen. The causes of diarrhoea may comprise a lot of physiological disorders (e.g. food allergy, monosaccharide malabsorption (Rowe et al., 1956), lactase deficiency (McMichael et al., 1965), or manifestation of several diseases (e.g. ulcerative colitis, Crohn' disease, diabetes, malaria) (Ahmed et al., 2014). However the most widespread and serious causes of diarrhoea are predominantly some gastrointestinal infections caused by a variety of bacterial, viral or parasitic pathogens (WHO, 2013).

1.1 Epidemiology of diarrhoeal diseases

In spite of that diarrhoeal diseases can be often known as a mild, self-limited and not life-treating conditions, globally (especially the infectious diarrhoeas) they represents a serious health problem and one of the leading cause of morbidity and mortality in the world (Lanata and Mendoza, 2012). It has been estimated that there are about 1.7 billion cases of infectious diarrhoeal disease every year, whereas the mostly affected are particularly young children in the developing world. Diarrhoea is therefore the predominating problem for paediatrics working in the developing countries, because the outbreaks of diarrhoea can be especially for vulnerable young children often fatal. Actually, it has been estimated that diarrhoea represents the second leading cause of death in children under the age of five worldwide. In 2013 the accounted annual mortality rate

for young children under five due to diarrhoea, was 760 000 deaths (WHO, 2013), which accounts for about 10% of whole children annual mortality (WHO/ Unicef, 2013). As we can see, affecting children's life is therefore what has made diarrhoea that dangerous and important for international health organizations to discuss and look for possibilities of prevention and treatment (WHO/Unicef, 2009). The death is in most of the cases caused by a loss of high amounts of fluids resulting in dehydration and subsequent systemic collapse. Dehydration is generally defined as the loss of water and electrolytes (sodium, chloride, potassium and bicarbonate) particularly in the liquid stool, but also through vomit, sweat, urine and breathing, which is not replaced adequately and thus the deficit of water and electrolytes develops (WHO, 2005).Despite the still high number of fatal cases in children, during the past decades the annual mortality rate has significantly declined (from original 4.5 million deaths in 1979), especially due to finding of effective methods to stop these cases of quick dehydration (WHO/ Unicef, 2004).

So the mortality is reducing during the time, however the morbidity has not yet declined and a still high number of the diarrhoeal cases occurs, particularly in Africa and South Asia where bouts of diarrhoea are more likely to result in death or other severe outcomes. Poor hygiene, lack access to safe water, open defecation and insufficient promotion of breastfeeding and malnutrition are the major factors influencing threat level to diarrhoea infection, whereas these two regions are probably most problematic from this point of view (WHO/Unicef, 2009). The absolutely leading country important to mention is India, where was estimated about 386 600 annual child deaths and the second African Niger where had been calculated about 151 700 similar cases (WHO, 2008). As said, what these regions connects most of all, according to diarrhoea, is low level of hygiene, especially due to contaminated water by indiscriminate or open defecation and consecutive non-established habits including for example consequential hand-washing. Children are more vulnerable, because among others they usually play in areas in which stools are found and then also their own stools tend to carry a higher pathogen load than adults (WHO/Unicef, 2008). Nearly 1 in 4 people in developing countries practises open defecation, whereas in South Asia it is 49% of the population and 28% in Sub-Saharan Africa. Improving access to safe water is therefore most important preventive measure. Between 1990 and 2006, amount of people in developing countries using improved water rose from 71% to 84%, however still almost 1 billion people lack access to improved drinking water sources (WHO/Unicef, 2009).

Whereas viral agents as the cause of diarrhoea are more prevalent in developed part of the world, the enteric bacterial and protozoan pathogens are predominating in the tropical regions where the most of developing countries occur (WGO, 2008). Within the bacterial pathogens, the important diarrhoea causing agents are e.g. *Campylobacter jejuni*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Shigella dysenteriae* or *Vibrio cholerae*. The viral agents represent Rotavirus and Norwalkvirus and the final protozoan *Entamoeba hystolitica* and *Giardia lamblia* (WGO, 2012) The further epidemiological review about certain of these pathogens is more in detail described in the chapter devoted to microorganisms.

1.2 Types of diarrhoeal outbreaks

Diarrhoea can be divided to three or four clinical types partially related to the causative agent and the use of its specific pathogenic mechanism. These types are chiefly characterized by the duration of diarrhoeal cases, occurrence of additional symptoms or the causative pathogens (WHO, 2005).

1.2.1 Acute diarrhoea

The outbreaks of acute diarrhoea can vary from mild and self-limited to those very serious and life-threatening in the cases when they are too severe. It is because there then often appears a high potential risk of the quick dehydration, especially in young children who usually require an immediate care in these cases (Diniz-Santos, 2006). Acute diarrhoea is generally defined as an episode of diarrhoea that lasts less than 14 days. Clinically, there are two specific groups in which the acute type of diarrhoea is usually divided to: acute watery and acute bloody diarrhoea (WHO, 1993).

Acute watery diarrhoea often lasts several hours or days and is characterized by presence of specific types of pathogens. These pathogens are often non-invasive microorganism that are especially active in the intestine and lead to diarrhoea through a variety of interactions with the intestinal mucosa. As the name indicates, the main symptom of this type is watery diarrhoea accompanied by eventual vomiting with no or just low fevers (WHO, 2012). According to health problems in developing countries, the leading pathogen represents Rotavirus that is responsible for almost half of all cases of

acute diarrhoea in children under five (WHO/Unicef, 2009). Within the bacteria domain, the most specific for acute non-bloody diarrhoea are *Vibrio cholera* and the epidemics of cholera, and the infectious Enterotoxigenic *E. coli*. The both bacteria are distinguished from the others by their ability to cause diarrhoea by production of specific enterotoxins. The severity of the diarrhoea and vomiting can lead to rapid electrolyte imbalance and consequent dehydration and death in some cases (John Wiley & Sons. 2009). It is possible to see first signs and symptoms that are fluently developing in the beginning of dehydration, which initially include thirst, restless or irritable behaviour, decreased skin turgor, sunken eyes, and in infans sunken fontanelle. In severe dehydration these effects usually become more pronounced and consequently may develop other health problems such as hypovolemic shock (WHO, 2005). It has been estimated that acute watery diarrhoea accounts for approximately 80% of all death caused by diarrhoea, especially due to dehydration (Mathabe et al., 2006).

Acute bloody diarrhoea or also dysentery is very specific type because of presence of blood and the mucus in the stool. Visible blood is the result of inflammatory disorder and the damage of the intestinal mucosa especially in the colon. This can cause elevated temperature, painful spasms of the intestinal muscles (cramping), swelling due to water leaking from capillaries of the intestine (edema), and further tissue damage by the body's immune cells and the chemicals, called cytokines, which they release to fight the infection. Amounts of blood, presence of mucus, pus or frequency of urges to defecate depends on type of pathogens that can be of bacterial, viral but also parasitic origin (John Wiley & Sons. 2009). However dysentery is generally characterized by two specific causing organisms and accordingly is divided to two types. Acute bloody diarrhoea caused by bacteria of the genus *Shigella* is so-called bacillary dysentery or shigellosis, which is the most common cause of children's severe cases of diarrhoea in developing countries. If it is the severe case the symptoms can include watery diarrhoea that contains blood or mucus, nausea or vomiting, stomach cramps, fever or in general high temperature. The second type of acute bloody diarrhoea due to protozoan amoebic *Entamoeba histolytica* and we talk about amoebic dysentery or amoebiasis that mainly occurs in tropical regions. The main symptoms are water diarrhoea which can contain blood, mucus or pus, nausea vomiting, abdominal pain, fever and chills, bleeding from rectum, loss of appetite and weight loss. The most dangerous in dysentery is in general potential sepsis, malnutrition and in some cases dehydration may also occur (WHO, 2005).

1.2.2 Persistent diarrhoea

If diarrhoea lasts more than two weeks, we can name it as persistent that is also in many cases very dangerous, usually not because of dehydration, but mostly because of malnutrition and serious non-intestinal infection (WHO, 2012). For example undernourished children and those with other illnesses, such as AIDS, are more likely to develop persistent diarrhoea which in turn, tends to worsen their condition. During persistent diarrhoea, decreased food intake, decreased nutrient absorption, and increased nutrient requirements often combine to cause weight loss and failure two grow: the child's nutritional status declines and any pre-existing malnutrition is made worse (WHO/Unicef, 2009).

1.2.3 Traveller's diarrhoea (TD)

TD is a term used for common illness affecting international travellers especially in developing countries and each year between 20-50% of them, an estimated 10 million persons, develop diarrhoea. It also contains at least three but mostly four to five loose or watery bowel movements each day and is usually accompanied by abdominal cramps, nausea, bloating, fever, urgency, vomiting and malaise. Bacterial pathogens cause approximately 80% of TD cases and the most common causative agent isolated in countries surveyed has been Enterotoxigenic *E. coli*. Besides ETEC and other bacterial pathogens, a variety of viral and parasitic enteric pathogens also are potential causative agents (CDCP, 2006). TD is not in most of the cases dangerous and life-threatening and the travellers usually recover within a few days with little or no treatment, however sometimes the symptoms can be severe enough to necessitate medical intervention and especially for those who have compromised immune system, TD is cause for significant concern and in rare instances conversely can become life-threatening (Ericsson et al., 2007)

1.3 Diarrhoea-causing bacteria

Now in this chapter I will describe in detail exclusively the bacterial pathogens we have tested in our study. These pathogens include strains of *E. coli*, *Salmonella enter*ica, *Enterococcus faecalis* and *Lysteria monocytogenes*. In a point of view that most of these pathogens cause diarrhoea by several mechanisms particularly affecting gastrointestinal tract, the first part of this chapter concisely describes how the normal composition of intestinal microflora looks like.

1.3.1 Normal gut flora

The indigenous microbiota that colonizes the gastrointestinal tract (GIT) tract comprises the resident, autochthonous members of the community and is also referred to as the commensal microbiota. The commensal relationship implies that one member of the association benefits while the other is unaffected. However, in most cases, this is a mutualistic relationship. The microbiota benefit from the host that provides a nutrient rich and hospitable environment, while the microbiota provide nutrients that would not otherwise be available to the host, regulate immune development, and create a barrier to infection from pathogens. These roles include synthesis of vitamins, degradation of xenobiotics, metabolism of bile and host hormones, immune development, and the competitive exclusion of pathogens. There are also transient (allochthonous) bacteria that are regularly ingested, but are unable to colonize the gut under normal conditions. The human faecal microbiota is dominated by two bacterial phyla: the *Firmicutes* and *Bacteroidetes*. Approximately 800 to 1 000 different bacterial species and more than 7 000 strains inhabit the human GI tract (Sadowsky and Whitman, 2011). In infants the resident flora of GIT are members of the genus *Bifidobacterium* or *Lactobacillus* species. The resident flora of GIT in adults includes facultative anaerobes of genera *Lactobacilli*, *Streptococcus*, *Clostridium*, *Veillonella*, *Bacteriodes*, *Fusobacterium* and *Coliforms*. The enteric bacteria include species of genera *Eschereschia*, *Proteus*, *Klebsiella* and *Enterobacter*. The microbial flora in the upper region of GIT is scanty (Kale and Bhusari, 2007). The stomach and duodenum have a relatively low microbial density, with 103 to 105 CFUs per gram of contents, and a population dominated by the *Lactobacillus*,

Streptococcus, and *Enterococcus* genera (Sadowsky and Whitman, 2011). The juices secreted by stomach makes the pH of gastric juice so low that existence of microorganisms associated with the lining of the organ is not possible. Similarly, enzymes of small intestine do not favour the organisms survived. It is the lower part of the small intestine and large intestine of GIT, where abundant indigenous microflora exists. The required temperature (37°C), availability of water and variety of nutrients makes large intestine most favourable environment for variety of microorganisms. Most of the microorganisms in the large intestine are anaerobic or facultative anaerobes (Kale and Bhusari, 2007). Complexity of the microbial community is greatest in the large intestine, where the density increases to 1010 to 1012 CFU per gram and the community is dominated by gram-positive genera, including *Clostridium*, *Bacillus*, *Ruminococcus*, and *Fusobacterium* (Sadowsky and Whitman, 2011).

1.3.3 *Escherichia coli*

The genus *Escherichia* is composed of motile or non-motile Gram-negative, facultatively anaerobic, non-spore forming, rod-shaped bacteria. There are six species in this genus: *Escherichia albertii*, *E. blattae*, *E. fergusonii*, *E. hermannii*, *E. vulneris* and finally the mostly type *E. Coli* (Murray et al., 2007).

Strains of *E. coli* are straight rods peritrichously flagellated, usually motile and often fimbriate. Some, especially those from extra-intestinal infection, may produce a polysaccharide capsule. Strains of *E. coli* and related Gram-negative coliform bacteria of the family *Enterobacteriaceae* predominate among the aerobic commensal flora in the gut of human beings and animals, thus they are present wherever there is faecal contamination, a phenomenon that is exploited by public health microbiologists as an indicator of faecal pollution of water sources, drinking water and food (Greenwood et al., 2007). Despite of the species as a nearly ubiquitous constituent of the bowel flora of healthy individuals, certain strains may cause extra-intestinal and intestinal infections in healthy as well as immunocompromised individuals (Murray et al., 2007). *E. coli* is therefore the most frequent causative pathogen in human bacterial infections affecting intestine or urinary tract (Kayser et al., 2005).

They grow over a wide range of temperature (15-45°C); some strains are more heat resistant than other members of the *Enterobacteriaceae* and may survive 60°C for 15

min or 55°C for 60 min. *E. coli* can be differentiated from other enteric Gram-negative bacteria by the ability to utilize certain sugars and by a range of other characteristic biochemical reactions, such as indole production and the formation of acid and gas from lactose and other carbohydrates (Greenwood et al., 2007).

Traditionally, *E. coli* isolates have been characterized by serotyping with lipopolysaccharide (LPS) or somatic (O) antigens, flagellar (H) and capsular (K) antigens. They represent the stable and reliable strain characteristics with 173 O antigens, 80 K antigens, and 56 H antigens, which can all be subdivided into partial antigens. The final number of *E. coli* serotypes is very high, 50 000 - 100 000 or more. In general, O and H antigens are the two major antigens of Gram-negative bacteria including *E. coli*. Serotypes from diarrhoeal diseases are mostly species specific, and could at present be used as epidemiological markers for bacterial clones equipped with special virulence markers, such as toxins and adhesions. Their O-antigen lipopolysaccharides may be regarded as virulence factors (Orskov F and Orskov I, 1992). Fimbrial antigens have also been described and like many other members of *Enterobacteriaceae*, *E. coli* may produce fimbriae, and strains may express both sex pili and several types of fimbrial structure. One of them can mediate adhesion to a wide range of human and animal cells that contain the sugar mannose and such adhesion might be involved in pathogenicity, for example by the filamentous protein structures resembling fimbriae that cause mannose-resistant haemagglutination.There is a good evidence to suggest that these proteins play an important part in the pathogenesis of diarrhoeal disease and urinary tract infection. They include for example colonization factor antigens (CFAs) or coli surface (CS) antigens expressed by enterotoxigenic *E. coli* (ETEC) causing human diarrhoea. Another ability by which is recognized pathogenic mechanism of *E. coli* strains is an expression of siderophores, such as enterobactin or also aerobactin, that readily remove ferric ions from mammalian iron transport proteins such as transferrin and lactoferrin (Greenwood et al., 2007).The identification of pathogenicity-associated islands (PAIs) revealed that "extra" genetic material exists in the chromosomes of pathogenic *E. coli*. Subsequently, it was realized that pathogenic *E. coli* can be divided into "pathotypes" based on the genes that they possess (or lack), the diseases that they can cause, and the hosts in which they are able to cause disease (Sadowsky and Whitman, 2011).

