STUDY OF NEEM BARK TANNINS FOR THE LEATHER INDUSTRY

By

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Dedicated to my beloved Mum

Frontispiece. Neem bark

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ABSTRACT

Neem bark (*Azadirachta indica*) contains a mixture of complex chemical components identified as:

One procyanidin trimer	C1	Epicatechin-[4α-8]-epicatechin epicatechin.
Four dimers	B1	Epicatechin-[4β-8]-catechin,
	B2	Epicatechin-[4β-8]-epicatechin,
	B3	Catechin-[4α-8]-catechin,
	B4	Catechin-[4α-8]-epicatechin.
Four well known monomers		(+)-Catechin,
		(-)-Epicatechin,
		(+)-Gallocatechin,
		(-)-Epigallocatechin.
Other compounds		Quercetin,
		Kaempferol,
		Fisetin and
		Gallic acid

These compounds were characterised by chromatographic and spectroscopic techniques, including by thiolytic degradation with toluene-α-thiol and using acetylated and methyl derivatives.

Structural elucidation of the cleavage products was by ^{1}H-NMR. The backbone of the isolated compounds consisted mainly of a mixture of flavan-3-ol units with 2R-configuration.

The purity of monomers, dimers and trimer was checked by HPLC on Lichrosorb RP-8 column.

The molecular weight distribution ranges between 250 and 5500 and the average molecular weight distribution was found to be 1550 by gel permeation chromatography.

Gas chromatography-mass spectroscopy demonstrated the complex nature of NBT molecular masses consist of monomeric, dimeric and trimeric units of procyanidin B and C types respectively.

IR and FT-IR spectroscopy results were consistent with the rest of the techniques used.

The hydrothermal stability of neem bark tannin tanned and crosslinked hide powder and leathers was found to be paralleled to mimosa tannin extract.

Studies of the physical properties of tanned and retanned leathers showed comparability of neem bark tannins with mimosa tannin extract.

ABBREVIATIONS

NBE	Neem bark extract
NBT	Neem bark tannins
HPLC	High performance liquid chromatography
^{1}H NMR	Proton nuclear magnetic resonance
GPC	Gel permeation chromatography
gc	Gas chromatography
gc-MS	Gas chromatography-mass spectrometry
IR	Infrared
FT-IR	Fourier transform infrared
ATR	Attenuated total reflectance
P Av.	Particle size average
SG	Specific gravity
cp	Centipoise
UV	Ultra violet
Me	Methyl
GIa	α-L-arabinose
GIb	Arabinofucoglucan
GIIa	Branched arabinofucoglucan
ara	Arabinose
Fr.	Fraction
RPLC	Reverse phase liquid chromatography
NPLC	Normal phase liquid chromatography
THF	Tetrahydrofuran
MS	Mass spectroscopy
Pcb	Printed circuit board
TLC	Thin layer chromatography
NMR	Nuclear magnetic resonance
D_6 DMSO	Hexadeutero dimethyl sulphoxide
Mol. wt. or MW	Molecular weight
Mn	Number average molecular weight
Mw	Weight average molecular weight

P_d	Polydispersity
rpm	Revolution per minute
Py	Pyrolysis
THM	Thermally assisted hydrolysis methylation
TMAH	Tetraethylammonium hydroxide
DSC	Differential scanning calorimetry
Ts	Shrinkage temperature
THPS	Tetrakis hydroxymethyl phosphonium sulphate
SLTC	Society of Leather Technologist and Chemists
BSLT	British School of Leather Technology
IUP	International Union (Physical)
ΔH_{ENDO}	Change in enthalpy (endothermic)
ΔTs	Change in shrinkage temperature
PMA	Pyromellitic dianhydride
C	Catechin
EC	Epicatechin
GC	Gallocatechin
EGC	Epigallocatechin
B1	Procyanidin EC-C
B2	Procyanidin EC-EC
B3	Procyanidin C-C
B4	Procyanidin C-EC
C1	Procyanidin EC-EC-EC
OSP	Official sampling position
ΔE_{ab}^*	Colour difference
L*	Lightness factor
a* and b*	Chromaticity co-ordinate factors
SATRA	Shoe and Allied Trades Research Association
i.d	Internal diameter
F	Frequency
df	Degree of freedom
Sig.	Significant
Rt.	Retention

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1. INTRODUCTION

The preferred modern tanning method is to use salts of chromium(III), presently used to tan about 90% of the world's production. Increasingly, this generic process has come under scrutiny by regulatory authorities, with the result that there is continuing pressure on tanners to limit discharges of chromium(III) into the environment[1]. This is not confined to the leather industries of the developed economies, it is happening all over the world. In some of the developing economies, including Pakistan, there is an additional factor to be considered: people use the waste chrome shavings and dust as fuel for cooking, with the result that the oxidised fly ash, containing chromium(VI), is causing cancers. Chromium(III) poses no environmental hazard, although it is accepted that sparingly soluble chromate salts are carcinogenic. These factors create an imperative to study the alternatives to chromium(III) tanning.

From an environmental standpoint, the obvious option is a low chrome offer tanning process. This is only a partial solution to the problem, because although there can be a greater than commensurate decrease in waste chrome by reducing the offer, this requires an increased level of process control and there is a practical lower limit to the chrome offer; to maintain shrinkage temperature above 100°C, the lowest chrome offer is 0.75% Cr_2O_3[1], so the health hazard associated with burning tanned waste is not diminished.

The unique feature of chromium(III) as a simple tanning agent is that it confers high hydrothermal stability to the leather; whilst this is not essential for all leathergoods purposes, it is critical for most applications, particularly for shoe upper leather, which is likely to encounter stringent conditions during the manufacture of shoes. Currently, there is only one established tanning method that can match this aspect of leather performance and that is semi-alum tannage, comprising tanning with natural polyphenolic plant extracts (vegetable tanning) and retanning with aluminium(III) salts. Semi-alum tanning is not new; it has been used but not

understood, from as long as 6000 years ago[2]. Conventional technology has two drawbacks: first, it is usually a two stage process, in which the first stage is vegetable tanning, conferring typical characteristics to the leather; second, it involves aluminium(III) salts which, for a variety of reasons, are perceived to be environmentally unacceptable.

To address this problem, this project is focused on tannins from the neem tree, indigenous to the Indian-subcontinent and found all over Pakistan.

1.1.1. PLANT POLYPHENOLS (vegetable tannins)

The word tannin has a long and well-established usage in the scientific literature. The importance of vegetable tannins to a range of scientific disciplines has been recognised for over 100 years[3], apart from their use for centuries in the art of leather manufacture. White[4] defined the term 'tannin' as "the substance, which converts the putrefiable hide or skin into imputrescible leather". Probably the most acceptable concise and simple definition is still that of Bate-Smith and Swain[5], who based their classification on the earlier ideas of White as "water soluble phenolic compounds having molecular weights between 500 and 3,000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatine and other proteins".

Scientifically and terminologically, 'plant polyphenols' is to be preferred as a description for this class of higher plant secondary metabolites[3]. In general, the term 'tannin' includes mixtures of polyphenolic substances that have limited solubility in water and tend to form supersaturated solution[6]. It is difficult to single out any phenolic compound and define it as the respective tannin of the plant material concerned. In view of this complexity in the nature of tannins, the term 'tannin extract' is used. It is known that plants synthesis different polyphenolic substances, some of which may contribute to the formation of tannins.

Freudenberg's classification of these vegetable tannins, based on their chemical nature and structural characteristics, into (i) hydrolysable tannins and (ii) condensed (or flavonoid) tannins[6] provides a convenient basis for chemical studies on these vegetable tannins. While the hydrolysable tannins undergo hydrolysis with mineral acids or enzymes, the condensed tannins, which are non-hydrolysable,

produce coloured solutions and/or precipitates, known as 'phlobaphenes or tannin reds', with these reagents.

The tannins are mostly amorphous, astringent in taste and feebly acidic in character. Aqueous tannin solutions or infusions are known in the trade as tan liquors.

1.1.1.1. Hydrolysable tannins

These are based on esters of phenol carboxylic acids (gallic acid and/or hexahydrodiphenic acid) with a central carbohydrate core. They are sub-divided into (i) gallotannins and (ii) ellagitannins. Gallotannins yield gallic acid and glucose on hydrolysis. The ellagitannins, on the other hand, produce ellagic acid in addition to gallic acid and glucose on hydrolysis.

1.1.1.2. Condensed tannins

Commercially, condensed tannins are more important from the leather manufacture point of view. Structurally related to flavonoids, these tannins are distributed widely in nature and constitute a heterogeneous group. The C_{15} skeleton of the flavonoid is made up of two distinct units, viz. 'A' ring (consisting of a C_6 unit) and 'B' ring (made up of C_6 -C_3 unit) as shown in Figure 1-1.

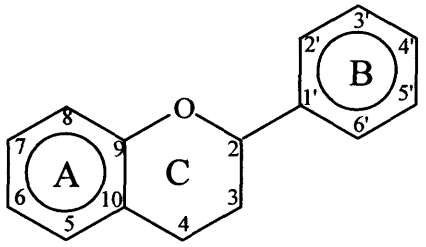

Figure 1-1: Basic flavonoid structure.

The flavonoids are further classified into flavan-3-ols, flavan-3, 4-diol and proanthocyanidins. To collectively define all the colourless substances isolated from plants that form anthocyanidin when heated with acid, Freudenberg and Weinges,[7] coined the term 'proanthocyanidins'. Weinges *et al.*[8] used the term leucoanthocyanidins, to refer to flavan-3, 4-diol for the monomeric procyanidin and the name "condensed proanthocyanidins" for the various flavan-3-ol dimers and higher oligomers. Some commercially important plant polyphenols are listed in Figure 1-2.

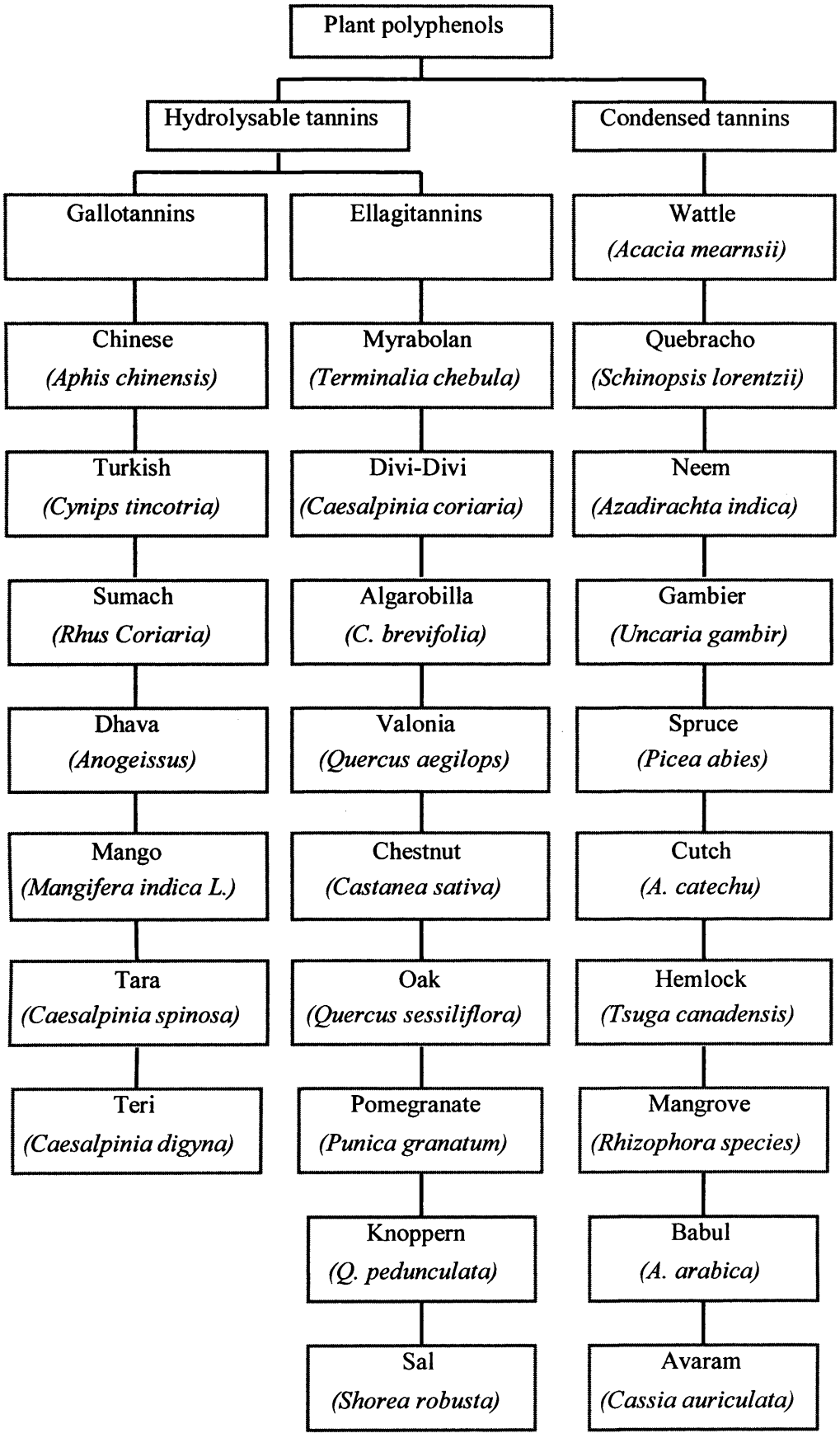

Figure 1-2: Classification of some commercially available plant polyphenols.

1.1.2. PROANTHOCYANIDINS

The terms proanthocyanidins, condensed tannins and polyflavonoids are generic names used to define the more abundant class of tannin[9]. The proanthocyanidins constitute the most ubiquitous group of all plant phenolics[10]. All of these compounds contain the familiar flavonoid skeleton, linked together in apparently infinite arrangements, depending upon the nature of the interflavonoid linkage, hydroxylation pattern, stereochemistry at carbons 2, 3, and 4 of the pyran ring, as well as the presence of additional substituents, (e.g., ester-linked gallic acid). The proanthocyanidins are oligomers and polymers consisting of chains of polyhydroxyflavan units. The most common monomer units are hydroxylated at C-3 and are listed in Table 1-1[11]. Hence, well over a hundred oligomeric and polymeric proanthocyanidins with subtle, but well-defined structural variations have been isolated from many different plant species. Their exceptional concentrations in the barks and heartwoods of a variety of tree species have resulted in their commercial extraction, with the initial objective of applying the extracts in leather manufacture[12].

The chemical literature concerning the structure and formation of the condensed tannins contains many speculative theories, and evidence was accumulated in the 1960's which favoured the view, originally suggested in the form of the "catechin hypothesis" by Freudenberg[13], that the complex polymeric structures of many of the condensed tannins are derived primarily by polycondensation of precursors of a flavonoid type. The generic term catechin was first used by Freudenberg to describe the colourless crystalline substances commonly located in plant tissues in association with the condensed tannins[14]. Later work has shown the catechins to be derivatives of the basic flavan-3-ol structure and nowadays these compounds, to avoid confusion with trivial names, are generally referred to as flavan-3-ols. Condensed tannins constitute a heterogeneous group, possessing the common characteristics of producing red colours 'phlobaphenes' from colourless substances which are present in the leaves, flowers, roots, barks and fruits of certain plants, upon treatment with hot dilute mineral acids in aqueous medium and may be astringent to the taste[15]. The most widely distributed of these proanthocyanidins in nature are the dimers and higher oligomers of the procyanidin B-group, which biosynthetic studies have shown[16a,b] are metabolised by reaction between (+)-catechin (1) or (-)-epicatechin (2) and the related carbocations (3) and (4) as shown in Figure 1-3. Each

of the four biosynthetic reactions yields one predominant procyanidin dimer (B1-to B4 respectively).

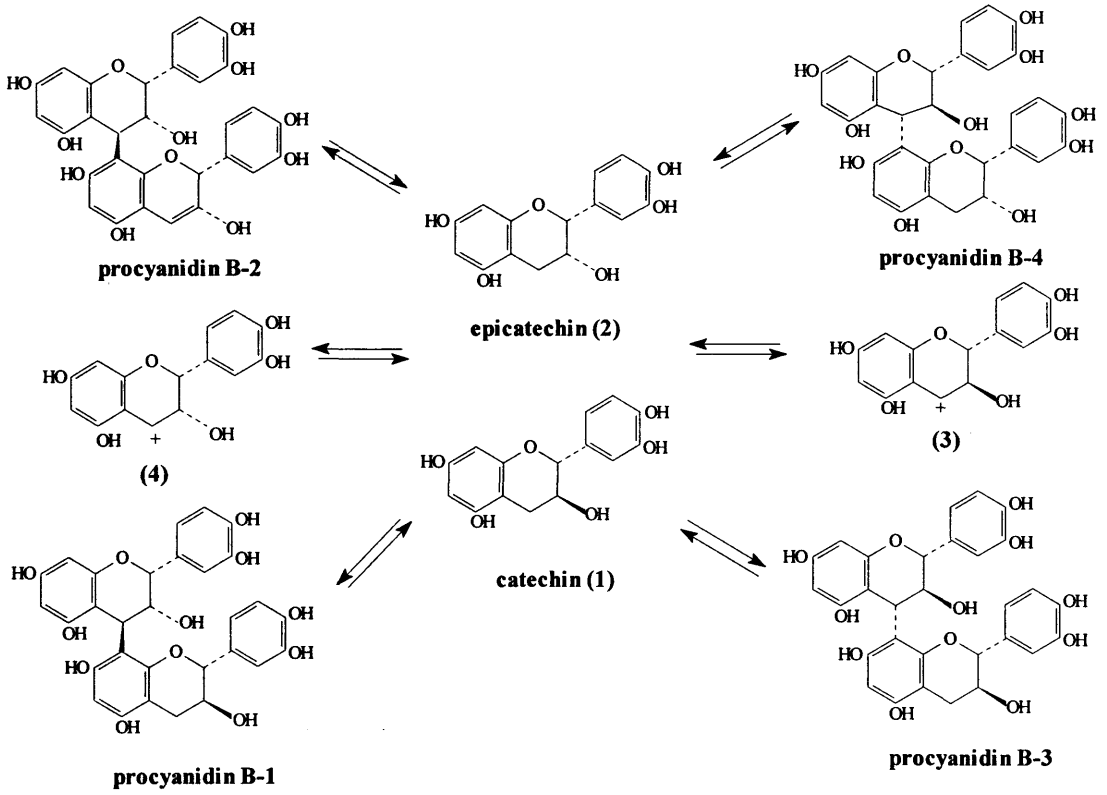

Figure 1-3: Biogenetically patterned procyanidin synthesis.

Table 1-1: Proanthocyanidins: Common monomer units and polymer nomenclature.

Monomers	Hyroxylation pattern	Polymers
Fisetinidol	3,7,3',4'	Profisetinidin
Robinetinidol	3,7,3',4',5'	Prorobinetinidin
Catechin	3,5,7,3',4'	Procyanidin
Epicatechin	3,5,7,3',4'	Procyanidin
Gallocatechin	3,5,7,3',4',5'	Prodelphinidin
Epigallocatechin	3,5,7,3',4',5'	Prodelphinidin

1.1.2.1. Structure and chemical properties of the condensed tannins

The condensed tannins are oligomers and polymers of polyhydroxyflavan-3-ol monomer units linked by acid-labile 4 → 6, 4 → 8 bonds. The polymer chains are irregular, sometimes branched, and often contain more than one type of configurational base unit. Condensed tannins are relatively unstable in aqueous solution and precipitate in mild acids or bases. The monomer units extensively rearrange in stronger acidic or basic solutions. The reactions are largely radical-mediated in basic solutions, to form highly rearranged and oxidatively coupled products. Condensed tannins complex strongly with metal ions, carbohydrates, and proteins.

Polymers with more than one structural base unit are quite common. Thus, procyanidin / prodelphinidin and profisetinidin / prorobinetinidin copolymers occur, as well as those with diastereoisomeric or enantiomeric monomer units; e.g., procyanidins based on both catechin and epicatechin or epicatechin and epicatechin in the same polymer chain[10].

A particularly interesting example of irregular polymers occurs in the Liguminosae and Anarcardiaceae where catechin or gallocatechin commonly form the terminal units of the chains, and fisetinidol or robinetinidol (or their enantiomers) form the extender units. Roux and Ferreira[12] coined the term 'angular' proanthocyanidins to describe this class of tannins. The constraint of the much greater nucleophilicity of the phloroglucinol pattern A-ring of the catechin or gallocatechin unit, compared with the resorcinol pattern A-ring of the chain extender units, leads exclusively to doubly substituted terminal units for the trimeric and higher oligomers. These are shown in Figures 1-4 and 1-5.

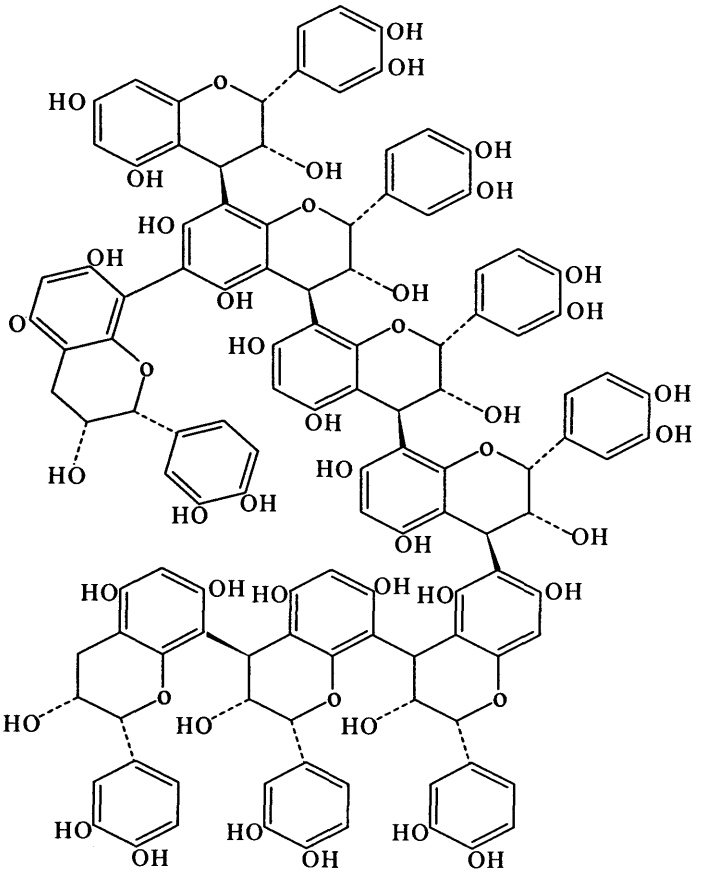

Figure 1-4. Representative structure of an epicatechin procyanidin oligomer.

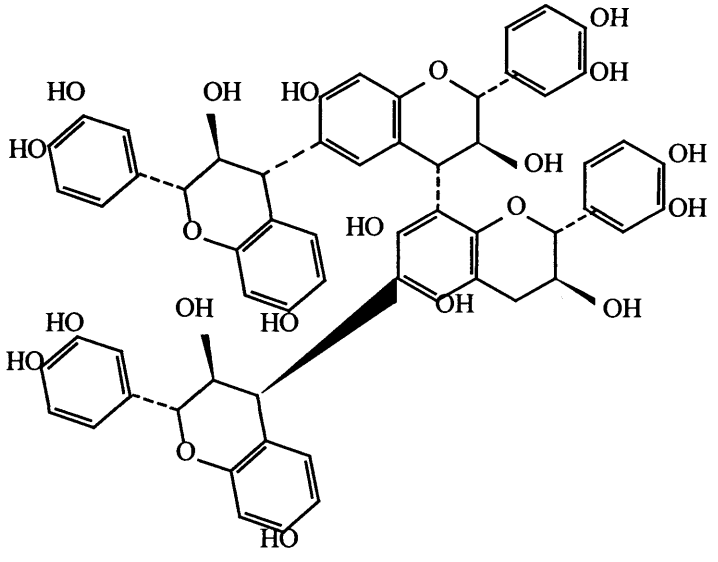

Figure 1-5. Representative structure of a fisetinidol-catechin profisetinidin oligomer showing the 'angular' chain terminating structures.

Condensed tannins are further subdivided based on the hydroxylation pattern of the monomeric flavanoid A and B rings. Table 1-2 describes the most common condensed tannins and their hydroxylation pattern.

Table 1-2: Hydroxylation pattern of condensed tannins.

Class	Hydroxylation pattern
Propelargonidin	3, 4', 5, 7
Procyanidin	3, 3', 4', 5, 7
Prodelphinidin	3, 3', 4', 5, 5', 7
Proguibourtinidin	3, 4', 7
Profisetinidin	3, 3', 4', 7
Prorobinetinidin	3, 3', 5', 7
Proteracacidin	3, 4', 7, 8
Promelacacidin	3, 3', 4', 7, 8
Proapigenidin	4', 5', 7
Proluteolinidin	3', 4', 5, 7

Stereochemistry of the monomer units can be either 2,3-cis or 2,3-trans and the associated monomers (-)-epicatechin and (+)- catechin are found in the bark.

The hydroxylation pattern of the flavonoid units in the condensed tannins plays an important role in determining the nature and rate of reaction of both the A and B rings and also influences the type and rates of reactions at the interflavonoid bond. In case of procyanidins, the 3', 4'-dihydroxyl group of the B-ring does not make that ring active to electrophilic substitution, so that polymerisation with formaldehyde does not take place at the B-ring under normal used conditions, when A-ring polymerisation can occur[17].

1.1.2.2. Properties and reactions of condensed tannins

The proanthocyanidins and flavan-3-ols are typical polyphenols that readily ionise in aqueous solution. The pKa for the first ionisation constant of epicatechin or procyanidin B-2 is 8.5, so their solutions are weakly acidic[18]. Titration of solutions of

the epicatechin polymer from *Chaenomeles chinensis* showed that titratable protons with a much lower pKa, equivalent to a carboxylic acid, also occurred, at a relative concentration of one per 40 or 50 monomer units[19]. The solubility of proanthocyanidin polymers is dependent on phenolate ion formation, as lowering solution pH reversibly precipitates procyanidin polymers. It has been shown that the epicatechin polymer has the ability to form an aluminium (III) -diphenolate complex that encapsulates the metal ion[20]; the polymer forms strong and often insoluble complexes with Al (III) and Fe (III).

Another important property of condensed tannins is their ability to complex strongly to carbohydrates and proteins (the origin of astringency). Such binding is quite powerful and difficult to reverse[21].

1.1.3. NEEM TREE

Neem tree (*Azadirachta Indica*) has been well known in the Indian-Subcontinent and neighbouring countries for more than 2,000 years[21], and for centuries the fruits, leaves, oil, bark and roots of the tree have been used in Ayurvedic and Unani medical treatments.

From an initial caution and scepticism, neem has now been universally accepted as a wonder tree. In the early 90's the importance of neem recognised by the US National Academy of Sciences, resulting in a 1992 report entitled[22] "Neem- a tree for solving global problems."

1.1.3.1. Taxonomy

The generic epithet Azadirachta is derived from Azad-darakth, (Persian: free tree), The Species Plantarum by Linnaeus in 1753 contains two representatives of the family Meliaceae, *Melia azadarch* and *Azadirachta indica.* Linnaeus renamed *Azadirachta indica* as *Melia azadirachta.* Adrien Henri Laurent De Jussieu[22] on account of its 'one seeded nut' separated neem from the genus Melia to Azadirachta. The three important classifications of the Meliaceae are those of Bentham[22-25] and Hooker who, in their *Genera Plantarum,* merged *Azadirachta* into the genus *Melia* and included it in the tribe *Melieae*[26]. Harms divided *Meliaceae* into three sub-families, Cedreloideae, Swietenioideae, and Melioideae. The sub-family *Melioideae* was further divided into six tribes. *Azadirachtieae* and *Melia* were put in the tribe

Melieae, the number of tribes in the sub-family *Melieae* was reduced to five, removing *Azadirachtieae* and putting *Azadirachta* and *Melia* in the same tribe *Melieae*[26, 28, 29].

Integrifolia (Syn. *A. excelsa; Melia excelsa*) the species of *Azadirachta* are synonyms of *Melia*[30].

Schmutterer described the taxonomy of neem as follows[21].

Order	*Rutales*
Suborder	*Rutineae*
Family	*Meliaceae (*Mahogany family)
Subfamily	*Meliodeae*
Tribe	*Melieae*
Genus	*Azadirachta*
Species	*Azadirachta indica.*

1.1.3.2. Chemical characters and the classification of the Rutales

There is general agreement between taxonomists that Burseraceae, Cneoraceae, Meliaceae, Rutaceae, and Simaroubaceae should be placed in the same order.

1.1.3.3. The chemical make-up of the Rutales

Table 1-3: Distribution of some classes of phenolics in the Rutales.[31-34].

Classes of constituents	Rutaceae	Meliaceae
Flavonoids	Flavonols, flavones, flavanones, and flavanonols, as glycosides and polymethoxylated derivatives. Phenylated and C-methylated flavonoid rather common	Quercetin and myricetin glycoside in leaves and flowers. Methylated flavones in leaves.
Tannins and their precursors[32]	Procyanidins and prodelphenidins common. Gallic and ellagic acids rare.	Catechins, proanthocyanidins, condensed tannins. Tannin content sometimes considerable

1.1.3.4. Geographical distribution

Neem tree is an attractive evergreen tree native to the Indian-Subcontinent, but cultivated throughout south-east Asia, Australia, East Africa, Fiji, Mauritius, many countries in Central and South America, the Caribbean, Puerto Rica, and the Virgin Islands. In its native environments, neem is generally found growing in mixed forests, and associated with other broadleaf species, such as *Acacia sp.* and *Dalbergia sissoo*. It grows in tropical to subtropical regions, semiarid to wet tropical regions, and from sea level to over 610 m (2000 ft). It is cultivated throughout the Indian sub-continent[35], but some[36] attribute its nativity to the dry forest areas of Sri Lanka, Malaysia, Indonesia, Thailand, and Burma. East Indian immigrants introduced neem to Mauritius, and it is thought that they took it to a number of African countries. It is widely cultivated on the African continent in Ethiopia, Somalia, Kenya, Tanzania, Mozambique, Mauritania, Togo, Ivory Coast, Chad, Nigeria, Cameroon, Guinea, China, Gambia, Sudan, Benin, Mali, Niger, Burkina Faso, and Senegal, particularly in rainfall-deficient regions[37]. Neem plantings are also abundant in Guatemala, Bolivia, Ecuador, Honduras, Argentina, Brazil, Cuba, Nicaragua, Dominican Republic, St. Lucia and Antigua and large numbers are now being planted in Haiti. In addition to the ongoing experimental cultivation of neem in Puerto Rico and the U.S.Virgin Islands, plantings in southern Florida are thriving, and the field cultivation of neem in Oklahoma, southern California, and Arizona has begun[38-41].

1.1.3.5. Ecology

Neem tree is famous for its drought resistance. Normally it thrives under sub-humid to semi-arid conditions and can be established in areas with an annual rainfall of 450-750 mm. Optimum growth is obtained in higher rainfall areas (1150 mm); 130 mm /year is sufficient for survival[36], but it needs 450 mm to grow successfully. It grows where maximum shade temperature may be as high as 49°C, but it does not stand excessive cold. Neem is frost-tender, specially in the seedling and sapling stages, but it is grown in frost zones of the sub-Himalayan tract by protecting seedlings during the winter with screens[39]. Neem is not a very sociable tree, does not grow well in pure stands (plantations in Africa often die out in 3 to 10 years) and is very competitive for water and soil nutrient; thus it does not grow well on marginal soils.

Neem seems to grow best in deep sandy soils that are well drained, but can grow in practically all sorts of soil; it thrives on black cotton soil in India and can do well even on clay. It does better than most species in dry localities, on sandy, stony shallow soils with a waterless subsoil or in places where there is a hard calcareous or clay pan (hardpan) not far from the surface. However, neem will grow much better if this hardpan is broken up before planting. Occasionally, neem will grow well on soils that appear to be sandy, but quickly dies out when roots hit a deep layer of dense clay. Neem can grow even on saline and on alkaline "usar" soils. Neem does not tolerate waterlogged soils, and does not do well on soils with impeded drainage and on soils subject to inundation. Growth is not good on poorly drained soils because the taproot tends to rot and the tree gradually dies.

A soil pH value of between 6.2 and 7.0 seems to be best for this tree, but pH 5.9 and 10 may also be tolerated under certain circumstances. In Australia neem thrives on soils with pH values from slightly alkaline to slightly acid[42]. The same applies to Nicaragua[43]. Neem leaf litter can change the pH value of the top layer of the soil under neem trees from 5.4 to 6.8 in West Africa (Nigeria); the pH value of neem leaves is 8.2[40].

Light is another important environmental factor for *Azadirachta indica's* growth. Although young seedlings are often raised under shade, mature trees need a lot of light. The net rate of photosynthesis, measured in Australia, was 10-17 μmol CO_2 m^{-2} s^{-1}, which is intermediate to high compared with tropical fruit trees[44]. To enable regular growth and the development of a broad crown with numerous flowers and fruits, the tree also needs sufficient space. Consequently, solitary neem trees are much more productive than others are in dense groups or plantations.

1.1.3.6. Botanical characteristics

Azadirachta indica is a fast growing plant that usually reaches a height of 15-20 m, and under very favourable conditions up to approximately 35-40 m. As a rule it is evergreen but under extreme circumstances, such as extended dry periods, it may shed most or nearly all of its leaves. The branches spread widely. The trunk is relatively short, straight and may reach a girth of 1.5-3.5 m the bark is hard, fissured or scaly, and whitish-grey to reddish-brown. The sapwood is greyish-white and the heart wood reddish when first exposed to the air, becoming reddish-brown after exposure.

1.1.3.7. Bark

The stem bark varies in appearance and thickness according to the age of the part of the tree and to some extent to environment and climate. Bark on the younger stems or branches is smooth, soft and greenish slightly brownish-red in colour. Occasionally it has alternating green and rusty green longitudinal bands. Barks taken from medium sized branches vary from 1.25-2.50 cm in thickness. The dead outer rind is rough, woody, very much fissured and peels off in fairly thick slices. In some old barks there is a narrow zone of dark yellowish brown, soft, suberous tissue within the outer 'woody' rind, as in root-barks. The exposed living bark has a rose-purple to purplish brown colour. Its thickness is about 0.25 cm or more. In a transverse section it appears differentiated into three regions.

(I) A comparatively narrow rose-purple to light purplish brown peripheral part.

(II) A whitish middle portion comprising slightly less than half the thickness of the living bark.

(III) A fairly thick innermost region occupying the greatest bulk and composed chiefly of secondary bast. This part appears tangentially laminated with alternating dull yellowish white and whitish striations. The entire officinal bark has a characteristic garlic like odour and bitter astringent taste. In the fresh condition the inner bark is more intensely bitter than the outer rosy or dark coloured tissue which on the other hand is more astringent.

In the young bark a hard outer rind is absent. The cork tissue forms the outer skin and is composed of twelve to fifteen or more rows of thin walled, narrow rectangular tangentially elongated cells. Of these, a few outer-most rows are compressed and thick walled. The cortex is composed of several rows of a fairly large oblong cells, most of which are loaded with starch grains. The others contain cubical, rectangular and polyhedral crystals. Scattered throughout the cortex are several groups of sclereid-like cells, with pitted but not very thick walls, each enclosing a wide lumen. Most of these cells are nearly of the same size and shape as the surrounding cortical cells, but a few are tangentially elongated. It is noteworthy that such sclereid like cells are not usually found in older barks. The inner bark forms the thickest region and consists of radial segments of bast alternating with medullary rays. Each segments of bast consists of tangential bands of phloem with collapsed elements in the

older portions alternating with groups of sclerenchyma. A few large secretory cavities are found scattered in the phloem. The phloem parenchyma cells contain starch.

The outer hard and 'woody' exfoliating rind that is considerably thick in old barks consists almost entirely of the dead elements of secondary bast, alternating with discontinuous tangential strips of compressed and hardened cork tissue. The former is composed of tiers of regularly arranged groups of sclerenchyma together with more or less collapsed thin walled phloem elements with reddish brown contents. Between the successive zones of cork tissue there may be a three or five or more tiers of sclerenchyma groups with intervening thin-walled and often collapsed elements of phloem. Each zone of cork tissue consists of several rows of regular thin-walled cells occasionally with a few compressed rows of thick walled cells towards its outer side[45].

1.1.3.8. Leaf

The unpaired, pinnate leaves are 20-40 cm long dark green leaflets, which number up to 31, and are approximately 3-8 cm long. The terminal leaflet is often missing. The petioles are short. Very young leaves are reddish to purplish in colour. The shape of mature leaflets is more or less asymmetric and their margins are dentate with the exception of the base of their basiscopal half, which is normally very strongly reduced and cuneate. The leaves contain 12-18% crude protein, 11-23% crude fibre, 43-67% N-free extract, 2-6% ether extract, 8-18% total ash calcium and 0.1-0.3% phosphorus[21].

1.1.3.9. Flowers

The white, fragrant flowers are arranged in axillary, normally more-or-less droop panicles that are up to 25 cm long. The inflorescence, which branch up to the third degree, bear approximately 150, and occasionally up to 250 flowers.[38] An individual flower is 5-6 mm long and 8-11 mm wide. The five petals are 5.0-5.5 mm long and 2 mm wide. Certain solitary growing neem trees seem to be unable to self-fertilise. There are ten glabrous anthers that are inserted at the base of the flowers. The nectar is annular and fused at the base of the ovary.

1.1.3.10. Seeds

The glabrous fruit are olive like drupes that vary in shape from elongate oval too nearly roundish and when ripe are 1.4-2.8 x1.0 x 1.5 cm. They are green when young and yellowish-green to yellow when mature.

The fruit skin (exocarp) is thin and the bittersweet pulp (mesocarp) is yellowish-white and very fibrous. The mesocarp is 0.3-0.5 cm thick. The white, hard 'shell' (endocarp) of the seed encloses one, rarely two and very rarely three elongated seeds (seed kernels) having a brown testa. The approximate size of seeds is 0.9-2.2 x 0.5-0.8 cm, and the 'seed kernels' are 0.8-1.6 x 0.4-0.5 cm[21].

1.1.4. METHODOLOGY

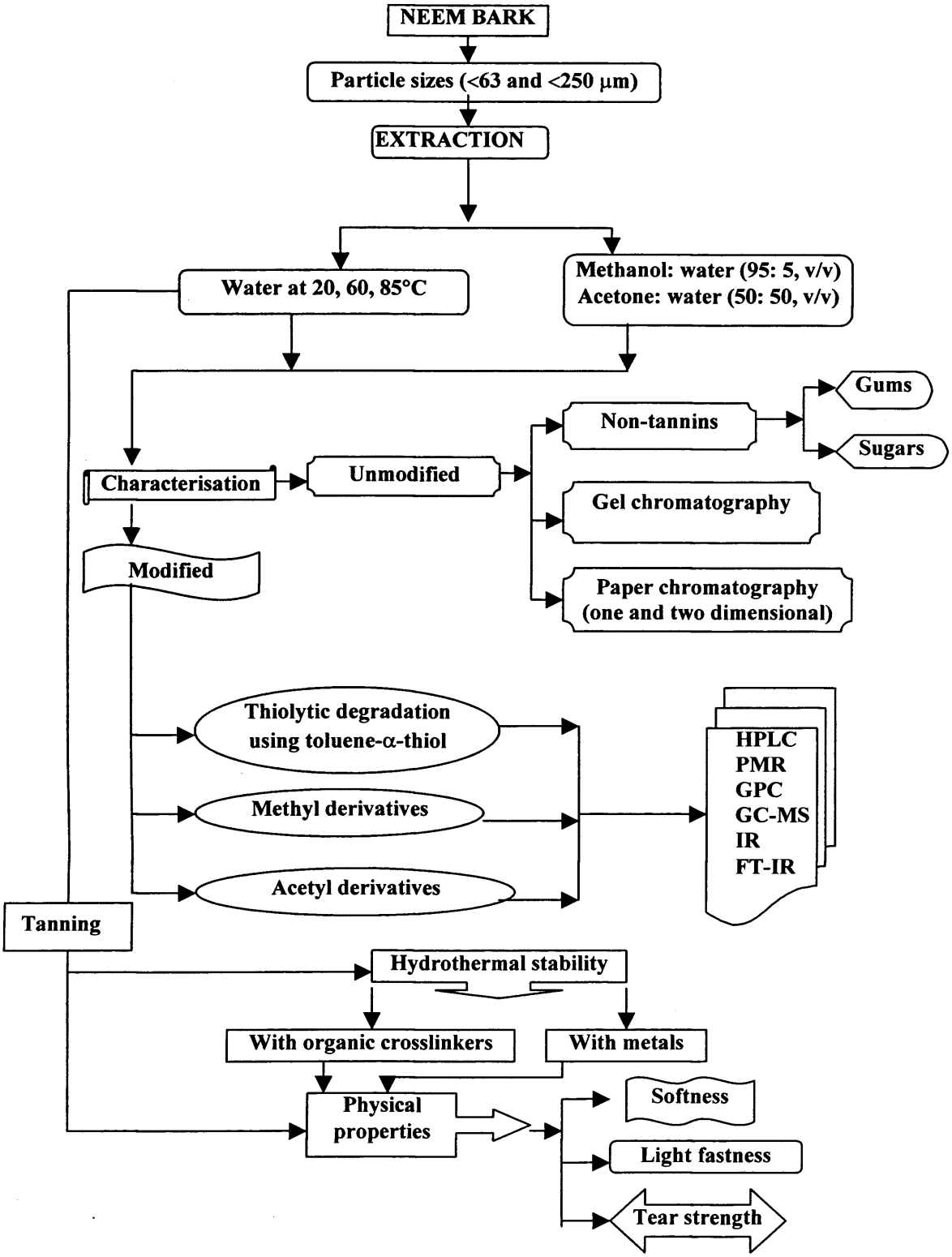

Figure 1-6: Methodology of the techniques for studying neem bark.

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CHAPTER 2

EXTRACTION

2.1. BACKGROUND

Extraction of tannins is probably the most difficult step to control in tannin production. The state and conditions of materials at the time of extraction may lead to large variations in the yields. Collection of samples, storage, drying, and grinding, together with the nature of solvent mixture, particle size and temperature chosen for extraction may alter the tannin chemical structures and extraction efficiency. The method of extraction used will determine not only the extract yield but also its composition and hence its chemical, physico-chemical, astringency properties, due to differences in solubility or extractibility of the complex tannin molecules or the polymeric nature present in a particular plant tissue. The method will also affect the proportion of tannins and non-tannins in the extract[1].

The particle sizes of samples, conditions of extraction, nature of extractant, and temperature of extraction are all-important variables. Solubilisation depends only partly on the solubility of the tannins in the extractant. Water is a good solvent for most tannins, but is not necessarily a good extraction solvent. The solvation of tannins must allow the disruption of the interactions with the insoluble matrix, polysaccharides, proteins, or other polymers. Solubilisation of tannins may thus be considered as a stripping reaction of plant materials[1].

2.2. THE EXTRACTIVES OF BARK

By the term 'extractives', one means such substances as can be dissolved from bark by means of organic solvents or water without their undergoing any changes. In bark, these substances are higher fatty acids and alcohols, resin acids, fats, waxes, hydrocarbons, terpenes, steroids, alkaloids, proteins, pigments, tannins, phlobaphenes, glycosides and carbohydrates.

Nearly all the groups of substances listed here are represented among the extractives from most types of bark. Although their total part by weight in the bark is large (in general, 20-40%) the different individual substances are, with a few

exceptions, present in such small amounts that their economically profitable isolation has not been possible.

Methods for isolation of the different groups of substances based upon their variations in solubility in organic solvents and water are as follows.

Table 2-1: Solubilities of extractives from bark.

Class of substances	Soluble in	Insoluble in
Phlobaphenes, sometimes also glycosides	Alcohol	Water, petroleum ether, ethyl ether
Tannins, simple sugars, glycosides etc.	Alcohol, water and acetone	Petroleum ether and ethyl ether

In successive extractions, the result depends upon the sequence of extractions. In practice, it is of course not possible to achieve a complete fractionation. A rather considerable overlapping has to be expected in successive extraction as well as in the precipitation of groups of substances from extracts by the employment of the other solvents.

2.2.1 The influence of external factors upon the extraction

Large differences usually appear when the results obtained by different authors from extractions of the same kind of bark are compared with one another. Actually, the results from extractions of bark are dependent upon so many external factors that it is probably practically impossible to achieve completely reproducible results. Such factors are, for example, the age and the growing conditions of the tree, the height at which the sample was taken from the trunk, the sampling method, the lapse of time between sampling and analysis, the treatment of the analytical material, and so on.

2.2.2. Colouring matter

Flavone, isoflavanone derivatives, anthocyanins and leucoanthocyanins constitute an important group of pigments, which are widely spread in plants. Many authors use the name "flavonoids" for all these compounds.

2.2.3. Flavonoid compounds

The derivatives of flavone, isoflavone, and flavanone occur naturally as yellow pigments, and they can, with respect to their general structure as shown in Figure 2-1, be derived from 2-or 3-phenylbenzo-γ-pyrone.

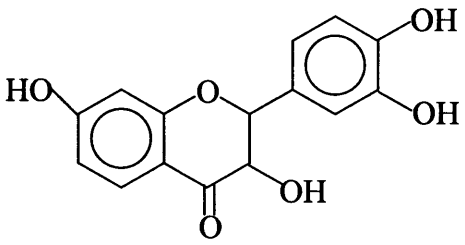

Figure 2-1: Basic structure of flavone.

Flavonoids are present in large amounts in different plant tissues, both as glycosides and in a free state. Some of them are colourless, such as for example the basic substance flavone. A visible colour appears when hydroxyl groups are present as substituents; mono, di, tri-saccharides can be connected to different positions in the aglycone.

2.2.4. Extraction solvents

For analytical purposes, the best extractants are aqueous organic solvent mixtures. The most largely used extractants are aqueous methanol and aqueous acetone. These mixtures are also among the best stripping solvents for vegetable tanned leather. Their action is far more effective than pure water, or methanol or acetone[2]. The proportion of water used, within the limits of 20 to 70 percent, has a limited influence on yield, but best results have usually been obtained with 50 percent water in a variety of solvent mixture[2,3]. These solvents in aqueous mixtures may be ranked in the following order of increasing efficiency: methanol< acetone< isopropanol < dioxane[1].

The majority of the compounds can be extracted with methanol, acetone, and ethanol. Alcohols are certainly the solvents of choice and methanol (lower boiling point) or mixtures of methanol and ethanol (1:1 v/v) are often preferred to ethanol. Aqueous alcoholic solutions (e.g. 50% methanol) may be used in order to avoid too much contamination with lipid material or to avoid the partial esterification of

phenolic acids. Thus phenolic acids as well as their glycosides are normally extracted with alcohol-water mixtures[1].

The value of aqueous methanol as a tannin extractant has been questioned by Haslam's group[4]. Aqueous acetone was shown to give higher yields of tannins in the extraction of various plant materials. The magnitude of the differences between these two solvents varies with the sample considered. Aqueous acetone gives 20 to 50 percent higher yields than aqueous methanol for proanthocyanidins.

One percent methanolic hydrochloric acid has sometimes been used for tannin extraction. Compared to methanol extraction, the acidic solvent increases the yields of proanthocyanidins solubilised from the grains of some sorghum varieties[5,6]. It cannot be excluded that these relatively high molecular weight proanthocyanidins originate from the acid-depolymerisation of insoluble proanthocyanidins. This same extractant has also been applied to oak and maple leaves, but extract yields were significantly lower than those obtained with aqueous methanol or aqueous acetone[7].

The temperature at which a tanning material is leached is important both from the standpoint of the amount of material extracted and the tanning quality of the extract. Raising the temperature up to the boiling point of solvent mixtures has a limited effect on the extraction of wood and barks by aqueous methanol and no effect on the extraction by aqueous acetone[3].

To avoid chemical alteration, conditions of extraction should be carefully considered. Methanol, even at room temperature, may cleave the depside linkage of gallotannins[8]. Higher extraction temperatures may result in the hydrolysis of tannins such as geraniin[9].

Water should be properly demineralised in order to limit autoxidation catalysis by transition metal ions; also polyphenol oxidases are released in the plant tissues during the oxidation process. Polyphenol oxidase inhibitors[10] or quinone-reducing agents such as ascorbic acid[11], cysteine, dithionite[12] or sulphur dioxide[13] may be used.

Due to the complex composition of plant tissue, tannins are often co-extracted with other phenolic and non-phenolic compounds such as proteins or polysaccharides. These non-tannins may eventually interfere with tannin assays.

It is also possible to separate small molecular weight phenols from tannins by solvent fractionation with diethyl ether; all hydrolysable tannins and proanthocyanidins dimers or oligomers will remain in the aqueous phase, whereas, the

small molecular weight phenols such as gallic acid or catechin will be found in the ether phase[14].

The methods of extraction and isolation of the polyphenols and phenolic acids are mainly based on the polarity, acidity and hydrogen-bonding capacity of the hydroxyl group(s) attached to the aromatic ring. The techniques used for the extraction of polyphenols have not changed significantly during the last three decades, although schemes for the quantitative extraction of the compounds have been elaborated[15].

Polyphenols occur in almost all plant parts and the method of isolation depends on the source of material, its water content and the type of compound being isolated. Ideally, fresh and uncontaminated plant tissue should be used and the material should be plunged as rapidly as possible into extractant.

Structure determination of various phlobatannins presents many difficulties. Chief of these has been the quantitative isolation in the pure state of those commercially important polyhydroxy compounds which constitute the major fraction of the extracts and which actually combine with the hide or skin in the tanning process.

Plant polyphenols extracts contain variable amounts of sugars, gums, salt and glycosides associated with the polyhydroxy tannin units. When determining the molecular structure or composition of the tannin, the separation and identification of these so called non-tans are also of fundamental importance[16].

The literature contains mention of dialysis[17] and solvent extraction[18] methods to increase the tannin content of extracts. Trimble[19], while investigating phlobatannins, introduced the use of acetone for removal of sugars. Russel[20] in his studies on flavinacols, used a method of salting out the commercial mimosa extract with sodium chloride to free it from sugars, extracting the dried material with acetone to free it from salt, and finally washing the crushed product with ether to remove simple organic impurities. He makes no claims as to the "purity" of the product, however, stating that the method is empirical, and that the tannin probably contains large amounts of impurities. He also purified the tannins through their lead salts[21], but gave no details of experimental procedure. He found this "purification" did not alter the elementary analyses in any way.

Corbett[22] found that ultrafiltration gave effective "purification" but the method was relatively unsatisfactory due to its tediousness and the low yields obtained.

William[23] successively used acetone, ethyl acetate and amyl acetate in that order, and also ethyl acetate alone on freshly cut wattle bark. He claimed a high degree of "purity" (94-96 %) by these methods, but his results have since been found to be erroneous and abnormally high for the methods employed[16]. Ninety five percent purity was also claimed for the product obtained from the cold water extraction.

Debate about the best solvent for tannins in plant tissues has been based on a limited number of studies of few plant species, and conclusions are not always consistent with one another. Aqueous methanol (boiling or at room temperature), acidic methanol, and aqueous acetone have been recommended by various authors. Usually one solvent is recommended without comparison with alternatives or without measurement or estimation of the extent of extraction achieved. The suitability of methanol as an extractant for hydrolysable tannins has been questioned[24,25] and its ability to extract condensed tannins from some plants also is low[26-28]. Many workers now use aqueous acetone for extraction of tannins, although it does not give high recoveries with all plants[29,30]. The presence of water in organic extractants can increase extraction of phenolics[16,4]. The choice of water content usually appears to be arbitrary between 0% to 50% and to follow precedents set by other workers analysing other plant species.

Extraction time has been studied even less rigorously. Times, from a few minutes to one or more days, have been used. Short extraction times aim to minimise chemical degradation of extracted tannins, and long times to maximise extraction, but seldom is the extent of extraction or degree of degradation quantified[31]. Similarly, few published methodologies make any recommendations about exposure to light, which might affect the stability of phenolics[32, 33].

From the above discussion it would seem that hitherto no satisfactory method for the quantitative separation of extract has been developed, and, above all, for quantitative fractionation of the extract, without causing condensation or oxidation of the tannins available.

We investigated the particle size, solvent, and temperature for extraction of tannins from the neem bark, including tannins and non-tannin phenolics. We observed

several trends reported earlier in the literature[5-21, 31-35] that probably have general applicability.

2.3. EXPERIMENTAL PROCEDURE

2.3.1. Extraction of neem bark tannin

2.3.1.1. Preparation of neem bark

The outer bark of neem used in this study was imported from India in October 1997. The woody part was carefully stripped and immediately cut into small pieces, which were ground by Apex grinder (NECO. Ltd, London).

Water and different organic solvents, such as methanol, acetone, and ethyl acetate in different proportions were used for the extraction of tannins from neem bark for analytical purposes.

The efficiency of the extraction ought to be increased by means of mechanical stirring, but it should be considered that mechanical stirring in contact with the air may allow the oxidation of catechins and proanthocyanidins to a certain extent[34].

2.3.1.2. Extraction with water at different temperatures

From 900g crude neem bark material the woody parts were removed carefully. The remaining bark (500 g.) was cut into small pieces and ground to a powder and screened to particle sizes <63μm and <250μm. Both sizes of particles were macerated with water at 20°C, 60°C and 85°C (8 x 250 ml) with continuous stirring over 24 hours. Subsequently, decantation and filtration separated the extract. The aqueous extracts were reduced under pressure at 30°C to a small volume (~500 ml) and gum was removed as follows.

The gum was precipitated from a dilute aqueous solution of the extract by the slow addition of excess ethanol (500 ml) during vigorous stirring. The gelatinous precipitate was centrifuged (3500 rpm, for 15 min) and then sucked to semi-dryness over 24 hours on a Buchner funnel. The product appeared reddish brown and obviously still contained tannin. It was redissolved with vigorous stirring in a minimum of cold water and the precipitation repeated. This process required 4-5 repetitions for the complete elimination of tannins.

All supernatant solutions from the precipitation were collected and the alcohol removed under reduced pressure at 30°C. The tannin was precipitated by the addition of excess lead acetate and the lead tannate was removed by centrifugation. An aliquot of the supernatant solution was evaporated to dryness under reduced pressure at 30°C and repeated by extracted with boiling methanol. The tannins were liberated from the lead tannate according to the lead-salt purification method[16], discussed below.

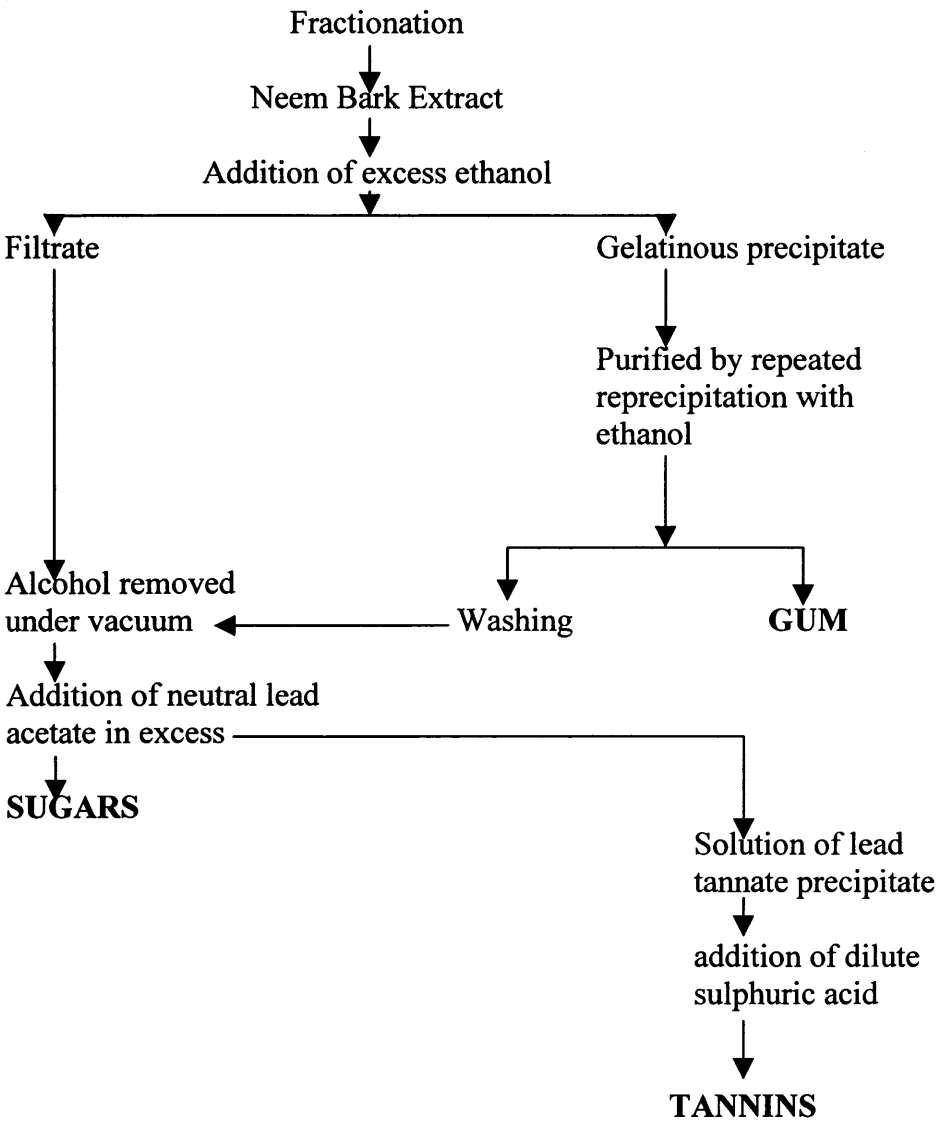

Figure 2-2: Scheme used for the neem bark tannins' purification.

2.3.1.3. The lead salt technique

Arata[34] and Russel [20] claimed to have carried out purification of tannins by formation of a lead salt, but did not give details. The method was also tried by Temple[35], using hydrogen sulphide to liberate the tannins from its lead derivative. He was however unable to free the tannin solution from the lead sulphide formed. Roux[1] attempted Temple's procedure but found only small quantities of tannin liberated by hydrogen sulphide treatment. In the presence of tannin the lead sulphide failed to centrifuge or filter off satisfactorily.

In the present study, for the separation of neem tannins and carbohydrate non-tannins, the aqueous and solvent mixture solutions, which resulted after the removal of the gums were used.

10 gms of gum free fraction of the extract of tannin plus sugar content were dissolved in 500 ml of distilled water. The extract solution was vigorously stirred and a strong solution of neutral lead acetate (10%) slowly added in excess. A clean white precipitate of lead tannate formed instantaneously. The lead tannate was centrifuged down and washed four or five times with small quantities of 1 % lead acetate solution on each occasion to ensure efficient washing. The supernatant solution and washings were kept for non-tannins investigation. Due to the semi-colloidal nature of the product, the lead tannate was carefully macerated with water on each occasion to ensure efficient washing. Under the above conditions the lead tannate appeared quite stable and showed no darkening in colour. The final product was suspended in water, and dilute sulphuric acid (0.2 M) slowly added. The pH decreased from 4.75 (natural pH) to 2.0, at about which point the aqueous solution assumed a light tannin like colour and the low solubility lead sulphate precipitated. The precipitates were easily centrifuged or filtered off. There now remained a tannin solution contaminated by small quantities of free sulphuric acid and traces of salts. With very vigorous stirring and careful addition of dilute NaOH (0.2 M), the pH was raised to about 4.0, well below the natural pH of neem bark extract. At this pH all the sulphuric acid should have been neutralised, and the solutions were taken to complete dryness under reduced pressure. The product was repeatedly extracted with hot absolute methanol, ethanol or acetone, which left a residue of the salts. The latter was separated off by centrifuging or filtering under anhydrous conditions and the filtrate was concentrated under reduced pressure at 30°C. Duplication of the above method does not effect

much improvement in "purity" as determined by the Official Method[36] of tannin analysis. Results are shown in Table 2-2 and 2-7.

2.4. RESULTS

Table 2-2: Effect of particle size on the extraction of neem bark with water at different temperatures. (Results on moisture-free basis)†.

Extraction Medium	Particle Size	Total Extractives (%)	Total Soluble (% of total extractives)	Insoluble (% of total extractives)
Water (20°C)	<63μm	32	86	14
	<250μm	34	88	12
Water (60°C)	<63μm	32	89	11
	<250μm	29	88	12
Water (85°C)	<63μm	31	96	4
	<250μm	28	94	6

Table 2-3: Analysis of different component contents of neem bark extracts†.

Extraction Medium	Particle Size	Insoluble (%)*	Tannin (%)*	Non-Tannin (%)*	Moisture (%)*	Purity (%)**
Water (20°C)	<63μm	14	18	57	11	21
	<250μm	12	17	59	12	19
Water (60°C)	<63μm	11	26	51	12	29
	<250μm	12	22	53	13	25
Water (85°C)	<63μm	4	55	39	2	57
	<250μm	6	51	41	2	54

* Percentage tannins, non-tannins, insoluble, and moisture were determined by Official Methods (SLT- 2/3a, 3b, 3c and 3d)[36].

** Purity is 100 times the tannin contents divided by the soluble extractives.

† All results are averages of duplicate analyses.

Table 2-4: Effect of particle size on the extraction of neem bark with methanol: water at ambient temperature[†].

Solvent	Particle Size	Total Extractives (%)	Total Soluble (% of total extractives)	Insoluble (% of total extractives)
Methanol: water	<63μm	37	95	5
(95:5v/v)	<250μm	29	94	6

Table 2-5: Analysis of different component contents of neem bark extract (methanol: water).

Extraction Medium	Particle Size	Insoluble (%)*	Tannin (%)*	Non-Tannin (%)*	Moisture (%)*	Purity (%)**
Methanol: water	<63μm	5	85	7	3	89
(95:5v/v)	<250μm	6	84	7	3	89

Table 2-6: Effect of particle size on the extraction of neem bark with acetone: water (50: 50 v/v) at ambient temperature[†].

Solvent	Particle Size	Total Extractives (%)	Total Soluble (% of total extractives)	Insoluble (% of total extractives)
Acetone: water	<63μm	34	78	22
(50:50 v/v)	<250μm	32	80	20

Table 2-7: Analysis of different component contents of neem bark extract (acetone: water).

Solvent	Particle Size	Insoluble (%)*	Tannin (%)*	Non-Tannin (%)*	Moisture (%)*	Purity (%)**
Acetone/water	<63μm	5	74	16	5	95
(50:50 v/v)	<250μm	6	72	18	4	90

* Percentage tannins, non-tannins, insolubles, and moisture were determined by Official Methods (SLT- 2/3a, 3b, 3c and 3d)[36].

** Purity is 100 times the tannin contents divided by the soluble extractives.

[†]All results are averages of duplicate analyses.

2.5. DISCUSSION

The results of these studies, showing a decrease in tannin percentage with low temperature, would suggest that a portion of tannins may be rendered insoluble due to polymerisation. It also shows large variations in the percentage of tannin and total solubles.

It is also evident from the data that the tannins are not easily extracted, as non-tannins decrease with increasing temperature and aqueous solvent systems. There is no significant difference found in the total extractives from the two particle sizes. The finer and more uniform preparation, ground to <63μm screen size gave a more complete extraction of total solubles and tannin compared to <250μm. Larger particle sizes were not considered because a clearer picture comes from the two smaller particle sizes.

It is usual practice in tannin extraction not to use very low temperature, to avoid gum gelatinisation. In neem bark extract, the major portion of polyphenolic tannin might have high molecular weight and is readily extracted with aqueous methanol and acetone but not with water at low temperature.

It is also observed that the total solubles content was found to decrease with increasing in the period of heating. There was an appreciable increase in the phenolic non-tannins. It can be seen from Tables 2-2, 2-4 and 2-6, that there is no significant difference in total extractive, about 2-3% when comparing particle size 63μm with 250μm, with one exception, when aqueous methanol used as extractant. 63μm gave 8% more yield than the coarser particle size.

The differential aqueous extractions yield different tan/non-tan ratios and different amounts of non-tannins, as also observed by Roux[43]; the cold and cool extracts were found to be clearer and lighter than hot extracted liquor.

From Table 2-3, it is evident that leaching at 20°C yields an extract of low tan/non-tan ratio. At that temperature almost the same amount of gum was obtained with as other higher temperatures and the largest amount of total salts was extracted and these were presumably mainly responsible for high non-tans as compared to the tannins extracted. On dry bark basis, the yield of extract at 20°C was found to be the lowest and total salt and gum contents were lower than those at higher temperatures.

As the temperature increases, the tannin increases, but the salt content decreases to almost half and there is a much increased tan/non-tan ratio.

Humphreys and Martin[44] established extraction methods employing sulphite solution, which secured high yields. The resultant extracts had very high non-tans, high ash and gave a dark coloured leather. This latter phenomenon was noted by the present author and found to be due mainly to excessive heat during extraction and concentration. It was felt that no extract of commercial quality could be prepared along the lines used by Humphreys and Martin, because the initial objective in the present work was the development, firstly, of an extraction method combining high yield with the minimum of heat damage to the tannin and, secondly, of treatment methods to improve the colour and solubility of the liquors obtained.

2.5.1. Statistical assessment

Certain statistical inferences may be derived from these basic results (Tables 2-2 to 2-7). Firstly, the effectiveness of water as an extraction medium may be judged at the three selected temperatures in conjunction with the two screen size standards used, before comparing the most effective of these results with methanol/water and acetone/water. For statistical purposes (e.g. regression and correlation) the 63μm and 250μm screen sizes adopted can assume dummy or dichotomous values; here, 0 and 1. For ease of reference the following tables of abbreviation are selectively used: Tables 2-8 and 2-9.

Table 2-8: Abbreviated identification of variables.

Solvent medium	Particle sizes		
	<63mm	**<250mm**	**Average**
Water (20°C)	W20°C P63	W20°C P250	W20°C PAv.
Water (60°C)	W60°C P63	W60°C P250	W60°C PAv.
Water (85°C)	W85°C P63	W85°C P250	W85°C PAve.
Methanol: water (95:5 v/v)	Methanol P63	Methanol P250	Methanol PAv.
Acetone:water (50: 50 v/v)	Acetone P63	Acetone P250	Acetone PAv.

Table 2-9: Dichotomous values for particle sizes.

Medium	Particle Size	Total Extractives (%)	Total Soluble(%)	Insoluble (%)
W20°C P63	0	32	86	14
W20°C P250	1	34	88	12
W60°C P63	0	32	89	11
W60°C P250	1	29	88	12
W85°C P63	0	31	96	4
W85°C P250	1	28	94	6
Methanol P63	0	37	95	5
Methanol P250	1	29	94	6
Acetone P63	0	34	78	22
Acetone P250	1	32	80	20

With regard to initial processes there was a significant degree of correlation between the particle size and total extraction. As seen in Figure 2-3, the extractives quantity declined in line with higher particle size (markedly so with methanol) in all cases except with water at 20°C. Excluding this aqueous case, the coefficient was 0.71, significant at the $P< 0.05$ level. There was no relationship apparent between particle size and % solubles.

