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In vitro growth-inhibitory effect of plant extracts and alkaloids against diarrheal and probiotic bacteria

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled *In vitro* growth-inhibitory effect of plant extracts and alkaloids against diarrheal and probiotic bacteria 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.

In Prague

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Abstract

Although symptom diarrhoea is treatable and preventable, every year more than 1.5 million people die from diarrhoeal diseases. The most common bacteria causing-diarrhoea are *Shigella* (low and middle-income countries) and *Salmonella* (developed countries). When the bacterial diarrhoea is diagnosed, generally causal treatment in form of antibiotics is applicated. The widespread misuse of antibiotics has contributed to the development of resistance in bacterial strains to the drugs. According to ethnobotanical, chemotaxonomic and structure-activity relationships (SARs) data, five alkaloids and six plant extracts were selected and tested for their antimicrobial effects by broth microdilution method. Two of five alkaloids chelerythrine and 6,6'-dihydroxythiobinupharidine produced growth-inhibitory activity against various pathogenic diarrhoeal bacteria. MIC values of chelerythrine against *B. cereus*, *C. perfringens, E. coli*, *L. monocytogenes* and *Y. enterocollica* were 8, 64, 128, 32 and 128 μg/mL, respectively. 6,6′-Dihydroxythiobinupharidine inhibited *B. cereus, C. perfringens, E. faecalis* and *Y. enterocollica* with slightly higher respective MICs of 64, 128, 256 and 256 μg/mL than chelerythrine. Of the six plant extracts tested, only *M. pteleifolia* showed antibacterial activity against *C. perfringens* with MIC = 512 μg/mL. Nevertheless, both chelerythrine and 6,6′-dihydroxythiobinupharidine were more toxic to beneficial than to diarrheal bacteria. Antibiotic ciprofloxacin produced selective antibacterial effect and inhibited growth of all pathogenic strains. To the best of our knowledge, the activity of tested plant extracts and alkaloids against various diarrhoeal and probiotic bacteria have been tested for the first time.

Key words: alkaloids, plant extracts, growth-inhibitory effects, chemotaxonomy, diarrheal bacteria, probiotic bacteria

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CLSI: Clinical and Laboratory Standards Institute

IHME: Institute for Health Metrics and Evaluation

MIC: minimum inhibitory concentration

ORS: Oral rehydration salts

SAR: structure-activity relationship

SI: selectivity index

TM: traditional medicine

WHO: World Health Organization

1. Introduction

Diarrhoea is the most widespread serious symptom of gastrointestinal disease signalling an infection in the intestinal tract. Frequent stools occur at least three times per day (or more than usual for the individual) as a loose, liquid passage (WHO 2013). High incidence of diarrhoeal illness can be caused by chemical agents, microorganisms (bacteria, virus), contaminated food, parasitic organisms or from person-to-person infection (caused by poor hygiene) (WHO 2017, Bellido-Blasco & Arnedo-Pena 2011). During the diarrhoeal episode, the body has lack of water and electrolytes, e.g.: sodium, chloride, potassium and bicarbonate (WHO 2017).

The prevalence of diseases with diarrhoea is higher in developing world, mainly where poor access to drinking water, inadequate sanitation, cleanliness, worse general health, and nutritional status is present. Around one billion people do not have safe drinking water, and approximately 2.5 billion people need adequate sanitation facilities. These unsatisfying hygienic environments allow diarrhoea-causing pathogens to spread more easily (UNICEF & WHO 2009). According to clinical syndromes, we can divide this symptom to three major syndromes: acute (watery), dysentery (with blood) and persistent diarrhoea (Casburn-Jones et al. 2004). The most common type is the acute (watery) with the loose and watery stool, mild symptoms self-resolve within one to two days. In the case of dysentery, more severe disease with presence of inflammation and blood is present. *Shigella* as the predominant cause of dysentery spreads mainly in low- or middle-income countries. On contrary, *Salmonella* as foodborne pathogen is main cause in high-income countries (Kotloff et al. 1999, Majowicz et al. 2010). Persistent diarrhoea comes with many causes, the most common include diet, medications, and chronic disease (Cleveland Clinic 2022).

1.1. Bacterial diarrhoea

Diarrhoea caused by bacterial infection requires higher alertness due to more dangerous, potential symptoms compared to gastroenteritis triggered by virus. Recognizing the pathology of infections and distinguish diarrhoeal cause from the less serious ones is recommended and required in clinical decision-making. Besides, the

treatment of patients involves determining whether to administer antibiotics or when to perform diagnostic stool tests (Akhondi & Simonsen 2023). Disease can range from mild discomfort to life-threatening, each person can experience it differently. Other signs of bacterial diarrhoea include nausea, vomiting, abdominal pain, fever, and dehydration (The Johns Hopkins University 2024). Bacterial infection in gut can be characterized also by dysbiosis, growth inhibition and decrease in the number of beneficial bacteria, which is the main ways of pathogens interaction in the gut (Bellido-Blasco & Arnedo-Pena 2011). Bacterial diarrhoea has various causes and incidence according to countries, level of public health, lifestyle, and diet. Even in case of the same infection, the disease can have different symptoms and consequences (Kim et al. 2019).

1.1.1. Epidemiology

Despite being treatable and preventable, 1.6 million people died from diarrhoeal diseases in 2017, one-third were children under five years old (446000 in 2016 and 525000 in 2017). This makes diarrhoea one of the largest killers of death of children, fifth and second leading cause worldwide and in developing countries, respectively (WHO 2017, IHME 2019). Global Enteric Multicentre Study identified *Shigella* as the leading diarrhoea-causing bacteria in children mainly in developing countries (99% of all cases), 165 million episodes occur each year (Kotloff et al. 1999, Zhang et al. 2014). Recently. insufficient data exists, in 1999 in the study by Kotloff et al. (1999) assumed, that infections by *Shigella* are responsible for 1.1 million deaths per year, 61% of them under the age of 5. Lately estimated global mortality of shigellosis in 2013 for children less than 5 years old were 34400 deaths (IHME 2013). In the developed countries, the most diarrhoeal diseases are spread by foodborne pathogens *Salmonella* and *Campylobacter*, which are found mainly in poultry, eggs, dairy products, fresh fruit, or vegetable (Silva et al. 2011, Pui et al. 2011). In 2010 *Salmonella* as non-typhoidal gastroenteritis is most frequent worldwide, with 93757000 cases, results in 155000 deaths per year. Diarrhoea has high morbidity rate in developing countries since salmonellosis relates to typhoidal fever (Majowicz et al. 2010).

