# Biological and chemical studies of medicinal plants

## *A: Chemistry and biological activities of medicinal plants from Mali*
## *B: Complement fixing polysaccharides from Chinese medicinal plant Codonopsis pilosula*

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# Contents

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## Abstract

The main purpose of this thesis was to evaluate the potential of two Malian medicinal plants and one Chinese cultivated medicinal plant in the treatment of immune related diseases. This thesis also aimed to promote the sustainable use of medicinal plant resources.

Seven purified pectic polysaccharides fractions were isolated from *Parkia biglobosa* bark. All of the fractions exhibited potent complement fixation activity, and fractions PBEII-I, PEBII-III and PBEII-IV also showed potent macrophage stimulating activity. The common structural features of these seven fractions are rhamnogalacturonan I (RG-I) backbone highly branched with arabinogalactan type I and/or type II (AG-I and/or AG-II) side chains. The homogalacturonan region may not present in fractions PBEII-III, PBEII-IV and PB100I-I due to the high ratio of rhamnose (Rha) to galacturonic acid (GalA). The higher yield and biological activities of fractions obtained from the 50% ethanol-water extract suggests that this extract could be more related to the medicinal activity than the 50$^{o}$C and 100$^{o}$C water extracts.

27 different crude extracts were obtained by boiling water extraction (BWE) and accelerated solvent extraction (ASE) from root bark, stem bark and leaves of *Terminalia macroptera*. None of the extracts are toxic against brine shrimp larvae in the test concentration. Significant correlations were found among enzyme inhibition (α-glucosidase, 15-lipoxygenase, xanthine oxidase), DPPH scavenging activity and total phenolic content, thus a screening of phenolic content in *T. macroptera* extracts will probably indicate the presence of compounds with enzyme inhibitory and antioxidant activities. Based on the results from principle component analysis, the ASE ethanol extracts of root bark and stem bark and the low molecular weight fraction of 50% ethanol-water extract of leaves showed the highest total biological activities, which indicated ASE has higher extraction efficiency than BWE. The results indicate that part of activities like antioxidant activity and enzyme inhibition activities are present in the high molecular weight part of our crude extracts. The observed enzyme inhibition activities, radical-scavenging properties and complement fixation activities may explain some of the traditional uses of *T. macroptera*, such as against diabetes and wound healing.

Fifteen purified pectic polysaccharide fractions were obtained from nine crude extracts of *T. macroptera* (root bark, stem bark and leaves) by using BWE and ASE. The root bark, leaves and stem bark are all good sources for fractions containing bioactive polysaccharides. But due to sustainability, it is prefer to use leaves rather than the other two plant parts, and then the dosage

by weight must be higher when using leaves. The results also indicated that BWE provide higher yields of crude extracts with comparable complement fixation activities to the crude extracts isolated with ASE. For the purpose of obtaining purified polysaccharide fractions, ASE was more efficient, as the method provided higher yields and higher complement fixation activity. The common structural features of these fifteen polysaccharide fractions are 1,4-linked galacturonan, interrupted by RG-I regions with AG-I and/or AG-II side chains. The most active polysaccharide fraction 100WTRBH-I-I, has different structural feature from other fractions. It has a long RG-I region with galactan side chains, not arabinogalactans. The structural differences present among these fifteen fractions are *Mw*, chemical compositions and position of side chains. The three most abundant fractions, 50WTRBH-II-I, 50WTSBH-II-I and 50WTLH-II-I were subjected to pectinase degradation; the results indicated that the activity of these three fractions was expressed mainly by their ramified regions.

Two purified polysaccharide fractions, 50WCP-II-I and 100WCP-II-I, were isolated from 50$^{o}$C and 100$^{o}$C water ASE extracts of cultivated roots of *Codonopsis pilosula* Nannf. *var. modesta* L.T.Shen. The structure studies of native and sub-fractions showed the 50WCP-II-I is a pectic polysaccharide fraction, with long homogalacturonan regions (some of the GalpA were methyl esterified), interrupted by short RG-I regions, the side chains (AG-I and AG-II) of RG-I region are attached on position 4 of Rha. The structural feature of fraction 100WCP-II-I is different from that of 50WCP-II-I, the AG-I side chains are attached on position 2 of GalA, and AG-II side chains are attached on position 4 of Rha in the latter.

We have compared the complement fixation activity of the different pectic polysaccharides obtained, and it became clear that, parameters as *Mw*, ramified regions (RG-I or branched galacturonan), side chains (arabinogalactan and/or galactan) and phenolic compounds, are important for the expression of complement fixation activity.

## List of papers

**Paper I**

Yuan-Feng Zou, Bing-Zhao Zhang, Kari Tvete Inngjerdingen, Hilde Barsett, Drissa Diallo, Terje Einar Michaelsen, Elnour El-zoubair, Berit Smestad Paulsen. Polysaccharides with immunomodulating properties from the bark of *Parkia biglobosa*. *Carbohydrate Polymers*, **2014**, 101: 457-463.

**Paper II**

Yuan-Feng Zou, Giang Thanh Thi Ho, Karl Egil Malterud, Nhat Hao Tran Le, Kari Tvete Inngjerdingen, Hilde Barsett, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Enzyme inhibition, antioxidant and immunomodulatory activities, and brine shrimp toxicity of extracts from the root bark, stem bark and leaves of *Terminalia macroptera*. Accepted for publication in *Journal of Ethnopharmacology*.

**Paper III**

Yuan-Feng Zou, Bing-Zhao Zhang, Kari Tvete Inngjerdingen, Hilde Barsett, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Complement activity of polysaccharides from three different plant parts of *Terminalia macroptera* extracted as healers do. Accepted for publication in *Journal of Ethnopharmacology*.

**Paper IV**

Yuan-Feng Zou, Bing-Zhao Zhang, Hilde Barsett, Kari Tvete Inngjerdingen, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Complement fixing polysaccharides from *Terminalia macroptera* root bark, stem bark and leaves. *Molecules,* **2014**, 19 (6): 7440-7458.

**Paper V**

Yuan-Feng Zou, Hilde Barsett, Giang Thanh Thi Ho, Kari Tvete Inngjerdingen, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Immunomodulating pectins from root bark, stem bark and leaves of the Malian medicinal tree *Terminalia macroptera,* structure activity relations. Accepted for publication in *Carbohydrate Research*.

**Paper VI**

Yuan-Feng Zou, Xing-Fu Chen, Karl Egil Malterud, Frode Rise, Hilde Barsett, Kari Tvete Inngjerdingen, Terje Einar Michaelsen, Berit Smestad Paulsen. Structural features and complement fixing activity of polysaccharides from *Codonopsis pilosula* Nannf. *var. modesta* L.T.Shen roots. Submitted to *Carbohydrate polymers*.

## List of Abbreviations

| | |
|---|---|
| α or β | Configuration of the anomeric site of the monosaccharide |
| 4-OMe-GlcA | 4-*O*-methylated glucuronic acid |
| 15-LO | 15-lipoxygenase |
| *f* | Furanose |
| *p* | Pyranose |
| AG-I | Arabinogalactan type I |
| AG-II | Arabinogalactan type II |
| Ara | Arabinose |
| ASE | Accelerated solvent extraction |
| BPII | Pectic polysaccharide from *Biophytum petersianum* |
| BWE | Boiling water extraction |
| DMSO | Dimethyl sulfoxide |
| DMT | Department of traditional medicine |
| DPPH | 1,1-diphenyl-1-picrylhydrazyl |
| EtOH | Ethanol |
| FPLC | Fast protein liquid chromatography |
| Fru | Fructose |
| Fuc | Fucose |
| GAE | Gallic acid equivalent |
| Gal | Galactose |
| GalA | Galacturonic acid |
| GC | Gas chromatography |
| GC-MS | Gas chromatography-Mass spectrometry |
| GF | Gel filtration |
| Glc | Glucose |
| GlcA | Glucuronic acid |
| HG | Homogalacturonan |
| HMW | High molecular weight |
| HMBC | Heteronuclear multiple bond correlation |

| | |
|---|---|
| HSQC | Heteronuclear single quantum correlation |
| $IC_{50}$ | Concentration needed for 50% inhibition |
| $ICH_{50}$ | Concentration needed for 50% inhibition of hemolysis |
| IEC | Ion exchange chromatography |
| KDO | 3-deoxy-D -*manno*-2-octulosonic acid |
| $LC_{50}$ | Concentration needed for 50% lethality |
| LMW | Low molecular weight |
| LPS | Lipopolysaccharide |
| Man | Mannose |
| *Mw* | Molecular weight |
| NMR | Nuclear magnetic resonance spectroscopy |
| NO | Nitric oxide |
| PCA | Principal Component analysis |
| RG-I | Rhamnogalacturonan type I |
| RG-II | Rhamnogalacturonan type II |
| Rha | Rhamnose |
| ROS | Reactive oxygen species |
| SEC | Size exclusion chromatography |
| TCC | Total carbohydrate content |
| TCM | Traditional Chinese medicine |
| TPC | Total phenolic content |
| WHO | World Health Organization |
| XG | Xylogalacturonan |
| XO | Xanthine oxidase |
| Xyl | Xylose |

# 1 Introduction

## 1.1 Traditional Medicine

Traditional medicines include herbal medicines composed of herbs, herbal materials, herbal preparations, and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations thereof. Traditional medicines may also use animal parts and/or minerals (WHO, 2002). They are used in every country in the world, and have been relied upon to support, promote, retain and regain human health for millennia (Li et al., 2008; Shi, et al., 2009; Sucher et al., 2008; Upton, 1999). Traditional Chinese medicine (TCM), for example, is a completely defined medical system running parallel to allopathic medicine which has been used successfully to diagnose, treat and prevent illness for over 2500 years (Holtz, 2007). Traditional medicines (products) are a part of the larger field of traditional medicine which includes procedures and practitioners, as well as products (WHO, 2002). In much of the developing world, 70–95% of the population relies on these traditional medicines for primary health care. In Mali, like many African countries 75% of the population depends on traditional medicine for primary health care (Imperato, 1981; WHO, 2002).

The traditional medicine is mainly based on plants, and most of these plants have never been investigated for their chemical composition or pharmacological properties. It is therefore interesting to study these plants to substantiate the traditional medical knowledge, and to evaluate their benefits, risks and limitations.

The Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, has collaborated with the Department of Traditional Medicine (DMT) in Mali since 1996. DMT is a collaborating center of the WHO for research in traditional medicine. The main objective of the collaboration is to assure that traditional medicine produced from local plants is complementary to conventional medicine. The main activities of DMT are registration of traditional healers and their use of medicinal plants, in addition to research and development of improved traditional medicines (ITMs). DMT has carried out many phytochemical, pharmacological and toxicological studies with the ultimate goal of providing effective and non-toxic medicine to the population. So far, twelve ITMs have been developed and seven of them are regarded as essential and effective medicines in Mali (Diallo & Paulsen, 2000).

In order to know more about the use of medicinal plants, ethnopharmacological studies have been performed in several areas in Mali (Diallo et al., 1999, 2002; Inngjerdingen et al., 2004; Togola et al., 2005; Grønhaug et al., 2008; Pham et al., 2011a). Leaf, stem bark, roots, root bark or whole plant from different medicinal plants or trees were used in different preparations for traditional use. As a result of the developing of ITMs, the demands of some of the medicinal plants and trees were significant increased. For example, in 2006, 9000 kg of raw material (roots) of *Vernonia kotschyana* was used to produce 37 770 bags of Gastrosedal in Mali (Inngjerdingen et al., 2012). For some of the medicinal trees such as *Terminalia macroptera* and *Parkia biglobosa* (Grønhaug et al., 2008; Pham et al., 2011a), leaves and bark are normally used in traditional Malian medicine as many ethnopharmacological surveys revealed. But if root bark from a tree is collected this can lead to serious damages to the tree being greater than if the stem bark or leaves are collected. Harvesting from the wild, the main source of raw material, is causing a growing concern that it might diminish the populations and lead to local extinctions. In China, the wild products of many medicinal plants also cannot meet the increasing demands, especially for some kinds of them as food and medicine, such as *Panax ginseng* and *Codonopsis pilosula*.

To protect biodiversity, we have to think about the sustainable use of plant resources. Although plants are renewable resources, we also need to protect them in order to conserve the biological diversity, and also meet the demands for treatment of severe ailments. There are some suggestions to be considered. First, the government should issue regulations or laws to regulate the sustainable use of plant resources. Second, the research institutions and pharmaceutic industries should do further studies on the use of plant resources to increase the utilization efficiency, such as highly extraction efficiency methods development. In this case, the amounts consumed should be decreased. Third, find replacement resources, such as use leaves to replace root bark or roots, and/or other abundant plants with similar pharmacological activities. The last but not least, develop the cultivated products to replace the wild products. Therefore, the aims of this study are to find some proofs of the viabilities of some of these suggestions (including better extraction methods, replace resources and cultivated products).

The most common way of preparing the traditional medicine is by making a decoction. The extracts contain both low molecular weight (LMW) and high molecular weight compounds. In 1805, morphine became the first pharmacologically active compound to be isolated in pure form

from *Papaver somniferum* (Sneader, 1996). The isolation, purification techniques, structure elucidation technical and biological assays of LMW compounds have grown steadily since that time. Elucidation of the pharmacological activity of macromolecules is important because the clinical efficacy of most medicinal plants cannot be explained by low molecular weight compounds alone (Yamada et al., 2009). The developing of structure elucidation techniques like GC-MS and NMR, and immunology assays promote the developing of polysaccharide analysis. The first report of a pure complex polysaccharide having biological activity came in 1984 (Stimpel et al., 1984), and after this many reports have been published on the chemical characteristics and biological activity of polysaccharides. Plant pectic polysaccharides isolated from crude water extracts have shown effects related to the immune system by different *in vitro* and *in vivo* test systems (Paulsen & Barsett, 2005).

### 1.2 Extraction techniques

The qualitative and quantitative studies of bioactive compounds from plant materials mostly rely on the selection of extraction method (Smith, 2003; Sasidharan et al., 2011). Extraction of plant materials can be performed by various extraction procedures. Several methods have been developed, such as Soxhlet extraction, ultrasound-assisted extraction, pulsed-electric field extraction, enzyme-assisted extraction, microwave-assisted extraction and pressurized liquid extraction (Azmir et al., 2013). Water decoction is the most common preparations used by the traditional healers in Mali (Inngjerdingen et al., 2004; Togola et al., 2005; Grønhaug et al., 2008; Pham et al., 2011a). Thus the boiling water extracts (BWE) should contain bioactive compounds present in the plant material. LMW compounds like steroids, terpenes, alkaloids and phenolic compounds will together with HMW substances, like polysaccharides, be isolated by the boiling water.

Soxhlet extraction (SE) has been widely used for extracting bioactive compounds from various natural sources (Azmir et al., 2013). To isolate polysaccharides, the plant material was pre-extracted by Soxhlet with organic solvents to remove low molecular weight and lipophilic compounds (Austarheim et al., 2012; Inngjerdingen et al., 2012, 2013). Based on our experience, SE requires large amounts of solvent and more than 20 h to complete extraction with each organic solvent.

Accelerated solvent extraction (ASE) is a pressurized liquid extraction method, which was first described in 1995 (Ezzell et al., 1995; Richter et al., 1995). Under elevated temperature and pressure, an extraction solvent can be used above its boiling point, but still remain in the liquid state, and thus increase the kinetics of the extraction process. In this case, solvent consumption and extraction time are significantly decreased (Wang et al., 2010). ASE has been applied for extracting components from environment samples (Olivella, 2006; Sporring et al., 2005; Wang et al., 2007; Xu et al., 2007), biological materials (Schäfer, 1998), plant materials (Chen et al., 2007; Chitescu et al., 2012; Hossain et al., 2011; Pearson et al., 2013; Zhang et al., 2013), dietary compounds (Morgan et al., 2002), feeds (Pecorelli et al., 2003; Sun et al., 2012), and food (Breithaupt 2004; Klejdus et al., 2004). However, reports on the use of ASE for polysaccharide extraction, were mainly from wood (Le Normand et al., 2014, 2012; Song et al., 2012, 2008) and have only recently been reported. Thus, it was of interest to investigate the isolation of bioactive polysaccharides from medicinal plants after extraction with ASE.

### 1.3 Plant pectic polysaccharides

Pectins are important structural components of non-graminaceous plant cell walls, and are the most structurally complex macromolecules in nature. The pectic polysaccharides comprise a class of galacturonic acid (GalA) containing polysaccharides that are abundant in the plant cell wall. It has been estimated that approximate 90% of the uronic acids in the wall derive from the Gal*p*A residues of pectic polysaccharides (Caffall & Mohnen, 2009). Pectins are generally known to consist of three regions, homogalacturonan (HG), rhamnogalacturonan I (RG-I) and substituted galacturonan like rhamnogalacturonan II (RG-II) and xylogalacturonan (XG). The composition of pectins, and other plant polysaccharides, varies with plant material, extraction processes and the growth conditions.

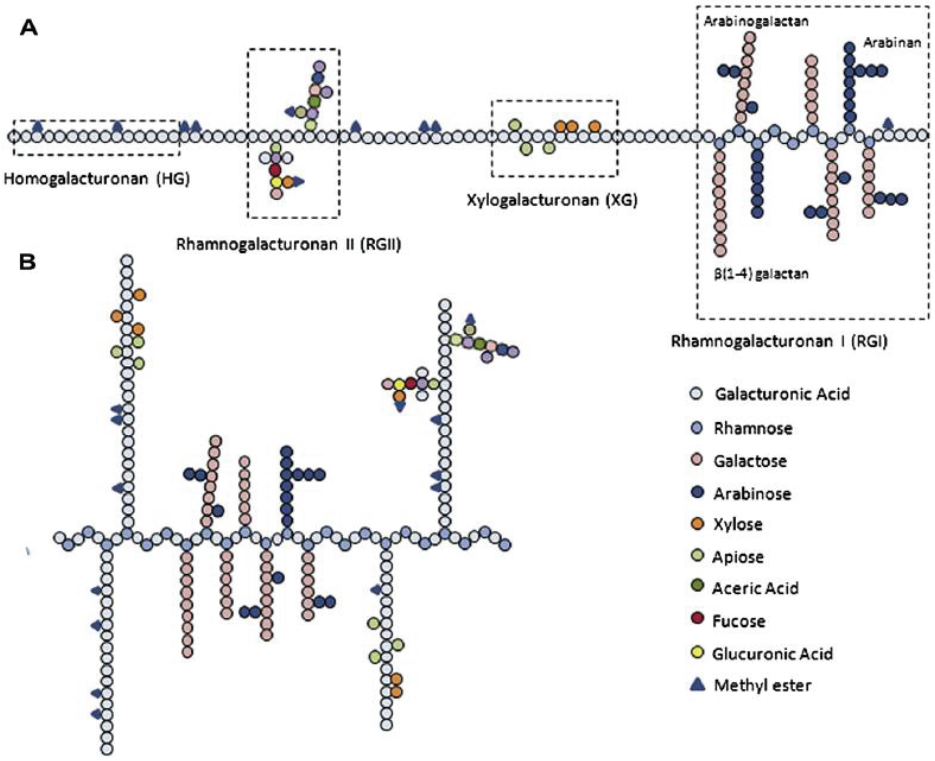


**Fig 1.1.** Schematic diagrams of the two proposed structures of pectin. A. The RG-I (hairy) region is considered attached to HG (smooth) regions which are partially methyl-esterified and also contain RG-II and XG. Typical neutral side chains of RG-I are linear galactans and arabinans, branched galactans and arabinans, and arabinogalactans. B. An alternative structure whereby HGs are side chains of RG-I (From Maxwell et al., 2012).

The structure of pectins has yet to be fully elucidated and is still under debate. There exists several models for describing the pectin structure, and currently there are two preferred proposed models, the "smooth and hairy region" (Fig 1.1A) and the "RG backbone" (Fig 1.1B) (Maxwell et al., 2012). In the "smooth and hairy region" model, the backbone of the pectic polysaccharide consists of smooth regions made up of HG and interrupted by hairy regions consisting of RG-I regions with neutral sugar chains. In the "RG backbone" model, the pectic polysaccharide consists of a RG-I backbone with HG and neutral sugar chains as side chains (Vincken et al., 2003).

### 1.3.1 Homogalacturonan

Homogalacturonan (HG) is a polymer of α-1,4-linked-D-GalA that can account for greater than 60% of pectins in the plant cell wall. Some of the Gal*p*A residues may be methyl-esterified at C-6 carboxyl or acetylated at the *O*-2 or *O*-3 (Ridley et al., 2001), depending on the plant sources.

### 1.3.2 Rhamnogalacturonan

Rhamnogalacturonan I (RG-I) contains a backbone of alternating 1,2-linked α-L-rhamnose (Rha) and 1,4-linked α-D-Gal*p*A residues. The RG-I are highly branched structures with neutral sugar (mainly D-galactose (Gal) and L- arabinose (Ara) residues) side chains (arabinans, galactans and arabinogalactans) attached to position 4 of Rha (Voragen et al., 2009). The highly branched nature of RG-I has made it known as the "hairy region" of the pectin, in contrast to HG regions which are known as the "smooth" regions. Arabinans have a backbone of 1,5-linked Ara, with branching points on position 2 or 3 of Ara. Galactans consist of β-1,4-linked-Gal residues (Fig. 1). The structure of the arabinogalactan side chains may be either of the type I (AG-I) or type II (AG-II). AG-I is basically composed of 1,4-linked Gal units, normally with substitutions of various size arabinans on position 3 of some of the Gal units. AG-II is more complex compared to AG-I and can be highly branched with 1,3,6-linked Gal as the branch point. AG-II consist of a galactan backbone with either 1,3-linked or 1,6-linked Gal as the main chain. Ara or arabinans can be bound to *O*-3 or *O*-6 of Gal. The side chains in RG-I from some plants may be esterified with phenolic acids (Levigne et al., 2004), and/or occasionally terminal α-Fuc, β-GlcA and 4-*O*-methyl-GlcA (Willats et al., 2001).