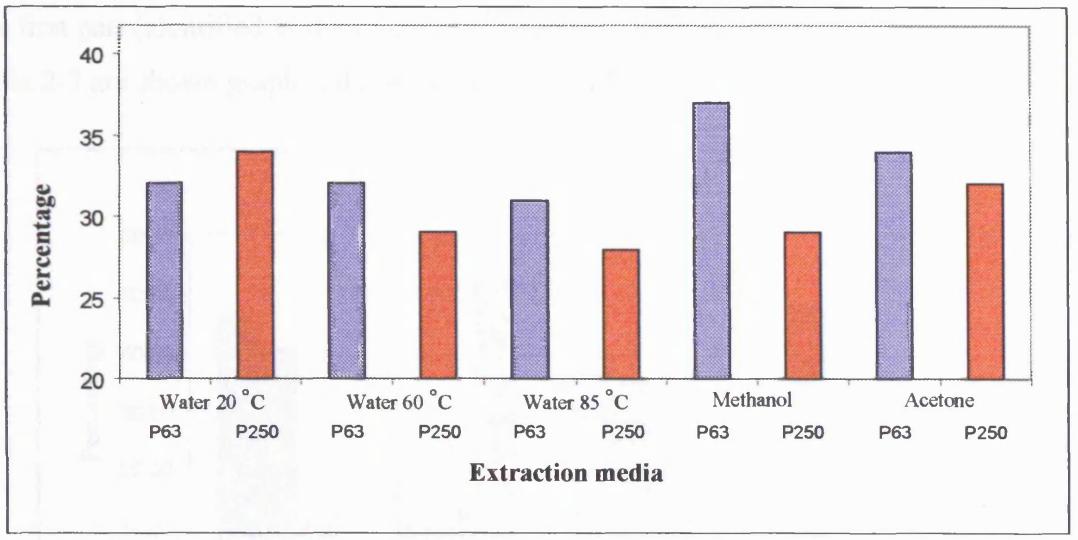

Figure 2-3: Extraction yields percentage as defined according to screen sizes.

In order to judge more effectively the influence of temperature and other inter-relationships the results from the averages of the two particle sizes are adopted. These are shown in Table 2-10, along with marginal change in results effected by the temperature change over the two ranges 20-60°C and 60-85°C.

Table 2-10: Average of particles sizes, marginal change per band of temperature and per degree (°C).

Water extraction media - Average of particle sizes 60μm and 250μm.					
Extraction media	**Insoluble (%)**	**Tannin (%)**	**Non-Tannin(%)**	**Moisture(%)**	**Purity (%)**
Results at stated temperature levels					
W20°C PAv.	13.0	17.5	58.0	11.5	20.0
W60°C PAv.	11.5	24.0	52.0	12.5	27.0
W85°C PAv.	4.0	55.0	39.0	2.0	57.0
Marginal change					
W20-60°C PAv.	-1.5	6.5	-6.0	1.0	7.0
W60-85°C PAv.	-7.5	31.0	-13.0	-10.5	30.0
Marginal change per degree (°C)					
W20-60°C PAv.	-0.04	0.16	-0.15	0.03	0.18
W60-85°C PAv.	-0.30	1.24	-0.52	-0.42	1.20

The first part (identified as the relative percentage of each variable) and third part of Table 2-3 are shown graphically as Figure 2-4 and Figure 2-5.

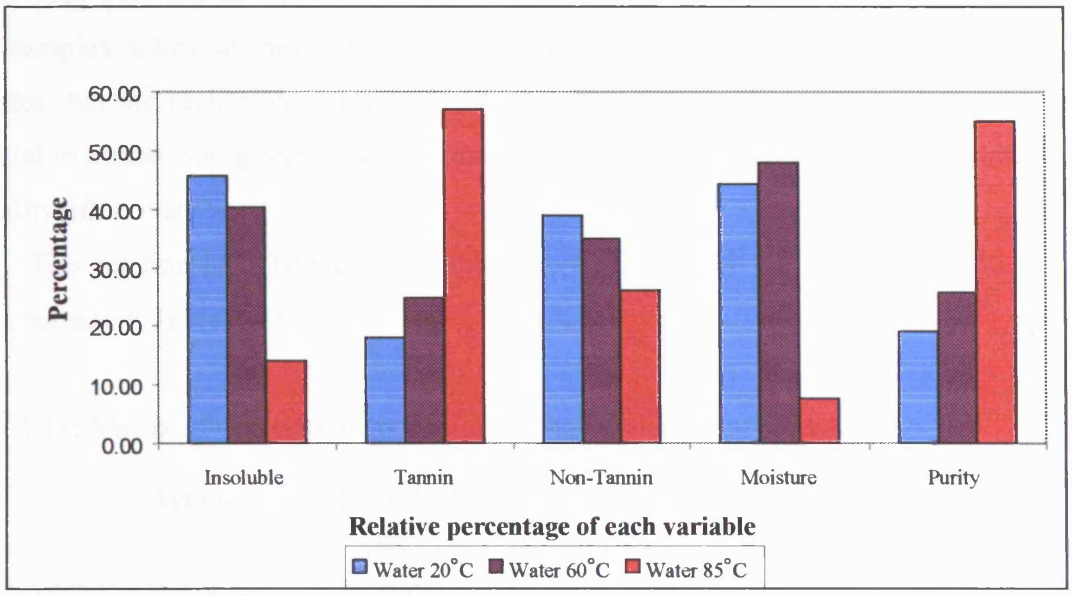

Figure 2-4: Variable percentage of each extraction method expressed as percentage of all extraction methods.

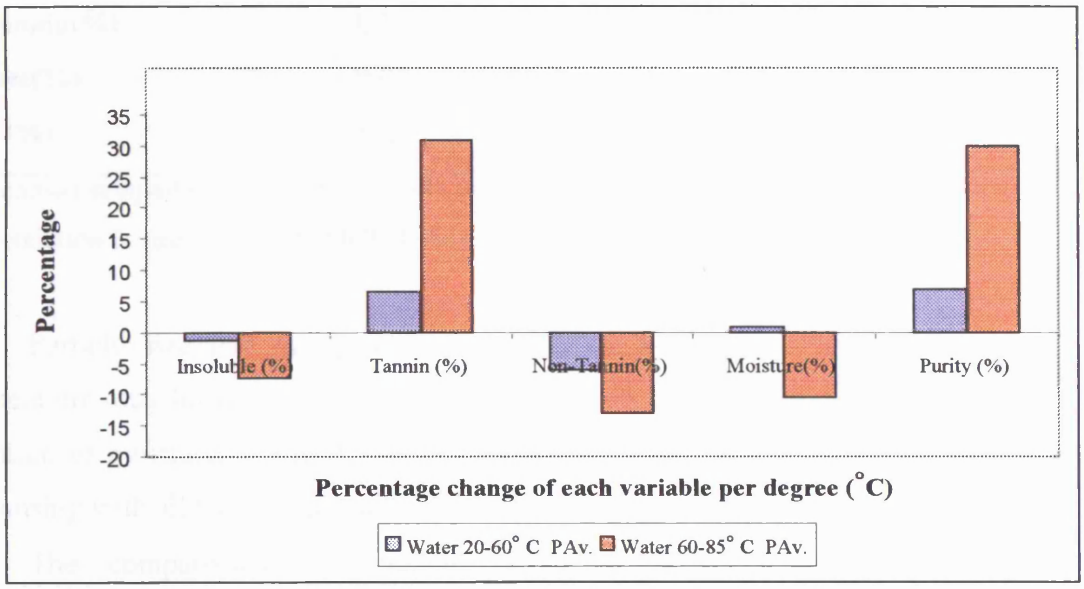

Figure 2-5: Percentage of each extraction method expressed as percentage change per degree (°C).

These data suggest that the temperature is a critical factor in a water-based process system. It is indicated, particularly from the results per unit of temperature, that the process is not strictly linear. The difference per degree is greater between 60 and 85 than between 20 and 60. The nature of the curve could be determined only with samples taken at more frequent temperature intervals, however the study indicates that the higher temperature yields the most favourable results and has the potential to allow setting marginal cost (energy use) against marginal benefit in terms of quality/effectiveness.

The relationships between variables were tested and are summarised in a matrix format in Table 2-11.

Table 2-11: Matrix of correlation coefficients (2-tailed significance tests).

	Temperature	**Particle Size**	**Insoluble (%)**	**Tannin (%)**	**Non-Tannin(%)**	**Moisture (%)**	**Purity (%)**
Temperature	**1.00**	1.00	0.03*	0.02*	0.01*	0.10	0.02*
Particle Size	0.00	**1.00**	0.93	0.86	0.80	0.89	0.85
Insoluble (%)	-0.86	0.05	**1.00**	0.00**	0.00**	0.01*	0.00**
Tannin (%)	0.88	-0.10	-0.98	**1.00**	0.00**	0.00**	0.00**
Non-Tannin(%)	-0.94	0.13	0.96	-0.99	**1.00**	0.01	0.00**
Moisture(%)	-0.73	0.07	0.93	-0.96	0.91	**1.00**	0.00**
Purity (%)	0.89	-0.10	-0.97	1.00	-0.99	-0.96	**1.00**

*Correlation is significant at the 0.05 level (2-tailed).

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

Particle size had no apparent influence on any of the measured results. Temperature was however significant at the $P< 0.05$ level for all factors with the exception of moisture, where it was not significant. Purity had a highly significant relationship with all factors, generally $P< 0.01$ level, except particle size.

The comparisons between the water at 85°C, methanol/water and acetone/water extraction media are given in Table 2-12 with the marginal differences tabulated. These data are shown in Figure 2-6, with each variable expressed as a percentage of the all variables, and Figure 2-7 comparing the differences between each extraction medium.

Table 2-12: Extraction media compared, based on average particle size (PAv.).

	Insoluble (%)	Tannin (%)	Non-Tannin (%)	Moisture (%)	Purity (%)
Water 85 °C/PAve	5	53	40	2	55.5
Methanol/ PAve	5.5	84.5	7	3	89
Acetone/ PAve	5.5	73	17	4.5	92.5
		Marginal change			
Methanol-Water	0.5	31.5	-33	1	33.5
Acetone-Water	0.5	20	-23	2.5	37
Acetone-Methanol	0	-11.5	10	1.5	3.5

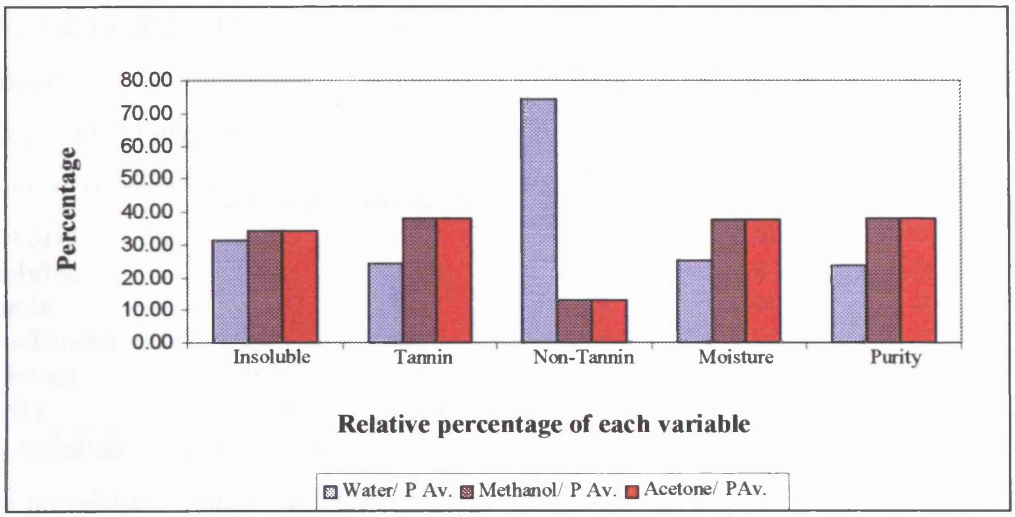

Figure 2-6: Variable percentage of each extraction method expressed as percentage of all extraction methods.

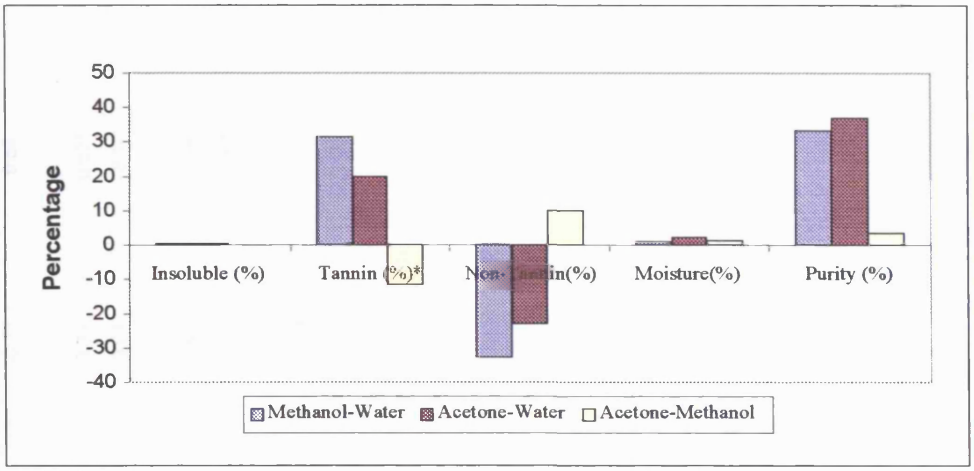

Figure 2-7: Difference between extraction media.

The statistical relationships between the variables are set out in matrix form in Tables 2-13 and 2-14. This is based on treating particle size as separate part of the analysis.

Table 2-13: Matrix of correlation coefficient (vertical and horizontal).

	Part Size.	Insoluble	Tannin	Non-Tannin	Moisture	Purity
Part Size.	**1.00**	0.02	0.87	0.93	0.77	0.88
Insoluble	0.89	**1.00**	0.70	0.63	0.79	0.65
Tannin	-0.09	0.20	**1.00**	0.00	0.29	0.02
Non-Tannin	0.05	-0.25	-1.00	**1.00**	0.23	0.01
Moisture	-0.16	0.14	0.52	-0.58	**1.00**	0.04
Purity	-0.08	0.24	0.90	-0.93	0.84	**1.00**

*Correlation is significant at the 0.05 level (2-tailed).

The correlation matrix indicates that particle size is statistically related only to the insoluble component significant at $P < 0.05$ level, which in turn is unrelated to the other components.

Figure 2-14: Correlation coefficient matrix of variables.

Correlations	Test	Extraction Medium	Particle Size	Insoluble (%)	Tannin (%)	Non-Tannin(%)	Moisture(%)	Purity (%)
Extraction Medium	**Pearson Correlation**	1.00	0.00	-0.86	0.88	-0.94	-0.73	0.89
	Sig. (2-tailed)		1.00	0.03*	0.02	0.01**	0.10	0.02*
Particle Size	**Pearson Correlation**	0.00	1.00	0.05	-0.10	0.13	0.07	-0.10
	Sig. (2-tailed)	1.00	.	0.93	0.86	0.80	0.89	0.85
Insoluble (%)	**Pearson Correlation**	-0.86	0.05	1.00	-0.98	0.96	0.93	-0.97
	Sig. (2-tailed)	0.03*	0.93	.	0.00**	0.00**	0.01*	0.00**
Tannin (%)	**Pearson Correlation**	0.88	-0.10	-0.98	1.00	-0.99	-0.96	1.00
	Sig. (2-tailed)	0.02*	0.86	0.00**	.	0.00**	0.00**	0.00**
Non-Tannin(%)	**Pearson Correlation**	-0.94	0.13	0.96	-0.99	1.00	0.91	-0.99
	Sig. (2-tailed)	0.01*	0.80	0.00**	0.00**	.	0.01*	0.00**
Moisture(%)	**Pearson Correlation**	-0.73	0.07	0.93	-0.96	0.91	1.00	-0.96
	Sig. (2-tailed)	0.10	0.89	0.01*	0.00**	0.01*	.	0.00**
Purity (%)	**Pearson Correlation**	0.89	-0.10	-0.97	1.00	-0.99	-0.96	1.00
	Sig. (2-tailed)	0.02*	0.85	0.00**	0.00**	0.00**	0.00**	.

*Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

2.6. THE PHYSICO-CHEMICAL PROPERTIES OF NEEM BARK EXTRACT

2.6.1. Colour and stability

The dry material is buff coloured. It is stable up to 246°C, after which charring starts to take place. The powder is hygroscopic. Exposure to sunlight gives rise to a gradual photochemical reddening of the material. Aqueous solution is stable, although slow auto-oxidative darkening may occur on exposure to light, by which it resembles mimosa extract in the raw state.

2.6.2. Solubility

The semi quantitative solubilities are based on solvents at 25°C. In all cases, an increase in temperature increases the solubility and darkens the colour of the extract.

Table 2-15: Solubility of neem bark tannins with different solvents.

Good solubility	Fair solubility	Poor solubility
Water	Butan-2 ol	Isopropyl alcohol
Dimethyl Formamide	Acetone	Acetic acid
Glycerol / water (1:10)	Ethyl acetate	n-Butyl alcohol
Ethylene Glycol		Chloroform
Acetone / water (1:1)		Hexane
Pyridine		Toluene
n-Propanol		
Methanol		
Ethanol		

The pH of an aqueous solution at a concentration of 13.5 w/v in distilled water is 4.65 at 20°C.

2.6.3. Acid and salt contents

Free Acids 39 mg equivalents/litre

Free Salts 35 mg equivalents/litre at pH 5.5 and 12 Bé

12 Bé is 1.089 SG.

2.6.4. Viscosity

Aqueous solutions of neem bark extract show relatively low viscosities up to 35% solids content*, but the viscosity increases exponentially with concentration beyond this level. The relationship of viscosity and concentration is shown in Figure 2-8 and that of viscosity and temperature is shown in Figure 2-9.

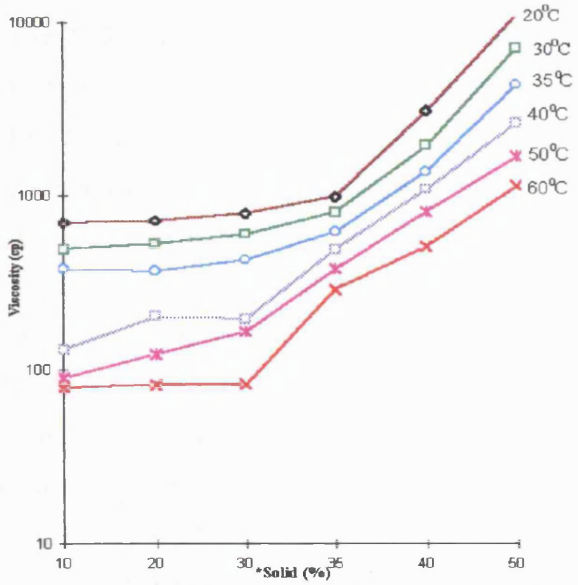

Figure 2-8: The relation between the viscosity of neem bark extract and solids content.

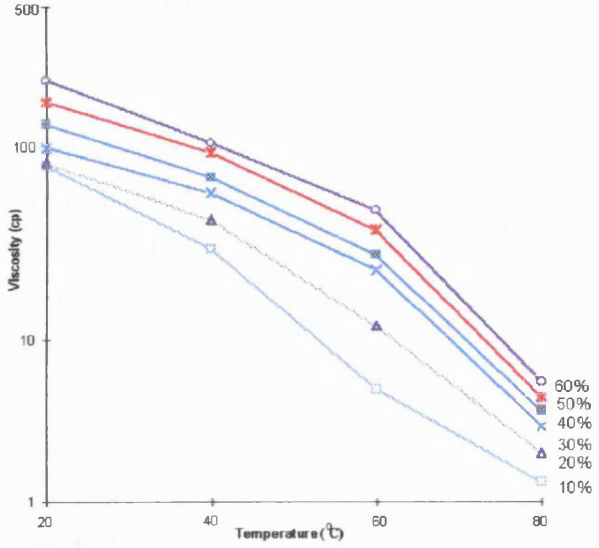

Figure 2-9: The relation between viscosity of neem bark extract and temperature at different solids content.

$$*\%\text{Solid} = \frac{\text{Weight of neem bark extract}}{\text{Weight of water}}$$

2.7. REFERENCES

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CHAPTER 3

CHEMISTRY OF NEEM BARK TANNINS

3.1. PAPER CHROMATOGRAPHY

3.1.1. INTRODUCTION

Paper chromatography has proved to be a powerful analytical technique when used in the study of plant extracts[1]. It has been applied extensively in the preliminary qualitative analysis of condensed tannin materials. Paper chromatographic methods are ideal for the analysis of the co-occurring flavonoid compounds, since these have a wide range of solubilities and are readily detected on chromatograms by means of specific spray reagents and in certain cases by their distinctive fluorescence in ultra-violet light.

White[2] first used one-dimensional chromatography for the study of quebracho extract; this has been followed by a number of publications, which will be discussed elsewhere.

In Bate-Smith's[3] view "It is likely that but for paper chromatography the plant phenolic group would not have come into being". There can be few branches of natural product chemistry where the applications of paper chromatography have proved so immediately prolific and where the speed and certainty of the results obtained have so simplified the task of the chemist and botanist engaged in research.

Paper chromatography is used routinely in almost all investigations of flavonoid compounds in plant materials. Several reasons account for its use. First the natural colour of most of the compounds in visible light (if not, in UV light) or the ease of development of colour in the case of others makes the compounds readily recognisable on paper. Secondly, it gives results quickly without difficulty and requires relatively inexpensive apparatus. In addition it is the best method for working with mixtures and with the small quantities in which flavonoids occur in plant materials. Finally, in contrast to column chromatography, it offers an excellent method of identification, as well as being adaptable to isolation[4].

3.1.2. TECHNIQUES

3.1.2.1. One-dimensional chromatography

One-dimensional chromatography, using strips or sheets of filter paper with a single developing solvent, is the method of choice for preliminary qualitative surveys of extracts. Only those solvents were used which give best and quick separation for the mixture at hand, in order to determine the R_f values, i.e., the ratio of the distance travelled by the solute to the distance travelled by the mobile phase; as a physical constant, for the final identification.

3.1.2.2. Two-dimensional chromatography

Two-dimensional paper chromatography, run on sheets of filter paper with two different solvents in succession at right angles to each other, is the best method for investigating all the components of plant extracts. This is particularly valuable in the study of flavonoid compounds in plants, since they occur almost always in mixtures of several similar components. In order to identify spots other than by R_f value, known flavonoids may be added to the extract and comparison of chromatograms with and without this addition carried out. While two dimensional chromatograms may be an end in themselves, such maps are often of great value as guides, if the next step involves isolation of a single component from the mixture.

3.1.2.3. A brief review of the application of one-dimensional paper chromatography to plant polyphenols

Bate-Smith[5] demonstrated the usefulness of paper chromatography for the identification of polyphenolic bodies in plant extracts. Bate-Smith and Westall[3] used a variety of C_6 and naturally occurring C_{15} phenolic compounds in order to study the relation between chromatographic behaviour and chemical structure. Under carefully controlled conditions, R_f values, accurate and reproducible to $\pm$ 0.02, could be obtained using butan-2-ol-acetic acid-water (4:1:5) solvent mixtures.

Bate-Smith[5] used the same solvent mixtures for examining the behaviour of flavanones, chalcones, flavanols, flavones, flavonols, and flavylium salts. He found that the R_f values obtained show the following regularities.

a) C_{15} compounds having the same hydroxyl groups have approximately the same R_f value and the R_f values are reduced by each additional hydroxyl group.

b) With the exception of rhamnose, the glycosidic combination of sugars with phenols causes approximately the same fall in R_f value as the addition of a hydroxyl group. With rhamnose either a slight rise or fall in R_f value is observed.

c) Methylation of a hydroxyl group causes a rise in R_f value, as a rule between one-thirds of the rise that would result from the loss of the hydroxyl group.

3.1.3. EXPERIMENTAL PROCEDURE

3.1.3.1. Paper chromatography of neem bark tannin

The previously described fractionation methods (Figure 2-2, section 2.1.3) will separate the extract qualitatively or almost quantitatively into sugars, gums and tannins. The homogeneity of each must be determined in order to establish what significance may be attached to degradation products or analytical figures obtained from any one of these fractions. The main interest in this study lies with the polyphenolic or true tannin fraction that, due to its amorphous nature and reputed high molecular weight, presents a complex problem.

The concept of purity or homogeneity, when dealing with highly condensed macromolecules, must naturally be regarded in its widest sense. Their similarity in chemical and physical properties and the additional lack of criteria of purity also complicate the investigation of what is obviously an amorphous heterogeneous mixture of compounds.

For the study of neem bark polyphenols one and two-dimensional chromatography and other analytical methods were used for investigating the complex polyphenols of the tannin fraction.

3.1.3.2. Preparation of material

As the lead salt method was the only technique giving a quantitative precipitation of the phenolic fraction, this method was first used for the preparation of "purified" tannin samples, from which the gums had previously been removed. The presence of a low proportion of sugars was later found not to affect the appearance of the chromatograms to any degree, but the removal of gums was considered essential, due to their colloidal nature and their marked ability to retain tannins. The tannins were dissolved in methanol (25% solution) and 5µl spotted on to the paper in small

quantities at a time with drying between each, to build up small spots not more than 0.5 cm. in diameter.

In some cases the Whatman No.1 paper was acetylated with acetic anhydride and perchloric acid in order to reduce the affinity of what was suspected to be the most highly condensed portion of the tannin. After acetylation, the paper was thoroughly washed and finally irrigated with water in the chromatographic apparatus for 24-36 hours. After thorough air drying, the acetylated paper was used in exactly the same way as the original chromatographic paper.

3.1.3.3. Experimental conditions

According to Consden, Gordon, and Martin[6], a number of precautions are essential for the accurate reproduction of R_f values. These include

(a) accurate temperature control, as equilibria in mixtures are sensitive to temperature fluctuations,

(b) the thorough equilibration of the solvent mixture at the temperature at which it is to be used,

(c) the exposure of the paper, with spots applied, to the aqueous phase of the solvent phase of the solvent-water mixture for 24 hours before the commencement of irrigation,

(d) the irrigation of the paper continues only to a fixed distance from the starting-line,

(e) variations in the paper and alterations to the solvent mixture (e.g. esterification in an alcohol-acid mixtures) may be controlled by running a control compound of known R_f value with each chromatogram.

In the present work (a), (b), and (d) were observed, and when butanol-acetic acid-water mixtures were used these were discarded and renewed after seven days use. The chromatograms were developed in each instance to a distance 16-25 cm from the starting line. All experiments were run at 21°C, using Whatman No.1 paper, which is thin and less absorbent than others in current use. The ultra-violet lamp used was equipped with 2537 and 3560 watt sources, with emission in the visible violet.

Upward and downward migrations were used for the continuous irrigation of the paper when necessary. The apparatus used was a simple 30 x 30 cm square jar fitted with a lid and with a narrow air space. The apparatus was maintained and operated in a constant temperature room (21°C).

Butanol-acetic acid-water (4:1:5) was used in a preliminary investigation as Bate-Smith[5] had shown that this solvent mixture was excellent for the separation and accurate reproduction of R_f values of the polyphenols.

3.1.4. RESULTS

Table 3.1-1: One-dimensional chromatography R_f values for neem bark extract (NBE) and tannins (NBT)

Materials	R_f	Colour appearance	Identify
NBE with water at 85°C	0.48	Brown	Condensed tannin
NBT extracted with water at 85°C	0.45	Brown	Condensed tannin
NBT extracted with methanol: water	0.47	Light brown	Condensed tannin
	0.49	Yellow fluorescent	
	0.55	Yellow fluorescent	
	0.75	Light yellow fluorescent	Fisetin
	0.89	Blue	
NBT extracted with ethyl acetate	0.55	Bright yellow	
	0.75	Blue zone	
	0.90	Blue	
	0.53	Yellow	

3.1.5. DISCUSSION

The developed paper chromatogram of raw neem bark extract and purified neem bark tannins showed the presence of a light brown relatively uniform streak from the point of application to about R_f 0.45 in ordinary light. The streak was far more pronounced and much darker in colour in the case of neem bark tannins (NBT) than for the raw neem bark extract (NBE), due to the oxidation of the former as shown in Figures 3.1-1 and 3.1-2.

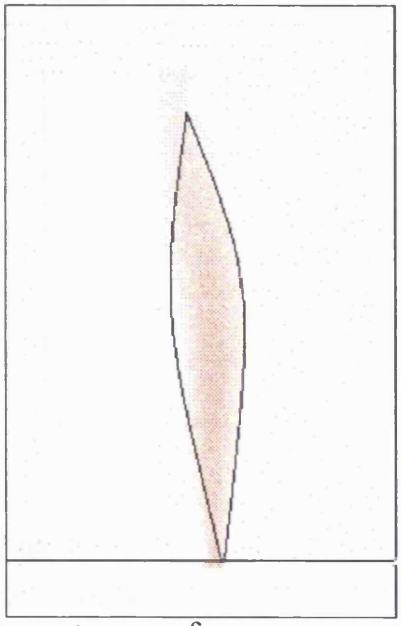

Figure 3.1-1: Paper chromatogram of aqueous extract of neem bark tannin.

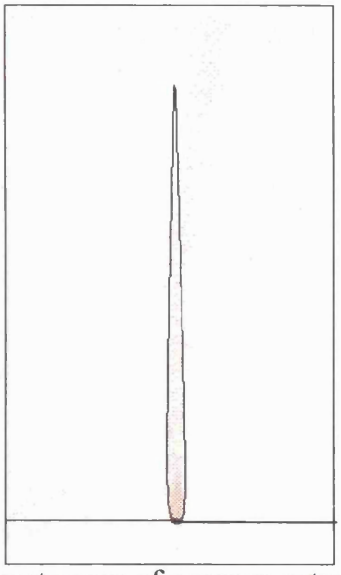

Figure 3.1-2: Paper chromatogram of aqueous extract of neem bark extract.

Primarily to investigate the number and orientation of hydroxyl groups in the B-ring of the flavonoid, two spray reagents were used (on an acetone extract of *Azadirachta Indica*): (i) ferric chloride-potassium ferricyanide and (ii) ammonical silver nitrate: o-and p-dihydroxy, o-trihydroxy and other easily oxidised phenols react with the former reagent give Prussian blue spots on a white background as shown in Figure 3.1-3 and are recorded as brown-black spots on a white background by the latter reagent as shown in Figure 3.1-4.

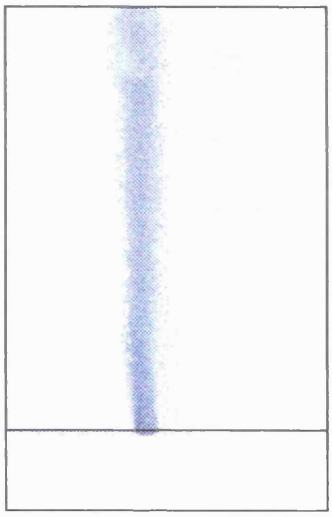

Figure 3.1-3: Paper chromatogram of acetone extract of neem bark tannin sprayed with ferric chloride-potassium ferricyanide.

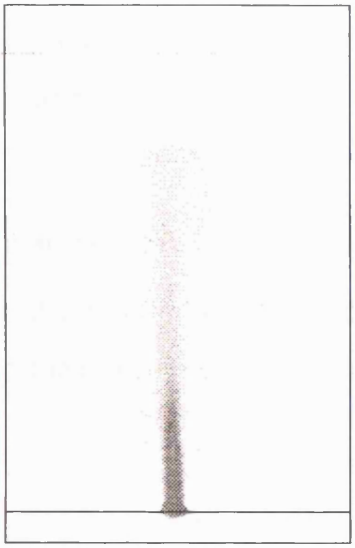

Figure 3.1-4: Paper chromatogram of neem bark tannin sprayed with ammonical silver nitrate.

Under ultra-violet light the presence of at least four different areas are visible. These were: (a) a light brown streak R_f 0.47, which appears to constitute the major portion of the extract and represents the condensed tannins, (b and c) weak yellow fluorescent spots R_f 0.49 and 0.55 (d) a bright yellowish green fluorescent spot, which was suspected to be fisetin R_f 0.75 and (e) a blue fluorescent spot R_f 0.89, which was intensified with ammonia vapour, see Figure 3.1-5.

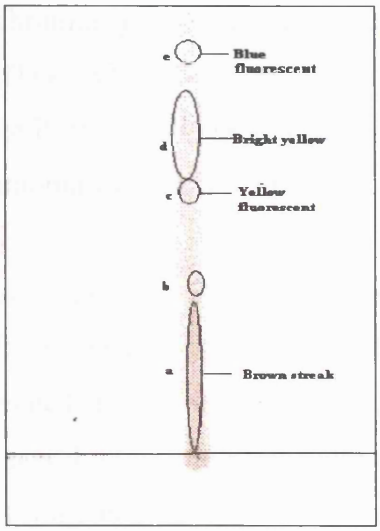

Figure 3.1-5: One-dimensional chromatogram of methanolic extract from neem bark extract.

From the observations, it was considered that a low proportion of fluorescent bodies accompanied the bulk of the condensed tannin, as the bright fluorescence under UV light is known to be a very sensitive property of compounds such as fisetin.

3.1.5.1. Identification of the associated fluorescent polyphenols

The separation of the compound with R_f 0.72, suspected to be fisetin, was attempted by Parkin and Gunnel's method[7] of separating fisetin from quebracho using concentrated sulphuric acid.

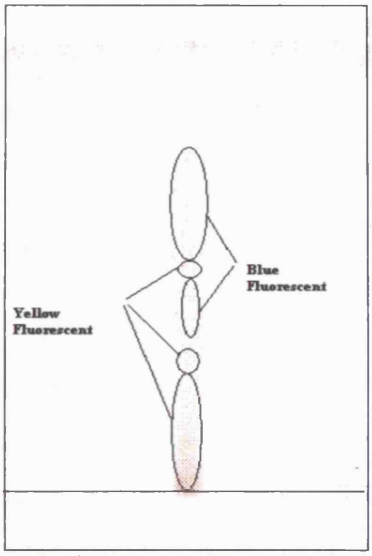

Figure 3.1-6: One-dimensional chromatogram of ethyl acetate extract neem bark extract.

A one-dimensional chromatogram of the ethyl acetate extract of the resulting solution appeared complex (UV-light), consisting of (a) a bright yellow fluorescent spot R_f 0.55, (b) a bluish spot R_f 0.72, followed by a long blue zone, the latter part of which fluoresced blue in ammonia vapour, R_f 0.90, as shown in Figure 3.1-6.

The other yellow fluorescent principle R_f 0.53 is apparently present in even lower concentration. The yellow fluorescence matches that of fisetin and it is possible that these compounds may be related. From the preceding discussion of Bate-Smith[8] on related C_{15} compounds, two possibilities might account for its lower value:

1. If both are aglycones, the more highly hydroxylated have lower R_f values than the less hydroxylated.
2. Glycosidation of the hydroxyl groups with glucose, in whatever position, usually results in a large decrease in R_f value.

As both resorcinol and gallic acid nuclei are present in neem bark tannin, the above isolation of these compounds suggests that strong sulphuric acid is capable of degrading the tannin to a limited extent.

All the fluorescence bodies are ether-soluble and could also be separated from the remainder of the tannins by the repeated extraction of strong aqueous solutions with ether[9].

3.1.5.2. Other solvent systems

The butanol: acetic acid: water (4:1:5 v/v), while giving excellent separations of the associated fluorescent bodies, was not equally effective for the tannin fraction. No effective separation could be obtained by one-dimensional chromatography.

3.1.5.3. Alcohol-water mixtures

Rockland, Blatt and Dunn[10] have used water-miscible and water-immiscible alcohols for the separation of amino acids. The presence of some acids (e.g. HCl) in completely miscible mixtures appeared undesirable, as two widely separated boundaries were formed, and in upward chromatography the amino acids did not migrate above the lower boundary. In the absence of acids, satisfactory resolution was obtained. From the previous work of Consden, Gordon and Martin[6], it appears that success in resolving amino-acids with water-miscible solvents depends on the

relatively low water content of papers not subjected to preliminary equilibration with aqueous solvents. Both of these groups of workers used various propyl, butyl and amyl alcohols mixed with varying proportions of water. With water miscible alcohols, the ratio alcohol: water 7:3 v/v appeared most satisfactory, while with water-miscible alcohols the water-saturated solution of the alcohol was usually employed.

These mixtures were applied to a study of the tannins and the effect of varying proportions of acids also determined. The chromatograms were run one-dimensionally and the efficiency of the solvent mixtures assessed by examining the developed paper chromatogram of the tannins well as determining the efficiency with which two catechin bodies were separated.

In this study of solvent mixtures the following conclusions were drawn:

(a) **Alcohol-water: (7:3 v/v)** mixtures and water-saturated alcohols were excellent for the separating phenols on one-dimensional chromatograms. The degree of separation varied with the various alcohols. Water-saturated sec-butanol and tert-amyl alcohol and tert-butanol-water (7:3v/v) mixtures were most efficient[9]. n-propanol: water, iso-propanol: water, ethanol-water and methanol-water (7: 3 v/v) were less efficient but still gave good separation of catechin bodies. Water-saturated n-butanol gave much the same tannin chromatogram when acetic acid was present in the developing solvent mixtures; the only exception being that the fluorescent spots appeared somewhat more diffuse.

(b) **Water-alcohol:** (7:3 v/v) mixtures in which the water predominates generally gave poor or no separation of catechin and gallocatechin, and it is presumed that the tannin constituents are also less satisfactorily separated. With neem bark tannins all of the yellow fluorescent material is left at the origin.

(c) **Acid mixture:** The presence of acetic and other acids in solvent mixtures used for chromatography is superfluous when only phenols are examined. Phenols of the C_6 and C_{15} classes of compounds travel as discrete, well separated spots in alcohol-water mixtures. When phenolic acids are present in the mixture under investigation, alcohol-water mixtures alone are not satisfactory, and the presence of an acid is essential to prevent trailing. The stronger acid in the solvent mixture suppresses the ionisation of the weaker organic acid, and thus enables it to move as a single unionised spot. Lugg and Overell[11] previously also observed this phenomenon in a study of carboxylic acids obtained from plant tissues.

For neem bark extract and tannins, the cresol-water mixtures were tried and it was observed that the extract and tannin constituents move very slowly, and gave very low R_f values, which appeared to be characteristic of all phenolic-water mixtures. Water saturated phenol gave excellent separation of both tannin and extract, when the spots were more widely separated and were far more individually distinct. This difficulty of low R_f values was overcome by irrigating the paper continuously with the fast-moving water saturated phenol mixture.

The effects of the following solvent systems were also studied:

i. Pyridine-water (7 : 3)
ii. Acetic acid-water (7 : 3)
iii. Formic acid-water (8 : 2)
iv. Butan-2-ol-acetic acid-water (4 : 2 : 5)
v. Butan-2-ol-formic acid-water (4 : 1 : 5)
vi. n-propanol-acetic acid-water(70 : 5 : 30)
vii. n-propanol-formic acid-water (7 : 1 : 3)
viii. iso-propanol-acetic acid-water (70 : 5 : 30)
ix. m-cresol-water (50 : 50)

Of the above, mixtures (i) and (ix) were totally unsuitable, as the phenols and tannins moved with the solvent front and low R_f values were noted. Solvent system (ii) was remarkable, in that it gave good separation of individual components, most of the spots were discrete and well separated as compared with other solvent systems, which gave only one big streak or two spots overlapping.

None of the above solvent systems separated the tannin constituents effectively except (v), although areas of higher concentration were indicated when chromatograms were developed with almost all the alcohol-water and the water saturated phenol solvent systems.

This one-dimensional study was then used as the basis for two-dimensional chromatography.

3.1.6 REFERENCES

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11. Lugg, W. and Overell, R., *Nature*, 160, 87, 1947.

3.1.7. TWO-DIMENSIONAL CHROMATOGRAPHY

Many workers have attempted fractionation of tannins by two-dimensional paper chromatography: Kirby *et al*[1-3], Hillis[4,5], Roux[6], Roux and Evelyn,[7-9] Asquith[10,11], Kilchner[12], Putnam and Gensler[13], Putnam and Bowles[14], Schmidt *et al*[15-22], Clarke and Levy[23], Evans et.al[24]., Torii and Furnya[25], Oshima and Nakabayashi[26], Bradfield and Bate-Smith[27], Kuntzel and Zissel[28], Kenten[29], Chang *et al*[30]., Boleda[31], Del Pezzo[32], Roberts and Wood[33].

These workers used different solvent systems. In the 1950's, work on both pyrogallol and catechol types of materials indicated the advantages of using specific solvent systems. The application of two dimensional paper chromatography on different materials has shown that the tannin extracts are all complex mixtures of several polyphenolic and related substances.

Although it is possible to find out the number of constituents by paper chromatography, the identification of the different separated constituents is not possible, unless the R_f values of the different constituents in the solvent systems used are known. But these can be known only if the different constituents are isolated in the pure form.

In this study the solvent combination used was butanol-acetic acid-water (14:1: 5, v/v) for the first run and water or 6% acetic acid for the second or vice versa. It is already well known that R_f values in butanol-acetic acid-water are dependent upon chemical structure[34] and structure also plays an important part in determining the R_f in water. The position occupied by a spot on a two-dimensional paper chromatogram therefore gives several clues to the structure of the substance responsible for the spot and it is the aim of this research to show how these considerations have assisted in the identification of some of the uncharacterised substances revealed on the paper chromatogram.

3.1.7.1. General relationships between R_f in 6% acetic acid and chemical structure

Before considering the several individual identifications which have resulted from this approach, it will be useful to discuss the various ways in which structure has been shown to affect R_f values in 6% acetic acid.

3.1.7.2. Influence of the number of phenolic hydroxyl groups

Table 3.1-2 shows that the introduction of an extra phenolic group into a molecule is associated with a decrease in the R_f values. In most cases, an increase in the number of hydroxyl groups goes with an increase in water solubility, so that the effect is unexpected, since increasing solubility usually means an increase in R_f value.

Table 3.1-2: R_f values of standard compounds in 6% acetic acid.

Substance	Phenolic groups	R_f
resorcinol	2	0.71
phloroglucinol	3	0.60
catechol	2	0.71
pyrogallol	2	0.67
gallic acid	3	0.45
(-)-epicatechin	4	0.33
(-)-epigallocatechin	5	0.27
(+)-catechin	4	0.45
(+)-gallocatechin	5	0.38

3.1.7.3. Planarity of the molecule

The R_f values of all phenolic substances with two hydroxylated benzene rings in 6% acetic acid or water are low, but whenever these two rings are in the same plane the R_f values becomes zero. This possibility had already been considered by Roux[34], but is now considered to be a near certainty. Examples of planar structures associated with zero R_f in water include: flavone, flavanols typified by quercetin and by anthocyanidins and chalcones. Conversely, substances of similar structure, but with their benzene rings not in the same plane have R_f values appreciably greater than zero [35,36]. This is shown convincingly in the case of (-)-epicatechin (R_f in water 0.33), which on the basis of a *cis*-conformation must have either structure (a) or structure (b) shown in Figure 3.1-7.

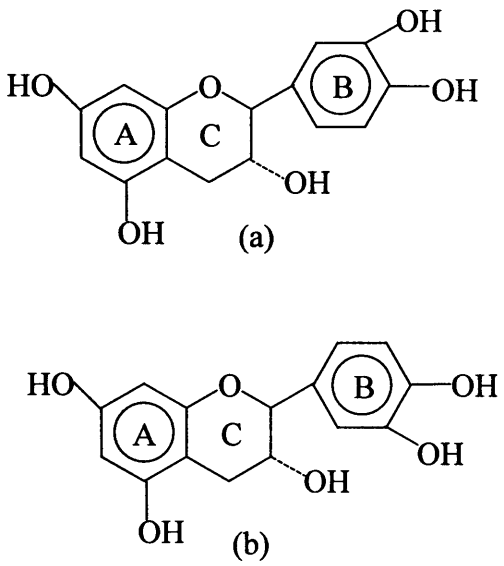

Figure 3.1-7: Structures of *cis*-conformations of epicatechin.

In neither of these conformations are the two benzene rings in the same plane, as in the case of catechin as well.

3.1.7.4. Separation of optically active isomers

As reported by Roberts and Wood[37], it has been claimed that the use of water as a chromatographic solvent effects the separation of such pairs of optically active isomers as (+)- and (-)-catechin.

3.1.7.5. Separation of *cis* and *trans* isomers

Williams[38] showed that the *cis*-form of derivatives of cinnamic acid has a higher R_f in 2% acetic acid than the corresponding *trans* form. This is of particular value for interpreting of the chromatograms of p-coumaric acid, caffeic acid and their depsides with quinic acid.

3.1.8. RESULTS

3.1.8.1: R_f values of bark of *Azadirachta Indica.*

Table 3.1-3: Effect of hydroxylation pattern on R_f values of *Azadirachta Indica.*

Compounds	Pattern of hydroxylation	R_f (butan-2-ol: acetic acid: water, 14: 1: 5)	R_f (acetic acid)
(-)-epicatechin	3,5,7,3',4'	0.65[a]	0.29[c]
(+)-catechin	3,5,7,3',4'	0.75[b]	0.40[a]
quercetin	3,5,7,3',4'	0.63[a]	-
(+)-gallocatechin	3,5,7,3',4',5'	0.48[b]	0.41[b]
(-)-epigallocatechin	3,5,7,3',4',5'	0.47[b]	0.30[c]
(+)-fisetinidol	3,7,3',4'	0.68[a]	-
(-)-robinetinidol	3,7,3',4',5'	0.44[b]	0.38[c]
kaempferol	3,5,7,4'	0.84[c]	0.78[c]
dimer-B2	epicatechin-epicatechin	0.63[b]	-
dimer-B3	catechin-catechin	0.50[b]	-
dimer-B1	epicatechin-catechin	0.55[b]	-
dimer-B4	catechin-epicatechin	0.56[b]	-
trimer-C2	-	-	-
trimer-C1	-	-	-
polymeric tannin	-	-	-

Spraying agents used

(a) Ammonical silver nitrate.

(b) Gibbs reagent (2,6-dibromobenzoquinone 4-chloroimide).

(c) p-toluene sulphonic acid.

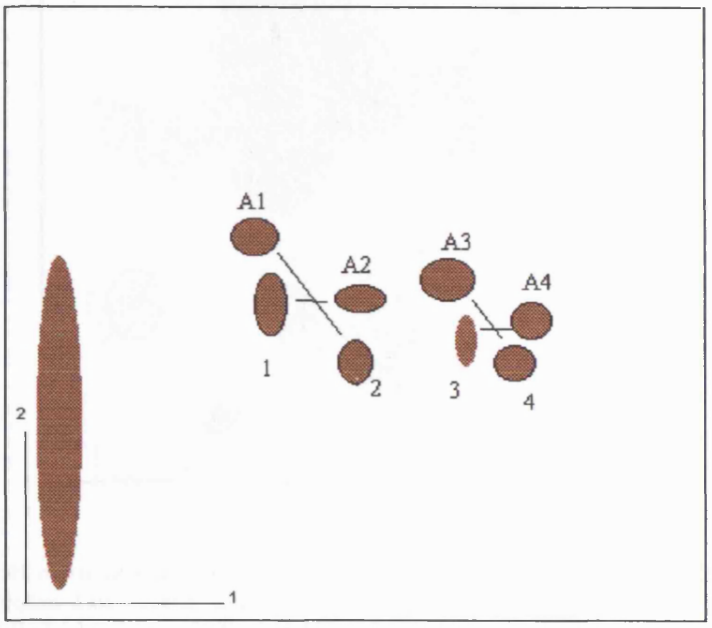

Figure 3.1-8: Two-dimensional chromatography of neem bark tannin.

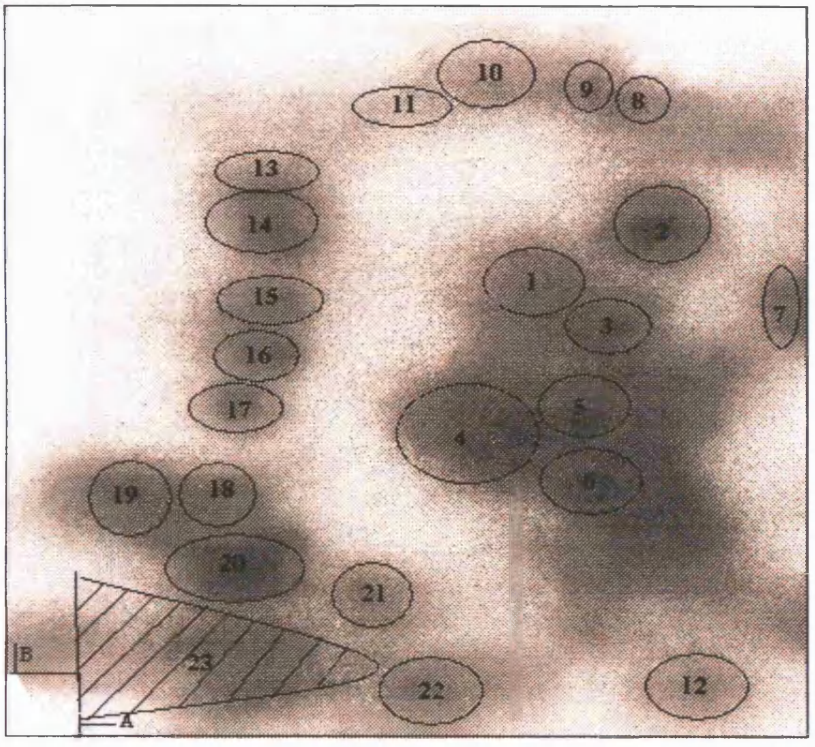

Figure 3.1-9: Paper chromatogram of acetone extract of neem bark extract. Solvent (A)-6% acetic acid; (B)-Butan-2-ol: acetic acid: water (14:1:5). Spraying reagent: ammonical silver nitrate. Compounds identified: 1, (-)-epicatechin; 2, (+)-catechin; 3, quercetin; 5, dihydrorobinetin; 6, robinetin; 7, (+)-epicatechin; 8, kaempferol; 9, (-)-epicatechin; 13, fisetin; 15, B2; 16, B4; 17, B3; 18, C2, 19, C1; 23, polymeric tannin, spot numbers 4, 10-12, 14, 20-22 have not been identified.

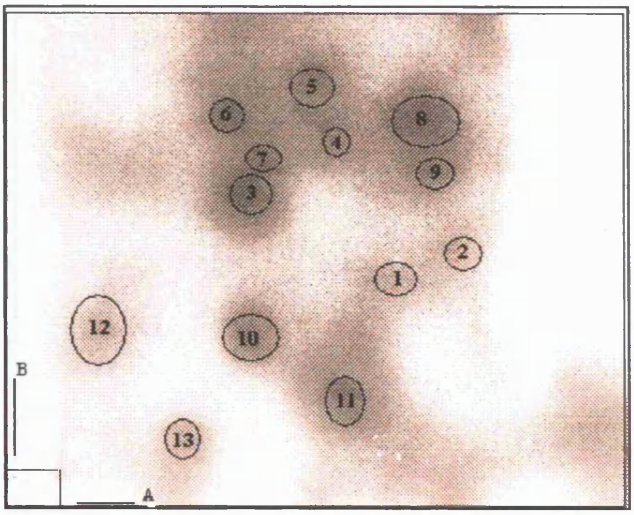

Figure 3.1-10. Paper chromatogram of aqueous extract (20°C) of neem bark tannin. Solvent (A)- 6% acetic acid; (B)-Butan-2-ol: acetic acid: water (14:1:5 v/v). Spraying reagent: Gibb's reagent. Compounds identified: 1. (-) -epigallocatechin; 2. (-)-robinetinidol; 3. (+)-gallocatechin; 4. (-)-epicatechin; 5. kaempferol; 6. fisetin; 7. (+)-epicatechin; 8.(+)-catechin; 9. quercetin; 10. robinetin; 11 and 13 are unknown; 12. polymeric tannins.

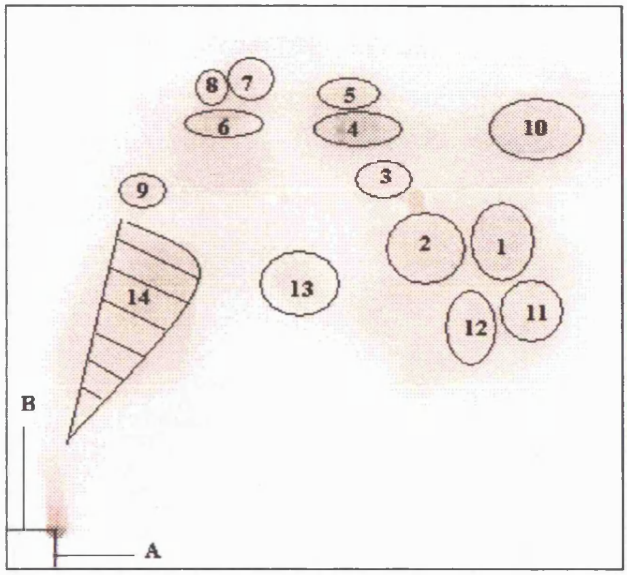

Figure 3.1-11. Paper chromatogram of aqueous extract (85°C) of neem bark tannin. Solvent (A)-6% acetic acid; (B)-Butan-2-ol: acetic acid: water (14:1:5 v/v). Spraying reagent: Gibb's reagent. Compounds identified: 1, (-)-epicatechin; 2, (+)-gallocatechin; 3, catechin; 4, (-)-epicatechin-3-gallate; 5, (+)-epicatechin-3-gallate; 6, 7, 8, are unknown; 9, fisetin; 10, kaempferol; 11, (-)-robinetinidol, 12 and 13 are unknown, 14, polymeric tannins.

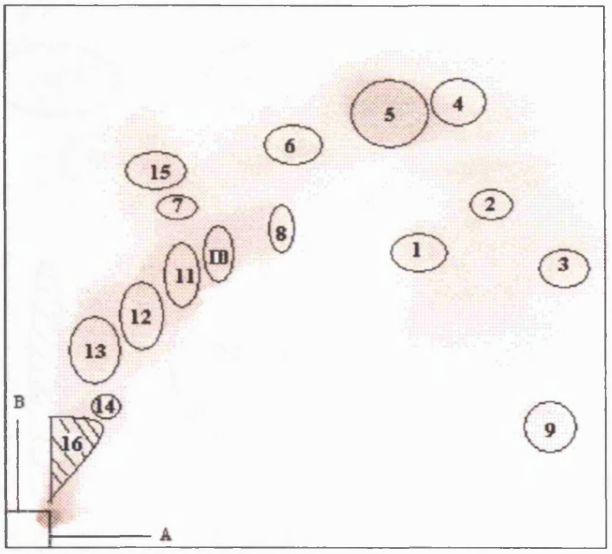

Figure 3.1-12: Paper chromatogram of methanol extract of neem bark tannin. Solvent (A)- 6% acetic acid; (B)-Butan-2-ol: acetic acid: water (14:1:5 v/v). Spraying reagent: Gibb's reagent. Compounds identified: 1, (+) -epicatechin; 2, (-)-epicatechin; 3, (+)-gallocatechin; 4, kaempferol; 5, (-)-epicatechin-3-gallate; 6, catechin; 7, quercetin; 8, B2; 9, unknown; 10, B4; 11, B1; 12, C1; 13, robinetin; 14, unknown; 15, (-)-epigallocatechin; 16, polymeric tannins.

Descending migration

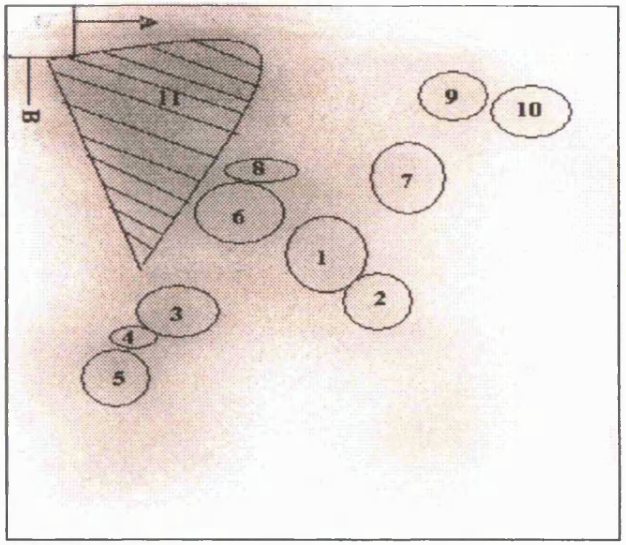

Figure 3.1-13: Paper chromatogram of acetone extract of neem bark tannin. Solent (A)- 6% acetic acid ; (B)- Butan-2-ol; acetic acid: water (14:1:5 v/v). Spraying reagent: Gibb's reagent. Compounds identified as: 1, B4; 2, (-)-epigallocatechin; 3, (+)-dihydrorobinetin; 4, (+)-gallocatechin; 5, (-)-epicatechin; 6, robinetin; 7, B3; 8, B1; 9, B3; 10, unknown 11, polymeric tannin.

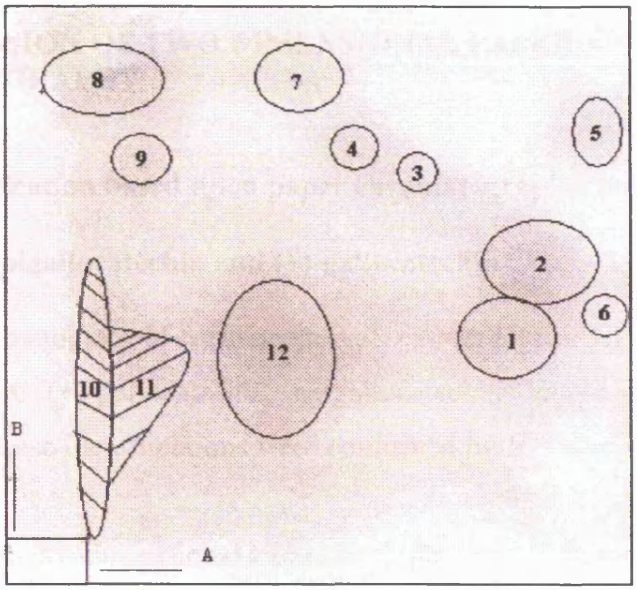

Figure 3.1-14: Paper chromatogram of ethyl acetate extract of neem bark tannin. Solvent (A)-6% acetic acid; (B)-Butan-2-ol: acetic acid: water (14:1:5 v/v). Spraying reagent: Gibb's reagent. Compounds identified as: 1, (-)-epicatechin; 2,(-)epigallocatechin; 3, (+)-catechin; 4, (-)-epicatechin-3-gallate; 5, kaempfarol; 6, (+)-leucorobinetindin; 7 and 8 are unknown; 9, fisetin; 10, (-)-robinetindol; 11 and 12 are polymeric tannins.

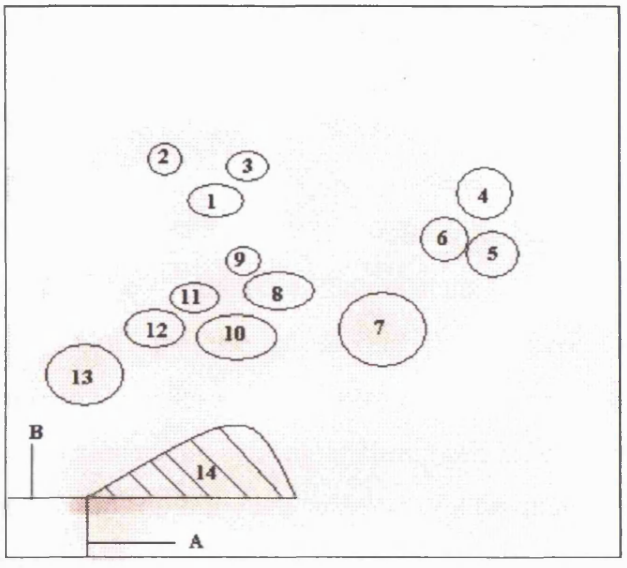

Figure 3.1-15: Paper chromatogram of aqueous extract (85°C) of neem bark tannin. Solvent (A)-6% acetic acid; (B)-Butan-2-ol: acetic acid: water (14:1:5 v/v). Spraying reagent: Gibb's reagent. Compounds identified: 1, unknown; 2, fisetin; 3, kaempferol; 4, (+)-epicatechin; 5, (-)-catechin; 6, gallocatechin; 7, (-)-robinetinidol; 8, (-)-epigallocatechin; 9, B4; 10, B2; 11, B3; 12, B1; 13, C2; 14, polymeric tannins.

3.1.9. DISCUSSION OF TWO DIMENSIONAL PAPER CHROMATOGRAPHY

3.1.9.1. Identification based upon paper chromatographic behaviour

3.1.9.1.1. (-)-epigallocatechin and (+)-gallocatechin

The paper chromatogram of fresh neem bark extract Figure 3.1-8 shows that spots 1, 2, 3 and 4 are (+)-gallocatechin, epigallocatechin, catechin and (-)-epicatechin respectively. These identifications were confirmed by R_f value with standard samples in this study.

The result of two-dimensional chromatography, with 6 % acetic acid as the second solvent, apparently complicated the situation, as it was found that an extract of raw extract, did not give double spots for all four components, but single spots appear as A1-A4 see Figure 3.1-8. The most likely interpretation is that spots A1-A4 are optically active forms of active components of tannins (catechin, epecatechin, gallocatechin and epigallocatechin). These identifications are fully in accordance with the principles of the dependence of the R_f values in butanol-acetic acid-water (14:1:5) on the number of phenolic groups in the molecule and are also consistent with a general similarity of epimeric differences between the catechins and gallocatechins.

Regarding the relationship of structure with R_f value, it would be expected that a compound of the structure suggested for one of these four forms of catechins and gallocatechins in 6 % acetic acid would be non-planar and that the pyran ring may be broken either by the course of oxidation or the molecule is more substantially planar.

According to Haslam[1] the use of solvent pairs offers the advantage of a separation based on different physical principles for each direction of development of the chromatogram. In dilute acetic acid separation, both differential absorption by the cellulose and differences in solubility are achieved. As far as organic phase separation is concerned, repeated partitioning of the solute takes place between the solvent and the microscopic stationary aqueous phase of the paper. Mobility of neem bark tannin components in water was found to be poor, which may be due to the non-planar configuration of flavonoids present in the bark, which permit adsorption (via hydroxyl groups) to the cellulose support at several points.

By spraying developing reagents, flavanols, flavan-3-ol, and polymeric species (dimer to oligomers) appeared more deeply coloured, which can be seen by ordinary daylight. Some of the R_f values are greater than 0.90, showing there are some hexahydroxy derivatives present. R_f values between 0.50 to 0.70 show that highly hydroxylated rings or side chains are present, which shows the isomeric nature of the neem bark tannin. Due to the early purification of tannins (see Figure 2-2 section 2.1.3), it was assumed those glycosidic groups, which usually decrease the R_f values, are negligible.

3.1.10. SUMMARY

The polyphenols (tannins) of NBE and NBT were separated into a number of constituents by paper chromatography (one and two dimensional).

The following constituents were identified

i. gallic acid
ii. fisetin
iii. (+)-catechin
iv. (-)-epicatechin
v. (+)-gallocatechin
vi. (-)-epigallocatechin
vii. quercetin
viii. kaempferol
ix. procyanidins B1, B2, B3, B4, C1, and C2
x. dihydrorobinetin
xi. robinetin
xii. robinetinidol
xiii. polymeric tannin.

3.1.11. REFERENCES

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CHAPTER 3.2

NEEM BARK NON-TANNINS

3.2.1. BACKGROUND

All tanning materials contain varying proportions of sugars, uronic acids and small phenolic bodies, which are generally classified as "non-tannins". The proportionate amount of non-tannins, and their chemical character, greatly influences the behaviour of the accompanying tannin toward the hide substance. The nature of non-tannins varies with the tanning materials themselves; they may change as a function of fermentation, oxidation, or other influences. The influence of non-tannin, as indicated above, is of importance in regulating the tanning behaviour of the tan liquor[1]. The nature of non-tannins associated with condensed tannins is still unknown. Few compounds have been identified from this heterogeneous group and therefore little is known of their function in the vegetable tanning process. White[2] identified glucose, arabinose and xylose as carbohydrate non-tannins constituents of quebracho extract. Phillips[3] and Tarboton[4] obtained reactions characteristic of pentoses, hexoses and uronic acids from black wattle non-tannins.

The non-tannins studies of bark of *azadirachta indica* were obtained from the primary fractionation see Section 2.1.3.

3.2.2. THE GUMS

Neem bark gum, is the salt of a complex polysaccharide acid. It has been in pharmaceutical use in the Indian subcontinent for many centuries[5]. The gum exudate from the bark was found to be a complex condensate of proteins and heteropolysaccharides. The proteins are linked tightly to the polysaccharides, which constitute the major components. The material is well protected against enzymatic attack and the usual chemical or physical techniques applied to separate the proteins from the polysaccharide matrix were not very successful[6, 7]. A variety of smaller components, however, have been identified after more drastic degradation methods. D-glucose, D-glucuronic acid, L-arabinose, L-fucose[5], mannose, xylose, rhamnose[7], D-glucosamine[8], the aldobiouronic acids[9], have been reported.

In the present investigation, they were best separated from the tannins and sugars by the addition of a large excess of ethanol, accompanied by vigorous stirring, to a 10% solution of the extract. The gum precipitates as a swollen gelatinous mass from the mother liquor; it is removed with difficulty, first by centrifugation, and subsequently by suction in a Buchner funnel.

The tannins are completely removed only by repeating the precipitation 6 or 7 times and their separation depends on the efficient removal of the mother liquor from the swollen material on each occasion. After eight hours extraction of the solid extract (7 g of < 63μm particle size) with 95% ethanol; the residue constitutes about 5-13% of the total solids present, depending on the extraction systems. Aqueous cold extraction gives more gum than aqueous organic solvent systems. According to the fractionation used (see Section. 2.3.3), which removes the sugars and tannins completely, the residue obtained remained stationary on one-and two-dimensional chromatograms.

Hydrolysis of the gum (10 g) with 1M sulphuric acid[10] for six hours gave evidence of the presence of water soluble but methanol insoluble barium salt, probably of a uronic acid residue. The identification of uronic acid in the form of aldobiuronic acid was obtained by the more drastic hydrolysis.

The equivalent weight of the gums was found by Mukharjee[5] to be > 1000 D, compared with 1080, found in the present study.