1.1.2. Bacteria causing diarrhoea

In general, main causative bacteria of infectious diarrhoea are *Bacillus cereus, Campylobacter jejuni, Clostridium perfringens, C. difficile, Enterococcus faecalis, E. faecium, Escherichia coli, Listeria monocytogenes, Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *V. parahaemolyticus* and *Yersinia enterocolitica* (Bellido-Blasco & Arnedo-Pena 2011, Casburn-Jones et al. 2004, Li et al. 2021, BC Centre for Disease Control 2024). Symptom dysentery (also gastroenteritis with blood) can be caused by bacteria, which invade bowel mucosa, cause inflammation and tissue damages, that result in bloody diarrhoea. The causative bacteria are *C. jejuni, Clostridia* spp.*, E. coli, Shigella* spp., *Salmonella* spp., *Yersinia* spp. or amoeba *Entamoeba histolytica* (called as amoebic shigellosis) (Jones 2004, WHO 2005). The following pathogens presented are according to the type of diarrhoea caused.

In acute watery *Bacillus cereus, C. jejuni, E. faecalis, E. faecium,* enterotoxigenic *E. coli, L. monocytogenes, Vibrio cholerae*, *V. parahaemolyticus* and *Yersinia enterocolitica* are prevalent bacteria. They usually invade gut without causing inflammation or mucosal destruction and mostly do not result in dysentery. Diarrhoea caused by these bacteria self-resolves within 5-10 days, however, sometimes other gastrointestinal symptoms is present, for example the blood in stool, fever, nausea, etc (Bellido-Blasco & Arnedo-Pena 2011, Casburn-Jones et al. 2004, Li et al. 2021, BC Centre for Disease Control 2024). Food-borne diarrhoea has more severe symptoms in general, the dysentery and inflammation in gut is present. They can be caused by not invasive enteroaggregative and enterohemorrhagic *E. coli* and *C. difficile*. During infection of mentioned bacteria, diarrhoea is watery with possible passage of blood or mucus, abdominal pain, and low-grade fever. Infections by invasive *Campylobacter* spp.*, Salmonella* spp., *Shigella* spp. and *Yersinia* spp. usually result in mucosal destruction. Compared with non-invasive bacteria, they can cause serious abdominal pain, more blood in stool and higher fever. It is reported that infection by food-borne bacteria may turn into persistent diarrhoea (Gaea & Fasano 2005, Navaneethan & Giannella 2008, Thielman & Guerrant 2004). In addition, the domination of several bacteria (mostly inflammation-causing) also plays role in shift of the microbiota composition from a mutualistic to a pro-carcinogenic. The bacteria with higher risk of development to pro-carcinogenic microbiome are *Shigella*, *Salmonella*, superoxide-

producing *E. faecalis*, enterohemorrhagic or enteroinvasive *E. coli*, *C. difficile, C. jejuni*, *L. monocytogenes* and *Y. enterocolitica*. The chronic diarrhoea with mentioned bacteria may result in persistent inflammation and promotion of intestinal cancer (Cheung 2017, Candela et al. 2014).

1.1.3. Beneficial bacteria in gut

In the human gastrointestinal tract, complex and dynamic population of microorganisms (gut microbiota) occurs. The gut microbiota performs essential functions in maintenance of health, including having protective, structural, and metabolic roles. Healthy microbiome brings benefits to host metabolic pathways, e.g., synthesis of essential vitamins, oligo-elements and biochemical pathways required for the fermentation of nondigestible substrates (mainly carbohydrates) which provide energy (Candela et al. 2014, Prakash et al. 2011). The healthy composition of gut microbiota is called eubiosis, which is defined as balance of gut microbiota with the prevalence of probiotic bacterial species, belonging mainly to phylum Firmicutes, Bacteroides and Actinobacteria. The dominant genera are Bacteroides, *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus*, whereas aerobes (facultative anaerobes) such as *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Proteus* are the subdominant genera (Guarner & Malagelada 2003, Thursby & Juge 2017). Every individual has several hundreds of species belonging to mentioned dominant phylum, with a particular combination of predominant species distinct from that found in others. In this state, physiology and metabolism of the host improve. Potential pathogenic species (e.g.: species from Enterobacteriaceae) are present in a very low percentage (Iebba et al. 2016). In the microbiota of infants fed with breast milk Bifidobacteria strain (Actinobacteria phylum) is the major components (Rinninella et al. 2019). Composition of the flora can vary under circumstances, for example, acute diarrhoeal illnesses, inappropriate antibiotic treatment, or by dietary interventions. Without these impacts, microbiota usually remains stable and possess a significant influence on the host during homeostasis and immunological response to disease (Thursby & Juge 2017).

We can call the beneficial bacterium a probiotic when consumed as a food supplement providing benefits to human health. These foods or supplements contain

live microorganisms, which maintain or improve the "good" bacteria (normal microflora) in the gut. In the diet, probiotics are in foods such as yoghurt, sauerkraut, prebiotics as food for human microflora consist of nourishments (typically in high-fibre diet). They are consumed to bring nourishment for the beneficial bacteria in gut. Prebiotics can be taken in foods for example whole grains, greens, bananas, garlic, onions, soybeans, and artichokes. Both probiotics and prebiotics can be added to diet as dietary supplements (Mayo clinic 2022). Adding them to diets can promote the health of gut. In infants also prevents gastrointestinal diseases in chronically ill children. *L. rhamnosus* strain GG has also been useful as a prophylaxis in undernourished children, individuals with acute diarrhoea who received the supplement (*L. casei, L rhamnosus, L. reuteri*) had significantly decreased duration of episode. Probiotics are also effective in acute diarrhoea caused by rotavirus infection, their use reduces excretion of the pathogen. Lastly, it was demonstrated that consumption can enhance specific responses to enhance immunological reaction, which prevents *S. typhi* colonisation (Guarner & Malagelada 2003).

1.1.4. Treatment

Prevention is essential to avoid infection, general recommendations are access to safe drinking water, improved sanitation, hand washing with soap, good personal and food hygiene, exclusive breastfeeding for the first six months of life, health education about how infections spread and possible rotavirus vaccination (WHO 2005, WHO 2017). Since the treatment of diarrhoea is prescribed, it can be distinguished based on its aim to symptomatic and causal. The symptomatic care (also called supportive care, therapy, or palliative treatment) cures only symptoms of disease, not its causes. The biggest danger of diarrhoea and intestinal diseases is dehydration, which can lead to death caused by large loss of a water and salt from the body. To reduce fatal consequences (mainly in children), water and nutrients should be given to as soon as possible. The low-cost rehydration therapy, zinc supplements and nutrient rich foods are recommended, they can be applied by oral rehydration salts (ORS - solution of sugar, salt, and clean water). ORS are absorbed in the small intestine, in case of serious dehydration or shock, the rehydration therapy is intravenously applied (WHO et al. 2005, WHO 2007). Another treatment used to stop symptoms are antimotility agents. They reduce the rate of passage of the contents through the intestine and slow down its

movement, which cause longer digestion of food in the stomach and ensures greater absorption of water in the body. As a result, the stool is firmer and passed less frequently (Whitehall 2012). However, therapy with motility inhibitors is not recommended for Shiga toxin-producing *E. coli, C. difficile* infections, and colitis. In addition, the effect in treatment of some infectious diarrhoea has not been proven yet, more study is needed to prove their safety in certain bacterial diarrhoea (Koo et al. 2007, Lübbert 2015).

