POLYPHENOLIC COMPOUNDS LIBERATED DURING COIR RETTING IN A BIO-REACTOR: SEPARATION, CHARACTERISATION AND POSSIBLE APPLICATIONS

T.R. Satyakeerthy

Thesis submitted in
partial fulfilment of the requirements
for the degree of

Doctor of Philosophy

under

**THE FACULTY OF
ENVIRONMENTAL STUDIES**

School of environmental studies
Cochin University of Science and Technology
Cochin - 682 016

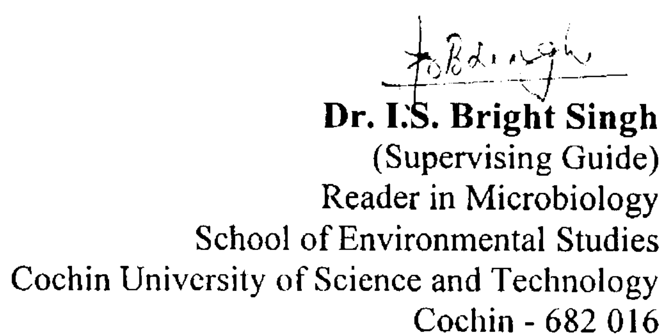
January, 1999

To the Almighty, my parents
and to the poor who are deprived
of a days meal

CERTIFICATE

This is to certify that the research work presented in this thesis entitled **'Polyphenolic Compounds Liberated during Coir Retting in a Bio-reactor: Separation, Characterisation and Possible Applications'** is based on the original work done by **Mr. T.R. Satyakeerthy** under my guidance in the School of Environmental Studies, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part of this work has previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Cochin - 682 016
22.01.1999



Dr. I.S. Bright Singh
(Supervising Guide)
Reader in Microbiology
School of Environmental Studies
Cochin University of Science and Technology
Cochin - 682 016

DECLARATION

I, hereby declare that the work presented in this Ph.D thesis entitled '**Polyphenolic Compounds Liberated during Coir Retting in a Bio-reactor: Separation, Characterisation and Possible Applications**' is based on the original work done by me under the guidance of Dr. I.S. Bright Singh, Reader in Microbiology, School of Environmental Studies, Cochin University of Science and Technology, Cochin - 682 016 and no part of this work has previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Cochin - 682 016
22.01.1999

T.R. Satyakeerthy

ACKNOWLEDGEMENTS

This piece of work was undertaken with the guidance of **Dr. I.S. Bright Singh** , Reader in Microbiology, School of Enviornmental Studies, Cochin University of Science and Technology who conceptualised the whole programme as part of the research project Coir Retting: Process upgradation and Pollution abatement through environmental biotechnology sponsored by Coir Board. I express my gratitude to my guide for his guidance, constant supervision and critical appraisal through out the reserarch period and the preparation of the thesis.

I also take this oppurtunity to thank **Dr. A. Mohandas**, Director, School of Environmental Studies for his cooperation, and for providing all the facilities required for the research.

The financial support by the **Coir Board**, Ministry of Commerce, Govt of India is gratefully and sincerely acknowledged.

I thank the former Chariman fo Coir Board **Shri. Joseph I.A.S**, the present Chairmen of Coir Board **Shri. Christy Fernandez, Dr.U.S.Sharma**, Director, CCRI, Kalavoor, **Smt. Anitha Das Ravindranath**, Scientific Officer, CCRI, Kalavoor for their keen interest in this work and for the valuable suggestions and support.

I thank **Dr.S.S.Kulkarni**, Scientist, Chemical Engg Division, NCL, Pune for his guidance, advices and the technical support provided for setting up and designing a reverse osmosis unit , the constant help given during the period of my research; **Shri. N.K.Chandrababu**, Assistant Director of Central Leather Research Institute, Adayar, Madras for the support and technical guidance rendered in testing the tanning property; **Shri. K.P. Janakiraman**, HOD, Textile Chemistry of SITRA, Coimbatore for the help and

support rendered in testing the mordanting property; **Dr. Shankaran**, Scientist, Kerala Forest Research Institute, Peechi, Kerala for helping me in testing the antifungal property and **Dr. Varma**, Scientist, Kerala Forest Research Institute, Peechi, Kerala for his valuable suggestions.

I would like to express my gratitude to **Shri. Joseph.J, Shri.Venkitesh, Shri. Somasundaram** of AVT, Vazhakulam in helping me with the HPLC analysis.

I am indebted to **Dr.M.C.Nair**, College of Agriculture, Vellayani, Trivandrum for providing certain fungal cultures for the study.

I also thank **Dr. Rosamma Philip**, Lecturer, School of Marine Biology for all help and valuable suggestions rendered during the period of research.

Smt. Meera Jan Abraham, Research Scholar, School of Marine Sciences spent quite a lot of time in helping me with the work on determining the antifouling property of polyphenols.

I express my sincere thanks to all the faculty members, technical staff and non-teaching staff of the school of Environmental Studies for their help and co-operation, especially to **Dr. Sivashankara Pillai**, Professor, **Dr. Sathyanathan**, techical officer and **Dr. Rajalekshmi Amma**, technical Assistant.

I am grateful to **Dr.Muraleedharan Pillai** and **Dr. Chandramohan**, Chemical Oceanography Dept, CUSAT for their valuable suggestions and help and **Ms. Siny K.P** of BTS, Aluva for all the assistantce rendered at various stages of this work.

I thank my colleagues **Mr.Narayanan.P. I, Mr. Harikrishnan.P, Mr.Sunilkumar, Mr.Abdul Khader, Mr.Radhakrishnan, Mrs.Saritha G Bhat, Ms. Kavitha Ramachandran, Ms. Cini Achuthan, Ms. Valsamma Joseph, Ms.Latha.C, Ms.Divya,**

Mr. Jayaprakash, Ms. Manjusha and Ms. Reshmi Varghese for their encouragement, support and companionship.

I thank **Mr. Gopinath, Mrs. Geetha Gopinath, Mr. Rajeevan, Mrs. Jyothi Rajeevan, Ms. Snehajyothi, Ms. Shanthi**, and all my family members for their love and encouragement.

I take this oppurtunity to thank my friends **Mr. Jayadev.K.B** and **Mr. Rajesh.C.K** for thei help and encouragement.

The services rendered by **Mr.Balan Panicker** for drawing the figures are thankfully acknowledged.

I thank **Mr. Biju V.George, Mr. Brilston Francis** and **Ms. Nisha Kurien** of BICS Communications for graphics.

And finally its time to express my heartfelt gratitude to all those special people in my life, who have contributed a great deal to help me complete this work.

T.R. Satyakeerthy

CONTENTS

Chapter 1.
Introduction.

1.1 Occurance and distribution of polyphenols in nature. 1

1.2 Bio-synthesis of polyphenols in plants 2

1.3 Functions, Physical, Biological and Chemical properties of polyphenols in nature. 5

1.4 Polyphenols in coconut husk and its liberation during retting. 10

1.5 Environmental effects of polyphenols from coconut husk during retting. 13

1.6 Development of Bio-reactor for retting coconut husk as an alternative method of retting in open waters. 14

1.7 Relevance of this study and its possible impact on coir industry and in the protection of environment. 15

Chapter 2
Pre-retting operation.

2.1 Introduction. 17

2.2 Materials and methods. 23

- 2.2.1 Optimum salinity for stripping polyphenols 23
- 2.2.2 Time required for maximum liberation of polyphenols. 25
- 2.2.3 Solvent extraction for the separation of polyphenols from ret liquor 25
- 2.2.4 Activated charcoal for the separation of polyphenols. 25
- 2.2.5 Membrane concentration 27
- 2.2.6. Designing, fabrication and standardisation of a polyphenol stripping device. 28

2.2.7. Liberation of a red coloured compound from husk during stripping of polyphenols. 29

2.2.8. Removal of the red coloured compound fron ret liquor. .... 30.

2.3 Results and Discussion. .... 30.

2.3.1. Liberation of polyphenols from husk and the time required by gentle suiting. 30

2.3.2 Separation of polyphenols fron ret liquor.. 31

2.3.3 Membanc concentration.

2.3.4 Polyphenols stripping device as the basic unit of coir retting bioreactor and its standardisation. 36

2.3.5 Liberation of the red coloured compound from husk during stripping of polyphenols. 36

2.3.6 Removal of the red coloured compound from ret liquor 38

2.4 Conclusion. 38

Chapter 3
Characteristics of polyphenols.

3.1 Introduction...

3.2 Materials and methods. 40

3.2.1 Shelf life of concentrated polyphenols. 41

3.2.2 Thin layer chromatographic separation of polyphenols and identification.. 42

3.2.3 Column chromatographic separation of the polyphenols and identification of the compound. 43

3.2.4 HPLC analysis of the concentrated polyphenols................................ 43

3.3 Results and Discussion. 44

3.3.1 Shelf life of cocentrated polyphenols. 44

3.3.2 Thin layer chromatographic separation of polyphenols and identification. 45

3.3.3 Column chromatographic separation of polyphenols. 47

3.3.4 HPLC analysis of the concentrated polyphenols. 48

3.4 Conclusion

Chapter 4
Possible Applications.