### 1.3.3 Substituted galacturonans

Substituted galacturonans contain a backbone of linear 1,4-linked Gal*p*A, substituted with various sugars (Fig 1.1). Rhamnogalacturonan II (RG-II), a highly complex branched low molecular mass (5~10 kDa) occurring with much less frequency than RG-I. The characteristic part of RG-II is the presence of unusual sugars in the side chains, such as apiose (Api), aceric acid (AceA), 2-OMe-Xyl, 2-OMe-Fuc, 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and 3-deoxy- D-*lyxo*-2-heptulosaric acid (Dha). The backbone of RG-II has been shown to be composed of at least seven 1,4-linked α-D-GalA residues, two structurally distinct disaccharides and two oligosaccharides chains are attached the backbone (Pérez et al., 2003).

Other substituted galacturonans have also been identified. Xylogalacturonans (XG) contain β-D-xylosyl (Xyl) residues attached to position 3 of the Gal*p*A backbone. The Gal*p*A residues of XG can be methyl-esterified as in HG (Vincken et al., 2003).

## 1.4 Biological activities

### 1.4.1 Free radicals and antioxidant activity

Free radicals are molecules with one or more unpaired electrons, and can be formed when oxygen interacts with certain molecules. Once formed these highly reactive radicals can start a chain reaction, like dominoes. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, or the cell membrane. Free radicals and other reactive oxygen species (ROS) are continuously produced in normal or pathological cell metabolism, but also derived from external sources such as exposure of radiation, smoking, air pollutants and industrial chemicals. Oxidative stress is a result of imbalance between the antioxidant defense system and the formation of ROS. It is believed to damage cell membranes and DNA, as well as membranes lipid peroxidation with subsequent decreases in membrane fluidity (Finkel & Holbrook, 2000; Melov et al., 2000). Oxidative damage may cause cell injury, death and exacerbate the development of several age-related chronic diseases including cancer, Alzheimer's disease, Parkinson's disease and heart disease (Raouf et al., 2000). An antioxidant is a substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell, 1990). Much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals. Numerous assays were developed to evaluate the antioxidant capacity of natural substances. A total antioxidant capacity assay using one chemical reaction seems to be rather unrealistic and not easy to come by, yet there are numerous published methods claiming to measure total antioxidant capacity *in vitro* using more than one methods (Huang et al., 2005; Frankel & Meyer 2000).

#### 1.4.1.1 Scavenging of the 1,1-diphenyl-1-picrylhydrazyl (DPPH) radical

The 1,1-diphenyl-1-picrylhydrazyl (DPPH) assay is a frequently used method to estimate antioxidant capacities in extracts and naturally occurring compounds. The assay is rapid and easy to perform, and based on the scavenging ability of antioxidants towards the stable DPPH radical by measuring the decrease of its absorbance at 517 nm methods (Wangensteen et al., 2004). The

DPPH assay was believed to involve hydrogen atom transfer (HAT) reaction (Huang et al., 2005). However, recent studies suggest that an electron transfer (ET) mechanism is favored in the reaction between phenols having a low $pK_a$ value and DPPH in strong hydrogen-bond-accepting solvents, such as methanol and ethanol (Foti et al., 2004; Litwinienko & Ingold, 2004).

#### 1.4.1.2 Inhibition of 15-lipoxygenase (15-LO)

Lipoxygenases are found widely in plants, fungi and animals, and are nonheme iron dioxygenases that catalyze the regioselective and stereospecific hydroperoxidation of polyunsaturated fatty acids (Brash, 1999; Choi et al., 2007). Lipoxygenases catalyze the oxygenation of arachidonic acid in both a regiospecific and a stereospecific fashion, producing hydroperoxide products primarily at the 5-, 8-, 12-, and 15- positions (Brash, 1999). 15-Lipoxygenase (15-LO) is an enzyme present in multiple systems that reacts with polyunsaturated fatty acids, producing active lipid metabolites which are involved in many diseases such as cancer, atherosclerosis and diabetes (Dobrian et al., 2011). The development of selective of 15-LO inhibitors may be useful to treat these diseases in the future. The 15-LO enzyme inhibition assay is normally carried out using linoleic acid as the substrate for soybean 15-LO. Results with linoleic acid and arachidonic acid as substrates for 15-LO are in good agreement, showing that the inexpensive and fairly stable linoleic acid is a suitable substrate in this test. Soybean 15-LO is easily available and inexpensive compared to mammalian one, and a good correlation for inhibitory activity toward the two enzymes has been shown, as well (Boyington, 1993; Gleason et al., 1995).

#### 1.4.1.3 Inhibition of xanthine oxidase (XO)

Xanthine oxidase (XO) is a form of xanthine oxidoreductase, a type of enzyme that generates reactive oxygen species, and has been demonstrated in various tissues including liver, skin, heart and intestine (Ardan et al., 2004). The primary role of this enzyme is the conversion of hypoxanthine to xanthine, xanthine to uric acid (Equation 1-4) (Flemmig et al., 2011).

$$\text{Hypoxanthine} + O_2 + H_2O \rightarrow \text{Xanthine} + H_2O_2 \quad (1)$$

$$\text{Xanthine} + 2O_2 + H_2O \rightarrow \text{Uric acid} + 2O_2^{\bullet -} + 2H^+ \quad (2)$$

$$\text{Xanthine} + O_2 + H_2O \rightarrow \text{Uric acid} + H_2O_2 \quad (3)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

Overproduction or insufficient excretion of uric acid in the blood can cause hyperuricemia, which is associated with gouty arthritis and uric acid nephrolithiasis (Tsai & Lee, 2014), but also been linked to cardiovascular diseases (Doehner & Landmesser 2011). Inhibition of xanthine oxidase (XO), an enzyme involved in purine metabolism (Ramallo et al., 2006) can reduce production of uric acid. Thus XO inhibitors are used for treatment of hyperuricemia and related medical conditions, including gout (Unno et al., 2004; Kong et al., 2002). Up to now, only a few XO inhibitors have been approved for such purposes due to side effects (Kong et al., 2000, 2002). Thus, safer and effective XO inhibitors are still needed. As seen from Eq. 1-4, the action of XO reduce molecular oxygen, leading to the formation of superoxide radical and hydrogen peroxide, which can cause oxidative stress. Therefore, the inhibition of XO may not only be beneficial to treat gout and hyperuricemia, but could also be useful to other diseases associated with oxidative stress.

### 1.4.2 Inhibition of α-glucosidase

Diabetes type 2 (*Diabetes mellitus*), by far the most common type of diabetes, is a metabolic disorder of multiple etiologies characterized by carbohydrate, lipid and protein metabolic disorders that include defects in insulin secretion, almost always with a major contribution of insulin resistance (Alberti & Zimmet, 1998). One therapeutic approach to treat diabetes is to decrease post- prandial hyperglycemia (Kumar et al., 2012). This is done by retarding and reducing the digestion and absorption of glucose through the inhibition of carbohydrate hydrolyzing enzymes such as α- glucosidase and α-amylase in the digestive tract. α-glucosidase is a membrane bound enzyme at the epithelium of the small intestine that catalyzes the final step of the digestive process of carbohydrates acting upon 1,4-alpha bonds and giving as a result glucose (Tundis et al., 2010). Inhibition of α-glucosidase delays the digestion of the carbohydrates, causing a reduction in the rate of glucose absorption. Acarbose is currently used as α-glucosidase inhibitor, but also induce side effects such as bloating, flatulence and diarrhea (Chakrabarti & Rajagopalan, 2002). Traditional plant treatments have been used throughout the world for the therapy of *Diabetes mellitus*. Among many medications and other alternative medicines, several herbs have been known to cure and control diabetes (Jung et al., 2006). Alkaloids, phenol, terpenoids, flavonoids, saponins, xanthones, polysaccharides and others compounds from medicinal plants have been reported to have anti-diabetic activity (Arif et al.,

2014). Therefore, natural α-glucosidase inhibitors from plants can be used as effective therapy for post prandial hyperglycemia with minimal side effects (Kumar et al., 2012).

### 1.4.3 Immunomodulating activity

An immunomodulator is a substance that either suppresses or activates the body's immune response. Many substances from medicinal plants have been reported with immunomodulating activities, both low molecular weight compounds like phenols, terpenoids, alkaloids (Pieters et al., 1999), and high molecular weight compounds like polysaccharides (Yamada & Kiyohara, 2007).

#### 1.4.3.1 Complement system

The complement system is composed of a large number of distinct plasma proteins that react with one another to opsonize pathogens and induce a series of inflammatory responses that help to fight infection. This system is essential for the operation of the innate as well as the adaptive immune defenses (Carroll, 2004). The complement is a cascade system, the component proteins can be activated through three separate pathways: the classical pathway, the alternative pathway, and the lectin pathway (Fig 1.2). The classical pathway is often referred to as "antibody-dependent" because it is strongly initiated by IgM or IgG cluster with complement component 1 (C1) (Richlin et al., 2010). The alternative pathway is directly activated from C3 by microorganisms or some activators such as lipopolysaccharide through an "antibody-independent" mechanism. In addition, another "antibody-independent" mannose-binding lectin (MBL) pathway has been established as the third activation pathway of complement system, and initiated from C4 (Turner, 1996).

Due to the important physiological role of the complement system, complement modulation, either inhibition or stimulation, is an interesting target for drug development (Alban et al., 2002). Many compounds from medicinal plants have been reported with complement activities, both LMW compounds (Pieters et al., 1999), and HMW compounds (Yamada & Kiyohara, 2007). Several complement activating polysaccharides and pectins have been isolated from bacteria, fungi and from hot water extract of medicinal plants (Paulsen & Barsett, 2005; Yamada & Kiyohara, 2007). Pectic polysaccharide fractions from several Malian medicinal plants have also been investigated for their effects on the complement system, and shown potent complement

fixing activities (Togola et al., 2008; Inngjerdingen et al., 2004, 2013, 2012; Grønhaug et al., 2010, 2011).

The complement fixation assay method A (Michaelsen et al., 2000) was designed to detect the activities of substances through the classical pathway, but it does not discriminated between activation and inhibition of the complement cascade because both result in inhibition of hemolysis (Alban et al., 2002). It is possible to distinguish between activation and inhibition of hemolysis by using ELISA methods for detection of C3 activation products (Michaelsen et al., 2000). A simple method to distinguish activation and inhibition is simply to vary the incubation time (Alban et al., 2002).

The majority of the plant pectic polysaccharides that have shown potent complement fixation activities contain the hairy regions of RG-I, with highly branched side chains or AG-I and AG-II structures plants (Paulsen & Barsett, 2005, Yamada & Kiyohara, 2007). The molecular size of the polymers might also be of importance regarding bioactivity. It has been reported that acidic polysaccharides with higher molecular weights appear to be more active in the complement assay than those with lower molecular weights (Grønhaug et al., 2010; Nergård et al., 2005; Togola et al., 2008). Other type of polysaccharides, like β-glucan, heterglycan and arabinans have also been shown effects on the complement system (Yamada & Kiyohara, 2007).

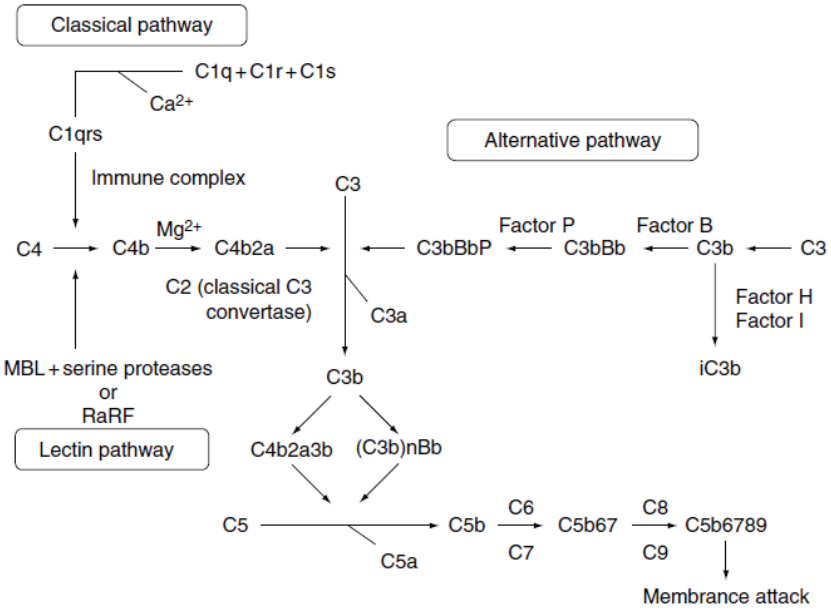


**Fig 1. 2.** Activation steps of the complement system (From Yamada & Kiyohara, 2007).

### 1.4.3.2 Macrophage stimulation

Macrophages are cells produced by the differentiation of monocytes in tissues, and function in both innate and adaptive immunity. As summarized by Schepetkin and Quinn (2006), plant-derived polysaccharides have been shown to exhibit a variety of beneficial pharmacological effects via their ability modulate macrophage immune function. Plant polysaccharides can activate macrophages via complement receptor (CR3), mannose receptor (MR), scavenger receptor (SR), Dectin-1 and Toll-link receptor 4 (TLR4) (Fig 1.3). Plant polysaccharides can also be phagocytosed, leading to activation of unknown intracellular targets. Activation of these transcription pathways induces expression of pro-inflammatory cytokines and inducible oxide synthase (iNOS). Pectic polysaccharide fractions from several Malian medicinal plants have been shown to induce nitric oxide (NO) production from macrophages (Austarheim et al., 2012; Inngjerdingen et al., 2008; Grønhaug et al., 2010, 2011).

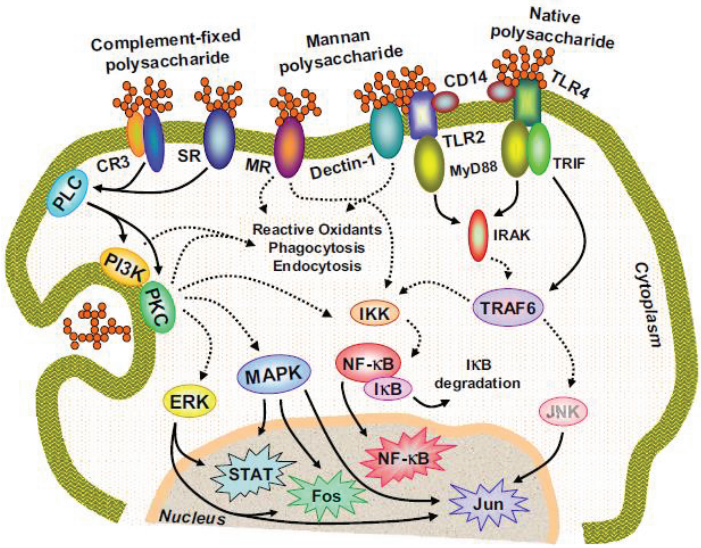


**Fig 1.3.** Schematic model illustrating potential signaling pathways involved in macrophage activation by botanical polysaccharides (From Schepetkin & Quinn, 2006).

## 1.5 Plants used in this study

The medicinal trees *Parkia biglobosa* and *Terminalia macroptera* were chosen as they are part of the battery of medicinal plants chosen by DMT, Bamako, as possible plants for production of ITMs. The plant *Codonopsis pilosula* Nannf.var.*modesta* L.T.Shen was chosen due to the fact that this cultivated product is one of the most popular ingredients in the traditional herbal medicines in China. All plants have traditional uses that may involve the immune system.

### 1.5.1 *Parkia biglobosa*

*P. biglobosa* (Fabaceae), sometimes called the African locust bean tree, is one of the grain legumes (Anderson & Pinto, 1985). *P. biglobosa* has a wide distribution across the Sudan and Guinea savanna ecological zones. The range extends from the western coast of Africa in Senegal across to Sudan, and found in nineteen African countries (Builders, 2014). *P. biglobosa* is a tree being up to 20 m high, bole stout, not buttressed, low-branching, bearing a large wide spreading crown, deciduous, flowering while leafless; flowers in pendulous capitula bearing also pendulous, large fruit pods (Grønhaug et al., 2008) (Fig 1.4).

**Fig 1.4.** *Parkia biglobosa* (Jacq.) R.Br. **(**Fabaceae**)**. The local name is Nere.

In traditional medicine in Mali, the stem bark of *P. biglobosa* is used most frequently, followed by the leaves and seeds (Diallo et al., 2002; Grønhaug et al., 2008). It is used as a remedy to cure a wide range of illnesses (Inngjerdingen et al., 2004; Modupe et al., 2011), such as external and internal wounds, headache, malaria, and cough. Most studies have focused on *P. biglobosa* seeds and seed products (Alabi et al., 2005; Elemo et al., 2011; Labia et al., 2007; Nnemeka et al., 2009; Chukwu et al., 2010; Compaoré et al., 2011). Known bioactive compounds such as sterols and triterpenes from the bark of *P. biglobosa* have been reported (Araujo et al., 1995; Tringali et al., 2000). The result of an acute toxicity study of the stem, leaf and root of *P. biglobosa* indicated that $LD_{50}$ fell within the range of 500~5000 mg/kg body weight confirming them to be only slightly toxic and hence not potentially dangerous (Builders, 2014).

To our knowledge, the isolation, purification and characterization of pectic polysaccharides from the bark of *P. biglobosa* and evaluation of immunomodulating activities have not been performed.

### 1.5.2 *Terminalia macroptera*

*T. macroptera* Guill. & Perr. (Combretaceae) is a tree that grows in West Africa, and it called "woloba" in Mali (Fig 1.5). The root bark, stem bark and leaves of the tree are used frequently in

traditional African folk medicine. In Mali, *T. macroptera* is used against a variety of ailments; about 31 different indications have been mentioned by the traditional healers in ethnopharmacological studies, such as wounds, hepatitis, diabetes, malaria, fever, cough, diarrhea as well as tuberculosis and skin diseases (Pham et al., 2011a; Sanon et al., 2003; Diallo et al., 2002). The stem bark and leaves are most commonly used against sores and wounds, pain, cough, tuberculosis and hepatitis (Pham et al., 2011a). The roots are used against hepatitis, gonorrhea and various infectious diseases, including *H. pylori*-associated diseases (Pham et al., 2011a; Silva et al., 2012, 2000, 1997, 1996). Flavonoids (Nongonierma et al., 1990, 1988, 1987), triterpenoids (Conrad et al., 2001a, 1998), ellagitannins (Pham et al., 2011b; Conrad et al., 2001b; Silva et al., 2000) and other phenolics (Conrad et al., 2001a), have been identified from different parts of *T. macroptera*.

**Fig 1.5.** *Terminalia macroptera* Guill. & Perr. (Combretaceae). The local name is woloba.

The methanol crude extract, ethanol crude extract and butanol extract, and some isolated compounds from leaves of *T. macroptera* have shown potent DPPH radical scavenging activity, XO and 15-LO inhibitory activities, and α-glucosidase inhibitory activity (Pham et al., 2014, 2011b). The toxicity investigation of crude extracts (dichloromethane, methanol, ethanol and butanol) and some isolated compounds from leaves of *T. macroptera* has been reported

previously (Pham et al. 2014). The results indicated that none of the extracts or the isolated compounds seemed to be very toxic in the brine shrimp assay compared with the positive control podophyllotoxin. However, these studies on leaves have not been performed on root bark and stem bark.

To our knowledge, the isolation, purification and characterization of pectic polysaccharides from the root bark, stem bark and leaves of *T. macroptera* and evaluation of immunomodulating activities have not been performed.

### 1.5.3 *Codonopsis pilosula*

Radix Codonopsis is one of the most popular ingredients in the traditional herbal medicines in China, Japan and Korea, consists of the root of *C. pilosula* (Franch.) Nannf.; *C. pilosula* Nannf.var.*modesta* L.T.Shen and *C. tangshen* Oliv (Campanulaceae). Jiuzhaigou County (Sichuan province, China) is one of the famous production areas of Radix Codonopsis which are considered as high quality products in the traditional medicine market in China (Zou et al., 2011a). The original plant is *C. pilosula* Nannf.var.*modesta* L.T.Shen which is mainly cultivated in high altitudes, with non-fertilization and non-pollution, and has a production of 5 000 tons every year.

Radix Codonopsis was used in traditional medicine to lower blood pressure and increase white blood cell count, and is reported to cure appetite loss and boost immunity (China Pharmacopoeia Committee, 2010). Radix Codonopsis was utilized primarily as a substitution for ginseng (*Panax ginseng*), called poor man's ginseng. Some reports indicated that the main components of Radix Codonopsis were sterols, triterpenes, lobetyolin (He et al, 2005), atractylenolide III (Wang et al, 1991), alkaloids and polysaccharides. Radix Codonopsis is an edible medicinal plant with abundant nutritive components, such as protein, essential amino acids and minerals (Bi et al, 2008).

Recently, several investigators reported that the polysaccharides extracted from Radix Codonopsis had several bioactivities such as significant increase of lymphocyte proliferation (Sun & Liu 2008), improvement of the compensatory hematopoiesis of spleen (Yang et al., 2005; Zhang et al., 2003), scavenging of oxygen free radicals (Li & Yang, 2001) and antitumor activities (Xin et al., 2012; Xu et al., 2012). Hot-water reflux extraction and ultrasound extraction techniques are the main extraction methods to isolate polysaccharides from Radix

Codonopsis in recent studies (Sun et al., 2010; Zou et al., 2011b). Most of the current reports on *C. pilosula* polysaccharides focused on their isolation, pharmacological activity and therapeutic effects. Only a few of the polysaccharides have been characterized, and most of them are neutral polysaccharides (Zhang et al., 2005; Zhang et al., 2010; Han et al., 2005; Ye et al., 2005). To our knowledge, only one antitumor pectic polysaccharide, CPP1b, was isolated from *C. pilosula* (Franch.) Nannf., and this has been well characterized (Yang et al., 2013), no study on the pectins from *C. pilosula* Nannf.var.*modesta* L.T.Shen has yet been published until now. The results from our previous study indicated that *C. pilosula* from Jiuzhaigou County contain high amounts of polysaccharides (Zou et al., 2011b); it was of interesting to further investigate the structure and activity details of polysaccharides from this plant.

