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In vitro growth-inhibitory effect of medicinal plants used in Cambodian traditional medicine for treatment of diarrhoea against intestinal bacterial pathogens

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled *"In vitro growth-inhibitory effect of medicinal plants used in Cambodian traditional medicine for treatment of diarrhoea against intestinal bacterial pathogens"* independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

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Abstract

Infectious diarrhoea is the most widespread and serious health problem. Worldwide this malady affects up to 5 billion people per year, and in developing countries every year causes up to 10 million deaths. Especially in developing countries it is leading cause of morbidity and mortality in infants and young children. Due to the increasing antibiotic resistance is necessary to find new pathways how to face it. An opportunity are medicinal plants traditionally used in indigenous Cambodian herbal medicine that have not been examined and studied in detail yet. For this purpose, Cambodian healers use mainly roots and bark of concrete medicinal plants.

The purpose of this study was to investigate the *in vitro* growth-inhibitory effect of plants traditionally used in Cambodian herbal medicine to cure gastrointestinal disorders, against the representatives of diarrhoea-causing bacteria. In order to assess that, the minimum inhibitory concentrations (MIC) of 12 ethanol extracts from collected 9 different species were determined against 12 intestinal pathogens. The impacts of the plant extracts on bacterial growth were evaluated by broth microdilution method. 10 out of 12 tested plant extracts showed growth-inhibitory effect against at least 1 out of 12 intestinal pathogens tested, whereas 5 plant extracts demonstrated interesting MICs. Namely, *Ancistrocladus tectorius* exhibited the highest antibacterial effect against *Bacillus cereus* (MIC = 64 μg/mL) and *Listeria monocytogenes* (MIC = 128 μg/mL); certain antimicrobial effects showed also *Shorea siamensis* against *B. cereus, Escherichia coli* and *Salmonella enterditis* (MIC = 256 μg/mL), *Bauhinia malabarica* against *E. coli* (MIC = 256 μg/mL), *Melastoma dodecandrum* against *E. coli* (MIC = 256 μg/mL), and *Breynia vitis-idaea* against *E. coli* and *Salmonella typhimurium* (MIC = 256 μg/mL). Additionally, antimicrobial effects of *Aganoneiron polymorphum, B. malabarica, B. vitis-idaea, Lagerstroemia cochinchinensis, Melastoma saigonense* and *S. siamensis* were tested in our study for the first time. These results suggest that *A. tectorius, B. malabarica, B. vitis-idaea, M. dodecandrum* and *S. siamensis* can be used for further research focused on identification of compounds with antibacterial effect against pathogens causing diarrhoea.

Key words: Antimicrobial activity, broth microdilution method, Cambodia, plant extracts, medicinal plants

Abstrakt

Infekční průjmová onemocnění jsou rozšířeným a vážným zdravotním problémem. V celosvětovém měřítku tato nemoc postihne až pět miliard lidí ročně, přičemž v rozvojových zemích jí podlehne každý rok až deset miliónů lidí. V těchto zemích je to také přední příčina onemocnění a úmrtnosti kojenců a malých dětí. Vzhledem k vzrůstající antibiotické rezistenci je potřeba najít nové způsoby, jak tomuto faktu čelit. Příležitost se nabízí v léčivých rostlinách tradičně používaných v kambodžské bylinné medicíně, jenž doposud nebyly detailně studovány a prozkoumány. Pro léčbu průjmových onemocnění jsou tradičními léčiteli v Kambodži využívány především kořeny a kůra jednotlivých léčivých rostlin.

Účelem této studie bylo prozkoumání *in vitro* inhibičního růstového vlivu rostlin tradičně používaných v Kambodžské bylinné medicíně při léčbě gastrointestinálních potíží, jež byly testovány vůči vybraným průjmovým bakteriím. Pro účel dosažení tohoto zjištění byly použity minimální inhibiční koncentrace (MIC) dvanácti ethanolových extraktů získaných z devíti rozdílných druhů, které byly determinovány proti dvanácti intestinálním patogenům. Účinky rostlinných extraktů na bakteriální růst byly hodnoceny bujónovou mikrodilutační metodou. Deset z dvanácti testovaných rostlinných extraktů prokázalo inhibiční efekt proti minimálně jednomu z dvanácti testovaných patogenů, přičemž pět extraktů demonstrovalo zajímavé MIC. Jmenovitě *Ancistrocladus tectorius* vykázal nejvyšší antibakteriální efekt proti bakteriím *Bacillus cereus* (MIC = 64 μg/mL) a *Listeria monocytogenes* (MIC = 128 μg/mL); určité antimikrobiální efekty projevila take *Shorea siamensis* proti bakteriím *B. cereus, Escherichia coli* a *Salmonella enterditis* (MIC = 256 μg/mL), *Bauhinia malabarica* proti *E. coli* (MIC = 256 μg/mL), *Melastoma dodecandrum* proti *E. coli* (MIC = 256 μg/mL), a *Breynia vitis-idaea* vůči bakteriím *E. coli* a *Salmonella typhimurium* (MIC = 256 μg/mL). Antimikrobiální efekt rostlin *Aganoneiron polymorphum, B. malabarica, B. vitis-idaea, Lagerstroemia cochinchinensis, Melastoma saigonense* a *S. siamensis* byl úplně poprvé testován právě v naší studii. Výsledky nasvědčují tomu, že *A. tectorius, B. malabarica, B. vitis-idaea, M. dodecandrum* a *S. siamensis* mohou být použity pro další výzkum zaměřený na identifikaci složek s antibakteriálním efektem vůči patogenům způsobujícím průjmová onemocnění.

Klíčová slova: antimikrobiální aktivita, bujónová mikrodilutační metoda, Kambodža, rostlinné extrakty, léčivé rostliny

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List of the abbreviations used in the thesis

aEPEC	atypical enteropathogenic *E. coli*
ATCC	American Type Culture Collection
CCM	Czech Collection of Microorganisms
CFA	colonizing factors
CLSI	Clinical and Laboratory Standards Institute
CZU	Czech University of Life Sciences Prague
DEC	diarrheagenic *Escherichia coli*
DMSO	dimethyl sulfoxide
DMSZ	Deutsche Sammlung von Microorganismen und Zellkulturen
EAEC	enteroaggregative *E. coli*
EHEC	enterohemorrhagic *E. coli*
EIEC	enteroinvasive *E. coli*
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
LPS	lipopolysaccharide
LT	heat-labile toxin
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
NCTC	The National Collections of Type Cultures
ST	heat stable toxins
STEC	Shiga toxin-producing enterohemorrhagic *E. coli*
tEPEC	typical enteropathogenic *E. coli*
TKM	Traditional Khmer Medicine
WCB	Wilkins-Chalgren broth

1. Introduction

1.1. Bacterial diarrhoea

Bacterial diarrhoea is serious and the most widespread case of gastrointestinal infections (WHO 2013). It is clinical symptom or health condition associated with disrupted function of gastrointestinal tract (WHO 2005). Although healthy adults can somehow face up to this malady, it becomes dangerous and more severe in case of indisposed individuals, elderly people and young children. Consequences are often serious and end by death. In order to properly prevent and treat this illness, it is important to know its aetiology. *Campylobacter jejuni*, *Clostridium* spp., *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp. and *Vibrio cholerae* can be mentioned as examples of important diarrheagenic bacteria that cause serious infections even in the developed world (Greenwood et al. 2012).

This malady is defined as a "passage of three or more loose or liquid stools per day". It can be spread by consumption of contaminated food or water, from one to another person or because of not sufficient hygiene. During dehydration, the body is deprived of water and electrolytes such as sodium, chloride, potassium and bicarbonate (WHO 2005). We can divide diarrhoea in to the three clinical types (according to the length and symptoms) – acute watery, acute bloody and chronical (WHO 2017).

Infectious diarrhoea generally a results from the disturbance in normal intestinal physiology, such as increased intestinal secretion of fluid and electrolytes activated by infection in small intestine and decrease absorption of fluid, electrolytes and nutrients in small and large intestine (Casburn-Jones & Farthing 2004).

Acute diarrhoea begins acutely and usually last for several hours or days and causes dehydration and undernutrition (WHO 1992). The passage of frequent loose or watery stools are without visible blood, whereas the vomiting and fever may be present (WGO 2012). The pathogens, often non-invasive microorganism active in the intestine, include *V. cholerae*, *E. coli* and *S. enterica* as well as rotavirus, which is the leading cause of this type of diarrhoea. Bacterial acute watery diarrhoea is caused by faecal-oral transmission of *E. coli* strains (Greenwood et al. 2007). In developing countries, this

enterotoxigenic bacterium is the second major cause of mortality among children under 5 years (Gomez-Duarte et al. 2013). Moreover, there is a potential risk of this inconvenience for travellers in countries where *E. coli* strains are endemic (Murray et al. 2007). *V. cholerae* have similar pathogenic mechanism as *E. coli*. It is endemic for regions in Africa and south-east Asia. Cholera is quite rare, but risk of dehydration is much higher (Kayser et al. 2005). Transmission is done by contaminated food and water (Greenwood et al. 2007). The nontyphoidal *S. enterica* is common in both develop and developing countries. Gastroenteritis caused by these strains is from contaminated eggs, chicken meat and dairy products.

