

NEW IMMUNE MODULATING COMPOUNDS
ISOLATED FROM A WOUND-HEALING PLANT
FROM MALI

“ *PARKIA BIGLOBOSA BARK* ”

THESIS FOR MASTER DEGREE

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1- Abstract

In this study, the polysaccharides contained in the powdered *Parkia biglobosa* bark were first extracted with 50% ethanol followed by 50⁰ C and 100⁰ C purified distilled water.

After ANX Sepharose-4 fast flow separation and because the acidic fractions were the most interesting part of these extractions, they were purified with gel filtration technique to limit the degree of impurities, which influence the accuracy of the tests as well as the efficiencies of some equipment.

After extraction and isolation of the acidic fractions, six samples were selected as follows: 2 fractions from 50⁰ C extract (acidic non-floor fraction No-2 or S 50-2 and acidic floor fraction no2 or S 50A f₂), 2 fractions from 100⁰ C extract (acidic S 100-i and S 100-ii), 100NaCl, and E-1. These fractions were studied in order to explore their biological activity using complement-fixation assay and to know the possible structure of their components which resemble that of the pectins. The samples S50-2, S100-i, S100-ii, and E1 were bioactive, so they were selected for further study.

N.B: Since S50-2 was the only fraction from 50⁰ C extract proceeded with to the end of this study, it was just written S50 in the whole process.

After the purification of the active samples; S50-2, S100i, S100ii and E1 using gel filtration chromatography, the sample S100i was divided into three fractions; S100i-1, S100i-2, and S100i-3 depending on phenol-sulfuric acid profile. In order to estimate the quantity of the sugars contained in the plant polysaccharides, methanolysis was used followed by derivatization method. The sugar contents obtained from the previously mentioned methods show the different amount of each monomer of polysaccharide present in these samples.

Studying of the polysaccharide linkage patterns revealed by the GC-MS chromatograms provides good knowledge of the structure of the polysaccharides which the *Parkia biglobosa* bark contains. The compositions of these polysaccharides were mainly homogalacturonan, arabinogalactan, rhamnogalactans, and xylogalactans, which comprise the main core of the typically bioactive pectins found in plants that have the capability to modulate the complement system.

Further, stimulation of macrophages by the polysaccharides in the samples was performed to measure the nitric acid release from these macrophages. The result of the test was positive and confirms the bioactivity of the polysaccharides present in the *Parkia biglobosa* bark.

2- Abbreviations and symbols

α	alpha anomer monosaccharide that has an –OH group at C ₁ , which has the same configuration as the sugar itself.
Abs	Absorbance
AG I	Arabinogalactan type I
AG II	Arabinogalactan type II
AGP	Arabinogalactanprotein
Ara	Arabinose
AUC	Area under the curve
β	Beta anomer monosaccharide that has an –OH group at C ₁ , which has an opposite configuration of the sugar itself.
BSA	Bovin Serum Albumin
C3a	Fragment of complement component C3
C3b	Fragment of complement component C3
C5a	Fragment of complement component C5
C5b	Fragment of complement component C5
D	D-sugar with a hydroxyl group attached to a chiral carbon projected to the right position in the Fischer-projection
Da	Dalton
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DMT	Département de Médecines Traditionnelles
<i>f</i>	furanose form, 5-ring
FID	flame ionization detector
FPLC	Fast protein liquid chromatography
Fuc	Fucose
Gal	Galactose
Gal A	Galacturonic Acid
GC	Gas chromatography
GC-MS	Gas chromatography – Mass Spectroscopy
Gls	Glucose
Glc A	Glucuronic Acid
HG	homogalacturonan
HMDS	hexamethyldisilazane
HR	the hairy region
ICH 50	inhibitory concentrations with 50% hemolysis
IL-1	interleukin 1
IR	Infra Red
L	L-sugar with a hydroxyl group attached to a chiral carbon projected to the left position in the Fischer-projection
LPS	Lipopolysaccharide
Man	Mannose
MASP	Mannose-binding lectine serine protease
MS	mass spectrometry
MBL	Mannose- binding lectine
Me	Methyl
MW	molecular weight
MWCO	Molecular weight cut off

N ₂	nitrogen gas
NO	nitrogen oxide
NO ₂ ⁻	nitrite
OH	hydroxyl group
<i>p</i>	Pyranose form, 6-ring
PMII	<i>Plantago major</i>
Pb	<i>Parkia biglobosa</i>
Pbb	<i>Parkia biglobosa</i> bark
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
Rha	Rhamnose
RI	Refractive index
Rpm	Rotation per minute
S50	acidic fraction (tubes 81-120) obtained from aqueous extract of <i>Parkia biglobosa</i> bark at 50 ⁰ C
S100i	acidic fraction (tubes 80-106) obtained from aqueous extract of <i>Parkia biglobosa</i> bark at 100 ⁰ C
S100i-1	fraction-1 (tubes 52-72) from S100i after gel filtration
S100i-2	fraction-2 (tubes 73-92) from S100i after gel filtration
S100i-3	fraction-3 (tubes 93-124) from S100i after gel filtration
S100ii	acidic fraction (tubes 107- 140) obtained from aqueous extract of <i>Parkia biglobosa</i> bark at 100 ⁰ C
SEC	size exclusion chromatography
T	Terminal
TCC	Terminal complement complex
TFA	Tri
TMCS	trimethylchlorsilane
TMS	Trimethylsilane
UV	ultra violet
V ₀	the void volume; eluting volume of the mobile phase in a chromatographic column
XG	xylogalacturonan
Xyl	Xylose

3 – Introduction:

3.1- Traditional medicine

The plant kingdom is still a mysterious world that humans are working day and night to explore its codes. This plant kingdom has provided the mankind with a diverse of benefits, which will last forever. Those of worth of mentioning are nutrients, leisure, security rescues as well as health maintaining substances. One of the crucial plant kingdom fields is the successive provision of its elements to the welfare of the human kind in the form of immune system strengthening, which is the main aim of this thesis.

Traditional medicine is one of the inherited cultures in most African lands from ancient eras. It has been born from the grieves and the poverty of the African natives and handed over from generation to generation. The concept of traditional medicine has been progressively changed time by time meanwhile, it has occupied a leading position in the modernized medication. Traditional medicine will proceed as a cheap and pure source of natural drugs as well as an attracting field that appeals for every researcher who pursues a comprehensive understanding of the products of the generous nature. The availability of the modernized technology has greatly boosted the ideas of the traditional healers and paved the way to better understanding of plant constituents included in these plants and the methods of extracting of such components and so far their therapeutical importance.

The African land, Mali, is one of the lands well known in the field of traditional medicine. Many of ethnopharmacological institutes have been established there to follow up the medicinal plants and to scientifically evaluate them according to the biological activity. *Parkia biglobosa* is one of the plants with a numerous traditional use in the land. Its leaves, seeds, roots as well as the bark were utilized on a wide scale; both for humans and livestock.

The *Parkia biglobosa* bark has been used traditionally to cure a wide range of illnesses e.g., external and internal wounds, headache, malaria, cough, urinary disorders, diabetes, blood pressure etc. Based on this traditional use, the plant has been subjected to an intensive investigation to explore the bioactive ingredients present in it. Recently, it was confirmed that polysaccharides of certain structures modulate the immune system and intensify its defense mechanism. The polysaccharides having such ability and modulation were of pectin type, which consists of different parts. These parts were further analyzed to evaluate their relative potencies in achieving of their curative effect depending on their unit structures and their molecular weight.

The understanding of the scientific properties and the chemical structure of the *Parkia biglobosa* tree becomes a field of interest for many researchers as well as herbalists. The purpose of this master thesis is focusing on the studying of the plant bark to scientifically elucidate and identify its properties which are capable of killing the organisms present in internal and external wounds.

3.2- Carbohydrates:

Carbohydrates are so far the class of organic compounds present in highest amounts around the world. Moreover carbohydrates comprise one of the most complicated and advantageous materials available today and therefore, no doubt that carbohydrate and the technologies related to them have introduced a crucial breakthrough in the development of new products ranging from foods, pharmaceuticals, textiles, papers, and biodegradable packaging materials.(Cui, 2000).

Carbohydrates are present in the higher classes of animals, plant cells, as well as some of the seaweeds. The presence of carbohydrates in the different cells functions as energy reserves e.g., starch, structural materials e.g., cellulose, chitin and protective substances.

Polysaccharides of some plant cell wall are elicitors of plant antibodies e.g., phytoalexins, other polysaccharides function as fragments for the synthesis of a protein i.e., protein inhibitor inducer factor that inhibits insect and microbial proteinase.

One of the benefits of carbohydrates in the cereal storage field is the ability of arabinoxylans to inhibit intercellular ice formation, thus ensuring winter survival of cereals.

Oligosaccharides conjugated to protein i.e., glycoproteins or to lipids i.e., glycolipids are important components of cell membranes and can be active in cell to cell recognition and signaling. Polysaccharides serve as information transfer agents e.g., nucleic acids.

Carbohydrates have generally the empirical formula $C_x(H_2O)_y$, hence the name carbohydrates or hydrates of carbon was given. The majority of the naturally occurring carbohydrates have the *D*-configuration. The famous *L*-configurations are the *L*- arabinose and *L*-galactose, which are present as carbohydrate units in polysaccharides.

According to nutritionists, carbohydrates can be divided into two classes:

- a- Available carbohydrates or those which are readily utilized and metabolized. They may be mono-, di-, oligo- or polysaccharides, e.g., glucose, fructose, lactose, dextrans, starch.
- b- Unavailable carbohydrates or those which are not utilized directly but instead they are broken down by symbiotic bacteria producing fatty acids i.e., they do not supply the host with direct carbohydrates. This includes structural polysaccharides of plant cell walls and many complex polysaccharides, e.g., cellulose, pectins, beta-glucans.

Carbohydrates comprise a wide range of molecules and can be classified according to their chemical structure into three main groups,(Cui 2000):

- 1- Low molecular weight mono- and disaccharides.
- 2- Intermediate molecular weight oligosaccharides.
- 3- High molecular weight polysaccharides.

Classification of polysaccharides:

Polysaccharides are condensation polymers in which glycosidic linkage is formed from the glycosyl moiety of hemiacetal (or hemiketal) and a hydroxyl group of another sugar unit, acting as an acceptor molecule or aglycone (Bruneton 1999; Cui 2000). Glycans is a general term given to polysaccharides in which large numbers of glycoses (monosaccharides) are mutually joined by O-glycosidic linkages. Polysaccharides may be linear or branched, and with the exception of cyclic polysaccharides known as cycloamyloses, there is a defined chain character from the non-reducing terminus (or termini) to one reducing terminus.

Based on the number of different monomers present, polysaccharides can be divided into two classes:

- Homopolysaccharides, consisting of only one kind of monosaccharides.
- Heteropolysaccharides, consisting of two or more kinds of monosaccharide units. Some polysaccharides are composed of sugar units only: they are known as neutral polysaccharides (e.g., amylose, amylopectin, cellulose). Polysaccharides containing sugar acids in their structure will carry negative charges, and, therefore, they are anionic polysaccharides.

Based on the type of sequence of sugar units, polysaccharides can be generally divided into three groups:

- Periodic type: amylose and cellulose
- Interrupted type: alginate and pectins
- Aperiodic type: oligosaccharide chain of glycoproteins, e.g., oligosaccharides in the human blood groups (ABO).

3.3- Plant Polysaccharides:

The plant cell wall is a dynamic and a complex macromolecular structure that changes through the life course of the cell. It is essential for the survival of the plant and it must be strong enough to support the plant and withstand the internal pressure of the cell as well as the interactions with the environment. The plant cell wall structure must adapt itself to cope with the development stage during cell extension and cell growth (Caffal & Mohnem, 2009).

The plant cell wall is a highly organized composite and it is composed of three components: the primary cell wall, the middle layer or lamella, and the secondary cell wall. This cell wall consists of different polysaccharides, proteins, and aromatic substances. The primary cell wall contains cellulose, hemicellulose, and pectin as the main polysaccharides. The secondary wall has generally less pectin, more cellulose and a different class of hemicellulose (β 1-4 xylans). The cell wall composes a network of strong rod-like molecules boundtogether by cross-linked glycans and embedded in a highly organized matrix of acidic polysaccharides (Varki, Cummlings, Esko, Freeze, Stanley, Bertozzi, Hart, Etzler, 2009).

The middle lamella forms the interface between the primary wall of adjacent cells, and finally; many cells emerge within the primary wall to form the secondary wall during

differentiation process. The molecular composition and arrangements of the wall polymers differ among species, individual cells, and even, among regions of the wall around a single protoplast. Polysaccharides are the principal component of the cell wall and form its main structural framework and understanding of the chemistry of the carbohydrates greatly enables understanding of many biological functions of the cell wall polysaccharides (Carpita & McCann, 2000).

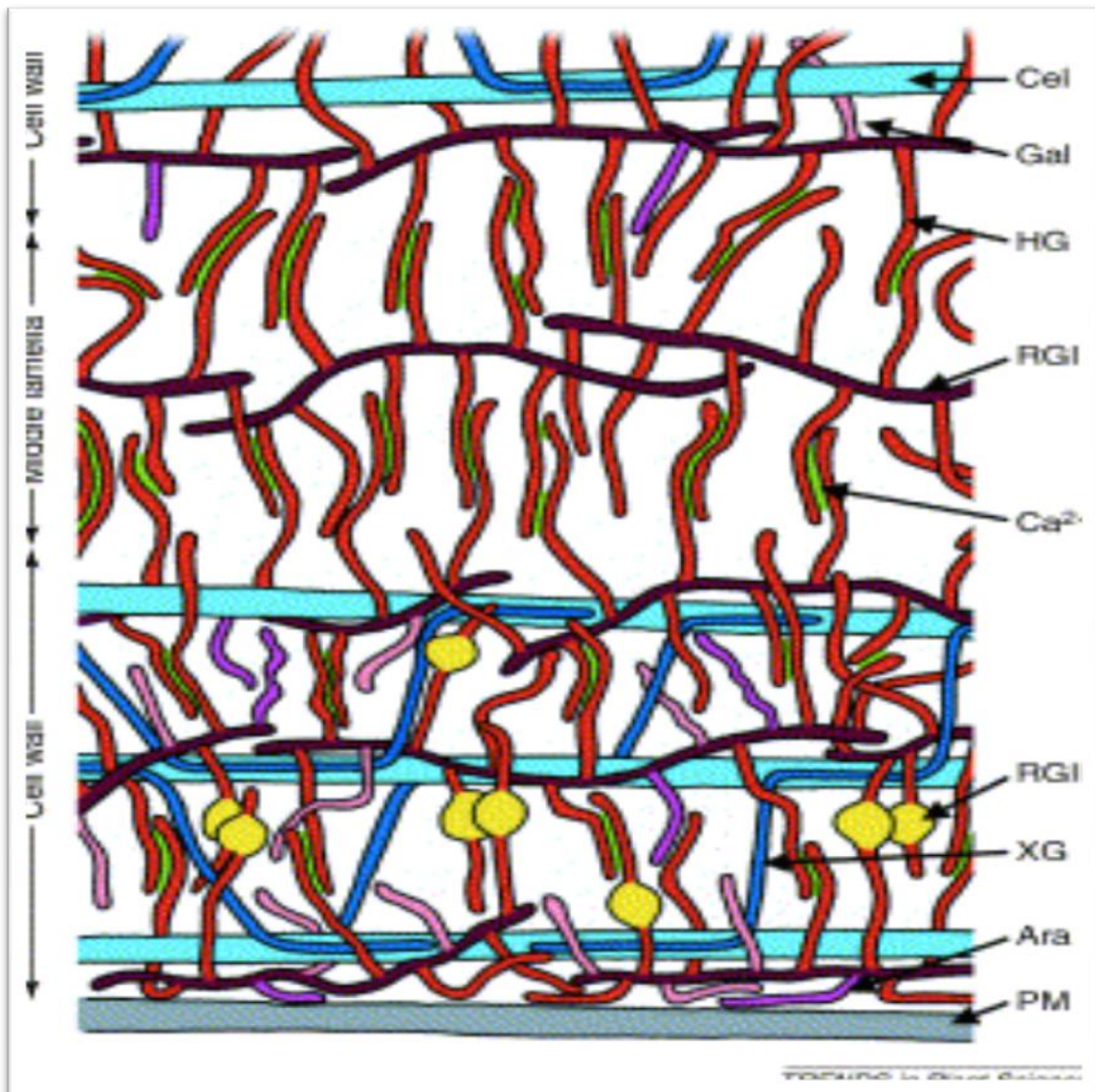


Figure (3.3) A structure shows the organization of polysaccharides in plant cell walls (Vincken et al, 2003)

Cel = cellulose, Gal = galactans, HG = homogalaturonan, RGI = Rhamnogalacturonan I, RG II = Rhamnogalacturonan II, XG = xyloglucan, Ara = arabinans, Ca₂ = calcium molecules and PM = plasma membrane.

3.3.1-Cellulose:

Is the most abundant polysaccharide in the plant's primary and secondary walls. It forms the basic structural material of the cell walls in all higher plants, some seaweed, and it is synthesized by few bacteria. Cellulose exists in a form of water-insoluble microfibrils, which are paracrystalline assemblies of several dozen glucan chains and each glucan chain may contain several thousand units of glucose. From the structural viewpoint, cellulose is probably the best-understood of all carbohydrates of the plant cell wall (Carpita & McCann, 2000; Grant, 1997)

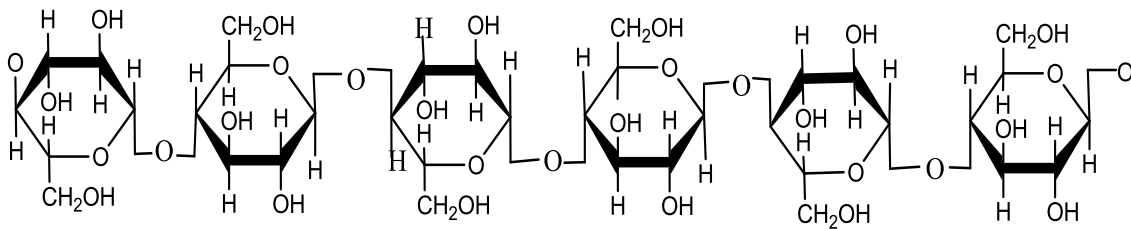


Figure (3.3.1) schematic structure of cellulose

3.3.2-Hemicelluloses:

Hemicellulose is a heterogeneous group of compounds that in plant-cell walls form part of the matrix within which cellulose fibers are embedded. Hemicellulose is composed of cross-linked glycans, which are polysaccharides. Hemicellulose contains many different sugar monomers, which can include xylose, mannose, galactose, rhamnose, and arabinose.

Hemicelluloses contain most of the D-pentose sugars, and occasionally small amounts of L-sugars as well. Xylose is always the sugar monomer present in the largest amount, but mannuronic acid and galacturonic acid also tend to be present.

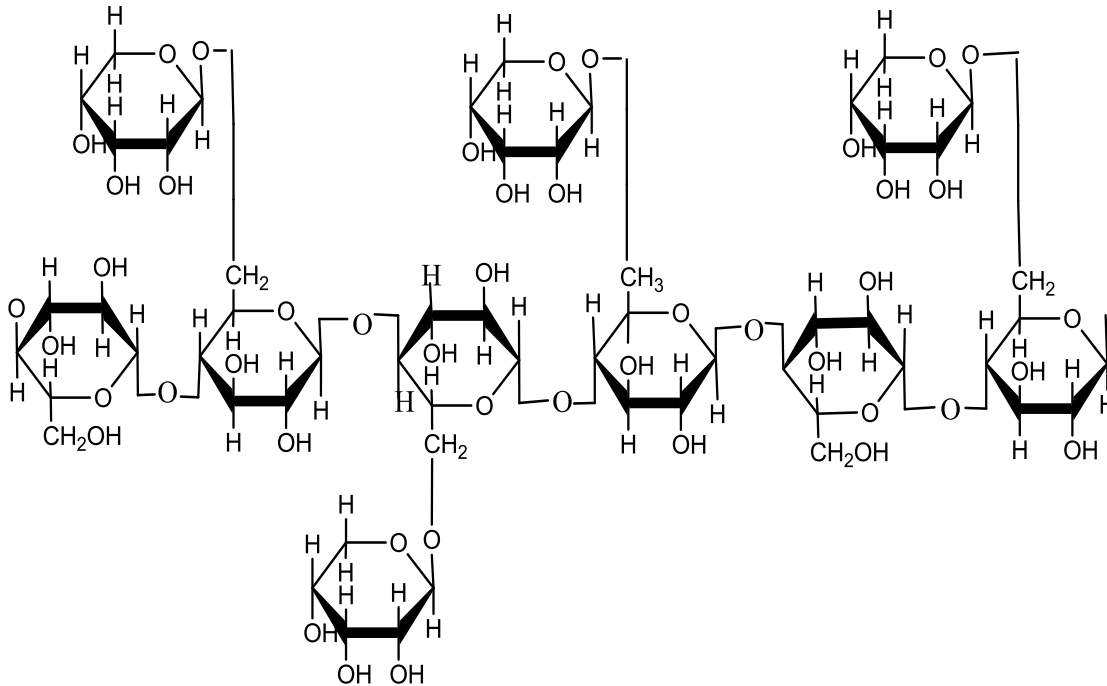


Figure (3.3.2) schematic structure of hemicellulose

3.3.3-Bioactive Pectic Polysaccharides:

Polysaccharides from plants have been the subject of studies for a very long time, which mainly focused on their physical properties, their chemical and physical modification and their application (Paulsen & Barsett, 2005). Since plants have been traditionally used worldwide to treat various kinds of illnesses including both external and internal wounds, modern science has revealed that many plants contain polysaccharides that possess biological activity of different kinds.

Different types of polysaccharides isolated from plants used in traditional medicine are identified for their activities on the complement system; e.g. arabinans, arabinogalactans and rhamnogalacturonans. (Yamada, & Kiyohara, 1999).

Similar types of polymers have also been shown to have effects on macrophages, T – lymphocytes and NK – cells. The majority of these polysaccharides exhibit also a mucoadhesive effect. They bind to the surface of cells, and can then be the cause of local effects seen in certain experiments. (Schidgall, Schnetz, Hensel, 2000)

The word pectin includes a wide range of associated polysaccharides from the cell walls and intercellular parts of plants. Pectins can generally be divided into neutral and acidic polymers. The term pectic substances is used to include associated neutral sugar side chains (arabinans, galactans and arabinogalactans), which are linked to rhamnogalacturonan segments within the pectin molecule. Other structural elements of pectin include rhamnogalacturonan, xylogalacturonan and apiogalacturonan. (Voragen et al.1995, 2001). D-galacturonic acid is the major sugar residue of which most pectins are composed of and it is present in an α - (1 \rightarrow 4) linked linear chain in which various proportions of the acid groups are esterified with methanol.

Native polysaccharides are compounds that mainly have the backbone of linear homogalacturonan (HG) chain, which is composed of 1,4-linked galacturonosyl (GalA)

residues and sometimes they contain carboxymethyl esters. This chain can bear rhamnogalacturonan-1 (RG-1) residues with a backbone of alternative 1,4-linked GalA and 1,2-linked rhamnopyranosyl (Rha) units. A neutral (1,3- and 1,3,5- linked arabinan and 1,4- and 1,3,4-linked and/or 1,3-, 1,6- and 1,3,6-linked arabinogalactans) and acidic oligosaccharides are attached to the rhamnose units at position 4. Compounds containing RG-1 and arabinogalactans are generally referred to as pectic hairy regions in which AG-1 and arabinan are the hairs (Inngjerdigen, Patel, Drissa & Paulsen, 2008).

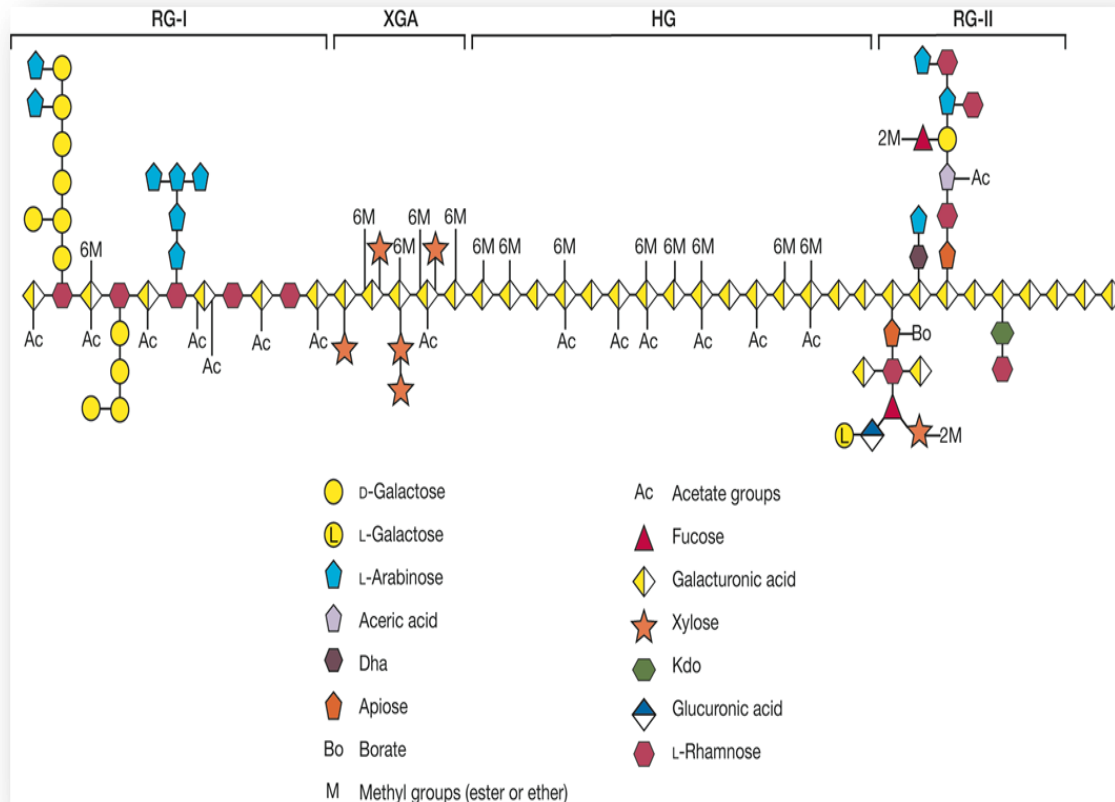


Figure (3.3.3) Schematic structure of pectin showing the three main pectic polysaccharides: rhamnogalacturonan I, rhamnogalacturonan II at each side of a homogalacturonan (HG) chain. A region of substituted galacturonan, known as xylogalacturonan (XGA), is also shown between the HG and the RG-I. 3-Deoxy- D- lyxo- heptulosaric acid (Dha) from (Essentials of Glycobiology).

