ETHNOPHARMACOLOGY, PHYTOCHEMISTRY AND BIOLOGICAL ACTIVITIES OF MALIAN MEDICINAL PLANTS

Thesis for the Degree of Philosophiae Doctor
PhD DEGREE

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Oslo, 2008
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Acknowledgements

This doctoral dissertation was carried out from May 2004 to June 2008 as a research project at the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo (Norway), and the Department of Traditional Medicine, National Institute of Research in Public Health, Bamako (Mali). The research has financially been supported by the Mali project. The Quota program provided living expenses for the last two and halt years. It is with eternal gratitude that I acknowledge the authorities of these institutions for the academic and financial support that was indispensable to the realisation of this research. The opportunity to undertake this doctoral research gave me true scientific knowledge, related to modern technology in the exploration of plants and their bioactivities, which I am grateful for.

I would like to express my sincere gratitude to Pr Drissa Diallo, for believing in me and giving me the opportunity to be part of this scientific evaluation of Malian medicinal plants through the Mali project, and for all the discussion and encouragements during the last four years. His kindness, receptiveness, and implication for the success of this project have been deeply appreciated.

A vital part of this process was meeting my main supervisor Pr Berit Smestad Paulsen, who as painstakingly supervised this dissertation. Her accessibility and commitment to this research project have made it a cherished experience for me. I am eternally grateful for the knowledge in the field of modern science of carbohydrates. My co-supervisor Hilde Barsett is also acknowledged for her enlightening contribution and discussion. Additional supervision readily and graciously came from Pr Karl Egil Malterud during the research of low molecular weight compounds. His interest and enlightening discussion have greatly enriched this dissertation. Pr Terje Michaelsen has kindly granted his time for discussion and useful comments on the evaluation of complement fixing ability of the samples. All of them are deeply and sincerely acknowledged for their painstaking contribution to the accomplishment of this research.

I further like to thank all my co-authors, particularly Dr Marit Inngjerdingen for her contribution during the evaluation of the immunomodulator properties of the samples and the proof reading of these parts of the draft, and Dr Helle Wangesteen for her tremendous effort during the structure
determination of the low molecular weight compounds and the drafting of the related paper. Working with both was truly inspirational.

Of particular mention is Dr Kari Inngjerdingen for the pleasant moments inside and outside the laboratory. A pleasant working atmosphere was granted by all my colleagues and students at the Department of Pharmaceutical Chemistry which I am thankful for. Particular gratitude is expressed to Ingvild Austrheim and Ragnild Kárvatn Evjenth for sharing several joyful moments outside the laboratory.

Additional support has consistently come from Idrissa Coulibaly and his wife Maimouna Doumbia and from Salif and Kari Mounkoro, they are acknowledged for the social affection that helped me carry on during the difficult moments. My sincere gratitude also goes to Dr Sekou Bah, Dr Ababacar Maiga, and MS Fanta Sangho for their care and friendship during the initial years of this process.

Finally, it is with eternal gratitude that I acknowledge the constant support and encouragement from my parents and family: to my dad, Le Vieux Dioko, the decision to undertake this doctoral research was partly enforced by your encouragements, you will always be associated with the glory. To my loving mother, Aïssata Koné, I am thankful for the strong spirit, you tirelessly inducted to me, and which guided me through the long path of this research. To my uncle and ante Bazan Togola and Aminata Sangaré, eternal thanks for the care and encouragements. Sincere and deep thanks are expressed to all my brothers and sisters for the strong support and encouragements that have immensely contributed to making this feat realizable.

Oslo February 2008

Adiaratou Togola
Abstract

This thesis presents the results of ethnopharmacological, phytochemical and biological activities investigations on *Opilia celtidifolia* (Guill. & Perr.) Endl. ex Walp (Opiliaceae), *Cola cordifolia* (Cav.) R. Br. (Sterculiaceae), and *Erythrina senegalensis* DC (Fabaceae), three plants used in traditional medicine in Mali to cure various types of diseases.

The first part of this thesis deals with the results of ethnopharmacological surveys performed to identify the traditional uses of these plants. These studies covered different areas of Mali (Siby Dioila, Kolokani, Dogonland, and Koutiala) and their results showed extended uses of the plants. *O. celtidifolia* was mostly identified by traditional healers as an anti-malaria, appetizer, and anti dermatitis plant and *C. cordifolia* was reported to cure gastritis, skin inflammation, wounds, malaria, high blood pressure, overweight and other diseases as well. The ethnopharmacological information of *E. senegalensis* was related mostly to amenorrhoea, bilharzias and other types of infections, malaria and jaundice. The fidelity level among traditional healers from the same area about the most reported diseases was calculated. A high healer agreement (75%) was found about the use of *O. celtidifolia* against dermatitis in Siby, against malaria in Dioila (61%), and abdominal pain (54%) in the same area. The use of *E. senegalensis* against amenorrhoea meets 21% of agreement between Dioila and Kolokani.

The second part of this thesis presents the results of structural and immunological properties investigations of pectic polysaccharide fractions isolated from the water 50 °C extract of *O. celtidifolia* and *C. cordifolia*. These plants were previously reported to be used to treat internal and external chronic wounds. It is known from the literature that the plants which are used against wounds and other immune related diseases in traditional medicine are likely to contain immunomodulatory compounds, and polysaccharides are largely reported to be responsible of these activities.

Two acidic polysaccharide fractions were isolated from the leaves extract of *O. celtidifolia*, Oc50A1 and Oc50A2. They showed, strong complement fixing ability, the ICH50 values were 0.5 and 0.9 μg/ml respectively; nitric oxide release ability from activated macrophages, a concentration of 100 μg/ml induced the release of about 7.3 μM of NO. The fractions induced 14
and 24% respectively of proliferation of T cells at 50 μg/ml and moderate B cell activation property at 100 μg/ml as well. Analysis of the monosaccharide composition of Oc50A1 and Oc50A2 showed the presence of arabinose (26.7 and 13.2%), galactose (31.5 and 28%) and galacturonic acid (5.3 and 7.8%) respectively. The Yariv test confirmed the presence of arabinogalactan type II in both fractions. Structural analyses did also show the presence of terminal and 1-4 linked galacturonic acid and terminal and 1-2 linked rhamnose. Endo-polygalacturonanase treatment was performed to isolate the heavily substituted parts of the polysaccharides. These parts contained the same monosaccharides in similar proportion, and showed stronger dose dependent complement fixing activity and also stimulated macrophages to release nitrite oxide.

A pectic polysaccharide Cc50-1 with a molecular weight between 1000 and 2000 kDa was isolated from *C. cordifolia*. Monosaccharide composition and linkages analysis showed the presence of unusual type of monomers. 20% of terminally and 3-linked 2-O-methyl galactose, 18% of terminally linked 4-O-methyl glucuronic acid and 2% of 2-O-methyl fucose were identified. These monomers seem to be linked to a RGI like backbone composed of 20% of 2,3- and 2,4-linked rhamnose almost equal amount, which alternate with 24% of 4-linked galacturonic acid some of which also carry side chains. This polysaccharide showed low complement fixing ability with an ICH50 value of 480 μg/ml. Viscosity measurement gave a Huggins constant value of K’=1.2 which indicated the presence of aggregated particles when Cc50-1 is dissolved in water based solvents.

In the last part of the thesis, results of phytochemical and biological investigations on low molecular weight compounds isolated from *E. senegalensis* are presented. A new pterocarpan named Erybraedin F and six known ones, erybraedin A, C, D, eryvarin K, phaseollin and shinpterocarpin and a flavone, carpachromene were isolated from the dichloromethane extracts of the root and stem bark. The pterocarpans showed 15-lipoxygenase enzyme inhibition activity. The IC50 values ranged between 32 and 82 μM. Erybraedin D (IC50 < 32) was stronger enzyme inhibitor than quercetin (IC50 = 42 μM). Erybraedin A, D, C and eryvarin K also showed positive inhibition results on *Staphylococcus aureus*. The antimicrobial activity of the compounds is in good agreement with literature data. These compounds although reported before in the genus
*Erythrina* are for the first time here reported in the species *E. senegalensis* and the first time reported to have 15-LO inhibition activity.
### List of abbreviations and symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AGI</td>
<td>Arabinogalactan type I</td>
</tr>
<tr>
<td>AGII</td>
<td>Arabinogalactan type II</td>
</tr>
<tr>
<td>AGP</td>
<td>Arabinogalactan protein</td>
</tr>
<tr>
<td>Ara</td>
<td>Arabinose</td>
</tr>
<tr>
<td>Cc50</td>
<td><em>Cola cordifolia</em> water 50 °C extract of the bark</td>
</tr>
<tr>
<td>Cc50-1</td>
<td>Acidic fraction isolated from Cc50 after anion exchange chromatography</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DMT</td>
<td>Department of Traditional Medicine (Département de Médecine Traditionnelle)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-Diamine Tetraacetic Acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>ES-MS</td>
<td>Electrospray-Mass Spectroscopy</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fucose</td>
</tr>
<tr>
<td>2-O-Me Fuc</td>
<td>2-O-methyl fucose</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>2-O-Me Gal</td>
<td>2-O-methyl galactose</td>
</tr>
<tr>
<td>GalA</td>
<td>Galacturonic acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectroscopy</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>4-O-Me GlcA</td>
<td>4-O-methyl glucuronic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HGA</td>
<td>Homogalacturonan</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Coherence</td>
</tr>
<tr>
<td>HMQC</td>
<td>Heteronuclear Multiple Quantum Coherence</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>ICH50</td>
<td>Inhibit Concentration of 50% of Haemolysis</td>
</tr>
<tr>
<td>ITM</td>
<td>Improved Traditional Medicine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LO</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>15-LO</td>
<td>15-lipoxygenase</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane Attack Complex</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannan Binding Lectin</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>1H-NMR</td>
<td>Proton-NMR</td>
</tr>
<tr>
<td>13C-NMR</td>
<td>Carbon-NMR</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Oc50</td>
<td><em>Opilia celtidifolia</em> water crude 50 °C extract</td>
</tr>
<tr>
<td>Oc50A1</td>
<td><em>Opilia celtidifolia</em> first acidic fraction isolated from Oc50 after anion exchange chromatography</td>
</tr>
<tr>
<td>Oc50A2</td>
<td><em>Opilia celtidifolia</em> second acidic fraction isolated from Oc50 after anion exchange</td>
</tr>
</tbody>
</table>
chromatography

Oc50A₁₁ High molecular weight fraction isolated after sephacryl separation of Oc50A₁
Oc50A₁₂ Low molecular weight fraction isolated after sephacryl separation of Oc50A₁
Oc50A₂₁ High molecular weight fraction isolated after sephacryl separation of Oc50A₂
Oc50A₂₂ Low molecular weight fraction isolated after sephacryl separation of Oc50A₂
Oc50A₁d₁ High molecular weight fraction isolated after endopolygalacturonanase digestion of Oc50A₁ and Biogel P30 separation
Oc50A₁d₂ Low molecular weight fraction isolated after endopolygalacturonanase digestion of Oc50A₁ fractionated on Biogel 30
Oc50A₂d₁ High molecular weight fraction isolated after endopolygalacturonanase digestion of Oc50A₂ and Biogel P30 separation
Oc50A₂d₂ Low molecular weight fraction isolated after endopolygalacturonanase digestion of Oc50A₂ and Biogel P30 separation
Oc50A₁₂H High molecular weight fraction obtained after arabinofuranosidase treatment of OC50A₁₂ and separation o Biogel P30

PE Pectin esterase
PG Endopolygalacturonanase
PMII Plantago major polysaccharide
RGI Rhamnogalacturonan type I
RGII Rhamnogalacturonan type II
Rha Rhamnose
RI Refractive Index
SEC Size Exclusion Chromatography
SEC-MALLs Size Exclusion Chromatography coupled with Multiangle Laser Light scattering
TLC Thin layer chromatography
TFA Trifluoric acid
WHO World Health Organisation
Xyl Xylose
XGA Xylogalacturonan
List of Papers

The results of this thesis are presented in the following publications:

**Paper I**

Togola Adiaratou; Diallo Drissa; Dembele Seydou; Barsett Hilde; Paulsen Berit Smestad

**Paper II**

Togola Adiaratou; Austarheim Ingvild; Theis Annette; Diallo Drissa; and Paulsen Berit Smestad; *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* (2008), 4: 6.

**Paper III**

Togola, Adiaratou; Inngjerdingen, Marit; Diallo, Drissa; Barsett, Hilde; Rolstad, Bent; Michaelsen, Terje E.; Paulsen, Berit Smestad; *Polysaccharides with complement fixing and macrophage stimulation activity from Opilia celtidifolia, isolation and partial characterization. Journal of Ethnopharmacology* (2008), 115(3) 423-431

**Paper IV**

Togola Adiaratou; Naess Knut Håkon; Diallo Drissa; Barsett Hilde; Michaelsen Terje E.; and Paulsen Berit Smestad; *A polysaccharide with 40% mono-O-methylated monosaccharides from the bark of Cola cordifolia (Sterculiaceae), a medicinal tree from Mali (West Africa). Carbohydrate Polymer* (2008), 73(2), 280-288

**Paper V**

Togola Adiaratou; Hedding Berte; Theis Annette; Wangensteen Helle; Paulsen Berit Smestad; Diallo Drissa; Malterud Karl Egil; *Prenylated flavonoid from Erythrina senegalensis and their biological activities. Submitted to Planta Medica*
Other related publication by the doctoral candidate

Omarsdottir, Sesselja; Petersen, Bent O.; Paulsen, Berit Smestad; Togola, Adiaratou; Duus, Jens O.; Olafsdottir, Elin S; **Structural characterization of novel lichen heteroglycans by NMR spectroscopy and methylation analysis.** *Carbohydrate Research* (2006), 341(14), 2449-2455.

1. Introduction

1.1. Traditional Medicine

Traditional medicine, having a long history, is defined as the total sum of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses. The terms complementary/alternative/non-conventional medicine are used interchangeably with traditional medicine in some countries (WHO, 2000).

In general considerations, practices of traditional medicine vary greatly from country to country, and from region to region, as they are influenced by factors such as culture, history, personal attitudes and philosophy. In many cases, their theory and application are quite different from those of conventional medicine. Long historical use of many practices of traditional medicine, including experience passed on from generation to generation, has demonstrated the safety and efficacy of traditional medicine. However, scientific research is needed to provide additional evidence of its safety and efficacy. The quantity and quality of the safety and efficacy data on traditional medicine is far from sufficient to meet the criteria needed to support its use worldwide (WHO, 2000).