Although *E. coli* is normally carried in the gut as a harmless commensal, it may therefore cause gastro-intestinal disease ranging in severity from mild, self-limiting diarrhoea to haemorrhagic colitis and associated, potentially life-threatening, haemolytic uremic syndrome. Such strains fall into at least five groups, each associated with specific serotypes and with different pathogenic mechanisms (Greenwood et al., 2007).

Enteropathogenic *E. coli* (EPEC): These bacteria cause epidemic or sporadic infant diarrhoeas, now rare in industrialized countries but still a main contributor to infant mortality in developing countries (Kayser et al., 2005). At the second international meeting on EPEC in São Paulo, Brazil 1995, EPEC were defined as diarrheagenic *E. coli* that produce a characteristic histopathology known as attaching-and-effacing on intestinal cells and that do not produce Verocytoxin (Donnenbergand Brett, 2013).

Full EPEC pathogenicity requires two genetic elements: the EPEC adherence factor (EAF) plasmid, which encodes most importantly the bundle-forming pilus (BFP), and the chromosomal locus of enterocyte effacement (LEE), which mediates the above mentioned attaching-and-effacing phenotype (Murray et al., 2007). EPEC are generally divided to typical (tEPEC) and atypical (aEPEC) groups based on the presence/absence of these virulence genes, thus whereas tEPEC possess EAF, aEPEC do not (Monaghan et al., 2013). As a specific virulence factor, EPEC uses special protein for attachment named intimin by which can bind directly the cytoplasm of intestinal epithelial cells (Greenwood et al., 2007).The infectious dose of EPEC varies depending on human age: infants require a presumably low dose, whereas adults require a dose in the range of 10^8 to 10^{10}organisms (Sadowsky and Whitman, 2011).The current model of typical EPEC infection begins with BFP-mediated localized adherence. Bacteria then deliver a translocated intimin receptor (Tir) to infect host cell by mean of LEE-encoded type III secretion. The Tir protein inserts into the host membrane, providing a receptor for the bacterial outer-membrane protein, intimin, also LEE encoded. Intimin-bound Tir is tyrosine phospohorylated in the host, beginning a cascade of signalling events that ultimately lead to reorganization of the host cytoskeleton around the bacteria. A number of effector proteins, delivered via the type III secretion, likely contribute to diarrheagenicity (Okekeet al., 2009). Together these processes also result in the characteristic EPEC pedestals with the intimately adherent organism damaging the mucosa and causing so-called an attaching and effacing lesion

resulting in subsequent loose of brush border microvilli of the intestine (Greenwood et al.,2007, Engleberg et al., 2012).

The symptoms of often severe, prolonged, and nonbloody diarrhoea, vomiting, and fever in infants or young toddlers are characteristic of EPEC illness. These infections have been therefore particularly associated with typical chronic diarrhoea where sequels may include malabsorption, malnutrition, weight loss, and growth retardation (Murray et al., 2007).

EPEC is considered to be an important pathogen, particularly due to their association with paediatric infection worldwide. EPEC infection have been reported to be the second most frequent cause of death among children and are responsible for nearly one in five child deaths worldwide. The symptoms of the disease are linked to high rates of morbidity and mortality in developing countries as well as serious if sporadic outbreaks in developed countries (Monaghan et al., 2013). EPEC is common in communities with poor hygiene where sporadic cases and frequent outbreaks occur in general community as well as in institutions (Greenwood et al., 2007). tEPEC correspond to EPEC of the classical serotypes representing important cause of diarrhoea in developing countries. In the 1970s and 1980s tEPEC serotypes were associated with disease in many parts of Africa, suggesting that EPEC was a predominant cause of diarrhoea at the time (Okeke et al., 2009). However before 1970, these organisms were also implicated in highly lethal nursery outbreaks in the United States and the United Kingdom. More recently, aEPEC have been implicated as enteric pathogens in the United States, including in several outbreaks of diarrheal disease. Since 1971 few epidemics of EPEC enteritis have been reported in the UK or the USA (Murray et al., 2007).

Enterotoxigenic *E. coli* (ETEC): The bacterium, which produces heat-labile *E. coli* enterotoxin (LT) and/or heat-stable *E. coli* enterotoxins (ST), or both, is an important cause of diarrhoea in developing countries, particularly among young children. ETEC also is a frequent cause of traveller's diarrhoea (Murray et al., 2007).

ETEC pathogenicity primarily derives from specific CFA fimbriae that allow these bacteria to attach themselves to small intestine epithelial cells, thus preventing their rapid removal by intestinal peristalsis. The enterotoxins and CFA are both determined by plasmid genes (Kayser et al., 2005). Heat-labile enterotoxin (LT) is by structure and the

mechanism, by which causes diarrhoea, closely related to the toxin produced by strains of *Vibrio cholerae*. There are two main forms termed LT-I and LT-II, which are consequently divided to other "sub-forms". These toxins have been associated with human, porcine and chicken infections. Although these toxins have a degree of structural variation, they are all subunit protein toxins comprising one A subunit (made up of two peptides A_1 and A_2) and five B subunits with molecular weights of 26 000-28 000 and 11 500-11 800 Da, respectively (Greenwood et al., 2007). As the name of the toxin indicates, heat stability is on a lower level and its inactivation comes already at 60°C after 30 min (Kayser et al., 2005). The B subunit binds to sugar residues of ganglioside GM_1 on the cell lining the villi and crypts of the small intestine (Greenwood et al., 2007). After entering the cell, Cholera-toxin (CT) is routed in a retrograde manner through the Golgi apparatus into the endoplasmic reticulum (ER). A specific amino acid sequence, KDEL, which is located within the A_2 subunit of the toxin, mirrors an epitope that is present in proteins that are typically retained in the ER and results in CT translocation from the Golgi to the ER by a shuttle protein known as ERD2. Cholera toxin co-opts the ER-associated degradation (ERAD) pathway to subsequently gain entry into the host cell cytosol. The ERAD pathway ensures that proteins transiting through the ER for secretion are properly folded. CT mimics a misfolded protein and is retrotranslocated into the cytosol, where typical ERAD targets would be degraded by the proteasome. Instead, the A_1 peptide of CT goes on to ADP-ribosylateadenylate cyclase, leading to production of cyclic AMP, which activates protein kinase A. Protein kinase A then phosphorylates cystic fibrosis transmembrane regulator, leading to Cl^- secretion (Viswanathan et al., 2008). In contrast to LTs, the heat-stable enterotoxin (ST) of *E. coli* has a low molecular weight which confers heat stability (ST can tolerante temperatures up to 100°C) and poor antigenicity (Kayser et al., 2005; Greenwood et al., 2007). There are two major classes, designated ST-I (ST_a) and ST-II (ST_b), whereas ST-II is distinguished from ST-I by its biological activity and its insolubility in methanol. Variants of ST-I have been associated with porcine and human infections. In comparison with LT, this toxin activates guanylate cyclise activity, resulting in an increase in the level of cyclic guanosine monophosphate (cGMP). The mechanism of secretion caused by ST-I, via cGMP, is not fully understood but calcium appears to play a role. ST-I is plasmid encoded, and these plasmids may also encode the genes for LT, adhesive factors and antibiotic resistance (Greenwood et al., 2007).

The most prominent symptoms of ETEC illness are diarrhoea and abdominal cramps, sometimes accompanied by nausea and headache, but usually with little vomiting or fever. Although ETEC is usually associated with relatively mild watery diarrhoea, illness in some recent ETEC outbreaks has been notable for its prolonged duration (Murray et al., 2007). In developing countries ETEC are a major cause of death in children under the age of 5 years. These strains also commonly cause diarrhoea in travellers visiting countries where ETEC are endemic (Greenwood et al., 2007).

Entero-invasive *E. coli* (EIEC): These bacteria can penetrate into the colonic mucosa, where they cause ulcerous, inflammatory lesion. The pathogenesis and clinical picture of EIEC infections are therefore the same as in *Shigella* representing bacterial dysentery, which cause disease by invading intestinal epithelium (Kayser et al., 2005; Greenwood et al., 2007).

Infection is by ingestion; only a small number of bacteria need to be swallowed as they are relatively resistant to gastric acid and bile, and pass readily into the large intestine where they multiply in the gut lumen. The bacteria pass through the overlying mucous layer, attach to the intestinal epithelial cells and are carried into the cell by endocytosis into an endocytic vacuole, which then lyses. After lysis of the vacuole the bacteria multiply within the epithelial cell and kill it. Spread to neighbouring cells leads to tissue destruction and consequent inflammation, which is the underlying cause of the symptoms of bacillary dysentery. Pathogenicity depends on both chromosomal and plasmid genes, whereas plasmid genes encode attributes for expression of outer membrane proteins necessary for invasion and insertion in the cell membrane, chromosomal genes include those pathogenic mechanisms required for the expression of long-chain LPS and those encoding an aerobactin-mediated iron-sequestering system (Greenwood et al., 2007).

The epidemiology and ecology of EIEC have been poorly studied, but there appears to be no evidence of an animal or environmental reservoir. Infections are usually food-borne but there is also evidence of cross-infection and survey suggest, that they cause about 5 % of all diarrhoeas in areas of poor hygiene (Greenwood et al., 2007). EIEC is however rare in developed countries (three large outbreaks reported in USA) and less common than ETEC or EPEC in the developing world (Murray et al., 2007).

Verocytotoxigenic *E. coli* (VTEC): Owing to similarity in structure between Verocytoxin (VT) and Shiga toxin expressed by *Shigella dysenteriae*, VTEC have also been termed Shiga toxin-producing *E. coli* (STEC) or sometimes also referred to as enterohaemorrhagic *E. coli* (EHEC) (Greenwood et al., 2007).The last mentioned term is due to these *E. coli* strains are causative pathogens in the haemorrhagic colitis and haemolytic-uremic syndrome (HUS) that occur in about 5% of VTEC infections, accompanied by acute renal failure, thrombocytopenia, and anaemia (Kayser et al., 2005).Strains of *E. coli* expressing a protein cytotoxic for Vero cells were discovered in 1977. Once epidemiologists were aware of VTEC, the importance of these bacteria in human disease became apparent and a link was established with these two diseases of previously unknown aetiology. Outbreaks were first recognized in the USA in 1982, and strains of VTEC belonging to serogroup O157 emerged as the major cause (Greenwood et al., 2007).

VTEC serotypes O157:H7 and O157: nonmotile (NM) produce one or more VT. Two distinct toxins, VT_1 and VT_2, also referred to as Shiga toxins, have been described. VTEC may produce either VT_1 or VT_2 or both toxins, whereas there are several variant forms of VT_2, including VT_2c, VT_2d, VT_2e, and VT_2f, which in one study were more frequently identified from asymptomatic carriers than HUS (Murray et al., 2007). As above mentioned, the biological properties, physical characteristics and antigenicity of VT are very similar to those of Shiga toxin produced by strains of S. *dysenteriae*, but the genes encoding VT are carried on a lambda-like bacteriophage, whereas those encoding Shiga toxin are located on the chromosome. Like Shiga toxin, VT_1 and VT_2 comprise A and B subunits. For both toxins the A subunit posses the biological activities of the toxin and the B subunits mediate specific binding and receptor-mediated uptake of the toxin. VT_1 and VT_2 bind to globotriosylceramide (Gb_3) molecules present on the surface of certain eukaryotic cells. In contrast VT_2 variant toxins bind to globotetraosylceramide (Gb_4). During the infection increase the number of ceramide receptors on the surface of eukaryotic cells, enhancing the binding of VT to these cells. Once bound to the eukaryotic cell surface, the holotoxin becomes internalized by host cell and remains active within endosomes. The toxin eventually reaches the Golgi apparatus by mechanisms as yet unknown. At some point within the host cell, the A sunbunit becomes enzymatically nicked to form portions A_1 and A_2; the A_1 portion of the toxin prevents protein synthesis and results in cell death (Greenwood et al., 2007). The presence of additional virulence

factors other than VT correlates with disease potential. The most important of these virulence factors are the intimin adhesion and the type III secretion system encoded on the LEE pathogenicity island(Murray et al., 2007).

VTEC infection can be therefore associated with range of clinical symptoms from mild, non-bloody diarrhoea to the severe manifestations of HUS, as mentioned condition characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (Kayser et al., 2005; Greenwood et al., 2007). Additional symptoms of *E. coli* O157:H7 infection include abdominal cramps and lack of a high fever. O157 VTEC strains are the most frequently identified diarrheagenic *E. coli* serotypes in North America and Europe. Each year an estimated 73,000 cases of illness and 60 deaths are caused by O157 STEC in the United States 157 STEC is thought to cause at least 80% of cases of HUS in North America and is recognized as a common cause of bloody diarrhoea in developed countries (Murray et al., 2007). Outbreaks of infection with VTEC have occurred in the community, in nursing homes for the elderly and in day care centres for young children. Children and elderly are thus mostly affected by several clinical manifestations of the disease and the most important source of infection is usually food (Greenwood et al., 2007).

1.3.4. *Salmonella enterica*

The genus Salmonella is composed of motile Gram-negative, facultatively anaerobic, non-spore-forming, rod-shaped bacteria. There are well over 2000 different antigenic types of Salmonella. They were originally classified as separate species, but it is now generally accepted that they represent serotypes of a single species, Salmonella enterica, which is further divided to six subspecies denoted as I to VI. Subspecies I strains (also referred as Salmonella enterica subspecies enterica) are commonly isolated from humans and warm-blooded animals, thus are mostly responsible for the typical enteric infections including diarrhoea commonly known as salmonellosis. Subspecies I further includes a lot of serotypes from which the most quotable are Enteritidis, Typhimurium, Typhi and Paratyphi. Its full designation in example of serotype Enteritidis is therefore Salmonella enterica subspecies enterica serotype Enteritidis, often abbreviated to Salmonella Enteritidis (Greenwood et al., 2007; Murray et al., 2007).

S. enterica is in general ubiquitous and hardy bacteria that can survive several weeks in a dry environment and several months in water, which therefore represents the main infectious source, especially for those causing typhoid fever (WHO, 2013; Engleberg et al., 2012). The normal habitat of S. enterica is the animal intestine, whereas subspecies I is the only one occurring in warm-blooded animals including human. All other subspecies (II-IV) are usually isolated from those who are coldblooded (Murray et al., 2007). To date, there are over 2300 serotypes identified within subspecies I. However, only a small fraction of the thousands of described subspecies I serotypes frequently cause disease in humans and domestic animals (Porwollik et al., 2004).Some strains, such as S. Typhimurium or S.Enteritidis, which are the two most prevalent serotypes of Salmonella, show a wide range of hosts and can be isolated from many different animal species including human (Liu et al, 2011). There are also a few other S. enterica serotypes so-called the host-adapted, which are much more restricted in the species they inhabit, differing in the symptoms of consequent illness. The high restriction of host adaption is probably due to their genetically monomorphic genomes and relatively low sequence diversity that were found. These pathogens particularly represent strains of Typhi and Paratyphi A, B and C serotypes responsible for typhoid fever (Zhang et al., 2014).