The pathogenicity of enterotoxigenic *E. coli* is due to the heat-labile (LT) and the heat stable (ST) toxins that stimulate the activity of adenylate and guanylate cyclase. Pathogenicity also originates from colonizing factors (CFA) that allow bacteria to attach themselves to small intestine epithelial cells. The enterotoxins and CFA are both determined by plasmide genes. *V. chloreae* is similarly attached to epithelial cells in the small intestine where it multiplies cholera toxin (CT) which is encoded on bacterial chromosome. *S. enterica* strains pass through the small bowel, attach to enterocytes of the ileum and colon and invasion proteins induce penetration of mucosal barrier (Kayser et al. 2005; Brooks et al. 2010).

Acute bloody diarrhoea, also called dysentery, is specific by presence of visible blood and mucus in the stools caused by damage of intestinal mucosa in the colon and nutrient losses. Pathogens responsible for this type of diarrhoea can be of bacterial, viral and parasitic origin (Wiley 2009). Bacillary dysentery is the most common cause of serious cases of children's bloody diarrhoea in developing parts of the world. Since it is usually caused by bacteria of the genus *Shigella*, this disease is also called shigellosis (UNICEF/WHO 2009). Shigellosis occurs worldwide but it is major problem in tropical areas of developing countries where it is endemic (Kayser et al. 2005). Every year, about 5 million cases require hospital treatment and approximately 600,000 people die due to this malady. Similar pathogenic mechanism has entero-invasive *E. coli*, however, the infections caused by this bacterium are comparably less serious causes (Greenwood et al. 2007). *C. jejuni* is another bacterium that causes acute bloody diarrhoea that commonly occurs in developed world (Brooks et al. 2010).

Shigella strains enter the terminal ileum and colon and are able to penetrate the colonic mucosa and thus cause local necrotic infections. Shigatoxin which is toxic

lipopolysaccharide inhibits protein synthesis and participates in the colonic epithelial damage. The detailed mechanisms of pathogenicity of *C. jejuni* are not known very well, however are very similar to *Shigella* spp. by invasive properties and enterotoxins expression. Endotoxic lipopolysaccharides have also been observed (Greenwood et al. 2007).

Diarrhoeal symptoms that begin acutely with or without blood that lasts at least two weeks are considered as persistent. The outbreaks are not directly life-threatening, but danger become serious in case of undernourished children and children with other illnesses. Their condition tends to by worsen, nutritional status declines and have higher chance to develop into persistent diarrhoea (UNICEF/WHO 2009). Chronical diarrhoea affects first and foremost young children in developing countries (WHO 2005). Frequently, the weight loss and malnutrition can be fatal. Persistent diarrhoeal infections are major mortality cause of infants below 2 years in developing countries (Rao 2020). This type of diarrhoea should not be confused with diarrhoea which is non-infectious but long-lasting and is caused by sensitivity to gluten or inherited metabolic disorders (WHO 1992).

Chronical diarrhoea is caused by enteropathogenic *E. coli* (EPEC) with peculiar pathogenic mechanisms. After ingestion strains tightly attach themselves to the epithelial cells of the small bowel by EPEC adhesion factor, inject toxic molecules into the enterocytes and damaging the mucosa (Kayser et al. 2005; Greenwood et al. 2007).

1.1.1. Current Epidemiology

Worldwide diarrhoea affects 3 to 5 billion people every year, and in developing countries, causes over 5 to 10 million deaths (Seupaul 2012). It is one of the leading causes of morbidity and mortality in infants and young children in developing countries and also in the world generally (Lanata & Mendoza 2012). During 2015, 1.32 million children among all ages and 499 thousands children below 5 years died due to diarrhoea. It still represents third largest cause of neonatal deaths (Rao 2020). The death is usually the result of dehydration (WHO 2005). Often affected countries are India, Malaysia, Niger, Nigeria, Pakistan, Philippines and Sub-Saharan Africa (Mokomane et al. 2018). More than half of cases are in Africa and South Asia. These two parts of the world are also home to more than 80 % of child deaths due to diarrhoea. The occurrence

of diarrhoea differs depending on the season and age. The highest incidence is in the first two years of life (UNICEF/WHO 2009). Bacterial pathogens causing serious infections are also common in developed world. For example, *C. jejuni* in United States bring about 2 million cases every year (Brooks et al. 2010).

Diarrhoeal pathogens have commonly similar route of transmission known as faecal-oral. Poor hygiene, polluted water, bad sanitation, contaminated food, and malnutrition are causes that spread diarrhoeagenic pathogens. Sum of these emergencies often bring diarrhoeal symptoms. The number of diarrhoeal incidences and the rate of their severity grows especially in developing areas and regions affected by natural disasters where people are accumulated in overcrowded shelters and camps. During last years, some progress is distinguishable due to the micronutrient supplementation, handwashing with soap and oral rehydration therapy. Since the disease can be less transmitted in the environment with high hygiene standards, the support of communities in better and safer practices of sanitation and hygiene and promotion of safe drinking water is essential. It is estimated that 88 % of diarrhoeal deaths worldwide are due to unsafe water, inappropriate sanitation, and poor hygiene. Use of sanitation facilities in community can significantly reduce disease transmission. Another functional barrier that can reduce incidence by over 40 % is washing hands with soap. Undernourished children tend to suffer more severely, therefore the adequate nutrition also plays a big role. Breastfeeding can greatly reduce the risk of infection because the breast milk contains nutrients, antioxidants, antibodies, and hormones needed for child's development. It is estimated that infants that are breastfed until two years of age have less severe illnesses than those who are not. Infants who are not breastfed have a sixfold higher risk of dying in first two months of life from infectious diseases including diarrhoea. Micronutrient supplementation is preventive and reduce mortality from 19 % to 54 %. Especially vitamin A and zinc can reduce complications connected with diarrhoea symptoms. From 100 million episodes of acute diarrhoea every year can result up to 600 thousands child deaths. Oral rehydration therapy prevents life-threatening dehydration and play important role as life-saving remedy not only for children diarrhoeal cure (UNICEF/WHO 2009). Most common causes of acute diarrhoea are infectious agents such as viruses, bacterial and parasitic organisms (Seupaul 2012). From the bacterial pathogens, *C. jejuni*, *E. faecalis*, *E. coli*, *L. monocytogenes*, *S.*

enterica, S. dysenteriae and *V. cholerae,* are important diarrhoea causing agents (WGO 2012). It is also estimated that more than 90 % of food poisoning every year is caused by *Staphylococcus aureus, Salmonella, C. perfringens, Campylobacter, L. monocytogenes, V. parahaemolyticus, Bacillus cereus,* and entero-pathogenic *E. coli* commonly found on raw foods (Wagner 2008). Some of these pathogens are described in detail in the chapter dedicated to intestinal bacterial pathogens.

Countries with lower level of income such as Cambodia has risk factors like lack of access to clean water, undernutrition, stunting and rotavirus vaccine availability. These risk factors increase the chance that person will develop a disease (Dadonaite et al. 2018). In year 1990 it was second leading disease, today hold ninth place in Cambodian disease scale. Latest data from year 2017 showed that 1,699 people in Cambodia died due to diarrhoea. It was 18.51 deaths per 100,000 individuals. In detail it was 840 persons over 70 years old, 345 persons in age group 50-69, 147 persons in 15-49, 37 persons in 5-14 and 300 children under 5 year of age (GBD 2018). Especially groups of oldest and youngest inhabitants are susceptible to infections caused by intestinal bacterial pathogens. In both groups *Shigella* spp. is leading bacterial pathogen. Whereas on second and third place it is cholera and *Campylobacter* spp. for group under 5 years, in case of people over 70 it is non-typhoidal *Salmonella* spp. and enterotoxigenic *E. coli*. For both group we can found three similar reasons of death – unsafe water source, unsafe sanitation and no access to handwashing facility. In group under five there are also another diarrhoea-associated risk factors – child growth failure, suboptimal breastfeeding, vitamin A deficiency, low birth rate, and short gestation and zinc deficiency. Sum of all already mentioned risk factors together with lack of access to essential treatment (e.g., only 35,2 % of children under 5 received oral rehydration therapy in year 2014) still makes from diarrhoea one of the leading health problem (Dadonaite et al. 2018).

1.2. Intestinal bacterial pathogens

The human gastrointestinal tract consists of the complex bacterial ecosystem counting over 400 different bacterial species of weight up to 6.8 kg together. The most common genera are *Bacillus, Bifidobacterium, Clostridium, Escherichia, Eubacterium,*

Fusobacterium, Kleibsiella, Lactobacillus Peptococcus, Peptostreptococcus, Staphylococcus, Streptococcus. These commensal microorganisms play a significant role in human health, especially due to their nutrients production, metabolic functions, and support of the digestive system. Gastrointestinal tract is in balance that can be disrupted by many factors – for example parasites, overgrowth of opportunistic pathogenic bacteria, illness, toxins, stress, poor hygiene and bad water or by improper diet. Most important functional food for digestive system are probiotics that can help to keep balance of gut microflora. Probiotics contain health-promoting bacteria with positive influence on the metabolic activity of the intestinal flora (English & Dean 2013). When gastrointestinal tract is not in balance it can lead in overgrowth of opportunistic pathogen. This situation can evoke diarrhoea, as well as presence of intestinal bacterial pathogens due to food poisoning or poor hygiene. These pathogens are described more in detail in following subchapter.