In Mali, like most African countries, traditional medicine is contrasted with conventional medicine. Most traditional medical theories have a social and religious character and emphasize prevention and holistic features. Traditional medical practices are usually characterized by a healer’s personal involvement, by secrecy and a reward system, while conventional medicine theory and practice show an almost opposite picture: asocial, irreligious, curative and organ-directed; professional detachment, public knowledge and until recently, free of charge. Despite this contrast, both systems having the same target as local communities had to come together in a complementary way since 80% of the world population still use traditional medicine as their first recourse (Diallo & Paulsen, 2000). A study from Kenya showed that patients had a clear sense of which diseases they would go to a western clinic for, and when they would visit a traditional healer (Van der Geest, 1997). Therefore, since 1978, the World Health Organization (WHO) has
been calling for more cooperation, even integration, of traditional medicine and biomedicine. The role of traditional medicine was viewed upon as an integral part of primary health care with its basic philosophy of self-reliance. National governments created departments of traditional medicines.

In Mali, the Institute of Phytotherapy was created in 1968. After many renewals, the establishment is now called the Department of Traditional Medicine (DMT). DMT is housed in the National Institute for Research in Public Health (INRSP), and is the collaborating centre of WHO for research of traditional medicine. The main objective of the collaboration is to ensure that traditional medicine is used complementary to conventional medicine in a rational way based on scientific observations and experiments, assuming that medicines can be developed or improved from local resources, in particular, medicinal plants. The main activities of the DMT are registration of traditional healers and their remedies, in addition to research and development of phytomedicines called Improved Traditional Medicine (ITMs) (Diallo & Paulsen, 2000).

It is estimated that less than 10% of the word’s genetic resources have been studied seriously as sources of medicine. Yet, from this small fraction, humanity has gained enormous benefits. We are grateful to the plant kingdom for such useful drugs like vinblastine from the African periwinkle, *Cathanranthus roseus*, used in the treatment of leukaemia, the cholinergic drug, physostigmine, used in the treatment of glaucoma, comes from the Calabar beans, *Physostigma venenosum*, which was used in south-eastern Nigeria as an “ordeal poison”. Curare for surgery, reserpine for high blood pressure, taxol for cancer, quinine and artemisinin for malaria are other examples of very important medicines derived from plants. The transformation of these drugs from noxious vegetable preparations often took several decades and involved many scientists and immense cost. Modern technology can now speed up the process, but it still takes about 15 years to develop a single pharmaceutical entity from discovery to market and at an estimated cost of between $150 and 300 million (Iwu, 2002). Both the cost and the time can be drastically reduced by developing medicinal plants as phytomedicines or standardized herbs, especially for developing countries which can not afford the process.

The phytoremedies are produced from local plants in their natural form or in the form of powder, infusion, ointments and syrups and are being standardized according to traditional administration regimes. So far, the DMT has developed twelve ITMs, and seven are now recognized as essential
and effective medicines in Mali (Diallo & Paulsen, 2000). These ITMs are: Balembo against cough, Dysenterial against dysentery, Gastroedal against ulcers and gastritis, Hepatisane against hepatitis, Laxia-cassia against constipation, and Malarial against malaria and Psorospermine against dermatitis (Figure 1.1.)

![Figure 1.1 Improved Traditional Medicines produced at the DMT](image)

### 1.2. Ethnopharmacology

The approach for drug development from plant resources depends on the aim. Different strategies will result in an herbal medicine or in an isolated active compound. The selection of a suitable plant for pharmacological study is a very important and decisive step. There are several ways in which this can be done, including traditional use, chemical content, toxicity, and randomised selection; it is also possible and often desirable and sometimes inevitable to use a combination of several criteria. Apart from the chosen strategy, searching databanks and scientific literature is crucial in finding active and/or toxic compounds that have already been identified and can also be used as criterion for choosing plants for example if the purpose is to find a new source (Rates, 2001). The most common strategy is the careful observation of the use of natural resources in folk medicine from different cultures; this is known as ethnobotany or ethnopharmacology.
From its original definition (River & Bruhn, 1979), as a multidisciplinary area of research, concerned with the observation, description and experimental investigation of indigenous drugs and their biological activities, the term ethnopharmacology has undergone only slight evolution in meaning; its contemporary definition addresses the interdisciplinary study of the physiological actions of plants, animals and other substances used in indigenous medicines of past and present culture (International Society Ethnopharmacology, 2005)

To perform research for development of ITMs, DMT, like other scientific research based on plants, uses ethnopharmacology in order to maintain the knowledge on medicinal plants and their use in traditional medicine. Traditional healers are the base line of these researches. A traditional healer is defined as a person with competence to practice traditional medicine. From 1968 to 1978 registration of traditional healers and medicinal plants was carried out and still continues in all the administrative regions in Mali by an interdisciplinary team. The competence of a healer is evaluated on the person's achievements on curing diseases and the results are essential for consideration of registering the person as a traditional healer. After being registered, DMT sets up a principle of collaboration with the traditional healer. The collaborating healer is not obliged to deliver samples of his medications to DMT, but if he wishes to do so the plants will be subjected to toxicological, pharmacological and phytochemical analyses, the results of which are given back to the healer. As a result of this collaboration, the healer is granted official recognition as a practitioner in traditional medicine and is provided with an identity card for traditional practitioners. Other traditional healers are also allowed to practice with no restriction, but they do not have a registration card. In some localities of Mali, the healers are grouped in association and have created gardens of medicinal plants (Diallo & Paulsen, 2000). All studies being undertaken between DMT and the traditional healers follow ethical aspects and rules set down by the local government as both DMT and the traditional healers are part of the health care system of Mali. Face-to-face interviews are used to collect information during ethnopharmacological surveys. No appointment is made in advance and only traditional healers, registered or not, being willing to participate, are interviewed. The data are analysed and usually, the promising identified plants which are the most reported ones are selected for further studies.
1.3. Plant Polysaccharides

The plant cell wall is a dynamic compartment that changes throughout the life of the cell. The primary cell wall is born in the cell plate during cell division and rapidly increases in surface area during cell expansion, in some cases by more than hundred fold. The middle lamella forms the interface between the primary wall of neighbouring cells. Finally, at differentiation, many cells elaborate within the primary wall a secondary cell wall, building complex structure uniquely suited to the cell’s function. The plant cell wall is a highly organized composite of many different polysaccharides, proteins, and aromatic substances (figure 1.2). The molecular composition and arrangements of the wall polymers differ among species, among individual cells, and even among regions of the wall around a single protoplast. Polysaccharides are the principal component of the cell wall and form its main structural framework. Knowledge of the chemistry of the carbohydrates will greatly facilitate understanding the many biological functions of the cell wall polysaccharides (Carpita & McCann, 2000).

Figure 1.2: The plant cell wall (Taiz, 2006)
1.3.1. **Cellulose**

Cellulose is the most abundant plant polysaccharide, accounting for 15 to 30% of the dry mass of all primary cell, and an even larger percentage of secondary walls. It forms the basic structural material of cell walls in all higher land plants, is also present in some seaweeds, and is synthesized by few bacterias, e.g., *Bacillus xylinum* (Aspinall, 1980). Cellulose exists in a form of water insoluble microfibrils, which are parachrystalline assemblies of several dozen 4-\(\beta\)-D glucan chains hydrogen-bound to one another along their length. Each glucan chain may contain several thousand units of glucose. From the structural viewpoint, cellulose is probably the best-understood of all carbohydrates of the plant cell wall (Carpita & McCann, 2000; Grant Reid, 1997)

1.3.2. **Hemicellulose**

Hemicellulose is a widely used, but archaic term, and used for all materials regardless of structure, extracted from the cell wall with alkali (Bacic *et al.*, 1988; Carpita & McCann, 2000). Hemicellulose is composed of cross-linking glycans. These are polysaccharides which can hydrogen-binds to cellulose microfibrils; they may coat microfibrils but are also long enough to span the distance between these latter and link them together to form a network. The two major cross-linking glycans of all primary cell walls of flowering plants are xyloglucans and glucuronoarabinoxylans (Carpita & McCann, 2000); mixed-linkage (\(\beta\)-3 and \(\beta\)-4)-glucan are also found in the primary cell wall. Four major types of glycans are isolated from secondary cell walls: 4-O-methyl glucuronoxylan accompanied by lesser amounts of glucomannans in the hardwoods and galactoglucomannan with minor amount of arabino-4-O-methylglucuronoxylan in the softwoods (Grant Reid, 1997).

1.3.3. **Pectic polysaccharides**

Pectins are a mixture of heterogeneous, branched, and highly hydrated polysaccharides rich in D-galacturonic acid. They have been defined classically as material extracted from the cell wall by calcium ion (\(\text{Ca}^{2+}\)) chelators such as ammonium oxalate, EDTA, EGTA, or cyclohexane diamine tetra acetate. Two fundamental constituents of pectins are homogalacturonan (HGA) and rhamnogalacturonan I (RGI). There are two kinds of structurally modified HGAs,
xylogalacturonan, and Rhamnogalacturonan II (RGII) (Carpita & McCann, 2000; Perez et al., 2003; Vincken et al., 2003b).

HGAs are essentially unbranched homopolymers of 4-α-D-Gal A groups. The GalA residues can be methyl esterified at position C-6 and carry acetyl on O-2 and O-3. The methyl esterification, in particular, has gained a lot of attention over the years in pectin chemistry, because it determines to a large extent the physical properties of pectin. Not only the amount of methyl esterification is important, but also their distribution. Blocks of more than 10 unesterified Gal A residues generally yield pectin molecules which are sensitive to Ca^{2+}-crosslinking (Daas et al., 2001).

In the literature, there are reports describing a HGA with β-D-Xylp- side chains. This polysaccharide is referred to as Xylogalacturonan (XGA). The side chains were thought to be constituted of a single unit of xylose (Schols, 1995), but a XGA with longer side chain was identify in soybean pectin (Huisman et al., 2001).

HGAs can contain clusters of four different (hetero-oligomeric) side chains with very particular sugar residues. These side chain, together with the approximately nine galacturonyl residues to which they are connected, are referred to as RGII (O’Neill et al., 2004; Ridley et al., 2001). RGII has the richest diversity of sugars and linkage structures known, including apiose, aceric acid (3-C’-carboxy-5-deoxy-L-xylose), 2-O-methyl fucose, 2-O-methyl xylose, Kdo (3-deoxy-D-manno-2-octulosonic acid), and Dha (3-deoxy-D-lyxo-2-heptulosaric acid) (Carpita & McCann, 2000). The name RGII is somewhat misleading, because it suggests that this structure contains a rhamnogalacturonan backbone just like RGI. However, the rhamnose residues are much less abundant in RG-II than RGI, and they are present in the side chains of RGII instead of in the backbone. Although the structure of RGII is highly conserved in plant, new structural details are continuously being added to this complex element (Ridley et al., 2001).

RGI is a heteropolymer of repeating 2-α-L-Rha-4-β-D-GalA disaccharide units and can contain as many as 100 repeats of disaccharides. Other polysaccharides composed mostly of neutral sugars such as arabinans, galactans, and highly branched type of arabinogalactans (AG) of various configuration and sizes, are attached primarily to O-4 of several of the rhamnose units of RGI (Carpita & McCann, 2000; Perez et al., 2003; Vincken et al., 2003b).
HGA, RGI, and RGII are extracted together, they are not separated by size exclusion chromatography (e.g. Superose 6 and 12) and an enzyme treatment is require to generated a separation of theses structures; it is then assumed that they are covalently linked to one another (Ridley et al., 2001). Several models of pectin structure proposed in the literature are in agreement with this hypothesis (Vincken et al., 2003b). HGA and RGII are likely to be covalently linked since they both have backbones composed of 4-linked α-D-GalpA residues and they are both solubilised by treating walls with endopolygalacturonanase (Ridley et al., 2001).

1.3.4. Arabinogalactans

In the primary cell wall, other polysaccharides composed mostly of neutral sugars such as highly branched arabinogalactan are present as RGI side chains. The macromolecular structure of RGI is often referred to as hairy regions with arabinogalactan side chains comprising the hairs (Carpita & McCann, 2000; Vincken et al., 2003a). There are two type of AG structures; type I AG are found only associated with pectins and are composed of 4-β-D galactan chains with mostly terminal arabinose units at the O-3 of galactose units. Type II AGs constitute a broad group of short 3- and 6-β-D-galactan chains connected to each other by 3- and 6-linked branch point residues. This type of AG is also frequently found bound to RGI structure or associated with specific proteins in a proteoglycan structure, called arabinogalactan proteins (AGPs). The protein part normally consists of high proportion of Hydroxyproline, serine, and alanine (Carpita & McCann, 2000). Both types are found to be linked through position 4 of the rhamnose unit of the pectin chain. One easy method to distinguish between the two types of AG is the ability of AGII to precipitate the Yariv reagent (Van Holst & Clarke, 1985) and this is frequently used to show the presence of AGII in polymers, and can also be used for quantitative assessment of the amount of this type of polymer in the total pectic complex (Paulsen, 2001).
1.4. Wounds

Wound is a general term for conditions in which there is interruption or damage to the structural integrity of the skin or the underlying tissues. It can include abrasion, abscesses, bites, burns, blisters, boils, bruises (contusions), fractures, gunshot, incisions, injuries, punctures, skin lesion, scores, scalds, sprains, tears, and ulcers (Onayade et al., 1996).

In Mali, external wounds often occur during rural activities by injury, and are commonly infected leading to complications such as gangrene if not properly treated. Whereas absence of bacteria is unhealthy, conversely, too many bacteria, particularly in a wound, result in infection and dehiscence. The bacterial concentration on a wound bed and the ability of skin grafts to adhere and survive are related. Early tissue adherence is critical for healing, as well as wound sterilization. It is known from the literature that chronic wound fluid inhibits or fails to stimulated cell proliferation (Bucalo et al., 1993).
Wound healing is a dynamic, interactive process involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells. A wound healing has three phases: inflammation, blood vessel formation or angiogenesis, and tissue modelling or regeneration (Singer & Clark, 1999). Both soluble and cellular factors (like complement and macrophages) play capital roles in wound healing processes.

One of the cell types involved, macrophages, usually arrive from the blood stream as monocytes in the inflammation phase and they differentiate into macrophages at the site of injury. They are attracted and activated by growth factors and cytokines secreted by platelets. However, in the absence of haemorrhage, platelets are not essential to wound healing. Macrophages are then attracted to the wound site by numerous vasoactive mediators and chemotactic factors that are generated by the coagulation and activated-complement pathways and by injured or activated parenchymal cells (Singer & Clark, 1999). In the inflammation phase macrophages bind to specific proteins of the extracellular matrix by their integrin receptors, an action that stimulates phagocytosis of micro-organisms and fragment of extracellular matrix (Brown, 1995). During reepithelialisation process macrophages secrete growth factors that stimulate the migration and proliferation of epidermal cells (figure 1.3).
Figure 1.4 role of macrophages in the inflammatory phase of a cutaneous wound three days after injury (Singer & Clark, 1999).

Growth factors thought to be necessary for cell movement into the wound are shown: TGF denote transforming growth factor; FGF: fibroblast growth factor; VEGF: vascular endothelial growth factor; PDGF: platelet-derived growth factor, IGF: insulin-like growth factor; and KGF: keratin growth factor.