3.3.3. A- Arabinans:

Arabinans that is found in plants are basically composed of L- arabinofuranosides. Arabinans may be linear or branched and primarily linked through positions 3 or 5. It is generally accepted that the core linkage of the arabinans are the 5- linkage and the branches occur at C-3 or C-2 but arabinans are most probably linked to the galactans in the pectic complex and released either by enzymatic action or weak acid hydrolysis.

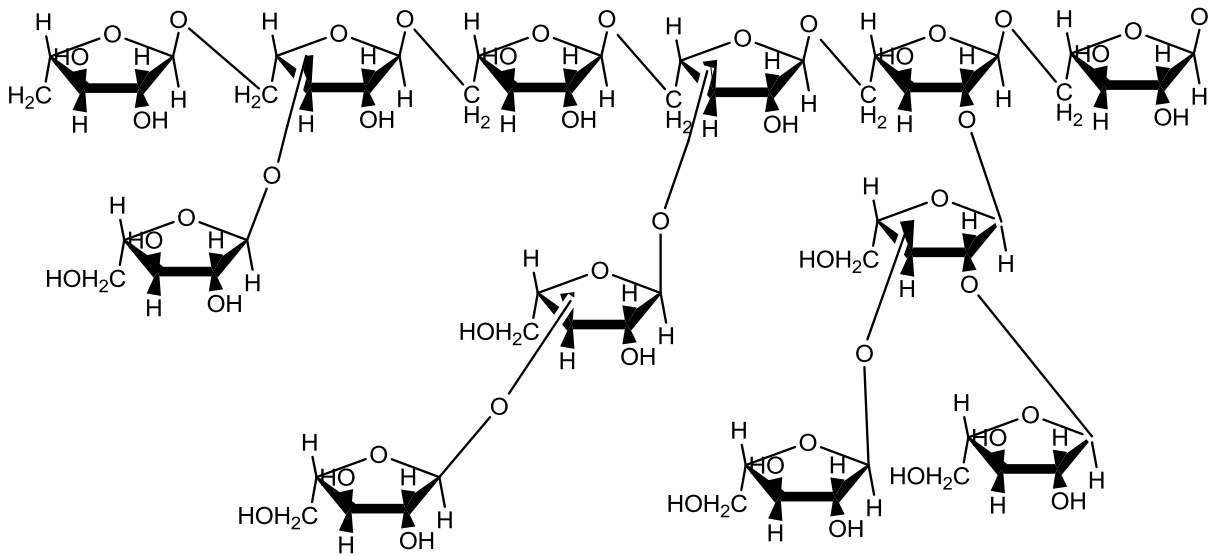


Figure (3.3.3-A) Schematic structure of highly branched α -L (1 \rightarrow 5) arabinan (Seymour & Knox, 2002).

3.3.3. B- Arabinogalactans type I and II (AG-I & AG-II):

The arabinogalactans have more frequently been reported for activity in various biological systems. They are often classified into three groups:

- a) Type I i.e. arabino-4-galactans
- b) Type II i.e. arabino-3,6- galactans, and
- c) Type III i.e. polysaccharides with arabinogalactans side chain or the real pectins.
(Clark, Anderson, Stone,1979)

Arabinogalactan I is composed of a β -1,4- linked galactan backbone with the side chains of arabinans basically linked through position 3 of the galactose units.

Arabinogalactan II core is mainly a galactan that have either 3 or 6 linkages in the main chain and is highly branched with the 1,3,6-linked galactose units at the branching points. Both types of arabinogalactans are found to be linked through position 4 of the rhamnose units of the pectic chain.

Arabinogalactan-II has the ability to form a red precipitate with the Yariv reagent while arabinogalactan-I has not and thus it is used to show the presence of AG-II in bioactive polymers.

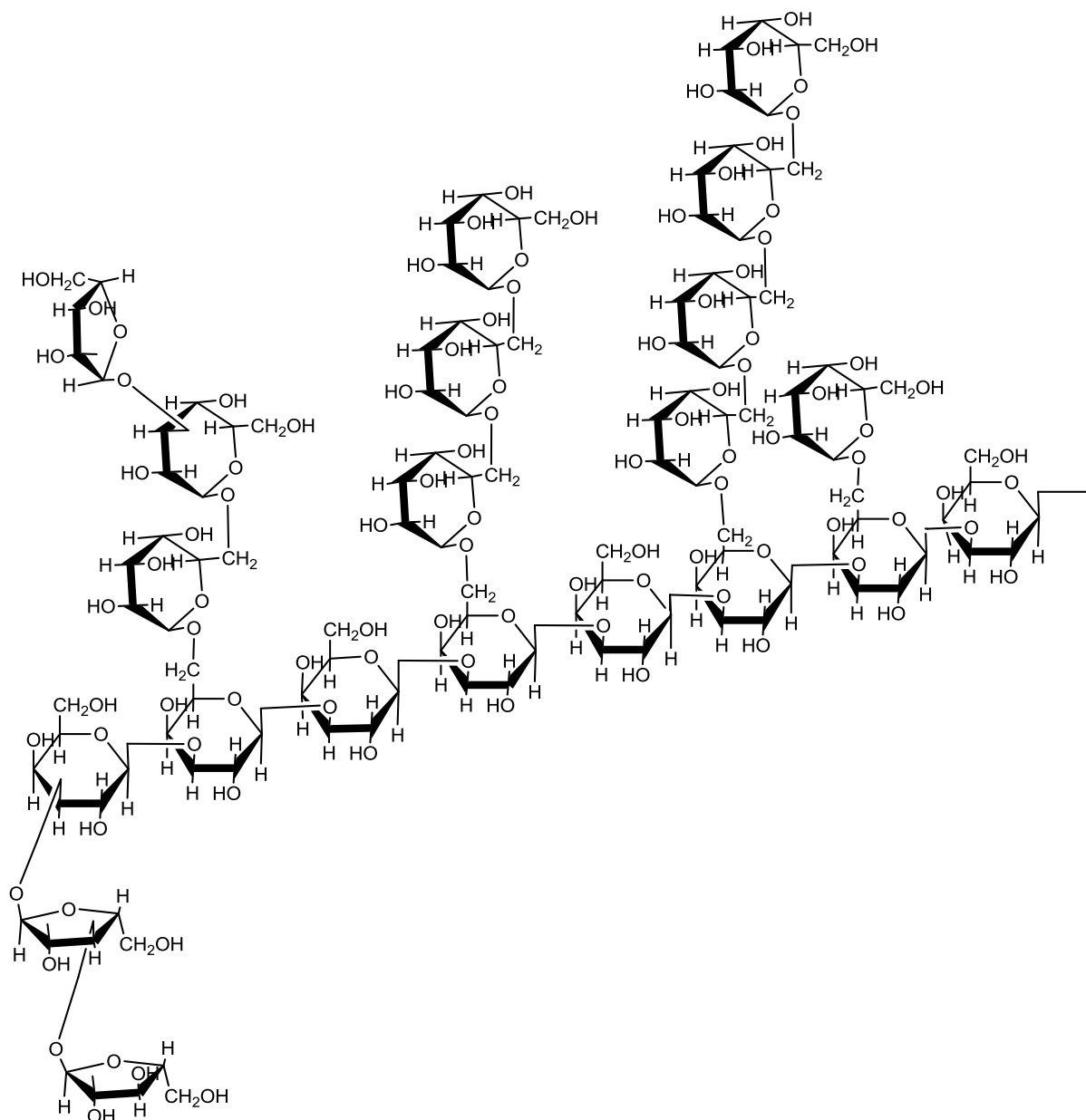


Figure (3.3.3-B) Schematic structure of type II β -(1, 3, 6) - arabinogalactan (Seymour & Knox, 2002).

3.3.3. C- Rhamnogalacturonans I (RhaGalA or RG-I):

It is a polymer that has a core of alternating α -1,4- linked D- galacturonic acid and α -1,2- L- rhamnose units. The rhamnose units in the alternating core were frequently found as branching points, primarily on position 4, carrying galactan and arabinan side chains of varying structures. The arabinogalactans attached to the rhamnose units are frequently found to be of the arabinogalactan type II. Both galactans with the main chain being 1,6 – linked and

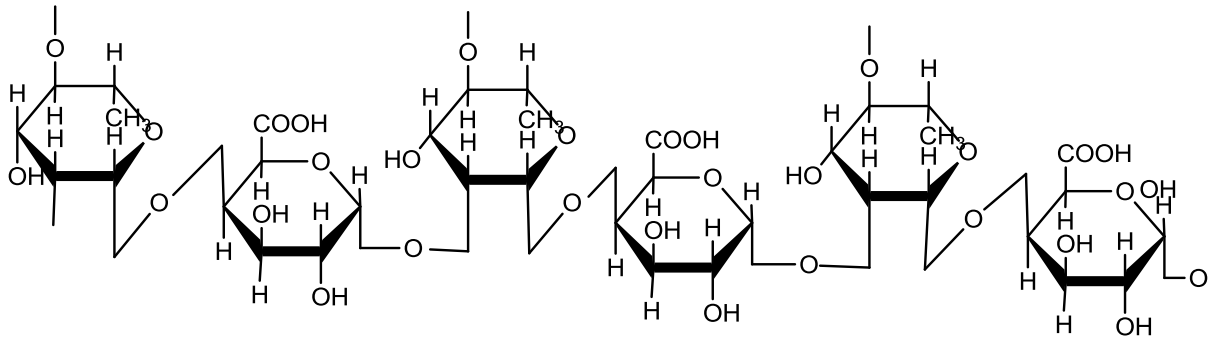


Figure (3.3.3-C) the alternating structure of rhamnogalacturonan segments of pectin. The acetyl groups may be substituted at O-2, O-3 or both O-2 and O-3 of the galacturonic acid residues (Seymour & Knox, 2002).

1,3- linked may be found for AG-II region, and hence, it is termed the hairy or the ramified region of the pectic polymer. In addition to the hairy region, the pectic polymer contains a smooth region, which only composed of α 1,4- galacturonic acid residues. This region can carry methyl ester groups and can be acetylated at positions 2 and/or 3. (Voragen, Daas, Schols, 2000).

3.3.3. D- Rhamnogalacturonan II (RhaGalA-II or RG-II):

It has a homogalacturonan backbone, which composed of 9-10 D- galacturonic acid units that are α -1,4- linked and four different oligosaccharide chains are attached through positions 3 and 4 of the uronic acid backbone. The most characteristic part of RG-II is the presence of the rare sugars 2-O-methylfucose, and 2-O-methylxylose, apiose, aceric acid, 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) and 3-deoxy-D-lyxo-2-heptulosaric acid (DHA) (O'Neill, Albersheim, Darvill, 1990) (Doco, Williams, Vidal, Pellerin, 1997)

3.3.3. E- Homogalacturonan (HG):

Homogalacturonan is a polymer of α -1,4-linked D-galacturonic acid. It comprises the largest part of pectins in the plant cell wall. Homogalacturonan residues may be methyl esterified at the C6 carboxyl or acetylated at the O-2 or O-3.

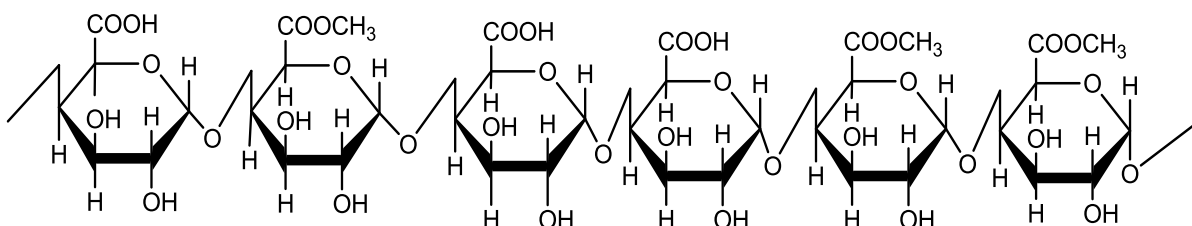


Figure (3.3.3-E) Homogalacturonan fragment of pectin. Acetyl groups may be present at O-2 and/or O-3 for some pectins (Seymour & Knox, 2002).

3.3.3. F- Xylogalacturonans (XGA):

Xylogalacturonan is a homogalacturonan i.e., the main chain is composed of α -1,4-D-galacturonic acid, substituted by D-xylose residues at the C3 of galacturonic acid backbone residues.

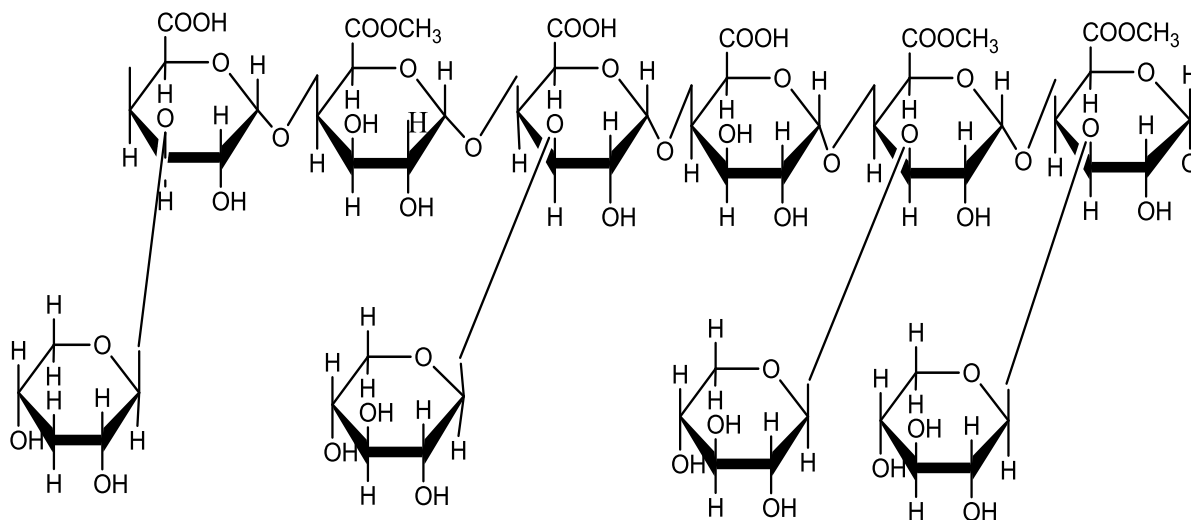


Figure (3.3.3-F) Schematic structure of a xylogalacturonan. The position of methyl esters is chosen arbitrarily (Seymour & Knox, 2002).

3.4 - The biological activity of plant polysaccharides:

Many of the naturally occurring compounds have the ability to interact and influence the vertebrate immune system. The resultant effects or responses may either boost or degrade certain host elements and, therefore, they are referred to as immune modulators. Many different kinds of polysaccharides were found to exhibit a diversity of immunological activities that include both of the adaptive and innate immune system.

Plant pectic polysaccharides have the capability to reinforce the immune system responses, which highlights the certain advantageous medical effects of these plants. Moreover, plant polysaccharides are therapeutically much safer compared with the synthetic compounds and they can be easily extracted from their sources with non-strenuous methods.

Since pectins are structurally composed of non-unique parts of polysaccharide, different studies were carried out to identify the most active part of its chain structure. The hairy region was found to have the highest activity than the corresponding original (non-hairy) pectin (Yamada & Kiyohara, 1999; Paulsen & Barsett, 2005).

Although it is difficult to confirm the importance of the structural activity relationship of the different kinds of pectic polysaccharides, but this relationship can often be expected. A study carried out by Lin, Z-b. reported that *Ganoderma lucidum* polysaccharides; a medicinal herb

in China, has basic β -glucan structure with β -1,3-linkages in the main chain of glucan and further β -1,6-branched points which are needed for immune-modulating and anti-tumor activities. This structure has a high molecular weight and was more effective than β -1,6-linkages alone. These findings raise the postulation of the effectiveness of the polysaccharides with higher molecular weights and vice-versa (Lin, Z-B, 2005).

The immune system of humans consists of many types of proteins, cells, organs and tissues, which interact collectively and dynamically to protect against diseases by identifying and killing pathogens. The human immune system adapts some pathogens by creating of antigen receptors on specific receptors i.e., B and T cells, which prepare the body to effectively defend itself in the future. Therefore, has the human immune system a combination of innate and adaptive immunity (Beck & Habicht, 1996)

The immune system protects the organism from pathogenic infections with different defensive layers of increasing specificity. In case a pathogen overcomes these barriers, the innate immune system will provide an immediate unspecific response. However, if the pathogen was able to evade the innate immune system, vertebrates have a third layer of protection; the adaptive immune system i.e., the immune system adapts its response to improve its recognition of the pathogen. This response will be retained after the pathogen has been eliminated in the form of an immunological memory and allows the adaptive immune system to act faster and stronger each time this pathogen is encountered (Mayer, 2006).

The innate immune system involves humoral and chemical barriers (inflammatory and complement system) and cellular barriers (leucocytes, phagocytes, dendritic cells and mast cells). In adaptive immunity, B cells are responsible for the humoral response while T cells are involved in cell-mediated immune response and as helper cells for B cells, too.

3.4.1- The complement system:

The complement system is an important component of our innate immunity. It consists of approximately 20 proteins that are present in normal human serum. The term complement refers to the ability of these proteins to complement (augment) the effect of other components of the immune system. The functions of the complement are:

- a- Lysis of cells such as bacteria, allograft and tumor cells.
- b- Generation of mediators that participate in inflammation.
- c- Opsonisation i.e., enhancement of phagocytosis.

Complement activation pathways:

Several complement components are proenzymes which might be cleaved to form active enzymes. Activation of the complement system can be initiated either by antigen-antibody complexes (classical pathway) or by a variety of molecules on pathogens and other foreign materials (alternative and lytic pathway).

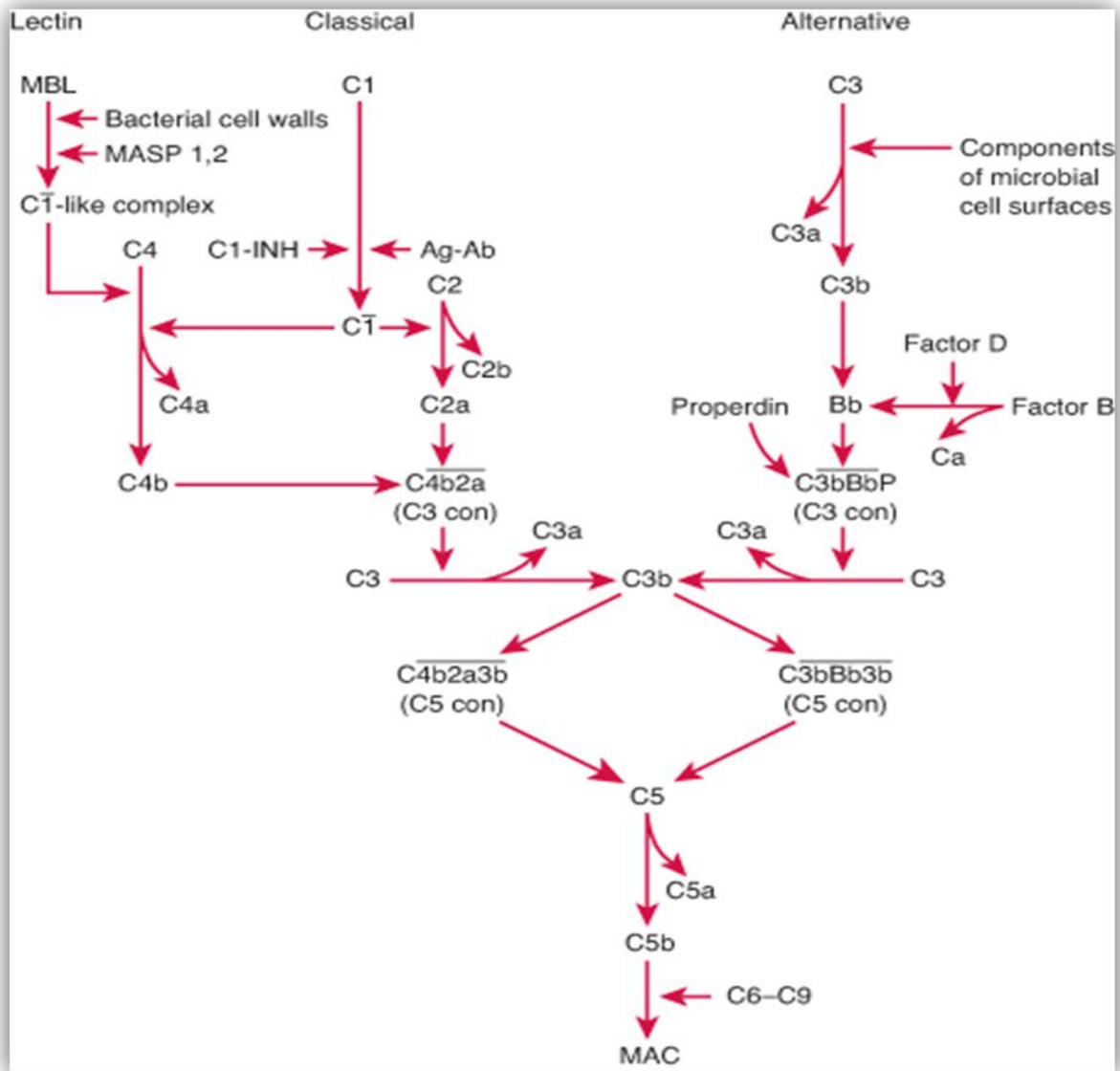


Figure (34.1) The three activation pathways of the complement system (Merck, 2005)

The classical, lytic, and alternative pathways converge into a final common pathway when C3 convertase (C3 con) cleaves C3 into C3a and C3b.

Ab = antibody; Ag = antigen; C1-INH = C1 inhibitor; MAC = membrane attack complex; MASP = MBL-associated serine protease; MBL = mannose-binding lectin; P = properdin.

The alternative and lytic pathways are important in the first time the body is infected by a micro-organism, because the antibody required to trigger the classic pathway is not yet present. All of the three pathways lead to the production of C3b, which is the central molecule of the complement cascade. C3b has two important functions:

- a- It combines with other complement components and generates C5 convertase, the enzyme that leads to the production of the membrane attack complex.
- b- It opsonises bacteria, because phagocytes have receptors for C3b on their surfaces (Lewinson & Jawetz, 2002)

The complement system is divided into four pathways each of which requires different protein components and each of these pathways is a series of sequential steps that proceed in a cascading fashion. The complement pathways are:

a- The Classical pathway (antibody dependant):

Antigen-antibody complexes activate C1, which is composed of three proteins: C1q, C1r and C1s, in the presence of calcium to form protease. This, in turn, cleaves C2 and C4 to form a C4b2a complex which is a classical C3 convertase that cleaves C3 molecules into two fragments; C3a and C3b. C3b forms a complex with C4b2a producing a new enzyme, C5 convertase C4b2a3b which cleaves C5 to form C5a and C5b. C5b binds to C6 and C7 to form a complex that increases with C8 and C9 to produce the membrane attack complex which causes cytolysis (Lewinson & Jawetz, 2002)

b- The lytic pathway (antibody independent):

In this pathway, the mannan-binding lectin MBL, also known as mannose binding protein, binds to the surface of microbes bearing mannan. This activates protease associated with MBL that cleaves C2 and C4 components in the same way to that of classical pathway but here lectin pathway bypasses the antibody requiring step and, thus, the pathway protect early the body during the infection before antibody is formed.

c- The alternative pathway (antibody independent):

Many unrelated cell surface substances, e.g. bacterial lipopolysaccharides, fungal cell walls and viral envelopes, can initiate the process by binding C3 and factor B. this complex is cleaved by protease to produce C3bBb. This acts as the alternative C3 convertase to generate more C3b (Dodds, 2002; Lewinson & Jawetz, 2002)

d- The Membrane attack complex pathway:

C5 convertase from the classical (C4b2a3b), lytic (C4b2a3b) or alternative (C3bBb3b) pathway cleaves C5 into C5a and C5b. C5 remains in the fluid phase and the C5b rapidly associates with C6 and C7 and inserts into the membrane. The C5b67 complex is referred to as the membrane attack complex (MAC). Subsequently C8 binds, followed by several molecules of C9. The C9 molecules form a pore in the membrane through which the cellular contents leak and lysis occur. Lysis is not an enzymatic process it is thought to be physical damage to the membrane. C5a generated in the lytic pathway has several potent biologic activities. It is the most potent anaphylotoxin. In addition, it is a chemotactic factor for neutrophils and stimulates the respiratory burst in them and it stimulates inflammatory cytokine production by macrophages. Its activities are controlled by inactivation by carboxypeptidase B (C3-INa) (Haggi, T, 2009).

3.4.2. Macrophages:

Macrophages are innate immune cells that play a critical role during the immune response and wound healing. They play a key role in normal tissue homeostasis, presentation of foreign and self antigens following infection or injury, pathogen clearance, resolution of inflammation and wound healing (Martinez Sica, Mantovani, Locati, 2008). They are derived from monocytes after they leave the blood circulation and enter the tissues; these monocytes become activated and differentiated into macrophages which locate throughout the body. They are important phagocytes, produce inflammatory cytokines, and can capture and present protein antigen to effector T-lymphocytes.