Complete hydrolysis of the gum, followed by partition chromatography and the preparation of crystalline derivatives, has shown that the gum contains L-arabinose, L-fucose, D-glactose, D-glucuronic acid and traces of D-xylose[5]. The ratio of D-glucose to L-arabinose was found to be 3:2.

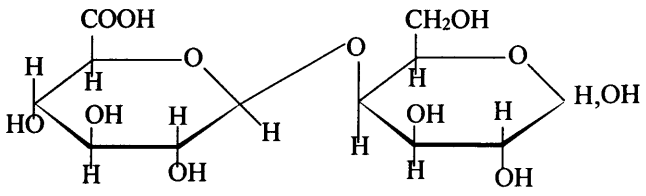


Figure 3.2-1: Aldobiuronic acid.

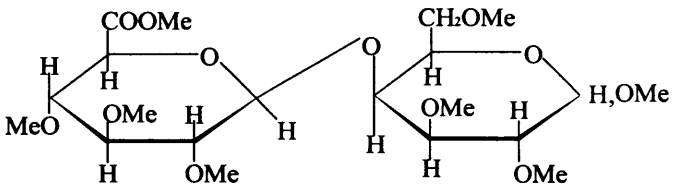


Figure 3.2-2: Octa-O-methyl derivative of aldobiuronic acid.

By mild acid hydrolysis the arabinose and fucose units were removed first[5]. The ease of removal of the arabinose units indicated that they were present in the gum in the furanose form[11].

More drastic hydrolysis of the gum affords D-galactose and an aldobiuronic acid, see Figure 3.2-1, composed of a unit of D-glucuronic acid and one of D-galactose.

The structure of the aldobiuronic acid has been established as follows. Upon methylation of the barium salt of aldobiuronic acid, an octa-O-methyl derivative was produced, Figure 3.2-2. Hydrolysis of this afforded equal amounts of 2,3,6-tri-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-glucuronic acid, the former being identified as the crystalline γ-lactose[11] and the latter as the 1,5-lactose 6-methyl ester[12]. These facts prove that the structure assigned to the aldobiuronic acid is correct. It is of interest to note that both lemon gum and grapefruit gum[13] produce aldobiuronic acid on hydrolysis[5].

3.2.2.1. Hydrolysis of the gum and characterisation of L-arabinose, L-fucose and D-galactose

A solution of the gum (30 g) in hot sulphuric acid (2 l, 0.05 M) was heated on a boiling water-bath for 22 hours, after that neutralised with barium carbonate, filtered and evaporated by using a rotary evaporator at < 30°C. The gum sample, dissolved in methanol: water mixture was applied to paper as described in section 3.2.3.1, using butan-2-ol-ethanol-water (5:1:4) as the irrigating solvent. Reference compounds were used and silver nitrate reagent[14] revealed the presence of arabinose, fucose and galactose. Results are shown in Table 3.2-1.

$$R_g\text{-value} = \frac{\text{distance substance travels from origin}}{\text{distance glucose travels from origin}} X\ 100$$

Table: 3.2-1: R_g values of hydrolysed gum.

Sugars	R_g (%)
Fucose	113
Galactose	88
Glucose	102
Glucosamine	71
Glucuronic acid	21

The gums in the bark extract have large molecular particles of remarkable swelling power which retain tannins due to occlusive and adsorptive effects, and which may seriously retard the tanning process. In concentrated aqueous solution they form mucilage and their presence probably contributes largely to the water retaining properties of the extract. Due to the insolubility of the gums in alcohols, they remain at the point of application on paper chromatograms of the whole extract which are developed with alcohol-water mixtures and, in view of their adsorptive power, their presence could result in the incorrect interpretation of chromatograms of the tannin fraction.

3.2.3. NEEM BARK SUGARS

The bark has been reported to contain several biologically active carbohydrates[15].

3.2.3.1. Polysaccharides GIa and GIb

GIa is composed of the following repeating unit:

-α-L-ara-(α-D-glu-1-4-α-D-glu*-1-6-α-L-ara**)$_5$
|
α-D-glu-α-L-fucose

* glu = glucose

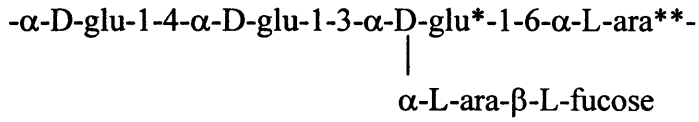
**ara= arabinose

GIa is composed of a repeating unit consisting of one residue of α-L-arabinose and five of α-D-glucose. The arabinose is linked (1-6) to one of the glucose

molecules, which are mutually linked (1-4). Glb is a branched arabinofucoglucan containing a main chain of (1-4) linked α-D-glucose molecules substituted in the 6 position with side chains of α-L-arabinose molecules and in the 4 position with 3-O-substituted fucose molecules[15].

3.2.3.2. Polysaccharides GIIa

GIIa is composed of the following repeating unit:



* glu = glucose

**ara= arabinose

GIIa is branched arabinofucoglucan, containing a main chain of (1-4)-linked α-D-glucose molecules, substituted in the 6-position with side chains of α-L-arabinose and β-L-fucose.

Two functionally different immunomodulators, one methanol insoluble, high molecular weight (> 10kD) saccharide-containing fraction[15] and the other, methanol soluble, low molecular weight (<10kD) fraction[16] has been isolated from aqueous bark extract.

The sugars were separated from the tannins by the lead salt method and remaining traces of polyphenolic materials were removed by shaking with hide powder. The main solution and the washings from the hide powder were concentrated under reduced pressure and taken to complete dryness. The sugars were extracted from the excess lead acetate with boiling methanol. The product was examined by chromatography.

3.2.4. EXPERIMENTAL

3.2.4.1. Paper chromatography of sugars of neem bark

One-dimensional chromatograms of the sugar fraction were run in mixtures of 60ml of butan-2-ol with 40 ml pyridine and 30 ml water. It took about 25 hours at room temperature of descending chromatography, then the chromatograms were dried in a

fume cupboard for about 4 hours. After complete drying the following developing reagents were used.

3.2.4.2. Spraying reagent

Two solutions were prepared:
[A]: 0.1 ml of a saturated solution of silver nitrate was added to 20 ml of acetone; if cloudiness appeared, a drop more water was added.
[B]: 2 g of sodium hydroxide were dissolved in a minimal volume of water (<1 ml) and made up to 100 ml with methanol.
The chromatogram was dipped in reagent A, dried and then sprayed with reagent B. This is a sensitive stain producing silver-brown spots with reducing carbohydrates at a sensitivity of about 1μg.

One-dimensional chromatograms of the sugar fraction were run in water-saturated butan-2-ol, using reference compounds and naphthoresorcinol-phosphoric acid as spraying reagent. The latter reagent was superior to all others for this purpose and does not affect the paper. Prolonged irrigation (18-24 hours) gave excellent separations of glucose, galactose, and fucose from the carbohydrate fraction of the neem bark extract. The fraction assumed to contain only fucose was further identified by hydrolysing 5 ml of the fucose solution in a sealed tube with 4 ml of 2.5M sulphuric acid for 6 hours on a water bath. The hydrolysis products were neutralised with barium carbonate and the final traces of acid removed by passing the hydrolysate through a cellulose column. The water was removed under reduced pressure and the concentrate examined by chromatography. Only glucose and fucose were present.

With the napthoresorcinol-phosphoric reagent, the presence of glucose, galactose and fucose could be established on one-dimensional chromatograms of the extract without prior separation of the tannins. The indications are, however, that this carbohydrate non-tan fraction constitutes about 15-50%, depending on which extraction system is used.

The neem bark carbohydrate moiety consists of arabinose, galactose and glucosamine in a molar ratio of 2.96:2.0:4.9[5].

3.2.5. DISCUSSION

The nature of the non-tannins is of some importance to the tanning industry. Due to lack of efficiency of separation methods hitherto, the effect of the small molecular carbohydrates (<10kD) on the tanning processing is as yet unknown. Large molecular particles (>10kD) are semi-colloidal in nature and tend to block the pores of fast filtering paper even in relatively dilute solution. The blocking of the inter-molecular spaces in the collagen structure, through which the tannins diffuse during the tanning process, is thus easily envisaged. The influence of the extraction temperature on the solubility of the gums and sugars also requires study. The presence of the gum in the extract also appears responsible for certain special solubility phenomena. In the concentrated aqueous extracts, due to their close association, the less soluble gums are probably held in solution by the tannins through inter molecular forces such as hydrogen bonding. On dilution all these constituents are predominantly hydrogen-bonded to the solvent and true solubility effects come into operation. The more soluble tannins may displace a proportion of the less soluble gums, resulting in the afore-mentioned phenomenon. At high dilution this displacement effect would obviously disappear.

3.2.6. SUMMARY

1. Neem bark extract non-tan consists of gums and sugars.
2. The gums give on hydrolysis D-glucose, D-galactose, L-fucose, L-arabinose and glucosamines. They thus appear identical with the natural exudate of the bark produced on injury or as a result of pathological conditions.
3. The sugars in the extract are glucose, galactose, glucuronic acid and fucose.

3.2.7. REFERENCES

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CHAPTER 3.3

GEL FILTERATION CHROMATOGRAPHY

3.3.1. SEPHADEX GEL CHROMATOGRAPHY OF BARK OF NEEM BARK TANNIN

3.3.2. INTRODUCTION

The cross-linked dextran gel, sephadex[1], has been widely used in the separation of substances with different molecular dimensions. The gel was originally prepared[2] as a material for use in preparative zone electrophoresis. However, it was later found[3] to be particularly useful as a molecular sieve. It is made up of beads formed by cross-linking dextran with epichlorohydrin. A partial structure[4] is shown in Figure 3.3-1

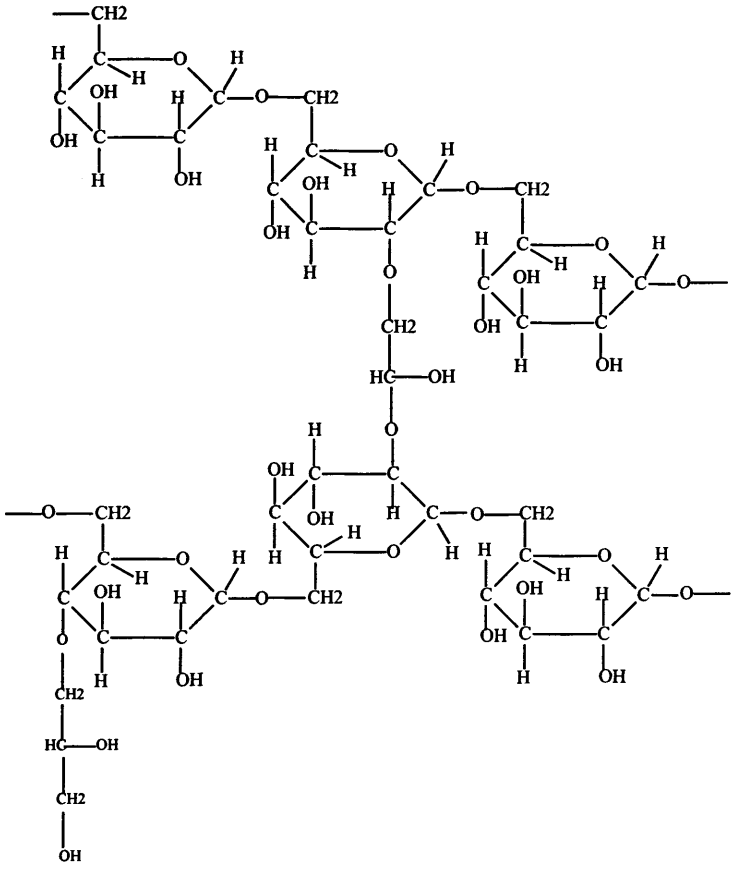

Figure 3.3-1: Partial structure of sephadex (dextran gel).

The fractionation range of the gels is dependent on the degree of cross-linking.[5] Consequently, a variety of sephadex gels have been produced with different degrees of cross-linking to cover most molecular weight ranges. For example, the higher cross-linked gels are better for separating low molecular weight species.

The sephadex G-series of gels are strongly hydrophilic in nature due to the high proportion of hydroxyl groups on the polysaccharide chains. Therefore, they swell in water and electrolyte solutions. However, they have limited use in organic solvents, where swelling is very often reduced considerably. To overcome this problem LH-20 and LH-60 sephadex gels have been produced: these are prepared by hydroxypropylation of sephadex G-25 and G-50 respectively. The hydroxypropyl groups are attached to the glucose units via an ether linkage. As a consequence of the increase in the ratio of carbon to hydroxyl groups, the gels are far more lipophilic than the G series and can be used with a wide range of organic solvents.

Since its introduction, sephadex has been employed as a medium for such operations as the separation of biological macromolecules[5-7] and peptides,[5] the desalting of proteins[5,6], the determination of macromolecule molecular weights[5] and structure[7,8], and for protein binding experiments[9-12], The ability of sephadex to adsorb certain molecules during filtration, results in greater elution volumes than would normally be expected for their size and shape[5,9,13-24].

The two main causes of retardation are adsorption or electrostatic interactions. The latter can occur with deionised water as the eluent, and is due to the presence of a relatively low proportion of charged carboxyl groups on the gel matrix. These may interact with cationic groups on a solute molecule, retarding its passage. Conversely positively charged species can be excluded from the matrix altogether as a result of this phenomenon and are eluted earlier than expected. Ion exchange effects can be removed completely by performing experiments with eluent containing a low concentration of electrolyte[23].

The second cause of retardation, adsorption by the gel, is a characteristic of aromatic molecules of which phenolic and polyphenolic substances form a large and significant group[13, 15-20, 22-24]. However, this behaviour is not unique to sephadex. Adsorption of phenolic compounds has been observed on other polysaccharide matrices such as cellulose[23] and starch[23]. Since the structures of these natural

polysaccharides are similar to that of sephadex one would expect that the mechanism for phenol binding would also be similar.

3.3.2.1. Gel filtration

The chromatographic bed is made up of a mobile phase and a stationary phase. The movement of the applied solute down the column, therefore, depends upon both the bulk flows of the mobile phase and the diffusion of solute molecules through the stationary phase. Separation of different molecules is usually a result of their different size and shape, which is reflected in their facility to pass through the pores of the gel grains.

If the molecules are too large to enter the grain pores then the solute will excluded from the gel and follow the bulk flow of the mobile phase. The smaller solute molecules will spend time in the stationary phase and hence will take longer to elute. The relative elution times then usually increase with the decreasing molecular weight of the species. Gel filtration separates molecules according to their size, so substances are eluted from columns of sephadex in order of decreasing molecular size as long as only molecular sieving effects occur.

3.3.3. EXPERIMENTAL PROCEDURE

3.3.3.1. Materials

3.3.3.2. Sephadex G-25 and LH-20

These are cross-linked dextrans obtained from the Pharmacia Company (Uppsala, Sweden). The handling and preparation of these substances is dealt with extensively in the chromatography sections 3.3.4 and 3.3.5.

3.3.3.3. Standard Chromatography column for sephadex

Column C 40/26 (45 x 3 i.d. mm).

3.3.3.4. Fraction collector

Fraction collector Model 2110 from Bio-Rad
Peristaltic Pump

3.3.4. SEPHADEX GEL CHROMATOGRAPHY

A straightforward chromatographic procedure[25] was adopted, involving downward elution of the samples and collected in fraction collector. Also, the aromatic nature of the molecules enabled elution to be monitored by UV detector.

3.3.5. METHODS

3.3.5.1. Packing the column

1. The eluent (ultrasonically degassed) was added to sephadex gel to make a fairly thick slurry. It was allowed to swell for three hours, after which time any fine particles were removed by decantation. Regarding the sephadex G-25 gel, to avoid any air bubbles forming in the column, it was placed under vacuum for a few minutes to degas the slurry.
2. The column was supported on a stand, and the 'dead-space' below the netting was filled by adding eluent down the column, closing the outlet tubing when the eluent level reached the level of the netting.
3. The column was packed by tilting it at an angle and pouring the slurry down the wall of the tube, thus avoiding trapping air bubbles. This was then adjusted to the vertical position. [It is important to pour the gel in one action, otherwise layering may occur, which would reduce the continuity of the gel and so affect the result]. The column was carefully filled up with eluent using a pipette, and the top-piece replaced. Eluent flow was started as quickly as possible to produce even sedimentation, passing at least five or six volumes through the column prior to use.
4. The experiment was kept at a constant temperature of 18.5 ±0.5 °C in a controlled room, in order to avoid any anomalies in flow or binding in the column due to temperature change. The eluent flow rate during experiments was maintained at approximately 2 ml min^{-1} by means of a peristaltic pump.

3.3.5.2. Application of the sample

1. Eluent was removed from the above the bed surface with a Pasteur pipette, leaving a few cm^3 of solvent to drain through the bed.

2. The sample was layered on top of the drained bed with a pipette, ensuring that the surface was not allowed to run dry.
3. After the sample had drained onto the bed, a few drops of eluent were applied to wash any remaining sample into the bed. The column was then refilled and the flow rate started as quickly as possible. Care was taken when applying the sample not to disturb the bed surface as this may result in an uneven flow of the sample through the column.

3.3.5.3. Sample concentration

The concentration of each sample was ~ 2 g in 1 ml of eluent.

3.3.5.4. Procedure

1. Eluent was passed through the column for approximately 30 minutes. An automatic sample collector collected the samples and the absorbance at 280 nm was immediately noted. The absorbance monitored was considered constant when there was no appreciable baseline drift on the chart recorder.
2. The sample was loaded onto the column, noting the time of application, taking care to refill and restart the flow to the column as quickly as possible.
3. The flow rate was found by measuring the time taken to fill a 15 ml fraction collector tube with eluent. Measurements were taken at the start and finish of each experiment to check the consistency of the flow.
4. The polyphenolic molecules are then eluted, observed as a series of peaks on the chart recorder as a function of time.

3.3.5.5. Analysis of data

The absorbance elution tracings obtained for the compounds were approximately symmetrical peaks, except for sephadex G-25 eluted compounds when broadening was recorded, which may be due to the size of molecules separated by elution. Graphs were made of absorbance versus fraction numbers as shown in Figure 3.3-4 and 3.3-5, for sephadex G-25 and LH-20 respectively.

3.3.6. Qualitative analysis of fraction (I) and fraction (II) using thin layer chromatography (TLC)

Thin layer chromatography, silica gel 60 F_{254} plates 10 x 5 cm 0.25 mm layer thickness (Merck, Germany) was used to separate the fraction components after passing through sephadex G-25. Fractions were developed using chloroform: methanol (500:1 v/v) mixtures in a sealed chamber (Shandon Unikit, UK) at ambient temperature. The plate was then air dried and visualised under UV light.

3.3.7. Separation and purification of neem bark tannins

Thompson *et al.*[25], have shown that the chromatography on sephadex is a good method for preparation and purification of flavonoid monomers and polymers. In the present investigation preliminary fractionation of the neem bark extract was achieved by the method given in Section 2.3.3. 12g neem bark tannin was chromatographed on sephadex G-25 column (acetone: water, 1:1 v/v) and separated into two fractions (II) and (I). Fraction II (10-18, 7.8gms) was collected and further chromatographed on sephadex LH-20 (ethanol, 95%) and separated into four fractions (IIa, IIb, IIc and IId) as shown in the Figure 3.3-2 and Table 3.3-1.

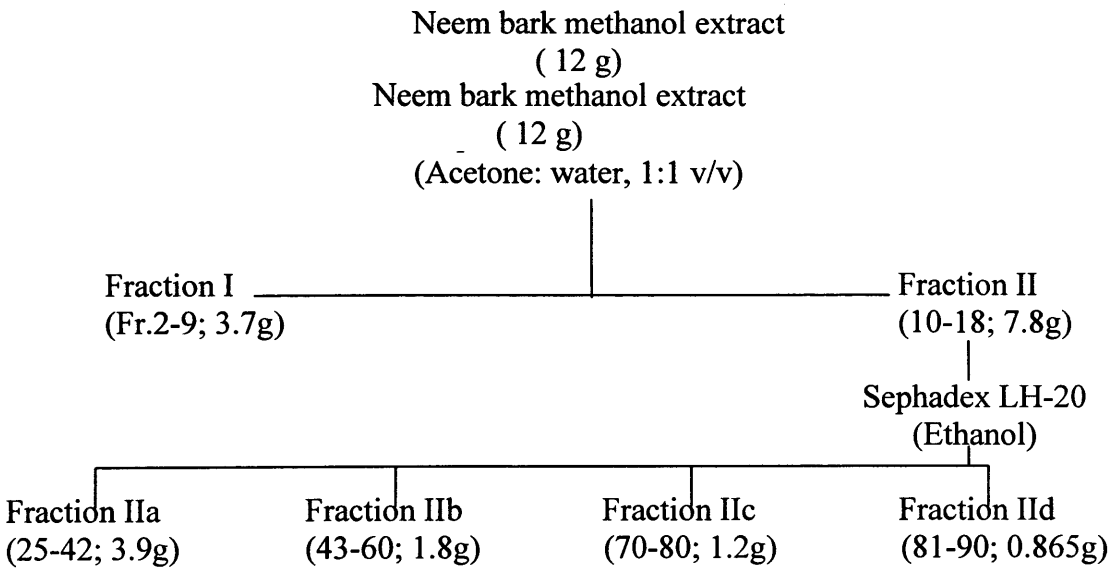

Figure 3.3-2: Fractionation of the methanol extract of neem bark tannin.

3.3.8. RESULTS

Table 3.3-1: Yield of fractions neem bark extract after purifying on sephadex G-25 and sephadex LH-20.

Fractions	Yield (% w/w)
I (7 x 15 ml)	31
II (8 x 15 ml)	65
IIa (17 x 15 ml)	50
IIb (17 x 15 ml)	23
IIc (10 x 15 ml)	15
IId (9 x 15 ml)	11

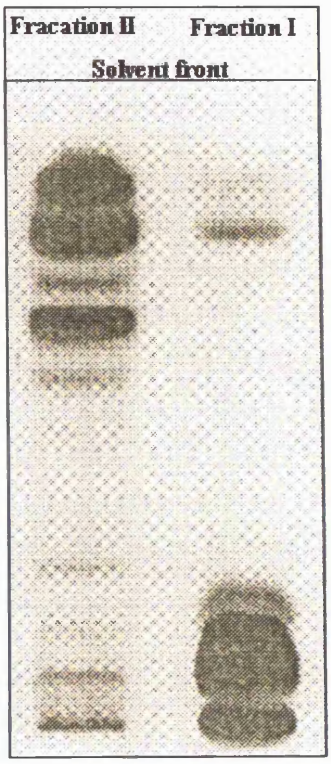

Figure 3.3-3: Thin layer chromatogram of NBT.

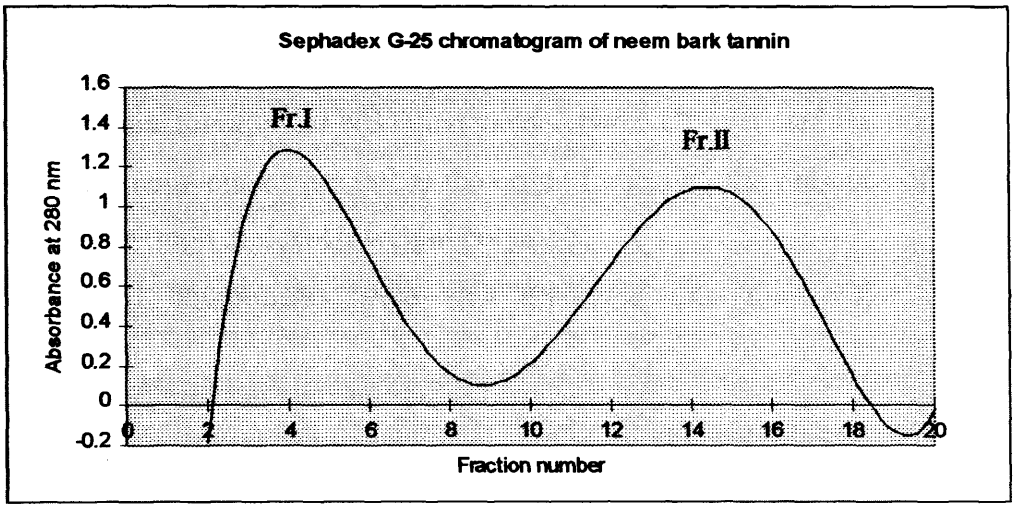

Figure 3.3-4. Sephadex G-25 fractogram of the methanol extract of bark of *Azadirachta Indica,* eluted with acetone: water, 1:1 v/v.

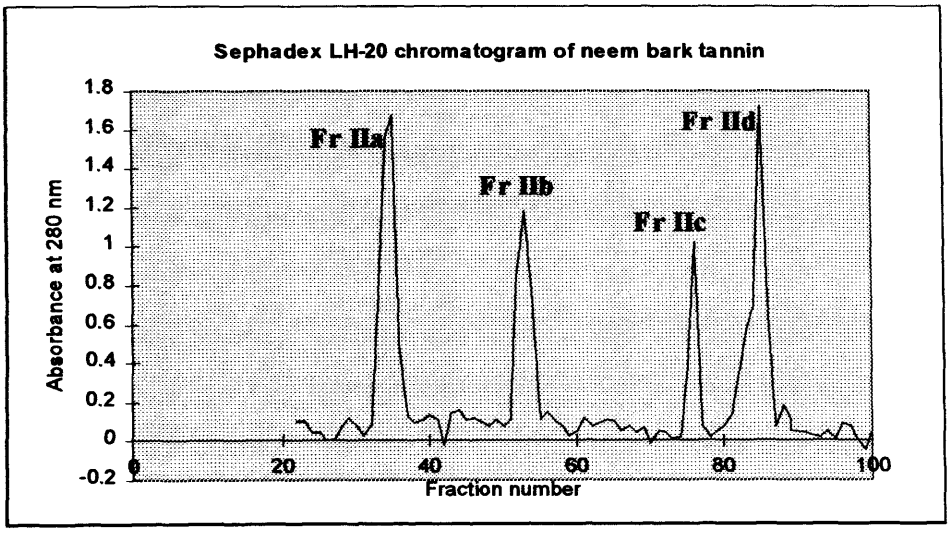

Figure 3.3-5. Sephadex LH-20 fractogram of the methanol extract of bark of *Azadirachta Indica,* eluted with ethanol.

3.3.9. SUMMARY

Sephadex chromatography of NBT first yielded two well separated fractions.

- Fraction I components are left at the origin indicating the presence of non-tannin materials or high molecular weight polymers.
- Fraction II components were well separated into four major components designated.

Fraction IIa

Fraction IIb

Fraction IIc

Fraction IId

All fractions were further analysed for the characterisation of neem bark tannin components.

3.3.10. REFERENCES

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CHAPTER 3.4

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.4.1. ANALYSIS OF BARK OF AZADIRACHTA INDICA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.4.2. INTRODUCTION

3.4.2.1. Analysis of flavonoids by high performance liquid chromatography

There has been a dramatic increase in the scope and versatility of this technique, due mainly to the availability of the necessary instrumentation and improvements in the efficiency and nature of column packing materials. It is noteworthy that in the flavonoid field, high performance liquid chromatography has been mainly used as an analytical technique, e.g. for quantitative determination of plant constituents, for checking the purity of natural samples and for the isolation of flavonoids on a preparative scale.

Natural plant constituents, with structures based on the aromatic heterocyle, 3-phenylbenzopyrone, are ideally suited for analysis by HPLC. The number and position of hydroxyl substituents and other derivatives can vary the basic complex structure. These variations cause changes in the way the compounds react in solvents and columns commonly used in HPLC, allowing separation and identification.

Since 1979, some pertinent and excellent reviews have been reported: Kingston[1], the application of HPLC to secondary metabolites; Adams and Nakanishi[2], selected examples of HPLC separation of natural products; Van Sumere et al[3], use of HPLC in the separation of plant phenolics; Roston and Kissinger[4], HPLC determination of phenolic acids of vegetable origin; Schwartz and von Elbe[5], HPLC of plant pigment; Rouseff and Ring[6], analysis of polymethoxylated flavones in citrus; Hrazdina[7], analyses of anthocyanins in fruits and beverage; and K. and Hostettmann[8], the application of HPLC techniques to flavonoids analysis.

3.4.2.2. Separation by silica gel columns

Various types of columns have been used for flavonoid analysis, using isocratic or gradient solvent systems. Early work was done on silica gel columns with and without derivatisation prior to analysis. Although a major advantage of HPLC is the lack of derivatisation prior to analysis, Hermann and co-workers cited several advantages to HPLC analysis of acetylated flavonoids. Among these were durability of the column, definite identification and quantitation, and isocratic separation of the flavonoids. The isocratic run does not require solvent-re-equilibration for each sample and this increases the rate of analysis.

Although silica gel columns have not been used often, they are well studied for the separation of non-polar or weakly polar flavonoid aglycones such as: polymethoxyelated flavones [9,10], isoflavones[11-14], and biflavonoids[11]. The normal phase liquid chromatography on LiChrosorb Si 60 of the acetate of numerous flavonoids permitted the application of flavonoid analysis to celery[15-17], orange juice [18,19], tomatoes[17, 20], plums[21], and cherries[22].

3.4.2.3. Reverse phase versus normal phase columns

By using the above-discussed packing materials, the stationary phase is less polar than the mobile phase and the procedure is called reverse phase chromatography (RPLC). Thus highly polar solutes exhibit shorter retention times than less polar solutes. The retention time of the same polymethoxylated flavones shows this difference between RPLC[23, 24] and normal phase HPLC (NPLC)[9]. However, with both types of columns, excellent separation was obtained in less than 30 minutes. The use of RPLC[25] for the resolution of isoflavones was criticised[12] for the observed band spreading, which would make the separation of multicomponent mixtures virtually impossible. A number of recent reports[26-29], however, have established a RPLC procedure for the separation and quantitation of the naturally occurring soybean isoflavone glycosides and aglycones, the non-sugar component of glycoside molecule that results from hydrolysis of the molecule.

3.4.2.4. Reverse-phase columns

In reverse phase columns, the stationary phases are prepared by bonding various organosilane molecules to the hydroxylic groups of a silica type surface. The most common of the organosilanes are octadecyltrichlorosilane, octyltrichlorosilane, and phenyltrichlorosilane. The simple procedures for preparing octadecylsilyl bonded stationary phases have been described [8,30,31] and may be employed, for example, for the preparation of preparative scale columns. Currently, there are a number of commercially available columns possessing a high degree of reproducibility.

Although the C_{18} or more specifically the μBondapak C_{18} column has been the dominant choice for RPLC of flavonoids, another type of column may be better dependent upon the class of flavonoid. In a comparison of LiChrosorb RP-18 and RP-8, Strack and Krause[32] obtained better resolution of glycosyl-flavone aglycones and glycosides on the RP-8 with a gradient methanol: acetic acid: water solvent system. Becker *et al.*[33], in the only reported use of LiChrosorb NH_2 column, achieved an excellent separation of isomeric O-glycosides of C-glycosylflavones. This type of column had been used for the separation of monosaccharides and for this reason chosen as the ideal phase for separation of C-glycosylflavones. There seemed to be no apparent differences in efficiency of separation of the flavonoids of *Silybum marianum* between the LiChrosorb RP-18 [34] and RP-8 [35, 36] column. However, two different solvent systems were used and an objective comparison cannot be made.

A μBondapak C_{18} with an acetonitrile: water solvent system failed to adequately separate methoxylated flavones, but a Zorbax C_8 column with a tetrahdrofuran: water system did[23].

The difference in columns with the same generic name and a lack of consistent specifications make the choice of reverse phase columns difficult. The initial choice of a column for a particular reverse phase separation would involve matching the type of substance (class of flavonoid) to be separated to the column capable of providing good retentively and selectivity characteristics towards the particular sample[37].

3.4.2.5. Aromatic acids and flavonoid separation

Extraction of phenolic from plant tissue usually means that both single ring phenolic and the flavonoids (C_6-C_3-C_6 carbon skeleton) are isolated in the same mixture. Two reports [38,39] advised carrying out prior separation of these groups for HPLC. Where a

μBondapak alkylphenyl column was used, some of the aromatic acids and phenols interfered with separation of the flavonoid[38]. However, RPLC on a μBondapak C_{18} column proved to be effective in the separation of both the plant phenols and flavanols of tobacco[40]. A gradient elution system was found to be most effective and necessary for the separation of the aromatic acids, phenols and flavonoid in the same mixture[41].

3.4.2.6. Solvent system

Methanol: water containing a small amount of acetic acid is one of the more commonly used solvent systems for RPLC of flavonoids. The addition of acetic or any acid improves the separation, but the amount of acid or pH of the eluting system is dependent upon the column's stability, as a Chrompack Nucleosiel C_{18} column ruptured probably due to a low pH[42]. In lieu of acetic acid, other compounds which have been used are: phosphoric acid [35,43-47], perchloric acid[48-50], potassium dihydrogen phosphate[40, 51-53], ammonium dihydrogen phosphate[54], and formic acid[55,56]. The absence of acid in the methanol: water is rare[57] but such a solvent system appears to be very successful in the separation of isoflavones[58-61].

The acetonitrile: water system was successful in the quantitative determination of naringin and hesperidin in citrus juice[62-65], and in the separation of both isomeric C-glycosylflavones[66] and isoflavones[67]. The acetonitrile: acetic acid: water solvent system was most popular for the separation of flavonoids of flowers[68, 69].

Other solvent systems used in RPLC are: tetrahydrofuran (THF): acetonitrile: water[9]: THF: water[70]; acetone: acetic acid: water[71-73]; methanol: dimethylformamide: acetic acid: water[74] and ethanol: acetic acid: water[75].

3.4.2.7. Effect of structure on retention

Wulf and Nagel[76] first discussed the effect of the structure of flavonoids on their elution behaviour. These data were confirmed and extended by Daigle and Conkerton[77]. In both cases a μBondapak C_{18} and a methanol: water solvent system was used. The influence of isomerisation and the glycosylation pattern of some C-cylcosylflavones on retention time was studied [78] and some of these findings were confirmed in a later report[79]. The chromatography behaviour of proanthocyanidins

was investigated using several packing materials (columns) [80, 81]. Lea concluded that RPLC offered the greatest potential for the separation of proanthocyanidins[81].

3.4.2.8. Detection

The high sensitivity of ultraviolet-visible detectors makes analysis of sub-microgram samples possible. The wavelength must be compatible with the solvent system and, of course, suitable for the compound to be detected. For the flavonols, flavonones, and isoflavones and their respective glycosides, the wavelength range between 254 nm and 280 nm is the most popular. A few researchers have also chosen the 340 nm to 360 nm range[82, 83]. The anthocyanins and proanthocyanidins were detected either in the range 520 nm to 546 nm or at 280 nm. The acetate derivatives of anthocyanidins were detected at 254 mn[84] and their chalcons at 340 nm[85]. The acetates of flavonols, and flavones and flavanones, however, were detected at 300 nm[86-92]. Rouseff and Ting[93, 94] employed a dual UV-fluorescence detector to determine the presence of interfering substances. The wavelength 313 nm applied to all five polymethoxylated flavones, since the impurities absorbed weakly at this wavelength. The C-glycosylflavones have been detected at a variety of wavelengths; 254 nm[78, 66], 270 nm[57], 312 nm[32], 330 nm[70], 335 nm[52], and 365 nm[79].

3.4.2.9. Qualitative analysis of flavonoids

In the HPLC analysis of complex mixtures, researchers have used more than retention time data to identify, positively or negatively, the compound of interest. However, off line techniques are relatively time consuming. Recently on line techniques such as HPLC UV/visible and HPLC/MS spectroscopy have been found, in some cases, to be sufficient to obtain positive identification of compounds with an overall reduction in analysis time[94].

Using detectors monitoring simultaneously, first at 280 nm and 525 nm, then 280 nm and 340 nm, enabled identification of the chalcones of malvidin 3-glucoside and malvidin 3,5-diglucoside. As a result, these chalcones could be collected in sufficient amounts to measure their UV/visible absorption spectra directly and to follow their conversion to their corresponding flavylium cations[85].

3.4.3. EXPERIMENTAL PROCEDURES FOR DERIVATISATION

3.4.3.1. Materials

Toluene-α-thiol

This product was obtained from Sigma Company (99 % purity) and used without further purification.

3.4.3.2. Methods

3.4.3.2.1. Derivatisation of methanol extract of neem bark.

Numerous analytical methods have contributed to our present knowledge of the structure of polymers. The development of spectroscopic methods, such as NMR spectroscopy and GC-MS principally, has made chemical methods of structure determination less useful at least for molecules of low molecular weight[95]. For polymers in general and proanthocyanidins (condensed tannins) in particular, chemical methods remain a powerful tool for their structure determination: polymers are degraded into their constituent units and analysed by the same techniques applied to low molecular weight molecules. The most used methods are colorimeteric assays, relying either on the reaction of the A-ring with an aromatic aldehyde (vanillin and dimethylaminocinnamaldehyde) or on their oxidative depolymerisation into anthocyanidins. Condensed tannins with 5,7-dihydroxy A-ring undergo facile cleavage under acidic or basic conditions[96]. Acid-catalysed degradations have given rise to the butanol/HCl assay in which the chain extender flavan-3-ol units are oxidised to give anthocyanidins absorbing in the visible region. The reaction mechanisms are complex, and the yields are not quantitative[97, 98].

3.4.3.2.2. Thiolysis

Depolymerisation reactions in the presence of nucleophiles are frequently employed for the structural analysis of condensed tannins[96]. Their structure, once established, can be used to determine the nature of the monomer units within the polymer. Numerous nucleophiles have been used including thiolacetic acid[99], benzene-*p*-sulphinic acid, benzenethiol[100], benzyl mercaptan (toluene-α-thiol), 2-mercaptoethanol[101] or phloroglucinol[102]. Various factors have dictated their choice, most particularly the ease of separation of the products and their recovery yields.

Phloroglucinol is sometimes preferred to the sulphur nucleophile because it is odourless, so it was used in the quantitative analysis of the condensed tannins by other chromatography methods. Finally, the use of a nucleophile limits the occurrence of side reactions that could affect recovery yields of the products.

3.4.3.2.3. Degradation with toluene-α-thiol

Each fractionated sample (30-50 mg) in ethanol (30 ml), mixed with toluene-α-thiol (11.25 ml) and acetic acid (6 ml), was refluxed under nitrogen at 95°C for 24-48 hours. Removal of the solvents at 30°C left an oily residue, which was dissolved in chloroform-propan-1-ol (4: 1 v/v) and chromatographed on a column of sephadex LH-20 (30 cm long and 1.5 cm diameter). Fractions were collected and dried under reduced pressure at 28°C then analysed by HPLC and GPC.

3.4.3.2.4. Acetyl derivative

Neem bark tannin peracetate was prepared[103] using the following method.
100 mg of neem bark tannin was dissolved in a mixture of 1 ml absolute pyridine and 1 ml distilled acetic anhydride. The solution was maintained for 24 hours at room temperature with constant stirring. Small pieces of ice were added to the solution, which was then left for few hours at 4°C (melting ice), during which time the neem bark tannin peracetate was precipitated. The solid was dissolved in a solvent mixture of chloroform-ethyl acetate (6:4 v/v) and applied to a silica gel column and eluted with the same solvent mixture. Chromatography was repeated three times to give the pure neem bark tannin peracetate (91.3 mg).

3.4.3.2.5. Methyl derivative

The methylation was carried out by the Freudenberg method[104]. The neem bark tannin (1.4 g) was dissolved in methanol (6 ml) and methylated with dimethyl sulphate (4 cm^3) and 50% KOH (4 cm^3). The reaction mixture was poured into water (100 cm^3), filtered by means of suction and crystallised from methanol.
The methyl derivative was also obtained by methylating the acetylated neem bark tannin derivative in the same manner as above.

3.4.4. EXPERIMENTAL PROCEDURE FOR HPLC

3.4.4.1. Materials

Acetone, ethyl acetate and acetic acid (BDH, UK) were of analytical grade. Methanol (Merck, Germany) used for the analysis was of HPLC grade. Procyanidins B2, B3 were kindly supplied by Prof. Haslam (Sheffield University): these compounds were used without further purification. (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin were obtained from Sigma (Sigma-Aldrich company Ltd. UK).

3.4.4.2. Apparatus

HPLC analysis was carried out using a reversed phase silica gel column LiChrosorb RP-8 (4.6 x 250 mm) (Merck, Darmstadt, Germany) with an HPLC 805 pump (Gilson Ltd.). The UV detector 115 (Gilson Ltd.) was a single compact unit consisting of the major components: a photometer subsystem, a low volume cassette-type cell, and an electronics processor; this unit is fitted with auto-sample collector JASCO AS 950 and computer.

3.4.4.3. Elution

Two solvents were used, mobile phase 5% v/v acetic acid/methanol (100/0→60/40, linear methanol increasing gradient of 2% v/v min^{-1}). The photodiode array detector was set at 280 nm. Sample injection volume was 10 µl, pressure was 120 kg cm^{-2}, flow rate was 1.1 ml min^{-1}.

3.4.4.4. Procedure

Before the solvent could be used in the experiment it was filtered and degassed (degassing is required to remove dissolved gases which may react with either the mobile or stationary phase). The detector lines and detector were then purged with the solvent and the system was equilibrated to the required temperature, 30°C. The desired wavelength was selected, 280 nm, and the flow rate adjusted to 1.1 ml min^{-1}. Once the required temperature was attained the recorder was switched on and when the base line was steady, the solution to be studied was prepared and a 10 µl sample immediately injected on the column. The traces were recorded and a further 10 µl sample was injected, to check the reproducibility of the column.

3.4.5. ISOLATION AND PURIFICATION OF NEEM BARK TANNIN USING TLC

3.4.5.1. Qualitative analysis of neem bark tannin components by TLC

Purified neem bark tannin (2 g) was dissolved in distilled water and filtered through activated charcoal for early purification. The charcoal treated NBT liquor was evaporated under vacuum at 30°C. Most of the original active material was recovered (98%). The purified NBT, 20 µl was applied on activated (105°C, 50 min) silica gel 60 F_{254} TLC plates, 20 x 5 cm, 0.25 mm layer thickness (Whatman, UK). The plates were developed with acetone: toluene (8: 2, v/v) in a sealed glass TLC chamber (Shandon Unikit, UK) at ambient temperature. The plates were then air dried and visualised under UV light.

3.4.5.2. Separation of NBT components by preparative TLC

Preheated at 105°C for 50 min, silica gel 60 F_{254} preparative TLC plates, 20 x 10 cm; 0.50 mm layer thickness (Whatman, UK) were used. A relatively large amount of sample (80 mg/ml) was applied on the plate with a glass pipette (2 ml). The plates were developed with the same conditions as in the TLC analysis. Procyanidins were visualised under UV light. Individual bands of NBT components were scraped from the preparative TLC plates and resuspended with 3 x 5ml of acetone or methanol. Solvents were removed under vacuum at 30°C using rotary evaporator.

The purified NBT components were used without further alteration for HPLC analysis. Methyl and acetyl derivatives of NBT components were used for other spectroscopic techniques.

3.4.6. RESULTS

The summarised results are set out in Table 3.4-1. Typical HPLC chromatograms are shown in Figures 3.4-1 to 3.4-6, the other details of Figures A1-1 to A1-12 and Tables A1-1 to A1-11 are set out in Appendix A.

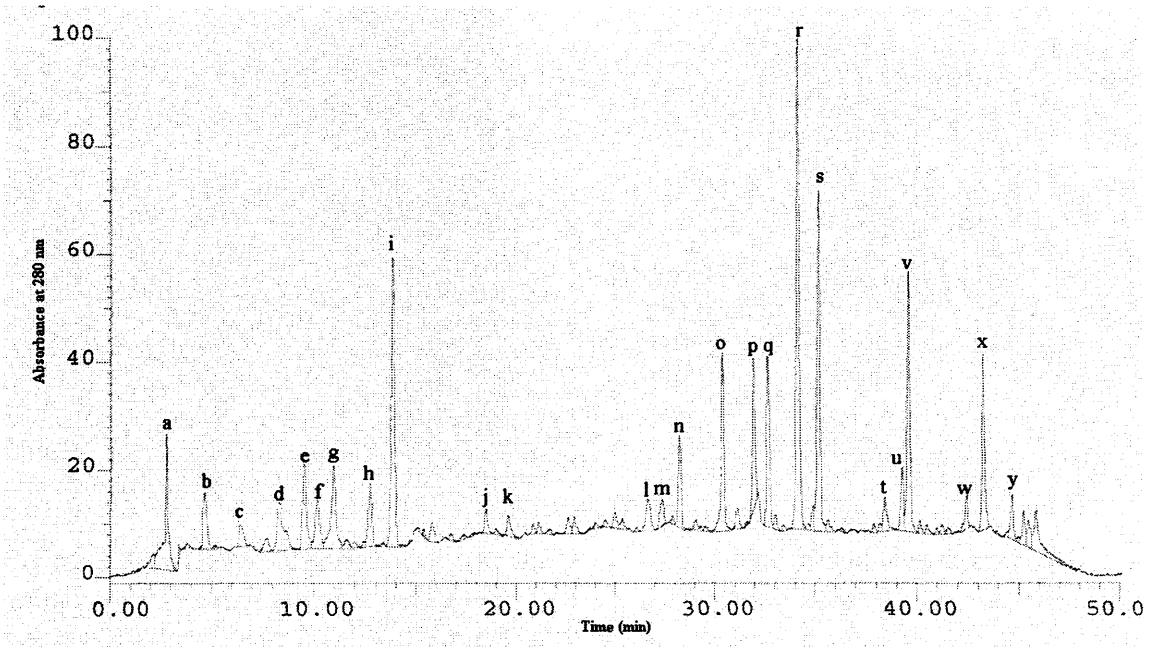

Figure 3.4-1: HPLC chromatogram of the thiolysis product of neem bark tannins extracted with acetone: water (1:1 v/v). Compounds of peaks a-y are identified and reported in Table A1-2 with retention times.

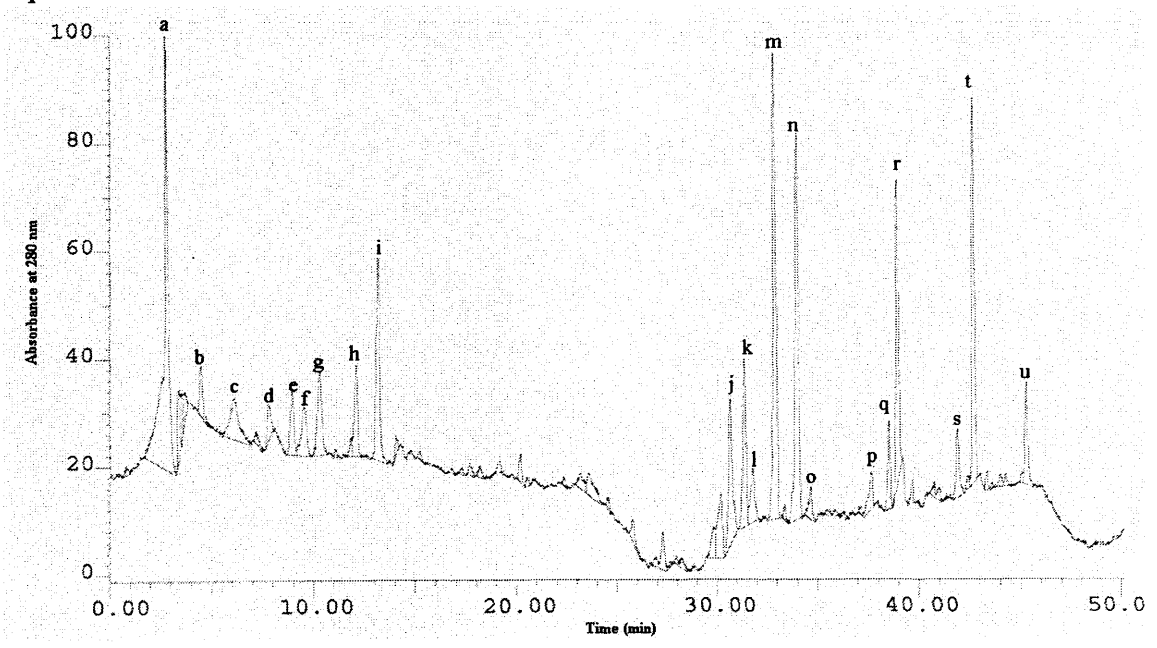

Figure 3.4-2: HPLC chromatogram of the thiolysis product of neem bark tannins extracted with water at 85°C. Compounds of peaks a-u are identified and reported in Table A1-2 with retention times.

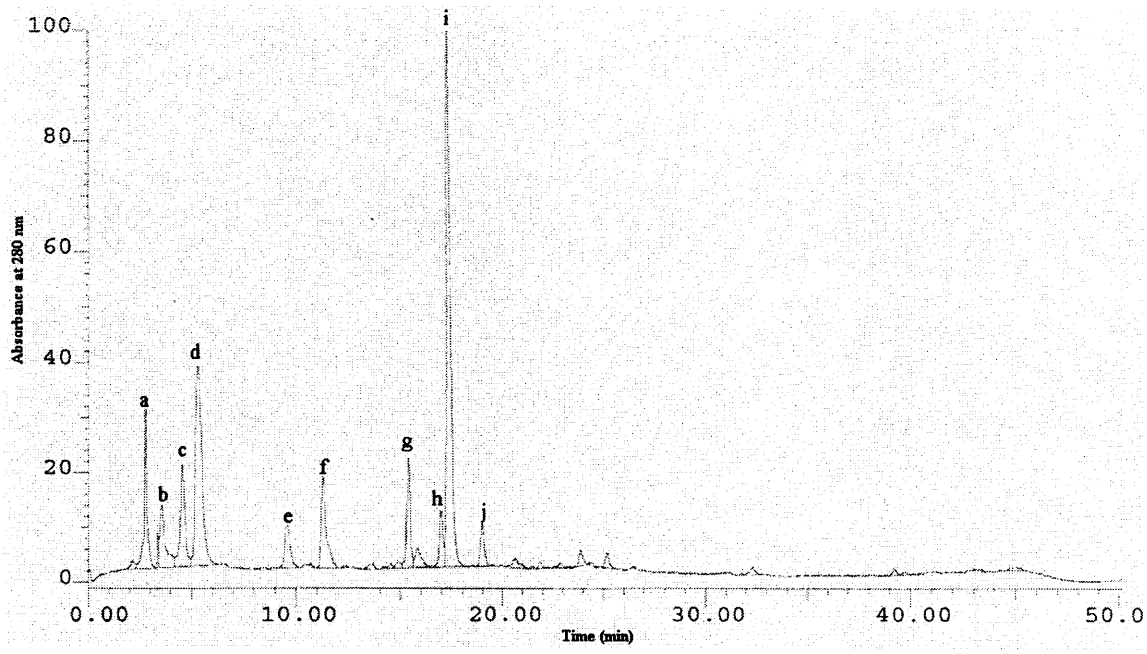

Figure 3.4-3: HPLC chromatogram of the thiolysis product of neem bark tannins extracted with ethyl acetate: water (90: 10 v/v). Compounds of peaks a-i are identified and reported in Table A1-1 with retention times.

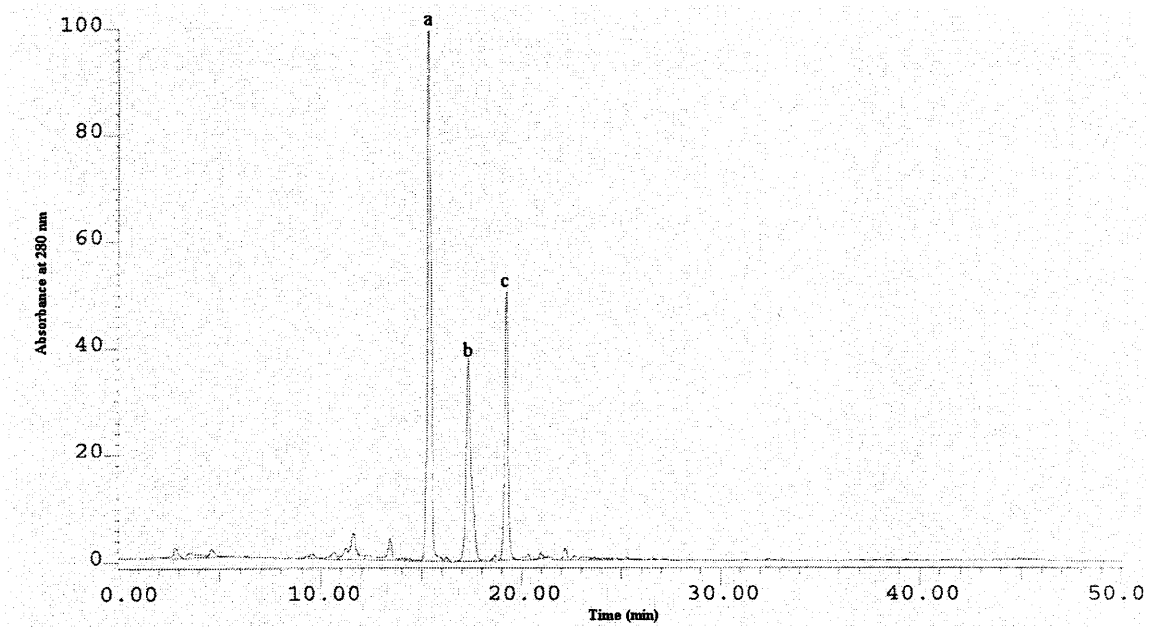

Figure 3.4-4: HPLC chromatogram of NBT procyanidin obtained by preparative TLC. Compound identified as procyanidin C1 trimer [EC (4β-8)-EC-EC].

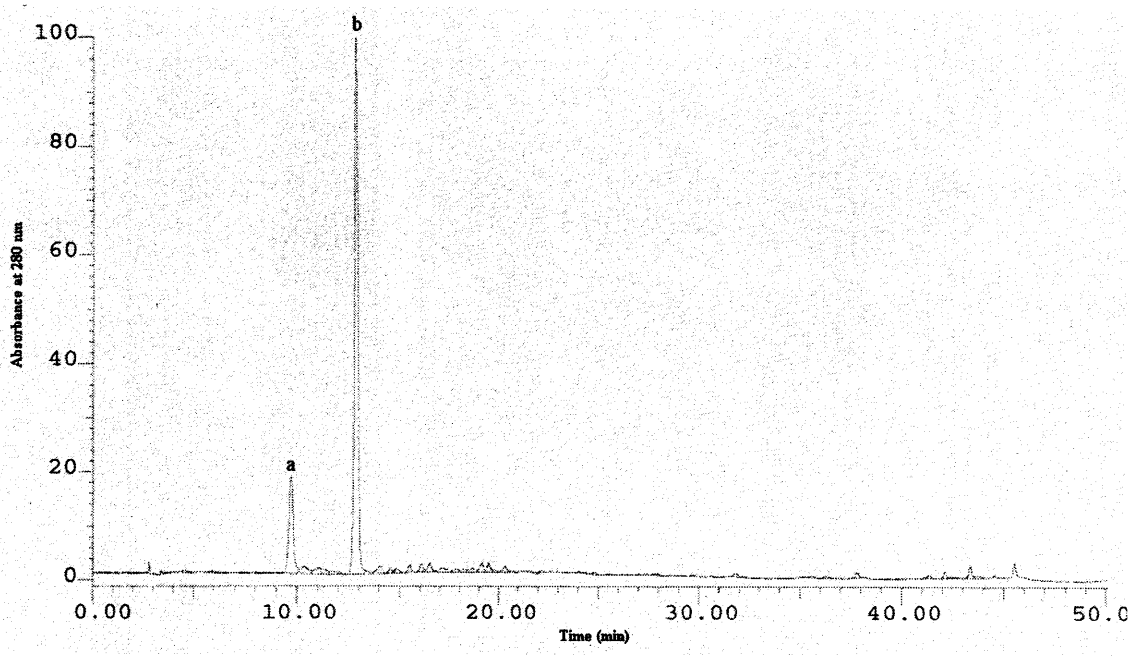

Figure 3.4-5: HPLC chromatogram of NBT procyanidin obtained by preparative TLC. Compound is identified as procyanidin B2.

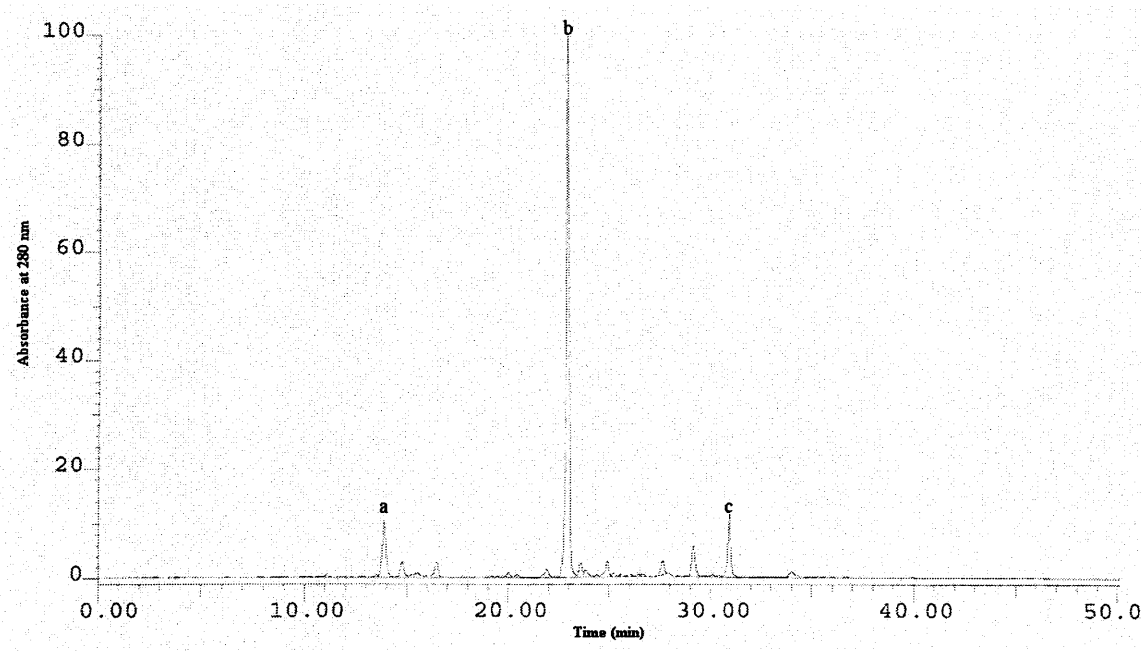

Figure 3.4-6: HPLC chromatogram of NBT procyanidin obtained by preparative TLC. Compound is identified as procyanidin B3.

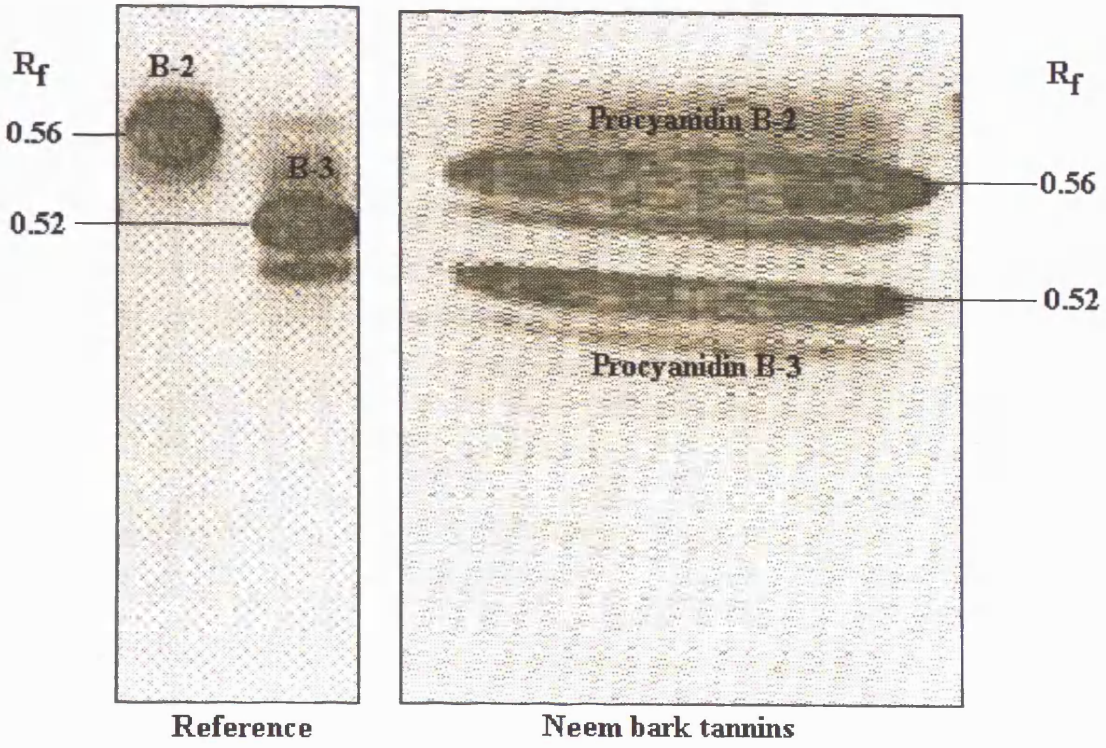

Figure 3.4-7: Thin layer chromatogram of procyanidins B-2 and B-3.

Table 3.4-1: Compounds of neem bark tannins separated on Lichrosorb RP-8 column.

Sample	Gallic acid	EGC	GC	C	EC	B1	B2	B3	B4	C1	Quercetin	Kaempferol	Trimeric	Tetrameric	Pentameric	Polymeric	Unknown
Acetone: water thiolysis product	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Aqueous (85 °C) thiolysis product	✓	✓	✓	✓	✓	✓	✓		✓	✓			✓	✓	✓	✓	✓
Ethyl acetate: water thiolysis product	✓		✓			✓	✓					✓					✓
Aqueous (85 °C)	✓		✓		✓		✓	✓		✓	✓		✓		✓	✓	✓
Ethyl acetate: water	✓				✓					✓					✓	✓	✓
Methanol: water	✓			✓	✓	✓	✓									✓	✓
Acetone: water	✓	✓		✓	✓	✓										✓	✓
Fraction I	✓					✓	✓									✓	✓
Fraction II	✓	✓			✓		✓	✓								✓	✓
Fraction II a	✓	✓		✓		✓					✓					✓	✓
Fraction II b	✓															✓	✓
Fraction II c	✓		✓		✓			✓	✓	✓	✓		✓		✓	✓	✓
Fraction II d	✓							✓			✓	✓					✓

✓ Major components of neem bark tannins,

✓ Minor components of neem bark tannins,

✓ Unidentified compounds of neem bark tannins.

3.4.7. DISCUSSION

Figures 3.4-1 to 3.4-3 show good separation and high resolution using HPLC. Twenty-five, twenty-one and ten peaks were observed, depending on the eluting solvent, corresponding to the compounds shown in Table A1-1-3 Appendix A. Twelve peaks are common to derivatised acetone and aqueous extracts. Quercetin and kaempferol were not resolved and identified from ethyl acetate extract, but both the compounds were well separated by the acetone and water extract at 85°C.

Gallic acid was the most rapidly eluted of all the compounds run. The rest of the results demonstrate that neem bark tannin is of procyanidin type, not gallated and containing epicatechin as the extension unit. Only the dimers B3 and B4, present in small quantities, contained catechin in their upper subunits. However, both catechin and epicatechin were found as initiation units. The presence of (-)-epigallocatechin and gallocatechin were also found in neem bark tannins. The higher oligomeric compounds were also separated.

It is clear from the results that, using nucleophilic substitution, significant separation of compounds is achieved; broader peaks and longer elution times were observed when neem bark tannin was not treated with the nucleophile. It is also noted that 5% acetic acid in 5% gradient solvent shortened the elution time and thereby complete separation of most of the compounds was achieved.

The major components within all the systems studied were: gallic acid, epicatechin, epigallocatechin, procyanidin B1, B2 and high molecular weight polymeric compounds, while epicatechin appears to be a terminal unit only.

The appearance of 'ghost' peaks, in the case of Figures A1.1, 5, and 6 in Appendix A1, has been observed, resulting from an injection of a single compound in a solvent different in composition to that of the chromatographic eluent. This effect has been described in the literature[105-107] and appears to be particularly prominent in the case of highly polar phenolic compounds such as procyanidins and condensed tannins, which is why a gradient was chosen carefully, using the procedure obtained by Snyder and co-workers[108-109].

From this work, which is supported by the literature, the retention times of the compounds with three hydroxyl groups on the B-ring (i.e. compounds with a pyrogallol group) are shorter than those of the corresponding compounds with two

hydroxyl groups (i.e. compounds with catechol group). The order of elution, from first to last, was as follow:

Elution order (first to last)	Compound	Configuration
1	(+)-gallocatechin	2R:3S
2	(-)-epigallocatechin	2R:3R
3	(+)-catechin	2R:3S
4	(-)-epicatechin	2R:3R

In addition to the substitution pattern of the B-ring polarity and the polarity of the compounds, the stereochemistry of hydroxyl at the C-3 position also influences the elution time. It has also been observed that R and S configuration may also affect the retention time, because (+)-gallocatechin which has 2R:3S configuration eluted earlier than (-)-epigallocatechin with 2R:3R configuration. In the same way (+)-catechin has 2R:3S configuration and has a shorter retention time than (-)-epicatechin 2R:3R configuration see Figures 3.5-8 to 3.5-11.

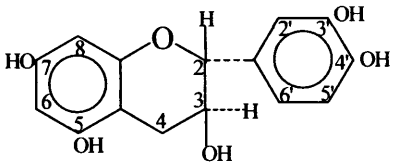

Figure 3.4-8: (+)-Catechin (2R: 3S)-3, 5, 7, 3', 4'-pentahydroxyflavan-3-ol.

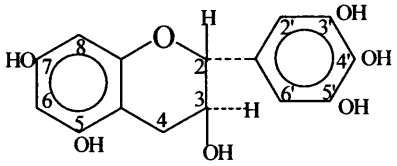

Figure 3.4-9: (+)-Gallocatechin (2R: 3S)-3, 5, 7, 3', 4', 5'-hexahydroxyflavan-3-ol.

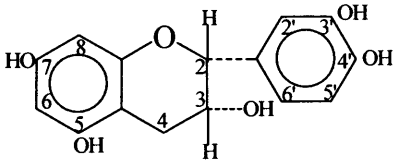

Figure3.4-10: (-)-Epicatechin (2R: 3R)-3, 5, 7, 3', 4'-pentahydroxy flavan-3-ol.

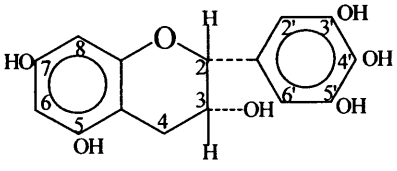

Figure 3.4-11: (-)-Epigallocatechin (2R: 3R)-3, 5, 7, 3' 4', 5' hexahydroxy flavan-3-ol.

