Structures, and immunomodulating and anti-ulcer activities of polysaccharides from Malian medicinal plants

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Oslo, November 2012
Ingvild Austarheim

Abstract

The main purpose of this thesis was to evaluate the potential of pectins from the Malian medicinal tree *Cola cordifolia* used in the treatment of gastric ulcer and wounds. This thesis is a small contribution to the ultimate goal of providing efficient none-toxic and inexpensive medicines for the Malian population.

The Department of Traditional Medicine, our collaborating partner in Bamako, wants to promote the use of renewable plant parts to guarantee a sustainable supply of medicinal plants. The structures and biological activities of the bark and leaf pectins were therefore compared in order to make recommendation on plant part substitution as debarking can damage or even kill the tree. We found that the pectins from the bark and leaves are structurally related. However, the leaf pectins are more polydisperse and heterogeneous compared to the pectins found in bark. Pectins from the bark were generally more active in the complement fixation test and the macrophage assay. Comparing the 50°C water extracts from bark and leaf in an experimental anti-ulcer model showed comparable and dose dependent inhibition of ulcer formation. However, a clinical trial is needed to evaluate the efficacy of the plant parts.

Powdered roots of *Vernonia kotschyana* are highly valued as the improved traditional medicine "Gastrosedal". The anti-ulcer activity of the medicine has previously been attributed to the presence of saponins. In this thesis, the anti-ulcer potential of 50°C and 100°C water extracts depleted of saponins, but high in inulin, 98% and 83% respectively, were evaluated in an experimental mouse model. The tested dose corresponded to the recommended daily intake of "Gastrosedal" and this dose showed a good inhibition of ulcer formation. We therefore concluded that inulin can also be responsible important for the anti-ulcer activity of "Gastrosedal".

In Mali, gastric ailments are rather common and contribute highly to morbidity in the country. In a previous study, *Helicobacter pylori* was found to be present in 95% of Malian patients with gastric ulcer. For future investigations and clinical trials, it was of interest to find a reliable and simple method for *H. pylori* detection. One stool and one serological based immunochromatographic method were tested, and the results showed that the sensitivity of these tests is too low in the Malian population. The low sensitivity was probably due to strain variability, in addition to high use of anti-malaria drugs, which might eradicate or lower the bio-burden of *H. pylori*.

List of papers

Paper I **Ingvild Austarheim, Bjørn E. Christensen, Ida K. Hegna, Bent O. Petersen, Jens O. Duus, Ragnar Bye, Terje E. Michaelsen, Drissa Diallo, Marit Inngjerdingen & Berit S. Paulsen**
Chemical and biological characterization of pectin-like polysaccharides from the bark of the Malian medicinal tree *Cola cordifolia*
Carbohydrate Polymers **89** (2012), 259–268.

Paper II **Ingvild Austarheim, Bjørn E. Christensen, Christian Thöle, Drissa Diallo, Marit Inngjerdingen & Berit S. Paulsen**
Chemical and biological characterizations of pectins from *Cola cordifolia* leaves
Manuscript

Paper III **Ingvild Austarheim, Haidara Mahamane, Rokia Sanogo, Adiaratou Togola, Mehdi Khaledabadi, Anne C. Vestrheim, Kari T. Inngjerdingen, Terje E. Michaelsen, Drissa Diallo & Berit S. Paulsen**
Anti-ulcer polysaccharides from *Cola cordifolia* bark and leaves
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Paper IV **Ingvild Austarheim, Cecilie S. Nergard, Rokia Sanogo, Drissa Diallo & Berit S. Paulsen**
Inulin-rich fractions from *Vernonia kotschyana* roots have anti-ulcer activity
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Paper V **Ingvild Austarheim, Kari T. Inngjerdingen, Adiaratou Togola, Drissa Diallo & Berit S. Paulsen**
Chromatographic immunoassays for *H. pylori* detection – are they reliable enough in Mali (West Africa)?
Accepted for publication in *PAN African Medical Journal* (2013)

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Paper VI **Kari T. Inngjerdingen, Selma Meskini, Ingvild Austarheim, N'Golo Ballo, Marit Inngjerdingen, Terje E. Michaelsen, Drissa Diallo & Berit S. Paulsen**
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Pectic polysaccharides isolated from Malian medicinal plants protects against Streptococcus pneumoniae in a mouse pneumococcal infection model
Submitted to *Scandinavian Journal of Immunology* (2012)

Paper VIII **Adiaratou Togola, Ingvild Austarheim, Annette Theis, Drissa Diallo & Berit S. Paulsen**
Ethnopharmacological uses of *Erythrina senegalensis*: a comparison of three areas in Mali, and a link between traditional knowledge and modern biological science
Journal of Ethnobiology and Ethnomedicine **4:6** (2008)

List of abbreviations and symbols

α or β	Configuration of the anomeric site of the monosaccharide
2-OMe-Gal	2-O-methylated galactose
4-OMe-GlcA	4-O-methylated glucuronic acid
f	Furanose
p	Pyranose
AAS	Atomic absorption spectroscopy
AEC	Anion exchange column
AFM	Atomic force microscopy
AG-I	Arabinogalactan type I (arabino-4-galactans)
AG-II	Arabinogalactan type II (arabino-3,6-galactans)
Ara	Arabinose
CC(..)	Polysaccharide fractions from bark of *C. cordifolia*
CC1P1	Polysaccharide containing Gal:Rha:GalA ratio 1:1:1
COSY	Correlation spectroscopy
DMT	Department of traditional medicine
DP	Degree of polymerization (Number of monosaccharides linked together)
EC	Electrochemical detection
EtOH	Ethanol
FPLC	Fast protein liquid chromatography (Pharmacia system)
Gal	Galactose
GalA	Galacturonic acid

GC	Gas chromatography
GI	Gastrointestinal
Glc	Glucose
HG	Homogalacturonan
HMBC	Heteronuclear multiple bond correlation
HPAEC-EC	High performance anion exchange column (Dionex system) with electrochemical detection
HSQC	Heteronuclear Single Quantum Correlation
ICH_{50}	Concentration needed for 50% inhibition of hemolysis (Complement fixation test)
IEC	Ion exchange column
Ig	Immunoglobulin
ITM	Improved traditional medicine
LCC(..)	Polysaccharide fractions from leaves of *C. cordifolia*
LPS	Lipopolysaccharide (endotoxin)
M_n	Number average molecular weight
M_w	Weighted average molecular weight
MALLS	Multi angle laser light scattering
MeOH	Methanol
MHS	Mark-Houwink-Sakurada plot
MS	Mass spectrometry
MW	Molecular weight
NMR	Nuclear magnetic resonance spectroscopy
NOESY	Nuclear Overhauser enhancement spectroscopy
PMII	Acidic pectin fraction from *Plantago major*
RG-I	Rhamnogalacturonan type I
RG-II	Rhamnogalacturonan type II
Rha	Rhamnose

RI	Refractive index detection
RI	Refractive index
SEC	Size exclusion chromatography
T	Terminal
Vk(..)	Fractions from *Vernonia kotschyana*
WHO	World health organization
Xyl	Xylose

1. Introduction

Traditional medicine is defined as the sum total of knowledge, skills and practices based on the theories, beliefs and experiences, indigenous to different cultures, that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (WHO, 2008). In most countries traditional medicine is better known under names like complementary, alternative or non-conventional medicine. However, for most developing countries traditional medicine is not *an alternative*, but the main supply of medicine for the population's primary health care. According to World Health Organization (WHO), 75% of the Malian population depends on traditional medicines and the interest in traditional medicine is growing (Robison & Zhang, 2011; Diallo & Paulsen, 2000). Mali is a poor and underdeveloped country, ranked as number 175 of 187 countries in the human development report of 2011 (Klugman, 2011). Hence the use of expensive imported medicines is difficult for most of the population, and it is therefore important that traditional medicine is used complementary to western medicines. In addition, Mali has a limited number of physicians, 1 for every 16 000, and a poorly developed infrastructure, especially in remote areas, which makes transportation of medicines difficult, thus resulting in low availability (Diallo & Paulsen, 2000). In 1994, a devaluation of the local currency happened over night, and the cost of the imported medicines doubled. Hence, western medication does not provide a medical system that is sufficient by its own due to the price, but must be acting side by side with traditional medicine. The traditional practitioners are higher in numbers, reaching 1 for every 500, and they diagnose and treat patients in addition to providing them with cheap and available traditional medicines. However, clearly, traditional medicine is not sufficient by itself to treat all conditions.

The best way of giving the population an appropriate primary health care is to assure that traditional medicine is used complementary to western medicine by developing more efficient and safe improved traditional medicines (ITMs). For this purpose the Department of Traditional Medicine (DMT), located in the capital Bamako in Mali, was established. DMT is now a collaborating center of WHO with the primary objective to establish a mechanism to assure that traditional medicine produced from local plants is complementary to western medicine. To achieve this goal, DMT is collaborating with the traditional practitioners. This collaboration is based on trust and the common goal to improve the health condition in the country and increase the knowledge on medicinal plants.

For the aim of learning more about the use of medicinal plants, several ethnomedical studies

have been carried out, especially in the period from 1968-1992 (Diallo & Paulsen, 2000). Traditional practitioners are the main informants of the DMT in the development of ITMs and for the basis of the ethnopharmacological studies. Studies on safety and efficacy of the traditional medicine are maintained by ethnopharmacological research which will provide additionally evidence for the uses, resulting in more reliable medicines. An ethnopharmacological investigation is, by definition, observation, identification, description and experimental investigation of the ingredients and the effects of indigenous drugs (Holmstedt & Bruhn, 1983). It is a truly interdisciplinary field of research which is important in the study of traditional medicine. So far, DMT has developed twelve ITMs and seven of them are regarded as essential medicines in Mali (Willcox *et al.*, 2012).

Typically, many types of molecules in the same plant are contributing to the observed biological activity. The most common way of preparing the traditional medicine is by making a decoction. Polar and semi polar low molecular weight substances like steroids, terpenes, alkaloids and phenolic compounds will together with macromolecules, like polysaccharides, be extracted into the boiling water. Normally, only the low molecular weight compounds are studied. However, polysaccharides are shown to possess immunomodulating properties which make them highly interesting as possible active substances in traditional medicine.

1.1 Plant polysaccharides

Polysaccharides are an important class of mainly plant derived polymers including cellulose, hemicellulose, pectins, starch and inulin. Their function in the plant is usually either structure or storage related. The molecular composition and arrangement in the plants differ among plant species.

Pectins are the most structurally complex family of polysaccharides in nature. They are found in the primary cell wall, as an interpenetrating matrix supporting cellulose microfibriles, together with hemicellulose and proteins. The precise chemical structure of pectin is under debate, although the structural elements of pectin are rather well described, see Fig. 1.1 (Coenen *et al.*, 2007). Pectins are galacturonic acid-rich polysaccharides including homogalacturonan, rhamnogalacturonan I and the substituted galacturonans; rhamnogalacturonan II (RG-II) and xylogalacturonan (XGA). It is generally believed that the HG, RG-I and RG-II are covalently cross-linked since harsh chemical treatments or digestion by pectin-degrading enzymes are required to separate (Ridley *et al.*, 2001; Mohnen, 2008).

The most abundant pectic polysaccharide is **homogalacturonan (HG),** a linear homopolysaccharide consisting of (1→4) α-galacturonans. Some of the carboxyl groups may be methyl-esterified, and depending on the source, the galacturonic acid (GalA) residues can also be acetylated in position 2 or 3. The methyl esterification might be present as blocks or the substitution may be randomly distributed. HG has been shown to be present in stretches of approximately 100 GalA residues in length (Yapo *et al.*, 2007).

Rhamnogalacturonan-I (RG-I) comprises a highly diverse population of developmentally regulated polymers (Willats *et al.*, 2001). RG-I is a group of pectic polysaccharides that contains a backbone of the repeating disaccharide →4)αGalA(1→2)-α-L-Rha-(1. The Rha residues are, depending on the plant source, substituted at C-4 with neutral and acidic mono or oligosaccharides. The highly branched nature of RG-I has made it known as the "hairy region" of the pectin, in contrast to HG domains which are known as the smooth regions. The side chains of RG-I can be arabinans, arabinogalactans, galactans or monomers of different types, see Fig. 1.2. Arabinans have a 1→5 linked arabinose backbone, with branching points consisting of 1→2,5 or 1→3,5 linked arabinose linked via *O*-2 or *O*-3, respectively, to linear arabinose side chains of varying size. Pure galactans consist of 1→4 linked galactose. Arabinogalactans can be divided into two subclasses, arabino-4-galactans (AG-I) and arabino-3,6-galactans (AG-II). AG-I has a 1→4 linked Gal backbone with branching via *O*-3 to linear arabinans of various size. AG-II is more complex compared to AG-I and can be highly branched with 1→3,6 linked Gal as branch points. AG-II has a galactan backbone consisting of 1→3 linked Gal as the main chain and often 1→6 linked Gal as side chains. Ara can be bound to *O*-3 or *O*-6 of Gal depending on where Ara is situated. The side chains may also contain terminal α-Fuc, β-GlcA, and 4-*O*-Me-β-GlcA residues (Mohnen, 2008). In addition the side chains can be esterified with ferulic acid (Ridley *et al.*, 2001).

Rhamnogalacturonan-II (RG-II) is a low molecular mass (5–10 kDa) pectic polysaccharide with a highly conserved sequence across plant species. The name RG-II is somewhat misleading, because it suggests that this structure contains a rhamnogalacturonan backbone like RG-I, but RG-II has a 1→4-linked GalA backbone. Two structurally distinct disaccharides and two oligosaccharides are attached the backbone, see Fig. 1.1 (Perez *et al.*, 2003).

Inulin belongs to a class of dietary fibers known as (2→1)-β-fructans with a degree of polymerization (DP) up to 100, and each chain can be terminated by a single glucose unit, depending of the type (Kelly, 2008). Inulin is typically found stored in roots or rhizomes as a source of energy (comparable to starch). Plants that store inulin, does normally not store starch. Plant families that normally store inulin are Asteraceae and Liliaceae.

1.2 Pectins as immunomodulators

The ability to modulate the immune response in an appropriate way can enhance the host's immune responses (Tzianabos, 2000). Polysaccharides capable of interacting with the immune system to up or down regulating specific aspects of the host response can be classified as immune modulators. Although few types of pectins have been rigorously studied, reports have revealed some of the structure-activity relationship of these molecules.

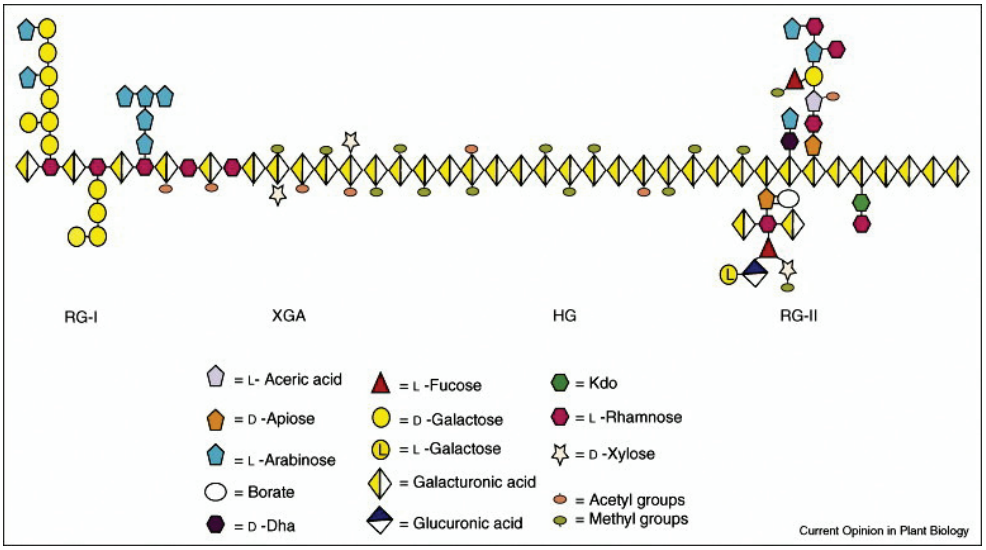

Figure 1.1: Schematic structure of pectin showing the four pectic polysaccharides homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) linked to each other. The representative pectin structure shown is not quantitatively accurate. From Mohnen (2008).