Wound healing plants often appear to have a common feature, containing material forming mucilage or gels when extracted with water, which indicates a content rich in polysaccharide material. These polysaccharides are also known as compounds having effects in the immune system. According to the traditional healers, a large number of patients who visit them suffer from internal wound like gastric ulcer, which is regarded as an important public health problem (Inngjerdingen et al., 2004). Collecting information about and investigation of plants used in ethnomedicine in the treatment of wounds is therefore of high priority.

1.5. The Immune system

The immune system of vertebrates, such as humans, consists of many types of proteins, cells, organs and tissues, which interact in an elaborate and dynamic network to protect against diseases by identifying and killing pathogens, and possibly prevent the development of tumour cells. As
part of this more complex immune response, the vertebrate immune system adapts particular pathogens by involving antigen specific receptors. The adaptation processes which engage these antigen receptors on B and T lymphocytes creates immunological memories and allows more effective protection during future encounters with the pathogens. This process of acquired immunity is the basis of vaccination. Therefore, the human immune system is a combination of innate and adaptive immunity (Beck & Habicht, 1996).

The immune system protects organisms from infection with several defence layers of increasing specificity. Most simple physical barriers prevent pathogens from entering the body. If a pathogen overcomes these barriers, the innate immune system provides an immediate, but unspecific response. This innate immune systems are found in all plants and animals (Litman et al., 2005). However, if the pathogens successfully evade the innate immune system, vertebrates possess a third layer of protection, the adaptive immune system. Here the immune system adapts its response to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger response each time this pathogen is encountered (Mayer, 2006). The innate immune system involves humoral and chemical barriers (inflammation and complement system) and cellular barriers (leucocytes, phagocytes, dendritic cells, mast cells). In adaptive immunity, B cells are responsible of the humoral response while T cells are involved in cell-mediated immune response and also as helper cells for the B cells.

This chapter will focus only on the complement system (which is one component of the humoral innate immunity) and macrophages (that are part of the cellular innate immunity).

1.5.1. The complement system

The complement system consists of approximately 20 proteins that are present in normal human (and in other animal) serum. The term complement refers to the ability of these proteins to complement (augment) the effects of other components of the immune system (antibody). Complement is an important component of our innate immunity. There are three main effects of the complement: the lysis of cells such as bacteria, allograft, and tumour cells; generation of mediators that participate in inflammation and attract neutrophils; and opsonisation, (enhancement of phagocytosis). Complement proteins are synthesised mainly by the liver.
Several components of the complement system are heat-labile; they are inactivated by heating serum at 56°C for 30 min (Dodds, 2002; Lewinson & Jawetz, 2002; Seelen et al., 2005).

**- Activation**

Several complement components are proenzymes which might be cleaved to form active enzymes. Activation of the complement system can be initiated either by antigen-antibody complexes (classical pathway) or by a variety of molecules on pathogens and other foreign materials (alternative and lectin pathway) (figure 1.4). The lectin and the alternative pathway are important the first time when infected by a micro-organism, because the antibody required to trigger the classic pathway is not present. The lectin and alternative pathway are therefore participants in the innate arm of the immune system. All three pathways lead to the production of C3b, the central molecule of the complement cascade. C3b has two important functions: 1) it combines with other complement component and generates C5 convertase, the enzyme that leads to the production of the membrane attack complex; 2) and it opsonises bacteria, because phagocytes have receptors for C3b on their surface (Lewinson & Jawetz, 2002).

In the classic pathway, antigen-antibody complexes activate C1 (C1 is composed of three proteins: C1q, C1r, C1s and calcium is require for its assembly and activation) to form a protease, which cleaves C2 and C4 to form a C4b,2a complex. The latter is classical C3 convertase, which cleaves C3 molecules into two fragments, C3a and C3b. C3b form a complex with C4b,2a producing a new enzyme, C5 convertase (C4b,2a,3b) which cleaves C5 to form C5a and C5b. C5b binds to C6 and C7 to form a complex that interacts with C8 and C9 to produce the membrane attract complex which causes cytolysis (Lewinson & Jawetz, 2002).

In the lectin pathway, mannan-binding lectin (MBL) (also known as mannose binding protein) binds to the surface of microbes bearing mannan. This activates proteases associated with MBL, much the same way as C1 is assembled, that cleaves C2 and C4 components. Note that this process bypasses the antibody requiring step and so is protective early in infection before antibody is formed.

In the alternative pathway, many unrelated cell surface substances, e.g., bacterial lipopolysaccharides (endotoxin), fungal cell walls, and viral envelopes, can initiate the process by biding C3 (H₂O) and factor B. This complex is cleaved by protease, factor D, to produce C3b,Bb.
This acts as the alternative C3 convertase to generate more C3b (Dodds, 2002; Lewinson & Jawetz, 2002)

- Regulation of the complement system

The Complement system is a powerful mediator system that in itself harbors major protective activities against pathogens, by its direct cytolytic, chemotactic, anaphylactic and opsonic activities. By these properties it is optimally suited to play a major role in the induction of acquired immune system by bringing antigens in optimal contact with the major players of acquired immunity namely B- and T-cells. However, the more negative aspect of complement action is its phlogistic effect on host tissue and cells leading to initiation and maintenance of inflammation and organ dysfunction. These types of aberrant functions of complement are found in many immune mediated diseases like systemic lupus erythematosus and rheumatoid arthritis and many renal diseases. In these conditions, it would be beneficial to control the degree of ongoing activation of the complement either by the use of naturally occurring complement inhibitor or by novel complement inhibitors specifically designed to control disease activity (Seelen et al., 2005)

The first regulatory step in the classical pathway is at the level of the antibody itself. The complement-binding site on the heavy chain of IgM and IgG is unavailable to the C1 component of complement if antigen is not bound to these antibodies. This means that complement is not activated by free IgM and IgG despite being present in the blood all the times. However when antigen binds to its specific antibody, a conformational shift occurs in IgM and IgG in such a way that the C1 component can bind and initiate the cascade. Several serum proteins regulate the complement system at different stages. C1 inhibitor is an important inhibitor of the classic pathway. It inactivates the protease activity of C1. Activation of the classic pathway proceeds past this point by generating sufficient C1 to overwhelm the inhibitor. The regulation of the alternative pathway is mediated by the binding of factor H to C3b and cleavage of this complex by factor I, a protease. This reduces the amount of C5 convertase. The protection of human cells from lysis by the membrane attack complex is mediated by decay-accelerating factor (DAF), a glycoprotein located on the surface of human cells. DAF acts by destabilizing C3 convertase and
C5 convertase. This prevents the formation of the membrane attack complex (Lewinson & Jawetz, 2002)

**Figure 1.5: The three activation pathways of the complement system (Merck, 2005)**

The classical, mannose-binding (MBL) and alternative pathways converge into a final pathway when C3 convertase (C3 conv) cleaves C3 into C3a and C3b. C1-INH = C1 inhibitor; MASP = mannose binding lectin associated serine protease; Ag = Antigen; Ab = Antibody; MAC= membrane attack complex
1.5.2. Macrophages

Macrophages play an important part in the immune response including wound healing; they act by means of number of different mechanisms: (a) directly by destroying bacteria, parasites, viruses and tumour cells; (b) indirectly by releasing mediator (interleukine-1, tumour necrosis factor-\(\alpha\), etc) which can activate other cells; (c) as accessory cells by processing antigen and presenting digested peptides to T lymphocytes; and (d) by repairing tissue damage (Xaus et al., 2001).

- Activation

Macrophages play these roles when they are activated. They are three different populations of activated macrophages with three distinct biological functions. The first and most well described is the classical activated macrophage whose role is as an effectors cell in Th1 cellular immune response, promoting inflammation, extra cellular matrix destruction and apoptosis. The second type of cells, the alternatively activated macrophage, appears to be activated by Th2-like phenotypic cells and appears to be involved in immunosuppression and tissue repair promoting cell proliferation and angiogenesis. The most recent addition to this list is the type II activated macrophages, which is anti-inflammatory and preferentially induces Th2 type humoral-immune responses to antigen (Mosser, 2003). Although all phenotypes are important components of both innate and adaptive immune systems, the classically activated macrophages tend to elicit chronic inflammation and tissue injury whereas the alternatively activated macrophages tend to resolve inflammation and facilitate wound healing (Gordon, 1999). Interferon-\(\gamma\) seems to be the most important activating stimulus of the classical activation in addition to lipopolysaccharide and peptidoglycans (Ma et al., 2003). Activated macrophages posses a markedly enhanced ability to kill and degrade intracellular micro organisms. This killing is accomplished by an increase in production of toxic oxygen species and induction of the inducible NO synthetase gene to produce NO (Mosser, 2003). In the presence of some Th2 cell cytokines, macrophages become activated in an alternative way to combat parasitic and extra cellular pathogens (Gordon, 1999).

- Nitrite oxide dependant killing

Binding of bacteria to macrophages, particularly binding via Toll-like receptors, results in the production of TNF-alpha, this acts in an autocrine manner to induce the expression of the
inducible nitric oxide synthetase gene (i-nos) resulting in the production of nitric oxide (NO). If the cells are also exposed to interferon gamma, additional NO will be produce. NO is toxic and can kill micro-organisms in the vicinity of the macrophages (Mayer, 2006).

- Regulation
Macrophages may form their own regulatory network to prevent a well-intentioned immune response from progressing to immunopathology. The switch-off of already activated macrophages is an active and controlled regulatory process. It can be achieved by many factors including the suppressive cytokine IL-10 (Gordon, 2003).

1.6. Immunostimulatory activity of plants polysaccharides

Immunostimulants are compounds leading to non-specific stimulation of the immune system. The effect may involve activation of the complement system and macrophages, and stimulation of T- or B-lymphocytes. Due to these effects, immunostimulants may be an alternative to conventional chemotherapy or prophylactics of infections. They may also be used in the treatment of cancer and for enhancing the healing of wounds (Wagner et al., 1985)

The complement system and macrophages have for long been considered as target for polysaccharides. A well known macrophage activator is a pectic arabinogalactan from *Echinacea purpurea* (Emmendörffer et al., 1999). This AG induced macrophage phagocytosis, and increased the production capacity of reactive oxygen intermediates as well as the production of cytokines. *Sedum telephium* and *Plantago major* are used in traditional medicine in the treatment of all kinds of inflammatory diseases in the skin and in wound healing. Pectin polysaccharides from *S. telephium* have anti-inflammatory activity (Sendl et al., 1993) and those from *P. major* have anti-complementary activity and activate macrophages (Samuelsen et al., 1996). Other anticomplementary pectin were isolated from the leaves of *Artemisia princeps* (Yamada et al., 1985b), the roots of *Angelica acutiloba* (Yamada et al., 1985a), and the leaves of *Panax ginseng* (Gao et al., 1989). A pectic polysaccharide with intestinal immune modulator activity was isolated from *Atractylodes lancea* (Yu et al., 2001). Acemannan isolated from the gel of *Aloe vera* is a well-known wound healing remedy in traditional medicine. Both fresh gel and acemannan are found to accelerate the wound healing process in rats (Chithra et al., 1998; Tizar et al., 1994). Alginates from *Ascophyllum nodosum* are also known immunostimulators. Such
polysaccharides stimulate monocytes to produce interferon-α, interleukin-6 and interleukin-1 (Otterli et al., 1991). Sulphated fucans from A. nodosum on the other hand inhibit the complement cascade and are therefore potential anti-inflammatory agents (Blondin et al., 1996). The use of these plants, as well as many Malian medicinal plants (Entada africana, Trichilia emetica, Cochlospermum tinctorium, Vernonia kotschyana, Biophytum petersianum and Glinus oppositifolius) against wounds, burns and various infections has to a large extent been assumed to be related to the content of complement fixing, mitogenic and macrophage activating polysaccharides (Diallo et al., 2001, 2002, 2003; Inngjerdingen et al., 2005, 2006; Nergard et al., 2004, 2005).

Infectious diseases, malaria, schistosomiasis, gastric ulcer, wounds of different origin and fungal diseases are common in Mali, an effective immune response is necessary to recover from these diseases. In addition to their immunostimulant properties, polysaccharides also represent a very interesting part of plants for development of phytoremedies due to their solubility in water, their low toxicity, their physical properties, and most importantly their availability in a matter of amount in crude water extract. Medicinal plants are basically used in the form of water extract in traditional medicine in Mali. A phytoremedy based on such type of compounds will be easy to produce and suitable for local population in Mali.
1.7. Plant secondary metabolites

A characteristic feature of higher plants is their capacity to synthesize an enormous variety of organic molecules. The production and accumulation of a wide variety of organic chemicals is one major mechanism by which plants defend themselves against herbivores, and attacks by microbial pathogens and invertebrate pests. Most of these chemicals are products of the secondary metabolism, originally thought to be waste product not needed by the plants for primary metabolic functions. It is, however, well known that their presence in different parts of the plant (root, bark, leaves etc) deters feeding by slugs, snails, insects and vertebrates, as well as attacks by viruses, bacteria and fungi (Wink, 1999). However, it was the potential use of plant secondary metabolites in health care and personal care products, and as lead compounds for development of novel drugs, that lead to a huge interest in their isolation and characterization from major plant species. At present, the total number of identified secondary metabolites exceeds 100,000. These can be grouped into three main chemical classes: Phenolic, Nitrogen containing compounds and terpenes (Wink & Schimmer, 1999). This chapter will discuss the implication of a group of phenolic compounds, the flavonoids, in human health as antimicrobial agents and lipoxygenase inhibitors.

1.7.1. Flavonoids as antimicrobial agents from plants

Flavonoids, hydroxylated phenolic substances as a C6-C3 unit linked to an aromatic ring (figure 1.6), are the best known group of polyphenols and comprise around 4000 of the 8000 known phenolics. The main bioactivities reported for flavonoids comprise antioxidant, antibacterial, vasodilatory, anticarcinogenic, anti-inflammatory, antihistaminic, antiviral and enzyme inhibitory properties (Cowan, 1999; Harborne, 1988).

The isoflavonoids represents an important and very distinctive subclass of flavonoids. These compounds are based on a 3-phenylchoman skeleton that is derived by acetyl migration mechanism from the 2-phenylchroman skeleton of flavonoids. Pterocarpsans, a sub class of isoflavonoids contain a tetracycle ring system derived from the basic isoflavonoid skeleton by an ether linkage between the 4 and 2′ positions (figure 1.6). They represents the second largest group of isoflavonoids after the isoflavones (Dewick, 1988). The majority of natural pterocarpans isolated have arisen from phytoalexin studies, using fungal or abiotically stressed plant tissues. Phytoalexins are substances with antimicrobial activity produce de novo by plants.
as a response to a stress factor, such as a fungal or bacterial invasion, or the influence of external abiotic agents, mainly heavy metal salts or UV radiation (Jimenez-Gonzalez et al., 2007). Since they are known to be synthesized by plants in response to microbial infection, it is not surprising that they have been found to be effective antimicrobial substances against a wide array of microorganism. These compounds are synthesized in healthy tissues surrounding the infection area but accumulated in the infected tissue, where their antimicrobial activity is needed. Pterocarpans work as bactericidal or bacteriostatic agents and several published hypothesis confirm that they exert their activity through a degradation of the cellular membrane of the microorganism (Jimenez-Gonzalez et al., 2007).