Salmonella grows on a wide range of relatively simple media and is distinguished from other member of the family Enterobacteriaceae by its biochemical characteristics and antigenic structure (Greenwood et al., 2007). Salmonella is classified according to its O antigens (LPS) and H antigens. Today, 57 O antigens and 117 H antigens have been identified within all described serotypes. Salmonella H antigens are expressed in different phases, whereas the most serotypes are diphasic, i.e. they express two flagella antigens, and a minor part are monophasic, i.e. express one flagella antigen Certain serotypes, such as S. Typhior Paratyphi C, biosynthesizes the virulence capsular polysaccharide also referred to as Vi antigen, thereby making differ from that of other serotypes of Salmonella. Vi antigen basically prevents the LPS antigens from binding of the specific antibodies the same way as the polysaccharide classically encapsulate the entire bacterium, thereby it can occasionally make detection of the O antigens difficult (Kaurand Jain, 2012).

For S. enterica to cause gastrointestinal disease, it must first reach its site of colonization by surviving the journey through the upper gastrointestinal tract. This includes

passing through the stomach, where it encounters a low pH and organic acids. Thus, an important trait of S. enterica is the ability to tolerate acids, which is accomplished by acid shock proteins encoded by the bacterium (Sadowsky and Whitman, 2011). In human volunteer experiments, a relatively large inoculum (106 to 108organisms) is required to infect those with normal gastric acid secretion, but the inoculum size is reduced 10 to 100 times when bicarbonate, which buffers the acidic pH of the stomach, is given along with the inoculum. Organisms pass through the small bowel to the distal ileum and colon where they penetrate the mucosal barrier. Bacteria can enter M cells and the apical membrane of the gut epithelial cells. Contact of salmonellae with cells in culture induces a dramatic "ruffling" of the plasma membrane, thus degeneration of the microvilli, which leads to uptake of the organisms within phagocytic vesicles. The entry of salmonellae into cells is an example of bacterial-mediated endocytosis (BME), the process which is directed by a type III secretion system encoded on a large region of the genome called Salmonella pathogenicity island 1 (SPI1). Salmonellae remain within vesicles, where are unusually resistant to the lysosomal contents of cells and to the antibacterial peptides (Engleberg et al., 2012).

Strains of S. enterica are generally categorized as typhoidal and nontyphoidal, corresponding to the disease syndrome with which they are associated, thus with the following pathogenic process as well (Murray et al., 2007).

S. Typhimurium and S. Enteritidis, belonging to the group of nontyphoidal serotypes, are characterized by the restriction and the focal inflammation in the gastrointestinal tract, causing an infection which is usually self-limiting (Kayser et al., 2005). Another thing that characterizes and differs them from nontyphoidal strains is the expression of type 1 fimbriae, which enable them to adhere to α-mannose-containing molecules on the microvilli of the ileal mucosa. For example strains of S.Enteritidis are thought to express at least three different fimbrial structures. However, they also use above-mentioned (SPI1), which encode adhesion mechanisms, including both a bacterial adhesion and the adhesion receptor, which is translocated into the host intestine. This process enables these bacteria to insert their own binding site into the gut, unlike fimbriae, which require host-derived binding sites located in the intestinal wall. For this serotype specific diarrhoea, begin after ileal penetration where inflammation of the ileal mucosa results in the efflux of water and electrolytes resulting in diarrhoea (Greenwood et al.,

2007). Nontyphoidal serotypes also express Salmonella enterotoxin (Stn), which is considered to be a putative virulence factor and causative agent of diarrhoea (Nakano M et al., 2012).

Manifest illness usually begins suddenly with diarrhoea, accompanied by headache, malaise and nausea. The incubation period is usually 8-48 h, the onset abrupt, and the clinical course short and self-limiting. The passage of two or three loose stools, which may be disregarded by the sufferer, to a severe and prostrating illness with the frequent passage of watery, green, offensive stools, fever, shivering, abdominal pain and, in the most severe case, dehydration leading to hypotension, cramps and renal failure. Vomiting is rarely a prominent feature. In most cases the acute stage is over within 2-3 days, although it may be prolonged (Greenwood et al., 2007). Although the disease is generally self-limited, patients may occasionally experience serious complications of extra-intestinal Salmonella infections, such as bacteraemia, which can result in meningitis, arthritis, or osteomyelitis, all associated with high mortality rate (Shkalim et al., 2012). Bacteraemia occurs in 1-4 % of immunocompetent patients, and 5-10 % of these persons will develop one of the extra-intestinal complication (Schrader et al., 2008).

The strains that cause gastroenteritis are usually transmitted by chicken meat, eggs, and dairy products. Unless care is taken in poultry farms, chicken eggs often become contaminated, both on their surface and within. Outbreaks are most frequent in summer months and are often related to contaminated egg or chicken salads (Engleberg et al., 2012). Transmission is predominantly foodborne, however other modes also include consumption of contaminated water, contact with infected animals and nosocomial exposure. In contrast to typhoidal infections, nontyphoidal have a worldwide distribution and represent a significant health problem in developing as well as in developed countries. There are an estimated 93.8 million episodes and 155 000 deaths each year attributable to nontyphoidal salmonellae. In developing countries, they are particularly an important cause of invasive disease, likely secondary to high prevalence of coexisting malnutrition, malaria and HIV infection (Haeuslerand Curtis, 2013). Due to this fact, in resource-constrained part of the world the mortality is much higher (18-24%) than in well-resourced countries (less than 2%) (Finn et al., 2010). In spite of the relatively lower mortality, S. Enteritidis and S. Typhimurium also represent very important causing agents in developed world, especially in Europe and the US, where they are the most common serotypes isolated from

human infections (Graziani et al., 2008). For example in the US, each year, an estimated 1.4 million cases of illness and 600 deaths are caused by nontyphoidal salmonellosis (Murray et al., 2007).

S. Typhi and S. Paratyphi A, B and C, belonging to the group of typhoidal serotypes, are characterized by causing an infection involving invasion of the bloodstream and various organs, the clinical symptom known as enteric fever. The clinical features tend to be more severe with S. Typhi (Typhoid fever) than with S. Paratyphi (Paratyphoid fever), whereas serotype Paratyphi B is a diverse serotype that is associated with both paratyphoid fever and gastroenteritis as well. After penetration of the ileal mucosa the organisms pass via the lymphatics to the mesenteric lymph nodes, whence after a period of multiplication they invade the bloodstream via the thoracic duct. The liver, gall bladder, spleen, kidney and bone marrow become infected during this primary bacteraemic phase in the first 7-10 days of the incubation period. After multiplication in these organs, bacilli pass into the blood, causing a second and heavier bacteraemia, the onset of which approximately coincides with that of fever and other signs of clinical illness (Greenwood et al., 2007). In contrast with nontyphoidal pathogenic mechanism, typhoid fever is marked by little intestinal inflammation and dissemination of the bacteria from the intestine to the reticuloendothelial system. Experimental evidence suggests that expression of an antiphagocytic capsule Vi antigen by typhoid fever–inducing salmonellae enables them to evade the intestinal inflammatory response and infect deeper tissue (Engleberg et al., 2012).

Enteric fever is a highly variable, non-specific illness. Typhoid fever and paratyphoid fever cannot be distinguished on clinical grounds. Incubation period vary from 3 to 50 day depending on size of the infective dose, but it is usually 2 weeks. Early symptoms include dry cough and epistaxis associated with anorexia, a dull continuous headache, abdominal tenderness and discomfort (Greenwood et al., 2007). Other frequent symptoms which often occur are malaise, chills, diarrhoea and consequent weight loss (Waddington et al., 2014). Severe intestinal haemorrhage due to ulceration of the Peyer's patches and intestinal perforation are serious complications that can occur at any stage of the illness (Kayser et al., 2005). Specific physical signs are frequently absent, but a relative bradycardia at the height of the fever, hepatomegaly and/or splenomegaly and often a rash of rose spots may also be present. Mild and asymptomatic infections are common

especially in endemic areas, where chronic infection can present with fever of many months duration, accompanied by chronic bacteraemia. S. Typhi and Paratyphi infection can lead to a variety of other rarer clinical entities including meningitis, septic arthritis and osteomyelitis (Greenwood et al., 2007; Waddington et al., 2014).

The infection is transmitted by ingestion of food or water contaminated by faecal or urinary carries excreting the bacterium. Individual level risk are therefore contaminated water supply, food bought from street vendors, the consumption of raw fruit and vegetables, and a history of contact with other cases of chronic carries (Polonsky et al., 2012). Typhoid fever remains an important public health problem in the world especially in impoverished countries. Globally the disease is estimated to cause 220 000 death and 22 million illnesses per year, predominantly in children of school-age or younger. Typhoid fever is one of the most etiological sources in many developing countries (Ochiai et al., 2012). In 2000, more than 90 % of the morbidity and mortality occurred in Asia (Ochiai et al., 2008). The cases of typhoid salmonellosis seen in developed countries occur sporadically and are usually imported by travellers, especially in northern and central Europe (Kayser et al., 2005). In developed countries, the incidence of cases and death has been greatly decreased by a combination of improved sanitation and hygiene, vaccines, and effective antimicrobial chemotherapy. The first two are difficult if not impossible to implement in many developing countries, and, unfortunately, the effectiveness of antimicrobial chemotherapy is also being eroded by the emergence of antibiotic resistance (Mirza et al., 2000).

1.3.5 *Enterococcus faecalis*

The members of the genus *Enterococcus*, belonging to the family *Enterococcaceae*, are Gram-positive, facultatively anaerobic, non-motile andnon-spore forming cocci that occur singly or arranged in pairs or as short chains. These microorganisms, now included in the genus *Enterococcus*, were related mainly to the "streptococci of faecal origin" or "enterococci" (Murray et al., 2007). They were considered for a long time to be a major division of the genus *Streptococcus*, however in 1984, many organisms were separated out into new two genera named as *Enterococcus* and *Lactococcus* (Facklam, 2002). In spite of this change, many publications still describe their classification together, using the three basic different methods. Primarily it is their ability to grow on blood and create different haemolytic patterns (α, β, γ-haemolysis); second

method is based on their antigenic groups denoted as (A-U); and the last comprises name of the species described by certain biochemical tests or on their DNA sequences. By using this, members of the genus *Enterococcus* were initially characterized as D streptococci group, which comprises all haemolysin patterns α, β and γ. There are many described species in this genus, however *E. faecalis* and *E. faecium*, are the most medically important due to their ability to cause wide range of diseases in humans such as urinary tract infections, wound infections, endocarditis, intra-abdominal abscesses and also bacteraemia (Engleberg et al., 2012).

E. faecalis is the most frequent enterococcal species isolated from human clinical specimens, representing 80-90% of all enterococcal infections. *E. faecalis*, as well as all other members of the genus, is however predominantly harmless commensal and normal inhabitant of the gastrointestinal and genitourinary tract of humans and animals, and as pathogen is usually characterized only as classical opportunist. Actually, enterococci occur relatively in high numbers in these guts and after potential defecation can survive in many diverse environments such as water, soil, plants, or food, and thus commonly with *E. coli*, they can be also used as indicators of faecal contamination and of hygienic quality of these environments (Murray et al., 2007)

E. faecalis and other enterococci are generally strong fermenters of carbohydrates resulting in production of lactic acid (Greenwood et al., 2007). In a comparison with other streptococci, they are very robust organisms that are able to grow in a wide range of pH and temperatures at (10-45°C), plus they have a resistance to bile and the high salt concentrations (6.5% NaCl) that are found in the intestine (Ranotkar S et al., 2014).

In a point of view of their pathogenicity, the main feature that differs pathogenic enterococci, including *E. faecalis*, from other streptococci groups is their intrinsic resistance to some antimicrobials commonly prescribed for Gram-positive cocci, such as cephalosporin, lincomycin, orcotrimoxazole (Medeiros et al., 2014). In addition, they have also the ability to acquire resistance genes via transposons or plasmidstransferred via conjugative elements, which may lead to uptake of novel resistance genes orextrinsic resistance. For example the most prevalent vancomycin resistance gene cluster in vancomycin-resistant enterococci (VRE) is found on these exogenously acquired plasmids and transposons. This way, they may also acquire absolute resistance to penicillins or

aminoglycoside, which normally show great synergistic effectivity (Engleberg et al., 2012). The whole pathogenic mechanism of *E. faecalis*is still not absolutely clear, however certain virulence factors of *E. faecalis* are thought to ease adherence of bacteria to the host cell membranes and to environmental surfaces, where these microorganisms can obtain nutrients and evade the host immune response. For example the extracellular surface protein, the cell wall associated protein, which serves as adhesin for the pathogen to host tissue colonization. Another cell surface protein present in *E. faecalis* is adhesion of collagen, which mediates the association of bacteria to host cell matrix proteins, such as collagen I and IV and laminin. The two major extracellular proteins classified as virulence factors of *E. faecalis* are gelatinase and cytolysin. Gelatinase hydrolyzes gelatine, casein, hemoglobin, and other bioactive compounds. The cytolysin is described as bacterial toxin with β-haemolytic properties in humans and with bacteriocin activity against other Gram-positive bacteria (Medeiros et al., 2014).

The infectious outbreaks of *E. faecalis* are most usual for nosocomial environments where the bacteria often affect hospital patients. These opportunistic pathogens are frequently recovered from patients who have received multiple courses of antibiotics, who have been hospitalized for prolonged periods or from those who are immunocompromised (Lins et al., 2013). Especially due to VRE and its antibiotic resistance, in 1990, this usually harmless bacterium, became one of the most feared infection in hospitals. Transmission of the pathogen is primarily via the hands of health care workers, but also can involve inanimate objects (Engleberg et al., 2012). As already mentioned above, *E. faecalis* is one of the agents particularly specified by causing serious diseases such as endocarditis or urinary tract infections. However, *E. faecalis* has been also implicated in food poisoning to a significant degree, resulting in classical symptoms including diarrhoea, and thus it is also considered to be the cause of gastroenteritis (Eley, 1996). Another disease, connected with *E. faecalis* infections and resulting in symptoms including diarrhoea, could be intra-abdominal infections (IAI), which represent the second common cause of severe sepsis and septic shock in intensive care unit. Although the predominant agent in IAI connected with these results is *E. coli*, and enterococci are more involved in secondary intra-abdominal abscesses, however all studies published to date have shown an increase in morbidity and, in some cases, in mortality when enterococci are isolated from the peritoneal fluid so they are considered to be also important agent (Dupont et al., 2011).

1.3.6 *Listeria monocytogenes*

Organisms of the genus *Listeria*, belonging to the genus *Listeriaceae*, are Gram-positive, facultatively anaerobic, non-spore forming peritrichously flagellated and quite motile (motile at 28°C by means of one to five peritrichous flagella but are much less motile at 37°C) rods that occur singly or in short chains(Murray et al., 2007). The genus contains six species, but almost all cases of human disease known as listeriosis are caused by *Listeria monocytogenes*. Other two species *L. Ivanovii* and *L. seeligeri* have been associated with only a very small number of human infections (Greenwood et al., 2007).