4.1 Introduction. 50

4.2 Materials and methods. 53

4.2.1 Mordanting property 53

4.2.2 Tanning property 54

4.2.3 Antifungal property. 56

4.2.4 Antibacterial property 58

4.2.5 Antifouling property 59

4.3 Results and Discussion 60

4.3.1 Mordanting property 60

4.3.2 Tanning property 61

4.3.3 Antifungal property 63

4.3.5 Antifouling property 65

4.4 Conclusion 66

Chapter 5
Conclusion. 67

References. 75

Abbreviations used

APHA	=	American Public Health Association
CLRI	=	Central Leather Research Institute
D	=	Diffusion coefficent
HIV	=	Human Immunodeficiency Virus
Hp	=	Horse Power
K	=	Mass transfer coefficient
NCL	=	National Chemical Laboratory
nm	=	Nanometre
PPT	=	Parts per thousand
ppm	=	Parts per million
psi	=	Pounds per square inch
SITRA	=	South Indian Textile Research Association
UV	=	Ultra Violet
ω	=	Angular velocity

CHAPTER 1

INTRODUCTION

Chapter 1
INTRODUCTION

1.1 Occurance and distribution of polyphenols in nature.

Polyphenolic compounds include groups of complex natural phenols which are mostly polyhydric , and are very often oxygen heterocycles. They are frequently coloured substances and constitute much of the pigmentation in nature and therefore widely distributed in plants. They are classified as the secondary metabolites produced in plants (Haslam, 1989). In plants they are variedly distributed and the polyphenols vary from plants to plants. Many polyphenolics occur in living cells in combined forms as glucosides and esters. In addition to natural polyphenols such as simple monocyclic phenols, chromones, depsides, quinones, coumerins, lignans, benzophenones and xanthones and flavanoids, lignins and tannins are important groups of polymeric polyphenolic materials of the class of phenolics that occur in combination with living cells. The term tannin dates from 1796 when it was first used (Seguin, 1796) to denote substances present in plant extract which possessed the property of converting animal skin to leather.

The results of several of researches quoted earlier have indicated the probable region of maximum accumulation or deposition of polyphenols in certain species. The work on the tannins of 'Quercus' species involving ringing experiments led Hathway (1959) to the conclusion that the pyrogallols formed in the leaves are translocated downwards by the sieve tube system to the cambium where they undergo oxidation through quinone, by the cambium polyphenol oxidase in the outer bark. However an explanation in better accord with the the recently obtained data is

that sucrose and related Oligosacharides are translocated and are converted insitu (Hillis, and Carle, 1960) together with the stored carbohydrate into the relevant polyphenols by the local enzyme system.

There are many factors that affect the rate and amount of accumulation of tannins and other polyphenols in the barks and wood of forest trees. Investigations show that the polyphenol content varies with the nutrition of the soil, wetness and dryness of soil, mild climate etc (Vogel, 1931). The bark of trees of *Acacia* species growing in high rainfall areas has been found to contain less tannin than that from trees grown in a lower rainfall area (Sherry, 1952). The hight factor has been quoted for black wattle bark, that near the top of the tree having appreciably less tannin than the bark (Williams, 1930). Sunlight plays an important role as a stimulant in the formation of the polyphenols in the plants (Hillis and Swain, 1959). A higher potassium content also helps in the production of polyphenols in plants. The *Sirex* unattacked sapwood of *Pines radiata* tree contains very small amounts of polyphenols, whereas the one which is attacked by *Sirex* is notable in the content of polyphenols (Hillis and Inoue, 1968) in the sapwood cells of the plant.

1.2 Bio-synthesis of polyphenols in plants

Polyphenolic compounds arise essentially by one of the three pathways: (a) the acetate-malonate pathway (b) the Shikimic acid pathways (c) combination of the both (the mixed pathway). Feeding experiments with labelled precursors indicate which of these pathways is operative in a given case. Recently the individual steps of the biosynthesis have been elaborated in a number of polyphenols by the realization of respective transformations by the isolated enzyme. It should be noted that

the various pathways could operate for the production of a class of polyphenols or even a single compound up to the organism concerned. From comparative view point, the acetate-malonate pathway is more common on bio-synthesis in micro-organisms and the shikimate pathway is more popular for higher plants.

(a) Acetate malonate pathway (Turner, 1971)

The primary building block in this pathway is the linear poly-ß-keto compound produced from an acetate and several malonates by a process analogous to fatty acid bio-synthesis. The presence of poly-ß-keto compounds as precursors in polyphenolic compound biosynthesis is greatly strengthened by the isolation of acylic polyketides, triacetic and tetracetic lactones from a strain of *Penecillium stiptatum* (Acker et. al, 1966).

(b) Shikimic acid pathway (Haslam, 1974)

A wide range of polyphenolic compounds originate from the essential amino acids, phenylalanine, tyrosine and tryptophane or the intermediates involved in their bio-synthesis from glucose, in which shikimic acid plays a key role.

1. C_6-C_1, C_6-C_2, and C_6-C_3, compounds (Pridham, 1968)

   C_6-C_3, and C_6-C_1, compounds oxygenated at 3,4,5 portions (eg. p-hydroxycinnamic acid, syringic acid, procatechuic acid, sinapic acid) are very common constituents especially in higher plants. C_6-C_2 (acetophenones and phenyl acetic acids are less common. Most of these compounds derived from phenylalanine and tyrosine through deamination followed by hydroxylation on the ring or degradation of the side chain or other modifications. These processess have been studied closely from the ground of enzymology (Hanson and Havir, 1972).

In several cases the direct production of some of these compounds from shikimic acid has been demonstrated (Dewick and Haslam, 1968). The formationn of procatechuic acid and gallic acid is closely related to the biogenesis of some tannins (Swain,1965).

2. Coumarins (Brown,1966 and Neish,1965)

   They are synthesised from trans-cinnamic acid via sequence of the reactions: ortho-hydroxylation, glycosylation,stereomutation and cyclization.

3. Lignins (Nord and Schubert,1967) and Lignans (Pridham,1965)

   The biosynthesis in lignins in general is by the radical polymerisation of cinnamic alcohols like trans-para-hydroxy cinnamic alcohol, trans-coniferyl alcohol and trans-sinapyl alcohol which are derived from the corresponding cinnamic acids. Lignin biosynthesis is supported by the polymerisation of phenyl propane monomers or peroxidases.

   Lignans comprise the dimers of all types formed by the oxidative coupling of p-hydroxyl phenyl propane units(Mc Credie, et. al, 1969).

(c) Mixed Pathway

1. Xanthones: The result of labelling experiments (Gupta and Lewis, 1971) seem to favour the proposal that Xanthones in higher plants are biosynthesised by the direct oxidative coupling of the hydroxybenzophenones derived from acetate and shikimate (Farkas and Pallos, 1967).

2. Flavanoids (Grisebach, 1965): The key compounds in the biosynthesis of flavanoids are chalcones. The interconversion between chalcones and flavanones occurs easily and these are converted to various flavanoid compounds by oxidation, reduction or numerous other secondary transformations. Polymerisation of catechins and flavan-3,4-diols lead to the formation of some sort of tannins (Weinges et. al, 1964).
3. Iso flavanoids (Grisebach, 1975): It is established that the isoflavones are formed by the 1,2-aryl migration of the corresponding flavones.
4. Neo flavanoids (Seshadri, 1972): A novel group of interesting compounds which have the carbon skeleton of 4-phenylcoumarin have been demonstrated to arise by nucleophilic attack of a phloroglucinol nucleus on a cinnamyl residue at the α–carbon of the side chain with the elimination of a suitable leaving group.
5. Stilbenes (Billek and Shimpl,1966): The ß-polyketo intermediates responsible for chalcone formation when subjected to another cyclisation(aldol type) with subsequent decarboxylation give stilbenes.

1.3 Functions, Physical, Biological and Chemical properties of polyphenols in nature.

The polyphenols in nature mainly accounting for the ones found in plants due to its wide distribution are found in the bark, leaf, flower and in fruits of plants. While some polyphenols render pigmentation, some others protect the plant from the invasion of insects and micoorganisms that destroy them.

The production of polyphenols after the attack of wood-wasp *Sirex noctilio* on *Pinus radiata* is responsible for the recovery of the tree from subsequent drying up (Hillis&Inoue,1968). Thus the tree is protected from the attack of the wasp by the production of polyphenols.

Certain tannins as reported by Haslam(1996), serve as drugs and if not properly maintained in the dietary intake can cause several diseases. It is also pointed out that there is a reduced risk of degenerative diseases by the consumption of beverages containing polyphenols in particular green tea and red wines both rich sources of polyphenols(Waterhouse,1995).

There are many medicinal plants which have polyphenolic metabolites as given by Haslam (1996) and they are:

1. Tree peony (*Paeonia lactifloa*) - Outer skin of the root; to cure disorders of blood stream. Principal polyphenolic metabolite - gallotannins.

2. Bear Berry (*Arctostaphylos uva - ursi*) - dried leaves; to cure ailments of bladder and urinary tract. Principal polyphenolic metabolite - gallotannins.

3. Rasp Berry (*Rubus idaeus*) - leaves and fruits; to cure digestive disorders. Principal polyphenolic metabolite - ellagitannins.

4. Hawthorn (*Crataegus sp*) - leaves and berries; used as an astringent for digestive system, diuretic etc. Principal polyphenolic metabolite - proanthocyanidins.

5. Meadow sweet (*Filipendula ullnaria*) - aerial parts of the plants, leaves and flowers used as an infusion; antirheumatic, antiinflamatory agent. Principal polyphenolic metabolite - ellagitannins.

The plant polyphenols are distinguished by the following general features.

(a) Water solubility:- In the natural state polyphenol interactions usually ensure some minimal solubility in aqueous media.

(b) Molecular weights:- Natural polyphenols encompass a substantial molecular weight range from 500 to 3000-4000.

(c) Structure and polyphenolic character:- Polyphenols, per 1000 relative molecular mass, possess some 12-16 phenolic groups and 5-7 aromatic rings.

(d) Intermolecular complexation:- In addition to giving usual phenolic reactions, they have the ability to precipitate some alkaloids, gelatin and other proteins from solution. These complexation reactions are of intrinsic scientific interest as studies in molecular recognition and as the basis of possible biological function.

(e) Biological and Chemical properties.

In additon to the general features plant polyphenols show various biological and chemical properties. Invitro testing has identified a wide range of potentially significant biological activities which are exhibited by natural polyphenols and a selection of these is shown below:

(i) Bactericidal action

(ii) Antihelminthic action

(iii) Inhibition of human simplex virus (HSV)

(iv) Antihepatoxic action.