![Flavonoid, Isoflavonoid, Flavone, Pterocarpan](image)

*Figure 1.6 Structure of some flavonoid subclasses*

**Infectious diseases**

In developing countries, infectious diseases remain the leading cause of death; according to WHO these illnesses are a significant cause of morbidity and mortality worldwide, accounting for approximately 50% of all death in tropical countries, partly as a result of the HIV/AIDS epidemic (WHO, 2003). Despite the availability of efficient treatment for most of them, their incidence tends to dramatically increase. Although, prevention and control of infectious diseases have been
so successful in the past half century that many people view these diseases as either a thing of the past or minor illnesses easily treated and cured, except among the very young, very old, or seriously ill. Those minor illnesses are still lethal in many countries in the world. Africa is besieged by a large number of infections, ranging from bacterial to parasitic infestation (NIAID, 1999). To the unsolved problem of the control of infectious diseases, drugs resistance brings an additional burden. It was a few years after the introduction of penicillin that scientists began to notice the emergence of a penicillin-resistant strain of *Staphylococcus aureus*. Since then, resistant strains of gonorrhea, dysentery-causing *Shigella* and *Salmonella* followed in the wake of *Staphylococcus* resistance. Multiresistant tuberculosis is no longer confined to any one country, or to those co-infected with HIV, but has appear in locations as diverse as Eastern Europe, Africa and Asia among health care workers and in the general population (WHO, 2003). Although some antibiotics still are active, the rapid progression of resistance suggests that many of these drugs may not be effective for much longer. The emergence of multiresistant drug-bacteria means that medication that once cost very little, must now be replaced with a drug a hundred times more expensive (WHO, 2003).

In addition to that, in recent years, the world has been shocked by the emergence of a variety of "new" infectious diseases. New diseases have emerged in developing countries where the current ones are still lethal. Ebola hemorrhagic fever, which was first described in 1976 in Zaire (now the Democratic Republic of the Congo), has particularly horrifying symptoms and a fatality rate of 50 to 90 percent. AIDS, which emerged simultaneously in the United States and Africa in the early 1980s, has become a global pandemic (NIAID, 1999).

Since the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobial remedies has been virtually nonexistent in most western societies, while local communities in developing countries that can not afford these antibiotics still rely on the use of plant extract. There are literally thousands of published scientific data from around the world describing the antimicrobial activities of plant extracts; among them, several species have ethnomedicinal data supporting their use to treat infectious diseases. Many of the plant species are native to tropical countries, due to the extraordinary biodiversity in these areas (Mahady, 2005). Plant components have not only proven to be active against drugs-resistant strains of microbes, they can also improve the activity of resistant drugs. Erybraedin A, a flavonoid isolated in many *Erythrina* species, showed
a high growth inhibitory potency against vancomycin resistant enterococci (VRE) and multiple-resistant *Staphylococcus aureus* (MRSA) and the combination of erythromycin and vancomycin acts either synergistically or additively against VRE and MRSA (Sato *et al.*, 2004). However, many of these medicinal plant extracts reported to have antibacterial activities have only been subjected to *in vitro* screening, and the vast majority of them has never been tested rigorously in animal models or controlled clinical trial; but it is now clear that the uses of plant extracts as phytomedicine against infectious diseases is based on their presence in those extracts.

1.7.2. Flavonoids as lipoxygenase inhibitors

Lipoxygenases (LOs) are a family of nonheme iron-containing dioxygenases able to induce enzymatic peroxidation of polyunsaturated fatty acids. LOs are widely distributed among plants and animals. In mammals, four types of LOs have been identified, namely 5-, 8-, 12-, and 15-LO, which insert dioxygen at C5, C8, C12, and C15 positions respectively of arachidonic acid (Takahashi *et al.*, 2005). Of particular interest is 15-LO, since it can also oxidize esterified fatty acids in biological membranes and lipoproteins, forming 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) from arachidonic acid and 13-hydroperoxy-octadecadienoic acid (13-HPODE) from linoleic acid (Brash, 1999; Lapenna *et al.*, 2003). Remarkably, 15-LO has been implicated for its specific oxidative effects in the pathogenesis of atherosclerosis (Cyrus *et al.*, 1999), in prostate cancer (Kelavkar *et al.*, 2001) and in spontaneous abortion (Dar *et al.*, 2001). Soybean lipoxygenase-1 (SLO) is a plant-derived 15-LO that efficiently catalyzes the oxidation of linoleic acid to 13-HPODE (Lapenna *et al.*, 2003). The reduction of Fe$^{3+}$ to its inactive form Fe$^{2+}$ and the scavenging of peroxyl radicals generated from LO-polyunsaturated fatty acid interaction may be involved in the pharmacological inhibition of enzymatic lipid peroxidation. Dietary constituents, drugs or phytomedicines that inhibit 15-LO may exhibit anti-atherogenic properties, thus protecting the cardiovascular system.

Because of structural and functional similarities with mammalian LOs, SLO is commonly used for both mechanistic and inhibitory studies to give indications for the behavior of a test substance in mammalian 15-LO. A good correlation for inhibitory activity toward the two enzymes has been shown (Lapenna *et al.*, 2003). But other studies showed that the usability of SLO as model for mammalian one is limited (Schewe *et al.*, 2001).
Inhibition of lipoxygenases occur in 3 different pathways, (I) reduction of Fe$^{3+}$ to the inactive Fe$^{2+}$, (II) scavenging of peroxyl radicals generated during the reaction, or (III) inhibiting 15-LO from binding the substrate. Flavonoids are known inhibitors of mammalian 15-lypoxigenase; both iron-chelating and reducing properties may be expected to be involved in the interaction with lipoxygenases. They may intercalate in the hydrophobic cavity of the active site located in the interior of the large domain of the enzyme, which is binding site for both hydrophobic substrate molecules and inhibitors (Brash, 1999).

1.8. Plants used in this study

1.8.1. Opilia celtidifolia

Opilia celtidifolia (Guill. & Perr.) Endl. ex Walp (Opiliaceae) locally know as “korôgué” or “warablé minkô” is a woody climber, spreading, heavily branched shrub or tree up to 10m high, common in fringing forest and savanna. It is widespread in the region from Senegal to Nigeria (West Africa) and dispersed over the dried part of tropical Africa (Burkill, 1997). Opilia celtidifolia is well known to the traditional healers in Mali as a remedy to cure several diseases; dermatitis (the term dermatitis is used by the healers as a common terminology for all
kinds of skin disorders) and malaria. The plant is also known as an appetizer, an abdominal pain killer and an intestinal worm cure (Togola et al., 2005).

According to the literature, decoction of the leaves is used as febrifuge in Ivory Coast. In Senegal it is used as a gargle, against dental abscesses, to treat oedema leprosy, acting as a purgative, and used against headache. A macerate left to stand overnight and strongly salted, taken on an empty stomach is meant to be particularly effective in expelling oxyuris worms from children (Burkill, 1997).

Little was found on biological studies of *O. celtidifolia* in the literature. Shihata et al. (Shihata et al., 1977) isolated saponins from the methanol extract and found antispasmodic and antihelmintic activities for these compounds. The authors also signaled the lack of information regarding the biological properties and possible therapeutic value of the plant. The effects shown above may explain both the use as an abdominal pain killer and against intestinal worms as found to be common uses in our survey.

1.8.2. *Cola cordifolia*

![Figure 1.8: Cola cordifolia](image)

The medicinal tree, *Cola cordifolia* (Cav.) R Br., Sterculiaceae, locally known as "N’tabanokò", is a large tree, 15-25 m high, with a short buttressed trunk and low degree of branching with a
dense crown growing on the savanna from Senegal to Mali (West Africa). Its heavy crown furnishes ample shade, thus the tree is an admirable “palaver” tree in Africa. The mature fruit is edible and is the main harvested plant part. It resembles that of the true Kola, *Cola nitida*, and the seeds are surrounded by a salmon-pink seed-coat which is sweet and pulpy. All parts of the tree, such as the leaves, roots, bark and seeds are used in traditional medicine. Burkill reports that the bark of the tree is used against constipation in Gambia, and against chest-affections and dysentery in Senegal, and the leaves are used as a remedy for eye-treatment. In Mali the leaves are used against gastric ulcer, malaria, malnutrition and head ache (Burkill, 2000). The bark is an internal and external wound healer; it is also used against constipation, overweight, high blood pressure, malaria and oedema. The wound healing activity was also previously reported, the decoction of the powder and/or the carbonized powder of the stem bark was used to treat old wounds. In that study, *C. cordifolia* was one of the fifteen most cited wound healing plants (Diallo *et al.*, 2002).

1.8.3 *Erythrina senegalensis*

*Figure 1.9: Erythrina senegalensis*

*Erythrina senegalensis* DC (Fabaceae), locally known as “nté”, is a tree that can be up to 15m high, usually much less, with a trunk with a corky bark and large red flowers, the plant is
distributed in tropical and subtropical region of Africa (Burkill, 1995). Extracts of the leaves, bark and roots are significantly used in traditional medicine to cure diseases such as amenorrhoea, urinary bilharzias and sterility (Togola et al., 2005).

According to the literature, in Gambia and Senegal the sap from the crushed leaves is applied to wounds for two or three days to promote healing. In Ghana and Nigeria the pounded bark and leaves are taken by women in a soup against barrenness. They are also used as enemas. In Mali the decoction of leaves is used to provoke diuretic activity and is taken against urinary bilharzias. In Senegal a macerate of the trunk-bark is taken internally for amenorrhoea and externally against headache and eye-troubles. In Ivory Coast the wood is chewed as an aphrodisiac (Burkill, 1995).

In addition to these traditional uses, positive biological activities such as antiplasmodial, analgesic and anti-inflammatory are also reported for this plant (Saidu et al., 2000)

Despite the many therapeutic uses of *Cola cordifolia* in Malian traditional medicine, no report could be found in literature on the identification of biologically active or other type of isolated compounds from this plant. Very few reports were found about *Opilia celtidifolia*, none were related to biologically active polysaccharides. In our search for immunomodulator compounds from Malian medicinal plants, ethnopharmacological surveys were performed in several areas of Mali to identify plants that are used in wound healing processes. *C. cordifolia* and *O. celtidifolia* were both reported by traditional healer to have potent wound healing ability. Theses results and the fact that none of these plants were being investigated for such type of compound before made them suitable for this study.

*Erythrina senegalensis* has gained lot of attention regarding the isolation of low molecular weight compound in the literature. The plant is largely studied with several biologically active isolated compounds. This plant was chosen as a suitable candidate for an eventual development of ITM against infectious diseases by the DMT. The evidence that the Malian *Erythrina* species contains those compounds was needed in order to develop an ITM from this plant. These reasons cited above made these plants suitable for this study and motivated our choices.
2. Aim of the study

The use of plants in traditional medicine is sustained by their biological activities either scientifically proven or not. In countries like Mali where the primary health care system of a majority of the population is traditional medicine, studies are necessary to assess the activity, the toxicity and the interaction of the plants with conventional medicine. Those plants have already been used by generations of Malian population, which may assess their efficacy and safety. The simultaneous use of plant extracts with conventional medicine, and the large environment degradation by the excessive use of plants without creation of new source of replacement for the species are new factors that need to be taken in to consideration to ensure the safety of the patients as well as the continuous use of the plants in the future. These studies take place in the objective framework of the DMT which is the development of new improved traditional medicine from cultivated sources. The present study is part of this main aim.

Immunostimulatory polysaccharides are present in several plants used in wound healing processes in traditional medicine. From previous published work, Malian medicinal plants contain biologically active polysaccharides (see chap 1.6). These polysaccharides might also be present in *Opilia celtidifolia* and *Cola cordifolia*. *Erythrina senegalensis* is also used in traditional medicine to treat infectious diseases. Low molecular weight compounds are likely responsible for these activities.

The aim of this study was therefore:

- To identify the uses of medicinal plants which were retained for the investigations as new ITMs, but were lacking of substantiated information on their use in traditional medicine
- To isolate and characterize biologically active polysaccharides from *Opilia celtidifolia* and *Cola cordifolia*
- To isolate, characterize and test the biological activities and toxicity of low molecular weight compounds from *Erythrina senegalensis*. 

3. Summary of results

3.1. Paper I and II

As the starting point of this research information were needed on the medicinal uses of the medicinal plants being the objects for further investigation. The traditional uses of *O. celtidifolia* against wounds was already reported (Diallo *et al.*, 2002; Inngjerdingen *et al.*, 2004). The aim of papers I and II of the present thesis was to identify other traditional uses of these plants. Other plants that are not investigated in this thesis were included in the ethnopharmacology survey, because they might be sources for new ITMs at the DMT. It is lacking substantiated information on their traditional uses as well. This made the survey necessary, but their results will not be discussed. The healers to be interviewed and the locations were selected randomly and no appointment was made prior to the visits. The survey gathered several medical indications for which these plants were used. The most frequently cited indications were malaria, abdominal pain and dermatitis for *O. celtidifolia*. Amenorrhea, malaria, abdominal pain, infections and wounds were some of the most reported diseases for *E. senegalensis* and these diseases are some of the most encountered ones in Mali. Abdominal pain as referred to by the traditional healer is rather a symptom encountered in different diseases with completely different manifestation. This latest result showed the traditional practice to be symptom directed. The leaves of *O. celtidifolia* and the roots and stem bark of *E. senegalensis* were the most frequent plant parts used. The majority of the remedies were prepared in the form of decoction in both cases and most of them were taken orally or by an external application as body bath, steam bath and as external application in the case of dermatitis and wounds.

The fidelity level among the healers from the same area was calculated. This was used to compare results from different area where the survey was performed. There was a high agreement among the traditional healers about the most reported uses of *O. celtidifolia*, 75% for the use against Dermatitis in Siby, 61% for malaria and 54% for abdominal pain in Dioila. The agreements were lower in the case of *E. senegalensis*, 21% for the use against amenorrhoea in Dioila and Kolokani.

A review of the literature on the chemistry and biological activities of the plants was performed. Although *O. celtidifolia* was widely used in traditional medicine in Mali, little information was
available on its biological activities and isolated compounds. Most of the studies on the activities were performed on total extracts. *E. senegalensis* was the most studied plant with the most reported isolated compounds. Several studies on antimicrobial activities were reported for these compounds.

3.2. Paper III

In this paper, results of a preliminary characterisation of polysaccharide fractions isolated from a water extract of the leaves of *O. celtidifolia* are presented. The leaves were chosen because most of the traditional used are reported on them. Water extracts, both 50 and 100°C, were prepared and tested for effect on the complement system. The 50°C extract, found to be the most abundant in polysaccharide material and also having higher activity in the complement system, was chosen for further studies. Two fractions (Oc50A1 and Oc50A2) were isolated after anion exchange chromatography. Both fractions showed potent dose dependant complement fixing activity *in vitro*. Their ICH50 values were 0.9 and 0.5 μg/ml respectively. A significant macrophages activation activity was observed (induction of nitric oxide release) as well. At doses of 100μg/ml both fractions induced about 7.2 μM of nitrite oxide.