In the causal treatment, the aim is to inhibit pathogenic bacteria, which cause the symptom. However, the symptomatic care is still needed to reduce effects of dehydration. Generally, acute syndromes commonly resolve themselves after 3-5 days, but targeted chemotherapy has been proven to be effective at managing the clinical symptoms and reducing the duration of diarrhoea to approximately 1.5 days. While maintaining the disease, it can help to bring balance of probiotic microbiota. The most widely used causal treatment in diarrhoea is application of antibiotics. Their discovery brought a revolution in the medical treatment seventy years ago. Seriously ill patients who have high frequency of stools (more than 6 per day), symptoms that have persisted for more than a week, fever, blood, an underlying immune deficiency, advanced age and significant comorbidities should be considered for empirical therapy (Lübbert 2015, Tribble 2017).

Before selecting appropriate antibiotic treatment, the causative bacteria should be indicated. Diarrhoea-causing bacteria can be recognized by incubation time. Disease caused by enterotoxins of *S. aureus* and *B. cereus* have short period 1-6 hours. *C. perfringens* or *B. cereus* producing diarrhoeal toxin are suspected when disease starts after 8-16 hours. Enterotoxigenic *E. coli*, *Salmonella*, *Shigella*, and *V. cholerae* are active after 16-72 hours (DuPont 2009, Kim et al. 2019). Another indication is travelling destination, which should be also considered because of regional variations in the prevalence of infections and bacterial resistance. The recommended initial line of treatment for acute watery diarrhoea is azithromycin with following doses per day: diarrhoea (500 mg), dysentery or with fever (1000 mg). Another option are levofloxacin and ciprofloxacin; however, they are losing their effectiveness due to rising fluoroquinolone resistance, especially in *Campylobacter* species. They are useful for treating acute watery diarrhoea (single doses of 500 and 750 mg), in febrile and

dysentery in areas with high rates of *Shigella* (500 mg once per 3 days, once with levofloxacin and twice with ciprofloxacin). Rifaximin (200 mg 3 times a day for 3 days) is an additional treatment option for acute watery diarrhoea. Use of loperamide in combination with antibiotic therapy bring advantages, reduce gastrointestinal symptoms and duration (Tribble 2017).

During the past few decades, the widespread misuse of antibiotics has contributed to the development of resistance in many bacterial strains (both Gram-positive and Gram-negative) to the drugs, posing a serious global problem for modern medical practice. Recently emerged resistant strains (many of which have acquired multidrug and antibiotic resistance) are considerable concern include diarrhoeal pathogens such as *Campylobacter* spp., *E. coli, E. faecalis, E. faecium, Salmonella* spp., *Shigella* spp. and *Staphylococcus aureus* (Wilson 2002). Treatment with unsuitable antibiotics can generate various side-effects (e.g.: dysbiosis, antibiotic-associated diarrhoea, …) (Mayo clinic 2021). Selective effect, which is antibacterial activity with low toxicity to intestinal probiotic bacteria, should be considered during exploring a novel antimicrobial agent.

1.2. Anti-diarrhoeal plant-derived products

Plants are the major contributors of natural products in drug discovery, and their classification based on the chemicals they produce is continually the centre of research. Plant-derived agents are important sources of novel compounds that can be used as direct chemotherapeutic treatment. Many methods have evolved toward the taxonomy of natural products, including chemotaxonomic classification. Chemotaxonomy determinate the taxonomic classification according to plant chemical constituents (especially secondary metabolites). This approach can help in the search for new plant sources of active compounds or for related plant-derived products with similar pharmaceutical potential (Singh & Geetanjali 2018). Seventy percents of the total number of small molecules drugs with antibacterial effects are related to natural products (Taneja & Qazi 2007), phenolics, terpenoids, essential oils, alkaloids, lectins, polypeptides and polyacetylenes are the major classes (Cowan 1999). Antibacterial activity was founded in 51 of 79 plant orders throughout the phylogenetic tree (mostly eudicots). Families Lamiaceae, Fabaceae and Asteraceae have the most often reported

effects, the most studied species are *Cinnamomum verum, Rosmarinus vulgaris* and *Thymus vulgaris* (Chassange 2017).

Many of plant-derived compounds, which have interesting biological activity possess direct medicinal application as drug scaffolds, that function as "leads" (model compounds) for drug synthesis or semi-synthesis (Balandrin 1993). The widely studied secondary metabolites of plants are alkaloids. They are classes of nitrogen based heterocyclic bioactive products naturally occurring in plants, which possess very large group of structurally diverse compounds. While they are quite prevalent in all the kingdoms of living things, plants account for four fifths of all known secondary metabolites (Masci et al. 2019). In drug development, their structures can help in discovery of novel drug using structure-activity relationship (SAR), which is the relationship between the structure and biological activity of a molecule. This approach provides a useful start point for drug discovery and reduces the time of developing new agent. It guides the acquisition or synthesis of desirable new compounds, as well as to further characterize existing molecules (CDD Vault 2023). Alkaloids were reported to possess bioactivity with the characteristics of broad-spectrum antibiotics but with a few of adverse effects and a low tendency to drug resistance (Yan et al. 2021). According to SAR, classes of alkaloids with several known compounds possessing anti-diarrhoeal effects are (iso)quinoline and sulphur-containing alkaloids (Kudera et al. 2020, Zhang et al. 2023).

Quinolines and isoquinolines consists of core with quinoline or isoquinoline, aromatic organic isomers with the chemical formulas C9H7N that differ in the position of the nitrogen in the ring structure (position 1 for quinoline and 2 for isoquinoline). Most of these compounds have a broad and significant biological activity. They play a role as precursors of antidiarrheal agents in drug development (Shang et al. 2018, Shang et al. 2020). The most known (iso)quinoline, which could be obtained from the medicinal plants used against diarrhoea are for example berberine and 8-hydroxyquinoline. Berberine, an alkaloid with selective antibacterial effects from the plant *Berberis aristata* is known since the ancient times, decoctions from plant's roots have been used for treatment of gastroenteritis in China and India since olden days. In Asia, berberine is sold in pure form as a cure against intestinal infectious diseases and diarrhoea (Berberine Hydrochloride Tablets, Northeast Pharmaceutical Group,