Macrophages are considered classically activated (M1) when stimulated by interferon (IFN)- γ or lipopolysaccharides (LPS) to release nitric oxide (NO), which is important for the killing of intracellular pathogens, and alternatively activated (M2) when stimulated by interleukin (IL)-4 or IL-13 (M2a) to produce IL-10, transforming growth factor (TGF)- β and arginase-1 (Arg-1), which are important for the killing of extracellular parasites (Martinez, Helming, Gordon, 2009). Macrophages are produced in large amounts and they survive in the presence of soluble factors, such as IFN- γ . They act by means of number of different mechanisms (Xaus et al, 2001):

- a- Directly, by destroying bacteria, parasites, viruses and tumor cells;
- b- Indirectly, by releasing mediators, such as interleukin-1, tumor necrosis factor- α , etc; which can activate other cells.
- c- As accessory cells, by processing antigen and presenting digested peptides to T-lymphocytes; and
- d- By repairing tissue damage.

3.5- Cutaneous wounds:

The primary function of the skin is to serve as a protective barrier against the environment. Loss of the integrity of large portions of the skin as a result of injury, abrasion, burns, skin lesion, ulcers or illness may leads to major disability or even death. Wound healing is a dynamic, interactive process involving soluble mediators, blood cells, extracellular matrix, and parenchymal cells. Wound healing has three phases: inflammation, tissue formation, and tissue remodeling that overlap in time (Singer, Clark, 1999)

Inflammation phase: tissue injury causes disruption of blood vessels and extravasation of blood constituents. The blood clot reestablishes hemostasis and provides a provisional extracellular matrix for cell migration. Platelets facilitate the formation of hemostatic plug and secrete several mediators of wound healing, such as platelet-derived growth factor, that attract and activate macrophages and fibroblasts.

Formation of granulation tissue phase: new stroma or granulation tissue, begins to invade the wound area approximately four days after injury where numerous capillaries supply the new stroma with its granular appearance. Macrophages, fibroblasts, and blood vessels move into

the wound space at the same time. The macrophages provide a continuing source of growth factors necessary to stimulate fibroblasts and angiogenesis; the fibroblasts produce the new extracellular matrix necessary to support cell growth; and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism (Singer & Clark, 1999).

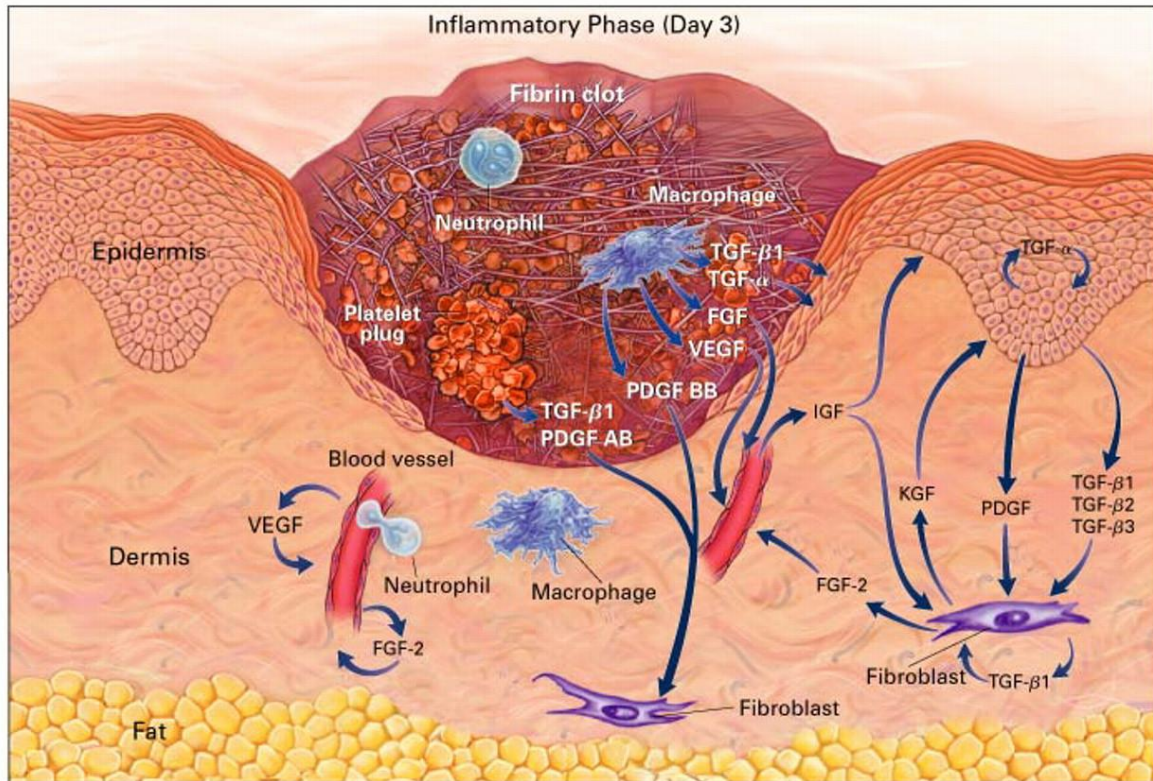


Photo (3.5) the growth factors thought to be necessary for cell movement into the wound (Singer & Clark, 1999).

TGF-β1, TGF-β2, and TGF-β3 denote transforming growth factor β1, β2, and β3, respectively; TGF-α transforming growth factor α; FGF fibroblast growth factor; VEGF vascular endothelial growth factor; PDGF, PDGF AB, and PDGF BB platelet-derived growth factor, platelet-derived growth factor AB, and platelet-derived growth factor BB, respectively; IGF insulin-like growth factor; and KGF keratinocyte growth factor.

3.6 - The plant used in this study (*Parkia biglobosa*):



Photo (3.6a) Parkia biglobosa tree (Societe Francaie d' Ethnopharmacologie)



Photo (3.6b) Leaves and flowers (Perlude Medicinal Plants)



Photo (3.6c) The green pods (Seed leaflet)

3.6.1- Synonym(s):

Inga biglobosa (Jacq.) Wild

Inga faeculifera Desv.

Mimosa biglobosa Jacq.

Parkia africana R. Br.

Parkia clappertoniana Keay

Parkia filicoidea Oliver

Parkia filicoidea Oliver Var. *glauca* Baker

Parkia intermedia Oliver

Parkia oliveri J.F. Macbr.

Prosopis faeculifera (Desv. ex W. Ham) Desv.

3.6.2- LOCAL NAMES:

English: monkey cutlass tree, Arber a farine, two ball nitta-tree, African

Locust bean, fern leaf

Trade name (dadawa, dawa-dawa, soumbal, soumbara)

Domain: *Eukaryota*

Kingdom: *Plantae*

Subkingdom: *Viridiaeplantae*

Phylum: *Magonoliphyta*

Subphylum: *Euphyllrophyta*

Infraphylum: *Radiatopses*

Class: *magnolipsida*

Subclass: *Rosidae*

Superorder: *Fabanae*

Order: *Fabales*

Family: *Fabaceae*

Subfamily: *Mimosoideae*

Tribe: *Mimoseae*

Genus: *Parkia*

Pacific epithet: *Biglobosa*

3.6.3-Botanical name: *Parkia biglobosa*

3.6.4- BOTANIC DESCRIPTION:

The African locust bean tree, *Parkia biglobosa* is a perennial tree legume with a height ranging from 7 to 20 meters with a large crown, although it can reach 30 meters under exceptional conditions. Its branches spread low down, it has amber gum exudes from wounds. The bark is grey brown, thick and with scaly fissured texture.

Leaves: are alternate, dark green, bipinnate to 30 cm long, pinnate up to 17 pairs with 13 -60 pairs of leaflets of distinctive shape and venation. It has no spines.

Peduncles: 10 – 35 cm long.

Capitula: 4.5 – 7 cm long and 3.5 – 6 cm in diameter, biglobose but the distal portion is much larger.

Flowers: are orange or red in color.

Corolla: 10 – 14 mm long, with very short lobes 1- 3 mm long, connate in the middle and free or connate at the base.

Pods: pink brown to dark brown when mature, about 45 cm long and 2 cm wide; it may contain up to 30 seeds embedded in a yellow pericarp.

Seeds: have a hard testa with large cotyledons forming about 70% of their weight

Parkia biglobosa grows in the savannah region of West Africa up to the southern edge of the Sahel zone 13⁰N. These trees are not normally cultivated but can be seen in population of two or more in the Savannah region. The *Parkia* tree play vital ecological role in cycling of nutrients from deep soils, by holding the soil particles to prevent soil erosion with the raid of roots. The trees also provide shades for farmers(Campbell-Platt 1983).

Parkia tree is used as timber for making pestles, mortars, bows, hoe handles and seats (Hagos 1962). The trees of the *Parkia* species are usually and carefully preserved by the inhabitants of the area where they grow because they are valuable sources of reliable food, especially the seeds which serves as sources of useful ingredients for consumption. It has been reported that the husks and bodes are good food for livestock(Doglas 1976). It has also been reported in early literature from the West Indies that *Parkia biglobosa* was apparently introduced in the 18th century from West Africa as a food plant. (Sabiiti, Cobbina, 1992)

3.6.5- PRODUCTS AND THEIR TRADITIONAL USES:

A- Food products:

Seeds are fermented to make dawadawa, a black, strong-smelling, tasty food high in protein. Dawadawa is rich in protein, lipids and vitamin B2. Fermented beans are rich in lysine.

The fat in the beans is nutritionally useful (approximately 60% is unsaturated).

Parkia biglobosa seeds are used as:

- 1- A coffee substitute.
- 2- Seeds embedded in a mealy pulp sometimes called dozim, that is high in energy value. It contains up to 29% crude protein and up to 60% saccharose, is rich in vitamin C and high in oil content.

The pulp of the tree is:

- 1- eaten raw or made into a refreshing drink and is
- 2- used as a sweetener.

The fruit provides emergency food during severe droughts, planks and carvings.

B- Medicine:

- 1- Bark is used as a mouthwash, vapor inhalant for toothache, or for ear complaints. It is macerated in baths for leprosy and used for bronchitis, pneumonia, skin infections, sores, ulcers, bilharzia, washes for fever, malaria, diarrhoea, violent

colic and vomiting, sterility, venereal diseases, guinea worm, oedema and rickets, and as a poison antidote.

- 2- Leaves are used in lotions for sore eyes, burns, haemorrhoids and toothache.
- 3- Seed is taken for tension, and pulp for fevers, as a diuretic and as a mild purgative.
- 4- Roots are used in a lotion for sore eyes.

C- Others:

- 1- Twigs are used to clean teeth; bark stains mouth red and contains saponins that clean teeth.
- 2- Tannin or dyestuff:
 - 1- Husks of pods mixed with indigo improve the luster of dye products.
 - 2- Seeds and bark contain tannin and so, it is used in tanning.
- 3- Poison: Bark and pods contain piscicides; the alkaloid parkine that occurs in pods and bark may be responsible for this effect.

3.6.6- Study of the biological activity of the stem bark of *Parkia biglobosa*:

Scientifically, the plant has elucidated a number of medical features not only in the field of traditional medicine, but also in the different trials and tests carried out. The extract of the tree's seeds showed an inhibitory effect towards platelets aggregation and secretion (Rendu et al, 1993). The fermented seeds of *Parkia biglobosa* was investigated in alloxan-induced diabetic rats and it had proved an antidiabetic property (Odetola, Akinlove, Egunjobi, Adekunli, Avoola, 2006). A third study with anethanol extract of *Parkia biglobosa* explicated anti-tumor and insecticidal effects (Spatafora, Tringali, 1996).

Parkia biglobosa bark was tested for its antibacterial activity against four strains of *Staphylococcus aureus* using alcoholic and aqueous extracts. The tests showed activity that is compared to gentamycin as a positive control. The phytochemical screening revealed the stem bark were rich in sterols, triterpenes, tannins, saponosides, anthrocyanides, coumarins, flavonoides and reducing compounds. (African journal of Traditional, complementary and Alternative Medicines, 2006)

Another phytochemical screening study (Journal of Medicinal Plants Research, 2009) confirmed that the stem bark's extract was active against *Pseudomonas aeruginosa*, a known difficult organism resistant to a wide range of antibacterial agents. These results justify the use of *Parkia biglobosa* by trado-medical practitioners in the treatment of wounds, burns, sores and ulcers.

Snake bites in rural Nigeria are commonly treated with plant extracts. *Parkia biglobosa* stem bark extract significantly protected the chick *biventer cervicis* muscle preparation from snake venom *Naja nigricolis* and significantly reduced the contractures of the preparation induced by venom. The extract protected egg embryos exposed to lethal concentrations of *Echis ocellatus* venom and completely blocked the haemorrhagic activity of the venom.

4. Methods and materials:

4.1- General methods:

4.1.1- Water quality:

Distilled water was used in the whole processes

4.1.2- Materials' weighing:

Bowl weight: OHAUS Portable advanced
Analysis weight: Sartorius BP221S

4.1.3- Filtration:

Glass fiber filter: Round filter glass fiber No. 8, D: 90 and 110 mm (Schleicher & Schull)
Micro filter: Durapore® Membrane Filters, 0, 22 µm GV (aqueous) (Millipore)
Sterile Millex® – AA 0,8 µm filter unit (Millipore)
Acro R 50A Device, 0.45 µm filter (Paul Gelman Laboratory)

Nutsj and vacuum apparatus were used in cases of big liquid amounts while a syringe or a filter unit was used in cases of little amounts.

4.1.4- Evacuation of gases:

The liquid-dissolved gases were evacuated by:
a- Helium bubbling in 10 - 15 minutes
b- Water jet vacuum in 15 - 30 minutes

4.1.5- Washing of Dialysis Membranes:

Principle:

The dialysis membranes should be washed before their use to remove and avoid any probable cellulose remedies.

Reagents:

2% NaOH solution
0, 05% sodium azide solution

Procedure:

- 1- The dialysis membranes were cut into the required lengths (50 – 60 cm) and rinsed thoroughly under a water tap.

- 2- The membranes were cooked for 10 minutes in a 2% NaOH solution, which was previously warmed.
- 3- The membranes were first rinsed thoroughly inside and outside with a water tap and then with a distilled water.
- 4- Membranes were cooked with distilled water for 10 minutes after that they were rinsed with distilled water
- 5- The membranes were then kept in 0, 05% sodium azide solution at 4⁰ C to prevent bacterial growth.

4.1.6- Dialysis of solutions:

Principle:

Dialysis is a simple process in which small solutes diffuse from a high concentrated solution to a low concentrated one across a semi permeable membrane until an equilibrium is reached. Since the porous membrane selectively allows smaller solutes to pass while retaining larger species, dialysis can effectively be used as a separation process based on size rejection. The conditions of dialysis can be controlled or manipulated to produce desired results for a variety of dialysis applications.

Selecting the Right Molecular Weight Cut-Off (MWCO):

Because the dialysis membrane consists of a spongy matrix of cross-linked polymers, the pore rating referred to as Molecular Weight Cut-Off (MWCO), is an indirect measure of the retention performance. More precisely, the membrane MWCO is determined as the solute size that is retained by at least 90%. However, since a solute's permeability is also dependent upon molecular shape, degree of hydration, ionic charge and polarity, it is recommended to select a MWCO that is half the size of the MW of the species to be retained and/or twice the size of the MW of the species intended to pass through.

Reagents:

Toluene
Saturated AgNO₃ solution

Procedure:

- 1- An end of the membrane was tied with a hug and a little amount of the sample solution was filled into the membrane to insure at the membrane is well tightened.
- 2- The membrane is then filled to the 2/3 of its length with the sample solution.
- 3- 2-3 drops of toluene were added as a preservative
- 4- Any air bubbles were removed from the membrane and the other end of the membrane was tied with another hug.
- 5- After the sample solution was distributed into many membranes, they were put into a large opening container filled with about ¾ volume degassed and distilled water.
- 6- A magnetic bar was placed into the container and the last was put over a magnetic shaker in order to provide a sustainable solution mixing around the membranes. To avoid membranes damage, three to four glass sticks were placed cross-over the magnetic bar.

- 7- Salts, in the form of chloride ions, were detected every time by addition of few drops of AgNO_3 to 2 ml of the sample solution. Dialysis came to an end when the 2 ml gave no more color.

4.1.7- Samples concentration:

A bulk volume of sample can be reduced by evaporating a part of it into a required volume.

Equipments:

- 1- Buchi Rotavapor- R
- 2- Round flask

Principle:

The rotary evaporation principle depends on separating products with different boiling points; it is the most common used method. The rotating flask generates an effective heat transfer for fast evaporation and prevents a local overheating whilst leading to a smooth mixing of the content.

Procedure:

- 1- The temperature of the distilled water in the water bath was adjusted to 40°C .
- 2- The proper pressure was selected by the aid of pressure regulator.
- 3- The sample was then evaporated by rotavapor under a tap pressure or a membrane pump.

4.1.8- Freeze-Drying or lyophilization

Principle:

To subject a material; e.g. solution, food, vaccines, etc. to quick-freezing followed by drying under high vacuum at a low temperature. Water is removed from the sample by sublimation when the sample freezes down under vacuum. A freeze-dried product will be kept for long periods at room temperature.

Equipments:

Methanol bath: Hetofrig (Heto Brikerød, Danmark)

Freeze – drier: Christ R Alpha 1- 6

Procedure:

- 1- Freezing: The sample was frozen under a continuous shaking in a methanol bath at -40°C . This provides a necessary condition for low temperature drying.

- 2- Vacuum: After freezing, the product is placed under vacuum. This enables the frozen solvent in the product to vaporize without passing through the liquid phase, a process known as sublimation.
- 3- Heat: Heat is applied to the frozen product to accelerate sublimation.
- 4- Condensation: Low-temperature condenser plates remove the vaporized solvent from the vacuum chamber by converting it back to a solid.
- 5- The drying process was carried out for about 24 hours.

4.1.9- Acid-washing of glass equipments:

Principle:

Concentrated acids are crucial for washing of glass equipments when they are used for analysis of carbohydrates. The purpose of glass equipment washing is to insure the removal of any carbohydrate contaminants, like cellulose and glucose, which may contaminate these equipments' cardboard packaging.

Reagents:

37% HCl

Procedure:

- 1- The concentrated acid is poured completely over the whole glass equipments in a glass beaker and kept in 30 minutes.
- 2- The equipments were rinsed thoroughly using a water tap.
- 3- The equipments were then rinsed thoroughly in distilled water
- 4- Finally, the equipments were dried using an incubator.

4.1.10- Ultra filtration:

Principle:

The idea of ultra filtration is based on a process that allows separation of different molecules depending on their molecular size. Molecules are passed through a membrane under a hydrostatic pressure that forces a liquid against a semi- permeable membrane. Molecules of high molecular weight are retained while water and low molecular weight solutes pass through the membrane.

The rate of passage depends on the pressure, concentration of the solute and the temperature of the molecules or solutes on either side, as well as the permeability of the membrane to each solute.

Separation of the low molecular weight polysaccharides by ultra filtration will allow the retaining and then recirculation of the high molecular weight polysaccharides while the low molecular weight polysaccharides are filtrated out.

Equipments:

Pump: Watson Marlow 520s

Filter:

Erlendmeyer flask

Membranes' hugs

Reagents:

Distilled water

0, 3 M NaOH solution

0, 1 M NaOH solution

Procedure:

- 1- Watson pump was pumped over the night in order to wash the filter.
- 2- The outlet tube of the filter was tided from the middle using a hug.
- 3- The extract of *Parkia biglobosa* bark was pumped the filter in a 5 minute period and the outlet tube was opened carefully so that the low molecular polysaccharides pumps out and collected in a flask.
- 4- The high molecular weight polysaccharides' outlet should be tided by a hug in order to obtain a sufficient high pressure to pump the solution through the filter and to allow the passage of few drops of the high molecular weight polysaccharides to be circulated into the filter.
- 5- An adequate amount of the filtrated high molecular polysaccharides No 1 was taken and ultra filtration was preceded with portion No2 likewise.
- 6- The ultra filtered amounts were concentrated in Nuchi apparatus.
- 7- Watson apparatus was washed with distilled water over the night and at the morning the 100⁰ C permeates was pumped and filtrated in the same procedure as for 50⁰ C.

Washing and storage of ultra filtration set:

- 1- The ultra filtration set was first washed with distilled water for 10 minutes keeping both outlets open.
- 2- The set was then washed with a non-circulated 0, 3 M Na OH solution; in order to eliminate all carbohydrates inside the apparatus. This process was carried on continuously until no more color of the pumped permeate was seen.
- 3- The set was, further, washed with 2 liter of a circulated 0, 3 M NaOH solution for 15 minutes.
- 4- NaOH solution was washed out using distilled water until the pumped out distilled water from both outlets becomes neutral (a pH paper was used).
- 5- Finally, 0, 1 M NaOH solution was pumped into the filter while all the outlets of the filter were completely closed and the set was stored at 4⁰ C.

4.2- Isolation of the polysaccharides in the plant sample material:

4.2.1- Ethanol extraction of the plant materials:

Principle:

When ethanol is used as extracting solvent, different amounts of non-polar constituents of the plant sample will dissolve into ethanol solution. Examples are: saponins, colored materials, and other low molecular organic components. It is, therefore, important to remove these components from the plant sample before liquid extraction process in order to prevent further analysis interference from these materials. The plant sample is extracted with methanol in a Soxhlet extractor, where ethanol recycles in a closed system.

Equipments:

Cooking pot (casserole)

Cooking plate: Wilfa

Termometer:

Soxhlet filter for sample materials

Soxhlet extractor

Buchi Rotavapor-R

Reagents:

98% ethanol

Distilled water

Procedure:

- 1- An amount of 500 g of the powdered plant material was weighed.
- 2- An amount of 4 liters (2 liters distilled water and 2 liters ethanol = 50% ethanol) was added into the weighed powdered plant material.
- 3- The plant material was then cooked in a Wilfa cooking pot at 50⁰ C for two hours.
- 4- The cooked plant material was divided into four equal plastic bottles and was centrifuged in a Multifuge 4 KR, Heræus, Kendro centrifuge for 10 minutes at 3550 cpm.
- 5- Supernatant which was dark brown, and the plant residue was cooked in 2 liter 50% ethanol (1 liter distilled water and 1 liter ethanol) for 1 hour at 60⁰ C.
- 6- The plant mixture was centrifuged, as in step 4 above.
- 7- Supernatant was separated in a flask and the plant residue was taken into the cooking pot with an amount of 2 liter distilled water for further extraction.
- 8- The ethanol extract was filtrated with a 15 cm Whatman filter paper and Nutsj apparatus under a water-tap vacuum pump.

4.2.2- Water extraction of the plant materials:

Principle:

When water uses as a solvent, the polar constituents dissolve in it. Some types of water-soluble polysaccharides will, therefore, dissolve in water depending on the water temperature. Consideration should be taken when extracting polysaccharides in hot water as some of polysaccharides can undergo decomposition at high temperatures.

Equipments:

Cooking pot (casserole)

Cooking plate: Wilfa

Thermometer:

Centrifuge: Multifuge 4KR, Heræus, Kendro

Water tap vacuum pump

Buchner flask and Buchner funnel

Buchi waterbath

Buchi Rotavapor-R

Round flask

Round filter:

Procedure for 50⁰ C water extract:

- 1- The plant residue of *Parkia biglobosa* bark from the ethanol extract with 2 liter of water was cooked at 50⁰ C for 2 hours. The temperature was controlled using a thermometer.
- 2- The plant material was centrifuged and supernatant was collected in a big flask (the color was intense dark brown).
- 3- The plant residue was mixed with another 2 liter distilled water and was cooked for another 2 hours at 50⁰ C.
- 4- The mixture was centrifuged and supernatant was added to the first portion while the residue was kept in a refrigerator for further extraction.
- 5- The extracted plant material was filtrated through a glass fiber filter using nutsj funnel.

Procedure for 100⁰ C water extract:

- 1- An amount of 2 liter distilled water was added to the residue of 50⁰ C water extraction and it was similarly preceded as the above procedure.
- 2- The temperature of extraction was 100⁰ C and was controlled by a thermometer.

Procedure:

- 1- The dialysis membranes were tied at one end with a clip and filled with a little amount of water to control the membrane was intact.
- 2- The membranes were put in a large beaker glass and filled to their 2/3 with the sample solution. 1-2 drops of toluene were added as a preservative.
- 3- Membranes were evacuated from air before they were tied at the other end. They were put into a big beaker glass with distilled water. The beaker glass was put into a shaking apparatus and a magnetic rod was placed into the beaker glass to insure a continuous shaking of the water.
- 4- Dialysis came to an end when 2ml of the distilled water gave no color with AgNO_3 solution.

4.2.3- Chromatographically Extraction Methods:

The ion exchange consists of an insoluble porous matrix where the charged sample groups are bound to. These charged groups of the sample can be exchanged by using other ions with the same charging. The principle of the ion-exchange chromatography is based on a reversible adsorption of the charged molecules in a sample into the stationary phase. The adsorbed molecules are then eluted from the stationary phase by using different solvents which pumped the ion-exchange column.

The separation process depends on the charged molecules, the number of the charged groups as well as the eluting solvent strength. (Amersham Biosciences)

4.2.3.1- ANX Sepharose® 4 fast flow: (Amersham Biosciences AB, 2000)

Principle:

ANX Sepharose 4 fast flow is a weak anion exchange type with a matrix that is based on cross-bound agarose. This gives the medium high mechanic and physical stabilities. The amount of agarose in the base matrix is lower; this results in a medium with high porosity making ANX Sepharose 4 fast flow particularly useful for the purification of high molecular-mass proteins. ANX sepharose 4 fast flow is well-suited for process-scale chromatography and can be run at high flow rates. The ion exchange group is a diethylaminopropyl group ($-\text{O}-\text{CH}_2\text{CH}(\text{OH})\text{CH}_2-\text{N}(\text{CH}_2\text{CH}_3)_2$).

During separation of samples which contain carbohydrates, the acidic ones will mostly be retarded on the column DEAE matrix because of their loaded negative groups without any competence for the chloride ions. These acidic carbohydrates will be easily eluted and separated by different Na Cl solutions. Increased acidic strength will need increased salt strength, too. The neutral amounts present in the sample do not react with ion-exchange materials and will indeed be separated with water.