Structural studies showed the polysaccharides fractions to contained high proportion of arabinogalactan types II, most likely arabinogalactan proteins due to the presence of hydroxyproline rich protein (1.4 and 0.9% respectively in Oc50A1 and Oc50A2).

The molecular weight determination demonstrated that Oc50A1 was composed of two polysaccharides of 160 and 37 kDa representing respectively 43 and 56% of the total fraction. Oc50A2 was mainly composed of a polysaccharide of 46 kDa which represented 82% of the total fraction, with an additional high molecular weight polysaccharide of 220 kDa represented only 15% of the material. Enzymatic degradation by endopolygalacturonanase and fractionation on a Biogel P30 column gave two sub fractions for each (Oc50A1d1, Oc50A1d2, Oc50A2d1 and Oc50A2d2). These fractions showed even higher complement fixing ability (ICH50 of 0.2 and 0.5 μg/ml for the most active ones) and they induced nitric oxide release from macrophages.

**Results of unpublished studies on *O. celtidifolia* Polysaccharides**

A new batch of Oc50A1 and Oc50A2 from the leaves of *O. celtidifolia* were prepared
These two fractions have shown high complement fixing ability and induction of NO release from activated macrophages as described in paper III. We next tested whether the polysaccharides could lead to an indirect proliferation of T cells, induced by an activation of dendritic cells, in a setup called Mixed Lymphocyte Reaction (MLR). First, a cell culture of dendritic cells was prestimulated with Oc50A1 or Oc50A2 for 24 hrs before purified T cells were added to the culture. The results in figure 3.1 show that these condition induced T cells proliferation. The estimated percentage of activity was 14 and 24% respectively at 50 μg/ml respectively.

**Figure 3.1: Proliferation of T cells induced through MLR**

Dendritic cells (DCs) were generated from PVG rat spleen monocytes by 6 days culture in IL-4 and GM-CSF. The DCs were then incubated overnight with LPS (200 ng/ml) or *Opilia celtidifolia* polysaccharides Oc50A1 or Oc50A2 (50 μg/ml). Purified rat T cells (from PVG rat spleen) were stained with CFSE, and added to the DCs at a ratio of 1:10 (DC/T cell). The cells were incubated for 3 days at 37°C, and proliferation analyzed by flow cytometry.

Since the determination of the weight average molecular weight showed both fractions to be composed of two polysaccharides, they were then separated on Sephacry S-400 into two subfractions called Oc50A1.1, Oc50A1.2, Oc50A2.1 and Oc50A2.2 respectively. The monosaccharide composition of the fractions is shown in table 3.1. Monosaccharide composition of the new batch was similar to those of the previous ones with minor differences in proportions. Arabinose and galactose were the main monosaccharides with a respective proportion in Oc50A1 and Oc50A2 of 0.6: 1 and 0.3: 1 while rhamnose and galacturonic acid were present at the proportion of 1: 1 and 1: 0.9 respectively in these same fractions. After gel filtration, arabinose and galactose having the proportion of 0.6: 1 and 0.7: 1 while the ratio rhamnose to galacturonic acid were 1.3: 1 and 0.9: 1 respectively in Oc50A1.1 and Oc50A1.2. Similar proportions were also found for Oc50A2.1 and Oc50A2.2. Compared to the previous batch, Glucose, glucuronic acid and 4-O-Methyl glucuronic
acid were present in higher amount in these samples. Fucose and mannose were present in minor amount. The fractions obtained after gel filtration presented very similar monosaccharide composition to their original fractions (table 3.1).

Table 3.1: Monosaccharides composition (% related to the total amount of sugar), effect in the complement system (ICH50) of the acidic fractions (new batch) gel filtration and arabinofuranosidase degraded fractions

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Fractions</th>
<th>Oc50A1</th>
<th>Oc50A1.1</th>
<th>Oc50A1.2</th>
<th>Oc50A1.2H</th>
<th>Oc50A2</th>
<th>Oc50A2.1</th>
<th>Oc50A2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara</td>
<td></td>
<td>16.8</td>
<td>19.2</td>
<td>20.4</td>
<td>7.9</td>
<td>7.8</td>
<td>12.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Rha</td>
<td></td>
<td>4.9</td>
<td>4.7</td>
<td>5.3</td>
<td>5.9</td>
<td>8.7</td>
<td>7.8</td>
<td>11.1</td>
</tr>
<tr>
<td>Fuc</td>
<td></td>
<td>1.3</td>
<td>1.3</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Xyl</td>
<td></td>
<td>3.0</td>
<td>3.0</td>
<td>2.8</td>
<td>2.5</td>
<td>3.0</td>
<td>1.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Man</td>
<td></td>
<td>5.5</td>
<td>7.1</td>
<td>5.6</td>
<td>9.5</td>
<td>4.8</td>
<td>4.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Gal</td>
<td></td>
<td>26.9</td>
<td>31.1</td>
<td>30.3</td>
<td>34.9</td>
<td>22.3</td>
<td>25.3</td>
<td>19.1</td>
</tr>
<tr>
<td>Glc</td>
<td></td>
<td>11.3</td>
<td>12.2</td>
<td>12.4</td>
<td>11.3</td>
<td>17.3</td>
<td>16.1</td>
<td>21.5</td>
</tr>
<tr>
<td>GlcA</td>
<td></td>
<td>14.0</td>
<td>9.3</td>
<td>5.9</td>
<td>6.8</td>
<td>18.6</td>
<td>11.8</td>
<td>12.0</td>
</tr>
<tr>
<td>GalA</td>
<td></td>
<td>4.8</td>
<td>3.6</td>
<td>5.8</td>
<td>11.5</td>
<td>9.3</td>
<td>11.6</td>
<td>12.5</td>
</tr>
<tr>
<td>4-O-Me-GlcA</td>
<td></td>
<td>11.5</td>
<td>8.3</td>
<td>10.5</td>
<td>8.7</td>
<td>7.3</td>
<td>7.1</td>
<td>5.7</td>
</tr>
<tr>
<td>ICH50 (µg/ml)</td>
<td></td>
<td>1.2</td>
<td>9.4</td>
<td>7.6</td>
<td>6.2</td>
<td>1.1</td>
<td>2.3</td>
<td>37.7</td>
</tr>
</tbody>
</table>

The complement fixing activities of the two series of extracted material were also similar, but the fractions obtained after gel filtration showed lower complement fixing activity than the original fractions. The ICH50 values ranged from 2.3 for the most active to 38µg/ml for the least active (table 3.1). The abilities to release NO from activated macrophages and to induce B cells proliferations were also tested. All fractions had the abilities to induce NO production to activated macrophages (figure 3.2). The original polymers fractions (Oc50A1 and Oc50A2) had slightly higher activities (6.5 and 5.4 µM respectively) than the gel filtration fractions (5.7 to 5.0 and 4.5 to 4.4 µM respectively for Oc50A1 and Oc50A2 sub fractions). A moderated mitogen activity on B cells was observed at the concentration of 100 µg/ml for all fractions (figure 3.3)
Figure 3.2: Nitrite oxide measure of the new batch acidic and gel filtration fractions of *O. celtidifolia*

The rat pleural macrophage cell line R2 (Wistar origin) was seeded into 96-well plates and incubated with various concentrations of the pectic polysaccharides, or LPS as a positive control, for 24 hrs at 37°C. The supernatants were harvested, and nitrite (a breakdown product of NO) was measured using the Griess Reagent System.

Figure 3.3: Proliferation of B cells of acidic and polygalacturonanase degraded fractions

B cells were isolated by positive selection from spleens of PVG rats using Dynabeads. After overnight culture, the cells were stained with the intracellular fluorescent dye CFSE (5 mM f.c.), and plated into round-bottom 96-wells (2x10^5 cells/well), and incubated with polysaccharides, or LPS as positive control (250ng/ml), for 5 days at 37°C. Proliferation is measured as a loss of CFSE fluorescence intensity in the cells as they divide. In the graphs, the mean fluorescence intensity of the control sample (medium alone) is set to 100, and the relative decrease in fluorescence in proliferating samples is calculated as: MFI sample/MFI control.
Enzymatic degradation

For structure and structure-biological activity relationship elucidation, Oc50A_{1.2} was chosen for enzyme degradation because of sufficient material available. The results obtained with this fraction can also be extrapolated to the Oc50A_{1.1} and the mother fraction Oc50A_{1} because of the similarities of monosaccharides compositions between these fractions.

Oc50A_{1.2} was treated with arabinofuranosidase and separated on Biogel P-30 into one high (Oc50A_{1.2}H) and one low molecular weight fraction which was not investigated any further. The monosaccharides composition of the high molecular weight material is given in table 3.1. The enzyme reduced the arabinose content of Oc50A_{1.2} more than 50% (from 20.4 to 7.9%) which is in good agreement with the result presented in paper III, were it was found that more than 50% of arabinose are terminally linked in the mother fraction (Oc50A_{1}). The enzyme treated fraction had slightly higher complement fixing activity (ICH_{50} value 6.2 ng/ml) than the original (ICH_{50} value 7.6 ng/ml) while the NO production ability was almost completely lost (1.8 μM of NO at 100 ng/ml data not shown). This result suggests that terminally linked arabinose is not essential for the complement fixing ability but may play an important role in the mechanism in the activation of macrophages.

These immunomodulatory polysaccharides are still under investigation for molecular weight determination, refined structure elucidation and structure activity relationship establishment using further enzymatic as well as chemical treatment followed by isolation and structural studies of isolated bioactive oligomers.
3.3. Paper IV

This study was aimed to identify the uses of *Cola Cordifolia* in traditional medicine, since the plant was not included in previous ethnopharmacological study, and to determine the structure and physical property (viscosity) of the complement fixing polysaccharide isolated from the water extract of the stem bark. The 50 °C extract of the bark (Cc50) was investigated in this study.

The results of the ethnopharmacological study showed that *C. cordifolia* was used against a variety of illnesses in Mali. Among these, abdominal pain, wounds, fever, headache, malaria, body pains, amenorrhoea, epilepsy, haemorrhoids, heart diseases and asthma were reported. Cc50 was attempted to fractionation by anion exchange chromatography and gave only one acidic fraction Cc50-1. The approximate molecular weight was estimated to be about 2000kDa. The structure of this polysaccharide was found to be a very complex novel type. The polymer contained approximately 40% of native methylated monomers identified as 2-O-Me Gal and 4-O-Me GlcA and traces of 2-O-Me Fuc, all these mostly terminally linked. The polysaccharide also contained 20% rhamnose being responsible for the main part of the branch points in the molecule and 24 % galacturonic acid being basically 4-linked, but also responsible for some branch points. This is an unusual type of polysaccharide and the first report of 2-O-methyl galactose as a monosaccharide component of polysaccharide from higher plant which probably has pectin like backbone. High amount of 4-O-methyl glucuronic acid was also present, although this has been identified in plant polysaccharides previously, a quantity as high as 18% was not seen before in polymer containing all these other monosaccharides mentioned above.

Cc50-1 was of viscous nature and then showed a poor solubility in water like solvents. Lack of good solubility as well as high viscosity can be caused both by the lipophilic sugars and uronic acids present in the polymer. Viscosity measurement showed possible formation of aggregates by using water as solvent. The natively methylated monomers were though to be partly responsible of the poor solubility which in is turn might explain the lower complement fixing activity observed. The polysaccharide showed a modest effect in the complement system having an ICH$_{50}$ value of 480µg/ml, compared to that of PMII from *Plantago major* used as a standard being 130µg/ml, measured in the same set up.
3.4. Paper V

The objective of this paper was to isolate and test the biological activities of compounds from the root and stem bark extracts of *Erythrina senegalensis*. Dichloromethane and methanol extracts were prepared from each plant parts. The root bark DCM extract showed antimicrobial activities against *Staphylococcus aureus* and *Candida albicans*, 15-Lipoxygenase inhibition activities and weak DPPH scavenging activities. A total of eight compounds, one new and seven known, were isolated from these extract. The new compound was isolated from the DCM extract of the root bark. Its structure was determined by NMR, which showed characteristic pattern of a pterocarpan. The molecular formula was C\textsubscript{21}H\textsubscript{20}O\textsubscript{5}. The HMQC/HMBC correlation and the comparison with other literature data of pterocarpans with similar substitution pattern allowed the establishment of the molecular structure to be 9-hydroxy-8-methoxy-6',6'-dimethylpyrano-[2',3':3,4]-pterocarpan. The compound was name Erybraedin F. In addition to this, six known pterocarpans, erybraedin A, C, D, eryvarin K, phaseollin, shinpterocarpin were also isolated from the same extract and the flavone carpachromene was isolated from the stem bark DCM extract. Their structures were identified by comparison of spectroscopic data with the literature. Based on the literature research in the database available on Scifinder, these compounds although previously reported from the genus *Erythrina*, are for the first time identified in *E. senegalensis*.

The isolated compounds were tested for biological activities. Erybraedin A, C, and D showed stronger 15-LO inhibition activities than quercetin, the positive control. Erybraedin D was the predominant 15-LO inhibitor with 61% of inhibition at 32\textmu M while quercetin showed and IC\textsubscript{50} value of 42\textmu M. Eryvarin K and carpachromene demonstrated moderate activities. Erybraedin A, C, and D and Eryvarin also showed antibacterial activities against *Staphylococcus aureus*, and Erybraedin A showed antifungal activity against *Candida albicans*. Carpachromene and erybraedin C showed moderate toxicity against brine shrimps.
4. General Discussion

4.1. Ethnopharmacological information

Plants are used as therapeutic resources all over the word since long time ago. They are used as herbal teas or other home made remedies, then they are considered as medicinal plants. They can be used as crude extracts or “standard enriched fractions” in pharmaceutical preparations, such as tinctures, fluid extracts, powder, pills and capsules, they are then considered as phytopharmaceutical preparations or herbal medicines. Finally, plants can be subjected to successive extraction and purification procedures to isolate the compounds of interest, which can themselves be active and used directly as a drug. Examples being quinine, digoxin and ergotamine, or they can be used as precursors (e.g. diosgenin) in hemisynthetic processes or as models for total synthesis, with well-defined pharmacological activity or structure–activity relationship studies determining a prototype drug (e.g. morphine) (Rates, 2001).