1.2.1. Gram-negative strains

Virulence factors are bacteria-associated molecules that are required to cause a disease while infecting humans. Virulence factors of gram-negative bacteria are encoded from mobile genetic elements and are known as exotoxins (e.g., cholera toxin, Shiga toxin). The spread is through horizontal gene transfer which converts harmless bacteria into dangerous pathogens (Abedon et al. 2009). Virulence determinants of gram-negative strains from group of endotoxins are mainly lipopolysaccharide (LPS) and capsular phenotypes and are encoded chromosomally (Cross 2008). Whereas they are rather resistant to penicillin and sulphonamide, their susceptibility to streptomycin, chloramphenicol and tetracycline is high. Resistance tends to be higher also due to impenetrable wall, inner and outer membrane, degradation enzymes and efflux pumps. Their ability to cause disease is almost in all systems in human organism such as digestive, nervous and urinary system and in bloodstream (Oliveira & Reygaert 2020).

Escherichia coli

E. coli is well known facultative anaerobic gram-negative rod belonging to the family Enterobacteriaceae which inhabits large intestine of humans and warm-blooded animals. Although these strains commonly invaded in the colon are usually harmless, they can infrequently cause disease. Poor countries in Africa, Asia and Latin America are most

touched regions with this disease. Lethal outcomes are more often due to poor living conditions. These strains are important etiological agents of diarrhoea. We can recognize six *E. coli* pathotypes commonly known as diarrheagenic *E. coli* (DEC). Namely, EPEC including watery diarrhoea of infants, enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC) including haemorrhagic colitis and haemolytic-uremic syndrome, enteroaggregative *E. coli* (EAEC) including persistent diarrhoea, enterotoxigenic *E. coli* (ETEC) including traveller's diarrhoea, and enteroinvasive *E. coli* (EIEC). Each of these pathotypes has specific virulence factors which can make hybrid strains combinations and possibly be more virulent. DEC infections are a serious health issue among children and adults in developing countries. These diarrheal episodes are associated with morbidity and mortality of children less than five years (Gomes et al. 2016).

EPEC cause infant diarrhoeas and lead to high infant mortality as a second most frequent cause of death among children under one year of age. Mainly in the urban areas it causes almost 40% of infant diarrhoea. The source of the bacteria is the faeces of infected human and animals. Transmission is by faecal-oral route. Food is contaminated directly by faeces or by untreated water. EPEC are divided to typical (tEPEC) and atypical (aEPEC) group. Dividing is based on the presence or absence of virulence genes. Specific virulence factor is a special protein intimin. The symptoms are watery diarrhoea, vomiting, fever. Outbreaks has seasonal distribution with peaks in warm months. Prevention is cooking and avoiding of recontamination of cooked meal (Greenwood et al. 2007; Wagner 2008; Gomes et al. 2016).

EHEC is STEC globally distributed foodborne pathogen. EHEC/STEC can cause infections from mild diarrhoea to severe haemorrhagic colitis and haemolytic uremic syndrome. Main reservoir are cattle but can be present in faeces of buffaloes, sheep, pigs, birds and fishes. EHEC/STEC is able to adhere and form biofilm on surfaces. Due to this ability it can easily survive in the soil, water, manure and on pastures. Possibility of its presence is higher in slaughterhouses. Hazardous is also using of chicken litter as an organic soil fertilizer for fruit and vegetables production. Part of prevention should target to animal handling (from farm to slaughter) and food safety during production and processing (Gomes et al. 2016).

EAEC is associated with cases of persistent diarrhoea of children, particularly in the areas where these strains are endemic. Main source of EAEC is contaminated food. Furthermore, EAEC causes diarrhoea in immunodeficient individuals and in travellers visiting less-developed countries. EAEC is able to bypass immune system and cause persistent infection and lead to malnutrition. Also the resistance to antibiotics has been proved (Okhuysen & DuPont 2010).

ETEC is a common cause of bacterial diarrhoeal disease. It is estimated to cause 840 million diarrhoeal cases per year in developing countries. Infection is induced via consumption of food and water contaminated by faecal bacteria. Although milder, the symptoms are comparable to cholera. Usually it is watery nonbloody diarrhoea, sometimes nausea and vomiting. Mortality is higher in malnourished individuals, elderly and children. ETEC produces ST (STa, STb) and LT toxins (LT-I, LT-II). STa and LT-I are more commonly associated with human disease (Murray et al. 2007). Heat stability of LT toxins is inactivated at 60°C after 30 min, whereas ST toxins can tolerate temperatures up to 100°C (Kayser et al. 2005).

EIEC is foodborne and causes 5 % of all diarrhoeas in developing regions or areas with nonsufficient hygiene (Greenwood et al. 2007). It is less common in the developing countries than ETEC and EPEC (Murray et al. 2007). This pathogenic form causes dysentery; therefore the symptoms are similar to those in shigellosis. However, the strains are nonmotile and have higher metabolic activity than *Shigella* strains (Lan et al. 2004).

Additionally, *E. coli* 0175:H7 is shigatoxin-producing enterohemorrhagic bacterial strain that has become major and worldwide food-borne pathogen. Main reservoir are ruminant animals (Ameer et al. 2021).

Salmonella enterica

S. enterica is gram-negative, flagellated, facultative anaerobic and rod-shaped bacteria belonging to the family Enterobacteridaceae. It is bacterium of gastrointestinal human and animal tracts. Common sources are high protein foods (meat, poultry, eggs, fish). Bacterial growth can be controlled by refrigeration below 4°C or destroyed by temperature above 65°C. Recontamination of processed food or insufficient cooking should be avoid. Symptoms are diarrhoea, vomiting and fever in range within 12-24

hours. Infection is called salmonellosis and treatment is done by antibiotics such as chloramphenicol (Wagner 2008).

Shigella spp.

The genus *Shigella* is nonmotile, non-spore forming, rod-shaped causative pathogen in bacterial dysentery (or shigellosis) belonging to the family Enterobacteridaceae. It is characterized by invasive properties with ability to penetrate colonic mucosa and cause local necrotic infections. Transmission is done directly, but more often indirectly (via food, drinking water of flies). It is pathologically active in humans only, thus humans are sole source of infection. It is estimated that shigellosis is responsible for over 164 million cases and 1 million deaths per year. Majority of cases caused by e.g., *Shigella flexneri* are in developing areas and cover mainly children. This genus produces toxic lipopolysaccharide (endotoxin) and LT shigatoxin (exotoxin). The dose of only few hundred of bacteria suffice to cause infection. Shigatoxin causes colonic epithelial damage. Antibiotics are used therapeutically, treatment is done by aminopenicillins, fluoroquinolones and cephalosporins (Jennison & Verma 2004; Kayser et al. 2005).

Vibrio spp.

This genus belonging to the family Vibrionaceae and consists of comma or spiral-shaped and monotrichously flagellated rods. *Vibrio* possess alkali tolerance (pH 9). Natural habitat including the ocean. Cholera which is caused by *V. cholerae* occurs in humans only. Disease develops when pathogen enter intestinal tract with food or drinking water (oral ingestion) in larger numbers. Many vibrios are killed by the hydrochloric acid in gastric juice. Multiplication and enterotoxins production is done in the proximal small intestine. Vaccine provides only moderated degree of protection for period of six months. Incubation period is for five days. Therapeutic focus on replacement of lost electrolytes and water, for treatment are used tetracyclines and combination of sulfamethoxazole and trimethoprim also called cotrimoxazole (Kayser et al. 2005).

V. parahaemolyticus is facultative anaerobic mobile bacteria. This organism requires salt for growth and thus is widely distributed in marine environment and is found on raw or cooked seafoods. It is sensitive to heat and cold. Storage of seafood should not exceed 4 °C, cooking and holding is important pursue with temperature above 60 °C. Symptoms of the disease are diarrhoea, cramps, vomiting, fever and headache (Wagner

2008). Antibiotics such as penicillin, tetracycline, and some antibiotics from groups of quinolones and cephalosporins are used for treatment (Wong et al. 2015).

Yersinia enterocolitica

Y. enterocolitica is facultatively anaerobic bacterium belonging to the family Enterobacteriaceae. It is pleomorphic and short rod with peritrichous flagellation. During lower temperatures it is motile, form colonies and is sensitive to acidity. It naturally occurs as parasite of rodents and most often is transmitted indirectly from animals to humans with food. Strains enter the lower intestinal tract and penetrate the mucosa. Most severely affected by yersiniosis are children. Symptoms resemble appendicitis. Death is rare. Treatment is done by cotrimoxazole and antibiotics from group of quinolones (Kayser et al. 2005; Wagner 2008).

1.2.2. Gram-positive strains

In gram-positive bacteria secretion of proteins is done across the single cell membrane (Cahoon & Freitag 2014). They do not have endotoxins, but presence of this bacteria can provoke an inflammatory response similar to gram-negative LPS. In their cell wall can be found teichoic acid. Susceptibility to penicillin and sulphonamide is high. On the other hand, sensitivity to streptomycin, chloramphenicol and tetracycline is quite low (Wilson et al. 2002).