3.4.8. SUMMARY

High performance liquid chromatography confirms the presence of quercetin, kaempferol, gallic acid, and procyanidins B1, B2, B3, B4, C1 and polymeric tannins in NBT. Elution order for NBT was found (+)-gallocatechin < (-)-epigallocatechin < (+)-catechin < (-)-epicatechin. It's also found that the R and S configuration may affect the retention time.

Within the total analysis, Fractions IIa to IId consist of:

- Fraction IIa: ECG, GC, procyanidin B1
- Fraction IIb: Polymeric tannins
- Fraction IIc: GC, EC, procyandins B3, B4, and C1
- Fraction IId: Procyanidin B3.
- All fractions contain gallic acid

3.4.9. REFERENCES

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CHAPTER 3.5

PROTON NUCLEAR MAGNETIC RESONANCE

3.5.1. PROTON NMR STUDY OF NEEM BARK TANNIN

3.5.2. INTRODUCTION

Structural complexity in the condensed tannins is centred principally on variations in hydroxylation patterns of the flavan chain extender units, the stereochemistry at the three chiral centres of the heterocyclic ring, the location and type of interflavanoid bond, and the structure of the terminal unit. A further level of structural diversity results from facile rearrangements of these compounds[1].

Proton nuclear magnetic resonance is routinely use for structure determination. The advent of ^{1}H NMR spectroscopy enabled Clark-Lewis and collaborators[2] to propose C-ring conformations approximating a half-chair, with the B-ring in an equatorial position, for a series of flavans with phenolic groups protected by methylation and with various heterocyclic ring substituents. Numerous ^{1}H NMR investigations have since then borne out these findings.

Conformational analysis of oligoflavonoids is in principle concerned with the conformation of the pyran heterocycle and with the phenomenon of conformational isomerism, owing to restricted rotation about the interflavanyl linkage.

3.5.3. EXPERIMENTAL PROCEDURE

NMR experiments were conducted using a Bruker AMX 400 spectrometer equipped with an Aspect X32 computer and a temperature probe. Spectra were obtained at a frequency of 400 MHz. All NMR studies were carried out on 0.6 ml samples, prepared in 5 mm standard NMR tubes.

Successive methylation and acetylation achieved purity of the neem bark tannin, with preparative thin layer chromatography separation, see in Section 3.4.3 and 3.4.5.2 The resultant methylated and peracetate derivatives were differentiated by ^{1}H NMR spectroscopy.

Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane. Splitting patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br).

3.5.4. RESULTS

Table 3.5-1 : ^{1}H NMR chemical shift assignments for neem derivatives.

Ring	Proton	Chemical shifts at 400 MHz in D_6 DMSO (ppm)	
		Major conformer	Minor conformer
C	H-C3	3.98 (s)	4.13 (m)
	H-C2	4.17 (s)	4.85 (s)
	H-C4	2.49 (m)	2.49 (m)
A	H-C6	5.70 (s)	5.70 (s)
	H-C8	5.87 (d, J=2.3)	5.87 (d, J=2.3)
B	H-C2'	6.87 (s)	6.63 (s)
	H-C6'	6.87 (s)	-
	H-C6'	6.64 (dd J=8.1)	-
	H-C5'	6.94 (s)	-
	CH_3OH	1.90-2.24	1.89-2.24

*s=singlet, bs= broad singlet, d=doublet, dd=double doublet, m=multiplet. For resolving coupling, J values are given in Hz.

Table 3.5-2: ^{1}H NMR chemical shift assignments of methyl triacetate derivative of [4,8: 4,8] all trans-bi-[(+)-epicatechin]-gallocatechin deca triacetate moieties in the triflavonoid of neem bark tannins.

Ring	Proton	Chemical shifts at 400 MHz in D_6 DMSO (ppm)
B	H-C2	6.63 (s)
	H-C6	6.63 (s)
C	OAc-H3	2.23 (s)
F	OAc-H3	1.89 (s)
	CH-2	2.48 (s)
I	OAc-H3	1.12 (s)
C or I	H-C4	3.98 (d)
	H-C4	4.71(d, J=9.6)
	H-C2	5.69 (s)
	H-C3	5.87 (s)
	H-C3	5.88 (d)

*s=singlet, bs= broad singlet, d=doublet. For resolving coupling, J values are given in Hz.

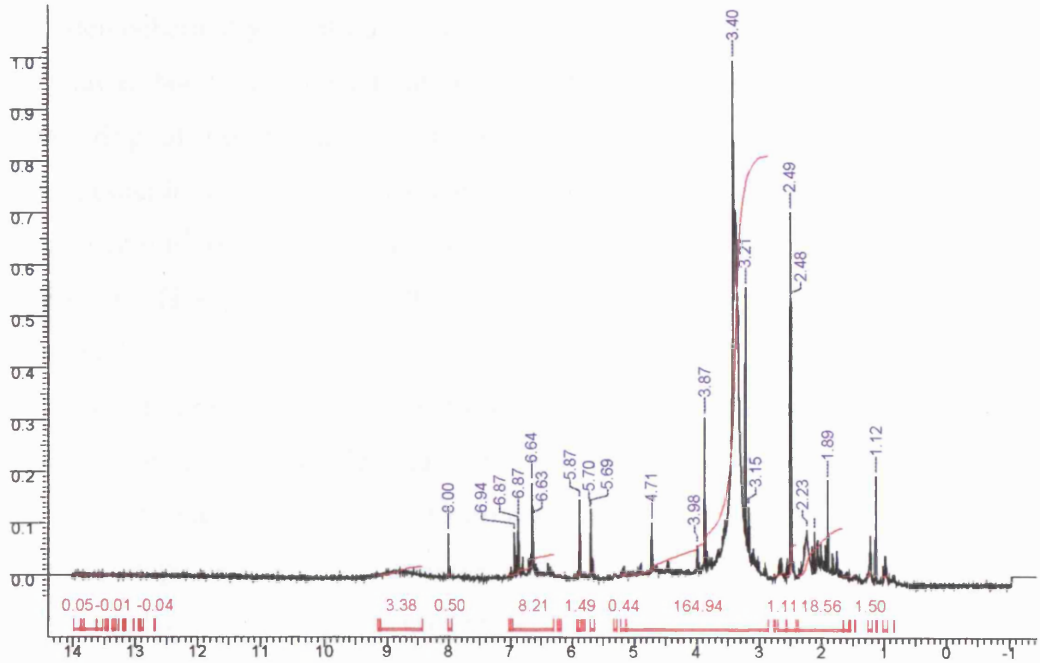

Figure 3.5-1: ^{1}H NMR spectrum of methyl derivative of NBT (aqueous extract) in D_6 DMSO.

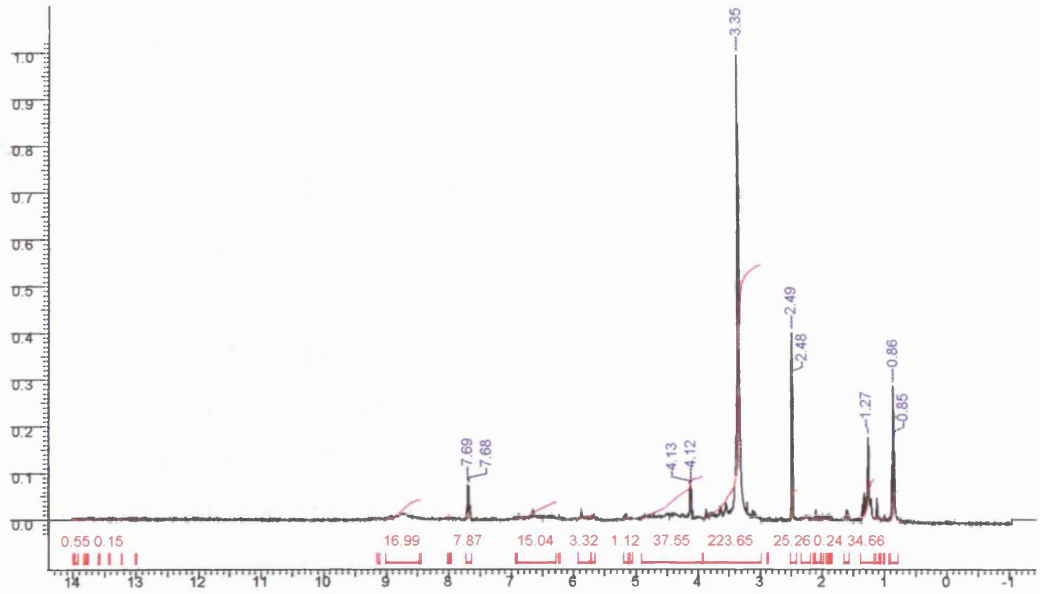

Figure 3.5-2: ^{1}H NMR spectrum of methyl derivative of neem bark tannins (acetone extract) in D_6 DMSO.

3.5.5. DISCUSSION

^{1}H NMR is used for the determination of structural features that relate to heterocyclic ring stereochemistry, configuration isomerism due to heterogeneity in location of the interflavan bond, restricted rotation about the interflavan bond and flexing of the pyran ring of neem bark tannin. Some previous work has been done on bark phytopesticide and the compounds, identified as 1-trigloyl-3-acetyl,11-methoxy azadirachtinin[3] ($C_{36}H_{46}O_{16}$; mol. wt. 734), nimbiol[4] ($C_{18}H_{24}O_2$; mol.wt. 272), and sugiol[5] ($C_{20}H_{28}O_2$; mol. wt. 300). But none of these reports mentioned tannins of neem bark.

It was assumed that dimers and oligomers dominate the neem bark tannin, so the structural features can be defined by proton NMR experiments. In neem bark tannin 2R absolute stereochemistry predominates. However, Kolodziej *et al*[16] mentioned natural compounds with 2S absolute stereochemistry do exist and are present the commercially important tannin quebracho.

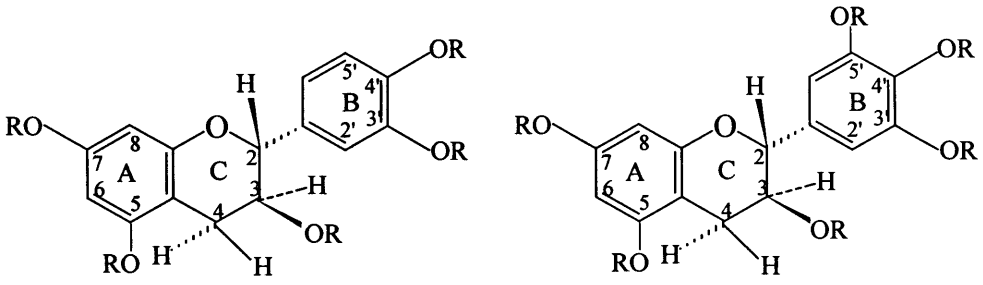

Figure 3.5-3: Structural features of neem bark tannin and sites for derivatisation: R may be H, CH_3, CH_3CO.

Allocations of the points of bonding at C-8 of the catechin and gallocatechin moieties are based on the chemical shift of the residual H-8 (see Table 3.5-2).

Examination of the different extract derivatives revealed the presence of trans-triflavonoid analogues (Figure 3.5-4). ^{1}H NMR spectra of these derivatives resemble those of the 2R series of triflavonoid procyanidins[7, 8].

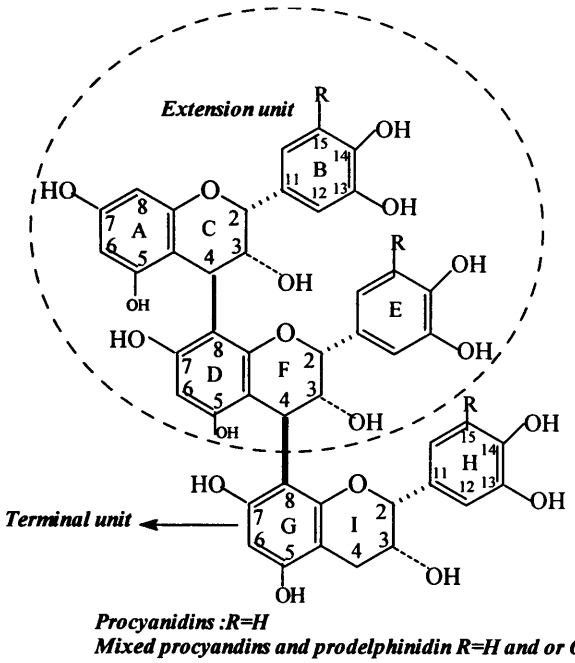

Figure 3.5-4: Triflavonoid from neem bark tannin and sites for derivatisation: R may be H, CH_3, CH_3CO.

Table 3.5-2 shows the major conformers of (+)-catechin, (-)-epicatechin and their derivatives, and the minor conformers of (+)-gallocatechin and (-)-epigallocatechin and their derivatives. The acetylated-methylated derivative of neem bark was identified as a triflavonoid; this was deduced from Figure 3.5-4, having a chemical shift of 0.85 ppm, although the analysis is subject to some uncertainty because of solvent and temperature[9]. The coupling constants ($J_{2,3}$ 10.0 and $J_{3,4}$ 8.75 Hz) indicate the 2,3-trans-3,4-trans orientation of the 'upper' unit of this compound. In the presence of (+)-catechin, condensation does not proceed significantly beyond the [4,8]-biflavonoid level, whereas with excess of the flavan-3,4-diols both [4,8]-bi- and tri-flavonoids result in sequence. Steric factors operating at the nucleophilic C-8 of (+)-catechin dominate the sequential nature of condensation up to triflavonoid level, as reported by Botha *et al*[7].

Soluble polymerics of neem bark tannin are composed of flavan-3-ol units. The majority of interflavan bonds comprise the C-4 to C-8 type. The hydroxylation patterns of the B-ring of the flavan-3-ol units or flavan-3,4-diols are 3', 4'-dihydroxy and the 3',4',5'-trihydroxy structures. Flavan-3-ol units possess the 2,3-trans (2R, 2S) stereochemistry. The interflavan bond is trans to the C-3 hydroxyl group as shown in Section 3.4.6.

The proton NMR spectrum in Figure 3.5-1 showed signals for the three protons of the catechol B-ring at 6.64 ppm (H-5', J=8.21 Hz), 5.87 ppm (H-6', J=1.49 Hz) and 5.69 ppm (H-2', J=1.49 Hz). The proton coupling constants of ortho and meta positions in aromatic ring systems are usually found in 6 to 8 Hz and 1 to 3 Hz range. In the present study, the ortho coupling was found to be 8.20 Hz and the meta coupling to be 1.87 Hz. These findings support the HPLC results presented in Section 3.4.5. The H-5' signal appeared at 6.64 ppm shifted upfield from the H-2' and H-6' signals due to the shielding effect of the OH-3' and OH-4' and due to the deshielding influence of the C-ring function on H-2' and H-6'. The A ring protons showed two doublets 6.63 ppm and 6.64 ppm, corresponding to H-6 and H-8, because the H-6 doublet consistently occurs at higher field than the H-8 due to the shielding effect of the OH-5 or OH-7. The coupling constant of the protons was 2.7 Hz and these results show that they are meta coupling. In the heterocyclic ring, the H-2, H-3 and H-4 show singlet signals at 4.71, 3.98 and 3.87 ppm respectively.

3.5.6. SUMMARY

- Neem bark tannin is dominated by 2R absolute stereochemistry
- The majority interflavan bonds comprise the C-4 to C-8 type, but some C-4 to C-6 linkages are probably present.
- Neem bark tannin consists of major conformers of (+)-catechin, (-)-epicatechin and minor conformers of (+)-gallocatechin and (-)-epigallocatechin.
- The acetylated-methylated derivative of neem bark was identified as a triflavonoid.
- The hydroxylation patterns of the B-ring of the flavan-3-ol or flavan 3,4-diol units are 3', 4'-dihydroxy and the 3', 4', 5'-trihydroxy structures.

3.5.7. REFERENCES

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CHAPTER 3.6

GEL PERMEATION CHROMATOGRAPHY

3.6.1. MOLECULAR WEIGHT PROFILE OF NEEM BARK TANNINS USING GEL PERMEATION CHROMATOGRAPHY

3.6.1.1. Gel permeation chromatography (GPC)

Gel permeation chromatography is normally used as an analytical procedure for separating small molecules by their difference in size and to obtain number average molecular weight (M_n), and the weight average molecular weight (M_w) or information on the molecular weight distribution of polymers. At times, however, it is also used for preparing various molecular weight fractions for further use. The raw data GPC curve is a molecular size distribution curve. If a concentration sensitive differential refractometer is used as a detector, the GPC curve is really a size distribution curve of weight concentration. With calibration, the raw data are converted to a molecular weight distribution curve and the respective molecular weight averages can be calculated[1].

3.6.1.2. GPC for condensed tannins

Standard techniques for estimating the molecular weights of condensed tannin polymers include: ^{13}C-NMR, vapour pressure osmometry and gel permeation chromatography.[2] Whereas ^{13}C-NMR[3] or vapour pressure osmometry[4,5] give an estimate of the number average molecular weight (M_n) of condensed tannin polymers, GPC gives both M_n and M_w. In addition, GPC gives a picture of discontinuities in the distribution of molecular weights. A sample solution is passed through the column and size sorting takes place in the packing material by an exclusion process, the largest molecules exiting from the column first, followed by those of decreasing size[6]. Analytical ultracentrifugation using GPC is perhaps the most convenient of analytical methods for many laboratories. Small sample sizes are required, which may be of the order of micrograms when using ultraviolet detection. Relatively rapid analysis times,

which vary from 10 to 20 minutes, are possible, depending on the number and types of columns used. The separation process in GPC is theoretically based solely on the entropic behaviour of solute between mobile and stationary phases. Molecular weight distribution data are obtained when the system is calibrated against monodisperse or well-characterised standards. Thus the molecular weight distribution is a secondary result limited by the availability of proper calibration on standards.

Several studies of bark tannins have used methyl ether derivatives (formed by repeated treatment with diazomethane); for example GPC of pine bark tannin[7,8]. However, methylation of polyphenols with diazomethane is a somewhat unsatisfactory procedure as it leads to a variable degree of methylation of the phenolic hydroxyls and some causes aromatic C- and O-methylation of the aliphatic 3-hydroxyl.

Polystyrene calibration standards are useful for the estimation of "apparent" molecular weights of polymers whose structures are not known or where standards with the appropriate structures are not available.

Karchesy *et al.*[2] suggested that the condensed tannins have to be derivatised as methyl ethers or peracetate in order to perform GPC analyses. His idea seems reasonable, because in their free phenolic forms, condensed tannins are likely to associate through hydrogen bonding and might exhibit higher molecular weight than they actually have; for this reason acetylated and nucleophilic derivatives of neem bark tannin were used in the present work for molecular weight profile.

Condensed tannins are polydisperse, so that in any one polymer extracted from a plant organ one can only consider average or statistical properties, as described by Porter[8]. This applies particularly to molecular weights.

GPC fractionates a polymer according to molecular size, so it seems reasonable to use polydispersity (P_d=*Mw/Mn*) which is a measure of the broadness or narrowness of the molecular weight distribution. Porter[8] described dispersities that are typically in the range of 1.5-3.0 for polymers isolated from fresh plant tissue. A value of P_d >2.0 is characteristic of polymers with some branched chains. He concluded further, from *Chaenomeles chinensis* studies, that polymers have a higher dispersity P_d >5.0 or even a double humped molecular weight distribution, when a small relative concentration of polymer occurs with chain length of several hundred units.

Molecular weight measurements of commercially significant black wattle and quebracho tannins have been done by Roux *et al*[9]. Black wattle tannins have a P_d of about 4.5 and are oligomers of up to about 10 units. Quebracho tannins have a P_d of about 6.5. Their average molecular weights and distributions are therefore similar to many sources of procyanidins and prodelphinidins.

Hergert[10] reported the molecular weight distribution of different wood water soluble and insoluble non-procyanthocyanidin tannin of red cedar wood, California redwood, western hemlock and red spruce bark using GPC: his results are summarised in Table 3.6-1.

Table 3.6-1: Molecular weight distribution of non-proanthocyanidins.

Non-proanthocyanidins	***Mn***	***Mw***	P_d
Red cedar wood			
Water soluble	687	862	1.25
Water insoluble	1575	4525	2.87
California red wood (acetylated)			
Water soluble	1105	1476	1.33
Water insoluble	977	1299	1.33
Western hemlock wood	2219	4223	1.90
Red spruce bark	1300	3554	2.73

3.6.2. EXPERIMENTAL PROCEDURE

Gel permeation chromatography analysis was carried out using a data collection unit PL-DCU (Polymer Laboratories), HPLC pump LC 1120 (Polymer Laboratories), UV detector 132 RI (Gilson Ltd.) and polymer 3μm Mixed E (300 x 7.5 mm) column (Merck, Germany). Samples for analysis were derivatives of NBT, as described in Section 3.4.3.

The samples were eluted with tetrahydrofuran (THF) OMNI SOLV (EM Merck) filtered through a PTEE membrane (0.47 μm) before use. The HPLC pump

was set at 4.4 MPa and a flow rate of 1.00 ml/minute. Polystyrene (Polymer Laboratories) was used as reference.

3.6.2.1. Calibration of GPC system

Williams *et al*[7] employed a series of μ-styragel columns (10^3 A° and 10^4 A°) with THF eluting solvent to analyse acetylated condensed tannins. Calibration was accomplished with higher molecular weight polystyrene standards. Other has employed similar techniques[10-14].

3.6.3. RESULTS

Figures 3.6-1 to 3.6-4 show the molecular weight distribution of neem bark tannins derivatives. The number average molecular weight (*Mn*) and the weight average molecular weight (*Mw*) of thiolysed neem bark tannins are set out in Table 3.6-2.

Table 3.6-2: Molecular weight distribution (based on the calibration by polystyrene standards).

Derivatives of NBT	*Mn*	*Mw*	P_d
α-toluene thiol derivatives of			
NBT extracted with water at 85 ℃	2090	3869	1.85
NBT extracted with acetone: water (1: 1, v/v)	92	177	1.92
Peracetate derivatives of			
NBT extracted with water at 85 ℃	58	345	5.92
NBT extracted with acetone: water (1: 1, v/v)	1700	3050	1.79
NBT extracted with methanol: water (95: 5 v/v)	2453	5499	2.24
NBT extracted with ethyl acetate	88	216	2.46
NBT Fraction I	1628	8889	5.46
NBT Fraction II	4626	17580	3.80
NBT Fraction IIa	396	885	2.23
NBT Fraction IIb	7029	14171	2.01
NBT Fraction IIc	92	104	1.13
NBT Fraction IId	5622	8985	1.60

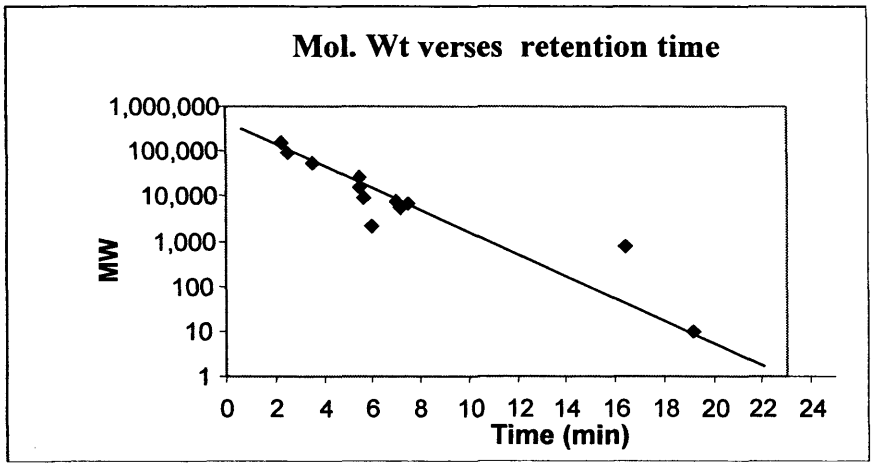

Figure 3.6-1: Calibration curve for GPC analysis with polystyrene standards.

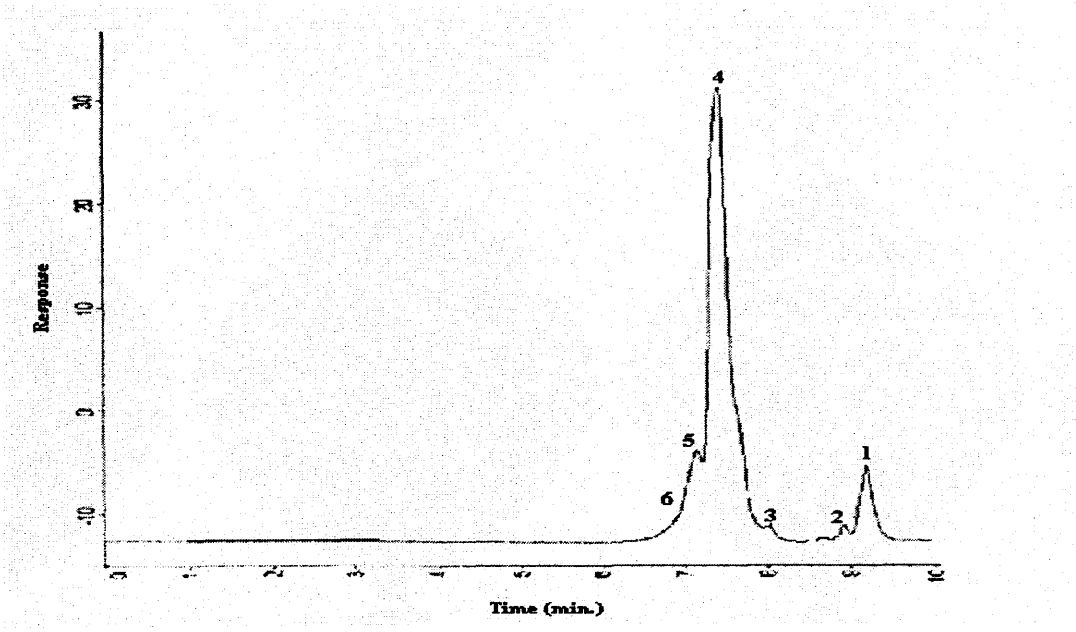

Figure 3.6-2: GPC of α-toluene thiol derivatives of neem bark tannin extracted with water at 85°C.

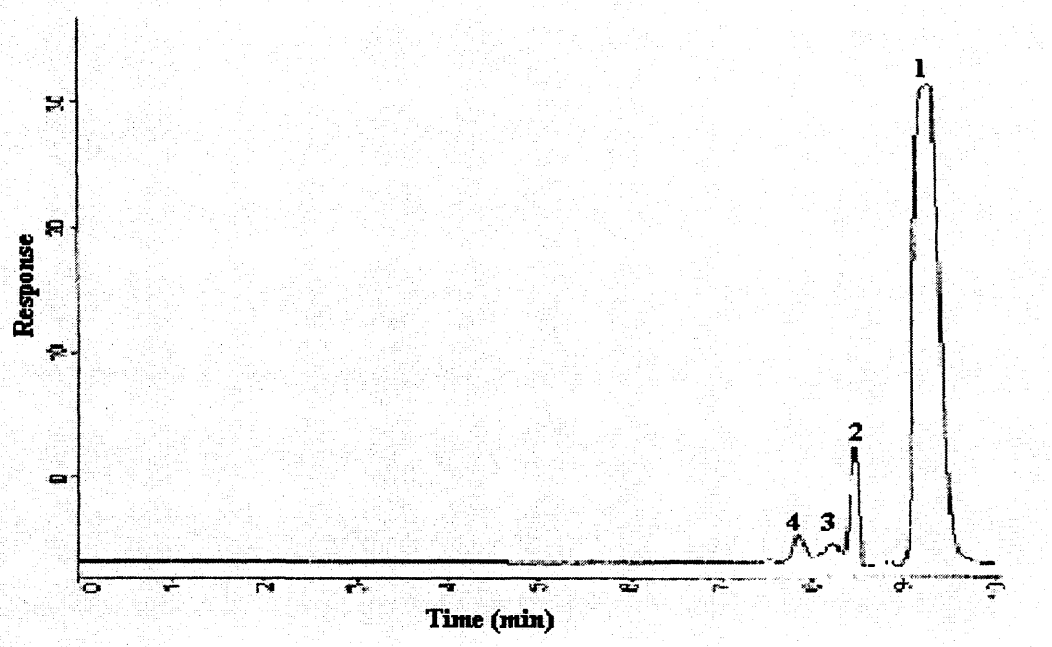

Figure 3.6-3: GPC of α-toluene thiol derivatives of neem bark tannin extracted by acetone: water.

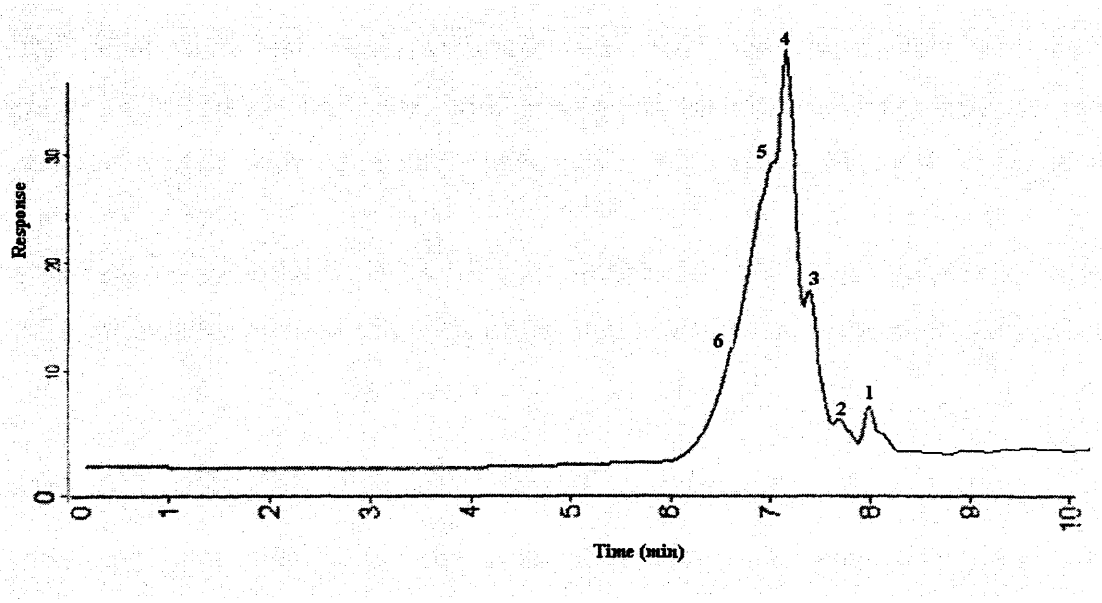

Figure 3.6-4: GPC of peracetate derivative of neem bark tannin extracted with water at 85°C.

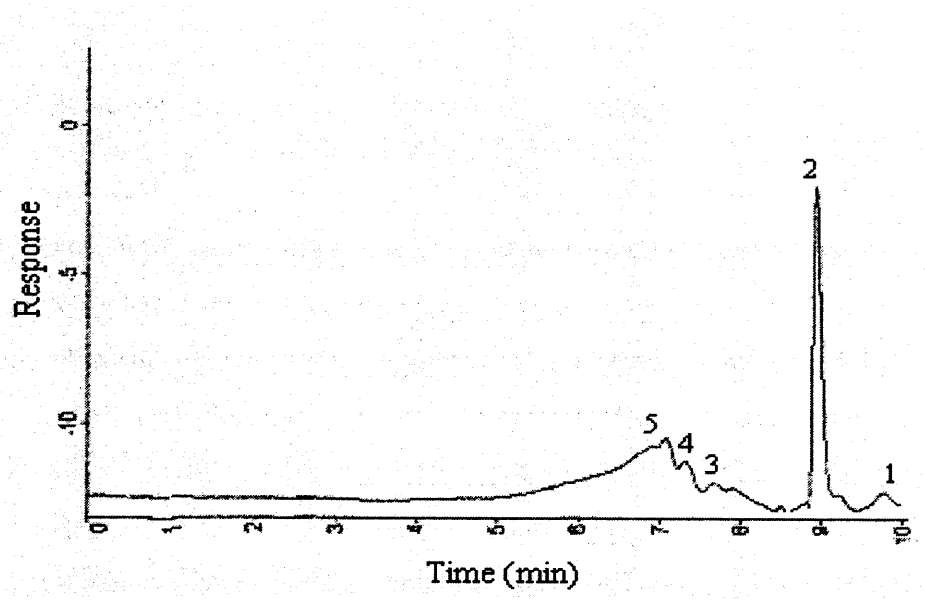

Figure 3.6-5: GPC of peracetate derivatives of neem bark tannins extracted by methanol: water.

3.6.4. DISCUSSION

In the present study, polystyrene standards were used to calibrate the GPC system (see Figure 3.6-1). System calibrated with polystyrene standards did not give the same molecular weight data for natural polymers. The existing literature explains the discrepancies observed when comparing the molecular weights of tannin that have been measured as acetate with polystyrene calibration. This situation points out that GPC is an indirect method of molecular weight analysis and its accuracy depends on the structural similarity of the standards and compounds analysed.

3.6.4.1. GPC analysis of thiolysed neem bark tannins

Considering molecular weight distribution, as shown in Figure 3.6-2, and 3.6-3 suggests NBT extracted with acetone/water derivatives gives low values of *Mn* and *Mw* compared to NBT extracted with water at 85°C, accounting for its low water solubility or presence of non-tannins materials. In addition, it is particularly noticeable that the degree of polymerisation is affected either by extraction methods or derivatisation, but there is no effect on polydispersity value, i.e., characterising of

polymer with the same basic units. Neem bark tannins consist predominantly of the tetramer.

3.6.4.2. GPC analysis of neem bark tannins peracetate

Figure 3.6-4 and 3.6-5 shows the molecular weight distribution of peracetate derivatives of NBT. Comparing four extraction systems indicates that organic solvent extraction polydispersity values are almost the same, i.e., about P_d 2.16, characterising polymers of some branched chains, tetramer or higher oligomers. Polydispersity of aqueous extraction indicates high value, characterising oligomers or large polymeric chain.

In this study, GPC was used to measure the molecular weight distribution of neem bark tannins only, but fractions of NBT were also investigated for characterising active components. It has been observed that polydispersity decreases from higher to lower values, as fraction I to IId see Table 3.6-2, which shows the varying and complex chemical nature of neem bark.

3.6.5. REFERENCES

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CHAPTER 3.7

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

3.7.1. GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF NEEM BARK TANINS

3.7.2. INTRODUCTION

So far the application and limitations of gs-MS have not been thoroughly investigated for the plant polyphenols. Rapid advancement in the field of pyrolysis gas chromatography mass spectrometry Py/gc/MS or gc-FT-IR analyses makes this technique more powerful for the structural study of the complex plant polyphenol component with merger of different chromatographic techniques. PY/gc-MS is well established for the study of lignin. Galletti[1] and Teresa[2] recently studied analytical pyrolysis and thermal assisted hydrolysis-methylation of wine tannin.

Pyrolysis mass spectrometry (PyMS) is a relative simple method to obtain information from plant tissue at the molecular level[3]. Phenolics of low masses are produced by pyrolysis of polyphenols at 500-1000°C in an inert atmosphere[1]; fragments are analysed by gc-MS and allow chemical characterisation of the original sample. When samples are pyrolysed in the presence of tetramethylammonium hydroxide (TMAH), a so-called thermally assisted hydrolysis-methylation (THM) occurs[4]. By THM, formerly called simultaneous pyrolysis methylation[5], hydroxyl groups are methylated, thus improving the gas chromatographic behaviour of more polar compounds.

3.7.3. EXPERIMENTAL PROCEDURE

3.7.3.1. Materials

Acetone (BDH, UK) was analytical grade.

Methylated neem bark tannin: The derivatisation of neem bark tannin was as described in section 3.4.3.

Preparation of samples:

All samples, 2 mg, were dissolved in 1 ml acetone and stored in a screw top sealed bottle prior to use.

3.7.3.2. Apparatus

3.7.3.2.1. Gas chromatograph

A gas chromatograph, Hewlett Packard 5890 Series II, was equipped with a BPX 5 (SGE) column (30 mm long x 0.32 mm, inner diameter, and film thickness 0.25 μm). The auto injector was a Hewlett Packard 7673.

3.7.3.2.2. Mass spectrometer

A Hewlett Packard 5971A mass selective detector ion trap mass spectrometer, with upgraded short transfer line, was operated under electron impact at 70 eV. Mass spectra were recorded in the range 250-800, and 350-1800 AMU (1 scan/s), using the same temperature programme as described in methods. A Hewlett-Packard ChemStation (DOS based), was used to generate the chromatograms. Peak identification was based on mass spectra.

3.7.3.3. Methods

10μl of each sample was injected using the auto sample injector for gc analysis. The injector temperature was 280°C in the split mode. The oven conditions were initially 50°C for 10 minutes heated at 5°C/min up to 300°C then held for 1 min, with helium carrier gas at 5-psi column head pressure. Standards were isolated from sorghum (kindly provided by Prof. Haslam).

3.7.4. RESULTS

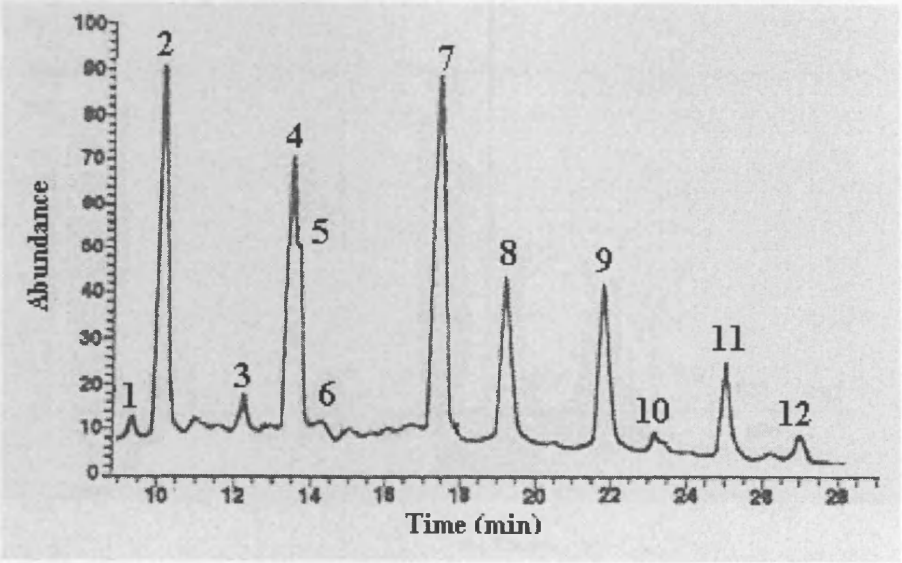

Figure 3.7-1: Gas chromatogram of methylated neem bark tannin extracted with water at 85°C.

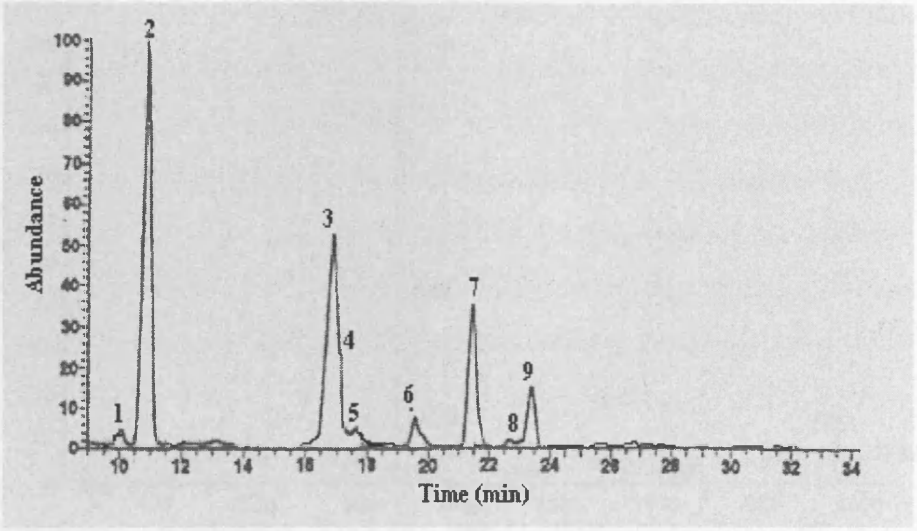

Figure 3.7-2:Gas chromatogram of methylated neem bark tannin extracted with acetone: water (1: 1v/v).

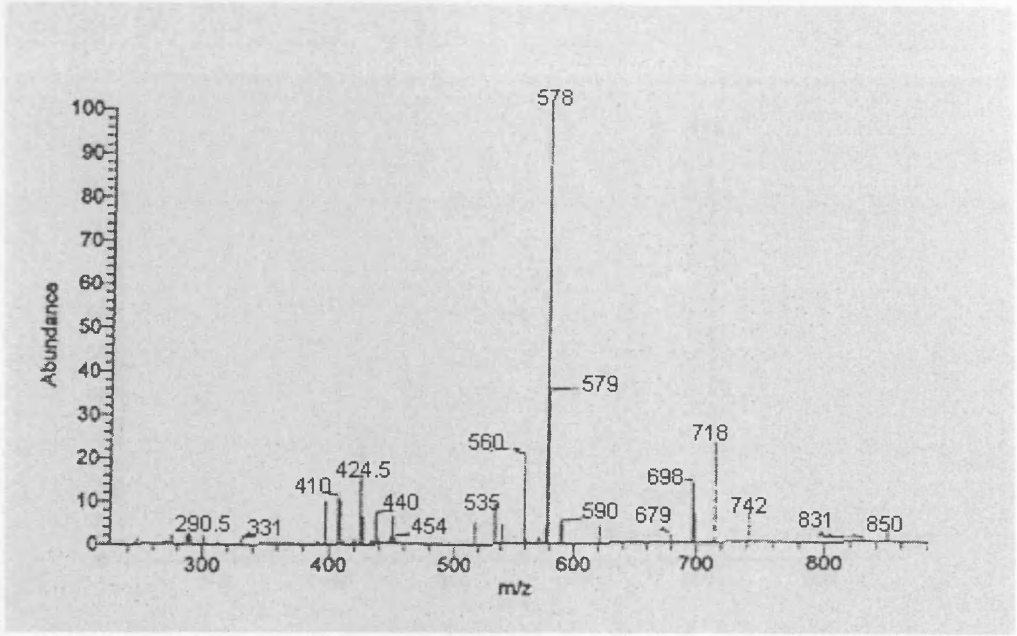

Figure 3.7-3: Mass spectrum of peak 2 of neem bark tannin extracted with water at 85°C.

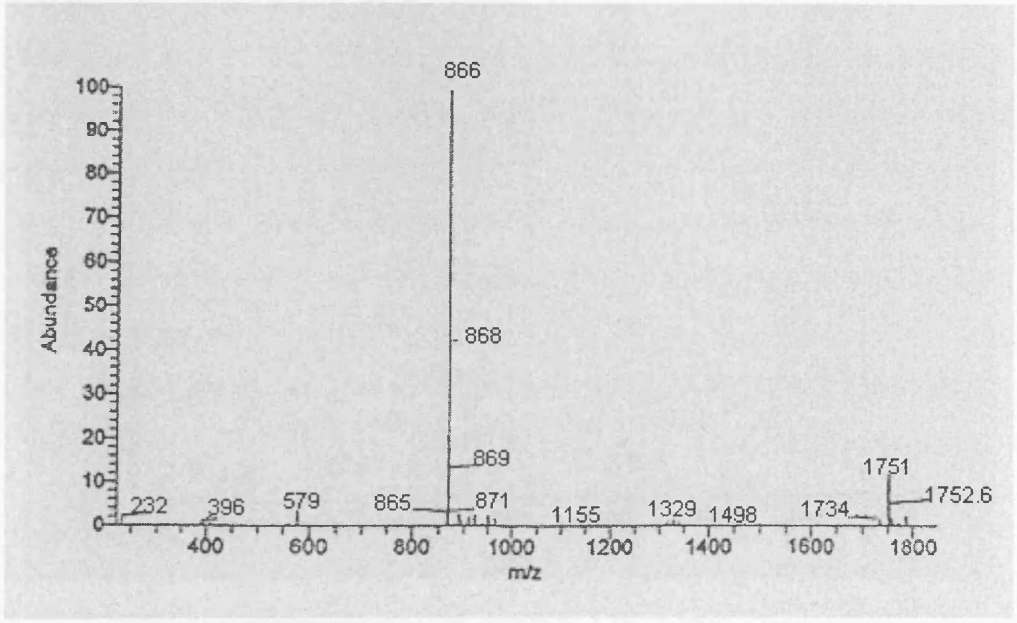

Figure 3.7-4: Mass spectrum of peak 2 of neem bark tannin extracted with acetone: water (1: 1v/v).

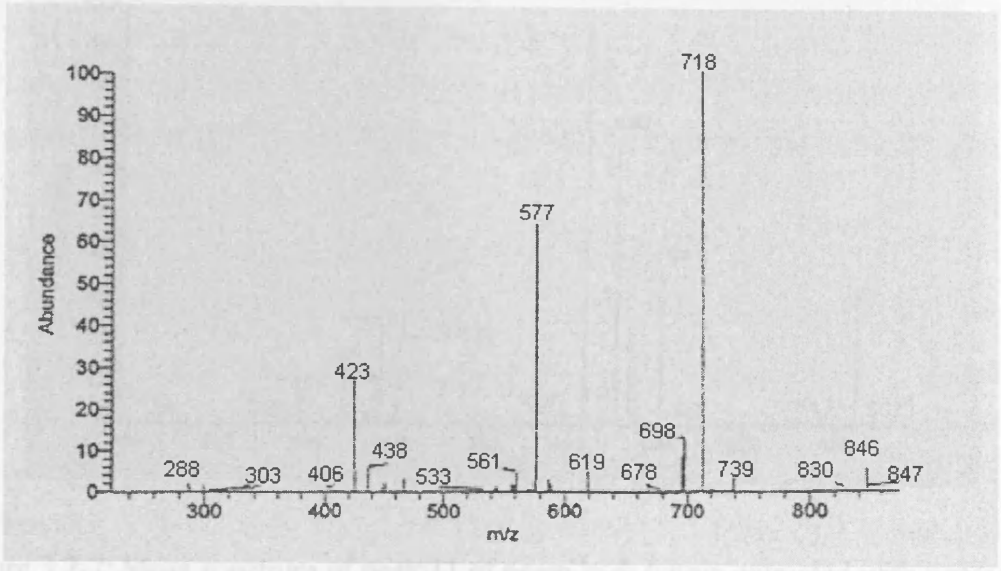

Figure 3.7-5: Mass spectrum of peak 7 of neem bark tannin extracted with water at 85°C.

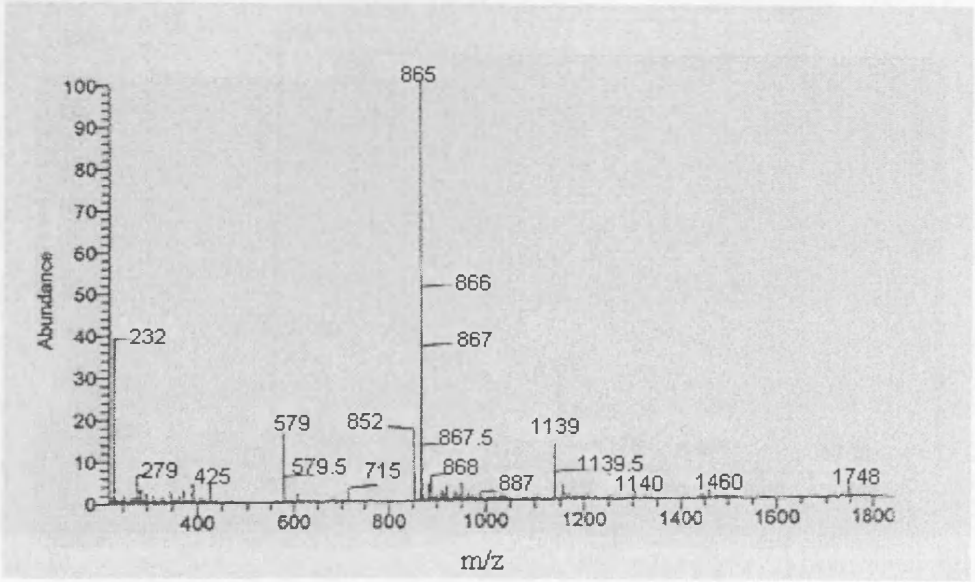

Figure 3.7-6: Mass spectrum of peak 7 of neem bark tannin extracted with acetone: water (1: 1v/v).

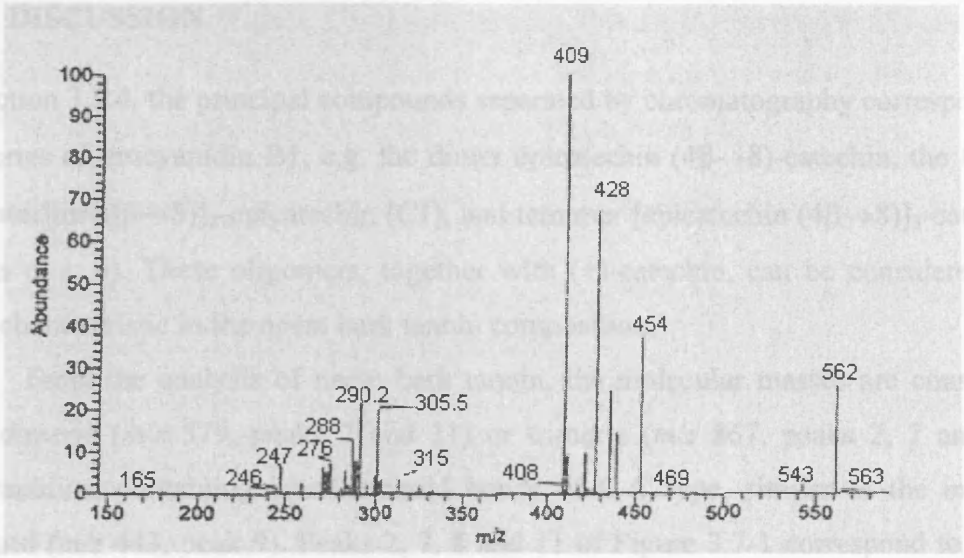

Figure 3.7-7: Mass spectrum of peak 11 of neem bark tannin extracted with water at 85°C.

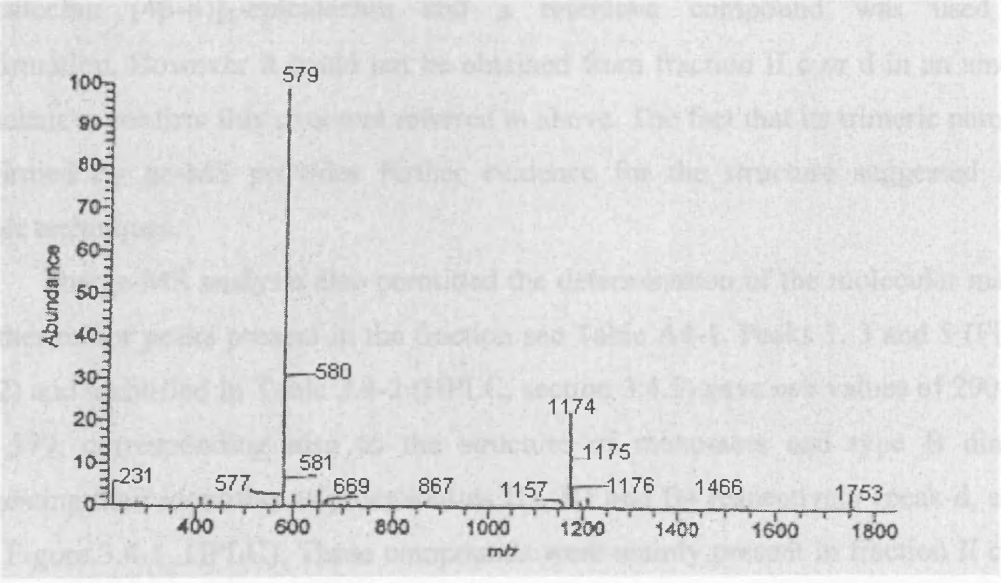

Figure 3.7-8: Mass spectrum of peak 11 of neem bark tannin extracted with acetone: water (1: 1v/v).

3.7.5. DISCUSSION

In Section 3.4.4, the principal compounds separated by chromatography correspond to the series of procyanidin B1, e.g. the dimer epicatechin (4β→8)-catechin, the trimer [epicatechin (4β→8)]$_2$-epicatechin (C1), and tetramer [epicatechin (4β→8)]$_3$-catechin (peaks e, s, u). These oligomers, together with (+)-catechin, can be considered the most characteristic in the neem bark tannin composition.

From the analysis of neem bark tannin, the molecular masses are consistent with dimeric (*m/z* 579, peaks 7 and 11) or trimeric (*m/z* 867, peaks 2, 7 and 12) procyanidins, containing interflavonoid bonds of C-C type, similar to the internal standard (*m/z* 443, peak 9). Peaks 2, 7, 8 and 11 of Figure 3.7-1 correspond to those numbered d, e, g and h in the HPLC chromatogram of Figure 3.4-1 (section 3.4.4). The mass spectra obtained confirm the identifications carried out from the thiolysis. Peak 9 of Figure 3.7-2 corresponds to peak 's' in the chromatogram of Figure 3.4-1 (HPLC): considering its retention time, this compound might be the trimer [epicatechin (4β-8)]$_2$-epicatechin and a reference compound was used for confirmation. However it could not be obtained from fraction II c or d in an amount sufficient to confirm this structure referred to above. The fact that its trimeric nature is confirmed by gc-MS provides further evidence for the structure suggested from earlier techniques.

The gc-MS analysis also permitted the determination of the molecular masses of other minor peaks present in the fraction see Table A4-1. Peaks 1, 3 and 5 (Figure 3.7-2) and identified in Table 3.4-2 (HPLC, section 3.4.5) gave *m/z* values of 290-355 and 579, corresponding also to the structure of monomers and type B dimers, suggesting their identities as procyanidins B3, B1 and B4 respectively (peak-d, e and g in Figure 3.4-1, HPLC). These compounds were mainly present in fraction II c and d, from which they were isolated and identified, but only the retention time could be used as a criterion for their identification in fraction II d. Peaks 6 and 8 (Figure 3.7-1) gave molecular ions at 865 and 577 respectively, two units lower than expected for the trimers and dimers with C-C interflavan linkage.

Peaks 2 and 6 (Figure 3.7-2) gave spectra with molecular ions at 881 and 593, respectively. It might be considered that these compounds are a procyanidin trimer and dimer in which one of the hydroxyl groups is substituted by a methoxyl.

3.7.6. SUMMARY

- The major peaks molecular mass (*m/z)* values were found to be 578 and 866, corresponding to procyanidin dimer and trimer.
- The minor peaks have molecular mass (*m/z)* values 360, and 578, corresponding to structures of monomers and type B dimers.

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CHAPTER 3.8

INFRARED SPECTROSCOPY

3.8.1. INFRARED SPECTROSCOPY OF NEEM BARK TANNINS

3.8.2. INTRODUCTION

Infrared radiation promotes transitions between rotational and vibrational energy levels of the molecules, so it involves examining the twisting, bending, rotating, and vibrational motions of atoms in a molecule. Upon interaction with infrared radiation, portions of the incident radiations are absorbed at specific wavelengths. The multiplicity of vibrations occurring simultaneously produces a highly complex absorption spectrum that is uniquely characteristic of the functional groups that make up the molecule and of the overall configuration of the molecule as well.

Spectral comparisons are normally made in dilute solutions, since a pure compound may crystallise in different forms, each with a characteristic solid phase spectrum but giving identical dilute solution spectra. Furthermore, optical isomers show identical spectra in solution, but racemates and enantiomers may give different spectra in the solid state owing to different packing arrangements within the crystal, and, therefore, no conclusions concerning the identify for enantiomers can be drawn.

Few investigations into the infrared spectroscopy of flavones, flavanols, flavanones and isoflavones are to be found. They are confined to the work of Hergert and Kurt[1], Inglett[2], Looker and Hanneman[3], Shaw and Simpson[4], Wagner[5], and Briggs and Colebrook[6]. These authors discussed refluxing solutions of salts (alkali metals and ammonia at pH 6.0 to 7.5) cause racemisation and/or conversion to the corresponding flavanol, flavanones and isoflavones.

3.8.3. EXPERIMENTAL PROCEDURE

3.8.3.1. Instrument

A Perkin-Elmer-781 infrared spectrophotometer was used, with the baseline adjusted to 100% at 4000 cm^{-1} with no sample in the optical paths. The instrument settings were: slit 3; scan time 6 min; noise filter 1; abscissa expansion 0.5. The spectrum of polystyrene was recorded over the range 4000 to 600 cm^{-1} for calibration purposes.

3.8.3.2. Preparation of KBr discs

The sample (1 part) and KBr (200 parts) were placed in the closed tube together with two 3/16" balls and vibrated for 5 minutes. Some authors have preferred to measure spectra in the solid state and others in Nujol, carbon tetrachloride, or dioxane. We have carried out our investigations using only KBr pellets, since most naturally occurring flavonoids are insoluble in the above-mentioned solvents.

3.8.4. RESULTS

Table 3.8-1: Frequencies of the main IR spectral peaks (cm^{-1}) of neem bark tannins.

Extract at 20°C	Extract at 60°C	Extract at 85°C	Methanol extraction	Acetone extraction	Ethyl acetate extraction
730			730	735	
795		780	790-760	770	
	800			800	
830	830	830-810	825		
875		855		845	
	1060	980	1070		
	1100	1115	1110		1110
1140	1140	1170-1160	1150	1150	
			1205		
1290	1285	1290	1290	1290	1290
1280-1215					
1470-1445	1450	1455	1450	1470-1450	1445
1530	1520	1520	1520-1540	1520-1550	1525
1630-1610	1615	1630-1620	1630-1610	1640-1610	1630-1610
				1720	
3500	3500	3500	3500	3500	

Table 3.8-2: Frequencies of the main IR spectral peaks (cm^{-1}) of neem bark tannin fractions.

Fraction I	FractionII	Fraction IIa	Fraction IIb	Fraction IIc	Fraction IId
745		730	730-760		630-720
780		780	780		780
					808
850-830		830	840		840
					895
				1050	1060-955
1120	1060-1035	1110			
1160	1130			1130	1160-1130
	1260-1210				1265
1300			1310		
1460	1480-1430	1450	1465	1490-1420	
1550-1530		1550-1530	1550-1535		
1650-1620		1615			
			1750		1710
	2990-2230			2960-2080	
3500	3500	3500		3500	3500

Table 3.8-3: Frequencies of the main IR spectral peaks (cm^{-1}) of monomers of polyphenols and commerical tannins.

Catechin	Epicatechin	Gallocatechin	Epigallocatechin	Mimosa	Quebracho
740	630-740	630-735	680-630	640-725	
780	795	770	770-735	780-750	780
	850-810	830-800	830-800	820	800
890	890-880	880		860	850
990-930	995-920	1040-990	1030	1045	980
		1150-1100	1150	1180-1135	1170-1120
		1295-1200	1295-1200		1290
	1365-1335	1390-1370			
	1495-1410	1470	1470	1470	1465
	1550-1530	1560-1520	1560-1525	1520	1520
1665-1610	1660-1620	1640-1615	1650-1615	1640	1610
1890	1890	1740	1740		
2980-2080		2945	2945		
3500	3500			3500	3500

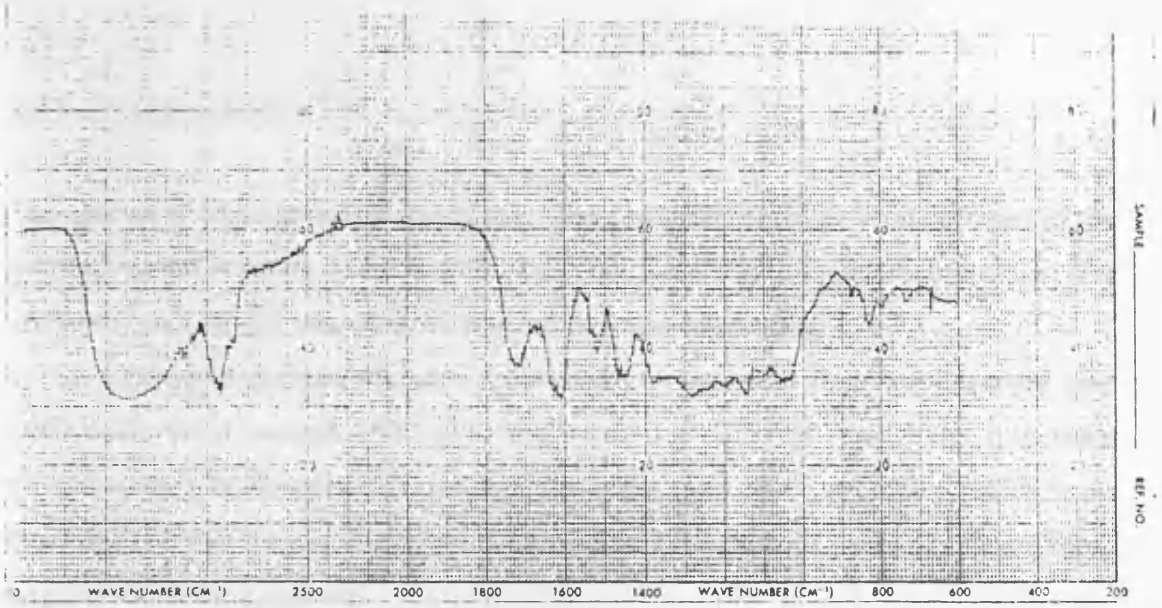

Figure 3.8-1: Infrared spectrum of neem bark tannins extracted with water at 20°C.

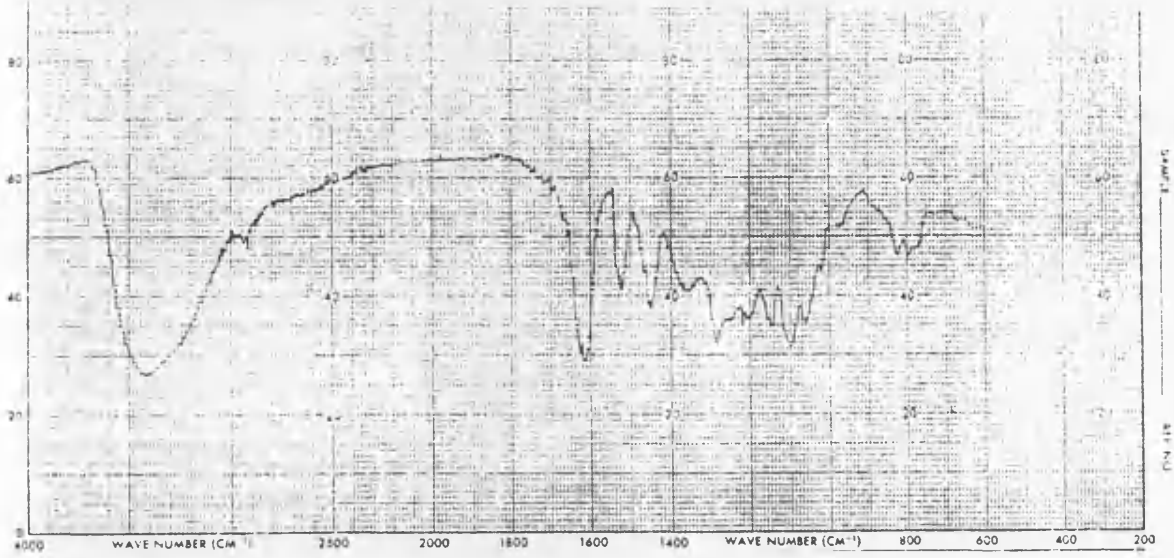

Figure 3.8-2: Infrared spectrum of neem bark tannins extracted with water at 60°C.

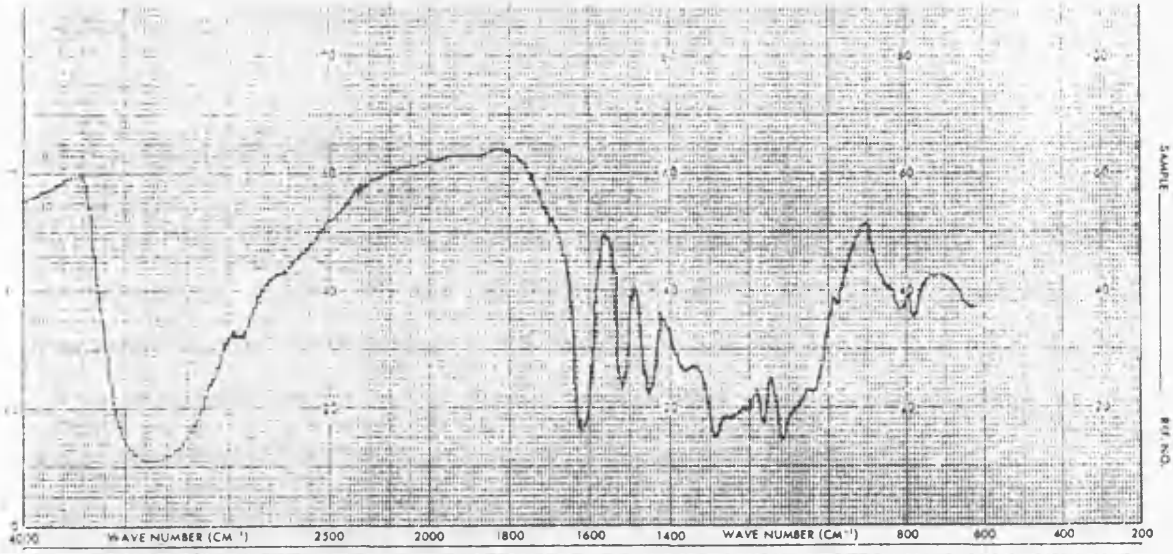

Figure 3.8-3: Infrared spectrum of neem bark tannins extracted with water at 85°C.

3.8.5. DISCUSSION

The spectra of neem bark tannin and other tanning materials have been obtained in the infrared region between 4,000 and 600 cm^{-1}, but the results obtained between 650 and 1650 cm^{-1} will be discussed since these are the most interesting.

Prominent skeletal vibration frequencies for aromatic rings are observed near 1600 cm^{-1}, from around 1500-1450 cm^{-1} and near 825-730 cm^{-1}. The respective vibrations may be described as quadrant stretching, semicircle stretching, and sextant bending. The two former vibrations consist of two components each, which can often be resolved in the spectrum.