Shenyang, China). The data from clinical testing has been reported, that berberine inhibits by approximately 70% the secretory responses of the heat-labile enterotoxins of *Vibrio cholerae* and *E. coli* in a rabbit ileum model (Sack & Froehlich 1982, Lahiri & Dutta 1967, Kokoska et al. 2019). Alkaloid 8-hydroxyquinoline can be isolated from plant *Sebastiana corniculata* (medicinal herb used in East Asia and South America). It has been showed that extracts, and pure compound possess selective antibacterial activity against food-borne bacteria *B. cereus*, *C. difficile, L. monocytogenes*, *S. typhimurium, S. sonnei* (Yang et al. 2013, Novakova et al. 2014). Chloroxine (synthetic analogue of 8-hydroxyquinoline) is used and prescribed under trade name Endiaron as a gut disinfectant used for infectious diarrhoea. Exhibits selective antimicrobial activity against the pathogens and does not affect the host indigenous microbiota. It is effective against bacteria, protozoa, yeast and funguses (Sanofi 2024). According to SAR, analogues of the (iso)quinoline alkaloid may possess antibacterial activity against diarrhoeal bacteria, so we decided to test structurally similar compounds. Selected compounds (chelerythrine, dihydrochelerythrine, neferine, yohimbine) have not been tested against diarrhoeal bacteria as much as above-mentioned alkaloids, so this thesis is focused more on the evaluation of their potential antibacterial activity. Chelerythrine and dihydrochelerythrine are alkaloids present in *Chelidonium majus*, they are benzophenanthridine alkaloid with potential reported antibacterial, anti-tumour and antiparasitic activities (Lin et al. 2020). Neferine is a bisbenzylisoquinoline alkaloid obtained from seed embryo of *Nelumbo nucifera.* This compound is reported to possess anticancer, antidepressant, antioxidant and anti-inflammatory properties (Asokan et al. 2018). Yohimbine is known as plant alkaloid with alpha-2-adrenergic blocking activity, which is used as a mydriatic and in the treatment of impotence. Yohimbine can be isolated from the bark of *Pausinystalia yohimbe*, which is traditionally used as aphrodisiac (WebMD 2020).

The sulphur-containing alkaloids (also called thioalkaloids) are class of bioactive products naturally occurring in aquatic plants. They displayed a variety of anti-proliferation, anti-virus, anti-inflammatory, antifungal and antioxidant activities (Okamura et al. 2015, Cullen et al. 1974). They can be obtained mainly from water plants and marine algae, sponges, cnidarians, etc. Nuphar alkaloids are a small family of bioactive terpene alkaloids with unusual bis(spirothiolane) structure, which can be isolated from *Nuphar japonicum, N. lutea* and *N. pumilum.* Extracts from *Nuphar* spp.

possessed immunosuppressive and anti-metastatic activity (Matsuda et al. 2003). The most known compound from mentioned class is 6,6′-dihydroxythiobinupharidine, which also showed antibacterial effect against various methicillin resistant *S. aureus* with MIC 1-4 μg/mL. This compound only exhibited small cross-resistance to norfloxacin-resistant *S. aureus*, which indicated that this compound may be a potent candidate or scaffold for novel antidiarhoeal agents (Okamura et al. 2015).

1.2.1. Medicinal plants containing alkaloids

Use of plants as a medicinal therapy can be traced back thousands of years across a wide variety of geographical regions and civilizations, and because of their great biochemical diversity, their usage is currently the subject of several studies. Chemotaxonomical approaches attempt to map the occurrence of biologically active alkaloids in plant species. The very known alkaloids used in pharmacy are opium alkaloids (morphine, noscapine, codeine, thebaine, papaverine) obtained from the mature capsules of *Papaver somniferum*. They are mainly used as analgesic, sedatives or psychotropics, the first information about their production is found on Sumerian clay tablets about 3000 BC (Shang et al. 2020). Recently, the most important compound used in pharmacy morphine, which was firstly isolated from the *P. somniferum* in the early 19th century. Next important and commonly used alkaloid is quinine which was isolated from the bark of the *Cinchona* spp in 1820, the crude bark is used in the treatment of malaria, the antimalarial quinoline-containing drugs such as chloroquine, quinine, and mefloquine are essential parts of our malaria chemotherapeutic arsenal (Foley & Tiley 1997). The medicinal plants possessing antibacterial activity against diarrhoea, which were reported in chemotaxonomical studies to contain bioactive (iso)quinolines and thioalkaloids are described in further detail.

Melicope semecarpifolia (Rutaceae) is evergreen dioecious with a height of 1-14 m trifoliated shrub or tree distributed in the Philipines and Taiwain, in mountains, hills or plains. Its roots are traditionally used as carminative medicine containing at least 16 active (iso)quinoline alkaloids, which display anti-inflammatory, analgesic, anti-bacterial, anti-tumour and other activities. Among them, furoquinolines evolitrine, melicarpine, kokusaginine, dictamnine, skimmianine, ayanine isolated from plant were also reported to have cytotoxic effects against colon cancer cell line (Chen et al. 2003,

Chou et al. 2005, Yao et al. 2020). *Tabernaemontana coronaria* (Apocynaceae) is a small tree or shrub with leaf and silvery-grey bark. White flowers bloom throughout nearly the whole year. The thin paste made from the flowers can be used as a local anaesthetic, vermicide, tooth ache reliever, to clear corneal opacity, to relieve burning eyes, and to treat wounds and skin diseases (Kumar et al. 2018). Species are used in traditional medicine for the treatment of abdominal pain, hypertension and sore throat. *Tabernaemontana* genus are a rich source of the monoterpene indole alkaloids and furoquinolines (dictamine skimmianine) used in antidiarrheal or anticancer therapy (Kumar et al. 2018, Athipornchai 2018, Fan et al. 2023). *Stephania glabra* (Menispermaceae) is a large, climbing shrub, indigenous to the lower parts of Indian Himalayan region. The plant usually grows in tropical and temperate lowlands up to the 2200 m. It has herbaceous vines, filamentous, axillary or found inflorescence and obovate and flattened drupes (Semwal & Semwal 2015) In traditional medicine tuber from plant treat a number of diseases such as diarrhoea, pyrexia, tuberculosis, dyspepsia, urinary troubles, abdominal ills and asthma. Plant mainly contains alkaloids and over 30 alkaloids such as bisbenzylisoquinolines, hasubanalactams, berberines and aporphines, which have been isolated from its tuber (Semwal & Semwal 2015, Semwal et al. 2010). *Nuphar* spp (*N. japonica, N. lutea* and *N. pumilum*) (Nymphaceae) grow as aquatic herbs with floating leaves, attached to ground by root system. They are native to northern temperate, some subtropical regions of Europe, northwest Africa, and western Asia. The plants develop from a rhizome with solitary and aerial inflorescence. The calyx comprises five sepals which are sulphur yellow and elliptic (Wiart 2013). Their rhizomes called Nuphar Rhizome have been used as a crude drug for the treatment of edema, irregular menstruation, hysteria with diuretics, and as a blood purifier and sedative (Okamura et al. 2015, Matsuda et al. 2003). Plant extracts are rich in alkaloids and polyphenolic compounds which have potential in therapeutic value. Indeed, the therapy-related actions discovered in recent studies of *N. lutea* extracts and individual sesquiterpene thioalkaloids include anti-inflammatory, antibacterial, antiviral, antifungal, antiparasitic and anticancer (Muduli et al. 2022).