*L. monocytogenes*is considered to be a widespread bacterium in nature that occurs in soil, surface water, plants, but it also appears as a part of the normal faecal flora of many mammals including chiefly animals, but also with some frequency (10%) healthy humans (Kayser et al., 2005). It is believed that the main route of bacterial transmission occurs through the consumption of contaminated food. They have a unique ability to survive and grow in harsh conditions such as wide pH range, high salt concentration, and refrigeration temperatures (-2 - -42 °C), which makes this pathogen a high concern to the food industry (Hernandez-MilianandPayeras-Cifre, 2014.).

Strains of *Listeria* species are divided into serotypes on the basis of somatic O and flagellar H antigens (Murray et al., 2007). On these bases, thirteen serotypes are recognized and can be further characterized by phenotypic or genotypic methods. Most cases of human listeriosis are caused by serotypes 4b, 1/2a and 1/2b, whereas large-food-borne outbreaks have been caused predominantly by serotypes 4b strains (Greenwood et al., 2007).

L. monocytogenes infects the human via the oral route through uptake of contaminated food products. It has been estimated that the infections may result if 10^6-10^9 pathogens enter the gastrointestinal tract with this food (Kayser et al., 2005).*L. monocytogenes* uses various host proteins, including some internalins to adhere and to invade the host epithelial cells. In the infected hosts, *L. monocytogenes* has the ability to induce its own entry into host epithelial cells. Once it is in the intracellular phagocytic vacuole, the bacteria secrete listeriolysins and phospholipases that allow it to lyse the vacuolar membrane and avoid the intracellular killing. Subsequently, adjacent cells have been invaded through plasma membrane protrusions and therefore cell-to-cell spread

occurs. Through this cycle, *L. monocytogenes* can move from one host cell to another, without being in the extracellular environment, thus escaping to the human T-cell immune system. If the immune system does not control the infection, after disruption the epithelial cell layer, the pathogen disseminates to the bloodstream and mesenteric lymph nodes. After that, it usually reaches the liver and spleen where it can subsequently replicate. Beside the intestine barrier, in addition there are generally in the body two other tight physiological barriers that *L. monocytogenes* can get across: the blood-brain barrier and the feto-placental barrier. The brain and placenta therefore represent the other usual organs that this pathogen reaches after the dissemination to the vessels (Hernandez-MilianandPayeras-Cifre, 2014.)

There are two forms of listeriosis caused by *L. monocytogenes*: non-invasive form that in immunocomponent individual develops as a febrile gastroenteritis and an invasive form that in immunocompromised hosts can manifest as septicaemia or meningocephalitis (Camejoet at., 2011). In those cases of febrile gastroenteritis in immunocomponent patients, illness typically occurs 24 hours after ingestion of sufficient inoculum of bacteria and usually lasts 2 days. Common symptoms include fever, watery diarrhoea, nausea, headache, and pain in joints and muscles. The infection is usually self-limited and the symptoms can disappear within a few days, and thus in a comparison with other diarrhoea causing pathogens, *L. monocytogenes* does not represent any dangerous agent. However the outbreaks of *L. monocytogenes* diarrhoea should be considered in investigations of gastroenteritis in which routine enteric pathogens have been ruled out(Murray et al., 2007).

Listeriosis is observed mainly in industrialized countries, where it can occur sporadically or epidemically. Fortunately, the incidence of listeriosis has been declining in these countries during the past decade, which is most likely due to the aggressive implementation of *Listeria* control measures by the food industry (Swaminathan and Gerner-Smidt, 2007).

1.4 Treatment of diarrhoea

The infectious diarrhoeal outbreaks can generally vary from those mild and self-limited which almost do not require any management of treatment to those very severe and life-treating which necessarily require any type of the cure. Severity, duration and the type of diarrhoeal outbreak always depends on the causing pathogen as well as on the nutritional and immune status of the patient. During the past decades, WHO and UNICEF treatment management has been particularly focused on those serious life-treating cases to primarily reduce the high mortality rate of diarrhoea, especially in children under the age of five in developing countries. The three main risk factors of infectious diarrhoeal outbreaks which affect human's life are predominantly dehydration, malnutrition and systemic infection. Dehydration is the major problem in outbreaks of acute types of diarrhoea and globally the leading participant of diarrhoeal childhood mortality. Malnutrition represents another problem particular in diseased children, which can be also in rare cases fatal, but in difference, it is more specific factor for diarrhoea with more prolonged duration, thus persistent diarrhoea. Final life-treating systemic infections are predominantly accompanied with pathogens causing bloody diarrhoea or dysentery. Focusing on these three risk factors has been the main objective of global management of treatment in diarrhoeal infectious outbreaks (WHO, 1993). One of the basics in treatment approach is therefore the adequate fluid and electrolyte replacement and maintenance, for which have been regularly used methods such as oral rehydration therapy, zinc and multivitamin supplementation, and dieting. Performance of selective faecal studies, consequent administration of selective antimicrobial therapy, or exploring for suitable and contraindicated antidiarrhoeal drugs is usually secondary in approach, except cholera, dysentery or serious systemic infections (Guerrant et al., 2001).

1.4.1 Oral rehydration therapy

Oral rehydration therapy (ORT) is the general indication of simple and inexpensive treatment method chiefly made up for preventing dehydration in cases of diarrhoeal outbreaks. For that, special glucose-electrolyte solutions named as oral rehydration salts (ORS) are administrated within the therapy. The general principle of the

ORS function is very simple: after oral ingestion of the glucose-electrolyte solution, it is absorbed in the small intestine even during copious diarrhoea, thus replacing the water and electrolytes lost in the faeces (WHO, 2005). The importance of fluid and salt administration and principles of its administration were undeveloped until 1940s, when the first glucose-electrolyte solution was used for the first time. In comparison to ORS, these were not used orally, but on the i.v. route only. The new history of ORT began in the late 1960s to early 1970s, when the concept of secretory diarrhoea was appreciated as the clinical entity and the physiology of orally administrated glucose-electrolyte solution had become already understood (McMahan and Dupont et al., 2007). Since that, ORT has been a cornerstone of treatment programmes of WHO and UNICEF, which have been recommending ORT to prevent and treat dehydration from diarrhoea irrespective of the cause or age group affected. It has been also estimated that by using ORS can be safely and effectively treated over 90% of cases of any type of diarrhoea. Due to this significant ability, ORS has substantially contributed to the dramatic global reduction in mortality from diarrhoeal diseases (WHO, 2005).

ORS solution is glucose-electrolyte mixture composed of sodium chloride, glucose anhydrous, potassium chloride, trisodium citrate dihydrate and sodium hydrogen carbonate. The two last mentioned compounds were added in 1984 with the aim of improving the stability ORS in hot and humid climates. In the beginning of usage, ORS provided a solution containing 90 mEg/l of sodium with a total osmolarity of 311 mOsm/l. During the time after that, numerous studies have been undertaken to develop an "improved" ORS, particularly to reduce this osmolarity of solution to avoid possible adverse effects of hypertonicity on net fluid absorption. After 20 years of research, an improved ORS solution, named as low osmolarity ORS solution, has been developed. After reduction of osmolarity value, low osmolarity ORS solution provided 75 mEg/l of sodium with a total osmolarity of 245 mOsm/l. The new ORS solution particularly reduced the incidence of vomiting (by 30%) and stool volume (by 20%), which was the significant progress (WHO/Unicef, 2006).

ORT could be qualified as highly suited technology to the primary health care approach in developing countries, where ORS are ordinarily delivered by village health workers and, usually with some guidance, practiced in homes mostly by mothers of ill children. Moreover, these mothers are also guided and taught, how to quickly make their

own home-prepared ORS "sugars and salts" with the use of available home fluids, when the packets of ORS are not available. In these instances, the preparation consists of overcooked water mixed with at least home sugars (glucose, sucrose) and salts (sodium chloride) partially substituting the ingredients of ORS. However, classical packet of ORS solution is normally composed of 1 litter of drinking water mixed with 20 mg of glucose, 3.5 mg of sodium chloride, 2.9 mg of citrate dihydrate and 2.5 mg of sodium hydrogen carbonate and 1.5 mg of potassium chloride (WHO, 1993).

For treatment of patients with acute types of diarrhoea, the amounts of classical ORS solution needed for rehydration may be estimated from patient's weight, or if not available then from his/her age. However, during the initial stages of therapy, general doses are estimated for adults to receive up to 750 ml per hour and for young children 20 ml per kg of body weight per hour. In extreme severe dehydration in cholera patients, such requirement of fluids can vary from 200 to 350ml per kg of body weight. In persistent diarrhoea, administration of ORT is not the prevailing treatment method, nevertheless, the regular take of ORS during the persistent diarrhoea can contribute to better weight gain, thereby can reduce the effect of diarrhoea on nutritional status in malnourished patients. Amounts are usually estimated from nutritional status and the degree of dehydration of the patient. When there is severe malnutrition, there is a recommendation to administrate modified ORS solution with less sodium, more potassium and added sugar to improve better nutrients absorption (WHO, 2005).

1.4.2 Zinc and multivitamin supplementation

Additional tool over ORS in reducing mortality due to diarrhoea, especially in children under 5 years of age, brings the administration of zinc and supplementation. Zinc is a mineral component having a wide spread deficiency among children in developing countries that occurs in most part of Latin America, Africa, the Middle East and South Asia (WHO, 2005). In human organism, zinc generally plays a lot of critical roles, mostly as a cofactor for numerous metalloenzymes located in cell membrane, required for its normal function and growth (Babu, 2013). Among others, zinc supposedly significantly contributes to the proper physiological function at the level of gastrointestinal system. It restores mucosal barrier integrity and enterocyte brush-border enzyme activity; it promotes

the production of antibodies and circulating lymphocytes against intestinal pathogens, and has a direct effect on ion channels, acting as a potassium channel blocker of 3-5-cAMP-mediated chlorine secretion. These aforesaid functions of zinc compel us to believe that multiple mechanisms may be involved in reducing stool output in diarrhoea, thus its administration could be proper for the treatment. In addition, during the diarrhoea, there is a significant loss of zinc, because it cannot be stored in the body, and nearly 50% of zinc excretion takes place through the gastrointestinal tract, and thus the demand of its replacing also contributes to the importance of its administration (Lazzerini and Ronfani, 2013). According to WHO, 10-20 mg of zinc supplementation per day significantly reduces the severity and duration of acute diarrhoea in children less than 5 years of age, whereas when these doses are taken for 10-14 days, it reduces the risk of incidence of diarrhoea for 2 to 3 months. The aforesaid doses are usually administered after initial four-hour rehydration in the management of acute outbreaks (WGO, 2008). In persistent diarrhoea, zinc is also administrated but in addition with another vitamins and minerals including vitamin A, copper, magnesium and folate. Recommended daily allowances are highest for vitamin A (400 mg per day) and magnesium (80 mg per day), whereas zinc is less (10 mg). All of the minerals and vitamins can be administrated by special multivitamin tablets, but if not available, it is recommended to administrate at least two of them, which should be received by patient each day for two weeks during the persistent diarrhoea (WHO, 2005). The usage of these multivitamins added to zinc and ORS is probably only due to the general belief of coexisting micronutrient deficiencies in diarrhoea. However, some investigations have announced, that it carries several disadvantages, such as the preparation is hyperosmolar due to the high concentration of various micronutrient solutes that increase the possibility of osmotic diarrhoea that can cause paradoxical worsening (Babu, 2013).

1.4.3 Antidiarrhoeal drugs

In addition to a solution of ORS to treat dehydration, there are many antidiarrhoeal drugs with a specific mechanisms, by which may help in reducing amount of fluid loss, frequency and consistency of stool, or shorten the clinical course of diarrhoea. However, these drugs are rather useful in self-limited diarrhoeal outbreaks predominating in developed countries or in cases of traveller’s diarrhoea, primarily to alleviate the progression of the disease and contribute to quicker recover (Manatsathit et at., 2002). In

life-treating acute diarrhoeas in developing world, the use of these drugs is not much implemented, because their value in treating these serious outbreaks has not been proven enough and their administration is usually too much costly. Actually within the management of childhood treatment, their usage is predominantly contraindicated, because some of them may evoke the dangerous paralysis of the gut, which can be fatal for the child. These antidiarrhoeals are generally denoted as adsorbens, antimotility drugs, and antisecretory drugs (WHO, 1993).

Adsorbens: These drugs are promoted for the treatment of diarrhoea on the basis of their ability to bind and inactivate bacterial toxins or other substances that cause diarrhoea, and their claim to protect the intestinal mucosa. They include e.g. kaolin, pectin, attapulgite, smectite, activated charcoal and cholestyramine (WHO, 2005). Their efficacy mostly depends on their potency to adsorb toxins, whereas some of them, for example smectite and attapulgite, are more effective in adsorbing than others. In clinical trials, they can increase stool consistency and decrease stool frequency, but cannot reduce the amount of fluid loss, and thus when prescribing these medications, it is important to maintain adequate hydration and proper diet, especially in the elderly. Commonly, they are mostly used in chronic diarrhoea and irritable bowel syndrome, never in more severe acute diarrhoeas (Manatsathit et at., 2002).

Antimotility drugs: These opiates and opiate like drugs, also denoted as antiperistaltics, have the ability to inhibit peristaltic movements of the bowel, by which reduce the frequency of stool passage. In addition, some of them may have mild pro-absorbtive and antisecretory activity. They include e.g. loperamide hydrochloride, diphenoxylate, codeine, tincture of opium and other opiates (WHO, 2005). These drugs may be helpful in uncomplicated diarrhoea of mild to moderate severity such as loperaminde for traveller's diarrhoea, however their use should be avoided in diarrhoea caused by invasive pathogens and in bloody or suspected inflammatory diarrhoea in fever patients, because in these cases, the enhanced intestinal static may enhance tissue invasion and prolong the infection by delaying elimination of the causative organisms (WGO, 2008). Moreover, in childhood diarrhoea, they can cause potentially fatal paralytic illeus, thus their use is in children and infants absolutely contraindicated (WHO, 2005).

Antisecretory drugs: As the name indicates, these drugs are reducing fluid loss by their ability to prevent an increased intestine secretion process. They work by a variety

of different mechanisms including inhibition of prostaglandins, and effects on AMP, calmodulin inhibition, inhibition of gut hormones or enkephalinase inhibition of chloride channels, by which can effectively prevent secretion. Nevertheless, many of them have to be administered in very high doses to give effective antisecretory effects *in vivo* (Manatsathit et at., 2002). Drugs with antisecretory activity include particularly bismuth subsalicyclate and racecadotril (WGO, 2008). Other agents with antisecretory activity could be e.g. phenothiazine, chlorpromazine, aspirin, indomethacin, lithium carbonate, and calmodulin inhibitors (Manatsathit et at., 2002). Bismuth subsalicyclate and racecadotril are drugs generally used for treatment of acute diarrhoea. Whereas almost all other antidiarrhoal agents are contraindicated in treatment of young children, these two drugs are licensed in many countries of the world and their administration is quite useful. For example for bismuth subsalicyclate is estimated, that when it is given every four hours, the decrease of stool output in children with acute diarrhoea is by about 30% (WHO, 2005). Racecadotril is one of the agents that cause antihypersecretory effect by inhibiting enkephalinase enzyme of chloride channels (Lehert et al., 2011). On the other hand the antisecretory effect of bismuth subsalicyclate is related to salicylate moiety (McMahan and Dupont et al., 2007). Among acute diarrhoea, bismuth subsalicyclate can be also useful for treating traveller's diarrhoea, where it can additionally alleviate in nausea or abdominal pain. In spite of the relatively positive efficacy of these antisecretory drugs, they are among other methods like ORS still rarely practical (WHO, 2005).