(v) Host mediated antitumour activity

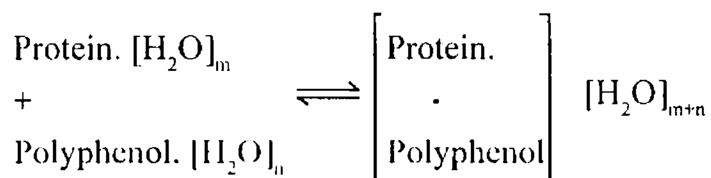
Although these studies have revealed important differences in pharmacological activity between individual polyphenols and between classes of different polyphenols overall they suggest some slectivity rather than high specificity towards particular biological targets. Thus Okuda (1993) and his collaborators (Kashiwada, et. al, 1992) demostrated significant inhibition of both the cytopathic effect of HIV and expression of HIV antigen in human lymphotropic virus type I (HTLV-1) - positive MT-4 cells by several hydrolysable tannins. Nevertheless, it is also clear that polyphenols have a number of physical and chemical properties - associated principally with the possession of a concatenation of phenolic nuclei within the molecule - in common. These properties, moreover, probably underly, at least in part their physiological and pharmacological activity.

Natural polyphenols can form complexes with metals and metal ions such as Iron, Vanadium, Manganese, Aluminium, Calcium etc. Iron is common to all life and the most abundant transition metal. There seems therefore a very good reason to think that natural polyphenols have the potential to modulate physiological reactions involving iron and other transition metals.

According to Halliwell (1995), plant phenols and polyphenols are possibly important as antioxidants and are known to lipid peroxidation and lipoxygenases in vitro, and information has been accumulated over the past few years demonstrating their ability to scavenge radicals such as hydroxyl, superoxide and peroxyl, which are known to be important in cellular peroxidant states.

Although the uses of polyphenols as medicinal agents are earlier seen, their action appear due to their ability to complex with proteins and polysaccarides. They thus

aid the healing of wounds, burns and inflamations. In doing so, they act to produce an impervious layer under which the natural healing can occur. This property to bind to proteins also, presumably accounts for the fact that polyphenols inhibit virtually every enzyme that are tested with *in vitro* (Loomis, 1974). In an earlier study of plant viral infection, Cadman (1960), suggested that polyphenolic extract of the leaf of raspberry probably act on most viruses by clumping the virus particles together into complexes which are largely uninfective. In later works, others have similarly deduced that viral inactivation, in vitro is directly attributable to preferential binding of the polyphenol to the protein coat of the virus (Konishi and Hotta, 1979). In a systematic study of the antiviral activity of a very wide range of natural products, Vaden Berghe et.al, (1985) concluded that polyphenols act principally by binding to the virus or the protein of the host cell membrane and thus arrest adsorption of the virus. The complexation of polyphenols with proteins is a specific example of the phenomenon of the molecular recognition



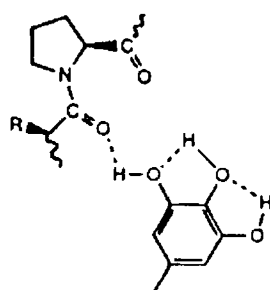
polyphenol-protein association

Proteins which are small and compact with a tightly folded secondary and tertiary structure have a poor affinity for polyphenols. Conversely, proline-rich proteins (e.g, gelatin, salivary proteins) which have an open, random coil type of confirmation have a high affinity for polyphenolic substrates.

The role of water and water solubility is one of the key factors in the phenomenon of polyphenol complexation. It is observed that where a polyphenol molecule is

highly water soluble, then there will be very little driving force towards complexation with proteins.

Hydrogen bonding is the second effect which dominates polyphenol complexation, and it is thought to be of importance because of its directionality and the overall strength derived from a multiplicity of hydrogen bonds radiating from the polyphenolic substrate. The carbonyl function in tertiary amides (such as polypeptides and caffeine) is a much more hydrogen bond acceptor than that in primary and secondary amides (Cheek and Lilley, 1988).



Reinforced Hydrogen bonding of a phenolic group with the tertiary carbonyl group of a prolyl peptide.

The reason for this improved binding has been suggested to be the poorer solvation of the tertiary amide function. The interaction of plant polyphenols with the proline rich protein collagen in skins forms the basis for the production of leather.

1.4 Polyphenol in coconut husk and its liberation during retting.

Coconut husk which represent the entire fibrous material enveloping the fruit of *Cocus nucifera* Linn. constituting both the exocarp and the mesocarp, is the raw material for the coir industry from where coir firber is extracted out. The elastic cellular cork like material forming the non-fibrous tissue of the husk is referred to

as the coconut pith and accounts for as much as 50-70% of the total weight of the husk; the remainder constitutes pectic substances, polyphenols etc (Bhat, 1969. 1000 husk yield about 82 kg coir. The age of the mesocarp is an important factor in the quality of fibre and so husks from near ripe to ripe nuts are reported to give best fibres.

The intense cultivation of the coconut tree is the reason for concentration of the coir industry along the coastal belt of Kerala.

Preliminary studies on the polyphenols that are present in coconut husk as stated earlier reveals tannic acid, protocatechuic acid, catechol, pyrogallol etc as the polyphenols.

The traditional method of natural retting practiced in India consists of soaking the husks in saline back waters. There are two methods of soaking the husk. The first method is to arrange the husks in coir nets into bundles and float them freely in the backwaters until they get soaked, become heavy and gradually sink to the bottom. The second method practiced is to steep the husk into pits dug within the reach of tidal action of the backwaters. Whatever be the practice, the principle involved is to steep the husk for retting in sea water or brackish water. Interestingly unlike other plant fibres which get released within a few days of retting, the coir fibre takes a longtime to get seperated from the binding material, the retting time varying from 4 to 12 months, depending on the area and the variety of yarns required to be produced. In fact, the quality of the product is judged by the area from which it is derived. According to a survey report (survey reports on Coir Industry, 1968),majority of the retters (67%) ret their husk for a period ranging from 7 to 9 months,

25% ret their husk for more than 9 months and the remainder (8%) do so for 2-6 months.

The principal change brought about in the plant tissue during retting is the break-down of pectic substances which form the chief chief constituent of the middle lamellae between the fibre cells and the cementing material. The biological retting of coconut husk differs from that of the other fibrous materials in that it is not confined to pectin decomposition alone but also extents to the disintegration of the phenolic cement binding the fibres together. One of the important observations made in the retting of husk was that polyphenols from the husks get constantly leached out into the surroundings (Jayashankar,1966). The relatively high percent-age of such polyphenols in coconut husk (Varrier and Moudgill,1947) has been pointed out by Menon & Pandalai (1958) as the very reason for the delay (4-12 months) in the completion of the retting process.

A proximate analysis of the husks best suited for retting and production of good fibre showed polyphenols and pectins to be the more important constituents as they represented 75-76 and 16-17g/Kg of husk materials (Jayashankar,1966 and Bhat,1969). For determining the end point of retting, it was considered essential to estimate residual polyphenols and pectins in the retting husks. Experimental re-sults indicated positive correlation between the progress of the extent of retting and the rate of disappearance of these compounds. The gradual fall in the high polyphenol content of the husks during the progress of retting led to ascertaining the nature of polyphenols present in the husk and the type of microflora associated with the proc-ess. As indicated earlier it is also assumed that the simple phenols may be found dispersed admist the built in units of plant polyphenols. The colour reactions with

alum, ferrous sulphate, bromine water and p-dimethylamino-benzaldehyde, the polyphenols of the husk were identified as catechin-like tannins(Jayashankar,1966).

The various factors affecting the retting of coconut husk are the salinity of water(Pandalai et.al,1957), periodic flushing of the ret liquor, which is correlated to the periodic tidal waves which helps in leaching out the polyphenols, crushing of the husk prior to retting for removing the polyphenols to reduce the retting period.

1.5 Environmental effects of polyphenols from coconut husk during retting

One of the striking features of Kerala state is the continuous chains of lagoons or backwaters existing along the coastal region. The backwaters support rich and diverse life forms and provide crucial nurseries for shrimps and fishes as well as habitat for oysters, clams and mussels which later enrich the ocean and make Kerala the principal exporter of marine products amongst the states of India. The shallow fringes of backwaters and the channels drawn from them are used for retting of coconut husk. Retting is brought about by the pectinolytic activity of microorganisms especially, bacteria, fungi and yeasts degrading the fibre binding material of the husks and liberating large quantities of organics and chemicals into the environment including, pectin, pentosans and polyphenols. Coir retting thus becomes a source of pollution to the backwaters of Kerala. From various experiments conducted for investigating and ascertaining the nature of pollution, it is found that there is no variation in temperature between the retting and non-retting areas, the population of fish in the retting areas is negligible when compared to non-retting areas due to the depletion of dissolved oxygen, the pH in the retting areas is between 4.2 to 5.6 as when compared to 7.1 to 7.8 of the non-retting areas. The

contents of phosphates, nitrates and hydrogen sulphides increase in the retting areas compared to the non-retting areas. According to a survey by Remani et.al, (1989) the fish population in the retting areas were 20Kg/hectare/month and 60.6Kg/hectare/month in the non-retting areas respectively. The benthic fauna scarcely exist in the retting zones due to the effect of polyphenols released from the husk.

In addition to the disturbances on the flora and fauna in the aquatic system, the polyphenols released during retting also causes skin diseases and irritations on the people who work in the retting yards. This irritation is due to the acidic nature of the polyphenols that are released from the husk in the natural environment.