Because of the reported traditional medicinal use of above-mentioned plants in treatment of diarrhoea and their content of active alkaloids, we decided to test the related plant species *M. pteleifolia*, *S. japonica*, *S. longa*, *T. bovina* and *T. divaricata*. *M. pteleifolia* (Rutaceae) is a shrub or tree with a height of 1-14 m. often grows in

mountains, hills or plains. Commonly is dioecious or rarely monoclinous. The leaves, stems and roots of the plant are used traditionally for the treatment of sore throat, rheumatism, eczema, dermatitis, bruises, or snakes' bites. It was reported to contain potentially active furoquinolines (Yao 2020). *S. japonica* (Menispermaceae) is a slender climbing plant species with peltate leaves native to tropical and subtropical Asia and Australia. Grows in hedges, secondary forests and along riverbanks at altitudes up to 2000 m. It can grow to heights of 10 m; the leaves are petiolate 3 to 12 cm long and can be glabrous or pilose (Macek et al. 2023). *S. longa* (Menispermaceae) as herbaceous liana, widely distributed in the south of China, is used in traditional medicine to treat fever, inflammation, and dysentery. This plant was reported to contain hasubanan-type alkaloids (Lin et al. 2022). *T. bovina* (Apocynaceae) is a shrub or small tree which grows to the height of 0.5-5 m tall. Grows in Southeast Asia, it is glabrous except for flowers. Petiole is 2-8 mm in length, leaf blade has deep green colour adaxially and pale green abaxially (Flora of China 1994). This plant was reported to contain aspidosperma-type alkaloids (Zhao et al. 2022). *T. divaricata* (Apocynaceae) is abundant in Southeast Asia and mainly distributed as a garden tree. The plant generally grows to a height of 1.5–1.8 metres and is dichotomously branched. Has large shiny leaves with deep green colour and about 15 cm in length and 5 cm in width. The waxy blossoms are found in small clusters on the stem tips. In Asia this plant is traditionally used for analgesic and antibacterial purpose (John & Cheriyan 2023).

1.2.2. Plants used in traditional medicine

About 80% of people on the planet are said to get their primary medical care from traditional (primarily herbal) medicine (Hamilton 2004). Ethnobotany is a branch of research that investigate the complex interactions between humans and plants to discover the various application including medicinal properties (Domingo-Fernandez et al. 2023). With the use of ethnobotanical knowledge, novel potential medicinal plants as a source of anti-diarrhoeal alkaloids can be obtained. Very wide use of herbal medicine is in Mainland Southeast Asia (formerly known as Indochina). This part of world has been influenced by Indian and Chinese culture. The challenging natural environment made people to gain extensive knowledge about preventing diseases, managing pain and health in various region resulting in the formation of comprehensive medical framework. Medicines, which include herbs, herbal preparations and products, are the

most widespread of traditional medicines (TM) (Liu 2021, J. De Boer & Cotingting 2014). In Cambodia, herbal TM is referred to as "yieb" and is widely used by the local population, the exact origins of traditional Khmer medicine (TKM) remain unclear. However, it is believed to have been formalised in from the Nokor Phnom period (Funan era) to the 9th century, during the Angkorian period. (Chassagne et al. 2017, Richman et al. 2010).

One of the plant species used in herbal TKM is *Helicteres isora* L. (The east Indian screw tree), (*Malvaceae*). This big shrub or small tree grows throughout India and SE Asia in dry deciduous woods up to 1500 meters on hill slopes. The leaves are ovate shaped, hairy leaves with serrate margins. In TKM this medicinal plant is used to treat several illnesses, its use in treatment of diarrhoea, dysentery, or other gastrointestinal complaints in traditional herbal systems in Cambodia according to literature (Savajol et al. 2011, Goya et al. 2021). The therapeutic values depend on bioactive compounds, which possess specific physiological action in the human. Preliminary qualitative studies on various extracts suggested presence of ascorbic acid, carotenoids, flavonoids, glycosides, phenolics, saponins and tannins in different parts of plant. In traditional medicine, plant extracts from bark, seed and root are known to cure diarrhoea, diabetes, snakebite, weakness, and skin ailments, applying the root juice topically can treat scabies and alleviate symptoms of cough, asthma, stomach aches, and other intestinal infections (Dayal et al. 2015, Venkatesh et al. 2007). According to the mentioned use of different parts of the plant in the treatment of diarrhoea, the leaves of *H. isora* will be tested in this study in an antibacterial test against intestinal bacteria.

2. Hypothesis

According to ethnobotanical, chemotaxonomic and SAR data, the alkaloids chelerythrine, dihydrochelerythrine, neferine, yohimbine, 6,6′-dihydroxythiobinupharidine and plant extracts from *H. isora*, *M. pteleifolia, S. longa, S. japonica, T. bovina, T. divaricata* may produce growth inhibitory activity against diarrhoeal bacteria.

Since the structurally similar plant-derived compounds possessed antibacterial effects against diarrhoeal bacteria with no harm to indigenous probiotic microbiota (e.g. berberine, 8-hydroxyquinoline), the certain alkaloids or plant extracts active against intestinal pathogenic bacteria may be potentially safe to probiotic bacteria.

3. Research questions

Which plant extracts and alkaloids will produce the strongest growth-inhibitory effect against diarrhoea-causing bacteria?

Which plant extracts and alkaloids inhibiting growth of diarrhoea-causing bacteria will not affect growth of probiotic bacteria?

4. Aims of the Thesis

The main aim of thesis was to evaluate *in vitro* growth-inhibitory effects of plant extracts and alkaloids chosen according to ethnobotanical data, chemotaxonomic and SARs against intestinal diarrhoea-causing and probiotic bacteria.

The specific objectives of thesis were:

a) determination of minimum inhibitory concentrations (MICs) of ethanol extract and alkaloids against diarrhoea-causing bacteria.

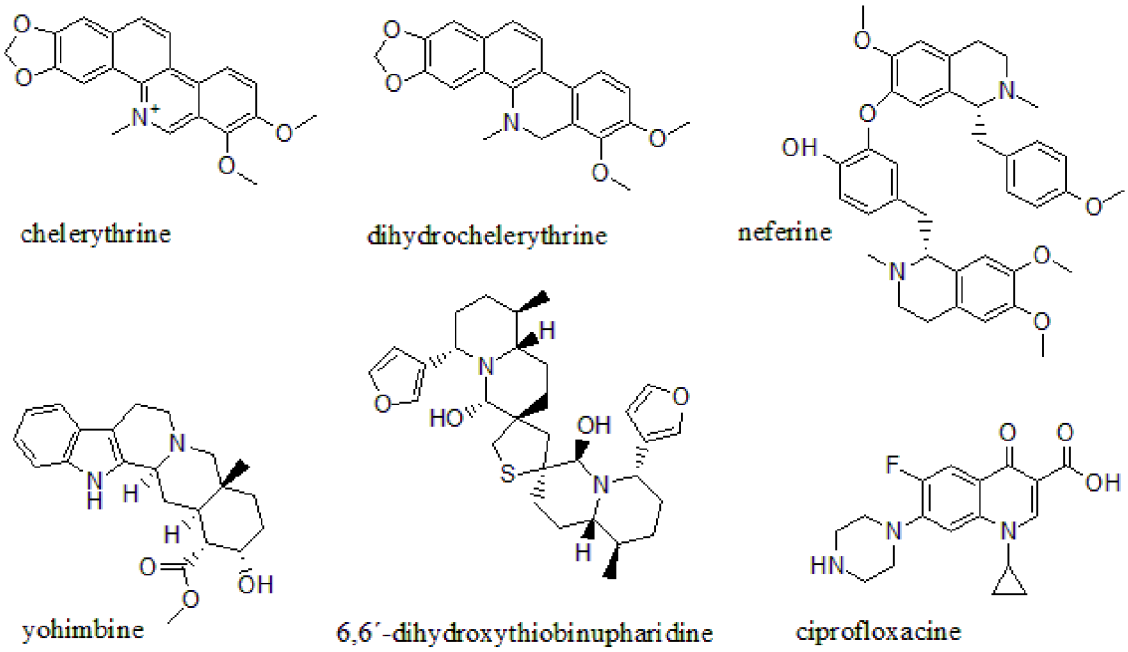
b) evaluation of selective effect of antibacterial alkaloids or extracts against diarrhoea-causing and probiotic bacteria.