1.4.4 Antimicrobials against diarrhoea causing pathogens

The use of antimicrobials for treatment in infectious diarrhoeal outbreaks is relatively rarely practiced and in general they should not be used routinely. The simple reason is that their administration is not profitable within the treatment management in most of the cases. Primarily there are not many antimicrobial agents enough effective against most of the organisms causing diarrhoea and a lot of them can conversely make the disease worse. In addition, use of antimicrobial drugs adds to the cost of treatment, risks adverse reactions and enhances the development of resistant bacteria, especially in empirical antimicrobial treatments (WHO, 1993).

Selecting an effective antimicrobials can minimize these problems, however it always requires precise knowledge of the pathogen and its likely sensitivity, which is information usually unavailable (WHO, 2005). This is especially true for cases of acute watery diarrhoea where if use of antimicrobial therapy, it almost always have to be done empirically, because the stool culture examination and subsequent selection of suitable drug are usually prolonged too much to use it in these self-limited outbreaks with a short duration. If the acute watery diarrhoea is severe and life-treating, especially for young children, the adequate rehydration therapy is always the main preference in a treatment process (Diniz-Santos et al., 2006). Although the pathogenic organism can be somehow estimated from the clinical symptoms, in most of the diarrhoeal outbreaks it is not possible to distinguish clinically episodes that might respond on antimicrobial activity from those unresponsive, such as ETEC infection from Rotavirus or *Cryptosporidium* (WHO, 2005).

The use of antimicrobial drugs within the treatment of diarrhoea is in general reliably helpful and usually required only in patients with bloody diarrhoea (shigellosis, amoebiasis), suspected cholera with severe dehydration, and in cases with serious non-intestinal infections. This restriction is especially related to childhood diarrhoea where use of antimicrobials is otherwise strictly forbidden (WHO, 1990). Bloody diarrhoea is often of prolonged duration, and thus the stool examination can be suitable method in this case to detect if the causing pathogen is *Shigella* spp. or *E. hystolitica*, for selection of effective antimicrobial agent. In cholera cases, the stool examination is not necessary, because it can be relatively easily clinically distinguished from other acute diarrhoeas, due to severe quick dehydration leading to hypovolaemic shock and also due to its typical endemic occurrence. In children, non-intestinal infectious outbreaks include pneumonia, sepsis, urinary tract infection or otitis (Guerrant et al., 2001; WHO, 2005). Other non-intestinal infection requiring antimicrobial therapy can be for example typhoid fever caused by *S.* Typhi. Antimicrobial therapy is also considered when diarrhoea has prolonged duration, thus in persistent diarrhoea, when the patient is severely malnourished or when his/her immune status is compromised. Other cases suitable for use of antimicrobials could represent traveller's diarrhoea outbreaks and the community - acquired secretory diarrhoeas, usually in cases when the pathogen is known (WGO, 2012).

All agents used for therapy of infectious diarrhoea of any aetiology can be commonly classified into ten classes of antibiotics: quinolones, macrolides, tetracyclines, sulfonamides, penicillins and aminoglycosides.

Quinolones: This class includes synthetic antibacterial agents, which are structurally aromatic compounds with the pyridine-β-carboxylic-acid nucleus. Compounds belonging to the subset fluoroquinolones are characterized by a fluorine atom attached to the central ring system. Quinolones have a selective and bacteriocidal effect by interfering with bacterial DNA replication. This make by specific substituents which produce great affinity to DNA gyrase and topoisomerase IV as well as allow penetration of the bacterial outer membrane. These drugs are generally used for Gram-negative as well as for Gram-positive bacteria with the effective results in both cases (Bryskier, 2005). The agents of this class used in therapy of infectious diarrhoeal outbreaks include ciprofloxacin, norfloxacin, levofloxacin and nalidixic acid.

Ciprofloxacin is a fluoroquinolone used for a broad antibacterial spectrum, however its largest effect is against Gram-negative bacteria (Sköld, 2011). It is the major agent routinely used in antimicrobial therapy in Shigellosis against *S. dysenteriae* in adults as well as in children. The drug can be also used in treatment of cholera, but exclusively in adults only. Further effect of the drug has been found against a lot of diarrhoea causing strains of *E. coli*, especially ETEC, EAEC, EIEC in cases of traveller's diarrhoea. The drug has also become very suitable agent in empirical treatment of acute diarrhoea in adults (WHO, 2005; WGO, 2008; Guerrant et al., 2001). Another fluoroquinolones, such as norfloxacin and levofloxacin, have also become useful for treatment of Shigellosis and against *E.coli* strains in traveller's diarrhoea (McMahan and Dupont et al., 2007).

Nalidixic acid is the only one non-fluorinated quinolone available and officially the first synthesized quinolone ever. It was shown to be an effective agent to use in several countries, particularly for empirical treatment of acute diarrhoea in adults and against typhoid fever, however its increasing resistance has compelled to synthesize more effective fluoroquinolones (Diniz-Santos et al., 2006). The agent is also used as the alternative way of treatment childhood Shigellosis (WHO, 1990).

Macrolides: This class include antibiotic drugs commonly characterized as hydrophobic molecules having a central 12- to 16- membered-ring lactone with few or no

double bonds and no nitrogen atom (Bryskier, 2005). Marcolides have a selective and bacteriostatic effect by inhibiting ribosome peptide synthesis of the bacterial cell. This makes by interfering with the binding of aminoacyl-tRNA to ribosome, thereby inhibits the peptide elongation. These drugs have a good effect against a rather broad spectrum of Gram-positive bacteria and also against a few Gram-negative bacteria (Sköld, 2011). The agents of this class used in therapy of infectious diarrhoeal outbreaks include azithromycin and erythromycin.

Azithromycin has been found to be an effective alternative for empirical treatment of acute diarrhoea in adults, especially due to its safety, comfortable once-daily posology and high cellular penetration. With fluoroquinolones, azithromycin has also shown similar clinical and bacteriological effectiveness in treatment of typhoid fever as well as in several cases of *C. jejuni* infections where fluoroquinolone-resistance is endemic (Diniz-Santos et al., 2006). This drug is another agent commonly used in cases of traveller's diarrhoea as well (McMahan and Dupont et al., 2007). Erythromycin is an effective option for the treatment of severe cases of cholera in young children who should not take tetracyclines or fluoroquinolones (Diniz-Santos et al., 2006).

Tetracyclines: This is a specific class of antibiotics originally isolated from bacteria of the genus Streptomyces. The name hints at their chemical structure, as they are four-membered polycyclic structures of the perhydronaphtanacene carboxamide type (Bryskier, 2005). Like Macrolides, tetracyclines also act bacteriostatically by reversibly inihibiting the bacterial ribosome peptide synthesis. This broad spectrum of antibiotics have positive effect against Gram-positive and Gram-negarive bacteria, as well as on non-bacterial, such as mycoplasma, protozoa (e.g.malaria) (Sköld, 2011). The agents of this class used in therapy of infectious diarrhoeal outbreaks include tetracycline and doxycycline. Both drugs have been administrated as the main agents for treatment of cholera with severe dehydration. Tetracycline had been used for adults and children, but it had subsequently showed very unfavourable side effects in young children, and thus for them, the administration of safer doxycycline began. However tetracyclines have been systematically used for a long time and a several resistant strains has arisen, and thus the alternative drugs like fluoroquinolones or TMP-SMX had to be administrated (WHO, 2005; Diniz-Santos et al., 2006).

Penicillins: This typical class belongs to the family β-lactams, structurally characterized by central β-lactam ring, attached by thiazolidine nucleus and a side chain at the position C-6. Penicillins act on peptidoglycan synthesis, causing lysis and cell death. This make by inhibiting one of the stages of necessary for the cross-linking of peptidoglycan, transpeptidation (Bryskier, 2005). Their effect is directed primarily against a limited number of bacteria, mostly Gram-positive cocci, whereas Gram-negative enterobacteria are unaffected at corresponding doses, supposedly due to their outer lipopolysaccharide layer (Sköld, 2011). The agents of this class used in therapy of infectious diarrhoeal outbreaks include penicillin and ampicillin. There has been found a great antibiotic synergy between penicillin or ampicillin with some aminoglycoside in treatment of infectious Listeriosis or in outbreaks of *E. faecalis* infection, thus they become cornerstone of the antimicrobial therapy in these cases (Greenwood et al., 2007; Engleberg et al., 2012). In addition, ampicillin represents another effective and safe choice for treatment Shigellosis in both adults and children, unfortunately resistance to this drug have quickly become frequent (WHO, 1993).

Cephalosporins: This class also belongs to the family β-lactams and with penicillins they are closely related. They differ from penicillins by having a six-membered, heterocyclic, sulphur containing ring (dihydrothiazine) attached to the four-membered betalactam ring (Sköld, 2011). The only agent of the class used for antibacterial treatment of diarrhoea is ceftriaxone. This drug has been shown as available alternative for treatment of Shigellosis in children, mostly because of its fewer adverse effects. Its effectiveness has been also demonstrated in both typhoid and non-typhoid salmonellosis. However, the clinical resolution of symptoms after its administration is relatively slower than with most of other drug, and to disadvantages contributes its high cost as well (WHO, 2005; Diniz-Santos et al., 2006).

Aminoglycosides: This is the class of antibiotics originally isolated from many different species of the bacterial genus *Streptomyces*. Aminoglycosides are structurally characterized as amino sugars divided to two groups: streptomycin and its derivates, and deoxystreptamines (Bryskier, 2005). Like macrolides and tetracyclines, they affect bacteria by inihibiting its ribosome peptide synthesis, but unlike them, aminoglycosides finally kill the bacteria. Aminoglycosides have a broad spectrum of activity, especially in Gram-negative bacteria (Sköld, 2011). The agents of this class used in therapy of infectious

diarrhoeal outbreaks include gentamicin and neomycin. Primarily as mentioned, aminoglycosides show a significant effect in synergy with penicillins in Listeriosis and *E. faecalis* infections (Greenwood et al., 2007; Engleberg et al., 2012). The use of gentamicin is recommended in combination with ampicillin for therapy of childhood diarrhoea with severe malnurtrition, when children are more vulnerable to serious infections (WHO, 2005). In neomycin, there are some evidences of treatment in childhood Shigellosis and in EPEC infectious outbreaks (McMahan and Dupont et al., 2007).

1.5 Plant-derived products for treatment diarrhoeal diseases

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of yearsand in a lot of rural areas, especially in developing countries, they continue to be used as the primary source of medicine. The use of medicinal plants for treatment of diarrhoeal diseases is common in most parts of the developing world, particularly in tropical areas, where the wide spread biodiversity of plants represents a big source of potential remedies. In most of these regions, cure of diarrhoeal outbreaks with plants, has beena routine component of the traditional medicine (Palombo, 2006).

Traditional medicine is the general term for the sum of total knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses.Practices of traditional medicine vary greatly from country to country and from region to region, as they are influenced by factors such as culture, history, personal attitudes and philosophy (WHO, 2000). Other terms used for description of traditional medicine are alternative, non-conventional, complementary, or holistic medicine (Rheumatology, 2014; NCFH, 2011). These terms are used chiefly in the developed countries where the modern medicine predominate, and as estimated the only 20 % of the population using traditional medicine are therefore represented as representatives of the alternative form of treatment. In the developing world, the situation is significantly different and the estimated percentage of the population routinely using tradition medicine is up to 80 %, thus it is the prime source

of health care (Rheumatology, 2014). For many people in the industrialised world, scientifically unfounded health methods in traditional medicine raises doubts about its useful effect. However, its long historical use including experience passed on from generation to generation, is contributing to belief of the efficacy and particularly the safety of traditional medicine (WHO, 2000).

Since the herbal remedies are known by having lesser side effects than the conventional drugs and are safer to use, the WHO has encouraged studies for the treatment and preventive of diarrhoea disease based on traditional practice.Numerous studies have validated the traditional use of antidiarrhoeal medicinal plants by investigating the biological activity of extracts of such plants, which have antispasmodic effects, delay intestinal transit, suppress gut motility, stimulate water adsorption or reduce electrolyte secretion (Offiah and Chikwendu, 1999). Moreover, the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics and antivirals and the fact that diarrhoeal diseases continue to be a major cause of morbidity and mortality throughout the world predominantly due to these pathogens, there is an increasing renewed interest in the discovery of novel plant-derived compounds that can be used to fight these diseases by their antimicrobial properties (Palombo, 2006).

1.5.1 Medicinal plants used in traditional medicine systems

The most renowned traditional medicine systems have their origin in South Asia in ancient China and India, where the implementation of plants to treat several diseases including those diarrhoea causing has always been the cornerstone of medicinal system (Rheumatology, 2014). Howeverbesides them, almost in all other parts of the world, thus in American continents, Africa and Europe, there have also been implemented traditional herbal healing systems sincetime immemorial (Palombo, 2006).

Traditional Chinese medicine (TCM) is a 5000 years old system of holistic treatment practises treating disorder of both the mind and the body. Beside herb therapy, other specific procedures involve for example acupuncture, massages etc. According to TCM, each type of diarrhoea can be treated either with a specific herb or, more commonly, a combination of herbs, each of which contributes to a cure for the disorder. One of the main herbs used to treat diarrhoea is Huang Lian - Golden thread rhizome, the powdered rhizome of *Coptischinensis*. This herb treats diarrhoea by having the tendency to rehydrate

and restore proper heat balance in the lower part of the body. Additionally, it supposedly clears damp-heat and has an antibacterial and antiviral effect. Other herbs like *Cuscutachinensis* (Chinese dodder), *Psoraleacorylifolia*, or *Amomumsubulatum* (Black cardamom)have the primer positive influence on function of the kidneys and spleen by which should indirectly improve the disordered gastrointestinal tract. A combination of herbs having a good synergistic effect on treatment of diarrhoea could within TCM represent special mixture of nine herbs: *Atractylodismacrocephalae, Codonopsispilosulae, Evodiarutaecarpa, Glycyrrhizauralensis, Myristicafragrans, Psoraleacorylifolia, Schisandrachinensis, Typhoniumgiganteum, Zingiberofficinale*. This combination of herbs is recommended because its components have astringent properties, causing tissues to contract and retain water, thus reducing diarrhoea (altMD.com Health & Wellness Community).

Indian Ayurveda is conceived as science of life and a comprehensive medical system that like TCM has been the traditional system of healthcare in India for more than 5000 years. This comprehensive medical system has been prescribed many preparations from Indian medicinal plants and their use to treat diarrhoeal diseases is also widespread (Thakurta et al., 2007). In Ayurveda, diarrhoea is known as Atisara and for the treatment, several plant species are used. The bark of the tree *Terminalia arjuna* (Arjuna) taken as a decoction; black nightshade leaves of the herb *Solanumnigrum* (Black Nightshade) taken in the form of an infusion with the juice of other liquids; the oil extracted from the seeds of the herb *Trachyspermumammi* (Bishop's Weed); the oil obtained from the herb *Anethumsowa* (Dill); all these plants are used for direct cure during the diarrhoea. In addition, eating of unripe or half-ripe fruit of *Aegle marmelos* (Bael) is recommended especially for diarrhoea accompanied by fever. For the prevention of diarrhoea, leaves of the tree *Acacia Arabica* (Babul) mixed with black cumin are mostly taken, however whatever other part of the tree can be also useful. Due to the beneficial and supportive effects during the outbreaks of diarrhoea, in Ayurveda there are taken other plants such as the buds of *Ficus bengalensis* (Banyan), the gum of the tree *Butea monosperma* (Butea), or the seeds of *Trigonella foenum-graecum* (Fenugreek) (Streetdirectory.com).