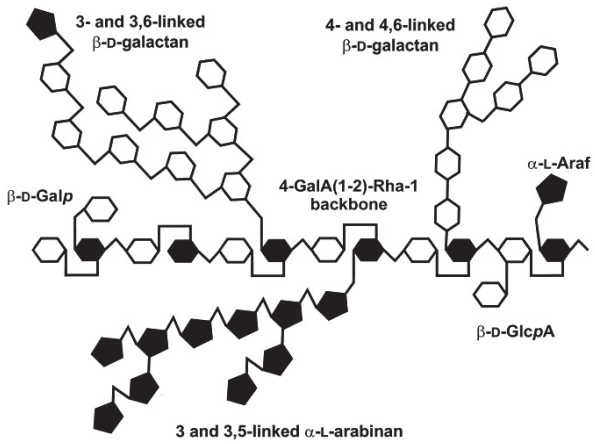

Figure 1.2: A model showing the major structural features of rhamnogalacturonan I. The backbone is composed of the disaccharide repeating unit [→4-α-D-Gal*p*A-(→2)-α-L-Rhap-(1→]. Branched and linear oligosaccharides composed predominantly of α-L-Ara*f* and β-D-Gal*p* residues are linked to C4 of some of the Rha*p* residues. Some of the Rha*p* residue may also be *O*-acetylated at C2 and/or C3. More than ten glycosyltransferase activities are required for the biosynthesis of RG-I. From Ridley *et al.* (2001).

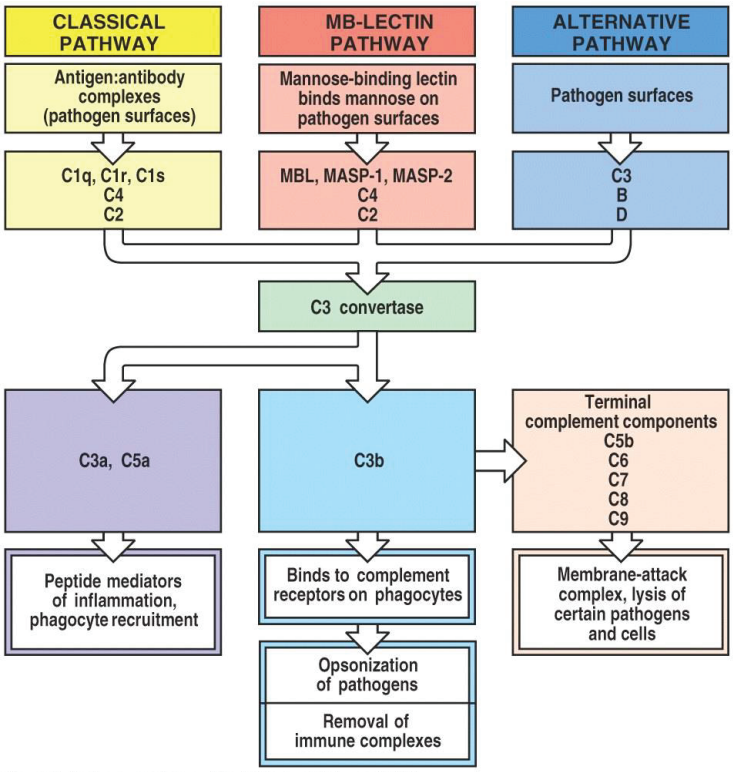

Figure 1.3: Overview of complement activation and function. The three known pathways that activate the complement cascade join at the formation of a C3 convertase. This complex cleaves C3 into components C3a and C3b, ultimately leading to pathogen opsonization, release of inflammatory mediators, and formation of terminal complement components. From Janeway *et al.* (2005).

1.2.1 Complement system

The complement is a cascade system of at least 20 serum glycoproteins that provides many of the effector functions of humoral (soluble factors) immunity and inflammation, including vasodilation, increased vascular permeability, phagocytosis and lysis of foreign cells. The complement plays an important role in the first line defense against infections and it holds important effector functions of the innate and the adaptive immune system. The complement is activated through the classical, mannose binding (lectin) or the alternative pathway, see Fig. 1.3. The classical pathway links to the adaptive immune system through binding of C1q to the Fc region of immune complexes. The alternative pathway is continuously and spontaneously activated in the blood, where C3b binds to hydroxyl groups (from carbohydrates) on the surface of bacteria and human cells. As C3b is randomly deposited, human cells have regulating proteins on their surface inactivating the cascade, see Fig. 1.3 (Janeway *et al.*, 2005).

It has been known for almost 40 years that complex polysaccharides can activate the complement (Snyderman & Pike, 1975). Pectins have been shown to activate the complement through the classical and alternative pathway. It is possible to distinguish between the classical and the alternative pathway, as the classical pathway requires both calcium and magnesium ions, whereas the alternative pathway requires magnesium ions only. Selective chelation of calcium ions in serum can be used to block the classical complement pathway while leaving the alternative pathway intact (Snyderman & Pike, 1975). The alternative pathway is inactivated by dilution of the complement source. Method A (Michaelsen *et al.*, 2000) uses a 1:70 dilution of serum which results in inactivation of the alternative pathway. There is not much information about activation of pectins through the mannose binding pathway. However, PM-II, an acidic pectic polysaccharide form *Plantago major*, did not activate the mannose-binding lectin pathway (unpublished results, personal communication Michaelsen, T). The experiment was carried out with a complement source from a person with an inactive mannose pathway and compared with results carried out with serum from a person with a functional mannose pathway.

The complement fixation assay does not discriminate between activation and inhibition of the complement cascade because both result in inhibition of hemolysis (Alban *et al.*, 2002). To distinguish between activation and inhibition it is possible to use ELISA methods for detection of C3 activation products (Michaelsen *et al.*, 2000). A simple method to distinguish activation and inhibition is simply to vary the incubation time. While activation requires time for building the cascade, inhibition happens immediately and it is therefore possible to distinguish the two mechanisms simply by omitting pre-incubation (Alban *et al.*, 2002). The basic mechanism of the pectic polysaccharides reported in the literature seems to be complement activation (Alban *et al.*, 2002).

Structure- activity studies suggest that the hairy regions of RG-I, with complex galactan or AG-II side chains attached are important for complement activity (Paulsen & Barsett, 2005; Yamada & Kiyohara, 2007). The size of these structures may also be important (Pangburn, 1989). Homogalacturonan regions of pectins found in *Angelica acutiloba* are shown to have an inhibiting or modulating activity of complement, see Fig. 1.4 (Yamada & Kiyohara, 2007).

1.2.2 Macrophage stimulation

Monocytes in the blood infiltrate and take residence in tissue and differentiate into macrophages. Their role is to remove microorganisms during infections and cellular and particle debris, in addition to interact with and stimulate lymphocytes (Janeway *et al.*, 2005). Plant polysaccharides are shown to interact specifically with pattern recognition receptors on macrophages via complement receptor 3 (CR3), mannose receptor (MR), scavenger receptor (SR), Dectin-1 or Toll-like receptor 4 (TLR4) (Schepetkin & Quinn, 2006). Plant polysaccharides can also be phagocytosed, leading to activation of unknown intracellular targets. Specifically, TLR4 has been identified as a receptor for acidic plant-derived polysaccharides (Kim *et al.*, 2007). Binding to TLR4 leads to the activation of transcriptional pathways leading to the production of

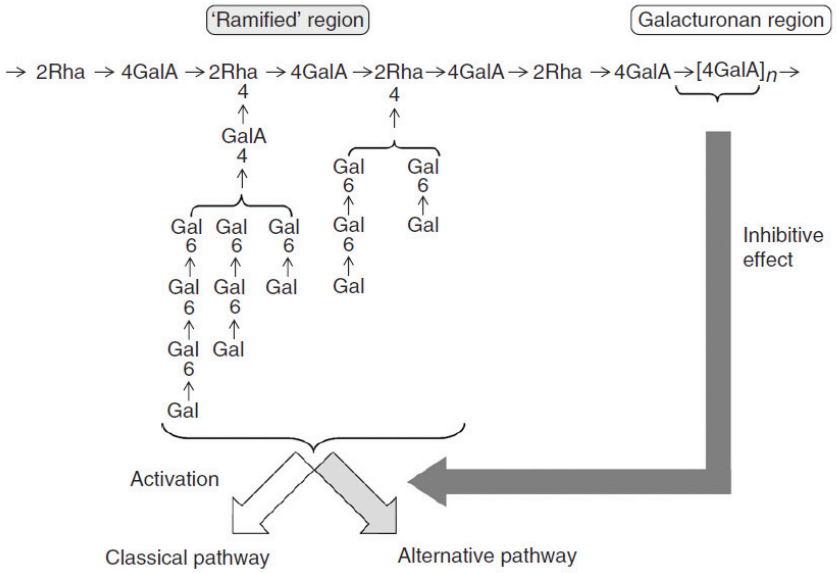

Figure 1.4: Structure requirements for complement activation of pectins from *Angelica acutiloba*. From Yamada & Kiyohara (2007).

pro-inflammatory cytokines and inducible nitric oxide synthase (Schepetkin & Quinn, 2006). In addition, complement activation can lead to activation of macrophages through complement receptors expressed on the surface of the phagocytes, see Fig. 1.5.Inngjerdingen *et al.* (2008) suggested that the presence of RG-I with AG-II side chains is part of the structural requirement of macrophage activation and that RG-II rich fractions did not activate macrophages.

1.3 Internal and external wounds

1.3.1 Gastric ulcer and *H. pylori* infection

H. pylori is recognized as major risk factor for developing gastritis and gastric ulcer. Infections with *H. pylori* are found worldwide in individuals of all ages, but are commonly acquired at an earlier age in developing countries (Wang & Peura, 2011). Individuals may be asymptomatic carriers of the disease, but the presence of *H. pylori* is highly correlated with underlying ailments causing dyspepsia. A previous study conducted in Mali on patients with gastric ulcer reported a *H. pylori* prevalence of 95% (Mourtala, 2000). In Mali, the prevalence of gastric ulcer in the population is reported to be 4.2% for men and 2.4% for women, and is probably higher for gastritis (Touré, 1989; Maïga *et al.*, 1995).

H. pylori is a non-invasive gram negative bacteria which uses locomotion to penetrate the vis-

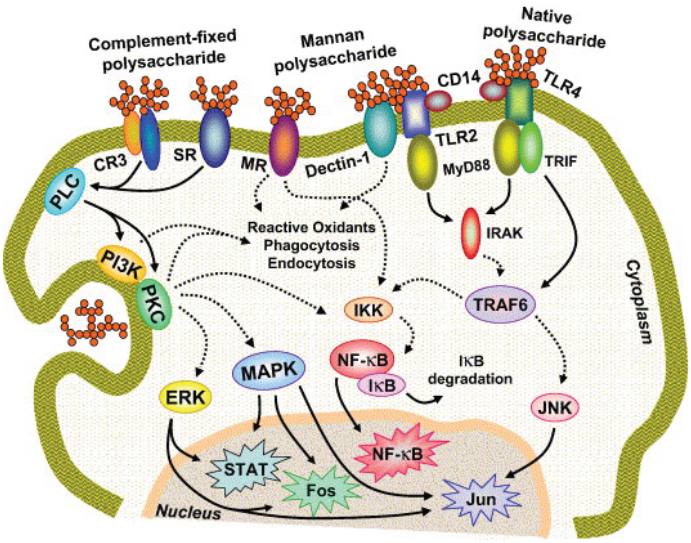

Figure 1.5: Schematic model illustrating potential signalling pathways involved in macrophage activation by botanical polysaccharides. From Schepetkin & Quinn (2006).

cous mucosa layer where it adheres to the mucus and colonizes. Soluble surface constituents, like LPS provoke pepsinogen release and trigger local inflammation. Urease, a soluble surface protein, is the primary chemoattractant to activate inflammatory cells, which again will release cytokines to promote inflammation. Anti-*H. pylori* antibodies (IgA and IgG) will often cross-react with glandular cells in the stomach, leading to destruction of gastric epithelia and ulceration. Unfortunately, presences of antibodies after *H. pylori* eradication do not provide protection towards re-infection. The bacteria also contribute, through a complex mechanism, to increased gastrin levels in the blood, which again contribute to a lower pH in the gastric fluid. A low pH contributes probably to the formation of metaplasia which again will increase the probability of getting gastric cancer. The presence of cytotoxin producing *H. pylori* is much higher in patients with ulcers (70%) than in patients with a silent infection (30%). It might seem that an active ulcer can modify the activity of *H. pylori* (Halter *et al.*, 1992).

The currently most effective treatment for peptic ulcer disease is a triple therapy regimen consisting of a proton pump inhibitor, such as omeprazole, and two antibiotics, clarithromycin and either amoxicillin or metronidazole. However, there is an increase in antibiotic resistance and in all countries, and unfortunately reinfection happens fast in countries with a high prevalence (Hunt *et al.*, 2010). For patients with severe ulcer and symptoms, conventional medicine is probably the best solution, but traditional medicine can provide pain relief from symptoms and give the patients a better quality of life.

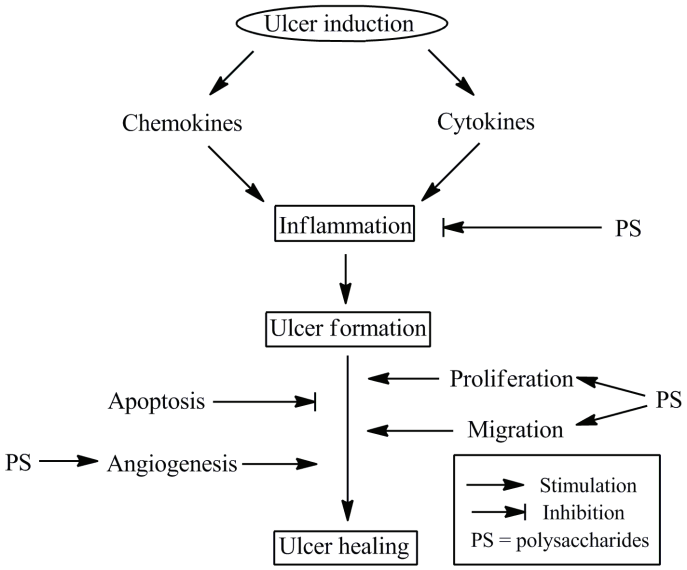

Figure 1.6: The pathogenesis of ulcer formation and healing, and the mechanism of how polysaccharides prevent ulcer induction through free radical scavenging, reduction of neutrophil infiltration and promotion of ulcer healing by stimulation of cell migration, proliferation and angiogenesis at the ulcer site. From Cho & Wang (2002).

1.3.2 Anti ulcer activity in *in vivo* experimental models

Immunomodulating polysaccharides from various plants have shown dose-dependent anti-ulcer activity in gastric lesions induced by necrotizing agents in experimental anti-ulcer models (Matsumoto *et al.*, 2002; Nergaard *et al.*, 2005*a*; Cipriani *et al.*, 2008, 2009). Anti-ulcer activity of acidic pectins from *Bupleurum falciparum* was reduced after pectinase treatment, indicating that HG regions are important for activity. Apple pectins consisting of 95% GalA showed no significant activity, indicating that the hairy regions are also important for activity (Yamada *et al.*, 1991). A direct correlation between immunomodulation and anti-ulcer activity of pectins has not been found.The mechanisms behind the anti-ulcer activity of acidic pectins from *Bupleurum falciparum* are suggested to be mucosal protective coating, anti-secretory activity of gastric acid and pepsin, in addition to radical scavenging activity. The mechanism did not involve endogenous prostaglandin production or increased mucus synthesis (Sun *et al.*, 1991; Matsumoto *et al.*, 1993). See Fig. 1.6 for an overview of possible mechanisms of how polysaccharides can prevent peptic ulcer formation.