**Fig 1.6**. *Codonopsis pilosula* Nannf.var.*modesta* L.T.Shen (Campanulaceae) and roots. The local name Chinese: 党参; pinyin: *dǎngshēn*.

## 2 Aim of the thesis

In Mali and China, like many developing countries, a high percentage of the population depends on traditional medicine for primary health care. Leaf, stem bark, roots, root bark or whole plant from different medicinal plants or trees were used in different preparations for traditional use. Harvesting from the wild, the main source of raw material, is causing a growing concern that it might diminish the populations and lead to local extinctions. Increase the utilization efficiency, find replacement resources and develop the cultivated products are the main suggestions to protect biodiversity and sustainable use of plant resources. Therefore, the aims of this study are find proofs of the viabilities of some of these suggestions (including better extraction methods, replace resources and cultivated products). Hot water extracts of medicinal plants are commonly used preparations, both LMW and HMW compounds are likely responsible for activities, and thus it was of interest to investigate the activities of both LMW and HMW parts of extracts from different plants and plant parts. Plant polysaccharides have previously been shown to act as potent immunomodulating agents; thus it is highly relevant to look for bioactive polysaccharides in plants used in traditional medicine.

The specific objectives of the study were:

- To isolate immunomodulating polysaccharides from bark of *P. biglobosa* (Paper I).
- To isolate different crude extracts using different extraction methods (boiling water extraction and accelerated solvent extraction) from different plant parts of *T. macroptera* (root bark, stem bark and leaves). Ultrafiltration should be applied for separation of LMW and HMW compounds, and biological activities of both LMW and HMW compounds should be evaluated and compared (Paper II).
- To isolate pectic polysaccharides from the different plant part of *T. macroptera* with different extraction methods based on complement fixation assay guided fractionation and purification (Paper III and IV).
- To study the structure-activity relations of the bioactive pectic polymers from 50 $^{o}$C water extracts (ASE) of different plant parts of *T. macroptera* (Paper V).
- To isolate bioactive pectic polysaccharides from cultivated roots of *C. pilosula* by using ASE, and study the structure-activity relations (Paper VI).

## 3 Summary of papers

### Paper I. Polysaccharides with immunomodulating properties from the bark of *Parkia biglobosa*

The aim of the paper was to isolate and study the structure of pectic polysaccharides from the bark of *P. biglobosa*. In order to remove LMW compounds, the powdered bark of *P. biglobosa* was pre-extracted with hexane, ethyl acetate and 96% methanol, respectively. The residue was further extracted with 50% ethanol-water, 50 $^{o}$C and 100 $^{o}$C water, respectively. After ultrafiltration, the HMW fractions of these crude extracts were denominated PBE, PB50 and PB100. Based on complement fixation assay guided fractionation and purification, seven purified pectic polysaccharides fractions were obtained by ion exchange chromatography and gel filtration. The chemical and structural characterizes were determined by GC, GC-MS and FPLC.

The monosaccharide compositions analyses indicated these seven purified fractions are typical pectic polysaccharides. The acidic fractions PBEII-I and PBEII-IV were the most active in the complement fixation assay, but the other fractions were also potent compared to the positive control BPII from *Biophytum petersianum*. Fractions PBEII-I and PBEII-IV were also the most potent fractions in stimulating macrophages to release nitric oxide. Structural studies showed that PBEII-I and PBEII-IV were pectic type polysaccharides, containing arabinogalactan type II structures. The observed differences in biological activities among the seven purified polysaccharide fractions are probably due to differences in monosaccharide compositions, linkage types and molecular sizes.

### Paper II. Enzyme inhibition, antioxidant and immunomodulatory activities, and brine shrimp toxicity of extracts from the root bark, stem bark and leaves of *Terminalia macroptera*

This study aimed to investigate the inhibition of the activities of α-glucosidase, 15-lipoxygenase and xanthine oxidase, DPPH scavenging activity, complement fixation activity and brine shrimp toxicity of different extracts obtained by boiling water extraction (BWE) and by ASE (Accelerated solvent extraction) with ethanol, ethanol-water and water as extractants from different plant parts of *T. macroptera*. 27 different crude extracts were obtained by BWE and ASE from root bark, stem bark and leaves of *T. macroptera*. The total phenolic and carbohydrate

contents and biological activities of these extracts were evaluated. Principal component analysis (PCA) was applied for total biological activities evaluation.

Several of the extracts from root bark, stem bark and leaves of *T. macroptera* obtained by BWE and ASE showed potent enzyme inhibition activities, radical-scavenging properties and complement fixation activities. None of the extracts are toxic against brine shrimp larvae in the test concentration. Based on the results from PCA, the ASE ethanol extracts of root bark and stem bark and the LMW fraction of the 50% ethanol-water extract of leaves showed the highest total biological activities. The boiling water extracts were less active, but the bark extracts showed activity as α-glucosidase inhibitors and radical scavengers, the leaf extract being less active. The observed enzyme inhibition activities, radical-scavenging properties and complement fixation activities may explain some of the traditional uses, such as against diabetes and wound healing, of this medicinal tree.

In this study, the boiling water extracts and ASE extracts were separated into LMW and HMW parts and their biological activities were determined. The results indicate that part of activities like antioxidant activity and enzyme inhibition activities are present in the HMW part of our crude extracts. This finding suggests that traditional phytochemistry studies on medicinal plants should also pay attention to HMW compounds.

### Paper III. Complement activity of polysaccharides from three different plant parts of *Terminalia macroptera* extracted as healers do

The aims of this study were comparing the properties of the polysaccharides among different plant parts, as well as relationship between the chemical characteristics and complement fixation activities when the plant material has been extracted as the traditional healers do, with boiling water directly. Five purified polysaccharide fractions, TRBD-I-I, TRBD-I-II, TSBD-II-I, TLD-I-I and TLD-II-I, were obtained from root bark, stem bark and leaves of *T. macroptera* by anion exchange chromatography and gel filtration. Chemical compositions were determined by GC of the TMS derivatives of the methyl-glycosides and the linkage determined after permethylation and GC-MS of the derived partly methylated alditol acetates. The bioactivity was determined by the complement fixation assay of the crude extracts and purified fractions. The acidic fraction TRBD-I-I isolated from the root bark was the most active of the fractions isolated.

Structural studies showed that all purified fractions are of pectic nature, containing RG-I backbone. AG-II side chains were present in all fractions except TRBD-I-II. The observed differences in complement fixation activities among the five purified polysaccharide fractions are probably due to differences in monosaccharide compositions, linkage types and molecular sizes. The crude extracts from root bark and stem bark have similar total activities, both higher than those from leaves. The root bark, leaves and stem bark are all good sources for fractions containing bioactive polysaccharides. But due to sustainability, it is prefer to use leaves rather than the other two plant parts, and then the dosage by weight must be higher when using leaves.

**Paper IV. Complement fixing polysaccharides from *Terminalia macroptera* root bark, stem bark and leaves**

The aims of this study were comparison of the properties of the polysaccharides from the different plant parts extracted by Accelerated Solvent Extractor. The root bark, stem bark and leaves of *Terminalia macroptera* were sequentially extracted with ethanol, 50% ethanol-water, and 50 $^{o}$C and 100 $^{o}$C water using an accelerated solvent extractor. Ten bioactive purified polysaccharide fractions were obtained from those crude extracts after anion exchange chromatography and gel filtration. The polysaccharides and their native extracts were characterized with respect to molecular weight, chemical compositions and effects in the complement assay.

The chemical compositions showed that the polysaccharides are of pectic nature. The results indicated that there was no great difference of the complement fixation activities in the crude extracts from the different plant parts when extracting with the Accelerated solvent extraction system. The purified polysaccharide fractions 100WTSBH-I-I and 100WTRBH-I-I isolated from the 100 $^{o}$C water extracts of stem and root bark respectively, showed the highest complement fixation activities. These two fractions have RG-I backbone, but only 100WTSBH-I-I contains side chains of both AG-I and AG-II. Based on the yield and activities of the fractions studied those from the root bark gave highest results, followed by those from leaves and stem bark. But in total, all plant materials are good sources for fractions containing bioactive polysaccharides.

**Paper V. Immunomodulating pectins from root bark, stem bark and leaves of the Malian medicinal tree *Terminalia macroptera,* structure activity relations**

The aims of this study were comparing the properties of the polysaccharides from different parts extracted by 50 $^{o}$C water using an accelerated solvent extractor, and study the structure-activities relations. The root bark, stem bark and leaves of *T. macroptera* were sequentially extracted with ethanol, 50% ethanol-water, and 50 $^{o}$C water using accelerated solvent extraction (ASE). Six bioactive purified pectic polysaccharide fractions were obtained from the 50 $^{o}$C crude water extracts after anion exchange chromatography and gel filtration.

The root bark, stem bark and leaves of *T. macroptera* were all good sources for fractions containing bioactive polysaccharides. The fraction 50WTRBH-I-I, being the most active fraction in the complement fixation test, has a highly ramified RG-I region with AG-II side chains. The most abundant fractions from each plant part, 50WTRBH-II-I, 50WTSBH-II-I and 50WTLH-II-I, were chosen for pectinase degradation. The degradation of pectinase revealed that the main features of these fractions are that of pectic polysaccharides, with hairy regions (RG-I regions) and homogalacturonan regions. The activity of the fractions obtained after pectinase degradation and separation by gel filtration showed that the highest molecular weight fractions, 50WTRBH-II-Ia, 50WTSBH-II-Ia and 50WTLH-II-Ia, had higher complement fixation activity than their respective native fractions. These results suggest that the complement fixation activities of these pectins are expressed mainly by their ramified regions.

**Paper VI. Structural features and complement fixing activity of polysaccharides from *Codonopsis pilosula* Nannf. *var. modesta* L.T.Shen roots**

The aims of this study are to isolate and study the structural features and complement fixation activities of pectic polysaccharides from cultivated roots of *C. pilosula* Nannf. *var. modesta* L.T.Shen. The roots of *C. pilosula* were sequentially extracted with ethanol, 50% ethanol-water, 50 $^{o}$C and 100 $^{o}$C water using an accelerated solvent extractor.

Two purified bioactive polysaccharide fractions, 50WCP-II-I and 100WCP-II-I, were obtained by ion exchange chromatography and gel filtration. These two polysaccharide fractions were characterized with respect to molecular weight, chemical compositions, structure and effects in the complement assay. Pectinase degradation treatments were applied to isolate the ramified

regions from these two fractions. The activity of the fractions obtained after pectinase degradation and separation by gel filtration showed that the highest molecular weight fractions, 50WCP-II-Ia and 100WCP-II-Ia, had higher complement fixation activity than their respective native fractions. These results suggest that the complement fixation activities of these pectins are expressed mainly by their ramified regions.

The structure studies of native and sub-fractions showed the 50WCP-II-I is a pectic polysaccharide fraction, with long HG region (some of the GalpA were methyl esterified), interrupted by short RG-I region, the side chains (AG-I and AG-II) of RG-I region are attached on position 4 of rhamnose. The structural features of fraction 100WCP-II-I are different from those of 50WCP-II-I where the AG-I side chains are attached on position 2 of GalA, and AG-II side chains are attached on position 4 of Rha.

# 4 Results and discussion

## 4.1 Extraction and isolation procedures

### 4.1.1 *Parkia biglobosa*

To our knowledge, the isolation, purification and characterization of pectic polysaccharides from the bark of *P. biglobosa* and evaluation of immunomodulating activities have not been performed. Pectic polysaccharides from bark of *P. biglobosa* (paper I) were extracted and purified according to the extraction and fractionation scheme, as described in Fig 4.1. The crude extracts PBE, PB50 and PB100 were applied to an ion exchange chromatography, and gave four high activity fractions PBEII, PB50II, PB100I and PB100II. PBEII was further fractionated by gel filtration and led to the isolation of four fractions, PBEII-I, PBEII-II, PBEII-III and PBEII-IV. PB50II, PB100I and PB100II were further fractionated by gel filtration and three active fractions were obtained; PB50II-I, PB100I-I and PB100II-I.

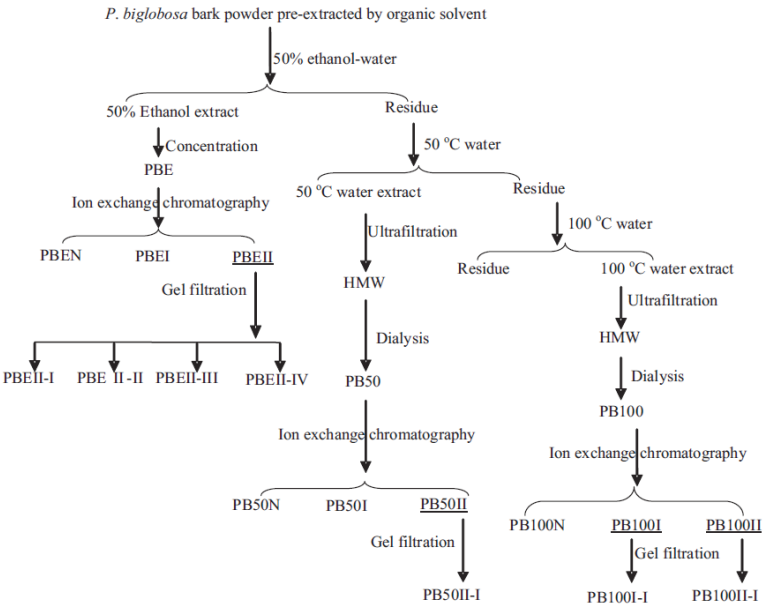


**Fig 4.1.** Extraction and fractionation scheme of the polysaccharides from the bark of *P. biglobosa* (underlined acidic fractions had high complement fixation activity and were fractionated for further studies).

### 4.1.2 *Terminalia macroptera*

Boiling water extracts of *T. macroptera*, administered orally, are the most common preparations of this plant used by the traditional healers in Mali. Organic solvent extracts and LMW compounds from the leaves of this plant have been investigated for biological activities (Pham et al., 2014). It was thus of interest to investigate bioactive compounds (both LMW and HMW compounds) in boiling water extracts from different plant parts of *T. macroptera*. As seen from Fig 4.2, three crude extracts (TRB, TSB and TL), three LMW fractions (TRBL, TSBL and TLL) were obtained follow the procedure (Paper II). Five bioactive purified polysaccharide fractions were isolated from HMW fractions by ion exchange chromatography and gel filtration (Paper III).

As mentioned in section 1.2, ASE has been applied for extracting components from environment samples, biological materials, plant materials, dietary compounds, feeds, and food. ASE has not been applied for extracting compounds from any plant parts of *T. macroptera*; therefore, it is of interest to extract bioactive compounds from different plant parts of *T. macroptera* using ASE.

The root bark, stem bark and leaves of *T. macroptera* were sequentially extracted with ethanol, 50% ethanol-water, and 50 $^{o}$C and 100 $^{o}$C water using accelerated solvent extractor. Ethanol extracts and LMW fractions of 50% ethanol-water, 50 $^{o}$C and 100 $^{o}$C water were obtained for further studies (Paper II). Based on complement fixation assay, ten bioactive purified polysaccharide fractions were obtained from HMW fraction of crude extracts after anion exchange chromatography and gel filtration (Paper IV and Paper V).

Generally, compared to the ASE water extraction, the BWE provide higher yields of crude water extracts (weight related to dry plant material) with comparable complement fixation activities. For the purpose of obtaining purified polysaccharide fractions, ASE was more efficient, as the method provided higher yields, and higher complement fixation activity (Paper III and Paper IV). For the content of total phenolic compounds and total carbohydrate in LMW and HMW fractions, ASE showed higher extraction efficacy than BWE as well (Paper II).

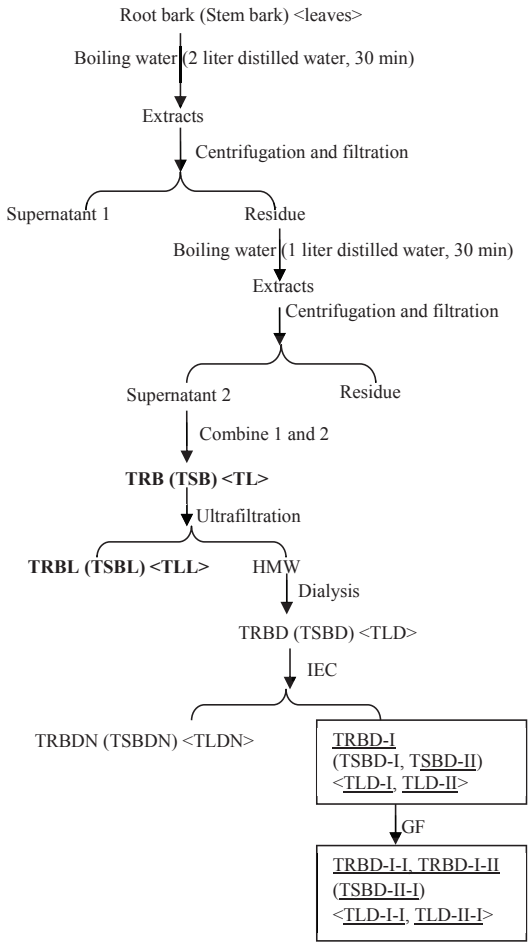


**Fig 4.2.** Extraction and fractionation scheme of polysaccharides extracted with boiling water (BWE) from root bark, stem bark or leaves of *T. macroptera* (underlined acidic fractions showed high complement fixation activity and were fractionated for further studies, IEC, ion exchange chromatography; GF, gel filtration).

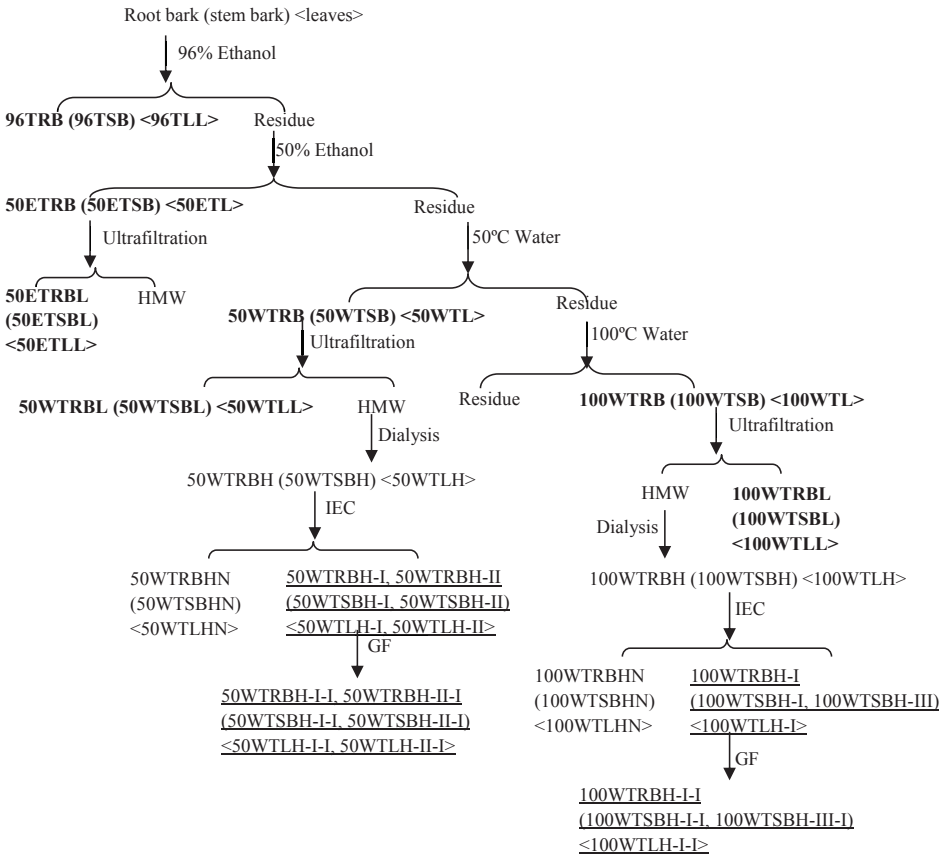


**Fig 4.3.** Extraction and fractionation scheme of polysaccharides extracted with accelerated solvent extraction (ASE) from root bark, stem bark or leaves of *T. macroptera* (underlined acidic fractions showed high complement fixation activity and were fractionated for further studies, IEC, ion exchange chromatography; GF, gel filtration).

### 4.1.3 *Codonopsis pilosula*

Hot-water reflux extraction and ultrasound extraction techniques are the main extraction methods to isolate polysaccharides from Radix Codonopsis in recent studies (Sun et al., 2010; Zou, et al., 2011b). ASE has not been applied for extracting pectic polysaccharides from *C. pilosula*; therefore, it is of interest to extract bioactive pectic polysaccharides from *C. pilosula* using ASE.

Cultivated roots of *C. pilosula* Nannf.var.*modesta* L.T.Shen from Jiuzhaigou county, Sichuan province, China, were sequentially extracted with ethanol, 50% ethanol-water, and 50 °C and 100 °C water using accelerated solvent extractor. The 50 °C and 100 °C water crude extracts (50WCP and 100WCP) were selected for futher purification due to high activity. Two purified polysaccharide fractions, 50WCP-II-I and 100WCP-II-I, were obtained based on complement fixation assay after ion exchange chromatography and gel filtration (Fig 4.4).