5. Methods

5.1. Compounds

Alkaloids chelerythrine, dihydrochelerythrine, neferine, yohimbine, and antibiotic ciprofloxacin were purchased from Sigma-Aldrich (Prague, Czech Republic). Thioalkaloid 6,6′-dihydroxythiobinupharidine from Sigma-Aldrich was received as a gift (mentioned in acknowledgements), dimethyl sulfoxide (DMSO) was used as a solvent to prepare the stock solutions of the phytochemicals. Ciprofloxacin was prepared using distilled water and was supplemented by 0.1N hydrochloric acid from Sigma-Aldrich (Prague, Czech Republic). The chemical structures of individual compounds tested are shown in Figure 1.

Figure 1. The chemical structures of the tested compounds



5.2. Plant samples

Plant species were chosen according to the ethnobotanical (traditional use in treatment of diarrhoea, dysentery, or other gastrointestinal complaints) and chemotaxonomical (presence of antibacterial alkaloids in related species) data, which are presented in Table 1 (Savajol et al. 2011, Goya et al. 2021).

Dried leaves from samples were milled and homogenized using Grindomix mill (Retsch, Haan, Germany), 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). Then the extracts were 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 solutions of the final concentrations 51.2 mg/ml and stored at -20°C until their use. The yields (%) of dry residues, sample selection method and collection places are given in Table 1.

Table 1. Ethnobotanical and chemotaxonomical data on tested plant materials

Species (Family)	Part	Collection place/geographical coordinates (EPSG 3857)	VSN	Sample selection method	Yield (%)
Helicteres isora L. (Malvaceae)	leaves	Kratie province, Phnom Sombok/ 12.5628965N, 106.0201076E	02631KBFR3	Ethnobotanical (bark, seeds and root juice reduce diarrhoea, dysentery, skin ailments, weakness)	11.8
Melicope pteleifolia (Champ. ex Benth.) (Rutaceae)	leaves	Mondulkiri province, Brou Sra/ 12.5649428N, 107.4204557E	02632KBFR4	Chemotaxonomical (furoquinolines eg. skimmianine found in leaves)	13.3
Stephania japonica (Thunb.) (Menispermaceae)	aerial part	Mondulkiri province, Putang village/ 12.4245864N, 107.1531116E	02629KBFRA	Chemotaxonomical (aporphines and protoberberines eg.: magnoflorine, palmatine found in leaves, stems and tubers)	25.7
Stephania longa Lour. (Menispermaceae)	aerial part	Mondulkiri province, Brou Sra/ 12.5653600N, 107.4232500E	02627KBFR8	Chemotaxonomical (aporphines and protoberberines eg.: magnoflorine, palmatine found in leaves, stems and tubers)	17.9
Tabernaemontana bovina Lour. (Apocynaceae)	leaves	Koh Trong, Kratie/ 12.4998994N, 105.9902036E.	02630KBFR2	Chemotaxonomical (acridones and furoquinolines eg.: arborinine, dicramnine in stem bark)	10.4
Tabernaemontana divaricata L. (Apocynaceae)	leaves	Mondulkiri province, Brou Sra/ 12.5682742N, 107.4171311E	02628KBFR9	Chemotaxonomical (acridones and furoquinolines eg.: arborinine, dicramnine in stem bark)	19.5

Footnote: VSN – Voucher specimen voucher, EPSG 3857 – map projection used for geographical coordinates

5.3. Bacteria

The following intestinal bacterial strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, United States), Czech Collection of Microorganisms (CCM, Brno, Czechia), German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Ten pathogenic bacterial strains were used in this study: *B. cereus* (ATCC 14579), *C. difficile* (DSMZ 12056), *C. perfringens* (DSMZ 11778), *E. coli* (ATCC 25922), *E. faecalis* (ATCC 29212), *E. faecium* (CCM 2308), *L. monocytogenes* (ATCC 7644), *Shigella flexneri* (ATCC 12022), *Salmonella enterica* ssp. *enterica* (ATCC 13076) and *Y. enterocolitica* (ATCC 9610). Medium Mueller-Hinton broth (Oxoid, Basingstoke, UK) was used for cultivation of *B. cereus, E. coli, L. monocytogenes* and *S. flexneri*. Bacteria *E. faecalis, E. faecium, S. enterica* ssp. *enterica* serovar *Enteritidis* were cultured in brain heart infusion (Oxoid, Basingstoke, UK). Anaerobic bacteria *C. difficile* and *C. perfrigens* were cultivated in Wilkins-Chalgren broth (Oxoid, Basingstoke, UK). Above mentioned bacteria were selected according to the described diversity of diarrheagenic gram-positive and gram-negative pathogens responsible for globally distributed foodborne, waterborne, and nosocomial infections according to Schirone et al (2019).

The following six probiotic bacterial strains, which belong to the dominant bacterial phyla in the human gut, were tested in this study: *Bifidobacterium adolescentis* (DSMZ 20087), *B. animalis* spp. *lactis* (DSMZ 10140), *Lactobacillus casei* (DSMZ 20011), *L. reuteri* (CCM 3625), and *L. rhamnosus* (CCM 7091). All these anaerobic strains considered as beneficial gut bacteria were culcured in Wilkins-Chalgren broth (Oxoid, Basingstoke, UK).

