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Ant-diarrheal Plants of Central Anatolia: Do They Inhibit Diarrhea-causing Bacteria?

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

ANTI-DIARRHEAL PLANTS OF CENTRAL ANATOLIA:
DO THEY INHIBIT DIARRHEA-CAUSING BACTERIA?

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Janna Leann Rose

2011

To: Dean Kenneth Furton
College of Arts and Sciences

This dissertation, written by Janna Leann Rose, and entitled Anti-Diarrheal Plants of Central Anatolia: Do they Inhibit Diarrhea-causing Bacteria?, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

_____
Suzanne Koptur

_____
Laura Ogden

_____
John Berry

_____
Steven Oberbauer

_____
Bradley Bennett, Major Professor

Date of Defense: June 27, 2011

The dissertation of Janna Leann Rose is approved.

_____
Dean Kenneth Furton
College of Arts and Sciences

_____
Interim Dean Kevin O'Shea
University Graduate School

Florida International University, 2011

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DEDICATION

For my brother, Michael, who never got over his shock and awe of nature.

May I spread that contagious sense of wonder to the people in my life.

ACKNOWLEDGMENTS

I am honored to have worked with many erudite specialists and motivating professors during the course of my dissertation research. In the laboratories at Florida International University, beginning with the Center for Ethnobiology and Natural Products Research (CENaP), I received detailed instruction from my advisor, Brad Bennett, his colleagues Horacio Priestap and Martin Quirke, and my dear graduate school sisters, Cassandra Quave and Angelle Bullard-Roberts.

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ABSTRACT OF THE DISSERTATION

ANTI-DIARRHEAL PLANTS OF CENTRAL ANATOLIA:

DO THEY INHIBIT DIARRHEA-CAUSING BACTERIA?

by

Janna Leann Rose

Florida International University, 2011

Miami, Florida

Professor Bradley Bennett, Major Professor

Infectious diarrhea results in 2 to 5 million deaths worldwide per year, and treatments that are safe, effective, and readily available are under investigation. The field of medicinal ethnobotany focuses on plants that are used by different cultural groups for treating various diseases and evaluates these plants for efficacy and cytotoxicity. In the present study, ethnobotanical research was conducted with Central Anatolian villagers in Turkey. Folk concepts and etiologies surrounding diarrhea were analyzed, as were salient plant-based remedies for diarrhea. Reviewing the literature, 91 plant species were described as anti-diarrheal in all of Turkey. In Central Anatolia, villagers described 35 species. For continued research via bactericidal and bacteriostatic bioassays, 15 plants were selected. Methanolic and aqueous extracts of medicinally used plant parts were evaluated for inhibitory properties against 10 diarrhea-causing bacteria in the first bioassay, and later 21 bacteria in a second assay utilizing spectrophotometry. The cytotoxic properties were also evaluated in an Alamar Blue Assay using HepG-2, PC-3, and SkMEL-5 human cell lines. While several extracts showed bactericidal and bacteriostatic properties, the methanolic extract of *R. canina* galls inhibited the most bacteria at the lowest concentrations. They were not cytotoxic. Thus, *R. canina* methanolic gall extracts were selected for bio-assay guided fractionation. Antibacterial

activity was maintained in the third fraction which was composed of almost pure ellagic acid. The bioassay was repeated with standard ellagic acid, and the polyphenol retained potency in inhibiting multiple bacterial strains. Several other extracts showed promise for safe, effective anti-bacterial remedies for diarrhea.

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DIARRHEAL DISEASE AND THE NEED FOR NEW THERAPIES

INTRODUCTION

Acute gastrointestinal disease has many causes, yet the most intense and deadliest etiologies involve viruses and virulent bacteria and their toxins (Marcos and DuPont, 2007). Four billion people suffer from cases of acute diarrhea each year, leading to 2 million deaths (WHO and UNICEF, 2004). Children under the age of five account for 1.8 million of these deaths (UNICEF and WHO, 2009).

Although diarrheal disease affects billions of people, it is inadequately studied because of its complex etiology, diverse causes, prevalence in emerging countries, and minimal financial incentives for the development of pharmaceutical treatments (Guerrant *et al.*, 2002). Regions of the world with the lowest Human Development Index (a measure of citizens' health, education, and living standards) inversely suffer from the highest diarrhea mortality rates (Sergio and Ponce de Leon, 2009). Consequently, WHO, UNICEF, various NGOs and funding agencies are promoting mucosal immunity research, vaccine exploration, and education campaigns for safe water acquisition, oral rehydration therapy (ORT), and effective diarrhea treatments (UNICEF and WHO, 2009).

Instead of treating diarrheal symptoms as natural pathways for ridding the body of disease, a new paradigm in the biomedical field calls for novel ways to treat the causes of infectious diarrhea. By treating the causes—viruses, bacteria, parasites—the severity of disease in patients is reduced, and the infectious agents cannot spread as rampantly

(Ericsson, 2008; Levine and Svennerholm, 2008). However, with concerns over antibiotic resistance, new ways of reducing the virulence of bacteria without necessarily killing them (which selects for resistance) is of vital importance, especially in species-rich microenvironments such as the human gut. Plants are common sources of therapies for diarrheal diseases, and analyzing their inhibitory properties against diarrhea-causing bacteria is a promising path toward effective treatments.

PLANT-BASED REMEDIES FOR DIARRHEA

Plants have been used as medicines by people for millennia. Today, the majority of people in the world use traditional medicines for their primary course of treatment because biomedical healthcare systems and pharmaceuticals are not available in most places. Thus, to improve health and to instill pride in traditional knowledge systems, several governments (e.g., China, India, and South Africa) are incorporating traditional healthcare practices into their national regimes (UNDP, 2010; UN EcoSoC, 2008; WHO, 2003).

Theories that Support Plant-Based Remedies for Diarrhea

Ethnobotanical theories support the effectiveness of plants for therapeutics, especially when treating gastrointestinal disease. Chemical ecology (Johns, 1990; 1996) and "foods-as-medicines" theories (Etkin, 2000; 2008; Pieroni and Price, 2006) examine the maintenance or restoration of human health through the nutritive and pharmaceutical activities of ingested plants. Foods-as-medicines theories blur the distinction between foods and medicines, as many foods serve therapeutic functions in cultural contexts. Johns (1996) distinguishes foods as substances ingested for energy and nutrients and non-

foods as plants materials that are ingested as teas, gums, dental brushes, or other non-nutritive supplements. Johns (1996) further argues that the physiological benefits of these non-nutritive plants and human behaviors for their ingestion are not yet fully understood.

Stemming from plant-herbivore studies, chemical ecology places humans in the chemical co-evolution of herbivory, or omnivory. As animals, humans have evolved senses to select appropriate plants for ingestion, digestive enzymes to acquire nutrients from plants, and behaviors or detoxifying enzymes to neutralize harmful plant chemicals (Johns, 1996). On the basis of dietary practices and experimentation, humans have evolved to ingest plants and other matter as preventative and therapeutic aids (Hart, 2005). Johns (1999) postulates that "fundamental forms of medicine involve the gastrointestinal tract" because of the immediate cognitive link between ingesting effective medicinal plants and relief of gastrointestinal ailments. This cognizant association could be one reason why all known pharmacopeias of the world possess remedies for gastrointestinal illness. Another reason is that gastrointestinal ailments are ubiquitous. Therefore, the need for treatments is universal. Like the skin and respiratory tract, the gut is continuously open to potential pathogens and toxins, resulting in the need for therapies as people inevitably fall ill (Balick and Cox, 1997).

Possible Mechanisms of Action for Anti-Diarrheal Plants

Plant chemicals may relieve diarrhea in numerous ways. Besides providing nutrients and generally increasing gastrointestinal health, plant chemicals can act and bind with a number of different cells in the GI environment, including human epithelial cells, human immune system cells, commensal flora, or pathogenic bacteria. Much plant-based anti-diarrheal research has analyzed the effects of phytochemicals on intestinal tissues

(e.g., Brijesh, *et al.*, 2006; Grover, *et al.*, 2002; Sagar, *et al.*, 2005; Shaphiullah, *et al.*, 2003; Shilpi, *et al.*, 2006; Teke, *et al.*, 2007). Using rodent models, extracts are evaluated for antispasmodic effects, gut motility suppression, or water and electrolyte reabsorption (e.g., Akindele and Adeyemi, 2006; Mbagwu and Adeyemi, 2008; Sairam, *et al.*, 2003; Thakurta, *et al.*, 2007) with tannins and flavonoids exhibiting promising results for water and electrolyte retention (Palombo, 2006). Astringent and pectin-rich plants often are used to treat diarrheal disease, as are opiates that slow smooth muscle contractions of the intestines (Lewis and Elvin-Lewis, 2003). However, these remedies that suppress intestinal function block the symptoms of diarrhea and not the causes.

Viruses, bacteria, and parasites are the major causes of infectious diarrhea, with bacteria leading to an estimated 2 to 4 billion cases of infectious diarrhea per year and 3 to 5 million deaths (Sanchez and Holmgren, 2005). Phytochemicals inhibit the growth and virulence of diarrhea-causing bacteria in many ways. When bacteria invade the intestines, they follow similar etiologies. The sequence, known as the five stages of pathogenicity (Mitchell, 1998), includes: 1) adherence to host tissue, 2) invasion or control of host tissues, 3) multiplication in host tissues or with nutrients from host tissues, 4) evasion of host defenses, and 5) damage and spread. Phytochemicals can inhibit bacterial growth or virulence at any of these five stages of pathogenicity.

For example, mucilaginous, astringent, and fibrous properties of some plants can mechanically prevent bacterial adhesion to host intestinal cells by direct competition between plant-derived lectins and bacterial membrane glycosides (Coutião Rodriguez, *et al.*, 2001; Rabbani, *et al.*, 2004). Oleanolic acid, ursolic acid, and betulinic acid from ethyl acetate extracts of *Chaenomeles speciosa* (Sweet) Nakai (Rosaceae) prevent the heat-labile enterotoxin of enterotoxigenic *E. coli* from binding to the appropriate host cell

receptor, preventing diarrhea in mice (Chen, *et al.*, 2007). As novel bioassays and technological advances are developed, the precise effects and mechanisms of action for plant-based remedies on bacterial pathogenicity is increasingly understood (Brijesh, *et al.*, 2006).

Diarrhea—Definitions and Types

Diarrhea is defined as an abrupt increase in number of bowel movements to three or more per day, with a concomitant increase in the volume of feces (Wolters, 2010). Some physicians define diarrhea as the passing of 250g of stool or more per day (Goljan, 2010). In high-volume diarrhea, fluids are lost as water molecules osmotically follow secreted ions (resulting from bacterial toxins) or excess salts and fats (from foods) into the lumen. The mucosa is not inflamed, so blood and leukocytes do not appear in the feces. Severe secretory diarrhea is often the result of enterotoxins, such as cholera toxin, or an inflammatory response (Wolters, 2010).

When pathogens such as *Shigella dysenteriae, Campylobacter jejuni,* or *Entamoeba histolytica* invade the intestinal epithelium, the result is a low-volume diarrhea with blood and leukocytes from the inflamed intestinal epithelium. This is known as dysentery or invasive diarrhea (Goljan, 2010). At the cellular level, intestinal mucosal cells respond to bacterial infection with edema of the superficial layers and inflammation of the lamina propria. As epithelial cells lyse, ulcers form and exude neutrophil-filled fluids. With the loss of goblet cells, mucin production halts and the protective mucus layer passes. Systemically, the results are fever, intestinal cramping, and diarrhea that contains sloughed-off bloody tissues, pus, and mucus. This is the most common result of bacterial infection of gut tissues (Fenoglio-Preiser, *et al.*, 1999).

Usually, tissues must be damaged or host defenses disrupted before bacteria can adhere to host intestinal tissues and establish infection. Damage might occur during previous infections, especially from viruses, or from bacterial products, such as toxins or enzymes (Jackson, *et al.* 1998). In the intestines, bacteria must reach epithelial cell surfaces to interact with specific target receptors that trigger cascade responses. Adherence to epithelial cells is more stable than adherence to mucus layers. Plus, adherence to epithelial cells allows for close proximity during toxin release and during nutrient uptake from damaged host cells (Jackson, *et al.* 1998).

Food poisoning is distinct from gastroenteritis. The former occurs when allocthonous (externally pre-made) toxins are ingested, and it is usually less virulent and of a shorter duration than gastroenteritis (Fenoglio-Preiser, *et al.*, 1999). Food poisoning also causes nausea and vomiting, which are associated with the stomach and esophagus. Gastroenteritis leads to additional diarrhea, which is associated with the intestines. Nonetheless, several bacteria that establish infections in the intestines simultaneously produce toxins to assist in colonization.

When large sections of the intestinal mucosa are damaged, it is essential for the tissue to repair itself quickly. If the barrier function of the mucosa is disrupted, invading pathogens can easily travel throughout the human body and establish infections elsewhere. To keep a semblance of a barrier, the extracellular matrix that lies beneath the epithelial tissues regulates the restitution of the mucosa (Fenoglio-Preiser, *et al.*, 1999). Undamaged epithelia cells form sheets that migrate rapidly over any damaged naked basement membrane, initially ignoring any defects. Once the membrane is restituted, cell proliferation is induced by growth factors. In an otherwise healthy individual, the intestinal epithelium can be completely repaired within a few days.

RECENT HIGHLIGHTS IN INFECTIOUS DIARRHEA RESEARCH

Several major discoveries in the past decade have altered research methods in infectious diarrheal diseases and treatments. Four findings relate to the present research and are discussed below. These include 1) the vital role of the human gut flora, or microbiota, in intestinal development and immunity, 2) the diversity and specificity of bacterial secretion and communication systems that lead to colonization and infection of the human gut, 3), the primary immune function of the intestines and connections between inflammation and gastrointestinal health, and 4) the crucial antigen-sampling areas in the intestine, called M cells, that are targeted by virulent bacteria.

Gut Flora and Illness Prevention

Research over the past decade has highlighted the complex interactions between human epithelial tissues and both nonpathogenic and virulent bacteria (Granger, 2001; Relman, 2001; Black and Lanata, 2007). Around 400 bacterial species occur in the healthy flora of the small intestine, including *Lactobacillus* and *Streptococcus* species and Enterobacteriaceae species (Turnbaugh, *et al.*, 2010). There are more bacteria near the distal (colon) end of the small intestine than the proximal (stomach) end, as there is a decrease in acidity further from the stomach. The colon has an even larger, more diverse population of bacteria than the small intestine (Fenoglio-Preiser, *et al.*, 1999). Normally, one third of feces weight is composed of bacteria (Parker, 2007).

The small intestine controls bacterial growth through the effects of gastric, pancreatic and biliary secretions, as well as copious amounts of mucus, constant motility, and very secure intercellular tight junctions in the apical *zona occludens* of the columnar epithelium of the villi (Fenoglio-Preiser, *et al.*, 1999). In the large intestine, bacteria

assist in breaking down fibrous materials and releasing remaining nutrients. Throughout the intestines, bacteria keep the mesenteric immune system in a prepared defensive state by maintaining immune responses, mucus production, and antimicrobial secretions (Clarke and Sperandio, 2005; Stecher and Hardt, 2011).

The normal, commensal flora of the gut wards off virulent bacteria by occupying specialized niches and usurping limited resources. Microenvironments are created by the villi and crypt formations of mucosal tissues, which line the lumen of the small intestine, and by the microvilli on the apical ends of epithelial cells. A dense glycocalyx covers microvilli in the lumen, which is covered by a thick mucus layer and then an unstirred aqueous layer. Hydrogen ions, bicarbonate, mucus, and secretory immunoglobulins are pumped into the unstirred water layer where they interact with bacteria, toxins, and lumen contents (Fenoglio-Preiser, *et al.*, 1999). These layers are barriers between bacteria and intestinal cells, but damaged tissues (from viral infection, toxins) lose the glycocalyx, mucus, and aqueous layers, thereby allowing virulent bacterial to attach and interact with the epithelium.

Phytochemicals alter the microenvironments of the gut. For example, tannic acid chelates iron, preventing iron-seeking bacteria from reaching critical levels of growth (Akiyama, *et al.*, 2001). Polyphenols are capable of altering microenvironments by quenching free radicals, inactivating carcinogens, and stimulating cellular antioxidant defenses while repressing the expression of stress and apoptosis pathways (Vattem and Shetty, 2005). By altering ionic concentrations in the microenvironment, phytochemicals can disrupt communication and signaling pathways between the host and bacteria, thereby preventing or decreasing the severity of infection.

Secretion Systems and Virulence Factors

The gut is the largest and most complex environment in the human body, and commensal and pathogenic bacteria in the gut are able to communicate with each other and with their host through quorum-sensing (QS) mechanisms (Walters and Sperandio 2006). Through the AI-3/epinephrine/norepinephrine signaling cascade, bacteria use human hormones to respond to available nutrients in their environment and to sense stress pathways and immune responses of the host. Through indole and LuxR homologs, bacteria are able to communicate intercellularly and determine the concentrations of related or unrelated species in the environment (Nostro, 2006). If a quota of related species is reached, signal-cascade responses are induced, often leading to the up-regulation of genes that encode virulence factors for motility, adherence, toxins, or secretion systems. In essence, the bacteria become pathogenic (Rendón, et al, 2007).

During the past decade, bacterial virulence factors and their delivery systems have been the focus of intense research (Anderson, 2006). Currently, six secretion systems and twin-arginine translocation proteins have been described in Gram negative bacteria, while the secretory (Sec) pathway has been examined in Gram positive bacteria. Several of the worst bacteria to cause gastrointestinal disease (*E. coli, Shigella, Salmonella,* and *Yersinia* spp.) utilize a Type III Secretion System (T3SS) to insert virulence factors directly into host epithelial cells (Deane, *et al.*, 2006; Hueck, 1998; Stuber, *et al.*, 2003). *Pseudomonas* spp. and other plant pathogens utilize the T3SS and virulence factors similar to human pathogenic bacteria (Deslandes, *et al.* 2003; Hauck, *et al.* 2003).

Generally, virulence factors are genes for production of bacterial proteins, toxins that disrupt host cellular activities, or protein signals that subvert normal host cell activities (Arbeloa, *et al.*, 2011; Dean, *et al.*, 2006). Some injected virulence factors manipulate the host cytoskeleton to surround the bacterial cell in a protective manner,

preventing detection of the bacteria by host immune defenses (García-del Portillo, *et al.*, 2008). Other virulence factors, such as those in enteropathogenic *E. coli, Salmonella, Helicobacter pylori, Staphylococcus*, and *Clostridium difficile*, interact with the host mitochondria (Kozjak-Pavlovic, *et al.*, 2008), thereby altering cellular metabolism in host tissues.

Further elucidation of these and other host-pathogen interactions could bring about effective points in the virulence and infection process where phytochemicals or other compounds could disrupt inter-bacterial communication, quorum sensing, virulence triggering, or secretion system construction and prohibit pathogenicity in gut bacteria (Nostro, 2006). More importantly, disrupting the pathogenicity of bacteria without killing them can prevent selection for resistant strains (Adonizio, *et al.* 2006).

Inflammation and Mucosal Immunity

The GI tract is the interface between the internal body and the external world. As such, it is continuously exposed to pathogens and dangerous chemicals and plays a critical role in generating and maintaining mucosal immunity (Cerf-Bensussan and Gaboriau-Routhiau, 2010). More than any other area of the body, the small intestine produces the most antibodies and contains the most lymphoid cells, resulting in impressive immunological activity (Fenoglio-Preiser, *et al.*, 1999). In the lamina propria of the mucosa, besides the usual connective tissues, blood vessels, nerves, muscles, lacteals, and lymph ducts, there are numerous immunoglobulin-containing plasma cells. Most contain Ig-A, but Ig-M, D, G, and E are also present. Within the villi of the mucosa, macrophages aggregate at the tips and send pseudopods into the epithelial lining to absorb apoptotic cells and monitor for invasion (Fenoglio-Preiser, *et al.*, 1999).

The digestive system plays a critical role in immune response, but also, it is quickly converted to a site of inflammation in response to ingested chemicals, microbes, and allergens. Chronic gastrointestinal diseases such as Guillain-Barré Syndrome are associated with previous *Campylobacter jejuni* infections (Vucic, *et al.* 2009), and bacterial overgrowth of the intestines and deconjugation of bile acids can lead to chronic inflammatory diarrhea (Binder, 2009). Research now shows that bacteria such as *E. coli* are able to induce inflammatory responses through toxins (Jackson, *et al.*, 1998), or read hormonal changes in already inflamed tissues (Sperandio, *et al.*, 2003), which trigger bacterial virulence factors and gut colonization. Other related bacteria most likely carry out similar responses. Anti-inflammatory and anti-oxidant phytochemicals can reduce the inflammation associated with bacterial infections and prevent secondary disease of the GI tract.

M Cells as Targets for Bacterial Invasion

With technological advances in physiology, specific cells in the intestines have been found to play a large role in human immunity and bacterial infection (Neutra, 1998). In Peyer´s patches of the ileum, unique M cells reside in the central dome of the follicle. The M cells have fewer, shorter microvilli called microfolds and less glycocalyx than their surrounding enterocyte neighbors, to which they do not form tight junctions (Owen, 1999). The M cells create thin apical "cytoplasmic rims" that are full of endocytic vesicles. Abundant glycoconjugates are available on the surface of M cells for cationic molecules or lectin-like microbial surface binding (Fenoglio-Preiser, *et al.*, 1999). The one-cell thick cytoplasmic rim of the M cell is all that stands between the lumen contents and lymphocytes. Antigens from the lumen are directly carried over to lymphoid tissues

where they can induce immune responses that can quickly reach systemic proportions, if necessary (Jang, *et al.*, 2004).

Although M cells are a quick, efficient way for antigen processing to occur, they are also the preferred route of entry for some pathogenic microbes. *Listeria, Vibrio, Salmonella, Shigella*, and rotoviruses all use M cells to invade the epithelium of the colon (Corr, *et al.*, 2008). *Yersinia enterocolitica* and *E. coli* strains that express invasin, a virulence factor that binds to host cell integrins, actively seek out and select M cells for invasion (Jang, *et al.*, 2004). Discovering that M cells are sites for specific receptors and trans-cytosis is imperative for developing ways to prevent and treat intestinal bacterial infection.

Combining Intestinal Immunity and Bacterial Virulence Studies in Anti-Diarrheal Plant Research

A combination of techniques that utilize intestinal immunity factors and bacterial virulence factors should lead to more refined bioassays and evaluation procedures for analyzing plant-based remedies. For example, when *E. coli* or *Yersinia* strains express the virulence factor invasin which allows them to seek out M cells in the intestines, plant compounds might be able to disrupt infection by competing for binding sites with invasin or altering the pH of the microenvironment and slightly but significantly denaturing the signaling protein for invasin production. Phytochemicals can inhibit pathogenicity of bacteria at several points along the five stages of pathogenicity.

ANTI-DIARRHEAL PLANTS OF CENTRAL ANATOLIA, TURKEY

During the past 40 years, ethnopharmacologists have been gathering information about medicinal plants in various regions of Turkey. Villagers have related a rich botanical knowledge that varies with the landscape. However, new social reforms in Turkey have made medicines and healthcare relatively accessible and virtually free to all Turkish citizens. As a result, Turkish knowledge systems involving diarrhea and how to treat it are in a state of flux (Yeşilada, *et al.*, 1999). Government hospitals and the World Health Organization (WHO) campaigned amongst Turkish villages to promote the use of hospitals and clinics in cases of childhood diarrhea as opposed to traditional treatments. With educational campaigns for oral-rehydration therapy (ORT) and improved awareness in clean food preparation, childhood cases of diarrhea dropped from more than 710,000 children in 1996 to 188,000 in 2000 (The Ministry of Health of Turkey, 2004).

Decreasing numbers in diarrheal morbidity and mortality are a welcomed accomplishment, and responsible organizations and individuals deserve applause. However, changes in health care practices at the level of the individual most likely lead to changes in the use of home remedies, such as plant-based remedies. Analyzing changes in knowledge of diarrheal disease and anti-diarrheal plants at the local level could lead to a better understanding of how people are relating to their health, their national healthcare system, and the plants in their environments.

Diarrheal Disease in Turkey

Neither precise estimates nor information pertaining to leading causes of diarrhea in Turkey are available. First, most cases of diarrhea are treated at home and are not reported to hospitals. Second, when a patient arrives at a hospital, the first concern is to treat dehydration and other diarrheal symptoms. Treatment of symptoms does not require

knowing the cause. Third, many hospitals or clinics are not equipped to culture and identify pathogenic microbes. Fourth, no mechanism is in place to track or analyze data.

Global reports from the WHO (UNICEF and WHO 2009; Kosek, *et al.*, 2003), the CDC (2008), and other sources (Guerrant, *et al.*, 2002; Zhang, 2008) identify the major causative agents for diarrhea in rural areas on a global scale to be *Campylobacter, Shigella, Salmonella, E. coli, Vibrio, Yersinia, Listeria, Staphylococcus, Clostridium,* and *Klebsiella*. The most prevalent cases of diarrhea that result from food poisioning are caused by *Salmonella enterica*. However, the prevalence of *Campylobacter* is increasing, as are cases of enterohemorrhagic *E.coli* (EHEC). Apart from food poisoning, infective colitis often results from *Yersinia enterocolitica, Campylobacter jejuni*, and *Shigella dysenteriae*. *Klebsiella, Pseudomonas*, and *Chlamydia* are probably the most common causes of chronic colitis (Fenoglio-Preiser, *et al.*, 1999).

Diarrheal disease etiologies in children under the age of five have been examined in hospitals in neighboring Bulgaria (Nedkova, *et al.*, 2008). Preliminary results showed that 60% of pediatric diarrhea patients in hospitals had an identifiable pathogen present in stool samples, some with both viruses and bacteria. Bacterial strains were identified in 20% of the samples, with strains of *Salmonella, E. coli*, *Shigella*, *Campylobacter*, and *Yersinia enterocolitica* present in order from most to least.