1.6 Development of bioreactor for retting coconut husk as an alternative method of retting in open waters.

The natural method of retting of coconut husk for extracting out fibre takes long period of 6-12 months. During the initial period much of the polyphenols from the husk get leberated into the aquatic system, this inhibiting the microbial process of retting and eliminating the flora and fauna from the retting grounds. (Menon and Pandalai, 1958). Inorder to combat pollution and to have total control over the retting process a bio-reactor has been developed as an alternative method (Anon, 1998). In this bioreactor the ret iquor generated during retting is microbially treated and circulated. Since polyphenols are the inhibitory compounds delaying the retting process they have to be removed faster prior to retting and for which a pre-retting operation has to be incorporated. Thus the fabrication of a bio-reactor as an alternative method for coir-retting has the advantage of extracting out polyphenols prior to retting and thus eliminating any environmental impairment due to the process.

1.7 Relevance of this study and its possible impact on coir industry and in the protection of environment.

The present study focuses attention on developing a viable technology which can be designated as the 'pre retting operation' which mainly includes stripping polyphenols prior to retting the husk in the reactor. Once the polyphenols are separated they have to be concentrated and the ret liquor thus generated can find various industrial applications. The polyphenols thus form a by- product of coir retting in the bioreactor and to make them a product of commercial importance their physicl, chemical and biological characteristics have to be worked out, besides their possible applications.

The study was carried out along with the development of coir retting bioreactor and thus was made an integral part of the technology. So far the coir pith generated during the fibre extraction was the only by-product which has already found its commercial relevance at various fronts. Like wise by upgradating retting process from a natural to the bioreactor mode it is now made possible to recover one more by-product which otherwise would have been lost in the open waters. A proper utilization of the ret liquor and the polyphenols contained in it adds to the revenue and partially bears the cost of running the bioreactor.

In the environmental perspective the present study has great relevance as it totally eliminate the possiblility of any environmental impairment due to retting. Through the pre-retting operation the polyphenols are now extractable in water and can be concentrated and can find various bio-medical and industrial applications. This forms the first steps towards the development of a clean technology in coir retting which forms the backbone of the coir industry. As is well known that a 'clean technology'

is well acceptable than an 'cleaning up technology' it is envisaged that the present work shall dramatically transform the coir industry from a traditional, polluting to a modern, environment friendly industry which attracts skilled labour and earns copious foreign exchange.

The work carried out with this as the focus is presented under three chapters such as a). The pre-retting operation b). Characteristics of ret liquor and c). Possible applications with a Conclusion and Bibliography.

CHAPTER 2

PRE-RETTING OPERATION

Chapter 2

PRE-RETTING OPERATION

2.1 Introduction

Retting of coconut husk in brackish water systems is an age old traditional practice carried out for the extraction of coir fibre (Bhat and Nambudri, 1971) for which the husk is steeped in water for 6-12 months (Menon and Pandalai, 1958)

During the initial period much of the polyphenols in the husk get liberated into the water leading to an overall inhibition of the microbial retting process and elimination of much of the fauna and flora from the retting grounds. In order to combat pollution and to have better control over the retting process, a bio-reactor has been developed, where the ret liquor generated during retting by microbial consortium is treated and re-cirulated. In this controlled retting process, a pre-retting operation has been incorporated in order to facilitate speedy removal of polyphenols before the microbial process sets in. This operation has two advantages.

1). It extracts the inhibitory compounds from the husk paving the way for faster retting and 2). These compounds can be recovered for serveral commercial applications. The present work has been undertaken to develop a viable technology for stripping, separation, concentration and storage of polyphenols from coconut husk. To facilitate the development of such a technology investigations were carried out to determine the optimum salinity of water and the time required for the liberation of polyphenols. The process of retting depends on the salinity of water in which it is

carried out (Pandalai et.al, 1957). Therefore various salinities were chosen for accessing the appropriate one at which maximum amount of polyphenols are liberated so that the microbial process of retting can set in soon after. For this the husk was seeped in water having varying salinities including distilled & tap water and the amount of polyphenols liberated was determined. Greater the liberation of polyphenols, faster would be the retting process.

The polyphenols that are liberated being polar in nature mixes with water and for their effective utilization they are to be separated. The usual methods available for separating such compounds are by using activated charcoal and 'flyash. Activated charcoal being highly porous provides larger surface area for adsorption of the polyphenols on to the surface thus removing the polyphenol content from the ret liquor. Similarly, flyash is also an effective adsorbant of polyphenols (Malhotra, 1979).

Besides these physical methods, chemical methods of extraction using various solvents depending on their polarity can also be employed. The polar nature of polyphenols, has to be taken into consideration for the development of any such method. Considering the higher molecular weight of polyphenols in ret liquor, reverse osmosis has also been proposed as a method for the separation of polyphenols from ret liquor.

Reverse osmosis is one of the modern membrane techniques which is being developed for the use in desalination and other ion separation process. (Bal and Lutade, 1979). Among the various polymers reported to be useful for reverse osmosis membrane separation, cellulose acetate is found to be one of the most satisfactory poly-

mer. Based on the requiremnt the membranes are cast in two different modules. They are (i) The flat membrance module (ii) the spiral wound module. The flat membrance module is mostly used for experimental purposes in the laboratory and the spiral wound module is used for commercial purposes. The flat membrane module consists of a single flat membrane of a particular size, supported by a backing material, which should be able to support the operating pressure without collapsing or exhibiting excessive creep under long term service and a membrane holding cell that can withstand operating pressures of 100-200 psi, corrosion and economic considerations (Sourirajan, 1970).

The pressure applied for a flat membrance module varies from 100-200 psi and this is obtained from high pressure pumps. The feed solution is pumped with the necessary operating pressure into the membrance holding cell. The membrance rejects the high molecular weight ions or molecules and the substances having low molecular weight in feed solution are allowed to pass through the membrane. The molecules that are allowed to pass through the membrane are called the 'permeates', and the molecules which are rejected or retained by the membrane in the cell are called 'concentrates'. The rate of the passage of the low molecular weight substance in the feed through the membrane is called the flux and is expressed in ml/min. In the case of flat membrane module, the flux would be very less as the effective area of the membrane involved in this reverse osmosis process is very less.

The special wound module consists of a membrane of larger surface area a porous product water-side backing material. The membrane is bonded along two sides at the end, and around the product water tube, forming a sealed envelop that encloses the backing material except at the product water tube open end. The brine side flow

spacer is placed on the membrane, and the several layers are then wrapped around the product water tube to form cylindrical module. In operation, the module is placed in the snug-fitting cylinderical pressure vessel. Feed water flows in at one end of the vessel, axially through the module in the passage provident by the coarse Brine-side spacer material, and out at the other end. Product water is collected in the product-water-side material, spirals inwards to the central tube and is collected there. The central tube is attached to a penetration in the end wall of the pressure vessel, and product water escapes through this penetration. Spiral wound modules have been made in many sizes with operating pressures of 600-2500 psi. In general, the module diametre varies from 2 to 4 inches and the length is 12 or 36 inches. With the spiral design, it is expected that 90 m^2 of membrane area can ultimately be accomodated in 1 m^3 of pressure vessel volume (sourirajan, 1970) The large membrane area, in spiral module, when compared with flat membrane module is its advantage for use in commercial applications.

The selection of membrane in any reverse osmosis process depends on the molecular weight of the feed solution. In general for the purpose of desalination (where reverse osmosis is mostly employed) the major characteristics that the membranes must possess include (a)the highest possible salt rejection with an adequate rate of permeation (b) the adequate rate of permeation should be obtainable under reasonable applied pressure and (c) this rate of permeation and the salt rejection rate should be maintained constant with an appreciable time limit under the continuous operation (Bal and Lutade, 1979)

Besides the membrane characteristics the backing material of a reverse osmosis membrane needs to have some basic requirements for satisfactory function of mod-

ule. They (i) should be able to support the operating pressure (ii) should have fine enough structure so that the membrane is not fixed onto the surface layers of the backing material (iii) should have sufficient porosity and large pore sizes so that the flow of product water through it does not cause an excessive pressure drop (iv) should be economical and (v) shoud have satisfactory life time. Fibrous plastics as backing materials is limited to operating pressures less than 200 psi whereas uniform silica granules held in place on a layer of felt paper by a synthetic material can operate at high pressure of 1500 psi (Sourirajan, 1970)

The effect of operating pressures in the reverse osmosis technique is also an important factor. When pressure is applied, the entire porous structure of the membrance compacts bringing the polymer segments still close together (Bal, 1992). This compaction results in further increasing the cross-links and decreasing the capillary spaces, and thus a still more dence surface as well as interior structure is formed. (Kopecek & Sourirajan , 1969). During this compaction, the dense surface layer remains intact while the spongy mass compacts thereby becomes progressively more dense and offers more resistance to the flow of liquid. This causes a 'flux decline'. Therefore it is necessary to maintain an optimum operating pressure for different polymer membranes.

In the present study, a flat membrance module was chosen for experimental purpose. The flat membrane module in this study consists of a membrance holding stainless steel cell having a holding capacity of 100 ml with an in-let and outlet.The membrane chosen was cellulose acetate -14 (CA-14) supplied by GSFC, Gujarat having an effective separation area of 47.75 cm^2. The operating pressure was 100 psi (7Kg/cm^2). The feed, was added through the inlet and the operating

pressure was applied from a compresser. The high molecular weight ion or molecules are rejected in the membrane holding cell, whereas the low molecular weight substances passes through the membrane and is collected as the permeate with a flux of 0.5 ml/min. The rejected high molecular weight polyphenols (>500) collected in the cell were lyophilised at -85°C for further use.

Interestingly unlike other plant fibres, which get released within a few days of retting, coir fibre takes a long time to get seperated from the binding material, the retting time varying from 4 to 12 months, depending on the area and the variety of yarns to be produced (Bhat and Nambudiri, 1971). This long period is attributed to the slow release of polyphenols from the coconut husk. (Menon, 1935). Thus the release of polyphenols in the natural system is a slow process, which depends on the total influx of seawater in an area. In order to speed up the whole process, the polyphenols, which remain as a hindrance for the microbial process of retting to set in has to be invariably removed from the system. For this experiments are to be carried out to estimate the amount of polyphenols liberated by gentle swirling of coconut husk over a magnetic stirrer for a certain period of time and the optimum salinity at which their is maximum liberation of polyphenols. At normal conditions, without applying any force, by gentle swirling, it took 96 hours for the maximum liberation of polyphenols using 5PPT sea water.