One of the objectives of the Department of Traditional Medicine in Mali is to study the plants that are used by traditional healers to treat illnesses in the local communities. There are several ways in which the selection of these plants for pharmacological studies can be done. These include traditional use, chemical content, toxicity, randomized selection or a combination of several methods. The study of the traditional uses of a plant is the most common strategy used (Rates, 2001). Ethnopharmacological surveys were performed in several areas in Mali to identify the traditional uses of the plants investigated in this study. Several diseases were reported against which the plants were used (paper I, II, IV). The agreement of traditional healers was high for the most reported uses (paper I). The leaves and root of *O. celtidifolia* were reported in Dogonland (Mali) to cure skin disorders (Inngjerdingen *et al.*, 2004), the leaves of the plant was also reported by traditional healers in Siby to cure dermatitis (paper I), the same plant part was also reported as wound healer in Bamako areas (Diallo *et al.*, 2002). *Erythrina senegalensis* is used in traditional medicine against infections (paper I and II), Magassouba *et al.* reported that *E. senegalensis* was the plant mostly reported to be used to treat infectious diseases in another study performed in Guinea (Magassouba *et al.*, 2007). According to traditional healers in Dioila and Dogonland, the leaves of *Cola cordifolia* are used against gastritis, the stem bark is used for treatment of internal and external wounds, the roots are used to treat skin inflammation (paper IV); *C. cordifolia* was
among the fifteen most cited plants to be used in wound healing processes in the Bamako area (Diallo et al., 2002). The fact that those plants are used by several traditional healers from different areas is a possible sign of their efficacy and safety. However, to produce phytoremedies from these plants that will be accepted as medicine and sold in drug stores, the existing and claimed biological activities need to be confirmed by scientific studies. As example, the improved traditional medicine “Gastroedal” is prepared from the root of *Vernonia kotschyan* and used against gastric ulcer. The water extract of this plant was claimed by traditional healers to cure ulcer during an ethnopharmacological survey, and polysaccharides isolated from the root water extract have shown anti-ulcer activity *in vivo* (Nergard et al., 2005b).

Malian traditional medicine abounds in medicinal plants, and the local communities, wherever they exist, still rely chiefly on herbal medicines which are now been recognized and standardized as essential medicine by the local government in Mali (Diallo & Paulsen, 2000). Herbal remedies still play a vital role in health care delivery systems, especially in remote places where clinics and hospitals are sparsely located and the availability of convention medicine is still limited due to poverty. In these communities, traditional herbalists and healers operate closer to the people, taking advantage of the biodiversity of plant species in such areas to cure various diseases and ailments. Although herbal medicine is well established in Mali and has been the part of cultures and traditions, not much information has been documented in scientific literature about Malian medicinal plants. Information on herbal medicine in this part of the world has been dominated by oral tradition. Since this information is held by elderly people, the need of documentation is urgent. The development of drugs from traditional medicine in the form of ITMs by the scientific community would help to increase availability of drugs to a large majority of the impoverished communities and would maintain the biodiversity through a cultivation of plants to face their extended use. Published data from these studies will contribute to the documentation of the traditional medicine.
4.2. Structures of isolated compounds

4.2.1. Polysaccharides

Water soluble polysaccharide fractions were isolated from *O. celtidifolia* and *C. cordifolia* according to figure 4.1. The fractionations were performed by a combination of gel filtration and anion exchange chromatography. The choice of fractions to be investigated was based on their complement fixing activity and the amount available. These fractions are highlighted in the schemes.

Figure 4.1: Extraction and isolation scheme of polysaccharides fractions from *O. celtidifolia* (Oc) and *C. cordifolia* (Cc)

Polysaccharides isolated from *O. celtidifolia* (Oc50A₁ and Oc50A₂) are highly soluble in water and have no tendency to form gels after dissolution. These properties are associated with highly branched structures (Bacic et al., 1996). The polymers were polydisperse and composed each of two polymers of different molecular weight as shown by SEC-MALLS analysis; one high molecular weight polysaccharide for each fraction of 160 and 220 kDa respectively, and smaller polymers with an average weight of 37 and 46 respectively in Oc50A₁ and Oc50A₂. Studies are in progress to isolate and determine the properties, both chemically and biologically, of both the high and low molecular weight polymers.
The results of the performed structural studies presented in paper III indicated the presence of glycosidic linkages characteristic of AGII type polymers in Oc50A1, Oc50A1d1, Oc50A2 and Oc50A2d1 (table 4.1) as major polysaccharide based on the proportion of the monosaccharides component of this structure. AGII usually occur as a complex proteoglycans or glycoproteins depending on the relative proportion of carbohydrate and protein (Gaspar et al., 2001). Or they occur as polysaccharides linked to the complex pectin structure through the rhamnose units in the pectin backbone. Oc50A1d1 fulfil the criteria that are generally set to define arabinogalactan-proteins (Paulsen & Barsett, 2005; Van Holst & Clarke, 1985), these are related to a high content of Ara and Gal (25% and 39% of the total carbohydrates respectively), the positive reaction with the Yariv β-glucosyl reagent, the 3-linked galactan backbone heavily branched with 6-linked side chains having terminal and 5-linked Ara residues linked to O-3 of the galactose units of the side chains; and the presence of hydroxy-rich proteins as well. Oc50A2d1 also presented the same criteria with lower amount of arabinose and galactose (14 and 38.5% respectively). These characteristics were described for the AGII polysaccharides isolated from the leaves of Plantago major and the root of Vernonia kotschyan (Nergard et al., 2004; Samuelsen et al., 1998). The protein portion of AGP is usually less than 10% (Gane, 1995), Oc50A1 and Oc50A2 contained 1.4 and 0.9% of protein respectively. Hydroxyproline (33%) was identified as the major amino acid in these protein polymers followed by alanine (26%), serine (12%) and threonine (9%). Samuelsen et al. (1998) identified 29% hydroxyproline, 15% alanine and 11% serine as major amino acids in Plantago major AGII.

Oc50A1d1 and Oc50A2d1, the high molecular weight fraction isolated after enzymatic treatment and gel filtration, also contained low proportion of 4-linked GalA and 2-linked Rha residues (table 4.1); the latest showed branching at O-3 of the rhamnose units. These might indicate that pectins are present as well, either covalently linked to or associated to the arabinogalactan. Up to now, it is still unclear whether AGII is part of the pectin complex or belong to an AGP or both. The two polysaccharides often seem to co-extract, and are subsequently difficult to separate from each other. These observations fuel speculations that the two are covalently linked (Vincken et al., 2003a).
Oc50A1d2 and Oc50A2d2, the low molecular weight fractions obtained after enzymatic treatment, contained higher proportion of rhamnose (10 and 20% respectively) and galacturonic acid (12 and 22% respectively). The rhamnose residues were 2-linked with branching point at position 4 for Oc50A1d2. Although these fractions also contained high amount of arabinose and galactose, they did not precipitate the Yariv reagent. Even though not all AGII structures precipitate with the Yariv-reagent (Yamada & Kiyohara, 1999), this property is one easy method to distinguish the AGII structure from AGI (Van Holst & Clarke, 1985) but needs to be combined with other structure determinations. These two fractions are suspected to contain be typical pectins with AGI side chains (Carpita & McCann, 2000) based on the methylation results.

In addition to the indicated presence of AGII and RGI structure component monosaccharides, 4- and 4,6-linked glucose was also present in all fractions; this might be associated to the terminally linked xylose in a xyloglucan structure. This type of polymer is present as major building material of primary cell wall of higher plants (Ebringerova et al., 2005). Based on the proportion of xylose and glucose in our fractions this type of polymer if it is present is in minor amounts.

Polysaccharide Cc50-1 was isolated as shown in figure 4.1 from Cc50, the water 50 °C extract of the bark of C. cordifolia (figure 4.1). In addition to its uses as wound healing agent, and it previous complement fixing ability, our interest in studying this extract was motivated by its apparent high viscosity. In addition to their biological properties, polysaccharides also present physical properties that are used in food industries. Most food polysaccharides are classified as water soluble polysaccharides either naturally present or purposely added to food systems to control functional properties and provide desired textures of food products. Their most functional properties are their water binding capacity and enhancing viscosity (Wang & W., 2005). The intrinsic viscosity of Cc50-1 was estimated; this measure the hydrodynamic volume occupied by the isolated polymer chain in a given solvent and depends primarily on the molecular structure (linear vs branched, rigid vs flexible) and molecular weight of polysaccharides as well as on the solvent quality (Wang & W., 2005). The polymer was found to form aggregated in water.
Table 4.1: Monosaccharides composition and linkages in isolated polysaccharides fractions from *O. celtidifolia* and *C. cordifolia*

<table>
<thead>
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<th>Monosaccharides</th>
<th>Deduced Linkages</th>
<th>Cc Cc50-1</th>
<th>Oc Oc50A1</th>
<th>Oc Oc50A2</th>
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</tr>
<tr>
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<td>4</td>
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Pectin is probably the most complex macromolecule in nature, because it can be composed as many as 20 different monosaccharides including their isomers (Vincken et al., 2003b). Rather than making all possible combinations, the nature provided a number of distinct polysaccharides, which together form pectins. Although the primary based pectin structure are somewhat largely elucidate, structural particularities based on the origin will still make differences between these types of polysaccharides. The structure of RGI carrying arabinan, galactan and arabinogalactan side chains appear to be the general figure which describes the pectin, and most of the isolated structures seem to fit in this structural description; but inside the general pattern, pectins present differences related to the type of monosaccharides, or their inter-molecular linkages.

Cc50-1 is a relatively polydisperse polysaccharide with a molecular weight about 2000 kDa. It most probably fit in the general definition of pectins but presents a particular monosaccharide composition not reported before. Preliminary structure characterisation indicates that the backbone of Cc50-1 is composed of a 4-linked GalA and 2-linked Rha. The latest carrying side chains at position 3 and 4 on different Rha units in almost equal proportion. Based on the results of periodate oxidation, GalA residues are also suspected to carry side chains, this is most likely to occur at position 3 (Tomoda & Satoh, 1977). The almost equal proportion of Rha and GalA (20 and 24% respectively) indicates the absence of homogalacturonan HGA region. This described structure resembles that of pectins, but contains either lesser galacturonan moieties than pectins or no galacturonan moiety at all. HGA is chains of 4-linked α-galacturonic acid, which may be esterified. IR analysis showed no esterification of Cc50-1. So far pectic substances from plant cell walls were always found to consist of smooth galacturonan regions interrupted by blocks of ramified rhamnogalacturonan regions, so-called hairy regions. The absence of homogalacturonan implies the inability of the pectic substance to participate in calcium cross-linking (Vincken et al., 2003b), which is the mechanism behind the gel formation property of pectin. The application areas of these gels are mainly in food industries. Jams, jellies etc, are some of the main uses of industrial pectin (Thibault & Ralet, 2003) and Cc50-1 is most likely unsuitable for this purpose.

In addition to the suspected pectin backbone, Cc50-1 contains unusual monomers (table 4.1); 20% of terminally-linked 2-O-methyl galactose was identified. Based on the literature research, natively methylated galactose is a rare component of pectic polysaccharides from medicinal
plants; to our knowledge, quantities as high as 20% of this monomer is here identified for the first time in higher plant polysaccharides. 3% of 3-\text{O}-methyl galactose was identified in water soluble polysaccharides from the medicinal plant \textit{Salvia officinalis}; these polysaccharides were arabinogalactans associated with galacturonan and/or rhamnogalacturonan backbone (Capek & Hribalova, 2004). Natively methylated galactose mostly occurs naturally in algae polysaccharides instead. 2-\text{O}-methyl, 3-\text{O}-methyl and 6-\text{O}-methyl galactose have been identified as component of \textit{Grateloupia} and \textit{Rhodophyta} species (Miller, 2005; Usov \textit{et al.}, 1997; Watt \textit{et al.}, 2002).

Cc50-1 also contained terminally linked 2-\text{O}-methyl fucose, this occurs as a monosaccharide component of RGII structures (Stasser & R., 2002), the search for such type of structure in Cc50-1 gave negative results (data not shown). No report was found in the literature that connects this monosaccharide to structurally defined RGI polysaccharides, but a polymer with galacturonosyl-2-rhamnose backbone was isolated from the apple fruit and was reported to contain 2-\text{O}-Me Fuc (Barret & Northcote, 1964).

Cc50-1 also contained 18% of terminally linked 4-\text{O}-methyl GlcA. This monomer is usually associated with xylose in glucuronoxylan type polysaccharides. Most of those have a single 4-\text{O}-methyl-glucuronic acid residue attached at position 2 of the main xylose chain. The glucuronic side chain may be present in both methylated and non-methylated forms (Ebringerova \textit{et al.}, 2005). Xylose was not identified as component of the Cc50-1 polysaccharide; the methylated glucuronic acid must then be associated to the pectin structure. This monosaccharide was also found in smaller proportion (3-5\%) associated with pectin structures of \textit{Glinus oppositifolius} (Inngjerdingen \textit{et al.}, 2007). It is also present (about 4.5\%) in polysaccharides isolated from \textit{O. celtidifolia} (paper III). Complete structural elucidation of Cc50-1 is in progress.
4.2.2. Low molecular weight compounds

A new pterocarpan erybraedin F and six known ones, erybraedin A, C, D, eryvarin K, phaseollin and shinpterocarpin were isolated from the root bark DCM extract in addition to a flavone, carpachromene isolated from the stem bark DCM extract of *E. senegalensis* (figure 4.2).

![Chemical structures of compounds](image)

**Figure 4.2: Structure of compounds isolated from Erythrina senegalensis**
Pterocarpan is derived from the basic structure of isoflavonoids, however, the systematic numbering presented in erybraedin F is used rather than that of simple isoflavonoids. For convenience, pterocarps are divided in pterocarps, 6a-hydroxypterocarps and pterocarpenes (Dewick, 1988). These isolated compounds belong to the subclass of pterocarpan. Most pterocarps have oxygenated substituents in the aromatic position C3, C9 and/or C8. Some others have and isoprene derived substituent next to an oxygenated aromatic position. Based on this, pterocarpan are considered as “simple” or “complex”. In simple pterocarps only short chain substituent are present on the usual tetracyclic system, and complex pterocarps carry cyclic or acyclic substituents of a higher complexity such as isopentenyl, modified furans, gem-dimethyl-chromenes, etc. (Jimenez-Gonzalez et al., 2007). Based on this classification, all the isolated pterocarpan here described are considered as complex. They all carry prenyl chain of different complexity. These pterocarps and carpachromene, although being reported before from the genus Erythrina, are for the first time isolated in the species E. senegalensis.

4.3. Biological Activities

4.3.1. Polysaccharides

Polysaccharides isolated from O. celtidifolia have shown high dose dependant activity in the complement system. The ICH50 values ranged between 0.2 to 4.4 µg/ml largely exceeding the activity of PMII as the positive control (ICH50 = 70.5µg/ml). The higher molecular weight fractions that contained AGII, isolated after polygalacturonanase digestion (Oc50A1d1 and Oc50A2d1) showed higher complement fixing activities than the original fractions and the lower molecular weight fractions (Oc50A1d2 and Oc50A2d2) had lower activity. This is consistent with the structure-complement fixing activity relationship described so far for highly branched polysaccharides. The ramified region of complement fixing pectin is proposed to be the active sites for expression of this activity.