Bacillus cereus

It is opportunistic pathogen with relatively low capacities for virulence belonging to the family Bacillaceae. It is aerobic, sporing and obligate rod bacterium. It is ubiquitous organism that can be found in all environments from where all infections originated. Gastroenteritis caused by this pathogen is mediated by two enterotoxins. The ST enterotoxin causes vomiting disease (emetic form) while LT enterotoxin causes diarrheal form of disease. The heat-labile enterotoxin leading to intense watery diarrhoea is similar to those produced by *E. coli* and *V. cholerae*. Emetic form of disease is caused by consumption of contaminated rice. Bacteria are killed during cooking, but heat-resistant spores not. If the cooked rice is not refrigerated, spores germinate. Thus heat-stabile enterotoxin is not destroyed and lead to intoxication. Second diarrheal form is true infection from ingestion of the bacteria that contaminated

food. Incubation period is longer. Manifestation are diarrhoea, nausea, cramps. Infections are rapid and genus is multiple-drug resistant. Penicillin and cephalosporins are not effective. Vancomycin, clindamycin, ciprofloxacin and gentamicin are used for treatment (Kayser et al. 2005; Murray et al. 2007).

Diarrheagenic clostridia

The genus of anaerobic bacteria belonging to the family Clostridiaceae is omnipresence in soil, water and sewage and form part of the normal microbial population in gastrointestinal tract of humans and animals. Clostridia are rods that are large, and spore-forming. Food poisoning, diarrhoea and colitis are clearly documented diseases caused by this genus. Capability to induce disease is caused by their ability to survive adverse environmental conditions through spore formation, rapid growth in a nutritionally enriched and oxygen-deprived environment and production of histolytic toxins, enterotoxins and neurotoxins. We can recognize two main diarrheagenic species – *C. difficil*e and *C. perfringens.*

C. difficile is toxin-producing bacteria responsible for antibiotic-associated gastrointestinal disease ranging from self-limited to severe diarrhoea and life-threatening colitis. Virulence of this strain is increasing as a result of mutation in gene regulating production of the enterotoxin and cytotoxin. Production of toxin is significantly increasing. *C. difficile* is resistant to fluoroquinolone antibiotics. Mild disease is treated by ampicillin. Severe diarrhoea or colitis require treatment with metronidazole or vancomycin.

C. perfringens is producent of a large number of toxins and hydrolytic enzymes. It also produces LT enterotoxin with impact on small intestine epithelium. Disease has short incubation period and manifests by abdominal cramps and watery diarrhoea with no fever, nausea or vomiting. Severe infections require high-dose penicillin therapy. Under adverse environmental conditions it forms spores that can survive for longer period. Spores can be heat-resistant and may survive cooking below 60 °C (Murray et al. 2007).

Diarrheagenic enterococci

Widespread bacterial genus belonging to the family Enterococcaceae is commonly found in the intestinal microbial flora of humans and animals. Bacteria is enteric, non-motile, catalase negative, arranged in short chains and pairs. Enterococci are commonly

referred as lactic acid bacteria due to its glucose fermentation with L-lactic acid. Clinically important species are *E. faecalis* and *E. faecium.* As a facultative anaerobe it proliferates both aerobically and anaerobically in broad temperatures range (10-45°C), in wide pH range (4.6-9.9) and in presence of high concentrations (6%) of NaCl and bile salts. Enterococci represent less than 1 % of the microflora of large bowel of human adults. Abundance decreases with increasing age. It is well adapted and capable to resist in variety of environmental stresses including heat, acid, oxidation, hyperosmolarity and UV irradiation. Very few clinical conditions can inhibit the growth of enterococci. They tolerate ethanol, detergents and prolonged desiccation. They have ability to degrade intestinal mucin and thus occupy deeper layers of the mucosa. Generally, it shows low level of pathogenicity. Strains frequently develop resistance to antibiotics which together with ability to adhere to tissues and form biofilms arrange their virulence. Antimicrobial therapy is complicated and consist of aminoglycoside and ampicillin or vancomycin combination (Pillar & Gilmore 2004; Kayser et al. 2005; Murray et al. 2007).

Listeria monocytogenes

This bacterium is the most significant human facultative intracellular pathogen. It is a short and nonbranching, gram-positive anaerobic rod belonging to the family Listeriaceae. *L. monocytogenes* can grow at temperatures from 1 °C to 45 °C and it is tolerant to high concentration of salt and wide pH range. It occurs in soil, water and vegetation. Leading source of infection is contaminated food (e.g., milk, cheese, raw vegetables). There is also possibility of human-to-human transmission, primarily transplacental from mother to child in utero or at birth. Occurrence of disease called listeriosis is mainly a concern to high-risk groups such as neonates, elderly people, pregnant women and individuals with defective in cell-mediated immunity (e.g., AIDS). It is possible to distinguish two forms of listeriosis: non-invasive and invasive. Non-invasive form appears as a febrile gastroenteritis and invasive form as meningocephalitis (Camejo et at. 2011). In healthy adults, death is rare. Mortality in the immunocompromised, very young patients and new-born is approximately 30 % (Wagner 2008). *L. monocytogenes* is the most common food pathogen (Temple & Nahata 2000). The only prevention for high-risk groups is to avoid eating raw or not well-cooked foods of animal origin and unwashed vegetable. It is also estimated that *L.*

monocytogenes can cause meningitis. Treatment is done by penicillin or ampicillin (Murray et al. 2007).

1.3. Antibacterial Treatment of Diarrhoea

Considering the therapy approaches applicable for diarrhoea, antibiotic treatment should be very last option. The first and most crucial step is prevention. Not only personal hygiene such as washing hands is capable practice how to avoid disease. Also, sufficient hygiene before, during and after handling foods can prevent contamination and recontamination (Wagner 2008).

Probiotics that have positive influence on the intestinal flora are favourable during the treatment and can enhance health condition of affected individual. Probiotics contain health-promoting bacteria (e.g., lactic-acid bacteria) with positive influence on the intestine microflora. Promising results in treating diarrhoea from probiotic strains shown predominantly *Lactobacillus* species that adhere to intestinal wall and improve microflora balance and normalizes faecal enzyme (English & Dean 2013).

Treatment should be done by oral rehydration solution, which is solution of water, salt and sugar (glucose-electrolyte solution). For many patients with severe diarrhoea it has been lifesaving therapy with principal of sodium-glucose cotransport. In last few decades the possibility of replacing glucose, which has traditionally been used as main substrate, has been explored. Replacing glucose with glucose polymer (e.g., rice starch) has advantage of osmolality solution and also drive active sodium absorption. Cereal based oral rehydration solution has significant advantage primarily in cholera treatment (Casburn-Jones & Farthing 2004). Oral rehydration therapy is major treatment method, however, it does not reduce volume or duration persistence of diarrhoea (Subbotina et al. 2003). If all mentioned methods fail, then the task of antibiotics is to treat diarrhoeal symptoms.

1.3.1. Antibiotics

Treatment by antibiotics play the predominant role in diarrhoeal infections control. Commercial antibiotics help reducing mortality (Manatsathit et al.2002) and shortening length of an illness (Diniz-Santos et al. 2006). However, application should be done

cautiously. Irrational use and incorrect dosage raises resistance to the drug. Risk of the side effects is also high, as well as inhibition of beneficial gut microflora (Kudera et al. 2020). Whereas cases with mild and improving symptoms do not need antibiotic treatment, use of antimicrobial agents is recommended in cases of dysenteric shigellosis, cholera, enterocolitis, non-cholera vibrios, prolonged yersinia infections and outbreaks of enteropathogenic *E. coli* diarrhoea in nurseries (Casburn-Jones & Farthing 2004).

The antibiotics of the following groups are most often used for treatment of diarrhoea: aminoglycosides (gentamicin, streptomycin, chloramphenicol), penicillins (ampicillin, penicillin), rifamycins (rifaximin), sulfonamides (sulfamethoxazole – in combination with trimethoprim create antibiotic called cotrimoxazole) (Werth 2020), cephalosporins (ceftriaxone), fluoroquinolones (ciprofloxacin), glycopeptides and lipoglycopeptides (vancomycin), tetracyclines (tetracycline) (Kudera et al. 2020). Antibiotics used for treatment of diarrhoea already mentioned in part "Intestinal bacterial pathogens" together with ciprofloxacin that was used in this research are described in more detail bellow.

Aminoglycosides are recommended to treat those bacterial infections that are serious - such as infections caused by gram-negative bacteria. They prevent bacteria from protein production which is necessary for growth and multiplication. Usually are injected into a vein or muscle because are poorly absorbed into the bloodstream after oral application. Chloramphenicol is used in case of serious infections caused by bacteria resistant to other antibiotics, commonly to salmonella infections and meningitis.

Cephalosporins are together with penicillins a subclass of antibiotics called beta-lactam (for their chemical structure of beta-lactam ring). Cephalosporins and penicillins are bacteriocins that work by preventing bacteria from forming protective cell wall. Disadvantage of this class is interaction with many other drugs that increases the effects and side effects of the other drugs.

Rifamycins stop genetic material production thereby causing bacteria death. Rifamixin is used to cure traveller's diarrhoea caused by *E. coli*.

Glycopeptides and lipoglycopeptides are used to treat serious infections caused by gram-positive bacteria by preventing them from cell wall forming likewise beta-lactams.

Sulfonamides are antibiotics effective against both gram-positive/negative bacteria by blocking folic acid production that is necessary for growth and multiplication. Cotrimoxazole is combination of sulfamethoxazole and trimethoprim effective against many bacteria including those one, which are resistant to other antibiotics.