1.3.3 Anti-adhesive activity towards *H. pylori*

H. pylori infection is initiated by adhesion to the gastric epithelia of the host. Adhesion is mediated by lectins bound to the surface of the bacteria. The lectins bind to complementary carbohydrates on the surface of the host tissue, and the bacteria can start colonizing at the adhesion site. To block tissue adhesion and colonization, soluble complementary polysaccharides like pectins can be administrated. These carbohydrates can bind to the bacterial lectins, which will lead to blocking of the adhesion site of the bacteria (Sharon & Ofek, 2002). Previously it has been shown that acidic polysaccharides from immature okra (*Abelmoschus esculentus*) inhibited adhesion of *H. pylori* to human gastric mucosa, but the polysaccharides were ineffective in an *in vivo* study of infected chicken broilers due to metabolism in the gastrointestinal system (Wittschier *et al.*, 2007). The structure important of *in vitro* inhibition is mainly highly charged pectins, with glucuronic acid as the main uronic acid. Anti-adhesive drugs are postulated to only be used prophylactic as a dissociation of bacteria already in the state of adherence with the host tissue seems unlikely (Wittschier *et al.*, 2007). Prophylactic use as a diet or in health promoting food could only be successful if the active compounds are not degraded in the gastrointestinal system.

1.3.4 Wound healing

In developing countries like Mali, injuries leading to wounds occur during farming activities. Numerous plants are used for the treatment of wounds (Diallo *et al.*, 2002).

The complexity of wound healing is a major problem when studying wound healing activities *in vitro*. Wound healing is an complex interplay between residential and infiltrating immune cell types and it may be divided into four phases: (i) coagulation and haemostasis; (ii) inflammation; (iii) proliferation; and (iv) wound remodelling with scar tissue formation (Burd & Huang, 2008). As wound healing is an immune-mediated process, it is possible that agents modulating the immune function, like pectins, have an effect on the reparative process. Immunomodulating pectins can be important in activation of macrophages, direct or through activation of the complement system, and has been shown in some model systems to contribute to wound healing (Werner & Grose, 2003; Rizk *et al.*, 2004). Chronic ulcers are known to have reduced levels of platelet derived growth factor, basic fibroblast growth factor, epidermal growth factor, and transforming growth factor β compared with acute wounds (Harding *et al.*, 2002). All of these growth factors are expressed by activated macrophages. Pectins with macrophage stimulating activity might therefore modulate the regenerative process of healing of chronic wounds. In addition, polysaccharides can have a direct keratinocyte proliferative activity, as seen by pectins from *Plantago major* in an *in vitro* scratch assay (Zubair *et al.*, 2012). Nutrition is also an important parameter to consider in wound healing. Lack of proteins and vitamin A and vitamin C is often correlated with a slow healing (MacKay & Miller, 2003). Since Mali is a one of the poorest countries in the world, nutritional status is often poor. The fruit of *C. cordifolia* contains vitamin C (Diop *et al.*, 1988), and can in this regard help people with a low vitamin C status.

Figure 1.7: *Cola cordifolia* (Cav.) R. Br. (Malvaceae). The local name is N'tabanokò.

1.4 *Cola cordifolia* (Cav.) R.Br (Malvaceae)

According to The Plant List (2012) *Cola cordifolia* (Cav.) R. Br. has recently changed family from Sterculiaeae to Malvaceae. *C. cordifolia* grows on the savannah in Senegal to Mali (West Africa) and is a large tree ranging between 15 and 25 m in height. It has a short buttressed trunk, low-branching and a dense crown, see Fig. 1.7 (Burkill, 2000). In Bamako, merchants are often seen trading their goods in the shadow provided by the tree. The mature fruit is edible and is a source of vitamin C (Diop *et al.*, 1988). All parts of the tree (roots, leaves, bark and seeds) are used in traditional medicine. In Mali, the bark and leaves are used to treat different types of wounds and stomach problems, pain, fever and diarrhea (Grønhaug *et al.*, 2008; Togola *et al.*, 2008; Austarheim *et al.*, 2012). In an extensive survey of wound healing plants in the Bamako region, 123 species were identified, and *C. cordifolia* was among the fifteen most cited wound healing plants identified (Diallo *et al.*, 2002). Previously, anti-ulcer activity was reported from related species like *Cola acuminata* (Diallo *et al.*, 1999). Burkill (2000) reports a wide use of the tree, treating ailments like chest affections, constipation, wounds and leprosy among others. In Senegal, the bark is used to treat bronchitis, abscesses and gangrene (Kerharo & Adam, 1974). According to Grønhaug *et al.* (2008), the two most common ways of preparing the traditional medicine were decoction and preparation of a powder. The decoction was used for a bath and/or to drink. The powder was suspended in water and used for a bath and/or as a drink, or it was thrown into fire and the smoke was inhaled.

Figure 1.8: *Vernonia kotschyana* roots and flowers.

1.5 *Vernonia kotschyana* Sch. Bip. ex Walp (Asteraceae)

The plant *Vernonia kotschyana* Sch. Bip. ex Walp. (Asteraceae) was renamed according to The Plant List (2012) to *Vernonia adoensis* var. *kotschyana* (Sch.Bip. ex Walp.) G.V.Pope. However, the first name is used throughout this thesis, since the new name has never been cited in the scientific literature and it is an unresolved name. Unresolved names are highly likely to be changed in the future (The Plant List 2012). *V. kotschyana* is a shrub growing in the savannah from Senegal to Nigeria across Africa to Ethiopia, see Fig. 1.8 (Burkill, 2000). *V. kotschyana* is highly valued in Mali (West-Africa) for the treatment of gastritis, stomach ulcers and wounds (Diallo *et al.*, 2002; Willcox *et al.*, 2012). The powdered roots of *V. kotschyana* are commercially available as an ITM, sold under the name "Gastrosedal". "Gastrosedal" is on the national list of essential drugs in Mali for treatment of gastritis and gastric ulcers. The efficacy of the medicine has been evaluated in two (uncontrolled) clinical trials. The first being an open clinical trial with 16 outpatients with gastric ulcers, 50% of the patients were relieved of symptoms and in 6 patients lesions had disappeared after ingestion of 6g powdered roots per day for 30 days (Touré, 1989). One year later, 47 patents with gastric ulcer were enrolled; 80% reported symptomatic improvement (Diallo *et al.*, 1990). Furthermore, the herbal medicine also shows good tolerability in Irwing screening and in the brine shrimp assay (Sanogo *et al.*, 1996). An experimental anti-ulcer rat model on various extracts from *V. kotschyana* roots showed a high protective activity. It was suggested that the steroidal saponins was the active principle (Germano *et al.*, 1996; Sanogo *et al.*, 1996). However, since the roots contain a high amount of inulin and immunomodulating pectins (Nergaard *et al.*, 2005*b*,*c*), these compounds are also suggested to explain parts of the activity of the plant.

2. Aims of the study

The ultimate goal for the research presented in this thesis is to provide efficient, non-toxic, available and affordable medicines to the population of Mali. The plants chosen for this thesis are *Cola cordifolia* and *Vernonia kotschyana*. These two plants, especially the latter, have long traditions in the treatment of wounds and gastric ulcers. Decoctions (hot water extracts) of these plants are commonly used preparations, and as plant polysaccharides are readily extracted into the decoction, it is highly relevant to examine for bioactive polysaccharides.

The specific objectives of the study were:

1. Compare the structure and immunomodulating activity of polysaccharides present in the bark and leaves of *C. cordifolia* to investigate whether or not plant part replacement can be recommended (Paper I and II).
2. To study the anti-ulcer activity in experimental rodent models of polysaccharide rich extracts from bark and leaves of *C. cordifolia* and the roots of *V. kotschyana* (Paper III and IV).
3. To perform ethnopharmacological surveys in order to provide more information on the traditional use of *C. cordifolia* (Paper III).
4. To find a simple method for *H. pylori* detection for further research on gastric ailments in Mali (Paper V).

3. Summary of papers

Paper I. Chemical and biological characterization of pectin-like polysaccharides from the bark of the Malian medicinal tree *Cola cordifolia*

The aim of the paper was to isolate and study the structure of pectins from the bark of *C. cordifolia*. In addition, the complement and macrophage activating properties of the pectin fractions were evaluated. A 50°C water extract was prepared from de-fatted *C. cordifolia* bark powder. The extract was fractionated on an ion exchange column to provide three fractions, CC1, CC2 and CC3. Unexpectedly, CC1 did not attach to the column despite a 47% uronic acid content. Interfering divalent ions were removed and CC1 was further purified to give CC1P1 and CC1P2. Structure elucidation was carried out by GC, GC/MS, SEC-MALLS, IR and NMR, which gave the structure of the relatively homogeneous CC1P1, 2→[α-D-Gal(1→3)]α-L-Rha(1→4)α-D-GalA(1→], with a molecular weight of M_w 135 kDa and a polydispersity index of 1.2. The presence of α-linked Gal and 1→2,3 linked Rha are unusual in RG-I structures. CC1P2 (1400 kDa), contained the same backbone, but in addition to T-α-Gal, α-4-OMe-GlcA and α-2-OMe-Gal were found as terminal units. CC1P1 shows a high complement-fixing activity, ICH_{50} being 2.2 times lower than the positive pectin control PMII (ICH_{50} appr. 71 µg/ml) while ICH_{50} of CC1P2 was 1.8 times lower. The simple structure of CC1P1 did not activate macrophages, while CC1P2 (100 µg/ml) showed the same potency as the positive controls PMII (100 µg/ml) and LPS (500 ng/ml). No cytotoxicity was detected.

Paper II. Chemical and biological characterizations of pectins from *Cola cordifolia* leaves

The main goal of this study was to investigate the structure-activity relationship of the pectins present in the leaves of *C. cordifolia*. A 50% EtOH, in addition to a 50°C and 100°C water extracts, named LCC50%, LCC50 and LCC100 respectively, were prepared from de-fatted *C. cordifolia* powdered leaves. Due to a high initial viscosity, LCC50 was not further fraction-

ated. LCC50% and LCC100 were fractionated by ion-exchange chromatography, giving the fractions denominated LCC50%A and LCC100A. These two fractions were fractionated on a MonoP column gave rise to heterogeneous and polydisperse fractions with Mw between 3 and 1300 kDa. Most fractions contained branches attached to O-3 of Rha and GalA. This suggests highly branched polymers with short side chains. Free acidic oligosaccharides (<3kD) consisting of 41% T-4-OMe-GlcA, 20% GalA, 5.6% Xyl, in addition to Ara, Rha and Gal were present in some of the fractions. Oligosaccharide analysis on a HPAEC-PAD showed three major oligomeric fragments (<3 kDa). The pectin fractions did not induce macrophages. However, all fractions showed complement fixing activity comparable to our positive control, acidic pectin from Plantago major, PMII. By comparing the pectins from the bark and leaves, we observe a structural relationship when it comes to molecular weight, types of side chains and linkages. The complement modulating and macrophage activating activities, which are thought to be important for the traditional use, are apparently lower for leaf pectins compared to bark pectins. LCC100A (from leaf) and CC1 (from bark) did not show any anti-adhesion towards H. pylori, and it was therefore concluded that the pectins does not possess anti-ulcer activity by hindering H. pylori attachment to the mucus.

Paper III. Anti-ulcer polysaccharides from *Cola cordifolia* bark and leaves

The main objective of this paper was to evaluate and compare the *in vivo* anti-ulcer activity of the leaves and bark from *C. cordifolia*. De-fatted, powdered, bark and leaves of *C. cordifolia* were extracted with 50°C water and subsequently characterized by GC, Yariv-precipitation and quantification of phenolic compounds. The bark contained more 2-OMe-Gal and less GalA compared to the leaves. Phenolic compounds were measured to be 2.2% (bark) and 18.8% (leaves), and both extracts were AG-II positive. Gastric ulcers were induced in rats by administrating 90% EtOH by gavage one hour after administration of the 50°C water extracts (0, 50 or 200mg/kg b.w.). The inhibition of ulcer formation was calculated based on lesion index (the sum of the lengths of all ulcers). The results showed that the bark and the leaves comprise a dose dependent anti-ulcer activity in (no statistical difference between the plant parts). To acquire more knowledge about the traditional use, an ethnopharmacological investigation was carried out including 26 traditional practitioners in Siby (a village near Bamako). Pain and wounds were the most cited indications.

Paper IV. Inulin-rich fractions from *Vernonia kotschyana* roots have anti-ulcer activity

The aim of this study was to evaluate the anti-ulcer potential of inulin rich extracts from roots of *V. kotschyana*. Previously, the anti-ulcer activity shown by *V. kotschyana* was attributed solely

to the saponins present. De-fatted root powder was extracted with 50°C water and subsequently with 100C water to give Vk50-I and Vk100-I. An inulin content of 98% and 83%, respectively, were found. In addition to inulin, Vk100-I contained approximately 15% pectins and minor amounts of phenolic compounds and proteins. Saponins were not detected. Vk50-I and Vk100-I were administrated 50 minutes before induction of gastric ulcers in mice with 0.3 M HCl-60% EtOH. Inhibition of ulcer formation was calculated based on lesion index. Vk50-I and Vk100-I significantly inhibited the formation of gastric lesions in mice in the concentration 100 mg/kg b.w. which corresponds to a daily intake of 15 g dried roots. In addition to the direct ulcer inhibiting ability, it is possible that water soluble polysaccharides have an indirect impact on the general health of the GI. Immunological activities were measured by complement fixation and macrophage activation. Vk50-I and Vk100-I did not show any activity in the mentioned assays. In addition, a simple toxicity study was carried out on brine shrimps which showed that toxic components were not present.

Paper V. Chromatographic immunoassays for *H. pylori* detection – are they reliable enough in Mali (West Africa)?

The aim of the paper was to find a simple method for *H. pylori* detection in addition to understand more about gastrointestinal (GI) related problems in Mali. *H. pylori* is often associated with GI diseases which are major reasons for morbidity in Mali. Twenty-nine volunteers with confirmed gastric ulcer by gastroscopy and 59 randomly selected volunteers were diagnosed by using the rapid serological test Clearview® *H. pylori*. The ImmunoCard STAT!® HpSA® test was applied on stool from 64 volunteers seeking help for gastrointestinal related ailments. An *H. pylori* prevalence of 20.7% was found among the individuals with confirmed gastric ulcer, 44% among the randomly selected volunteers and 13.4% in individuals with gastrointestinal related ailments. According to what is already known about the etiology of gastric ailments and the prevalence of *H. pylori* in neighboring countries, the infection rates in our study appear strikingly low. This might indicate that Clearview® *H. pylori* and ImmunoCard STAT!® HpSA® have low sensitivities in the populations studied. Strain variability and use of anti-malarial drugs may be an explanation. The tests need to be properly evaluated in Mali before they can be relied upon as diagnostic tools.

4. Results and discussion

4.1 Ethnopharmacological research (Paper III)

During the years 1998-2008 four ethnopharmacological surveys on the medicinal tree, *Cola cordifolia*, were carried out in Siby, Dioila and the Dogonland (Diallo *et al.*, 2002; Grønhaug *et al.*, 2008; Togola *et al.*, 2008, Paper III). The first study, Diallo *et al.* (2002), identified *C. cordifolia* as one of the fifteen most cited wound healing plants (out of 123 plants) in the Bamako region. It was therefore of interest to acquire more knowledge about the use of this particular tree. In 2008, Togola et al. reported that the tree was used against abdominal pain, wounds and fever, while Grønhaug *et al.* (2008) reported pain, fever and diarrhea as the main areas for treatment. In the third paper of this thesis (Paper III), the most cited indications were pain and wounds/dermatitis. The ethnomedical information obtained from the three studies

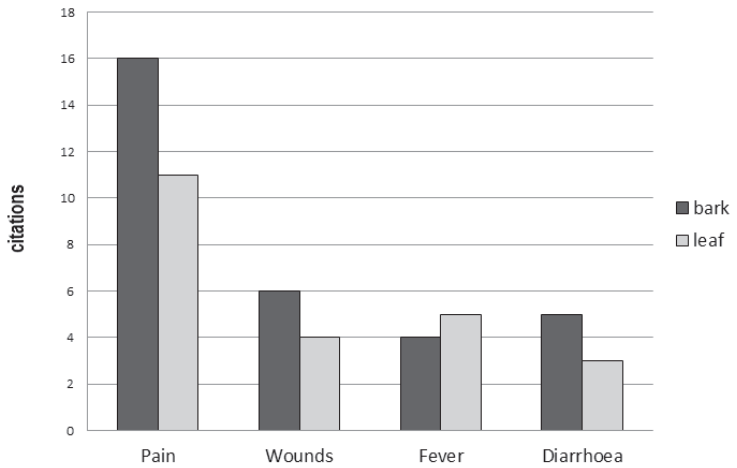

Figure 4.1: The combined ethnomedical results of *C. cordifolia* bark and leaves.

mentioned above (Diallo *et al.*, 2002; Grønhaug *et al.*, 2008; Togola *et al.*, 2008, Paper III) was combined in Fig. 4.1. The results showed that there was not a big difference in the traditional uses between the leaves and bark. Similarities in the use of the two plant parts indicate that plant part substitution should be possible.