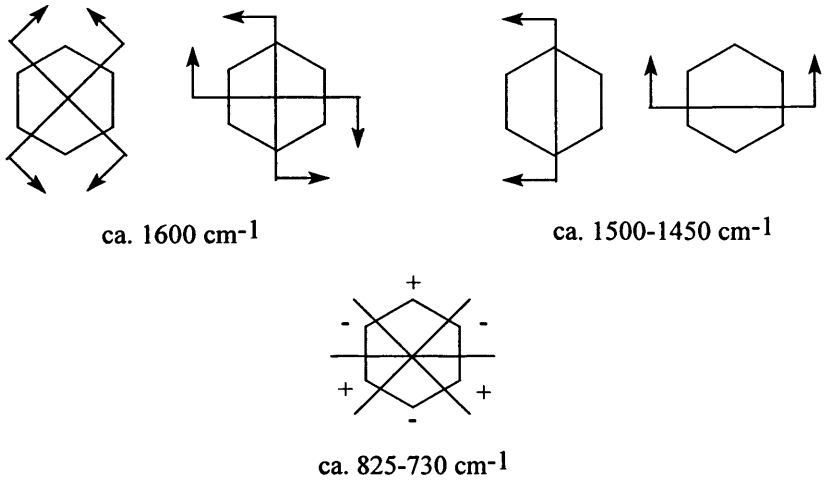

Figure 3.8-4: Possible vibrations in the aromatic rings.

3.8.5.1. The 1550-1535 cm^{-1}region

1550-1535 cm^{-1}region contains a single peak in the spectrum commonly attributed to the stretching modes of the aromatic B-ring of catechin, epicatechin, and procyanidins: this can been seen in all spectra, except Figures A5-2 to A5-6 and A5-20 in Appendix A-5, where double peaks were noted, corresponding to the gallocatechin and epigallocatechin aromatic B-ring deformation.

In the 1550-1530 cm^{-1} region, doublets were noted in Fraction I, II, IIa, only apparent in the spectra of neem bark tannin containing about 45% prodelphinidin unit (i.e., gallocatechin). This is consistent with the observation that only a single band is

present in the spectrum of the mixed dimers. However, the IR spectrum of Fr. IIc shows some degree of broadening in the band at about 1485-1430 cm^{-1}, which is aromatic ring C-C stretching at low energy (lower wavenumber) than olefinic C=C stretch.

The upper component of this doublet is usually above 1470 $cm^{-1}$for stretching and deformation of C-C in the aromatic ring: 1510-1470 cm^{-1} for, ortho-, and meta-substitution: 1525-1480 cm^{-1} for para-substitution. The lower component of the doublet absorbs at 1465-1430 cm^{-1} for, ortho- and meta-substitution and 1420-1400 cm^{-1} for para-substitution. In the case of flavonoids of neem bark tannin, these wavenumbers represent the stretching of the aromatic B-rings of catechin and gallocatechin units.

The distinction of singlet and doublet bands in these regions is due to an extra hydroxyl group in the B-ring of gallocatechin. The IR spectra of polymeric proanthocyanidins of known structures indicate that these distinguishing vibrational characteristics are equally applicable to the polymers[7], present in the IR spectra of Fr. I, II and IIa, which contain predominantly prodelphinidins polymers. The rest of the IR spectra show a singlet in the region of 1550-1520 cm^{-1}, due to procyanidins polymers.

3.8.5.2. The 800-725 cm^{-1} region

According to Foo[7] this region indicates the B-ring hydroxylation pattern where the out-of-plane deformations of the hydrogen atoms of the aromatic rings absorb, since the deformation frequency is known to be determined by the position rather than the nature of the substitutions[8]. Most importantly, in the neem bark tannin spectrum of the mixed dimer where the upper unit is gallocatechin and the lower unit is catechin, the absorption bands at around 780 and 740 cm^{-1} are of about the same order of intensity (see Figures 3.8-1, 2 and A5-1, 4, 6, 9-11, 16-18 in Appendix A5). These bands are weak but are a clear demonstration of the presence of condensed tannins, according to their B-ring hydroxylation pattern. The intensities observed for the respective bands in the infrared spectra of the neem bark tannin and other vegetable materials correspond to Figure 3.8-4, which shows possible vibrations i.e., stretching, twisting and bending of aromatic ring at certain frequencies.

3.8.5.3. Catechin and gallocatechin configuration

The pattern of catechin and gallocatechin configuration was observed in IR studies see Section 3.4.6. The nature of the configuration can be seen in the 800-780 cm^{-1} region (see Figures 3.8-2, 3 and A5-2, 4, 9-11, 13, 17, 18). The presence of neighbouring bands, particularly the procyanidins bands 780-760 cm^{-1}, tends to obscure this weak band, but shows both *cis* and *trans*-type configuration. The discussion is confined to these two regions because the differences in the vibrational frequencies in these regions are simply related to the structures of condensed tannins.

The second most prominent band in the 1640-1595 cm^{-1} region shows the aromatic A ring C-C stretching. The position of this band varies slightly with the stereochemistry of the A-ring, as has been noted by Rosenkrantz and Skogstrom[9]. These stereochemical shifts show parallelisms with the two regions discussed above. Several other bands characteristic of neem bark tannin and other tanning materials are listed in Tables 3.8-1 to 3.8-4.

All the spectra of neem bark tannins from different extraction systems show the same absorption band patterns as the methanol and ethyl acetate extracts (Figures A5-2, 3). In general, the spectra indicate a resemblance between NBT and quebracho. The fractions of neem bark tannin show similar absorption bands, but Fr. II and IIc show bands at 2080-3500 cm-1 region, which characterise C-H stretching of aromatic type compounds and also O-H stretching of carboxylic acids.

3.8.6. REFERENCES

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CHAPTER 3.9

FOURIER TRANSFORM INFRARED (ATTENUATED TOTAL REFECTANCE) STUDY OF NEEM BARK TANNINS

3.9.2. INTRODUCTION

3.9.2.1. Fourier-Transform Infrared Spectroscopy

In any spectroscopic technique, a polychromatic beam of radiation must be acted upon so that each frequency of the radiation is somehow differentiated from the other frequencies, in order that the intensity of each frequency, or, more precisely, resolution, can be measured. Conventionally, passing the beam through a prism or reflecting it from a grating so that each frequency is distinguished spatially carries out this differentiation. In FT-IR, the use of an interferometer rather than a monochromator gives important advantages for the measurement of infrared spectra.

The basic component of most FT-IR spectroscope is the Michelson interferometer[1]. This is not the only interferometer used in FT-IR, but it is employed more often than other designs.

3.9.2.1.1. FT-IR Advantages

There are three advantages to the technique[2], which make FT-IR superior to conventional infrared spectroscopy.

3.9.2.1.1.1. Fellgett's Advantage

This represents the fundamental advantage over dispersive spectrometers, in that the information from all frequencies in the spectrum is gathered simultaneously, a given interferogram can be measured in the time that it takes to measure one resolution by a slit spectrometer with the same source, detector and optical throughput.

3.9.2.1.1.2. Jacquinot's Advantage

This advantage represents the improvement in the optical throughput. Whereas the only radiation reaching the detector of a dispersive spectrometer has passed through the entrance and exit slits of the monchromator, there is no obstruction in an interferometer other than the mirrors.

3.9.2.1.1.3. Conne's Advantage

This advantage is found for certain instruments that use a laser to monitor the optical retardation. Accurate and precise measurement in the interferogram yields accurate frequency assignment to the spectral values. This does not give an improvement to signal-to-noise ratio over dispersive spectrometers, but does give large advantages in terms of frequency accuracy of the measured spectrum.

3.9.2.1.2. Reflectance methods

Reflectance techniques can be used for samples, which are difficult to analyse, by normal transmittance methods. Reflectance methods can be divided into two categories: internal reflectance measurements can be made by using an attenuated total reflectance (ATR) cell in contact with the sample, while there are also external reflectance measurements which involve an infrared beam being reflected directly from the sample surface.

3.9.2.1.2.1. Attenuated Total Reflectance (ATR) Spectroscopy

Attenuated total reflectance (ATR) spectroscopy utilises the phenomenon of total internal reflectance. A beam of radiation entering a crystal will undergo total internal reflection when the angle of incidence at the interface between the sample and the crystal is greater than the critical angle[2]. The critical angle is a function of the refractive indices of the two surfaces. The beam penetrates a fraction of a wavelength beyond the reflecting surface and when a material which selectively absorbs radiation is in close contact with the reflecting surface, the beam loses energy at the wavelength where the material absorbs. The resultant attenuated radiation is measured and plotted as a function of the wavelength by the spectrometer and gives rise to the absorption spectral characteristics of the sample[3].

Yunnus[4] has reported the FT-IR spectra of collagen and assigned the major bands for amides 1650 cm^{-1}, 1540 cm^{-1}, 1236 cm^{-1}, and 1260 cm^{-1}; COO^- group, 1403 cm^{-1}, CH_2 and CH_3, 1447 cm^{-1}, in the range from 2000 to 400 cm^{-1}.

This study revealed that this technique could provide overall information on molecular changes during interaction between the substrate and vegetable tannin material: in another way this technique can be used to investigate penetration of vegetable tanning material into skins/hides after different intervals and with process variations.

3.9.3. EXPERIMENTAL PROCEDURE

3.9.3.1. Materials

NBT treated leathers were obtained from different extracts applied to sheepskin pieces (11 x 13 cm), using the recipe process recepie as described in Section B 1-6 Appendix B. Samples were split to the thickness 0.75mm.

3.9.3.2. Instrument

The spectroscope was a Mattson Polaris, fitted with Attenuated Total Reflectance (ATR) sampling device. Spectra were acquired over the wavelength range 800-4000 cm^{-1}: the spectrum of the sample holder was used as background blank.

3.9.4. RESULTS

Figures 3.9-1 to 3 and A6-4 to 5 (Appendix A-6) show the FT-IR (ATR) spectra obtained from neem bark tanned leathers. The FT-IR (ATR) bands assignments, reported in Table 3.9-1, were made according to literature data[4,5].

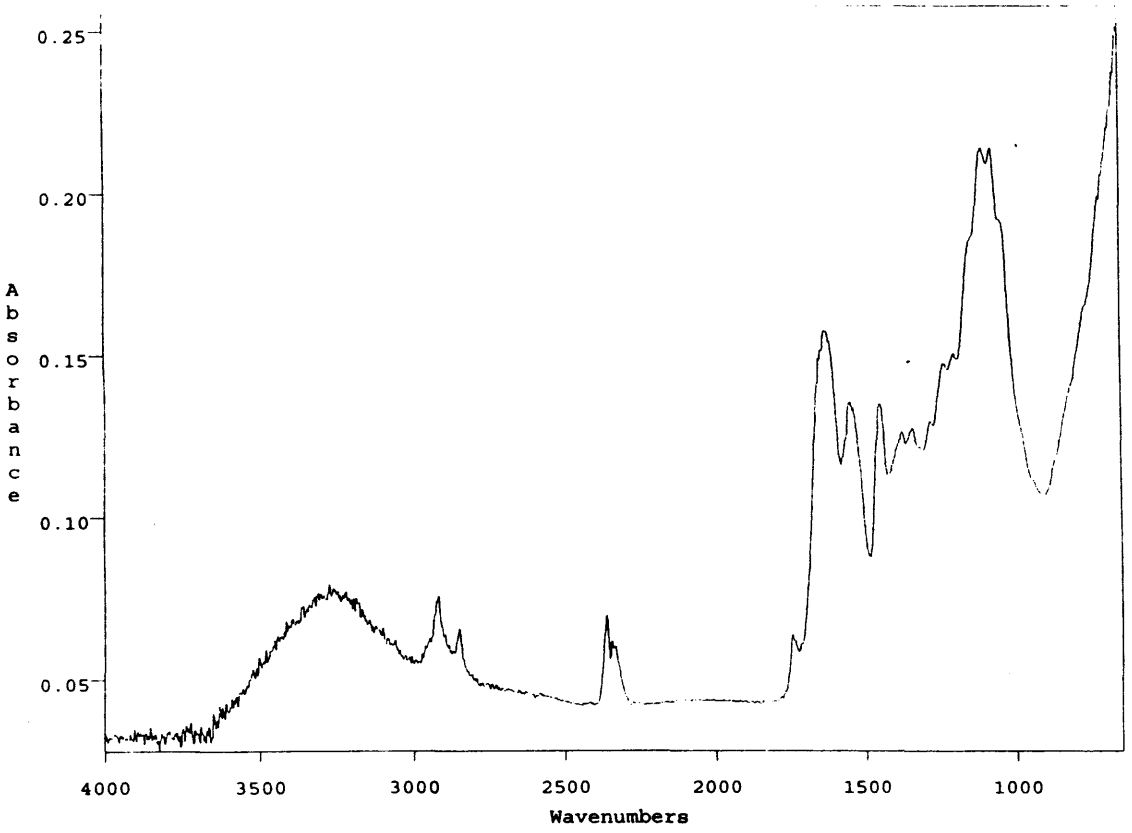

Figure 3.9-1: FT-IR spectrum of NBT tanned leather extracted with water at 85°C.

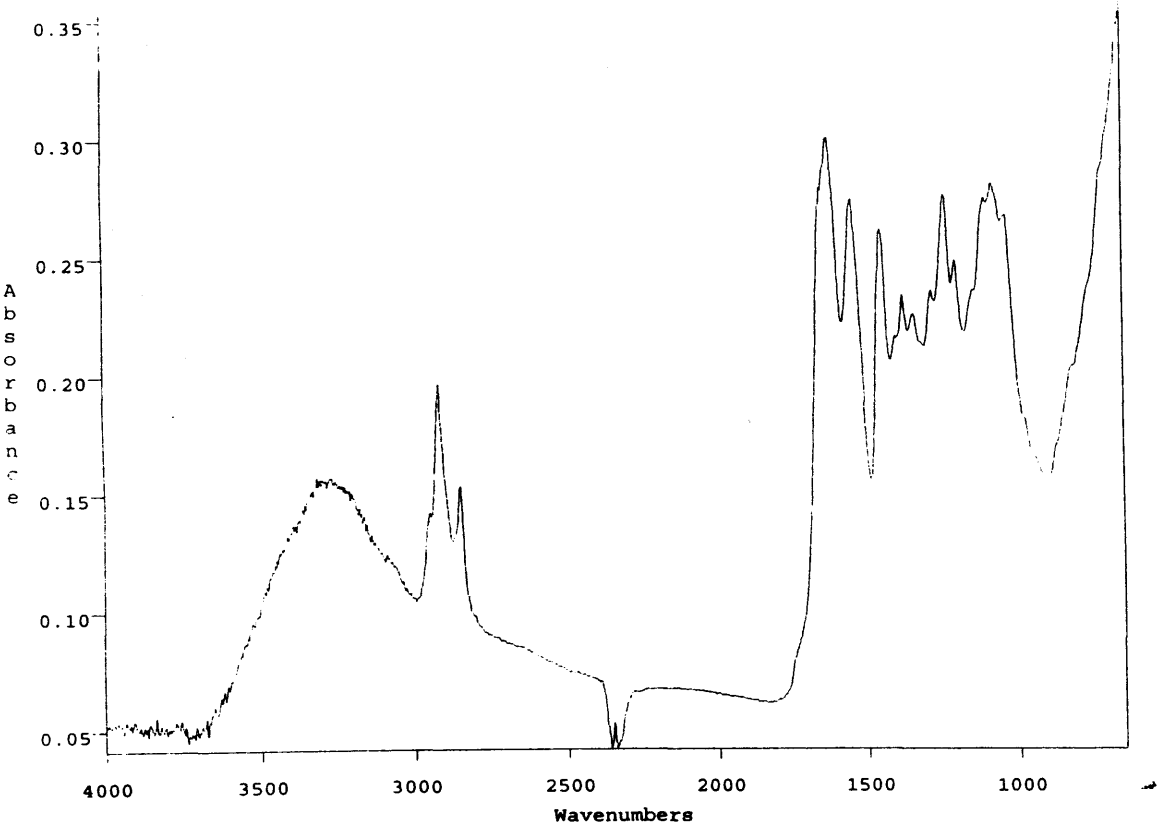

Figure 3.9-2: FT-IR spectrum of NBT tanned leather extracted with methanol: water.

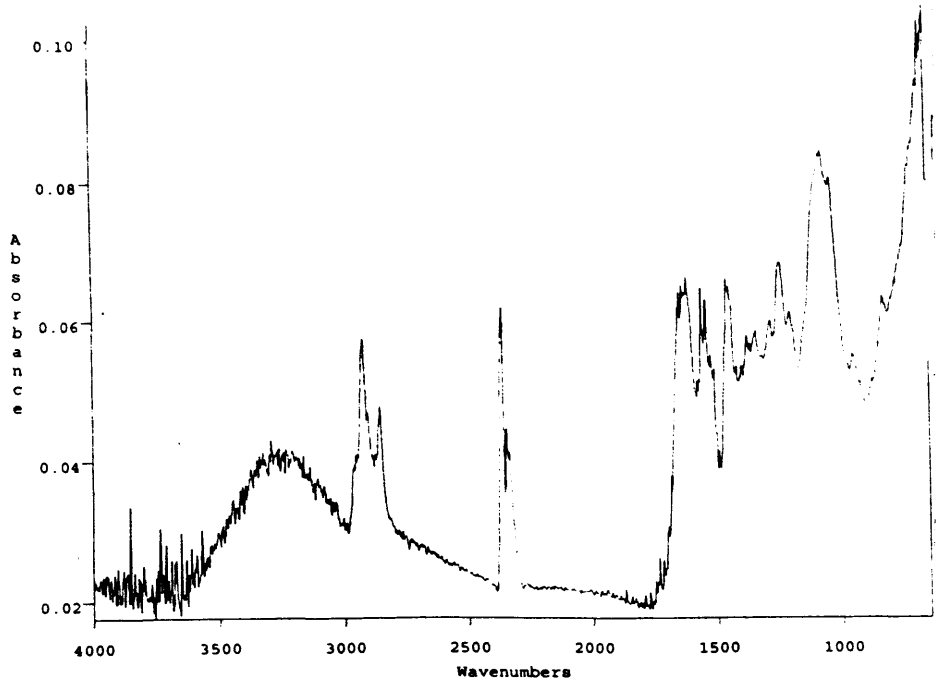

Figure 3.9-3: FT-IR spectrum of NBT tanned leather extracted with acetone: water.

Table 3.9-1: Assignments of FT-IR (ATR) band frequencies of neem bark tannins.

Band Frequencies (cm^{-1})	**Assignment**
725-915	Aromatic C-H out of plane deformation
910	C=C stretching in alkenes
970	OH out of plane deformation (trans)
1035	Aromatic C-H in plane deformation
1085	C-O deformation, secondary alcohol and aliphatic ether
1140	Aromatic C-H in plane deformation
1200-800	O-H stretching alcohols
1265-1229	O-H stretching phenolics
1270-1230	Aromatic ring with C-O stretching
1370	C-H deformation (symmetric)
1430	Aromatic skeletal vibrations
1470	C-H deformation (asymmetric)
1510-1605	Aromatic skeletal vibrations
1660-1715	Carbonyl stretching
1780-1670	C-O stretching
2920-2820	OH stretching in methylene groups
1200-800	O-H stretching alcohols
3600-3200	OH stretching (hydrogen bonded)

3.9.5. DISCUSSION

FT-IR (ATR) spectra of NBT tanned leathers showed considerable complexity. In general, the fingerprint region of the spectra (1500-800 cm^{-1}) provides similar information to conventional IR spectroscopy. The only major differences appear at 1780 cm^{-1}; this band might be assigned to carbonyl groups. High intensity is manifested by the neem bark tannin samples, indicating the presence of many functional groups, which causes overlap of the bands at the same frequency see Figures 3.9-2, and 3.9-3.

Increases in the absorption band intensities corresponding to alkanes RCH=CHR (815-885 cm-1), phenolic O-H (1235-1265 cm-1), carbonyl C=O (1670-1780 cm-1), alcoholic O-H and methyl and methylene groups (2850-2960 cm-1) are also observed in the IR spectra of neem bark tannin.

3.9.6. SUMMARY

- Results are consistent with the IR spectra, but high intensities were found indicating complexity of NBT functional groups.

3.9.7. REFERENCES

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CHAPTER 4

SHRINKAGE TEMPERATURE STUDY OF NBT TANNED LEATHERS

4.2. BACKGROUND

Shrinking is a phenomenon associated with dimensional changes of hide/skin or leather when subjected to heating. This can be measured by observing the point at which a specimen shrinks, when it is held in water, heated at a rate of 2°C per minute. This is the conventionally measured shrinkage temperature, which varies when the hide or skin is tanned, due to the process of cross-linking. The shrinkage temperature of a specimen in water as the heating medium is taken as a measure of hydrothermal stability and the boil test (viz., test for curling of leather in boiling water) is routinely used to assess the completion of chrome tanning.

The phase transition process involves the conversion of the crystalline triple helical collagen structure to an amorphous random coil form[1]; this change in the three dimensional fibre refers to the endothermic helix to coil transition, which occurs at a very slow rate under ambient conditions[2]. It has been extensively analysed by a number of workers in recent years using differential scanning calorimetry (DSC) [3-6].

From the tanners' point of view, the thermal shrinking of hide/skin and leather is associated with changes in dimension as well as in area and apparent volume. In the case of collagen fibres, raw or tanned, the shrinking is associated with a decrease in length and increase in thickness[7,8]. An increase in the real volume of skin and collagen matrix due to shrinking[1,9] has also been reported.

4.2.1. Shrinking phenomena

Different views and theories have been expressed on the shrinking phenomena. Earlier, shrinking of collagen fibres, when heated in a medium like water, was considered to occur when thermal agitation was sufficient to overcome the interchain cohesive forces and this caused collagen to revert to a stable form, i.e., the chain bundles melted and collapsed[10].

Weir[9] expressed the view that shrinking was caused when intramolecular forces were greater than the intermolecular forces of proteinaceous material. This view was based on the observation that the shrinking of collagen did not occur abruptly at a particular temperature but occurred over a range of temperature, i.e. the shrinking was viewed for the first time as a rate process. During shrinking, he observed an increase in real volume of approximately 1% and this increase in volume was irreversible, but the increase in the apparent volume below the transition range was completely reversible. He supported his views further by carrying out shrinkage measurements of specimens in thermal equilibrium with water at various temperatures and showed that the rate of shrinking of collagen was directly related to the temperature of the heating medium.

Weir and Carter[13] expressed the view that all cross-linkages of collagen and skin did not have equal strength and the number of such linkages might not be equal in any given region. A small region containing a few linkages of low energy would act as favourable sites for initiation of shrinkage, i.e. as a shrinking nucleus. The weak spot gets saturated with water because of thermal agitation, with the result that, as the temperature is raised, more and more bonds would be broken, causing a weakening of the protein structure and hence the nucleus would proceed to a more stable (shrunk) state by folding. The shrinking nuclei might occur at random locations throughout and the shrinking initiation at such spots might occur at any time; once the shrinkage was initiated, it would propagate internally and longitudinally until shrinking was complete.

Flory *et al.*[1, 14-16] considered that shrinking was a melting phenomenon of the oriented crystalline structure of native collagen and that collagen behaved like any other crystalline polymeric material; its equilibrium melting temperature (T_m), at which native crystalline and shrunken or amorphous state could co-exist, is lowered by diluents[15]. The super heating of collagen in the heating medium was necessary for external manifestation of the shrinkage, as witnessed by the shrinkage temperature[1]. This is because in native collagen the amorphous and crystalline phases do not coexist in juxtaposition along the length of the fibres. Flory *et al*[15]. also showed that there was a small but unmistakable latent volume change on shrinking collagen, which showed partial recovery during cooling. This led Flory to conclude that the native shrunken

state transformation of collagen is a transition phase involving a melting phenomenon rather than a rate process.

The difficulty in eliminating time effects, and especially the recognition that a component of collagen may be disordered before shrinkage, should not, however, be regarded as evidence that the process underlying shrinkage is not a melting or first order phase transition. The fact that shrinking involves the fusion of the crystalline state has been amply documented; X-ray diffraction studies showed that the original crystalline reflections disappear on shrinking and are replaced by amorphous halos[15,16]; optical birefringence of collagen was also found to disappear on shrinking[17, 18].

The hydrothermal stability of native collagen, however, is also governed by factors such as the co-operative contribution mechanism[23] of surrounding tissues and other types of linkages that might be present in the collagen of specific species[11, 24-26] particularly in the region of the non-crosslinked domain[23].

4.2.2. Influence of tannage on shrinkage temperature (Ts)

Recently the stability of leather tanned with mineral salts was investigated by Covington[27], who showed that the reaction kinetics of the tanning process is important in determining the hydrothermal stability. Covington *et al.*[28] supported the view that only intra- and/or inter-triple helix hydrogen bonds break as the protein superstructure collapses at different rates, depending on the nature of the synthetic crosslinks; those do not break during shrinking. This assumed that the same crosslinks break during shrinking, irrespective of the tanning treatment and environment.

Vegetable tannins, aldehyde, mineral tanning agents like chrome, aluminium, zirconium, synthetic tannins and fish oil are some well known tanning agents used in the leather industry. Some of the less commonly known but effective tanning agents are p-benzoquinone and similar quinones, sulphonyl chlorides, silicate, certain resins and polymeric compounds like dialdehyde starch, basic titanium and cerium salts and isocyanates. In addition, irradiation also introduces crosslinks in collagen.

Mostly, treatment with tanning agents raises the Ts of collagen because of the introduction of crosslinks[11,29-36], but tannages like fish oil and sulphonyl bromide are exceptions to this rule, although these produce a leathering effect. Different tannages

influence the shrinking behaviour in different ways, since the crosslinkages incorporated and the reactive groups involved on collagen are not the same.

Covington *et al.*[37] found that activation process of collagen shrinking consists of breaking key crosslinks in the collagen molecule and the process is rate dependent. They concluded that shrinkage temperature is determined by the effectiveness of a tanning molecule to produce high molecular weight crosslinked moieties, which in turn depends on the kinetic stability of the interaction between the tanning molecule and the protein sidechains.

Weir[9] showed that Cr (III) salts markedly increases the enthalpy, by which the free energy of activation value increases and so the value of the shrinkage temperature increases. But for other tanning materials, such as iron, uranium, vanadium, and formaldehyde, he found that the enthalpy is decreased compared to raw collagen. In his studies shrinking phenomena of vegetable tanned collagen are not mentioned.

4.2.3. Effect of mineral salts on shrinkage temperature

There is significant increase in the shrinkage temperature of vegetable tanned hide powder retanned with chrome or aluminium relative to tanned hide powder. This effect, semi metal tanning, was viewed by Hernandez *et al.*[38] as extended multiple interaction between vegetable tanning material and substrate created by crosslinks but, more importantly, a rigid matrix within the collagen interfibrils. In this way, it has a positive effect on both the crystallinity and the effective co-operating unit, which leads to an increased shrinkage temperature as shown in Table 4-2. Covington[39] analysed the synergistic interaction between plant polyphenols and the aluminium, which arises from one of the following options:

Collagen-Al-veg-Al-collagen

Collagen-veg-Al-veg-collagen

Collagen-veg-Al-collagen.

He suggested that applying the aluminium salt before the vegetable tannin produces only moderate shrinkage temperature, characteristic of aluminium alone. Therefore, the first and third options are unlikely. The most probable mechanism is for the aluminium (III) to crosslink the vegetable tannin[38]. In effect, the crosslinking polyphenols on collagen matrix stablises the collagen by a multiplicity of connected hydrogen bonds in the new macromolecule.

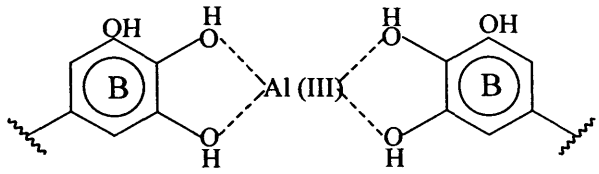
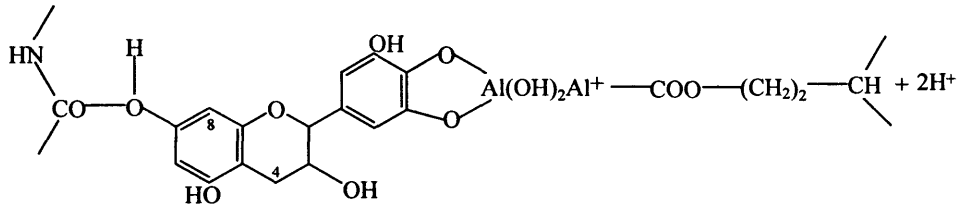


Figure 4-1: Possible crosslink of B-ring of mimosa with Al (III)

Slabbert's[40] model postulated the mixed crosslinking system as shown in Figure 4-2, that was based on the work of Sykes *et al*[41], who showed that aluminium can complex with mixed phenolate and carboxylate groups.



Mimosa -Al crosslink

Figure: 4-2 Postulated model of binding of metal and vegetable tannin

Table 4-1: Effect of combination tannages on hydrothermal stability[42].

Main tannage	Retannage	Ts_v (°C)	Ts_r (°C)	ΔTs (°C)
Vegetable	Aluminium[11]	76	117	41
PMA*	Aluminium[12]	89	105	16
Vegetable	Cobalt (II)	86	112	26
Vegetable	Fe (II)	86	111	25
Vegetable	Manganese(II)	86	94	8
Vegetable	Nickel(II)	86	107	21
Vegetable	Methanal[13]	87	108	21
Titanium	Vegetable[14]	84	106	22

Ts_v = Shrinkage temperature of tanned leather.
Ts_r = Shrinkage temperature of retanned leather.
PMA*= Pyromellitic dianhydride.

4.2.4. Effect of aldehyde retannage on shrinkage temperature

4.2.4.1. Formaldehyde retanning

The increases in shrinkage temperature of HCHO retanned hide powder relative to tanned hide powder are +6.2 and +8.1°C for neem bark and mimosa tanned hide powder respectively. The formaldehyde tanning reaction occurs at amino groups of collagen

$$\text{Collagen-NH}_2 + \text{HCHO} \rightarrow \text{Collagen-NH-CH}_2\text{OH}$$

The N-hydroxymethyl group is highly reactive and cross-linking can occur at a second amino group:

$$\text{Collagen-NH-CH}_2\text{OH} + \text{H}_2\text{N-Collagen} \rightarrow \text{Collagen-NH-CH}_2\text{-HN-Collagen}$$

Covington[39] has suggested, by this way, that the shrinkage temperature can be raised to 80-85°C and that crosslinking is relatively inefficient, because of polymeric nature of formaldehyde species. The presence of polyhydroxy species and their reaction with skin produces white, spongy, hydrophilic leather.

Glyoxal is another type of aldehydic agent, which can be used for tanning leather[43].

4.2.5. Differential scanning calorimetry (DSC) temperature profiles

In the present study, an attempt has been made to study the effect of neem bark pretanned, tanned and retanned leather/hide powder on shrinkage temperature and the enthalpy of the shrinking transition. In discussing the data, from thermogram see Figure 4-3, reference is made to the following elements.

4.2.5.1. Onset temperature

This is the temperature at which the slope of the thermogram first departs from the base line on heating. This corresponds to the first indication that a change in the sample is starting to take place.

4.2.5.2. Peak temperature

This is the temperature at which the temperature differential between sample and reference is greatest; the transformation taking place in the sample is almost complete at this temperature. For compounds undergoing fusion, the temperature at this point is reported as the compound's melting point.

4.2.5.3. Extrapolated onset temperature

This is the temperature obtained by extrapolation of the base line and the straight line portion on the low temperature side of the peak. This point represents the starting temperature for the major portion of the transformation.

4.2.5.4. Midpoint temperature

This is the arithmetic mean of the extrapolated onset and peak temperatures.

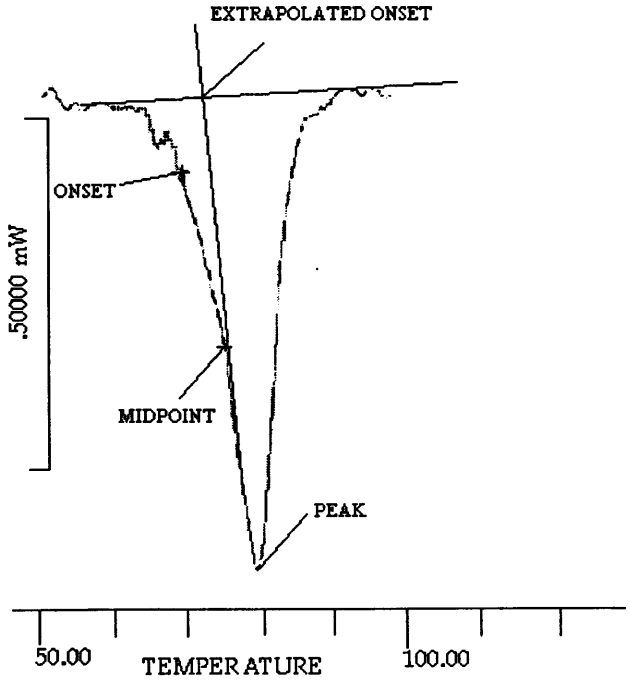


Figure 4-3: A typical example of DSC traces.

4.3. EXPERIMENTAL PROCEDURE

4.3.1. Materials and reagents

4.3.1.1. Neem bark tannins

The process described in Section 2.1.3.1 was used to obtain the neem bark tannins.

4.3.1.2. Other tanning materials

4.3.1.2.1. Mimosa

Untreated mimosa was kindly provided by Forestal Mimosa Limited (London) and was used without further alteration.

4.3.1.2.2. Chrome tan powder

Received as the commercially available chrome tan powder (chromium sulphate, 33% basic, 25% Cr_2O_3, Hodgson Chemicals, UK).

4.3.1.2.3. Basyntan P

Received as the commercially available phenolic condensate Basyntan P (BASF, Germany), 54-56% concentration, acid value approx. 200, pH 3.8-4.0.

4.3.1.2.4. Aluminium chloride

Received as the commercially available basic aluminium chloride (65% basic, 22-25% Al_2O_3 Lutan B, BASF, Germany).

4.3.1.2.5. Tetrakis hydroxymethyl phosphonium sulphate (THPS)

Received as the commercially available THPS (Albrite AD, Albrite UK).

4.3.1.2.6. 5-Hydroxymethyl-1-azo-3, 7-dioxabicyclo (3,3,0) octane

Received as the commercially available oxazolidine (Neosyn TX, Hodgson Chemicals, UK), active content 97%, pH analytical solution 8.0-9.0.

4.3.1.2.7. Formaldehyde (30 w/w)

Analytical grade formaldehyde (30 w/w), BDH (UK).

4.3.1.2.8. Glyoxal (pyruvaldehyde)

This was analytical grade methyl glyoxal, density 1.17 g/ml, and 40% aqueous solution. pH 4.0. Sigma Chemicals (UK).

4.3.1.3. SLTC Official hide powder

SLTC Official hide powder Batch B-5 was obtained from BLC The Leather Technology Centre, Northampton: moisture 18.9%, ash 0.26%, pH 6.3.

4.3.1.4. Skins for pilot scale

Pickled sheepskins, which were commercially manufactured in the UK were obtained from BSLT raw material department.

4.3.2. Methods

4.3.2.1. Tanning Experiments

4.3.2.1.1. Laboratory scale experiments

4.3.2.1.1.1. Tannage of hide powder

Hide powder was soaked in deionised water (20°C) overnight, drained and the moisture content (%) was determined. Hide powder was then tanned with neem bark tannins and mimosa according to the recipe shown in section B.1.3. Appendix B.

4.3.2.1.1.2. Tanning of hide powder with neem bark/mimosa by using pretanning agents

Hide powder was soaked in deionised water (20°C) overnight, drained and the moisture content (%) was determined. Hide powder was then pretanned with either phenol based syntan, THPS, chromium(III) salts, or methyl glyoxal, then tanned with NBT or mimosa, according to recipes shown in section B.1.4. Appendix. B.

4.3.2.1.1.3. Retanning of hide powder

Tanned hide powder with neem bark or mimosa was retanned with chrome, aluminium, methyl glyoxal, THPS or oxazolidine according to the recipes shown in Appendix B.1.5.

4.3.2.1.2. Pilot scale tanning experiments

Sheepskin leathers were made using NBT or mimosa, retanned with TX and dyed, as mentioned in Section B.1.6. Appendix B.

4.3.2.2. Differential scanning calorimetric studies

Hide powder (5-12mg) was sealed in aluminium capsules fitting into the chamber of the differential scanning calorimeter (Metler TA3000). Thermal changes were measured using a punctured capsule as a reference. Heating was within the temperature range 5-120°C, at a constant rate of 5°C min^{-1}. The total energy input, ΔH_{ENDO}, during the observed shrinking reaction was calculated from the integrated area of the endothermic profile. In these cases the samples were weighed before and after the thermal change, to determined the total energy in Joules per gram of sample.

4.3.2.3. Shrinkage temperature of hide powder tanned and retanned with various tanning reagents

Shrinkage temperature measurements were made using an apparatus as described by Bavington[44] and SLC[45] 406 and SLP 18/IUP 16.

4.4. RESULTS

Hide powder pretanned with different tanning agent was analysed by DSC set out in Table 4-3; the DSC thermograms are shown in Figures B-1 to B-20 in Appendix B.

Table 4-2: Effect of pretannage and retannage on shrinkage temperature relative to NBT and mimosa tanned hide powder.

Effect of pretannage on ΔTs (°C)		
Tanning materials	**Neem bark**	**Mimosa**
Phenolic based syntan	-14.3	-6.8
THPS	-18.9	-1.7
Chromium	-15.1	-6.4
Glyoxal	-15.0	-4.0
Effect of retannage on ΔTs (°C)		
Chrome	+19.9	+18.9
Aluminium	+12.0	+11.0
Glyoxal	-14.2	-18.0
THPS	0	-2.5
HCHO	+6.2	+8.1
Oxazolidine (Cr pretan)	+22.3	+22.2
Oxazolidine	+16.8	+19.0

Table 4-3: Differential scanning calorimeter parameters for shrinking of tanned and retanned hide powder.

	Onset temperature (°C)	Peak temperature (°C)	Extrapolated peak (°C)	Mid point temperature (°C)	Total energy ΔH_{ENDO} (J/g)
			Tanned		
Neem bark tannin	84	94	86	90	38
Mimosa	83	94	85	90	32
			Pretanned + Tanned		
Phenol based syntan + neem bark	70	80	71	76	13
Phenol based syntan + Mimosa	76	82	79	80	14
THPS + neem bark	65	68	66	67	4
THPS + mimosa	81	83	82	83	32
Chromium + neem bark	69	74	71	73	10
Chromium + mimosa	77	84	80	82	10
Glyoxal + neem bark	69	75	71	73	19
Glyoxal + mimosa	79	83	80	82	7
			Tanned +Retanned		
Neem bark+Chrome	104	112	103	108	77
Mimosa +Chrome	102	113	101	107	80
Neem bark+Al	96	108	99	104	63
Mimosa+Al	95	110	102	106	60
Neem bark+Glyoxal	70	74	71	73	15
Mimosa+Glyoxal	65	70	67	67	11
NBT+THPS	84	104	96	100	35
Mimosa+THPS	81	93	89	91	28
NBT+HCHO	90	92	91	92	41
Mimosa+HCHO	91	92	91	92	40
NBT+Oxazolidine (Cr pretan)	106	114	110	112	44
Mimosa+Oxazolidine (Cr pretan)	105	112	108	110	48
NBT+Oxazolidine	101	110	107	109	41
Mimosa+Oxazolidine	102	110	106	108	40

4.5. DISCUSSION

4.5.5. Effect of pretannage on shrinkage temperature of neem bark tannin or mimosa tanned hide powder

There is only 1°C difference in shrinkage temperature between neem bark and mimosa tanned hide powder, but pretannage has adverse affects on the shrinkage temperatures of both tannages. The changes in shrinkage temperatures relative to tanned hide powders are set out in Table 4-2. Pretanning systems are usually made use of in the vegetable tanning industry for increasing penetration; this is particularly the case when strong liquors are used, to avoid the drawn grain caused by over tanning of the surface, which slows down further penetration. But it seems from these results that there is an adverse effect of pre-treatment to the hide powder prior to tannage, despite, using the appropriate pH and temperature. This may be due to the early capture of the crosslink sites of substrate by the pretanning agents, which hindered the further formation of crosslinks.

THPS as pretanning agent does not have much effect on mimosa tannage, but the shrinkage temperature of neem bark tanned leather is reduced by 19°C. Comparing the two tanning systems (i.e., neem bark and mimosa), pretannage affects neem bark tanning more than mimosa. In the case of phenolic based syntan, THPS, Cr salt, and glyoxal used as pretanning agents, shrinkage temperatures fell by 8, 17, 9 and 11°C respectively compared to mimosa.

4.5.5.1. Effect of retannage on shrinkage temperature of neem bark and mimosa

The results are better in the case of hide powder, pretanned with Cr salts and retanned with organic crosslinker oxazolidine in both the tannage systems used. The changes in shrinkage temperatures ($\Delta T°C$) relative to neem bark or mimosa tanned hide powder show almost the same pattern for all types of retanning agents, see Table 4-2.

4.5.5.2. Effect of aldehyde retannage on shrinkage temperature

4.5.5.2.1. Formaldehyde retanning

The increases in shrinkage temperature of HCHO retanned hide powder relative to tanned hide powder are +6.2 and +8.1 °C for neem bark and mimosa tanned hide powder respectively.

4.5.5.2.2. Glyoxal

The results show relative changes in shrinkage temperature of -14.2 and -18 °C for neem bark tannin and mimosa respectively see Figure 4-5. The most likely reason of this fall in shrinkage temperature might be the pH, higher pH is required for the fixation of this reagent, but which is not suitable for the vegetable material, because at high pH stripping takes place.

4.5.5.2.3. Oxazolidine retanning

The results shows highest shrinkage temperature values for both tanning materials compared to other retanning agents. Relative increases in temperatures are listed in Table 4-2. All vegetable tannins react with collagen at the side chain amino groups and at the dipolar groups within the protein peptide links by hydrogen bonding. In addition, condensed tannins (i.e. neem bark and mimosa) can form covalent bonds with collagen by reaction via quinoid species. This reaction is favoured at elevated pH, so the conditions of the retannage with oxazolidins enhance the hydrothermal stability. The fused ring structure of mimosa or neem bark is the prerequisite for the crosslinking reaction with oxazolidine[46].

Oxazolidine effectively reacts as an aldehyde[47]. It can be seen that the results of oxazolidine retannage of NBT and mimosa hide powder are almost the same. The variation lies in the degree to which the interaction is synergistic[39].

4.5.5.3. Tetrakis hydroxymethyl phosphonium sulphate (THPS)

The results show no effect on neem bark whereas the shrinkage temperature of mimosa tanned hide powder drops 2.5°C relative to vegetable tanned hide powder Figure 4-2. The reaction depends on pH and temperature. The final pH of the process was 6.5, which is the starting point of stripping vegetable tannin material in aqueous

medium. The temperature of the tanning process is important. The effect is to accelerate the reaction of the THPS salt[46] with vegetable materials.

4.6. Isothermal titration microcalorimetry (ITC)

Isothermal titration microcalorimetry (ITC) has the advantage over other thermodynamic analysis techniques in that the signal measured is heat, allowing many different systems to be studied without labelling, tagging or otherwise modifying the reactants. Furthermore, it measures the enthalpy change of the reaction directly, allowing complete thermodynamic characterisation of the interaction between protein and metals[48].

An attempt was made to measure the heat of reaction between soluble collagen and neem bark tannin, using ITC at Glasgow University.

4.6.1 Experiments

Measurements were carried out using Microcal (ITC) instrument (Microcal, UK). All experiments were performed with a sample temperature of 25°C. The temperature controlled jacket surrounding the sample and reference cell was kept 10°C cooler than the experimental temperature in order that the machine may respond to both exothermic and endothermic heats. Both soluble collagen (Sigma Ltd.) and NBT were microfiltered and degassed before the experiment. 2 mg/ml soluble collagen was placed in the sample cell and 20 mg/ml NBT added to it by controlled injections in the cell. It was not possible to increase the collagen concentration significantly because of the limited solubility of collagen. 100 mM potassium chloride was used to control the ionic strength.

4.6.2 RESULTS

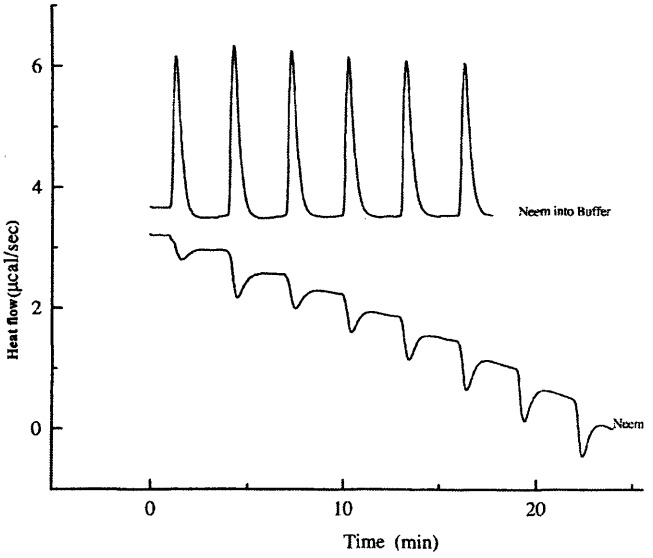


Figure 4-4: ITC thermogram of neem bark tannin base line adjustment.

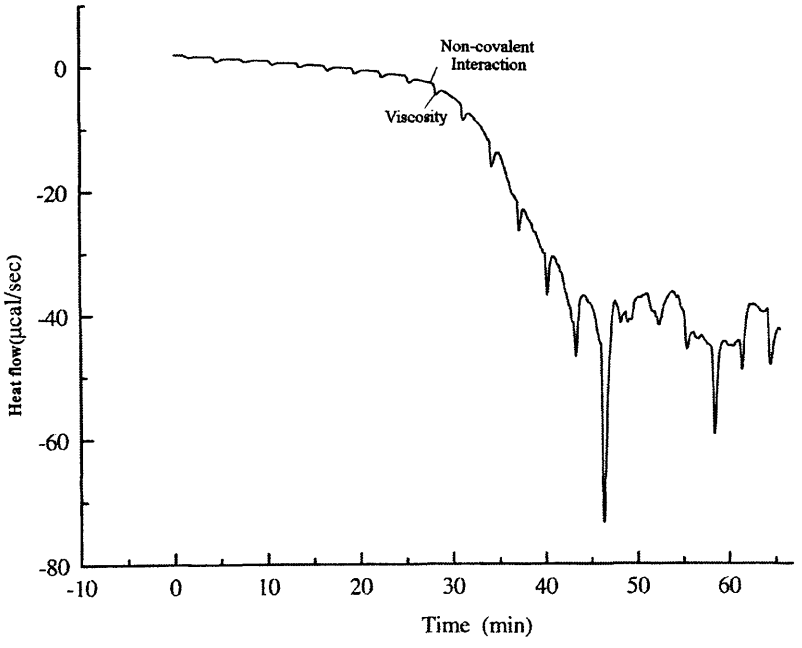


Figure 4-5: ITC thermogram of soluble collagen and neem bark tannin.

4.6.3. DISCUSSION

Figure 4-4 shows the baseline for the NBT and Figure 4-5 shows that interaction between soluble collagen and NBT is very complicated. It might be a combination of multiple effects, including coordination, folding of the molecular backbone, disruption and formation of hydrogen bonds, the breaking of the hydration layer of polyphenol ions, the hydrophobic effect of NBT interactions, Van der Waals interactions, etc.

It was assumed that the use of ITC might demonstrate the contribution of the interaction between proteins and tannins, but this appears not to be the case.

It was suggest by Cooper[49] that heat flow values just below 0 μcal /sec might indicate non-covalent bond interaction, and values decline further due to viscosity changes.

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CHAPTER 5

PHYSICAL PROPERTIES

5.1. PHYSICAL TESTING

5.1.1. INTRODUCTION

The most important factor influencing physical properties of leather is the original interwoven fibre structure of the hide/skin[1]. In general, the mechanical properties of leather are not only determined by the physical structure but also by the chemical composition and the mechanical work done on it[2]. In the present study, three important physical tests were carried out, which are related to appearance, touch and strength of leather i.e., lightfastness, softness and tear strength.

The interwoven structure of leather affects the physical properties, especially those measured by testing samples in a particular direction. To accommodate such variations, sampling positions were chosen. They are summarised as follows.

- Official sampling position (OSP)
- Shoulder
- Belly

Samples were chosen from parallel and perpendicular positions to the backbone where appropriate.

Most physical properties vary with the temperature and the moisture content of the leather: Mitton[3] has shown that some physical testing results decreased with temperature and increased with moisture content. It follows that, in order to make comparative studies of these physical properties, the relative humidity and temperature of the atmosphere should be kept constant.

5.2. STUDY OF NEEM BARK TANNED LEATHER SOFTNESS

5.2.1. INTRODUCTION

The traditional method for determining leather softness involves a hand grading by experienced tanners. Such a judgement is subjective and although it can be argued that this subjectivity allows a skilled grader to take account of subtle variations in the leathers, a more objective evaluation of leather softness would be invaluable[4].

Guy[5] used a number of tests, including dynamic bending to determine leather softness; his approach was considered to be too complex except for research and other workers have tended to accept the limitations associated with using a single parameter to measure leather softness. As far as leather compressibility is concerned, Moore[6] modified a Schieffer compressometer to measure the relative performance of deformation. This method was then modified by Ornes[7] and then later by Stubbings and Eisenfelder[8] to measure fullness and temper of vegetable tanned leather. Livesey and Owen[9] used a simple bending test similar to the Pierce flexometer[10], as described by Conabere[11], to investigate flexural rigidity of textile. The vertical loop test[12] and cylinder compression test[13] both correlated with leather softness. The drawback of these methods is that they require samples to be taken from the leather to be tested.

Inter and intra variations in skins are reported by Mitton[3]. Climate, age, sex, breed, health, and nourishment of the animal affect these variations. Softness is also affected by these variations.

The British Leather Confederation has developed a method for the non-destructive measurement of leather softness. They designed a portable device designated Official Method IUP 36[14].

5.2.2. EXPERIMENTAL PROCEDURE

5.2.2.1. Methods

This test was performed with a ST 300 BLC softness tester; the 25mm ring was used. In this instrument a disc shaped portion of the leather under test is clamped onto an aperture, a fixed load applied to its centre and the amount of deformation produced is measured.

5.2.2.2. Leathers used for physical testing.

The leathers used in this work were prepared in the BSLT tannery in laboratory drums, according to the recipes set out in Section B.1.5 & B 1.6 in Appendix B.
Neem bark/mimosa tanned leathers
Neem bark/mimosa+oxazolidine retanned leathers
Neem bark/mimosa +dyed leathers.

5.2.2.3. Conditioning (SLP3)

The specimens subjected to any physical investigation were conditioned for 48 hours before proceeding to any testing; specimens were stored under a relative humidity of 65 ± 2% at 20°C (± 2°C). All the physical tests were performed in the same standard atmosphere, unless otherwise specified.

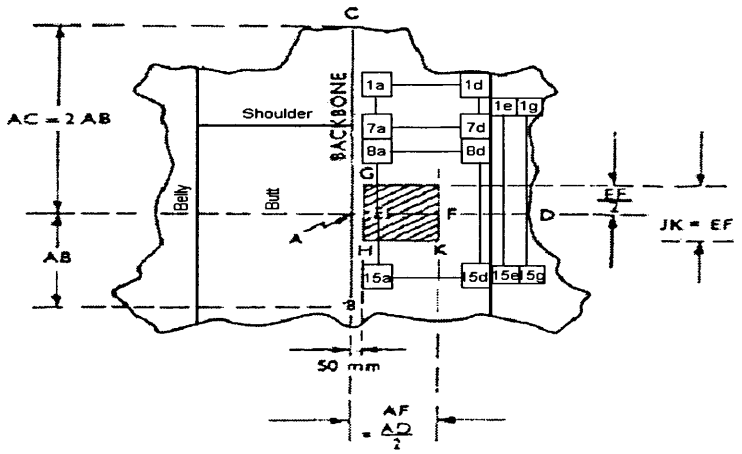

Figure 5.2-1: Selected sampling positions for softness test.

The neem bark and mimosa tanned, retanned and dyed leathers are described in Section B.1.1.

5.2.2.4. Statistical assessment

Statistical analysis used was analysis of variance (ANOVA) and Post Hoc Tukey tests (multiple comparison).

5.2.3. RESULTS

The results are also set out in Tables C1-1 to C1-6 in Appendix C1.

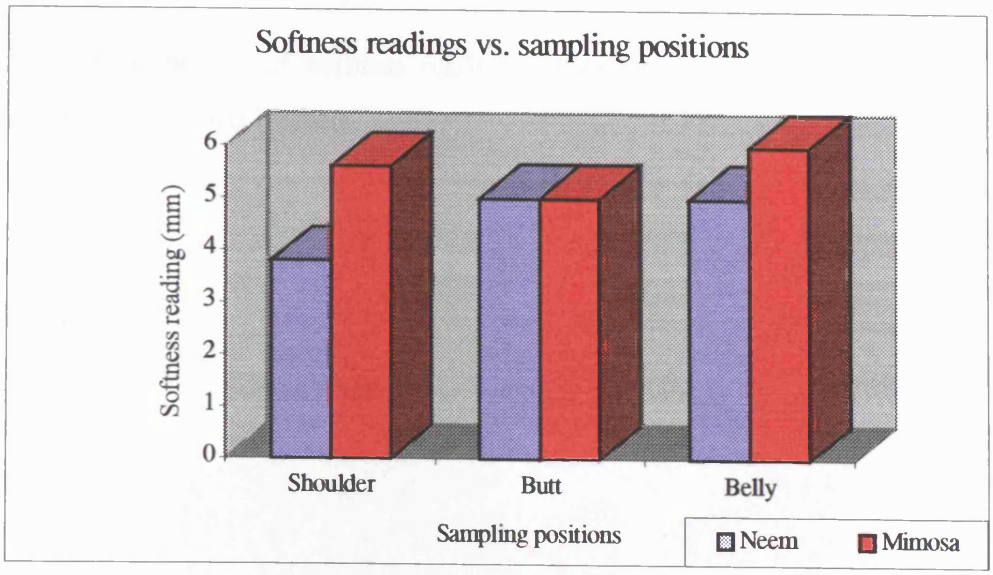

Figure 5.2-3: Comparison of softness readings of neem bark tannin and mimosa tanned leathers.

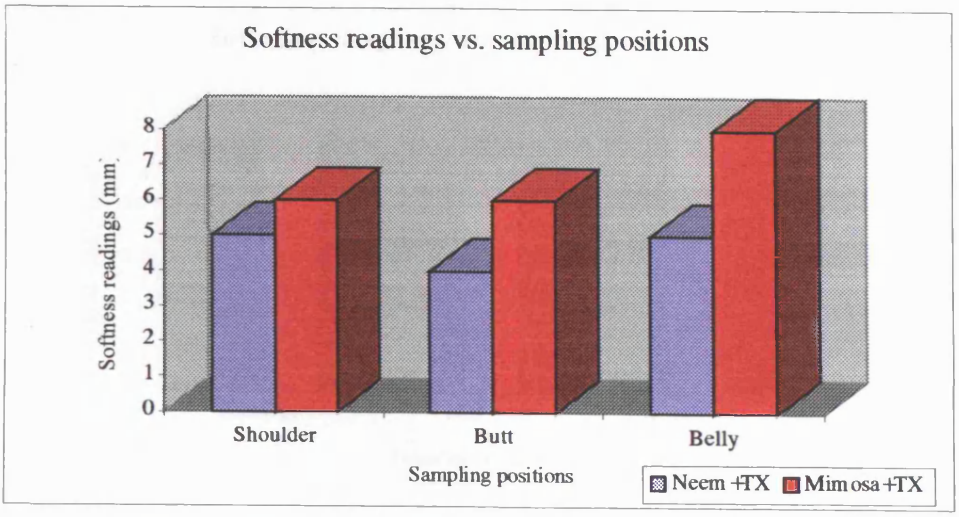

Figure 5.2-4: Comparison of softness readings of oxazolidine retanned neem bark tannin and mimosa tanned leathers.

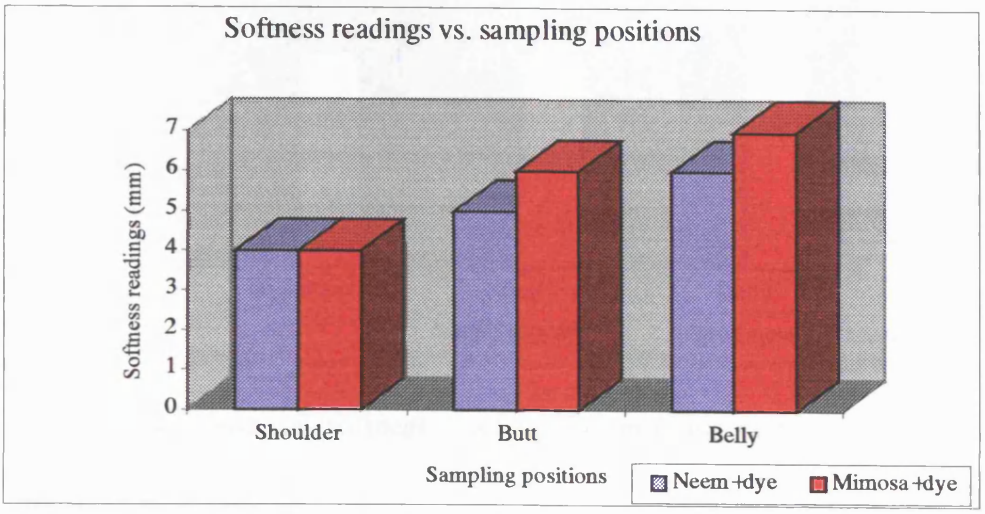

Figure 5.2-5: Comparison of softness readings of dyed neem bark tannin and mimosa tanned leathers.

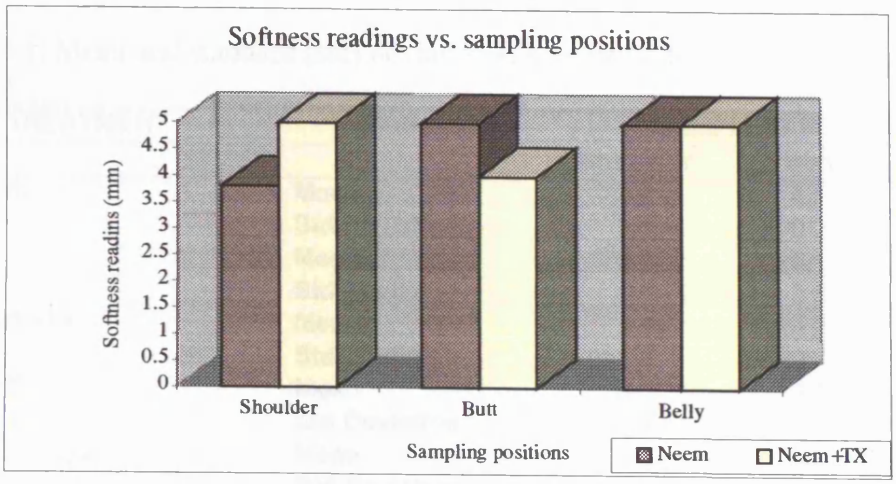

Figure 5.2-6: Comparison of softness readings of neem bark tanned and retanned leathers.

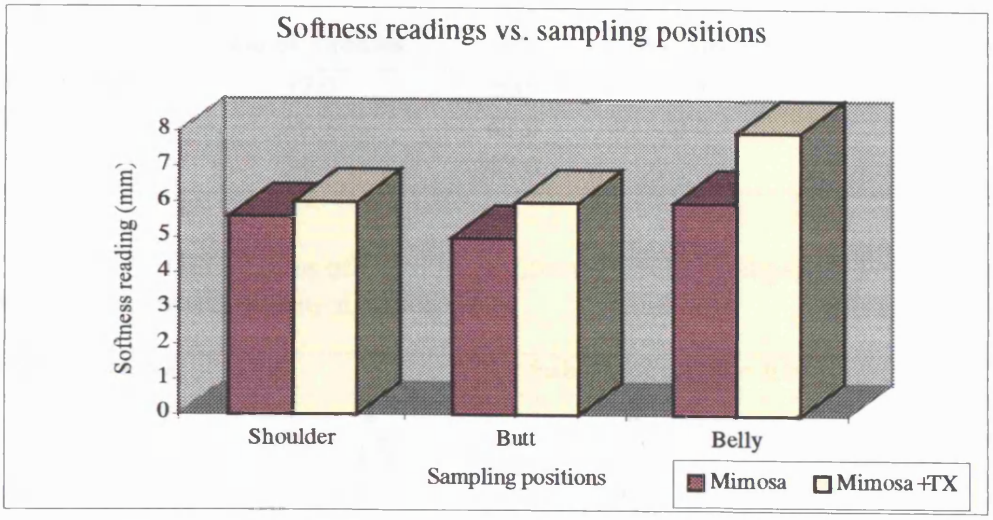

Figure 5.2-7: Comparison of softness readings of mimosa tanned and retanned leathers.

5.2.3.1. Statistical analysis of softness

Table 5.2-1: Mean and standard (std) deviation values for softness test.

TREATMENT	Statistics	SAMPLING POSITIONS		
		Shoulder	Butt	Belly
Neem bark	**Mean**	3.7	4.1	4.0
	Std Deviation	0.4	0.2	0.6
Mimosa	**Mean**	5.5	5.1	6.3
	Std Deviation	0.5	0.6	0.8
Neem bark+TX	**Mean**	4.7	4.2	4.3
	Std Deviation	0.8	0.7	0.4
Mimosa+TX	**Mean**	6.3	6.1	7.2
	Std Deviation	0.8	0.8	0.8
Neem bark +dye	**Mean**	3.9	4.8	5.3
	Std Deviation	0.5	0.5	0.6
Mimosa +dye	**Mean**	4.5	5.1	6.4
	Std Deviation	0.6	0.4	0.6

Table 5.2-2: Analysis of variance of shoulder positions (parallel and perpendicular) for softness test.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	39.0	5.0	7.8	21.8	0.00
Within Groups	15.1	42.0	0.4		
Total	54.1	47.0			

Table 5.2-3: Tukey HSD values of shoulder positions (parallel and perpendicular) for softness test, 8 measurements in all cases.

TREATMENT	Subset for alpha = 0.05		
	1	2	3
Neem bark	4.0		
Neem bark +TX	4.0	4.0	
Neem +dye	4.0	4.0	
Mimosa		5.0	4.7
Mimosa +TX			5.5
Mimosa +dye			
Sig.	0	0	0.1

Table 5.2-4: ANOVA values of butt positions (parallel and perpendicular) for softness test.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20.3	5.0	4.0	13.1	0.00
Within Groups	13.3	43.0	0		
Total	33.6	48.0			

Table 5.2-5: Tukey HSD of butt positions positions (parallel and perpendicular) for softness test.

TREATMENT	N	Subset for alpha = 0.05		
		1	2	3
Neem bark	8.0	4.1		
Neem bark+TX	8.0	4.2		
Neem +dye	8.0	4.8	4.8	
Mimosa	9.0		5.1	
Mimosa +Tx	8.0		5.1	
Mimosa +dye	8.0			6.1
Sig.		0.1	0.9	1.0

Means for groups in homogeneous subsets are displayed.

Table 5.2-6: ANOVA values of belly positions (parallel and perpendicular) for softness test.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	109.0	5.0	22.0	52.0	0.00
Within Groups	31.0	75.0	0		
Total	140.0	80.0			

Table 5.2-7: Tukey HSD of belly positions (parallel and perpendicular) for softness test.

TREATMENT	Subset for alpha = 0.05			
	1	2	3	4
Neem bark	4.0			
Neem bark+TX	4.3			
Neem +dye		5.3		
Mimosa			6.3	
Mimosa +dye			6.4	
Mimosa+TX				7.2
Sig.	0.7	1.0	1.0	1.0

Means for groups in homogeneous subsets are displayed.

5.2.4. DISCUSSION

Figure 5.2-3 shows the comparison of softness readings of neem bark and mimosa tanned leather and it shows mimosa tanned leather is softer than neem tanned. Figure 5.2-4 shows the improvements in softness when NBT/mimosa tanned leathers were treated with oxazolidine. The dyed leather also shows a similar pattern, see Figure 5.2-5.

The distributions of softness values depending on their sampling positions are shown in Figures 5.2-8 to 5.2-10. The mimosa leather softness values, presented as a three dimensional surface, demonstrate that the softness of mimosa tanned, retanned leathers is better than neem bark tannin, but as far as equal distribution of softness values is concerned, it is better in neem tanned and retanned leather. Figures 5.2-6 and 5.2-7 show the comparative softness values within each tanning agent these indicate that there is no significant difference between leathers neem bark tanned and retanned with TX, whereas mimosa softness values increase after using TX.

5.2.4.1. Statistical assessment

The ANOVA for 99 percentile values for the F distribution indicates that there is a significant effect of different treatments (i.e.tanning agents) on softness. The Post Hoc test for shoulder, butt and belly positions (parallel and perpendicular to the backbone) shows mimosa tanned and retanned leathers are softer than neem bark tanned leather at $P < 0.001$ level.

Tanning treatments had a significant effect on softness at all sampling positions (using ANOVA, at $P < 0.001$ level). It should be noted that in the Tables 5.3-3, 5 and 7, the matrices indicate a statistically significant difference at $P < 0.05$ level.

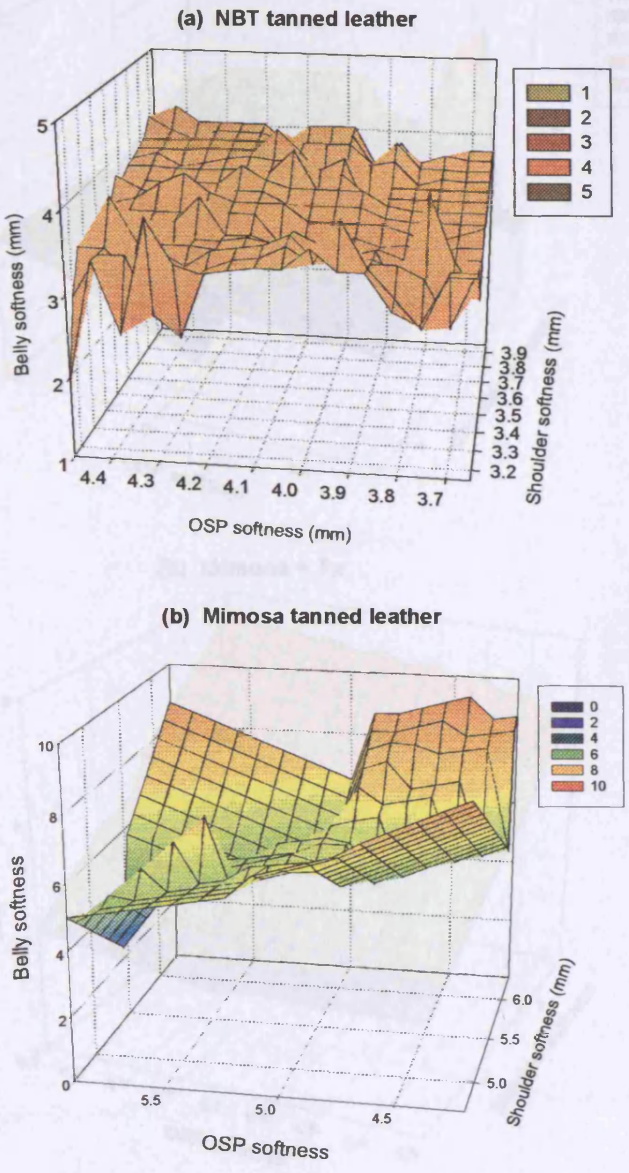

Figure 5.2-8 (a): Distribution of softness values for neem tanned leather.
(b): Distribution of softness values for mimosa tanned leather.