More distant geographically, a study in diarrheal etiologies in children in Egypt (El-Mohamady, *et al.*, 2006) identified causative agents in 46% of children sampled, with 17% of all children suffering from rotavirus, 20.7% of children infected with bacteria, 10.7% had *Cryptosporidium*, a Nile parasite, and 6.1% had a combination of causative agents. Bacteria included enterotoxigenic *E. coli* (10.8%), *Campylobacter jejuni* or *C.*

coli (5.6%), *Shigella flexneri, S. dysenteriae,* or *S.boydii* (2%), *Aeromonas hydrophila* (1.1%), *Salmonella* (0.6%), and *Vibrio fluvialis* (0.6%).

Overall, the most likely bacterial causes of diarrheal disease in Turkey are pathogenic *E. coli* strains, *Salmonella*, *Shigella*, *Campylobacter*, *Staphylococcus, Yersinia*, and possibly *Vibrio, Listeria, Aeromonas*, and *Klebsiella.*

The Phytogeography of Turkey

Turkey straddles Europe and Asia, with Africa just across the Mediterranean. Three large ecosystems converge here (Davis, 1965). Sclerophyllous Mediterranean forests cover western mountains in Turkey. In the south, the Syrian-Iraqi desert influences coniferous and deciduous montane forests. To the northeast, the Russo-Iranian mountains are covered in deciduous (at times euxine-colchic) forests (Olson, *et al.*, 2001). At the center of these varied mountain forests, a central plateau, described as semiarid steppe (BSk type in Peel, *et al.*, 2007), gradually inclines in elevation from sea level in the west to 1700 meters in the east.

The plateau has cold, wet winters and dry, hot summers. Annual precipitation averages 400mm in the central plateau and southeast desert borderlands, but along the coasts of Turkey, precipitation doubles (Ergener, 2002). Turkey is a temperate nation with rich biodiversity. Of the estimated 9,000 to 10,000 species of vascular plants in Turkey, a third of the species are endemic (Kaya and Raynal, 2001). More than 400 new Turkish plant species have been described in the past 30 years (Güner *et al.*, 2001), illustrating the need for further research in Turkish botany and pharmacognosy.

Anti-diarrheal Plants of Turkey

Many Turkish medicinal plant species, including anti-diarrheal plants, have been recorded in the past few decades (Ertuğ, 2000; Fujita, *et al.*, 1995; Honda, *et al.*, 1996; Özgökçe and Özçelik, 2004; Sezik, *et al.*, 1991; Sezik, *et al.*, 1992; Sezik, *et al.*, 1997; Sezik, *et al.*, 2001; Tabata, *et al.*, 1994; Tuzlacı and Aymaz, 2001; Tuzlacı and Tolon, 2000; Yeşilada, *et al.*, 1993; Yeşilada, *et al.*, 1995; Yeşilada, *et al.*, 1999). Consistent between reports, plants for gastrointestinal ailments comprise about a third of most regional pharmacopeias in Turkey, including Central Anatolia. Anti-diarrheal plants often belong to the well-represented Lamiaceae, Rosaceae, and Asteraceae, which are congruent with the semiarid steppe climate of Central Anatolia. Species in Lamiaceae comprise 22% of gastrointestinal remedies recorded in all of Turkey, while species in Rosaceae compose 12%, and those in Asteraceae compose 10% (personal data, Chapter II). Previous investigation (summarized in Duke, 1997; Lewis and Elvin-Lewis, 2003) of the medicinal use of these families records anti-oxidant and anesthetic properties in essential oils of Lamiaceae, soothing pectin and astringent tannins in Rosaceae, and anti-inflammatory sesquiterpenes in Asteraceae. However, how these plants might inhibit pathogenic bacteria in the gut is still to be determined.

DISSERTATION RESEARCH

Many studies on the effectiveness of anti-diarrheal plants have examined the effects of plant chemicals on gastrointestinal tissues in mouse or rat models (see Palombo 2006 for a review) or on standard *E. coli* and *Staphylococcus aureus* strains of bacteria (Cowan, 1999). Until the present study, little has been done to screen anti-diarrheal plants for inhibitory properties against diarrhea-causing bacteria such as *Shigella,*

Yersinia, Enterococcus, Klebsiella, Listeria, Vibrio, or *Salmonella* species. In the current study, bioassays using a wide range of diarrhea-causing bacteria were performed to evaluate the antibacterial properties of fifteen anti-diarrheal plants from Central Anatolia, Turkey.

From September 2008 to September 2009, I conducted ethnobotanical research in Central Anatolia with the following four objectives: The first was to determine the specific folk concepts, classifications, and etiologies of diarrheal diseases perceived by Turkish villagers of Central Anatolia and to identify botanical treatments that villagers used. I wanted to understand why villagers used the plants that they did and whether the present use of plants differed from plants reported in previous Turkish ethnobotanical studies. The second objective was to analyze the botanical remedies for bactericidal and bacteriostatic properties, especially in relation to infectious bacteria that cause diarrhea. The third aim was to evaluate the safety of plant-based remedies for human consumption. Plants commonly consumed as foods, spices, or medicines often are assumed to be safe, but cytotoxicity screenings can test dose-dependent toxicity and cellular responses to phytochemicals. Following the first three objectives of the research, my final aim was to isolate and identify the bioactive compound(s) from the extract that inhibited the most bacteria at the lowest concentrations with little human cytotoxicity.

Two hypotheses were formulated to address the first objective of understanding folk concepts of diarrheal disease and its treatment. H1a. Rural inhabitants of Central Anatolia treat diarrhea with plant-based remedies, and H1b. The anti-diarrheal pharmacopeia changes over time. To evaluate the antibacterial properties of the individual botanical treatments, two more hypotheses were devised. H2a. Each Central Anatolian anti-diarrheal plant is bacteriostatic against each bacterial strain. H2b. Each

Central Anatolian anti-diarrheal plant is bactericidal against each bacterial strain. For the third objective involving cytotoxicity, I hypothesized: H3. Each Central Anatolian anti-diarrheal plant shows no toxicity to the cell lines used in the Alamar Blue Cytotoxicity Assay. Finally, I hypothesized: H4. The combined inhibitory and toxicity data support the traditional use of Central Anatolian anti-diarrheal plants.

In chapter II, I address the first objective of my research. As biomedical definitions of diarrhea may differ from the conceptualizations of Turkish villagers, I employed free-listing techniques and saliency rankings to assess disease concepts, causes, and classifications, and I conducted an ethnobotanical survey to determine botanical remedies. The third chapter describes laboratory work conducted in Turkey with collaborators at my sponsoring institution, Gazi University. Bactericidal and bacteriostatic properties of 34 extracts from 15 Turkish plants used to treat diarrhea were determined in assays that included ten strains of diarrhea-causing bacteria. The fourth chapter describes a second antibacterial assay with a panel of twenty-one bacterial strains conducted at Florida International University and a cytotoxicity assay utilizing Alamar Blue to determine the cytotoxicity of the fifteen plants under investigation. The laboratory analysis led to the further investigation of one plant part, the galls from *Rosa canina* L. (Rosaceae), as it inhibited the most bacteria at the lowest minimum inhibitory concentrations (MICs). The fifth chapter details the bioassay-guided fractionation of the methanolic extract from *R. canina* galls and the resulting chemical identification of ellagic acid as the chemical responsible for bacterial inhibition.

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Chapter II

DISEASE CONCEPTS AND ETHNOBOTANY OF DIARRHEAL DISEASES IN CENTRAL ANATOLIA

ABSTRACT

Acute gastrointestinal illness is a common, life-threatening complication for rural villagers in developing countries such as Turkey. My study identifies and describes the classification schemes surrounding acute gastrointestinal illness and its folk etiologies and treatments among Central Anatolian villagers. I conducted informal small group interviews with rural Turkish villagers in the spring of 2009 and used scenarios, recall, free-listing, and ranked saliency techniques to determine the shared knowledge that residents held about diarrheal disease. The perceived causes of illness included germ theory, food preparation, evil eye, hot/cold values, and other folk theories. Common treatments included foods immediately available in the home as well as plants collected nearby. Central Anatolian villagers had a malleable, shifting understanding of gastrointestinal disease, influenced by recent biomedical education campaigns along with traditional village beliefs and practices. These findings are evidence of a culture dealing with change at local, national, and global levels.

Keywords: Ranked Saliency, Acute Gastrointestinal Illness, Diarrhea, Traditional Health, Turkey, Central Anatolia

INTRODUCTION

Diarrheal disease in Turkey, as in most parts of the world, is ever-present and, at times, life-threatening. Worldwide, complications from diarrhea such as dehydration and malnutrition lead to the deaths of an estimated 2 to 5 million people annually, with 1.8 million being children under the age of five (WHO and UNICEF 2004). Morbidity affects even more children, as each child averages 3.3 episodes of diarrhea per year. In Turkey, 10% of infant mortality is caused by diarrhea (Ergener 2002), and uncounted others fall ill yet recover.

Episodes of diarrhea in children lead to vicious cycles of malnutrition, stunted development, and susceptibility to other pathogens (Simeon and Grantham-McGregor 1990). In some regions of Turkey, the Ministry of Health provides medicines, iron supplements, and nutritious foods in school lunches to combat malnutrition and helminthic diarrhea (PCD 2000). Diarrhea and its effects decrease performance measures in students (Ulukanlığıl and Seyrek 2004), which is a national concern.

Around the world, researchers have surveyed plant-based remedies for gastrointestinal disease (e.g., Mexico—Berlin and Berlin 1996; Heinrich, Rimpler, and Barrera 1992, India—Tetali et al. 2009, Nigeria—Agunua et al. 2005, and South Africa—Fawole et al. 2009; Mathabe et al. 2006). Plants are commonly used for treating infectious diarrhea and other gastrointestinal illnesses in these regions, as reliance on biomedical knowledge and pharmaceutical drugs is not always feasible. The same holds true in rural Turkey, although national healthcare clinics are working to provide biomedical services to all regions of the nation. These changes alter medicinal plant use and knowledge.

Health Care in Turkish Villages

In 1920, Turkey was one of the first countries in the world to develop a national health ministry (Aydın 1997). The importance of health was symbolized in bodies of post-WWI Turkish citizens and in the figuratively ailing Ottoman sultanate. As new regimes overthrew the old, a political idea of the *yeni adam* (new man) came into the public imagination (Dole 2004). This idealized man was healthy, hygienic, rational, scientific, and proud to identify himself as a Turk (not an ethnic subgroup). With the 1940's Kemalist nation-building regime came "a society based upon science, rationality and reason…a society free from, by implication, unscientific, and irrational religio-political authority" (Dole 2004:258). The new health initiative emphasized personal hygiene, medical doctors, new pharmaceuticals, and the right to health. However, healthcare clinics took several decades to reach rural Turkish villages and are still sparse in eastern regions.

In many Turkish villages, mothers are usually the first to dispense treatments when illness strikes. They use plants, foods, or other items available around the home. Plant-based remedies for diarrhea are ubiquitous in rural Turkey, with 30% or more of the pharmacopeias consisting of treatments for gastrointestinal ailments (Honda et al. 1996; Yesilada et al. 1995). Mothers react to illnesses using their knowledge of past experiences and socially-derived concepts about the disease. Some of their disease concepts have been influenced by biomedical experiences such as doctor visits or participation in clinical education programs. Women discuss situations with their friends and neighbors and glean information for future ordeals. The resulting social system of health and disease is similar to other cultural systems (religion, politics, economics, or

kinship) in that symbolic meanings, social values, and normative behaviors are used to construct knowledge that is shared by the community (Kleinman 1978).

Disease concepts are folk etiologies that describe why a person becomes ill, the symptoms they experience, and the proper response and treatment (Hughes 1968). Disease concepts involving diarrhea might include biomedical germ theory, Mediterranean cold-hot beliefs, or novel blended theories. People describe symptoms using personally meaningful words and perceptions, and they seek treatments from a range of locally available health specialists including doctors, nurses, herbalists, wise old ladies, imams, and other traditional healers.

In the past, traditional healers were abundant in Turkey. Today, healers are difficult to locate because of political tensions. There are several different specialties, including Koranic healers who are gifted at holy prayers and blessings, either spoken or written (Eyüboğlu 1987). The *cinci hoca* (genie master) or *üfürükçü* (anger man) employ spiritual therapies to resolve problems resulting from *cin* (jinn, genie, or spirit) possession or harassment. Other healers included the *ocaklı* (miracle worker), the *kurşuncu* (lead pourer), the *evliya* (saint-like person), and the *aktar* (herbalist) (Dole 2004). Knowledge of each specialty is passed down along familial lines. Today, a*ktarlar* (herbalists) own family-run shops, which supply herbs, tonics, powders, prepared remedies, pastes, and healing balms. Some shops also import European botanical medicines. However, the national government requires herbal shops to maintain pharmaceutical licenses if they are to administer drugs and give advice on remedies.

Turkish villagers have ambivalent attitudes toward herbal remedies. Since plants bear an association with past medical schools and herbaria, plant-based remedies are tenuously legitimated in Turkey. However, in public attitudes, association with

traditional healing practices antiquates the rigor of herbal remedies and belittles their use and application despite the significant contribution of medicinal plants to people's health. In the 1970s and 1980s, a growing number of pharmaceutical professors in Turkey and Japan began collaborative research on traditional Turkish medicines. Lists of vouchered plants, their preparations, and their treatment procedures were tabulated and published in Turkish and international journals as a way to salvage some of the invaluable Turkish traditional knowledge. For example, Tabata, Honda and Sezik state:

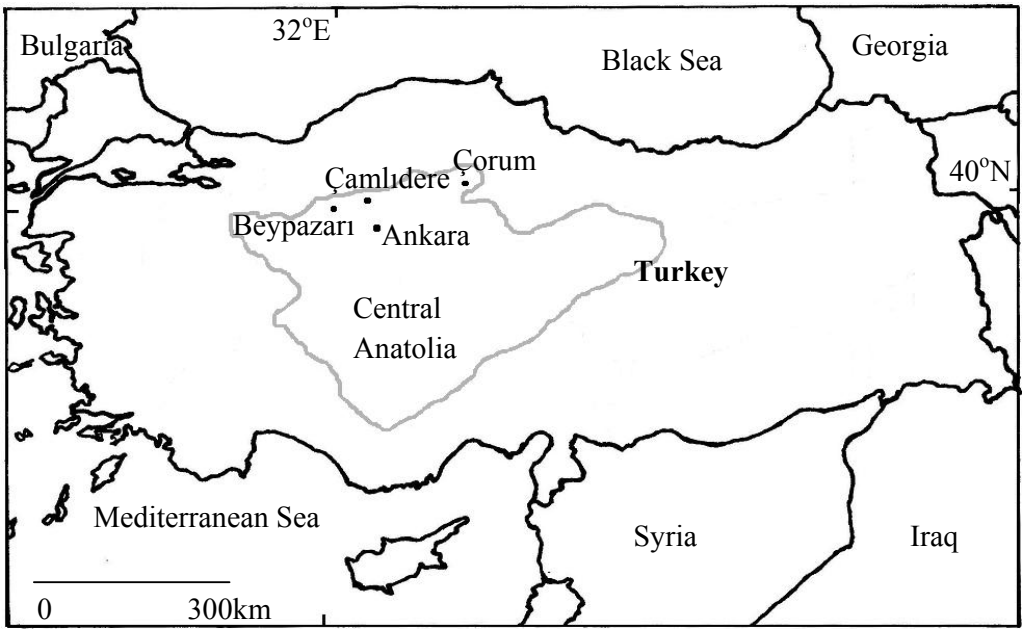
> Villagers are the only source of correct and practical information on folk medicine. Other knowledge is generally based on Islamic medicine or European books of phytotherapy. There have been few studies that disclosed the accumulation of folk medicine in villages, and no analytical investigation on such knowledge has yet been carried out [1988:11].

If traditional Turkish knowledge is considered backward and affiliated with religion, Turkish researchers worry that medicinal plant knowledge, since it is considered traditional, will be left by the wayside as the country's politics focus on western, biomedical healthcare. Today, every Turkish citizen has access to subsidized healthcare and medications. Although numbers of doctors, dentists, and hospitals are still low (one doctor for 852 citizens, one dentist for 3900 citizens, and one hospital bed for 396 citizens), the numbers are steadily growing (Ergener 2002). Ethnopharmacologists are concerned that people will opt for biomedical care and forget age-old, regional plant-based remedies (Yeşilada et al. 1999).

THE FIELD SITE: TURKEY

For centuries, Turkey has been a physical and cultural bridge between Asia and Europe (Figure 2.1). Today, Turkey is continuing to act as a moderator between the East and West (Göle 2008). World leaders view Turkey as a successful, secular country with a growing economy, a young work force, and much to offer in world politics (Dismorr 2008; Fuller 2008).

Figure 2.1 Map of Turkey, Central Anatolia outlined in gray



Turkey is situated on the Eastern edges of the Mediterranean and Aegean Seas and the Southern edge of the Black Sea. Neighboring countries include Greece, Bulgaria, Georgia, Armenia, Iran, Iraq, and Syria. While a small portion of Turkey lies in Europe (the 24,378 km^2 of Thrace), the rest lies within Asia (Anatolia, 790,200 km^2). Turkey ranges from 40°N to 42°N in latitude and 25°E to 45°E in longitude.

Anatolia is a high plateau bounded on all sides by mountain ranges. Elevation ranges from sea level in the west and steadily rises to 1700m in the east. Anatolia is

divided into six phytogeographic regions (Davis 1965), including Central Anatolia. Central Anatolia ranges from 30°E to 38°E longitude and 37°N to 41°N latitude. In the WWF Terrestrial Ecoregion terms, the biome consists of steppe interspersed with mixed conifer and deciduous forests or solely deciduous forests (Olson et al. 2001). According to the Köppen-Geiger climate classification system (Peel, Finlayson and McMahon 2007), the region is predominantly semi-arid steppe (BSk), with a middle latitude temperature range. The climate is hot and dry from July to September. Temperatures average 25°C with rainfall less than 10mm a month (Atalay 2002). Winters are cold and wet, with average temperatures of 0°C from December to February. Rainfall is 40mm per month from October through June (Atalay 2002), with annual precipitation at 414mm in Ankara (Ergener 2002).

Demographics and Livelihoods

Turkey has a population of 72.5 million, according to the 2009 census (Turkish Ministry of the Interior 2010). Three-quarters of the population live in cities (Ergener 2002). Turkey's economy relies on exports of steel and concrete and on agricultural products such as cotton, wool, hazelnuts, apricots, pistachios, citrus, and olives (Ergener 2002). Agriculture is the primary mode of subsistence for Central Anatolian villagers, with approximately 25% of Turkey's wheat grown in this region (Ergener 2002). Other major crops in the plains of Central Anatolia include barley, oats, lentils, and chickpeas. Sheep are the most common domesticated animal in Turkey (60%), but cows and goats are also raised for meat, yoghurt, cheese, and milk (Ergener 2002).

Language

Turkish is the official language of Turkey. It is a Ural-Altaic language (Laffont 1995), related to other Turkic languages of Central Asia (Kyrgyzh, Tatar, Azerbaijani, Turkmen, Uyghur). Common characteristics of Turkic languages are agglutination, vowel harmony, non-gendered nouns, and verbs occurring at the end of sentences. During the Ottoman Empire, Arabic script was used, but a reform in 1928 led to the implementation of a phonetic variety of the Latin alphabet which is used currently. Today, 77 million people speak Turkish worldwide (Ergener 2002).

History

Turkish lands encompass trade routes, water supplies, mountain passes, fertile fields, and natural migratory pathways, over which several peoples and empires have fought throughout the millennia. In 1923, Turkey became a republic. Ever since, Turkey's political drive has pushed blatantly for modernity. Under the guidance of the remarkable Kemal Atatürk, social, political, and economic institutions were completely overhauled in the 1920s, including the health sector (Mango 2002).

Turks first ask about hometowns when meeting a new acquaintance. Rootedness to a fatherland or hometown is very important in Turkish culture (Delaney 1991). Over 30,000 villages dot the countryside of Turkey and each is distinct.

Village Research Sites

My study took place amongst Turkish villagers in Central Anatolia, in the towns and surrounding rural lands of Beypazarı, Çorum, and Çamlıdere (Figure 2.3). Beypazarı ($40^{o}10'$N by $31^{o}55'$E) is a town of about 34,000 people and a province that covers 1814

km^2 and another 16,400 people. Since it is near Ankara (100 km west), the village is in a dynamic relationship with the capital, promoting day trips and shopping sprees for urbanites in the village market. Beypazarı is home to two museums, many silver shops, and is well known for its sweet carrots.

Çorum is a large city of 212,000 people on the northeastern edge of Central Anatolia (40^{o}32'N and 34^{o}57'E). It is 244 km east of Ankara on a high plateau (800m), with mountains blocking northern passage to the Black Sea. The province of Çorum covers 12,800 km^2 with 580,000 people who work in agricultural or industrial sectors. Çorum is famous for its dried chickpeas (*leblebi)*.

Çamlıdere is located 100 km northwest of Ankara (40^{o}29'N and 32^{o}29'E). It is known for its petrified forests, rolling hills, and a beautiful lake that is the source of drinking water for Ankara. About 6300 people live in the town while 15,339 people live within the district of 633 km^2.

Figure 2.3 Survey participants near Çamlıdere

Of importance, Turkish villagers, not city-dwellers, were interviewed for this study. Urban lifestyles are very different from village life, although numerous similarities exist in basic beliefs and cultural nuances. Village life is considered closed, endogamous,

and self-sufficient, and the disjunction between rural and urban Turks is socially marked in daily conversations, TV shows, and even manners of greeting or presenting oneself.

Most villagers in Central Anatolia are Sunni Muslims (with a heritage descended from Fatima, a daughter of Mohamed the Prophet). However, Alevi Muslims are influential in eastern portions of Central Anatolia. The Alevi are a special sect of Muslims who maintain some Turkic Central Asian traditions of mysticism, nomadism and shamanism. Many Sunni do not consider the Alevi to be truly Muslim; this is a source of political tension in Turkish society.

Aims of Study

This study aimed to 1) determine folk concepts and classification of diarrheal diseases in Central Anatolia, 2) identify perceived causes of diarrhea (i.e., folk etiologies), 3) determine botanical treatments for diarrhea, and 4) determine whether the present use of plants differs from plants reported in previous Turkish ethnobotanical studies. Hypotheses included: H1. Rural inhabitants of Central Anatolia treat diarrhea with plant-based remedies, and H2. The Central Anatolian anti-diarrheal pharmacopeia changes over time.

METHODS

Interviews

To gather folk concepts, classifications, and etiologies of diarrheal diseases, I conducted informal, semi-structured interviews with Turkish villagers in Central Anatolia. Fieldwork was undertaken for one year (from September 2008 to 2009) with the assistance of a J. William Fulbright Foreign Scholarship Award and the Turkish

Fulbright Commission. I selected the capital city of Ankara, Turkey, for permanent residence because of its central location and because my sponsoring institution, Gazi University, was situated in Ankara.

Prior informed consent was obtained verbally for each interview session (FIU IRB Approval No. 082508-01). Ethical guidelines of the American Anthropology Association and the Society for Economic Botany were followed during the course of research.

I visited Çorum, Çamlıdere, and Beypazarı multiple times during the Spring (April to June) of 2009. As it was important to establish contacts before visiting a village, these areas were selected because of collaborators (Evrim Özünel and Sema Demir) who were from these areas and because professors (İlhan Gübüz and Ekrem Sezik) in the Pharmacy Department at Gazi University had access from previous studies.

In the villages and surrounding rural areas, I first visited the market or the mayor's home to drink tea, discuss research goals, and conduct an interview with the family. Using snowball sampling techniques, other homes in the area or tables in the markets were visited. Culturally, it was difficult to interview people of the opposite sex and to interview people individually. Thus, most interviews were held in small groups of two to seven women.

Interviews were conducted in Turkish. Collaborators were present to assist in the flow of conversation and later interpretation. Semi-structured questionnaires (Appendix 1 and 2) were translated, checked for cultural relevancy, and back-translated with the assistance of Turkish collaborators. Questions were asked about diarrhea, illness scenarios (e.g., If your child has diarrhea, what do you do?) and plant-based treatments.

Free-listing exercises and saliency ranking techniques (Alexiades 1996, Nolan 2001, Quinlan 2010) were performed with every group interview. While saliency is

normally calculated for individual informants, the formula was modified to analyze a small group's comments and listings. The formula for saliency ranking is

$$s_i = \frac{\text{inverse rank order of item}}{\text{N of items listed}}$$

Each group's free-listed items were ranked in order and then inversely valuated according to the total number of items listed for that question or concept. Then the composite saliency (S) for every listed item from all groups was calculated to determine the intracultural saliency of each item.

$$\text{Composite S} = \frac{\sum s_i}{N_i}$$

Items with the highest composite saliency values are those that villagers are most likely to think about first. Free listing exercises were conducted to discover the most prominent names for diarrhea, the types and classification of diarrhea, culturally relevant causes of illness, and preferences for botanical treatments.

Demographics

The sex, age, marital status, parental status, and grandparental status were collected from one person in each group interview. The person's time of residence in the village was recorded along with other locations of residence and time spent elsewhere. Class and ethnicity were not recorded, as these categories are not locally relevant.

Literature Review of Turkish Ethnobotanical Resources

To better understand folk illnesses and plant-based remedies, I conducted a literature review of Turkish resources. While several articles involving Turkish medicinal plants are available in international journals, I reviewed reports that were difficult to

acquire while in residence at Gazi University. I only included publications that reported vouchers and expert botanical identifications (Ertuğ, 2000; Fujita, et al., 1995; Honda, et al., 1996; Özgökçe and Özçelik, 2004; Sezik, et al., 1991; Sezik, et al., 1992; Sezik, et al., 1997; Sezik, et al., 2001; Simsek, et al., 2004; Tabata, et al., 1994; Tuzlacı and Aymaz, 2001; Tuzlacı and Tolon, 2000; Yeşilada, et al., 1993; Yeşilada, et al., 1995; Yeşilada, et al., 1999).