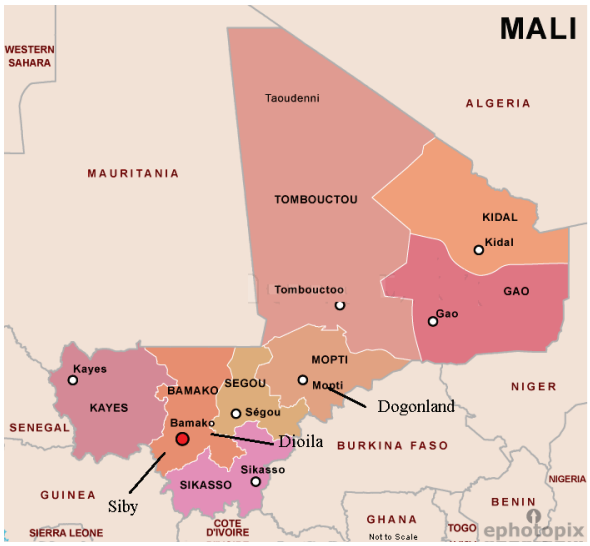

Figure 4.2: Map of Mali. The village Siby lies 50 km southwest of the capital, Bamako. The surveys of Grønhaug *et al.* (2008) were carried out in Siby and Dioila. The study of Togola *et al.* (2008) was carried out in Dioila and Dogonland, and the study in Paper III was carried out solely in Siby.

The main way of preparing the medicine of the two plant parts in the three surveys was extraction with boiling water (decoction). In addition to low molecular weight polar and semi-polar substances like alkaloids, saponins and flavonoids, also high molecular weight substances like polysaccharides are extracted by the hot water. For healing of wounds and gastric ulcers, it is probably highly relevant to look for potentially active polysaccharides as these molecules can have an immunomodulating activity in addition to adhering to the surface of the wound and have an effect as protecting remedy.

4.2 Isolation and purification of the pectic polymers

Water soluble polysaccharides from *C. cordifolia* described in Paper I and II were extracted and purified according to the flow schemes, see Fig. 4.3. Water extracts for the anti-ulcer experiments in rat and mouse models in Paper III and IV were purified according to the flow scheme in Fig. 4.4. CCbark50 and CCleaf50 in Fig. 4.4 must not be confused with any of the fractions in Fig. 4.3.

Initial investigations of the polysaccharides isolated from the bark of *C. cordifolia* have previously been carried out by Togola *et al.* (2008) and Næss (2003). They found that a 50°C water extract was more active in the complement fixing test compared to the 100°C water extract. It was therefore decided to focus on the 50°C water extract in Paper I.

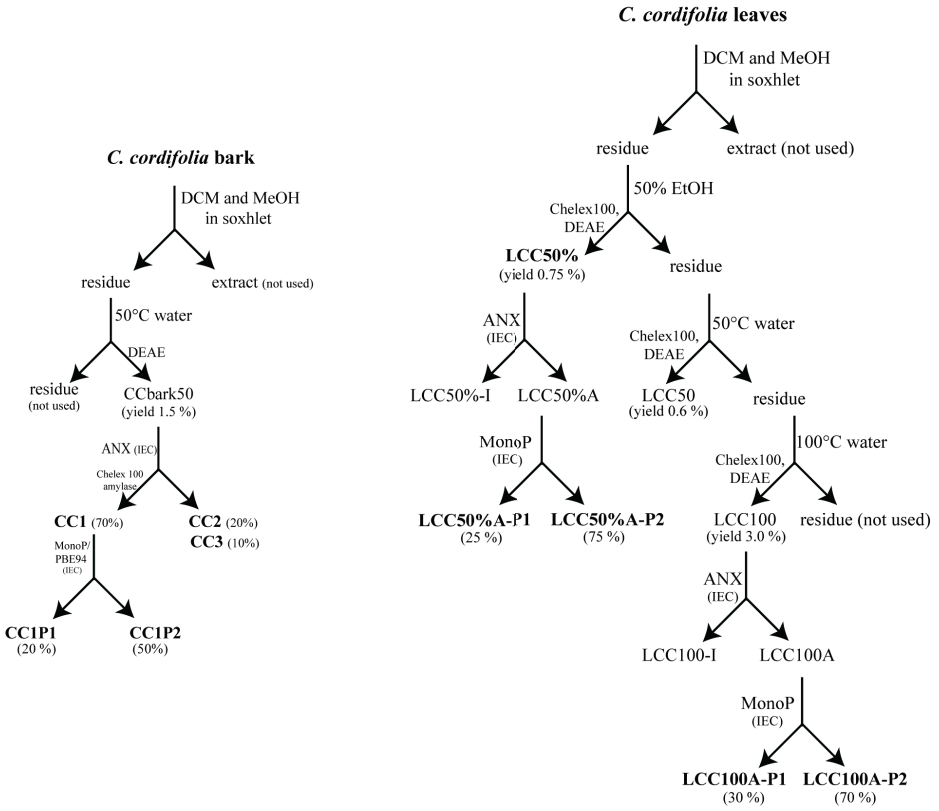

Figure 4.3: Fractionation scheme of *C. cordifolia* bark and leaves as described in Paper I and II.

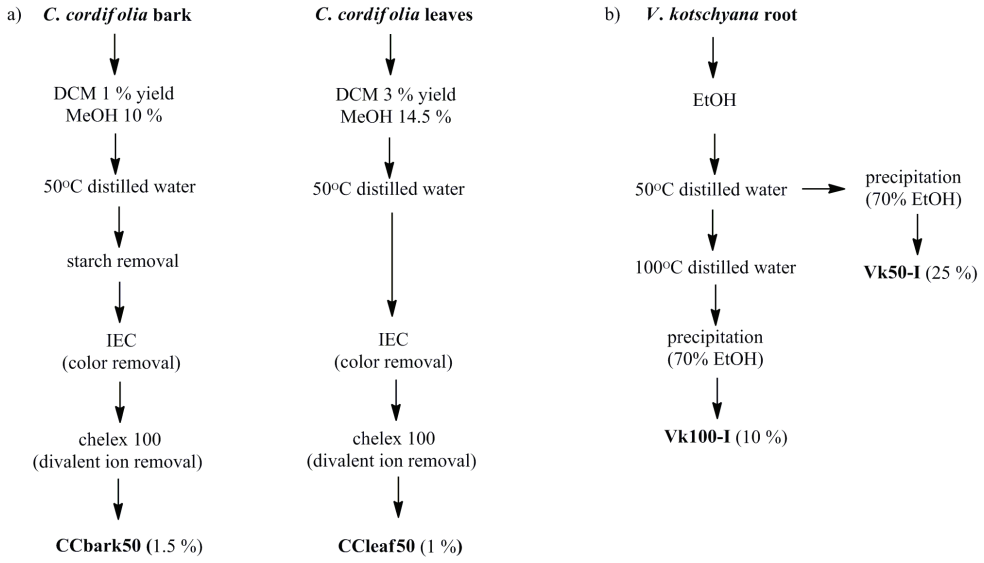

Figure 4.4: Extraction and purification of water extracts used for experimental anti-ulcer model (Paper III and IV).

The polysaccharides from leaves (Paper II) had not been investigated before, and it was therefore decided to extract the leaves with 50% EtOH, 50°C and 100°C water. The polysaccharide rich fractions obtained were named LCC50%, LCC50 and LCC100 respectively. LCC50 had a similar monosaccharide composition as LCC100, but was even more viscous. It was therefore decided not to further purify the 50°C leaf extract, see Fig. 4.3.

Papers III and IV focus on the anti-ulcer activity of pectins from the bark and leaves from *C. cordifolia* and the roots from *V. kotschyana*, respectively. Since we were primarily interested in the medical activities contributed by the pectins present, the plant materials were de-fatted with organic solvents prior to water extraction, see Fig. 4.4. Extraction with DCM and MeOH removes hydrophobic and semi-polar substances.

Togola *et al.* (2008) had problems reducing the uronic acids in connection with the linkage studies of the polysaccharides from the bark of *C. cordifolia*. It was shown by IR that the uronic acids not were esterified. Since this was the case, the presence of divalent ions like Ca^{2+} and Mg^{2+} may have created ionic cross-linkages of carboxyl groups in chains. Strong ionic cross-linkages may have hindered the reduction of the uronic acids. An intermediate reaction with carbodiimide is necessary to convert the uronic acids to lactones, so that the lactones can be reduced in a second step by sodium borodeuteride ($NaBD_4$) (Kim & Carpita, 1992). Successful reduction of the pectins from *C. cordifolia* bark was achieved in Paper I by first removing the divalent ions by passing the extracts through a solid phase chelator, Chelex 100. The cross-linking of the pectins before removal of divalent ions was visualized by atomic force microscopy (AFM), see Fig. 4.5 AFM. AFM has previously been used to image individual pectin molecules and to study their aggregation (Morris *et al.*, 2011).

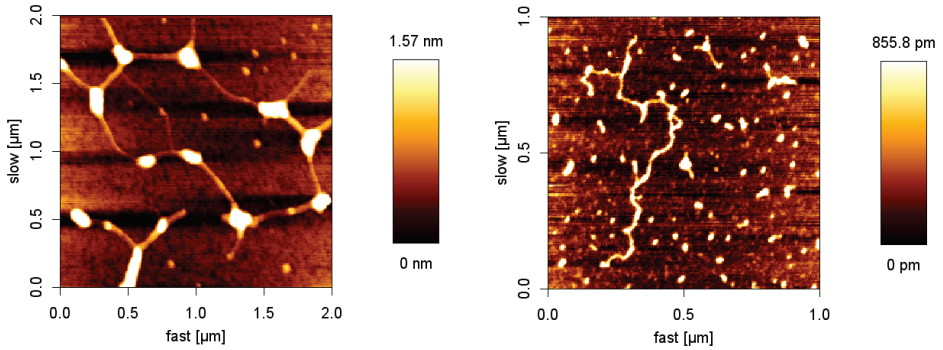

Figure 4.5: Atomic force microscopy. Topography images of (left) CC1 before divalent ion removal (2.0 x 2.0 µm), (right) CC1 after Chelex (1.0 x 1.0 µm).

High viscosity was also observed in the leaf fractions (Paper II). Divalent ions were therefore removed prior to fractionation, but unfortunately the removal of the divalent ions was not as efficient as reported for the bark extracts (Paper I) and the extracts therefore remained viscous. This may be due to a higher initial viscosity of the polymer fractions in addition to the fact that the extracts were not first separated on the ANX-IEC as was done for the bark fraction, but passed directly through the Chelex column in an earlier stage of fractionation, see Fig. 4.3.

4.2.1 Structures of pectins from the bark of *C. cordifolia* (Paper I)

The purified fractions CC1P1 and CC1P2 were analyzed for monosaccharide contents and type of linkages present using GC and GC-MS (see table 1) as well as analyses by various NMR techniques. CC2 was analyzed for monosaccharide content and linkage types only.

As seen from the linkage analysis, table 1, CC1P1 consists of T-Gal, 1→2,3 linked Rha and 1→4 linked GalA in the ratio 1:1:1. Due to the simplicity of the structure, it was possible to unambiguously deduce the whole structure by NMR, see Fig. 4.6. A M_w of 135 kDa corresponds to n= 40 in Fig. 4.6(a).

The spin system for each sugar residue of CC1P1 was assigned according to the COSY spectrum, Fig. 4.7, with assistant/confirmative information from the TOCSY (spectra not shown). The sequence of the linkages of sugar residues was inferred from the HMBC. The anomeric configuration of the monomers was based on measurements of coupling constants and comparison of the chemical shifts with data from literature (Duus *et al.*, 2000; Bock *et al.*, 1984; Bock & Thøgersen, 1983). We found that the three monomers had α configuration. Gal is normally found to be present as β configuration. However, lately, similar types of pectins were found in flaxseed hulls. The structure had 1→2,3 linked Rha in the RG-I backbone and monomeric terminal αGal (in addition to other monosaccharides) attached to *O*-3 of Rha flaxseed hulls (Qian *et al.*, 2012).

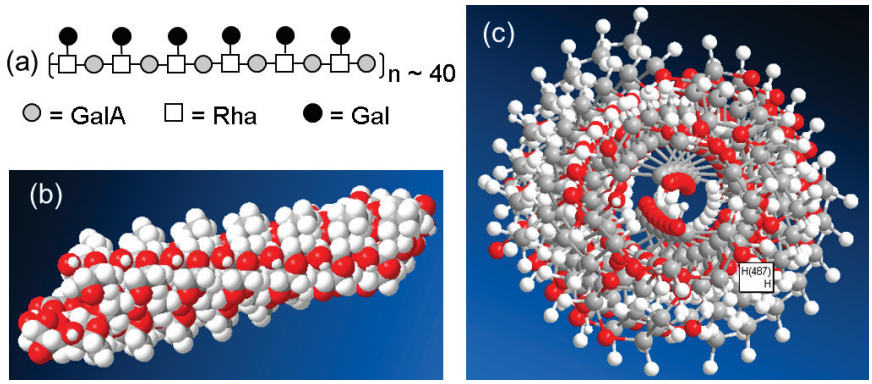

Figure 4.6: (a) Proposed structure of CC1P1. (b) and (c) Hypothetical drawings of the low energy conform of CC1P1, [2→)[α-D-Gal(1→3)]α-L-Rha(1→4)α-D-GalA(1→]$_{20}$.

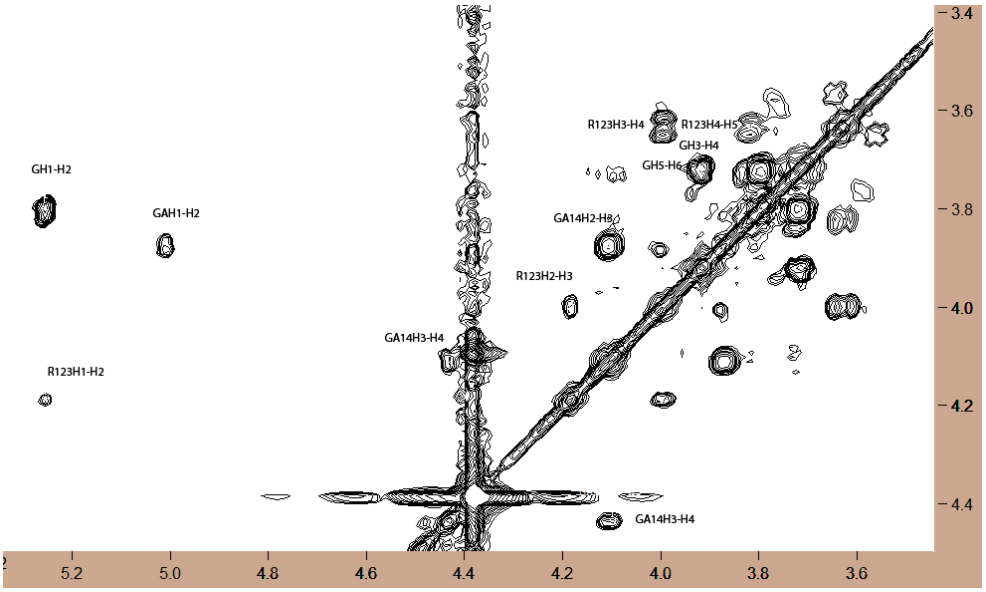

Figure 4.7: COSY spectrum of CC1P1. Abbreviations: The two last letter/numbers indicate the location of the proton (H). The first letter(s) indicates the monosaccharide; G=Gal, GA=GalA, R=Rha.