Traditional healers from American continents, thus Indians or the Native Americans have thousands of years long history of herbal medicine as well. In most instances, plants must be prepared using exacting methods and if used otherwise, the

results may ineffectual if not dangerous or life threatening. Among Indians of northern California the most frequently used remedy for diarrhoea has been a tea of *Prunusserotina* (blackberry) roots. The same tree have been used by the Mohegans against dysentery, in the difference that they have used juice of the naturally fermented ripe wild fruits of the tree which then drank. Dysentery has been also treated by the Pawnee, Omaha, and Dakota tribes with the tea of boiled roots of the plants belonging to the genus *Rubus* (Black Raspberries). Catawbas have treated dysentery with the tea of Star Grass leaves for change. Very unusual method to treat diarrhoea have been used by the Menominees, when they boiled the inner bark of the shrub *Cornussanguinea*(Dogwood) and passed the warm solution into the rectum with a naturally made rectal syringe. The warmed solution supposedly had a positive effect on the function of the colon and helped in decreasing fluid secretion (Mantaca: American Indian Council).

1.5.2 Phytotherapeuticals and dietary supplements

Botanical dietary supplements, also called botanical nutraceuticals or herbals, can be best defined as plant-derived materials with medical benefits aimed at disease prevention or treatment that go beyond satisfying basic nutritional requirements (Raskin et al., 2002).

At the present time, one of the potential possibilities how to improve the function of gastrointestinal tract, and thus prevent or cure whatever disorders such as diarrhoea, represents the modification of intestinal ecosystem by the intake of dietary components such as probiotics and prebiotics. Whereas probiotics are defined as a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance and prevents engagement of pathogenic microorganisms or compete with those present, prebiotics represent the essential nutrients required by these commensals (Vaisman et al., 2010). The whole definition of prebiotic is that it is a "non-digestible food" ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of or a limited number of bacteria in the colon, and thus improving host health. Prebiotics are usually, but not exclusively, non-digestible oligosaccharides, from which the most frequently studied are fructoologosaccharides (FOS). FOS consist of polymers of glucose and fructose monomers in varying ratios. Shorter chain lengths ($n < 9$) of FOS are referred to as oligofructose and medium chain lengths ($n = 3\text{-}60$) are referred to as inulin (Whelan et al., 2001). These components are specific by their resistance to

digestive enzymes in the human gut causing its indigestibility, whereas for colonic microflora represent fermentable material. The fermentation generally results in bifidogenic and pH-lowering effects causing inhibition of certain strains of potentially pathogenic bacteria thereby can among others prevent diarrhoea. This hypothesis have been confirmed particularly by certain *in vitro* studies showing that a symbiotic combination of inulin plus oligofructose with *Lactobacillus plantarum* and *Bifidobacteriumbifidum* increased the growth of bifidobacteria but inhibited human pathogenic strains such as *C. jejuni*, *E. coli*, and *S. enteridis* more than other carbohydrate tested (de Vrese and Marteau, 2007). Although at least no studies *in vivo* have shown any significant improvement of prebiotic administration especially during the acute outbreaks of diarrhoea, some other studies have shown its contribution mainly as a preventive approach and suitable supplement for treatment of mild and prolonged diarrhoeal outbreaks, especially because the estimated amount of time needed in order to detect significant effect of prebiotics on gut microbiota was at least two weeks (Vaisman et al., 2010).

In a point of view that the most of the prebiotic carbohydrates such as inulin are originally plant-derived components occurring, there is still a big interest in finding new plants representing their potential source. Beside FOS, additionally the raffinose family of oligosaccharides, resistant starch (the type that is not absorbed in the gastrointestinal tract) and some polysaccharides found in plant cell walls, such as xylans and pectins, have also been recognized as potential prebiotic carbohydrates. Nowadays industrially most often extracted prebiotic is an inulin from *Chicoriumintibus L.* (Chicory) distributed in tubes as a food supplement. However there are much more plant potential sources; e.g. *Abelmoschus esculentus* L. Moench (okra), *Allium ampeloprasum* L. (leek), *A. cepa* L. (onion) or *A. cepa* L. var. *aggregatum* (shallot), *A. sativum* (garlic), *Helianthus tuberosus* L. (Jerusalem artichoke), among vegetables; *Artocarpus heterophyllus* Lam (jack fruit), *Borassus flabellifer* L. (palm fruit), *Hylocerus* species (dragon fruit), *Prunus persica* L. Batsch (nectarine)or among fruits; and *Dahlia* species (dahliya), *Dioscorea esculenta* Burk. (gembili), or *Smallanthus sonchifoliu* (yacon) among root and tuber crops (Dwivedi et al., 2014).

Beside prebiotics, there are a lot of botanical dietary supplements particularly distributed in the developed countries as commercial products to prevent, treat or just alleviate the course of rather not serious diarrhoeal cases. These herbal products are generally recommended to maintain intestinal health as part of balanced diet, improve gut motility, neutralise bacterial infections, or treat inflammation. These may include e.g. *Althaea officinalis* (Marshmallow), *Artemisia absinthium* (Wormwood), *Echinacea sp.*, *Garcinia mangostana* (Mangosteen), *Hordeum vulgare* (Barley Grass Juice Powder), *Hydrastis canadensis* (Goldenseal), *Olea sp.* (Olive Leaf Extract), *Phyllanthusamarus* (KeelaNelli) (Regenerative nutrition; altMD.com Health & Wellness Community).

1.5.3 Plant compounds affecting growth of diarrhoea-causing microorganisms

During the process of evolution, the plants have developed the ability to synthesize a wide range of aromatic substances fulfilling the specific functions in their metabolism, whereas one of them is to protect the plant against attacks of various microbial pathogens. These substances, usually released as secondary metabolites, therefore often possess antimicrobial properties and represent the suitable source of novel antibacterial agents. Plant-derived compounds with these properties can be divided to couple of classes such as phenolics, or terpenoids (Cowan M. 1999).

Phenolics: These compounds are structurally characterized by the presence of at least one aromatic ring substituted by at least one hydroxyl group, free or engaged in another function: ether, ester, or glycoside (Bruneton, 1999). They generally possess antibacterial properties and in certain studies they have a significant activity against certain diarrhoeal causing pathogens. For example, the extracts from Amazonian plants *Diclinanona calycina*, *Ipomoea alba* and *Moronobea coccinea* have shown *in vitro* growth-inhibitory effect against *E. faecalis* with MICs≤ 40 μg/mL (de Castilho et al., 2014). Flavonoid rutin have shown very high activity (MIC 16 μg/mL) against *E. coli* and (MIC 8 μg/mL) against *E. faecalis* (Orhan et al., 2010). Finally, antimicrobial polyphenolics occurred in aqueous extract from stem bark of *Roureopsisobliquifoliata* have been responsible for inhibiting growth of *V. cholerae* and *S. dysenteriae* (MICs 31.25μg/mL).

Terpenoids: These plant secondary metabolites are from the point of view their chemical structure basically characterized by the variable number of head-to-tail condensed isoprene units. Their antibacterial activity against diarrhoea causing pathogens demonstrates a number of studies (Bruneton, 1999). Triterpenoids isolated from the African tree *Combretum imberbe* produced significant *in vitro* antibacterial activity against *E. coli* with MICs16 µg/ml, and *E. faecalis* with MICs 24µg/ml (Angeh et al., 2007). Dichlormethane and ethanol leaf extracts from South African trees *Rapanea melanophloeos* and *Maesa lanceolata* have showed the credible antibacterial activity (MIC 98 µg/mL) against *S. flexneri*, whereas saponins have been reportedly considered to be the major bioactive components of the extracts (Madikizela et al., 2012). Methanol and aqueous extracts of Schizandrae Fructus from *Schizandra chinensis* have shown great antibacterial effectivity (MIC 15.6µg/mL) against *S.* Paratyphi A, (MIC 31.3µg/mL) against S. Enteridis (Lee et al., 2006). Some antimicrobial active nortriterpenoids have been isolated from *Schizandra* species and thus can be considered to be also active agents in this case (Xiao et al., 2006).

Hypotesis

In a point of view that plants and their constituents have always had a leading position in treatment of diarrhoeal diseases within most of the traditional medicinal systems in the world and their visible antidiarrhoeal efficacy has been also exploited in the developed world in a form of dietary supplements, we can expect that systematic microbiological and photochemical research of plant derived products, such as extracts, essential oils and their constituents, can lead to discovery of effective antidiarrhoeal low-cost agents suitable for administration in certain especially tropical areas where diarrhoea represents a significant problem and the conventional medications are not available.

Aim of the thesis

The aim of this study is evaluation of *in vitro* growth inhibitory effect of plant derived extracts and their compounds against potentially pathogenic microorganisms causing diarrhoea.

Materials and Methods

2.2 Plant material and extracts preparation

Four plant species selected according to their antimicrobial effects described in the literature, were purchased from commercial sources. Dried samples were homogenized using Grindomix mill (Retsch, Haan, Germany) and 15 g of dry matter from each sample was extracted for 24 hours in 450 ml 80% ethanol at room temperature using laboratory shaker (GFL, Burgwedel, Germany). The extracts were then filtered and concentrated using rotary evaporator (Büchi Labortechnik, Flawil, Switzerland) *in vacuo* at 40°C. Dried residues were subsequently diluted in DMSO to obtain stock solution of the final concentration 51.2 mg/mL and stored at -20°C until their use. The yields (%) of dry residues are given in Table 1.

Table 1.Summary of data on plant materials tested

Species	Family	Plant part	Source	Yield (%)
Alpinia oxyphylla Miq.	Zingiberaceae	Seeds	Seedville (Green, Ohio, USA)	10.67
Berberis aristata DC.	*Berberidaceae*	Roots	Aramacs (Delhi, India)	10.87
Emblica officinalis Gaertn.	*Euphorbiaceae*	Roots	Aramacs (Delhi, India)	45.93
Santalum album L.	*Santalaceae*	Hardwood	Zdeněk Pazdera - Wendys (Miličín, CZ)	15.88

2.2 Chemicals

In this study, we tested twelve commercially obtained plant-derived compounds: aloe emodin, antrachinon, capsaicin, curcumin, naringenin, norhydrogenic acid, pterostilbene, zinc pyrithion, resveratrol, rhein, rutin, sanguinarine (Sigma-Aldrich, Prague, CZ). The stock solutions were prepared by dissolving compounds in dimethyl sulfoxide (Lach-Ner, Neratovice, CZ) at a concentration of 12,800 μg ml^{-1} and then stored

at -20°C. Tetracycline (Sigma-Aldrich, Prague, CZ) was dissolved at an appropriate concentration in ethanol (Lach-Ner, Neratovice, CZ) prior to testing.

2.3 Bacterial strains and growth media

The antimicrobial activity was evaluated against nine bacterial American Typical Culture Collection (ATCC) strains, and they were selected as representatives of both classes of Gram-positive (*Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644) and Gram-negative (*Escherichia coli* ATCC 25922, *Salmonella enteritca*ATCC 13076) bacteria. The standard ATCC strains were purchased from Oxoid (Basingstoke, UK). All bacteria were tested in Mueller-Hinton broth (Oxoid, Basingstoke, UK). For *E. faecalis*, Mueller-Hinton broth was enriched with 1 % of glucose (Sigma-Aldrich, Prague, CZ). The sensitivity of standard strains to tetracycline had been checked.

2.4 Antimicrobial assay

The *in vitro* antimicrobial activity was determined by the broth microdilution method using 96-well microtiter plates according to CLSI guidelines (2007), modified according to the recommendations previously proposed for more effective assessment of the anti-infective potential of natural products (Cos et al. 2006). The samples were 2-fold diluted in Mueller-Hinton broth (100 μl) in a ranges of 0.125-128 and 0.5-512 μg ml^{-1} for compounds and extracts respectively. Bacterial cultures were diluted to contain 2.5-3 × 104 CFU ml^{-1} in microtiter plate, each well was subsequently inoculated with the suspension and microplates were incubated at 37°C for 24 h. Growth of microorganisms were estimated visually as turbidity and more accurately determined by measuring the optical density by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. Minimum inhibitory concentrations (MICs) were calculated based on the density of the growth control and were expressed as the lowest concentrations, which showed at least 80% reduction of microorganisms' growth, compared to that of the compound-free growth control. Tetracycline has been tested as positive antibiotic control in concentration range 0.25-32 μg ml-1. All samples were tested as three independent experiments, each was carried out in triplicate and the results are presented as the mean of MICs obtained from these experiments.

Results and discussion

The results showed that with exception of ethanol extract from hardwood of *Santalum album*, which produced moderate antibacterial effect against *E. feacalis* (MIC = 256 μg/mL) and *L. monocytogenes* (MIC = 512 μg/mL), none of the plants tested exhibited significant growth-inhibitory effect against diarrhoea causing microorganisms in this study. When comparing the antibacterial activity of all compounds tested, the best results were obtained for zinc pyrithione and sanguinarine (Tab. 1), whereas zinc pyrithinone had shown the absolutely strongest bacterial growth-inhibitory effect with MICs 4 μg/mL for all tested bacteria, closely followed by sanguinarine with MIC values ranging from 16 to 32 μg/mL. With only exception of *E. coli*, zinc pyrition had additionally shown higher effectivity than the positive antibiotic control (tetracyclin) against all other bacterial strains. Gram-positive bacteria demonstrated the stronger susceptibility to sanguinarine, in case of *E.* faecalis even comparable with tetracycline.

Table 1: *In vitro* growth-inhibitory effects of the selected compounds against diarrhoea causing bacteria

Compounds	**Bacterial strain/MIC [a] (μg/ml)**			
	Salmonella enteritca ATCC 13076	*Escherichia* coli ATCC 25922	*Listeria monocytogenes* ATCC 7644	*Enterococcus faecalis* ATCC 29212
Aloe emodin	>128	>128	>128	>128
Antrachinon	>128	>128	>128	>128
Capsaicin	>128	>128	>128	>128
Curcumin	>128	>128	>128	>128
Naringenin	>128	>128	>128	>128
Norhydrogenic acid	>128	>128	>128	64
Pterostilbene	>128	>128	>128	64
Zinc pyrithione	4	4	4	4
Resveratrol	>128	>128	>128	64
Rhein	>128	>128	>128	32
Rutin	>128	>128	>128	128

Sanguinarine	32	32	16	16
Tetracycline [b]	8	0.5	8	16

[a]Minimum inhibitory concentration, [b]positive antibiotic reference standard (μg/ml)

Rhein, pterostilbene and norhydrogenic acid possessed a moderate antibacterial effect with MIC values ranging from 32 to 64 μg/mL against E. faecalis. The rest of the compounds had shown no antibacterial inhibitory effect at concentration 128 μg/mL.