Botanical Specimens

Following interviews, medicinal plants were gathered from fields, gardens, and roadsides. I recorded GPS coordinates and habitat information for each plant specimen. For taxonomic identification, I used the 11 volumes of the *Flora of Turkey* (Davis 1965, Güner, et al. 2001) along with the taxonomic expertise of Zeki Aytaç and Ufuk Özbek from the Botany Department at Gazi University. The Angiosperm Phylogeny Group (APG III 2009, Stevens 2001 onwards) was used for familial nomenclature. I prepared and deposited 43 voucher specimens in GAZI.

RESULTS

Interviews

Over 130 participants (Table 2.1) were interviewed in 46 small-group sessions. Key informants and collaborators in research included two doctoral graduate students (Sema Demir and Evrim Özçelik) from Gazi University's Folklore Department.

Table 2.1 Demographic results for group interview participants

Age	Gender	Marital Status	Parental Status	Residency
Range: 26 to 72	Female 87%	Married 87%	Parent 91%	Local 52%
		Widowed 7%		
Ave = 46.3	Male 13%	Single 2%	Grand-parent 37%	15+ yrs Resident 91%
		No Reply 4%		

From the ranking and free listing exercises, several ideas and concepts about diarrhea were obtained. The common name for diarrhea (*ishal*) did not differentiate between desired diarrhea, as in the case of constipation, and undesirable diarrhea, as in illness. Therefore, I learned to use alternative, locally appropriate words for undesired diarrhea (Table 2.2). These referred to *amel* (the deed) or *bozulma* (broken) intestines. Another name was descriptive of the sound of diarrhea (*Cır cır*, pronounced jur jur). Inappropriate names were not gathered systematically.

Table 2.2 Common names for diarrhea

Turkish Names for Diarrhea	English Translation
İshal	Diarrhea
Amel	The Deed
Cır cır	Onomatopoeia
Bozulma	Broken
Ötürük	After constipation -- desirable

When people were asked to free list the types of diarrhea they knew, their responses overwhelmingly ranked poisonous diarrhea (*zehirli ishal*) first (Table 2.3). Participants animatedly discussed how horrible and deadly it was. Symptoms included black or green diarrhea, with occasional vomiting or fever. Even though many cases of

diarrhea were normal and not poisonous, the poisonous type was feared because of the unknown source of poison, severity of disease, and high rate of mortality.

Table 2.3 Ranked salient types of diarrheal disease

Turkish Terms	Translated Terms	Saliency	Symptoms
zehirli ishal	Poison	1.647	Green or black, deadly, sometimes fever or vomiting
kanlı olur	Bloody	0.677	Red blood in stool, weakness
Sulu	Watery	0.147	High volume, colorless
Normal	Normal	0.118	Cramping, powerful urge

When asked about causes of diarrheal disease, villagers mentioned 22 possibilities. There was high saliency in the first seven causes mentioned (Table 2.4) and less consensus in the remainder. Interviewees mentioned being cold or having cold feet as the primary cause of diarrhea and illness in general. Causes associated with germ theory were highly ranked and included concepts such as dirty hands when preparing food, microbes in food or water, food that had spoiled, and dirty water. Sun poisoning and eating oily foods were ranked next in saliency for causes of diarrhea.

Table 2.4 Ranked causes of diarrhea (22 total)

Rank	Top 10 Causes	Saliency
1	Being cold, feet are cold	0.472
2	Dirty food preparation	0.306
3	Microbes	0.278
4	Spoiled food	0.250
5	Drink unhealthy water	0.222
6	Heat, sun poisoning	0.194
7	Eat oily foods	0.181
8	Eat Dried Apricots, Prunes	0.069
9	Evil Eye	0.042
9	Sit in dirt	0.042
9	Eat green chickpeas	0.042

9	Broken inside	0.042
9	From things we eat	0.042
9	Eat junk food—nuts, chocolate	0.042
9	Don't eat bread with sour things	0.042
9	When salt and water lost from body	0.042
10	Sugar overdose in children	0.028
10	Teething babies	0.028
10	Drink tea with milk	0.028
10	Eat dried beans	0.028
11	Eat too much *pestili*, or dried fruit roll	0.014
11	Eat unhealthy foods	0.014

The free-listing of salient treatments of diarrhea identified 44 plant-based remedies, including foods and wild plants (Table 2.5). The differentiation between foods and wild plants was acquired from interviewees. Food plants were called by their specific household names (lemons were *limonlar*), but wild plants were called *yabanıl* (wild, uncultivated) or deemed *yayla'dan* (from the countryside, summer grazing lands, or nearby fields). Therefore, the translated term wild was used to indicate uncultivated plants collected outside in a variety of locations.

Many women listed food items that were readily available in their homes for treating diarrhea. Half the groups only listed food items (e.g., potatoes, rice, coffee), and 38% of the groups mentioned both foods and wild plants used to treat diarrhea. Of these, 58% preferred to use wild plants instead of foods when treating diarrhea, and the remaining 42% preferred food-based treatments. Only 8% of the interview groups listed solely wild plants for treatments, and 4% went straight to a doctor without using any plant-based remedies. All participants said that after a day or two with no improvement, they would take a child suffering from diarrhea to a nearby clinic or doctor.

Table 2.5 Ranked treatments for diarrhea (44 total)

Rank	Treatments	Saliency
1	Boiled Potato	0.677
2	Coffee Powder (with lemon, salt)	0.647
3	*Pirinç*, or watery rice soup	0.588
4	Cola syrup or flat soda	0.459
5	Yogurt, *Torba yoğurt*, *Ayran*	0.406
6	*Övez* or *Yuvaz*, *Sorbus domestica* fruits	0.288
7	Dry tea (with honey)	0.282
8	Salt-Sugar-Water Solution (ORT)	0.247
9	Lemon wedge with salt	0.241
10	Mint-Honey-Lemon Water	0.212
11	*İğde, Elaeagnus angustifolia*	0.200
12	*Muz*, banana	0.171
13	*Pestil*, dried *Cornus mas* fruit paste	0.159
13	Lemon and vinegar mix	0.159
14	Water, ample amounts	0.147
15	*Leblebi*, or dry roasted chickpeas	0.130
16	Aspirin, with cola or coffee	0.124
17	Lemon, eaten	0.118
18	*Maya*, yeast, drunk or eaten	0.106
19	*Şeftali*, peach juice or peel	0.100
20	*Ihlamur*, *Tilia* spp.	0.094
20	*Karaçalı*, *Ulex europaeus*	0.094
21	*Yavşan otu*, *Artemisia vulgaris*	0.077
22	Garlic cloves, swallowed	0.047
23	*Oğul otu*, *Melissa officinalis*	0.041
23	*Papatya*, chamomile varieties	0.041
23	Apple, peeled and boiled	0.041
23	*Nışasta*, starch, drunk	0.041
23	*Acı gevendikeni*, *Astragalus* spp.	0.041
24	*Isırgan*, *Urtica dioica*	0.035
24	*Parı yavşan*, *Teucrium polium*	0.035
24	*Kekik*, oregano or thyme	0.035
24	Hot water from local natural spring	0.035
24	Breast milk for baby	0.035
24	*Un*, flour, drunk	0.035
24	Honey, in cold water	0.035
24	Warm feet	0.035
24	Salt in bag, on stomach	0.035
24	Butter biscuits	0.035
24	Salted crackers	0.035
24	Dried apricots	0.035
24	Coffee *lokum*, Turkish delight	0.035
24	Bread, with butter and fruit jam	0.035

24	Apple vinegar	0.035

Women rarely gave coffee to their children during diarrheal episodes, but they often treated themselves or their spouses with a spoonful of coffee powder, usually mixed with lemon and salt. A similar mixture of powdered tea leaves and honey was used, as were mixtures of lemon juice and salts or vinegar and lemons.

The ten most salient treatments for diarrhea included commonly available household food items. However, almost every interview discussed one or more plants collected in fields or along roadways. While not sown outright, a few plants such as *ısırgan* (stinging nettle), *papatya* (chamomile), and *kekik* (oregano or thyme) were fostered to grow in their places of germination, including backyard gardens and orchards. A list of plants collected from the outdoors is available in Table 2.6. Herbs were also purchased in markets or as spices in grocery stores, but these were not collected as voucher specimens or bulk samples.

Table 2.6 Plants collected outdoors, ranked by saliency, 13 of 21 plants

Turkish Name	**English Name**	**Latin Name**	**Family**
övez	service tree	*Sorbus domestica* L.	Rosaceae
nane	mint	*Mentha longifolia* (L.) Huds.	Lamiaceae
iğde	oleaster	*Elaeagnus angustifolia* L.	Elaeagnaceae
pestil	cornelian cherry	*Cornus mas* L.	Cornaceae
ıhlamur	linden tree	*Tilia cordata* Mill., *T. tomentosa* Moench	Malvaceae
karaçalı	gorse, furze	*Ulex europaeus* L.	Fabaceae
yavşan otu	mugwort	*Artemisia vulgaris* L.	Asteraceae
oğul otu	lemon balm	*Melissa officinalis* L.	Lamiaceae
papatya	chamomile	*Anthemis tinctoria* L., *A. coelopoda* Boiss.	Asteraceae
gevendikeni	vetch	*Astragalus* spp.	Fabaceae
par yavşanı	germander	*Teucrium polium* L.	Lamiaceae
kekik	oregano, thyme	*Origanum* spp., *Thymus zygoides* Griseb.	Lamiaceae
ısırgan	stinging nettle	*Urtica dioica* L.	Urticaceae

All the plants were prepared as infusions, decoctions or teas. Fruits such as *övez* and *iğde* were eaten fresh or boiled and drunk. *Pestil* was boiled, mashed, and dried as a fruit roll-up for storage until winter months. It was either eaten or melted in hot water as a tea. All of the plants were collected and available for sale in local markets in dried forms, just as people were able to collect the plants for themselves from the nearby landscape.

Literature Review of Plants

The literature review identified 155 different uses of 91 species of plants from 33 families to treat gastrointestinal illnesses. Since each literary source had its own method of categorizing illnesses or use patterns, categories such as diarrhea, colitis, abdominal pain, intestinal pain, abdominal cramping, stomachache, stomach upset, gastrointestinal ailments, and nausea were tabulated. Of these citations, 77 uses of 53 species from 25 plant families directly mentioned diarrhea as an illness and use category (Appendix 3).

Botanical Specimens

Forty-three vouchers of 35 plant species from 20 families were collected during fieldwork with informants. Voucher specimens were collected during flowering season; fruits were collected when available.

DISCUSSION

Folk Concepts of Diarrheal Diseases

Types of Diarrhea

The most salient type of diarrhea among the villagers was the green or black poisonous kind. The symptoms of poisonous diarrhea are similar to those described in biomedical text books as bacteria-induced diarrhea, caused by small intestinal infections and toxins from *Salmonella*, *E. coli, Shigella, Yersinia* and *Campylobacter* species (Fenoglio-Preiser et al. 1999). The green color occurs because of un-processed green bile secretions from the upper small intestines which normally turn brown during transit. The black color results from blood that is acidified, as in the acidic environment of the upper small intestine (Navaneethan and Giannella 2011).

Bloody diarrhea can be linked to bacterial infection of the intestines, as well. Most cases of bacillary dysentery, caused by *Shigella* species, lead to blood in the stool as bacteria lyse and kill the epithelial lining of the intestines (Fernandez and Sansonetti 2003). Watery diarrhea can be caused by too many salts or fats in the colon (from a big meal). A more serious form of watery diarrhea is caused by bacterial infection and toxin release, such as toxins produced by *E. coli*, *Vibrio cholerae*, *V. parahaemolyticus,* or *Campylobacter jejuni*. In neighboring Bulgaria, the most common causes of diarrhea in children are rotaviruses (31%), followed by bacteria (20%), with *Salmonella, E. coli, Shigella, Campylobacter,* and *Yersinia* species comprising the most cases, in that order (Nedkova et al. 2008). Considering that 20% of global deaths of children under the age of five (Zhang 2008), and 10% of infant deaths in Turkey (Ergener 2002) are caused by infectious diarrhea, the fear of poisonous diarrhea in villages is understandable.

Causes of Diarrhea

The most salient cause of diarrhea for village women was cold feet. Throughout Turkey, wearing shoes and slippers is believed to ward off illness, as is keeping the body warm in general. Guests are given slippers and tea upon arrival, and outdoor shoes are not permitted inside the living area of a home. When asked about the use of slippers, some people stated that a lot of heat is lost by the feet and head, so they must be covered. Women also mentioned the need to protect themselves from cold winds, some with names, which blow over the Central Anatolian plains and bear good or bad influences into the region.

The second through fifth most salient causes of diarrhea were related to biomedical germ theory. Women often mentioned and quoted national healthcare education campaigns in which they were taught healthy hand washing techniques and food storage practices. Bad water was also discussed frequently as dirty water opposes religious notions of purity. Some villages were well-known for their healing waters, with springs linked to activities of past saints. In these locations, villagers maintained large waterworks for people (and animals) to stop and drink. Healthy water was described as a critical resource in both mundane and religious matters.

Sun poisoning or sunstroke was discussed as a cause of diarrhea, and several plants in Turkish pharmacopeias are used to treat it. Since most villagers have agricultural livelihoods, sunstroke affects many people. Most field workers are women, and women remain covered for cultural reasons and to prevent sunburn and chapped skin from winds. The month of fasting, *Ramadan*, proves a difficult period for laborers when it falls during the agricultural season, as sunstroke becomes more prevalent.

Other causes of diarrhea mentioned by villagers included consumption of certain foods or practices such as sitting in the dirt or not drinking hot tea after a big meal. Evil eye was also cited as a cause of diarrhea and a source of a numerous afflictions in Turkish culture. Glass beads that resemble blue eyes (*mavi boncuklar* or blue beads) are frequently worn as protection from jealous glances and evil stares. The bead is thought to take the cursing glance in place of the person wearing it. If one is caught without protection, the evil eye can bring on difficulties such as diarrhea.

Treatments for diarrhea

Since many cases of diarrhea are self-limiting, treatments such as bland foods usually are sufficient in sustaining the sufferer through a bout. Villagers commonly gave a boiled potato to a person with diarrhea. The potato was thought to bind up contents of the intestines, making them less watery and adding bulk. In biomedical reports, small quantities of starchy or fibrous foods relieve cramping during diarrhea (Lewis and Elvin-Lewis 2003). The adult treatments involving coffee grounds, lemons, salts, vinegar, or dried tea leaves probably lead to a drastic increase in acidity of the GI tract and possibly act as a diuretic, an intestinal muscle stimulant, or denature bacterial toxins to end an infection in the gut.

A watery rice soup preparation was described in health education campaigns, as were sugar-salt solutions. Women explained that these treatments were often used to treat children's diarrhea, with good results. Yoghurt, a staple food in the Turkish diet, was used to settle upset stomachs as well as diarrhea. Varieties of mints were found in the fields or roadsides and were often collected for teas or infusions. People ate 10-15 *iğde* (*Elaeagnus angustifolia*) fruits to treat diarrhea and stored them for use in winter.

Comparing the results of the ethnobotanical literature review with the plants listed by villagers in this study, seven plants were mentioned (*Camellia sinensis, Cicer arietinum, Coffea arabica, Cornus mas, Sorbus domestica, Teucrium polium*, and *Urtica dioica*). The literature review covered all of Turkey, while the present research covered only Central Anatolia. Turkish phytogeography is diverse, and several plant species do not occur in multiple regions. Only two previous studies covered the pharmacopeias of Central Anatolia (Honda, et al., 1996, Sezik, et al. 2001) and of Ankara (Simsek, et al. 2004). Besides the seven plants mentioned above, these sources listed eight other botanical treatments for diarrhea: *Rhus coriaria* L., *Viscum album* L., *Rumex patientia* L., *Punica granatum* L., *Cydonia oblonga* L., *Pyrus ealaeagnifolia* L., *Rosa canina* L, and *Lycopersicon esculentum* Mill.

While previous researchers collected entire repertoires of medicinal plants for all diseases, I asked specifically about diarrheal diseases and related treatments. Also, I geared my questions to collect information on all types of botanical remedies, including foods and wild plants, as I wanted to know exactly how local women treated diarrhea. Other ethnobotanical studies might have focused more on wild plants. Nonetheless, plants used as treatments today are from similar families as those reported in the literature, although over half of the published plants used in the past were not mentioned in the recent village interviews.

Women who collected plants tended to be older (40+ years). They viewed a collecting trip as a wonderful way to spend a spring or autumn day outdoors, when plants would be ripe for harvest. Women went out with friends and children to locations where specific plants and trees were known to grow. Locales included narrow forest plots that lined fields outside the villages, fencerows, or road edges along expansive non-

agricultural plains. Some women also nurtured opportunistic plants in their gardens, orchards, or backyards. For the town markets, the men of Beypazarı worked together to collect wild plants while the women sat in the market, tending booths.

When I asked about specialists who might know of herbal remedies, interviewees responded that such specialists existed in the past but died 10 to 15 years ago. If they wanted specialist knowledge now, they consulted popular books on herbal remedies, of which there were many for purchase at bookstores, or they might look on-line at Turkish herbal websites. Many women noted that it was just as easy to go to the clinic or call a doctor as to find an herbalist nearby. Younger women in group discussions knew fewer plant-based remedies (wild or food plants) and often argued within groups that water, oral rehydration therapy (ORT), and doctor visits were the best ways to treat diarrhea, especially for children. This aligned with WHO and government health campaign information.

Turkish women are dealing with changes in healthcare regimes at the local, national, and even global levels. Women who attend health education campaigns gain knowledge that shapes their perceptions of disease and, by extension, their practices in treating it. With more local clinics available, women are able to visit doctors more easily. Nationally, the government has worked for years to instill in its citizens the need for better healthcare, which translates directly into biomedical healthcare. Doctors and staff with biomedical training are esteemed and their advice is highly regarded by villagers. Turkey has been working to expand its healthcare system for decades, to improve its citizens' health and also to meet global EU, IMF, and other multilateral corporations' expectations. Additionally, diarrhea was one of the leading topics for social health reform of the UN and UNICEF in the 1990s. As such, diarrheal disease and its management

have received considerable attention in countries such as Turkey. The effects of these global, multilateral forces can be seen in local village women's changing ethnobotanical knowledge and practices.

CONCLUSIONS

Central Anatolian villagers have a malleable, shifting understanding of gastrointestinal disease, influenced by recent biomedical education campaigns along with traditional village beliefs and practices. With the implementation and growth of federal health care programs and UN health education campaigns in Turkey, mothers have the option, and are encouraged, to seek medical care in cases of acute diarrhea instead of using home remedies. These and other factors are altering the folk classifications for gastrointestinal diseases as well as the ethnobotanical knowledge used to treat diarrhea in rural communities.

Currently, villagers include biomedical paradigms such as microbe theory, food preparation, and dirty water along with folk theories involving the evil eye or hot/cold values as salient causes of diarrheal illness. Common treatments include foods immediately available in the home as well as plants collected nearby. Therapies may also involve prayers or blessings from powerful Koranic scriptures.

As is common in ethnobotanical studies, older participants (>40yrs) knew more treatments for diarrhea in general, including more wild (*yabanıl*) plant-based remedies. They readily offered a variety of treatments in group discussions. Several had sheets of *pestil* ready for use, and others sold plants as herbs and spices in the market. This difference in age might be explained by older mothers having greater mobility, as their children are older and able to collect plants for or with them. As older women with more

children, they also have a more established position in the family, which enables them to leave the home more freely.

A minority of younger participants opined that no home treatment of any sort except water or ORT should be used in the case of diarrhea, especially for children. They preferred to immediately go to the clinic and seek professional help. However, most women (96%) agreed that some plant-based remedy, whether food or not, should be used to treat diarrhea. Women also said that they discussed these topics with their peers and family members, and they saw infomercials during their favorite television shows advocating for healthy Turkish children. Younger women have grown up going to school, seeing the doctor, watching television, and generally accruing more experience with foreign commodities and livelihoods. The older women grew up working more in the fields. They were more intimately exposed to the plants lining the roadways and field edges and have a different relationship with the land in their village.

Today, traditional knowledge in Turkey is treated ambivalently. Sometimes traditions are berated for being backward. At other times, traditions are deemed the heritage and birthright of every Turk. Often, anthropologists define traditional knowledge as a knowledge system preceding colonization or as a social perception of old-versus-new ways of life. Turkish definitions of traditions involve constant use and change. Turkish traditions are equated to air (Glassie 1993). Everyone in a close-knit group has to breathe, and eventually, they exchange air by inhaling and exhaling. People take what they need from the air (ideas, nourishment, or artistic styles), change it, and release it. Understanding Turkish perceptions of traditional knowledge is important for better understanding the acceptance and expectations of constantly changing trends and trajectories in local knowledge, including medicinal plant knowledge. Change is normal,

unsurprising, and even anticipated in this context, albeit constrained somewhat by cultural values.

When considering diarrheal disease concepts, Turkish villagers act and change at a local or individual level while they are informed by national and global media to treat diarrhea in specific ways. While some women still know of wild or food medicinal plants used to treat diarrhea, the actual use of plants and transmittance of this knowledge appears to be waning as the national healthcare system becomes entrenched in the daily lives of Turkish villagers. Understanding this multi-level process is a first step in addressing the future needs of Turkish villagers and re-setting ethnobotanical theory to address these concerns.

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Chapter III

COMPARATIVE *IN-VITRO* BACTERICIDAL AND BACTERIOSTATIC ACTIVITY OF ANTI-DIARRHEAL PLANTS OF CENTRAL ANATOLIA

ABSTRACT

In vitro bactericidal and bacteriostatic properties of methanol and aqueous extracts of 15 anti-diarrheal plants used in Central Anatolia were evaluated against 10 diarrhea-causing bacteria. Gram negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enteriditis*, *Shigella dysenteriae,* and *Vibrio cholerae,* as well as Gram positive *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus cereus*, and *B. subtilis* were used in a microdilution broth bioassay. Clinical and Laboratory Standards Institute (CLSI) protocols were used to determine percent inhibition and minimum inhibition concentrations (MICs). Minimum bacteriostatic inhibition concentrations (MBSs) and minimum bactericidal concentrations (MBCs) were determined as μg/mL. The MBCs were defined as the concentration at which bacteria ceased to grow and were no longer viable. The MBSs were further counted as colony forming units (CFU)/mL and were defined as extract concentrations that disrupted macroscopic growth but did not kill the bacteria. Ninety-five MICs were 64μg/mL or less, with 21 MICs at 32μg/mL or less. *Vibrio cholerae, Staphylococcus aureus,* and *Enterococcus faecalis* showed the most susceptibility to plant extracts, particularly those of *Rosa canina*, *Cydonia oblonga*, *Hypericum perforatum*, *Rhus coriaria*, and *Rumex patientia*. In summary, nine anti-diarrheal plants from Central Anatolia had bacteriostatic activity against bacterial strains that cause acute gastrointestinal illness.

INTRODUCTION

Acute gastrointestinal disease (or diarrhea) is a significant threat to children under the age of five throughout the world. At least 2 million people die each year from diarrhea, and 90% of these deaths are children under the age of five (WHO 2009). Finding a prevention or treatment that is accessible and affordable to rural populations could alleviate some of these deaths.

Acute gastrointestinal disease is a significant problem for children in rural and peri-urban sites in Turkey (The Ministry of Health of Turkey 2004). Turkish WHO and government organizations promote Oral Rehydration Therapy (ORT) to treat diarrhea. While ORT decreases mortality, it alleviates symptoms of diarrhea and not their causes. Ineffectual therapies lead to chronic malnutrition, lowered school performance, and death (Ulukanlığıl and Seyrek, 2004). Moreover, ORT does not decrease morbidity, with children averaging 3.2 cases of diarrhea per year (Parashar, et al. 2003).

Diarrhea often is treated in homes with infusions or decoctions of wild or cultivated plants. A review of the Turkish ethnobotanical literature identified 91 species of plants from 35 families used to treat diarrhea (Chapter II). The present study identified 35 anti-diarrheal plant species from 20 families that are used in Central Anatolia, a semiarid steppe region in the center of the country. Of these species, 15 were evaluated for their anti-bacterial properties. These were selected on the basis of their availability, their flowering and fruiting seasons, and whether previous research had been conducted on their antibacterial properties. Because these plants were ingested regularly by locals, their toxicity was thought to be negligible by ethnopharmacologists (Sezik, et al., 2001). Later evaluation with an Alamar Blue Cytotoxicity Assay (Chapter IV) tested these assumptions.

Pathogens that commonly cause diarrhea in Turkey are not databased, but 10 used in this study were chosen because they are: 1) common gut flora that can become pathogenic under certain conditions (*E.coli*, *Klebsiella pneumoniae*), 2) severe infectious agents (*Vibrio cholerae, Shigella dysenteriae*) which cause epidemics with high mortality rates, 3) difficult pathogens to treat because of innate and acquired antibiotic resistance (*Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa*), 4) common causes of food poisoning (*Salmonella enterica, Bacillus cereus*), and 5) common soil pathogens (*B. subtilis*). All are capable of toxifying or infecting the GI tract, albeit in different ways, and bringing on diarrhea and other complications.

Here, the antibacterial properties of each plant extract are evaluated against 10 diarrhea-causing bacteria. The primary questions addressed are: Do crude extracts from anti-diarrheal plants of Turkish villages exhibit bacteriostatic activity against bacteria strains that commonly cause diarrhea? Do crude extracts from anti-diarrheal plants of Turkish villages exhibit bactericidal activity against bacteria strains that commonly cause diarrhea? Does the activity of methanolic extracts differ from that of aqueous extracts (that are analogous to traditional preparations)? I tested the following hypotheses: H1. each Central Anatolian anti-diarrheal plant is bacteriostatic against each bacterial strain, and H2. each Central Anatolian anti-diarrheal plant is bactericidal against each bacterial strain.