		Paper I			Paper II			
		CC1		CC2	LCC50%A		LCC100A	
		P1	P2		P1	P2	P1	P2
Ara	T*f*		1.6	15.9	7.5	1.6	5	3.8
	1→3*f*			5.7				
	1→5[a]			9.6	4.4		1	5.9
	1→3,5[a]			3.3	1.8		1	0
	1→2,5[a]			2.7	2.4		1.2	2.8
Rha	T[b]			2.7	1	1		
	1→2			3.2	1	0.7	1	3
	1→3				0.3	0.6	0.5	0.4
	1→2,3	32	15.3	1.1	9.8	10.2	9	4.4
	1→2,4		8.2	1.5	3.7	7.4	1.7	3.3
Xyl	T				1.3		0.6	
	1→4				1.4		0.6	
Gal	T	31	7.9	3.3	8	7.5	7.2	4.6
	1→3			5.2	1.3	2.1	1	1.4
	1→4		13	1.8	6	7.1	1.1	3.7
	1→6			3.2	1.3	0.4		
	1→3,6			17.8	3.9	0.9	1.2	
2-OMe-Gal	Tp		6.7			3.5		
4-OMe-GlcA	Tp		14.9	6.6	8.2	21.5	2.3	5.1
GalA	1→4	35	23.9	11.5	29	19.5	58.5	50.7
	1→3,4		5.7		4	12.7	4	4
	1→2,4		1.3		1.6	2	1	1.5

[a] It is not possible to distinguish between 1→5 linked Ara*f* and 1→4 Ara*p*

[b] The conformation is pyranose if otherwise not stated.

Table 4.1: Linkage analysis. GC-MS of methylated alditol-acetates of selected pectin fractions from the bark (CC1P1, CC1P2 and CC2) and the leaves (LCC50%A-P1, LCC50%A-P2, LCC100A-P1 and LCC100A-P2).

The $^{3}J^{CH}$ HMBC couplings confirmed a RG-I backbone consisting of [→4)αGalA (1→2) αRha(1→]. All terminal α-Gal were linked via *O*-3 to α-Rha (spectra not shown). This information was also inferred by the 3D HSQC-NOESY spectrum, see Fig. 4.8. The NOE signals can bee seen in a distance up to 5Å under the right circumstances. The HSQC-NOESY gave additional information about the three-dimensional (3D) structure, as it provides distance constrains between the protons that are located on average less than 5Å from each other. In general, NOE peaks with a high intensity are located closer together than the weaker correlations. The two protons bound to the carbons participating in the glycoside linkage will give medium intensity, as the protons are situated approximately 2.4Å away from each other, as calculated in Chem BioDraw ultra 13.0, see correlations marked with green rings in Fig. 4.8. Correlations giving information about distance constrains in the 3D structure of CC1P1 are marked by blue circles on Fig. 4.8. The information obtained from the distance constrains is illustrated on Fig. 4.9.

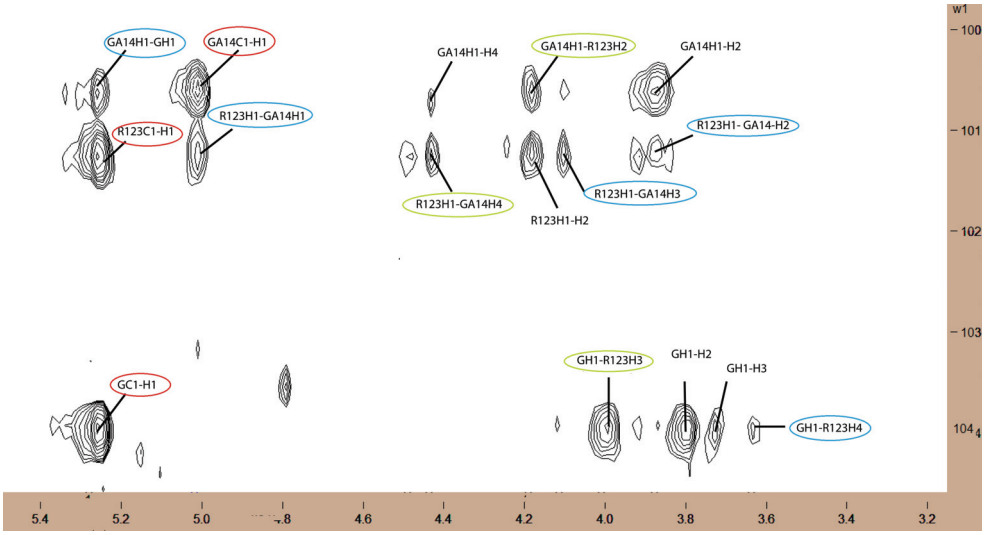

Figure 4.8: HSQC-NOESY (3D) spectra of the anomeric signals of CC1P1. The red circles indicate pure HSQC ($^{1}J^{CH}$ correlations), the green circles indicate NOE constrains between the protons that are located adjacent to the carbons participating in the same glycoside linkage, blue circles indicate NOE signals giving information about distance restrains closer than 5Å. Abbreviations: The two last letter/numbers indicate the location of the proton (H) or the carbon (C). The first letter(s) indicates the monosaccharide; G= Gal, GA=GalA, R=Rha.

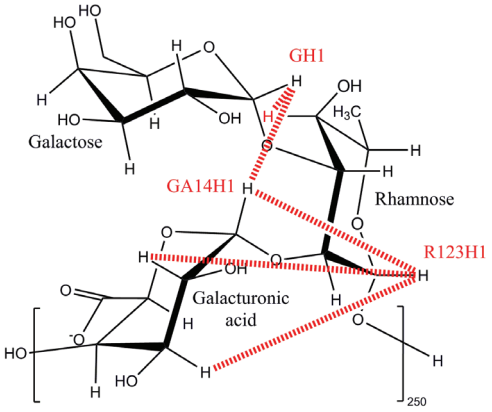

Figure 4.9: NOE distance constrains found in CC1P1

Fig. 4.9 shows that GalA is probably tilted more so that H3 of GalA is closer to H1 of Rha. Rha must also be turned to the left to have a conformation responding to what is seen in the HSQC-NOESY spectrum. As can be seen from Fig. 4.6 (b), the methyl groups of Rha are facing directly outwards making the surface more hydrophobic. However, the surface is still negatively charged due to GalA. SEC-MALLS results indicate that CC1P1 is almost homogeneous with a PDI (M_w/M_n) of 1.2. The MHS plot (intrinsic viscosity as a function of molar mass) showed that CC1P1 is rather stiff, comparable to alginate. The stiffness is attributed to the RG-I backbone.

CC1P2, eluting after CC1P1 (MonoP column), had a more complex nature consisting of 15% 4-OMe-GlcA and 6.7% 2-OMe-Gal. We see from the non linear conformation plot obtained by SEC-MALLS analysis, that CC1P2 was heterogeneous, meaning that it consists of subpopulations with different extensions of side chains. This fraction was also subjected to NMR analysis, but due to its complexity, we were not able to deduce the whole structure. HMBC was difficult to record, so the sequence of the linkages of sugar residues was inferred by the HSQC-NOESY and the NOESY. Rha and GalA were clearly alternating as seen from the inter-residue connectivities of Rha H1 - GalA H4, and GalA H1 - Rha H2. Strong NOEs were observed between 2-OMe-Gal H1 and 1→2,3 linked Rha H3. 2-OMe-Gal is therefore deduced to be linked directly to the backbone via *O*-3 of Rha, see Fig. 4.10. Correlations between H1 of 1→2,3 linked Rha and H1 of 4-OMe-GlcA might indicate that 4-OMe-GlcA is not directly linked to Rha, but to the adjacent GalA in the RG-I backbone. We identified three different spin systems for 1→2,3 linked Rha. This indicates that 1→2,3 linked Rha is present in three different electronic surroundings, presumably meaning that the unit is attached to different monomers. We could not unambiguously find all the three Rha and connect them to neighboring subunits. However, the Rha spin system found to have correlations with H1 of 2-OMe-Gal, also have correlation with 4-OMe-GlcA. This indicates that 2-OMe-Gal and 4-OMe-GlcA are probably linked to adjacent monomers, Rha and Gal A respectively. Capek *et al.* (1987) and Renard *et al.* (1999) have previously found terminal GlcA directly linked to *O*-3 of 1→3,4 linked GalA in the RG-I backbone. This supports our theory that Rha and GalA have both *O*-3 linked side chains, where 4-OMe-GlcA is linked to GalA. However, it is more 4-OMe-GlcA (14.9%) present than 1→3,4 linked GalA (6%) which indicates that T-4-OMe-GlcA also has to be present linked to an other monosaccharide. This unit is probably not Rha as we did not see any correlations between these units. H1 of Gal1→4 shows correlations with Rha H3. Therefore, it is possible that T-4-OMe-GlcA may be linked to 1→4 Gal. Due to a crowded spectrum in the 4 ppm region, we did not succeed to determine correlations between 4-OMe-GlcA and Gal.

We could not detect correlations between H1 and H4 of GalA, suggesting that homogalacturonan (HG) was not present. NMR is not a very sensitive method, so we cannot exclude small amounts of HG. A tentative suggestion of the overall structure of CC1P2 can be found in Fig. 4.10. How the monomers, as found linked together by NMR, are distributed on the RG-I backbone is difficult to deduce. They can be randomly distributed along the RG-I chain, they can be found in blocks and some elements can even be present in different populations of the polymers as seen by SEC-MALLS.

In order to determine the structure in more detail, selective degradation, separation and analy-

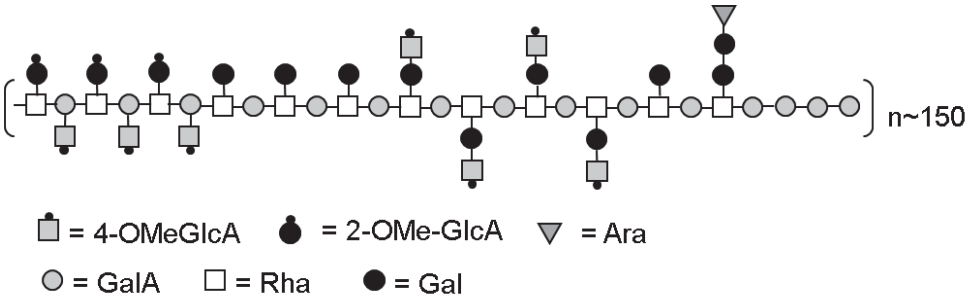

Figure 4.10: Hypothetical drawings of CC1P2 based on linkage analysis (GC and GC-MS) and NMR. One monomer on the figure correlates to a content of 2% in the fraction.

ses of the oligomers obtained can reveal more about the true structure. It was tried to degrade CC1P2 with rhamnogalacturonase without success. These enzymes are often difficult to purify and unfortunately not possible to buy. Chemical β-degradation of the RG-I backbone can be an alternative. However, adding an electron withdrawing group on the carboxylic acid of GalA, enabling β-degradation (i.e. trans elimination of the glycoside binding on C4 of GalA), was shown to be difficult because conventional methods for methyl esterification of carboxylic groups use DMSO as solvent (Deng *et al.*, 2006). Unfortunately, CC1P2 did not dissolve well enough in DMSO for esterification of the carboxylic groups. Production of an intermolecular lactone between hydroxyl (OH) at C4 and the carboxylic acid of GalA with carbodiimide was therefore tried. This resulted in clear degradation of the molecule, providing a reduction in M_w from 1400 kDa to approximately 90 kDa (unpublished results from SEC-MALLS). Surprisingly, analysis on a Bio-LC (HPAEC-EC) gave results which were difficult to be reproduced Fig. 4.11.

CC2 is structurally not related to CC1P1 and CC1P2. CC2 contains a high degree of arabinans and AG-II side chains, see Fig.4.12, and therefore resembles pectin structures commonly found in medicinal plants (Paulsen & Barsett, 2005). SEC-MALLS showed a compact, but flexible nature due to the long side chains. The M_W was determined to be 63 kDa, corresponding to approximately 350 monomers, or the structure in Fig. 4.12 repeated approximately seven times. The AG-II side chains were heavily branched with 17.8% 1→3,6 Gal. Normally, terminal monosaccharides of AG-II are Ara or Gal, but GlcA and 4-OMe-GlcA residues can also be present (Mohnen, 2008). Presence of GlcA and 4-OMe-GlcA as terminal units on the surface of the AG-II side chains of CC2 is highly likely. Due to large branches and a relatively low amount of GalA (11.5%), the viscosity of CC2 was low. The protein content of CC2 was 1.3 % ±0.2% (unpublished results), and CC2 is therefore probably not an arabinogalactan protein.

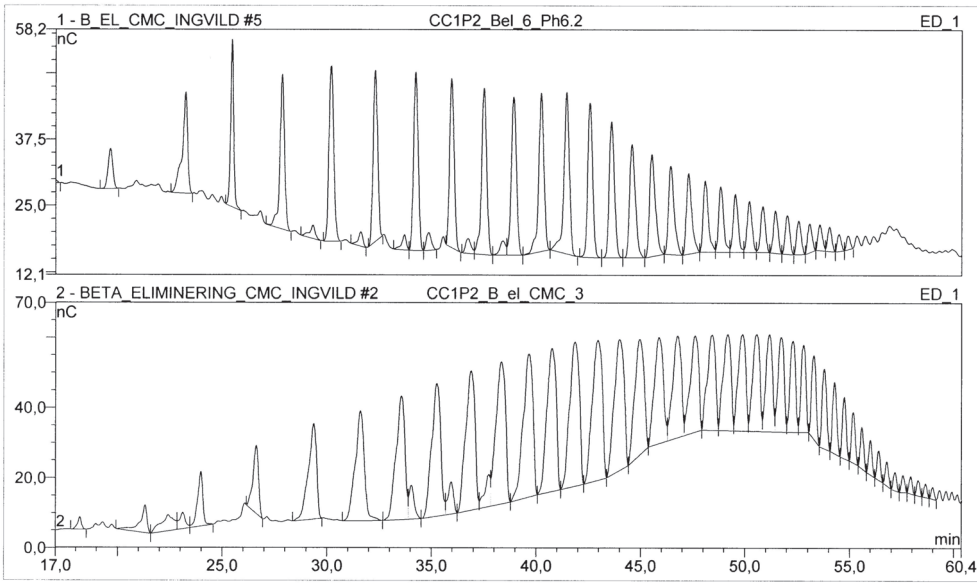

Figure 4.11: HPAEC-EC profile from β-elimination of CC1P2.

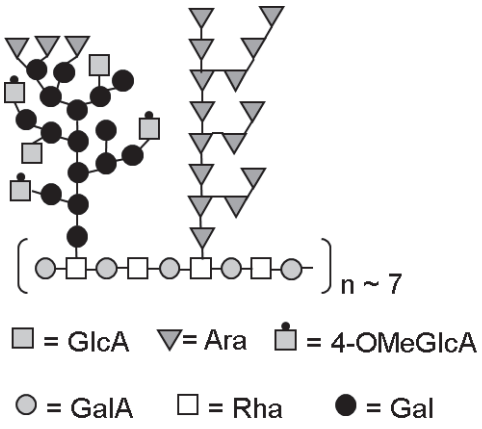

Figure 4.12: A hypothetical structure of CC2 based on linkage analysis and methanolysis. One monomer on the figure correlates to a content of 2% in the fraction.