Gradient elution using ANX Sepharose® 4 fast flow:

Purpose:

This method was used as a fast and minor scale type to separate the *Parkia biglobosa* contents of carbohydrates to be used for further qualitative survey. This method is based on using gradient elution with solutions of different ion strengths.

Equipments:

ANX Sepharose® 4 fast flow column material (Amersham Pharmacia Biotech)

Packed column: Pharmacia

Internal Diameter: 5 cm

Height: 30 cm

Column Volume: approximately 540 ml (height 27.5 cm, i.d. 5cm)

Fractions collector: Pharmacia LKB SuperFrac

Collecting Tubes: centrifuge tubes RB, 15 ml (Heger AS)

Acro® 50A Device with 5µm Versapor® Membrane (Pall Corporation)

Sterile Millex-AA 0,8 µm filter unit (Millipore)

10 ml syringe (Terumo)

Nutsj and vacuum pump apparatus

Büchi Rotavapor- R

Spectra/Pro® Membrane dialysis membranes, MWCO 3500

Erlenmyer flask

Glass rod

Reagents:

Distilled water, 4L

1 M NaCl, 2L

2M NaCl, 2.5 L

1M Sodium Acetate adjusted to pH 3 with concentrated HCl, 0.5L

Sodium Azide 0.05%, 1L

2M Na OH, 0.5 L

Saturated AgNO₃

Washing of the pre-packed column:

- 1- The flow rate was adjusted first using Perimax pump to 2ml/1 minute
- 2- The pre-packed column was washed with approximately 2 column-volumes distilled and degassed water to pack up the column.
- 3- An amount of 1.5 column volume 2M NaCl was run through the column with 2ml/1min to insure the domination of Cl⁻.
- 4- Excess NaCl in the column was washed with distilled and degassed water using the same flow rate. The salt was over when no more salt was detected using 2 drops of AgNO₃ solution.

Application of elude and sample solution:

- 1- 500 ml of the sample was filtered and degassed with water jet vacuum.
- 2- The sample solution was run through the column with 1ml/1minute.
- 3- The neutral polysaccharides were eluded with about 2 column volume distilled and degassed water with a flow rate of 2ml/1minute. The neutral polysaccharide was fractioned into tubes.
- 4- The acidic fraction was eluded with 2M NaCl with a flow rate of 2m/1minute according to the following formula:

$$C/t = (C_0 R) / V_0$$

where:

C = is the NaCl concentration in the mixed chamber at time t.

T = is the elution time.

C₀ = the outlet concentration from the NaCl chamber.

R = the flow rate (1ml/1min) from the salt solution to the mixed chamber.

V₀ = the starting volume in the mixed chamber.

- 5- 180 tubes with 6ml were collected.
- 6- Each rack was first tested for polysaccharides presence using phenol- sulfuric Acid test in the following manner: tubes numbers 10, 30,50,70,90,110, 130,150. To find out the polysaccharide ranges. The tubes which contain the polysaccharides were then added together. The collected amount was dialyzed, concentrated and freeze-dried.
- 7- The neutral content was also dialyzed, concentrated, and freeze-dried.
- 8- The column was run for 2 times with 2M NaCl to elude the more acidic polysaccharide compounds.

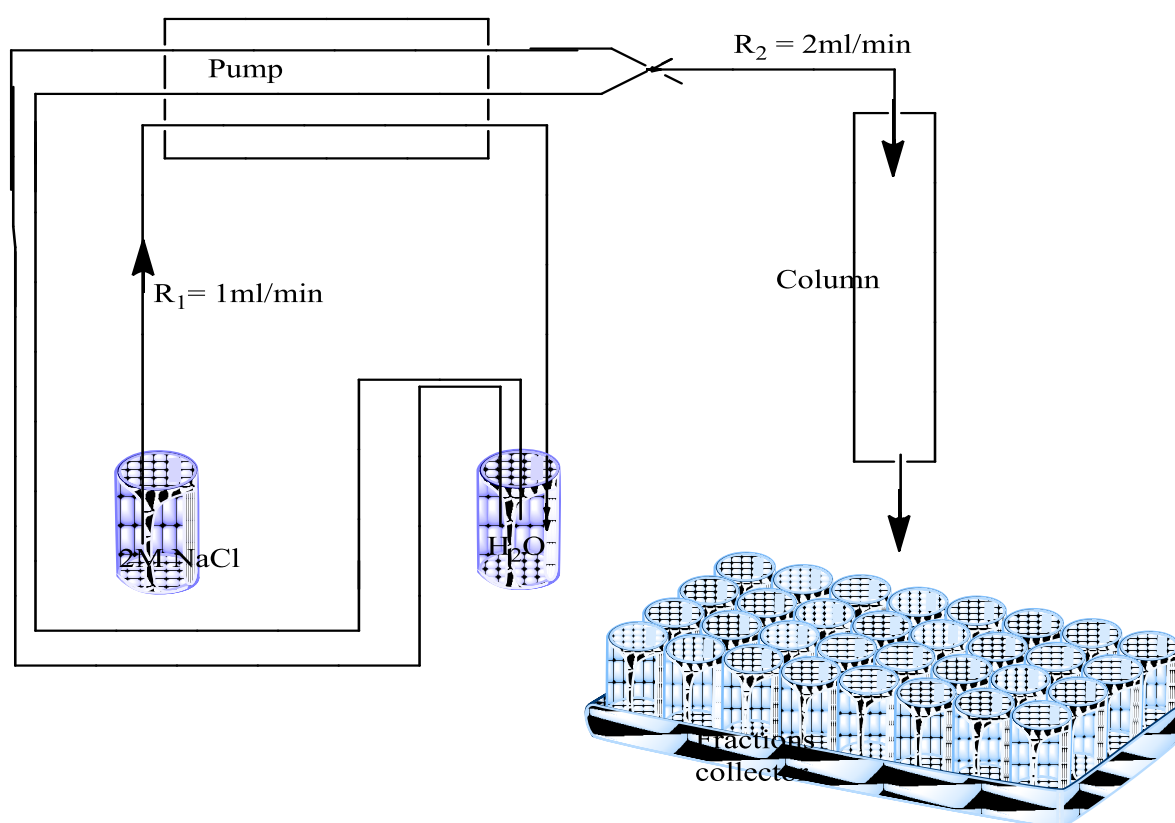


Figure (4.2.3.1) Schematic illustration for the linear NaCl gradient method.

Washing and regeneration of the column:

- 1- The column was turned-up down.
- 2- It was eluted with 1, 5 of its volume with 1M Na OH with a flow rate of 2ml/1minute.
- 3- It was regenerated with 2 of its volume with 2M NaCl with a flow rate of 2ml/1minutes.
- 4- The column was eluted with distilled water with a flow rate of 2ml/1minute until no more turbidity (opaque) was checked with 2 drops of AgNO₃ solution.

- 5- The column was conserved with 0.05% NaN₃ by running of the solution through the column with a flow rate of 2ml/1minute and it was kept at 4⁰ C.

4.2.3.2- Fast Protein Liquid Chromatography (FPLC):

Principle:

The theory of FPLC is based on gel filtration principle. It is originally formulated to purify substances or to separate proteins by ion exchange technique. The column that is used in FPLC can separate the macromolecules according to their size, hydrophobicity or charging. The technique is quite suitable for the purification and separation of polysaccharides as well as the estimation of the molecular weight distribution of these polysaccharides.

Equipments:

ÄKTA-FPLC

Data program: UNICONR Version 4.0

Detector: RID-10A, Shimadzu®

Writing machine: REC 112

Pump: P-920

Monitor: UPC-900

Injection: Valve Inv-970, Superloop, 10 ml

Fraction collector: Frac-900

Collection tubes: centrifuge tubes SB 7ml

Filter: Pall® Acrodisc® 32 with more than 0, 45 µm Supor® membranes

Plastic syringes: BD 1ml

Distilled and degassed water

Procedure:

- 1- 1 mg of the sample was dissolved in 1 ml distilled water; the solution was injected into the superloop Inv-907 valve.
- 2- Samples were eluted with a flow rate of 1ml/minute and were collected in 5ml-tubes.
- 3- The tubes contents were tested for their polysaccharide amounts using phenol-sulfuric acid test.
- 4- Fractions were added together according to their profile monster.

4.2.3.3- Sephacryl S-200 Gel Filtration (Size exclusion chromatography, SEC):

SEC is a widely used technique for the purification and analysis of synthetic and biological polymers, such as proteins, polysaccharides and nucleic acid (IUPAC)

Gel filtration is almost used to fractionate and separate multiple components in a sample depending on the differences in their size. The goal may be:

- i- To isolate one or more of the components.
- ii- To determine molecular weight, or
- iii- To analyze the molecular weight distribution in the sample.

Sephacryl S-200 has a gel matrix medium consists, usually, of cross-linked polymer of methylenebisacrylamide, allyl dextran or agarose and filter under low pressure. This will promote a great mechanical strength that enables the elution of samples with a high speed; in addition, it has a separation zone between 1000-80000 D. The medium is a porous matrix, which is chemically and physically stable, and inert.

The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the outer mobile phase particles. Samples are eluted isocratically, i.e. there is no need to use different buffers during the separation.

If a sample is applied into the column, the mobile phase (buffer) and the sample diffuse i.e., partitions in and out of the pores of the matrix. Smaller molecules move further into the matrix and so stay longer on the column. As buffer passes continuously through the column, molecules that are larger than the pores of the matrix are unable to diffuse into the pores and pass the column faster than smaller ones.

The objectives of this method include:

- 1- The method offers a good separation of large molecules from the small molecules using a minimal volume of elute;
- 2- Various solutions can be applied without interfering with the filtration process keeping the biological activity of the particles to be separated unchanged;
- 3- With size exclusion chromatography there are short and well defined separation times together with narrow bands, which lead to good sensitivity.
- 4- There is also no sample loss because solutes don't interact with the stationary phase.

The method has some drawbacks such as obtaining of a limited number of bands because the time scale of the chromatogram is short, and there has to be a difference in molecular mass to have a good resolution. (Skoog, 2006)

Reagents:

10 mM NaCl filtered 0.45 μ m HA (Millipore)

0.05 % NaN₃

Equipments:

Column: XK25, Sephacryl S-200, Amersham Bioscience

Diameter: 2.6 cm

Length: 90 cm

Pump: pump p-1, Pharmacia Biotech

Flow rate: x 1

Fractions collector: SuperFrac

Micro filter: 0.45 μ m (Millipore)

Conditions:

Elution's flow rate: 0.5 – 1ml/min

Applied volume: 10 – 20 ml

Sample's concentration: 15 – 20 mg/ml

Volume = $\pi \cdot R^2 \cdot H = 3.14 \times (1.3 \text{ cm})^2 \times 90 \text{ cm} = 477.6 \text{ ml}$

Elution liquid: distilled water

The total amount of distilled water needed to wash the column was calculated as follows:

Collected amount of liquid (ml) /rotation = 0.7 ml/ min

Amount of ml / hour = 0.7ml x60 min = 42 ml

Amount of ml / day = 42 ml x 24 hours = 1008 ml

Procedure:

- 1- About 102 mg of the sample Pb S50, 143 mg of Pb S100i-1 and 414 mg of Pb S100ii were dissolved in 15 ml, 15 ml and 20 ml distilled water, respectively, and they were filtered through 0.1 μ m Acrodisc syringe filter.
- 2- The flow rate of the pump was adjusted to 0.5 ml/min and the samples were pumped into the Sephacryl S-200 column.
- 3- The flow rate was re-adjusted to 1ml/min and the samples were eluted with distilled water.
- 4- 60 tubes of each sample fractions (5 ml/tube) were collected.
- 5- The carbohydrate profiles were determined using the Phenol-sulfuric acid test.
- 6- Samples were finally concentrated and freeze-dried.

4.3- Qualitative and quantitative tests for carbohydrate contents:

As a quite heterogeneous group, the physical and chemical differences of carbohydrates will give rise to different properties, such as solubilities, reactivities and susceptibility to digestive enzymes. (Cui, 2005)

4.3.1- Phenol – Sulfuric acid Assay:

Reaction Theory:

Carbohydrates undergo a range of reactions in the presence of strong acids and heat. The results of these reactions are the so-called the furan derivatives. i.e. furanaldehyde and hydroxymethyl furaldehyde. A dehydration reaction exists first followed by the formation of furan derivatives. These derivatives, in turn, either condense with themselves or with phenolic compounds to produce dark colored complexes. The existed complex color absorbs UV – VI light and the absorbance is proportional to the carbohydrate concentration in a linear pattern.

Reagents:

4% phenol

Concentrated H₂SO₄

Equipments:

Finnpipette® 40 - 200µl (Labsystems)

Gilson Microman® pipette 100µl

Gilson Microman® pistons and tips

Microtest Plate 96 – Well flat bottom (Sarstedt, Inc.)

BIO – RAD Model 3550 Microplate reader

Glass test tubes

Vortex mixer: (Fisons)

Gloves and eye- glass

Procedure:

- 1- 200µl 4% phenol solution is added to a glass test tube containing 100µl of a clear sample solution.
- 2- 1 ml of concentrated H₂SO₄ is added in a rapid stream to the glass test tube.
- 3- The mixture thoroughly combined using a vortex mixer and allowed to stand for 30 minutes.
- 4- 100µl from each tube was transferred to its equivalent well in the Microtest plate.

5- The carbohydrate absorbance was read at 490 nm using a spectrophotometer.

Applicability:

This Assay is widely used to determine the total concentration for both reducing and non-reducing carbohydrates in a sample. It is advantageous in that:

- i- The reagents are of low cost.
- ii- The reagents are readily available.
- iii- The required equipment is minimal.
- iv- It is simple.
- v- It can be used to quantify monosaccharides, oligosaccharides and polysaccharides.
- vi- The assay provides the most accurate results when applied to samples containing just one type of carbohydrate. (Steve W. Cui. Food Carbohydrate. Pp.72)

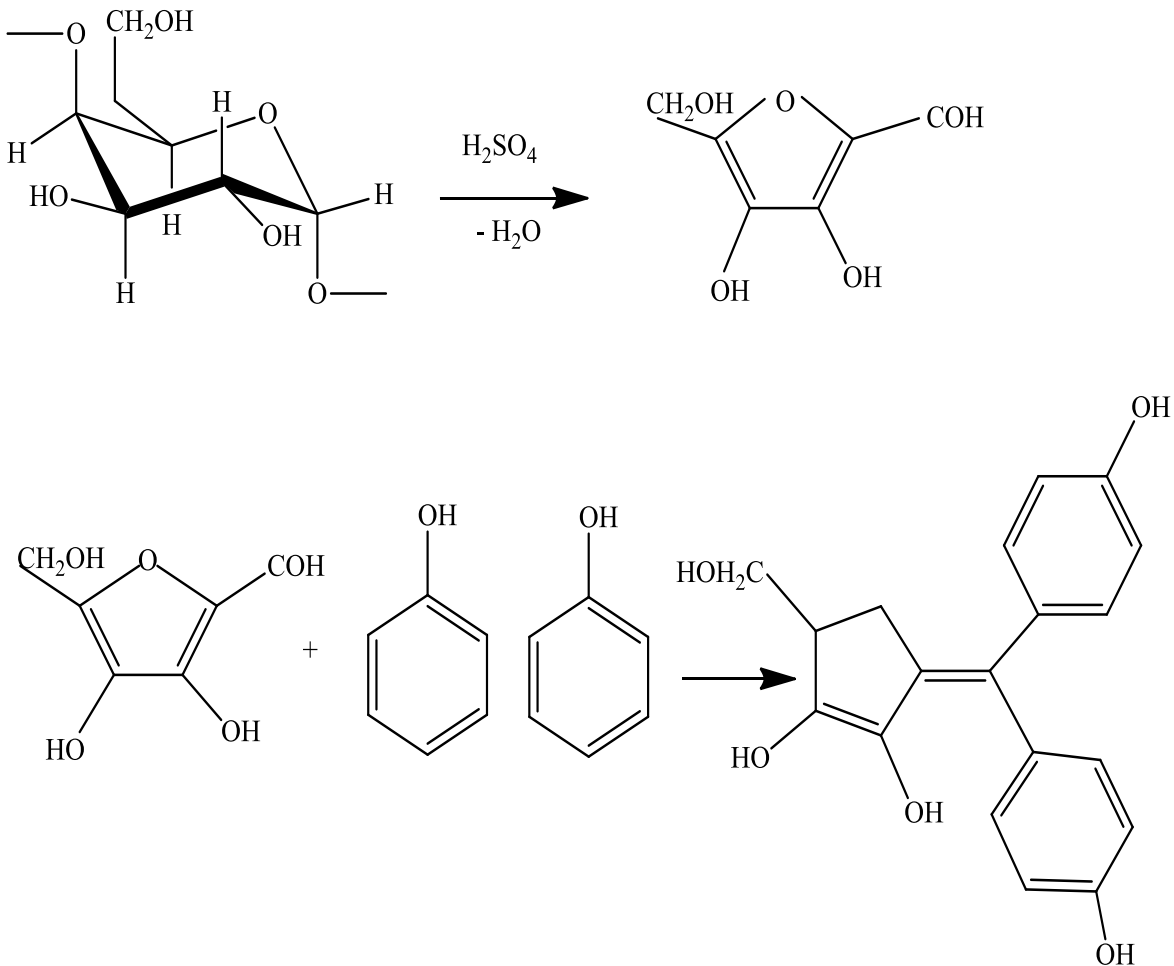


Figure (4.3.1) Phenol-Sulfuric acid Assay (Dubois et al 1956)

Polysaccharides react with conc. sulfuric acid to form anhydrosugar, which condenses with two phenol molecules to give a yellow complex.

4.3.2- Methanolysis:

Principle:

The different polysaccharides should be degraded into their simplest monosaccharides in order to examine their different sugar contents. This examination is carried out using methanolysis which results in the formation of methyl glycosides. The test is carried out in an acidic, water-free and a methanol-containing medium. In such conditions, the glycosidic linkages between carbohydrates monomers will break down into their simplest monosaccharides. A methyl glycoside is formed from OH-group at C1 while a methyl ester will be formed from the COOH group at C6. The rate of reaction is dependent on the anomeric configuration, position of the glycosidic linkage and the identity of the monosaccharide.

Mannitol was used as an internal standard in this procedure as it is a solid substance, present in highly purified form, moreover, it is a carbohydrate with chemical similarity to the polysaccharides monomers, and it gives a single chromatographic peak different from other components. It is also thought to be stable under the analytical conditions ([Jamieson & Reid 1974](#)).

The end products of methylated polysaccharides are useful for the: determination of sequences, the determination of branching patterns and the determining location of substitutes.

Reagents:

4M HCl in MeOH

1 µg/µl mannitol in 1M HCl in MeOH

Water-free MeOH

Equipments:

Supelco tubes with covers

P₂O₅ exicator

Transfer pipette®, 100 µl

SMI capillaries (blue)

Incubator: Functionline (Heraeus Instruments)

N₂-gas setup with a heating unit

N.B: All glass equipments were washed with concentrated HCl

Procedure:

- 1- 1 mg of each sample was weighed into a glass vial and the vials were dried using a stream of P_2O_5 gas in 24 hours.
- 2- 1 ml 4M HCL in methanol was added into the samples.
- 3- 100 μ l of 1 μ g/ μ l mannitol was added as an internal standard.
- 4- The samples were placed in an incubator for about 24 hours at 80⁰ C. The vials' covers were locked properly after 15 minutes to prevent any sample evaporation.
- 5- The samples were dried under stream of N_2 gas.
- 6- 200 μ l water-free methanol was added followed by drying under N_2 gas.
- 7- Step 6 was repeated twice.
- 8- The samples were dried under vacuum using P_2O_5 exiacator for one hour before TMS derivatizations.

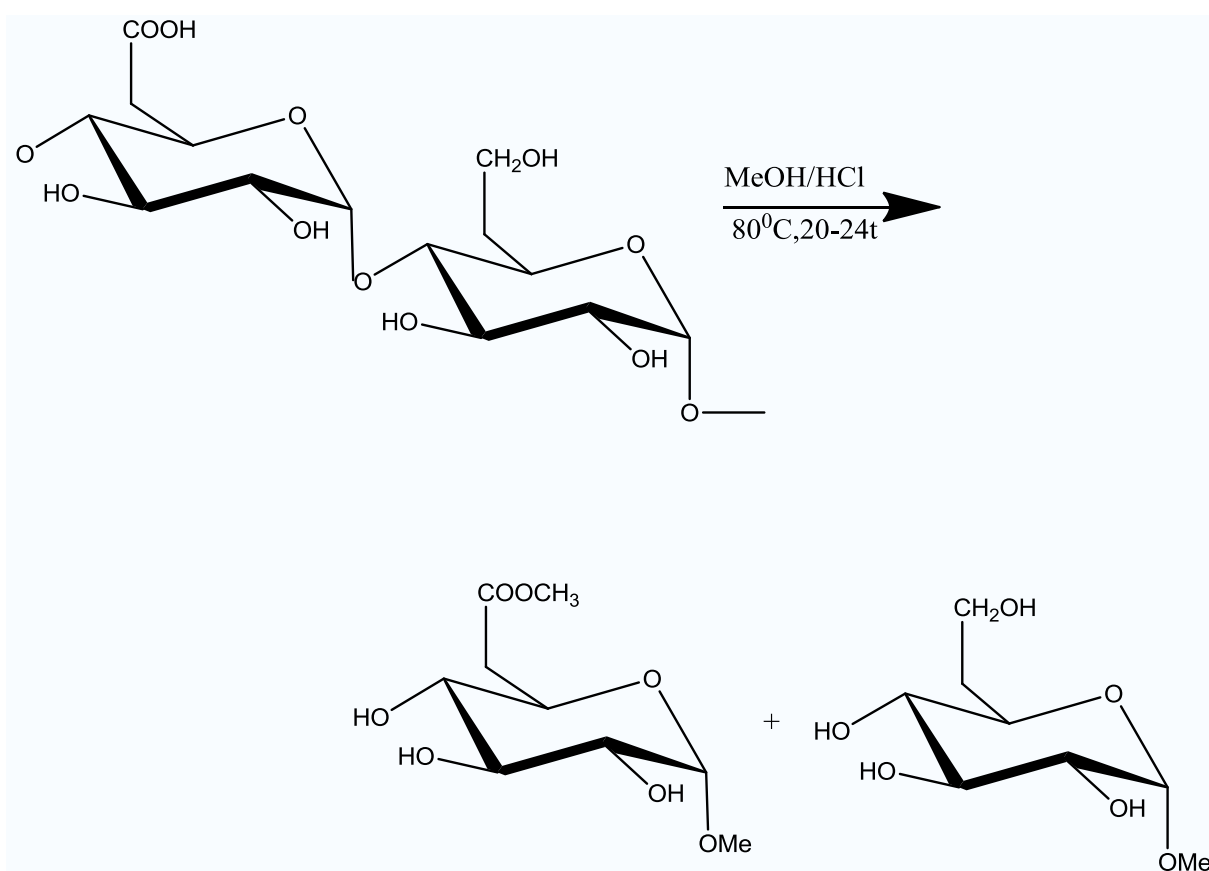


Figure (4.3.2) Methanolysis of the glycosidic linkage between an acidic and a neutral monosaccharide in a polysaccharide chain.

4.3.3- TMS Derivatization:

Principle:

Organic functional groups as carboxylic acid, hydroxyl, phenol and amino groups reduce the volatility of substances; therefore, they cause surface adsorption for these substances. When these substances are eluted, broad tops as well as tail-formation resulted from such groups,

which cause GC analysis problems. The resultant tops and tail-formation can be avoided by derivatization method. Derivatization results in volatile and thermal stable products that are much suitable for GC analysis.

Given that monosaccharides are polar compounds and of low volatility, they must be derivatized prior to analysis. The aim of derivatization is to convert the free hydroxyl groups into volatile and stable TMS derivatives by hexamethyl disilazane (HMDS) and trimethylchlorosilane (TMS) reaction

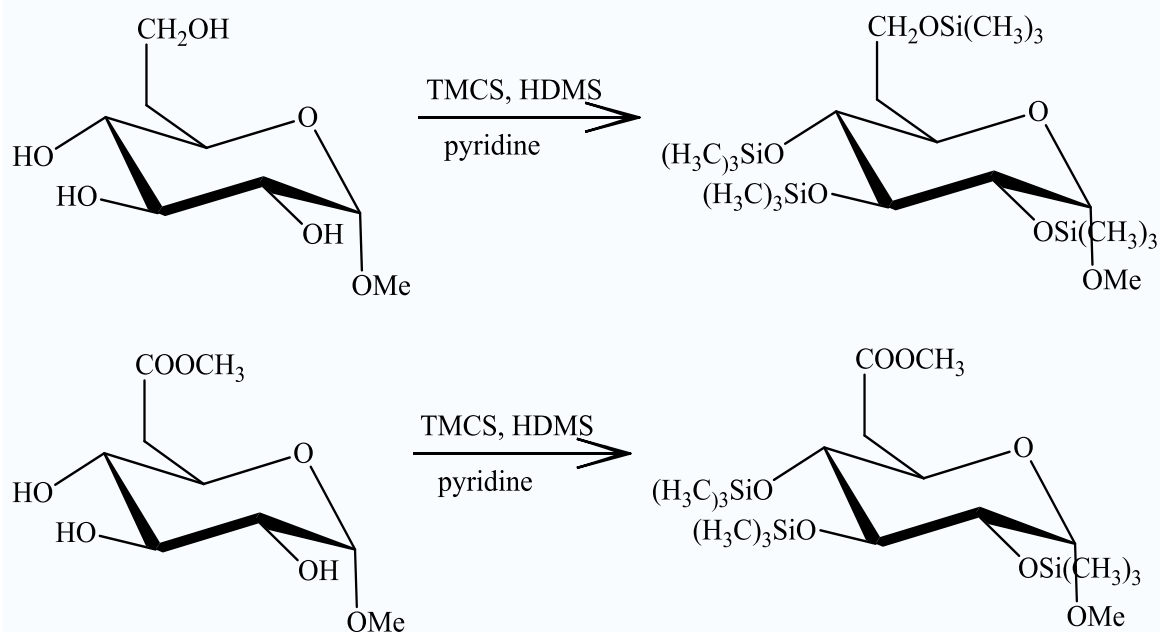


Figure (4.3.3) A reaction between TMS reagent and a methyl glycoside monosaccharide with/without methyl ester group (Sogn 1998).