METHODS

Plant materials

I collected the 15 plants in this study in their flowering and fruiting seasons between March and July in 2009. Determinations and names follow the Flora of Turkey (Davis, 1965, Güner, et al. 2001). Experts in the Botany Department at Gazi University

assisted in plant identification when necessary. The Angiosperm Phylogeny Group III (2009, Stevens 2001 onwards) was used for familial nomenclature. Voucher specimens for all species were deposited in GAZI. Botanical data for the tested plants are included in Table 3.1.

Table 3.1 Codes used for methanol and aqueous extracts.
Two parts ([1],[2]) used for RP and RoC.

Code	Latin Name	Family	Local Name	Parts used
AM	*Achillea millefolium* L.	Asteraceae	Amelotu, Ayvadene	Herb
AC	*Ajuga chamaepitys* (L.) Schreb. subsp. *laevigata* (Banks & Sol.) P.H.Davis	Lamiaceae	Kiraçotu	Herb
CO	*Cydonia oblonga* Mill.	Rosaceae	Ayva	Leaves
HP	*Hypericum perforatum* L.	Hypericaceae	Sarı kantoron	Aerial Parts
ML	*Mentha longifolia* Huds.	Lamiaceae	Yarpuz	Herb
PR	*Papaver rhoeas* L.	Papaveraceae	Gelincik	Aerial Parts
RhC	*Rhus coriaria* L.	Anacardiaceae	Sumak, somak	Fruits
RoC	*Rosa canina* L.	Rosaceae	Kuşburnu	Fruits[1], Galls[2]
RP	*Rumex patientia* L.	Polygonaceae	Enikmancar	Leaves[1], Fruits[2]
TF	*Tussilago farfara* L.	Asteraceae	Derekabalağı	Leaves
TP	*Teucrium polium* L.	Lamiaceae	Acı yavşan, Oğlanotu	Herb
TT	*Tribulus terrestris* L.	Zygophyllaceae	Dadaşotu	Herb
UD	*Urtica dioica* L.	Urticaceae	Isırgan	Leaves
VAA	*Viscum album* L.	Santalaceae	Güveltekotu	Herb
VAC	*Vitex agnus-castus* L.	Lamiaceae	Ayıt	Fruits

From various field sites, I collected bulk samples (over 100g dry weight) and recorded habitat information, including GPS coordinates. I left a few plants in village areas for future populations and local use, but I collected multiple individuals for each species. To prevent cross-contamination, I isolated plant materials from dirt and kept plant species separated. Bulk materials were divided into ethnobotanically-relevant parts (leaves, stems, flowers, herbs) and dried in the open, in the lab for 48-72 hours. After drying, plant materials were stored in large plastic bags in an acquisitions storage unit in the Pharmaceuticals building at Gazi University.

Before extraction, I pulverized dried plant samples with a mortar and pestle. Methanolic extracts of all plant samples were made by soaking 1g plant material in 20ml methanol for 72hrs, with daily agitation. To mimic traditional preparations, I prepared aqueous extracts by infusion (distilled water and plant material held at 80˚C for 20min) at the ratio of 1g plant material to 15mL distilled water.

Methanolic extracts were vacuum filtered (Weißband 0.00007gr, Carl Schleicher & Schüll no18089) and rotary-evaporated (Büchni Rotovapor R-200). Extracts were further dried in a sealed vacuum desiccant dome. Aqueous extracts were filtered, frozen at -70˚C and lyophilized (Lyolab C, LSL SecFroid). Main stocks of all extracts were stored in glass vials at 4˚C in the Gazi University Pharmacognosy Laboratory. Extracts were re-constituted at 1mg dried extract to 1mL excipient. Dimethylsulphoxide (DMSO) was used as the vehicle for methanolic extracts, and H_2O for aqueous extracts. The stocks were sterile-filtered using 0.22µm Millipore filters (MA 01730, USA), and were stored in the dark at 4˚C.

Materials Preparation

Sterilized plant extracts (1-34) were concentrated at 1024 µg/ml for use in the microdilution assay. Stock solutions of reference antibiotics were prepared in solvents according to the CLSI (Barry, et al. 1999). Anti-bacterial controls (ampicillin, ciprofloxacin, gentamicin, nitrofurantoin, azithromicin, vancomycin, and trimethoprim-sulfamethoxazole) were dissolved in phosphate buffer solution (ampicillin**,** nitrofurantoin, pH: 8.0; 0.1 mol mL), in water (gentamicin, ciprofloxacin, vancomycin, trimethoprim-sulfamethoxazole), or in 95% ethanol and medium (azithromicin).

Bacterial Assay

Bacterial strains (Table 3.2) belonged to the American Type Culture Collections (ATCC), the Culture Collection of the Refik Saydam Central Hygiene Institute (RSKK), and the NRRL, now the USDA Agricultural Research Service (ARS). Mueller Hinton Broth (MHB, Difco) and Mueller Hinton Agar (MHA, Oxoid) were used for growing and diluting the bacteria suspensions (Özçelik et al. 2005). The microorganism suspensions used for inoculation were prepared at 5 x 10^5 CFU/ml by determining the McFarland 0.5 turbidity (1.5 x 10^8 CFU/ml) and diluting. Bacteria were in log phase of growth at the time of preparation.

Table 3.2 Bacterial Strains and Brief Descriptions

1	*Escherichia coli*	ATCC 35218	- Rod, motile
2	*Pseudomonas aeruginosa*	ATCC 10145	- Rod, unipolar motility
3	*Klebsiella pneumoniae*	RSKK 574	- Rod, non-motile, encapsulated
4	*Salmonella enteriditis*	RSKK 538	- Rod, motile
5	*Shigella dysenteriae*	RSKK 851	- Rod, non-motile
6	*Vibrio cholerae*	RSKK 96023	- Comma, polar flagellum
7	*Staphylococcus aureus*	ATCC 25923	+ Clustered Coccus, non-motile
8	*Enterococcus faecalis*	ATCC 29212	+ 2-Coccus, non-motile
9	*Bacillus cereus*	NRRL B3711	+ Rod, Endospore
10	*Bacillus subtilis*	ATCC 6633	+ Rod, Endospore

In a clear 96-well plate, each well was filled with Mueller-Hinton broth. Extract solutions were added to the first row to make a final concentration of 512µg/ml. Then I serially diluted the extracts across the remainder of the plate to constitute a range of concentrations from 512µg/ml to 0.25µg/ml. Control wells were included for positive growth, negative growth, vehicle influence, and sterility of media. Wells were inoculated with 10µl of appropriate bacterial suspensions. All organisms, controls, and extracts were tested in triplicate. The 96-well plates were incubated at 35ºC overnight. The lowest

concentration (MIC) of the extracts that completely inhibited visible, macroscopic growth was determined, as described in Özçelik, et al. (2008). When no growth was observed in a well, 10µl of the well contents were transferred onto an agar plate. When macroscopic growth was not observed in liquid medium but growth was seen on agar plates, I recorded the concentration as bacteriostatic. Viable cells counts were determined as CFU/ml. The effect was deemed bactericidal when growth was observed in neither the broth nor the agar plate.

RESULTS

Twenty different extracts had MBSs of 32µg/ml or less (Table 3.3). Bactericidal concentrations (MBCs) were high (512-256µg/ml) in most cases. The *R. canina* methanolic gall extract was bactericidal at 128µg/ml and bacteriostatic at 16µg/ml. In this instance, the CFU was reduced to 9.0×10^3, an order of magnitude less than the initial 5.0×10^5 concentration of bacterial cells.

Table 3.3 Extracts with MBSs of 32µg/ml or less and the inhibited bacteria

Bacterium	Plant Name	Solvent	MBC	MBS	CFU/ml
V. cholerae	*Rosa canina-galls*	Methanol	128	16	9.0×10^3
S. aureus	*Ajuga chamaepitys*	Methanol	512	32	2.4×10^4
S. aureus	*Cydonia oblonga*	Methanol	512	4	1.6×10^4
S. aureus	*Cydonia oblonga*	Aqueous	512	8	3.5×10^4
S. aureus	*Hypericum perforatum*	Methanol	512	16	2.7×10^4
S. aureus	*Hypericum perforatum*	Aqueous	512	8	3.7×10^4
S. aureus	*Rhus coriaria*	Methanol	>512	8	1.1×10^4
S. aureus	*Rosa canina-galls*	Methanol	>512	16	2.4×10^4
S. aureus	*Rosa canina-galls*	Aqueous	>512	4	3.9×10^4
S. aureus	*Rumex patientia-fruit*	Methanol	>512	8	2.4×10^4
S. aureus	*Rumex patientia-fruit*	Aqueous	>512	32	3.1×10^4
E. faecalis	*Teucrium polium*	Methanol	256	32	3.6×10^4

E. faecalis	*Teucrium polium*	Aqueous	256	32	$1.8x10^4$
E. faecalis	*Tribulus terrestris*	Methanol	256	32	$1.8x10^4$
E. faecalis	*Tribulus terrestris*	Aqueous	256	32	$1.7x10^4$
E. faecalis	*Urtica dioica*	Methanol	256	32	$1.9x10^4$
E. faecalis	*Urtica dioica*	Aqueous	256	32	$3.3x10^4$
E. faecalis	*Viscum album*	Methanol	256	32	$2.8x10^4$
E. faecalis	*Viscum album*	Aqueous	256	32	$3.9x10^4$
E. faecalis	*Vitex agnus-castus*	Methanol	256	32	$3.5x10^4$
E. faecalis	*Vitex agnus-castus*	Aqueous	256	32	$2.0x10^4$

V. cholerae=Vibrio cholerae; *S. aureus=Staphylococcus aureus*; *E. faecalis=Enterococcus faecalis*

Methanolic and aqueous extracts of *Teucrium polium, Tribulus terrestris, Urtica dioica, Viscum album,* and *Vitex agnus-castus* inhibited *Enterococcus faecalis* at MBSs of 32µg/ml and MBCs at 256µg/ml, with a decrease in CFUs. Six plants inhibited *S. aureus* at MBSs ranging from 4µg/ml to 64µg/ml (Table 3.4). *Cydonia oblonga* methanolic leaf extract was bacteriostatic at 4µg/ml against *S. aureus*, while the aqueous extract was bacteriostatic at 8µg/ml against *S. aureus*. These MBSs are at clinically relevant concentrations, similar to pharmaceutical drug concentrations.

Table 3.4 MICs (µg/ml) of six extracts against *Staphylococcus aureus*

	Rumex patientia	*Rosa canina*	*Rhus coriaria*	*Hypericum perforatum*	*Cydonia oblonga*	*Ajuga chamaepitys*
	Fruits	Galls	Fruits	Herb	Leaves	Herb
Aqueous	32	4	16	8	8	64
Methanolic	8	16	8	16	4	32

Rhus coriaria fruit extracts inhibited *S. aureus* at 8µg/ml in methanolic extract and 16µg/ml in aqueous extract. The aqueous extract of *Ajuga chamepitys* inhibited *S. aureus* at 64µg/ml and the methanolic extract inhibited *S. aureus* at 32µg/ml. Galls of *R. canina*

are effective against *S. aureus* and *V. cholerae*, and rosehips showed similar yet less potent activity. The methanolic extract of *R. canina* galls had MBS concentrations of 16µg/ml and MBC concentrations at 128µg/ml against *V. cholerae* (Table 3.5).

Table 3.5 MICs (µg/ml) of *Rosa canina* extracts against *Vibrio cholerae*

	Galls		Fruits	
	Methanol	Aqueous	Methanol	Aqueous
MBC	128	512	512	512
MBS	16	64	64	64

Of the crude extracts tested in this study, 28% inhibited bacteria at or below 64µg/ml, and 6% inhibited bacteria at or below 32µg/ml. Nine plants (*Rosa canina, Cydonia oblonga, Ajuga chamaepitys, Rhus coriaria, Rumex patientia, Teucrium polium, Tribulus terrestris, Urtica dioica,* and *Viscum album*) are of further interest against *E. coli, Vibrio cholerae*, *Staphylococcus aureus,* and *Enterococcus faecalis*.

DISCUSSION

Phytochemicals that exhibit bacteriostatic instead of bactericidal properties can disrupt pathogenicity and halt disease without selecting for resistance in bacteria. In this study, the severe pathogen *V. cholerae* was inhibited by methanolic extracts of *R. canina* galls at MBSs of 16µg/ml and MBCs of 128µg/ml. *Enterococcus faecalis* was inhibited by aqueous and methanolic extracts of *T. polium, T. terrestris, U. dioica, V. album,* and *V. agnus-castus* at MBSs of 32µg/ml and MBCs of 256µg/ml. *Enterococcus faecalis* has several innate resistance factors for disrupting the efficacy of various antibiotics, including last-resort antibiotics such as vancomycin (Garrity 2004). It is a major concern in nosocomial settings, as is *S.aureus,* another bacteria with increasing antibiotic

resistance. Methanolic extracts of *C. oblonga* and *R. canina* galls showed bacteriostatic activity at 4µg/ml, while bactericidal activity was not observed until extract concentrations reached 512µg/ml or more (Table 3.3). The aqueous extracts of *C. oblonga* and *H. perforatum* had MBSs of 8µg/ml, as did the methanolic extracts of *R. coriaria* and *R. patientia* fruit. MBCs remained high, at or above 512µg/ml.

Cydonia oblonga, a member of Rosaceae, has high concentrations of pectins and polyphenolic compounds such as highly astringent hydrolyzable tannins. In Portugal, an HPLC analysis revealed high polyphenolic content of *C. oblonga* leaves (Oliveira, et al. 2007). Previous research has analyzed fruit pulp and peels of quince fruits for antibacterial activity, with acetone extracts of pulp showing inhibition of *S. aureus* in a disc diffusion assay (Fattouch, et al. 2007).

Several previous studies have analyzed *R. coriaria* spice for antibacterial properties against *Bacillus, Listeria, E. coli, Salmonella, Staphylococcus, Shigella, Klebsiella, Branhamella, and Pseudomonas* with varying effects (Fazeli, et al. 2007, Khalil, 1996, Sokmen, et al. 1999).

Ajuga chamaepitys is a plant endemic to eastern Turkey. No previous research on the antibacterial properties of *A. chamaepitys* was found in the published literature. Antiviral and antifungal properties of *A. chamaepitys* have been published (Orhan, et al. 2009), and research on the relief of colitis with the use of teupolioside, a phenylpropanoid glycoside harvested from *A. reptans,* was shown to slow intestinal motility and fecal transit (DiPaola, et al. 2009).

Many species of Lamiaceae are used in circum-Mediterranean regions. Researchers analyzed synergistic capabilities of *T. polium* with various pharmaceutical antibiotics against *S. aureus* (Darwish, et al. 2002) and *P. aeruginosa* (Aburjai, et al.

2001). Ethyl acetate extracts of the leaves of *V. agnus-castus* inhibited methicillin-resistant *Staphylococcus aureus*, carbapenem-resistant *Acinetobacter* spp., and Enterobacteriaceae species at 0.312, 0.625, and 0.625mg/ml respectfully (Arokiyaraj, et al. 2009). Menthol isolated from *M. longifolia* leaves in Iraq inhibited *S. aureus* and *S. mutans* at 15.6µg/ml and *S. faecalis, S. pyogenis,* and *L. acidophilus* at 31.2µg/ml (Al-Bayati 2009). In the current study, *M. longifolia* had MICs of 128µg/ml or above, but *T. polium* and *V. agnus-castus* inhibited *E. faecalis* at 32µg/ml.

A second important plant family in this region is the Asteraceae. In this study, *Achillea millefolium* and *Tussilago farfara* were evaluated. In Siberia, where *T. farfara* is used as an antiseptic and antiphlogistic, ethanolic extracts of *T. farfara* had an MIC of 62.50mg/ml against *S. aureus* (Kokoska, et al. 2002). *Achillea millefolium* exhibited antibacterial activity at high concentrations, but the essential oils were found to be more active than aqueous or methanolic extracts (Stojanovic, et al. 2005). In some villages in Turkey, *A. millefolium* is known as *amelotu* or "the deed weed," which refers to the vernacular term for diarrhea, *amel* ("the deed").

While several studies have analyzed rosehips for antioxidant properties, only one previous publication was found evaluating antibacterial activities. *Rosa canina* seeds from Scotland were shown to inhibit *E. coli* at 0.10mg/ml (Yashodharan, et al. 2002).

Other plants commonly used to treat diarrhea include the rind of *Punica granatum*, *Sorbus domesticus*, and other Rosaceae species as well as *Cornus mas* L. (Cornaceae). The ripe fruits of these plants were not available for collection at the time of study. Plants in the Rosaceae are known for their astringency, which affects the mucosal lining of the gut and tightness of cell junctions. Mucus is a critical attachment and nourishment factor for the pathogenicity of many gastrointestinitis-inducing bacteria. Rosaceous fruits also

often contain pectin, which adds bulk to the intestinal contents and facilitates proper muscle movement. Pectins and astringent coumpounds are known to affect the human gut and treat symptoms of diarrhea (Lewis and Elvin-Lewis 2003).

CONCLUSION

Results showed 95 MBSs at 64µg/ml or below. Twenty-one MBSs were read at 32µg/ml or less, yet their MBCs were much higher, at 256 µg/ml or more. Thus, plant extracts were inhibiting bacterial growth without killing the bacteria. Such bacteriostatic effects are desirable in novel treatments against bacteria. Since bacteria are only inhibited in bacteriostatc circumstances and not killed, resistant strains evolve much less rapidly.

Of the clinically relevant MICs, with MBSs of 32µg/ml or less, nine of the extracts were aqueous while 12 were methanolic. Since traditional uses of plants usually prescribe aqueous preparations, the relatively high effectiveness of aqueous extracts in this study supports the continued use of these plants and their reliability in treating infectious diarrhea.

Of special significance, the galls from the *R. canina* showed low MICs against 6 of the 10 bacteria. Galls form from a complex interaction between an insect, in this case, and a host plant part. The novel up-/down-regulation of genes in this process could lead to a change in anabolic pathways or new chemicals.

This study included only 15 of the 35 non-domesticated plant species collected in Central Anatolia. Throughout Turkey, 91 plant species were recorded as medicinal for diarrhea. Further research for biologically active chemical constituents, cytotoxicity levels, and mechanisms of action could lead to a readily-available treatment for acute gastroenteritis in the region.

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Chapter IV

BACTERIAL INHIBITION AND CYTOTOXIC PROPERTIES OF PLANTS USED TO TREAT DIARRHEA

ABSTRACT

I evaluated 15 Central Anatolian plants for their safety and efficacy in inhibiting the growth of 21 bacterial strains that commonly infect the human intestinal tract and cause diarrhea. I carried out ethnobotanical research in Central Anatolia for one year, beginning in September 2008. Following interviews and a literature review, I collected plants used to treat diarrhea. Voucher specimens were deposited in Gazi University´s Herbarium (GAZI). I collected plant materials and processed them in bulk, with methanolic and aqueous extracts prepared from medicinally-relevant parts of the plants for a total of 34 extracts. I used a microtiter broth dilution assay (CLSI protocol) to evaluate antibacterial activity and an Alamar Blue cytotoxicity assay to evaluate the cytotoxicity of the plants for human cells. The percent inhibition was used to calculate the effectiveness of the 34 extracts in inhibiting one or more of the 21 bacteria. *Rosa canina*, *Hypericum perforatum*, *Vitex agnus-castus*, and *Rhus coriaria* showed significant inhibition at low concentrations (32, 16, 8, and 4μg/ml) against 8 bacteria (*Enterococcus faecalis, Klebsiella pneumoniae, Listeria monocytogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella flexneri, Vibrio parahaemolyticus,* and *Yersinia pseudotuberculosis*). Further evaluation of these Turkish plants could lead to an easily accessible, readily available plant-based remedy for diarrhea in the region.

INTRODUCTION

Plants are commonly used to treat gastrointestinal problems and often are the first line of defense against diarrhea or gastroenteritis. Acute gastrointestinal disease (or diarrhea) is a major concern for children under the age of five in rural and peri-urban areas all over the world (WHO 2009). Bacteria cause 2 to 4 billion cases of infectious diarrhea every year, leading to 3 to 5 million deaths in developing countries (Sanchez and Holmgren 2005). Infectious diarrhea disproportionately affects residents of less affluent countries (Guerrant, et al. 2002).

In rural Turkey, diarrhea usually is treated in homes with infusions or decoctions of wild or cultivated plants (Chapter II). Turkish ethnobotanical literature lists 91 species of plants from 35 families that are used to treat diarrhea. Research conducted in this study recorded 35 plant species from 20 families that are used to treat diarrhea in Central Anatolia, a semiarid steppe region in Turkey (Peel, et al. 2007). Fifteen of these species are herein evaluated for their safety and anti-bacterial properties.

Previous research on anti-diarrheal plants generally focused on phytochemical properties to slow motility, decrease spasms, and increase water and electrolyte readsorption in the intestines (e.g., Palombo 2006). In addition to human physiological changes, plant compounds might interrupt the attachment of bacteria to intestinal epithelia or unfavorably alter microenvironments for pathogenic bacterial growth. Blocking or disrupting bacterial pathogenicity treats the cause of infectious diarrhea, not just the symptoms, and therefore decreases the severity of infection in a patient and prevents the spread of the infectious agent (Levine and Svennerholm 2008).

The aim of this study was to evaluate the anti-bacterial and cytotoxic properties of anti-diarrheal plants from Central Anatolia. The three primary questions addressed here

are: Do crude extracts from plants that are used to treat diarrhea in rural Central Anatolia exhibit antibacterial activity against bacteria strains that commonly cause diarrhea? Do these plant extracts have acceptable levels of cytotoxicity and are therefore safe for human ingestion? Do the activities of aqueous and methanolic extracts of the same plant differ?

Four hypotheses were tested. H1. Each Central Anatolian anti-diarrheal plant inhibits each bacterial strain. H2. There is no difference in inhibitory concentration between more traditional aqueous extracts of a plant and methanolic extracts of the same plant. H3. Each Central Anatolian anti-diarrheal plant shows no toxicity to the cell lines used in the Alamar Blue Cytotoxicity Assay. H4. The combined inhibitory and toxicity data support the traditional use of Central Anatolian anti-diarrheal plants.

METHODS

Plant Extract Preparation

I prepared crude plant extracts from dried bulk plant specimens collected in Turkey during ethnobotanical field work (IRB Approval #082508-01). Plants were identified by using the Flora of Turkey and the East Aegean Islands (Davis 1965, Güner, et al. 2001). I deposited vouchers in GAZI in Ankara, Turkey. Methanolic and aqueous extractions were made from medicinally-used parts of 15 plants, with two parts used in two plants and one part used in the remaining thirteen. In total, 34 crude extracts were screened in the cytotoxicity and anti-bacterial assays (Table 4.1).

Table 4.1 Plant extracts listed with Codes, Local Names, Family Names, and Parts Used. Note that two plant parts ([1],[2]) were used for Rumex patientia and Rosa canina.

Code	Latin Name	Family	Local Name	Parts used
AM	*Achillea millefolium* L.	Asteraceae	Amelotu, Ayvadene	Herb
AC	*Ajuga chamaepitys* (L.) Schreb. subsp. *laevigata* (Banks & Sol.) P.H.Davis	Lamiaceae	Kiraçotu	Herb
CO	*Cydonia oblonga* Mill.	Rosaceae	Ayva	Leaves
HP	*Hypericum perforatum* L.	Hypericaceae	Sarı kantoron	Aerial Parts
ML	*Mentha longifolia* Huds.	Lamiaceae	Yarpuz	Herb
PR	*Papaver rhoeas* L.	Papaveraceae	Gelincik	Aerial Parts
RhC	*Rhus coriaria* L.	Anacardiaceae	Sumak, somak	Fruits
RoC	*Rosa canina* L.	Rosaceae	Kuşburnu	Fruits[1], Galls[2]
RP	*Rumex patientia* L.	Polygonaceae	Enikmancar	Leaves[1], Fruits[2]
TF	*Tussilago farfara* L.	Asteraceae	Derekabalağı	Leaves
TP	*Teucrium polium* L.	Lamiaceae	Acı yavşan, Oğlanotu, Merven	Herb
TT	*Tribulus terrestris* L.	Zygophyllaceae	Dadaşotu	Herb
UD	*Urtica dioica* L.	Urticaceae	Isırgan	Leaves
VAA	*Viscum album* L.	Santalaceae	Güveltekotu	Herb
VAC	*Vitex agnus-castus* L.	Lamiaceae	Ayıt	Fruits

I made methanolic extracts by soaking homogenized plant materials in methanol (1g/20 ml) for 72hr, with daily agitation. Analogous to traditional preparations, I prepared aqueous extracts by infusion (distilled water and plant material held at 80˚C for 20 minutes) at the ratio of 1g plant material to 15ml distilled water, so that plant materials were completely submerged during the procedure.

Methanolic extracts were vacuum-filtered (Weißband 0.00007gr, Carl Schleicher & Schüll no18089), rotary-evaporated (Büchni Rotovapor R-200), and dried in a sealed vacuum desiccant dome. Aqueous extracts were filtered, frozen (-80˚C) and lyophilized (Lyolab C LSL SecFroid). Main stocks of all extracts were stored in glass vials at -5 to 4˚C. I re-constituted dried plant extracts at 10mg/ml in dimethylsulphoxide

(DMSO) for methanol extracts and phosphate-buffered saline solution (PBS) for aqueous extracts. The final concentrations of DMSO and PBS solvents constituted less than 5% of total well volume, thereby preventing false positives. The re-constituted extracts were sterile-filtered (0.2 microns, Corning Incorporated 431222) and stored in amber glass vials at -5˚C.

Anti-Bacterial Assay

Twenty-one bacteria strains (Table 4.2) were established on agar plates and grown in Cation-Adjusted Mueller Hinton Broth (CAMHB, Difco) at 37ºC according to the Clinical Laboratory Standards Institute (CLSI) protocol M26-A (Barry, et al. 1999). Growth curves for each bacterial strain were determined by kinetic readings (every 10 min/24 hrs) on a BioTek Powerwave Spectrophotometer with incubator and shaker capabilities. Each strain was inoculated in log phase and analyzed at its peak log phase of growth.