Therefore to enhance the speed of the retting process and to make it economically viable, a polyphenol stripping device was designed for maximum liberation of polyphenols using 5 PPT sea water thereby reducing the time period for retting. This polyphenol stripping device thus refers that the application of pressure on husk gives a maximum liberation of polyphenols (Speech delivered by Chairman,

Coir Board, Coir Vol. XXXV, B. No. 1, 1991). Thus the pre-retting operation consists of two distinct processes like mechanical crushing of the husk and stripping of polyphenols using a stripping device.

During the stripping of polyphenols in the stripping device, a red coloured compound was found to be liberated into water especially when no more liberation of polyphenols could be observed. Spectrophotomatically, this compound or derivative show different absorption peaks under UV and Visible region indicating that the compound is altogether different from polyphenols. In order to estimate the extent of liberation of this red coloured compound and the time required for its maximum liberation, experiments were conducted. Besides a simple mechanism by which the red coloured compound can be separated from the ret liquor also was evolved.

This chapter deals with the development of a viable technique of stripping polyphenols from coconut husk, development of a polyphenol stripping device, methods of concentration of polyphenols and lyophilization. Limited details of a red coloured compound liberated during stripping polyphenols and its removal from the ret liquor are also dealt with.

2.2 Materials and Methods

2.2.1 Optimum salinity for stripping polyphenols

For determining the optimum salinity required for the liberation of polyphenols, 30 gm fresh coconut husk drawn from middle portion was soaked in 500 ml water having varying salinities such as 5,10,15 and 20 parts per thousand (PPT) and also in tap water and in distilled water in a beaker fitted with a

perspex plateform. The whole preparation was placed on a magnetic stirrer for 48 hours at 3000 rpm. At the end of each experiment, the total polyphenols in the ret liquor was estimated by the method followed in APHA,(1989) where 1ml ret liquor was reacted with 2ml sodium taarate reagent followed by 0.2ml Folin-Phenol reagent, made up the volume to 10ml with distilled water and maintained for 30 minutes for colour development. The blue colour was read in a Hitachi UV-Vis spectrophotometer at 700nm. Tannic acid was used as the standard

Total proteins in the ret liquor was estimated by the standard Biuret method following Gornall et.al, (1949) by reacting 1ml sample with 2ml NaOH and 8ml biuret reagent. The blue colouration developed was read at 540 nm using a Hitachi UV-Vis spectrometer. Bovine Serum albumin (Sigma Chemical Co.) was rum as the standard.

Total sugars were estimated following Roe (1955) using Anthrone reagent. To an aliquot of 1ml sample 10ml Anthrone reagent was added and the mixture was heated in a water bath for 10 to 15 minutes, cooled in dark at room temperature and the absorbancy read in a Hitachi UV-Vis spectophotometer. Glucose (Hi-media Laboratories, Bombay) was run as the standard.

Total lipids were estimated following the sulpho-phosphovanillin method of Barnes and Blackstock (1973). In this method 10 ml ret liquor was mixed with 10 ml aliquot of 2:1 mixture of chloroform-methanol and 2 ml 0.9% aqueous NaCl in a separating funnel and after thorough agitation the preparation was allowed to stand for 30 minutes. From the clear biphasic layer formed the lower phase was removed and the same quantity of chloroform was added to

make up the volume. From the extract 0.5 ml sample was dried in a descicator and added 0.5 ml concentrated H_2SO_4 and maintained in a water bath at 60°C, cooled at room temparature and added 5 ml vanillin reagent and allowed to stand for 30 minutes. The absorbacy of the pink colour developed was measured at 520 nm. A standard was run employing the same method using cholestrol.

2.2.2 Time required for maximum liberation of Polyphenols

For determining the minimum time required for liberation of polyphenols 30 gm husk drawn from the middle portion of a coconut husk was soaked in water having a salinity of 5PPT (this salinity was found to be the optimum for the liberation of polyphenols) in a beaker fitted with a perspex platform. The whole preparation was stirred over a magnetic stirrer for a period of 120 hours, and the amount of polyphenols liberated was determined as mentioned earlier.

2.2.3. Solvent extraction for the seperation of polyphenols from ret liquor

Polyphenols being slightly polar and organic they can be desorbed using various organic solvents like diethyl ether, benzene, hexane, chloroform, carbon tetrachloride, petroleum ether etc. 10 ml of ret liquor was strirred with the 10 ml of the solvents one after the other for 30 minutes at 3000 rpm. The solvent phase was seperated using a seperating funnel and evaporated in a previously weighed empty beaker in an oven at 60°C. The quantity of the polyphenols extracted was estimated gravimetrically.

2.2.4. Activated charcoal for the separation of polyphenols

To evolve a viable method for separating polyphenols from ret liquor, activated charcoal was tried as an absorbing agent. It is known that the adsorption/ desorption of polyphenols is pH dependant (Bhatt et. al, 1983). Hence a set of

experiment were carried out to determine the optimum pH of the ret liquor at which the adsorption could take place. Ret liquor for this experiment was prepared by soaking a fresh coconut husk in water having 5PPT for 24 hours. The fluid was maintained at -20°C

2.2.4.a. pH optima for adsorption.

The pH of 10 ml aliquits of the ret liquor was adjusted within a range of 1-7 using 1 N HCl/NaOH. To each aliquot 500 mg activated charcoal (Merck 17505) was added and stirred over a magnetic stirrer at 3000 rpm for 30 minutes. The supernatant was decanted and centrifuged at 3000 rpm for 10 minutes to free it from activated charcoal. Total polyphenol content of the ret liquor treated with activated charcoal and that without the treatment of activated charcoal was determined using the methods described earlier and from this the pH at which maximum absorption achieved was recorded.

2.2.4.b. Optimum normality for desorption

For further utilization of the adsorbed polyphenols they have to be effectivity desorbed from the activated charcoal. NaOH having affinity for polyphenols was used for desorption. To find out the optimum normality of NaOH at which desorption takes place, 1.0 gm of polyphenol bound activated charcoal was agitated with 20 ml. aliquots of 0.1, 0.5, 1, 2 and 3N NaOH for 30. minutes over a magnetic stirrer at 3000 rpm. The supernatant was decanted of centrifuged at 3000 rpm for 10 minutes. The total polyphenols available in the supernant was measured to determine the extent of desorption using the method described earlier.

2.2.4.c Organic solvent as desorbant

Organic solvent were used for the separation of phenolics as phenols are organic indicating its affinity for various solvents. Therefore organic solvents such as ethyl alcohol, methanol, acetone, petroleum ether, hexane, carbon tetrachloride (depending on their polarity) were tried for desorbing the polyphenols from the activated charcoal. Activated charcoal (500mg) containing known quantity of adsorbed polyphenol was agitated with 20 ml each of organic solvent over a magnetic stirrer at 3000 rpm for 30 minutes. The Supernatant was collected and centrifuged, dried at 60°C and the desorbed polyphenols was estimated gravimetrically.

2.2.5 Membrane Concentration

Finally reverse osmosis was investigated for the concentration of polyphenols from ret liquor using a stirred flat membrane holder with cellulose acetate (CA-14) membrane, (supplied by NCL, pune). The flat membrane holder as shown in Fig. 2.1 consisted of a mettalic sietz filter holder with a meshed support. A known quantity of ret liquor was poured into the module and connected to a presssure control device, whereby a constant pressure of 100 psi was applied while keeping it on a magnetic stirrer and stirring at 550 rpm. After a period of 5 hours, the total polyphenol, protein, sugars and lipids of the permeate and the concentrate were determined following the methods mentioned above and the rejection of the membrane for each species was determined. After each run, the membrane was washed with distilled water and stored at 4°C until the next trial. The rejection is defined as

$$R = 100 \times (1 - C_p/C_f)$$

Where Cp is the permeate and C_f is the corresponding feed side concentration.

The concentrated ret liquor was lyophilized using a bench top lyophilizer (FTS system Inc. USA). The proportion of polyphenol in the lyophilized mass was determined following the method mentioned earlier.

2.2.6. Designing, fabrication and standardization of a polyphenol stripping denice.

Polyphenol stripping device consists of a tank of 32 cm^2 base and 40 cm height. A perforated platform is positioned 10 cms up above the base to support the husk. From the bottom of the tank an outlet pipe with 2 cm diameter connects with a 0.25 HP monoblock electric pump which facilitates drawing water forcefully from the bottom and to discharge at the top, there by a strong circulation of water at a turnover of 40 litres per minute can be generated. Through another outlet pipe fixed at the opposite side ret liquor can be drained off for concentration of polyphenols (Fig. 2.2). The tank was made with fibreglass, pipes with PVC and valves with polypropylene to avoid corrosion.

Freshly split fresh coconut husk crushed mechanically using a hammer was arranged in the tank inverted and the tank was filled with 5PPT sea water. The husk was allowed to soak for 6 hrs and the pump was operated subsequently. Sampling of ret liquor was carried out once in 6 hours for the determination of polyphenol content. The polyphenol content was determined spectrophotometrically following (APHA, 1989) when the polyphenol content was found to attain stationary phase the ret liquor was drained off and the tank was filled with fresh 5 PPT sea water and the stripping of polyphenols

repeated.

2.2.7. Liberation of a red coloured compound from husk during stripping of polyphenols.

During the standarization of stripping polyphenols, a red coloured compound was found to be liberated into water especially during the second stage of polyphenol stripping when no more liberation of polyphenols occured. This compound exhibited different absorption peaks under UV-Visible region indicating that it was altogether different from polyphenols. Inorder to estimate the extent of liberation of the coloured compound and the time required for its maximum liberation the following experiments were conducted.