Reagents:

TMS reagent: trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS) and water-free pyridine in a ratio of 1:2:5.

Equipments:

Transferpipette, 100 μ l (Brand)

SMI (acid-washed)

WirliMixer (Votrex mixer) (Fisons)

Procedure:

- 1- 200µl TMS reagent is added to the samples and mixed thoroughly.
- 2- The samples are left for a while, at least 30 minutes before they were analyzed by gas chromatography.

N.B: samples which couldn't be driven the same day were not derivatized.

4.3.4- Gas-Liquid chromatography (GC):

It is a technique used in separate and analyzing compounds in a mixture based on either their degree of interaction with a liquid stationary phase or their affinity for it.

The driving phase (mobile phase) is usually a carrier gas that is inert, like helium, or a non reactive gas such as nitrogen, while the stationary phase is a microscopic layer of liquid or polymer on an inert solid support placed inside a column. Hydrogen has been the most efficient and provided the best separation; however, helium has a large range of flow rates that are comparable to hydrogen in efficiency, in addition helium is non-flammable and works with a greater number of detectors. Therefore, helium is the most common carrier gas used. (Pavia, Lampman, Kriz, Engel, 2006).

The gaseous compounds being analyzed interact with the walls of the column in a manner that causes each compound to elute at a different time, known as the retention time of the compound. Liquid analyte is injected into the column using a microsyringe to be driven by the moving gas. The rate at which the molecules progress along the column depends on certain parameter, i.e. the strength of adsorption, which in turn depends on the type of molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end of the column at different retention times.

Temperature control inside the column is crucial; reducing the temperature produces the greatest level of separation, but can result in very long elution time. Desired separation can be obtained by either temperature programming or electronic pressure control which modifies the flow rate during the analysis, aiding in faster run times while keeping acceptable levels of separation.

The flame ionization detector (FID) is the most commonly used detector. It is sensitive to a wide range of components, primarily hydrocarbons, and works over a wide range of concentrations. Quantification requires using an internal standard and a formulation of response factor (RF). Response factors are used to correct for disparate GC response to monosaccharides and losses arising from hydrolysis and derivatization.

Equipments:

Gas chromatography: GC 6000 VEGA SERIES 2 (CARLO ERBA INSTRUMENTS)

Programming unit: ICU 600

Integrator: C- R6A CHROMATOPAC (Shimadzu)

Detector: Flame ionization detector (FID)

Injector: Split: Splitless

Column: DB-5 (J & W Scientific)

“Fused silica” capillary column

Length: 30 m

Internal diameter: 0,32 mm

Film thickness: 0,25 μm

Driving gas: Helium

Flow: column: 1.8 ml/min (37,6 cm/sec)

Split flow: \approx 11 ml/min

Split ratio: 1:6

Injection volume: 0,5 – 1,0 μl

Injector temperature: 260⁰ C

Detector temperature: 310⁰ C

Temperature programming: 140⁰ C $\xrightarrow{1^0 \text{ C/min}}$ 170⁰ C $\xrightarrow{6^0 \text{ C/min}}$ 250⁰ C

4.3.5- Linkage pattern determination of the monosaccharides present in the polysaccharides (Cui 2005, Kim & Carpita 1992):

4.3.5-1- Carboxylic acid reduction:

It is an assay that is used to differentiate between neutral, uronic and methoxylated sugars as well as to distinguish among methylated sugars with symmetrical substitutions that result in identical spectra. Using sodium borodeuteride (NaBD₄) as a reductant will result in derivatives that can be identified by a fragmentation pattern at the C-1 that is characterized to only neutral sugars. Carboxylic esters are reduced to their 6, 6-dideuteriosugars, which can then be distinguished from their neutral ones as fragments with M⁺+2; where M⁺ is the mass of the neutral sugar and the +2 is the added mass from the D₂. The free uronic acids are activated using carbodiimide and reduced with NaBD₄ to produce total uronic acids.

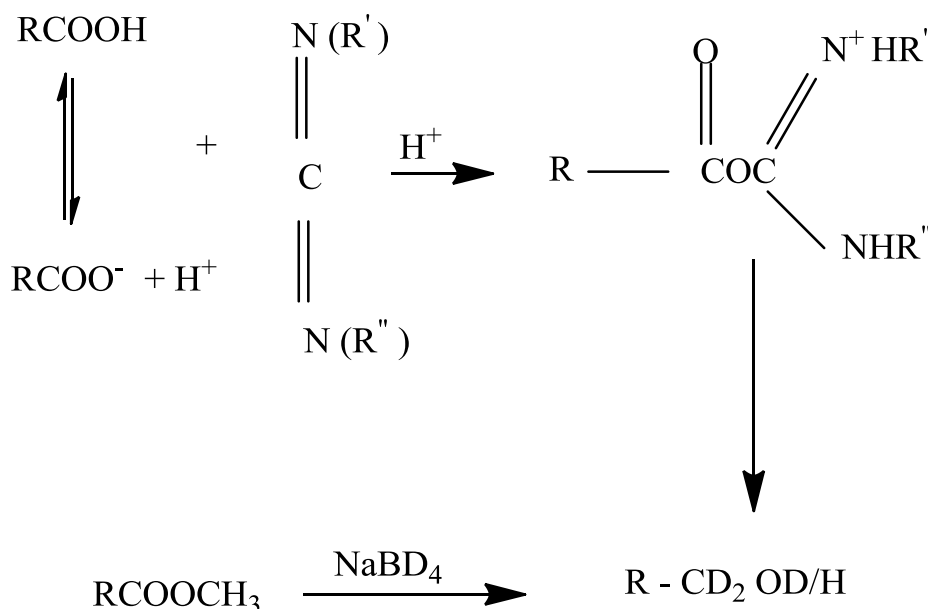


Figure (4.3.51) Activation and reduction of carboxylic acid group in a sugar containing uronic acid.

Reagents:

2-[N- Morpholino] ethane sulphonic acid (MES): Sigma Cat. No. M-8250

1-cyclohexyl- 3 (2- morpholino) carbodiimide – metho-p- toluene sulphonate (Carbodiimide): Aldrich Cat. No C 10, 640-2

Tris [hydroxymethyl] aminomethane (TRIZMA base): Sigma Cat. No T 1503

0.05M Sodium hydroxide: analytic reagent. Mallinckrodt Cat. No 7708

Glacial acetic acid: BDH Aistar grade Cat. No. 45001

Octanal

Distilled water

Equipments:

Methylation tubes with covers

Glass pipette with plastic balloon

Glass vials

MS2 mixer (IKA®)

Transfer pipettes, 100µl & 200µl

SMI Capillaries (blue)

Spectra/Por® 3 Dialysis membrane, MWCO 3500, d: 11.5mm (Spectrum Laboratories)

Pasteur pipettes with plastic balloon

P₂ O₅ exiator

Ice

pH paper

Procedure:

- 1- Samples of 2 mg were dissolved in distilled water, 200µl 0.2 M MES was added and 400µl of 500mg/ml freshly prepared carbodiimide in distilled water was added, too. The mixture was vortexed and incubated for 3 hours at 25⁰ C.
- 2- 1 ml of 2M TRIZMA was added followed by 4 drops of octanal (as an antifoam). The mixture was cooled in ice. 1 ml of 70mg/ml freshly prepared sodium borodeuteride in 0,05M NaOH was added. The whole mixture was incubated overnight at 4⁰ C in the cooling room.
- 3- The excess reductant was destroyed by slowly adding 200µl + 200µl + 100µl of glacial acetic acid, keeping the pH of the mixture basic.
- 4- The samples' mixtures were transferred into dialysis membranes and dialyzed for about 24 hours. To insure maximum dialysis, the dialysis water was changed regularly.
- 5- The samples were freeze-dried and kept in a P₂ O₅ exiacator for 1 hour.

4.3.5-2- Methylation Analysis using NaOH:

(Cui 2005, Kim, & Carpita, N.C 1992)

It is used in the determination of carbohydrates structure analysis for a long period and it has been proved to be a powerful method for this purpose. This analysis consists of two major steps: a chemical derivatization and a gas-liquid chromatography-mass spectroscopy (GC-MS). The substitution monster of the O-acetyl group in the partially methylated alditol acetate will reflect the nature of the glycosidic linkage together with the ring form of the polysaccharides.

Principle:

In the methylation reaction all free hydroxyl groups of the polysaccharides are converted into methoxyls in the presence of a strong basic medium followed by methylating reagent. The presence of the strong basic medium will aid in the formation of OH^- and carbanion from (DMSO), which ionize the free hydroxyl groups from the reduced carboxylic acids. These

free hydroxyl groups will deprotonated and subsequently become methylated by the addition of methyl iodide (CH_3I) reagent.

Reagents:

Water-free methanol

Dimethylsulfoxide (DMSO)

Dry sodium hydroxide pellets

Methyliodide

Sodium thiosulfate pentahydrate

Chloroform

Distilled water

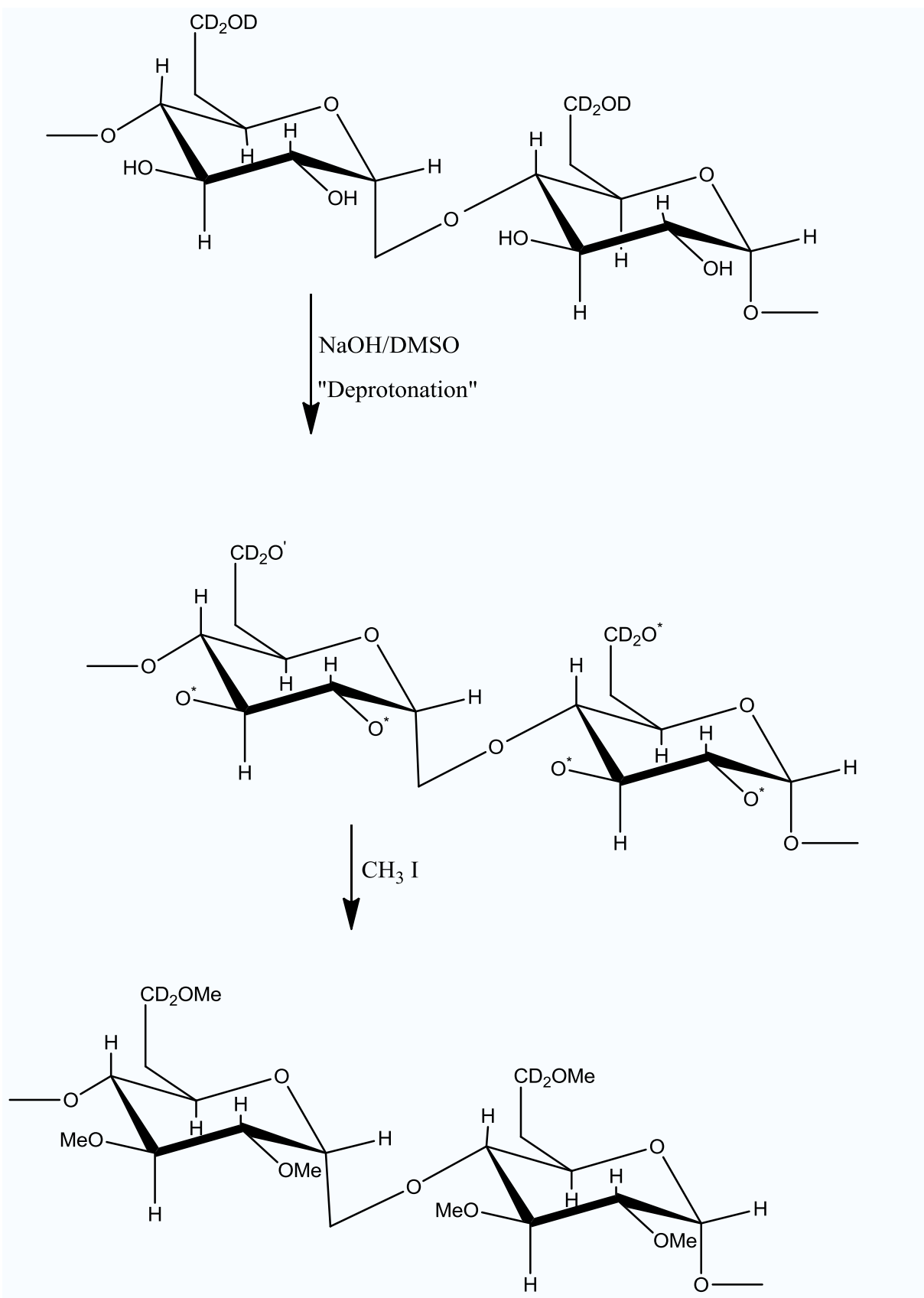


Figure (4.3.5.2) Methylation of hydroxyl groups of polysaccharides.

Equipments:

Transfer pipette®, 100µl (Brand)

SMI Capillaries

N₂ gas apparatus with a warm unit: Reacti-Therm™ III Heating Module (Pierce)

Glass pipette with a plastic balloon

Orbital shaker: Vibrax VXR (IKA Labortechnik)

Agatmortar with a pestle

Glass vials

MS2 minishaker (IKA)

Centrifuge: Heraeus Multifuge 4KR

Pasteur pipettes with a plastic balloon

Methylation's glass tubes with covers.

All glass equipments were previously washed with HCl

Procedure:

- 1- 500 µl of DMSO was added to the dry samples of the reduced carboxylic acids' polysaccharides. The tubes were covered, fixed to an orbital shaker and shake for more than 20 minutes at 120 rpm to dissolve glycans.
- 2- Slurry of NaOH was prepared by adding 4 pellets of NaOH to 2 ml DMSO solution.
- 3- 500 µl from this slurry was added to each sample using the SMI glass pipette. The slurry was dropped straight into the glycan solution.
- 4- The tubes were flushed with N₂-gas, capped and shake for 1 hour.
- 5- 100 µl of methyl iodide was added and stirred for 10 minutes in a fume hood, 100 µl of methyl iodide was again added and shake for 10 minutes. At last 200 µl of methyl iodide was added and shake for 20 minutes.
- 6- 10 ml of freshly prepared 100 mg/ml sodium thiosulphate in distilled water followed by 2ml dichlormethane were added to each sample. The samples were capped and vortexed for about 1 minute and were centrifuged to separate the two phases.
- 7- The aqueous (upper) phase was removed and the lower phase was washed with 3x 5 ml water and the lower phases were combined.
- 8- The lower phase was finally dried by a stream of dry nitrogen.

4.3.5-3- Hydrolysis:

Principle:

In the presence of a strong acid and heat, the glycosidic linkages between the monosaccharide residues in polysaccharides cleave (Cui 2005). The cleavage of these susceptible linkages accomplishes respectively according to the time course of temperature, in addition, long exposure to hot concentrated acid may degrade the samples. The resultant of acidic hydrolysis on the poly-methyl-ethers is the breakdown of the intern-glycosidic linkages leaving the methyl-ether bonds intact.

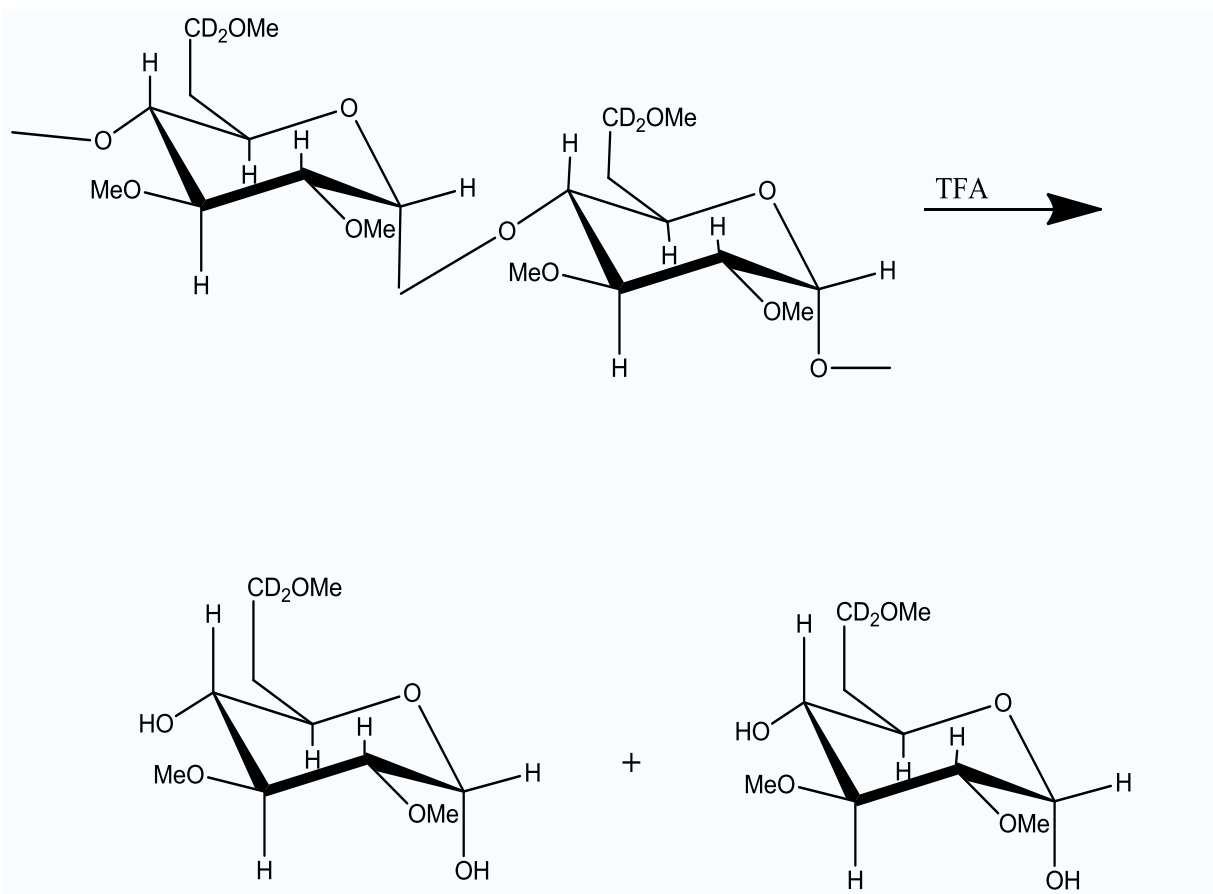


Figure (4.3.5.3) Hydrolysis of methylated polysaccharides.

Reagent:

2,5 M Trifluoroacetic acid (TFA)

Equipments:

Transfer pipette®, 100 µl (Brand)

SMI Capillaries (blue) washed with HCl

N₂-gas apparatus

Incubator: Functionline (Heraeus Instruments)

Procedure:

- 1- 500 µl of 2,5 M TFA was added to the methylated samples and they were flushed with N₂-gas.
- 2- Samples were hydrolyzed in an incubator for 2 hours at 100 – 102⁰ C.
- 3- Samples were then cooled to 30⁰ C and evaporated to dryness with a stream of nitrogen.

4.3.5-4- Reduction:

(Kim, & Carpita, 1992)

Principle:

The ring structure of monosaccharides cleaves at C1 to form alditols as a result of cyclic hemiacetal reduction.

Reagents:

2M aqueous ammonia (NH₃.H₂O)

Sodium borodeutride (NaBD₄)

Ice Acetic Acid

5% Acetic Acid in Methanol

Water- free Methanol

Equipments:

Incubator: Functionline, Heraeus Instruments

N₂- gas apparatus

Minishaker: MS2, IKA

Ultrasonic water-bath: Bransonic 220

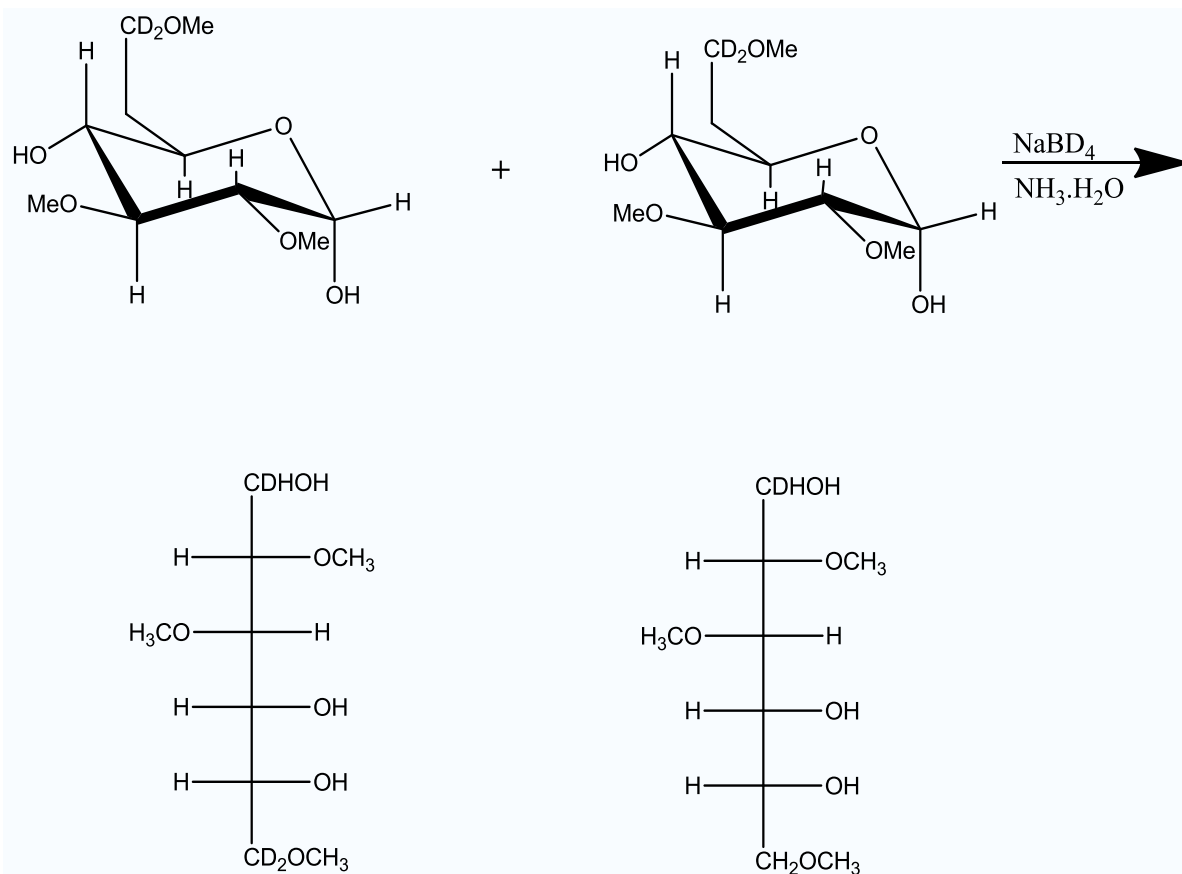


Figure (4.3.5.4) Reduction of hydroxyl groups at C-1.

Procedure:

- 1- The dried samples' residues were dissolved in 500 μ l of 2M NH₃·H₂O followed by 500 μ l of 1M freshly prepared sodium borodeutride in 2M aqueous ammonia. Solutions were shake for 1 minute and incubated at 60^o C for 1 hour.
- 2- Excess NaBH₄ was carefully destroyed with 3x 50 μ l ice acetic acid, a little amount of methanol was added to facilitate the evaporation of acetic acid, and the samples were dried under a stream of nitrogen gas.
- 3- Samples were evaporated using 2 x 2.5 ml of 5% acetic acid in methanol and 5 x 2.5 ml methanol in order to remove boric acid crystals.

4.3.5-5- Acetylation:

Principle:

Hydroxyl groups are formed after the completion of hydrolysis and reduction phases of the methylated samples. The resultant hydroxyl groups are further acetylated with acetic acid anhydride. The acetylated polysaccharides are extracted with dichloromethane and finally dissolved in methanol for later GC-MS analysis.

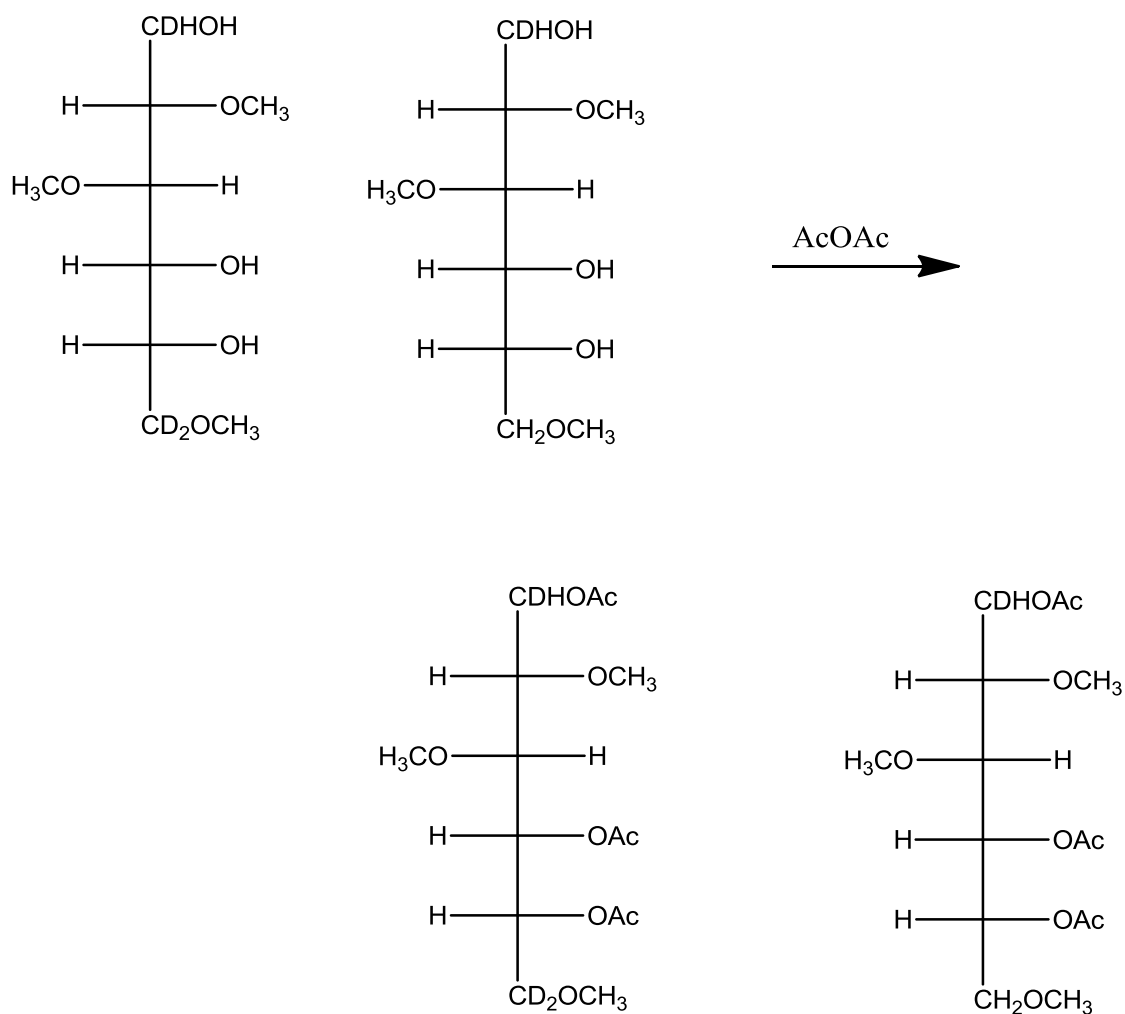


Figure (4.3.5.5) Acetylation using acetic acid anhydride.