Most of the complement fixing arabinogalactans isolated from medicinal herbs are characterized as AGII. As many complement fixing pectic polysaccharides contain AGII side chains, it is postulated that these carbohydrates are also responsible for expression of the activity (Yamada &
Kiyohara, 1999). These factors explain at the same time the higher activities of Oc50A$_{1d1}$ and Oc50A$_{3d1}$ and the lower activities of Oc50A$_{1d2}$ and Oc50A$_{3d2}$ than their original molecules.

The fractions have also shown macrophage stimulation properties as they release nitric oxide (NO). This was also in a dose dependant manner. At 100$\mu$g/ml the original fractions (Oc50A$_{1}$ and Oc50A$_{2}$) released 7.2 and 7.3$\mu$M of NO respectively. Contrary to the complement fixing ability, the lower molecular weight fractions showed slightly higher activity than the high molecular weight ones. The amount of NO released ranged between 6.3 to 7.1$\mu$M. This slight difference might give an indication that the complement fixing activity is not expressed by the same active site as the macrophages activation activity. This result was confirmed after arabinofuranosidase treatment which showed that terminal linked arabinofuranosidase are not important for the complement fixing ability, this result was reported before in the literature (Nergard et al., 2005a).

In the opposite, they play an important role in macrophages activation activity; for this reason, this activity was considerably decreased after removal of the terminally linked arabinose units. As reported above, AGII polysaccharides are known as having complement fixing ability, but not all AGII have macrophage activating properties. The AGII isolated from Vernonia kotschyana have shown a high complement fixing ability, but were unable to activate macrophages to release NO (Nergard et al., 2005b)

Preliminary results on the abilities of O. celtidifolia to activate other immunological system are presented in section 3.2. The polysaccharide fractions showed the abilities to activated T cells through activation of dendritic cells, and also demonstrated moderate B cell proliferation activities. Investigations on the link between these biological activities and the structure of the purified polysaccharides are on going.

Polysaccharide Cc50-1 showed moderate activity in the complement system (ICH$_{50} = 480\mu$g/ml) compared to O. celtidifolia polysaccharide fractions. The crude extract which generate Cc50 showed four to five fold stronger activity (ICH$_{50} = 100 \mu$g/ml). Viscosity measurement results showed that Cc50 forms aggregates with water like solvents, the fact that a fully dissolution could not be obtained using the water-based buffer used to dissolve the sample in the complement assay might also have affected the activity. But a most probable explanation of this low activity could lie in the fact that the structural feature of Cc50-1 does not resemble that of any complement
fixing polysaccharide reported so far based on its monosaccharides composition. Other components present in the crude extract might be responsible of the higher activity of this extract.

4.3.2. Low molecular weight compounds

The pterocarps erybraedin A, C, D, and eryvarin K, and carpachromene were isolated from the root and stem bark DCM extract of *E. senegalensis*. All these compounds were 15-lipoxygenase inhibitors, the IC$_{50}$ values range from 32 to 82 µM. Erybraedin D demonstrated the strongest activity and erybraedin C had similar activity as quercetin (IC$_{50}$ = 41 and 42 respectively). The enzyme inhibition activity was specific at least for the case of erybraedin C because this compound showed no activity on the adenosine deaminase enzyme. This is a new property reported for these compounds. From the literature they are known as antibacterial compounds, but their ability to inhibit lipoxygenase enzyme was not reported.

The possibility that oxidative modification of low-density lipoproteins (LDL) might be a key step in the genesis of the atherosclerosclerotic lesion was suggested long time ago. 15-LO has been implicated in oxidation of LDL. This process is believed to be important for the development of atherosclerosis. In principle, any enzyme system that generates free radicals could contribute to LDL oxidation; in vitro studies have identified a long list of candidates, including NADPH oxidase, myeloperoxidase, P450, the mitochondrial electron transport system, xanthine oxidase, and lipoxygenases (Steinberg, 1999). Today, there is direct and specific evidence for a role of lipoxygenases in atherogenesis (Cyrus et al., 1999). Inhibition of soybean 15-LO is generally regarded as predictive for inhibition of the mammalian enzyme.

The DCM root bark extract and all the isolated pterocarpan showed a positive antibacterial activity against *S. aureus*, in a TLC bioautography test or disc diffusion test. Erybraedin A also showed an inhibition of *Candida albicans*. The antibacterial activity of erybraedin A, C and D was reported before in the literature (Mitscher et al., 1988; Nkengfack et al., 1995; Rukachaisirikul et al., 2007; Sato et al., 2004; Wanjala et al., 2002). All these 3 compound have shown antibacterial activity against *S. aureus* with an minimal inhibit concentration (MIC) of 13.6, 12.6, 78.3 µg/ml respectively (Nkengfack et al., 1995). Erybraedin A showed a strong antifungal activity against yeast spores (Wanjala et al., 2002), a high growth inhibitory potency
against vancomycin resistant enterococci (VRE) with a MIC value of 1.56-3.13 μg/ml; and against multiresistant Staphylococcus aureus (MRSA) at 3.13-6.25 μg/ml. These antibacterial activities were based on bacteriostatic action. The authors also showed that the combination of erybraedin A and vancomycin acts either synergistically or additively against VRE and MRSA; the fractional inhibitory concentration index against VRE ranged from 0.5 to 1 μg and against MRSA, from 0.5 to 0.7 μg (Sato et al., 2004).

The compounds were not tested for DPPH scavenging activity as the activities of the crude extracts were low.
5. Concluding Remark

The results presented in this thesis contribute to the knowledge of ethnopharmacological uses, structures of immunomodulatory polysaccharides and biological activities of low molecular weight compounds from Malian medicinal plants.

From the ethnopharmacological information, the large and extended use of plants in Mali was noticed. Several diseases were reported against which the plants investigated were used; the fact that there is few scientific available data to support these activities has also been noticed. It can be assumed that not all the reported activities will be scientifically demonstrated; in this case, the reason why traditional healers use these plants will always be a subject of discussion, but most of the time, the most reported diseases find scientific explanations in laboratories. The presence of immunomodulating polysaccharides in Malian medicinal plant can at least be related to their medicinal effects reported by traditional healers.

Among immunomodulating pectins there are differences in potencies expressed by individual pectins, Oc50A1 and Oc50A2 have a strong effect on the complement system and macrophages, they also showed moderate T cell and B cell activation properties. The mechanism of these effects has not been investigated in our studies and little is also known about that in literature. The determination of the refine structures of these polysaccharides will most probably give several hypotheses about their active site. Structural evaluation of immunomodulatory pectins always suggested that the ramified regions are greatly implicated in the expression of these activities. The structural feature of Cc50-1 will bring an additional specificity of this statement that not only side chains linked to RG-1 structure are important, but the nature (monosaccharides composition and length) of these side chains is also important for at least the complement fixing activity, since Cc50-1 is only composed of ramified region and has weak activity in this system.

The isolation of antibacterial and 15-LO inhibitor compounds from *E. senegalensis* could not be directly predicted from the results of our ethnopharmacological surveys, since the uses against infectious diseases were not the mostly reported use, but the combination of literature research was found necessary. These compounds have certainly other health implications that need to be investigated in further studies.
6. Methodology

6.1. Extraction and isolation procedures

6.1.1. Polysaccharides

The plant materials were first extracted with organic solvents (dichloromethane, chloroform, ethanol and methanol) using Soxhlet to remove lipophilic and low molecular weight compounds. Each extract was kept separately for further studies (paper IV). The residuum was air dried at room temperature and extracted with water at 50 and 100 °C for isolation of polysaccharides. The water extracts were dialysed prior to further purification by chromatographic methods. Due to the presence of coloured compounds, the crude water extract was fractionated on Biogel P6 and eluted with water to obtain less coloured material which mainly was of lower molecular weight (Paper III). Polysaccharide fractions were isolated by anion exchange chromatography, eluted with a gradient of sodium chloride solution in water (paper III, IV). When a refined peak was not obtained, further fractionation was performed by gel filtration on Sephacry S-400 (Pharmacia) (chapter 3.2.).

6.1.2. Low molecular weigh compounds

The plant material was extracted with dichloromethane using Soxhlet for several hours and the extract was dried under vacuum. Fractionation was performed by flash chromatography using a VersaPak™ C18 Cartridge column (40 x 150 mm), follow by a separation on a Chromatotron with Silica gel 60 PF254 Gipshaltig (Merk) as stationary phase and by preparative HPLC on a ProStar Polaris instrument with a Microsorb 60-8 C18 (21.4 x 250 mm) column. TLC analysis were performed on Silica gel 60 F254 (Merck) aluminium plates for fractions determination; after elution the plates were sprayed with ceric reagent [Ce(SO₄)₂ 1% in 10% aqueous H₂SO₄] and to 100°C for 5 min (paper V)

6.2. Quantification of fraction components

6.2.1 Polysaccharides

The presence of carbohydrates in fractions was monitored by the phenol-sulphuric acid test. This is a colorimetric determination of sugars, their methyl derivatives, oligosaccharides and
polysaccharides. The method is simple, rapid, and sensitive, and gives reproducible results. The colour produced is permanent and the method does not required special conditions (Dubois et al., 1956). GC analysis was performed after methanolysis to identify the monosaccharides and their quantity. The samples were hydrolysed with 4M HCl in methanol at 80°C for 24h followed by trimethylsilylation and GC analysis. Identification and quantification of the monosaccharides were performed on the basis of standards and mannitol as an internal standard (paper III and IV).

6.2.2. Proteins

The protein contents of the acidic fractions were determined as described by Lowry (Lowry et al., 1951) using bovine serum albumin (BSA) as standard. The amino acid composition was determined using a Biocal JC 5000 automatic amino acid analyser after hydrolysis of the samples. Protein determination by the method of Lowry and co-workers was used because of its simplicity, sensitivity, and precision. Other substances presents in the fractions might interfere with this measurement. The most effective and generally used methods involve isolation of the protein by acid precipitation which removes all the interfering substances. It is also know that most of the interfering substances are of low molecular weight, the extraction and isolation process of the polysaccharide fractions tested by this method might remove some of these interfering compounds. For the purpose of this study the method described by Lowry and co workers was found suitable.

6.2.3. Phenols

The total amount of phenolic compounds of the acidic fractions was determined according to the Folin-Ciocalteu (FC) assay (Singleton & Rossi, 1965), using ferulic acid was used as standard (paper III and IV). The total phenolic content was determined as ferulic acid equivalents. The FC method is always referred to as a determination of total phenolic content. During the reaction, electron transfer (oxidation-reduction) takes place between a phenolic or an oxidized substance and the phosphomolybdic phosphotungstic acid complex (the FC reagent). The reduction of the latest gives formation of a blue complex that can be detected at 765nm. The total fraction is used for this measurement; products like lipids might interfere with the results. The FC method does
not detect only phenols; the reagent can be reduced by other oxidized substances like ascorbic or uric acid.

6.3. Structure analysis

6.3.1. Reduction of uronic acid

In this method carboxylic esters are reduced with sodium borodeuteride to generate 6,6'-dideuterio-sugars, which can then be distinguished from neutral sugars by GC-MS by the presence of fragment with $M^+ + 2$ in masses compared to fragments obtained from native neutral monosaccharides. Sample are dissolved in imidazole buffer, and reduced with sodium borodeuteride. After neutralisation and dialysis, the polysaccharides are dissolved in 2-(N-morpholino) ethane sulphonic acid (MES) buffer (Ph 4.75). 2M Tris (hydroxyl methyl amino-methane) pH 9.0 is added and the activated uronic acids are reduced with sodium borodeuteride (Kim & Carpita, 1992). Dialysis was required for removal of salts; therefore this method is not suited for oligosaccharide analysis (paper III and IV).

6.3.2. Methylation and Ethylation

In this method, the free hydroxyl groups in the carbohydrates are de-protonated and methylated. Then the glycosidic linkages are hydrolysed, the partially methylated monosaccharides are reduced to alditols and those hydroxyl groups originally involved in linkages are finally acetylated (paper III and IV).

The deprotonation of the carbohydrate hydroxyl groups are performed using a suspension of sodium hydroxide in DMSO for the formation of the sodium methyl sulphinyl carbanion. Polysaccharides are chemically methylated or ethylated with excess of methyl iodide or ethyl iodide at every free hydroxyl group (Carpita & McCann, 2000; McConville et al., 1990). The ethylation follows the same procedure as the methylation except methyl iodide is replaced by ethyl iodide. The carbon atoms that participate in glycosidic linkages and in the rings not having free hydroxyl group are not methylated. Excess of reagent and by-products are removed from the solution by extraction with chloroform and sodium thiosulphate in water (McConville et al., 1990).
The methylated polysaccharides are hydrolyzed into their components of partly methylated monosaccharides. Hydrolysis was performed using trifluoroacetic acid (TFA); the use of TFA is preferred because the acid remaining can be eliminated by volatilisation. These resulting components are reduced and acetylated. However, because linkage determination of certain derivatives depends on the ability to differentiate the top of the molecule from its bottom, the reducing agent borodeuteride (sodium borodeuteride in ammonia) was used to label C-1. Introduction of deuterium in C-1 made differentiation between primary hydroxyl groups on C-1 and C6 possible after GC-MS. The partly methylated/ethylated alditols are then acetylated using ethyl acetate and acetic anhydride with perchloric acid as catalyst. An acidic catalyse is preferred because acid breaks the borate-alditols complex that tends to form during the reduction step. After acetylation, the conversion of excess acetic anhydre into acetic acid was catalysed with 1-methylimidazole (Harris et al., 1984). Finally, the methylated or ethylated alditol acetates were extracted into chloroform, dissolved in methanol after evaporation of chloroform and analysed by GC-MS.

6.3.3. GC and GC-MS

Analysis of sugar monomers in complex a matrix was performed using mass spectrometry (MS) after prior on-line gas chromatography (GC) separation (paper III and IV). Although current instrument can perform both electron impact (EI) and chemical ionisation (CI) with both positive and negative ion detection capabilities, most GC-MS analyses of sugars are still performed with EI in positive detection mode. These instruments are simple to operate and maintain, and are run by windows-based PCs (Fox, 2002). For GC analysis, generally neutral and amino sugars are converted to trimethylsilyl or acetate derivatives, often after destruction of the anomeric center. Interpretation of chromatograms, thus, becomes simpler and quantification more easer. However, leaving out this anomeric destruction step makes for a simpler sample preparation.

6.3.4. NMR, MS

$^1$H-NMR and $^{13}$C-NMR spectroscopy were performed for structure elucidation of low molecular weight compounds. The spectra were recorded in deuterated chloroform and methanol and acetone on a Varian Gemini-200 instrument (Palo Atta, USA) at 200MHz and 50MHz respectively. EI-MS was accomplished on a micromass Prospeq Q instrument (paper V).
6.3.5. Enzymatic degradation

Polygalacturonase (PG) hydrolyses α-4-linkage between two adjacent galacturonic acid residues within the pectin backbone. As such, this is a key enzyme responsible for depolymerisation of pectin. The exact substrate requirement for PG action is still subject to debate, but it is generally acknowledged that the enzyme will cleave only between de-esterified galacturonic acid residues. This raises the possibility of synergy between PG and pectin esterase (PE) enzyme in degradation of pectin, the action of pectin esterase generating blocks of de-esterified galacturonic acid that in turn act as sites for PG action. PG normally has a pH optimum in the acid range (pH 4-6) and can be either exo- or endo-acting (Tucker & Seymour, 2002). The polysaccharide fractions from *O. celtidifolia* were degraded using endo-PG, this was not used in combination with PE but a solution of NaOH was used as de-esterification agent for 2 hours at room temperature.