5.4. Antibacterial *in vitro* essay

The *in vitro* antimicrobial activity was determined by the broth microdilution method using 96-well microtiter plates according to CLSI guidelines (CLSI 2024, CLSI 2018), with modification based on recommendation proposed for effective assessment of the antibacterial potential of natural products (Cos et al. 2006). The samples were 2-fold diluted in Mueller-Hinton, Brain Heart or Wilkins-Chalgren broth (100 μl) in a

range of 0.125-1024 and 0.5-512 μg/mL for plant alkaloids and extracts, respectively. Bacterial cultures were diluted to contain 2.5-3 × 10^4 colony-forming unit per ml in microtiter plate. Each well was afterward inoculated with the suspension. Microplates were incubated at 37°C for 24 h. Growth was estimated visually as turbidity and determined by measuring the optical density by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were calculated based on the density of the growth control and were expressed as the lowest concentrations, which showed at least 80% growth reduction of microorganisms, compared to the compound-free growth control. Ciprofloxacin has been tested as positive antibiotic control in concentration range 0.0125 - 8 μg/mL. All samples were tested as 3 independent experiments, each was carried out in triplicate. The mode and median were used for the final value calculation when the triplicate endpoints were within the two- and three-dilution range, respectively. The means of MIC for probiotic and diarrhoeal bacteria (MIC_{PB} and MIC_{DB} respectively) defined for a certain type of strain was used for calculation of selectivity index (SI) between activities against probiotic and diarrheagenic strains. SI was calculated for 6,6′-dihydroxythiobinupharidine, chelerythrine and antibiotic ciprofloxacin using formula: $SI = \log (MIC_{PB}/MIC_{DB})$. Values greater than maximum of tested concentrations were replaced with 1024, 256 and 8 μg/mL for 6,6′-dihydroxythiobinupharidine, chelerythrine and ciprofloxacin. Values of MIC lower than minimum of tested concentrations were replaced with one series lower concentration.

6. Results and discussion

6.1. Growth-inhibitory effect against diarrheal bacteria

As a result of this study, one of six plant extracts and two of five compounds produced certain degree of *in vitro* growth-inhibitory effect against diarrheal bacteria. The data on susceptibility of tested pathogenic bacteria are summarized in Table 2. Among all plant extracts tested, only *Melicope pteleifolia* possessed antibacterial activity against *C. perfringens* with MIC = 512 μg/mL. Although antibacterial activity of the plant has previously been reported (Liao 2012), according to our best knowledge, this is the first report on its inhibitory effect against *C. perfringens*. Extracts from species *Stephania, Tabernaemontana*. and *Helicteres isora* did not show any antibacterial effects against diarrhoeal bacteria tested. In previous studies, an aqueous extract from leaves of *S. japonica* have been reported to have a weak activity against *S. aureus* (MIC = 2800 μg/mL) and *E. faecalis* (MIC = 2800 μg/mL) (Kishnarao & Rajeswari 2023). Besides the fact that different solvent and plant part was used (aqueous extract from leaves), the significantly higher concentrations were tested in previously published study. According to Kokoska et al. (2019) samples with MICs higher than 1000 μg/ml for mixtures and 100 μg/ml for pure compounds should strictly be evaluated as no active and excluded from further experiments. In correspondence with this recommendation, our samples were evaluated as not active. In term of symptomatic treatment, petroleum ether soluble fraction of methanolic extract from *S. japonica* was reported to have antidiarrheal effects on castor oil induced diarrhoea at the dose of 250 and 500 mg/kg body weight of mice (Bokshi et al. 2013). However, another effect than direct antimicrobial action can be responsible for antidiarrheal activity observed in previously published study. Although growth-inhibitory activity of hasubanan-type alkaloid eletefine isolated from aerial plant of *S. longa* has been reported against *S. aureus* (MIC = 50 μg/mL) (Lin et al. 2023), we did not observe any antimicrobial activity for extract of this species. In the disc diffusion method, the latex of *T. divaricata* showed activity against *E. faecalis* at the concentration of ~ 25 000 μg/disk (Raju et al. 2021). Since we tested activity of extract from this species at significantly lower concentration, we did not detect any antibacterial effect. According

to our best knowledge, this is the first study testing antibacterial activity of *T. bovina* against diarrhoeal pathogens. In TM in Southeast Asia, bark, fruit, seed and root juice of *H. isora* are used against diarrhoeal disease (Dayal et al. 2015). Previously tested acetone extract of dried fruits demonstrated antibacterial activities against *B. cereus, E. coli* and *E. faecalis* with MIC 400-800 μg/mL (Shriram et al. 2010). The different part used in this study can be responsible for variances of the results.

Out of five tested alkaloids, chelerythrine and 6,6′-dihydroxythiobinupharidine showed antibacterial activity. Isoquinoline alkaloid chelerythrine inhibited growth of *B. cereus*, *C. perfringens, E. coli*, *L. monocytogenes* and *Y. enterocollica* with MIC values of 8, 64, 128, 32 and 128 μg/mL, respectively. Chelerythrine has previously been reported to be effective against *E. coli* (MIC = 25 μg/mL) and *S. aureus* (MIC = 12.5 μg/mL) (Miao et al. 2010). Although the methodology of the previously mentioned study is not described in substantial details, it varied from methodology of our assay in several parameters such as media for cultivation of bacteria (Baird–Parker agar broth). As far as we know, there are no data on *in vitro* antibacterial activity of chelerythrine against *B. cereus*, *C. perfringens*, *L. monocytogenes* and *Y. enterocollica*, which we have reported in this study. Thioalkaloid 6,6′-dihydroxythiobinupharidine inhibited *B. cereus, C. perfringens, E. faecalis* and *Y. enterocollica* with slightly higher respective MICs of 64, 128, 256 and 256 μg/mL than chelerythrine. According to Okamura et al. (2015) 6,6′-dihydroxythiobinupharidine produced antibacterial effects against various methicillin and norfloxacine resistant strains of *S. aureus* with MIC 1-4 μg/mL as well as against *E. faecalis* (MIC = 4 μg/mL) and *E. faecium* (MIC = 4 μg/mL). Variations in susceptibility of *E. faecium* can be due to the different strains used (ATCC 19434). The fact that *E. faecalis* strain was cultivated in different growth medium (brain heart infusion) than the same strain of this bacterium (grown in Mueller-Hinton broth) may contribute to the significantly different results observed in both studies. This observation can be supported by findings of Nayak et al. (2002) who reported influence of growth media on *E. faecalis* and *E. faecium* resistance to antibiotics. Except the study of Cullen et al (1974), who reported antifungal action of 6,6′-dihydroxythiobinupharidine against *Histoplasma capsulatum* and *Blastomyces dermatitis* (MICs = 100 μg/mL), data on its activity against other microorganisms are missing. In this study, alkaloids dihydrochelerythrine, neferine and yohimbine did not produced antibacterial activity against diarrhoeal bacteria tested (MICs > 128 μg/mL).

Previously, dihydrochelerythrine was reported to produce effects against *E. coli* (MIC = 640 μg/mL) and *S. aureus* (MIC = 320 μg/mL) (Xue et al. 2017), which are higher concentrations than those tested in our study. Alkaloid neferine was reported as one of the active compounds found in seed embryos of *Nelumbo nucifera.* Extract from rhizome of this species is traditionally used as antibacterial and antidiarrheal drug (Bhardwaj & Modi 2015). To the best of our knowledge, this is the first study of testing antibacterial effects of neferine against diarrhoeal bacteria. In this study, the yohimbine did not produce any antibacterial activity. Nevertheless, this compound was reported to inhibit growth of *E. coli* and *E. faecalis* (MIC = 2 μg/mL) (Özçelik et al. 2011). In the above-mentioned study, they tested different strain of *E. coli* (ATCC 25922) and the same strain of *E. faecalis* (ATCC 29212). Similarly, as in case of 6,6´-dihydroxythiobinupharidine, the fact that *E. faecalis* strain was cultivated in different growth medium (brain heart infusion) than the same strain of this bacterium (grown in Mueller-Hinton broth) may contribute to the significantly different results observed in both studies.