In a point of view of about mentioned facts, E. faecalis was the most susceptible bacterium, which's growth was successfully affected by six of all used compounds with MIC values ranging from 4 to 64 μg/mL. Moreover, E. faecalis was the most resistant strain to the tetracycline. The rest of the bacteria were more resistant against compounds tested, only with the exception of the most effective zinc pyrithione and sanguinarine.

According to our best knowledge there are almost no reports on antibacterial effect of zinc pyrithione (ZPT) against diarrhoea causing pathogens only with a few exceptions. For example, (Wang et al., 2010) performed some antibacterial assays with ZPT which has shown antibacterial activity against E. coli with MIC 400μg/mL. In comparison to our results, these values demonstrate really much lower inhibitory effect, however we consider that for above mentioned study had been used the different macrodilution method, which is unable to show sufficiently exact results as the microdilution in our case. Furthermore, (Polson et al.,) have undertaken the antibacterial assay with the extracts from root of *Polyalthia nemoralis* (the natural plant source of pyrithione compounds(Lewis and Ausubel, 2006) and with ZPT as a single compound, and have found very high antibacterial effect either against *E. coli* with MICs 78.1 μg/mL with extract and 2.44 μg/mL with single ZPT. This is corresponding with our results showing significant in vitro growth-inhibitory effect of ZPT against E. coli (MIC = 4 μg/mL).Also our findings on significant inhibitory activity of sanguinarine (MIC range 16-32 μg/mL) are well corresponding with a several previous reports. For example, on the basis of the antibacterial tests, (Hamoud et al., 2015) have reported that sanguinarine provides a strong antibacterial activity against Gram-positive as well as Gram-negative bacteria. MIC value against E. coli was 4μg/mL in this case, demonstrating its high activity. In another study, (Miao et al., 2011) have these result of sanguinarin significant activity against *E. coli* confirmed by testing several structurally different modifications of the compound.

In spite of that both zinc pyrithion and sanguinarine have provided a significant antibacterial activity against all selected diarrhoea causing pathongens *in vitro*, their usage as the alternative antibiotic drugs in diarrhoeal outbreaks in vivo is contingent on their safety status, and thus the consideration of their potential toxicological features will be the main object of the further discussion.

Zinc pyrithioneis quite wide spread drug approved by Food and Drug Administration in the USA and worldwide used as the microbicidal agent in clinical antiseptic products and cosmetic consumer products especially including antidandruff shampoos (Lamore et al., 2010). As all other zinc-based compounds, it generally represents an important cutaneous therapeutic agent designed for photoprotective, antimicrobial and anti-inflammatory intervention. In a point of view of these properties it has been for example also found as a potentially effective antipsoriatic drug (Lamore and Wondrak, 2011). However, to use this compound as the alternative antibiotic agent to fight diarrhoea causing pathogens, it almost always requires administration via oral route, which can be quite often problematic due to its potential internal toxicity. In certain studies practiced on animals, the internal toxicity of ZPT has been studied by its oral administration. For example,(SCCS, 2013) has published that after oral administration to rats the median lethal doses (LD50) for ZPT had been ranging from 92 to 266 mg/kg. For comparison, for example in tetracycline orally received by rats, value of LD50 was determined to 6443 mg/kg (ScholAR Chemistry, 2009). In another orally administrated widespread drug aspirin, the LD50 values in rats were about 200mg/kg (Toxins© UC regents, LHS living by Chemistry, 2004.). As we can see, the range in ZPT reaches the lowest value but it can be also comparable with aspirin, the drug commonly internally administrated. The studies published by, (SCCS, 2013) based on the above mentioned LD50, have denoted ZPT as the drug may cause acute oral toxicity. The same publisher has also provided another study where the animal species (rat, mouse, dog etc.) have been exposed for 10 days to feed containing 250 ppm of ZPT. The consequences were characterized by weight loss and decreased hind-limb muscle mass of the tested animals. One of the positives of ZPT also mentioned by, (SCCS, 2013) based on another studies, is that its excretion from the body has been found to be relatively rapid, principally via the urine where accounted amount was found for about 73% of the administered ZPT dose, which seems to contribute to its certain safety.(Skoulis et al., 1993) support ZPT potential safety by in vitro study showing its lack to any genotoxic activity and in addition another *in vitro* study undertaken by

(Tailler et al., 2012),has proved its potential antineoplastic activity in case of acute myeloid leukemia. Although we could not find any study directly describing safety of orally received ZPT and some rather give advise to avoid it, there is still an open space to concider and perform another investigations. For example the natural source of pyrithione compounds *Polyalthia nemoralisis* traditional herb commonly used in China to treat stomachaches, thus should be investigated which role these compounds play in their preparations and if there may be potentially some posibility of its higher safety and efficacy in synergy with some other natural bioactiv compounds. Nevertheless, also in the case of usage as a single drug, it is often important to realise that the amounts of received antibacterial drugs are among other depended on the level of the agent's efficacy. In a point of view that ZTP has shown that low values of MIC, there may be some hope that the required amounts of the drug would not have to be that high to produce clinical toxicological effect.

Sanguinarine is the benzophenanthridine alkaloid, which can be isolated from several plant species, particularly from *Papaveraceae* family: e.g. *Sanguinaria Canadensis*, *Macleaya cordata*, or *Eschscholziacalifornica*. It is a compound that exhibits pronounced antimicrobial, antifungal, antiviral and anti-inflammatory activity. To affect pathogenic cells, sanguinarine exhibits a strong DNA intercalating activity. Due to these antimicrobial properties and ability to inhibit bacterial attachment to solid substrates, sanguinarine had been used in toothpastes and mouth-washes (Hamoud et al., 2015). However subsequently certain researchers began suspecting that sanguinarine caused the formation of white lesions called oral leukoplakia, which can in same rare cases result in oral cancer, and thus sanguinarine occurred in toothpaste named as Viadent had to be consequently excluded (The Ohio State University, 2012).This is quite inconsistent with the study of (Ahmad et al., 2000) proving the potential anticancer activity of sanguinarine and some literature researchers also affirm that association between Viandent and leukoplakia is spurious (Proenca-Afonso I, 2008). As in case of ZPT, oral use of sanguinarine is also inescapable to treat gastrointestinal infections. What differs sanguinarine from ZPT is that its defence effects is generally not aimed on microbial pathogens only, but it also possess supposedly toxic effects against insects or vertebrates potentially including human as well. (Schmeller et al., 1997) performed study on its median lethal dose in mice who received the drug by i.v. route and the results were about16 mg/kg. If the drug is received by i.v. route it usually shows lower amounts of

lethal dose, however in comparison with other commonly administrated drugs the number is despite that still significantly low. Nevertheless, these tests on animals, such as rats and mouse, cannot be always strictly applied on the case of humans or other animals. It is confirmed by (Flesar et al., 2010) who have written that sanguinarine is a commercially available feed additive, registered as Macleaya extract (0.7 to 2.5% of sanguinarine) commonly administrated for various species of animals to improve the taste, and it seems that no visible unhealthy consequences have been described. In addition, (Lee K et al., 2015) mention in their study that dietary sanguinarine has been shown to improve growth, performance and altered various biological and physiological parameters in broiler chickens. In a point of view of this and additionally with lots of studies such as (Hamoud et al., 2015) that report strong synergistic antibacterial activity of sanguinarine with various conventional antibiotics, we can consider that sanguinarine could be potentially safe and effective drug administrated as a minor component of the higher complex antibacterial mixture or as the food supplement possessing antibacterial activity.

Conclusions

Among 4 ethanol extracts and 12 compounds tested in this study, two agents (zinc pyrithione and sanguinarine) have shown the significant growth-inhibitory effect against all diarrhoea-causing bacteria tested. The antibacterial potential of zinc pyrithion, a microbicidal agent frequently used in clinical antiseptic and cosmetic consumer products (e.g. antidandruff shampoos), has previously been tested against microorganisms causing dermatological diseases. However, its antimicrobial effect against other classes of pathogens is poorly known, therefore, this study is one of the first providing the information about its significant *in vitro* antibacterial efficacy against diarrhoea causing bacteria. In Sanguinarine, conversely high number of previous studies has proven its antibacterial activity also including diarrhoea causing bacteria especially *E. coli*. We can therefore conclude that, zinc pyrithione and sanguinarine have been found as drugs theoretically suitable as the alternatives to antibiotics against several diarrhoea-causing pathogens. Unfortunately, there is lack of information on their internal safety, and thus the following investigations should be primarily aimed to find eventual ways and possibilities for the safe rote of their administration. Moreover, evaluation of their effects on other microorganisms causing infectious diarrhoea can be recommended.

References

Ahmad N, Gupta S, Husain M, Heiskanen K, Mukhtar H. 2000. Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versusnormal cells. Clinical Cancer Research 6. 1524-1528.)

Ahmed A, McGaw L, Moodley N, Naidoo V, Eloff J. 2014. Cytotoxic, antimicrobial, antioxidant, antilipoxygenase activities and phenolic composition ofOzoroa and Searsia species (Anacardiaceae) used in South African traditional medicine for treating diarrhoea. South African Journal of Botany 95. 9-18.

altMD.com Health & Wellness Community. .Traditional Chinese Medicine (TCM) for Diarrhea. Available at http://www.altmd.com: Accessed

altMD.com Health & Wellness Community. Herbal Remedies for Acute Gastritis. Available at http://www.altmd.com: Accessed

Angeh J, Huang X, Sattler I, Swan G, Dahse H, Haertl A, Eloff J. 2007. Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid fromCombretum imberbe (Combretaceae). Journal of Ethnopharmacology 110. 56-60.

Babu T. 2013. The ideal zinc preparation in the management of acute diarrhea in children. Indian Journal of Pharmacology 45. 539-539.

Bruneton J. 1999. Pharmacognosy, Phytochemistry, Medicinal Plants. New Yourk: Springer. 1119 p.

Bryskier A. 2005. Antimicrobial Agents: Antibacterials and Antifungals (2nd Edition). Washington, D.C.: ASM Press. 1458p.

Camejo A, Carvalho F, Reis O, Leitao E, Sousa S, Cabanes D. 2011. The arsenal of virulence factors deployed by Listeria monocytogenes to promote its cell infection cycle.Virulence 2.379-394.

Centers for Disease Control and Prevention (CDCP). 2006. Travelers' Diarrhea. Avaible at http://www.cdc.gov: Accessed 2006-11-21

Cowan M. 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews 12. 564-+.

de Castilho A, Correa da Silva J, Coury Saraceni C, Collantes Diaz I, Barradas Paciencia M, Varella A, Suffredini I. 2014. In vitro activity of Amazon plant extracts against Enterococcus faecalis. Brazilian Journal of Microbiology 45. 769-779.

de Vrese M, Marteau P. 2007. Probiotics and prebiotics: Effects on diarrhea. Journal of Nutrition 137. 803S-811S.

Diniz-Santos, D, Silva, L, Silva, N. 2006. Antibiotics for the empirical treatment of acute infectious diarrhea in children. Brazilian Journal of Infectious Diseases 10. 217-227

Donnenberg M, Brett F. 2013. Combating enteropathogenic Escherichia coli (EPEC) infections: the way forvard.Trends in Microbiology 21.317-319.

Dupont H, Friggeri A, Touzeau J, Airapetian N, Tinturier F, Lobjoie E, Lorne E, Hijazi M, Regimbeau J, Mahjoub Y. 2011. Enterococci increase the morbidity and mortality associated with severe intra-abdominal infections in elderly patients hospitalized in the intensive care unit. Journal of Antimicrobial Chemotherapy 66. 2379-2385.

Dwivedi S, Sahrawat K, Puppala N, Ortiz R. 2014. Plant prebiotics and human health: Biotechnology to breed prebiotic-rich nutritious food crops. ELECTRONIC JOURNAL OF BIOTECHNOLOGY 17. 238-245.

Eley A. 1996. Microbial Food Poisoning. London: Springer Science & Business Media. 211p.

Engleberg N, Dermody T, DiRita V. 2012. Schaechter's Mechanisms of Microbial Disease. Philadephia: Lippincott Williams & Wilkins. 862p.

Ericsson C, DuPont H, Steffen R. 2007. Travelers' Diarrhea (2nd Edition). Shelton: PMPH USA, Ltd. 301p.

Facklam R. 2002. What happened to the streptococci: Overview of taxonomic and nomenclature ganges. Clinical Microbiology Reviews 15. 613.

Finn A, Curtis N, Pollard A. 2010. Hot topics in infection and immunity in children VI. New Yourk: Springer. 206p.

Fisher-Walker CL, Black RE. 2010. “Diarrhoea morbidity and mortality in older children, adolescents and adults. Epidemiology and Infection 138. 1215-1226

Flesar J, Havlik J, Kloucek P, Rada V, Titera D, Bednar M, Stropnicky M, Kokoska L. 2010. In vitro growth-inhibitory effect of plant-derived extracts and compounds against Paenibacilluslarvae and their acute oral toxicity to adult honey bees. Veterinary Microbiology 145. 129-133.

Graziani C, Busani L, Dionisi A, Lucarelli C, Owczarek S, Ricci A, Mancin M, Caprioli A, Luzzi I. 2008. Antimicrobial resistance in Salmonella entericaserovarTyphimurium from human and animal sources in Italy.Veterinary Microbiology 128.414-418.

Greenwood D, Slack R, Peutherer J, Barer M. 2007. Medical microbiology:a guide to microbial infections: pathogenesis, immunity, laboratory, diagnosis and control (17th edition). Churchill Livingstone, Elsevier. 752p.

Guerrant R, Van Gilder T, Steiner T, Thielman N, Slutsker L, Tauxe R, Hennessy T, Griffin P, DuPont H, Sack R, Tarr P, Neill M, Nachamkin I, Reller L, Osterholm M,

Bennish M, Pickering L. 2001. Practice guidelines for the management of infectious diarrhoea. Clinical Infectious Diseases 32. 331-351.

Gupta S. K., Keck J., Ram P. K., Crump J. A., Miller M. A., Mintz E. D. 2007 Analysis of data gaps pertaining to enterotoxigenic Escherichia coli infections in low and medium human development index countries. Epidemiology and Infection 136. 721-738.

Haeusler G, Curtis N. 2013. Non-typhoidal Salmonella in Children: Microbiology, Epidemiology and Treatment. Hot Topics in Infection and Immunity in Children IX 764. 13-26.

Hamoud R, Reichling J, Wink M. 2015. Synergistic antibacterial activity of the combination of the alkaloid sanguinarine with EDTAand the antibiotic streptomycin against multidrug resistant bakteria. Journal of Pharmacy and Pharmacology 67. 264-273.

Hernandez-Milian A, Payeras-Cifre A. 2014. What Is New in Listeriosis?. Biomed Research International. 358051

John Wiley & Sons. 2009. Novartis Foundation Symposia : Acute Diarrhoea in Childhood. New Jersey: John Wiley & Sons. 387p.

Kale V, Bhusari K. 2007.Applied Microbiology. Mumbai: Himalaya Publishing House. 419p.

Kaur J, Jain S. 2012. Vi antigen of Salmonella enetricaserovarTyphi - biosynthesis, regulation and its use as vaccine candidate. Central European Journal of Biology 7.825-838.

Kayser FH, Bienz KA, Eckert J, Zingernagel RM. 2005. Medical microbiology. Stuttgart: Georg ThiemeVerlag. 698p.