In order to differentiate the red coloured compound from the polyphenols of the husk, the ret liquor was initially scanned under UV and Visible ranges of wave lengths in a Hitachi spectrophometer with 5PPT sea water as blank (Fig. 2.3). Two peaks were obtained at two different wave lengths of which one was in the UV range (190-360nm) range and the other in the visible range (360-480nm) and this was further used for assessing the concentration of the compound. For polyphenols maximum absorbancy under UV range of 190 to 360 nm and for the red coloured compound maximum absorbance under visible range at 360 - 480 nm were used for assessing their respective concentration.

The experiments were conducted in two stages with the measurement of absorbancy under both UV and Visible ranges and the duration required for the liberation of both polyphenols and the red coloured compound were determined.

2.2.8. Removal of the red coloured compound from ret liquor.

When the ret liquor generated during the second stage of stripping was maintained open in a beaker, settlement of the compound as flocs and the clearing of the liquor were noticed. Meanwhile an aliquot of liquor when maintained air tight was found to remain as such without any change. This observation led to the formulation of the concept of separating the compound by agitation facilitating adequate separation. The following experiment was carried out to assess the extent of separation of the the compound, duration required, changes in the concentration of polyphenols, pH and salinity changes during floculation.

About 650 ml aliquots of the ret liquor generated during the second stage of polyphenol stripping was maintained in 1000 ml beaker and agitated using an electric stirrer at 100 rpm for 24 hours. Once in three hours total polyphenol content absorbancy under UV range (190-360 mm) and Visible range (360-480 mm) pH and salinity were monitored. As control ret liquor was maintained in screw caped bottles air tight and were subjected for the above analysis.

2.3. Results and Discussion

2.3.1 Liberation of polyphenols from husk and the time required by gentle swirling.

The extent of liberation of total polyphenols, lipids, sugars and proteins from coconut husk into distilled water, tap water and sea water at varying salinities all at neutral pH is summarised in Fig. 2.4. From this it could be observed that maximum liberation of polyphenols occured at 5 PPT and at this salinity the liberation of lipids, sugars and proteins remained comparitively low. It was

observed that 96 hours were required for attaining the maximum release of polyphenols into water at 5 PPT with mild swirling (Fig. 2.5).

The liberation of polyphenols from coconut husk during the initial phase of retting in backwaters has been reported by Jayashankar (1966). However, it has not been known at what salinity the maximum liberation occurs and the time required for the liberation to be completed. In the present study 5 PPT was demostrated to be the optimum salinity for the release of polyphenols and 96 hours were required for the maximum liberation under gentle swirling which is comparable to the movement of water in the natural environment. For further removal of polyphenols from the husk it is essential to maintain an effective circulation of water mechanically and thereby considerable force can be applied on the husk for releasing the polyphenols. Shortening of the time required for stripping polyphenols to a couple of hours is essential in terms of the viability of the technology. For this a polyphonol stripping device was developed.

2.3.2 Seperation of polyphenols from ret liquor

2.3.2.a Solvent Extraction

Results of the experiment conducted to examine the feasibility of solvent extraction of polyphenols directly into organic solvents from ret liquor are summarized in Table 2.1. The highest percent of extraction was only 2.9 ± 1.8 in diethl ether, with very low values in all other solvents.

2.3.2.b Activated carbon adsorption:

As activated charcoal has been reported to be a good adsorbent of polyphenols, experiments were conducted to examine the possibiity of using it to seperate polyphenols from ret liquor. Though the maximum adsorption (98.9%) was at pH 1, upto 96.3% adsorption was found even at pH 7. It is interesting to note that at the normal pH of the ret liquor (6.5 ± 0.2) prepared in 5PPT sea water, 96% of adsorption of polyphenols could be achieved with activated charcoal (Table 2.2).

Once removed from ret liquor b y adsorbing on to activated charcoal, the polyphenolic compounds have to be desorbed by a suitable solvent for its effective utilization for various applications. Sodium hydroxide solutions with varying normalities and various organic solvents were tried as the desorbents. The desorption of polyphenols in NaOH solution was negligible and the highest desorption obtained was only 0.034% at 1 N NaOH (Fig. 2.6). The percent desorption of polyphenols from activated charcoal with six organic solvents is summarised in Table 2.3. It is evident that none of the solvents used were able to effectively desorb the polyphenols, the percent decorption in all instances was less than 0.5%, with maximum desorption seen in ethyl alcohol (0.44 ± 0.13%).

2.3.3 Membrane concentration

Considering the inadequacies of the above methods for the effective separation of polyphenols from ret liquor, another technique such as reverse osmosis

was investigated. On subjecting the ret liquor prepared in both distilled water and in 5PPT sea water for concentration with a loose reverse osmosis membrane more than 95% rejection of polyphenols, lipids, sugars and proteins could be obtained (Table 2.4 and 2.5). In both instances, the flux was 0.5 ml/min in the experimental set up.

Concentrated ret liquor was lyoplilised and analysed for their total polyphenol content. From (Table 2.6) it was found to have 495 ± 38 mg/g polyphenols in case of ret liquor prepared in distilled water and 302 ± 94.5 mg/g in case of ret liquor prepared in 5 PPT, sea water.

Removal of polyphenols from ret liquor and their concentration are the two essential steps towards their utilization for any commercial purpose. Activated charcoal has already been reported to be a good adsorbent for phenolics by Zogaski and Fouri (1977) and, for the removal of polyphenolics from ret liquor also this could be a fairly good adsorbent. The extent of adsorption of polyphenols by activated charcoal is pH dependant (Bhat et. al, 1983) and in the present study maximum adsorption (98.87%) was obtained at pH 1.

However, even at the normal pH of ret liquor (pH 6.5 ± 0.2) the adsorption was well above 96% indicating that the effect of pH variations on adsorption was small. This is a negligible difference considering the requirement of acid for the pH adjustment.

Desorption of polyphenols from the adsorbed state into a solvent is essential for utilizing it for any commercial purpose. But it was observed that the adsorption of polyphenols to activated charcoal was an irreversible process and neither treatment with alkali nor with different organic solvents could desorb

it effectively. This may be due to the polar nature of the polyphenols. Solvent extaction to recover the polyphenolics directly from ret liquor also was not successful. (2.89 ± 1.8% in diethyl ether)

Reverse osmosis was found to be a feasible alternative for the concentration of polyphenols from ret liquor. A cellulose acetate based membrane was used for this application since adsorption of the polyphenols, proteins and lipids present in the ret liquor is expected to be less compared to polyamide based membranes. The pH and temperature of the ret liquor is also suitable for cellulose acetate membranes.

The membrane was cast from a dope solution containing 19% celluose acetate (from GSFC, R & D centre) in a 2:1 mixture of Acetone : Formamide. The membrane was not annealed and hence has comparitively low rejection for salts while retaining rejection for higher molecular weight organics. This rejection profile is as essential condition for the concentration for polyphenols from ret liquor prepared with 5 PPT saline water and distilled water as an extractant as it reduces the increase in osmotic pressure due to rejected salt.

The ret liquor concentrated could be lyophilized effectively. The lyophilized mass obtained from the ret liquor prepared in distilled water contained comparatively higher quantity of polyphenols (494.8 ± 37.68 mg/g) than the one obtained from the ret liquor prepared in 5PPT seawater (301.60 ± 94.5 mg/g). This is because of salting out of the nonphenolic impurities like proteins which were retained in the membrane, consequently the saline recovered polyphenols happened to be more pure. For large scale processes spray drying could be investigated after suitable concentration.

In short, polyphenols from fresh coconut husk can be extracted bya process known as 'pre-retting operation' which consists of crushing the husk and steeping in diluted sea water having a salinity of 5 PPT for 96 hours under gentle swirling. Ret liquor thus generated can be concentrated by reverse osmosis and the concentrated mass can be lyophilized and maintained.

The stirred cell was operated in the laminer regime ($\omega r^2 = 14{,}962$). This mass transfer coefficient (Blatt, 1970) can then be estimated assuming the diffusion coefficient 'D' of the rejected species in water to be similar to that of sucrose ($\sim 5 \times 10^{-6}$ cm^2/sec.)using the formula given below.

$$K = 0.253 \times \frac{D}{r} \left(\frac{\omega r^2}{\upsilon}\right)^{0.55} \times \left(\frac{\upsilon}{D}\right)^{0.33}$$

where r = 2.6 cm (radius of membrane)

$$\upsilon = \frac{\mu}{\rho} = \frac{0.01 \text{ poise}}{1 \text{ gm/cm}^3}$$

The value of the mass transfer coefficient K is found to be 1.18×10^{-3} cm/sec, which is similar in magnitude to that achievable in commercially available tangential flow membrane modules.

Inspite of operating at a relatively low pressure, the membrane productivity was ~13 Lmh. This permeability rate along with the mass transfer coefficient reported above can be used to estimate the required membrane area using standard theoretical treatments. It is encouraging to note that both membrane rejection (~95% for various organics) and productivity were constant over repeated trials as shown in Table 2.4 and 2.5. The membrane productivity did not decrease significantly even over a volume concentration factor of 10; which may be due to the relatively low absolute concentrations of the rejected species.

2.3.4 Polyphenol stripping devices as the basic unit of coir retting bioreactor and its standardization.

The polyphenol stripping device designed and fabricated functions as the basic unit of coir retting bioreactor where subsequent to the stripping of polyphenols the husk can be retted also by inoculating with the retting microbial cultures.

Table 2.7 summarises the extent of polyphenol stripping achieved during the final stage of treatment. It could be seen that the maximum liberation of polyphenols occured with in 24 hours and the concentration declined subsequently. The extent of liberation of polyphenols in the second stage of stripping summarised in Table 2.8. It was noticed that right from the zero hour onwards the concentration of polyphenols got declined.