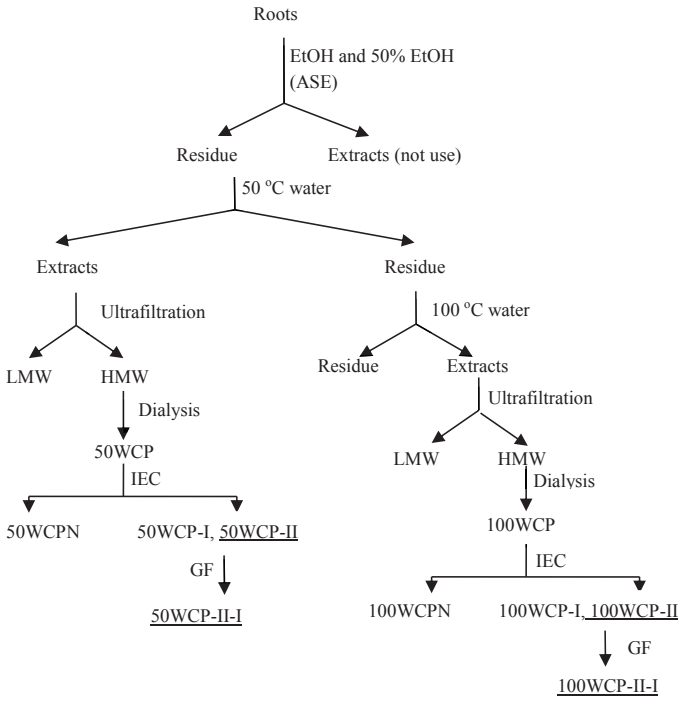


**Fig 4.4.** Extraction and fractionation scheme of polysaccharides extracted by ASE from roots of *C. pilosula* (IEC, ion exchange chromatography; GF, gel filtration).

## 4.2 Chemical characteristics

### 4.2.1 Crude extracts from *T. macroptera* (Paper II)

The total phenolic content (TPC) and total carbohydrate content (TCC) of 27 samples (Bold marked fractions in Fig 4.2 and Fig 4.3) from different parts of *T. macroptera* were determined by using the Folin-Ciocalteu method and the phenol-sulfuric acid method. TPC values were

expressed as gallic acid equivalent (g GAE)/100 g plant extracts, and TCC values were expressed as glucose equivalent (g Glc)/100 g plant extracts. The results are given in Table 4.1. Of the boiling water extracts, the root bark gave the highest TPC and the stem bark gave the highest TCC. Of the ASE extracts, the 96% ethanol crude extracts of root bark gave the highest TPC and the 100 $^{o}$C water crude extracts gave the highest TCC. In general, 96% ethanol crude extracts from ASE gave higher TPC and 100 $^{o}$C water crude extracts gave higher TCC (not in leaves extracts) than BWE extracts. The leaves contain lower TPC and TCC compared to root bark and stem bark, but still considerable amounts. Both in crude extracts from BWE and ASE, the original fractions contain higher amount of TPC and TCC than their LMW fractions, which shows that a considerable amount of TPC and TCC were present in the HMW fractions. The phenolic compounds present in HMW fractions most are probably due to the phenolic compounds that are present as cross-links between polysaccharides in cell wall (Bunzel et al., 2004), and/or being linked as ester to Ara and Gal in pectins (Livigne et al., 2004). It is thus of interest to investigate phenolic compounds and carbohydrate in the HMW fractions.

**Table 4.1** Total phenolic content and total carbohydrate content of extracts from root bark, stem bark and leaves of *T. macroptera*

| | TPC [a] | TCC [b] | | TPC | TCC | | TPC | TCC |
|---|---|---|---|---|---|---|---|---|
| TRB | 30.7±0.8 | 38.4±1.7 | TSB | 24.7±1.8 | 47.0±0.8 | TL | 15.4±0.1 | 38.4±5.7 |
| TRBL | 13.0±0.2 | 27.1±4.1 | TSBL | 26±0.7 | 22.7±3.5 | TLL | 6.0±0.3 | 30.8±3.8 |
| 96TRB | 34.9±1.1 | 36.9±0.8 | 96TSB | 31.0±0.7 | 31.2±2.7 | 96TL | 19.7±0.4 | 29.1±4.1 |
| 50ETRB | 32.5±0.6 | 32.9±3 | 50ETSB | 32.1±0.4 | 63.6±2.6 | 50ETL | 18.8±0.5 | 37.5±1.2 |
| 50ETRBL | 16.2±0.1 | 27.3±4.6 | 50ETSBL | 9.0±0.1 | 20.5±3.0 | 50ETLL | 27.3±0.6 | 33.3±3.7 |
| 50WTRB | 13.6±0.3 | 29.3±2.7 | 50WTSB | 17.3±0.1 | 43.4±2.9 | 50WTL | 7.1±0.2 | 24.1±1.7 |
| 50WTRBL | 6.7±0.2 | 12.8±0.3 | 50WTSBL | 8.7±0.1 | 21.8±3.4 | 50WTLL | 1.0±0.1 | 16.1±0.4 |
| 100WTRB | 10.3±1.1 | 82.8±4.3 | 100WTSB | 5.7±0.5 | 73.7±3.5 | 100WTL | 16.6±0.3 | 36.0±3.1 |
| 100WTRBL | 2.7±0.1 | 20.8±1 | 100WTSBL | 3.9±0.1 | 12.7±2.5 | 100WTLL | 2.4±0.1 | 17.1±0.7 |

[a] TPC values were expressed as gallic acid equivalent (g GAE)/100 g plant extracts, GAE±S.D.
[b] TCC values were expressed as glucose equivalent (g Glc)/100 g plant extracts, Glc±S.D.

### 4.2.2 Structural characteristics of isolated pectic polymers

#### 4.2.2.1 Polysaccharides from *P. biglobosa* (Paper I)

Seven purified polysaccharide fractions were isolated from the 50% ethanol-water, 50$^{o}$C and 100$^{o}$C water extract from the bark of *P. biglobosa* (Fig 4.1). The chemical and structural characteristics were determined by GC and GC-MS, the linkage units of the polysaccharide fractions are shown in Table 4.2. These seven polysaccharide fraction had slightly different

monosaccharide compositions, but all contained monosaccharides typical for pectic polysaccharides.

**Table 4.2** The linkages (mol%) of the monosaccharides present in the purified fractions from the bark of *P. biglobosa,* determined by GC-MS after methylation

| | | PBEII-I | PBEII-II | PBEII-III | PBEII-IV | PB50II-I | PB100I-I | PB100II-I |
|---|---|---|---|---|---|---|---|---|
| Ara | T*f* | 4.5 | 12.4 | 19.7 | 14.8 | 3.1 | 11.0 | 7.8 |
| | 1,2 *f* | n.d. | n.d. | 1.0 | 1.5 | n.d. | n.d. | n.d. |
| | 1,3 *f* | n.d. | 0.7 | 1.4 | 1.8 | n.d. | 0.7 | n.d. |
| | 1,5 *f* | 4.5 | 14.0 | 8.3 | 5.3 | 5.5 | 9.1 | 10.0 |
| | 1,3, 5 *f* | 1.3 | 2.7 | 2.4 | 2.3 | 0.6 | 3.6 | 2.3 |
| | 1,2, 5 *f* | n.d. | n.d. | n.d. | n.d. | n.d. | 2.2 | 1.0 |
| Rha | T*p* | 1.0 | 3.1 | 8.7 | 7.2 | 2.4 | Trace | 1.7 |
| | 1,3 *p* | n.d. | n.d. | 2.7 | n.d. | n.d. | 5.3 | n.d. |
| | 1,2 *p* | n.d. | n.d. | 9.1 | 16.1 | 5.3 | Trace | 4.0 |
| | 1,2, 4 *p* | 1.6 | 5.1 | 5.2 | 5.8 | 0.7 | 3.3 | 1.6 |
| Xyl | T*p* | n.d. | n.d. | n.d. | n.d. | n.d. | 3.9 | n.d. |
| | 1,4 *p* | n.d. | n.d. | n.d. | n.d. | n.d. | 20.1 | n.d. |
| Gal | T*p* | 1.3 | 2.3 | 4.4 | 2.9 | 7.2 | 1.9 | 6.9 |
| | 1,4 *p* | 0.5 | n.d. | 0.7 | n.d. | n.d. | 4.5[b] | n.d. |
| | 1,3 *p* | 14.3 | 5.3 | 3.0 | 2.4 | 6.8 | 3.0 | 4.1 |
| | 1,6 *p* | 1.9 | 4.7 | 3.2 | 3.6 | 3.2 | 0.6 | 3.0 |
| | 1,2, 4 *p* | n.d. | n.d. | 1.8 | n.d. | n.d. | n.d. | n.d. |
| | 1,3, 4 *p* | 12.2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | 1,3, 6 *p* | 11.8 | 18.6 | 11.3 | 7.6 | 5.6 | 4.2 | 4.0 |
| | 1,3, 4, 6 *p* | 0.8 | 2.5 | 2.9 | 7.8 | n.d. | n.d. | n.d. |
| Glc | T*p* | 2.6 | 0.6 | n.d. | 1.4 | 1.9 | 0.9[a] | 1.4 |
| | 1,3 *p* | n.d. | n.d. | 1.0 | 1.8 | n.d. | 3.8 | n.d. |
| | 1,4 *p* | 0.7 | n.d. | 0.5 | 2.0 | 5.7 | n.d. | 3.0 |
| | 1,6 *p* | 6.6 | n.d. | n.d. | 0.6 | 0.8 | n.d. | 0.6 |
| | 1,4, 6 *p* | 10.8 | n.d. | 0.6 | 2.6 | 1.6 | n.d. | 1.1 |
| | 1,2, 6 *p* | 1.0 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| GlcA | T*p* | 0.9 | 0.6 | 1.5 | 0.9 | Trace | n.d. | 3.4 |
| | 1,4 *p* | 13.4 | 11.4 | 2.9 | 2.5 | 18.6 | n.d. | 7.1 |
| GalA | T*p* | 1.7 | n.d. | n.d. | n.d. | 5.4 | n.d. | 5.5 |
| | 1,4 *p* | 5.7 | 10.5 | 4.6 | 3.9 | 18.3 | n.d. | 23.6 |
| | 1,3, 4*p* | n.d. | 2.3 | 1.3 | 0.6 | 2.4 | n.d. | 1.0 |
| *Mw* | ( kDa) | 303.7 | 91.6 | 23.3 | 5.9 | 275.4 | 37.5 | 746.5 |

[a] May contain trace T-GlcA

[b] May contain trace 1→4 GalA

n.d. = not detected

The polysaccharide fractions from 50% ethanol extracts (PBEI~PBEIV) and PB100I-I from 100 $^{o}$C water extracts contain high amount of Ara and Gal, and low amount of GalA. These indicate that the structural features of these fractions are a short backbone (1,4-linked GalA and 1,2-linked and/or 1,2,4-linked Rha) highly branched with arabinogalactan (AG-I and/or AG-II), arabinan and/or galactan. The fraction PBEII-IV has a low *Mw* (5.9 kDa), with side chains that may be esterified with phenolic acids (Levigne et al., 2004), since 2.4% of phenols were detected. Fractions PB50II-I from 50 $^{o}$C water extracts and 100PBII-I from 100 $^{o}$C water extracts contain higher amount of GalA compared to other five polysaccharide fractions. The structural features of these two fractions are as 1,4-linked galacturonans, interrupted by RG-I region, and RG-I regions that are branched with arabinogalactan (AG-II), arabinan and/or galactan. These two fractions contain similar linkage types, albeit in different amounts.

#### 4.2.2.2 Polysaccharides from *T. macroptera* (Paper III ~ Paper V)

According to paper III, five pectic polysaccharide fractions were obtained from root bark, stem bark and leaves of *T. macroptera* using BWE (Table 4.3). The main structural feature of all fractions are similar, having 1,4-linked galacturonan, with a few branch points in position 3 of GalA. The Rha units are basically 1,2-linked, with a few branch points on position 4. The low ratio of Rha (including 1, 2-linked and 1, 2, 4-linked Rha) to GalA (including 1, 4-linked and 1, 3, 4-linked GalA) indicated that the backbone of the polysaccharide fractions consist of shorter RG-I structures, and longer homogalacturonan regions. These features have certain similarities with pectins that are composed of areas with hairy or ramified and smoother regions (Vincken et al., 2003).

TRBD-I-I contain longer RG-I region compared with the other purified fractions, since a higher ratio of 1,2 Rha to 1,4-linked GalA was found in TRBD-I-I. The presence of 1,3-linked Gal and 1,3,6-linked Gal indicate the presence of AG-II structures in fractions TRBD-I-I, TSBD-II-I, TLD-I-I and TLD-II-I, also showed by positive reactions in the Yariv test. The occurrence of AG-II in the fraction TRBD-I-II could not be detected by the Yariv-test although 1,3,6-linked Gal was present. This may be due to the fact that too short chains of 1,3 linked Gal is present in this polymer (Paulsen et al., 2014). 1, 4-linked Gal was only found in the fraction TRBD-I-II which may indicated the presence of the AG-I in this fraction only (Paulsen & Barsett, 2005). High amount of phenolic compounds (30.8%) were found in fraction TRBD-I-II, indicating that the side chains of TRBD-I-II may be esterified with phenolic acids (Levigne et al., 2004).

**Table 4.3** The linkages (mol%) of the monosaccharides present in the purified fractions from boiling water extracts of root bark, stem bark and leaves of *T. macroptera,* determined by GC-MS after methylation

| | | TRBD-I-I | TRBD-I-II | TSBD-II-I | TLD-I-I | TLD-II-I |
|---|---|---|---|---|---|---|
| Ara | T*f* | 6.3 | 7.9 | 6.4 | 18.8 | 5.7 |
| | 1,2*f* | 0.7 | 0.2 | 0.1 | 1.3 | 0.5 |
| | 1,3*f* | 1.2 | n.d. | 0.6 | 1.0 | 0.2 |
| | 1,5*f* | 4.7 | 3.1 | 2.3 | 9.8 | 4.0 |
| | 1,3,5*f* | 1.6 | 0.4 | 2.8 | 4.6 | 2.6 |
| Rha | T*p* | 2.5 | 5.8 | 3.1 | 0.8 | 1.5 |
| | 1,2*p* | 11.6 | 3.3 | 8.0 | 3.5 | 6.3 |
| | 1,2,4*p* | 3.0 | 1.1 | 2.0 | 1.0 | 2.2 |
| Xyl | 1,4*p* | 5.8 | 6.2 | 3.6 | 0.0 | 0.4 |
| Man | 1,3,6*p* | 2.7 | n.d. | 1.7 | n.d. | n.d. |
| Gal | T*p* | 3.4 | 4.5 | 4.5 | 5.3 | 1.8 |
| | 1,4*p* | n.d. | 0.8 | n.d. | n.d. | n.d. |
| | 1,3*p* | 3.4 | 1.0 | 1.6 | 5.3 | 1.9 |
| | 1,6*p* | 4.1 | 8.8 | 2.8 | 2.9 | n.d. |
| | 1,2,4*p* | 0.3 | 0.2 | 0.5 | n.d. | n.d. |
| | 1,3,6*p* | 7.8 | 1.8 | 3.2 | 15.5 | 4.1 |
| | 1,3,4,6*p* | 0.9 | 0.2 | 0.4 | 1.9 | 1.0 |
| Glc | T*p* | 0.8 | 9.2 | 0.4 | n.d. | n.d. |
| | 1,3*p* | 0.5 | 0.6 | 1.8 | 0.3 | n.d. |
| | 1,4*p* | n.d. | 8.5 | n.d. | 0.5 | 0.3 |
| | 1,6*p* | 0.4 | 8.2 | 0.8 | 0.1 | 0.8 |
| | 1,4,6*p* | n.d. | 0.6 | 1.0 | 0.2 | n.d. |
| GlcA | T*p* | 1.2 | 1.6 | 1.1 | n.d. | 0.9 |
| | 1,4*p* | 2.7 | 1.4 | 3.2 | n.d. | 4.5 |
| GalA | T*p* | 0.6 | n.d. | n.d. | n.d. | 1.9 |
| | 1,4*p* | 28.5 | 15.3 | 41.5 | 24.9 | 54.2 |
| | 1,3,4*p* | 3.3 | 5.4 | 4.7 | 1.4 | 3.6 |
| *Mw* | (kDa) | 71.5 | 5.5 | 23.3 | 115.9 | 23.3 |

n.d. not detected

As shown in Table 4.4, six purified pectic polysaccharide fractions were isolated from 50 $^{o}$C water extracts (by ASE) from root bark, stem bark and leaves of *T. macroptera* (Paper V). The methylation results for the samples studied indicated that the main structural feature is the 1,4-linked galacturonan, with a varying degree of branch points in position 3 of GalA. The Rha units are basically 1,2-linked, with some being branched on position 4. The Gal and Ara present have the normal type of linkages that are found in the arabinogalactan type II (AG-II) as branch chains (Paulsen et al., 2014). The presence of 1,3-linked Gal and 1,3,6-linked Gal show the presence of AG-II structures in all fractions, concomitant with the positive reaction in the Yariv test. These features have similarities with pectins that are composed of areas with hairy and smoother regions, and the different amount of linkages among fractions may lead to length variation of some of the structural regions (Vincken et al., 2003). The Xyl is mainly present as 1,4-linked

units which may exist as a xylan in the cell wall (Waldron & Faulds, 2007). GlcA appearing in these six fractions are terminally and 1,4-linked units. Terminal GlcA might be directly linked to position 3 of 1,4-linked GalA in the RG-I backbone, or may also be a part of the AG-II side chains (Capek et al., 1987; Renard et al., 1999). Sub-fraction 50WTLH-I-I contains higher amount of Glc compared to other fractions, which most probably comes from starch as the iodine test gave a positive reaction. This could be due to an association between the pectic moiety and the starch being so tight that these polymers could not be separated by the ion exchange chromatography and gel filtration methods used in this study.

The fractions 50WTRBH-II-I, 50WTSBH-II-I and 50WTLH-II-I were chosen for enzymatic degradation. Highly branched Gal units (1,3,4-linked and 1,3,6-linked) found in 50WTRBH-II-Ia indicated the presence of both AG-I and AG-II side chains in fraction 50WTRBH-II-Ia. The analyses of the native fraction and sub-fraction 50WRBH-II-Ia show that the native fraction consists of long HG region and a main RG-I region with AG-I and AG-II side chains.

After pectinase treatment, three main fractions were obtained from the native fraction 50WTLH-II-I. The highest *Mw* sub-fraction 50WTLH-II-Ia consists of an RG-I region with AG-I and AG-II side chains, and/or an RG-II region due to the presence of KDO. The analyses of the native fraction and sub-fractions suggest that the main feature of 50WTLH-II-I is 1,4-linked galacturonan, being interrupted by RG-I regions and/or RG-II regions.

For the fraction 50WSBH-II-I the main active site is 50WSBH-II-Ia, with very low *Mw* (a small RG-I region). These findings suggest the main structural feature of 50WSBH-II-I is a long HG region, with only a small RG-I region (Fig 4.5).