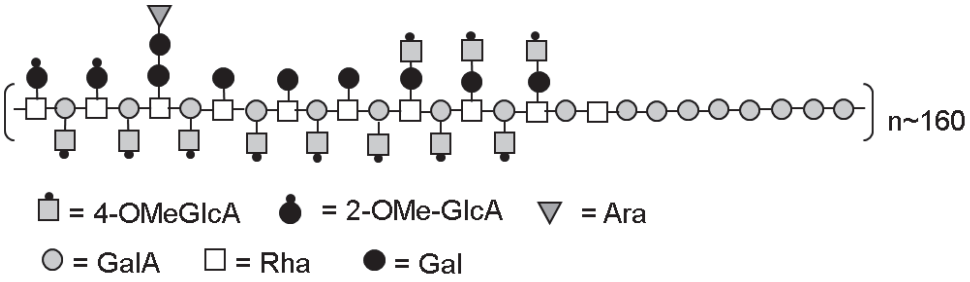

Figure 4.13: A hypothetical structure of LCC50A-P2 based on linkage analysis (GC and GC-MS).One monomer on the figure correlates to a content of 2% in the fraction.

4.2.2 Structures of pectins from the bark of *C. cordifolia* (Paper II)

The water extract from the leaves was fractionated according to Fig 4.3.Isolating pectin fractions from the leaves proved to be more challenging than purifying bark fractions. The viscosity was higher and the fractions were more polydisperse and also more heterogeneous. Compared to the bark, the leaves are constantly renewed and they are therefore also in a more dynamic state of development when it comes to pectin synthesis and remodeling. Pectins have a direct role in cell wall rheology and stoma functions, and they work as plasticizer in regulation of Ca^{2+} mediated interactions of HG (Harholt *et al.*, 2010). Considering that the bark and leaves have different biological functions in the two plant parts, it is not surprising that the pectins present in leaves are structurally dissimilar, even though the pectins origin from the same genetic material.

Deduced from the linkage analysis, arabinan side chains are present in LCC50%A-P1, LCC100A-P1 and LCC100A-P2 as seen from the presence of terminal Ara, in addition to 1→5, 1→3,5 and 1→2,5 linked Ara (Table1). In addition to arabinan side chains, LCC50%A-P1 contains AG-II as shown by the presence of 1→3,6 Gal, 1→3Gal, 1→6Gal and terminal Ara*f* in addition to a positive Yariv precipitation.

As calculated from SEC-RI results (Paper II), LCC50%A-P1 and LCC100A-P1 contained approximately 25% and 15% free oligosaccharides respectively. The HPAEC-EC chromatogram from the oligosaccharides with a size of less than 3kDa isolated of LCC50%A-P1 and LCC100A-P1 showed a presence of three overlapping peaks. Methanolysis of LCC50%A-P1 <3 kDa showed a presence of 22% Ara, 9.8% Rha, 9.8% Xyl, 5.8% Gal, 6.4% 4-OMe-GlcA, 34.7% GalA and 11.5% Glc. Linkage analysis was not carried out. As the mother fraction LCC50%A-P1 (>3kDa) were depleted from Xyl after removal of oligosaccharides, we conclude that Xyl was not present as part of xylogalacturonans, and only present as oligomers (see 4.1).

LCC100A-P2 was analyzed by NMR. Due to heterogeneity, high viscosity and presence of complex arabinans the spectra gave poor resolution. This fraction was therefore not suitable for NMR analysis.

4.2.3 Comparing pectins from bark and leaves of *C. cordifolia* (Paper I and II)

The pectic fractions derived from *C. cordifolia* diverge structurally from pectins commonly found in medicinal plants from Mali (Grønhaug *et al.*, 2010, 2011; Inngjerdingen *et al.*, 2007; Nergaard *et al.*, 2005*c*; Diallo *et al.*, 2001). Generally, the pectins present in the bark and leaf of *C. cordifolia* differ from these by having RG-I backbone with the rather uncommon 1→2,3 linked Rha instead of the more common 1→2,4 linked Rha. In addition,they have a high frequency of short side chains consisting of the monomers T-4-OMe-GlcA, T-Gal or T-2-OMe-Gal. Infrared spectroscopy (IR) analysis did not show any absorption in the relevant areas corresponding to esters for bark and leaf polysaccharide fractions, thus the free uronic acids are responsible for the resulting cross-linkages present caused by divalent ions.

The leaf fractions had a higher content of HG compared to bark fractions and also a higher viscosity. However, pectins from both plant parts have a higher viscosity than what should be expected. A reason for this may be that the short monomeric side chains cannot provide steric hindering of the HG cross-linking. In addition, the 4-OMe-GlcA terminal units may also participate in crosslinking with divalent ions. Pectins from the vegetable okra or lady's fingers (*Abelmoschus esculentus)* are similar in structure compared to pectins found in *C. cordifolia*. The vegetable is well known for the high viscosity of the fruit juice.

Despite the general similarities between the pectins present in the bark and leaves, it was not possible to follow the same fractionation scheme for polysaccharides from the two plant parts. The leaf polysaccharide fractions LCC100-I and LCC50%-I, that should correspond to the main bark fraction CC1, diverged from CC1 in addition to precipitate in solution. It was therefore not possible to follow the same fractionation scheme which makes it difficult to directly compare fractions.

The most purified fractions from the bark were generally more homogeneous and less polydisperse compared to those from the leaves. In addition, free oligosaccharides were not present in the bark. These differences can be due to the fact that the leaves are in constant construction, while the bark is more in a steady-state concerning biosynthesis of pectins.

The leaf fraction LCC50%A-P2 was structurally similar to CC1P2. However, they differ in the amount of 2-OMe-Gal, which seems to be more abundant in CC1P2, and of 4-OMe-GlcA, which is present in higher amounts in LCC50%A-P2. In addition, branching on GalA is more abundant in LCC50%A-P2, which might be related to the presence of 4-OMe-GlcA (as this unit is probably linked to *O*-3 on GalA). The AG-II rich fraction CC2, present as one of the main fractions in the bark, was not found i the leaves.

In Paper III de-fatted plant material from bark and leaves are extracted with 50°C water to produce the extracts CCbark50 and CCleaf50 (see Fig. 4.4). The monosaccharide compositions determined by GC showed similarities between the two polymers, but CCbark50 contained more 2-OMe-Gal, 4-OMe-GlcA, Rha and less GalA. The higher amounts of GalA, in addition

to lower amounts of Rha in the leaf polysaccharide, suggest a presence of less RG-I and more HG. The presence of HG, which can participate in cross-linkages with divalent ions, may be the reason for why the polysaccharide CCleaf50 had a much higher viscosity than CCbark50.

The phenol-content of CCbark50 and CCleaf50 was evaluated since phenolic substances can interfere with immunological assays. A high phenolic content can often indicate presence of tannins. Tannins are incompatible with many of the ingredients in the *in-vitro* assays, i.e. with metal salts and proteins. The leaves showed 10 times higher phenolic content compared to the bark (18.8 and 2.2% respectively).

4.3 Immunomodulating properties and structural requirements

Polysaccharides capable of interacting with the immune system by up- or down-regulating specific aspects of the host response can be classified as immune modulators. Polysaccharides like pectins and β-glucans have been reported to display a variety of immune modulating activities (Schepetkin & Quinn, 2006; Goodridge *et al.*, 2009; Yamada & Kiyohara, 2007). More detailed information about immunomodulating activity is found in §1.2. The purified pectins from the bark and leaf of *C. cordifolia* (Paper I and II) were investigated and tested for immune modulation by complement fixation and macrophage stimulation, see Fig. 4.14. The polysaccharides CCbark50 and CCleaf50 (Paper III) were tested for complement fixation abilities only.

4.3.1 Complement fixation activity

CC1P1 from bark (1:1:1 ratio of Rha:GalA:Gal), shows the highest complement activating activity of all polysaccharide fractions tested. Previously, it was suggested that the hairy regions of RG-I, with complex galactans or AG-II side chains attached, are important for activity (Paulsen & Barsett, 2005; Yamada & Kiyohara, 2007). CC1P1 has only T-Gal attached to the RG-I backbone, but still shows a fairly high activity (three times more active than PMII). This means that complex galactans are not an absolute requirement as monomeric side chains of T-Gal also shows activity.

CC1P2 has a high frequency of short side chains, see Fig. 4.10. However, the terminal groups consisting of acidic 4-OMe-GlcA make the surface negatively, and this seems to reduce the complement fixing activity compared to CC1P1.

In theory, CC2 has the structural requirements important for exhibiting a very high activity in the complement system. However, as also seen for CC1P2, we believe that the negative charged terminal groups reduce the activity. Looking at the tentative structure, see Fig. 4.12, the Gal units are covered by acidic sugars like 4-OMe-GlcA and GlcA, which gives the side chains an acidic surface. This is in agreement with what suggested by Yamada & Kiyohara (1999) that

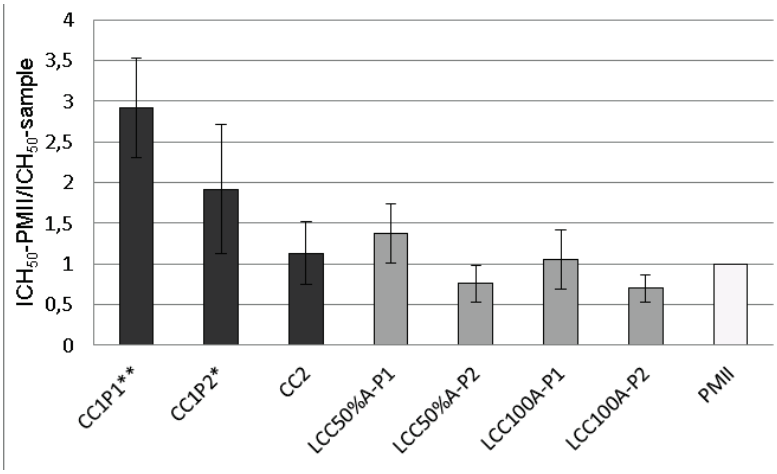

Figure 4.14: Complement fixation. The bars show ICH_{50}-PMII divided on ICH_{50}-sample and thus shows how active each individual test sample is compared to the positive control, PMII. These results are based on three separate experiments. **p<0.01, *p<0.05 compared to PMII. Brown bars represent bark fractions, green bars represent leaf fractions.

high complement fixing activity seen for pectins is due to neutral side chains attached to the RG-I backbone. The AG-II side chains of CC2 are not neutral and will therefore probably be less active than the corresponding neutral AG-II side chains.

LCC50%A-P2, see tentative structure in Fig. 4.13, has a highly branched structure with mono or disaccharides attached to the RG-I backbone, and is structurally similar to CC1P2. However, LCC50%A-P2 contains even higher amounts of negatively charged uronic acids facing the surface compared to CC1P2. The complement fixing activity is lower for LCC50%A-P2 compared to CC1P2, which is in agreement with our theory that a negative surface down regulates the activity. In addition, LCC50%A-P2 contains HG which have been shown to reduce the activity (Yamada & Kiyohara, 2007). LCC50%A-P1 has a higher activity compared to LCC50%A-P2 ($p<0.05$). This may be due to the presence of AG-II structures and arabinans in LCC50%A-P1, which can increase the activity and a lower amounts of acidic monosaccharides in terminal positions. The activity of the oligosaccharides present in LCC50%A-P1 shows no activity in the complement assay. LCC50%A-P1>3 kDa has not been tested, but the activity is probably higher than what reported for LCC50%A-P1.

CCbark50 and CCleaf50 (Paper III) were also evaluated in the complement fixation assay. Interestingly, CCleaf50 did not show any activity, see Fig. 4.15, while the ICH_{50} of CCbark50 was 50 µg/ml. We attribute the low activity of CCleaf50 to interference of polyphenols which were present at a concentration of 18.8%. BPII was used as a positive control instead of PMII used in Paper I and II. PMII normally have an ICH_{50} value of 70 µg/ml (Paper I), which makes CCbark50 equal or more active compared to PMII.

LPS is shown to activate the complement through the alternative pathway, but since we use a

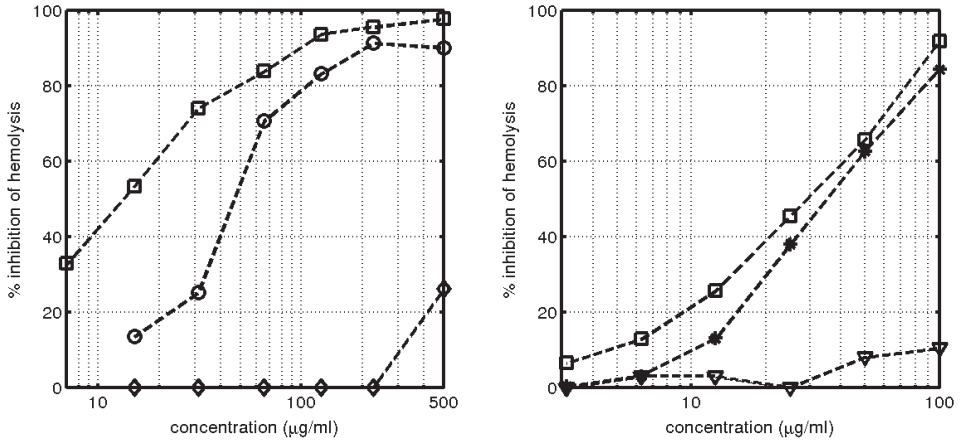

Figure 4.15: Complement fixation. Concentration dependent activity of (left) CCbark50 (○) and CCleaf50 (◊); (right) influence of LPS on the inhibition of hemolysis. BPII (□), BPII added LPS (10 µg/ml) (∗) and pure LPS (▽). BPII from *B. petersianum* (□) was used as a positive control.

1:70 dilution of human serum (the source of complement) (Michaelsen *et al.*, 2000) the alternative pathway will be inactive. Our results see Fig. 4.15, show that interference with LPS is practically non-existing and removal of LPS prior complement fixation is therefore unnecessary when using Method A (Michaelsen *et al.*, 2000).

4.3.2 Macrophage induction

CC1P1 did not induce macrophage activity, while 10 µg/ml of CC1P2 induced nitric oxide release in comparable amounts to that of LPS 0.5 µg/ml, see Fig. 4.16. Apparently, the short side chains of single Gal monomers in CC1P1 are not sufficient for stimulation. However, CC1P2 also have short side chains, but the activity is high. This may be due to the presence of negatively charged terminal 4-OMe-GlcA. Inngjerdingen *et al.* (2008) suggested that the presence of arabinogalactan side chains is part of the structural requirements for the induction of macrophage response.

LPS was not removed from CC1P2 prior to macrophage co-incubation. However, we find it unlikely that the observed activity is due to presence of LPS contamination since CC1P1 did not induce macrophage activation. CC1P1 and CC1P2 are obtained in the same fractionation step, which normally should result in the same contamination rate of LPS. All leaf fractions, LCC50%A-P1, LCC50%A-P2, LCC100A-P1 and LCC100A-P2 were passed through a Detoxy-Gel™column (polymyxin B). We could not detect any macrophage stimulation of any of these fractions. Detoxy-Gel™is not recommended for LPS removal from pectins, because insufficient LPS removal from pectins has been observed (personal communication Samuelsen,

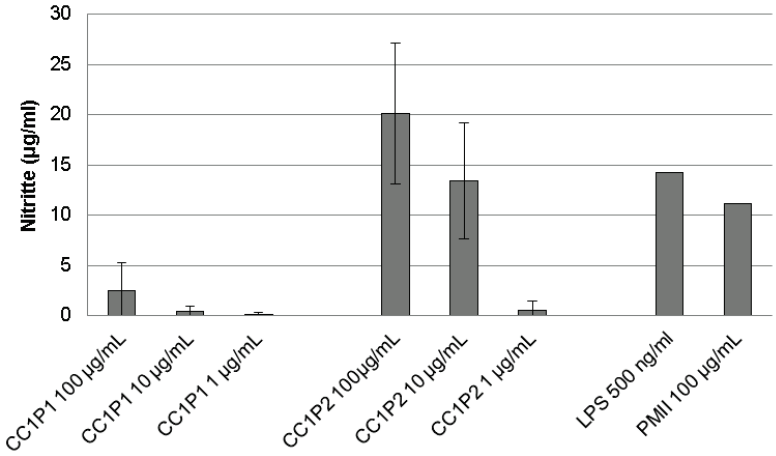

Figure 4.16: Stimulation of macrophages with extracts from *C. cordifolia* bark. NO(g) liberated from activated macrophages is naturally broken down to nitritte which is measured by colorimetric detection. A representative result is given as mean ±SD. LPS and PMII are present as positive controls.