The data clearly indicated that maximum liberation of polyphenols was achieved with in 24 hours and for the recovery of the compound this was the right time to draw off the fluid. Subsequent maintenance of the liquor in the reactor lead to the degradation of the polyphenolics while a progressive declining in the concentration of the polyphenolics was taking place during the later part of first stage of stripping and also during the entire second stage. Interestingly intensity of the colour of the liquid was found to be increasing gradually. This was resolved in the next set of experiments.

2.3.5 Liberation of the red coloured compound from husk during stripping of polyphenols

As seen in Fig. 2.3, the ret liquor contained two compounds having two distinct absorption peaks in the UV region (190-360 nm) and the invisible region (360-480 nm). The compounds which showed absorbancy in the UV region

corresponded to polyphenols (TALLS, Pyrogallot etc.) and that in the visible region corresponded to the group of red coloured compound.

Table 2.9a and 2.9b summarises the concentration of polyphenolics and the red coloured compound (in terms of absorbancy) liberated during the first and second stage of stripping for a period of 36 and 48 hours respectively. It is well clear that during the first stage of stripping the polyphenolic compounds registered a progressive increase in the concentraton for 24 hours after which it declined. Meanwhile, the liberation of the coloured compound which was progressive in the first stage continued even to the second stage for another 36 hours. Precisely the liberation of polyphenoles with in 24 hours of stripping (Stage 1) and for the completion of the liberation of coloured compound it required altogether 60 to 72 hours depending on the quality of husk. The extent of liberation of polyphenols and the red coloured compound during the second stage of stripping are summerised in Table 2.10 in terms of absorbancy in UV and visible ranges. Clearly during this period no more liberation of polyphenols took place and at the same time there was progressive release of red coloured compound for 36 hours.

In short, during the first stage of stripping, polyphenolics could be extracted and during the second stage the red coloured compound. The technique of seperating polyphenols from ret liquor by reverse osmosis was standardized and a method had to be evolved to remove the red coloured compound from ret liquor generated during second stage of stripping.

2.3.6 Removal of the red coloured compound from ret liquor

During the 24 hour long flocculation experiment there was the removal of the red coloured compound, as shown by the reduction in absorbancy in the visible range, and also visibly in the form of brown coloured flocs. (Table 2.11). Interestingly in the controls maintained there was very little change in the absorbancy and no visible flocculation could be observed. Along with removal of red coloured compound during agitation polyphenol content was also found to be reducing drastically as indicated in the absorbancy under UV range. pH and salinity stood almost constant at 7.8 and 6PPT respectively during the flocculation. By this simple technique more than 88 % of the red coloured compound which remained in the soluble form could be separated.

2.4 Conclusion

The polyphenols that are liberated during the initial phase of retting are responsible for much of the environmental impairment caused during retting and also they inhibit and prolong the onset of microbial process of retting delaying the whole process for 6 to 12 months. In a bioreactor which was conceptualized and developed (Anon, 1998) for process upgradation and pollution abatement it was essential to have a component with which the polyphenols could be extracted with in the shortest period possible, paving the way for the onset of microbial process of retting. Now by employing the polyphenol stripping device developed it is possible to stripp off the polyphenols from the crushed coconut husk into 5PPT saline at room temperature within a period of 24 hours. Polyphenols from this ret liquor can be effectively concentrated by a flat membrane reverse osmosis module. This concentrated mass can be lyophilized and made available for commercial application. During the

second stage of polyphenol stripping a red coloured compound was found to be liberated which was different spectrophotometrically from the polyphenols. This compound eventhough was not identified was able to be seperated from the liquor by flocculation. The water which was recovered after seperation of polyphenols by reverse osmosis and after flocculation by the removal of the red coloured compound was fit to be reused for stripping polyphenols afresh. The polyphenolic compounds shall find commercial application making the whole process commercially viable.

Table 2.1 PERCENT EXTRACTION OF POLYPHENOL BY ORGANIC SOLVENTS DIRECTLY FROM RET LIQUOR

Sl. No.	Concentration of Polyphenols in ret liquor (mg/ml)	Percentage extraction of polyphenols in the organic solvents such as					
		Diethyl ether	Benzene	Hexane	Chloroform	Carbon tetra Chloride	Petroleum ether
1	12.43	1.86	1.70	1.11	0.51	0.66	0.10
2.	8.32	2.6	1.8	1.21	0.12	0.45	0.10
3.	3.75	0.93	0.53	0.39	0.15	0.24	0.10
4.	2.09	2.98	2.6	1.39	0.58	0.88	0.18
5.	4.34	6.28	4.24	2.56	0.20	0.55	0.010
6.	8.32	2.69	1.68	1.22	0.29	0.72	0.03
Mean	6.54 ±3.49	2.89 ±1.8	2.15 ±1.13	1.31 ±0.64	0.22 ±0.17	0.47 ±0.20	0.08 ±0.055

Table 2.2 OPTIMUM PH OF ADSORPTION OF POLYPHENOLS TO ACTIVATED CHARCOAL

Serial Number	Concentration of Polyphenols in ret liquor (mg/ml)	Percentage adsorption of polyphenols to activated charcoal						
		1	2	3	4	5	6	7
1	1.415	98.65	98.43	98.17	97.95	98.3	97.66	96.6
2.	1.415	99.74	99.68	99.29	98.93	98.51	94.2	94.06
3.	2.473	99.82	99.64	99.63	99.33	98.82	98.49	97.78
4.	3.335	96.48	96.25	96.16	95.95	95.5	95.12	94.93
5.	3.335	98.77	96.91	96.73	96.64	96.19	95.21	95.94
6.	3.68	99.89	99.82	99.73	99.59	99.32	98.91	98.61
Mean	6.54 ±0.91	98.89 ±1.18	98.46 ±1.41	98.29 ±1.36	98.07 ±1.37	97.77 ±1.41	96.60 ±1.73	96.32 ±1.72

Table 2.3 Percent desorption of polyphenols from activated charcoal into various solvents

Serial Number	Concentration of Polyphenols adsorbed to the activated charcoal(mg/g)	Percentage of desorption of polyphenol into solvents such as					
		Ethyl alcahol	Acetone	Methanol	Hexane	Carbon tetra Chloride	Petroleum ether
1	74.05	0.42	0.35	0.34	0.064	0.037	0.010
2.	121.81	0.45	0.37	0.32	0.016	0.011	0.00
3.	83.07	0.46	0.39	0.35	0.050	0.01	0.00
4.	47.57	0.44	0.31	0.13	0.01	0.00	0.00
5.	59.46	0.68	0.28	0.21	0.09	0.04	0.00
6.	44.31	0.21	0.17	0.11	0.021	0.00	0.00
Mean	75.04 ±26.23	0.44 ±0.13	0.31 ±0.073	0.25 ±0.09	0.04 ±0.02	0.016 ±0.016	0.00 ±0.00

Table 2.4 Percent rejection of polyphenols sugars, proteins, lipids from ret liquor prepared in distilled water and flux removal by reverse osmosis.

Sl.No.	Polyphenols		Sugars		Proteins		Lipids	
	Flux ml/min	% removal	Flux ml/min	% removal	Flux ml/min	% removal	Flux ml/min	% removal
1.	0.5	91.4	0.5	98	0.5	97.4	0.5	98.2
2.	0.5	94.6	0.5	96.64	0.5	94.0	0.5	96.99
3.	0.5	95.5	0.5	95.2	0.5	94.01	0.5	97.15
4.	0.5	97.4	0.5	93.7	0.5	98.2	0.5	99.04
5.	0.5	95.24	0.5	96.04	0.5	92.25	0.5	98.71
Mean	0.5	94.82 ±1.95	0.5	95.91 ±1.43	0.5	95.17 ±1.96	0.5	98.01 ±0.82

Table 2.5 PERCENT REJECTION OF POLYPHENOLS SUGARS, PROTEINS, LIPIDS FROM RET LIQUOR PREPARED IN 5PPT SALINE WATER AND PERCEUTAGE REMOVAL BY REVERSE OSMOSIS.

Sl.No.	Polyphenols		Sugars		Proteins		Lipids	
	Flux ml/min	% rejection	Flux ml/min	% rejection	Flux ml/min	% rejection	Flux ml/min	% rejection
1.	0.5	92.4	0.5	61.53	0.5	94.71	0.5	89.58
2.	0.5	96.17	0.5	97.68	0.5	97.28	0.5	99.86
3.	0.5	95.17	0.5	97.4	0.5	97.19	0.5	99.90
4.	0.5	95.98	0.5	97.3	0.5	98.59	0.5	99.84
5.	0.5	96.69	0.5	92.83	0.5	94.66	0.5	99.90
Mean	0.5	95.28 ±1.52	0.5	89.34 ±14.02	0.5	96.48 ±1.55	0.5	97.81 ±4.1

Table 2.6 POLYPHENOL CONTENT IN THE LYOPHILIZED RET LIQUOR CONCENTRATE.