Reagents:

1-methylimidazol
 Acetic acid anhydride
 Dichloroform
 Distilled water

Equipments:

Minishaker MS2, IKA
 Centrifuge: MSE
 Acid-washed supelco tubes with covers
 Acid-washed glass equipments
 N₂- gass apparatus

Procedure:

- 1- 200 μ l 1-methylimidazol was added to the reduced residue followed by 2 ml of acetic acid anhydride. The mixture was mixed thoroughly with a mini shaker and left for 10 minutes.
- 2- Excess acetic acid anhydride was destroyed with 10 ml water; the mixture was then mixed and left for 10 minutes.
- 3- The partially methylated and acetylated polysaccharides were extracted twice with 1 ml dichloromethane after a thorough mixing and centrifugation.
- 4- The lower (organic) phases were collected and double-washed with 5 ml water. The aqueous phase, which contains some impurities can both affect the GC-MS apparatus and influence the analysis procedure.
- 5- The residue was dissolved in 100 μ l methanol and analyzed using GC-MS.

4.3.5.6- GC-MS (Pedersen-Bjergaard and Rasmussen 2004):

Principle:

This combined technique is used for explanation and determination of the monosaccharides obtained from the methylation phase using a mass-spectrum detector (MS). The method highlights information about the structures of the different carbohydrate units. The monosaccharides will separate into the GC column followed by their ionization with an electron beam under vacuum inside the mass spectrometer. The ionized molecules will break down into small fragments and, hence, could be detected and determined according to their mass/charge relationship. The mass spectrum is a plotting sum of the relative ions intensities and their m/z values that is characteristic for each monosaccharide molecule.

This phase of analysis was carried out by Finn Tønnesen at pharmaceutical chemistry department, School of Pharmacy, The Oslo University.

Equipments:

GC-MS: GC 8000 series
Detector: Fisons Instruments, MD 800
Injection: Split (1: 10)
Column: FactorFOUR™, VF-1 ms
Film thickness: 0.25 μ m
Inner diameter: 0,25 cm
Length: 30 cm
Temperature zone: 80 – 280⁰ C
Injection's temperature: 250⁰ C
Data programming: Masslab
Important ions: 45, 47, 117, 118, 162, 162, 189, 190, 205, 207, 233, 234, 235, 261, 262, 305 and 307
Temperature programming:
80⁰ C (5 min) -----20⁰ C/min→ 170⁰ C ----0.5⁰ C/min→ 200⁰ C ----30⁰ C/min→ 280⁰ C (20 min).

4.3.6- Immune modulator activity:

4.3.6.1- the complement-fixation test:

Principle:

Polysaccharides interfere with the complement system in a way it may either activate or deactivate the system. Both effects lead to reduction in the hemolysis of the sensitized red blood cells (SRBC) obtained from a sheep, which were previously sensitized with a rabbit antibody. A complex is formed by the interaction of the cells and the antibody that leads to activation of complement cascade.

Polysaccharides that modulate the complement system will modulate the cells through their binding to the complement factors and reduce the ability of the complement cascade to form the terminal complement complex (TCC) that is responsible for the hemolysis of red blood cells.

The resultant reduction in the red blood cells color is measured at a definite wave length. In this test, a human serum was used as complement source.

Reagents:

Blood cell:	Sheep red blood cells “white 161”, prepared on 06.08.09
Antibody:	Virion 9020 Amboceptor (sheep red blood cell haemolysin), diluted 1:10 in Veronal/BSA/ NaN_3
Complement:	ECG (human serum), prepared on 14.02.08
Complement buffer:	Veronal Buffer saline for (complement Fixation Test, pH 7, 2) 1.67% ml Bovine Serum Albumin (BSA) 30% 0, 5 ml Sodium azide This buffer will contain 2mg/ml BSA and 0.02% NaN_3 and it was prepared on 11.05.09 and autoclaved
Phosphate buffer:	salt water (PBS) M/150, pH 7, 4; prepared on 22.01.08 and autoclaved
Standard:	Plantago major II, 1 mg/ml 0, 9% NaCl solution
	Sterile distilled water.

Equipments:

	Finnpipette® with tips.
	Reagent tubes.
	Round and flat microtiter plates containing 96 wells.
	Micro tubes
Whirlimixer:	(Heigar Intermed).
Centrifuge:	Heraeus Multifuge 3SR + Centrifuge
Incubator with a shaking plate:	Thermax, 37 ⁰ C.
Micro plate reader:	Thermomax

Procedure:

A- Washing of the sheep blood cells:

- 1- The supernatant of the sheep bloodstock was removed into a clean tube (to be replaced to the blood stock afterwards)
- 2- 500 µl was taken from the packed blood cells by a micro titer plate
- 3- 2ml (2000µl) phosphate buffer was first added to the blood cells and the mixture was mixed thoroughly, then another 4ml (4000µl) of phosphate buffer was added
- 4- The mixture was centrifuged in Martin Christ centrifuge and the supernatant was decanted
- 5- The above steps (3 & 4) were repeated
- 6- The precipitate of the blood cells was washed once with complement- buffer as in step (3) above and was centrifuged

B- Sensitization of the sheep blood cells:

- 1- 15µl Amboceptor 9020 was added to 5,925ml Veronal/BSA buffer and 60µl of the washed sheep blood cell was then added and thoroughly mixed in glass tube
- 2- The glass tube was well fixed onto the grill plate and was incubated in Nicht Drehen incubator at 37⁰ C for 30 minutes
- 3- The mixture was centrifuged and washed twice with phosphate buffer and once with complement-buffer as described in step (2) and (5) above. Supernatant of each centrifugation was decanted
- 4- An amount of 5,940ml of complement-buffer was added to the blood cells precipitate to make a 1% solution of the blood cells.

C- Dilution of the samples:

- 1- 1mg of each sample was weighed
- 2- The weight was dissolved in 1ml veronal/BSA buffer (complement-buffer) to give a stock solution of 1mg/ml
- 3- A dilution table was prepared as follows:

Tube	Concentration		
1	500 µg/ml	300 µl veronal/BSA buffer	300 µl of stock solution
2	250 µg/ml	300 µl veronal/BSA buffer	300 µl from tube No 1
3	125 µg/ml	300 µl veronal/BSA buffer	300 µl from tube No 2
4	65,5 µg/ml	300 µl veronal/BSA buffer	300 µl from tube No 3
5	31,3 µg/ml	300 µl veronal/BSA buffer	300 µl from tube No 4
6	15,6 µg/ml	300 µl veronal/BSA buffer	300 µl from tube No 5

Table (4.3.6.1a) showing the 2-fold dilution of Parkia biglobosa samples and PMII.

Tube	Concentration		
1	500 µg/ml	300 µl veronal/BSA buffer	300 µl of stock solution
2	125 µg/ml	300 µl veronal/BSA buffer	100 µl from tube No 1
3	31 µg/ml	300 µl veronal/BSA buffer	100 µl from tube No 2
4	8 µg/ml	300 µl veronal/BSA buffer	100 µl from tube No 3
5	2 µg/ml	300 µl veronal/BSA buffer	100 µl from tube No 4
6	0,5 µg/ml	300 µl veronal/BSA buffer	100 µl from tube No 5

Table (4.3.6.1b) showing the 4-fold dilution of *Parkia biglobosa* samples and PMII.

4- Standard solution of 5 mg/ml PMII was prepared as follows:

a- Concentration of the standard was 5 mg/ml

b- Calculation of the standard fraction to be diluted:

5 mg = 1 ml or 3 mg = 600 µl

From the formula: $C1.V2 = C2.V1$

5 mg/300 µg = 1ml/Xml eller 5000 µg/300 µg = 1000 µl/X µl

therefore: $X\mu\text{l} = 1000 \times 300 / 5000 = 60 \mu\text{l}$

c- The amount of the buffer to be added to the standard was:

600 µl – 60 µl = 540 µl

D- Dilution of the complement:

Proportion Complement: Buffer	Complement (µl)	Veronal/BSA-buffer (µl)
1:30	10	290
1:40	10	390
1:50	10	490
1:60	10	590
1:70	10	690
1:80	10	790
1:90	10	890
1:100	10	990

Table (4.3.6.1d) the titration curve of the complement.

5- Calculation of the complement and buffer (1:95%):

Total of amount of buffer needed for 88 wells (84 for the samples and 4 wells for 50% lysis) = $88 \times 50\mu\text{l} = 4400 \mu\text{l}$ (5000 μl to be used)

The amount of complement = $5000 \mu\text{l} / 95 = 52,63 \mu\text{l}$ (approx. 53 μl)

The amount of buffer = $5000 \mu\text{l} - 53 \mu\text{l} = 4947 \mu\text{l}$

Procedure:

- 1- 15 μl of each diluted sample and standard was put into its specific well of a 96- well plate. Two parallel wells were made for each sample and standard. Four wells with 50 μl veronal/BSA buffer and another four wells with distilled water for 100% lysis were made as control.
- 2- 50 μl of the diluted complement was rapidly added into each plate well with the exception of the four wells standing for the 100% lysis. The plate was covered with a wide plate cover to prevent evaporation and it was incubated at 37⁰ C for 30 minutes with continuous shaking.
- 3- 50 μl of the sensitized sheep blood cells was added to each plate well and the plate was incubated at 37⁰ C for 30 minutes under continuous shaking.
- 4- The plate was taken from the incubator and centrifuged at 1650 rpm in 5 minutes.
- 5- 100 μl was taken from each plate well and transferred into a 96 flat-well plate. The plate was again centrifuged, in order to remove any air bubbles, at 1650 rpm for 5 minutes.
- 6- The absorbance of each well was read at 405 nm.

Reading of absorbance:

The absorbance was read at 405 nm.

Calculations:

Lysis degree was determined according to the following formula:

$$(\text{Abs}_{\text{control}} / \text{Abs}_{\text{dist. water}}) \times 100\%$$

where:

$\text{Abs}_{\text{control}}$ represents absorbance of the wells with just complement and the red blood cells

$\text{Abs}_{\text{dist. water}}$ represents absorbance with 100% lysis of the red blood cells

In this test, the lysis degree indicates the degree in which the complement itself can lysate the red blood cells and, in this test, it should be about 50%.

The preventive (fixative) effect of the sample on the complement system was calculated by the following formula:

$$[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100\%$$

A sample with a good complement fixation effect will give a small hemolysis and, therefore, a small absorbance at 405 nm.

4.3.6.2- Measurement of released-NO from macrophages:

When monophages leave the blood stream and enter the tissues, they become activated and differentiated into macrophages. When a microphage is activated by effector T helper lymphocytes (called Th1cells), the IFN-gamma molecules of that cell bind IFN-gamma receptors on the macrophage and later is activated to be enabled to produce more hydrolytic lysosomal enzymes, nitric oxide (NO), and toxic oxygen radicals that destroy the microorganisms within the phagososomes and phagolysosomes (Gary Kaiser, 2008). Activated macrophages release NO, which play an important role in tissues vasodilatation and increasing of vascular permeability.

Nitric oxide (NO) is a lipid- and water-soluble radical gas, which is an important physiological messenger and a signal transmitter in many biological systems. Nitic oxide is a short-lived gas that can diffuse freely through cells and interact with thiol groups on cysteines and glutathiones or protein heme groups (Lisardo, Zeini, Traves & Hortelano, 2005).

Principle:

Microphages release NO and other mediators on activation with Th1 lymphocytes. The released NO breaks down into two major products; one of these two compounds is the nitrite (NO_2^-). Nitrite is a stable and a non-volatile compound that explicit macrophages activation. This activation together with (NO_2^-) release is the foundation upon which the Griess- reagent system is based on. The reaction involves the formation of NO_2^- , which forms a colored azo-compound with sulfanilamide and N-(1-naphthyl)ethylenediamine in an acidic medium. The resultant colored solution is measured at 540nm.

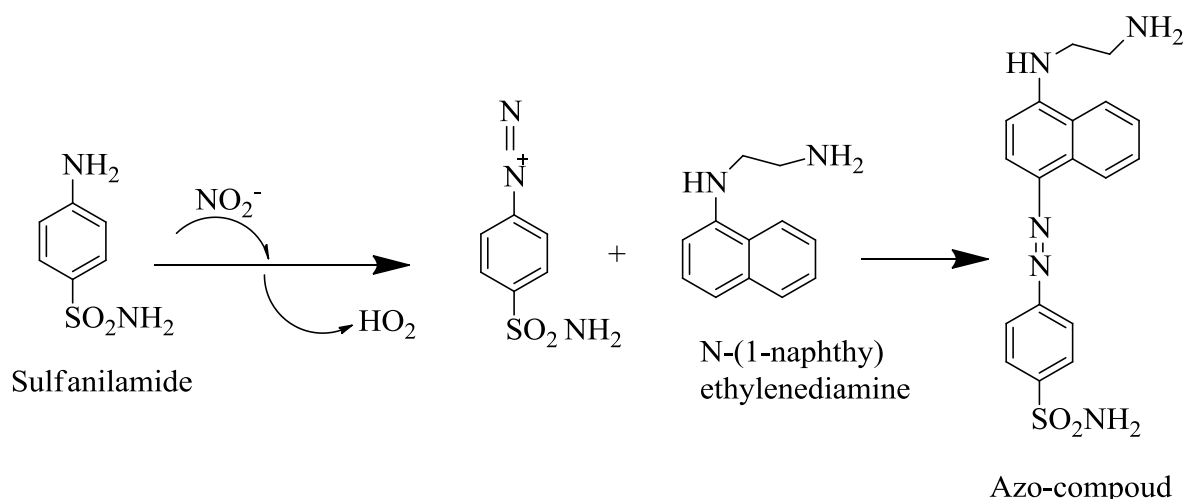


Figure (4.3.6.2) the chemical reaction combining NO_2^- with Griess-reagent system. The produced azo compound is measured at 540nm.

Reagents:

Culture medium: RPMI 1640 (added 10% ox serum, 1% penicillin/streptomycin, 2mM L-glutamine, and $5 \cdot 10^{-5}$ m 2-mercaptoethanol).

Griess-reagent A: 1% sulfanilamide (in dist. Water).

Griess-reagent B: N-(1-naphthyl)ethylenediamine (NED) in 2.5 % phosphoric acid.

Cells line: R2- macrophages obtained from a rat macrophages.

Positive control: Lipopolysaccharide (LPS), PMII from *Plantago major*.

Equipments:

Cell counter: Coulter counter

Centrifuge: Rotana 460 R (Beckman)

Cell incubator with CO₂-control

Absorbance reader: Titertek multi scan

One flat and one rounded bottom microtiter plate.

Isotone II: 0.099% NaCl solution

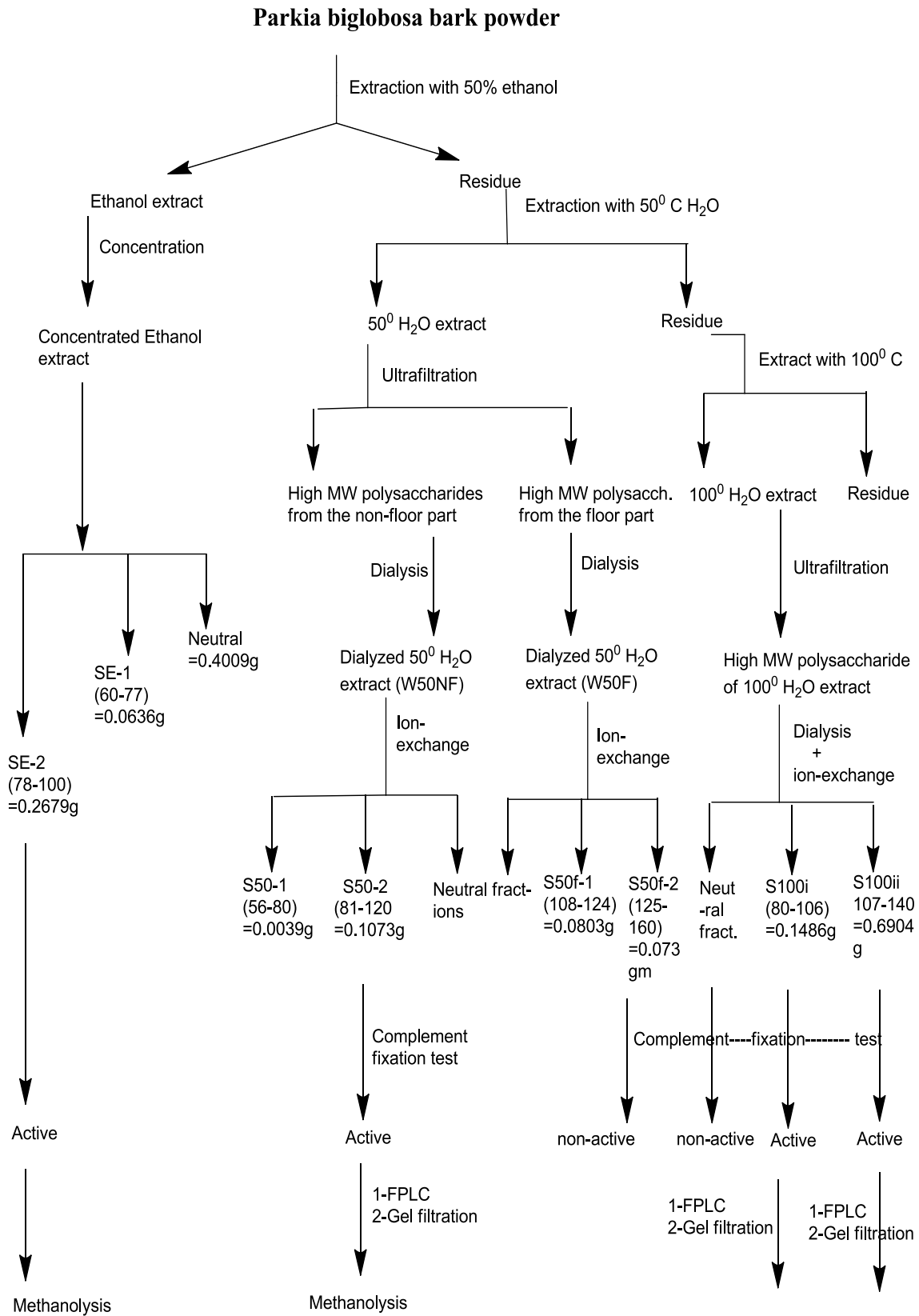
Procedure:

A standard curve for nitrite must be done for every assay due to color changing.

1. Macrophages were cultured from a cell culture.
2. 20µl of a cell suspension was added to 10 ml isotonic NaCl solution and the cells were counted using a cell counter.
3. Cells were spun down at 1300 rpm in 5 minutes.
4. The cells were resuspended in a culture medium until their concentration was 500000 cell/ml.
5. The samples were diluted as follows: 5 mg/ml, 500µg/ml, and 50µg/ml.
6. 2 µl of each dilution was added into a 2-parallel flat bottom well of the microtiter. Another 2 parallel wells containing 5 µl (10 ng/µl) LPS and 5 µl (2 mg/ml) PMII were used as positive control; while a pure medium was used as a negative control.
7. 100 µl (= 50000 cells) of the cell suspension was added to each well. The final concentrations of the samples were: 100µg/ml, 10µg/ml, and 1 µg/ml.
8. The cells were incubated at 37⁰ C with a 4% CO₂ in about 24 hours.
9. The supernatant was transferred into a rounded bottom microtiter plate and centrifuged at 1400 rpm in 2 minutes.
10. 50µl of the supernatant was transferred into a flat bottom microtiter plate.
11. A standard curve of a 2-fold dilution of 100mM NaNO₂ was constructed (100, 50, 25, 12.5, 6.25, 3.13, and 1.56). These dilutions were titrated as the same way as in point 6 above.
12. 50µl Griess reagent was added to each well and the plate was kept in a dark place for 10 minutes
13. 50µl of Griess reagent B was added to each well.
14. The absorbance of the generated color was measured at 540nm.

5. Results and discussion:

5.1 - Schematic chart:



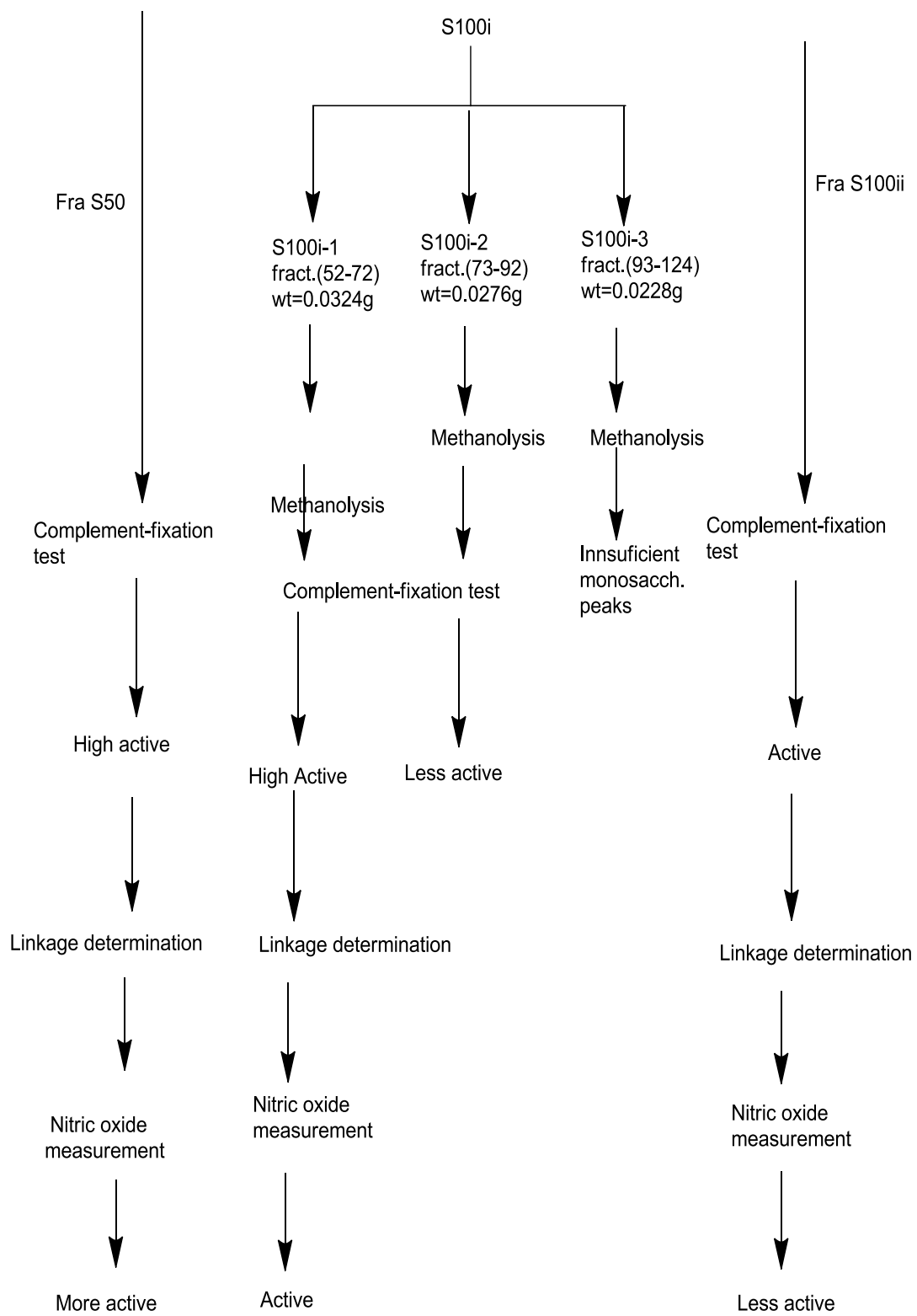


Figure (5.1) a chart illustrating the processes included in the extraction, separation, and analysis of polysaccharides from the *Parkia biglobosa* bark.

5.2- Introduction:

The uses and utilities of the different parts of the African Locust bean tree are well known for the most inhabitants of the Sub-Sahara regions of the African continent. The seeds are used for feeding due to the high protein content as well as the high mineral amounts. The fruits and leaves are edible as well. Because the plant leaves are annually green, they are heavily browsed by livestock. Twigs and bark are used to clean the teeth and as mouth wash.