A preliminary broth dilution assay (Amsterdam 1996, Isenberg 2004) was used to evaluate all 34 extracts and to determine the five that were most active. All extracts were serially diluted in a 96-well plate so that concentrations ranged from 512µg/ml to 8µg/ml. Controls included positive and negative growth controls as well as DMSO and PBS solvent controls. The antibiotics ampicillin, ciprofloxacin, nitrofurantoin, azithromicin, and vancomycin were used as positive controls.

Table 4.2 Bacteria strains listed with codes (abbreviations) and standards

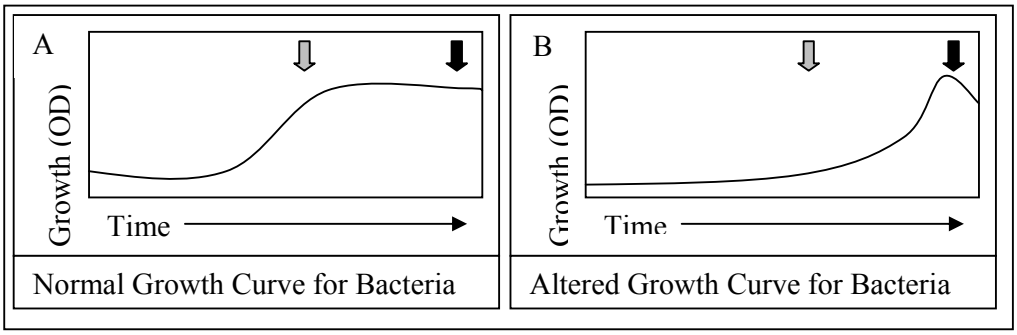
#	Code	Bacteria	Standard
1	Bs-1	*Bacillus subtilis*	ATCC 6051
2	Ec-0	*Escherichia coli*	Nat OH5 alpha
3	Ec-1	*Escherichia coli*	ATCC 10836
4	Ef-1	*Enterococcus faecalis*	ATCC 19433
5	Kp-1	*Klebsiella pneumoniae*	ATCC BAA-1705
6	Lm-1	*Listeria monocytogenes*	ATCC 19115
7	Lm-2	*Listeria monocytogenes*	ATCC19015
8	Pa-1	*Pseudomonas aeruginosa*	PA 01
9	Se-1	*Salmonella enterica*	ATCC 14028
10	St-4	*Salmonella enterica typhimurium*	Plano Lab, UM
11	Sa-1	*Staphylococcus aureus*	Mathee Lab, FIU
12	Sa-2	*Staphylococcus aureus*	ATCC 10882
13	Sa-3	*Staphylococcus aureus*	ATCC 25923
14	Sa-4	*Staphyloccocus aureus*	ATCC 29213
15	Sf-1	*Shigella flexneri* (1a)	ATCC 9199
16	Ss-1	*Shigella sonnei*	ATCC 25931
17	Ye-1	*Yersinia enterocolitica*	8081c w/o pYv
18	Ye-2	*Yersinia enterocolitica*	O18 8081v w/ pYv
19	Yu-1	*Yersinia pseudotuberculosis*	III YP KmR
20	Yu-2	*Yersinia pseudotuberculosis*	ATCC 6903
21	Vp-1	*Vibrio parahaemolyticus*	ATCC 17802

Bacteria were grown to log phase, concentrated to McFarland Standard 0.5 turbidity, and then diluted to 5 x 10^5 colony-forming units (CFU)/ml. Serial dilution colony counts were performed to evaluate bacterial concentrations. Assays were completed in triplicate. After incubation at 37˚C, plates were read for the absence or presence of macroscopic growth in wells at the peak of log growth for each strain and at 18 hours (or 24 hours for slow-growing *Yersinia* spp.).

After the preliminary screening, the five extracts that showed the most inhibition against the most bacteria were run through a second analysis involving kinetic optical density (OD) readings at 600nm wavelength (BioTek Powerwave Spectrophotometer). Readings were taken so that minor shifts in growth curves brought about by extracts could

be analyzed. For instance, in Figure 4.1, Box A shows a normal growth curve, while Box B shows a growth curve of bacteria growing in the presence of a plant extract. More sensitive data were acquired by taking multiple readings and comparing bacterial growth at peak log phases instead of at one standard 18 or 24hr reading.

Figure 4.1 Differing growth curve patterns betwen Box A and Box B with optimal reading time at peak of logarithmic growth versus later at 18 or 24 hours



Kinetic OD readings were taken (10min/24hr) for the top five extracts and five antibiotic controls. The peaks of the bacterial growth control triplicates were averaged and used to calculate % inhibition with the following formula (Quave, et al. 2008).

$$\%\ \text{Inhibition} = \left[\left[1 - \left(\frac{\text{OD}_{\text{t}^{\text{peak}}} - \text{OD}_{\text{t}^{\text{initial}}}}{\text{OD}_{\text{gc}^{\text{peak}}} - \text{OD}_{\text{gc}^{\text{initial}}}}\right)\right]\right] \times 100$$

The difference in initial and log peak optical density (OD) readings of the treatment group were divided by the difference in initial and log peak OD readings of the growth control for that bacterial strain. The resulting number was then subtracted from one and multiplied by 100 to create a percentage.

The MIC_{50} (50% of bacteria growth inhibited) and MIC_{90} (90% inhibited) were then tabulated using the percent inhibitions of each extract. When MIC_{50} or MIC_{90} were

at or below 64 μg/ml, a one-way ANOVA with Tukey post-hoc tests was performed using PASW SPSS software to analyze the significant differences ($p<0.05$) in OD readings between concentrations of the extract for a specific bacterial strain.

Cytotoxicity Assay

I performed a cytotoxicity assay to evaluate the relative safety of the crude extracts by determining whether the extracts disrupt human cellular metabolism. The Alamar Blue Assay measures cellular metabolic activity in a time- and concentration-dependent manner. Unlike the MTT Assay, cells are kept alive and handling of cells and potential contamination is minimized (Gloeckner, et al. 2001). As a dye, Alamar Blue is conveniently stable, non-radioactive, and non-toxic. It is composed of the blue, non-fluorescent indicator resazurin which is naturally, continuously, and reversibly reduced by living cells to red, fluorescent resorufin (O'Brien, et al. 2000). The reduction can be measured quantitatively, with higher absorbance levels correlating with higher metabolic activity.

Human cell lines included prostate adenocarcinoma PC-3 (ATCC CRL-1435), skin melanoma SKMEL-5 (ATCC HTB-70), and hepatocellular carcinoma HEP G-2 (ATCC CRL-11997). Since the liver filters ingested materials such as phytochemicals, any inhibition of the HEP G-2 cell line was taken into special consideration.

I established the HEP G-2 cell line in Eagle's Medium (ATCC 30-2003) with 10% Fetal Bovine Serum (Sigma-Aldrich F0926) and 1% Antibiotic and Antimycotic Solution (100x, Sigma A 5955). The HEP G-2 cells grew to confluency in 4-5 days. I established the PC-3 and SKMEL-5 cell lines in HyClone RPMI-1640 Medium (Thermo Scientific SH 30027.01) with 10% Fetal Bovine Serum and 1% Antibiotic and Antimycotic

Solution. PC-3 cells grew quickly to confluency in 1-2 days while the SKMEL-5 cells took slightly longer (3-4 days). All cell lines were incubated at 37˚C with 100% humidity and 5% carbon dioxide.

I added cells to flat-bottomed 96-well plates with suitable medium and allowed them to grow to confluency. For a preliminary screening of the 34 extracts and 3 cell lines, I pipette plant extracts into wells to make 200, 100, 50, and 25µg/ml concentrations in duplicate. Cells grew in the presence of the extracts for 18 hours. As the extracts are colored and might prevent accurate dye readings, I removed spent media from the wells and added fresh media with 10% Alamar Blue (AbD Serotec BUF012B). After waiting 4 hours (O'Brien, et al. 2000), I graded well color on a scale from 1 to 5. Red was scored 1(reduced and viable), 2 was reddish purple, 3 was purple, 4 was bluish-purple, and 5 was blue (nonviable).

From these data, I used a more rigorous screening to evaluate extracts that showed inhibition at concentrations of 100µg/ml or less against one or multiple cell lines. In the second phase of testing, I serially diluted extracts from 512µg/ml to 0.5µg/ml in wells with fresh confluent cells. The last well was left as a control. Other controls included DMSO, PBS, media, and camptothecin, a known lethal phytochemical for human cells.

After 18 hours, I removed spent media and added fresh media with 10% Alamar Blue. After 4 hours and 18 hours, the plates were read in a Bio-Tek Synergy HT Spectrophotometer at M595λ. The following formula was used to determine the difference in growth between absorbance at 595nm of the treatment group, or the wells with extracts, and absorbance at 595nm of the control group, or untreated wells showing normal growth for each cell line. Percent viability was calculated for each concentration

of each extract and then triplicates were averaged. Note that percent viability (used in the cytotoxicity assay) is the inverse of percent inhibition (used in the anti-bacterial assay).

$$\text{Viability} = \left(\frac{\text{Abs}_{595}}{\text{AbsControl}_{595}}\right) \times 100$$

RESULTS

Anti-Bacterial Assay

The first anti-bacterial screening identified 20 of the 34 crude extracts that inhibited at least one strain of bacteria (Table 4.3).

Table 4.3 Results from the Initial Bioassay showing MICs (μg/ml) of any crude extracts that exhibited bacterial inhibition against one or more bacterial pathogens

	Bacteria													
Extracts	**Bs**	**Ec**	**Ef**	**Kp**	**Lm**	**Pa**	**Se**	**St**	**Sa**	**Sf**	**Ss**	**Vp**	**Ye**	**Yu**
AC met		512							256			256	512	
AM met		256			256							512	128	
CO met		512												
HP met	256	128	64		64				64	512	256	32	128	
ML met		256							256			128		
PR met	256	128			256						256	128		
RhC met	256	8			64				256	512	64		64	64
RoCF met	128	512			128						512	32		64
RoCF aq														256
RoCG met		128			64	64		512	256	128	256	16	256	256
RPF met		512			128				256		512			256
RPL met	256				128							512		
TF met	128	512			64				256			256		256
TF aq												128		
TP met	256	512			32				128			256		256
TP aq												512		
TT met	128	256			64							256		
UD met	256	128			32				256			256		
VAA met	64	256			16				256					128
VAC met	256	128			16		64		256		512	32	256	

MICs are the lowest 2 out of triplicates. Abbreviations for plants and bacteria names can be found in List of Plants (Table 4.1) and List of Bacteria (Table 4.2). Cells left blank when no inhibition was observed within the concentration range.

Using these data (Table 4.3), the five extracts that inhibited the most bacteria at the lowest concentrations were selected for further investigation in the secondary phase of the anti-bacterial assay. Methanolic extracts of *Hypericum perforatum, Rhus coriaria, Rosa canina* fruits, *R. canina* galls, and *Vitex agnus-castus* were selected because these plant extracts inhibited a total of 9, 8, 6, 10, and 8 bacteria strains, respectively. The resulting MIC_{50} and MIC_{90} of the five extracts and the five antibiotic controls are tallied in Table 4.4 below.

Table 4.4 MIC_{50} and MIC_{90} for the five most-inhibitive extracts and five antibiotics for clinically relevant comparisons

		Five Plant Extracts					Five Antibiotics				
Bacteria		**HP**	**RhC**	**RoCF**	**RoCG**	**VAC**	**Cipro**	**Azithro**	**Amp**	**Nitro**	**Vanco**
Bs-1	**MIC50**	128	64		4	128	0.5	< 0.25	<0.25	16	< 0.25
	MIC90		512			512	1	0.5	0.5	32	0.5
Ec-0	**MIC50**	512	512	512	256		0.25	0.5	4	< 0.25	32
	MIC90				512		0.5	4	8	0.25	128
Ec-1	**MIC50**		512		64	512	< 0.25	< 0.25	1	4	64
	MIC90				512		0.25	0.5	2	8	256
Ef-1	**MIC50**	64	256		256	256	1	< 0.25	1	16	0.25
	MIC90	512			512		4	0.5	2	64	0.5
Kp-1	**MIC50**	256	256	128	64	64	128	4		128	128
	MIC90				256		256	16		256	512
Lm-1	**MIC50**	512	256	512	128	256	1	0.5	1	16	16
	MIC90		512		512	512	8	1	2	32	128
Lm-2	**MIC50**	64	128	512	128	64	< 0.25	< 0.25	< 0.25	8	0.25
	MIC90	128	256		256	128	1	< 0.25	< 0.25	16	0.5
Pa-1	**MIC50**	128	32	128	8	128	1	0.5	512	256	128
	MIC90	512	256	512	128	512	4	2		512	
Sa-1	**MIC50**	128	256	512	64	64	0.5	< 0.25	< 0.25	16	0.5
	MIC90	512	512		512	512	2	0.25	< 0.25	32	1
Sa-2	**MIC50**	256	256		128	256	0.25	< 0.25	< 0.25	16	0.5
	MIC90	512	512		512	512	2	< 0.25	0.25	32	1
Sa-3	**MIC50**	256	256	256	128	256	2	0.25	< 0.25	16	1
	MIC90	512	512	512	512	512	8	0.5	< 0.25	32	2
Sa-4	**MIC50**	256	256	256	128	256	1	0.25	0.5	16	0.5
	MIC90	512	512	512	512	512	4	0.5	1	32	1

Se-1	**MIC50**		512		128	512	< 0.25	0.5	1	8	128
	MIC90				256		< 0.25	1	2	16	256
Sf-1	**MIC50**	128	64	256	16	128	< 0.25	< 0.25	2	4	4
	MIC90		512		128	256	< 0.25	< 0.25	4	8	8
Ss-1	**MIC50**	512	256	512	128	128	< 0.25	0.5	1	4	16
	MIC90				512		< 0.25	1	2	>512	128
St-4	**MIC50**	128	128	256	16	128	< 0.25	0.5	0.5	4	64
	MIC90				512		< 0.25	1	1	16	256
Vp-1	**MIC50**		128	256	16	512	0.25	< 0.25	1	4	16
	MIC90		512		64		0.5	< 0.25	4	8	32
Ye-1	**MIC50**	64	64	64	8	64	< 0.25	0.25	4	1	4
	MIC90		512	512	256	512	< 0.25	0.5	8	32	128
Ye-2	**MIC50**	128	128	128	64	256	0.25	< 0.25	8	8	8
	MIC90		512		128	512	0.5	< 0.25	16	16	256
Yu-1	**MIC50**	64	256	64	128	32	0.25	1	0.25	64	16
	MIC90	256	512	512	512	512	0.5	4	0.5		256
Yu-2	**MIC50**	512	256	512	128	512	0.5	0.5	< 0.25	32	8
	MIC90		512		512		1	4	< 0.25	64	512

Abbreviations for plants and bacteria names can be found in List of Plants (Table 4.1) and List of Bacteria (Table 4.2). Blank cells denote no observed inhibition within concentration range.

When MICs of 64µg/ml or lower were reached (Table 4.4), the data were analyzed statistically for significant differences between concentrations using separate ANOVAs with Tukey post-hoc tests for each extract and bacterial strain (Table 4.5). Methanolic crude extracts of *H. perforatum*, a plant often used as a mood elevator, inhibited *L. monocytogenes* at 32µg/ml and *Y. pseudotuberculosis* at 16µg/ml. Methanolic crude extract of *R. coriaria* inhibited *B. subtilis* at 256µg/ml, but it also inhibited *P. aeruginosa* and *S. flexneri* at low concentrations of 16µg/ml. Methanolic extract of *V. agnus-castus* inhibited three bacteria (*K. pneumonia, L. monocytogenes*, and *S. aureus*) at 32µg/ml and *Y. pseudotuberculosis* at 16µg/ml.

Methanolic extracts of *R. canina* were prepared from rosehips and galls of rosehips produced by cynipid wasps. The rosehip methanolic extract inhibited *Y. pseudotuberculosis* at 256µg/ml. The gall extract did not significantly inhibit *Y.*

pseudotuberculosis in this assay. However, methanolic crude gall extract inhibited several other bacteria. *Yersinia enterocolitica, S. enterica typhimurium, K. pneumonia,* and *B. subtilis* were inhibited at 256µg/ml. *Staphylococcus aureus* was inhibited by methanolic extract of *R. canina* galls at 128µg/ml, while *E. coli* growth was inhibited at 64µg/ml. In even smaller concentrations, *R. canina* methanolic gall extract inhibited *E. faecalis* and *S. flexneri* at 32µg/ml, *V. parahaemolytics* at 8µg/ml, and *P. aeruginosa* at 4µg/ml.

Table 4.5 Significant MIC (µg/ml) results statistically analyzed by ANOVA and Tukey post-hoc tests for top five extracts and inhibited bacterial pathogens

Top 5 Extracts	**Bacteria**											
	Bs	**Ec**	**Ef**	**Kp**	**Lm**	**Pa**	**Sa**	**Sf**	**St**	**Vp**	**Ye**	**Yu**
HP					32							16
RhC	256					16		16				
RoCF												256
RoCG	256	64	32	256		4	128	32	256	8	256	
VAC				32	32		32					16

Each extract and bacteria were analyzed separately from other extracts and bacteria. Differences in bacterial growth (OD) between extract concentrations were significant at $p<0.05$. Abbreviations for plants and bacteria names can be found in List of Plants (Table 4.1) and List of Bacteria (Table 4.2). Blank cells denote no significant inhibition within concentration range.

Because the methanolic extracts of *R. canina* showed such high rates of inhibition at low concentratios for 10 of the 21 bacteria in this study, they were selected for further analysis by bioassay-guided fractionation (see Chapter V).

Significant differences in $[OD_{Tpeak} - OD_{Tinitial}]$ were compared between concentrations for each extract that showed inhibition of growth in specific bacteria. The differences were further analyzed by Tukey post-hoc tests to determine significant differences ($p<0.05$) and plotted for visual clarity.

Cytotoxicity Assay

The majority of the extracts showed no cytotoxicity toward the tested human cells. However, 13 out of 34 showed some form of toxicity in the initial screening, when blue-red color changes were judged by eye (Table 4.5). Prostate (PC-3) and skin melanoma (SkMEL-5) cell lines were more sensitive than the hepatocytes (HepG-2) to the plant extracts, especially the methanolic extracts. Although DMSO was the solvent vehicle for the methanolic extracts, the controls showed no inhibitory effect of DMSO in the cell lines. The DMSO was kept to <5% of total well volume. For hepatocytes, methanolic extracts of *H. perforatum, R. coriaria, R. patientia* fruits, and *V. agnus-castus* showed inhibition at 25µg/ml and 5µg/ml. Extracts that showed inhibition at 100µg/ml or less in the preliminary screening were evaluated in the more elaborate secondary screening process.

Table 4.5 Results of the Preliminary Alamar Blue Assay showing any IC's of 100µg/ml or less. (Non-toxic results are not shown).

Plant	**Solvent**	**Extract #**	**PC-3**	**SkMEL-5**	**HepG-2**
Ajuga chamaepitys	MeOH	1	100	50	200
Achillea millefolium	MeOH	3	100	50	50
Hypericum perforatum	MeOH	7	25	5	25
Mentha longifolia	MeOH	9	100	5	200
Rhus coriaria	MeOH	13	5	5	25
Rosa canina galls	MeOH	17	50	100	200
Rumex patientia fruits	MeOH	19	100	50	25
Rumex patientia leaves	MeOH	21	200	25	200
Tribulus terrestris	MeOH	27	50	5	50
Tribulus terrestris	Aq	28	25	100	50
Urtica dioica	MeOH	29	100	5	50
Vitex agnus-castus	MeOH	31	5	5	5
Viscum album	MeOH	33	200	50	100
DMSO		35	200	200	200
PBS		36	200	200	200
CAMPTOTHECIN		37	5	50	5

Although the aqueous extract of *T. terrestris* inhibited PC-3 cells (78.4% viability at 32µg/ml and 85.3% viability at 64µg/ml), these differences were not significant at $p<0.05$. *Urtica dioica* methanolic extract showed minor inhibition of PC-3 cell growth with 82.6% viability at 64µg/ml and 89.3% viability at 32µg/ml. *Vitex agnus-castus* methanolic extract showed slight inhibition of PC-3 cells with 85.9% viability at 32µg/ml and 86.6% viability at 64µg/ml. However, the overall inhibition of human cell lines was negligible in the secondary cytotoxicity screening that utilized spectrophotometric readings. In fact, the extracts seemed to help the human cells grow, with most viability percentages over 100% and no MIC_{50} or MIC_{90} reached.

DISCUSSION

Anti-bacterial Properties

Of the fifteen Turkish anti-diarrheal plants assayed in this study, all inhibited, to some degree, one or more bacterial strains. Besides the top five plants analyzed, three methanolic extracts inhibited *L. monocytogenes* at low concentrations. *T. polium* and *U. dioica* inhibited *L. monocytogenes* at 32µg/ml while *V. album* inhibited *L. monocytogenes* at 16µg/ml. These three plants were commonly used to treat diarrhea and a wide variety of gastrointestinal ailments and auto-immune complaints. The severity of diarrhea has been linked to increases in interleukins and other inflammatory signals in the intestines. The mistletoe *V. album* grows on oak, pear, and other tree species in Central Anatolia. Locals and botanists (Türe, et al. 2010) reported that *V. album* harvested from different trees bear different medicinal or chemical properties. Following local use patterns, only *V. album* from pear trees was used to prepare extracts.

Hypericum perforatum exhibits a variety of biological activities, including activity against Gram positive bacteria and MRSA, but the plant's anti-bacterial properties are not exhaustively known (Saddiqe, et al. 2010). In part, the variation in reports of antibacterial activity might be the result of seasonal changes in chemical concentrations of *H. perforatum* (Borchardt, et al. 2008). Previous research has analyzed the essential oils of Serbian *H. perforatum* against several Gram negative and Gram positive bacteria to great effect (Saroglou, et al. 2007). In the present study, methanolic extractions of *H. perforatum* inhibited Gram negative *L. monocytogenes* at 32µg/ml and *Y. pseudotuberculosis* at 16µg/ml.

Rhus coriaria is commonly used as sumac spice throughout much of the Middle East. Methanolic extract of *R. coriaria* inhibited *P. aeruginosa* and *S. flexneri* at 16µg/ml. Both *P. aeruginosa* and *S. flexneri* are infectious agents with growing resistance to prophylactic treatments, and new treatments would prove beneficial. Previous research of antibacterial properties of aqueous extracts of *R. coriaria* ripened and unripened fruits showed inhibition of mostly Gram positive bacteria including *S. aureus*, as well as some Gram negative bacteria including *E. coli* and *Proteus* sp. (Nasar-Abbasa and Halkman 2004). Other investigations showed inhibition of *Moraxella catarrhalis* (syn. *Branhamella catarrhalis*) (Sokmen, et al. 1999), as well as MRSA at high concentrations (Abu-Shanab, et al. 2005), and other Gram positive strains (Khalil 1996).

The methanolic extract of *V. agnus-castus* fruits inhibited *K. pneumoniae, S. aureus,* and *L. monocytogenes* at 32µg/ml. *Klebsiella pneumonia*e is particularly difficult to inhibit. Also, in this study *V. agnus-castus* inhibited *Y. pseudotuberculosis* at 16µg/ml, a clinically relevant concentration. Previous studies of *V. agnus-castus* in India showed that ethyl acetate extracts of the leaves inhibited MRSA (Arokiyaraj, et al. 2009).

Hexanic and dichloromethanic extracts of *Vitex trifolia* are cytotoxic against several cancer lines in (Hernández, et al. 1999). Several South African *Vitex* species inhibited the growth of Gram positive bacteria more than Gram negative bacteria (Nyiligira, et al. 2008). More hydrophobic preparations of leaves were used in previous studies, while more hydrophilic preparations of the fruits were used in the present research.

Methanolic extracts of *R. canina* fruits inhibited *Y. pseudotuberculosis* at 256µg/ml. Previous research on the antibacterial properties of *R. canina* showed that methanolic extracts of seeds inhibited *E. coli* (ATCC 8110), while hexane and dichloromethane extracts showed no inhibition (Kumarasamy, et al. 2002). Another study examined the use of common antibiotics (ß-Lactams) with *R. canina* in treating MRSA, with synergistic results (Shiota, et al. 2000).

The antibacterial properties of *R. canina* galls have not been previously reported nor analyzed in bioassays. Galls result from complex biochemical interactions between an insect, parasites to that insect, the plant host, and possible microorganisms. The signaling and biochemical changes are not fully understood. In this study, methanolic extracts of *R. canina* galls inhibited several bacteria: *P. aeruginosa* at 4µg/ml, *V. parahaemolyticus* at 8µg/ml, *E. faecalis* and *S. flexneri* at 32µg/ml. With intrinsic resistance to a variety of antibiotics, *P. aeruginosa* and *E. faecalis* constitute serious concerns as nosocomial infections. *Vibrio parahaemolyticus* and *S. flexneri* play a significant role in severe diarrhea epidemics. *Rosa canina* galls show promise as possible antibacterial agents. Similarly, in southeastern Turkey, villagers commonly used galls from oak trees, induced by a closely related wasp, to treat diarrhea.

Cytotoxicity

Since the plants under study are commonly ingested by people as spices or for medicines, their cytotoxicity levels were expected to be low. In the preliminary cytotoxicity assay, read as changes in dye color, 13 of 34 plant extracts showed inhibition or death to one or more human cell lines. However, in the secondary cytotoxicity assay in which a spectrophotometer read color wavelengths of the dye, growth was not significantly inhibited in the human cell lines.

In vivo, the ingestion of phytochemicals commonly alters their chemical composition. The acidity of the stomach and secretions of the intestines, pancreas and liver can change the ionic valences or bonded structures of phytochemicals. Enzymes known as cytochrome P450s (CYPs) in humans, plants, bacteria, and all life forms metabolize and activate thousands of exogenous compounds, and are known to interact with phytochemicals (Budzinski, et al. 2007). Drug-transport proteins like P-glycoproteins (P-gps) efflux foreign chemicals back into the lumen (Nair, et al, 2007). Humans are well-equipped to metabolize plant compounds. Also, most of the plants used in traditional Turkish villages were extracted with water, which pulls hydrophilic chemicals out of the plants. The non-toxic aqueous extracts are more representative of what was actually ingested in villages than the methanolic extracts.