Tetracyclines are used to treat broad spectrum of bacterial infections. Usually are taken orally but should be given by injection as well.

Fluoroquinolones are broad-spectrum antibiotics against variety of infections and can be taken orally, intravenously or as eye drops. These antibiotics are interfering with bacterial DNA that leads to death of bacteria.

Ciprofloxacin, which belongs to fluoroquinolones, is applied in the treatment of serious gastrointestinal infections of bacterial origin (Werth 2020). It is a bactericidal antibiotic able to inhibits DNA replication due to DNA topoisomerase and DNA-gyrase inhibition. Among fluoroquinolones, it is the most potent antibiotics against Enterobacteridaceae. Oral application usually achieves 70-80% absorption but is available intravenously too. Mild adverse effects are predominantly gastrointestinal disruptions such as nausea. Serious side effects include for example peripheral neuropathy, hyper/hypoglycaemia, photosensitivity and tendonitis. In developing countries and mostly in Europe it is prescribed in neonates for life-threating infections (Thai et al. 2020).

1.3.2. Plant-Derived Antimicrobials

For care and support of gastrointestinal tract and management of diarrhoea, there are nowadays commercially available antibacterially active plant extracts and derivatives of their constituents in form of capsules, liquids, tinctures and tablets (Kokoška et al. 2018). Although the therapeutic effect of these products is often based on antimotility and antisecretory activities, the antimicrobial action is an important part of functionality of herbal medicines as well (Kudera et al. 2020). For example, *Coptis sinensis* rhizomes extract is commonly sold at Asian markets under the name Huang Lian Su Tablets (Hubei Minkang Pharmaceutical, Yichang, China), North American markets are offering roots and rhizomes extracts of *Berberis* spp. (Berberis Formula, Seroyal, Richmond Hill, Canada) and *Coptis* spp. (Solaray Goldenseal Root Capsules, Nutraceutical Corporation, Park City, USA). Antimicrobial activity of these products is caused by isoquinoline alkaloid berberine. Also, berberine is in Asia sold in pure form

as Berberine Hydrochloride Tablets (Northeast Pharmaceutical Group, Shenyang, China). Additionally. there are extracts of plants containing tannins available in European countries such as *Uncaria gambir* and *Quercus* spp. (e.g., Gambir Extract Powder, ZhongYun Biotech, Shaanxi, China; Solaray White Oak Bark Capsules, Nutraceutical Corporation, Park City, USA). They have antiseptic and astringent effects and are used for treatment of mild acute diarrhoea. At European markets, for example the tannin albuminate-based products derived from *Caesalpinia spinosa* and *Rhus* spp. are commercially available. Trade names are Tannacomp and Tannalbin (Medice Arzneimittel Putter, Iserlohn, Germany). Combination of those two preparations have been shown as effective prophylactics especially for travellers' diarrhoea. Tasectan (Novintethical Pharma, Lugano, Switzerland) and Gelenterum (Angelini, Ancona, Italy) are in the form of gelatine tannate that can control and reduce the symptoms associated with infants, children and adult's diarrhoea. In the form of liquid and tablets there is an active substance bismuth subsalicylate sold under the name Pepto-Bismol (The Procter & Gamble Company, Cincinnati, USA) with positive influence on stomach and gastrointestinal tract. *Glycyrrhiza glabra* root extract is in form of capsules traded under the name GutGard (Natural Remedies, Bangalore, India) that reduces abdominal fullness, pain and nausea (Kokoška et al. 2018). Last example of plant-derived product is from the Amazon River basin, where the locals are extensively using red tree sap derived from *Croton* spp., which is commercially available as Zangrado (Rainforest Phytoceuticals, Delmar, New York, USA) (Palombo 2006).

1.4. Cambodian Traditional Medicine

The beginnings of Traditional Khmer Medicine (TKM) are not clear but there is strong evidence to claim that this indigenous medicine developed during Angkor period and was inspired by theories and practices from Ayurvedic and Chinese medicine system. Angkorian seat of traditional medicine was apparently the temple Neak Poan. Main media of transmission of Khmer medical knowledge represent manuscripts written in the Pali language on palm leaf. TKM consist of two major diagnostic techniques – clinical (questioning and examination of the patient) and magico-religious (divination and supernatural illness explanations). Also, the prevention of disease is as important as cure. Apprenticeships, personal research and traditional community or family

knowledge are sources of practitioners' skills. For all types of healers the term "kru khmer" is used. This person provides remedies in forms of dried plant medicine (mixture of dry wood, twigs, leaves and roots), liquid medicine (ground-up plant), steeped medicine (raw materials steeped in alcohol), powders (materials ground into a powder given as a powder, pill or capsule), loose ingredients (roots, twigs, seeds) and topical medicines (for external application). Those healers who make own remedies either collect raw materials themselves, employ people to collect them or buy ingredients. Others only diagnose the patient and not provide remedies but information what patient need to find in forest or buy at market. Khmer Rouge regime banned western teachings and so traditional medicine were the only available medical care. After the fall of this era traditional healers from all over Cambodia were invited to Phnom Penh to share traditional knowledge. This was first inventory od medicinal plants created in Khmer language by group of "kru khmer" working with National Centre of Traditional Medicine in Phnom Penh.

Due to the difficulties accessing public health providers, poverty, corruption and the healthcare system itself has Cambodia one of the world's lowest rates of use of a government health care system. A large percentage of the rural population who have no cash income or who live at a distance from the nearest settlement still depend on traditional remedies (Ashwell & Walston 2008).

1.4.1. Cambodian Herbal Medicine

According to the Royal Government of Cambodia (2014) and most recent estimates of the Forestry Administration's International Cooperation Project on Biodiversity and Environmental Conservation, there can be found more than 4,500 vascular plant species in Cambodia. A relatively little research has been published about Cambodian medicinal plant remedies so quantities as well as diversity of medicinal plants used in traditional medicine is quite unclear. However, from what was estimated so far, medicinal plant species counted over 1,000 species which is about 40% of known native flora in Cambodia (Ashwell & Walston 2008; Savajol et al. 2011). Unfortunately, increasing pressure on land and forest causing rapid deforestation and reducing of local sources of medicinal plants (Savajol et al. 2011). Khmer pharmacopoeia consist of trees, shrubs and sub-shrubs, climbing lianas, palms, herbs, aquatic species and bamboo epiphytes. Habitat of medicinal plants is wide-range from dense forest to narrow

habitats such as beaches, mangroves, limestone areas and flooded forests. Majority of Cambodian native medicinal plants can be found in narrow habitats (Ashwell & Walston 2008; Chassagne et al. 2016). The most dominant plant families are Fabaceae followed by Malvaceae and Poaceae. Other not so frequent but still important families are Annonaceae, Apocynaceae, Combretaceae, Dilleniaceae, Dipterocarpaceae, Euphorbiaceae, Lamiaceae, Phyllanthaceae, Rubiaceae, Rutaceae, Vitaceae and Zingiberaceae. Most often used form of remedies are multi-ingredient recipes that seem to be preferred by all traditional healers. Method of extraction unquestionably is decoction administered orally or in form of (steam) baths and inhalations (Chassagne et al. 2016). The most effective plant part by many traditional healers are roots. Roots also predominant at medicinal markets because are highly demand (Ashwell & Walston 2008; Chassagne et al. 2016). In case of direct gathering local users usually take only small parts of roots and bark from individual plants because they know importance of sustainable harvest with respect to future harvesting (Ashwell & Walston 2008). Most frequent disease cured by natural remedies is diarrhoea followed by cold/fever, post-partum and malaria (Chassagne et al. 2016). Considering the treatment of diarrhoea, Khmer people mostly use roots and bark for the herbal medicine preparation. Leaves, flowers, fruit and resin are used less frequently. Although tannins are probably among the most common substances in these plants due to their astringent antidiarrheal effect, other bioactive compounds with antibacterial activities are expected as well (Robles 2014).

1.4.2. Plants Used for Treatment of Diarrhoea

Already pharmacologically evaluated antidiarrheal plants are *Anacardium occidentale, Careya arborea, Chromolaena odorata, Dipterocarpus intricatus, Psidium guajava* and *Terminalia nigrovenulosa*. Antibacterial activity against gastrointestinal bacterial pathogens was also reported for *Antidesma ghaesembilla, Helicteres angustifolia* and *Shorea obtuse*. Furthermore, according to Cambodian people, *Anogeissus acuminata* and *Xylia xylocarpa* were mentioned as traditional antidiarrheal medicinal species (Chassagne et al. 2016).

A. occidentale (Anacardiaceae) is also known as cashew tree. This tree originated from South America and usually is grown for fruits known as cashew apples and nuts. The cashew kernels are used for curing diarrhoea in some parts of the world (Orwa et al.

2009). Root and bark can be also decocted and drunk (Chassagne et al. 2016). Antidiarrheal properties are caused through inhibition of hyper-secretion, enteropooling and gastro-intestinal motility (Omoboyowa et al. 2018).

C. arborea (Lecythidaceae) is tree that grows up to 15 m and its flowers and younf leaves are eaten as vegetable. Bark is by Cambodian people traditionally grilled, decocted and drunk (Chassagne et al. 2016). Study done with bark methanol extract and castor oil-induced diarrhoea in mice showed significantly reduced number of defecation and thus support traditional anti-diarrhoeal use of this plant (Rahman et al. 2003).