The plant has elucidated a number of medical features not only in the field of traditional medicine, but also in the different trials and tests carried out. The extract of the tree's seeds showed an inhibitory effect towards platelets aggregation and secretion. The fermented seeds of *Parkia biglobosa* was investigated in alloxan-induced diabetic rats and it had proved anti-diabetic property. A third study with an ethanol extract of *Parkia biglobosa* explicated anti-tumor and insecticidal effects.

All of these trials for the different parts of the plant were encouraging for more investigations and hopeful expectations to exploring more of the plant medical advantages and uses in the near future. The plant possesses different phytochemical properties and has a very well understanding traditional knowledge among many African nations.

The most traditionally applications and uses of the *Parkia biglobosa* bark are:

Body pains and aches, asthma, headache, fever, cough, internal wounds and infections, hemorrhoids, insomnia, dysentery associated with abdominal pains, mouth lesions, hypertension, paralysis, diabetes, syphilis, etc.

The *Parkia biglobosa* bark was collected in Mali, the bark was already powdered and Soxhlet extracted, the total amount of the powdered bark was 625 g of which 500 g was used in this master thesis.

5.3- Extraction with ethanol 50% and hot water:

500 g from the powdered *Parkia biglobosa* bark was extracted with 50% ethanol at 50⁰ C for 2 hours and centrifuged. The residue was then extracted with water at 50⁰ and 100⁰ C for 2 hours, respectively. The extractions were filtered through gauze and a glass fiber filter. Extractions were ultra-filtered to exclude the low molecular weight polysaccharides and to remove the salts that were present. During ultra filtration process, an opening (fissure) occurred to the outlet of the circulated high molecular polysaccharides which resulted in the leakage of polysaccharides solution into the floor. This amount was collected and labeled as 50⁰ C floor part while the first portion was labeled 50⁰ C non-flour part.

To ensure salts removal, AgNO₃ solution was used to detect the presence of the salts as they form a white color with AgNO₃. Extractions were finally concentrated at 40⁰ C under vacuum with an evaporator to approximately 600 ml each. The color of the concentrates was intense brown.

5.4-Ion-exchange chromatography:

Polysaccharides from the different types of *Parkia biglobosa* bark concentrates that were isolated from water extracts were further applied into ANX Sephacryl 4 column to separate these polysaccharides of the bark.

The purpose with the use of ion-exchange chromatography was to separate the neutral polysaccharides from the acidic ones as the acidic polysaccharides were the most relevant for the experiments being working for i.e., understanding of the relationship between the traditional uses of the *Parkia biglobosa* bark in the treatment of various types of wounds in Mali. This traditional use can only be rationalized, justified and confirmed by the complement-fixation bioactivity tests.

Since the tested plant bark contains various kinds of polysaccharides, it was, therefore, necessary to elute these polysaccharides depending on their chemical characteristics by using different elution solvents of different properties that suit the elution of the polysaccharides.

During separation of the samples that contain carbohydrates, the acidic polysaccharides will mostly be retarded on the ANX column's matrix because of their loaded negative groups without any competence for the chloride ions. These acidic carbohydrates will be easily eluted and separated by different NaCl solutions. To completely elute the acidic polysaccharides present in the plant sample, it was practically needed to use Na Cl. Increased acidic strength will need increased salt strength, too. The neutral polysaccharides present in the sample do not react with ion-exchange materials and will indeed be separated with distilled water, while a linear 0-2 M Na Cl gradient was the proper solvent and method for the elution of the acidic polysaccharides.

Depending on the polysaccharide profiles obtained using the phenol-sulfuric acid test, the different polysaccharide fractions were added together, concentrated and freeze-dried. The following elution fractions were obtained:

- i- Pb50N (Neutral), PbS50-1, and PbS50-2 (Acidic fractions) from the Non-floor part of 50⁰ C water extract.
- ii- Pb N 50f (Neutral), Pb S50 f₁, and Pb S50 f₂ (Acidic fractions) from the floor part of 50⁰ C water extract.
- iii- Pb 100N (Neutral), Pb S100-1, Pb S100-2 (Acidic fractions), and Pb 100NaCl eluent of the 100⁰ C water extract
- iv- Pb N E (Neutral fraction of ethanol extract), Pb E Nacl gradient of ethanol extract or Pb E-1, and Nacl elution of ethanol extract or PbE-2.

1mg from fractions Pb S 50-2, Pb S 50A f₂, Pb S 100-1, Pb S 100-2, Pb 100NaCl, and E-1 was weighed for complement-fixation bioactivity test. Fractions Pb S 50 f₂ and Pb 100NaCl were devoid of bioactivity and were then excluded.

N.B: Since S50-2 was the only fraction from 50⁰ C extract proceeded with to the end of this study, it was just written S50 in the whole process.

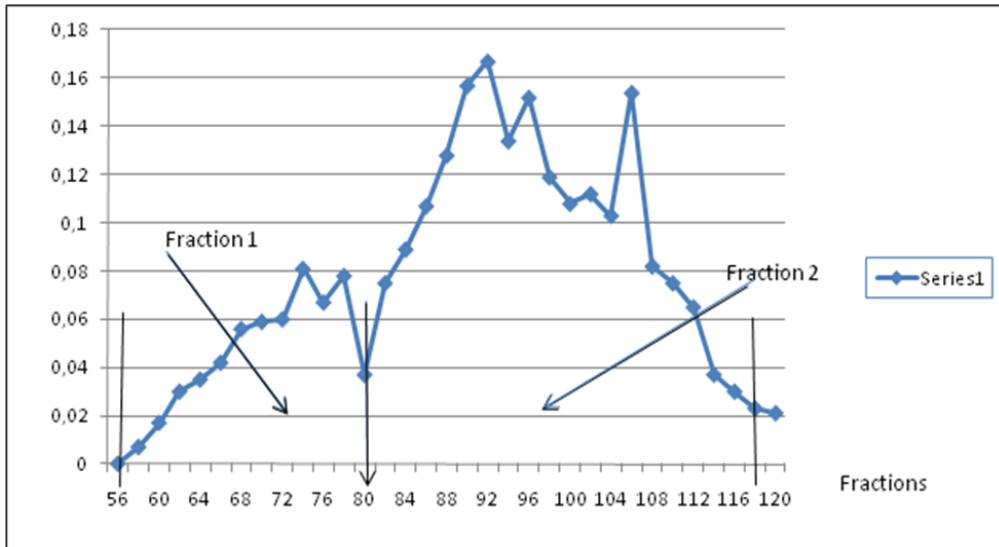


Figure (5.4a) Pbb, acidic fractions (S50-i) & (S50-ii) from 50⁰C water extraction using ANX Sephacryl-4 column.

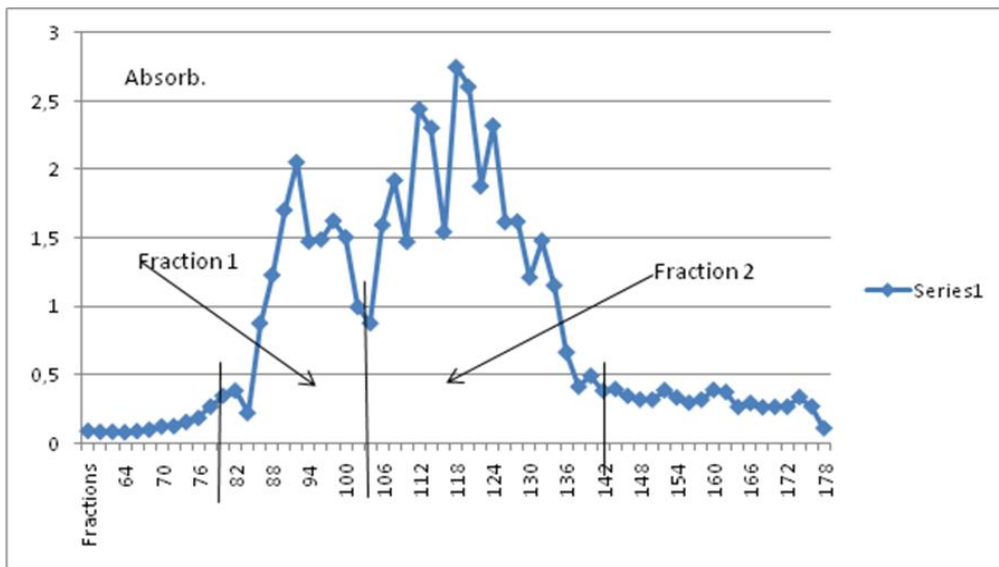


Figure (5.4b) Pbb, acidic fraction-1 (S100i) & fraction-2 (S100ii) from 100⁰ C water extraction using ANX Sephacryl-4 column.

5.5- Fast Protein Liquid Chromatography (FPLC):

Molecular weights distribution of the samples was determined using the fast protein liquid chromatography. This method depends on separation of substances based on their molecular size. The molecules with large sizes will not pass into the column matrix and, therefore, they

will be carried out by the mobile phase directly through the void volume (V_0). The retention times for the different polysaccharides will be inversely proportional to the polysaccharide unit sizes, i.e., as the polysaccharide unit sizes increase, their retention times decrease. Dextran standard curve with a known molecular weight was used to determine the regression line. The conditions used for dextran curve were the same as for this test and the function of the regression line was applied to calculate the molecular weight distribution of the samples. S50, S100i, S100ii and E1 were applied into FPLC to, firstly, separate the available polysaccharides and then determine the molecular weight distributions of them.

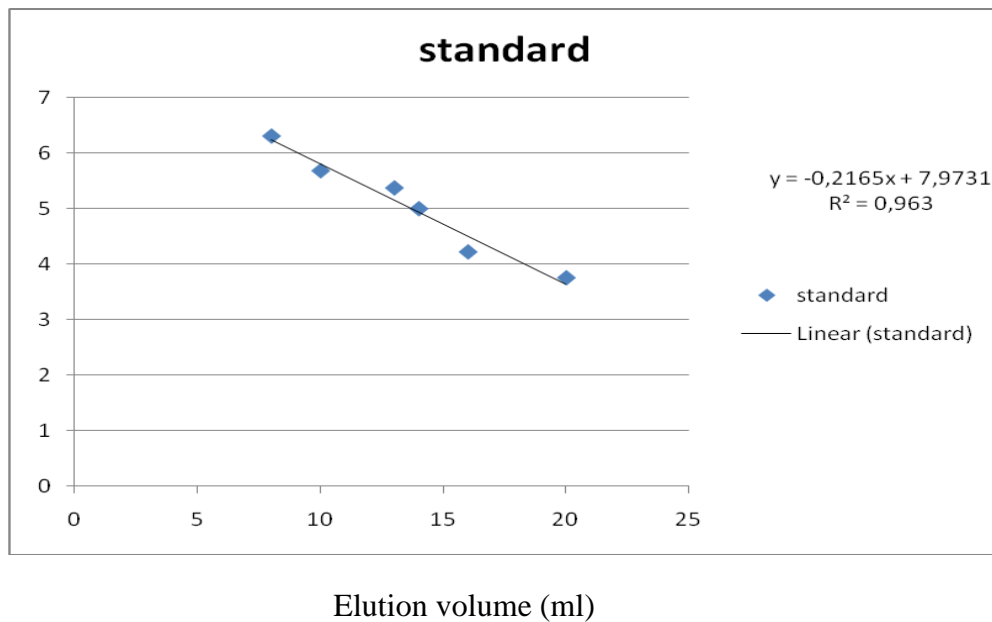


Figure (5.5a) Standard curve for dextrans with a known molecular weight

Sample name	From molecular Weight (Da)	To molecular Weight (Da)	Maximum top molecular weight (Da)
S 50	2023252	101637	275455
S 100 I	2023252	22780	37502
S 100 ii	20233252	61738	746534

Table (5.5b) Overview over the estimated molecular weight intervals of the samples S50, S100i & and S100ii. The distance from MW to MW is the whole interval.

From the (table 5.5b above), one can find out that the resultant molecular weight intervals of the different samples S50, S100-I and S100-ii comprise a wide range that extends from 22780 to 2023252 Da. This wide range indicates that the present polysaccharide samples have different structures and may be of high molecular weight polysaccharides. The method is an estimation one, which does not inform of the proper molecular structure present in the samples nor the appropriate molecular weight. Instead, it points to the starting point and the range where the different intervals are. Furthermore, it can provide the suitable chromatographic method to isolate the high molecular weight polysaccharides contained in these samples depending on their elution volume.

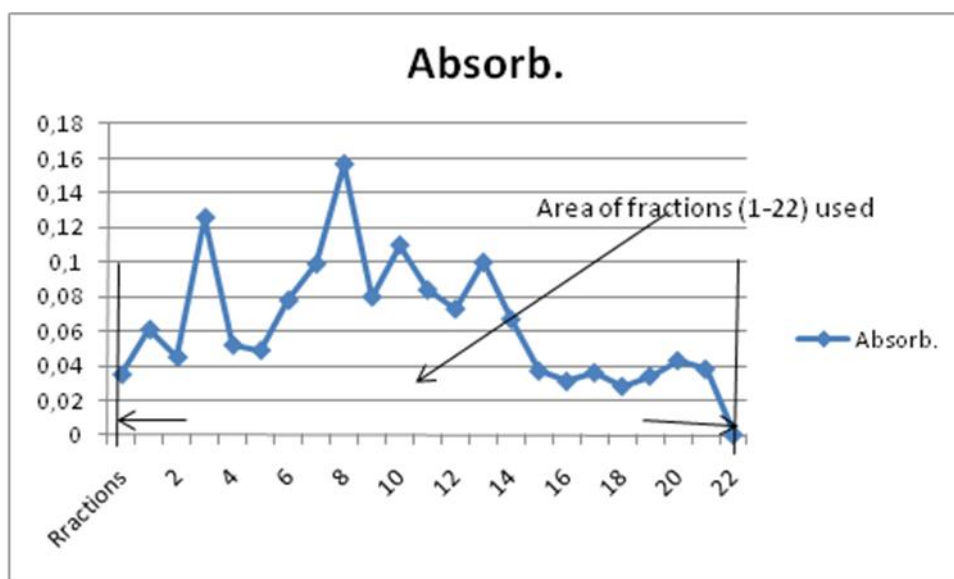


Figure (5.5c) Pbb, S50 after FPLC using Superose-6 column.

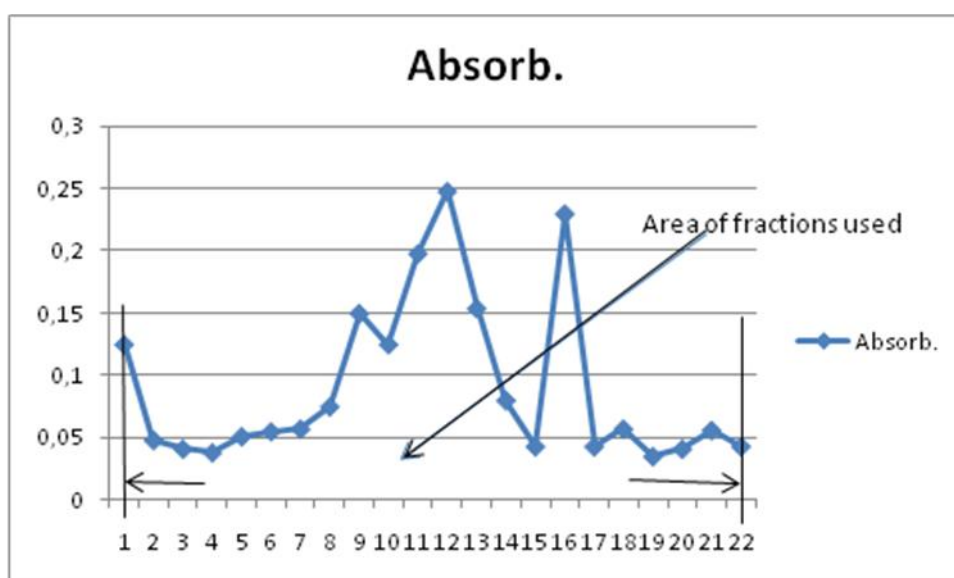


Figure (5.5d) Pbb, S100-i after FPLC using Superose-6 column.

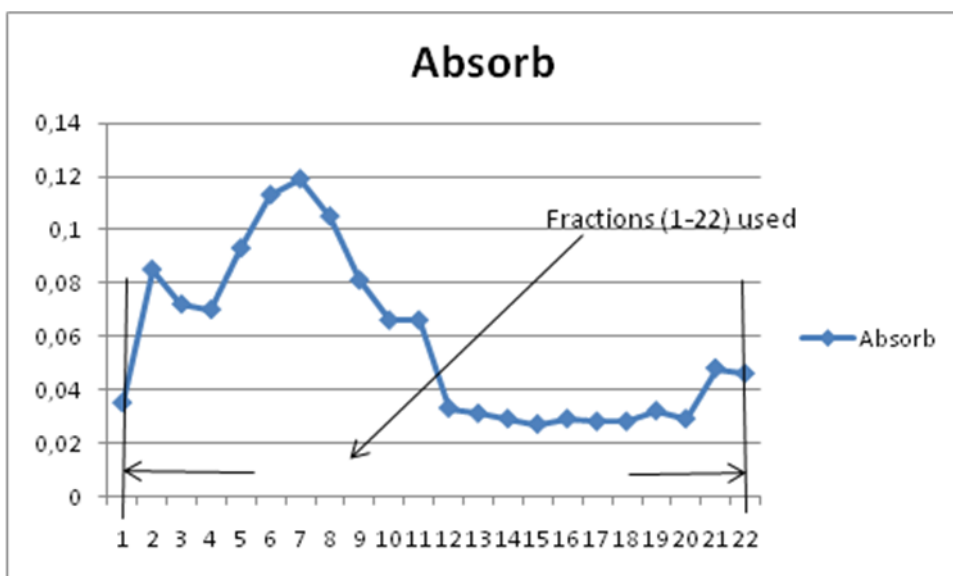


Figure (5.5e) Pbb, S100-ii after FPLC using Superose-6 column

5.6- Gel filtration chromatography:

Samples obtained from ion-exchange chromatography were tested for their bioactivity using the complement-fixation test. These samples were: S 50, S 50f, S 100i, S 100ii, 100 NaCl and E1. Due to the large amount of impurities which has interfered with and affected the complement-fixation bioactivity test results, it was then necessary to purify these samples to an extent that can limit the impurities and minimize their influence to the efficiency of further complement-fixation test and the related experiments.

Gel filtration was the suitable chromatographic technique for such purification of these samples. Gel filtration has a large extraction capacity for the separation of carbohydrate substances. This large capacity enables the isolation of the interested polysaccharides with a limited contamination. The type of the matrix present in the gel filtration chromatography promotes a great mechanical strength that enables the elution of samples with a high speed; in addition, it has a separation zone between 1000-80000 D. These advantages are highly recommended for the separation of polysaccharides as they contain large molecular weight units.

If a sample is applied into the column, the mobile phase (buffer) as well as the sample diffuse i.e., partitions in and out of the pores of the matrix. Smaller molecules move further into the matrix and so stay longer on the column. As buffer passes continuously through the column, molecules that are larger than the pores of the matrix are unable to diffuse into the pores and pass the column faster than smaller ones.

After the samples were gel-filtered, the carbohydrate profiles were determined using the phenol-sulfuric acid test and the related fractions were added together. These fractions were concentrated, freeze-dried and marked S50, S100i-1, S100i-2, S100i-3 and S100ii.

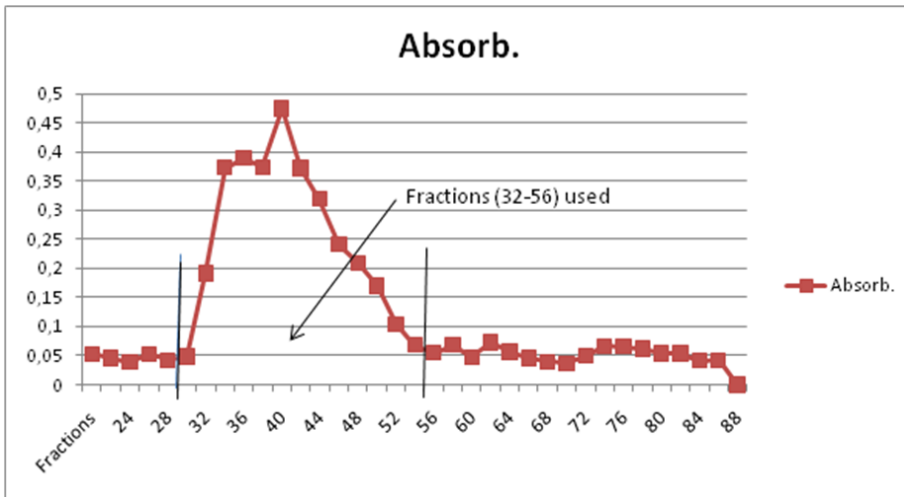


Figure (5.6a) Pbb, S50, after Sephacryl-S200

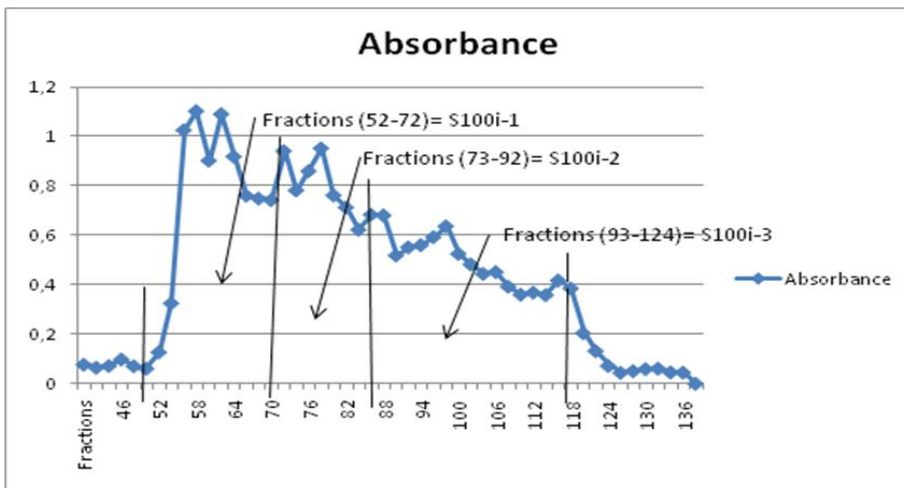


Figure (5.6b) Pbb, S100-I, after Sepharyl-S200

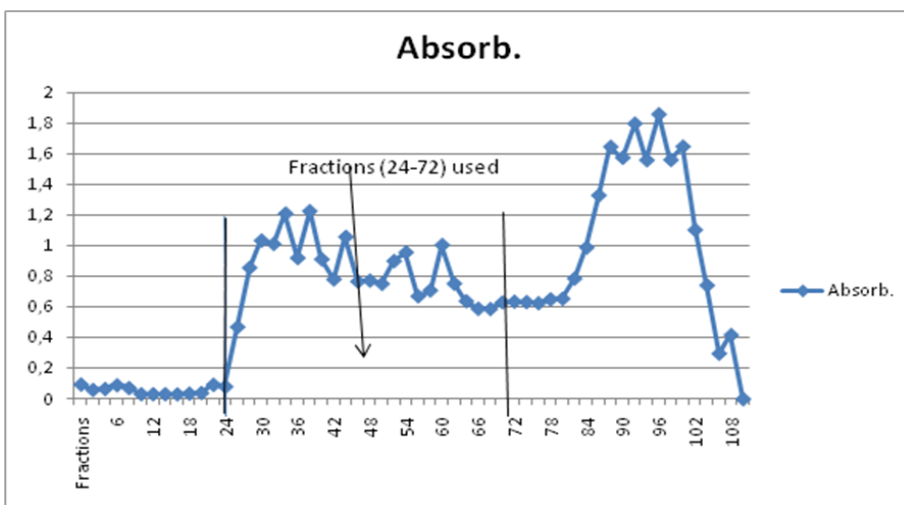


Figure (5.6c) Pbb, S100-ii, after Sepharyl-S200

5.7- Carbohydrate analysis:

The monosaccharide compositions for the examined samples S50, S100i-1, S100i-2, S100i-3 and S100ii were determined using GC analysis after the samples were methanolized and TMS derivatized according to methods (4.3.2 & 4.3.3) respectively.

Provided that mannitol meets the characteristics for an internal standard that was used to standardize the different monosaccharide structures, the different monosaccharide peaks are obtained depending on their retention times. The analysis of these peaks will elucidate a specific structure that is identical for each monosaccharide molecule in the chromatogram.