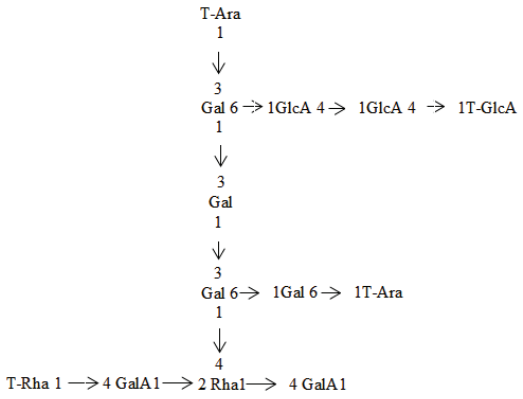


**Fig 4.5.** Proposed structure of fraction 50WTSBH-II-Ia.

**Table 4.4** The linkages (mol%) of the monosaccharides present in the purified fractions from 50 °C water extracts of root bark, stem bark and leaves of *T. macroptera* and fractions obtained after pectinase treatment, determined by GC-MS after methylation

| | | 50WTRBH-I-I | 50WTRBH-II-I | 50WTRBH-II-Ia | 50WTSBH-I-I | 50WTSBH-II-I | 50WTSBH-II-Ia | 50WTLH-I-I | 50WTLH-II-I | 50WTLH-II-Ia |
|---|---|---|---|---|---|---|---|---|---|---|
| Ara | T*f* | 11.8 | 5.4 | 8.4 | 10.1 | 6.9 | 13.8 | 8.9 | 4.4 | 9.8 |
| | 1,2*f* | 0.1 | n.d. | n.d. | 0.8 | 0.2 | n.d. | 1.0 | 0.7 | n.d. |
| | 1,3*f* | 0.8 | n.d. | n.d. | 1.0 | 0.4 | n.d. | 0.7 | 0.4 | n.d. |
| | 1,5*f* | 6.2 | 1.0 | 1.3 | 2.6 | 1.8 | n.d. | 3.3 | 3.7 | 2.4 |
| | 1,3,5*f* | 0.7 | 0.7 | n.d. | 0.8 | 1.4 | n.d. | 1.3 | 2.0 | n.d. |
| Rha | T*p* | 2.2 | 3.4 | 3.0 | 1.3 | 6.5 | 6.3 | 0.6 | 1.0 | 3.3 |
| | 1,2*p* | 0.7 | 22.5 | 1.5 | 0.7 | 9.9 | n.d. | 0.2 | 9.2 | 2.1 |
| | 1,2,4*p* | 0.7 | 2.9 | 3.7 | 2.2 | 1.8 | 5.9 | 1.6 | 2.0 | 1.7 |
| Xyl | T*p* | 0.1 | n.d. | n.d. | 0.2 | 0.8 | n.d. | n.d. | n.d. | n.d. |
| | 1,4*p* | 6.3 | 2.3 | n.d. | 21.2 | 3.6 | n.d. | 34.7 | 1.5 | n.d. |
| Man | 1,3,6*p* | n.d. | n.d. | n.d. | 2.6 | n.d. | n.d. | 2.7 | n.d. | n.d. |
| Gal | T*p* | 2.5 | 1.5 | 1.6 | 4.6 | 6.1 | n.d. | 3.1 | 1.4 | 3.5 |
| | 1,4*p* | 0.3 | 1.0 | 2.5 | 2.0 | 0.6 | n.d. | 1.5 | n.d. | n.d. |
| | 1,3*p* | 3.4 | 2.5 | 2.4 | 4.6 | 1.9 | 7.6 | 3.5 | 1.9 | 2.7 |
| | 1,6*p* | 29.2 | 3.5 | 4.6 | 7.2 | 4.0 | 11.1 | 3.3 | 1.6 | 3.9 |
| | 1,3,4*p* | n.d. | Trace | 14.3 | n.d. | n.d. | n.d. | n.d. | Trace | n.d. |
| | 1,3,6*p* | 12.4 | 6.1 | 10.5 | 14.8 | 3.4 | 20.7 | 7.6 | 4.3 | 8.3 |
| | 1,3,4,6*p* | 0.5 | 0.3 | 3.4 | 1.4 | 0.2 | n.d. | 1.8 | 0.9 | 3.9 |
| Glc | T*p* | n.d. | 0.4 | 3.2 | 0.9 | n.d. | n.d. | n.d. | n.d. | 2.0 |
| | 1,3*p* | 0.6 | n.d. | n.d. | 0.9 | 1.5 | n.d. | n.d. | n.d. | n.d. |
| | 1,4*p* | 0.8 | 0.6 | n.d. | 0.6 | n.d. | n.d. | 5.1 | 0.2 | n.d. |
| | 1,6 *p* | 0.7 | 0.3 | n.d. | 0.3 | 1.0 | n.d. | 0.7 | 0.2 | n.d. |
| | 1,4,6*p* | n.d. | n.d. | n.d. | 0.5 | 0.2 | n.d. | 1.1 | 0.8 | n.d. |
| GlcA | T*p* | 1.1 | 0.8 | 12.8 | 1.7 | 0.9 | 5.5 | 0.9 | 2.2 | 0.6 |
| | 1,4*p* | 1.0 | 2.4 | 3.4 | 1.1 | 2.6 | 12.1 | n.d. | 3.7 | 11.3 |
| GalA | T*p* | n.d. | 1.7 | 1.4 | n.d. | n.d. | n.d. | n.d. | 0.4 | 0.9 |
| | 1,4*p* | 17.0 | 35.3 | 10.4 | 11.9 | 35.9 | 11.1 | 15.8 | 51.4 | 39.4 |
| | 1,3,4*p* | n.d. | 2.2 | n.d. | 3.2 | 7.7 | n.d. | n.d. | 2.3 | 2.1 |
| *Mw* | (kDa) | 136.1 | 115.9 | 19.1 | 19.8 | 23.2 | 2.0 | 19.8 | 220.3 | 19.1 |

According to paper IV, the most active fractions from each plant part, 100WTRBH-I-I from root bark, 100WTSBH-I-I from stem bark and 100WTLH-I-I from leaves, were selected for linkage analysis. The main structural feature of 100WTSBH-I-I and 100WTLH-I-I are similar, having 1,4-linked galacturonan, with a few branch points in position 3 of GalA. The Rha units are 1,2-linked, with branch points on position 4. The low ratio of Rha to GalA indicate that the backbone of the polysaccharide fractions consist of shorter RG-I structures, and longer HG regions. AG-I and AG-II side chains were found in both fractions. The structural feature of 100WTRBH-I-I was quite different from other two fractions. The main structural feature of 100WTRBH-I-I is the presence of RG-I, but without AG-I/II since Ara is absent and only a small amount of Gal is found. The higher ratio of 1,2-linked Rha to 1,4-linked GalA indicate that the backbone of the 100WTRBH-I-I consists of longer RG-I structures, and shorter homogalacturonan regions. High amount of Glc, mainly as 1,4-linked and small amount of 1,4,6-linked may indicate that they come from starch as positive reactions were observed in the iodine test.

**Table 4.5** The linkages (mol%) of the monosaccharides present in the purified fractions from 100 $^{o}$C water extracts of root bark, stem bark and leaves of *T. macroptera,* determined by GC-MS after methylation

| | | 100WTRBH-I-I | 100WTSBH-I-I | 100WTLH-I-I |
|---|---|---|---|---|
| Ara | T*f* | n.d. | 10.2 | 14.6 |
| | 1,2*f* | n.d. | 0.1 | 1.2 |
| | 1,3*f* | n.d. | 0.2 | 0.7 |
| | 1,5*f* | n.d. | 4.3 | 6.7 |
| | 1,3,5*f* | n.d. | 1.6 | 2.1 |
| Rha | T*p* | n.d. | 1.5 | 1.3 |
| | 1,3*p* | 1.3 | 0.1 | 0.1 |
| | 1,2*p* | 21.3 | 0.5 | n.d. |
| | 1,2,4*p* | 1.0 | 2.2 | 1.7 |
| Xyl | T*p* | n.d. | n.d. | Trace |
| | 1,4*p* | n.d. | 1.5 | 23.8 |
| Gal | T*p* | 1.2 | 3.4 | 2.1 |
| | 1,4*p* | n.d. | 0.5 | 3.3 |
| | 1,3*p* | 2.1 | 1.2 | 4.8 |
| | 1,6*p* | n.d. | 9.9 | 2.1 |
| | 1,3,6*p* | 0.4 | 3.1 | 10.7 |
| | 1,3,4,6*p* | n.d. | 0.6 | 0.3 |
| Glc | 1,3*p* | n.d. | 0.7 | 2.3 |
| | 1,4*p* | 21.8 | 13.9 | 3.5 |
| | 1,6*p* | 4.0 | 3.8 | 0.7 |
| | 1,4,6*p* | Trace | 0.4 | 0.5 |
| GlcA | T*p* | n.d. | 1.0 | 0.3 |
| | 1,4*p* | n.d. | 0.7 | n.d. |
| GalA | T*p* | 1.3 | n.d. | n.d. |
| | 1,4*p* | 44.6 | 34.6 | 12.3 |
| | 1,3,4*p* | n.d. | 2.3 | 0.1 |
| *Mw* | (kDa) | 491.8 | 98.7 | 136.1 |

In all three plant parts, purified polysaccharide fractions from ASE generally have higher yields and *Mw* compared to those from BWE. Lower yields of purified active polysaccharide fractions were obtained from BWE, most probably due to more of low molecular weight compounds being present in the BWE crude extracts making the purification process more difficult. The higher *Mw* may be due to high pressure during ASE, making aggregation of polysaccharide molecules, and/or that in BWE processing enzymes causing degradation of polysaccharides are active. The polysaccharide fractions from BWE and ASE have similar structural features, a long HG region, interrupted by RG-I region, branched with AG-I and/or AG-II side chains. Apart from similarities, there are differences between BWE and ASE as well. They have various amounts of different linkage types, and leading to different structural details (such as different length of AG-I and AG-II side chains). For example, higher amount of Xyl were found in polysaccharides fractions from ASE than BWE.

#### 4.2.2.3 Polysaccharides from *C. pilosula* (Paper VI)

In paper VI, the two most abundant pectic polysaccharides, 50WCP-II-I and 100WCP-II-I were isolated from roots of *C. pilosula* using ASE. Fractions 50WCP-II-I and 100WCP-II-I were characterized by 1D and 2D NMR spectroscopy and comparison with chemical shift values from the literature (Yang et al., 2013; Hromádková et al., 2014; Košťálová et al., 2013), and by monosaccharide composition and linkage types (Table 4.6). The monosaccharide composition and linkage analysis indicate that these two fractions are fairly similar. Fraction 100WCP-II-I had somewhat better solubility (25 mg/mL) than fraction 50WCP-II-I (15 mg/mL).

The analyses after pectinase treatment gave ramified regions of these two fractions. The main sub-fractions from their parent fraction were subjected for monosaccharide compositions and linkage analysis (Table 4.6). The fraction 50WCP-II-Ia consists of a 1,4-linked galacturonan with a high degree of branching on position 2. The Rha is present as 1,2-linked units with branching on position 4. Gal and Ara are present as typical linkage units for AG-II, which also was proven with a positive Yariv reaction. The presence of 1,4-linked Gal may indicate the presence of AG-I. These features indicate the 50WCP-II-Ia consists of an RG-I backbone with AG-I and AG-II side chains. The linkage units of 100WCP-II-Ia are different from 50WCP-II-Ia. The Rha is present as 1,2-linked only. 1,3-linked Gal, 1,6-linked Gal and 1,3,6-linked Gal were not detected in 100WCP-II-Ia indicating the absence of AG-II, this fraction gave also a negative reaction in the Yariv test. The proposed feature of 100WCP-II-Ia is a 1,4-linked galacturonan,

interrupted by 1,2-linked Rha, with side chains AG-I substituted on position 2 of GalA.

**Table 4.6** The linkages (mol%) of the monosaccharides present in native fractions and fractions obtained by gel filtration after pectinase treatment, determined by GC-MS after methylation

| | | 50WCP-II-I | 50WCP-II-Ia | 100WCP-II-I | 100WCP-II-Ia |
|---|---|---|---|---|---|
| Ara | T*f* | 4.7 | 6.6 | 5.6 | 8.0 |
| | 1,5*f* | 0.9 | 0.2 | 1.2 | n.d. |
| | 1,3,5*f* | 0.2 | 0.1 | 0.7 | n.d. |
| Rha | 1,2*p* | 4.0 | 0.7 | 4.3 | 3.5 |
| | 1,2,4*p* | 1.7 | 2.2 | 2.1 | n.d. |
| Gal | T*p* | 3.5 | 4.8 | 1.9 | 4.9 |
| | 1,3*p* | 0.9 | 0.4 | 1.2 | n.d. |
| | 1,4*p* | 3.3 | 14.9 | 5.8 | 12.6 |
| | 1,6*p* | 1.1 | 0.7 | 0.6 | n.d. |
| | 1,2,4*p* | n.d. | n.d. | 1.0 | 2.7 |
| | 1,3,6*p* | 2.3 | 0.9 | 1.8 | n.d. |
| Glc | T*p* | 1.3 | 3.0 | 1.9 | 0.5 |
| | 1,4*p* | 1.3 | 3.3 | 0.4 | 5.5 |
| GlcA | 1,4*p* | 1.3 | 3.0 | 1.2 | 2.8 |
| GalA | T*p* | 0.8 | 1.6 | 0.8 | n.d. |
| | 1,4*p* | 67.7 | 38.6 | 66.4 | 38.0 |
| | 1,3,4*p* | 1.0 | n.d. | 0.3 | n.d. |
| | 1,2,4*p* | 2.0 | 10.3 | 1.4 | 11.2 |
| *Mw* | (kDa) | 71.6 | 17.3 | 53.2 | 17.3 |

From the $^{1}H$ and $^{13}C$ one-dimensional spectra and the HSQC two-dimensional spectrum (Fig 4.6), most of the signals could be assigned. Some signals from minor sugars could not be observed. We found three monomers, GalA, Rha and Ara; all had α configuration, while Gal is found to be present as β configuration. The intense $^{1}H$ signal at δ 3.82 ppm, the $^{13}C$ signal at δ 55.7 ppm and cross peaks at δ 55.7/3.82 (-$OCH_3$ in Fig 4.6) and δ 74.2/4.70 ppm (B5 in Fig 4.6A and K5 in Fig 4.6B) in the HSQC spectrum and cross peaks at δ 173.6/3.82 and 173.6/5.11 ppm in the HMBC spectrum (data not shown) indicate methyl esterified α-D-Gal*p*A. In the downfield region of $^{13}C$ NMR spectrum, signals at δ 173.6 and 177.7 ppm (Fig 4.6A-b and Fig 4.6B-b) were assigned to the carboxyl groups of methyl esterified and non-methyl esterified α-D-Gal*p*A, respectively (Yang et al., 2013). Integration of signals at δ 4.70 ppm (H5 of non-esterified α-D-Gal*p*A), δ 4.98 ppm (H1 of esterified α-D-Gal*p*A) and δ 5.12 ppm (H1 of non-esterified α-D-Gal*p*A and H5 of esterified α-D-Gal*p*A), indicates a ratio between non-esterified α-D-Gal*p*A and esterified α-D-Gal*p*A about 2:1(50WCP-II-I) and 1.25:1(100WCP-II-I). Thus, there is a higher degree of esterification in the 100 °C water extracts than in the 50 °C water extracts.

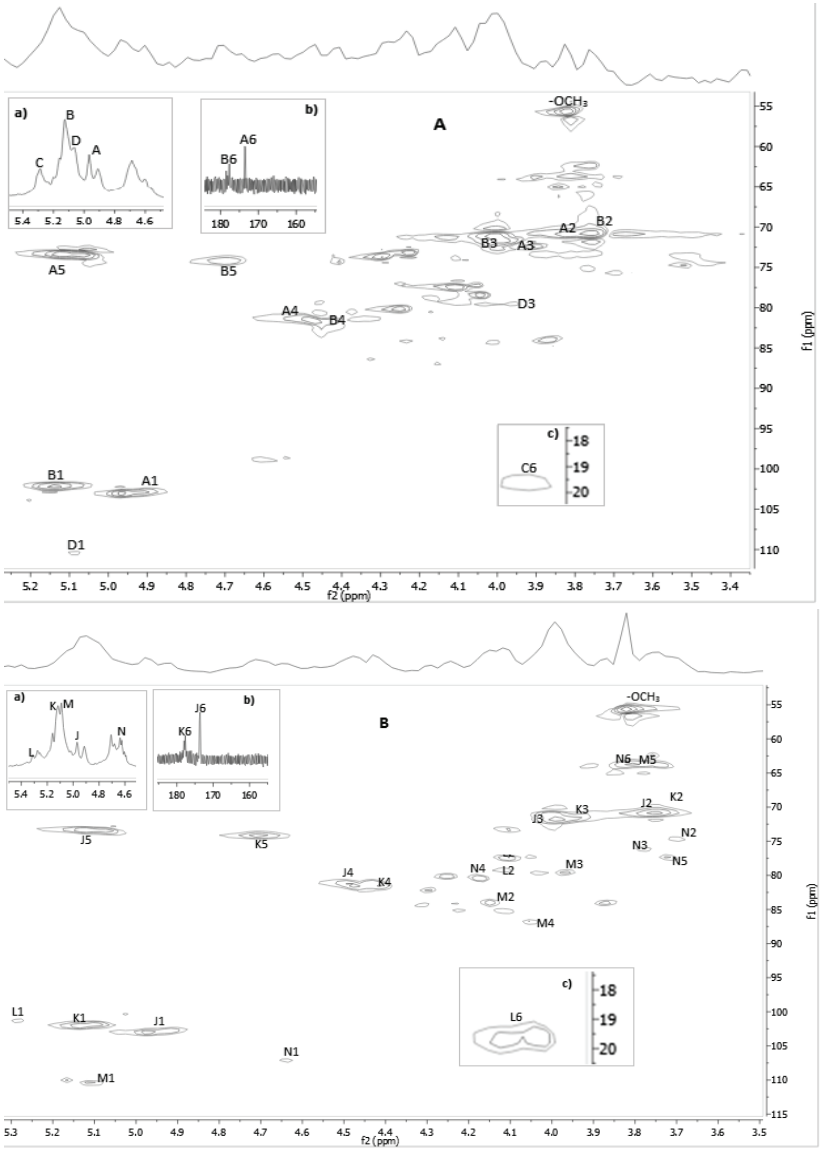


**Fig 4.6.** $^{13}C/^{1}H$ HSQC NMR spectrum of 50WCP-II-I (A) and 100WCP-II-I (B). The insets represent the enlargement of (a) anomeric region (δ 4.5-5.5 ppm), (b) downfield region of $^{13}C$ NMR spectrum (160-180 ppm), (c) methyl group of rhamnose.

Based on monosaccharide compositions, linkage analyses (both before and after pectinase treatment) and 1D and 2D NMR spectrum, the structure of 50WCP-II-I was proposed as shown in Fig. 4.7. The proposed structural feature of fraction 50WCP-II-I is 1,4-linked galacturonan (some of residues are methyl esterified), interrupted by ramified region. The ramified region (Fig 4.7a) consists of alternating 1,4-linked α-D-Gal*p*A and 1,2-linked α-L-Rha*p*, with branching AG-I/AG-II side chains on position 4 of α-L-Rha*p*. The ramified region also contains 1,2,4-linked α-D-Gal*p*A, branched by terminal linked monosaccharides on position 2.

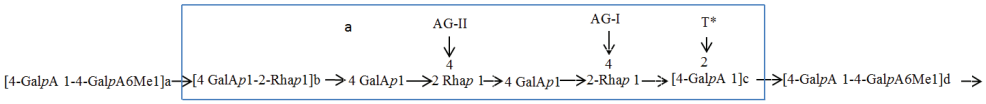


**Fig 4.7.** Proposed structure of 50WCP-II-I (* Terminal linked units of GlcA or Glc or Gal or Ara).

The structure of 100WCP-II-I was proposed as shown in Fig. 4 using same methods as fraction 50WCP-II-I. The proposed structure of fraction 100WCP-II-I is 1,4-linked galacturonan (some of residues are methyl esterified), interrupted by ramified regions. The ramified region (Fig 4.8a) consists of alternating 1,4-linked α-D-Gal*p*A (with/without methyl esterified) and 1,2-linked α-L-Rha*p*, with branching AG-I side chain and terminal linked monosaccharides on position 2 of 1,4-linked α-D-Gal*p*A. The ramified region (Fig 4.8b) contains alternating 1,4-linked α-D-Gal*p*A and 1,2-linked α-L-Rha*p*, with side chain AG-II branched on position 4 of α-L-Rha*p*.

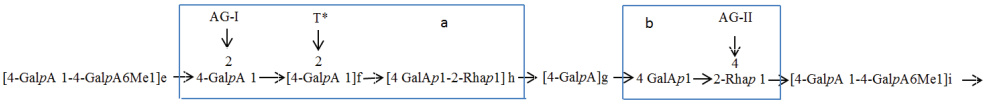


**Fig 4.8.** Proposed structure of 100WCP-II-I (* Terminal linked units of GlcA or Glc or Gal or Ara).

## 4.3 Biological activities and chemical-activity relationships

### 4.3.1 Crude extracts from *T. macroptera* (Paper II)

According to Paper II, 27 different crude extracts were investigated their enzymes inhibition, antioxidant and immunomodulation activities, and brine shrimp toxicity. The results are given in Table 4.7. 96TRB showed the strongest α-glucosidase (same as 50ETRB) and XO inhibition activities compared to other crude extracts, and 50ETLL exhibited the highest 15-LO inhibition activity. The most potent DPPH scavenging activity was found in fraction 96TSB, while the

highest complement fixation activity was present in fraction 50ETL. α-Glucosidase inhibition was, however, strong in all these extracts.

**Table 4.7** Inhibition of α-glucosidase, 15-LO, and XO, DPPH radical scavenging and complement fixation activity of extracts from root bark, stem bark and leaves of *T. macroptera*

| | α-Glucosidase $IC_{50}$±S.D.[a] | 15-LO $IC_{50}$±S.D.[a] | XO $IC_{50}$±S.D.[a] | DPPH $IC_{50}$±S.D.[a] | Complement fixation $IC_{50}$±S.D.[a] |
|---|---|---|---|---|---|
| TRB | 1.6±0.1 | 58.8±2.3 | 145±5 | 6.2±0.3 | 43.6±5.2 |
| TRBL | 5.3±0.3 | 118±6 | >250 | 10.9±0.4 | 30.1±2.8 |
| 96TRB | 0.5±0.1 | 53.7±2.9 | 26.0±5.2 | 5.3±0.1 | 166±2 |
| 50ETRB | 0.5±0.1 | 70.0±2.2 | 138±4 | 5.1±0.7 | 70.1±5.2 |
| 50ETRBL | 3.3±0.2 | 106±15 | >250 | 11.8±0.6 | 61.2±1.2 |
| 50WTRB | 3±0.1 | >167 | >250 | 21.3±3.2 | 34.1±3.4 |
| 50WTRBL | 22.8±0.3 | >167 | >250 | 38.5±1.3 | 142±6 |
| 100WTRB | 4.1±0.2 | >167 | >250 | 39.6±2.5 | 63.5±2.7 |
| 100WTRBL | >100 | >167 | >250 | >167 | >250 |
| TSB | 1.1±0.1 | 64.1±3.0 | >250 | 8.0±0.3 | 56.9±1.9 |
| TSBL | 0.9±0.1 | 56.5±3.2 | 133±6 | 9.0±0.4 | 31.6±3.5 |
| 96TSB | 0.6±0.1 | 33.3±2.4 | 43.7±5.1 | 4.6±0.4 | >250 |
| 50ETSB | 1.3±0.1 | 57.6±2.5 | 167±6 | 8.3±0.4 | 44.6±3.2 |
| 50ETSBL | >100 | >167 | >250 | 20.5±1.0 | >250 |
| 50WTSB | 2.9±0.1 | >167 | >250 | 39.0±1.1 | 37.1±4.8 |
| 50WTSBL | 12.8±0.1 | >167 | >250 | 33.3±0.5 | 45.1±2.8 |
| 100WTSB | 10.2±2.2 | >167 | >250 | 58.5±1.7 | 30.5±6.4 |
| 100WTSBL | >100 | >167 | >250 | >167 | 151±5 |
| TL | 2.5±0.2 | 51.3±0.9 | 249±30 | 17.2±0.6 | 65.2±4.0 |
| TLL | 18.5±1.5 | 116±4 | >250 | 17.7±0.2 | 45.9±1.6 |
| 96TL | 1.7±0.1 | 73.9±2.7 | 47.6±5.3 | 7.5±0.7 | >250 |
| 50ETL | 0.7±0.1 | 54.5±2 | 86.5±9.0 | 5.4±0.2 | 25.2±1.8 |
| 50ETLL | 0.6±0.1 | 36.6±1.2 | 48.2±4.4 | 5.3±0.1 | 52.1±3.0 |
| 50WTL | 3.3±0.1 | >167 | >250 | 48.5±1 | 66.3±4.1 |
| 50WTLL | >100 | >167 | >250 | >167 | >250 |
| 100WTL | 2.5±0.5 | 142±34 | >250 | 20.9±0.7 | 64.9±7.2 |
| 100WTLL | >100 | >167 | >250 | >167 | 66.1±1.4 |
| Acarbose [b] | 105±4 | | | | |
| Quercetin [c] | | 32.1±2.4 | 2.7±0.1 | 3.9±0.2 | |
| BPII [d] | | | | | 14.5±1.5 |

[a] The $IC_{50}$ value (µg/mL) given in the table are presented as mean ± standard deviation (n=3).
[b] Positive control of α-Glucosidase inhibition assay.
[c] Positive control of 15-LO, XO inhibition assays and DPPH scavenging assay.
[d] Positive control of complement fixation assay, a high active polysaccharide from *B. petersianum*.