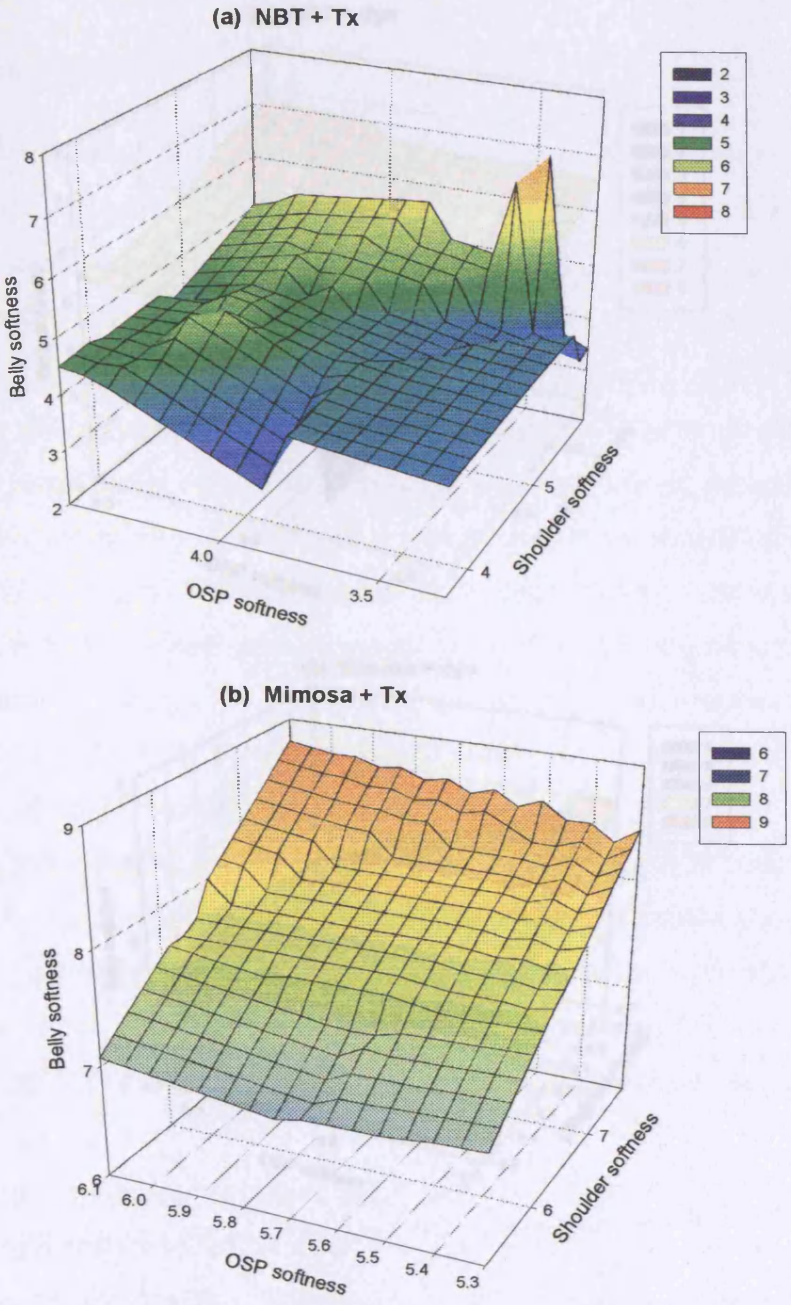

Figure 5.2-9 (a): Distribution of softness values for neem bark tannin + TX.
(b): Distribution of softness values for mimosa + TX.

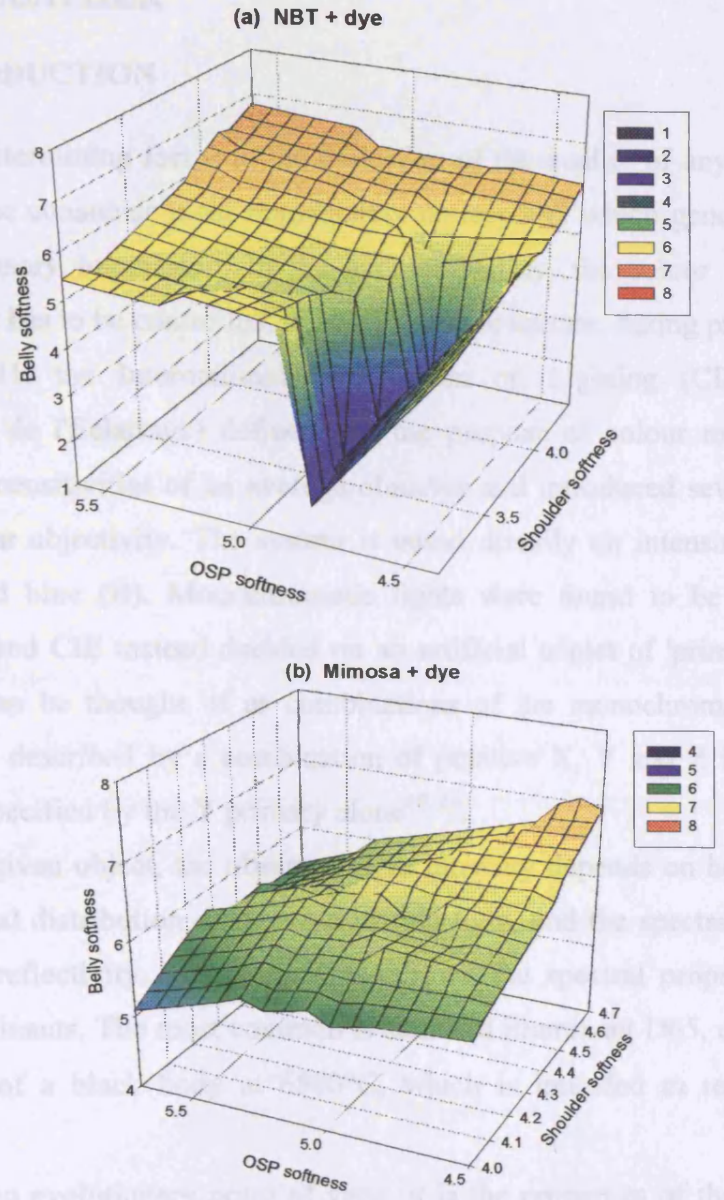

Figure 5.2-10: (a) Distribution of softness values for neem bark tannin + dye.
:(b) Distribution of softness values for mimosa + dye.

5.3. ASSESSMENT OF LIGHT FASTNESS OF NEEM BARK TANNED LEATHER

5.3.1. INTRODUCTION

Colour is a determining factor in the definition of the quality of any product: it is a feature that the consumer most immediately notices and which generally influences subjective sensory impression. In the case of leather, the colour is an important parameter that has to be controlled, together with the texture, during processing.

In 1931, the International Commission on Lighting (CIE, Commission Internationale de l'Eclairage) defined, for the purpose of colour measurement, the cone spectral sensitivities of an average observer and introduced several methods to describe colour objectivity. The system is based directly on intensities of red (R) , green (G) and blue (B). Monochromatic lights were found to be mathematically inconvenient and CIE instead decided on an artificial triplet of 'primary light', X, Y and Z, that can be thought of as combinations of the monochromatic lights. Any colour can be described by a combination of positive X, Y and Z intensity values. Lightness is specified by the Y primary alone[15, 16].

For a given object, the observed light intensity depends on both the intensity and the spectral distribution of the illuminating light, and the spectral distribution of the object is reflectivity. Therefore, CIE defined the spectral properties of several standard illuminants. The most common is standard illuminant D65, corresponding to the radiation of a black body at 6500°C, which is intended to represent average daylight[15].

From an evolutionary point of view, it is the properties of the object that are relevant, not the properties of illumination. The human vision system developed a way to correct for the quality of illumination and to preserve differences in hue. A white paper is perceived as white, even in reddish evening light. To allow similar possibilities in colour measurement, CIE defined several more human-related, less illumination-dependent measures of colour, of which the most common is L*, a* b* as shown in Figure 5.3-1.

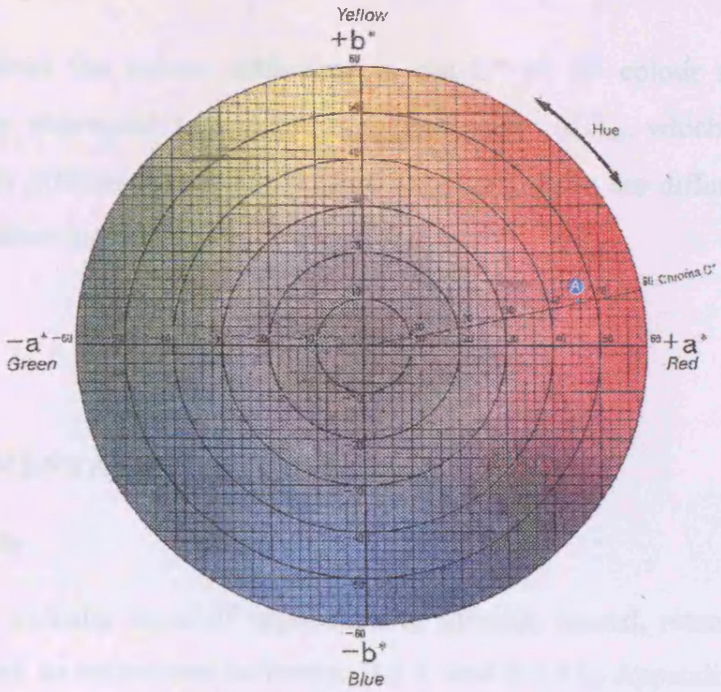

Figure 5.3-1: L*, a* and b* colour chart (hue and chroma)

L*, a* and b* colour values are therefore more suitable for direct comparison with sensory data[16]. Conventional colour meters usually provide readings in both the XYZ and L* a* b* colour spaces (see Figure 5.3-1).

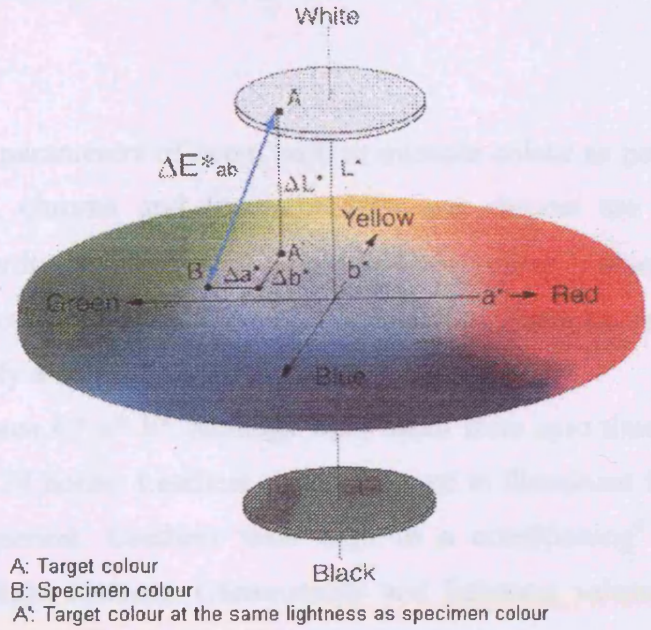

Figure 5.3-2: Colour difference (ΔE^*_{ab}) in the L* a* b* colour space.

Figure 5.3-2 shows the colour difference in the L* a* b* colour space. Colour difference can be expressed as a single numerical value ΔE^*_{ab}, which indicates the size of the colour difference, but not in what way the colours are different. ΔE^*_{ab}, is defined by the following equation.

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

5.3.2. EXPERIMENTAL PROCEDURE

5.3.2.1. Materials

7.2 cm diameter circular discs of neem bark or mimosa tanned, retanned and dyed leathers were used, as mentioned in Section B.1.5. and B.1.6 in Appendix B.

5.3.2.2. Apparatus

A xenon lamp was used for the xenotest. A chroma meter, Minolta (Osaka, Japan) was used to measure the sample's colorimeteric parameters. This instrument readout can be set to any CIE units. The white reference used was according to DIN 5033 (white reference model Weiß-Standard LZM 076).

5.3.2.3. Method

The colorimetric parameters of neem bark or mimosa colour as perceived have three dimensions: hue, chroma and lightness. Hue and chroma are specified by two chromaticity co-ordinates, a* (red □ green) and b* (yellow □ blue). Since these two coordinates cannot describe a colour completely, a lightness factor (L) must be included to identify a colour precisely.

Colour space L* a* b* readings were taken from zero time (starting time) to 144 hours, every 24 hours. Leathers were subjected to illuminant D65 (Xenon lamp) throughout this period. Leathers were kept in a conditioning[4] room during the investigation of light fastness. Chromaticity and lightness values of both types of leather tested were compared using contour graphs, see Figures 5.3-4 and 5.3-5. Statistical analysis used was Pearson correlation (2 tailed) and Post Hoc Tukey tests.

5.3.3. RESULTS

Results are also set out in Tables C2-1 to C2-3 Appendix C 2.

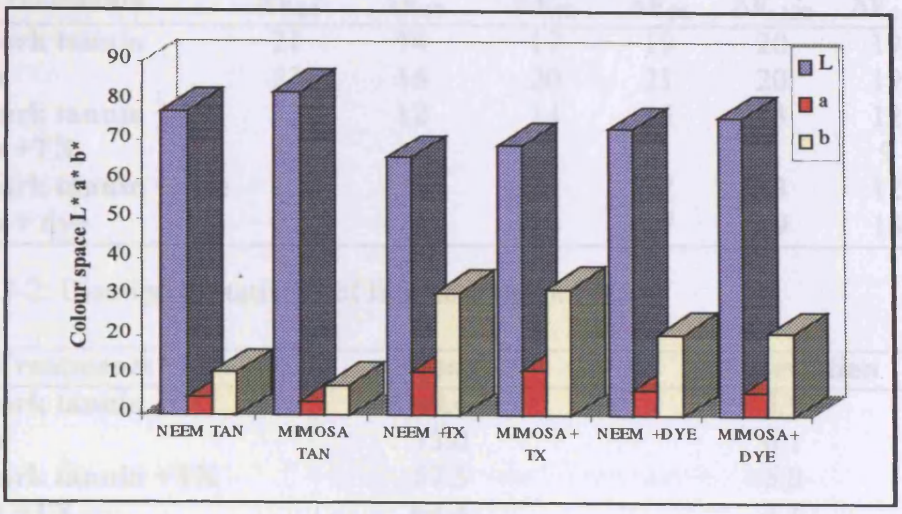

Figure 5.3-3: L* a* b* colour space at zero time of leathers.

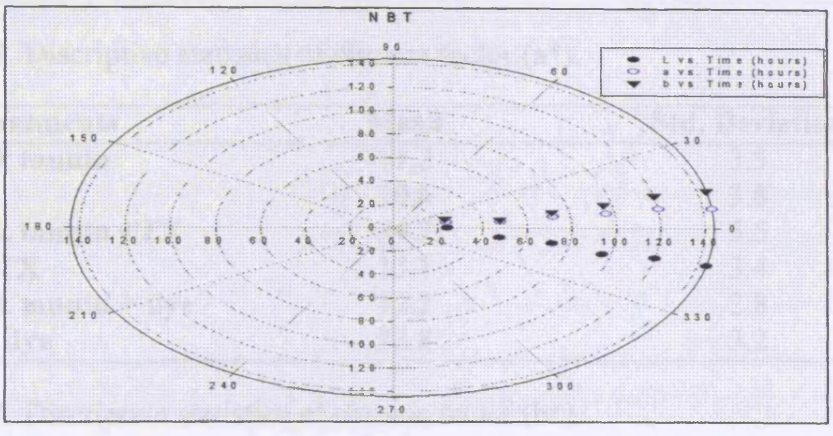

Figure 5.3-4: Chromaticity and lightness of neem bark tanned leather.

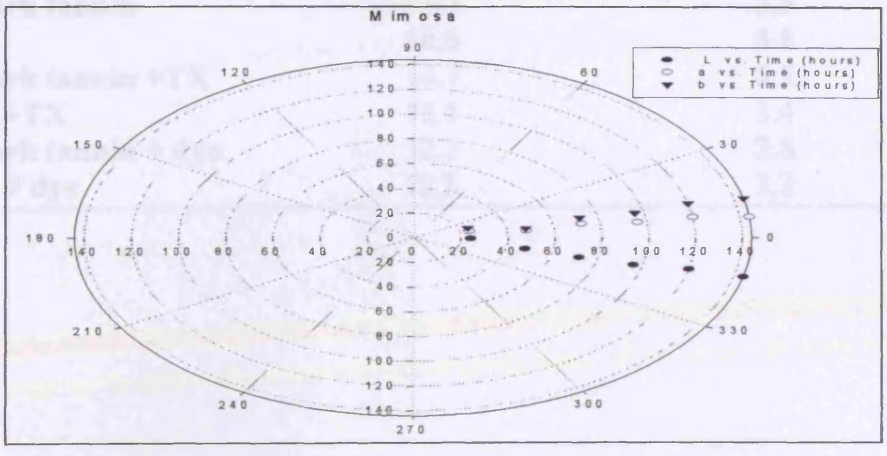

Figure 5.3-5: Chromaticity and lightness of mimosa tanned leather.

Table 5.3-1: ΔE values at different intervals of time in the xenotest.

Treatments	ΔE_{24}	ΔE_{48}	ΔE_{72}	ΔE_{96}	ΔE_{120}	ΔE_{144}
Neem bark tannin	21	14	17	19	20	19
Mimosa	23	16	20	21	20	19
Neem bark tannin +TX	13	12	14	15	13	12
Mimosa +TX	10	9	9	9	8	9
Neem bark tannin + dye	15	9	13	17	13	12
Mimosa + dye	15	12	18	17	19	18

Table 5.3-2: Discriptive statistics of lightness factor (L*).

Treatments	Mean	Std. Deviation
Neem bark tannin	69.4	5.9
Mimosa	73.0	6.1
Neem bark tannin +TX	57.5	5.0
Mimosa +TX	64.4	3.1
Neem bark tannin + dye	66.3	5.6
Mimosa + dye	66.8	6.4

Table 5.3-3: Discriptive statistics of chroma factor (a*).

Treatments	Mean	Std. Deviation
Neem bark tannin	11.2	3.5
Mimosa	10.6	3.8
Neem bark tannin +TX	14.7	5.5
Mimosa +TX	13.1	3.4
Neem bark tannin + dye	12.2	2.8
Mimosa + dye	12.8	3.2

Table 5.3-4: Discriptive statistics of chroma factor (b*).

Treatments	Mean	Std. Deviation
Neem bark tannin	11.2	3.5
Mimosa	10.6	3.8
Neem bark tannin +TX	14.7	5.5
Mimosa +TX	13.1	3.4
Neem bark tannin + dye	12.2	2.8
Mimosa + dye	12.8	3.2

Table 5.3-5: ANOVA of L* a* b* values for neem bark/mimosa leathers (tanned/retanned).

	L*				
	Sum of Squares	**Df**	**Mean Square**	**F**	**Sig.**
Between Groups	1016.5	6.0	169.4	5.9	0.000
Within Groups	1008.5	35.0	28.8		
Total	2025.1	41.0			
	a*				
Between Groups	229.44	6.00	38.24	3.65	0.006
Within Groups	366.47	35.00	10.47		
Total	595.90	41.00			
	b*				
Between Groups	635.32	6.00	105.89	3.32	0.011
Within Groups	1116.39	35.00	31.90		
Total	1751.71	41.00			

Table 5.3-6: Tukey HSD L* a* b* values for neem bark/mimosa leathers (tanned/retanned).

Tukey HSD L*		
Time (hours)	**Subset for alpha = 0.05**	
	1	**2**
120	61.43	
144	62.75	
96	62.98	
72	63.63	
48	65.13	65.13
24		73.35
0		74.3
Sig.	0.05	0.04
Tukey HSD a*		
0	7.3	
24	11.5	11.5
144	12.5	12.5
48	13.1	13.1
96		13.8
120		13.9
72		15.1
Sig.	0.057	0.041
Tukey HSD b*		
0	20.8	
48	23.7	
144	25.3	25.3
96	25.6	25.6
72	25.9	25.9
120	26.2	26.2
24		34.5
Sig.		0.048

5.3.4. DISCUSSION

Understanding the potential influence of light on vegetable tanning materials and leather requires an understanding of their variability with time. It is evident from Figures 5.3-4 and 5.3-5, that the variability can be significant, even in a relatively short time of light exposure, which presents difficulties for predicting the exact influence either by light or an environment, in which leather is placed.

Figure 5.3-3 shows the colour values at zero time for both types of tannage: There is no significant difference comparing neem bark and mimosa leathers. The lightness factor (L*) of mimosa leather is less affected when subjected to light than neem bark, whereas chromaticity a* and b* move away from the achromatic centre at the same rate as shown in Figures 5.3-4 and 5.3-5. The change in chroma a* values of neem bark retanned leather is more than mimosa retanned leather, which is observed as darkening of the leather.

5.3.4.1. Statistical assessment

Statistical data of Pearson correlation shows that there is no signifiance difference in light fastness properties when comparing neem bark and mimosa tanned leather at different intervals of time. In the case of lightness factor (L*), there is high correlation between all types of leathers. The Tukey Post Hoc tests, at a 95% confidence level, show same results. It indicates colour attributes are not easily distinguished, with respect to the size of the colour differences. It should be noted that large hue differences are more easily identifiable than smaller ones and a constant hue is more identifiable when the entire colour difference is small, see Table 5.3-1. It should be noted that Table 5.3-6 indicates statistically no significant difference at $P< 0.05$ level in both types of leathers.

5.4. TEAR STRENGTH OF NEEM BARK TANNED LEATHER

5.4.1. INTRODUCTION

A common mode of mechanical failure of footwear and clothing in wear is tearing. Consequently, a number of laboratory test methods have been developed to measure the tear resistance of materials in the textile, plastics, paper, leather and shoemaking industries.

The tear test has been used to quantify the difference in strength between grain and its underlying corium. In terms of tearing energy, the grain is weaker than the corium. The tear testing of leather is a good example of trying to predict the performance of a material in wear from simple relatively rapid laboratory tests. Usually, the maximum load reached in the test is taken as the parameter indicating likely performance.

It has been generally accepted that the loads required to propagate a tear or the maximum tearing load are measures of the tear resistance of the material. However, Hole and McIntyre[17] have postulated that a better criterion of resistance to tearing is the energy required initiating the tear.

It was shown by Covington *et al.*[18] that the influence of fatliquors on tear strength is considerable, but this variable was not included in these experiments.

Stather and Schmidt[19] stated that tanning by different methods caused a lowering of tear strength, in relation to that of delimed pelt. On the other hand, Mao and Roddy[20] stated that, in experiments carried out on single fibres from the same skin, the original strength was retained after tannages.

5.4.2. EXPERIMENTAL PROCEDURE

5.4.2.1. Methods

The trouser tear test (ALCA E-10, SATRA method PM-30) consists of propagating a tear from a slit cut in the narrow side of a rectangular specimen and parallel to its long axis. The slit is terminated by a circular hole 4.8 mm in diameter (Figure 5.4-1a). The cut ends are held in the jaws of the extensometer and the initial tearing occurs in the plane of the tearing force (Figure 5.4-1b). The tear resistance is quoted as the average tearing load, which is the total work done in tearing divided by the total distance torn.

Prior to testing, the thickness of the specimens was measured on the projected line of the tear and the average values were calculated and are set out in Table C3-4 Appendix C.

Tearing was carried out on an Instron Tensometer at a rate of 50 mm min^{-1}.

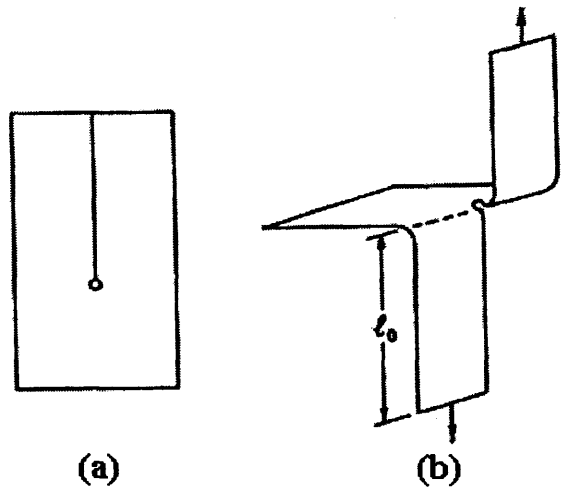

Figure 5.4-1: The trouser tear samples of SATRA PM-30.

The results in this study were obtained from duplicate, measurements on leathers made from similar skins, tanned, retanned, dyed and fatliquored in the conventional way, as described in section B.1.1 Appendix B.

5.4.2.2. Apparatus

1. Instron Tensometer1026 machine
2. Standard thickness gauge

5.4.2.3. Thickness (SLP4)

Thickness was measured by placing the leather in the dial micrometer thickness gauge with 500gf/cm^2 applied pressure on the presser foot. The load was applied gently to avoid any external fluctuation in pressure. The dial was graduated to 0.01mm.
Statistical analysis used was Tukey B (Homogenous subset)

5.4.2.4. Selected sampling positions for tear test

The sampling plan used was the same as given in Figure 5.2-1 Section 5.3.2.2.

5.4.3. RESULTS

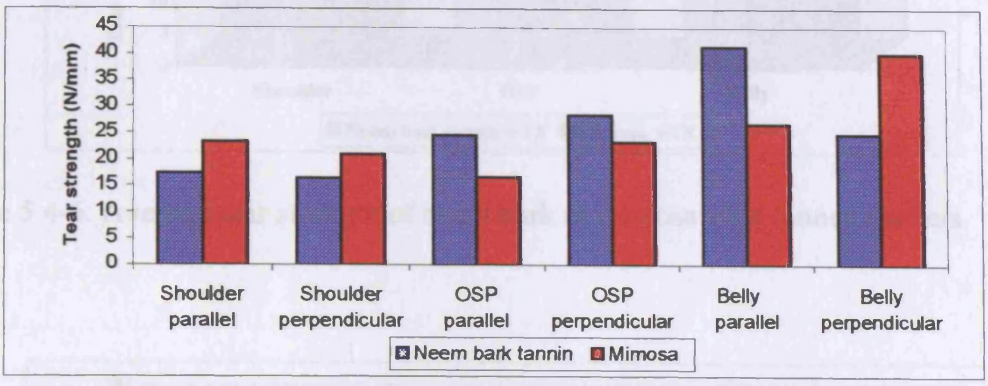

Figure 5.4-3: Tear strength of neem bark or mimosa tanned leathers.

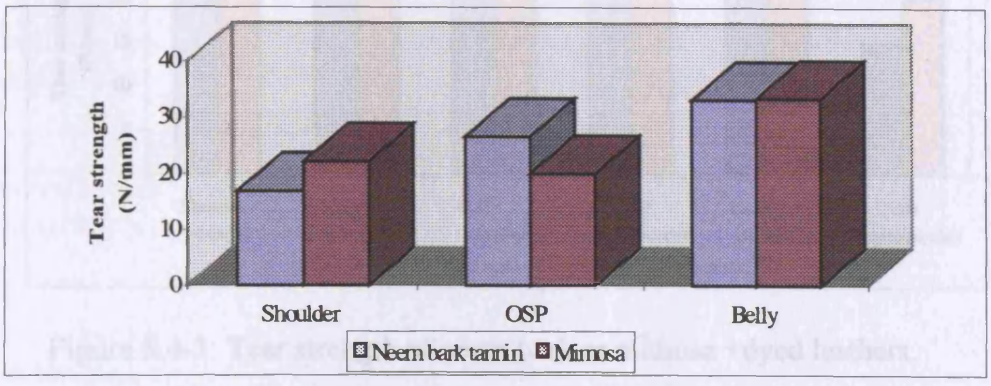

Figure 5.4-4: Average tear strength of neem bark or mimosa tanned leathers.

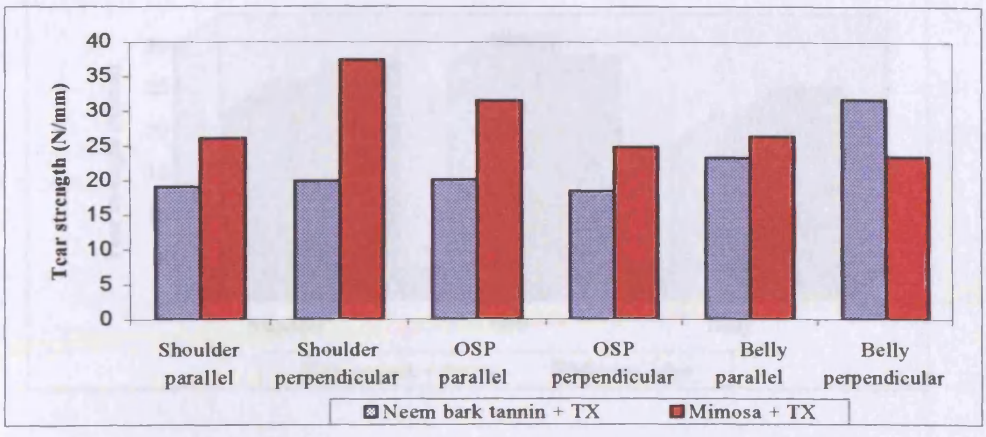

Figure 5.4-5: Tear strength of neem bark or mimosa +TX leathers.

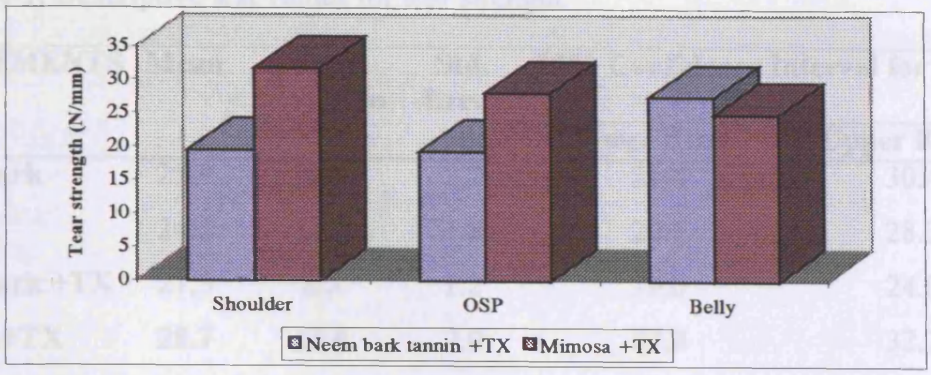

Figure 5.4-6: Average tear strength of neem bark or mimosa +TX tanned leathers.

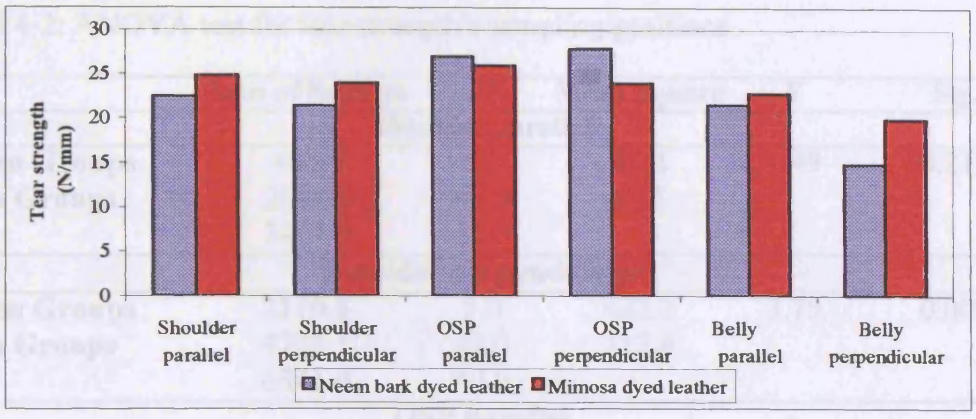

Figure 5.4-7: Tear strength of neem bark or mimosa +dyed leathers.

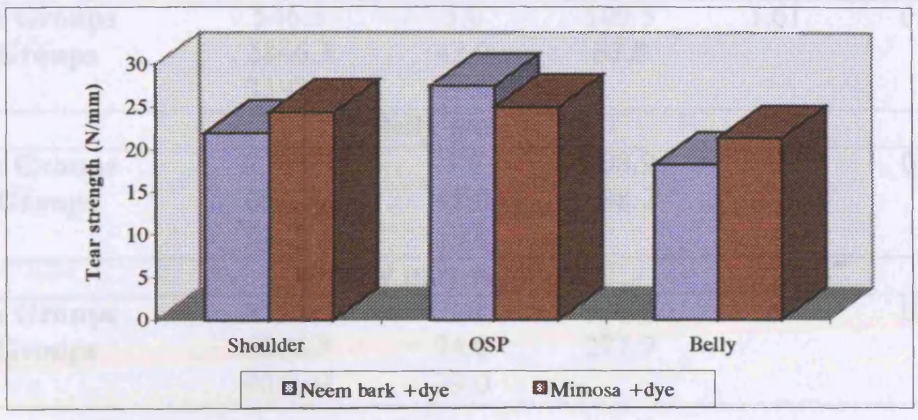

Figure 5.4-8: Average tear strength of neem bark or mimosa tanned + dyed leathers.

Table 5.4-1: Descriptive test values for tear strength.

TREATMENTS	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
				Lower Bound	Upper Bound
Neem bark	25.9	15.4	2.2	21.4	30.4
Mimosa	24.6	12.5	1.8	20.9	28.3
Neem bark +TX	21.5	8.5	1.2	19.0	24.0
Mimosa+TX	28.7	13.4	2.0	24.8	32.7
Neem bark+dye	23.0	8.6	1.3	20.5	25.5
Mimosa+dye	23.7	6.2	0.9	21.9	25.6
Total	24.6	11.4	0.7	23.2	25.9

Table 5.4-2: ANOVA test for tear strength's sampling positions.

	Sum of Squares	df	Mean Square	F	Sig.
Shoulder parallel					
Between Groups	463.9	5.0	92.8	1.49	0.212
Within Groups	2998.0	48.0	62.5		
Total	3461.9	53.0			
Shoulder perpendicular					
Between Groups	2110.8	5.0	422.2	3.75	0.007
Within Groups	4731.1	42.0	112.6		
Total	6841.9	47.0			
OSP parallel					
Between Groups	1059.9	5.0	212.0	3.49	0.010
Within Groups	2549.9	42.0	60.7		
Total	3609.8	47.0			
OSP perpendicular					
Between Groups	546.5	5.0	109.3	1.61	0.178
Within Groups	2846.3	42.0	67.8		
Total	3392.8	47.0			
Belly parallel					
Between Groups	2544.3	5.0	508.9	3.681	0.007
Within Groups	6635.2	48.0	138.2		
Total	9179.5	53.0			
Belly perpendicular					
Between Groups	2530.2	5.0	506.0	1.861	0.139
Within Groups	6526.7	24.0	271.9		
Total	9056.9	29.0			

Table 5.4-3: Homogeneous subsets (Tukey B) of shoulder positions for tear strength.

Shoulder parallel to the backbone		
TREATMENT	**Subset for alpha = 0.05**	
	1	
Neem bark	17.34	
Neem+TX	19.09	
Neem+dye	22.11	
Mimosa	23.28	
Mimosa+dye	23.67	
Mimosa+TX	26.1	
Sig.	0.097	
Shoulder perpendicular to the backbone		
	1	**2**
Neem bark	16.44	
Mimosa	19.90	
Neem+TX	20.94	
Neem+dye	23.00	23.00
Mimosa+dye	24.63	24.63
Mimosa+TX		37.40
Sig.	0.639	0.094

Table 5.4-4: Homogeneous subsets (Tukey B) of OSP positions for tear strength.

OSP parallel to the backbone		
TREATMENT	**Subset for alpha = 0.05**	
	1	**2**
Mimosa	16.71	
Neem+TX	20.14	20.14
Neem bark	24.68	24.68
Neem+dye	25.00	25.00
Mimosa+dye	26.50	26.50
Mimosa+TX		31.54
Sig.	0.144	0.058
OSP perpendicular to the backbone		
	1	
Neem+TX	18.50	
Mimosa	23.33	
Mimosa+TX	24.84	
Mimosa+dye	25.63	
Neem+dye	28.25	
Neem bark	28.53	
Sig.	0.168	

Table 5.4-5: Homogeneous subsets (Tukey B) of belly positions for tear strength.

Belly parallel to the backbone		
TREATMENT	**Subset for alpha = 0.05**	
	1	**2**
Mimosa+dye	20.78	
Neem+dye	22.11	
Neem+TX	23.28	
Mimosa+TX	26.38	26.38
Mimosa	26.81	26.81
Neem bark		41.36
Sig.	0.884	0.093
Belly perpendicular to the backbone		
	1	
Neem+dye	14.60	
Mimosa+dye	20.20	
Mimosa+TX	25.40	
Neem bark	26.76	
Neem+TX	31.96	
Mimosa	43.70	
Sig.	0.094	

5.4.4. DISCUSSION

The histograms of average values parallel and perpendicular to the backbone values show that there is no significant difference in tear strength of neem bark and mimosa tanned leather, see Figures 5.4-4, 6 and 8. The distribution curves, see Figures C-1 to C6 Appendix C, show that the cumulative % frequency of the tear strength has same distribution.

The results indicate that dyes play some role in the strength of fibres, see Figure 5.4-7, 8. The tear strength values decrease in both the neem bark or mimosa tanned leathers after dyeing. The values indicate that the tear strength is affected either by the dyes or the dyeing process relative to neem bark or mimosa tanned leather. The effect is believed to be due to the tanning effect of dyes. The distribution curves, Figures 5.4-9, 10, show the same distribution frequency and cumulative % for tear values for both types dyed leathers, see Table C3-5 Appendix C.

Variation in the tear strength from these two of tanning materials reflects that tear strength does not depend on a single factor i.e., tannage, because leather is affected by various other factors, such as the relative thickness of the leather, the tannage itself, acidity and salt contents of the liquors used, fatliquoring and the mechanical operations which directly influence the texture and strength of leather.

5.4.4.1. Statistical assessment

Statistical data of Tukey B analysis shows that there is no significant difference of tear strength, comparing sampling positions in neem bark and mimosa leathers. It should be noted that Table 5.3-6 indicates statistically no difference at $P < 0.05$ level.

5.5. SUMMARY

- NBT leather is generally not as soft as mimosa tanned leather.
- There is no significant difference in light fastness between NBT tanned and mimosa tanned leathers.
- There is no significant difference in tear strength between NBT tanned and mimosa tanned leathers.

5.6. REFERENCES

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CHAPTER 6

CONCLUSIONS

From the present investigation on the neem bark the following facts have been established.

- Neem bark tannin has chemical similarities to quebracho, but is also comparable in its tanning reactions to mimosa tannin, the market leader in this sector.
- Extraction was applied to two particle sizes, but in the range investigated there is no effect of particle size on extraction efficiency and effectiveness.
- The polyphenols extractable from neem bark appear to be a mixture of proanthocyanidins and procyanidin based polymers, but the precise constitution depends on the different solvent systems that can be used.
- High temperature extraction yields 55% extractive using an aqueous system, but the colour of tannin was darker.
- The best extracting solvent system was found to be acetone: water, yielding 72% tannins, with a purity of 95%.
- Some minor phenols, such as fisetin, kaempferol, robinetinidol, fisetinidol and high polymeric tannins were identified in the mixture, as part of the non-tans content.
- Non-tannins of neem bark are mainly gums, hydrolysable to give D-glucose, D-galactose, L-fucose, L-arabinose.
- HPLC confirms the presence of quercetin, kaempferol, gallic acid and procyanidins B1, B2, B3, B4, C1 and polymeric tannins. It was also found that R and S configuration affects the retention time in this analytical method.
- Examination of the different neem bark extract derivatives by ^{1}H NMR revealed the presence of trans-triflavonoid analogues, consisting of a 2R series of triflavonoid procyanidins.
- Further proof is the monomeric, dimeric and trimeric units of molecular masses, for procyanidins B and C type of NBT, confirmed by gc-MS.
- Molecular weight distribution has been found by GPC to be in the range 250-5500 and the average molecular weight of neem bark tannin is 1350-1500, indicating a dominance of 4 to 5 flavonoid units in the polymers.

- Functional groups assigned by IR and FT-IR (ATR) are also consistent with the rest of the techniques used.
- The hydrothermal stability of neem bark tanned, retanned hide powder was found to be equivalent to mimosa tannins. The physical properties of leather treated with neem bark tannin revealed that it does not produced leather as soft as mimosa tanned leather. But it was found that there is no significant difference in light fastness and tear strength properties of neem bark tanned leather compared to mimosa tanned leathers.
- The analytical studies and tanning trials indicate that neem bark tannin has the potential to be used as a commercial alternative to the more common condensed tannins, quebracho and mimosa.
- More importantly, these results offer the possibility of useful tannins being made available to the developing economies based on an indigenous natural, renewable resource. The effect would be to reduce their dependency on importing vegetable tannin and paying in hard currency.

6.1. FUTURE WORK AND OVERVIEW

Extraction systems carried out in the present work highlight the importance of purification and stability of tannins. There is a need to develop a new method of extraction system, which not only enhances the tannin quality, but also isolates tannins rapidly and cost effectively, for instance:
Rapid extraction by using ultrasound
Enzyme assisted extraction system, which might accelerate the rate of extraction and improve the quality of the tannin.

As the structures of NBT have been elucidated, it has become possible to undertake detailed studies of how tannin and protein structure influence the interaction. We know the procyanidin dimers and trimer structures and stereochemistry, but we do not know precisely how these individual components interact with protein and which particular dimers or oligomers stabilise the structure of protein. Further studies of tannin-protein interactions in these complex mixtures are needed.

To understand the chemical, structural and physical behaviour of tannin materials and their interaction with protein, it is necessary to study the hydrophobic nature of the tanning material, which is responsible for the shrinkage temperature, using modern experimental techniques such as nuclear magnetic resonance.

Hydrothermal stability should be seen in light of the geometry and solvating processes of the substrate and the tanning materials, either organic or mineral salts.

There is a need to increase our knowledge about the biodegradation of condensed tannins, which can lead to the overall understanding and commercial use of these tannins. This will facilitate the application of tannin degrading enzymes or genes encoding them in strategies for improving industrial and livestock production and utilisation of tannin rich waste biomaterials.

APPENDICES

APPENDIX A

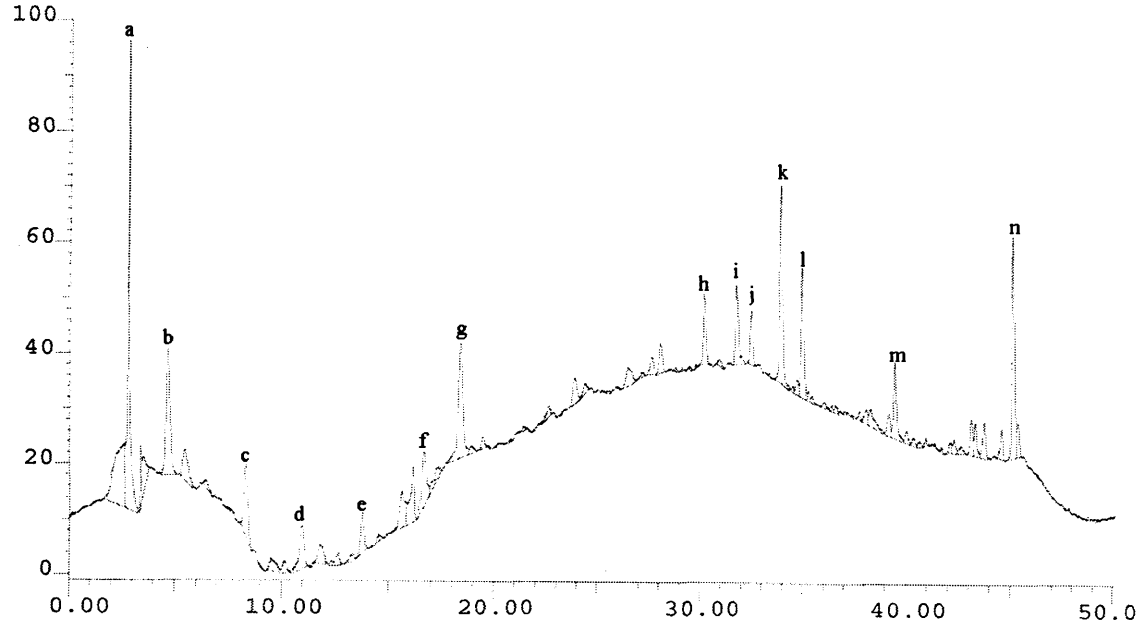


Figure A1-1: HPLC chromatogram of neem bark extracted with water at 85°C. Compounds of peaks a-n are identified and reported in Table A1-3 with retention times.

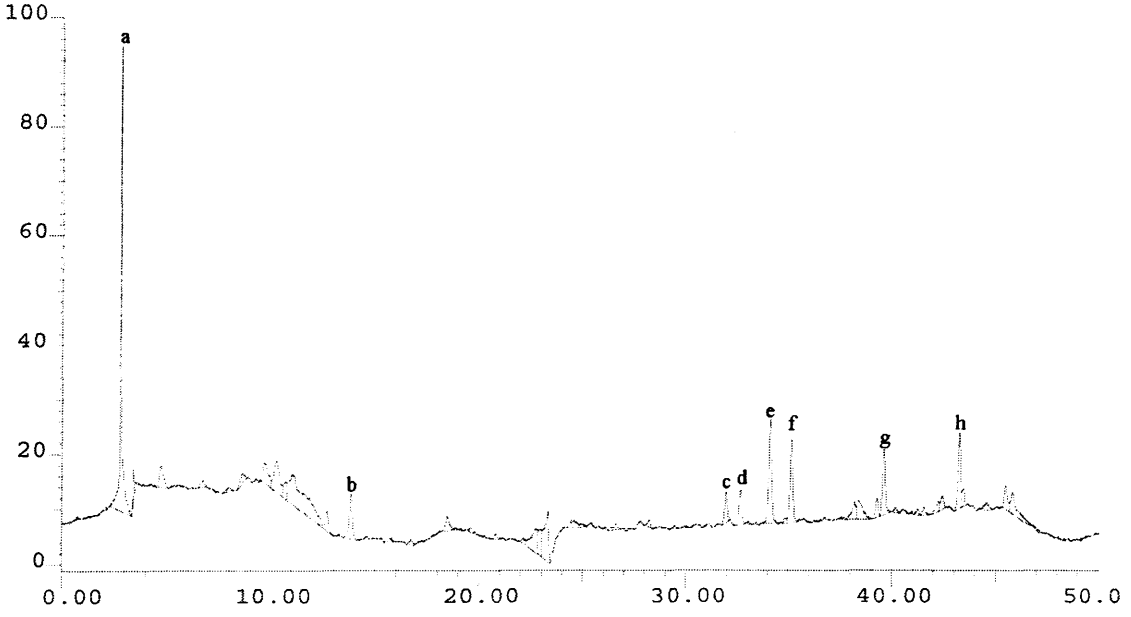


Figure A1-2: HPLC chromatogram of neem bark extracted with ethyl acetate. Compounds of peaks a-h are identified and reported in Table A1-4 with retention times.

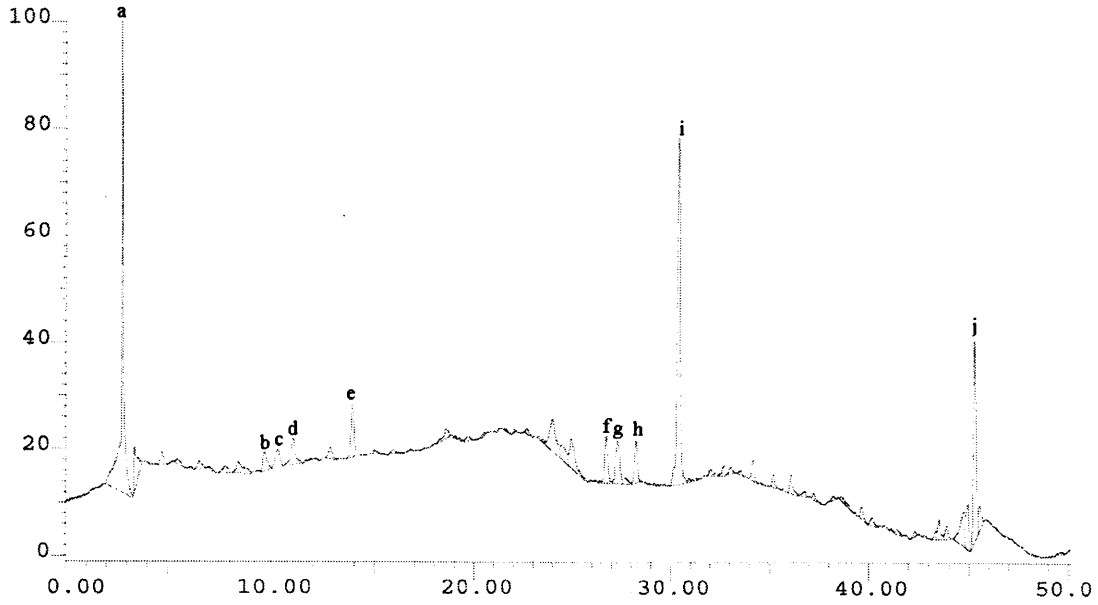


Figure A1-3: HPLC chromatogram of neem bark extracted with methanol: water (95:5 v/v). Compounds of peaks a-h are identified and reported in Table A1-5 with retention times.

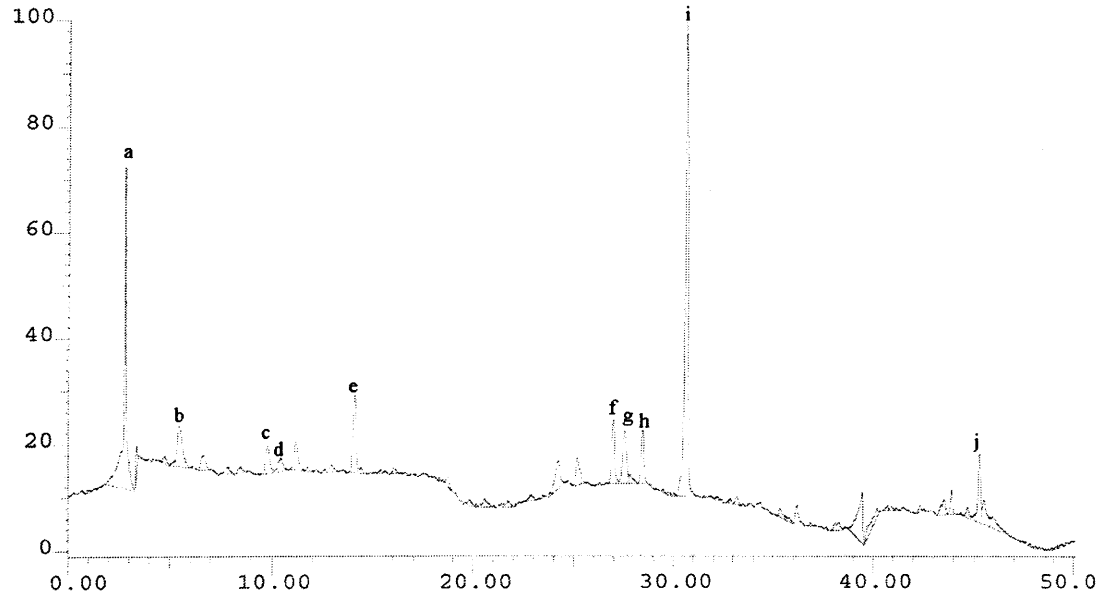


Figure A1-4: HPLC chromatogram of neem bark extracted with acetone: water (1:1 v/v). Compounds of peaks a-j are identified and reported in Table A1-6 with retention times.

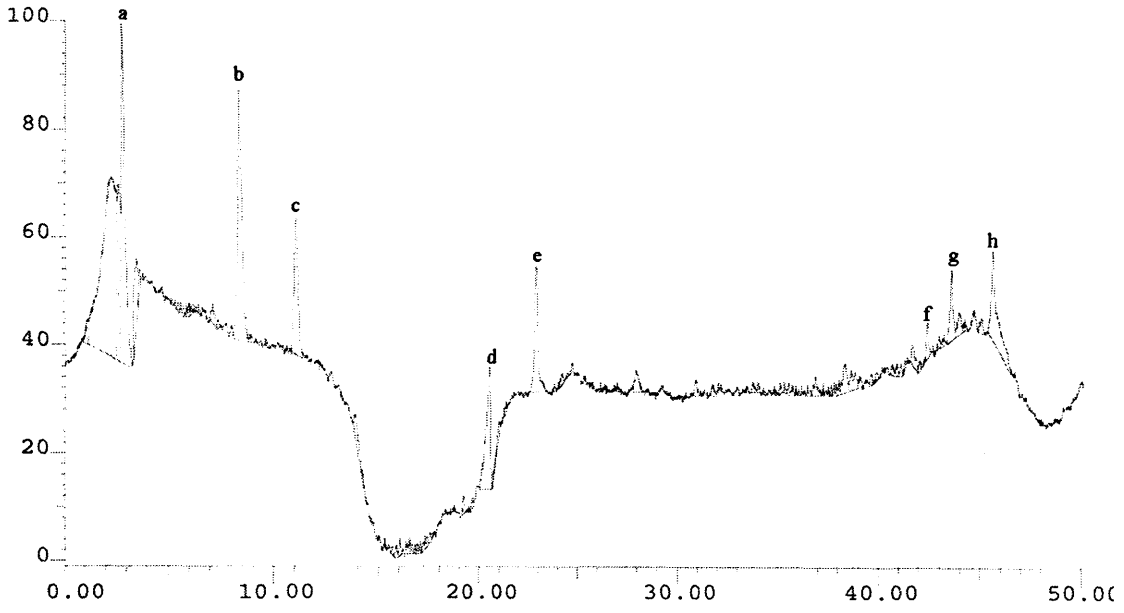


Figure A1-5: HPLC chromatogram of Fraction I. Compounds of peaks are identified and reported in Table A1-7 with retention times.

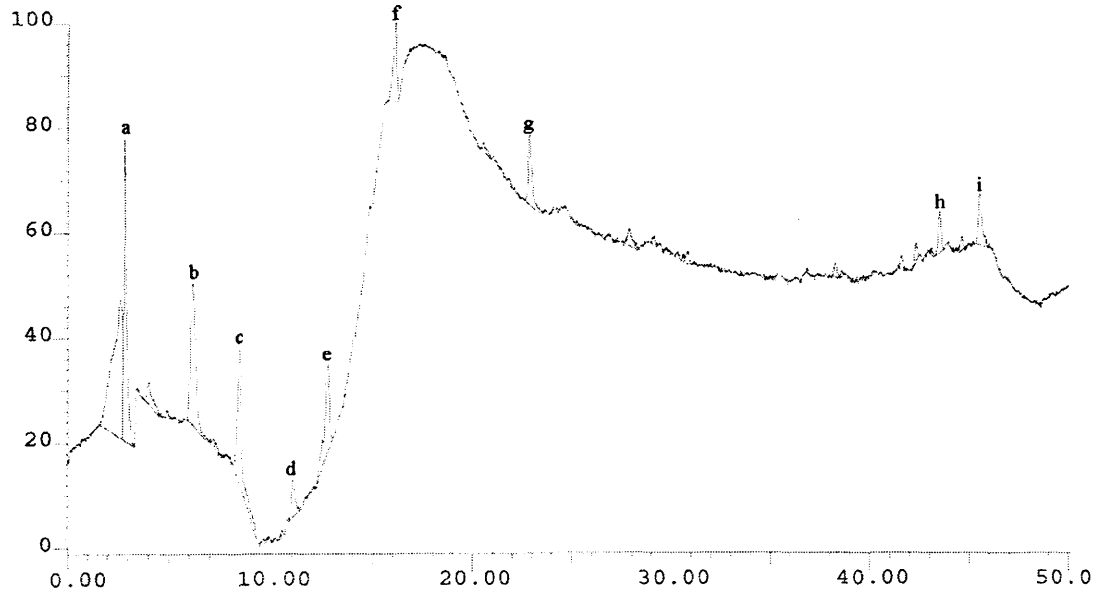


Figure A1-6: HPLC chromatogram of Fraction II. Compounds of peaks a-i are identified and reported in Table A1-8 with retention times.

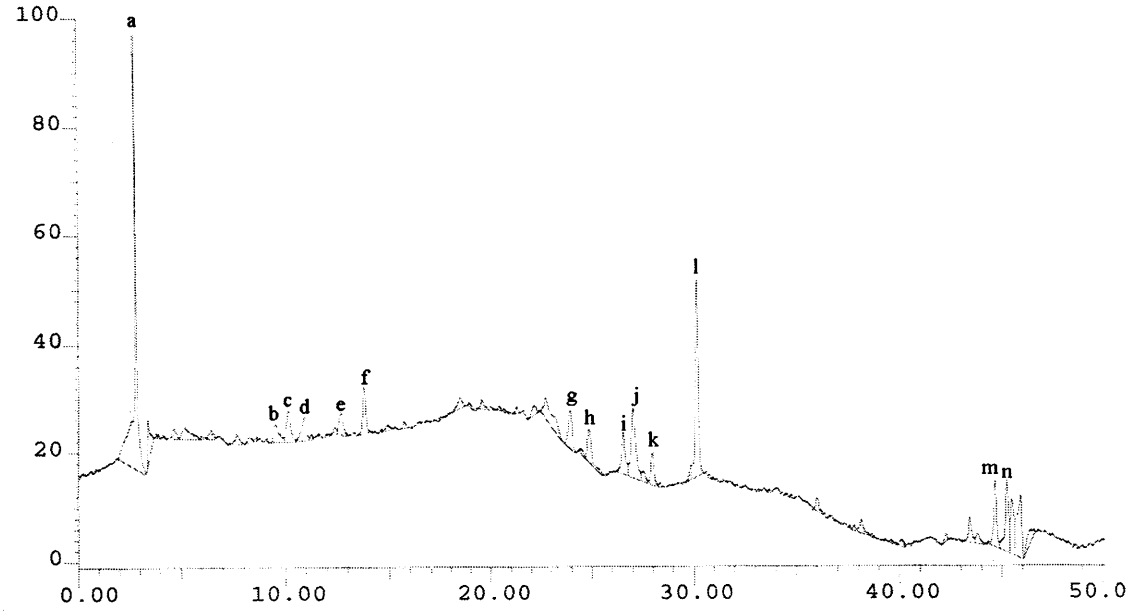


Figure A1-7: HPLC chromatogram of Fraction IIa. Compounds of peaks a-n are identified and reported in Table A1-9 with retention times.

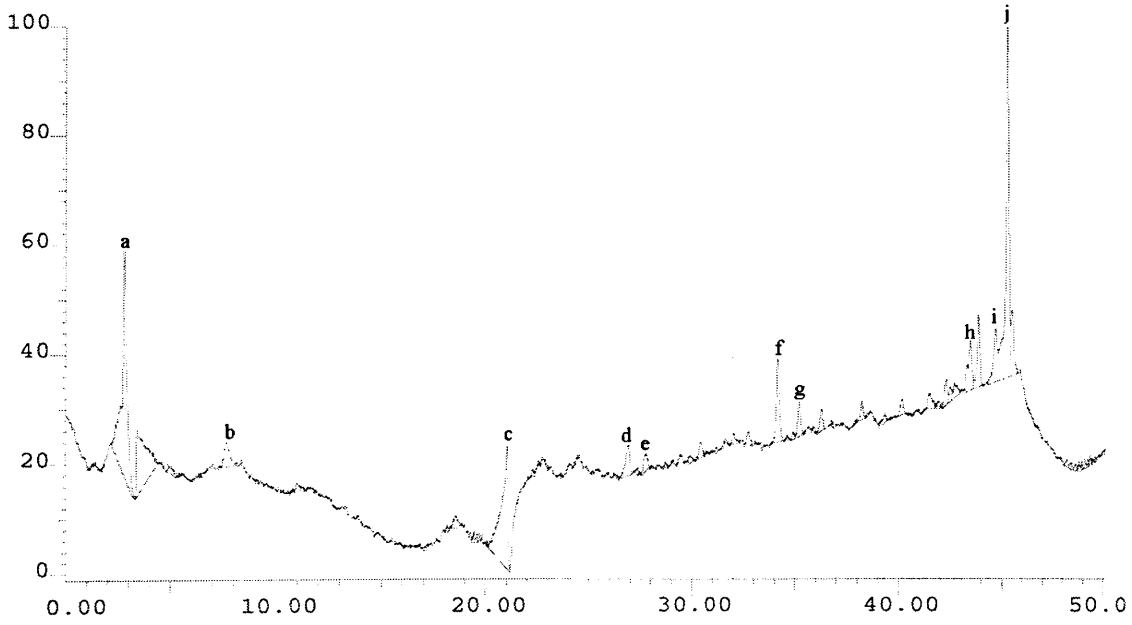


Figure A1-8: HPLC chromatogram of Fraction IIb. Compounds of peaks a-j are identified and reported in Table A1-10 with retention times.

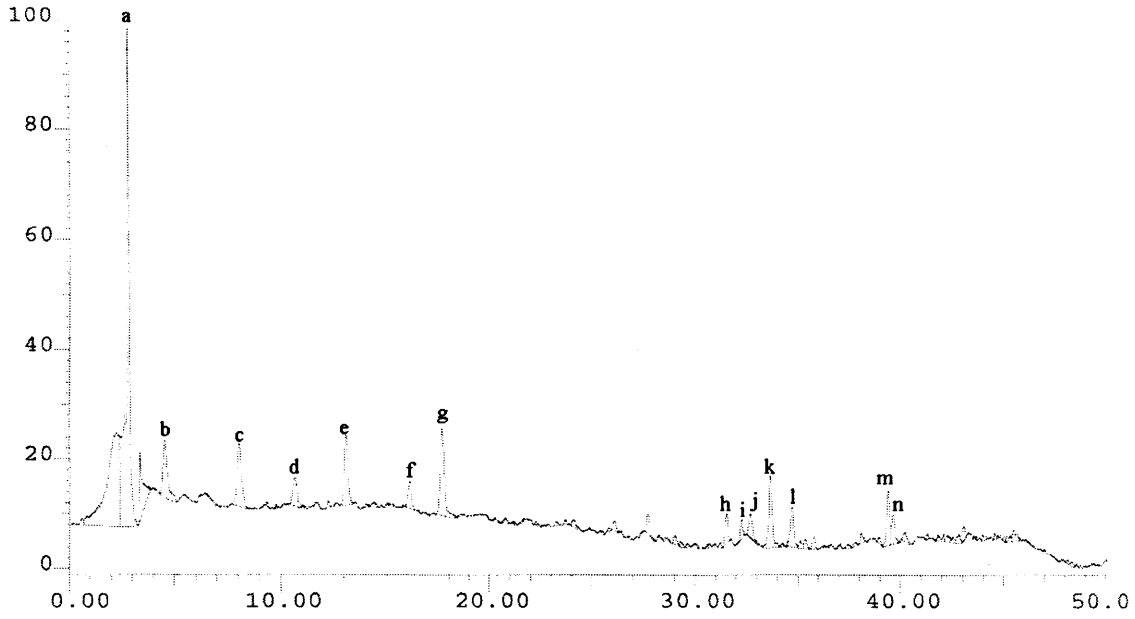


Figure A1-9: HPLC chromatogram of Fraction IIc. Compounds of peaks a-n are identified and reported in Table A1-11 with retention times.

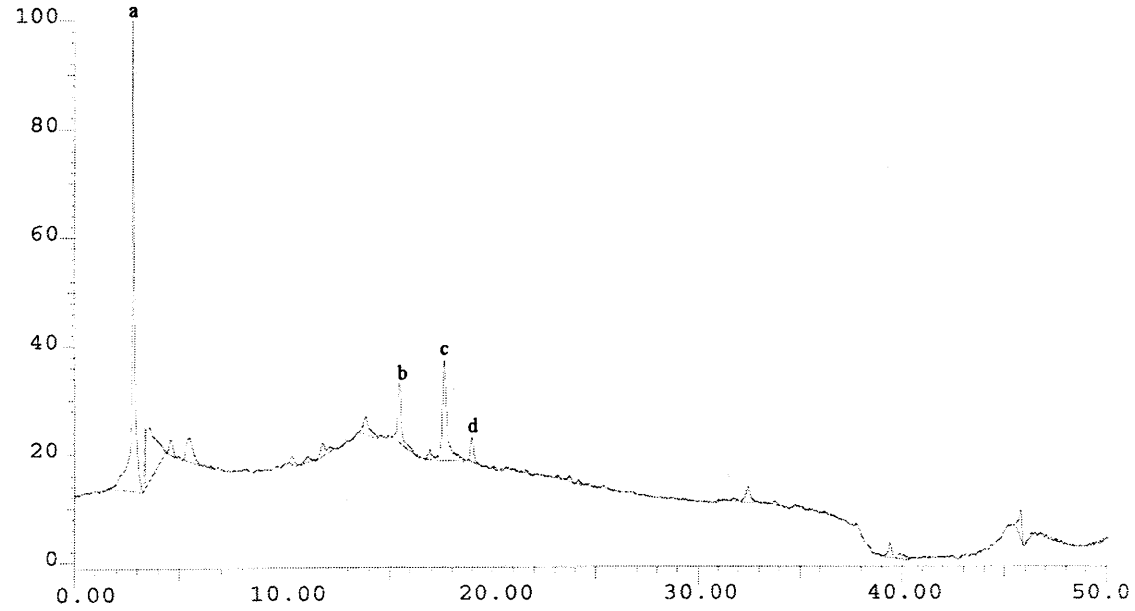


Figure A1-10 HPLC chromatogram of Fraction IId. Compounds of peaks a-d are identified and reported in Table A1-12 with retention times.

Table A1-1: Compounds retention times and of the thiolysis product of NBT extracted with acetone: water (1:1 v/v).