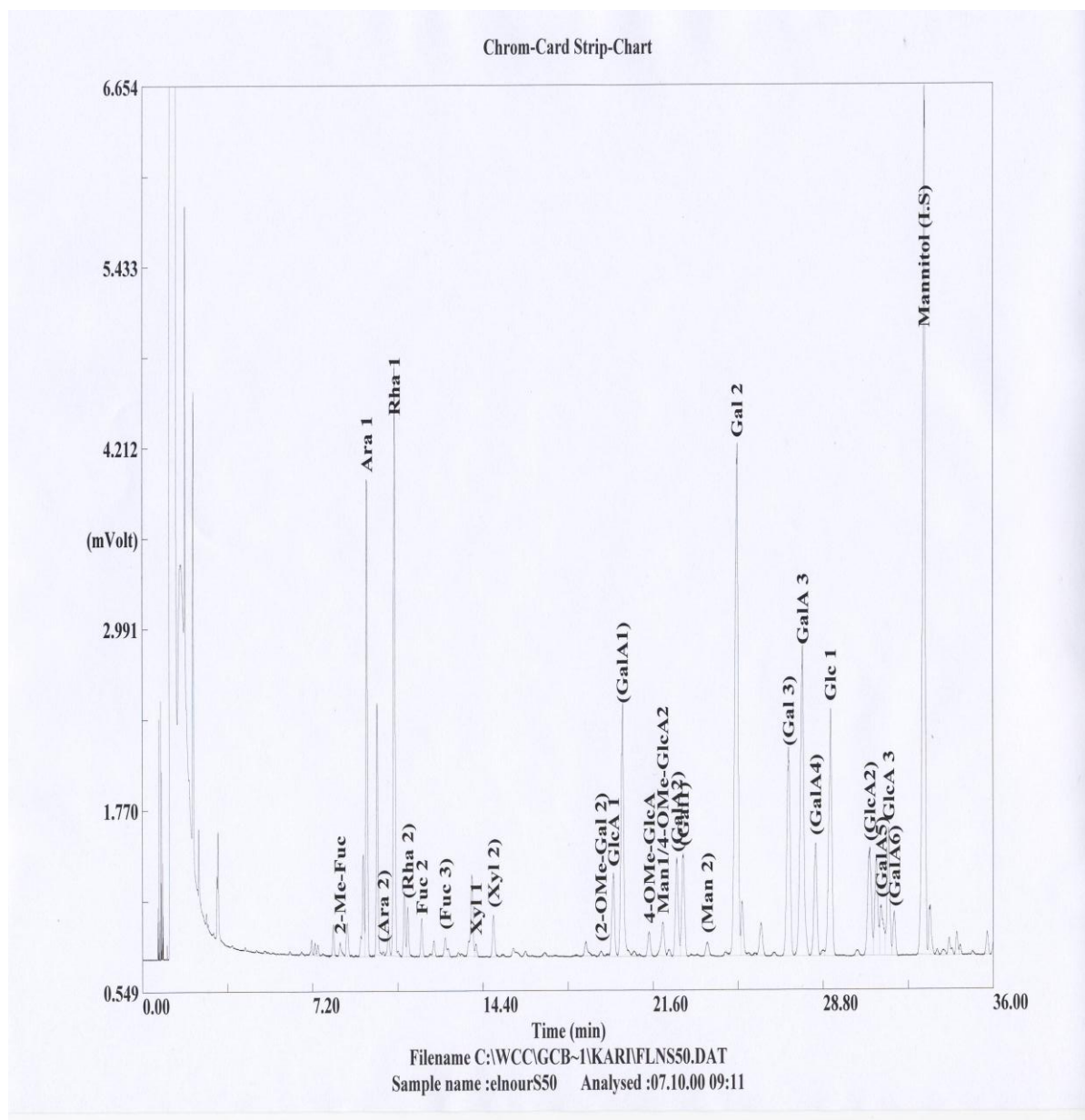


Figure (5.7a) shows a GC chromatogram for a polysaccharide composition for the fraction S50. Mannitol has been used as a standard.

The chromatogram (figure 5.7a above) of S50 sample shows a high amount of galacturonic acid (26,2%) followed by galactose (22,8%), glucuronic acid (18,3%), glucose (10%), arabinose (9,7%), and rhamnose (8,5%) respectively.

On the other hand, the chromatogram of S100i-1 showed that the amount of arabinose (26,6%) was the highest followed by xylose, galactose, and rhamnose. Meanwhile, the amount of galacturonic acid (30,1%) was higher than amounts of arabinose (21,2%), galactose (18%), glucuronic acid (10,5%), rhamnose (7,3%), and glucose (6,1%) respectively.

Table (5.7b) below reflects the percentage distribution of the different monosaccharides present in *Parkia biglobosa* bark samples. A remarkable amount of xylose was found in S100i-1 being 25% of the sample content. It is also of worth to mention that all of the samples contain glucuronic acid.

Monosaccharide Type	S 50	S 100i-1	S 100i-2	S 100ii
Arabinose	9,7	26,6	35,8	21,2
Rhamnose	8,5	8,6	9,2	7,3
Fucose	0,8	0,2	0,4	0,3
Xylose	0,3	24,1	17,9	0,2
Mannose	1,5	2,8	0,0	0,0
Galactose	22,8	14,1	10,4	18,0
Glucose	10,1	4,7	8,1	6,1
Glucuronic Acid	18,3	5,8	5,1	10,5
Galacturonic acid	26,2	5,0	6,7	30,1
4-O-Me-GlcA	1,5	5,5	4,9	1,3

Table (5.7b) shows the distribution of monosaccharides in the polysaccharide fractions in relation to the total sugar amount.

5.8-- Monosaccharide structures and their linkage patterns' positions:

GC-MS analysis was a helpful and supportive method for the revealing of the different structures of the most interested monosaccharides.

Because partial acid hydrolysis of fully methylated polysaccharides often permits useful information on the positions at which the oligosaccharides were linked in the original polysaccharide, the analysis of the given fragments obtained from GC-MS together with their understanding not only enabled in the formulation of the different monosaccharides structure but also in the knowledge of the positions of the fragmentation pattern. The objection with knowing the different structures was to limit every monosaccharide patterns type followed by the calculation of the total area under the curve for them.

It is sometimes difficult to extract all the information from the available chromatogram due to some practical difficulties. For example, some molecules locate in the same zone with other molecules and this can cause an overlap in AUC calculations. Also, the presence of impurities can greatly affect the analysis of polysaccharides present in the sample. Moreover, incomplete methylation or reduction can underestimate the available results of the polysaccharides.

The linkage pattern positions of *Parkia biglobosa* bark samples; S50, S100i-1, and S100ii were determined using carboxylic acid reduction, which was followed by methylation, hydrolysis, reduction, acetylation, and GC-MS analysis.

Table (5.8) shows the results and the distribution of the different monosaccharide molecules present in the samples of *Parkia biglobosa* bark. The table reflects also the linkage types of these molecules. The available amount of each linkage type was calculated based on the area under the curve (AUC) of each monosaccharide peak divided by the total amount of these areas under the curves of these cleaved monosaccharides. The obtained number was multiplied by the amount of the AUC for the individual monosaccharide present in the GC chromatogram. The percentage of each monosaccharide's linkage pattern was finally included based on the context of the total percentages of the sugar types obtained from methanolysis.

Linkage proportion in the sample S50:

The fraction consists of terminal arabinose (3.1%), Ara1, 5 (5, 5%), and traces of Ara 1,3,5 and Ara 1,2,5. This can indicate the presence of arabinan where the main chain consists of 1,5-linked arabinose and side chains of 1,2,5-linked arabinose and 1,3,5-linked arabinose bound through O-2 and O-3 positions. Arabinan is a key element in the structures of both arabinogalactan-1 and II.

Rhamnose is a main element in the structure of rhamnogalacturonan-1 where it alternates with galacturonic acid (1→4). Rhamnose is also present in rhamnogalacturonan-II as a branched side chain. The presence of terminal, 1,2,5-, and 1,3,5-linked arabinan and 1,4-linked galactose give an obvious indication of the presence of AG-II, while the presence of 1,4-linked rhamnose, and 1,4-linked galacturonic acid indicates the presence of rhamnogalacturonan. Rhamnogalacturonan together with arabinogalactan forms rhamnogalacturonan-I, which is the hairy region of pectins polysaccharides

Monosaccharides and their linkage type		S 50	S 100i-1	S 100ii
Ara	<i>Terminal_f (1→)</i>	3,1	12,3	7,8
	<i>1,3_f</i>	X	0,8	X
	<i>1,5_f</i>	5,5	9,3	10,0
	<i>1,3,5_f</i>	0,6	0,3	2,3
	<i>1,2,5_f</i>	0,4	4,2	1,0
Rha	<i>Terminal_p (1→)</i>	2,4	X	1,7
	<i>1,2_p</i>	5,3	<i>Trace</i>	4,0
	<i>1,3_p</i>	X	7,5	X
	<i>1,2,4_p</i>	0,7	1,0	1,6
Xyl	<i>Terminal_p (1→)</i>	0,3	3,9	0,2
	<i>1,4_p</i>	X	20,1	X
Glu	<i>Terminal_p (1→)</i>	1,9	4,7	1,4
	<i>1,4_p</i>	5,7	X	3,0
	<i>1,6_p</i>	0,8	X	0,6
	<i>1,4,6_p</i>	1,6	X	1,1
GluA	<i>Terminal_p (1→)</i>	X	-	3,4
	<i>1,4_p</i>	18,6	-	7,1
Gal	<i>Terminal_p (1→)</i>	7,2	2,4	6,9
	<i>1,3_p</i>	6,8	7,4	4,1
	<i>1,6_p</i>	3,2	4,3	3,0
	<i>1,3,6_p</i>	5,6		4,0
GalA	<i>Terminal_f (1→)</i>	5,4	-	5,5
	<i>1,3_p</i>	X	-	23,6
	<i>1,4_p</i>	18,3	-	X
	<i>1,3,4_p</i>	2,4	-	1,0

Table (5.8) the distribution percentage of the different monosaccharide molecules of the samples S50, S100i-1, and S100ii together with their linkage pattern types.

Although a trace of terminal xylose persists in the sample, this amount can indicate the presence of xylogalacturonan. Galacturonic acid is present in a large amount and this can mean the galacturonic acid forms the long chain of homogalacturonan (HGA).

The sample contains many monosaccharide terminals and this can raise the postulation of the presence of a large part of a hairy region polysaccharide in the sample; if one considers the non-optimal conditions that may associate with the sample concentration.

Linkage proportion in the sample S100i-1:

The carboxylic acids contained in this sample were not reduced to free uronic acids, therefore, the linkage patterns of the acidic monosaccharides were not available.

The sample contains much amount of terminal arabinose, moderate amount of 1,5-linked arabinose as well as 1,2,5-linked arabinose. Arabinose presents as a branch in either arabinogalactan-1; through O-2 or O-3, or in arabinogalactan-II in position O-3 or O-6. The presence of 1, 3- and 1, 3, 6-linked galactose strongly suggests the presence of both arabinogalactan-1 and arabinogalactan-II in the sample.

Also, the sample contains much of 1, 4-linked xylose, a moderate amount of terminal xylose and 1, 4-linked glucose, which may indicate the presence of xyloglucan where xylose is attached to glucose at position 6.

Although the carboxylic acids were not reduced in this sample, one cannot exclude the presence of xylogalacturonan as galacturonic acid is present in the sample during methanolysis process.

As mentioned above, the absence of galacturonic acid cannot emphasize the absence of rhamnogalacturonan-I and rhamnogalacturonan-II. The presence of 1, 2-linked rhamnose as well as 1,2,4-linked rhamnose can indicate the presence of RG-I and RG-II where rhamnose is branched at position 4. The presence of 1, 3-linked rhamnose can indicate the attachment of arabinose to position 3 of rhamnose.

Linkage proportion in the sample S100ii:

The sample contains terminal arabinose, 1, 5-linked arabinose, 1,2,5- and 1,3,5-linked arabinose. The sample contains 1, 3-, 1,6- and 1,3,6-linked galactose and their presence together with those of arabinose form the backbone of arabinogalactan-I and II; where arabinose is branched at positions 2 and 3 and galactose is branched at positions 3 and 6. Since rhamnogalacturonan's main chain consists of alternating galacturonic acid and rhamnose (1→4) and (1→2) and since the sample lacks (1→4)-linked galacturonic acid, can one say the sample does not consist of rhamnogalacturonan-I. The sample can contain rhamnogalacturonan-II due to the presence of 1, 3-linked galacturonic acid as a branching point for either arabinose or rhamnose.

5.9- Immune modulating effect:

The fractions S50, S50f, S100i, S100ii, 100 NaCl, and E1 were tested for the complement-fixation bioactivity. The dilution used in this test was a 2-fold dilution. Although, the high presence of impurities affected the obtained results, samples S50, S100i, S100ii and E1 elucidated complement bioactivity and was decided to proceed with these samples. The value of the PMII as a positive control was too high and the lysis percentage was 71%. Data produced from the complement test was not reliable, too. Accordingly, the test was repeated. Due the high content of impurities in the samples, it was necessary to subject the samples into more purification process which enables one to obtain reasonable results in the future. The purification process was carried out using the gel-filtration method. After gel-filtration, the polysaccharides obtained from E1 sample were of low-weight polysaccharides and it was excluded. The fraction S100i-I was divided into three fractions; S100i-1, S100i-2, and S100i3, hown by the phenol-sulfuric acid test profile. After methanolysis, S100i-3 was excluded due to insufficiency polysaccharide content in the obtained chromatogram.

The results of the second trial showed that samples S50, S100i-1, and S100ii were active while the forth sample; S100i-2 was inactive, therefore, it was excluded. Because the ICH50 values for S50, S100i-1, and S100ii were above 50%, and ICH50 was high, the test was decided to be repeated.

Complement inhibition of *Parkia biglobosa* polysaccharides

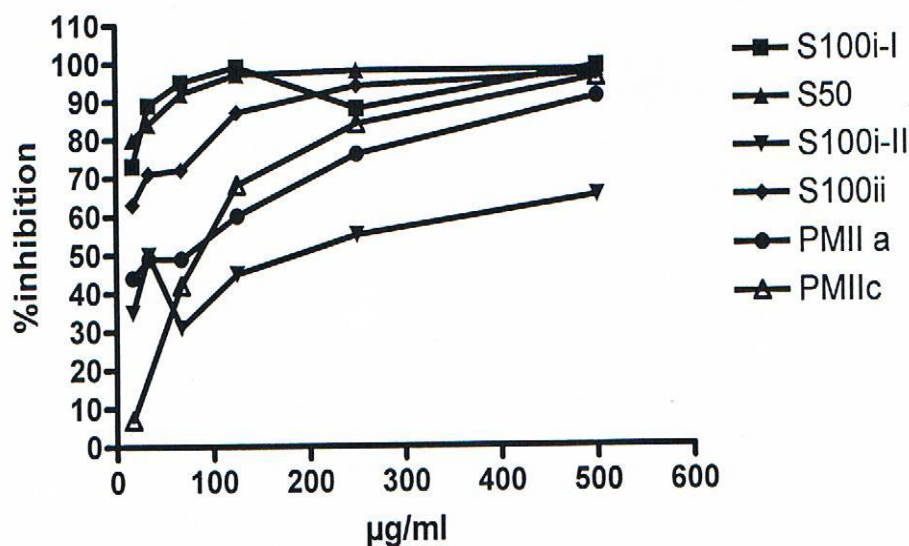


Figure (5.9a) the 2nd complement-fixation test. The vertical axis shows the % prevention of the extracts while the horizontal axis shows the conc. of the samples.

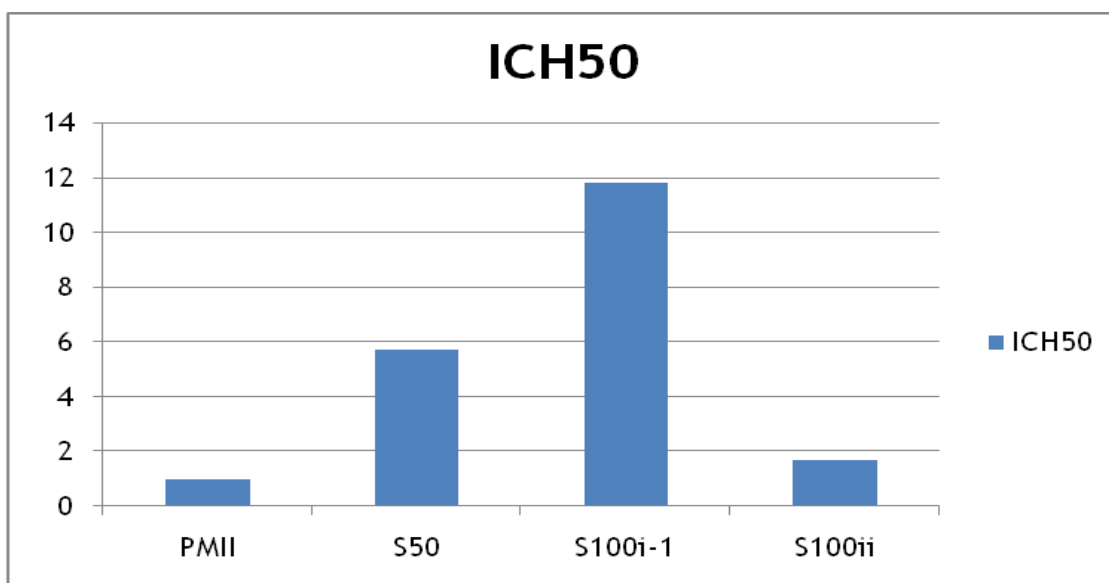


Figure (5.9b) the % of inhibition of the samples calculated as $PMII / sample$. $PMII$ was set to 1. The results for S50, S100i-1, and S100ii from the second complement test are shown as calculated and a high value shows high activity relative to the activity of $PMII$.

In the diagram (5.9b above), it is clearly shown that the biological activity for S100i-1 was the highest followed by S50 and then S100ii compared to $PMII$.

In the third trial, a 4-folds dilution was used, since ICH50 was not clearly obtained using 2-folds dilution. This may point out that the concentration at which 50% hemolysis present at a lower concentration than 15.1 $\mu\text{g/ml}$ and the plant has a potent effect on the complement activity

Samples	ICH50 for the second complement test	ICH50 for the third complement test
S50	70,6	87,7
S100i-1	34,1	52,2
S100ii	343,8	311
$PMII$	404,5	349,4

Table (5.9) ICH_{50} values ($\mu\text{l/ml}$) for the samples and $PMII$

From the following complement-fixation diagram (figure 5.9c), the ICH_{50} s for the tested polysaccharides were under 50%.

From the comparative inhibition percentage diagram (5.9d), the sample S100i-1 was the most active compared to $PMII$ followed by S50, and then S100ii.

The results of the last test were acceptable because samples activities were correlated with the previous results and they are almost alike. The ICH_{50} of $PMII$ as a control was reliable, too.

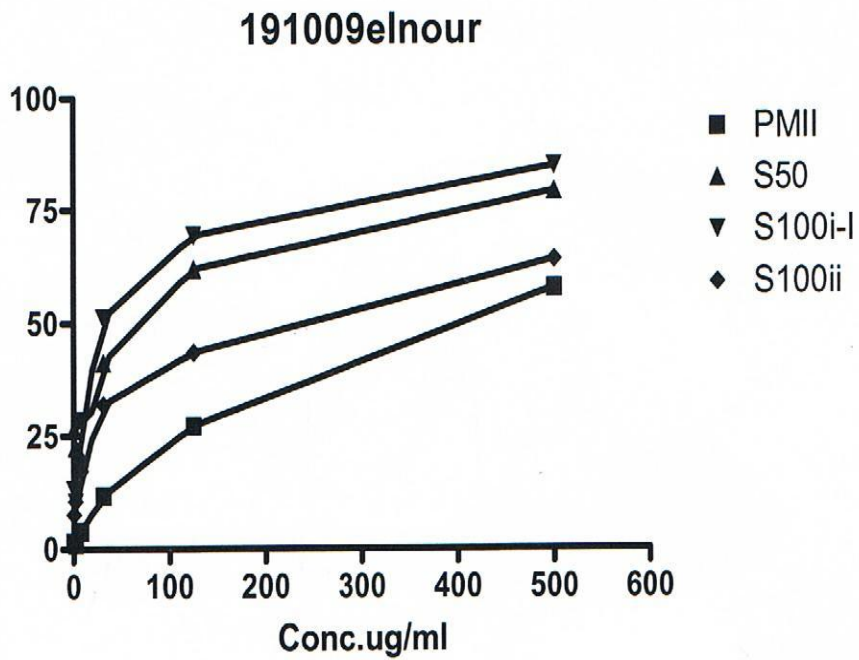


Figure (5.9c) the 3rd complement-fixation test. The vertical axis shows the % prevention of the extracts while the horizontal axis shows the conc. of the samples.

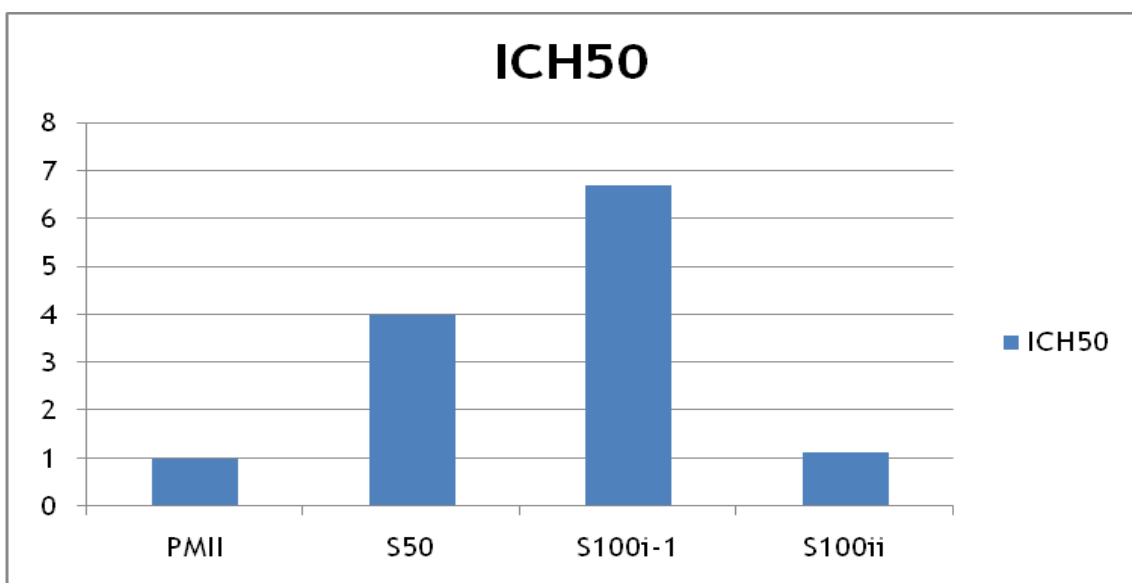


Figure (5.9d) Activity calculated as activity of PM II divided by activity of sample, and the results for S50, S100i-1, and S100ii from the last complement test is given in the figure.

5.10- Stimulation of Macrophages:

This test was carried out by Dr Kari Inngjerdingen at the Royal Hospital, Oslo.

Macrophages function as phagocytes and produce nitric oxide, growth factors and cytokines upon activation. The functions of these effectors are important in the immune response against an infection which may often accompany a wound. The production of nitric oxide (NO) was measured after treatment of macrophages with fractions isolated from *Parkia biglobosa* bark. Macrophages from the mouse macrophage cell line Raw 264,7 were utilized.

The pectin fraction PMII from the leaves of *Plantago major* L., previously shown to be a highly biological active polysaccharide was used as a positive control, in addition to LPS.

All samples produced NO amounts from macrophages stimulation that was related to the samples concentration used. The NO release by the samples compared with that of the standard PMII was as follows: S50 was relatively higher than PMII followed by S100i-1, which was rather similar to PMII, while S100ii was the lowest.

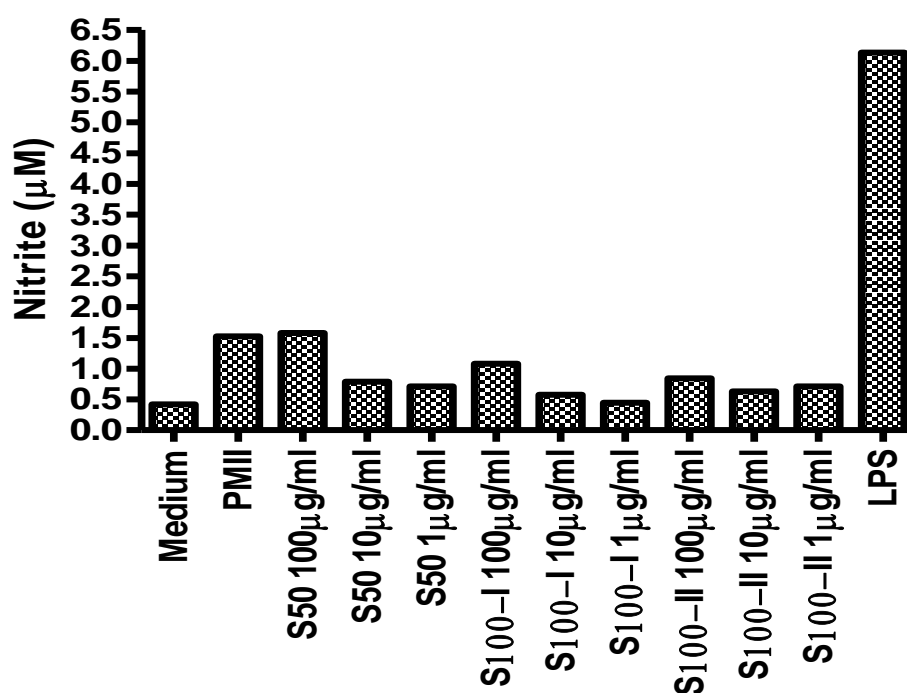


Figure (5.10) stimulation of macrophages. Measurement of nitric oxide released from macrophages after overnight stimulation with polysaccharide fractions. The mouse macrophage cell line Raw 264,7. LPS was used as positive control, in addition to the pectin fraction PMII from the leaves of *Plantago major* L.

6. Conclusions:

The *Parkia biglobosa* bark has been used traditionally in Mali to cure a wide range of illnesses e.g., external and internal wounds, headache, malaria, cough, urinary disorders, diabetes, blood pressure etc. The aim of this study was to find a logical correlation between the use of *Parkia biglobosa* bark in the treatment of wounds by the Malian healers and modern science, which is based on scientific findings and demonstrations.

The results of the complement-fixation bioactivity as well as the macrophage stimulation tests carried out to elucidate the ability of the investigated polysaccharides to modulate the function of the immune system were highly active and typical of the characteristics of the bioactive pectins. Although, the sample S100i-1 was highly active regarding the complement-fixation bioactivity test followed by S50 and S100ii, the results obtained from the stimulation of macrophages showed sample S50 to be the most potent in releasing nitric oxide followed by sample S100i-1 and sample S100ii.

The study of the polysaccharide linkage patterns revealed by the GC-MS chromatograms reflected the structure of the polysaccharides which the *Parkia biglobosa* bark contains. The compositions of these polysaccharides were mainly homogalacturonan, arabinogalactan, rhamnogalactans, and xylogalactans. These obtained structures are the building blocks of pectins that comprise the main core of the typically bioactive pectins found in plants that have the capability to modulate the complement system.

The results obtained from this study confirm and justify the traditional usage of the *Parkia biglobosa* bark by the African natives in general, and specially by the Malian healers in the treatment of diverse diseases including management and treatment of both internal and external wounds.

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