As mentioned in section 4.2.1, the TPC and TCC of these crude extracts were determined as well, as shown in Table 4.1. A direct correlation analysis among the five biological assays, as well as among TPC, TCC and the biological activities of 27 different extracts was carried out by Pearson's correlation analysis. The results of correlation analysis are shown in Table 4.8. As shown in Table 4.8, α-glucosidase, 15-LO and XO inhibition and DPPH scavenging activity were highly correlated with TPC. Wangensteen and co-authors (2004) also found the TPC has significant correlations with DPPH scavenging activity and 15-LO inhibition activity. In our study, the results of the correlation analyses indicate that the α-glucosidase, 15-LO and XO inhibition and DPPH scavenging activities were expressed mainly by the phenolic compounds. These findings are reasonable, since isolated phenolic compounds from leaves of *T. macroptera* have shown potent 15-LO, XO and α-glucosidase inhibition and DPPH scavenging activities (Pham et al., 2011b, 2014). Thus a screening of phenolic content in *T. macroptera* extracts will probably indicate the presence of compounds with enzyme inhibitory and antioxidant activities.

Generally, the original extracts have higher TPC and TCC than their LMW fraction, as well as activities. Since TPC is response for the enzymes inhibition and DPPH scavenging activities, it is reasonable that the amount of TPC is lower in LMW fractions as well as having lower activities. The TCC in HMW fractions are most probably due to polysaccharides, thus on would expect that the original fractions have higher complement fixation activities than their LMW fractions. Various plant-derived polysaccharides have shown high complement fixation activity (Paulsen & Barsett, 2005); it was of interest to further investigate the polysaccharides in the HMW fractions.

**Table 4.8** Pearson's correlation coefficients (*R*) of biological assays (α-glucosidase, 15-LO and XO inhibition, DPPH scavenging, complement fixation), total phenolic content (TPC) and total carbohydrate content (TCC) of extracts from root bark; stem bark and leaves of *T. macroptera*.

| | TPC | TCC | α-Glucosidase | 15-LO | XO | DPPH | Complement |
|---|---|---|---|---|---|---|---|
| TPC | 1 | 0.279 | 0.861** | 0.827** | 0.677** | 0.895** | 0.127 |
| TCC | | 1 | 0.148 | 0.136 | 0.038 | 0.135 | 0.365 |
| α-Glucosidase | | | 1 | 0.807** | 0.786** | 0.908** | 0.063 |
| LO | | | | 1 | 0.707** | 0.896** | 0.069 |
| XO | | | | | 1 | 0.758** | -0.222 |
| DPPH | | | | | | 1 | 0.095 |
| Complement | | | | | | | 1 |

** Correlation is significant at the 0.01 level (2-tailed).

In our study, five different biological assays were investigated. As shown in Table 4, there are complex relations among the five biological assays. The principal component analysis (PCA) is known to reduce dimensionality of a data set while simultaneously retaining the information present in the original data (Huang et al., 2013). In this case, PCA was applied to process the $IC_{50}$ values for comparative biological activities profiling of different extracts from root bark, stem bark and leaves from *T. macroptera*. The results are given in Table 4.9.

**Table 4.9** Comprehensive principal value and rank of extracts from *T. macroptera*

| | $F_1$ | $F_2$ | F |
|---|---|---|---|
| 96TRB | 7.46 | -1.52 | 4.79 |
| 96TSB | 7.06 | -1.45 | 4.53 |
| 50ETLL | 6.22 | 0.21 | 4.32 |
| 50ETRB | 4.32 | 0.11 | 2.99 |
| 50ETL | 4.14 | 2.14 | 3.31 |
| 96TL | 2.56 | -1.78 | 1.37 |
| TSBL | 2.23 | 1.42 | 1.84 |
| TRB | 2.07 | 0.71 | 1.58 |
| 50ETSB | 1.68 | 0.65 | 1.30 |
| TSB | 1.23 | 0.41 | 0.94 |
| TL | 0.31 | -0.01 | 0.21 |
| 50ETRBL | -0.81 | 0.13 | -0.53 |
| TRBL | -0.96 | 1.58 | -0.31 |
| 100TL | -1.40 | -0.02 | -0.96 |
| TLL | -1.63 | 0.53 | -1.01 |
| 50TRB | -2.18 | 1.11 | -1.26 |
| 50TSB | -2.46 | 0.87 | -1.50 |
| 50TL | -2.60 | -0.17 | -1.82 |
| 100TRB | -2.62 | -0.11 | -1.82 |
| 50ETSBL | -2.67 | -1.46 | -2.15 |
| 50TSBL | -2.79 | 0.44 | -1.82 |
| 50TRBL | -2.91 | -0.88 | -2.19 |
| 100TSB | -2.93 | 1.34 | -1.72 |
| 100TLL | -3.32 | -0.22 | -2.33 |
| 100TSBL | -3.33 | -0.96 | -2.50 |
| 100TRBL | -3.33 | -1.53 | -2.62 |
| 50TLL | -3.33 | -1.53 | -2.62 |

Table 4.9 shows the comprehensive principle value, the higher principal value indicates higher total activities. 96TRB has the highest principal value among 27 different extracts, followed by

extracts 96TSB and 50ETLL, while the lowest principal value was found in 100TRBL and 50TLL. For sustainability, LMW fraction from 50% ethanol-water extracts of leaves obtained by ASE seems preferable. It is, however, noteworthy that some of the hot water extracts have activity in several of the assays. This might be seen as a rationale for the traditional medicinal use of the plant (Pham et al. 2011a). Some of the active constituents of the leaves have been identified (Pham et al. 2011b; Pham et al. 2014). In future work it would be of interest to analyze further active compounds present in all crude extracts.

The $LC_{50}$ values against brine shrimp larvae for the tested 27 extracts were much higher than 100 µg/mL. The extracts demonstrated low toxicity against brine shrimp larvae compared to the positive control, podophyllotoxin (80% lethality at 50 µg/mL).Thus, none of the 27 different extracts are toxic against brine shrimp larvae in the test concentrations. These findings are similar to the results obtained from leaves (Pham et al., 2014).

### 4.3.2 Polysaccharides

Compounds that are capable to suppress or activate the body's immune response can be classified as immunomodulators. Plant-derived polysaccharides, such as pectins, *β*-glucan, heterglycan and arabinans, have been reported to show a variety of immunomodulation activities, affecting both the adaptive and innate immune systems (Paulsen & Barsett, 2005, Yamada & Kiyohara, 2007). More information about immunomodulation activity can be found in section 1.4.3. All purified polysaccharide fractions from *P. biglobosa*, *T. macroptera* and *C. pilosula* were investigated for complement fixation activity, and only four polysaccharide fractions (PBEII-I~PBEII-IV) were tested for macrophage stimulating activity.

#### 4.3.2.1 Complement fixation activity

Regarding effects of pectic polysaccharides on the complement system, there are various reports that the ramified rhamnogalacturonan region has a more potent activity compared to their parent pectin, and that oligogalacturonides have weak or negligible activities. In our study, we found that after pectinase treatment, ramified rhamnogalacturonan regions (50WTRBH-II-Ia, 50WTSBH-II-Ia, 50WTLH-II-Ia and 50WCP-II-Ia) exhibited high activities, while other low *Mw* sub-fractions have weak activities. Yamada and Kiyohara (2007) have suggested that the potent complement fixation activity of the ramified regions is due to a combination of the rhamnogalacturonan core and the neutral sugar chains. However, we found one ramified region,

but not rhamnogalacturonan core, a 1,4-linked galacturonan with 1,4-linked galactan side chains branched on position 2 of GalA (100WCP-II-Ia). As seen in Table 4.10, fraction 100WCP-II-Ia has high complement fixation activity. Yamada and Kiyohara (2007) further proposed that a particular arabino-3,6-galactan structure (AG-II) linked to a rhamnogalacturonan core is necessary for the expression of the complementary fixation activity. In our study, many polysaccharide fractions have AG-II structures exhibiting high complement fixation activity (Table 4.10). However, fraction 100WCP-II-Ib obtained after pectinase treatment of 100WCP-II-I isolated from *C. pilosula*, has no activity in complement fixation assay. The low activity may be due to the low molecular weight. Two pectic polysaccharides isolated from *Opilia celtidifolia*, Oc50A1.I.pur and Oc50A1.II.pur, have similar linkage types, but different *Mw*. Oc50A1.I.pur has higher complement fixation activity and *Mw* than those of Oc50A1.II.pur (Grønhaug et al., 2010). In our study, 50WCP-II-I has higher activity and *Mw* than those of 100WCP-II-I. These findings indicated the *Mw* is an important factor for the expression of complement fixation activity, and the higher *Mw* the higher activity. However, this trend is not always present; many proofs can be found in Table 4.10. Among the seven polysaccharide fractions from *P. biglobosa*, the highest *Mw* fraction PB100II-I exhibited the lowest activity. Therefore, the conclusion should be *Mw* is an important factor, but chemical compositions and structure are also responsible for the expression of complement fixation activity.

As mentioned above, AG-II structure is necessary for the expression of the complementary fixation activity. However, the polysaccharide fractions with AG-II structure in our study still show different activities. For example, fraction 50WTRBH-II-Ia and 50WTLH-II-Ia both have same *Mw* and contain AG-II, but 50WTRBH-II-Ia exhibited higher activity than 50WTLH-II-Ia. This may be due to the fact that AG-I structure was found in 50WTRBH-II-Ia, but not in 50WTLH-II-Ia. Another example can be found between fractions TLD-II-I and 50WTSBH-II-I, 50WTSBH-II-I exhibits higher activity, and contains both AG-I and AG-II structures. These findings indicate the AG-I structure also is a part of the active site of pectic polysaccharides. These can be further proved by the fact that fractions only contain AG-I structure still have high activity, such as fractions PBEII-III, TRBD-I-II and 100WCP-II-Ia. Many polysaccharides with AG-I structure from medicinal plants exhibiting high complement fixation activity have been reported as well (Paulsen & Barsett, 2005).

**Table 4.10** Complement fixation activity, *Mw*, structure fragments and phenolic contents of pectic polysaccharide fractions from *P. biglobosa*, *T. macroptera* and *C. pilosula*

| Original plant | Fractions | $ICH_{50}$(μg/mL) | *Mw*(kDa) | AG-I | AG-II [b] | Yariv test [c] | Phenols (%) |
|---|---|---|---|---|---|---|---|
| *P. biglobosa* | PBEII-I | 4.5 | 303.7 | + | + | + | n.d. |
| | PBEII-II | 28.5 | 91.6 | - | + | + | n.d. |
| | PBEII-III | 31.2 | 23.3 | + | + | - | n.d. |
| | PBEII-IV | 5.2 | 5.9 | - | + | + | 2.4 |
| | PB50II-I | 13.1 | 275.4 | - | + | n.k. | n.k. |
| | PB100I-I | 7.8 | 37.5 | + | + | n.k. | n.k. |
| | PB100II-I | 46.8 | 746.5 | - | + | n.k. | n.k. |
| *T. macroptera* | TRBD-I-I | 9.6 | 71.5 | - | + | + | n.d. |
| | TRBD-I-II | 68.9 | 5.5 | + | - | - | 30.8 |
| | TSBD-II-I | 65.5 | 23.3 | - | + | + | 3.1 |
| | TLD-I-I | 84.5 | 115.9 | - | + | + | 0.9 |
| | TLD-II-I | 74.9 | 23.3 | - | + | + | 0.4 |
| | 50WTRBH-I-I | 5.8 | 136.1 | + | + | + | n.d. |
| | 50WTRBH-II-I | 12.5 | 115.9 | + | + | + | n.d. |
| | 50WTRBH-II-Ia | 7.0 | 19.1 | + | + | + | n.k. |
| | 50WTRBH-II-Ib | 261.5 | 1.6 | n.k. | - | - | n.k. |
| | 50WTRBH-II-Ic | >500 | 0.8 | n.k. | - | - | n.k. |
| | 100WTRBH-I-I | 3.0 | 491.8 | - | - | - | n.d. |
| | 50WTSBH-I-I | 22.6 | 19.8 | + | + | + | 0.4 |
| | 50WTSBH-II-I | 25.1 | 23.2 | + | + | + | n.d. |
| | 50WTSBH-II-Ia | 12.4 | 2.0 | - | - | - | n.k. |
| | 50WTSBH-II-Ib | 34.5 | 1.5 | n.k. | - | - | n.k. |
| | 50WTSBH-II-Ic | >500 | 0.9 | n.k. | - | - | n.k. |
| | 100WTSBH-I-I | 3.1 | 98.7 | + | + | + | n.d. |
| | 50WTLH-I-I | 40.5 | 19.8 | + | + | + | 2.1 |
| | 50WTLH-II-I | 11.6 | 220.3 | + | + | + | n.d. |
| | 50WTLH-II-Ia | 8.7 | 19.1 | - | + | + | n.k. |
| | 50WTLH-II-Ib | 36.4 | 14.2 | n.k. | - | - | n.k. |
| | 50WTLH-II-Ic | >500 | 11.0 | n.k. | - | - | n.k. |
| | 100WTLH-I-I | 8.9 | 136.1 | + | + | + | 0.6 |
| *C. pilosula* | 50WCP-II-I | 28.8 | 71.6 | + | + | + | 1.4 |
| | 50WCP-II-Ia | 5.9 | 17.3 | + | + | + | n.k. |
| | 50WCP-II-Ib | >500 | 0.3 | - | - | - | n.k. |
| | 100WCP-II-I | 29.1 | 53.2 | + | + | + | 0.7 |
| | 100WCP-II-Ia | 11.1 | 17.3 | + | - | - | n.k. |
| | 100WCP-II-Ib | >500 | 1.3 | - | + | + | n.k. |
| | 100WCP-II-Ic | >500 | 0.2 | - | - | - | n.k. |
| Positive control | BPII [a] | 13.1 | 31.0 | - | + | + | n.k. |

[a] Positive control, BPII, a high active polysaccharide from *B. petersianum*

[b] Proposed by presence of 1,3-linked, 1,6-linked and 1,3,6-linked Gal

[c] Detected by the Yariv reagent. +, positive reaction; -, negative reaction.

n.d., not detected; n.k., not known; +, present; -, absent.

From the chemical point of view, AG-II is present in the fractions marked "+" in the Table 4.10, but not all these interact with the Yariv reagent. This may be due to the fact that these fractions contain short chains of 1,3-linked Gal (Kitazawa et al., 2013; Paulsen et al., 2014). Due to the

very low concentration of protein (<1%) in these fractions, it is dubious whether arabinogalactan proteins (AGPs) are present, but some of these fractions may contain a complex between pectins and AGPs in the manner described by Tan et al (2013). In our study, we found some polysaccharide fractions with high phenolic content, such as PBEII-IV, TRBD-I-II, 50WTLH-I-I and 50WCP-II-I. The phenolic compounds present in polysaccharide fractions are most probably due to the phenolic compounds that cross-links between polysaccharides in cell wall (Bunzel et al., 2004), and/or are linked as ester to Ara and Gal in pectins (Livigne et al., 2004). Many phenolic compounds from medicinal plants have been reported to have complement activities (Pieters et al., 1999). In our study, we found the presence of phenolic compounds both increase and decrease the complement fixation activity. For instance, fractions TSBD-II-I and TLD-II-I all have AG-II structure and same *Mw*, but fraction TSBD-II-I has a higher phenolic content and exhibits higher activity than fraction TLD-II-I. However, other example may indicate the presence of phenolic compound decrease the activity. As shown in Table 4.10, 50WTSBH-I-I and 50WTLH-I-I all have AG-I and AG-II structures and with same *Mw*, but fraction 50WTLH-I-I has higher phenolic content, but exhibits lower activity than fraction 50WTSBH-I-I. Therefore, we cannot easily conclude the importance of the effects of total phenolic compounds in our polysaccharide fractions. The effects may differ between various phenolic types and linked positions. It is of interest with further studies on types of phenolic compounds and linked position to polysaccharide fractions in order to find the role of these for the bioactivity.

The most active polysaccharide fraction 100WTRBH-I-I, has different structural features from the other fractions. Ara, AG-I and AG-II structures, and phenolic compounds were not detected in this fraction, but it has a long RG-I region with galactan side chains. In this case, Ara residues are not required for the expression of complement fixation activity of fraction 100WTRNH-I-I. These results are in accordance with the suggestions by Yamada and Kiyohara (2007).

In summary, regarding effects of pectic polysaccharides on the complement system, parameters as *Mw*, ramified regions (RG-I or branched galacturonan), side chains (arabinogalactan and/or galactan) and phenolic compounds, are important for the expression of the activity.

#### 4.3.2.2 Macrophage stimulating activity (Paper I)

As shown in Fig 4.9, fractions PBEII-I, PBEII-II and PBEII-IV showed statistical significant stimulating effects on NO release from macrophages at a concentration of 100 μg/mL. The fraction PBEII-IV induced the strongest release of NO, followed by PBEII-I and PBEII-II.

Inngjerdingen et al. (2008) suggest that the presence of arabinogalactan side chains is part of the structural requirements for the induction of macrophage response. In our study, all four fractions contain arabinogalactan in different amounts. The results indicate there is no correlation between amounts of arabinogalactan (Ara and Gal) and induction of macrophage response.

LPS, a potent stimulator of cells of the monocytic lineages, is a well-known contaminator in biological material. Fraction PBEII-III did not show any activity in this test system, and would probably contain LPS in similar concentrations as the active fractions due to the same isolation and purification procedures being used. It is therefore reasonable to anticipate that LPS is not responsible for the effect seen in the active fractions.

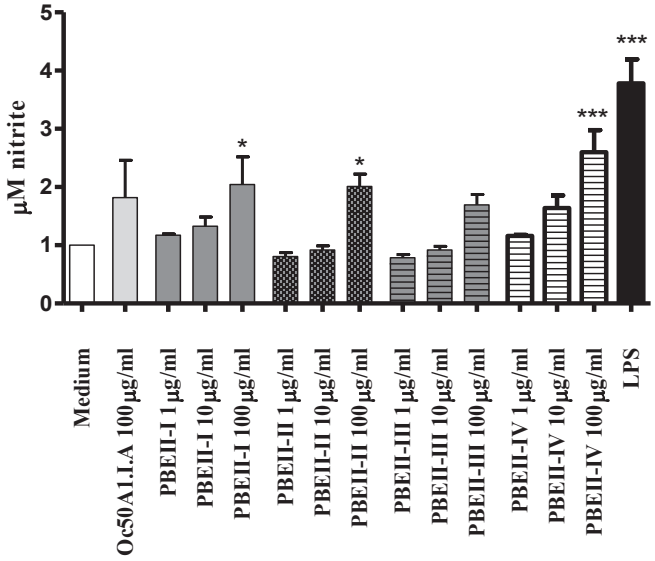


**Fig 4.9.** Measurement of nitric oxide release from macrophages after stimulation with isolated polysaccharide fractions from 50% ethanol-water extract of *P. biglobosa* bark. The polysaccharide fraction Oc50A1.I.A from *Opilia celtidifolia* and LPS were used as positive controls, and medium alone as negative control. Data are presented as the mean of three independent experiments ± SD. *$p<0.05$, ***$p<0.001$.

# 5 Conclusions

In Mali and China, like many developing countries, a high percentage of the population depends on traditional medicine for primary health care. Leaf, stem bark, roots, root bark or whole plant from different medicinal plants or trees were used in different preparations for traditional purposes. Harvesting from the wild, the main source of raw material, is causing a growing concern that it might diminish the populations and lead to local extinctions. The aims of this study are promote the sustainable use of medicinal plant resources, and investigate biological activities of interesting compounds from different plants and plant parts.

Based on complement fixation assay guided fractionation and purification, seven purified pectic polysaccharides fractions were obtained from *P. biglobosa* bark. The acidic fractions PBEII-I and PBEII-IV were the most active in the complement fixation assay, but the other fractions were also potent compared to the positive control BPII. Fractions PBEII-I and PBEII-IV were also the most potent fractions in stimulating macrophages to release nitric oxide. The common structural features of these seven fractions are the RG-I backbone being highly branched with arabinogalactan (AG-I and/or AG-II) as side chains. HG regions may not present in fractions PBEII-III, PBEII-IV and PB100I-I due to the high ratio of Rha to GalA. The higher yield and biological activities of fractions obtained from the 50% ethanol-water extract suggests that this extract could be more related to the medicinal activity than the 50$^{o}$C and 100$^{o}$C water extracts.

27 different crude extracts were obtained by BWE and ASE from root bark, stem bark and leaves of *T. macroptera*. None of the extracts are toxic against brine shrimp larvae in the test concentration. Based on the results from PCA, the ASE ethanol extracts of root bark and stem bark and the LMW fraction of the 50% ethanol-water extract of leaves showed the highest total biological activities, which indicated ASE has higher extraction efficiency than BWE. The results indicate that some activities like antioxidant activity and enzyme inhibition activities are present in the HMW part of our crude extracts. These finding suggest that traditional phytochemistry studies on medicinal plants should also pay attention on HMW compounds.