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	(+)-gallocatechin	4.45
c	(-)-epigallocatechin	6.30
d	catechin (4α-8)-catechin; B3	8.15
e	epicatechin (4β--8)-catechin; B1	9.30
f	(+)-catechin	10.15
g	catechin (4α-8)-epicatechin; B4	10.45
h	epicatechin (4β--8)-epicatechin, B2	11.30
i	(-)-epicatechin	13.45
j	quercetin	18.15
k	kaempferol	19.45
l	unknown	26.30
m	unknown	27.30
n	unknown	28.00
o	unknown	30.15
p	unknown	31.45
q	unknown	32.20
r	trimeric	34.00
s	epicatechin (4β--8)-epicatechin-epicatechin; C1	35.00
t	unknown	38.15
u	[epicatechin (4β--8)]$_3$-catechin	39.00
v	pentameric	39.15
w	polymeric	42.15
x	polymeric	43.00
y	polymeric	45.30

Table A1-2: Compounds and retention times of the thiolysis product of NBT extracted with water 85°C.

Peak	Possible compound	Retention time
a	gallic acid	2.45
b	(+)-gallocatechin	4.45
c	(-)-epigallocatechin	6.30
d	catechin (4α-8)-catechin; B3	8.15
e	epicatechin n (4β--8)-catechin; B1	9.30
f	(+)-catechin	10.15
g	catechin (4α-8)-epicatechin; B4	10.45
h	epicatechin (4β--8)-epicatechin, B2	11.30
i	(-)-epicatechin	13.45
j	unknown	30.15
k	unknown	31.15
l	unknown	31.30
m	unknown	32.45
n	trimeric	33.30
o	epicatechin (4β--8)-epicatechin-epicatechin; C1	34.30
p	unknown	37.15
q	unknown	38.15
r	tetrameric	39.00
s	polymeric	42.00
t	polymeric	42.45
u	polymeric	45.15

Table A1-3: Compounds and retention times of the thiolysis product of neem bark tannin extracted with ethyl acetate: water (90: 10 v/v).

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	unknown	3.15
c	gallocatechin	4.30
d	unknown	5.15
e	epicatechin n (4β--8)-catechin; B1	9.14
f	epicatechin (4β--8)-epicatechin, B2	11.15
g	unknown	15.20
h	unknown	17.00
i	unknown	17.15
j	kaempferol	19.00

Table A1-4: Compounds and retention times of neem bark tannin extracted with water at 85°C

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	gallocatchin	4.45
c	catechin (4α-8)-catechin; B3	8.15
d	epicatechin (4β--8)-epicatechin, B2	11.00
e	(-)-epicatechin	14.00
f	unknown	16.30
g	quercetin	18.30
h	unknown	30.00
i	unknown	31.45
j	unknown	32.30
k	trimeric	34.00
l	epicatechin (4β-8)-epicatechin-epicatechin; C1	35.00
m	pentameric	39.30
n	polymeric	45.15

Table A1-5: Compounds and retention times of neem bark tannin extracted with ethyl acetate.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	(-)-epicatechin	14.00
c	unknown	32.00
d	unknown	32.45
e	trimeric	34.00
f	epicatechin (4β--8)-epicatechin-epicatechin; C1	35.10
g	pentameric	39.30
h	polymeric	43.15

Table A1-6: Compounds and retention times of neem bark tannins extracted with methanol: water.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	epicatechin (4β--8)-catechin; B1	9.30
c	(+)-catechin	10.30
d	epicatechin (4β--8)-epicatechin, B2	11.05
e	(-)-epicatechin	14.00
f	unknown	27.00
g	unknown	27.30
h	unknown	28.15
i	unknown	30.30
j	polymeric	45.15

Table A1-7: Compounds and retention times of neem bark tannin extracted with acetone: water.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	(-)-epigallocatechin	5.45
c	epicatechin (4β--8)-catechin; B1	9.45
d	(+)-catechin	10.30
e	(-)-epicatechin	14.00
f	unknown	27.00
g	unknown	27.30
h	unknown	28.30
i	unknown	31.45
j	polymeric	45.15

Table A1-8: Compounds and retention times of Fraction I of neem bark tannin.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	epicatechin (4β--8)-catechin; B1	9.00
c	epicatechin (4β--8)-epicatechin, B2	11.30
d	unknown	20.30
e	unknown	22.45
f	polymeric	42.05
g	polymeric	43.15
h	polymeric	46.00

Table A1-9: Compounds and retention times of Fraction II of neem bark tannin.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	(-)-epigallocatechin	6.30
c	catechin (4α-8)-catechin; B3	8.45
d	epicatechin (4β--8)-epicatechin, B2	11.00
e	(-)-epicatechin	15.00
f	unknown	16.05
g	unknown	23.10
h	polymeric	43.45
i	polymeric	46.00

Table A1-10: Compounds and retention times of Fraction IIa of neem bark tannin.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	procyanidin B1	9.30
c	catechin	9.45
d	unknown	12.15
e	(-)-epicatechin	13.45
f	unknown	24.00
g	quercetin	24.45
h	unknown	26.15
i	unknown	27.00
j	unknown	28.00
k	unknown	30.00
l	polymeric	43.30
m	polymeric	44.30
n	polymeric	45.10

Table A1-11: Compounds and retention times of Fraction IIb of neem bark tannin.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	unknown	7.30
c	unknown	21.00
d	unknown	26.45
e	unknown	27.30
f	unknown	34.00
g	unknown	35.00
h	polymeric	43.15
i	polymeric	44.30
J	polymeric	45.15

Table A1-12: Compounds and retention times of Fraction IIc of neem bark tannin.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	(+)-gallocatechin	4.45
c	catechin (4α-8)-catechin; B3	8.15
d	catechin (4α-8)-epicatechin; B4	10.45
e	(-)-epicatechin	13.15
f	unknown	16.15
g	quercetin	17.45
h	unknown	31.30
i	unknown	32.00
j	unknown	32.30
k	trimeric	33.30
l	epicatechin (4β--8)-epicatechin-epicatechin; C1	34.50
m	pentameric	39.30
n	polymeric	39.45

Table A1-13: Compounds and retention times of Fraction IId of neem bark tannin.

Peak	Possible compound	Retention time (min)
a	gallic acid	2.45
b	unknown	15.45
c	quercetin	18.00
d	kaempferol	19.10

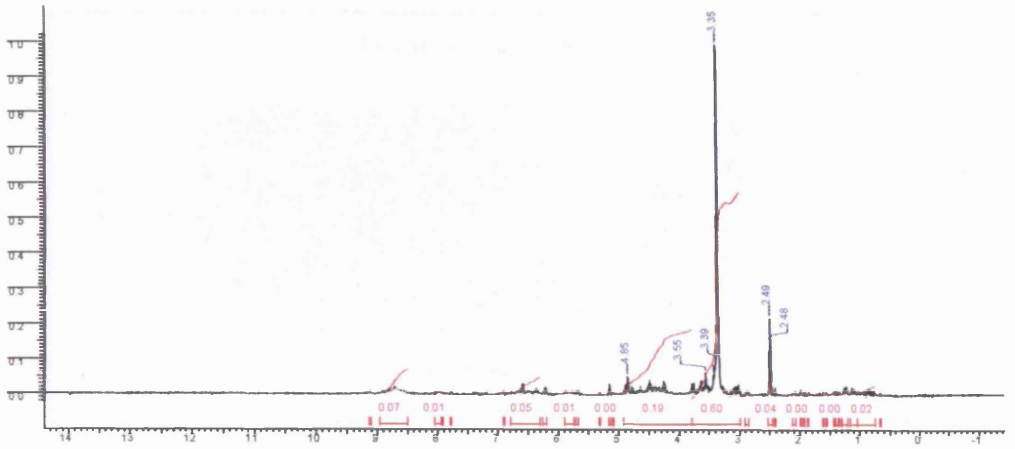


Figure A2-1: ^{1}H NMR spectrum of methyl derivative of neem bark (methanol extract) in D_6 DMSO

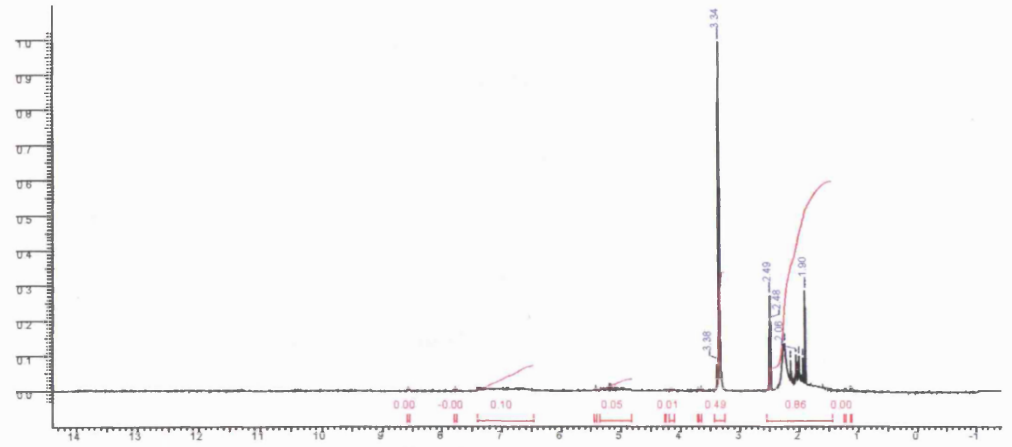


Figure A2-2: ^{1}H NMR spectrum of deca-acetate derivative of neem bark (methanol extract) in D_6 DMSO

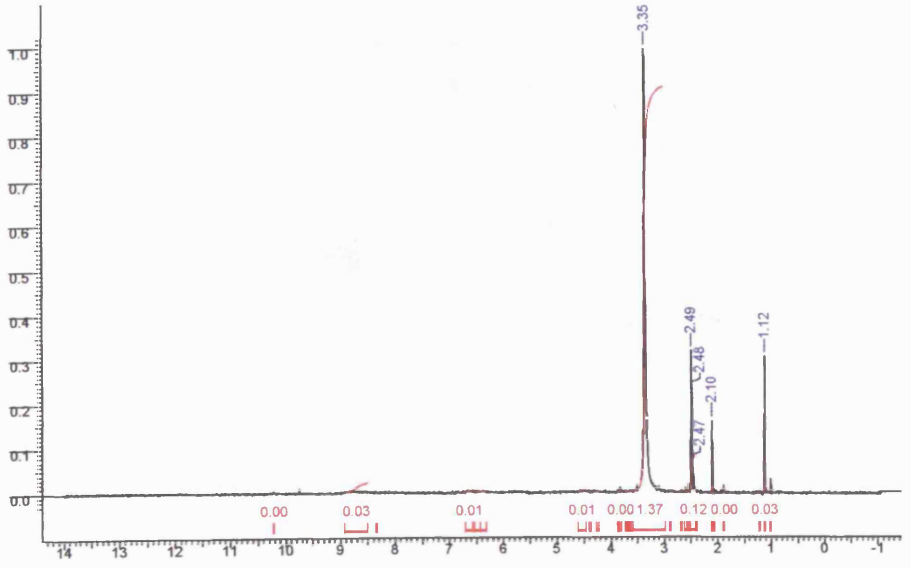


Figure A2-3: ^{1}H NMR spectrum of methyl derivative of neem bark (ethyl acetate extract) in D_6 DMSO

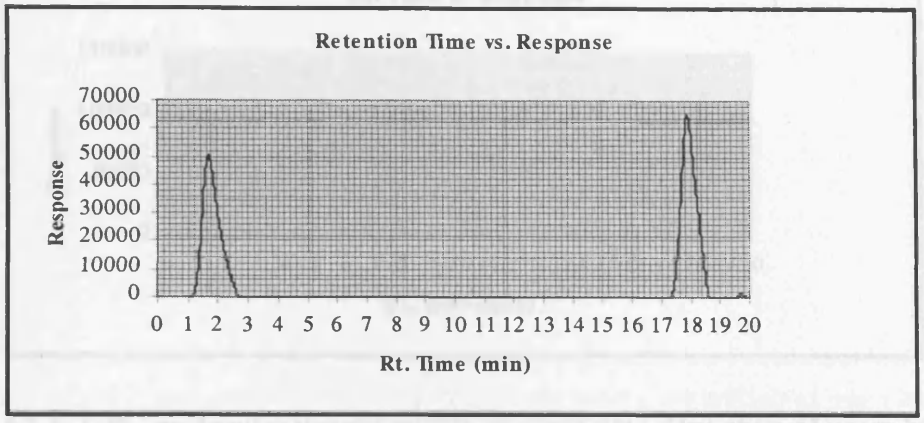


Figure A3-1: GPC molecular weight profile of peracetate derivative of neem bark tannin (acetone extract).

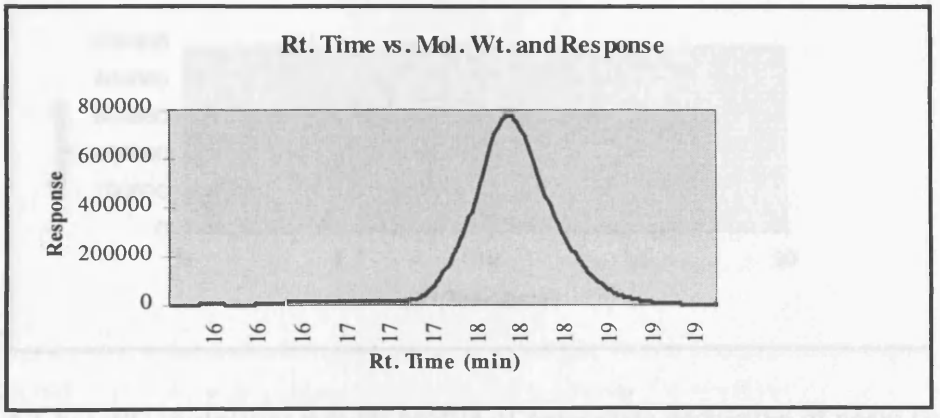


Figure A3-2: GPC molecular weight profile of peracetate derivative of neem bark (methanol extract).

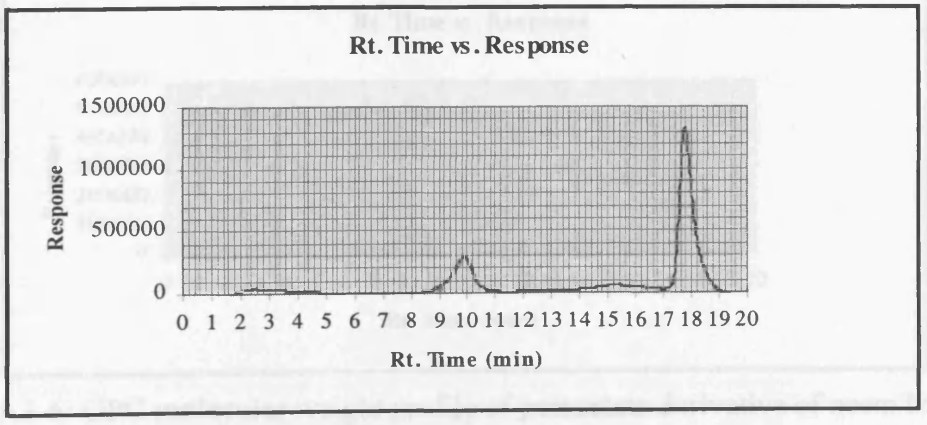


Figure A3-3: GPC molecular weight profile of peracetate derivative of neem bark (ethyl acetate extract).

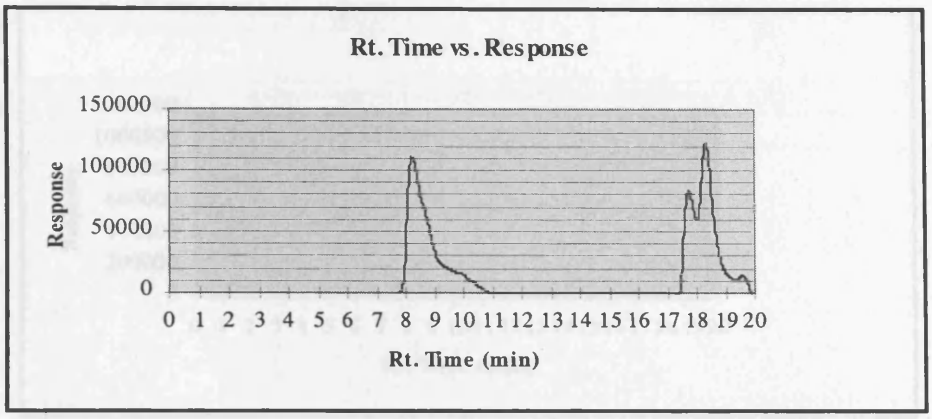


Figure A3-4: GPC molecular weight profile of peracetate derivative of neem bark (Fraction I)

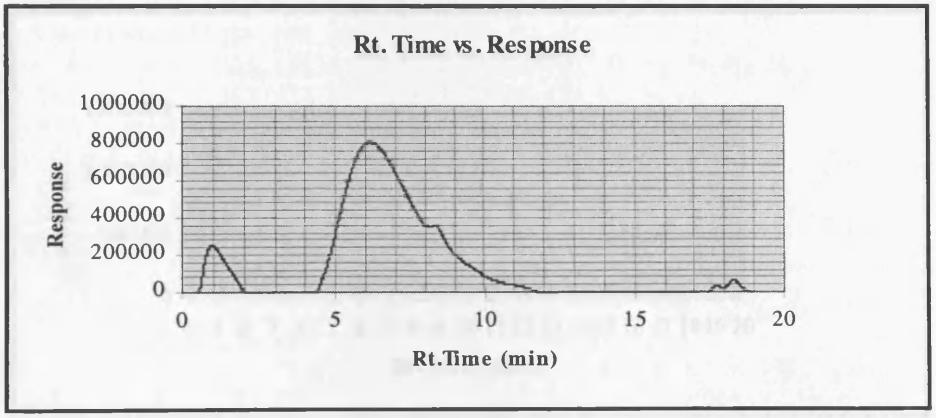


Figure A3-5: GPC molecular weight profile of peracetate derivative of neem bark (Fraction II)

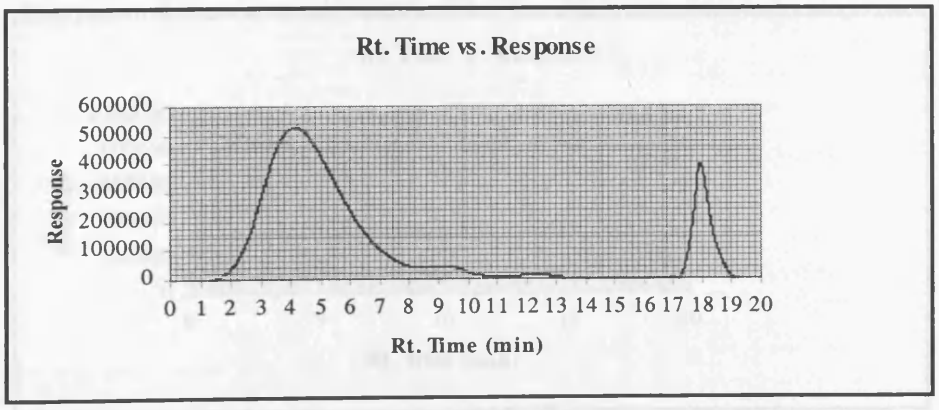


Figure A3-6: GPC molecular weight profile of peracetate derivative of neem bark (Fraction II a)

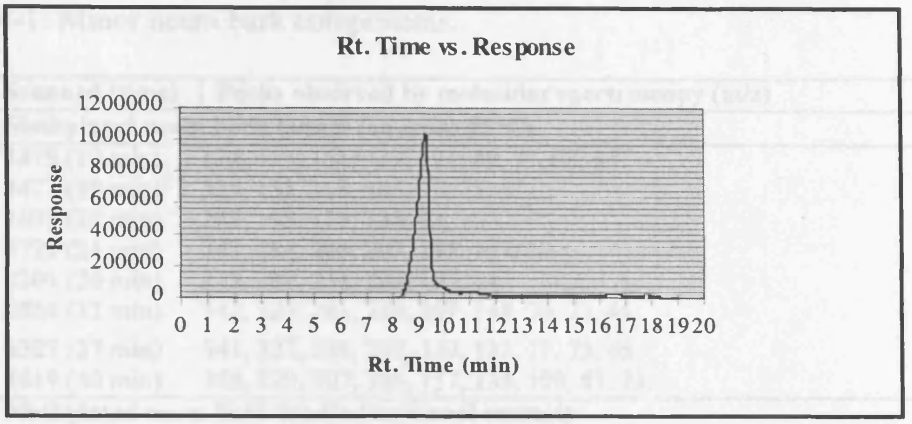


Figure A3-7: GPC molecular weight profile of peracetate derivative of neem bark (Fraction II b)

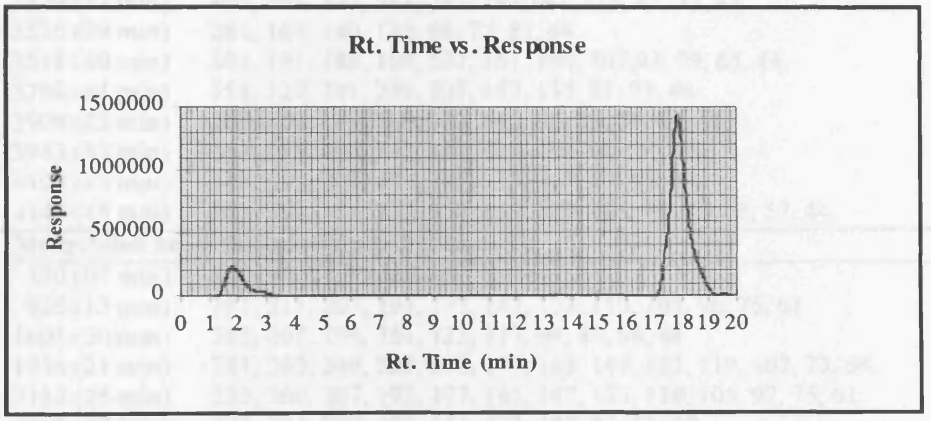


Figure A3-8: GPC molecular weight profile of peracetate derivative of neem bark (Fraction IIc)

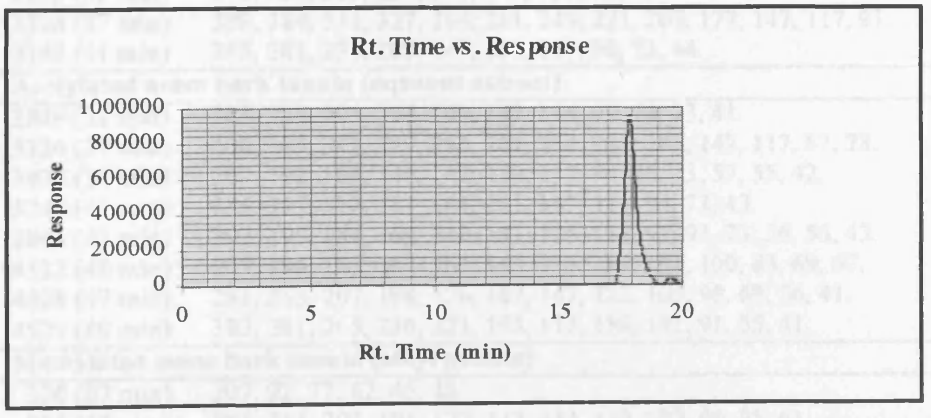


Figure A3-9: GPC molecular weight profile of peracetate derivative of neem bark (Fraction IId)

Table A4-1: Minor neem bark components.

gc peaks	Scanned (time)	Peaks observed by molecular spectroscopy (m/z)
	Methylated neem bark tannin (aqueous 85°C)	
1	1475 (19 min)	172, 153, 151, 133, 121, 89, 77, 63, 45.
2	1477 (19 min)	325, 151, 133, 105, 77, 75, 47.
3	1635 (20 min)	209, 193, 179, 133, 93.
4	1722 (21 min)	342, 281, 265, 207, 193, 167, 69.
5	2201 (26 min)	272, 267, 251, 207, 193, 81.
6	2854 (32 min)	341, 325, 281, 239, 207, 148, 79, 73, 44.
7	3327 (37 min)	341, 327, 281, 207, 147, 133, 77, 73, 45.
8	3619 (40 min)	355, 329, 207, 186, 157, 135, 109, 81, 73.
	Methylated neem bark tannin (methanol extract)	
1	1151 (15 min)	226, 101, 98, 83, 59, 58, 43.
2	1724 (21 min)	281, 207, 87.
3	2304 (27 min)	355, 341, 267, 251, 205, 191, 163, 127, 87.
4	2831 (32 min)	153, 133, 127, 95, 81, 75, 67, 55, 44.
5	2854 (32 min)	341, 325, 251, 207, 191, 179, 147, 127, 81, 73, 45.
6	3329 (37 min)	400, 341, 327, 281, 221, 193,147, 115, 85, 73, 45.
7	3526 (39 min)	281, 169, 140, 122, 94, 77, 51, 44.
8	3618 (40 min)	203, 191, 186, 169, 157, 151, 140, 107,93, 79, 65, 44.
9	3752 (41 min)	355, 327, 281, 221, 207, 147, 135, 81, 73, 44
10	3908 (43 min)	207, 202, 173, 149, 133, 110, 98, 82, 77, 44, 41.
11	3983 (43 min)	341, 219, 190, 173, 152, 127, 110, 98, 79, 57.
12	4104 (45 min)	341, 281, 207, 191, 147, 123, 117, 77, 73, 44.
13	4145 (45 min)	235, 202, 174, 173, 156, 135, 125, 108, 95, 79, 73, 57, 44.
	Methylated neem bark tannin (ethyl acetate)	
1	320 (07 min)	207, 92, 77, 62, 45, 43
2	925 (13 min)	281, 211, 207, 191, 177, 147, 133, 119, 103, 96, 75, 61
3	1605 (20 min)	285, 207, 191, 151, 133, 117, 99, 83, 64, 44
4	1734 (21 min)	281, 265, 249, 235, 193, 177, 163, 147, 133, 119, 103, 73, 68.
5	2132 (25 min)	225, 209, 207, 193, 177, 165, 147, 133, 119, 105, 97, 75, 61.
6	3368 (37 min)	355, 281, 207, 173, 151, 123, 109, 81, 53, 50
	Acetylated neem bark tannin (acetone extract)	
1	1144 (15 min)	101, 98, 83, 59, 56, 43, 41.
2	1707 (27 min)	281, 267, 207, 191, 55.
3	2297 (27 min)	356, 267, 207, 193, 135, 121.
4	2845 (32 min)	341, 325, 263, 224, 191, 147, 133, 73, 45.
5	3328 (37 min)	399, 384, 341, 327, 284, 281, 249, 221, 207, 177, 147, 117, 91.
6	3742 (41 min)	355, 281, 221, 207, 147, 133, 107, 96, 73, 44.
	Acetylated neem bark tannin (aqueous extract)	
1	2849 (32 min)	341, 325, 225, 207, 191, 147, 126, 97, 74, 73, 41.
2	3324 (37 min)	400, 383, 341, 327, 284, 281, 252, 221, 207, 147, 117, 87, 73.
3	3474 (38 min)	207, 192, 163, 146, 142, 130, 117, 94, 86, 73, 57, 55, 42.
4	3743 (41 min)	385, 355, 327, 281, 267, 221, 147, 111, 94, 73, 43.
5	3894 (43 min)	207, 195, 184, 169, 149, 141, 125, 117, 97, 91, 73, 56, 55, 43.
6	4122 (45 min)	207, 196, 181, 167, 163, 153, 135, 119, 107, 100, 83, 69, 67.
7	4328 (47 min)	281, 253, 207, 198, 176, 167, 147, 123, 105, 96, 69, 56, 41.
8	4529 (49 min)	383, 281, 265, 236, 221, 193, 175, 150, 105, 91, 55, 41.
	Methylated neem bark tannin (ethyl acetate)	
1	320 (07 min)	207, 92, 77, 62, 45, 43
2	925 (13 min)	281, 211, 207, 191, 177, 147, 133, 119, 103, 96, 75, 61
3	1605 (20 min)	285, 207, 191, 151, 133, 117, 99, 83, 64, 44
4	1734 (21 min)	281, 265, 249, 235, 193, 177, 163, 147, 133, 119, 103, 73, 68.
5	2132 (25 min)	225, 209, 207, 193, 177, 165, 147, 133, 119, 105, 97, 75, 61.
6	3368 (37 min)	355, 281, 207, 173, 151, 123, 109, 81, 53, 50

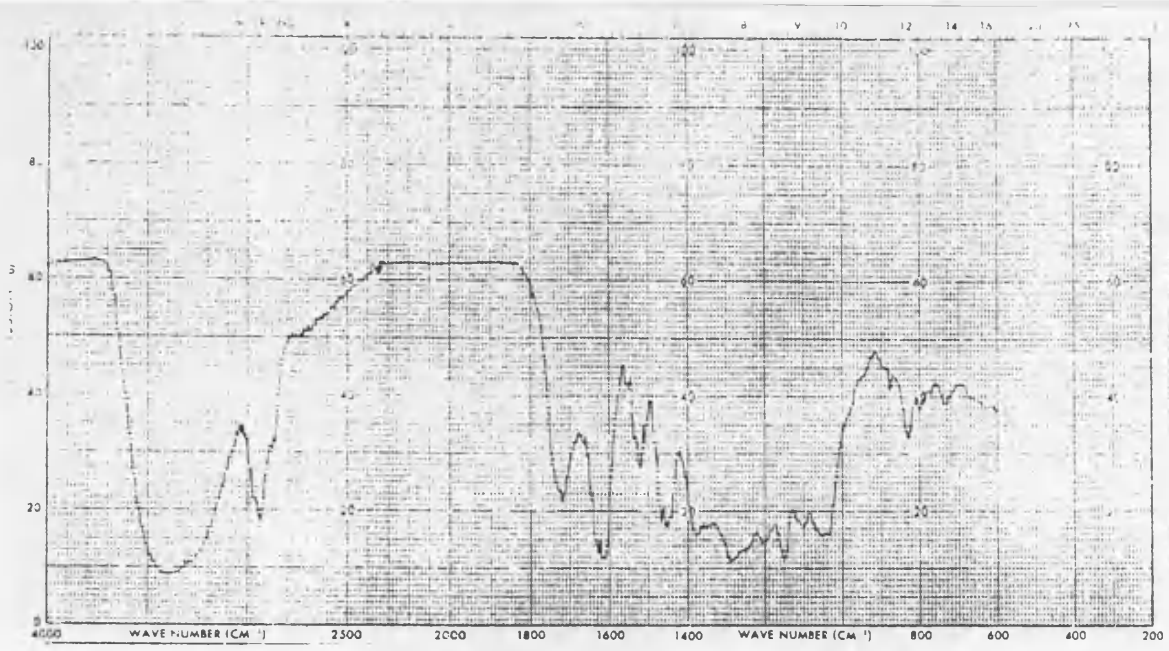


Figure A5-1: Infrared spectrum of neem bark tannins extracted with 50% acetone.

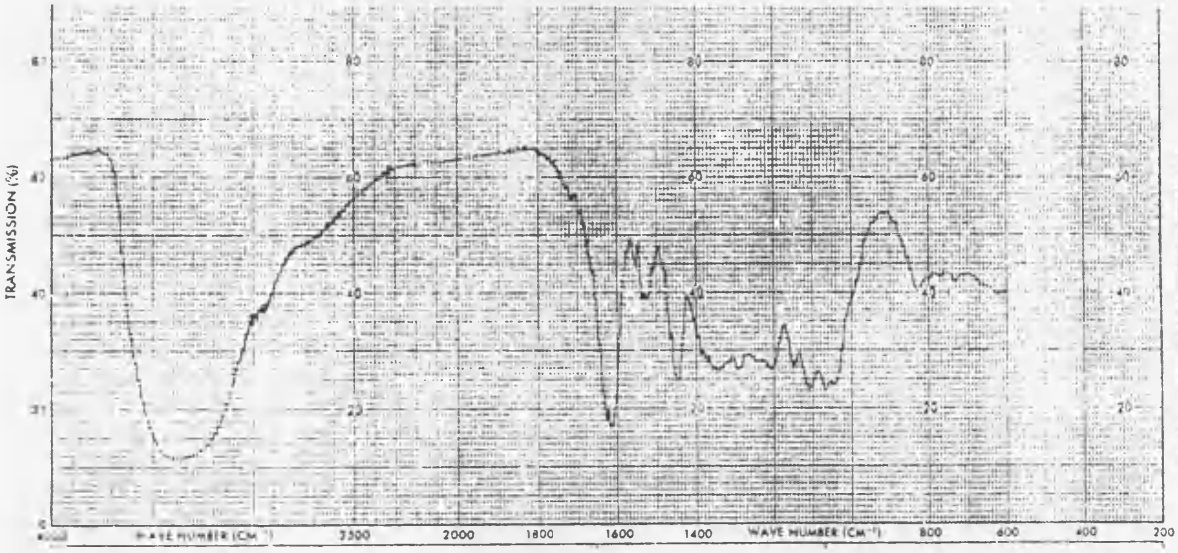


Figure A5-2: Infrared spectrum of neem bark tannins extracted with 95% methanol.

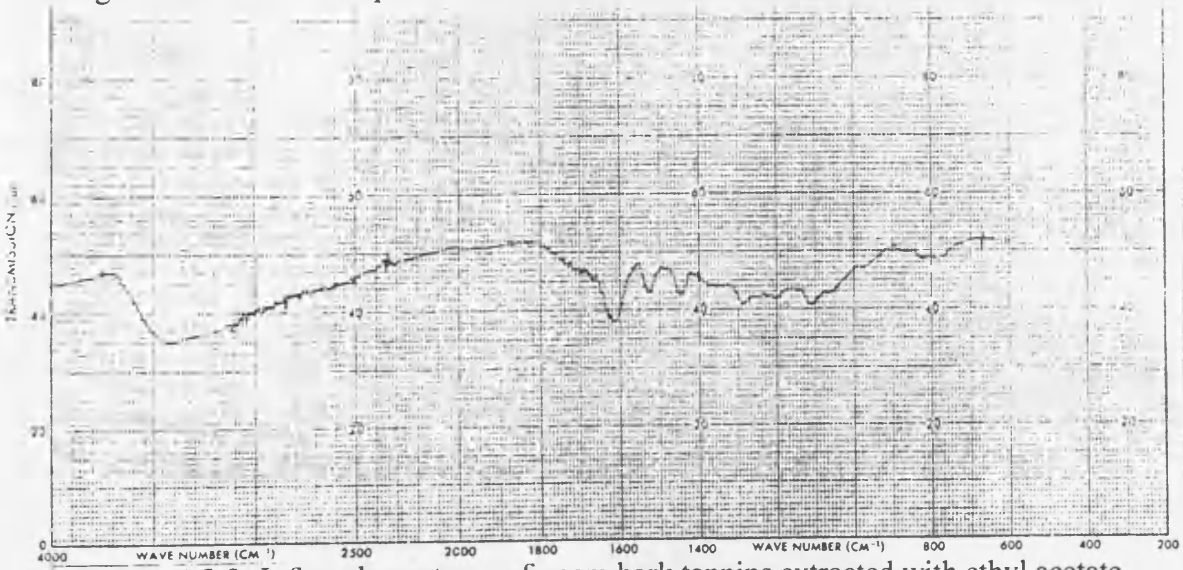


Figure A5-3: Infrared spectrum of neem bark tannins extracted with ethyl acetate.

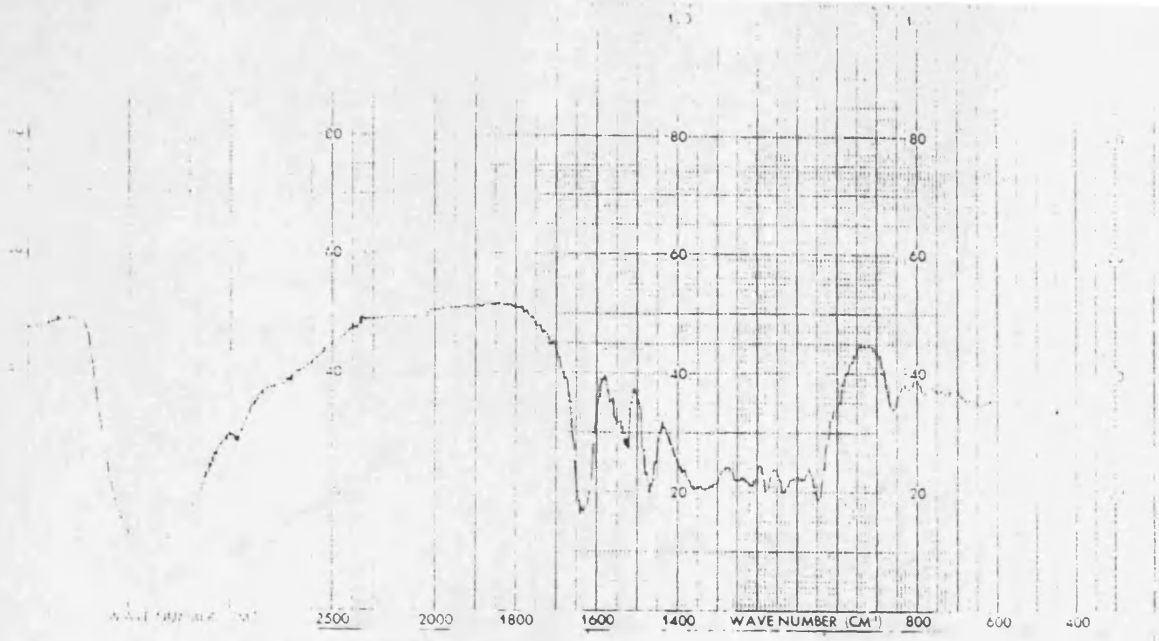


Figure A5-4: Infrared spectrum of neem bark tannins (Fraction I)

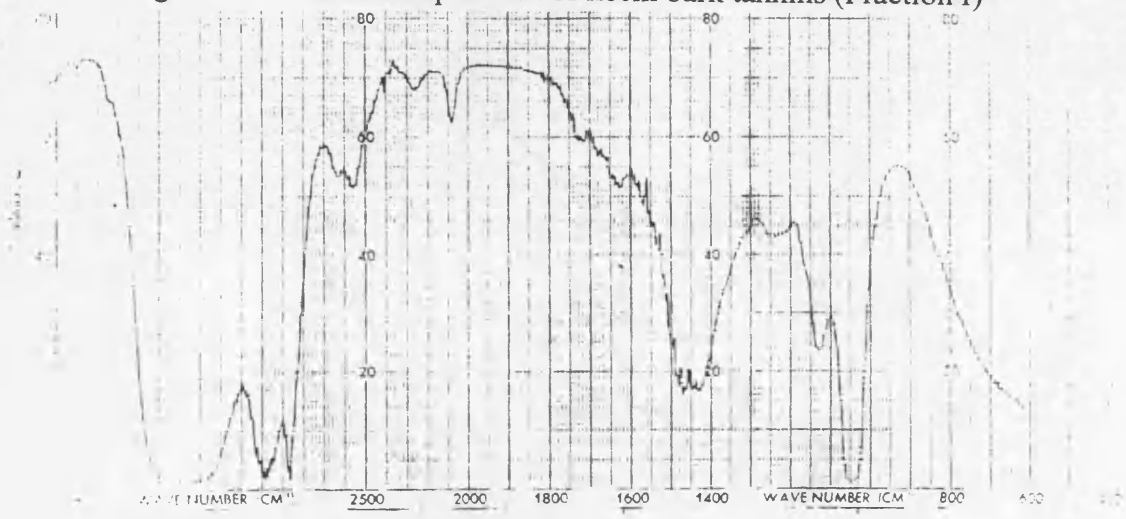


Figure A5-5: Infrared spectrum of neem bark tannins (Fraction II).

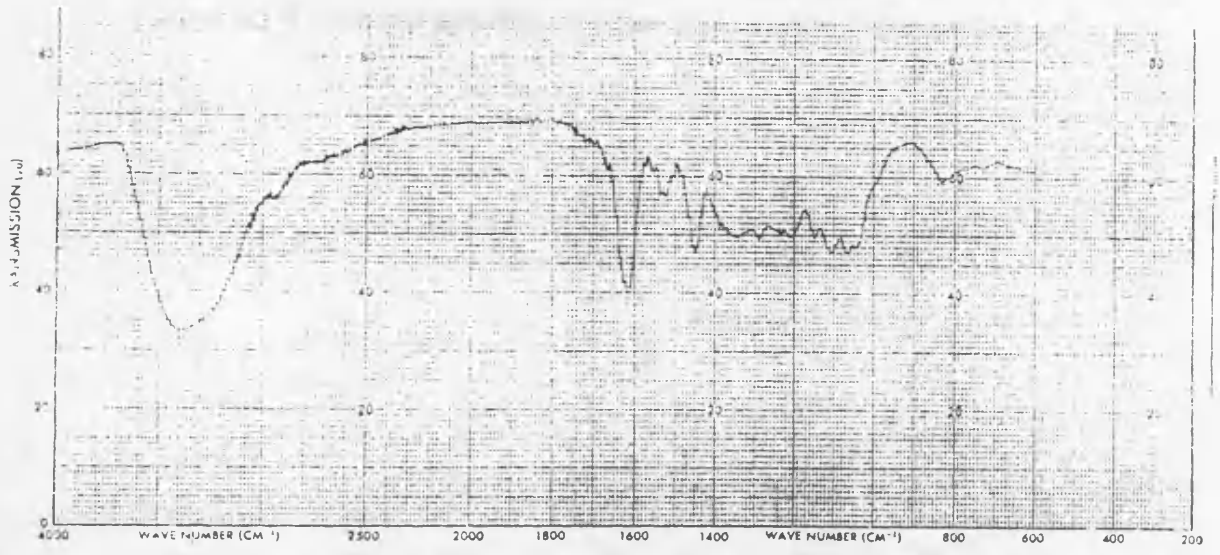


Figure A5-6: Infrared spectrum of neem bark tannins (Fraction IIa).

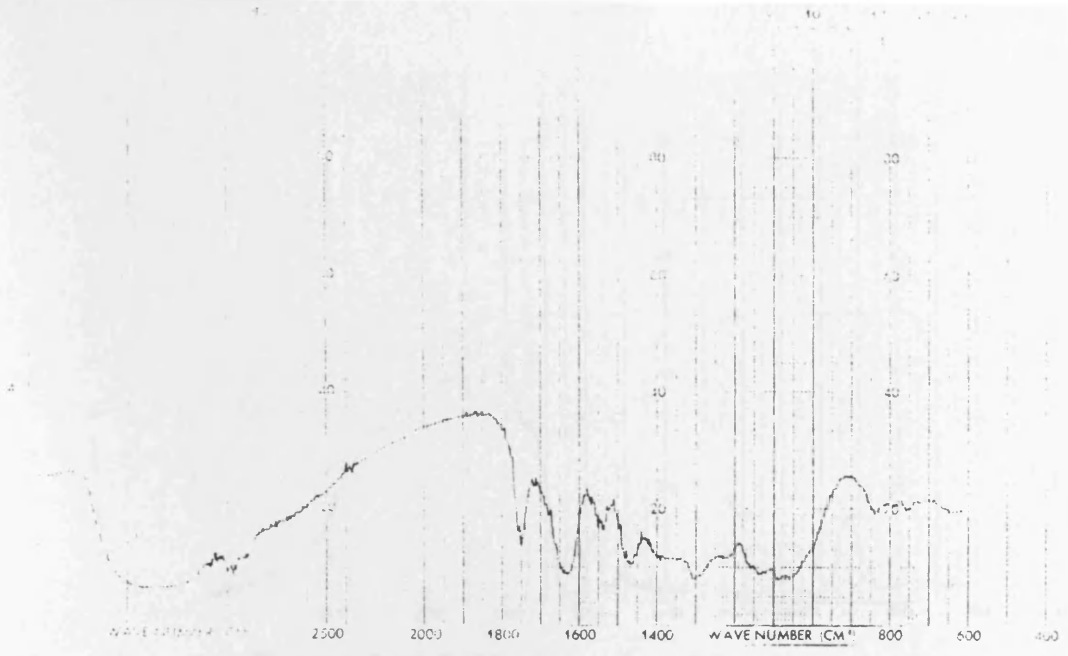


Figure A5-7: Infrared spectrum of neem bark tannins (Fraction II b)

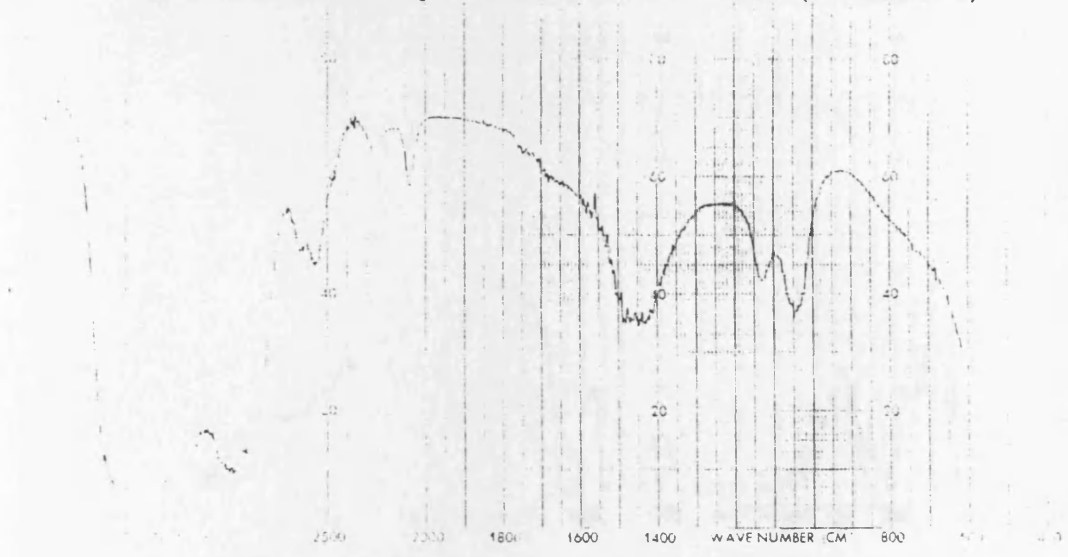


Figure A5-8: Infrared spectrum of neem bark tannins (Fraction II c)

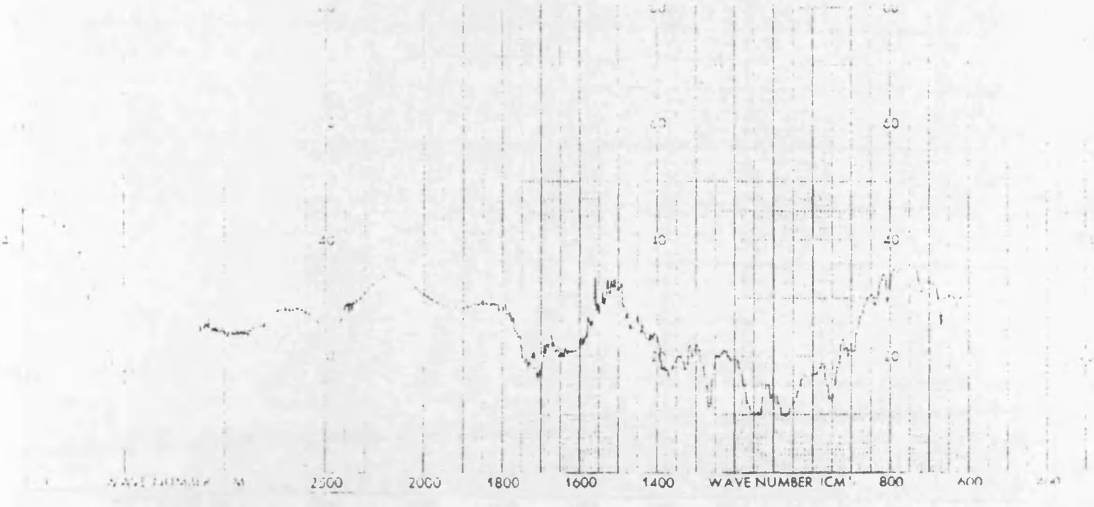


Figure A5-9: Infrared spectrum of neem bark tannins (Fraction II d)

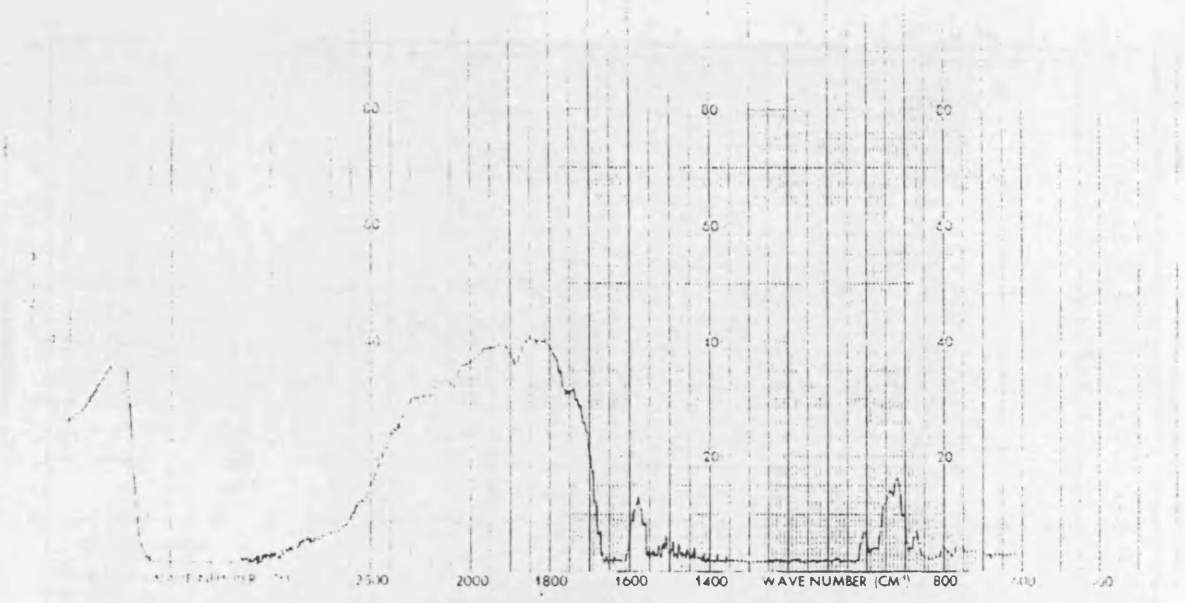


Figure A5-10: Infrared spectrum of catechin.

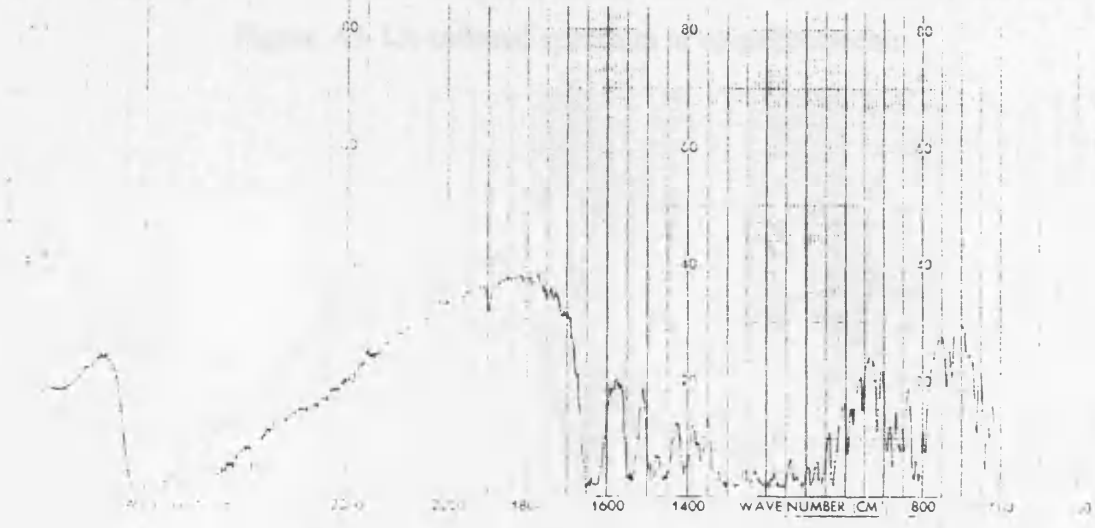


Figure A5-11: Infrared spectrum of epicatechin.

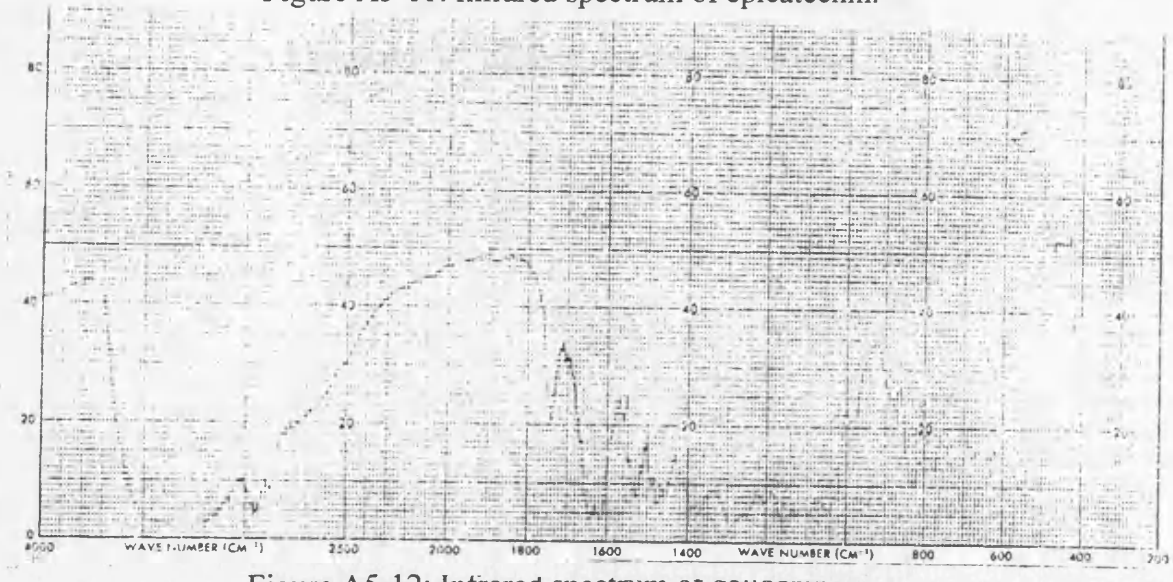


Figure A5-12: Infrared spectrum of gallocatechin.

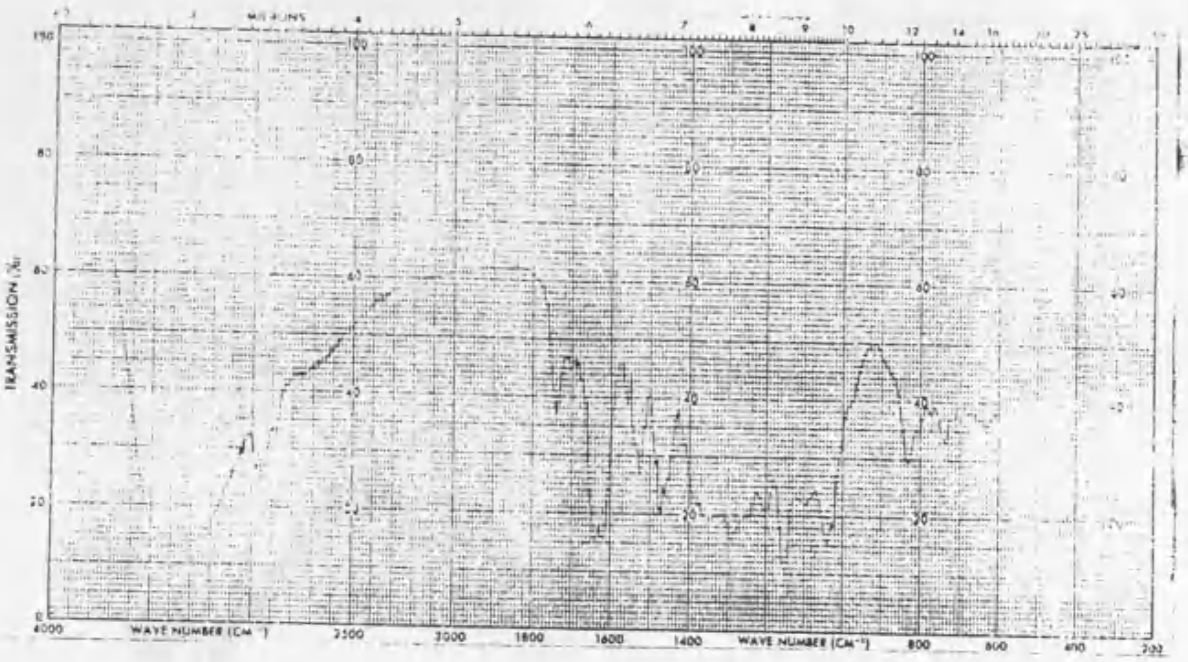


Figure A5-13: Infrared spectrum of epigallocatechin.

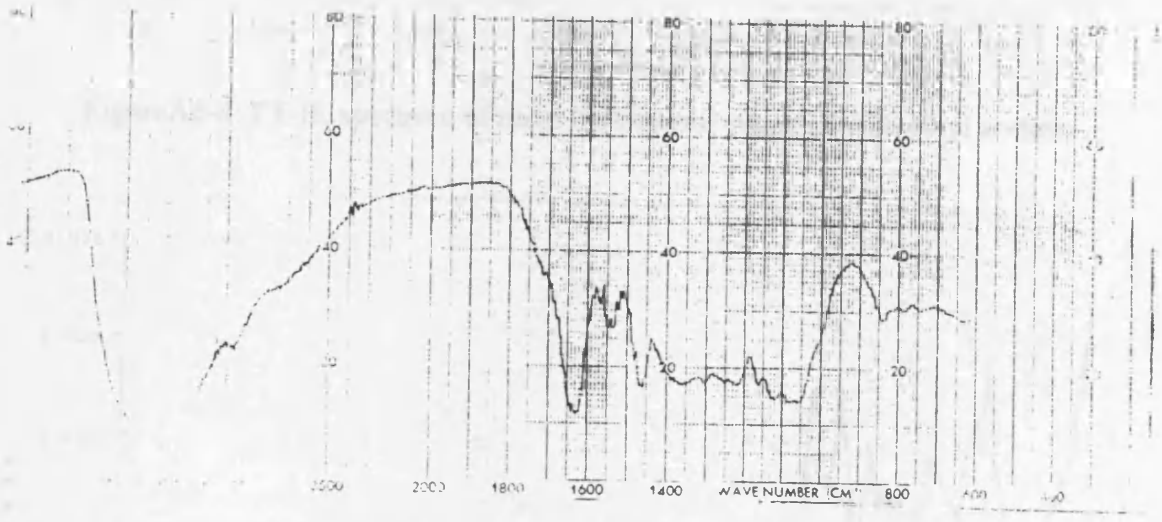


Figure A5-14: Infrared spectrum of mimosa.

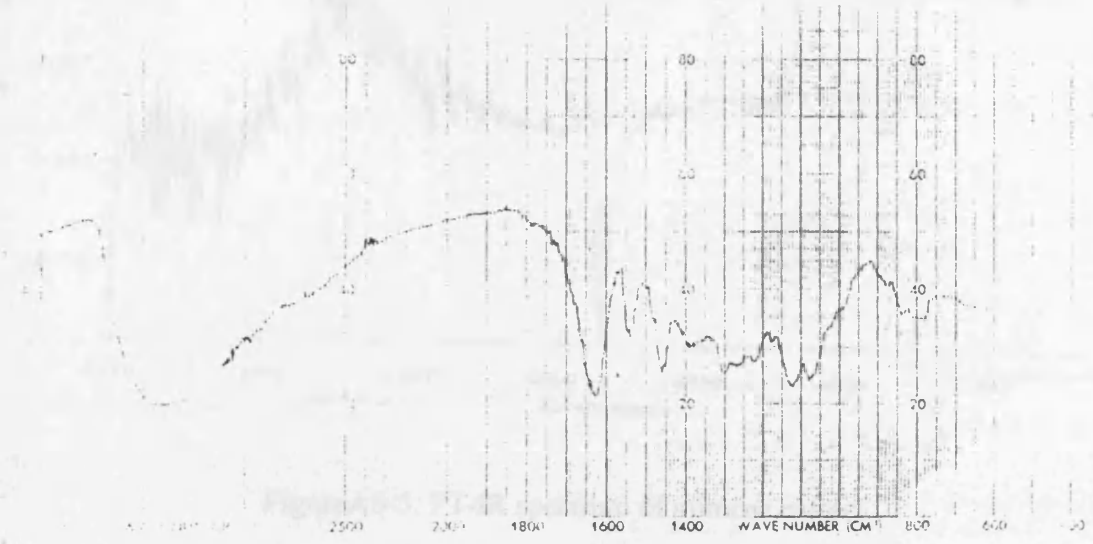
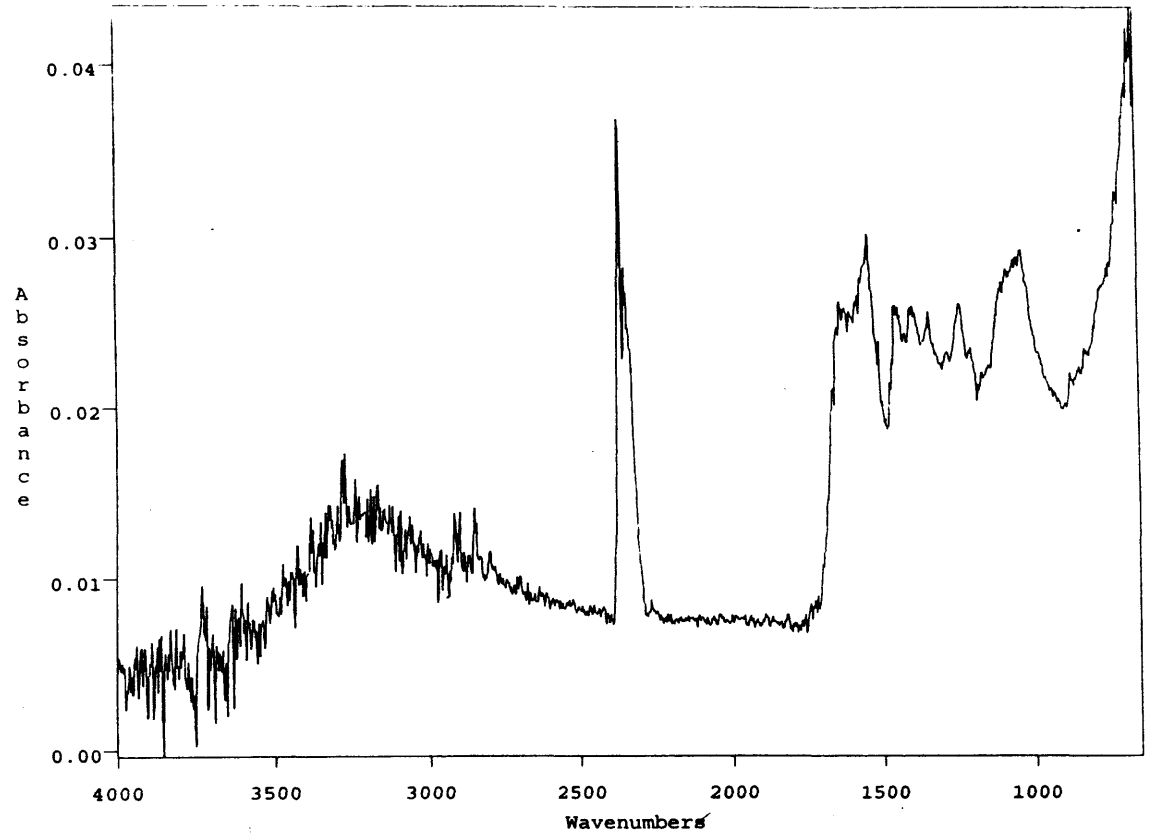
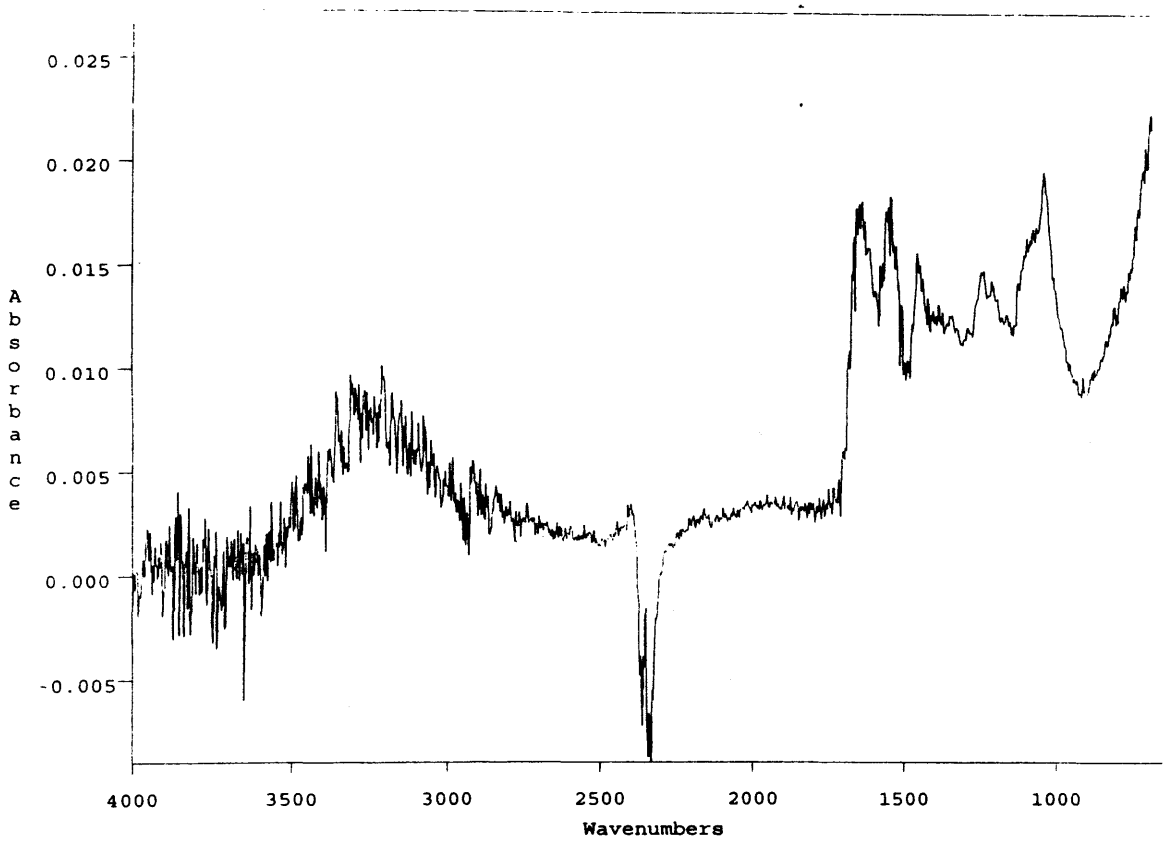


Figure A5-15: Infrared spectrum of quebracho.