6.3.6. Precipitation with Yariv β-glucosyl reagent

The amount of Arabinogalactan-protein in a plant extract can be quantified by single radial diffusion in agarose gels containing a dye known as the β-glucosyl-Yariv reagent [1,3,5-tri-(β-glucosyloxy-phenylazo)-2,4,6-trihydroxybenzène] which specifically interacts with and precipitates arabinogalactan-proteins. The reagent forms a coloured precipitate with AGII structures. The reaction was performed by radial diffusion in an agar gel. There is a linear relationship between the amount of AGII in the sample and the area of the coloured precipitation in the gel (Van Holst & Clarke, 1985). The mechanism of the reaction has yet not been established. Therefore other methods must be performed to confirm the presence of arabinogalactans; this was done by linkage study after methylation and GC-MS analysis.

6.3.7. Periodate oxidation

Polysaccharides have the potential to react with oxidation reagents, and this could be used to elucidate structural information. Vincinal-glycols can react with periodic acid or its salts to form two aldehyde groups upon the cleavage of the carbon chain. The reactions quantitatively consume molar equivalents of periodate and form formic acid. A quantitative determination of periodate consumed and formic acid formed combined with the information on the sugar units surviving the oxidation reactions provide clues to the nature of the glycosidic linkages and other structural
features of the polysaccharide. The periodate concentration can be measure by titrimetric or by spectrophotometric methods. Formic acid can be determined by direct titration with standard alkali or indirectly by the liberation of iodine from a solution of potassium iodide and iodate (Cui, 2005) (paper IV).

6.4. Estimation of molecular weight distribution

The average molecular weights of the polysaccharides were determined using SEC-MALLs analysis which was performed at the University of Nottingham, United Kingdoms (paper III). The measurement of the angular dependence of the total intensity of light scattered by solutions of polysaccharides provides a direct and absolute way of measuring their weight average molecular weight. The combined effect of the SEC column can provide clear fractionated samples to the light scattering cell, facilitating measurement of molecular weight (Harding, 2005). For molecular weight determination in our laboratory, SEC chromatography coupled with refractive index (RI) detection using dextrans of different molecular weights as calibration standards was performed. The latest gives an approximate average molecular weight due to the fact that the conformation of the investigated polysaccharides could be different from that of dextran which is mainly composed of 1-6 liked glucose units with very few ramifications; while pectic type polysaccharide are usually heavily substituted compounds. A better procedure would be a confirmation of the molecular size using other methods such us SEC-MALLS or sedimentation equilibrium.

6.5. Viscosity measurement

The viscosity of a polymer solution is directly related to the relative size and shape of the polymer molecules. Viscometry is also one of the most widely used methods for the characterization of polysaccharide molecular weight because it requires minimal instrumentation. The experiments can be easily carried out and data interpretation is simple. This simply requires the measurement of relative viscosities and polymer concentrations for a series of dilute solution. Intrinsic viscosity can lead to the calculation of molecular weight, a calibration with a series of known molecular weight polymers (ideally monodisperse substances) is then used for the Mark-Houwink constants determination (Wang & Cui, 2005) (paper IV).
6.6. Biological activities

6.6.1. Complement fixing test

In this test, samples were dissolved in veronal buffer and incubated with serum containing intact complement proteins for activation or inhibition of the complement cascade. Prior to addition, the serum was diluted to a concentration giving about 50% lysis for ensuring optimal sensitivity of the test system. Following incubation, indicator cells which were sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibodies were added. Thus, indicator cells were immune complexes that activated the remaining complement proteins, the indicator cells were lysed to some extents. The anti complementary activity of the sample could be determined from the degree of haemolysis relative to that of the medium alone (Michaelsen et al., 2000). The validity of a test was evaluated using the positive control PMII of known complement fixing activity (Samuelsen et al., 1996). In this test a general consumption of complement is observed, this might be due to either activation or inhibition of the complement system. Therefore the test can not differentiate between the two (paper III and IV).

6.6.2. Macrophage activation

Polysaccharides were tested for stimulatory activity on rat macrophages. The activation was determined as release of nitric oxide, which is quickly broken down to nitrite. Macrophages were plated at a density of 0.5x10^6 cells/ml in 96-well flat bottomed plates and incubated in medium alone, the positive control LPS, or medium containing various concentration of polysaccharide fraction. The supernatant was then harvested and the amount of nitric oxide (NO) released by macrophages was quantified by measuring the amount of nitrite (NO\textsubscript{2}^-), a stable breakdown product of NO. NaNO\textsubscript{2} titration curves were used as a standard (paper III) (Promega, 2005).

6.6.3. Antimicrobial activities

Antibacterial activity was determined by the disc diffusion method (Rukachaisirikul et al., 2007). Disk diffusion methods comprise the placing of filter paper disks containing test compound on agar plate surfaces previously inoculated with the test organism. The chemical then diffuse into the agar and inhibits the growth of the organism. The crude extract and isolated compounds were
tested against *Staphylococcus aureus* (ATCC 25923 Culti-loops®). Bacterial suspension (10⁶ to 10⁷ CFU (colony forming unit)/ml) was spread on Muller Hilton agar plates. Sterile filter paper discs (6mm in diameter) were impregnated with test samples and placed on the inoculated plates. After incubation for 24h inhibition zones were measured. Gentamicin was used as positive control.

The activity against *Candida albicans* (ATCC 10231 Culti-loops®) was tested by bioautography on thin-layer plates. This method is described as quick and versatile for detection of antifungal activity in natural compounds. It allows the identification of active compounds in crude extracts (Hadacek & Greger, 2000). Test samples were applied as small spots on TLC glass plates (Kieselgel 60 F₂₅₄, Merck). The TLC plate was homogeneously sprayed with yeast extract agar (Merck) broth containing *C. albicans* conidia (approx. 10⁵ CFU/ml). The plate was incubated for 24h in a moist chamber at 37°C. At the end of incubation, the plate was sprayed with the Cell Proliferation Kit (MTT- Roche) solution. The inhibition zones were measured (Paper V).

### 6.6.4 Inhibition of 15-Lipoxygenase

Lipoxygenase activity was measured in borate buffer solution (0.2 M pH 9.0) by the increase in absorbance at 234 nm from 30 to 90 s after addition of the enzyme, using linoleic acid as substrate. DMSO alone was added as uninhibited control in control experiment and test substances were added as DMSO solutions. To ensure constant enzyme activity throughout the experiment, the enzyme solution was kept on ice, and controls were measured at regular intervals. Quercetin was used as positive control. Calculation of enzyme activity was carried out as previously described (Lyckander & Malterud, 1992) and IC₅₀ values were determined by linear interpolation between the measuring points closest to 50% activity. Values are expressed as means ± SD. Student’s *t*-test was employed for determination of statistical significance using *P* values of 0.05 or less as a criterion for significant inhibition (Paper V).

### 6.6.5. Toxicity on Brine shrimp larvae

Brine shrimp Artemia eggs (Novo Temia, Germany) were incubated in salt water (Aqua-Biosal) in a Petri dish divided into compartments that allowed larvae to swim from one side to another. The compartment with the eggs was covered in order to keep the eggs in a dark environment. The next compartment was illuminated in order to attract shrimps. After 48h, the phototropic shrimp
were collected by pipette and incubated with test samples dissolved in DMSO under illumination for 24h at room temperature. At the end of incubation, survivors were counted and the mortality percentage was determined in relation to the number of larvae killed by the samples and the total number of larvae (Lima et al., 2002) (Paper V).
References


Annexe: About Mali

- Motto: "Un peuple, un but, une foi" "One people, one goal, one faith"
- Anthem: Pour l'Afrique et pour toi, Mali "For Africa and for You, Mali"
- Capital (and largest city): Bamako
- Official language French
- Area 1,240,192 km² (the world 24th biggest country), 1.6% of the land is covered by water
- Population (July 2005 estimate) 13,518,000 (world’s 65th largest population), density: 11/km²
- GDP (2005 estimate) Total: $14.400 billion, Per capita: $1,154

Mali, officially the Republic of Mali, is a landlocked nation in Western Africa. It is the seventh largest country in Africa. It borders Algeria on the north, Niger on the East, Burkina Faso and Ivory Coast on the South, Guinea on the South-West, and Senegal and Mauritania on the West. Its straight border on the north stretches into the centre of the Sahara, while the country's South, where the majority of inhabitants live, features the Niger and Senegal rivers. Formerly French Sudan, the country is now named after the Mali Empire. The name of the country comes from the Bambara (national language) word for hippopotamus, the name of its capital city, Bamako comes from the Bambara word meaning "crocodile swamp". Mali is divided into eight regions and one district (capital). The regions and district are: Gao, Kayes, Kidal, Koulikoro, Mopti, Ségou, Sikasso, Tombouctou (Timbuktu) and Bamako (capital district)

- History of Mali
The Mandé peoples settled in the Sahel (including present-day Mali), and formed a succession of Sahelian kingdoms, including the Ghana Empire, the Mali Empire, and the Songhai Empire. Mali's early history is dominated by these three famed West African empires. Timbuktu was a key city in these empires as an outpost for trans-Saharan trade and a center for scholarship.

**Early History 700 - 1591**

The Ghana, Mali or Malinké, and Songhai empires arose in the area then known as the western Sudan, a vast region of savanna between the Sahara Desert to the North and the tropical rain forests along the Guinean coast to the South. All were characterized by strong leadership and kingdom-based societies. None had rigid geopolitical boundaries or ethnic identities.

The Ghana Empire began possibly as early as the fifth century, and was a powerful trading state between 700 and 1075 dominated by the Soninke people. From its capital in Kumbi Saleh (situated in the actual Maritania) on the edge of the desert, the empire expanded throughout South-Eastern (actual) Mauritania, South-Western (actual) Mali, and Northern (actual) Senegal. The Soninké kings never fully adopted Islam, but the empire had good relations with Muslim traders. Nevertheless, the Ghana Empire fell in 1078 as a result of invasions by the Almoravids, nomadic Muslim Berbers who expanded and spread Islam throughout North-West Africa in the late eleventh century. Kumbi Saleh was destroyed in 1203 by a former vassal state, the anti-Muslim Sosso Kingdom, which ultimately controlled the southern portions of the former Ghana Empire.

The Mali Empire began with the Kingdom of Mali on the upper Niger River in the 11th century, expanding rapidly in the 13th century under the leadership of the King Sundiata Keita. Sundiata led a revolt against the Soso king and then unified a vast region of the Western Sudan into the Mali Empire. It reached its height about 1325, when it conquered Timbuktu and Gao and extended over a large area centered in the upper Niger and encompassed numerous vassal kingdoms and provinces. Under the Mali Empire, the ancient trading cities of Djenné and Tombouctou (often seen as Timbuktu) were centers of both trade and Islamic learning. The empire declined as a result of court intrigue and disputes over the succession. Vassal provinces revolted in the late fourteenth century, and the Songhai Empire ultimately supplanted the Mali Empire in the fifteenth century.

In the late fourteenth century, the Songhai gradually gained independence from the Mali Empire and expanded its borders, ultimately submerging the entire Eastern part of the Mali Empire from its center in Gao during the period 1465-1530. Tombouctou and Djenné prospered once again, as the rulers actively promoted Islam. The empire eventually collapsed as a result of both internal and external pressures, including a Moroccan invasion in 1591. The fall of the Songhai Empire marked the end of the region’s role as a trading crossroads. Following the establishment of sea routes by the European powers, the trans-Saharan trade routes lost their significance.

**After the Empires, 1591 – to now**

After the collapse of the Songhai Empire, no single state controlled the region. Several small successor kingdoms arose. Among the most notable that were located in what is now Mali were: the Bambara empire or Kingdom of Segu, the Kingdom of Kaarta, the Kenedougou Kingdom, the...
The Ancient Mali was located in one of the world’s most lucrative trade routes, the so-called trans-Saharan trade routes, Timbuktu was the trade center. Camels would carry salt from the Northern mines in the desert (middle-east) to be traded for gold and other goods such as kola nuts and grain from the Southern part of the Mali Empire. But nowadays, Mali is one of the poorest countries in the world. The economic performance is fragile, characterized by a vulnerability to climatic conditions, fluctuating terms of trade, dependence on ports in neighboring countries. Mali produces cotton, cereals and rice. Although locally produced rice now provides competition to imported Asian rice, Mali's primary export is cotton. Livestock export and industry (producing vegetable and cottonseed oils, and textiles) have experienced growth. Although most of Mali is desert or semi-desert, the Niger River is a potential irrigation source. Exports are in three primary sector products (56% gold, 27% cotton, 5% livestock). The mining industry in Mali has recently attracted renewed interest and investment from foreign companies. Gold and phosphate are the only minerals mined in Mali although deposits of copper and diamonds do also exist. The emergence of gold as Mali’s leading export product since 1999 has helped mitigate some of the negative impact of the cotton and Côte d’Ivoire crises (Mali’s importation products used to come through the ports of this neighboring country). The development of the oil industry is important due to the country’s dependence on the importation of all petroleum products from neighboring
states. Between 1987 and 1995, Mali implemented an economic adjustment program that resulted in economic growth and a reduction in financial imbalances. This was reflected in the increased GDP growth rates (9.6% in 2002) and decreased inflation, made up of agriculture 37.8%, industry 26.4% and services 35.9%.

- Demographics of Mali

The Ethnic groups in Mali are mostly, Mande 50% (Bambara, Malinke, Soninke), Peulh (Fula/Fulani) 17%, Voltaic 12%, Songhai 6%, Touareg and Moor 10%, other 5%

- Culture of Mali

Approximately 90% of Malians follow Sunni Islam, but not always to the exclusion of traditional religious beliefs and practices. Muslims have their own educational systems, leading in some cases to the equivalent of baccalaureate and doctoral studies. An increasingly large number of Muslims make the pilgrimage to Mecca and study in Arab countries. Christians comprise about 1% of the population, although under French colonial rule the introduction of missionaries into predominantly Islamic areas was encouraged. Literacy in French, the colonial language, is low and is concentrated in the urban areas. However, about 60% of the population is literate, although not necessarily in French: many are literate in Bamanakan (the largest spoken language), which has its own alphabet known as “N’Ko”. Others are also literate in Arabic, having attended Koranic school. One of the oldest universities in the world, the Sankore University in Timbuktu, dates to the 1400's.

The music of Mali is best known outside of Africa for kora virtuoso Toumani Diabaté, and the late guitarist Ali Farka Touré.