Totally, fifteen purified pectic polysaccharide fractions were obtained from nine crude extracts from *T. macroptera* (root bark, stem bark and leaves) by using BWE and ASE. The results indicated that BWE provide higher yields of crude extracts with comparable complement fixation activities to the crude extracts isolated with ASE. For the purpose of obtaining purified polysaccharide fractions, ASE was more efficient, as the method provided higher yields, higher

*Mw* polysaccharides, and higher complement fixation activity. The common structural features of these fifteen polysaccharide fractions are 1,4-linked galacturonan, interrupted by RG-I regions with AG-I and/or AG-II side chains. The most active polysaccharide fraction 100WTRBH-I-I, has different structural features from the other fractions. Ara, AG-I and AG-II structures, and phenolic compounds were not detected in this fraction, but it has a long RG-I region with galactan side chains. The structural differences present among these fifteen are *Mw*, amounts of linkage units and position of side chains. These differences may explain the variation of activity in the polysaccharide fractions. The root bark, leaves and stem bark are all good sources for fractions containing bioactive polysaccharides. But due to sustainability, it is preferable to use leaves rather than the other two plant parts, and then the dosage by weight must be higher when using leaves.

Two purified polysaccharide fractions, 50WCP-II-I and 100WCP-II-I, were isolated from 50$^{o}$C and 100$^{o}$C water ASE extracts of cultivated roots of *C. pilosula*. The structure studies of native and sub-fractions showed the 50WCP-II-I is a pectic polysaccharide fraction, with long HG region (some of the GalpA were methyl esterified), interrupted by short RG-I region, the side chains (AG-I and AG-II) of RG-I region are attached on position 4 of Rha. The structural feature of fraction 100WCP-II-I is different from those of 50WCP-II-I, the AG-I side chains are attached on position 2 of GalA, and AG-II side chains are attached on position 4 of Rha in the latter.

We have compared the complement fixation activity of the different pectic polysaccharides obtained, and it became clear that, parameters as *Mw*, ramified regions (RG-I or branched galacturonan), side chains (arabinogalactan and/or galactan) and phenolic compounds, are important for the expression of complement fixation activity.

The observed enzyme inhibition activities, radical-scavenging properties and complement fixation activities may explain some of the traditional uses of *T. macroptera*, such as against diabetes and wound healing. Significant correlations were found among enzyme inhibition (α-glucosidase, 15-LO, XO), DPPH scavenging activity and TPC, thus a screening of phenolic content in *T. macroptera* extracts will probably indicate the presence of compounds with enzyme inhibitory and antioxidant activities. In future work it would be of interest to analyze further active phenolic compounds present in all crude extracts. Future work on immunomodulating polysaccharides obtained would be aimed at degrading by different enzymes and more detailed studies of the structure-activity relationships.

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## Papers

**Paper I**

Yuan-Feng Zou, Bing-Zhao Zhang, Kari Tvete Inngjerdingen, Hilde Barsett, Drissa Diallo, Terje Einar Michaelsen, Elnour El-zoubair, Berit Smestad Paulsen. Polysaccharides with immunomodulating properties from the bark of *Parkia biglobosa*. *Carbohydrate Polymers*, **2014**, 101: 457-463.

**Paper II**

Yuan-Feng Zou, Giang Thanh Thi Ho, Karl Egil Malterud, Nhat Hao Tran Le, Kari Tvete Inngjerdingen, Hilde Barsett, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Enzyme inhibition, antioxidant and immunomodulatory activities, and brine shrimp toxicity of extracts from the root bark, stem bark and leaves of *Terminalia macroptera*. Accepted for publication in *Journal of Ethnopharmacology*.

**Paper III**

Yuan-Feng Zou, Bing-Zhao Zhang, Kari Tvete Inngjerdingen, Hilde Barsett, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Complement activity of polysaccharides from three different plant parts of *Terminalia macroptera* extracted as healers do. Accepted for publication in *Journal of Ethnopharmacology*.

**Paper IV**

Yuan-Feng Zou, Bing-Zhao Zhang, Hilde Barsett, Kari Tvete Inngjerdingen, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Complement fixing polysaccharides from *Terminalia macroptera* root bark, stem bark and leaves. *Molecules,* **2014**, 19 (6): 7440-7458.

**Paper V**

Yuan-Feng Zou, Hilde Barsett, Giang Thanh Thi Ho, Kari Tvete Inngjerdingen, Drissa Diallo, Terje Einar Michaelsen, Berit Smestad Paulsen. Immunomodulating pectins from root bark, stem bark and leaves of the Malian medicinal tree *Terminalia macroptera,* structure activity relations. Accepted for publication in *Carbohydrate Research*.

**Paper VI**

Yuan-Feng Zou, Xing-Fu Chen, Karl Egil Malterud, Frode Rise, Hilde Barsett, Kari Tvete Inngjerdingen, Terje Einar Michaelsen, Berit Smestad Paulsen. Structural features and complement fixing activity of polysaccharides from *Codonopsis pilosula* Nannf. *var. modesta* L.T.Shen roots. Submitted to *Carbohydrate polymers*.

# Paper I

## Polysaccharides with immunomodulating properties from the bark of *Parkia biglobosa*

# Paper II

**Enzyme inhibition, antioxidant and immunomodulatory activities, and brine shrimp toxicity of extracts from the root bark, stem bark and leaves of *Terminalia macroptera*.**

# Paper III

## Complement activity of polysaccharides from three different plant parts of *Terminalia macroptera* extracted as healers do.

# Paper IV

**Complement fixing polysaccharides from *Terminalia macroptera* root bark, stem bark and leaves.**

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*Article*

# Complement Fixing Polysaccharides from *Terminalia macroptera* Root Bark, Stem Bark and Leaves

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**Abstract:** The root bark, stem bark and leaves of *Terminalia macroptera* were sequentially extracted with ethanol, 50% ethanol-water, and 50 °C and 100 °C water using an accelerated solvent extractor. Ten bioactive purified polysaccharide fractions were obtained from those crude extracts after anion exchange chromatography and gel filtration. The polysaccharides and their native extracts were characterized with respect to molecular weight, chemical compositions and effects in the complement assay. The chemical compositions showed that the polysaccharides are of pectic nature. The results indicated that there was no great difference of the complement fixation activities in the crude extracts from the different plant parts when extracting with the accelerated solvent extraction system. The purified polysaccharide fractions 100WTSBH-I-I and 100WTRBH-I-I isolated from the 100 °C water extracts of stem and root bark respectively, showed the highest complement fixation activities. These two fractions have rhamnogalacturonan type I backbone, but only 100WTSBH-I-I contains side chains of both arabinogalactan type I and II. Based on the yield and activities of the fractions studied those from the root bark gave highest results, followed by those from leaves and stem bark. But in total, all plant materials are good sources for fractions containing bioactive polysaccharides.

**Keywords:** *Terminalia macroptera*; accelerated solvent extraction; polysaccharides; complement fixation activity

## Abbreviations

AG-I, arabinogalactan type I; AG-II, arabinogalactan type II; Ara, arabinose; ASE, accelerated solvent extraction; DCM, dichloromethane; EtOH, ethanol; Gal, galactose; GalA, galacturonic acid; Glc, glucose; GlcA, glucuronic acid; GF, gel filtration; HMW, high molecular weight; IEC, ion exchange chromatography; LMW, low molecular weight; Man, mannose; MeOH, methanol; RG-I, rhamnogalacturonan type I; Rha, rhamnose; Xyl, xylose.

## 1. Introduction

*Terminalia macroptera* Guill. & Perr. (Combretaceae) is a tree, up to 20 m high, which occurs widely in West Africa. In Mali *T. macroptera* is used against a variety of ailments, and more than 30 different indications have been mentioned by the traditional healers in ethnopharmacological studies. The stem bark and leaves are most commonly used against sores and wounds, pain, cough, tuberculosis and hepatitis [1]. The roots are used against hepatitis, gonorrhea and various infectious diseases, including *H. pylori*-associated diseases [1–5]. Flavonoids [6–8], triterpenoids [9,10], ellagitannins [11] and related phenolics [3,9,12], have been identified from different parts of *T. macroptera*. Ellagitannins are known antimicrobial compounds which may be related to the use of the leaves against wounds and infections [13]. Water decoctions of *T. macroptera*, administered orally, are the most common preparations used by the traditional healers in Mali [1]. Plant polysaccharides isolated from crude water extracts have shown effects related to the immune system by different *in vitro* and *in vivo* test systems [14]. The chemical characteristics and biological activities of polysaccharides, especially those from plants used in the treatment of wounds, ulcer and cancer have been reported [15–19]. Thus, it was of interest to investigate the bioactive polysaccharides from water extracts of *T. macroptera*.

Traditionally in laboratory studies, low molecular weight and lipophilic compounds are extracted from plant material by the Soxhlet extraction method. Accelerated solvent extraction (ASE) for these type of compounds was first described in 1995 [20,21], and the method has grown steadily in use since that time [22]. Under elevated temperature and pressure, an extraction solvent can be used above its boiling point but still remain in the liquid state, and thus increasing the kinetics of the extraction process. In this case, solvent consumption and extraction times are significantly decreased [23]. ASE has been applied for extracting components from environment samples [24–27], biological materials [28], plant materials [29–33], dietary compounds[34], feeds [35,36], and food [37,38]. However, reports on the use of ASE for polysaccharide extraction mainly from woods [39–41] have only recently been reported. Thus, it was of interest to investigate the isolation of bioactive polysaccharides from medicinal plant by ASE.

Root bark, stem bark and leaves are used in traditional medicine in Mali against several ailments; among those are illnesses where the immune system is involved quite frequent. Due to the use of all plant parts, it was relevant to investigate if polysaccharides from the three different plant parts had similar type of bioactivity and structural features. If this was the case, the recommendations should be to

rather use the leaves than the other plant parts as this will lead to a more sustainable use of the tree. If e.g., roots are overused, eradication of trees may be the result. Therefore, in this study, ASE was employed to extract polysaccharides from root bark, stem bark and leaves from *T. macroptera*. The aims of this study are comparison of the properties of the polysaccharides from the different plant parts, as well as relationships between the chemical characteristics and complement fixation activities. Crude polysaccharide extracts were obtained and further purified, the chemical characteristics and complement fixation activities of polysaccharide fractions were evaluated, and the results from the three different plant parts were compared.

## 2. Results and Discussion

### *2.1. Crude Extracts*

#### 2.1.1. Yields

The yields of the crude water extracts from the root bark (50WTRBH and 100WTRBH) obtained from ASE described, were 0.6% and 2.5% respectively. The yields of the crude water extracts from stem bark (50WTSBH and 100WTSBH) were 0.5% and 1.3%. The yields of the crude water extracts from leaves (50WTLH and 100WTLH) were 0.3% and 0.6%. All of the yields given in brackets in Table 1 are related to the dried, powdered materials. Extraction with ASE showed that the root bark gave higher yields of crude extracts than what was obtained from the stem bark and leaves.

**Table 1.** Monosaccharide compositions of crude water extracts from root bark, stem bark and leaves of *T. macroptera*.

| | 50WTRBH | 100WTRBH | 50WTSBH | 100WTSBH | 50WTLH | 100WTLH |
|---|---|---|---|---|---|---|
| Ara [a] | 5.9 | 0.4 | 14.0 | 0.6 | 15.3 | 10.2 |
| Rha [a] | 11.8 | 0.6 | 9.5 | 1.3 | 7.7 | 2.4 |
| Xyl [a] | 2.1 | 0.3 | 4.7 | 0.7 | 3.3 | 2.9 |
| Man [a] | 1.0 | 0.2 | 0.6 | Trace | 2.0 | 7.8 |
| Gal [a] | 20.2 | 1.1 | 23.7 | 1.3 | 18.5 | 8.4 |
| Glc [a] | 36.5 | 97.4 | 23.3 | 92.1 | 14.8 | 49.8 |
| GlcA [a] | 1.0 | n.d. | 4.3 | Trace | 2.6 | Trace |
| GalA [a] | 21.4 | Trace | 19.9 | 4.0 | 35.8 | 18.4 |
| Yield (% w/w) [b] | 0.6 | 2.5 | 0.5 | 1.3 | 0.3 | 0.6 |
| Presence of starch | + | + | + | + | + | + |

[a] mol% related to total content of the monosaccharides Ara, Rha, Xyl, Man, Gal, Glc, GlcA and GalA; [b] yield related to the dried, powdered materials; n.d. not detected.

#### 2.1.2. Chemical Compositions

After methanolysis, the monosaccharide compositions of the crude water extracts were analyzed by GC as the TMS derivatives of the methyl-glycosides. As can be seen from Table 1, the monosaccharide compositions of extracts from 50 °C water have similarities, but also differences. The compositions of the polymers in 50WTRBH and 50WTSBH and 50WTLH are quite similar, containing the neutral monosaccharides arabinose (Ara), rhamnose (Rha), galactose (Gal) and glucose (Glc), albeit in different

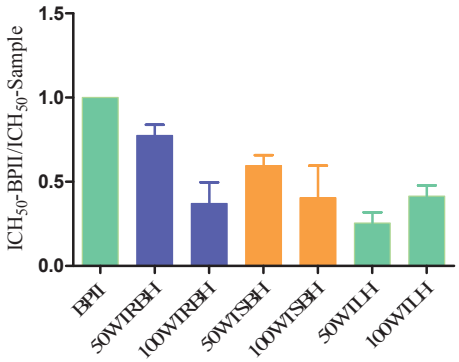
amounts. In addition to the neutral monosaccharides, the 50WTRBH, 50WTSBH and 50WTLH also contain galacturonic acid (GalA). The crude extracts 100WTRBH and 100WTSBH contain more than 90 mol% of Glc. The glucose detected in the crude extracts most probably comes from starch as the iodine test gave a strong positive reaction (Table 1).

2.1.3. Complement Fixation Activity

The complement system is an important part of the immune defense, such as primary defense against bacterial invasions and viral infections. Complement fixating activity of polysaccharides from plants has previously been shown as an indicator for effects on the immune system [42,43].

As can be seen from Figure 1, the crude water extracts from ASE of root bark, stem bark and leaves showed potent human complement fixation activities *in vitro*. The activity of the crude water extract 50WRB was slightly higher than other crude extracts, but all have slightly lower activities than the very active, positive control BPII. The crude water extracts from 100 °C of root bark (100WTRBH), stem bark (100WTSBH) and leaves (100WTLH) showed similar complement fixation activities.

**Figure 1.** Complement-fixating activities of the crude water extracts obtained by ASE from root bark, stem bark and leaves of *T. macroptera* related to positive control (BP II from *Biophytum petersianum*). $ICH_{50}$-BPII/ $ICH_{50}$-Sample shows how active each individual test sample is compared to the positive control BPII.




*2.2. Studies on Purified Polysaccharide Fractions*

The crude extracts were further purified by ion exchange chromatography and the isolated sub-fractions with activity in the complement fixation assay, were subjected to gel filtration. The purified fractions thus obtained where the objects for further studies as given below.

2.2.1. Yields

Three active purified polysaccharide fractions, 50WTRBH-I-I, 50WRBH-II-I and 100WRBH-I-I, were obtained from root bark crude extracts. The stem bark crude water extracts gave four active fractions; 50WTSBH-I-I and 50WTSBH-II-I were isolated from 50WTSBH and two 100WTSBH-I-I and 100WTSBH-III-I from 100WTSBH. Using the same purification procedure for the leaf

polysaccharides the following fractions were obtained: two from 50WTLH (50WTLH-I-I and 50WTLH-II-I) and one from 100WTLH (100WTLH-I-I). The total yields of purified polysaccharide fractions from stem bark were higher than those from root bark and leaves. All of the yields given in Table 2 are based on the dried, powdered materials.

#### 2.2.2. Chemical Compositions

The characterization of the purified polysaccharide fractions are given in Table 2. All the purified fractions contain the monosaccharides that are typical constituents in pectic polysaccharides (Table 2). The presences of AG-II in the root bark polysaccharides 50WTRBH-I-I and 50WTRBH-II-I and all the purified active polysaccharide fractions from the stem bark and leaves were identified by the Yariv-test. Purification of the polysaccharide fractions gave a huge reduction in the content of Glc. Still some purified fractions (100WTRBH-I-I, 100WTSBH-I-I, 100WTSBH-III-I and 100WTLH-I-I) from 100 °C water extracts have rather high amounts of Glc which most probably comes from starch, as positive reactions were observed in the iodine test. The monosaccharide compositions of the purified polysaccharide fraction 100WTRBH-I-I were quite different from other fractions, such as absence of Ara, Xyl and GlcA, and higher amount of Glc.

The Bio-Rad protein assay showed negligible amounts (<1%) of protein present in the fractions. Phenolic compounds were found in fraction 100WTSBH-III-I (3.0%), determined by the Folin-Ciocalteu assay, while only minor amounts were present in fractions 50WTSBH-I-I, 50WTLH-I-I and 100WTLH-I-I. Totally, the phenolic contents of the purified fractions from stem bark were slightly higher than those from leaves, while none was detected in the fractions from root bark.

#### 2.2.3. Molecular Weight Distribution

Size exclusion chromatography, using dextran standards, was employed to determine the average $M_W$ of the purified fractions. The highest molecular weight was found in the fraction 100WRB-I-I (491.8 kDa), while the lowest molecular weight (19.8 kDa) was found in the fractions 50WTLH-I-I and 50WTSBH-I-I. Generally, the molecular weights of most of the purified fractions from root bark and leaves are higher than those from stem bark.

#### 2.2.4. Complement Fixation Activity

As can be seen from Figure 2, the purified polysaccharide fractions showed potent human complement fixation activities *in vitro*. After fractionation, all purified polysaccharide fractions from the root bark, showed higher activity compared to the positive control BPII, the fraction 100WTRBH-I-I being the most potent. The fractions from stem bark were potent, but only fraction 100WTSBH-I-I was more active on weight basis than BPII. Considering the purified fractions from leaves, 50WTLH-II-I and 100WTLH-I-I showed higher activity than BPII, and 100WTLH-I-I was the most potent one.

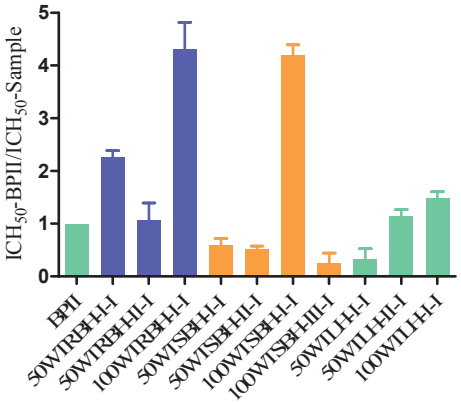
Totally, among the purified fractions, 100WTRBH-I-I and 100WTSBH-I-I, were the most potent fractions, showing almost the same activity. The other fractions from root bark were more active than the fractions from stem bark. The fractions 50WTRBH-I-I and 100WTRBH-I-I from root bark showed both higher activities than the purified fractions from leaves.

**Table 2.** Characterizations of polysaccharide fractions isolated from root bark, stem bark and leaves of *T. macroptera* after ion exchange chromatography and gel filtration.

| | 50WTRBH-I-I | 50WTRBH-II-I | 100WTRBH-I-I | 50WTSBH-I-I | 50WTSBH-II-I | 100WTSBH-I-I | 100WTSBH-III-I | 50WTLH-I-I | 50WTLH-II-I | 100WTLH-I-I |
|---|---|---|---|---|---|---|---|---|---|---|
| Ara [a] | 19.6 | 7.1 | n.d. | 15.4 | 10.8 | 16.4 | 9.7 | 15.2 | 11.7 | 25.4 |
| Rha [a] | 3.5 | 29.1 | 23.6 | 4.3 | 18.4 | 4.3 | 14.4 | 2.3 | 12.5 | 3.1 |
| Xyl [a] | 6.4 | 2.3 | n.d. | 21.4 | 4.3 | 1.6 | 4.2 | 34.8 | 1.5 | 23.9 |
| Man [a] | 1.0 | 2.2 | 1.0 | 2.6 | 0.4 | 0.8 | n.d. | 2.7 | 0.3 | 4.1 |
| Gal [a] | 48.3 | 15.3 | 3.7 | 34.7 | 16.2 | 19.3 | 7.5 | 21.2 | 10.7 | 23.6 |
| Glc [a] | 2.1 | 1.6 | 25.8 | 3.6 | 2.7 | 19.0 | 9.5 | 7.1 | 2.2 | 7.2 |
| GlcA [a] | 2.1 | 3.1 | n.d. | 2.8 | 3.4 | 1.7 | 4.9 | 0.9 | 5.9 | 0.3 |
| GalA [a] | 17.0 | 39.2 | 45.9 | 15.1 | 43.6 | 36.9 | 49.9 | 15.8 | 55.2 | 12.4 |
| Yield (% w/w) [b] | 0.001 | 0.01 | 0.01 | 0.003 | 0.014 | 0.003 | 0.033 | 0.003 | 0.016 | 0.01 |
| Mw (kDa) | 136.1 | 115.9 | 491.8 | 19.8 | 23.2 | 98.7 | 44.2 | 19.8 | 220.3 | 136.1 |
| The Yariv test [c] | ++ | ++ | - | + | ++ | ++ | ++ | + | ++ | + |
| Presence of starch | - | - | + | - | - | + | + | + | - | + |
| Protein (% w/w) | 0.1 | n.d. | n.d. | 0.9 | n.d. | n.d. | 0.7 | 0.3 | 0.2 | n.d. |
| Phenols (% w/w) [d] | n.d. | n.d. | n.d. | 0.4 | n.d. | n.d. | 3.0 | 2.1 | n.d. | 0.6 |

[a] mol% related to total content of the monosaccharides Ara, Rha, Xyl, Man, Gal, Glc, GlcA and GalA; [b] yield related to the dried, powdered material; [c] The presence of arabinogalactans type II (AG-II) was identified by precipitation with the β-glycosyl Yariv reagent; [d] The total phenolic content is expressed as ferulic acid equivalents; n.d. not detected.

**Figure 2.** Complement-fixating activities of purified polysaccharide fractions obtained from root bark, stem bark and leaves of *T. macroptera* related to positive control BPII. $ICH_{50}$-BPII/ $ICH_{50}$-Sample shows how active each individual test sample is compared to the positive control BPII.