C. odorata (Asteraceae) is a multi-stemmed perennial herb and is also known as Siam weed. Is used as soil fertility improvement plant in slash and burn system. Leaves are crushed with sugar and water and root is decocted (Chassagne et al. 2016). Leaves showed antibacterial activity against *S. faecalis* (Irobi 1992) and roots provides significant antibacterial activity against *E. coli* and *S. typhimurium* (Wafo et al. 2011).

D. intricatus (Dipterocarpaceae) is tree that is important source of resin that usually is drunk (Chassagne et al. 2016). In study of Sireeratawong et al. (2012), the resin undoubtedly decreased the movement of stomach content to the small intestine. The resin is also used for torch production.

Bark and wood from an evergreen woody *P. guajava* (Myrtaceae) are by Cambodian people grilled on fire, decocted and drunk. Leaves can be crushed with sugar and eaten (Chassagne et al. 2016). *P. guajava* is native to Mexico but extends through Asia as well. In Mexico, leaf decoction is used to treat digestive suffering associated with severe diarrhoea especially in rainy season when is this disease frequent. As an antidiarrheal agent, it is used all over the tropics (Gutiérrez 2008). *P. guajava* is also consumed as a fruit.

T. nigrovenulosa (Combretaceae) is tree up to 15 m tall which bark with tannin contain is used in Cambodian traditional medicine to cure diarrhoea (Chassagne et al. 2016). Different authors described traditional antimicrobial use for most of *Terminalia* spp. which showed strong activity against *S. typhimurium*, *S. dysenteriae* and *V. cholerae* (Silva & Serrano 2015).

A. ghaesembilla (Phyllanthaceae) is small tree from Indian Subcontinent capable to inhibit the growth of diarrhoea-causing bacteria (Sakunpak & Panichayupakaranant 2012). Traditional practice is application of decoction of bark and wood (Chassagne et al. 2016).

2. Hypothesis

In Cambodia, there are a number of plant species that are used by local people to cure diarrhoeal symptoms. Since many of these species have not been examined and studied in detail yet, there is a belief that these plants are hypothetically antimicrobially active against intestinal bacterial pathogens which cause diarrhoea.

3. Aims of the Thesis

The main aim of thesis was to evaluate *in vitro* growth-inhibitory effects of extracts derived from medicinal plants used in traditional Cambodian medicine for treatment of diarrhoea against representatives of diarrhoea-causing intestinal bacteria.

The specific objectives of this thesis:

a) The collection, identification and processing of Cambodian medicinal plants used to treat diarrhoea;
b) The determination of minimum inhibitory concentrations (MICs) of ethanol extracts from plant parts used in Cambodian traditional medicine against diarrhoea-causing intestinal bacterial pathogens.

4. Methods

4.1. Plant materials

The plant species were selected based on data in literature (Van Duong 1993; Padua et al. 1999; Dy Phon 2000; Padua et al. 2001; Padua et al. 2003; Kham 2004) and internet sources on their use in traditional medicine for diarrhoea treatment. Reference specimen sheets with botanical descriptions, natural habitat, traditional name, and illustrations were elaborated. The plants were verified by Bc. Marie Koryťáková and Ing. Tomáš Kudera. These plants were lately confirmed by local experts from National Centre of Traditional Medicine and Dr. Samnang Nuong from Royal University of Agriculture, Department of Food Science and Technology, Faculty of Agro-Industry. All samples were collected during March and April 2019 in Cambodia, namely in Phnom Penh (Orrusey market), Stung Treng, Ratanakiri and Mondulkiri provinces (Figure 1). All the collected plant materials were air-dried at room temperature and subsequently send by post to CZU, where further laboratory processing and testing took place. For all plants, the voucher specimens were collected and deposited in the herbarium of the Department of Botany and Plant Physiology of the Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences Prague. List of collected plant species is shown in Table 1.

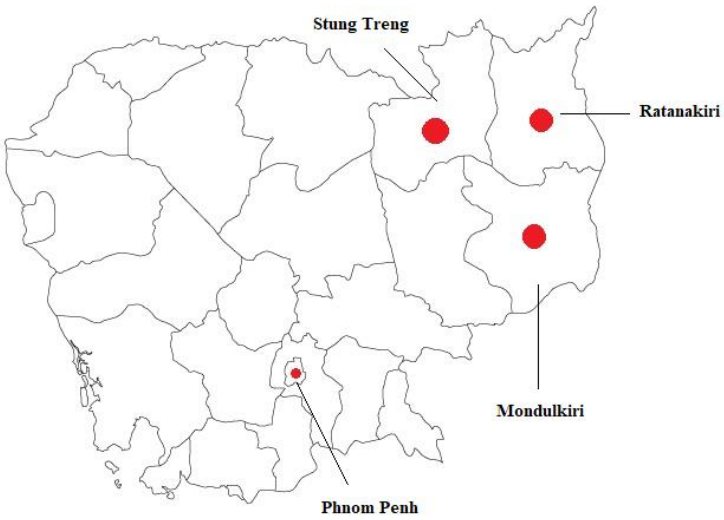

Figure 1: Map of Cambodia with marked places (red spots) of samples collections

Table 1. Ethnobotanical data on Cambodian medicinal plants

Latin name (Family)	Traditional name	Place of collection	Voucher specimen number	Part of plant used	Yield (%)
Aganonerion polymorphum (Apocynaceae)	Kaot Prum	Sen Monorom	02559KBFRC	whole plant	19.7
Ancistrocladus tectorius (Ancistrocladaceae)	Saraburi	Banlung	02560KBFR4	leaves	16
Bauhinia malabarica (Leguminosae)	Tchung-Po	Sen Monorom	02562KBFR6	leaves	11.2
				bark	14.7
Breynia vitis-ideae (Phyllantaceae)	Pneak Briep	Orrusey Market	02563KBFR7	wood, bark	9.9
Ixora nigricans (Rubiaceae)	Pkã-Mã-Chool Pæk	Banlung	02565KBFR9	leaves	10.8
Lagerstroemia cochinchinensis (Lythraceae)	Kouar	Stung Treng	02566KBFRA	bark	2.8
Melastoma dodecancrum (Melastomataceae)	-[a]	Sen Monorom	02568KBFRC	bark	12.7
				leaves, flower buds	9.9
Melastoma saigonense (Melastomataceae)	Mongkri	Orrusey Market	02569KBFRD	wooden stem	7.3
				leaves, flower buds	17.3
Shorea siamensis (Dipterocarpaceae)	Phchok Reang	Stung Treng	02571KBFR6	bark	5.8

Footnotes: [a] not known

4.2. Plant extract preparation

Air-dried samples were homogenized by Grindomix mill (Retsch, Haan, DE). Specific weight of 15 g of dry matter from each sample was extracted for 24 h in 450 ml 80% ethanol (Penta, Prague, CZ) at room temperature. Laboratory shaker (GFL, Burgwedel, DE) was used for extraction of active compounds in samples into the ethanol. The extracts were then filtered and concentrated using rotary evaporator (Büchi Lavortechnik, Flawil, CZ) *in vacuo* at 40°C. Dried residues were subsequently diluted in 100% dimethyl sulfoxide (DMSO) (Penta, Prague, CZ) to obtain stock solution of the final concentration 51.2 mg/ml and stored in Eppendorf Tubes 2.0 ml at -20°C until their use. The yields (%) of dry residues were calculated (Table 1).

4.3. Microorganisms and growth media

The antibacterial activity was evaluated against twelve bacterial representatives gram-positive/gram-negative and aerobic/anaerobic strains. The standard American Type Culture Collection (ATCC) strains were obtained from Oxoid (Basingstoke, UK), Deutsche Sammlung von Microorganismen und Zellkulturen (DMSZ) from German Resource Centre for Biological Material (Braunschweig, DE), The National Collections of Type Cultures (NCTC) from Culture Collections of Public Health England, and Czech Collection of Microorganisms (CCM) from Masaryk University. Following representatives of diarrheagenic bacteria were used: *B. cereus ATCC* 11778, *C. difficile* DSMZ 12056, *C. perfringens* DSMZ 11778, *E. coli* 0175:H7-VT (N) NCTC 1290, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212/51299, *L. monocytogenes* ATCC 7644, *S. enteridis* ATCC 13076, *S. flexneri* ATCC 12022, *S. typhimurium* ATCC 14028, *V. parahaemolyticus* ATTC 17802 and *Y. enterocolitica* ATCC 9610. As the maintenance and growth medium, Mueller-Hinton broth (MHB) (Oxoid, Basingstoke, UK) was used for bacterial that grow aerobically (*E. faecalis* supp. 1% glucose, *V. parahaemolyticus* supp. 3% NaCl). Wilkins-Chalgren broth (WCB), (Oxoid, Basingstoke, UK) was used for anaerobic bacteria.