FigureA6-4: FT-IR spectrum of neem bark tannin extracted with ethyl acetate.



FigureA6-5: FT-IR spectrum of mimosa extract.

APPENDIX B

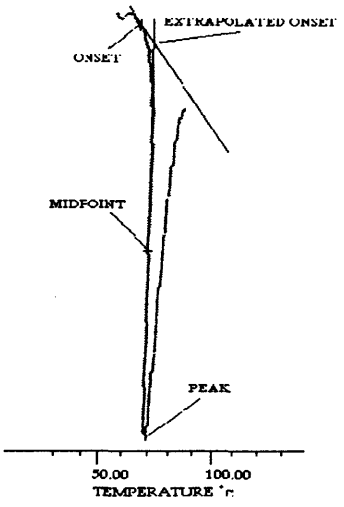


Figure B-1: DSC thermogram for the THPS pretanned hide powder tanned with neem bark.

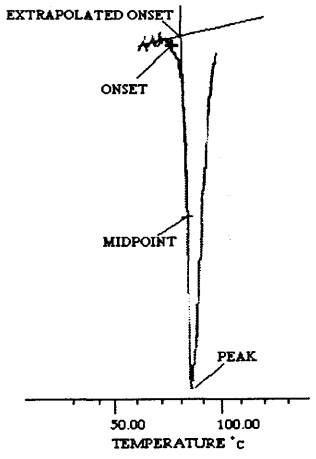


Figure B-2: DSC thermogram for the THPS pretanned hide powder, tanned with mimosa.

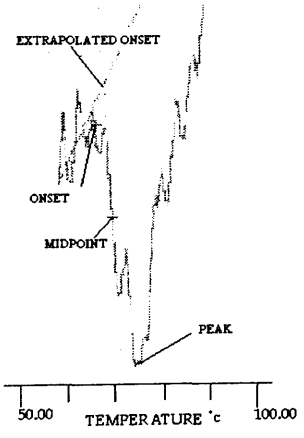


Figure B-3: DSC thermogram for the chromium pretanned hide powder, tanned with neem bark .

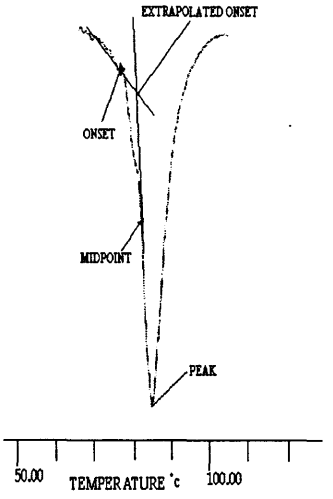


Figure B-4: DSC thermogram for the chromium pretanned hide powder, tanned with mimosa.

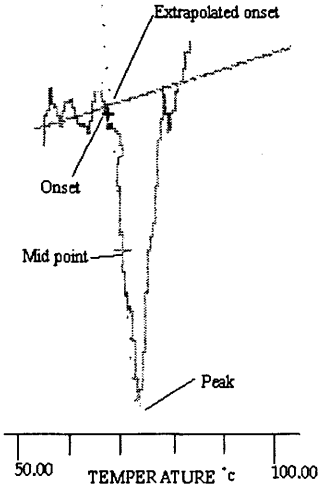


Figure B-5: DSC thermogram for the glyoxal pretanned hide powder, tanned with neem bark.

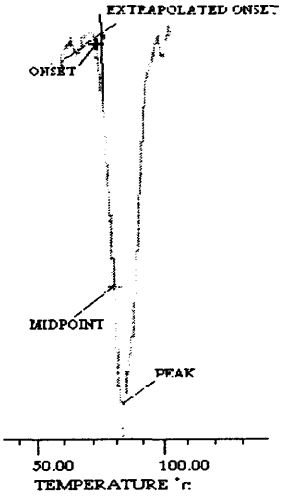


Figure B-6: DSC thermogram for the glyoxal pretanned hide powder, tanned with mimosa.

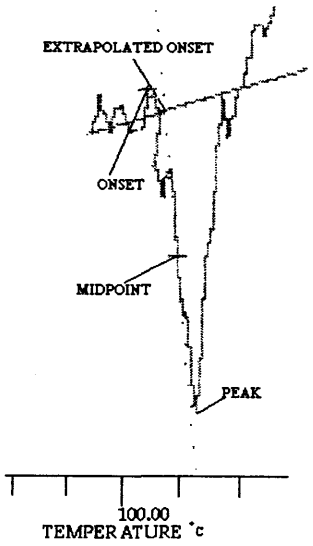


Figure B-7: DSC thermogram for shrinking of hide powder tanned with neem bark, chrome retanned.

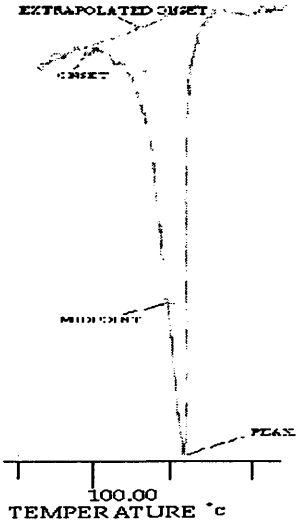


Figure B-8: DSC thermogram for shrinking of hide powder tanned with mimosa, chrome retanned.

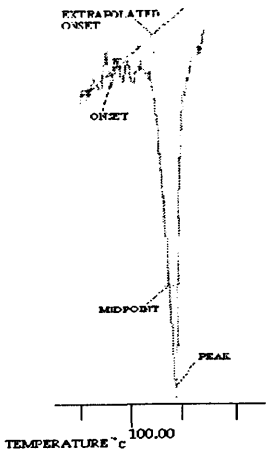


Figure B-9: DSC thermogram for shrinking of hide powder tanned with neem bark, aluminium retanned.

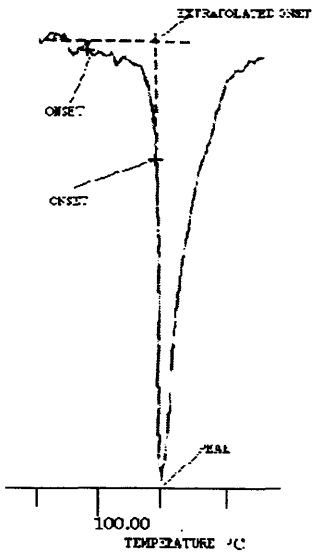


Figure B-10: DSC thermogram for shrinking of hide powder tanned with mimosa, aluminium retanned.

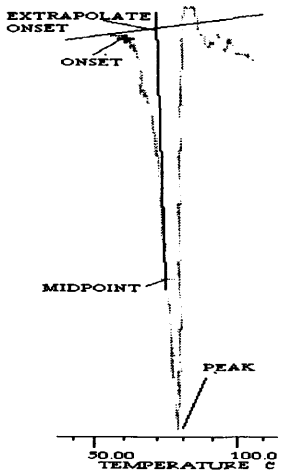


Figure B-11: DSC thermogram for shrinking of hide powder tanned with neem bark, glyoxal retanned.

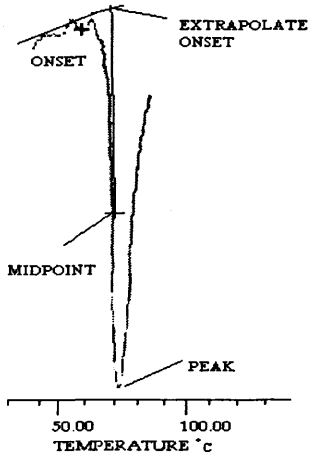


Figure B-12: DSC thermogram for shrinking of hide powder tanned with mimosa, glyoxal retanned.

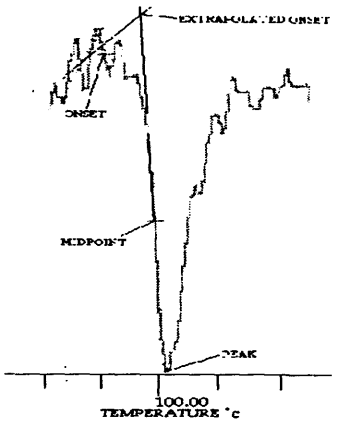


Figure B-13: DSC thermogram for shrinking of hide powder tanned with neem bark, THPS retanned.

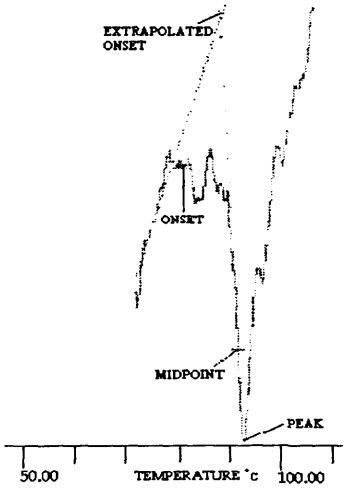


Figure B-14: DSC thermogram for shrinking of powder tanned with mimosa, THPS retanned hide.

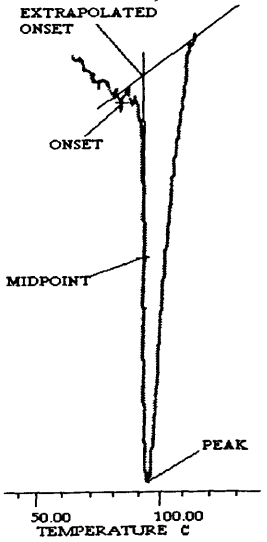


Figure B-15: DSC thermogram for shrinking of hide powder tanned with neem bark, HCHO retanned.

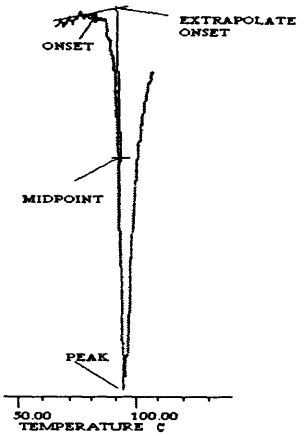


Figure B-16: DSC thermogram for shrinking of hide powder tanned with mimosa, HCHO retanned.

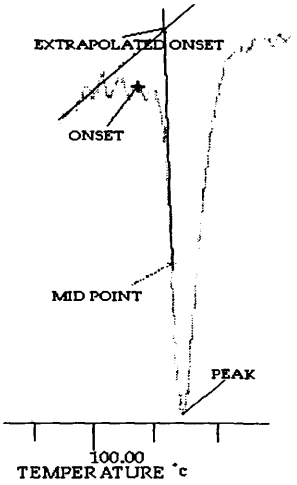


Figure B-17: DSC thermogram for shrinking of hide powder (pretan with chrome) tanned with neem bark, oxazolidine retanned.

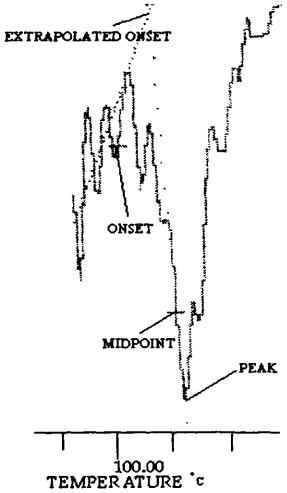


Figure B-18: DSC thermogram for shrinking of hide powder (pretan with chrome) tanned with mimosa, oxazolidine retanned.

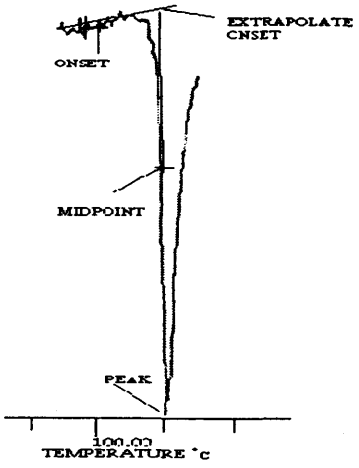


Figure B-19: DSC thermogram for shrinking of hide powder tanned with neem bark, oxazolidine retanned

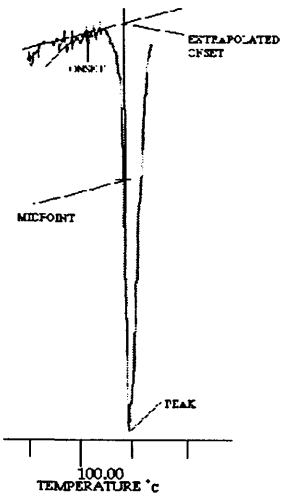


Figure B-20: DSC thermogram for shrinking of hide powder tanned with mimosa, oxazolidine retanned

B.1.1. RECIPES FOR THE PROCESSES

B.1.2. Vegetable tanning of hide powder

Offers on hide powder weight.

Soaked hide powder (100 g) in 250-cm^3 overnight, drained and processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Pickling					
	Water (distilled)	80	20		
	Sodium chloride	8		10	
	Formic acid (1:10)	0.2		60	pH 4.8
	Sulphuric acid (1:10)	0.1		60	pH 2.8
Drained and immediately dried by suction using Buchner flask then air-dried. Parts of sample used for rest of the processes.					
pH adjustment of hide powder	Water	80	20	5	
	Sodium chloride	5			
	Sodium bicarbonate (1:10)	0.5		30	pH 4.5
	Sodium bicarbonate (1:10)	0.1		60	pH 4.8
				120	Stand
				30	pH 5.0
Drain					
Tanning					
	Water (distilled)	25	25		
	Antimould A5	1			
	Neem bark tannin or mimosa	5		30	
		10		60	
		10	35	120	pH 3.8

For one or the other experiments, neem bark tannin or mimosa tanned sample were washed with distilled water, drained, wrapped with plastic sheet and held in a freezer at -4°C.

B.1.3. Phenolic based syntan used for pretanning

Offer on hide powder weight.
Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Pretanning					
	Water	60	20		
	Sodium chloride	5		5	
	Sodium bicarbonate (1:10)	0.1		30	pH 4.4
	Sodium bicarbonate (1:10)	0.05		30	pH 4.8
	Basyntan P			30	
Tanning	Neem bark tannin or mimosa	10		60	
	Neem bark tannin or mimosa	10		120	Leave overnight
				10	
	Formic acid	0.01		20	

B.1.3.2. THPS used for pretanning

Offers on hide powder weight.
Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Pretanning					
	Water	60	20		
	Sodium chloride	5		5	
	Sodium bicarbonate (1:10)	0.1		30	pH 4.0
	THPS (Albrite AD)	4		120	pH 4.0
	Sodium bicarbonate (1:10)	0.5	35	30	pH 6.8
Tanning	Neem bark tannin or mimosa	10		60	
	Neem bark tannin or mimosa	10		120	Leave overnight
				15	pH 6.0
	Formic acid	0.01		30	pH 5.5

B.1.3.3. Chromium was used for pretanning

Offers on hide powder weight.
Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Pretanning					
	Water	60	20		
	Sodium chloride	5		5	
	Sulphuric acid (1:10)	0.1		30	pH 2.9
	Chrome powder			30	
Tanning	Neem bark tannin or mimosa	10		60	
	Neem bark tannin or mimosa	10		120	Leave overnight
				10	pH 5.0
	Formic acid	0.01		20	pH 4.0

B.1.3.4. Glyoxal used for pretanning

Offers on hide powder weight.
Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Pretanning					
	Water	60	20		
	Sodium chloride	5		5	
	Sodium bicarbonate (1:10)	0.1		30	pH 4.4
	Sodium bicarbonate (1:10)	0.07		30	pH 5.2
	Methyl glyoxal	5		30	
Tanning	Neem bark tannin or mimosa	10		60	pH 6.0
	Neem bark tannin or mimosa	10		120	Leave overnight
				30	Leave overnight

For differential scanning calorimetry experiments, pretanned neem bark tannin or mimosa tanned sample were washed with distilled water drained and, wrapped with plastic sheet and held in a freezer at -4°C.

B.1.4. Retannage

B.1.4.1. Semi-chrome hide powder

Offers on tanned hide powder weight.
Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Retanning	Water	60	35		
	Sodium chloride	3		30	pH 4.8
	Formic acid	0.1		60	Leave overnight
	Sodium sulphite	0.01		30	pH 4.0
	Chrome	3	40	60	
				60	

B.1.4.2. Semi-aluminium hide powder

Offers on tanned hide powder weight.
Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Retanning	Water	60	35		
	Sodium chloride	3		30	pH 4.8
	Sodium acetate	0.1		30	pH 3.8
	Lutan B	3		120	Leave overnight
				60	pH 4.2

B.1.4.3. Glyoxal retanned hide powder

Offers on tanned hide powder weight.
Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Retanning	Water	60	45		
	Sodium chloride	3		30	pH 4.8
	Sodium sulphite (1:20)	0.1		30	pH 5.2
	Glyoxal	3		120	Leave overnight
			50	60	pH 4.2
	Sodium bicarbonate	0.2	50	30	6.7

B.1.4.4. THPS retanned hide powder

Offer on tanned hide powder weight.

Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Retanning	Water	60	35		
	Sodium chloride	3		30	pH 4.8
	THPS (Albrite AD)	4	55	45	
	Sodium bicarbonate (1:10)	0.5		120	pH 5.1
					Leave overnight
	Sodium bicarbonate	0.4	55	30	pH 6.0
					Leave overnight
	Sodium bicarbonate	0.1		30	pH 6.7

B.1.4.5. Formaldehyde retanned hide powder

Offer on tanned hide powder weight.

Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Retanning	Water	60	35		
	Sodium sulphite (1:20)	0.1		30	pH 4.6
	Formaldehyde	3		120	Leave overnight
	Sodium bicarbonate (1:10)	0.2		120	pH 5.3
					Leave overnight
	Sodium bicarbonate	0.1	60	30	pH 5.0

B.1.4.6. Oxazolidine retanned hide powder

Offer on tanned hide powder weight.

Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Retanning	Water	60	35		
	Sodium sulphite (1:10)	0.1		60	pH 5.1
	Neosyn TX	5	40	240	
	Sodium bicarbonate (1:10)	0.2	60	300	pH 6.2

B.1.4.7. Oxazolidine retanned hide powder (pretanned with chromium)

Offers on tanned hide powder weight.

Hide powder was soaked overnight in distilled water at 20°C, then drained before further processing as follows.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Retanning	Water	60	35		
	Sodium sulphite (1:10)	0.1		60	pH 5.1
	Neosyn TX	5	40	240	
	Sodium bicarbonate (1:10)	0.2	60	300	pH 6.1

B.1.5. Pilot scale-tanning experiments

Three commercially pickled sheepskins (weight 3.2 kgs)

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Degreasing					
	Paraffin liquid	100	35	120	
Drain					
Hand scudding					Blunt knife used
	Water	150	25		
	Sodium chloride	10			
	Supralan UF	1		60	
Drain					
Wash	Water	80	20		
	Sodium chloride	5		30	Leave overnight
Drain					
Tanning					
	Water	50	20		pH 3.4
	Sodium chloride	8		10	
	Sodium bicarbonate (1:10)	0.2		60	pH 5.7
	Lactic acid	0.2		60	pH 4.9
				120	Stand
	Antimould A5	0.8		30	
	Neem bark tannin or mimosa	10		60	
	Neem bark tannin or mimosa	10		180	Leave overnight
					Noted Ts every hour
Fatliquoring	Remsynol ESA	2	55	30	
	Formic acid	0.5		10	
Wash	Water	200	20	10	
Rinse				5	Horse up
Sam					
Set out					
Shaving					0.9-1.0 mm
Dried ,stake and toggle					

B.1.6. Retannage of neem bark tannin or mimosa tanned sheepskins with oxazolidine

Offers on shaved weight*

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Wetting back					
	Water	300	35	15	pH 4.7
Drain					
Rinse				5	
Retanning					
	Water	100	35		
	Neosyn TX	5		15	
			60	240	pH 6.2
Drain					Noted Ts every hour
Wash					
	Water	200	35	10	
Drain					
Rinse				5	
Fatliquoring					
	Water	100	55		
	Remsynol ESA	2		45	
	Formic acid	0.8		30	
Wash					
	Water	100	20	10	
Rinse					Hourse up
Samm					
Set out					
Hang dried					
Stake and toggle					

*NBT leather weight 875 gm: Mimosa leather weight 745 gm.

B.1.7. Dyeing of neem bark tannin or mimosa tanned leather.

Operation	Chemicals	%	Temp (°C)	Running Time (min)	Comments
Wet back					
	Water	200	25	10	
Dyeing					
	Water	100	35	5	
	Sellafast Yellow G	2	45	45	
			55	10	
	Remsynol ESA	2		30	
	Formic acid	0.8		10	
Wash					
	Water	100	20	10	
Rinse				5	
Horse up					
Samm					
Set out					
Hang dried					
Stake and toggle					

*NBT leather weight 867 gm: Mimosa leather weight 767 gm.

APPENDIX C

APPENDIX C1: Softness.

Table C 1-1: Average softness values of neem bark tanned leather, according to sampling plan.

Sample Positions	Shoulder				Average (mm)
	a	b	c	d	
1	3.1	3.4	3.6	5.7	4.0
2	3.5	3.1	3.7	4.3	3.7
3	3.0	3.3	3.5	3.5	3.3
4	2.5	3.2	3.1	3.8	3.2
5	3.3	3.7	3.3	3.5	3.5
6	4.0	4.6	3.5	3.2	3.8
7	3.7	4.1	3.6	3.4	3.7

Sampling Positions	Butt				Average (mm)
	a	b	c	d	
8	4.5	4.7	3.8	4.1	4.3
9	4.7	4.3	3.8	5.1	4.5
10	4.2	4.2	4.1	4.2	4.2
11	4.4	4.0	3.9	4.2	4.1
12	4.1	4.3	3.7	3.5	3.9
13	3.6	3.8	3.7	3.5	3.7
14	5.6	3.4	3.7	4.0	4.2
15	4.4	4.2	3.5	4.7	4.2

Sampling Positions	Belly			Average (mm)
	e	r	g	
1	4.0	4.0	4.1	4.0
2	3.9	4.1	3.1	3.7
3	3.3	3.6	3.2	3.4
4	3.9	3.2	3.2	3.4
5	3.4	2.8	3.4	3.2
6	3.8	3.5	3.6	3.6
7	3.0	4.2	4.2	3.8
8	3.5	4.8	4.0	4.1
9	3.9	5.0	4.1	4.3
10	3.9	3.2	3.4	3.5
11	4.9	4.0	3.5	4.1
12	4.1	3.9	3.5	3.8
13	4.1	5.2	5.0	4.8
14	3.5	4.7	5.3	4.5
15	4.9	4.8	5.9	5.2

Sampling position numbers and letters are defined in Figure 5.2-1.

Table C 1-2: Average softness values of mimosa tanned leather.

Sampling Positions	Shoulder				Average (mm)
	a	b	c	d	
1	6.1	5.7	5.3	6.1	5.8
2	4.0	4.2	4.7	5.8	4.7
3	6.8	6.1	4.2	4.9	5.5
4	5.8	5.5	5.1	5.5	5.5
5	7.2	5.4	5.8	6.8	6.3
6	5.7	5.2	4.9	5.0	5.2
7	6.2	5.1	5.1	5.o	5.4

Sampling Positions	Butt				Average (mm)
	a	b	c	d	
8	5.7	5.2	5.8	4.9	5.4
9	6.0	5.9	5.8	5.9	5.9
10	6.4	5.2	6.0	4.9	5.6
11	4.1	4.1	4.2	4.4	4.2
12	4.5	5.5	5.7	5.8	5.4
13	5.8	4.4	4.4	3.8	4.6
14	4.1	4.2	4.2	5.5	4.5
15	5.5	3.8	4.5	5.5	4.8

Sampling Positions	Belly			Average (mm)
	e	f	g	
1	5.8	5.9	4.9	5.5
2	5.2	5.0	4.7	5.0
3	4.7	5.1	4.7	4.8
4	6.7	6.3	6.8	6.6
5	6.8	7.8	7.9	7.5
6	5.3	6.8	6.8	6.3
7	4.9	6.3	6.9	6.0
8	6.8	7.8	7.9	7.5
9	6.0	6.8	7.8	6.9
10	5.2	5.5	6.8	5.8
11	4.5	5.1	7.2	5.6
12	6.2	7.2	7.9	7.1
13	4.2	5.2	6.1	5.2
14	6.0	5.9	7.2	6.4
15	6.0	7.2	7.2	6.8

Sampling position numbers and letters are defined in Figure 5.2-1.

Table C 1-3: Average softness values of neem bark tannin + TX leather.

Sampling positions	Shoulder				Average (mm)
	a	b	c	d	
1	6.2	5.5	6.2	5.7	5.9
2	5.5	5.1	5.6	5.6	5.5
3	4.5	4.5	5.1	3.9	4.5
4	8.9	4.0	4.0	4.1	5.3
5	4.5	3.8	4.5	4.2	4.3
6	3.7	3.9	3.5	3.7	3.7
7	3.6	3.3	3.8	4.1	3.7

Sampling positions	Butt				Average (mm)
	a	b	c	d	
8	4.7	4	4.3	4.3	4.3
9	4.3	4.3	4.4	4.3	4.3
10	4.6	3.4	4.1	3.8	4.0
11	4.1	5.1	4.2	4.5	4.5
12	3.4	3.4	3.7	4.7	3.8
13	4.1	4.0	4.3	5.0	4.4
14	4.0	3.6	2.8	2.9	3.3
15	3.4	3.4	3.8	4.4	3.8

Sampling positions	Belly			Average (mm)
	e	f	g	
1	5.0	4.9	6.9	5.6
2	4.3	5.0	4.3	4.5
3	4.3	4.3	5.5	4.7
4	4.4	4.7	4.9	4.7
5	4.0	3.5	3.9	3.8
6	3.6	4.8	4.9	4.4
7	3.2	4.4	4.5	4.0
8	4.2	3.3	5.3	4.3
9	5.1	4.2	5.3	4.9
10	3.7	4.5	3.9	4.0
11	4.7	3.9	4.0	4.2
12	4.8	4.0	4.2	4.3
13	4.2	3.6	4.2	4.0
14	4.0	4.3	4.4	4.2
15	4.7	4.5	5.6	4.9

Sampling position numbers and letters are defined in Figure 5.2-1.

Table C 1-4: Average softness values of mimosa +TX leather.

Sampling positions	Shoulder				Average (mm)
	a	b	c	d	
1	5.3	5.9	8	7.9	6.8
2	7.8	7.6	7.2	8.0	7.7
3	4.8	7.7	7.9	6.9	6.8
4	5.8	5.5	6.5	7.3	6.3
5	5.9	5.0	5.9	7.1	6.0
6	5.0	4.6	5.1	6.5	5.3
7	5.5	5.0	5.5	7.2	5.8

Sampling positions	Butt				Average (mm)
	a	b	c	d	
8	4.7	5.9	5.6	6.5	5.7
9	4.5	6.3	6.4	7.2	6.1
10	5.8	6.0	4.0	6.4	5.6
11	5.8	5.9	5.8	6.0	5.9
12	5.8	5.4	6.0	6.7	6.0
13	6.8	6.0	4.5	6.0	5.8
14	4.8	5.0	6.0	5.4	5.3
15	5.5	5.0	5.5	7.4	5.9

Sampling positions	Belly			Average (mm)
	e	f	g	
1	7.5	7.9	8.2	7.9
2	8.5	8.8	9.0	8.8
3	8.1	8.2	7.5	7.9
4	8.1	7.5	7.4	7.7
5	7.3	7.5	7.6	7.5
6	6.7	7.1	6.9	6.9
7	6.8	6.4	8.6	7.3
8	7.1	7.9	7.8	7.6
9	7.2	7.0	6.2	6.8
10	6.5	5.9	6.4	6.3
11	6.2	5.3	7.1	6.2
12	6.8	6.4	7.6	6.9
13	5.6	5.9	6.7	6.1
14	6.5	6.9	7.2	6.9
15	6.8	7.8	8.8	7.8

Sampling position numbers and letters are defined in Figure 5.2-1.

Table C 1-5: Average softness values of neem bark tannin +dye leather.

Sampling position	Shoulder				Average (mm)
	a	b	c	d	
1	2.9	3.2	3.2	3.2	3.1
2	3.3	3.7	3.9	3.9	3.7
3	3.6	3.6	3.3	3.6	3.5
4	3.7	3.7	3.7	4.0	3.8
5	4.6	4.2	4.2	4.1	4.3
6	4.3	4.1	4.5	4.6	4.4
7	4.9	3.6	3.6	4.0	4.0

Sampling positions	Butt				Average (mm)
	a	b	c	d	
8	4.1	3.8	3.9	5.7	4.4
9	4.8	3.9	4.3	4.8	4.5
10	4.7	4.7	3.9	4.7	4.5
11	3.7	4.5	4.0	5.3	4.4
12	3.6	4.8	4.7	4.5	4.4
13	3.3	5.1	4.9	4.8	4.5
14	3.0	6.8	5.6	6.1	5.4
15	5.0	5.7	6.0	5.6	5.6

Sampling Positions	Belly			Average (mm)
	e	f	g	
1	3.7	4.7	6.5	5.0
2	4.0	3.9	5.8	4.6
3	4.0	3.9	6.0	4.6
4	4.1	4.8	8.0	5.6
5	4.3	5.0	7.6	5.6
6	5.3	5.2	8.7	6.4
7	4.9	4.8	8.1	5.9
8	3.7	6.9	5.9	5.5
9	4.8	5.6	3.4	4.6
10	4.8	6.2	5.1	5.4
11	5.4	6.9	5.5	6.0
12	4.3	5.3	4.7	4.8
13	4.9	4.7	4.6	4.8
14	5.6	5.3	5.1	5.3
15	5.0	4.8	4.7	4.9

Sampling position numbers and letters are defined in Figure 5.2-1.

Table C 1-6: Average softness values of mimosa + dye leather.

Sampling positions	Shoulder				Average (mm)
	a	b	c	d	
1	3.5	3.7	4.7	4.4	4.1
2	4.1	3.9	3.8	4.0	4.0
3	4.4	4.5	4.5	5.4	4.7
4	3.8	4.1	4.2	4.2	4.1
5	4.2	4.2	4.3	4.2	4.2
6	3.6	5.0	4.2	4.1	4.2
7	4.2	5.0	4.1	4.9	4.6

Sampling positions	Butt				Average (mm)
	a	b	c	d	
8	6.3	5.5	4.4	6.5	5.7
9	5.2	5.0	6.0	5.2	5.4
10	5.0	5.1	6.1	5.9	5.5
11	4.8	4.9	5.0	5.7	5.1
12	4.0	4.8	5.0	5.5	4.8
13	3.8	3.9	4.8	5.6	4.5
14	4.0	4.5	4.7	5.6	4.7
15	3.9	5.0	6.2	6.0	5.3

Sampling positions	Belly			Average (mm)
	e	f	g	
1	3.9	6.0	6.5	5.5
2	3.9	5.7	5.8	5.1
3	5.3	5.9	6.0	5.7
4	5.5	5.5	8.0	6.3
5	5.3	4.5	7.6	5.8
6	4.9	4.8	8.7	6.1
7	6.0	5.9	8.1	6.7
8	6.4	6.4	5.9	6.2
9	6.5	6.6	8.0	7.0
10	5.7	6.0	8.9	6.9
11	6.4	6.2	8.0	6.9
12	6.0	5.9	7.6	6.5
13	7.1	6.5	6.9	6.8
14	5.9	6.0	8.0	6.6
15	6.5	7.9	8.5	7.6

Sampling position numbers and letters are defined in Figure 5.2-1.

Table C 1-7: Post Hoc (multiple comparison) test for softness of shoulder positions (parallel and perpendicular to the backbone)

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
TREATMENT (I)	TREATMENT (J)				Lower Bound	Upper Bound
Neem bark tannin	Mimosa	-1.8	0.299	0.000	-2.7	-0.9
	Neem bark +TX	-1.0	0.299	0.028	-1.9	-0.1
	Mimosa + TX	-2.6	0.299	0.000	-3.5	-1.7
	Neem + dye	-0.2	0.299	0.980	-1.1	0.7
	Mimosa + dye	-0.8	0.299	0.134	-1.7	0.1
Mimosa	Neem bark tannin	1.8	0.299	0.000	0.9	2.7
	Neem bark +TX	0.8	0.299	0.085	-0.1	1.7
	Mimosa + TX	-0.8	0.299	0.085	-1.7	0.1
	Neem + dye	1.6	0.299	0.000	0.7	2.5
	Mimosa + dye	1.0	0.299	0.016	0.1	1.9
Neem bark +TX	Neem bark tannin	1.0	0.299	0.028	0.1	1.9
	Mimosa	-0.8	0.299	0.085	-1.7	0.1
	Mimosa + TX	-1.7	0.299	0.000	-2.5	-0.8
	Neem + dye	0.7	0.299	0.146	-0.1	1.6
	Mimosa + dye	0.2	0.299	0.985	-0.7	1.1
Mimosa + TX	Neem bark tannin	2.6	0.299	0.000	1.7	3.5
	Mimosa	0.8	0.299	0.085	-0.1	1.7
	Neem bark +TX	1.7	0.299	0.000	0.8	2.5
	Neem + dye	2.4	0.299	0.000	1.5	3.3
	Mimosa + dye	1.9	0.299	0.000	1.0	2.7
Neem + dye	Neem bark tannin	0.2	0.299	0.980	-0.7	1.1
	Mimosa	-1.6	0.299	0.000	-2.5	-0.7
	Neem bark +TX	-0.7	0.299	0.146	-1.6	0.1
	Mimosa + TX	-2.4	0.299	0.000	-3.3	-1.5
	Mimosa + dye	-0.6	0.299	0.454	-1.4	0.3
Mimosa + dye	Neem bark tannin	0.8	0.299	0.134	-0.1	1.7
	Mimosa	-1.0	0.299	0.016	-1.9	-0.1
	Neem bark +TX	-0.2	0.299	0.985	-1.1	0.7
	Mimosa + TX	-1.9	0.299	0.000	-2.7	-1.0
	Neem + dye	0.6	0.299	0.454	-0.3	1.4

The mean difference is significant at the 0.05 level.

Table C 1-8: Post Hoc (multiple comparison) test for softness of butt positions (parallel and perpendicular to the backbone).

TREATMENT (I)	TREATMENT (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Neem bark tannin	Mimosa	-1.0	0.3	0.012	-1.8	-0.1
	Neem bark +TX	-0.1	0.3	0.999	-0.9	0.7
	Mimosa + TX	-2.0	0.3	0.000	-2.8	-1.1
	Neem + dye	-0.7	0.3	0.155	-1.5	0.1
	Mimosa + dye	-1.0	0.3	0.010	-1.8	-0.2
Mimosa	Neem bark tannin	1.0	0.3	0.012	0.1	1.8
	Neem bark +TX	0.8	0.3	0.036	0.0	1.6
	Mimosa + TX	-1.0	0.3	0.007	-1.8	-0.2
	Neem + dye	0.3	0.3	0.918	-0.5	1.1
	Mimosa + dye	0.0	0.3	1.000	-0.9	0.8
Neem bark +TX	Neem bark tannin	0.1	0.3	0.999	-0.7	0.9
	Mimosa	-0.8	0.3	0.036	-1.6	0.0
	Mimosa + TX	-1.9	0.3	0.000	-2.7	-1.0
	Neem + dye	-0.6	0.3	0.323	-1.4	0.3
	Mimosa + dye	-0.9	0.3	0.030	-1.7	-0.1
Mimosa + TX	Neem bark tannin	2.0	0.3	0.000	1.1	2.8
	Mimosa	1.0	0.3	0.007	0.2	1.8
	Neem bark +TX	1.9	0.3	0.000	1.0	2.7
	Neem + dye	1.3	0.3	0.001	0.4	2.1
	Mimosa + dye	1.0	0.3	0.015	0.1	1.8
Neem + dye	Neem bark tannin	0.7	0.3	0.155	-0.1	1.5
	Mimosa	-0.3	0.3	0.918	-1.1	0.5
	Neem bark +TX	0.6	0.3	0.323	-0.3	1.4
	Mimosa + TX	-1.3	0.3	0.001	-2.1	-0.4
	Mimosa + dye	-0.3	0.3	0.869	-1.1	0.5
Mimosa + dye	Neem bark tannin	1.0	0.3	0.010	0.2	1.8
	Mimosa	0.0	0.3	1.000	-0.8	0.9
	Neem bark +TX	0.9	0.3	0.030	0.1	1.7
	Mimosa + TX	-1.0	0.3	0.015	-1.8	-0.1
	Neem + dye	0.3	0.3	0.869	-0.5	1.1

The mean difference is significant at the 0.05 level.

Table C 1-9: Post Hoc (multiple comparison) test for softness of belly positions (parallel and perpendicular to the backbone).

TREATMENT (I)	TREATMENT (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Neem bark tannin	Mimosa	-2.4	0.3	0.000	-3.1	-1.6
	Neem bark +TX	-0.4	0.3	0.712	-1.1	0.4
	Mimosa + TX	-3.2	0.2	0.000	-3.9	-2.5
	Neem + dye	-1.3	0.2	0.000	-2.0	-0.6
	Mimosa + dye	-2.5	0.2	0.000	-3.2	-1.7
Mimosa	Neem bark tannin	2.4	0.3	0.000	1.6	3.1
	Neem bark +TX	2.0	0.3	0.000	1.3	2.7
	Mimosa + TX	-0.8	0.2	0.013	-1.6	-0.1
	Neem + dye	1.1	0.2	0.001	0.3	1.8
	Mimosa + dye	-0.1	0.2	0.999	-0.8	0.6
Neem bark +TX	Neem bark tannin	0.4	0.3	0.712	-0.4	1.1
	Mimosa	-2.0	0.3	0.000	-2.7	-1.3
	Mimosa + TX	-2.9	0.2	0.000	-3.6	-2.1
	Neem + dye	-0.9	0.2	0.004	-1.7	-0.2
	Mimosa + dye	-2.1	0.2	0.000	-2.8	-1.4
Mimosa + TX	Neem bark tannin	3.2	0.2	0.000	2.5	3.9
	Mimosa	0.8	0.2	0.013	0.1	1.6
	Neem bark +TX	2.9	0.2	0.000	2.1	3.6
	Neem + dye	1.9	0.2	0.000	1.2	2.6
	Mimosa + dye	0.8	0.2	0.034	0.0	1.5
Neem + dye	Neem bark tannin	1.3	0.2	0.000	0.6	2.0
	Mimosa	-1.1	0.2	0.001	-1.8	-0.3
	Neem bark +TX	0.9	0.2	0.004	0.2	1.7
	Mimosa + TX	-1.9	0.2	0.000	-2.6	-1.2
	Mimosa + dye	-1.2	0.2	0.000	-1.9	-0.4
Mimosa + dye	Neem bark tannin	2.5	0.2	0.000	1.7	3.2
	Mimosa	0.1	0.2	0.999	-0.6	0.8
	Neem bark +TX	2.1	0.2	0.000	1.4	2.8
	Mimosa + TX	-0.8	0.2	0.034	-1.5	0.0
	Neem + dye	1.2	0.2	0.000	0.4	1.9

The mean difference is significant at the 0.05 level.

APPENDIX C2: Light fastness.
Table C.2-1: L* a* b* colour space values.

0 hours	L	a	b
Neem tan	77.7	4.4	11.0
Mimosa tan	82.7	3.7	7.8
Neem +TX	66.0	11.3	31.5
Mimosa + TX	69.2	11.6	32.5
Neem + dye	73.6	6.7	20.8
Mimosa + dye	76.6	6.3	21.1
24 hours			
Neem tan	77.9	16.1	28.9
Mimosa tan	80.9	16.4	26.7
Neem +TX	63.1	3.6	41.4
Mimosa + TX	68.6	6.3	41.2
Neem + dye	74.7	13.9	33.9
Mimosa + dye	74.9	12.4	35.0
48 hours			
Neem tan	68.0	10.8	19.3
Mimosa tan	71.7	10.5	16.6
Neem +TX	56.1	17.4	30.4
Mimosa + TX	62.3	15.3	29.3
Neem + dye	65.8	11.5	22.4
Mimosa + dye	66.9	12.9	24.0
72 hours			
Neem tan	67.4	12.2	21.8
Mimosa tan	69.7	12.5	20.5
Neem +TX	55.0	19.2	31.6
Mimosa + TX	62.3	16.7	29.4
Neem + dye	63.7	13.6	24.8
Mimosa + dye	63.7	16.2	27.5
96 hours			
Neem tan	64.3	11.8	22.9
Mimosa tan	67.6	10.9	20.7
Neem +TX	52.9	17.6	29.8
Mimosa + TX	61.9	14.6	28.0
Neem + dye	60.2	15.4	27.8
Mimosa + dye	61.7	12.6	24.5
120 hours			
Neem tan	65.4	12.3	24.3
Mimosa tan	69.4	10.7	21.1
Neem +TX	54.8	17.8	30.8
Mimosa + TX	63.2	14.3	27.2
Neem + dye	63.0	12.8	25.5
Mimosa + dye	62.1	15.2	28.5
144 hours			
Neem tan	64.9	11.1	23.8
Mimosa tan	69.1	9.3	20.3
Neem +TX	54.9	16.3	29.8
Mimosa + TX	63.2	12.7	25.3
Neem + dye	63.0	11.7	24.8
Mimosa + dye	61.4	14.0	27.8

Table C.2-2: Light fastness from zero time to 144 hours.

TREATMENTS	ΔL_{24}	ΔL_{48}	ΔL_{72}	ΔL_{96}	ΔL_{120}	ΔL_{144}
Neem tan	0	-10	-10	-13	-12	-13
Mimosa tan	-2	-11	-13	-15	-13	-14
Neem +TX	-3	-10	-11	-13	-11	-11
Mimosa + TX	-1	-7	-7	-7	-6	-6
Neem + dye	1	-8	-10	-13	-11	-11
Mimosa + dye	-2	-10	-13	-15	-15	-15
	Δa_{24}	Δa_{48}	Δa_{72}	Δa_{96}	Δa_{120}	Δa_{144}
Neem tan	12	6	8	7	8	7
Mimosa tan	13	7	9	7	7	6
Neem +TX	-8	6	8	6	7	5
Mimosa + TX	-5	4	5	3	3	1
Neem + dye	7	5	7	9	6	5
Mimosa + dye	6	7	10	6	9	8
	Δb_{24}	Δb_{48}	Δb_{72}	Δb_{96}	Δb_{120}	Δb_{144}
Neem tan	18	8	11	12	13	13
Mimosa tan	19	9	13	13	13	13
Neem +TX	10	-1	0	-2	-1	-2
Mimosa + TX	9	-3	-3	-5	-5	-7
Neem + dye	13	2	4	7	5	4
Mimosa + dye	14	3	6	3	7	7

Table C.2-3: Light fastness from zero time to 144 hours.

Square values	$(\Delta L_{24})^2$	$(\Delta L_{48})^2$	$(\Delta L_{72})^2$	$(\Delta L_{96})^2$	$(\Delta L_{120})^2$	$(\Delta L_{144})^2$
Neem tan	0	94	106	180	151	164
Mimosa tan	3	121	169	228	177	185
Neem +TX	8	98	121	172	125	123
Mimosa + TX	0	48	48	53	36	36
Neem + dye	1	61	98	180	112	112
Mimosa + dye	3	94	166	222	210	231
	$(\Delta a_{24})^2$	$(\Delta a_{48})^2$	$(\Delta a_{72})^2$	$(\Delta a_{96})^2$	$(\Delta a_{120})^2$	$(\Delta a_{144})^2$
Neem tan	137	41	61	55	62	45
Mimosa tan	161	46	77	52	49	31
Neem +TX	59	37	62	40	42	25
Mimosa + TX	28	14	26	9	7	1
Neem + dye	52	23	48	76	37	25
Mimosa + dye	37	44	98	40	79	59
	$(\Delta b_{24})^2$	$(\Delta b_{48})^2$	$(\Delta b_{72})^2$	$(\Delta b_{96})^2$	$(\Delta b_{120})^2$	$(\Delta b_{144})^2$
Neem tan	320	69	117	142	177	164
Mimosa tan	357	77	161	166	177	156
Neem +TX	98	1	0	3	0	3
Mimosa + TX	76	10	10	20	28	52
Neem + dye	172	3	16	49	22	16
Mimosa + dye	193	8	41	12	55	45

Table C.2-4: Pearson correlation for lightness factor (L*) values.

Treatments		Neem bark tannin	Mimosa	Neem bark tannin +TX	Mimosa +TX	Neem bark tannin + dye	Mimosa + dye
Neem bark tannin	**Pearson Correlation**	1.00	0.99	0.98	0.95	0.99	0.99
	Sig. (2-tailed)	-	**0.00	**0.00	**0.00	**0.00	**0.00
Mimosa	**Pearson Correlation**	0.99	1.00	0.99	0.97	0.98	0.99
	Sig. (2-tailed)	**0.00	-	**0.00	**0.00	**0.00	**0.00
Neem bark tannin +TX	**Pearson Correlation**	0.98	0.99	1.00	0.97	0.97	0.98
	Sig. (2-tailed)	**0.00	**0.00	-	**0.00	**0.00	**0.00
Mimosa +TX	**Pearson Correlation**	0.95	0.97	0.97	1.00	0.95	0.93
	Sig. (2-tailed)	**0.00	**0.00	**0.00	-	**0.00	0.00
Neem bark tannin + dye	**Pearson Correlation**	0.99	0.98	0.97	0.95	1.00	0.98
	Sig. (2-tailed)	**0.00	**0.00	**0.00	**0.00	-	0.00
Mimosa + dye	**Pearson Correlation**	0.99	0.99	0.98	0.93	0.98	1.00
	Sig. (2-tailed)	**0.00	**0.00	**0.00	**0.00	**0.00	-

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

Table C.2-5: Pearson correlation for chroma factor (a*) values.

Treatments		Neem bark tannin	Mimosa	Neem bark tannin +TX	Mimosa +TX	Neem bark tannin + dye	Mimosa + dye
Neem bark tannin	**Pearson Correlation**	1.00	0.98	-0.20	-0.25	0.86	0.72
	Sig. (2-tailed)	-	**0.00	0.67	0.58	*0.01	0.07
Mimosa	**Pearson Correlation**	0.98	1.00	-0.27	-0.27	0.82	0.65
	Sig. (2-tailed)	**0.00	-	0.56	0.55	*0.02	0.11
Neem bark tannin +TX	**Pearson Correlation**	-0.20	-0.27	1.00	0.97	0.16	0.48
	Sig. (2-tailed)	0.67	0.56	-	**0.00	0.73	0.27
Mimosa +TX	**Pearson Correlation**	-0.25	-0.27	0.97	1.00	0.10	0.41
	Sig. (2-tailed)	0.58	0.55	0.00	-	0.82	0.36
Neem bark tannin + dye	**Pearson Correlation**	0.86	0.82	0.16	0.10	1.00	0.75
	Sig. (2-tailed)	*0.01	0.02	0.73	*0.82	-	0.05
Mimosa + dye	**Pearson Correlation**	0.72	0.65	0.48	0.41	0.75	1.00
	Sig. (2-tailed)	0.07	0.11	0.27	0.36	0.05	-

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

*Correlation is significant at the 0.05 level (2-tailed).

Table C.2-6: Pearson correlation for chroma factor (b*) values.

Treatments		Neem bark tannin	Mimosa	Neem bark tannin +TX	Mimosa +TX	Neem bark tannin + dye	Mimosa + dye
Neem bark tannin	**Pearson Correlation**	1.00	0.99	0.48	0.21	0.84	0.89
	Sig. (2-tailed)	-	**0.00	0.27	0.66	*0.02	**0.01
Mimosa	**Pearson Correlation**	0.99	1.00	0.50	0.24	0.86	0.88
	Sig. (2-tailed)	**0.00	-	0.25	0.61	*0.01	**0.01
Neem bark tannin +TX	**Pearson Correlation**	0.48	0.50	1.00	0.95	0.79	0.77
	Sig. (2-tailed)	0.27	0.25	-	**0.00	*0.04	*0.04
Mimosa +TX	**Pearson Correlation**	0.21	0.24	0.95	1.00	0.62	0.53
	Sig. (2-tailed)	0.66	0.61	*0.00	-	0.14	0.22
Neem bark tannin + dye	**Pearson Correlation**	0.84	0.86	0.79	0.62	1.00	0.87
	Sig. (2-tailed)	*0.02	*0.01	*0.04	0.14	.	*0.01
Mimosa + dye	**Pearson Correlation**	0.89	0.88	0.77	0.53	0.87	1.00
	Sig. (2-tailed)	**0.01	**0.01	0.04	0.22	*0.01	.

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

*Correlation is significant at the 0.05 level (2-tailed).

Table C 3-1: Tear strength (N/mm) and thickness (mm) of neem bark and mimosa tanned leathers.

	Neem bark tanned leather		Mimosa tanned leather	
Sampling positions	**Thickness (mm)**	**Tear strength (N/mm)r**	**Thickness (mm)**	**Tear strength (N/mm)r**
Shoulder (‖)	1.2	21	1.3	30
Shoulder (‖)	1.1	14	1.5	23
Shoulder (‖)	1.4	17	1.5	24
Shoulder (‖)	1.1	25	1.4	26
Shoulder (‖)	1.1	12	1.4	27
Shoulder (‖)	1.5	14	1.6	22
Shoulder (‖)	1.4	17	1.4	24
Shoulder (‖)	1.4	12	1.5	13
Shoulder (‖)	1.2	24	1.4	21
Shoulder (⊥)	1.8	6	1.4	19
Shoulder (⊥)	1.8	4	1.3	18
Shoulder (⊥)	1.4	5	1.5	22
Shoulder (⊥)	1.1	37	1.2	17
Shoulder (⊥)	1.1	33	1.5	22
Shoulder (⊥)	1.5	11	1.2	31
Shoulder (⊥)	1.2	13	1.4	24
Shoulder (⊥)	1.4	24	1.4	15
OSP (‖)	1.4	17	1.4	11
OSP (‖)	1.4	20	1.2	24
OSP (‖)	1.1	47	1.3	10
OSP (‖)	1.2	32	1.3	17
OSP (‖)	1.4	25	1.3	20
OSP (‖)	1.4	20	1.5	17
OSP (‖)	1.2	24	1.3	15
OSP (‖)	1.1	13	1.4	20
OSP (⊥)	1.1	39	1.5	24
OSP (⊥)	1.2	33	1.4	19
OSP (⊥)	1.2	25	1.5	20
OSP (⊥)	1.0	25	1.4	21
OSP (⊥)	1.1	21	1.5	36
OSP (⊥)	1.1	33	1.4	18
OSP (⊥)	1.1	29	1.3	22
OSP (⊥)	1.6	24	1.4	26
Belly (‖)	1.0	78	1.4	34
Belly (‖)	1.1	51	1.3	13
Belly (‖)	1.1	54	1.2	25
Belly (‖)	1.1	48	1.2	31
Belly (‖)	1.1	44	1.4	25
Belly (‖)	1.6	24	1.3	25
Belly (‖)	1.5	32	1.2	28
Belly (‖)	1.0	23	1.4	31
Belly (‖)	1.1	18	1.2	30
Belly (⊥)	1.1	65	1.2	33
Belly (⊥)	1.1	8	1.3	36
Belly (⊥)	1.3	21	1.2	40
Belly (⊥)	1.3	23	1.2	15
Belly (⊥)	1.6	18	1.0	95

(‖) Parallel (⊥) perpendicular.

Table C 3-2: Tear strength (N/mm) and thickness (mm) of neem bark and mimosa retanned leathers with NeosynTX.

	Neem bark tanned +TX leather		Mimosa tanned +TX leather	
Sampling positions	**Thickness (mm)**	**Tear strength (N/mm)r**	**Thickness (mm)**	**Tear strength (N/mm)r**
Shoulder (∥)	1.7	5	1.3	29
Shoulder (∥)	1.0	26	1.5	19
Shoulder (∥)	1.5	18	1.2	22
Shoulder (∥)	1.6	12	1.3	27
Shoulder (∥)	1.3	19	1.2	29
Shoulder (∥)	1.1	37	1.3	23
Shoulder (∥)	1.8	13	1.4	25
Shoulder (∥)	1.5	31	1.3	28
Shoulder (∥)	1.4	12	1.2	33
Shoulder (⊥)	1.2	30	1.4	14
Shoulder (⊥)	1.6	16	1.2	41
Shoulder (⊥)	1.4	17	1.4	45
Shoulder (⊥)	1.3	14	1.2	24
Shoulder (⊥)	1.3	17	1.2	31
Shoulder (⊥)	1.3	20	1.6	34
Shoulder (⊥)	1.3	30	1.1	74
Shoulder (⊥)	1.4	15	1.2	37
OSP (∥)	1.3	23	1.3	28
OSP (∥)	1.3	23	1.4	28
OSP (∥)	1.4	18	1.5	52
OSP (∥)	1.6	13	1.5	40
OSP (∥)	1.2	25	1.5	25
OSP (∥)	1.3	24	1.4	29
OSP (∥)	1.6	12	1.8	27
OSP (∥)	1.3	23	1.6	25
OSP (⊥)	1.3	15	1.7	25
OSP (⊥)	1.4	20	1.4	28
OSP (⊥)	1.4	29	1.6	12
OSP (⊥)	1.4	23	1.4	60
OSP (⊥)	1.4	13	1.5	21
OSP (⊥)	1.2	17	1.3	8
OSP (⊥)	1.3	20	1.5	15
OSP (⊥)	1.3	12	1.4	30
Belly (∥)	1.6	19	1.3	71
Belly (∥)	1.7	17	1.3	21
Belly (∥)	1.3	18	1.3	21
Belly (∥)	1.2	24	1.4	25
Belly (∥)	1.3	26	1.6	19
Belly (∥)	1.6	19	1.4	21
Belly (∥)	1.5	20	1.5	17
Belly (∥)	1.2	22	1.5	23
Belly (∥)	1.1	45	1.4	19
Belly (⊥)	1.1	40	1.4	33
Belly (⊥)	1.3	20	1.4	18
Belly (⊥)	1.0	45	1.3	23
Belly (⊥)	1.2	20	1.4	30
Belly (⊥)	1.1	34	1.6	23

(∥) Parallel (⊥) perpendicular.

Table C 3-3: Tear strength (N/mm) and thickness (mm) of neem bark and mimosa dyed leathers.

	Neem bark dyed leather		Mimosa dyed leather	
Sampling positions	**Thickness (mm)**	**Tear strength (N/mm)r**	**Thickness (mm)**	**Tear strength (N/mm)r**
Shoulder (‖)	1.2	24	1.9	20
Shoulder (‖)	1.3	10	2.4	21
Shoulder (‖)	1.7	5	1.7	22
Shoulder (‖)	1.2	10	1.9	18
Shoulder (‖)	1.1	37	1.7	21
Shoulder (‖)	1.1	25	1.6	31
Shoulder (‖)	1.7	22	1.4	32
Shoulder (‖)	1.4	21	1.3	31
Shoulder (‖)	1.3	45	1.7	17
Shoulder (⊥)	1.3	27	1.3	28
Shoulder (⊥)	1.8	17	1.5	29
Shoulder (⊥)	1.7	18	1.1	20
Shoulder (⊥)	1.8	12	1.1	25
Shoulder (⊥)	1.6	28	1.9	17
Shoulder (⊥)	1.2	42	1.4	34
Shoulder (⊥)	1.8	26	1.3	24
Shoulder (⊥)	1.7	14	1.4	20
OSP (‖)	1.5	14	1.3	28
OSP (‖)	1.2	30	1.3	20
OSP (‖)	1.2	27	1.4	13
OSP (‖)	1.2	30	2.1	36
OSP (‖)	1.2	24	1.8	40
OSP (‖)	1.1	31	1.3	28
OSP (‖)	1.2	28	1.4	25
OSP (‖)	1.4	16	1.3	22
OSP (⊥)	1.3	29	1.3	26
OSP (⊥)	1.6	25	1.4	25
OSP (⊥)	1.5	29	1.3	26
OSP (⊥)	1.4	34	1.3	27
OSP (⊥)	1.5	25	1.3	17
OSP (⊥)	1.4	33	1.2	23
OSP (⊥)	1.4	31	1.3	25
OSP (⊥)	1.3	20	1.0	36
Belly (‖)	1.6	26	1.1	28
Belly (‖)	1.5	24	1.5	16
Belly (‖)	1.3	18	1.5	23
Belly (‖)	1.4	26	1.3	25
Belly (‖)	1.6	13	1.7	25
Belly (‖)	1.4	24	1.4	17
Belly (‖)	1.4	32	1.8	12
Belly (‖)	1.3	19	1.6	18
Belly (‖)	1.3	17	1.6	23
Belly (⊥)	1.5	19	1.2	29
Belly (⊥)	1.6	15	1.3	16
Belly (⊥)	1.8	10	1.2	19
Belly (⊥)	1.3	18	1.3	21
Belly (⊥)	1.4	11	1.3	16

(‖) Parallel (⊥) perpendicular.

Table C 3-4: Average tear strength (N/mm).

Tear strength average(N/mm)		
Sample positions	**Neem bark tanned leather**	**Mimosa tanned leather**
Shoulder (‖)	17	23
Shoulder (⊥)	16	21
OSP (‖)	25	17
OSP (⊥)	29	23
Belly (‖)	41	27
Belly (⊥)	25	40
	Neem bark+TX	**Mimosa+TX**
Shoulder (‖)	19	26
Shoulder (⊥)	20	37
OSP (‖)	20	32
OSP (⊥)	19	25
Belly (‖)	23	26
Belly (⊥)	32	24
	Neem bark dyed leather	**Mimosa dyed leather**
Shoulder (‖)	23	25
Shoulder (⊥)	21	24
OSP (‖)	27	26
OSP (⊥)	28	24
Belly (‖)	22	23
Belly (⊥)	15	20

(‖) Parallel (⊥) perpendicular.

Table C 3-5: Distribution curve values for neem bark tannin or mimosa leathers.

Neem bark tanned leather			**Mimosa tanned leather**		
Tear strength (N/mm)	**Frequency**	**Cumulative (%)**	**Tear strength (N/mm)**	**Frequency**	**Cumulative (%)**
16.4	1	16.67	16.7	1	16.60
28.9	4	83.33	28.4	4	83.30
More	1	100.00	More	1	100.00
Neem bark tanned leather + TX			**Mimosa tanned leather + TX**		
18.5	1	16.67	23.5	1	16.67
25.1	4	83.33	30.45	3	66.67
More	1	100.00	More	2	100.00
Neem bark tanned leather + dye			**Mimosa tanned leather + dye**		
14.9	1	16.67	19.9	1	16.67
21.4	1	33.33	22.95	1	33.33
More	4	100.00	More	4	100.00

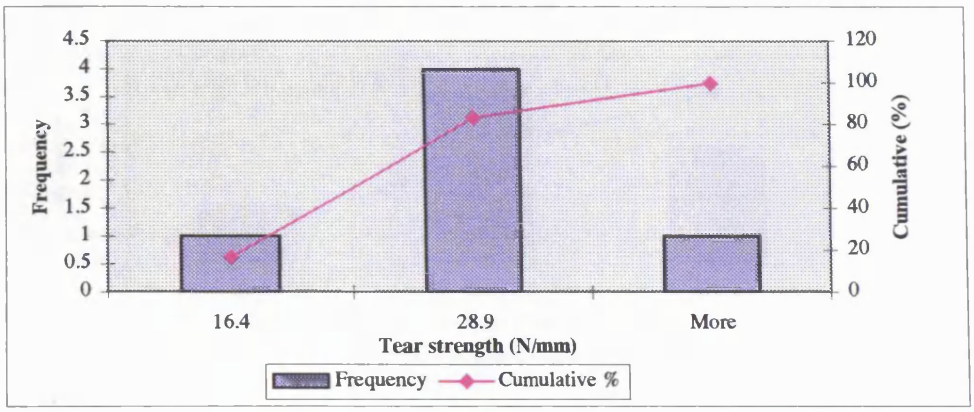


Figure C3-1: Frequency distribution curve for neem bark tanned leather.

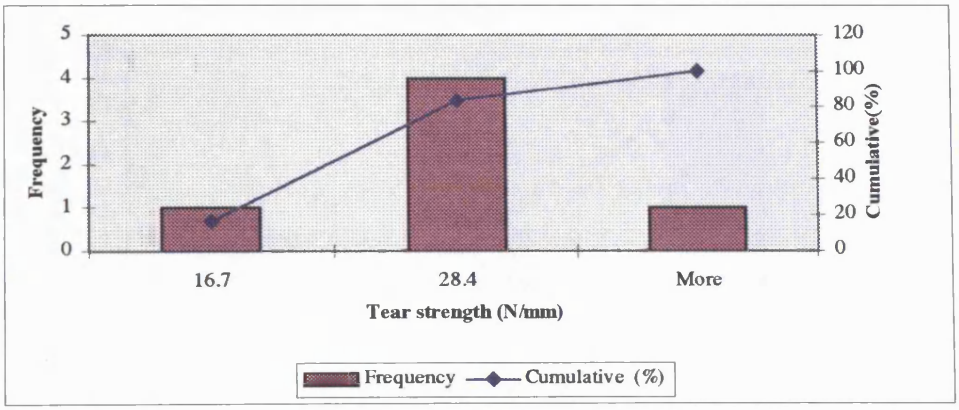


Figure C3-2: Frequency distribution curve for mimosa tanned leather.

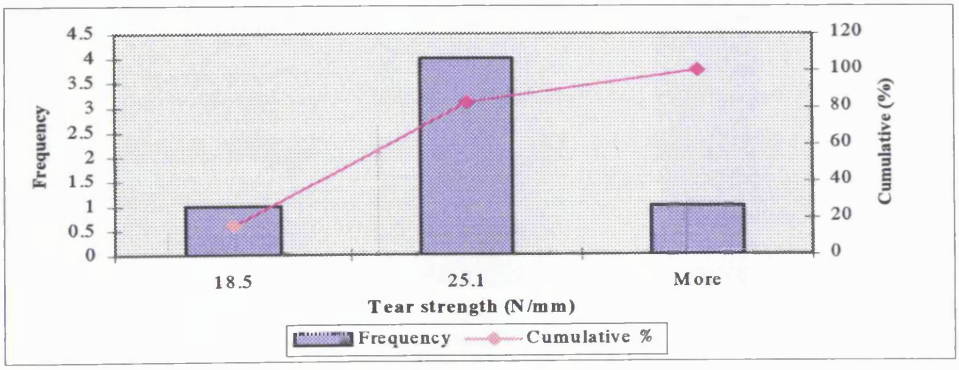


Figure C3-3: Frequency distribution curve for neem bark tannin + Neosyn TX retanned leather.

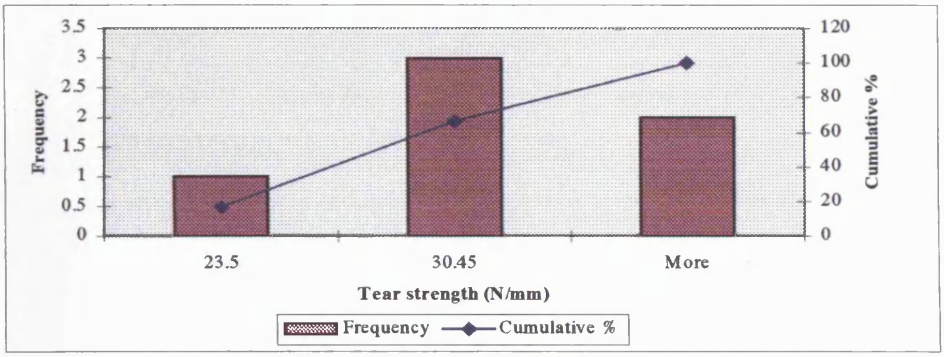


Figure C3-4: Frequency distribution curve for mimosa tannin+Neosyn TX retanned leather.

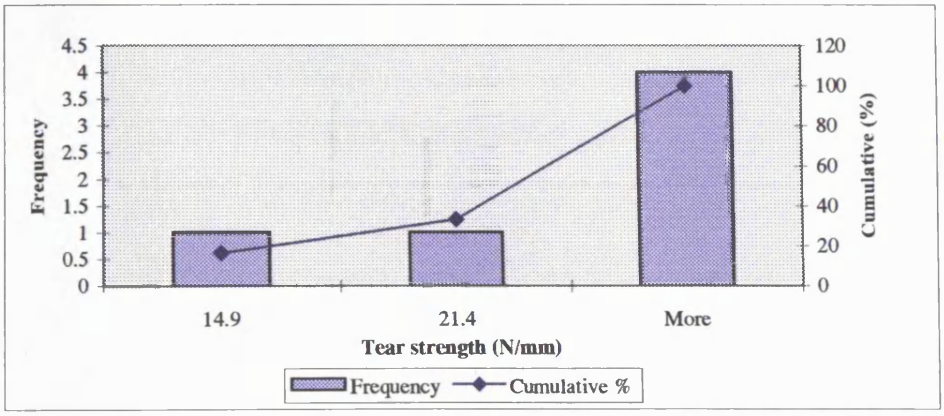


Figure C3-5: Frequency distribution curve for neem bark tanned dyed leather.

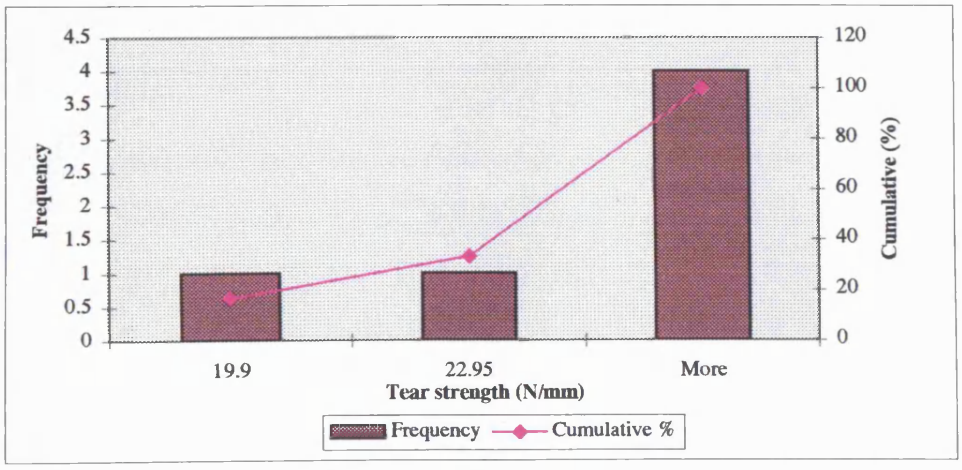


Figure C3-6: Frequency distribution curves for mimosa tanned dyed leather