Table 2. *In vitro* growth-inhibitory effect of plant extracts and alkaloids against diarrheal bacteria

Antibacterial agent	Bacterial strain/Minimum inhibitory concentration (MIC) in µg/mL									
	BC	CD	CP	EC	EF	EFA	LM	SE	SF	YE
Plant extracts										
Helicteres isora	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
Melicope pteleifolia	>512	>512	**512**	>512	>512	>512	>512	>512	>512	>512
Stephania japonica	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
Stephania longa	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
Tabernaemontana bovina	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
Tabernaemontana divaricata	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512
Compounds										
6,6′-dihydroxythiobinupharidine	**64**	>1024	**128**	>1024	**256**	>1024	>1024	>1024	>1024	**256**
chelerythrine	**8**	>128	**64**	**128**	>128	>128	**32**	>128	>128	**128**
dihydrochelerythrine	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
neferine	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
yohimbine	>128	>128	>128	>128	>128	>128	>128	>128	>128	>128
Positive antibiotic control										
ciprofloxacin	**<0.25**	**8**	**8**	**<0.25**	**2**	**4**	**2**	**<0.25**	**<0.25**	**<0.25**

Footnote: values of MICs produced by tested agents in bold, BC: *Bacillus cereus*, CD: *Clostridium difficile*, CP: *C. perfringens.* EC: *Escherichia coli*, EF: *Enteroccocus faecalis*, EFA: *E. faecium*, LM: *Listeria monocytogenes*, SE: *Salmonella enteridis*, SF: *Shigella flexnerii*, YE: *Yersinia enterocollica.*

6.2. Selectivity against probiotic and diarrheal bacteria

In the Table 3, there are data on susceptibility of probiotic bacteria to active alkaloids chelerythrine, 6,6′-dihydroxythiobinupharidine and antibiotic ciprofloxacin. SI was calculated between probiotic and diarrhoeagenic bacteria. Among all agents tested, ciprofloxacin showed selective antibacterial activities against the pathogens with relatively low activity against probiotic strains (SI = 0.391). Chelerythrine was more toxic to beneficial bacteria (SI = -1.133) than 6,6′-dihydroxythiobinupharidine (SI = -0.614). *L. reuterii*, which was the most resistant, was inhibited with. respective MICs of 256, 32 and >8 μg/mL by 6,6′-dihydroxythiobinupharidine, chelerythrine and ciprofloxacin. 6,6′-Dihydroxythiobinupharidine possessed highest MIC against *B. adolescentis* and *L. reuterii* with respective MIC = 256 and 4 μg/mL. According to our best knowledge, *in vitro* testing of above-mentioned alkaloids against probiotic intestinal bacteria was performed for the first time.

Table 3. *In vitro* growth-inhibitory effect of antibacterial alkaloids against probiotic bacteria and their selectivity against diarrheal bacteria

Antibacterial agent	Bacterial strain/ Minimum inhibitory concentration (μg/mL)					MIC_{DB}	MIC_{PB}	SI
	BA	BAN	LC	LR	LRH			
6,6′-dihydroxythiobinupharidine	256	64	128	256	128	684.8	166.4	-0.614
chelerythrine	8	4	8	32	8	163	12	-1.133
Positive antibiotic control								
ciprofloxacin	0.125	<0.0156	4	>8	8	2.463	4.03	0.391

Footnote: BA - *Bifidobacterium adolescentis,* BAN - *B. animalis,* LC – *Lactobacillus casei,* LR – *L. reuterii,* LRH – *L. rhamnosus,* MIC_{DB} – mean MIC against diarrhoeal bacteria, MIC_{PB} – mean MIC values against beneficial strains, SI – selectivity index between probiotic and diarrheagenic strains

7. Conclusions

In this study, *in vitro* growth-inhibitory effects of plant extracts and alkaloids against intestinal diarrhoea-causing and probiotic bacteria were tested. Compounds and plant species were chosen according to ethnobotanical and chemotaxonomic data and SARs. As a result, chelerythrine and 6,6′-dihydroxythiobinupharidine produced activity against several bacterial strains, whereas *M. pteleifolia* inhibited growth of *C. perfringens* only. Nevertheless, both chelerythrine and 6,6′-dihydroxythiobinupharidine were more toxic to beneficial than to diarrheal bacteria. To the best of our knowledge, the activity of tested plant extracts and alkaloids against diarrhoeal and probiotic bacteria have been tested for the first time. Since none for the samples tested possessed selective antibacterial effect towards diarrhoea-causing and probiotic bacteria comparable with ciprofloxacin, which was assayed as positive antibiotic control with reported selective antibacterial effect, tested alkaloids should not be considered as candidates for a novel selective treatment against bacterial diarrhoea. Nevertheless, certain agents tested (chelerythrine and 6,6'-dihydroxythiobinupharidine) produced *in vitro* growth-inhibitory effect against diarrhoea causing bacteria worth of further investigation, which should be focused on broader spectrum of stains tested, including clinical isolates, as well as on determination of mechanism of their antibacterial action.

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List of the Appendices:

Appendix 1. Photos of plant species collected in Cambodia

F**igure 2.** *Helicteres isora* L. and **Figure 3** *Melicope pteleifolia* (Champ. ex Benth.)

Figure 4 *Stephania japonica* (Thunb.) and **Figure 5** *Stephania longa* Lour.

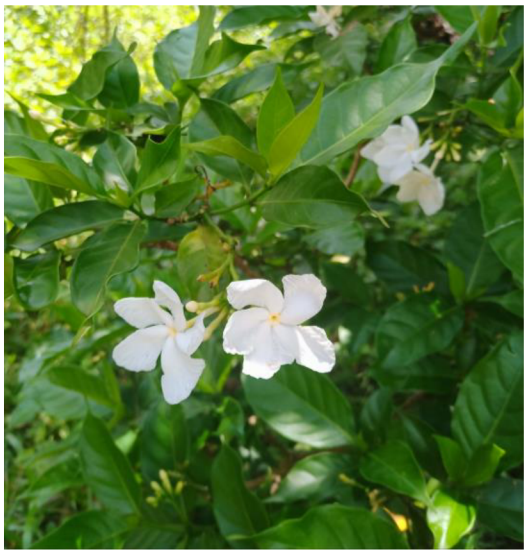

Figure 6 *Tabernaemontana bovina* Lour. and **Figure 7** *Tabernaemontana divaricata* L.

Figure 8 and **Figure 9:** Photos of plant samples collection in Cambodia