A.B.). However, as none of the fractions showed any activity, LPS was not present in interfering amounts.

4.4 Anti-ulcer activity of polysaccharide rich extracts from *V. kotschyana* and *C. cordifolia* (Paper III and IV)

Since gastric ulcer is regarded as an important public health problem in Mali, DMT wanted to focus on the traditional use of *C. cordifolia* as an anti-ulcer medicine. Polysaccharide rich extracts from *C. cordifolia* bark and leaves, see Fig. 4.4, (Paper III) were therefore tested for anti-ulcer activity in a preliminary acute experimental rodent model. The polysaccharide rich fractions CCbark50 and CCleaf50 showed a similar and dose dependent inhibition of ulcer formation (50 and 200mg/kg). The HCl/EtOH induced ulcer model is commonly used for screening of anti-ulcer activity of herbal drugs. Mechanisms that have been proposed for anti-ulcer activity of pectins include mucosal protective coating, anti-secretory activity of gastric acid and pepsin in addition to radical scavenging activity (Matsumoto *et al.*, 2002). Karaya gums (E-417) isolated from *Sterculia* species from the same family as *C. cordifolia*, Malvaceae, contains pectins with similar carbohydrate composition (Singh & Chauhan, 2011). Cross-linking of karaya gums with divalent ions was found to create structures that delay stomach emptying (Singh & Chauhan, 2011; Singh *et al.*, 2010). Residual pectins in the stomach when the necrotizing agent is administered may physically hinder the creation of lesions by coating the epithelial cells or diluting the necrotizing agent. Delay of stomach emptying could therefore also be a possible anti-ulcer mechanism of *C. cordifolia* pectins.

The acute anti-ulcer rodent model is probably not the best model for studying anti-ulcer activity as chronic *H. pylori* infection is the main factor for developing gastric ulcer. A standardized mouse model of *H. pylori* infection (Lee *et al.*, 1997) could probably be a better screening method. However, *H. pylori* anti-adhesion studies of fractions from the leaves and bark (LCC100A and CC1 respectively, Paper II) showed no significant activity, and direct antibacterial activity is unlikely since anti-microbial activity of pectins has not been reported before. The traditional use as an anti-ulcer medicine is therefore probably not explained by a direct activity on *H. pylori* colonization, and an immunomodulating activity is more likely. In theory, *H. pylori* anti-adhesive herbal drugs can only be used prophylactic, as a dissociation of the bacteria already in the state of adherence with the host tissue seems highly unlikely (Wittschier *et al.*, 2007). Anti-adhesive traditional medicines would therefore have to be used for lifetime, starting at a young age. The fact that most people in Mali are already infected with *H.pylori*, anti-adhesion is probably not a functional mechanism.

Roots of *V. kotschyana* are well known for anti-ulcer activity (more detailed information about the use is found in the introduction; subsection *Vernonia kotschyana*). Previously, saponins were claimed to be the active components responsible for the anti-ulcer activity observed of the roots (Germano *et al.*, 1996; Sanogo *et al.*, 1996). We wanted to examine if the polysaccharides also may be an active component contributing to the anti-ulcer activity (Paper IV). 50°C and 100°C water extracts named VK50-I and Vk100-I (see Fig. 4.4) were highly active at a dose of 100 mg/kg, corresponding to the recommended daily dose of root powder of 15 g per person (Germano *et al.*, 1996). We therefore concluded that inulin also contribute to the activity in the acute experimental ulcer model. However, whether or not this assay is relevant for chronic ulcer in humans remains in our opinion, controversial. A mechanism for anti-ulcer activity of inulin which is not evaluated in this model, is the bifidogenic activity of inulin. This activity might contribute to a better microbial flora in the intestine which again will provide relief of dyspeptic symptoms. The amount of soluble dietary fiber intake in Mali through fruits and vegetables are low (Hall *et al.*, 2009), which may explain why inulin supplements can relieve the symptoms. Pectins from *C. cordifolia* will probably not work as prebiotics since studies on prebiotic-function of the structurally related karaya gum showed that the gum was not degraded by a variety of intestinal bacteria (Salyers *et al.*, 1977). However, it can act as a mechanical laxative.

Through interviews with 59 randomly selected persons in the Bamako region (Paper V), we observed that the majority (61%) experienced gastrointestinal symptoms. This reflects the high degree of gastrointestinal related ailments in the population of Mali. Natural products lowering the dyspeptic symptoms, may be highly relevant for further research because rapid recurrence and antibiotic resistance are increasing problems in countries with a high prevalence of *H. pylori* (Ahuja & Sharma, 2002). Also, since Mali is ranked as one of the poorest countries in the world, medicinal plants are the main option for most of the primary health care of the population. It is therefore highly important to carry out more research on traditional medicines used for these types of ailments.

4.5 Gastric ailments and diagnosing *H. pylori* (Paper V)

Gastrointestinal ailments are major reasons for morbidity in Mali. The prevalence of gastric ulcer in the population is reported to be 4.2% for men and 2.4% for women, and is probably higher for gastritis (Touré, 1989; Maïga *et al.*, 1995). Since *H. pylori* is recognized as the major risk factor for developing gastritis and gastric ulcer, diagnosing *H. pylori* in a none-invasive manner can be a valuable diagnostic tool. Rapid tests for *H. pylori* detection are important tools for future plans on clinical studies on gastric ulcer and traditional medicine. For this reason, Paper V focuses on rapid tests for *H. pylori* diagnosis.

To evaluate Clearview® immunochromatographic rapid tests, we used whole blood from patients with confirmed gastric ulcer by gastroscopy. 21% (confidence level 95%: 6;35) of the patients were positive based on the rapid test. This number is presumable too low as patients with confirmed gastric ulcer normally have a *H. pylori* prevalence of 60-100% (Kuipers *et al.*, 1995). Previously, a study carried out in Mali by Mourtala (2000) found that *H. pylori* was present in 95% of the gastric ulcer lesions. The detection techniques used were biopsy and histology. Based on this, we concluded that the serological test Clearview® cannot be used in Mali. We therefore tested a stool based rapid test (ImmunoCard STAT! antigen test) on patients suffering from unknown gastric ailments. Only 14% tested positive on *H. pylori*, a result which is unlikely to be true as research in neighboring countries has revealed a prevalence of more than 80% (diagnosed by biopsy) in patients with dyspepsia.

According to the manufacturer of the stool test, it is a possibility that the stool test does not respond to all subgroups of *H. pylori* as there is a remarkable genetic diversity (Varbanova *et al.*, 2011). In addition, anti-malarial drugs may lower the amount of *H. pylori* present in the stomach, which again will result in lower amounts of antigens in the faces (stool). We therefore conclude that Clearview®hp and ImmunoCard STAT!hp rapid tests should not be used in Mali.

4.6 Conclusions

According to WHO, 75% of the Malian population depends on traditional medicines. Gastric ulcers and wounds are major health problems in Mali, and this thesis focuses on two plants used for these ailments, *C. cordifolia* and *V. kotschyana*. Traditional plant medicines are normally prepared as a decoction, extracting water soluble components. Polysaccharides are highly soluble in hot water and will therefore also be present in the prepared medicines. The fact that polysaccharides often express immunomodulating activities make them likely to be parts of the claimed activities of a medicinal plant, especially when it comes to healing of gastric ulcers and wounds. The polysaccharides can be the working components of the plant medicines, alone or in synergies with water soluble low molecular weight substances like saponins, flavonoids, alkaloids and terpenes.

In this thesis, a detailed study of pectin structures found in *C. cordifolia* bark and leaves were related to complement fixation, macrophage activation and anti-ulcer activity. Uncommon pectin structures with RG-I backbone having a high degree of branches *O*-3 linked to Rha and GalA with mono or dimeric side-chains consisting of a high degree of 4-OMe-GlcA, will give a highly negative surface for a majority of the fractions. Pectins isolated from the bark and leaves showed similarities in the structure. However, some differences in the amount of T-2-OMe-Gal and 4-OMe-GlcA, and HG proved to be important for immunomodulating activities and physiochemical properties. The bark fractions had generally higher complement fixing activity, in addition to a higher potential of activating macrophages. The observed difference in activity may be due to these relatively small structure differences, but cross-linking of HG blocs in pectins from leaves may be an indirect parameter for altered activity. CCbark50 and CCleaf50 showed similar anti-ulcer activity in an experimental mouse model in a dose dependent way. 50°C and 100°C water extracts from the roots of *V. kotschyana* (high content of inulin) were also subjected to anti-ulcer experiments and the extracts showed a high anti-ulcer activity with doses corresponding to the recommended daily intake of the root.

H. pylori is regarded as the main factor for dyspepsia, gastritis and gastric ulcer development. The next step in evaluating the anti-ulcer activity of medicinal plants used for ulcer treatment will be to carry out new clinical trails. Simple rapid-tests were evaluated for *H. pylori* diagnosis. Unfortunately, the tests showed low sensitivities in the Malian population, probably due to strain variability and use of anti-malarial drugs.

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Papers

Paper I **Ingvild Austarheim, Bjørn E. Christensen, Ida K. Hegna, Bent O. Petersen, Jens O. Duus, Ragnar Bye, Terje E. Michaelsen, Drissa Diallo, Marit Inngjerdingen & Berit S. Paulsen**
Chemical and biological characterization of pectin-like polysaccharides from the bark of the Malian medicinal tree *Cola cordifolia*
Carbohydrate Polymers **89** (2012), 259–268.

Paper II **Ingvild Austarheim, Bjørn E. Christensen, Christian Thule, Drissa Diallo, Marit Inngjerdingen & Berit S. Paulsen**
Chemical and biological characterizations of pectins from *Cola cordifolia* leaves
Manuscript

Paper III **Ingvild Austarheim, Haidara Mahamane, Rokia Sanogo, Adiaratou Togola, Mehdi Khaledabadi, Anne C. Vestrheim, Kari T. Inngjerdingen, Terje E. Michaelsen, Drissa Diallo & Berit S. Paulsen**
Anti-ulcer polysaccharides from *Cola cordifolia* bark and leaves
Journal of Ethnopharmacology **143** (2012), 221–227.

Paper IV **Ingvild Austarheim, Cecilie S. Nergard, Rokia Sanogo, Drissa Diallo & Berit S. Paulsen**
Inulin-rich fractions from *Vernonia kotschyana* roots have anti-ulcer activity
Journal of Ethnopharmacology **144** (2012), 82–85.

Paper V **Ingvild Austarheim, Kari T. Inngjerdingen, Adiaratou Togola, Drissa Diallo & Berit S. Paulsen**
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Paper V

Chromatographic immunoassays for *H. pylori* detection – are they reliable enough in Mali (West Africa)?

Chromatographic immunoassays for Helicobacter pylori detection – are they reliable in Mali, West Africa?

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***Keywords*:** stool test, serological test, rapid tests

Abstract

Background
Gastrointestinal diseases are major reasons for morbidity in Mali. As *Helicobacter pylori* is known to play a major role in gastritis and gastric ulcer we wanted to find a simple method for detection.

Methods
Twenty-nine volunteers with confirmed gastric ulcer by gastroscopy and 59 randomly selected volunteers were diagnosed by using the rapid serological test Clearview® H. pylori. The ImmunoCard STAT!® HpSA® test was applied on stool from 65 volunteers seeking help for gastrointestinal related ailments.

Results
A *Helicobacter pylori* prevalence of 21% was found among the individuals with confirmed gastric ulcer, 44% among the randomly selected volunteers and 14% in individuals with gastrointestinal related ailments.

Conclusions
According to what is already known about the aetiology of gastric ailments and the prevalence of *Helicobacter pylori* in neighboring countries, the infection rates in our study appear strikingly low. This might indicate that Clearview® *H. pylori* and ImmunoCard STAT!® HpSA® have low sensitivities in the populations studied. Strain variability of *H. pylori* may be an explanation. The tests need to be properly evaluated in Mali before they can be relied upon as diagnostic tools.

Introduction

Gastritis and other gastrointestinal (GI) disorders are important causes of morbidity in the African population [1]. In Mali, the prevalence of gastric ulcer in the population is reported to be 4.2% for men and 2.4% for women, and is probably higher for gastritis and non-ulcer dyspepsia [2, 3]. Medicinal plants are popular remedies for treating gastrointestinal ailments in Mali, as approximately 75% of the population mainly use plant based medicines for their primary health care [2, 4, 5]. It is important to increase the knowledge behind the aetiology of gastritis, gastric ulcer and GI-related problems in Mali for future research on Malian traditional medicines used for these ailments, and also in order to give patients the correct treatment.The presence of *Helicobacter pylori* in the population is one important factor needing assessment, as *H. pylori* is a known factor contributing to dysfunction of the GI.

Individuals may be asymptomatic carriers of the disease, but the presence of *H. pylori* is highly correlated with underlying ailments causing dyspepsia. Infections with *H. pylori* are found worldwide in individuals of all ages, but are commonly acquired at an earlier age in developing countries [6]. A previous study conducted in Mali on patients with gastric ulcer reported an *H. pylori* prevalence of 95% [7]. The prevalence of *H. pylori* in dyspeptic patients in other West African countries, diagnosed by biopsy and urease test, is reported to be 97% in Ghana [8], 91.3% in Ivory Coast [9], and 72-91% in Nigeria [10, 11]. In Senegal a prevalence of 100% was found in patients with ulcers or gastritis by urea breath test and histological findings [12].

Rapid stool- or serology-based chromatographic immunoassays were chosen in this study, as we wanted to diagnose patients in Mali with a simple, non-invasive technique.

Methods and material

Volunteers with confirmed gastric ulcer

Twenty-nine volunteers diagnosed with gastric ulcer, 18 (62%) male and 11 (38%) female, confirmed by gastroscopy at Cabinet Medical "Les étoiles" at Gabriel Touré hospital, Bamako, were tested by the chromatographic immune based assay Clearview® *H. pylori* (Alere Inc., Waltham, MA). Nine different ethnic groups were included; Bambara (31%), Peulh (28%), Sénoufo, Malinké, Somono, Dogon, Dafing, Sonrhaï and Minianka. The average age was 41 years (age 27 – 65 years). *H. pylori* antibodies (IgG) present in fresh whole blood were analysed according to the manufacturer's instructions. Briefly, a thin lancet was used to puncture the skin of the finger and 50 µl whole blood and one drop of buffer was dispensed into the round window of the test device. The assay was carried out at room temperature (30°C), and was read after 10 minutes. A pink-red line, even very weak, was

considered as a positive result. All volunteers gave informed consent, confirmed by a signature.

Randomly selected volunteers

The chromatographic immune based assay Clearview® *H. pylori* was used to detect *H. pylori* antibodies (IgG) present in fresh whole blood of 59 volunteers. The volunteers were randomly recruited from three different areas of Bamako, Mali; Hippodrome, Daoudabougou and Sotuba. The group included 31 (53%) female and 28 (47%) male with an average age of 36 years (age 18 - 65 years). Fourteen different ethnic groups were involved; Bambara (24%), Malinké (14%), Bozo, Dafing, Haoussa, Kakolo, Peulh, Sarakolé, Sénoufo, Sonrhaï, Tamasheq, Fofana, Sangare and Minianka. Whole blood was analysed as described under subsection 2.1. All volunteers gave informed consent, confirmed by a signature or a fingerprint.