CONCLUSIONS

To answer the tested hypotheses: H1. Several Central Anatolian anti-diarrheal plants inhibited different strains of bacteria. H2. There was a marked difference in bioactivity between methanolic and aqueous extracts, with methanolic extracts inhibiting bacteria more frequently and at much lower concentrations than traditionally used

aqueous extracts. H3. Central Anatolian anti-diarrheal plants showed insignificant cytotoxicity to the cell lines in the Alamar Blue Cytotoxicity Assay, and are therefore safe for consumption. Regarding whether the data support the use of traditional medicines in Central Anatolia: H4. While several methanolic extracts inhibited bacteria, the more traditional aqueous extracts showed little inhibition when compared to methanolic extract activity. However, cytotoxicity levels were low. Therefore, plants were safe to ingest as treatments. The assay in this study did not detect anti-bacterial activity in aqueous extracts, but a different assay could elucidate bioactivities of aqueous extracts.

Plant extracts that showed the highest rates of inhibition against bacteria included *H. perforatum, R. coriaria, R. canina,* and *V. agnus-castus*. The methanolic extracts of these plants inhibited bacteria at concentrations as low as 4µg/ml (MIC of *R. canina* galls against *P. aeruginosa*). Further analysis of the *R. canina* galls has been undertaken and will be described in the following chapter.

Overall, seven plants used in traditional Turkish pharmacopeias to treat diarrhea showed antibacterial properties at clinically relevant levels (≤ 32µg/ml). Also, the plants appear safe for consumption. Promising results include the effectiveness of *U. dioica, T. polium*, and *V. album* in inhibiting *L. monocytogenes*. *Vitex agnus-castus* showed high inhibition in *K. pneumoniae*, which is a difficult bacteria to inhibit. Future studies that examine the effectiveness of *R. canina* galls or *R. coriaria* fruits against *P. aeruginosa* would benefit many patients, as would the study of *R. canina* gall extracts in inhibiting *V. parahaemolyticus*. Several results show promise for future work in bacterial inhibition of Turkish anti-diarrheal plants.

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Chapter V

IDENTIFICATION OF ELLAGIC ACID AS THE ANTI-BACTERIAL COMPONENT OF *ROSA CANINA* L. (DOG ROSE) GALLS

ABSTRACT

In the spring of 2009, I conducted ethnobotanical fieldwork with villagers of Central Anatolia, Turkey, and gathered information about plant-based remedies used to treat diarrhea. Fifteen of these plants were later evaluated in two microtiter-broth dilution assays for bacterial inhibition. Of the plants collected and tested, the crude methanolic extracts of galls from *Rosa canina* L. (Rosaceae) showed the lowest minimum inhibitory concentrations (MICs) (from 4 to 64μg/ml) against eight bacteria strains. Methanolic extracts of *R. canina* hips (fruits) were less potent (from 128 to 512μg/ml). Methanolic gall and fruit extracts were then tested for cytotoxicity and found to exhibit acceptable inhibitory concentrations (ICs) with liver, prostate, and skin cancer cell lines. Finally, *R. canina* fruit and gall extracts were fractionated by high-performance liquid chromatography (HPLC) and analyzed via bioassay-guided fractionation. Using ElectroSpray Ionization Mass Spectrometry (ESI-MS) the active fraction was shown to contain almost pure ellagic acid, a common yet under-studied phytoalexin—a plant defense compound that could be used for human medicines.

INTRODUCTION

For thirty years, international NGOs have worked to halt the millions of deaths per year resulting from diarrheal disease (WHO and UNICEF 2004, WHO 2009). Since the 1980s, the Turkish Ministry of Health has addressed this issue by teaming with WHO and other agencies to sponsor education campaigns for treating diarrhea (The Ministry of Health of Turkey 2004). Most efforts prescribe clean water, hygienic food preparations, and oral-rehydration-therapy (ORT) for treating symptoms. When diarrhea occurs, it usually is first treated at home by a family caregiver with plant-based remedies.

In previous ethnobotanical fieldwork, I described and analyzed the efficacy and cytotoxicity of plant-based remedies for treating diarrhea in rural areas of Turkey (Chapters II, III and IV). The methanolic extract of *R. canina* galls inhibited more bacteria strains at lower doses and was selected for further chemical analysis and bioassay-guided fractionation.

Turkish villagers commonly use *R. canina* to treat a variety of gastrointestinal disorders. The achene-filled pseudofruits of *R. canina* are called *kuşburnu* in Turkish, or rosehips in English. Rosehips from *R. canina* are used to treat abdominal pain, diarrhea, hemorrhoids, stomach aches, and kidney stones (Sezik et al., 2001, Tuzlacı and Aymaz, 2001, Yeşilada et al., 1995, Yeşilada et al., 1999). Rosehips are steeped in hot water and drunk as a tea, but infusions or decoctions of flowers, roots, root bark, and root tumors also are employed. Informants in the region of Çamlidere (100 km north of Ankara) described the use of *R. canina* galls, or fruit tumors, of *R. canina* for therapeutic infusions to treat diarrhea. *Rosa canina* vouchers (JR0023 and JR0032) were deposited in GAZI.

Rosa canina is a perennial, long-lived shrub which grows throughout the Northern Hemisphere (Shorthouse, 2005). It likely originated in Central Asia, as the area retains

the highest levels of diversity for several roses, including *R. canina* (Krussmann, 1982). Rosehips have been evaluated for their anti-nociceptive and anti-inflammatory properties in Turkey (Orhan et al., 2007). However, no publications analyzing the medicinal uses of the galls have been found.

Galls are induced in rosehips by cynipid wasps of the *Diplolepis* genus. Each species of wasp induces galls in different parts of the host plant, creating different morphological features (Redfern and Shirley, 2002). Galls used in this study occured in *R. canina* rosehips; only *Diplolepis fructuum* Rübsaamen (Hymenoptera: Cynipidae) is known to infect rosehips in Turkey. As much as 90% of the rosehips in areas of Central Anatolia are infected by *D. fructuum* each year, leading to economic hardships in the rosehip industry (Güçlü et al., 2008).

The interactions between gall-inducing wasps and their host species are not fully understood (Randolph, 2005), yet after eggs are deposited, large amounts of glycerolipids and phosphoglycerides are sequestered to the nutritive cells adjacent to the egg (Bayer, 1994). Nutritive cells of immature *Diplolepis spinosa* galls on *Rosa rugosa* displayed zones of inhibition when grown on nutrient agar plates seeded with *E.coli* or *Staphylococcus aureus* (Barrett et al., 1998). When grown in the same conditions, normal stem tissue from *R. rugosa* showed no bacterial inhibition.

Rosa canina galls were selected for analysis in this study because of their significant inhibition of multiple bacteria strains. The novelty of studying the biochemical products of an intriguing ecological relationship also led to this choice. The goal was to isolate and identify the chemical compound(s) responsible for the bioactivity demonstrated by *R. canina* gall extracts in previous antibacterial assays.

METHODS

Extract preparation

Using Clinical and Laboratory Standards Institute (CLSI) guidelines (Barry, et al. 1999), I prepared crude extracts from bulk samples collected during field work in Turkey (IRB Approval No. 082508-01). Galls and rosehips were collected from multiple individual plants. I made aqueous extracts via infusion (1g/15ml), filtration, freezing, and lyophilizing. Methanol extracts were made via a 72hr soak (1g/20ml) with agitation, filtration, rotary evaporation, and desiccation. Dried methanolic extracts were reconstituted in DMSO (10mg/ml) and aqueous extracts were reconstituted in PBS Solution (10mg/ml). Stocks were sterile filtered (0.2µm) and stored at -20^{o} or -5º C.

Anti-Bacterial Assays

Two microdilution assays were performed. The first assay used 10 bacterial strains from Turkish (Refik Saydam Kültür Koleksiyonu, RSKK) and American (American Type Culture Collection and Northern Regional Research Laboratory) institutes. Gram-negative strains included *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 10145), *Klebsiella pneumoniae* (RSKK 574), *Salmonella enteriditis* (RSKK 538), *Shigella dysenteriae* (RSKK 851), and *Vibrio cholerae* Ojawa (RSKK 96023). Gram-positive strains included *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (NRRL B–3711), and *Bacillus subtilis* (ATCC 6633). Bacteria cultures were grown in Cation-Adjusted Mueller Hinton Broth at 35ºC.

I serially diluted extracts in 96-well plates with concentrations ranging from 512µg/mL to 0.25µg/mL with controls for positive growth, media, vehicles, and negative

growth with antibiotics ampicillin, ciprofloxacin, gentamicin, nitrofurantoin, azithromicin, vancomycin, and TMP-SMX (Barry, et al. 1999). Bacteria were grown to log phase and suspended at 5 x 10^5 colony-forming units (CFU)/ml. Plates were incubated overnight. Assays were completed in triplicate. Wells displaying inhibition or no visible growth were further tested for bactericidal (MBC) or bacteriostatic (MBS) properties. From clear wells, 10µl of well contents were transferred to Mueller Hinton agar plates. After overnight incubation, CFU/ml were counted. If no new growth occurred, the previous extract concentration was deemed bactericidal. If bacteria grew, the extract concentration was bacteriostatic.

For the second assay, 21 strains of diarrhea-causing bacteria were challenged (Chapter IV). Bacteria were grown in Cation-Adjusted Mueller Hinton broth at 37^{o}C. Extracts were serially diluted from 512µg/mL to 0.25µg/mL in 96-well plates with growth, media, vehicle and antibiotic (ampicillin, ciprofloxacin, nitrofurantoin, azithromicin, and vancomycin) negative controls in triplicate. Bacteria were grown to log phase and diluted to 5 x 10^5 CFU/ml. The plates were read for optical density (OD) at 600 nm wavelength (Biotek PowerWave Spectrophotometer) at initial and peak times for each bacterial strain. Results were calculated as percent inhibitions and minimum inhibitory concentrations (MICs) for 50% lethality and 90% lethality. Statistical analyses were performed on PASW SPSS software. One-way ANOVAs with Tukey post-hoc tests were used to evaluate significant differences in categorical data, with significance set at $p<0.05$.

Cytotoxicity Assay

An Alamar Blue Cytotoxicity Assay (O'Brien et al., 2000) was used to determine the concentrations of crude extracts which inhibited human cell growth. Human cell lines included the prostate adenocarcinoma PC-3 (ATCC CRL-1435), the skin melanoma SKMEL-5 (ATCC HTB-70), and the hepatocellular carcinoma HEP G-2 (ATCC CRL-11997). Cells were grown in appropriate RPMI and Eagle's Medium, with 10% Fetal Bovine Serum and 1% antibiotics.

Cells were grown to confluence at 37^{o}C with 100% humidity and 5% carbon dioxide. Extracts were introduced in triplicate at a range of 512µg/mL to 4µg/mL in white, flat-bottomed 96-well plates and incubated for 18 hours. Spent media was removed and replaced with fresh broth containing 10% Alamar Blue (AbD Serotec BUF012B). As per the manufacturer's instructions, the plates were read after 4 hours of incubation in a Biotek Spectrophotometer at 595nm. Cytotoxicity was expressed as percent viability and statistically analyzed using 2-tailed t tests on PASW SPSS software.

Bio-assay Guided Fractionation

The HPLC analysis was performed on a Thermo Spectra-System HPLC apparatus, using a reverse phase C18 column. The gradient system was phase A: H_2O; phase B: acetonitrile. Flow rate was 1ml/min. This procedure was used to analyze the methanolic crude extracts of *R. canina* galls and fruits.

The methanolic crude extract of *R. canina* galls was separated into four fractions using HPLC (liquid phase = 0% ACN:100% H_2O to 100% ACN:0% H_2O). Each fraction was reconstituted in DMSO at 50g/ml and tested against only the bacteria that were inhibited in previous assays. Further spectroscopic analyses were conducted using

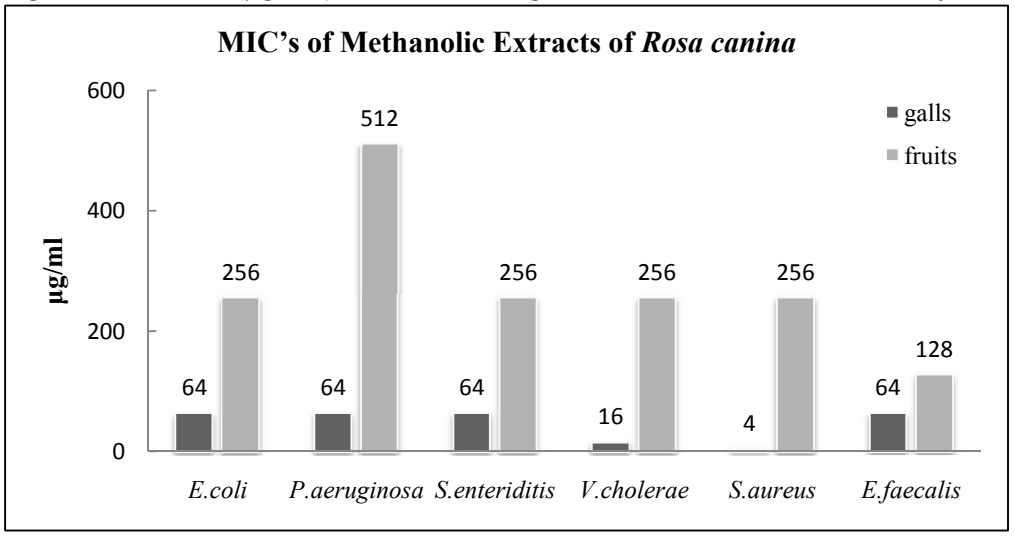
HPLC/UV/ESI-MSn with positive and negative ESI (ThermoFinnigan LCQ with electrospray ionization). The HPLC (Agilent 1100 series binary pump) was run with a Waters XTerra MS C18 column with a Phenomenex C18 guard column (2x4mm). The mobile phase was A: 0.2% acetic acid in H_2O; B: 0.2% acetic acid in Methanol. For ion detections, an Agilent 1100 G1314A UV/V was used with wavelengths at 254 and 280nm. After chemical analyses determined that the major constituent peak of the third fraction was ellagic acid, and the third fraction was consistently inhibiting the bacteria, another assay using pure ellagic acid (Sigma-Aldrich E2250) was conducted to determine the MICs of the pure compound. Ellagic acid precipitated into crystal form in aqueous broths but dissolved with slight heating.

RESULTS

Antibacterial Assay

The first anti-bacterial assay showed that *R. canina* gall methanolic extracts inhibited six bacteria (*E. coli, P. aeruginosa, S. enteriditis, Vibrio cholerae, Staphylococcus aureus,* and *Enterococcus faecalis*) at low concentrations (Figure 5.1). Clinical variants of these bacteria are antibiotic resistant.

Figure 5.1 MIC's (µg/mL) of Rose Galls against Bacteria from First Bioassay



In the second bioassay, *R. canina* galls and fruits inhibited fourteen strains of bacteria (Table 5.1). *Pseudomonas aeruginosa* was the most sensitive to methanolic rose gall extracts at 4µg/ml MIC, and *S. flexneri* was inhibited at 8µg/ml. *Vibrio parahaemolyticus* also was sensitive to methanolic rosehip extracts (32µg/ml MIC) and methanolic rose gall extracts (16µg/ml MIC). Methanolic extracts also showed inhibition against *L. monocytogenes* (rosehips at 128µg/ml MIC and galls at 64µg/ml MIC). *Enterococcus faecalis* and *S. enterica typhimurium* were inhibited at 32µg/ml. Other bacteria were inhibited at concentrations of 64µg/ml or above.

Table 5.1 Secondary assay MICs (µg/mL) of *Rosa canina* fruit and gall extracts

Extract	Bacterial Pathogens													
	Bs	**Ec**	**Ef**	**Kp**	**Lm**	**Pa**	**Se**	**St**	**Sa**	**Sf**	**Ss**	**Vp**	**Ye**	**Yu**
RoCF met	128	512			128						512	32		64
RoCF aq														256
RoCG met	256	64	32	256	64	4	128	32	256	8	256	16	256	64

Blank cells denote no inhibition.
RoCF=*Rosa canina* Fruits, RoCG=*Rosa canina* Galls; met=methanolic, aq=aqueous
Bacteria coded: Bs=*Bacillus subtilis*; Ec=*Escherichica coli*; Ef=*Enterococcus faecalis*; Kp=*Klebsiells pneumoniae*; Lm=*Listeria monocytogenes*; Pa=*Pseudomonas aeruginosa*; Se=*Salmonella enterica*; St=*S. enterica typhimurium*; Sa=*Staphylococcus aureus*; Sf=*Shigella flexneri*; Ss=*Shigella sonnei*; Vp=*Vibrio parahaemolyticus*; Ye=*Yersinia enterocoliticus*; Yu=*Y. pseudotuberculosis*

Cytotoxicity Assay

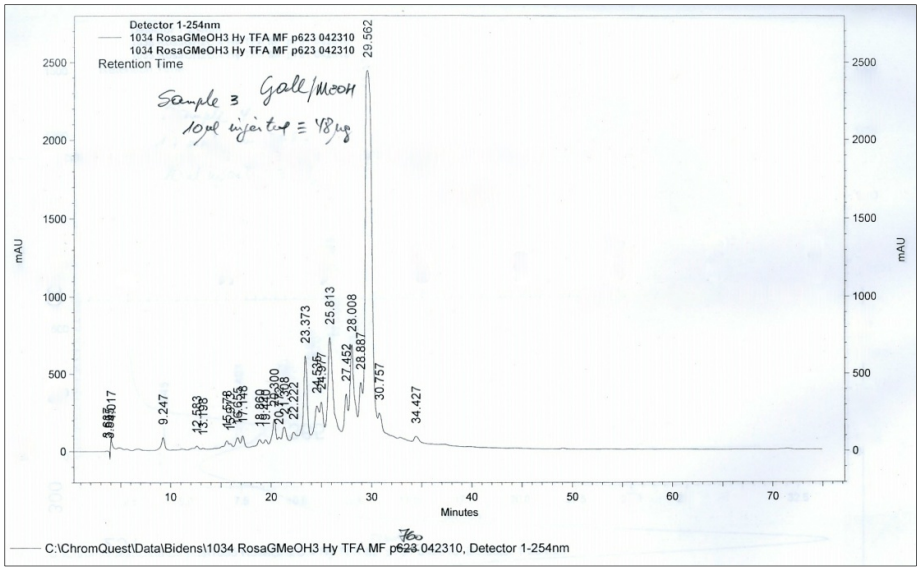
In the preliminary Alamar Blue assay, methanolic extracts of *R.canina* galls showed cytotoxicity of the prostate cell line at 50µg/ml and of the skin melanoma cell line at 100µg/ml. There was no cytotoxicity indicated for the hepatic HepG-2 cell line. Camptothecin showed toxicity in all cell lines at 5µg/ml. Considering the liver is the site for foreign chemical reduction and neutralization in the human body, inhibition of HepG-2 was considered a better indicator of cytotoxicity after ingestion of plant materials than inhibition of Sk-Mel or PC-3 cell lines.

Further evaluation of cytotoxic activity showed that aqueous and methanolic extracts of *R. canina* did not significantly inhibit human cell-line growth. The optical density (OD) readings for cell lines with *R. canina* extracts showed higher metabolic rates because of the growth and increase of cells. Therefore, after cytotoxicity assays, the *R. canina* extracts were considered ideal for further evaluation of their bacterial inhibition properties.

Bio-assay Guided Fractionation

Several peaks were seen in the HPLC of the *R. canina* gall methanolic extract. The peak at 29.562 min predominated the composition of the extract (Figure 5.2). The methanolic crude extract of *R. canina* galls was split into four fractions, with the first the most hydrophilic and the last the most hydrophobic. The third fraction showed inhibition in the repeated bioassays and was considered to hold the active component.

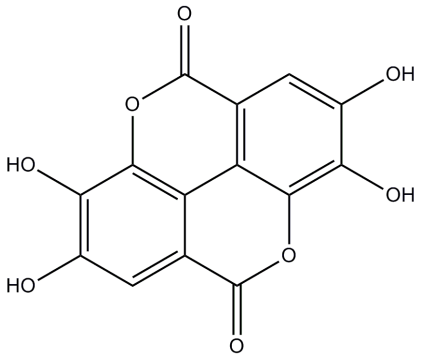
Figure 5.2 HPLC chromatogram of the methanolic *R. canina* gall extract



Fraction 3, which correlated with the peak retention time of 29.562 min. in the initial chromatogram, was further analyzed by HPLC/UV/ESI-MSn. A compound of molecular weight (MW) 302 was most abundant, with minor amounts of MW 434 and 464 monoglycosides of the MW 302 aglycone. Other compounds included larger compounds (MW 788, 936, and 1118) which most likely correlate with glycosides of gallic acid or ellagic acid, and might be gallotannins or ellagitannins. With UV analysis, ions characteristic of ellagic acid (Figure 5.3) were identified (m/z 284, 257, 229, 201,

and 185). The (-)ESI-MS/MS spectra did not match quercetin (MW 302.25), hesperetin (MW 302), morin (MW 302.25), or homoeriodictyol spectra (MW 302.29). Ellagic acid is a polyphenolic compound. Its molecular formula is $C_{14}H_6O_8$, MW 302, with IUPAC name 2,3,7,8-Tetrahydroxy-chromeno [5,4,3-cde]chromene-5, 10-dione.

Figure 5.3 Chemical structure of ellagic acid



After ellagic acid was determined by MS-ESI, the bioassay was repeated using an ellagic acid standard (Table 5.2). *Escherichia coli* Nat OH5 alpha and *Y. enterocolitica* O18 8081v with pYv were inhibited by ellagic acid at MIC_{50} 32µg/ml. *Yersinia enterocolitica* 8081c without pYv was minimally inhibited by ellagic acid, as was the case with *R. canina* extracts. At 64µg/ml, *L. monocytogenes* (ATCC19015) was inhibited more than 50%, as was *Y. pseudotuberculosis* III YP KmR. The PA 01 strain of *P. aeruginosa* showed MIC_{90} of 256µg/ml and MIC_{50} of 8µg/ml, a concentration that is low enough to be considered for clinical medications. Several bacterial strains were inhibited at 8µg/ml, including *S. aureus* (ATCC 29213) with an average percent inhibition of 78.5%, *V. parahaemolyticus* (ATCC 17802) with an average 75.9% inhibition, and *S. flexneri* (ATCC 9199) with an average 78.8% inhibition. The closely related *S. sonnei* (ATCC 25931), which often is less virulent than *S. flexneri*, was not inhibited by the ellagic acid except at high concentrations (512µg/ml).

Table 5.2 Bioassay Results (μg/ml) using Standard Ellagic acid

Bacterial Strains Previously Inhibited by *Rosa canina* gall MeOH Extracts	Pure Ellagic Acid MICs	
	MIC_{90}	**MIC_{50}**
Ec-0	512	32
Lm-2	128	64
Pa-1	256	8
Sa-4	256	<8
Sf-1	16	<8
Ss-1	512	-
St-4	512	128
Vp-1	16	<8
Ye-1	512	-
Ye-2	128	32
Yu-1	128	64

DISCUSSION

Ellagic acid explains inhibition of several diarrhea-causing bacteria in methanolic rose gall extract. Ellagic acid is a biphenyl lactone found in fruits and nuts, including fruits of the Rosaceae family such as strawberries, raspberries, and blackberries (Vattem and Shetty, 2005). Previous research has evaluated its antioxidant (Barch et al., 1995, Zhang et al., 1993), anti-carcinogenic (Chen et al., 2003, Kauer et al., 1997, Loarca-Pina et al., 1998, Narayanan et al., 1999, Teel et al., 1986), and anti-inflammatory properties (Gerritsen et al., 1995). With free radical scavenging, ellagic acid directly alleviates oxidative stress in cellular environments, but it can also stimulate cell pathways for reducing agents and increase antioxidant enzyme responses (Vattem and Shetty, 2005). As a biphenyl, ellagic acid is similar in structure to cell signaling molecules, is slightly hydrophobic and able to insert into lipid membranes, and acts as a chelator for sequestering metal ions (Vattem and Shetty 2005).

As a weak acid, ellagic acid can disassociate cell membranes, disrupt electrostatic gradients, reconfigure membrane proteins and receptors, and interrupt cell functions such as motility, nutrient uptake, ATP generation, and basic metabolism (Vattem and Shetty, 2005). Researchers have investigated the antibacterial properties of ellagic acid against *Staphylococcus aureus* (Akiyama et al., 2001), in combination with clove compounds against oral pathogens such as *Porphyromonas gingivalis, Streptococcus mutans, Actinomyces viscosus*, and *Prevotella intermedia* (Cai and Wu, 1996), and in synergistic combination with rosemary and cranberry compounds to prevent *Helicobacter pylori* urease activity (Lin et al., 2005) and to protect DNA from mutagenic toxins (Vattem et al., 2006). Like other polyphenolic compounds, ellagic acid increases the efficacy of other antioxidants synergistically (Shetty and Wahlqvist, 2004). Ellagic acid was found to significantly prevent biofilm formation of *E. coli w*ithout any bactericidal activity (Hancock et al., 2010). When a proton from ellagic acid's carboxyl or hydroxyl groups acidifies the microenvironments of bacteria, the overall ion charge is altered, allowing partially hydrophobic ellagic acid to insert into bacterial membranes and reconfigure enzymes used for bacterial pathogenicity such as urease (Lin et al., 2005).