Lamore S, Cabello C, Wondrak G. 2010. The topical antimicrobial zinc pyrithione is a heat shock response inducer that causes DNA damage and PARP-dependent energy crisis in human skin cells. Cell Stress & Chaperones 15. 309-322.

Lamore, S, Wondrak, G. 2011. Zinc pyrithione impairs zinc homeostasis and upregulates stress response gene expression in reconstructed human epidermis. Biometals 24. 875-890.

Lanata C. F., Mendoza W. 2012. Improving diarrhoea estimates. Institute de InvestigaciónNutricional A.P.- 18-0191, Lima-18 PERU. 48p.

Lazzerini M, Ronfani L. 2013. Oral zinc for treating diarrhoea in children. Cochrane Database of Systematic Reviews. CD005436

Lee K, Kim J, Oh S, Kang C, An B. 2015. Effects of Dietary Sanguinarine on Growth Performance, Relative Organ Weight, Cecal Microflora, Serum Cholesterol Level and Meat Quality in Broiler Chickens. Journal of Poultry Science 52. 15-22.

Lee, M, Kwon, H, Kwon, D, Park, H, Sohn, D, Kim, Y, eo, S, Kang, H, Kim, S, Lee, J. 2006. Antibacterial activity of medicinal herb extracts against Salmonella. International Journal of Food Microbiology 111. 270-275.

Lehert P, Cheron G, Alvarez Calatayud G, Cezard J, Gutierrez Castrellon P, Melendez Garcia J, Santos M, Savitha M. 2011. Racecadotril for childhood gastroenteritis: an individual patient data meta-analysis. Digestive and liver disease 43. 707-713.

Lewis K, Ausubel F. 2006. Prospects for plant-derived antibacterials. Nature Biotechnology 24. 1504-1507. John Wiley & Sons

Lins R, Andrade A, Hirata R, Wilson M, Lewis M, Williams D, Fidel R. 2013.Antimicrobial resistance and virulence traits of Enterococcus faecalis from primary endodontic infections.Journal of Dentistry 41.779-786.

Liu F, Kariyawasam S, Jayarao B, Barrangou R, Gerner-Smidt P, Ribot, E, Knabel S, Dudley E. 2011. Subtyping Salmonella entericaSerovarEnteritidis Isolates from Different Sources by Using Sequence Typing Based on Virulence Genes and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs).Applied and Environmental Microbiology 77. 4520-4526.

Liu L., Oza S., Hogan D., Perin J., Rudan I., Lawn J. E. 2014. Global, regional and national cause of child mortality in 2000-13, with projections to inform post-2015 priorities: an updated systematic analysis. Lancet 385, 430–440.

Madikizela B, Ndhlala A, Finnie J, Van Staden J. 2012. Ethnopharmacological study of plants from Pondoland used against diarrhoea. Journal of Ethnopharmacology 141. 61-71.

Manatsathit S, Dupont H, Farthing M, Kositchaiwat C, Leelakusolvong S, Ramakrishna B, Sabra A, Speelman P, Surangsrirat S. 2002. Guideline for the management of acute diarrhea in adults. Journal of Gastroenterology and Hepatology 17. S54-S71.

Mantaca, American Indian Council.. Indian medicine. Available at http://www.manataka.org: Accessed

Mathabe M, Nikolova R, Nyazema N. 2006. Antibacterial activities of medicinal plants used for the treatment of diarrhoea in Limpopo Province, South Africa. Journal of Ethnofarmacology 105. 286-293.

McMahan Z, Dupont H. 2007. Review article: the history of acute infectious diarrhoea management - from poorly focused empiricism to fluid therapy and modern pharmacotherapy. Alimentary Pharmacology & Therapeutics 25.759-769.

McMichael H, Webb J, Dawson A. 1965. LACTASE DEFICIENCY IN ADULTS - A CAUSE OF FUNCTIONAL DIARRHOEA. Lancet 1. 717-&.

Medeiros A, Pereira R, Oliveira D, Martins P, d'Azevedo P, Van der Sand S, Frazzon J, Frazzon A. 2014. Molecular detection of virulence factors among food and clinical

Enterococcus faecalis strains in South Brazil. Brazilian Journal of Microbiology 45.327-332.

Miao F, Yang X, Zhou L, Hu H, Zheng F, Ding X, Sun D, Zhou C, Sun W. 2011. Structural modification of sanguinarine and chelerythrine and their antibacterial aktivity. Natural Product Research 25. 863-875.

Mirza, S, Kariuki, S, Mamun, K, Beeching, N, Hart, C. 2000. Analysis of plasmid and chromosomal DNA of multidrug-resistant Salmonella entericaserovartyphi from Asia.Journal of Clinical Microbiology 38.1449-1452.

Monaghan A, Byrne B, Fanning S, Sweeney T, McDowell D, Bolton D. 2013. Serotypes and virulence profiles of atypical enteropathogenic Escherichia coli (EPEC) isolated from bovine farms and abattoirs. Journal of Applied Microbiology 114.595-603.

Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pffaler MA. 2007. Manual of clinical microbiology (9th edition). Washington, DC: ASM Press. 2399p.

Nacional center for farmworker health (NCFH). 2011. Folk Medicine and Traditional Healing. Available at https://s6-dallas.accountservergroup.com: Accessed 2011

Nakano M, Yamasaki E, Ichinose A, Shimohata T, Takahashi A, Akada J, Nakamura K, Moss J, Hirayama T, Kurazono H. 2012. Salmonella enterotoxin (Stn) regulates membrane composition and integrity. Disease Models & Mechanisms 5.515-521.

Offiah V, Chikwendu U. 1999. Antidiarrhoeal effects of Ocimum gratissimum leaf extract in experimental animals. Journal of Ethnopharmacology 68. 327–330.

Ochiai L, Khan M, Sahastrabuddhe S, Wierzba, T. 2012. Epidemiology of typhoid fever.International Journal of Infectious Diseases 16.E29-E29.

Ochiai R, Acosta C, Danovaro-Holliday M, Baiqing D, Lhattacharya S, Agtini M, Bhutta Z, Canh D, Ali M, Shin S, Wain J, Page A, Albert M, Farrari J, Abu-Elyazeed R, Pang T, Galindo C, von Seidlein L, Clemens J. 2008. A study of typhoid fever in five Asian countries: disease burden and implications forcontrols. Bulletion of the World Health Organization 86.260-268.

Okeke I. 2009.Diarrheagenic Escherichia coli in sub-Saharan Africa: status, uncertainties and necessities. Journal of Infection in Developing Countries 3.817-842.

Orhan D, Ozcelik B, Ozgen S, Ergun F. 2010. Antibacterial, antifungal, and antiviral activities of some flavonoids. Microbiological Research 165. 496-504.

Orskov F, Orskov I. 1992.ESCHERICHIA-COLI SEROTYPING AND DISEASE IN MAN AND ANIMALS. Canadian Journal Of Microbiology 38. 699-704.

Palombo E. 2006. Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: Modes of action and effects on intestinal function. Phytotherapy Research 20. 717-724.

Pediatric Emergency Medicine Practice. 2010. Pediatric Emergency Medicine Practice Acute Gastroenteritis – An Update 7: 1 – 26

Polonsky J, Martinez-Pino I, Nackers F, Chonzi P, Manangazira P, Van Herp M, Maes P, Porten K, Luquero F. 2014. Descriptive Epidemiology of Typhoid Fever during an Epidemic in Harare, Zimbabwe, 2012.Plos One 9: e114702.

Polson G, Koh B, Zhang J, Kanagsundarm Y, Kien Y, Everett M, Chang Sung, Choi S. . The Natural Occurrence of Zinc Pyrithione (ZnPT) Antimicrobial.

Porwollik S, Boyd E, Cho, C, Cheng P, Flore, L, Proctor E, McClelland M. 2004. Characterization of Salmonella enterica subspecies I genovars by use of microarrays.Journal of Bacteriology 186. 5883-5898.

Proenca-Afonso I. 2008. The effects of sanguinarine on normal oral epithelial cells in vitro [MSc.]. Texas: The Texas AM University System Health Science Center. 72p.

Ranotkar S, Kumar P, Zutshi S, Prashanth K, Bezbaruah B, Anand J, Lahkar M. 2014. Vancomycin-resistant enterococci: Troublemaker of the 21st century. Journal of Global Antimicrobial Resistance 2.205-212.

Raskin I, Ribnicky D, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno D, Ripoll C, Yakoby N, O'Neal J, Cornwell T, Pastor I, Fridlender B. 2002. Plants and human health in the twenty-first century. Trends in Biotechnology 20. 522-531.

Regenerative nutrition. Natural Supplements for Diarrhoea. Available at http://www.regenerativenutrition.com: Accessed

Rheumatology. 2014. Looking in the past for the future. Available at http://rheumatology.oxfordjournals.org: Accessed 2014

Rowe A, Uyeyama K, Young E. 1956. DIARRHEA CAUSED BY FOOD ALLERGY. Journal of Alergy 27. 424-436.

Sadowsky M., Whitman R. 2011. The Fecal Bacteria.Washington, DC: ASM Press. 329p.

Scientific Committe on Consumer Safety (SCCS). 2013. Opinion on zinc pyrithione COLIPA n°P81. p98.

Shkalim V, Amir A, Samra Z, Amir J. 2012. Characteristics of non-typhi Salmonella gastroenteritis associated with bacteremia in infants and young children. Infection 40.285-289.

Schmeller, T, LatzBruning, B, Wink, M. 1997. Biochemical activities of berberine, palmatine and sanguinarine mediating chemical defence against microorganisms and herbivores. Phytochemistry 44. 257-266.

ScholAR Chemistry. 2009. Material Safety Data Sheet: Tetracycline. Available at www.Scholarchemistry,com: Accessed: 2009-1-23

Schrader K, Fernandez-Castro A, Cheung W, Crandall C, Abbott S. 2008.Evaluation of commercial antisera for Salmonella serotyping.Journal of Clinical Microbiology 46.685-688.

Sköld O. 2011. Blackwell Handbooks of Behavioral Neuroscience, Volume 3: Antibiotics and Antibiotic Resistance. New Jersey: John Wiley & Sons. 223p.

Skoulis N, Barbee S, Jacobsonikram D, Putman D, San R. 1993. EVALUATION OF THE GENOTOXIC POTENTIAL OF ZINC PYRITHIONE IN THESALMONELLA MUTAGENICITY (AMES) ASSAY, CHO/HGPRT GENE MUTATION ASSAY ANDMOUSE MICRONUCLEUS ASSAY. Journal of Applied Toxicology 13. 283-289.

Streetdirectory.com. . Herbal and Ayurvedic Treatment for Diarrhea. Available at http://www.streetdirectory.com: Accessed

Swaminathan B, Gerner-Smidt P. 2007. The epidemiology of human listeriosis.Microbes and Infection 9.1236-1243.

Tailler M, Senovilla L., Lainey E, Thepot S, Metivier D, Sebert M, Baud V, Billot K, Fenaux P, Galluzzi L, Boehrer S, Kroemer G, Kepp O. 2012. Antineoplastic activity of ouabain and pyrithione zinc in acute myeloid leukemia. ONCOGENE 31. 3536-3546.

Tate J, Burton A, Boschi-Pinto C, Steele A, Duque J. 2011. 2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis. Lancet Infectious Diseases 12 , 136–141.

Thakurta P, Bhowmik P, Mukherjee S, Hajra T, Patra A, Bag P. 2007. Antibacterial, antisecretory and antihemorrhagic activity of Azadirachta indica used to treat cholera and diarrhea in India. Journal of Ethnopharmacology 111. 607-612.

The Ohio State University. 2012. Dentists see legacy of discontinued ingredients in patients‘ mouths. Available at http://researchnews.osu.edu/archive/viadent.htm. Accessed 2012

Toxins© UC regents, LHS living by Chemistry. 2004. Lethal Dose Table. Available at http://whs.rocklinusd.org: Accessed 2004

Vaisman N, Press J, Leibovitz E, Boehm G, Barak V. 2010. Short-Term Effect of Prebiotics Administration on Stool Characteristics and Serum Cytokines Dynamics in Very Young Children with Acute Diarrhea. Nutrients 2. 683-692

Viswanathan V, Hodges K, Hecht G. 2008. Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea. Nature Reviews Microbiology 7. 110-119

Waddington C, Darton T, Pollard A. 2014. The challenge of enteric fever.Journal of Infection 68.S38-S50.

Wang Y, Yang J, Fei Z, Chen F, Zhong M. 2010. Preparation and Antibacterial Performance of Zinc Pyrithione/Montmorillonite. Journal of Inorganic Materials 25. 512-516.

Whelan K, Gibso, G, Judd P, Taylor M. 2001. The role of probiotics and prebiotics in the management of diarrhoea associated with enteral tube feeding. Journal of Human Nutrition and Dietetics.

WHO & UNICEF. 2004. Clinical management of acute diarrhoea. Available at http://www.who.int: Accessed 2014

WHO & UNICEF. 2006. Oral rehydration salts: production of the new ORS. 2006. Geneva: WHO Document Production Services. 89p.

WHO & UNICEF. 2008. Progress on Drinking-water and Sanitation: special focus on sanitation. Available at http://www.who.int: Accessed 2008

WHO & UNICEF. 2013. Ending Preventable Child Deaths from Pneumonia and Diarrhoea by 2025: The integrated Global Action Plan for Pneumonia and Diarrhoea (GAPPD). World Health Organization & United Nations Children's Fund. 56p.

WHO& UNICEF. 2008. Progress on Drinking Water and Sanitation: Special focus on sanitation. UNICEF, New York. 77p.

WHO& UNICEF. 2009. Diarrhoea: Why children are still dying and what can be done. World Health Organization & United Nations Children's Fund. 68p.

WHO. 1990. The rational use of drugs in the management of acute diarrhoea in children. Geneva : World Health Organization. 71 p.

WHO. 1993. The Management and prevention of diarrhoea : practical guidelines. Geneva: World Health Organization. 50p.

WHO. 2000. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. Geneva : World Health Organization. 74p.

WHO. 2005. The Treating of diarrhoea: manual for physicians and other senior health workers. WHO Library Cataloguing-in-Publication Data. 50p.

WHO. 2008. Global burden of disease, 2004 update. Available at http://www.who.int: Accessed 2008

WHO. 2013. Media centre: Diarrhoeal disease. Available at http://www.who.int: Accessed 2013

World Gastroenterology Organisation (WGO). 2008. Acute diarrhea. Available at http://www.worldgastroenterology.org: Accessed 2008-3

World Gastroenterology Organisation (WGO). 2012. Acute diarrhea in adults and children: a global perspective. Available at http://www.worldgastroenterology.org: Accessed 2008-2

Xiao W, Pu J, Chang Y, Li X, Huang S, Yang L, Li L, Lu Y, Zheng Y, Li R, Zheng Q, Sun H. 2006. Sphenadilactones A and B, two novel nortriterpenoids from Schisandra sphenanthera. ORGANIC LETTERS 8. 1475-1478.

Zhang L, Xiao D, Pang B, Zhang Q, Zhou H, Zhang L, Zhang J, Kan B. 2014. The Core Proteome and Pan Proteome of Salmonella Paratyphi A Epidemic Strains.Plos One 9: e89197.