4.4. Antimicrobial assay

The growth-inhibitory activities of the tested plant extracts against aerobic and anaerobic bacterial strains were evaluated by the broth microdilution method using 96-well microtiter plates, following the protocols of Clinical and Laboratory Standards Institute (2015) and Hecht et al. (1999), respectively. For the effective assessment of the anti-infective potential of natural products, slight modifications were implemented according to Cos et al. (2006). Prior to testing, the strains that grow aerobically were sub-cultured in the appropriate media at 37 °C (*Y. enterocolitica* at 30 °C) for 24 h. Clostridia were cultured at 37 °C for 48 h using Whitley A35 Anaerobic Workstation (Don Whitley Scientific, Bingley, UK). The anaerobic conditions were created by the supply of a standard anaerobic gas mixture of 10% H_2, 10% CO_2, and 80% N_2 (Linde Gas, Prague, Czech Republic). *C. jejuni* was incubated at 37 °C for 48 h under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2). The extracts were diluted 2-fold in appropriate growth media using the Freedom EVO 100 automated pipetting platform (Tecan, Männedorf, Switzerland) and multichannel pipette (Eppendorf, Hamburg, Germany) (initial concentration of 512 μg/mL) in case of aerobic and anaerobic bacteria, respectively. After the bacterial cultures reached an inoculum density of 1.5×10^8 CFU/mL by 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, Czech Republic), the 96-well plates were inoculated (5 μL/well). Bacterial cultures in microplates were incubated by employing the same protocols as used for their cultivation prior to the test. The optical density of the cultures was measured at 405 nm ($OD_{450\ nm}$) using a Cytation 3 Imaging Reader (BioTek, Winooski, VT, USA) before and after the growth. The lowest concentration (μg/mL) of the extracts at which the bacterial growth was inhibited by ≥80% was defined as minimum inhibitory concentration (MIC). Ciprofloxacin (Sigma-Aldrich, Prague, Czech Republic), antibiotic commonly recommended for the treatment of infectious diarrhoea (Casburn-Jones & Farthing 2004), was dissolved in distilled water and used as positive control. All tests were performed as three independent experiments each carried out in triplicate. The mode and median were used for the final MIC value calculation when the triplicate endpoints were within the two- and three-dilution range, respectively. As a result of experiments performed without dissolved extracts and ciprofloxacin, DMSO

(both from Sigma-Aldrich, Prague, Czech Republic) and distilled water did not inhibit bacterial growth of any strain at the tested concentrations (≤1%).

5. Results

In this study, a total of 12 ethanol extracts from 9 different species belonging to 8 different families were examined for their *in vitro* antibacterial activity. Results disclosed that 10 plant extracts showed growth-inhibitory effect against at least 1 out of 12 intestinal pathogens tested at a concentration ranging from 64 to 512 μg/mL. Leave extract of *A. tectorius* produced the strongest anti-bacterial effects, whereas *B. cereus* (MIC = 64 μg/mL) and *L. monocytogenes* (MIC = 128 μg/mL) were the most susceptible bacteria, followed by *C. difficile* and *C. perfringens* (MIC = 512 μg/mL). Another extract with significant growth-inhibitory effect against multiple strains was from bark of *S. siamensis*, which showed inhibition of *B. cereus, E. coli, S. enterditis* (MIC = 256 μg/mL) and *L. monocytogenes* (MIC = 512 μg/mL). At higher MIC tested (512 μg/mL), the following four strains were sensitive to *M. saigonense* extract from leaves with flower buds: *B. cereus, C. difficile, L. monocytogenes* and *V. parahaemolyticus*. Extract from the bark of *B. malabarica* showed moderate inhibitory activity observed against *E. coli* (MIC = 256 μg/mL), followed by weak activity against *B. cereus* and *V. parahaemolyticus* (MIC = 512 μg/mL). Bark extract from *M. dodecandrum* inhibited *E. coli* (MIC = 256 μg/mL) and *V. parahaemolyticus* (MIC = 512 μg/mL), whereas different extract from leaves with flower buds from same plant species proved inhibition of *C. difficile, S. typhimurium* and *V. parahaemolyticus* (MIC = 512 μg/mL). Extract from wood and bark of *B. vitis-idaea* inhibited *E. coli* and *S. typhimurium* (MIC = 256 μg/mL). The whole plant extract from *A. polymorphum* showed positive results against *C. difficile* and *E. coli* (MIC = 512 μg/mL). *I. nigricans* leave extract produced weak antibacterial effect only against *C. difficile* (MIC = 512 μg/mL) as well as bark extract of *L. cochinchinensis* against *V. parahaemolyticus* (MIC = 512 μg/mL). *B. malabarica* (leaves) and *M. saigonense* (wooden stem) extracts did not exhibit growth-inhibitory effect against any of the strains at concentrations tested. Most susceptible were *E. coli, B. cereus, V. parahaemolyticus, C. difficile, L. monocytogenes, S. typhimurium, S. enterditis* and *C. perfringens*, respectively. *E. faecalis, E. coli* 0175:H7, *S. flexneri* and *Y. enterocolitica* were not affected by extracts tested. In correspondence with the data on standard antibiotic sensitivity of tested

bacterial strains, ciprofloxacin produced MICs in a range of 0.016-16 μg/mL. Results are presented in Table 2.

Table 2. *In vitro* growth-inhibitory activities of Cambodian plant extracts against bacteria causing diarrhoea

Plant samples	**Plant used**	**Bacterial strain/MIC[a] (µg/mL)**					
		B. cereus	*C. difficile*	*C. perfringens*	*E. faecalis*	*E. coli*	*E. coli* 0175:H7
A. polymorphum	whole plant	-[b]	512	-	-	512	-
A. tectorius	leaves	64	512	512	-	-	-
B. malabarica	bark	512	-	-	-	256	-
	leaves	-	-	-	-	-	-
B. vitis-idaea	wood with bark	-	-	-	-	256	-
I. nigricans	leaves	-	512	-	-	-	-
L. cochinchinensis	bark	-	-	-	-	-	-
M. dodecandrum	bark	-	-	-	-	256	-
	leaves with flower buds	-	512	-	-	-	-
M. saigonense	wooden stem	-	-	-	-	-	-
	leaves with flower buds	512	512	-	-	-	-
S. siamensis	bark	256	-	-	-	256	-
CIP[c]		1	16	1	2	0.062	0.016

Table 2. *In vitro* growth-inhibitory activities of Cambodian plant extracts against bacteria causing diarrhoea (continued)

Plant samples	**Plant used**	**Bacterial strain/MIC (µg/mL)**					
		L. monocytogenes	*S. flexneri*	*S. enteritidis*	*S. Typhimurium*	*V. parahaemolyticus*	*Y. enterocolitica*
A. polymorphum	whole plant	-	-	-	-	-	-
A. tectorius	leaves	128	-	-	-	-	-
B. malabarica	bark	-	-	-	-	512	-
	leaves	-	-	-	-	-	-
B. vitis-idaea	wood with bark	-	-	-	256	-	-
I. nigricans	leaves	-	-	-	-	-	-
L. cochinchinensis	bark	-	-	-	-	512	-
M. dodecandrum	bark	-	-	-	-	512	-
	leaves with f. b.	-	-	-	512	512	-
M. saigonense	wooden stem	-	-	-	-	-	-
	leaves with f. b.	512	-	-	-	512	-
S. siamensis	bark	512	-	256	-	-	-
CIP		4	0.016	0.031	0.031	0.062	0.125

Footnotes: [a] minimum inhibitory concentration; [b] not active (MIC >512 µg/mL); [c] ciprofloxacin were used as positive control

6. Discussion

In this study, leaf extract of *A. tectorius* showed the strongest antimicrobial effect especially against *B. cereus*, followed by inhibition of *L. monocytogenes*, *C. difficile* and *C. perfringens*. These results correspond with the previously published data on *in vitro* antimicrobial activities of this plant, showing growth-inhibition of *B. cereus* in the zone of 11 mm by 1000 μg/disc of its leaf methanol extract (Wiart et al. 2004). The phytochemicals that are most probably playing a substantial role in revealing such activity are naphthylisoquinoline alkaloids such as ancistrocladinine, 6-0-methylhamateine, and hamatinine, that have been isolated from the methanol and ethanol leaf extracts of this plant and their broad-spectrum of *in vitro* bioactivities have been broadly discussed (Jiang et al. 2013; Bringmann et al. 2016).

Bark extract from *S. siamensis* showed antimicrobial activity against *B. cereus*, *E. coli*, *S. enterditis* and *L. monocytogenes*. To the best of our knowledge, there are no previous studies reporting antimicrobial effect of this plant species. However, the ethanol extract from bark of stem of related species *Shorea roxburghii* has been reported to show inhibitory activity due to the studied content of oligomer diptoindonesin D against *B. cereus* and *E. coli* with MICs 18.75 and 100 μg/mL, respectively (Kanokorn et al. 2019). Antimicrobial activity was also observed against mentioned two bacterial strains by ethanol extract from bark of *Shorea robusta* in study of Marandi et al. (2016), where phytochemical screening of bioactive components showed the presence of tannins as the main candidates contributing to the antibacterial activities revealed by this species.