Previous chemical research on rose galls has investigated the nutritional properties of the galls with respect to the needs of growing larvae (Hartley and Lawton, 1992, St. John and Shorthouse, 2000) and possible hormonal changes in the galls when compared to non-galled tissues (Schönrogge et al., 1998). A chemical analysis that focuses on medicinal, antibacterial properties of rose galls logically focuses on plant defense compounds. Injury to a plant leads to up-regulation of defense chemicals, known as the Hypersensitivity Response (HR). After trauma induces HR, secondary pathways might lead to Induced Systemic Resistance (ISR), which is a response to insect or herbivore

damage. Microbes initiate the Hypersensitivity Response HR and later induce the host's Systemic Acquired Resistance (SAR).

Derived from the shikimate and acetate-malonate biosynthetic pathways (Strack, 1997), ellagic acid is commonly elicited by SAR, or the microbe-induced response. Yet, in a gall, an insect is supposedly initiating the plant response. The rose might produce ellagic acid to prevent bacterial infection, even though *Diplolepis* wasps maintain a sterile gall environment (Randolph, 2005) by ovipositing and hatching between plant cells and delaying defecation until just before pupation in the spring. Randolph (2005) and Shorthouse (2005) postulate that there is a microbe in the egg, larva, or ovipositor of the female wasp which induces changes in plant phenolic compound production. Bacterial infection of wasps is exemplified in *Wolbachia*-induced parthenogenesis (Plantard, 1999), as females predominate the population.

Oak leaves with cynipid wasp galls have increased levels of tannins correlated with higher species diversity and abundance of wasps (Taper and Case, 1987). Tannins were hypothesized to protect cynipid larvae from fungal attack. More recent research found that concentrations of phenolic compounds, including tannins, increased in Cecidomyiidae galled leaves, and this prevented foliverous insects from eating the leaves later in the season (Pascual-Alvarado, et al., 2008). Tannins are closely related to ellagic acid, which forms hydrolysable ester bonds with glucosides in plant tissues, forming complex ellagitannins.

A possible explanation for ellagic acid production in response to galls is the control for oxidative stress from larvae chewing through cells while maintaining photosynthetic rates. In Brazil, researchers found decreased concentrations of chlorophylls and carotenoids in galled tissues but increased numbers of plastoglobules,

allowing for thylakoid membrane recovery and maintenance of comparable maximum electron transport rates (ETR_{MAX}) in galled tissues (Oliveira, et al. 2011).

Cynipid *Andricus palustris* wasp galls alter the composition and distribution of two types of host plant cell membrane lipids—glycerolipids and phosphoglycerides (Bayer, 1994). Since polyphenolic compounds like ellagic acid are able to acidify cellular environments, alter electrochemical gradients, and embed within lipid membranes, perhaps ellagic acid functions as a signal or regulator for nutrient sink activities in the galled host tissues while also preventing infection or herbivory. Ellagic acid can prevent tumorigenic, mutagenic, and carcinogenic activity in human cells, and might be used in a similar manner in plant cells.

Better understanding the functions and properties of plant chemicals in their original plant cell environment and later in the human (or other herbivore) body would facilitate medicinal research on beneficial phytochemicals. Whether ellagic acid production in rose galls is induced by an insect, an unknown microbe, or the innate plant defense response, it has multiple potential benefits for treating and preventing human infectious disease as well as plant pathogens and herbivory. Gall ecology might also be used to increase desirable compounds for the biopharmaceutical or herbal supplement industry.

CONCLUSIONS

Rosa canina galls are used in decoctions to treat gastrointestinal illness and diarrhea in Central Anatolian villages. While rosehips are a common medicinal remedy for a variety of ailments, the galls of the rosehips are not used as often. After evaluation in antibacterial bioassays involving 10 and 21 bacteria and a cytotoxicity screening, the methanolic extracts of *R. canina* galls, and not rosehips, were able to significantly inhibit bacterial growth in *B. subtilis, E. coli, P. aeruginosa*, *S. aureus, S. flexneri, S. enterica typhimurium, V. cholerae, V. parahaemolyticus,* and *Y. enterocolitica.*

Using bioassay-guided fractionation techniques, an antibacterial fraction was identified and found to be primarily composed of ellagic acid when analyzed by ESI-MS. The bioassay was performed again with 100% ellagic acid against previously inhibited bacteria, with comparable results to the MIC's of the methanolic extract of *R. canina* galls. Bacteria that were highly inhibited by ellagic acid (at 8μg/ml or lower MIC_{50}'s) included *V. parahaemolyticus, P. aeruginosa, L. monocytogenes,* and *S. aureus*.

The preceding laboratory research was the culmination of a process of ethnobotanical inquiry. Such techniques are promising for the discovery of effective, safe plants that can be used to treat bacterial infectious diseases forthright, or with chemical isolation and manipulation after overarching mechanisms of action are described.

Future research with *R. canina* galls will involve further evaluation of the biochemistry of the galls and fruits to better understand the intricate relationships between cynipid wasps, roses, and possible microbes. How these relationships induce defensive chemicals, or phytoalexins, that are inhibitory of bacterial growth should be further investigated, along with the *in vivo* functions of ellagic acid within plants.

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APPENDICES

Appendix 1: Survey Instrument in Turkish

Türkiye'de ishal için kullanilan bitkisel

Merhaba. Bize yardim ettiginiz için çok tesekkür ederiz. Biz Ankara Gazi Üniversitesi'nde ögrenciyiz. Bu bilgiyi okul kimliklerimizden kontrol edebilirsiniz. Lütfen asagidaki sorulara sizin için uygun olan cevabi veriniz. Sizden herhangi bir kimlik bilgisi istemiyoruz. Sadece düsüncelerinizi yazmanizi istiyoruz.

Yasiniz:
Cinsiyetiniz:
Memleketiniz:
Medeni Haliniz: Evli Bekâr Bosanmis Dul Diger
Mesleginiz:
Çocuklariniz var mi? Evet Hayir
Torunlariniz var mi? Evet Hayir
Kaç senedir burada yasiyorsunuz?
Buradan önce baska bir yerde yasadiniz mi?
Eger yanitiniz evetse kaç yil baska yerde yasadiginizi belirtiniz.
Etnik kökeniniz nedir?

Çalisma alanimiz Türkiye'de ishal için kullanilan bitkisel tedavi yöntemleri ve diger dogal yöntemlerle ilgili uygulamalar. Asagidaki sorulari cevaplarsaniz çok seviniriz.

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1. "Ishal" disinda bu hastalik için kullandiginiz baska sözcükler var mi? Varsa neler?
2. Sizce neden insanlar ishal olur?
3. Bildiginiz ishal çesitleri var mi? Varsa bu farklari belirtebiliri misiniz?
4. Daha önce ishal tedavisi için hiç bitkisel ya da dogal baska bir yönteme basvurdunuz mu?

Cevabiniz "hayir"sa anketimiz burada bitmistir. Tesekkür ederiz.

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5. Ishal tedavisinde kullandiginiz bitki ve yiyecekleri nelerdir?
6. Sizce ishale en iyi gelen bitki nedir?
7. Bu bitkiye kolaylikla ulasabiliyor musunuz? Ulasamiyorsaniz ikinci tercihiniz nedir?

8. Bu bitkinin ishale iyi geldigini nereden ögrendiniz? (büyüklerinizden, annenizden,
babanizdan, dergilerden, televizyondan, diger)
9. Ishal hakkinda bildiklerinizi baskalarina da anlatiyor musunuz? (Örnegin: Komsunuzun
çocugu hasta olsa ona da bunu tavsiye eder misiniz?) Yanitiniz evetse bunu en çok nerelerde
kullaniyorsunuz.
10. Bu bitkileri kendiniz mi topluyorsunuz?
Yanitiniz hayirsa lütfen 18. Soruya geçiniz. Evetse 11. soruyla devam ediniz.

11. Hangi bitkileri topluyorsunuz?
12. Bitki toplamaya tek basiniza mi yoksa grup halinde mi gidiyorsunuz?
Birileriyle birlikte gidiyorsaniz kimlerle gittiginizi belirtiniz.
13. Genellikle bu bitkileri toplamak için nereye gidersiniz?
14. Yilin hangi zamani bu bitkileri toplarsiniz?
15. Bitkinin hangi parçasini toplarsiniz?
16. Topladiginiz bitkileri nasil saklarsiniz. (Kurutarak, asarak, konserve yaparak diger)
17. Topladiklarinizi kimler kullanir. (müsteriler, aile vs)
18. Eger kullanacaginiz bitkiyi kendiniz toplamiyorsaniz nereden elde edersiniz? (arkadaslardan,
aktardan, diger)
19. Bitkiyi tedavi amaçli kullanima nasil hazirliyorsunuz? (kaynatarak, pisirerek vs)
20. Ilaci hazirlarken ne kadar bitki kullaniyorsunuz?
21. Hasta kisi bu tedaviyi ne kadar zaman uygulamali?
22. Hiç bu konuda bir uzmana gider misiniz? Ne zaman?
23. Eger ishal hastasi bir çocugu ya da kendi çocugunuzu görürseniz tedavi olarak ne yaparsiniz?
24. Bir çocuga uyguladiginiz tedavi ile kendinize ya da daha yasli birisine uyguladiginiz tedavi
arasinda bir fark var mi?
25. Bu konuda söylemek istediginiz baska bir seyler var mi?

Anketimiz bitti tesekkür ederiz.

Appendix 2: Survey Instrument Back-translated into English

Hello. Thank you for assisting us in our research today. We are graduate students from Gazi University in Ankara, and we would like to ask you some questions. You may speak with us if you like, but you are under no obligation. Please feel free to ask us questions as well. Your answers will be recorded, but we will not record your name or any contact information so that you will remain anonymous in our research.

Age:
Nationality:
Village or Hometown:
Marital Status: Married Single Divorced Widowed Other
Occupation:
Do you have children? Yes No
Do you have grandchildren? Yes No
How long have you lived here?
If you've lived somewhere else, where was it?
If you've lived somewhere else, how many years were you there?
What ethnicity would you call yourself?

The following questions ask about herbal remedies used to treat diarrhea.

1. What is a common term you use for "diarrhea"? Do you use other names?
2. When someone you know gets diarrhea, what do you do?
3. Are there different kinds of diarrhea? What would they be?
4. Have you ever used a plant-based remedy to treat diarrhea?

If you answered "no" to question 4, then there are no more questions. Thank you for your time.

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5. Which plants or foods do you use to treat diarrhea?
6. What is the best herb or food for treating diarrhea?
7. Is your favorite herb easy to obtain? If you can't find it, is there something else you use?
8. Where did you learn about these herbs? (books, your parents, your friends, TV, Other)
9. If you learn that a friend of yours has diarrhea, what would you advise? (Or, if one of your children is sick with diarrhea, what would you do?)
10. Do you collect anti-diarrheal plants?
    If no, please skip to question 18. If yes, continue to question 11.
11. Which plants do you collect?
12. Do you collect these plants alone or in a group? With who would you go?
13. Generally, from where do you collect this plant?

14. In what season do you collect it?
15. Which parts of the plant do you collect?
16. How do you store it once it's collected? (dry, cook, don't do anything to it)
17. Do you share the plant with anyone? (Neighbors, Family, etc...)
18. If you do not collect the plant yourself, from where do you get it? (Friends, Store, other...)
19. How do you prepare this plant if someone is sick with diarrhea? (steam, tea, etc...)
20. How much of the plant or its preparation do you give to the patient?
21. For how long do you give the plant or its preparation to the patient?
22. Do you ever go to the doctor to treat diarrhea? When?
23. If a child is sick with diarrhea, do they get sick in a different way?
24. Is a child given the same plants and doses?
25. Do you have anything else you'd like to add?

Thank you so much for your help.

Appendix 3. Literature Review Results for Turkish Anti-Diarrheal Plants

Species (Family)	**Common name(s)**	**Voucher or Locale**	**Part Used**	**Preparation**	**Source**	**Area**
Achillea millefolium L. (Asteraceae)	Amelotu	13	Fl	Diarrhea; Infusion, internal	Yeşilada, et al. 1993	Mediterranean
Achillea millefolium L. ssp. m*illefolium* (Asteraceae)	Akbaşotu, Ayvadanası	12	Hb	For abdominal pain, diarrhea, eaten fresh or dried, decoction	Honda, et al. 1996	West Anatolia
Adiantum capillus-veneris L. (Pteridaceae)	İshalotu	8600049	Lf	Leaf pounded, mixed with flour, given to calf for diarrhea	Tabata et al, 1988	Artvin, Borçka, Muratlı
Ajuga chamaepitys (L.) Schreb. ssp *chia* Arcang. var. *ciliata* Banks & Sol. (Lamiaceae)	Kiraçotu	25	Hb	For diarrhea, internal disease, hemorrhoids, internal, decoction	Yeşilada, et al. 1995	South Anatolia, Taurus Mts
Asparagus acutifolius L. (Asparagaceae)	Zamparna	3	Rt	Dysentery, severe diarrhea; Decoction, keep one night in cool place, internal	Yeşilada, et al. 1993	Mediterranean
Bellis perennis L. (Asteraceae)	Koyun gözü	24, 30, 43	Fl	For diarrhea, as diuretic, purgative, inf, internal	Özgökçe & Özçelik 2004	East Anatolia
Camellia sinensis (L.) Kuntze (Theaceae)	Çay	4, 13	Lf	To stop diarrhea; a teaspoonful of tea leaves is drunk	Yeşilada, et al. 1999	Northwest Anatolia

Camellia sinensis (L.) Kuntze (Theaceae)		13	Lf	For diarrhea, pounded with honey	Yeşilada, et al. 1995	South Anatolia, Taurus Mts
Camellia sinensis (L.) Kuntze (Theaceae)	Çay	94407	Lf	Powdered black tea leaves eaten	Honda, et al. 1996	Central Anatolia, Kayseri, Akkışla, Ortaköy
Capsella bursa-pastoris (L.) Medik (Brassicaceae)	Çobançantası	513	All	For diarrhea (dysmenorrhea), infusion, internal	Aslan, *et al.* 2007	Izmir (Ödemiş)
Cedrus libani A. Rich. (Pinaceae)	Sedir ağacı	9	Tr	For abdominal pain, diarrhea, external poultice, internal, one drop in glass of water	Yeşilada, et al. 1995	South Anatolia, Taurus Mts
Celtis australis L. (Cannabaceae)	Kara çıtlık	43	Fr, Lf	For diarrhea, especially children, decoction, eaten before meals, internal	Tuzlacı & Sadikoğlu 2007	Koçarlı (Aydın)
Centaurea cyanus L. (Asteraceae)	Mavi süpürge çiçeği		Fl	For diarrhea, infusion 3 x 1, internal	Tuzlacı & Alparslan 2007	Babaeski (Kırklareli)
Centaurium erythraea Rafn. ssp. t*urcicum* (Velen.) Melderis (Gentianaceae)	Kantariye	54	All	For diarrhea, whole plant used in decoction, internal	Tuzlacı & Tolon 2000	Şile (Istanbul)
Centaurium pulchellum (Sw.) Druce (Gentianaceae)	Kantaron	54	All	For diarrhea, whole plant used in decoction, internal	Tuzlacı & Tolon 2000	Şile (Istanbul)

Cicer arietinum L. (Fabaceae)	Leblebi, Nohut	94386	Bn	Roasted beans eaten, leblebi for diarrhea	Honda, et al. 1996	Central Anatolia, Kayseri, Akkışla, Ortaköy
Coffea arabica L. (Rubiaceae)	Kahve	94386	Fr	Crushed, powdered beans eaten to treat diarrhea	*Honda, et al. 1996*	Central Anatolia, Kayseri, Akkışla, Ortaköy
Cornus mas L. (Cornaceae)	Kızılcık		Fr	For diarrhea, decoction, internal	Tuzlacı & Alparslan 2007	Babaeski (Kırklareli)
Cornus mas L. (Cornaceae)	Kızılcık pestili, Kızılcık ekşısı	15, 17	Fr	For diarrhea, internal, fruits boiled to paste, spread on cloth, dried in sun. Dried sheets for winter. Fresh or boiled fruits	Sezik, et al. 1997	East Anatolia
Cornus mas L. (Cornaceae)	Kızılcık	13	Fr	For diarrhea, internal, boiled and condensed to paste	Fujita, et al. 1995	Mid & West Black Sea
Cornus mas L. (Cornaceae)	Kiren	15	Fr	To treat diarrhea; stewed fruits are eaten	Yeşilada, et al. 1999	Northwest Anatolia
Cota austriaca (Jacq.) Sch.Bip. (Asteraceae)	Akbabatça	6	Fl	For abdominal pain, diarrhea, infusion, tea	Honda, et al. 1996	West Anatolia
Cydonia oblonga Mill. (Rosaceae)	Ayva	2	Lf	For diarrhea; decoction is used as tea	Sezik, et al. 2001	Central Anatolia
Cydonia oblonga Mill. (Rosaceae)	Ayva	4	Lf	For diarrhea, internal, decoction	Sezik, et al. 1997	East Anatolia
Cydonia oblonga Mill. (Rosaceae)	Ayva	4	Fr	To stop diarrhea; fruits are eaten	Yeşilada, et al. 1999	Northwest Anatolia

Cydonia oblonga Mill. (Rosaceae)	Ayva	8	Lf	For common colds, flu, diarrhea; fresh leaves are collected early in summer, on June20th, hung on rope, dried in shade, used in winter. Infusion, tea.	Yeşilada, et al. 1999	Northwest Anatolia
Daphne pontica L. (Thymelaeaceae)	Tasma	12	Bk	To stop diarrhea; the bark of the stem is applied to the abdomen and kept until dried	Yeşilada, et al. 1999	Northwest Anatolia
Ficus carica L. (Moraceae)	Kuru ıncır	2	Fr	For diarrhea, internal with milk	Sezik, et al. 1997	East Anatolia
Glycyrrhiza glabra L. (Fabaceae)	Sus	2	Rt	For diarrhea, or cough, internal, decoction	Sezik, et al. 1997	East Anatolia
Glycyrrhiza sp. (Fabaceae)	Süs	9000378	Rt	Root decocted, taken for cough and diarrhea	Tabata, et al 1993	Ağrı, Tutak, Geçimli
Helichrysum plicatum ssp. *polyphyllum* P.H. Davis & Kupicha (Asteraceae)	Kaymak çiçeği		Fl	For diarrhea, intestinal disease; internal, decoction, 1 glass a day, for 2-3 days	Sezik, et al. 1997	East Anatolia
Hypericum perforatum L. (Hypericaceae)	Kantıron, Kangran otu	1	Fl	For diarrhea, decoction, internal	Tuzlacı & Alparslan 2007	Babaeski (Kırklareli)
Hypericum perforatum L. (Hypericaceae)	Kantaron çiçeği	516	Fl	For diarrhea (arteriosclerosis, parasites, ulcers)	Aslan, et al. 2007	Izmir (Ödemiş)
Juniperus drupacea Labill. (Cupressaceae)	Andız	7	Tr	For diarrhea, according to patient's age,	Yeşilada, et al. 1995	South Anatolia, Taurus Mts

Lycopersicon esculentum Mill. (Solanaceae)	Domates	94567	Fr	Fruit juice drunk to stop diarrhea	Honda, et al. 1996	Central Anatolia, Konya, Halkapınar, Büyük Doğan
Mespilus germanica L. (Rosaceae)		22	Fr	For diarrhea; dried fruits are eaten	Yeşilada, et al. 1999	Northwest Anatolia
Micromeria myrtifolia Boiss. & Hohen. (Lamiaceae)	Amelotu	537	Ae	For diarrhea, infusion, internal	Aslan, et al. 2007	Izmir (Ödemiş)
Papaver rhoeas L. (Papaveraceae)	Gelincik	1	Fl	For diarrhea, infusion, internal	Tuzlacı & Alparslan 2007	Babaeski (Kırklareli)
Papaver rhoeas L. (Papaveraceae)	Gelincik	22	Fl	For diarrhea, infusion, internal	Tuzlacı & Sadikoğlu 2007	Koçarlı (Aydın)
Pinus brutia Ten. (Pinaceae)	Çam	4	Fr	For diarrhea; decoction kept overnight in cool place, internal	Yeşilada, et al. 1993	Mediterranean
Pinus brutia Ten. (Pinaceae)	Çam	10	Tr	For bloating and diarrhea; add olive oil, onion; external, on abdomen	Yeşilada, et al. 1993	Mediterranean
Pinus nigra J. F. Arnold ssp. *pallasiana* (Lamb.) Holmboe (Pinaceae)	Çam kabuğu	12	Bk	For diarrhea, ground on stone mortar, mixed with yogurt, eaten	Yeşilada, et al. 1995	South Anatolia, Taurus Mts

Pinus nigra J. F. Arnold ssp. *pallasiana* (Lamb.) Holmboe (Pinaceae)	Bise	4	Tr	For abdominal pain, diarrhea, internal, one drop in glass of water	Yeşilada, et al. 1995	South Anatolia, Taurus Mts
Pinus sp. (Pinaceae)	Çam Katranı	8600274	Tr	Small pieces of onion mixed with tar and olive oil for diarrhea; applied to abdomen for cold in stomach	Tabata et al, 1988	Konya, Akseki, Çimi köy, Kuyu District
Pinus sp. (Pinaceae)	Karacaotu	9100406	Tr	Tar applied on abdomen, or one drop in glass of water, drunk against abdominal pain or diarrhea	Tabata, et al 1993	Konya, Hadım, Dolhanlar
Punica granatum L. (Punicaceae)	Nar	10	Pe	For diarrhea; dried rind of the fruit is ingested	Sezik, et al. 2001	Central Anatolia
Pyracantha coccinea M. Roem. (Rosaceae)	Yemişen	91	Lf	For diarrhea (in humans or animals), decoction, internal, 1 x 1, used cold	Tuzlacı & Aymaz 2001	Gönen (Balıkesir)
Pyrus elaeagnifolia Pall. (Rosaceae)	Kirahlatı	9	Fr	For diarrhea, ingest dried fruits	Honda, et al. 1996	West Anatolia
Pyrus elaeagnifolia Pall. (Rosaceae)	Çördük	23	Fr	For diarrhea, dried, eaten, intenra	Fujita, et al. 1995	Mid & West Black Sea
Pyrus elaeagnifolia Pallas (Rosaceae)	Ahlat	10	Fr	For diarrhea; stewed fruits	Sezik, et al. 2001	Central Anatolia

Pyrus elaeagnifolia Pallas (Rosaceae)	Bozahlat	32	Fr	For diarrhea; fresh or dried fruits are eaten or decoction as tea	Sezik, et al. 2001	Central Anatolia
Pyrus spinosa Forssk. (Rosaceae)	Ahlat		Fr	For diarrhea, eaten fresh	Tuzlacı & Bulut 2007	Ezine (Çanakkale)
Quercus coccifera L. (Fagaceae)	Bodur ağaç, Pelit, Pinar	1	Br	For diarrhea, decoction, internal, 1 x 1	Tuzlacı & Sadikoğlu 2007	Koçarlı (Aydın)
Quercus ithaburensis Decne. ssp. *macrolepis* (Kotschy) Hedge & Yalt. (Fagaceae)	Meşe pelit	46	Sd	For diarrhea, crush and cook seeds, internal	Tuzlacı & Sadikoğlu 2007	Koçarlı (Aydın)
Quercus petraea (Matt.) Liebl. ssp. *iberica* (Steven *ex* M. Bieb.) Krassiln. (Fagaceae)	Meşe	35	Bk	For diarrhea, decoction, internal	Tuzlacı & Alparslan 2007	Babaeski (Kırklareli)
Quercus sp. (Fagaceae)	Meşe	9000084	Gl	Gall or *mesemazisi* pounded and 5-10 g of powder mixed with albumen, orally for diarrhea	Tabata, et al 1993	Muğla, Göktepe, Taşlı, Osman Kara
Rheum ribes L. (Polygonaceae)	Iskin, Ribes	9, 11, 46	Rt	For diabetes, diarrhea, anthelmintic, decoction, tea	Özgökçe & Özçelik 2004	East Anatolia

Rheum ribes L. (Polygonaceae)		36, 44	Sd	For diarrhea, decoction, internal	Özgökçe & Özçelik 2004	East Anatolia
Rheum ribes L. (Polygonaceae)	Işgın	1	Rt	Ulcer, Diarrhea, Anthelmentic; Decoction, internal	Tabata, et al. 1994	East Anatolia
Rheum ribes L. (Polygonaceae)	Işgın, Revas	5	Rt	Diarrhea in animals; Poultice, internal	Tabata, et al. 1994	East Anatolia
Rheum ribes L. (Polygonaceae)	Işgın	2	Rt, Sd	Diarrhea; Decoction, internal	Tabata, et al. 1994	East Anatolia
Rhus coriaria L. (Anacardiaceae)	Sumak	1	Fr	For diarrhea—powdered fruits sprinkled on boiled egg, ingested	Sezik, et al. 2001	Central Anatolia
Rhus coriaria L. (Anacardiaceae)	Somak		Fr	For dysentery, diarrhea, boiled with water and sat, eaten	Honda, et al. 1996	West Anatolia
Rosa canina L. (Rosaceae)		37, 41	Tr, Rt	For hemorrhoids and diarrhea; decoction as tea	Sezik, et al. 2001	Central Anatolia
Rosa canina L. (Rosaceae)	Sıtmagülu, Kuşburnu	17	Fr	For diarrhea (malaria, hemorrhoids, hepatitis, stomach ache, bronchitis), decoction, internal	Tuzlacı & Aymaz 2001	Gönen (Balıkesir)
Rosa canina L. (Rosaceae)	Kuşburnu	11	Fl	For abdominal pain, diarrhea, decoction	Yeşilada, et al. 1999	Northwest Anatolia
Rosa canina L. *x R. heckeliana* Tratt. (Rosaceae)	İtburnu	15	Rt	For abdominal pain, diarrhea, decoction	Yeşilada, et al. 1995	South Anatolia, Taurus Mts