Volunteers with diverse gastric ailments

The chromatographic immune based assay ImmunoCard STAT!® HpSA® Stool antigen test (Meridian Bioscience Inc., Cincinnati, OH) was used to detect *H. pylori* antigens present in the stool of 65 volunteers. The stool samples were given at the INRSP (Institut National de Recherche en Santé Publique, Bamako) due to ailments like infections (33%), dyspepsia (16%) and other stomach related ailments. The group included 33 (52%) female and 31 (48%) male, and with an average age of 33 years (age 2 – 70 years). Thirteen different ethnic groups were included; Sarakolé (23%), Peulh (20%), Bambara (19%), Sénoufo, Dogon, Malinké, Kasonké, Kakolo, Maure, Minianka, Diawando, Sonhraï and Somono. All stool samples had a pasty consistency and they were analysed at room temperature (25°C) two hours after stool sampling, according to the manufacturer's guidelines. Briefly, four drops of diluted stool were dispensed into the round window of the test device and the result was read after five minutes of incubation at room temperature. The appearance of a pink-red band in the reading window, even very weak, was considered as a positive result. All volunteers gave an informed consent for bacteriological research on stool samples.

Results

Volunteers with confirmed gastric ulcer

H. pylori antibodies were detected in 21% of the 29 volunteers diagnosed with gastric ulcer by gastroscopy (Table 1). These individuals were tested directly after endoscopy, and had not been through *H. pylori* eradicating regime. No association with a positive detection of *H. pylori* and gender, age or ethnic group was found (Appendix A).

Randomly selected volunteers

H. pylori antibodies were detected in 44% of the randomly selected volunteers (Table 1). No association between *H. pylori* positive individuals, gender, age or ethnic group was found. Nine (15.2%) individuals reported that they had previously undergone gastroscopy; out of these two were *H. pylori* positive in the present study. Thirty-six (61%) of the volunteers reported that they from time to time experienced dyspepsia, mostly due to Gastroesophageal Reflux Disease (GARD), and of these individuals 36% were *H. pylori* positive. Among the volunteers who did not experience GI symptoms, 54% were *H. pylori* positive. Four of the volunteers knew that they had recently been taking antibiotics, and of these three were *H. pylori* positive. See Appendix B for additional data on gender, age, ethnicity and GI related problems.

Volunteers with diverse gastric ailments

H. pylori antigens were present in 14% of the volunteers suffering from diverse gastric ailments (Table 1). Of the nine individuals that were *H. pylori* positive, five were tested for gastrointestinal infections problems. None of the dyspeptic individuals tested positive (Appendix C).

Table 1: Results obtained from serological and stool based tests in *Helicobacter pylori* detection.

	Positive (%)	**95% CI**	**n**
Confirmed gastric ulcer (serology based)	21	(6;35)	29
Randomly selected volunteers (serology based)	44	(31;57)	59
Gastric ailments (stool based)	14	(5;22)	65

95% CI; 95% Confidence interval, n; No of volunteers.

Table 2: Test sensitivities of the utilized serological and stool based tests as given by the manufacturer.		
	Test sensitivity	95% CI[a]
Clearview® (serology based)	93.0	(87.1;96.7)
ImmunoCard STAT!® HpSA® (stool based)	96.1	(86.5;99.5)
95% CI; 95% Confidence interval.		

Discussion

According to the manufacturer, Clearview® *H. pylori* is regarded as a sensitive test (Table 2), and a negative result should therefore indicate that the person truly is negative. In the present study we found that only 21% of the individuals with confirmed gastric ulcer tested positive for *H. pylori* by using Clearview® *H. pylori*. This number is probably too low to be realistic as it does not correlate with a previous investigation carried out in Mali in year 2000. Biopsies taken from 121 patients diagnosed with gastric ulcer by endoscopy confirmed that *H. pylori* were present in 95% of the cases [7].The population studied came from the same area (Bamako) and the gastric ulcer was diagnosed by endoscopy in the same manner as in the present study. The culture and habits have not changed much in Mali the last decade, which means that the populations studied should be comparable. It would therefore be expected that most of the individuals diagnosed with gastric ulcer in the current study should be *H. pylori* positive.

A low (41%) prevalence was also shown by Clearview® *H. pylori* among the randomly selected volunteers. The *H. pylori* sero-prevalence of healthy individuals in nearby countries is reported to be much higher, 80 – 85% in Nigeria [13] and 75.4% in Benin [14]. In addition, random studies in Africa have shown a seroprevalence of 80% [1]. Factors that seem to play a role in the infection rate are low-quality drinking water, lack of basic hygiene, habit of eating from the same plate as well as poor diet and overcrowding [11, 15].These factors are similar between Mali and the surrounding countries, making a comparable prevalence highly likely. To our comprehension, the most obvious reason to explain the low detection of *H. pylori* is that Clearview® *H. pylori* has a low sensitivity in the population studied in Mali. Sensitivity is not an intrinsic property of an assay [16], and it is therefore possible that the sensitivity experienced in the population studied here, is not the same as for the population studied by the manufacturer.

A low prevalence of *H. pylori* (14%) was also found by the ImmunoCard STAT!® HpSA® stool test. A good practice in diagnosing *H. pylori* with antigen tests is that antibiotics should be stopped at least four weeks and anti-secretory therapy at least two weeks before testing, as these medications negatively affects the sensitivity [11]. In Mali malaria is endemic, and anti-malaria drugs might have a negative effect on the sensitivity of the test, as these drugs might eradicate or decrease the presence of *H. pylori* [17, 18]. Most of the individuals in our study have a low educational level, and it was therefore difficult to know if they had been taking any interfering medications. *H. pylori* is, however, not easily eradicated [19], and self-medication is probably not sufficient for total removal of the bacteria, but potentially enough to lower the amount of stool antigens to make the outcome negative.

According to Meridian Bioscience, it is a possibility that the stool test does not respond to all subgroups of *H. pylori.* There is a remarkable genetic diversity of *H. pylori* worldwide, the number of genetic variability of *H. pylori* exceeding 2 to 5-fold the variability of other bacterial pathogens. Substantial diversity has recently also been described for the important virulence factor cagA [20]. This means that ImmunoCard STAT!® HpSA®, which uses monoclonal antibodies for detection, might have a lower sensitivity towards antigens produced by some strains. Calvet et al. [21] reported a sensitivity of 69-74% for ImmunoCard STAT!® HpSA® on patients with dyspeptic symptoms. This is much lower then what reported by the manufacturer (Table 2).

A weakness in the present study might be that the serological and the stool tests were not carried out on the same individuals, thereby making it difficult to compare these methods directly. However, our results suggest that both assays have a considerably lower sensitivity in Mali than reported by the manufacturers. A follow-up study is needed in order to give any definite conclusions.
It is noteworthy that the majority (61%) of the randomly selected volunteers experienced gastrointestinal symptoms. This reflects the high degree of GI related ailments experienced by the population of Mali. Natural products which reduce the density of *H. pylori* colonization and mucus adherence [22], and thereby lower the dyspeptic symptoms, might be highly relevant for further research as rapid recurrence and antibiotic resistance are increasing problems in countries with high a prevalence of *H. pylori* [23]. In addition, as Mali is ranked as one of the poorest countries in the world, the population has medicinal plants as the main option for most of primary health care. It is therefore highly important to carry out more research on traditional medicines used for these types of ailments, and also to have access to simple and non-expensive diagnostically methods reliable for the Malian population.

Conclusion:

Rapid immunoassays for *H. pylori* detection need to be properly evaluated in the respective countries where they are to be used before they can be relied upon as diagnostic tools. Interfering circumstances such as medications and strain variability should be addressed in greater detail. Due to endemic malaria, strain variability and self medication, it is a possibility that the stool based test looses some of its sensitivity. As self medication is difficult to avoid, it is probably wise to choose a different test system for *H. pylori* identification in Mali.

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Appendices A-C

Appendix A. Confirmed gastric ulcer by gastroscopy (serological test, Clearview® *H. pylori*)

Result	Sex	Age	Ethnic group
pos	F	49	Bambara
pos	M	35	Bambara
pos	M	37	d.ma
pos	M	38	Bambara
pos	M	38	Minianka
pos	M	65	Peulh
neg	F	27	Dogon
neg	F	29	Bambara
neg	F	32	Sénoufo
neg	F	47	Bambara
neg	F	47	Malinké
neg	F	49	Bambara
neg	F	50	Bambara
neg	F	51	Bambara
neg	F	52	Peulh
neg	F	55	Peulh
neg	M	23	Forgeron
neg	M	24	Peulh
neg	M	27	Sonrhaï
neg	M	28	Peulh
neg	M	32	Bambara
neg	M	35	Dafing
neg	M	40	Peulh
neg	M	40	Peulh
neg	M	41	Sénoufo
neg	M	42	Sénoufo
neg	M	43	Malinké
neg	M	46	Somono
neg	M	63	Peulh

[a] data is missing

Appendix B. Randomly selected volunteers (serological test, Clearview® *H. pylori)*					
Result	**Sex**	**Age**	**Ethnic group**	**Gastroscopy in the past**	**Gastric problems and treatment**
pos	F	20	Malinké	no	Sometimes. Uses aspirin, diclophenac, paracetamol.
pos	F	23	Bozo	no	Painful when eating, not frequently.
pos	F	24	Tamasheq	no	Reflux problems, not every week.
pos	F	25	Sonrhaï	no	Burning feeling in upper gut.
pos	F	26	Sarakolé	no	No problems.
pos	F	33	d.m[a]	no	Gastric problems. Uses metronidazol, amoxicillin and omeproazole.
pos	F	33	Sonrhaï	no	No problems.
pos	F	34	Peuhl	no	Stomach ache once a month. Uses the traditional drug Filafinh (a carbonized powder of a tree).
pos	F	36	Malinké	no	Reflux when she eats a lot, or not eating at all. Uses amoxicillin and antacid.
pos	F	43	Fofana	no	Reflux when hungry. Uses Gastrosedal twice a week
pos	F	44	Sarakolé	no	Reflux, not frequently. Uses antacid.
pos	F	50	Sarakolé	2003	Reflux when eating a lot or not at all. Perhaps ulcer. Uses omeprazole or lansoprazole, and antacid.
pos	F	52	d.m	yes	Omeprazole and antacid.
pos	M	20	d.m	no	No problems.
pos	M	20	Dafing	no	No problems.
pos	M	20	Sonrhaï	no	No problems.
pos	M	20	Sonrhaï	no	No problems.
pos	M	27	Bambara	no	No problems.
pos	M	27	d.m	no	Reflux when hungry, eating helps.
pos	M	27	Sénoufo	no	Pain when hungry. Has used antacid.
pos	M	27	Sénoufo	no	No problems.
pos	M	35	Sangaré	no	No problems.

pos	M	39	Sénoufo	no	Chronic constipation, uses *Laxa cassia* twice a week
pos	M	47	Bambara	no	No problems.
pos	M	49	Sénoufo	no	No problems.
pos	M	50	Peuhl	no	No problems.
neg	F	25	Bozo	2003	Did not have gastric ulcer.
neg	F	25	Fofana	no	No problems.
neg	F	25	Minianka	no	Reflux when eating greasy food.
neg	F	27	Sarakolé	2008	Perhaps ulcer. Uses antacid and antibiotic when the pain starts.
neg	F	28	Malinké	no	No problems.
neg	F	29	Bambara	2003	Conventional treatment 2003, now Gastrosedal.
neg	F	29	Sonrhaï	2003	Conventional treatment for gastric ulcer in 2003.
neg	F	30	Peuhl	no	Possible gastritis. Uses omeprazole and antacid.
neg	F	34	Sonrhaï	no	Reflux problems, use antacid.
neg	F	35	Malinké	no	Reflux when eating a lot or not at all. Uses antacid.
neg	F	41	Haoussa	no	Reflux, when eating a lot, or not at all. Uses antacid when problems (not frequently).
neg	F	42	Sarakolé	no	No problems.
neg	F	48	Bambara	no	No problems.
neg	F	48	Bambara	no	Reflux not frequently. Uses Gastrosedal.
neg	F	48	Malinké	no	No problems.
neg	F	51	Malinké	no	Reflux when eating a lot or not at all. Uses antacid.
neg	F	62	Kakolo	2007	Reflux, not frequently. Uses antacid.
neg	F	65	Sarakolé	2006	Reflux, twice every month, when eating a lot or not at all.
neg	F	70	Samôgô	no	Possible a gastric ulcer, uses antacid.
neg	M	18	Bambara	no	No problems.
neg	M	18	Bambara	no	No problems.
neg	M	19	Sonrhaï	no	No problems.
neg	M	21	Bambara	no	No problems.

neg	M	22	Bambara	no	Stomach ache, but not ulcer. Uses aspirin.
neg	M	22	Bambara	no	No problems.
neg	M	26	Minianka	no	Reflux once a week. Uses the traditional drug Diababoulouba.
neg	M	28	Sénoufo	no	Reflux problems, 3 times a week, no treatment.
neg	M	41	Bambara	no	Reflux problems when hungry. Uses Gastrosedal twice a week.
neg	M	50	Peuhl	no	Reflux when not eating during Ramadan.
neg	M	53	Bambara	no	No problems.
neg	M	56	Malinké	no	Conventional treatment for gastric ulcer.
neg	M	60	Bambara	1998	Conventional treatment for gastric ulcer in 1998, but has still problems.
neg	M	63	Peuhl	2008	Reflux, not frequently. Does not have ulcer. Uses traditional medicine.
[a] data is missing					

Appendix C. Volunteers seeking help for diverse gastric ailments (stool test, ImmunoCard STAT!® HpSA®).

Result	Sex	Age	Ethnic group	Reason for giving stool
pos	F	24	Sarakolé	Possible infection
pos	F	37	Peulh	d.m[a]
pos	F	40	Kasonké	Possible infection
pos	F	50	Bambara	Possible infection
pos	F	52	Peulh	Dysenteri
pos	F	70	Bambara	Possible infection
pos	F	70	Kakolo	d.m
pos	M	64	Sarakolé	Possible infection
pos	M	d.m.	Kasonké	Prurigo
neg	d.m.	d.m.	d.m	Urticaria
neg	F	4	Sarakolé	Abdominal pain
neg	F	8	Sonhraï	d.m
neg	F	9	Sarakolé	Abdominal pain
neg	F	11	Sarakolé	Abdominal pain
neg	F	15	Sarakolé	Abdominal pain
neg	F	16	Bambara	Dermatosis
neg	F	17	Sarakolé	Possible infection
neg	F	18	Dogon	Abdominal pain
neg	F	22	Dogon	Possible infection
neg	F	24	Sarakolé	Possible parasitic disease
neg	F	25	Kakolo	Dermatosis
neg	F	29	Peulh	d.m
neg	F	30	Kasonké	d.m
neg	F	30	Peulh	Possible infection
neg	F	31	Sarakolé	Possible infection
neg	F	35	Malinké	Leukorrhea
neg	F	35	Sarakolé	Possible infection
neg	F	37	Bambara	Possible infection
neg	F	39	Bambara	d.m
neg	F	40	Sarakolé	Abdominal pain
neg	F	41	Peulh	Possible infection
neg	F	43	Peulh	d.m
neg	F	45	Peulh	Constipation
neg	F	47	Malinké	Possible infection

neg	F	62	Sarakolé	Constipation
neg	M	2	Peulh	Circle cell anemia
neg	M	4	Sarakolé	Possible infection
neg	M	6	Maure	Possible infection
neg	M	6	Sarakolé	d.m
neg	M	8	Peulh	d.m
neg	M	9	Malinké	Abdominal pain
neg	M	17	Bambara	Abdominal pain
neg	M	20	Bambara	Possible infection
neg	M	21	Senoufo	d.m
neg	M	24	Peulh	d.m
neg	M	27	Minianka	d.m
neg	M	28	Bambara	Abdominal pain
neg	M	28	Dogon	Possible parasitic disease
neg	M	30	d.m	d.m
neg	M	31	Somono	Abdominal pain
neg	M	34	Dogon	d.m
neg	M	34	Sarakolé	d.m
neg	M	34	Sonhraï	Possible infection
neg	M	36	Malinké	d.m
neg	M	39	Peulh	d.m
neg	M	49	Bambara	d.m
neg	M	52	Sarakolé	d.m
neg	M	53	Bambara	Possible infection
neg	M	58	Bambara	d.m
neg	M	63	Kassonké	Diarrhoea
neg	M	70	Peulh	Abdominal pain
neg	M	d.m.	Bambara	Possible infection
neg	M	d.m.	Peulh	Possible infection
neg	M	d.m.	Sénoufo	Abdominal pain

[a] data is missing