Extract from leaves with flower buds of *M. saigonense* exhibited moderate antimicrobial activity against *B. cereus*, *C. difficile*, *L. monocytogenes* and *V. parahaemolyticus*. Wooden stem was also tested but with negative results. According to our best knowledge, there are no previous studies reporting the antimicrobial effect of this plant. Nevertheless, various previously published studies on related species *Melastoma malabathicum* reported inhibitory activity of its flower and leaf extract against *B. cereus* and *V. parahaemolyticus* (Keng-Chong et al. 2011); whereas related *Melastoma candidum* leaf extract proved antimicrobial activity against *B. cereus L. monocytogenes* and *S. typhimurium* (Yuan-Chuen et al. 2008). In phytochemical study

of *M. malabathicum*, ursolic acid, 2alfa-hydroxyursolic acid, asiatic acid, beta-sitosterol 3-O-beta-D-glucopyranoside, flavonols and ellagic acid were found to be active against some of the tested microorganisms (Keng-Chong et al. 2011).

In present study, bark extract from *M. dodecandrum* significantly inhibited *E. coli* and moderately *V. parahaemolyticus*, while extracts from leaves with flower buds from the same plant species showed inhibition of *C. difficile*, *S. typhimurium* and *V. parahaemolyticus*. For *E. coli* MIC found was 256 μg/mL while in study of Liu et al. (2014) the MIC was > 200 μg/mL and evaluated by authors as not active. However, in mentioned study *M. dodecandrum* proved significant inhibition of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and, in general, the extract from this plant was evaluated as one of the most active. Authors also mentioned that tannins play a main role for the bacterial growth inhibition in case of this this species.

Bark extract from *B. malabarica* exhibited inhibitory activity against *E. coli*, *B. cereus* and *V. parahaemolyticus*. Leaves were also tested but with negative results. Despite the fact that there are no previously published studies about antimicrobial activities of this plant, we can make a comparison with other studies about related species. According to the study of Ahmed et al. (2012), *Bauhinia bowkeri* showed antimicrobial activity against *E. coli*. Other studies reported about inhibitory activity against *E. coli* in case of *Bauhinia galpinii* leaf extracts (Ahmed et al. 2019; Erhabor et al. 2020), *Bauhinia petersiana* leaf extracts (Ahmed et al. 2012), *Bauhinia purpurea* leaf (Negi et al. 2012) and bark extracts (Avinast et al. 2011), *Bauhinia tomentosa* leaf extracts (Ramar et al. 2018) and *Bauhinia variegata* leaf extracts (Dhaka & Punia 2012). In study of Ahmed et al. (2019), the following phytochemicals were isolated from *B. galpinii* and their antimicrobial activities were proven in mentioned study against all the microbial strains therein tested. Quercetin and myricetin (active against genera *Bacillus*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, also against *E. coli* and *Vibrio cholerae*); new flavone isoetin 2′-methyl ether (that was isolated but due to low quality of this isolated compound MIC could not be determined); quercetin-3-O-beta-galactopyranoside and myricetin-3-O-beta-galactopyranoside (less antimicrobially active than quercetin and myricetin due to the presence of sugar moieties).

In our study, wood and bark extract of *B. vitis-idaea* showed antimicrobial activity against *E. coli* and *S. typhimurium*. According to our best knowledge, this is a first

report on the antibacterial activity of this species. However, our results can be supported by antimicrobial study of related *Breynia cernua*, which demonstrated inhibition of both *E. coli* and *S. typhimurium*. In previously published study, active compounds found in this plant showing growth-inhibition of *E. coli* were mainly flavonoids and tannins in stem bark and stem heartwood; and saponins, sterols and triterpenoids contained in stem heartwood. In case of *S. typhimurium* both stem bark and heartwood proved antibacterial activity of flavonoids and tannins, together with saponins, sterols and triterpenoid contained in heartwood (Khan & Omoloso 2008).

Extract from the whole plant of *A. polymorphum* showed moderate antimicrobial activity against *C. difficile* and *E. coli*. To the best of our knowledge, there are no previous studies about antimicrobial effect of this plant against *C. difficile*, but our results can be partially supported by other two studies dealing with nanoparticle formulations of the extracts derived from this plant. In these studies, inhibitory activities of leaf extract of *A. polymorphum* against *E. coli* with application of silver (Doan et al. 2019) and cuprum (Tran & Doan 2019) nanoparticles have been proven. Nevertheless, there is no literature about phytochemicals contained neither in this nor related species.

Leave extract of *I. nigricans* produced moderate inhibition of *C. difficile*. Our result can be supported by study of Annapurna et al. (2003) on related species *Ixora coccinea*, where leaf ether and methanol extract showed antimicrobial activity against *Clostridium* spp. in the disk diffusion test. The phytochemical screening of extracts revealed the presence alkaloids, flavonoids, phenols, sapogenins, sterols, and terpenes.

Tested bark extract of *L. cochinchinensis* showed moderate antimicrobial activity against *V. paraheamolyticus* only. To the best of our knowledge, there are no previously published studies about this plant and its antimicrobial activity. Despite this fact, we can compare studies of related species *Lagerstroemia speciosa* and its antimicrobial and antibacterial properties against different intestinal pathogens. Laruan et al. (2013) used modified Kirby-Bauer technique and found that methanol extract of *L. speciosa* is highly antibacterial active against *E. coli* and *S. aureus*. Another experiment of Bhaumik et al. (2014) tested methanol, ethanol and chloroform extracts from fruit of *L. speciosa* using agar well diffusion method. They found that all extracts showed good and moderated antimicrobial activity against *S. aureus* and *E. coli*. Antibacterial phytochemicals contained in *L. speciosa* are derivates of ellagic acid called ellagitannins

(Chan et al. 2014). In different study on related species *Lagerstroemia indica*, authors detected the presence of anthraquinones, terpenoids, flavonoids, saponins, tannins, alkaloids and cardiac glycosides (Ajaib et al. 2016).

7. Conclusions

In this study 10 of 12 ethanol extracts of Cambodian medicinal plants traditionally used to cure diarrhoea showed *in vitro* growth-inhibitory effects against at least 1 out of 12 representatives of intestinal bacterial pathogens. Together we observed inhibition of 8 out of 12 tested bacterial strains. The highest antibacterial effect showed *A. tectorius* against *B. cereus* followed by inhibition of *L. monocytogenes*. Significant antibacterial effect exhibited *B. malabarica* (bark), *B. vitis-idaea* (wood with bark), *M. dodecandrum* (bark) and *S. siamensis* (bark). In addition, *A. polymorphum* (whole plant), *I. nigricans* (leaves), *L. cochinchinensis* (bark), *M. dodecandrum* (leaves with flower buds extract) and *M. saigonense* (leaves with flower buds extract) produced moderate antibacterial effects. To our best knowledge, antimicrobial activity of 6 plant species in our study were tested for the first time. Namely, *A. polymorphum*, *B. malabarica*, *B. vitis-idaea*, *L. cochinchinensis*, *M. saigonense* and *S. siamensis*. The most susceptible microorganisms tested were *B. cereus*, *L. monocytogenes*, *E. coli*, *S. enterditis* and *S. typhimurium*, respectively. These results can be therefore helpful in further research targeting the development of new pharmaceutical and food applications effective for treatment of diarrhoea. However, further pharmacological and toxicological evaluation is needed before it can achieve clinical application.

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Appendix: Pictures from research

Figure 1: Orrusey market, Phnom Penh

Figure 2: Visitation in National Centre of Traditional Medicine in Phnom Penh

Figure 3: Plant sheets confirmation by local experts from National Centre of Traditional Medicine

Figure 4: Members of our expedition with local healer and guide Mr. Khram

Figure 5: Collection of medicinal plants with Mr. Khram

Figure 6: Consultation of medicinal plants spots with locals

Figure 7: *A. tectorius* (source: wikimedia)

Figure 8: *B. malabarica* (source: wikimedia)

Figure 9: *M. saigonense* (source: Useful Tropical Plants)

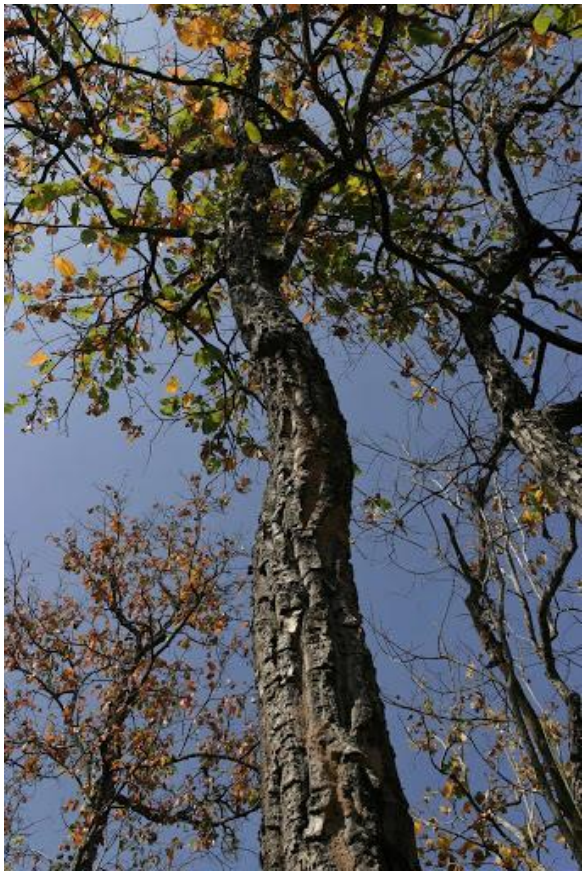

Figure 10: *S. siamensis* (source: Useful Tropical Plants)

Figure 11: Filtrated ethanol extracts