Rosa sempervirens L. (Rosaceae)	Sıtmagülu, Kuşburnu	17	Fr	For diarrhea (malaria, hemorrhoids, hepatitis, stomach ache, bronchitis), decoction, internal	Tuzlacı & Aymaz 2001	Gönen (Balıkesir)
Rubus canescens DC. (Rosaceae)	Karantı, Karamuk	17	Fr	For diarrhea (antiemetic, hemorrhoids, anaemia, asthenopia, woundes), fruits eaten	Tuzlacı & Aymaz 2001	Gönen (Balıkesir)
Rubus sp. (Rosaceae)	Böğürtlencik	8600294	Rt	Decoction taken for diarrhea	Tabata et al, 1988	South Anatolia, Taurus Mts, Antalya, Elmalı, Büyük Söğle
Rubus ulmifolius Schott (Rosaceae)	Karantı, Karamuk	17	Fr	For diarrhea (antiemetic, hemorrhoids, anaemia, asthenopia, woundes), fruits eaten	Tuzlacı & Aymaz 2001	Gönen (Balıkesir)
Rumex conglomeratus Murray (Polygonaceae)	İlabada	11	Sd	For diarrhea, decoction, internal	Yeşilada, et al. 1993	Mediterranean
Rumex patienta L. (Polygonaceae)	Enikmancar	23, 17, 18	Sd	To stop diarrhea, decoction of seeds, leaf used Guylek, Efelek	Sımsek, et al.2004	Ankara area
Salvia fruticosa Mill. (Lamiaceae)	Ada çayı, Moşapla	21	Lf	For stomach ailment, diarrhea, decoction, internal	Tuzlacı & Bulut 2007	Ezine (Çanakkale)
Sorbus domestica L. (Rosaceae)	Övez	25	Fr	For diarrhea, 5-10 pieces of fresh fruit eaten	Sımsek, et al.2004	Ankara area
Sorbus domestica L. (Rosaceae)	Hurma		Frt	For diarrhea, internal, eaten	Sezik, et al. 1997	East Anatolia

Sorbus domestica L. (Rosaceae)	Uvaz	2, 4	Fr	To stop diarrhea; red fruits are eaten	Yeşilada, et al. 1999	Northwest Anatolia
Sorghum cernuum Willd. (Poaceae)	Gilgil	25	Fr	For diarrhea, roasted, millet eaten	Yeşilada, et al. 1995	South Anatolia, Taurus Mts
Stachys sp. (Lamiaceae)		94578	Hb	Decoction used as tea against colitis	Honda, et al. 1996	Konya, Halkapınar, Büyük Doğan
Teucrium polium L. (Lamiaceae)	Merven	2	Hb	For diarrhea, internal, decoction, also hemorrhoids	Sezik, et al 1997	East Anatolia
Teucrium polium L. (Lamiaceae)	Acı yavşan	18	Hb	For abdominal pain, diarrhea, high fever, decoction, internal	Yeşilada, et al. 1995	South Anatolia, Taurus Mts
Tribulus terrestris L. (Zygophyllaceae)	Dadaşotu	28	Hb	For diarrhea, internal, decoction	Sezik, et al. 1997	East Anatolia
Unknown (Poaceae)	Gilgil, Süpürgedarısı	9000213	Sd	Seeds roasted, milled, mixed with poultice of *komec*, *Malva neglecta*, taken for diarrhea	Tabata, et al 1993	Maraş, Andırın, Darıovası, Ballarobası mah, Dede bal
Urtica dioica L., *U. urens* L. (Urticaceae)	Gezerek, Yığınç	8	Lf	Diarrhea; Tea, internal	Tabata, et al. 1994	East Anatolia

Urtica sp. (Urticaceae)	Gezerek yığınç	8600147	Lf	Decoction for diarrhea	Tabata et al, 1988	Northwest Anatolia, Bitlis, Sibek, Aridağ, Yanıkçay
Urtica urens L. (Urticaceae)	Isırgan otu	all	Lf	For diarrhea, tea, internal	Özgökçe & Özçelik 2004	East Anatolia
Verbascum sp. (Scrophulariaceae)	Mosi jehri	8600065	Rt	Root boiled, cooled, 1 Tbs taken every AM 1-2 days for diarrhea	Tabata et al, 1988	East Anatolia, Van, Gevaş
Vicia ervilia (L.) Willd. (Fabaceae)	Burçak	9	Sd	For diarrhea, pounded with honeycomb and eggs, pills	Honda, et al. 1996	West Anatolia
Viscum album L. var. *album* (Santalaceae)	Güveltek otu	10	Hb	For diarrhea; plant collected from pear tree is dried and pounded with honey and ingested	Sezik, et al. 2001	Central Anatolia
Vitex agnus-castus L. (Lamiaceae)	Ayıt	29	Fr, Lf	For diarrhea, internal, Leaves for nausea, headache	Tuzlacı & Bulut 2007	Ezine (Çanakkale)

Ae=Aerial parts, All=Whole plant, Bk=Bark, Bn=Bean, Br=Branches, Fl=Flowers, Fr=Fruits, Hb=Herb, Lf=Leaves, Pe=Pericarp, Rt=Root, Sd=Seed, Tr=Tar

Appendix 4. Antibacterial Activity of Extracts (1-34) and Controls (MICs in µg/ml, minimum bactericidal/-static (MBC)/(MBS) concentrations in µg/ml, and plated CFU's)

	Gram negative					
	Escherichia coli ATCC 35218			*Pseudomonas aeruginosa* ATCC 10145		
Extracts	**MIC**	**MBC/MBS**	**CFU/mL**	**MIC**	**MBC/MBS**	**CFU/mL**
1. AC MeOH	128	512/128	$1.1x10^5$	64	-/≥128	$1.8x10^5$
2. AC H_2O	128	512/≥128	$1.2x10^5$	64	-/≥128	$6.5x10^4$
3. AM MeOH	128	512/128	$7.8x10^4$	64	-/≥128	$3.1x10^4$
4. AM H_2O	128	-/≥128	TNC	64	-/≥128	$1.1x10^5$
5. CO MeOH	128	512/128	$1.8x10^5$	64	-/≥128	$4.9x10^4$
6. CO H_2O	128	512/≥128	$9.2x10^4$	64	-/≥128	$4.5x10^4$
7. HP MeOH	128	512/128	$1.7x10^5$	64	-/≥128	$4.0x10^4$
8. HP H_2O	128	512/≥128	$7.0x10^4$	64	-/≥128	$3.0x10^5$
9. ML MeOH	128	512/128	$2.7x10^5$	64	-/≥128	$4.7x10^4$
10. ML H_2O	128	512/≥128	$1.9x10^5$	128	-/≥128	$7.4x10^4$
11. PR MeOH	128	512/128	$8.2x10^5$	128	-/≥128	$6.2x10^4$
12. PR H_2O	128	512/≥128	$7.2x10^4$	128	-/≥128	$5.4x10^4$
13. RhC MeOH	128	-/128	TNC	128	-/≥128	$3.0x10^4$
14. RhC H_2O	128	-/≥128	TNC	128	-/≥128	$4.9x10^4$
15. RoC MeOH	128	-/128	TNC	128	-/≥128	$2.6x10^4$
16. RoC H_2O	128	-/≥128	TNC	128	-/≥128	$5.5x10^4$
17. RoCG MeOH	64	-/≥64	TNC	64	128/64	$1.5x10^4$
18. RoCG H_2O	128	512/128	$8.0x10^4$	128	-/128	$2.0x10^4$
19. RPF MeOH	128	512/≥128	$9.5x10^4$	128	-/128	$9.0x10^4$
20. RPF H_2O	128	512/128	$6.0x10^4$	128	-/≥128	TNC
21. RPL MeOH	128	512/≥128	$1.0x10^4$	128	-/≥128	TNC
22. RPL H_2O	128	512/128	$5.4x10^4$	128	-/≥128	$6.4x10^4$
23. TF MeOH	128	-/≥128	TNC	64	-/≥64	TNC
24. TF H_2O	128	512/128	$1.9x10^4$	128	-/≥128	TNC
25. TP MeOH	128	512/≥128	$9.2x10^4$	64	≥128/-	-
26. TP H_2O	128	512/128	$1.7x10^5$	64	≥128/-	-
27. TT MeOH	128	-/≥128	TNC	64	≥256/≥64	-
28. TT H_2O	128	-/128	TNC	64	256/≥64	-
29. UD MeOH	128	-/≥128	TNC	64	≥128/-	-
30. UD H_2O	128	-/128	TNC	64	256/≥64	-
31. VAA MeO	128	512/≥128	$2.5x10^5$	64	256/≥64	-
32. VAA H_2O	128	512/128	$6.0x10^4$	64	≥128/64	-
33. VAC MeOH	64	≥128/≥64	$2.3x10^4$	64	256/≥64	-
34. VAC H_2O	64	-/≥64	TNC	64	≥128/64	-
Ciprofloxacin	0.12			1		
Gentamicin	-			0.5		
TMP-SMX	2			-		
Nitrofurantoin	-			-		
Azithromicin	-			-		

MeOH: methanolic extract; H_2O: water extract

Appendix 4. Antibacterial Activity of Extracts (1-34) and Controls

	Gram negative					
	Klebsiella pneumoniae RSKK 574			*Salmonella enteriditis* RSKK 538		
Extracts	**MIC**	**MBC/MBS**	**CFU/mL**	**MIC**	**MBC/MBS**	**CFU/mL**
1. AC MeOH	64	≥128/64	$4.6x10^3$	128	≥256/128	$7.4x10^3$
2. AC H_2O	64	≥128/64	$7.6x10^3$	128	≥256/128	$8.1x10^3$
3. AM MeOH	64	≥128/64	$6.5x10^3$	128	≥256/128	$6.4x10^3$
4. AM H_2O	64	≥128/64	$5.9x10^3$	128	≥256/128	$6.0x10^3$
5. CO MeOH	64	≥128/64	$1.1x10^3$	128	≥256/128	$9.0x10^2$
6. CO H_2O	64	≥128/64	$8.5x10^3$	128	≥256/128	$3.7x10^3$
7. HP MeOH	64	≥128/64	$8.2x10^3$	128	≥256/128	$3.4x10^3$
8. HP H_2O	64	≥128/64	$8.3x10^3$	128	≥256/128	$9.1x10^3$
9. ML MeOH	128	≥128/64	$3.7x10^3$	128	≥256/128	$1.2x10^4$
10. ML H_2O	128	≥256/128	$3.9x10^3$	128	≥256/128	$5.4x10^3$
11. PR MeOH	128	≥256/128	$2.8x10^3$	128	≥256/128	$5.1x10^3$
12. PR H_2O	128	≥256/128	$2.0x10^3$	128	≥256/128	$2.4x10^3$
13. RhC MeOH	128	≥256/128	$4.2x10^3$	128	≥256/128	$4.6x10^3$
14. RhC H_2O	128	≥256/128	$1.0x10^4$	128	≥256/128	$6.8x10^3$
15. RoC MeOH	128	≥256/128	$3.2x10^3$	128	≥256/128	$8.8x10^3$
16. RoC H_2O	128	≥256/128	$2.9x10^3$	128	≥256/128	$6.1x10^3$
17. RoCG MeOH	128	≥256/128	$9.4x10^3$	64	≥256/128	$3.2x10^4$
18. RoCG H_2O	128	≥256/128	$4.6x10^3$	128	≥256/128	$1.5x10^4$
19. RPF MeOH	128	≥256/128	$8.0x10^3$	128	≥256/128	$7.8x10^3$
20. RPF H_2O	128	≥256/128	$3.3x10^3$	128	≥256/128	$1.6x10^4$
21. RPL MeOH	128	≥256/128	$5.0x10^3$	128	≥256/128	$1.4x10^4$
22. RPL H_2O	128	≥256/128	$5.4x10^3$	128	≥256/128	$1.1x10^4$
23. TF MeOH	128	≥256/128	$3.9x10^3$	128	≥256/128	$1.1x10^4$
24. TF H_2O	128	≥256/128	$7.8x10^3$	128	≥256/128	$6.7x10^3$
25. TP MeOH	128	≥128/-	-	128	≥256/128	$2.7x10^4$
26. TP H_2O	128	≥128/-	-	128	≥256/128	$9.1x10^3$
27. TT MeOH	128	512/128	$7.1x10^3$	128	≥256/128	$2.7x10^4$
28. TT H_2O	128	512/128	$1.0x10^4$	128	≥256/128	$2.4x10^4$
29. UD MeOH	128	≥128/-	-	128	≥256/128	$1.5x10^4$
30. UD H_2O	128	≥256/128	$6.6x10^3$	128	≥256/128	$1.4x10^4$
31. VAA MeO	128	≥256/128	$3.7x10^3$	128	≥256/128	$2.0x10^4$
32. VAA H_2O	128	128/-	$1.1x10^4$	128	≥256/128	$1.3x10^4$
33. VAC MeOH	128	128/-	$7.5x10^3$	128	≥256/128	$1.3x10^4$
34. VAC H_2O	128	128/-	$8.5x10^3$	128	≥256/128	$5.8x10^3$
Ciprofloxacin	0.5			0.5		
Gentamicin	-			-		
TMP-SMX	1			1		
Nitrofurantoin	-			0.25		
Azithromycin	-			0.5		

MeOH: methanolic extract; H_2O: water extract

Appendix 4. Antibacterial Activity of Extracts (1-34) and Controls

	Gram negative					
	Shigella dysenteriae RSKK 851			*Vibrio cholerae* ojawa RSKK 96023		
Extracts	**MIC**	**MBC/MBS**	**CFU/mL**	**MIC**	**MBC/MBS**	**CFU/mL**
1. AC MeOH	64	256/128	$6.4x10^3$	64	128/≥64	$2.3x10^4$
2. AC H_2O	128	256/128	$9.9x10^3$	64	128/≥64	$2.3x10^4$
3. AM MeOH	128	256/128	$2.1x10^4$	64	128/≥64	$2.9x10^4$
4. AM H_2O	128	256/128	$1.4x10^4$	64	128/≥64	$2.5x10^4$
5. CO MeOH	128	256/128	$6.7x10^3$	64	128/≥64	$2.4x10^4$
6. CO H_2O	128	256/128	$9.4x10^3$	64	128/≥64	$3.1x10^4$
7. HP MeOH	128	256/128	$2.1x10^4$	64	128/≥64	$8.2x10^3$
8. HP H_2O	128	256/128	$1.7x10^4$	64	128/≥64	$2.1x10^4$
9. ML MeOH	128	256/128	$1.9x10^4$	64	512/≥64	$2.2x10^4$
10. ML H_2O	128	256/128	$2.0x10^4$	64	512/≥64	$1.0x10^4$
11. PR MeOH	128	256/128	$1.2x10^4$	64	512/≥64	$2.2x10^4$
12. PR H_2O	128	256/128	$2.4x10^4$	64	512/≥64	$1.4x10^4$
13. RhC MeOH	128	256/128	$2.6x10^4$	64	512/≥64	$1.2x10^4$
14. RhC H_2O	128	256/128	$2.5x10^4$	64	512/≥64	$9.8x10^3$
15. RoC MeOH	128	256/128	$2.8x10^4$	64	512/≥64	$1.1x10^4$
16. RoC H_2O	128	256/128	$1.9x10^4$	64	512/≥64	$1.2x10^4$
17. RoCG MeOH	128	256/128	$1.4x10^4$	16	≥128/≥16	$9.0x10^3$
18. RoCG H_2O	128	256/128	-	64	≥64/-	-
19. RPF MeOH	128	256/128	-	64	≥64/-	-
20. RPF H_2O	128	256/128	$1.3x10^4$	64	≥64/-	-
21. RPL MeOH	128	256/128	$7.3x10^3$	128	512/128	$1.8x10^4$
22. RPL H_2O	128	256/128	$2.1x10^4$	128	512/128	$1.7x10^4$
23. TF MeOH	128	256/128	$2.4x10^4$	128	512/128	TNC
24. TF H_2O	128	256/128	TNC	128	512/128	$1.5x10^4$
25. TP MeOH	128	256/128	$3.5x10^4$	128	512/128	$2.7x10^4$
26. TP H_2O	128	256/128	$3.6x10^4$	128	512/128	$2.1x10^4$
27. TT MeOH	128	256/128	$2.7x10^4$	128	512/128	$1.1x10^4$
28. TT H_2O	128	256/128	$3.8x10^4$	128	512/128	$2.1x10^4$
29. UD MeOH	128	256/128	$3.3x10^4$	128	512/128	$1.3x10^4$
30. UD H_2O	128	256/128	$3.0x10^4$	128	512/128	$8.5x10^3$
31. VAA MeO	128	256/128	$2.9x10^4$	128	512/128	$2.1x10^4$
32. VAA H_2O	128	256/128	$3.7x10^4$	128	512/128	$2.1x10^4$
33. VAC MeOH	128	256/128	$1.7x10^4$	128	512/128	$1.1x10^4$
34. VAC H_2O	128	256/128	$2.1x10^4$	128	512/128	$1.7x10^4$
Ciprofloxacin	-			-		
Gentamicin	-			-		
TMP-SMX	1			1		
Nitrofurantoin	0.5			0.25		
Azithromycin	0.5			1		

MeOH: methanolic extract; H_2O: water extract

Appendix 4. Antibacterial Activity of Extracts (1-34) and Controls

	Gram positive					
	Staphylococcus aureus ATCC 25923			*Enterococcus faecalis* ATCC 29212		
Extracts	**MIC**	**MBC/MBS**	**CFU/mL**	**MIC**	**MBC/MBS**	**CFU/mL**
1. AC MeOH	32	512/≥32	$2.4x10^4$	64	128/64	$3.6x10^4$
2. AC H_2O	64	512/≥64	$2.7x10^4$	64	128/64	$4.1x10^4$
3. AM MeOH	64	512/≥64	$3.3x10^4$	64	128/64	$4.4x10^4$
4. AM H_2O	128	512/≥128	$2.1x10^4$	64	128/64	$7.8x10^4$
5. CO MeOH	4	512/≥4	$1.6x10^4$	64	128/64	$4.1x10^4$
6. CO H_2O	8	512/≥8	$3.5x10^4$	128	512/128	$8.8x10^3$
7. HP MeOH	16	512/≥16	$2.7x10^4$	128	512/128	$3.8x10^3$
8. HP H_2O	8	512/≥8	$3.7x10^4$	128	512/128	$1.8x10^4$
9. ML MeOH	128	512/≥128	$2.0x10^4$	128	512/128	$4.5x10^4$
10. ML H_2O	128	512/≥128	$1.4x10^4$	128	512/128	$5.4x10^4$
11. PR MeOH	128	-/≥128	$8.0x10^3$	128	512/128	$2.8x10^4$
12. PR H_2O	128	-/≥128	$1.5x10^4$	128	512/128	$7.0x10^4$
13. RhC MeOH	8	-/≥8	$1.1x10^4$	128	512/128	$5.7x10^4$
14. RhC H_2O	32	-/≥32	$8.5x10^4$	128	512/128	$4.9x10^4$
15. RoC MeOH	32	-/≥32	$8.6x10^4$	128	512/128	$4.7x10^4$
16. RoC H_2O	32	-/≥32	$3.4x10^4$	128	512/128	$5.0x10^4$
17. RoCG MeOH	16	-/≥16	$2.4x10^4$	64	512/≥64	$3.42x10^4$
18. RoCG H_2O	4	-/≥4	$3.9x10^4$	64	512/≥64	$3.8x10^4$
19. RPF MeOH	8	-/≥8	$2.4x10^4$	64	512/≥64	$4.6x10^4$
20. RPF H_2O	32	-/≥32	$3.1x10^4$	64	512/≥64	$1.2x10^4$
21. RPL MeOH	128	-/≥128	$1.7x10^4$	64	512/≥64	$3.0x10^4$
22. RPL H_2O	128	-/≥128	$1.8x10^4$	64	512/≥64	$3.4x10^4$
23. TF MeOH	128	-/≥128	$1.7x10^4$	64	512/≥64	$3.9x10^4$
24. TF H_2O	128	-/≥128	$2.2x10^4$	64	512/≥64	$4.0x10^4$
25. TP MeOH	128	-/≥128	$1.5x10^4$	32	256/≥32	$3.6x10^4$
26. TP H_2O	128	-/≥128	$1.4x10^4$	32	256/≥32	$1.8x10^4$
27. TT MeOH	128	-/≥128	$3.5x10^4$	32	256/≥32	$1.8x10^4$
28. TT H_2O	128	-/≥128	$4.2x10^4$	32	256/≥32	$1.7x10^4$
29. UD MeOH	128	-/≥128	$2.3x10^4$	32	256/≥32	$1.9x10^4$
30. UD H_2O	128	-/≥128	$3.3x10^4$	32	256/≥32	$3.3x10^4$
31. VAA MeO	128	-/≥128	$2.8x10^4$	32	256/≥32	$2.8x10^4$
32. VAA H_2O	128	-/≥128	$4.1x10^4$	32	256/≥32	$3.9x10^4$
33. VAC MeOH	128	-/≥128	$3.0x10^4$	32	256/≥32	$3.5x10^4$
34. VAC H_2O	128	-/≥128	$1.8x10^4$	32	256/≥32	$2.0x10^4$
Ampicillin	0.12			1		
Ciprofloxacin	0.5			0.5		
Gentamicin	1			1		
Vancomycin	0.12			-		
TMP-SMX	-			-		

MeOH: methanolic extract; H_2O: water extract

Appendix 4. Antibacterial Activity of Extracts (1-34) and Controls

	Gram positive					
	Bacillus cereus NRRL B–3711			*Bacillus subtilis* ATCC 6633		
Extracts	**MIC**	**MBC/MBS**	**CFU/mL**	**MIC**	**MBC/MBS**	**CFU/mL**
1. AC MeOH	64	-/≥64	$4.7x10^3$	128	256/128	
2. AC H_2O	128	≥128/-	-	128	256/128	
3. AM MeOH	128	-/≥128	TNC	128	256/128	
4. AM H_2O	256	-/≥256	$3.5x10^3$	128	256/128	
5. CO MeOH	128	-/≥128	$5.4x10^3$	128	256/128	
6. CO H_2O	128	-/≥128	TNC	128	256/128	
7. HP MeOH	128	-/≥128	TNC	128	256/128	
8. HP H_2O	128	-/≥128	TNC	64	128/64	
9. ML MeOH	128	-/≥128	$1.6x10^3$	64	128/64	
10. ML H_2O	256	-/≥128	$2.1x10^3$	128	256/128	
11. PR MeOH	128	-/≥128	$3.6x10^3$	64	128/64	
12. PR H_2O	128	-/≥128	$2.0x10^3$	64	128/64	
13. RhC MeOH	128	-/≥128	$2.2x10^3$	128	256/128	
14. RhC H_2O	128	-/≥128	TNC	64	128/64	
15. RoC MeOH	128	-/≥128	$2.8x10^3$	128	256/128	
16. RoC H_2O	256	-/≥256	$3.7x10^3$	128	256/128	
17. RoCG MeOH	128	-/≥128	$1.2x10^3$	128	256/128	
18. RoCG H_2O	128	-/≥128	$3.2x10^3$	128	256/128	
19. RPF MeOH	128	-/≥128	$3.2x10^3$	128	256/128	
20. RPF H_2O	128	-/≥128	$3.1x10^3$	128	256/128	
21. RPL MeOH	128	-/≥128	$1.5x10^3$	128	256/128	
22. RPL H_2O	256	-/≥256	$3.1x10^3$	128	256/128	
23. TF MeOH	128	-/≥128	$2.1x10^3$	128	256/128	
24. TF H_2O	128	-/≥128	$1.4x10^3$	128	256/128	
25. TP MeOH	128	-/≥128	$6.2x10^3$	128	256/128	
26. TP H_2O	128	-/≥128	$7.3x10^3$	128	256/128	
27. TT MeOH	128	-/≥128	$5.4x10^3$	128	256/128	
28. TT H_2O	256	-/≥256	$4.2x10^3$	128	256/128	
29. UD MeOH	128	-/≥128	$7.1x10^3$	128	256/128	
30. UD H_2O	128	-/≥128	$4.5x10^3$	128	256/128	
31. VAA MeO	128	-/≥128	$3.6x10^3$	128	256/128	
32. VAA H_2O	128	-/≥128	$7.5x10^3$	128	256/128	
33. VAC MeOH	128	-/≥128	$3.5x10^3$	128	256/128	
34. VAC H_2O	256	-/≥256	$4.4x10^3$	128	256/128	
Ampicillin	0.12			0.12		
Ciprofloxacin	0.25			0.25		
Gentamicin	0.5			0.5		
Vancomycin	-			-		
TMP-SMX	0.25			0.12		

MeOH: methanolic extract; H_2O: water extract

Empty cells denote lost plates.

VITA

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2011	Women in Botany Award

SELECTED PUBLICATIONS AND PRESENTATIONS

Rose, Janna. 2011. In Press. The Talloires Declaration: Global Networking and Local Action. Chapter in Deborah Gallagher (Ed.) Environmental Leadership: A Reference Handbook. Sage Publications, Thousand Oaks, CA.

Rose, Janna. 2010. Antibacterial and Cytotoxic Properties of Turkish Anti-Diarrheal Plants. MBRS RISE & MARC U*STAR Student Symposium, Miami, FL: November 4-5, 2010.

Rose, Janna. 2010. Bactericidal and Bacteriostatic Properties of Crude Extracts from Rural Turkish Anti-Diarrheal Plants. ICAAC—Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, MA: September 12-15, 2010.

Rose, Janna. 2010. The Reported Use of Dog Rose Galls (*Rosa canina* L.) to Treat Human Gastroenteritis. Botanical Society of America, Providence, RI: August 1-5, 2010.

Rose, Janna, Sema Demir, Evrim Özunel, and Brad Bennett. 2010. Concepts of Gastrointestinal Disease and its Treatment among Rural Turkish Villagers. Society for Economic Botany Annual Meeting, Xalapa, Veracruz, Mexico: June 6-10, 2010. Awarded Honorable Mention for Society's Morton Award.

Rose, Janna, Ílhan Gürbüz, Berrin Özçelik, and Brad Bennett. 2010. Bactericidal and Bacteriostatic Effects of 15 Anti-Diarrheal Plants from Central Anatolia. Society for Economic Botany Annual Meeting, Xalapa, Veracruz, Mexico: June 6-10, 2010.