2.2.5. Linkage Analysis of the Polysaccharide Fractions

The most active fractions from each plant part, 100WTRBH-I-I from root bark, 100WTSBH-I-I from stem bark and 100WTLH-I-I from leaves, were selected for linkage analysis. In order to determine the nature of the glycosidic linkages of the different monosaccharides in the purified fractions, permethylation of the reduced polymers was performed, partially O-methylated alditol acetates (PMAAs) were prepared and subjected to GC-MS. The results are given in Table 3.

The main structural feature of 100WTSBH-I-I and 100WTLH-I-I are similar, having 1,4-linked galacturonan, with a few branch points in position 3 of GalA. The Rha units are 1,2-linked, with branch points on position 4. The low ratio of Rha to GalA indicate that the backbone of the polysaccharide fractions consist of shorter RG-I structures, and longer homogalacturonan regions. The Gal and Ara present have the normal type of linkages that are found in the AG II side chain [44]. The presence of 1,3-linked and 1,3,6-linked Gal indicate the presence of AG-II structures in these two fractions, as they gave positive reactions in the Yariv test. The presence of 1,4-linked Gal may indicate the presence of AG-I in these two fractions [14]. The Xyl is present as 1,4-linked unit in fraction 100WTL-I-I, and lower amount was present in 100WTSBH-I-I. These features have certain similarities with pectins that are composed of areas with hairy or ramified and smoother regions [45]. GlcA appearing in these two fractions are mainly terminally linked units. Terminal GlcA might be directly linked to position 3 of 1,4-linked GalA in the RG-I backbone, or may also be a part of the AG-II side chains [46,47].

The structural feature of 100WTRBH-I-I was quite different from other two fractions. The main structural feature of 100WTRBH-I-I is the presence of RG-I, but without AG-I/II since Ara is absent and only a low amount of Gal is found. The higher ratio of 1,2-linked Rha to 1,4-linked GalA indicate that the backbone of the 100WTRBH-I-I consists of longer RG-I structures, and shorter homogalacturonan regions. High amount of Glc, mainly as 1,4-linked and small amount of 1,4,6-linked may indicate that they come from starch as positive reactions were observed in the iodine test. This could be due to a

physical binding between RG-I backbone with a few Gal units and starch bound so tightly that they could not be separated by the method used.

**Table 3.** The linkages (mol%) of the monosaccharides present in the most active purified fractions from root bark, stem bark and leaves of T. macroptera determined by GC–MS after methylation.

| | | 100WTRBH-I-I | 100WTSBH-I-I | 100WTLH-I-I |
|---|---|---|---|---|
| Ara | T*f* | n.d. | 10.2 | 14.6 |
| | 1,2*f* | n.d. | 0.1 | 1.2 |
| | 1,3*f* | n.d. | 0.2 | 0.7 |
| | 1,5*f* | n.d. | 4.3 | 6.7 |
| | 1,3,5*f* | n.d. | 1.6 | 2.1 |
| Rha | T*p* | n.d. | 1.5 | 1.3 |
| | 1,3*p* | 1.3 | 0.1 | 0.1 |
| | 1,2*p* | 21.3 | 0.5 | n.d. |
| | 1,2,4*p* | 1.0 | 2.2 | 1.7 |
| Xyl | T*p* | n.d. | n.d. | Trace |
| | 1,4*p* | n.d. | 1.5 | 23.8 |
| Gal | T*p* | 1.2 | 3.4 | 2.1 |
| | 1,4*p* | n.d. | 0.5 | 3.3 |
| | 1,3*p* | 2.1 | 1.2 | 4.8 |
| | 1,6*p* | n.d. | 9.9 | 2.1 |
| | 1,3,6*p* | 0.4 | 3.1 | 10.7 |
| | 1,3,4,6*p* | n.d. | 0.6 | 0.3 |
| Glc | 1,3*p* | n.d. | 0.7 | 2.3 |
| | 1,4*p* | 21.8 | 13.9 | 3.5 |
| | 1,6*p* | 4.0 | 3.8 | 0.7 |
| | 1,4,6*p* | Trace | 0.4 | 0.5 |
| GlcA | T*p* | n.d. | 1.0 | 0.3 |
| | 1,4*p* | n.d. | 0.7 | n.d. |
| GalA | T*p* | 1.3 | n.d. | n.d. |
| | 1,4*p* | 44.6 | 34.6 | 12.3 |
| | 1,3,4*p* | n.d. | 2.3 | 0.1 |

### *2.3. Discussion*

*T. macroptera* root bark, stem bark and leaves are used against a variety of ailments, such as against sores and wounds, pain, cough, tuberculosis and hepatitis [1]. Medicinal plants used for wound healing often appear to be rich in polysaccharides, which may be responsible of their wound healing properties [48]. The ASE is a highly efficient extraction method which significantly reduce the extraction time and amount of solvents used. It was therefore of interest to study the structure and biological activity of polysaccharides from root bark, stem bark and leaves of *T. macroptera* obtained by ASE.

The chemical and biological characteristics of crude water extracts from different plant parts had some similarities, but also differences. All the crude water extracts showed similar potent complement

fixation activities. All the crude extracts were fractionated by ion exchange chromatography and gel filtration as described, and led to the isolation of ten active sub-fractions with different molecular weights. The chemical compositions of these sub-fractions were quite different (Table 2), but all contained monosaccharides typical for pectic type polysaccharides. The purification procedure induces a huge amount of Glc in all fractions, which lead the increase of complement fixation activities after removal of the glucose polymers. But still some Glc polymer were present in some of the fractions, this could be due to a RG-I backbone this could be due to a RG-I backbone with a few Gal units attached in addition to starch, that physically bound tightly to the polymer and could not be removed by the method used.

It has been reported that acidic polysaccharides with higher molecular weights appear to be more active in the complement assay than those with lower molecular weights [49–51]. Among the ten sub-fractions in our present study, fraction 100WTRBH-I-I with the highest molecular weight exhibited the highest activity, but the other fractions with different molecular weight did not follow this trend.

In addition to molecular weight differences, the type of monosaccharide linkages might be another reason for the influence on the complement fixation activity. Pectins are generally known to be composed of linear homogalacturonan (HG) regions and branched rhamnogalacturonan (RG) I and II regions [52]. The side chains of RG-I consist usually of arabinogalactan (AG) type I and/or II, as well as arabinan and galactan. The branched regions of the pectins are thought to be related to their immunomodulating activities. Polysaccharides rich in AG-II have shown effects in a number of biological assays [14,18,49,53]. The results of the Yariv-test showed that all purified active fractions contain AG-II structures, except 100WTRBH-I-I from the root bark. The fraction 100WTLH-I-I is highly ramified compared to other two fractions. As the RG-I backbone generally consists of alternating units of Rha and GalA, the low Rha to GalA ratio indicates that the backbone of the polysaccharide fractions consist of shorter RG-I structures, and longer homogalacturonan regions. RG-I regi ith a few Gal units attached in addition to starch, that physically bound tightly to the polymer and could not be removed by the method used.

It has been reported that acidic polysaccharides with higher molecular weights appear to be more active in the complement assay than those with lower molecular weights [49–51]. Among the ten sub-fractions in our present study, fraction 100WTRBH-I-I with the highest molecular weight exhibited the highest activity, but the other fractions with different molecular weight did not follow this trend.

In addition to molecular weight differences, the type of monosaccharide linkages might be another reason for the influence on the complement fixation activity. Pectins are generally ons were present in all fractions, but with different lengths. Sub-fraction 100WTRBH-I-I may contain longer RG-I region than other sub-fractions as deducted from the linkages shown in Table 3. Sub-fractions 100WTSBH-I-I and 100WTLH-I-I may contain AG-I due to presence of 1,4-linked Gal units. The structural feature of positive control BPII is 1,4-linked galacturonan with branching on position 3, interrupted by RG-I region, side chains (AG-II) branched on position 4 of Rha [54]. The main structural features of 100WTSBH-I-I and 100WTLH-I-I are similar, having 1,4-linked galacturonan with branching on position 3, interrupted by RG-I region, side chains (AG-I and AG-II) branched on position 4 of Rha. The structural feature of fraction 100WTRBH-I-I is 1,4-linked galacturonan, interrupted by a long RG-I region, side chains (galactan) branched on position 4 of Rha. These similarities between BPII and our polysaccharide fractions may explain the possibility of our samples exhibited potent

complement fixation activities. In addition, our samples, 100WTRBH-I-I (491.8 kDa), 100WTSBH-I-I (98.7 kDa) and 100WTLH-I-I (136.1 kDa), has higher *Mw* than BPII (31kDa), which may explain why they showed stronger complement fixation activities than BPII.

Various immunomodulating polysaccharides isolated from plants (*Opilia celtidifolia*, *Vernonia kotschyana, Brassica oleracea*) also contain protein and phenolic compounds [51,55,56]. Many phenolic compounds were showed with potent complement activity, as reviewed by Pieters [57]. The presence of phenolic substances in the purified fractions might be due to ferulic acid being linked as ester to Ara and Gal in pectins [58]. The sub-fractions 100WTSBH-III-I and 50WTLH-I-I contain higher amounts of protein and phenolic compounds than other sub-fractions, which may explain part of the observed complement fixation activity in the lower molecular weight sub-fractions.

## 3. Experimental

### *3.1. Plant Material*

The root bark, stem bark and leaves of *T. macroptera* were collected in Mali, December 2011, and identified by the Department of Traditional Medicine (DMT), Mali. A voucher specimen is deposited at the herbarium of DMT (Voucher No. 2468/DMT). The plant material was washed, cut into small pieces, dried and pulverized to a fine powder by a mechanical grinder.

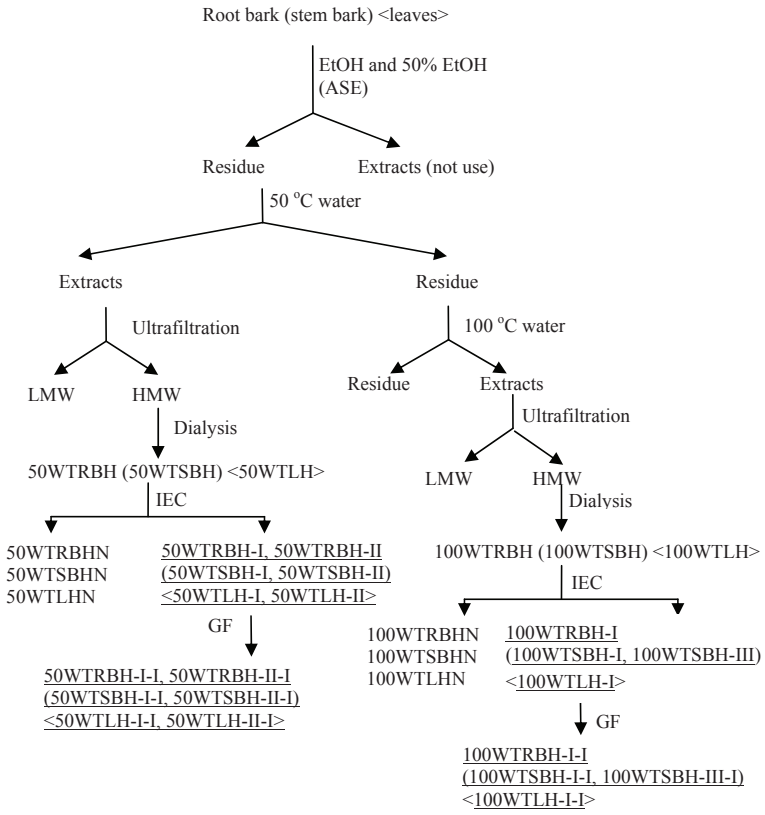
### *3.2. Extraction of Polysaccharides*

ASE was performed on a Dionex ASE350 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA, USA). Powdered root bark, stem bark or leaves (200 g of each) were weighed and mixed with diatomaceous earth (50 g), and then packed in eight 100 mL stainless steel cells. The extractions were performed at 1,500 psi, with 5 min heating, 5 min static time, and a 60 s purge for a total of three cycles. In order to remove low molecular weight compounds, the cells were pre-extracted twice with 96% ethanol (EtOH) at 70 °C. The cells were further extracted twice with 50% ethanol-water at 70 °C, followed by distilled water of 50 °C and 100 °C. After extraction the water extracts were subjected to ultrafiltration (cut off 5,000 Da), and the high molecular weight (HMW) fraction was concentrated and dialyzed at cut-off 3,500 Da, lyophilized and kept for further studies. The crude water extracts were named 50WRBH and 100WRBH for the root bark, and 50WSBH and 100WSBH for the stem bark, and 50WTLH and 100WTLH for leaves (Figure 3). These fractions were subjected to determination of monosaccharide composition, starch content investigation and complement fixation assay.

### *3.3. Fractionation and Characterization of Polysaccharides*

The crude extracts were further fractionated by anion exchange and gel filtration. All purified fractions were subjected to determination of their chemical and biological characteristics.

**Figure 3.** Extraction and fractionation scheme of polysaccharides extracted by accelerated solvent extraction (ASE) from root bark, stem bark and leaves of *T. macroptera* (IEC, ion exchange chromatography; GF, gel filtration; underlined acidic fractions showed high complement fixation activity and were fractionated for further studies).




3.3.1. Ion Exchange Chromatography and Gel Filtration

The crude extracts from ASE were filtered through 0.45 μm filters and applied to an anion exchange column packed with ANX Sepharose™ 4 Fast Flow (high sub) (GE Healthcare, Uppsala, Sweden). The neutral fractions were eluted with distilled water at (2 mL/min), while the acidic fractions were eluted with a linear NaCl gradient in water (0–1.5 M) at 2 mL/min. The carbohydrate elution profiles were monitored using the phenol-sulfuric acid method [59]. The related fractions were pooled, dialyzed at cut-off 3500 Da against distilled water for removal of NaCl, and lyophilized (Figure 3)

The acidic fractions marked in Figure 3 were dissolved in elution buffer (10 mM NaCl), filtered through a Millipore filter (0.45 μm), and subjected to gel filtration after application on a Hiload™ 26/60 Superdex™ 200 prep grade column (GE Healthcare) combined with the Äkta system (FPLC, Pharmacia Äkta, Amersham Pharmacia Biotech, Uppsala, Sweden), and eluted with 10 mM NaCl at 1.0 mL/min. Fractions were pooled based on their elution profiles, as determined by the phenol-sulfuric acid method, dialyzed and lyophilized.

3.3.2. Determination of Monosaccharide Composition

The monosaccharide compositions of the crude extracts and purified fractions were determined by gas chromatography of the trimethylsilylated (TMS) derivatives of the methyl-glycosides obtained after methanolysis with 3 M hydrochloric acid in anhydrous methanol for 24 h at 80 °C [60–62]. Mannitol was used as an internal standard. The TMS derivatives were analyzed by capillary gas chromatography on a Focus GC (Thermo Scientific, Milan, Italy).

3.3.3. Test for the Presence of Starch

The presence of starch in the fractions was tested by adding two drops of an aqueous iodine-potassium-iodide solution to the samples [63]. A positive reaction gives a dark bluish color. Starch was used as a positive control.

3.3.4. Molecular Weight Determination

The homogeneity and molecular weight of the purified polysaccharide fractions were determined by size exclusion chromatography on a Hiload™ 16/60 Superdex™ 200 prep grade column (GE Healthcare) combined with the Äkta system (FPLC, Pharmacia Äkta). Dextran polymers (Pharmacia) B512 (5.6 kDa), T8360 (19 kDa), T250 (233 kDa) and T500 (475 kDa) were used as calibration standards. Approximately 5 mg of the samples were dissolved in 2 mL of 10 mM NaCl buffer and filtered through a Millipore filter (0.45 μm) and applied to the column. The samples were eluted with 10 mM NaCl at 0.5 mL/min, collecting 2 mL fractions. The eluent was detected with a Shimadzu RI detector. The retention volume was converted to molecular weight by using the standards.

3.3.5. Precipitation with the Yariv β-Glucosyl Reagent

Precipitation with the Yariv β-glucosyl reagent was performed on the samples as described by van Holst and Clarke [64]. The Yariv β-glucosyl reagent forms a colored precipitate with compounds containing AG-II structures. A solution of Arabic gum in water (1 mg/mL) was used as a positive control.

3.3.6. Determination of Phenolic Content

The total amount of phenolic compounds in the purified polysaccharide fractions were quantitatively determined using the Folin-Ciocalteu assay [65]. 200 microliter sample (1 mg/mL) dissolved in water (three replicates) was added the same amount of Folin-Ciocalteu's phenol reagent (1:1 in water, Merck/Kebo), mixed and left for 3 min at room temperature. 200 microliter of 1 M $Na_2CO_3$ was added; the tubes were mixed and allowed to stand for 1 h. The absorbance was measured at 750 nm. A standard curve was plotted using ferulic acid. The total phenolic content was determined as ferulic acid equivalents (FA/sample) × 100%.

### 3.3.7. Determination of Protein Content

The protein content of the polysaccharide fractions was determined by the Bio-Rad protein assay, based on the method of Bradford (Bio-Rad, Hercules, CA, USA) [66]. The standard procedure for microliter plates was used with bovine serum albumin (BSA) as a protein standard in a concentration range from 15 to 500 μg/mL. The Bio-Rad protein assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs.

### 3.3.8. Complement Fixation Assay

The complement system is an important part of the innate immune system which also cooperates with the adaptive immune system in many ways [67]. Complement among other things play a direct part in the defence, such as primary defence against bacterial invasions and viral infections. Complement fixating activity of polysaccharides from plants has previously been used as an indicator for effect on the immune system [42,43]. Many resent publications support the notion that pectins convey at least some of the beneficial effect of medicinal plants and that this might be based on the interaction with the immune system [18].

The complement fixation test is based on inhibition of hemolysis of antibody sensitized sheep red blood cells (SRBC) by human sera as described by Michaelsen *et al.* (Method A) [42]. It is a quick, highly reproducible assay performed in microtiter plates with many samples analysed simultaneously and with positive control. BPII, a highly active pectic polysaccharide from the aerial parts of *Biophytum petersianum* Klotzsch (also known as *B. umbraculum*) [68], was used as a positive control. The indicator system in the assay is inhibition of haemolysis induced by human complement. Samples showing inhibition in the assay is thus having a direct effect on the human immune system. Inhibition of lysis induced by the test samples was calculated by the formula [(Acontrol-Atest)/Acontrol] × 100%. From these data, a dose-response curve was created to calculate the concentration of test sample giving 50% inhibition of lysis ($ICH_{50}$). A low $ICH_{50}$ value means a high complement fixation activity. The activity of all the polysaccharide fractions are given as the $ICH_{50}$ value of the positive control BP-II divided on the $ICH_{50}$ value of the sample.

### 3.3.9. Determination of Glycosidic Linkages

Glycosidic linkage elucidation was performed by methylation studies. The most active fractions from each plant part were selected for methylation. Prior to methylation, the free uronic acids were reduced with $NaBD_4$ to their corresponding neutral sugars. After reduction of the polymers, methylation, hydrolysis, reduction and acetylation [69] were carried out. The derivatives were analyzed by GC-MS using a GCMS-QP2010 (Shimadzu, Kyoto, Japan) attached to a Restek Rxi-5MS (30 m; 0.25 mm i.d.; 0.25 μm film) column. The injector temperature was 280 °C, the ion source temperature 200 °C and the interface temperature 300 °C. The column temperature was 80 °C when injected, then increased with 10 °C/min to 140 °C, followed by 4 °C/min to 210 °C and then 20 °C/min to 300 °C. Helium was the carrier gas (pressure control: 80 kPa.) The compound at each peak was characterized by an interpretation

of the retention times and the characteristic mass spectra. The estimation of the relative amounts of each linkage type was related to the total amount of each monosaccharide type as determined by methanolysis. Effective carbon-response factors were applied for quantification [70].

## 4. Conclusions

The root bark, stem bark and leaves of *T. macroptera* were traditionally used against wounds and various infection diseases. All these ailments involve the immune system. Ten purified polysaccharide fractions were isolated from six different crude water extracts from root bark, stem bark and leaves. These polysaccharide fractions all exhibited potent complement fixation activities. The complement system plays a direct part in the defense, such as primary defense against bacterial invasions and viral infections. Therefore, the traditional use of this tree to against wounds and various infection diseases may be, at least partly, connected to the complement system. Comparing the polysaccharide fractions from different plant parts, it is clear that they have some similarities when it comes to biological activity and structures, although some differences are present. In summary, the root bark, stem bark and leaves are all good sources for fractions containing bioactive polysaccharides. But due to sustainability of the tree *T. macroptera*, the authors would recommend that leaves should be used as a traditional remedy against illnesses where the immune system is involved instead of root bark or stem bark, but then higher dosage by weight has to be used. However, before plant part replacement should be recommended, it is important to also address the secondary metabolites as possible active components. Moreover, further studies should be focused on the active sites of the bioactive polysaccharides connected to the complement system, as well as in other biological assay.

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## Author Contributions

Yuan-Feng Zou, Kari Tvete Inngjerdingen and Berit Smestad Paulsen participated in desining the study. Plant material was collected by Drissa Diallo; complement fixation assay was conducted by Terje Einar Michaelsen. Data was collected by Yuan-Feng Zou and Bing-Zhao Zhang, and analyzed by Yuan-Feng Zou and Berit Smestad Paulsen. Manuscript was written by Yuan-Feng Zou, Berit Smestad Paulsen and Hilde Barsett.

## Conflicts of Interest

The authors declare no conflict of interest.

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*Sample Availability*: Samples of polysaccharide fractions are available from the authors.

# Paper V

**Immunomodulating pectins from root bark, stem bark and leaves of the Malian medicinal tree *Terminalia macroptera*, structure activity relations.**

## Paper VI

**Structural features and complement fixing activity of polysaccharides from *Codonopsis pilosula* Nannf. *var. modesta* L.T.Shen roots.**