Sl.No.	Polyphenols (mg/g)	
	Distilled water	5PPT sea water
1	516 mg/g	179 mg/g.
2	476 mg/g	301 mg/g.
3.	436 mg/g	238 mg/g .
4.	498 mg/g	459 mg/g.
5.	548 mg/g	331 mg/g.
Mean	494.8 mg/g ±37.68	301.6 mg/g. ±94.5

Table 2.7 LIBERATION OF POLYPHENOLS FROM COCONUT HUSK DURING STRIPPING IN THE POLYPHENOL STRIPPING DEVICE - STAGE1

	Concentration of polyphenols (mg/ml) during - stage 1				
Sl.No.	12 Hrs	18 Hrs	24 Hrs	30 Hrs	36 Hrs
1.	1.612	3.150	2.227	1.796	1.673
2.	0.664	0.775	1.993	1.735	1.476
3.	0.674	2.461	2.436	1.636	1.538
4.	0.775	0.898	1.058	0.787	0.726
5.	0.824	0.898	1.12	0.640	0.627
Mean	0.9102 ±0.356	1.6364 ±0.980	1.7668 ±0.571	1.3188 ±0.499	1.208 ±0.439

Table 2.8 LIBERATION OF POLYPHENOLS FROM COCONUT HUSK DURING STRIPPING IN THE POLYPHENOL STRIPPING DEVICE - STAGE2

Sl. No.	0 Hrs	12 Hrs	24 Hrs	36 Hrs	48 Hrs
1	1.427	1.403	0.906	0.812	0.0664
2.	0.516	0.445	0.332	0.812	0.073
3.	0.529	0.393	0.356	0.307	0.233
4.	0.381	0.369	0.332	0.332	0.295
5.	0.640	0.381	0.369	0.320	0.307
Mean	0.6986 ±0.373	0.6002 ±0.402	0.4698 ±0.223	0.3762 ±0.241	0.3144 ±0.193

Table 2.9a CONCENTRATION OF POLYPHENOLS AND THE RED COLOURED COMPOUND IN TERMS OF ABSORBANCY UNDER UV-VISIBLE RANGES AND THE CORRESPONDING COLOURIMETRIC VALUES OF POLYPHENOLS.

Stage1												
	Absorbancy under UV range (190-360) nm and concentration of Polyphenols											
	1		2		3		Mean		Absorbancy under visible range (360-480)nm			
Hrs	Absor-bance	Conc. mg/ml	Absor-bance	Conc. mg/ml	Absor-bance	Conc. mg/ml	Absor-bance	Conc mg/ml	1	2	3	Mean
12	0.562	0.674	0.554	0.775	0.889	0.824	0.668	0.757	0.293	0.417	0.222	0.311
18	0.758	0.461	0.638	0.898	0.934	0.898	0.777	1.419	0.322	0.480	0.272	0.311
24	1.127	2.436	0.739	1.058	0.953	1.12	0.940	1.538	0.327	0.485	0.464	0.425
30	1.145	1.636	0.565	0.787	0.806	0.640	0.839	1.021	0.485	0.553	0.388	0.475
36	0.654	1.538	0.529	0.726	0.606	0.627	0.629	0.963	0.487	0.572	0.312	0.457

Table 2.9 b CONCENTRATION OF POLYPHENOLS AND THE RED COLOURED COMPOUND IN TERMS OF ABSORBANCY UNDER UV-VISIBLE RANGES AND THE CORRESPONDING COLOURIMETRIC VALUES OF POLYPHENOLS.

Stage2												
	Absorbancy under UV range (190-360) nm and concentration of Polyphenols											
	1		2		3		Mean		Absorbancy under visible range (360-480)nm			
Hrs	Absor-bance	Conc. mg/ml	Absor-bance	Conc. mg/ml	Absor-bance	Conc. mg/ml	Absor-bance	Conc mg/ml	1	2	3	Mean
0	1.009	0.529	1.028	0.381	1.041	0.640	1.026	0.516	0.170	0.253	0.149	0.191
12	0.927	0.393	0.879	0.369	1.024	0.381	0.943	0.381	0.510	0.364	0.159	0.344
24	0.874	0.356	0.831	0.332	1.024	0.369	0.910	0.352	0.613	0.415	0.189	0.406
36	0.771	0.307	0.692	0.332	1.016	0.320	0.826	0.319	0.609	0.529	0.208	0.449
48	0.646	0.233	0.615	0.295	0.992	0.307	0.754	0.278	0.195	0.610	0.258	0.354

Table 2.10 CONCENTRATION OF POLYPHENOLS AND THE RED COLOURED COMPOUND IN TERMS OF ABSORBANCY UNDER UV-VISIBLE RANGES DURING THE SECOND STAGE OF STRIPPING

Hrs	Absorbancy under UV range (190-360) nm						Absorbancy under visible range (360-480)nm					
	1	2	3	4	5	Mean	1	2	3	4	5	Mean
0	0.933	1.163	1.009	1.028	1.041	1.035	0.286	0.226	0.170	0.253	0.149	0.216
12	0.808	1.121	0.927	0.879	1.024	0.952	0.744	0.258	0.510	0.364	0.159	0.407
24	0.799	1.083	0.875	0.831	1.024	0.922	0.967	0.348	0.613	0.415	0.189	0.506
36	0.460	1.802	0.771	0.692	1.016	0.804	1.264	0.385	0.609	0.529	0.208	0.599
48	0.014	1.051	0.646	0.615	0.992	0.661	0.540	0.388	0.195	0.610	0.258	0.398

Table 2.11 QUANTITATIVE VARIATIONS IN POLYPHENOLS RED COLOURED COMPOUND AND PH DURING FLOCULATION OF THE RET LIQUOR GENERATED DURING SECOND STAGE OF STRIPPING

Hour	Concentration of polyphenols		Absorbance UV 180-360		Absorbance Visible 360-480		PH Variations	
	S	C	S	C	S	C	S	C
0	0.1669	0.1669	0.084	0.084	0.372	0.372	7.940	7.940
3	0.1497	0.2188	0.000	0.0128	0.160	0.2978	7.41	7.47
6	0.0906	0.2628	0.000	0.0150	0.1344	0.3128	7.67	7.534
9	0.0710	0.3072	0.000	0.006	0.1208	0.2984	7.74	7.642
12	0.0785	0.312	0.000	0.0032	0.1222	0.2706	7.85	7.604
15	0.0563	0.3096	0.000	0.0016	0.1064	0.2764	7.94	7.49
18	0.0580	0.3346	0.000	0.0074	0.0634	0.2892	8.023	7.466
21	0.0451	0.2876	0.000	0.0004	0.0422	0.2114	7.876	7.498
24	-	-	-	-	0.0420	0.2132	7.834	7.494

'S' - Sample 'C' - Control

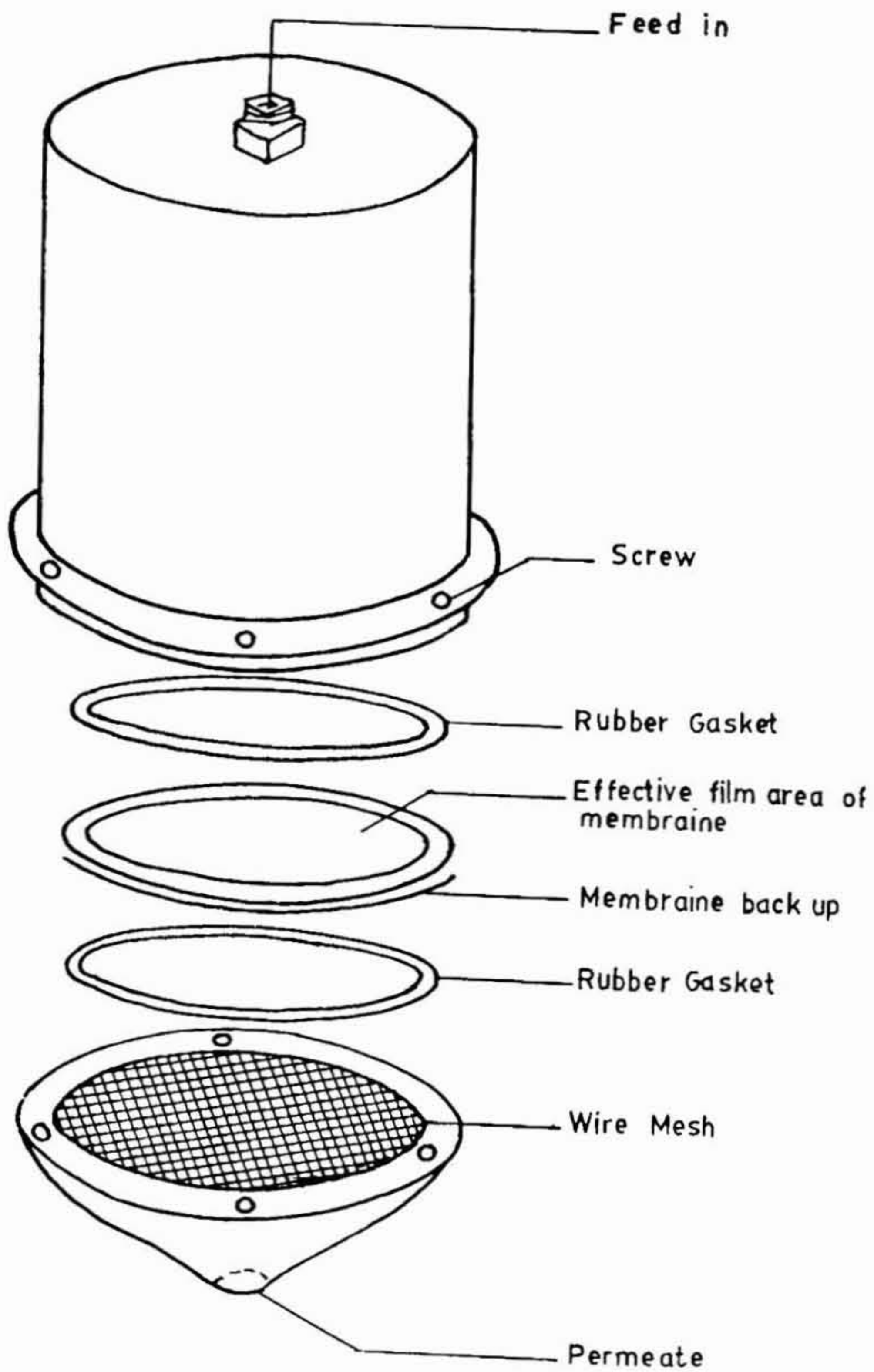


Fig. 2.1 Flat Membrane Holder

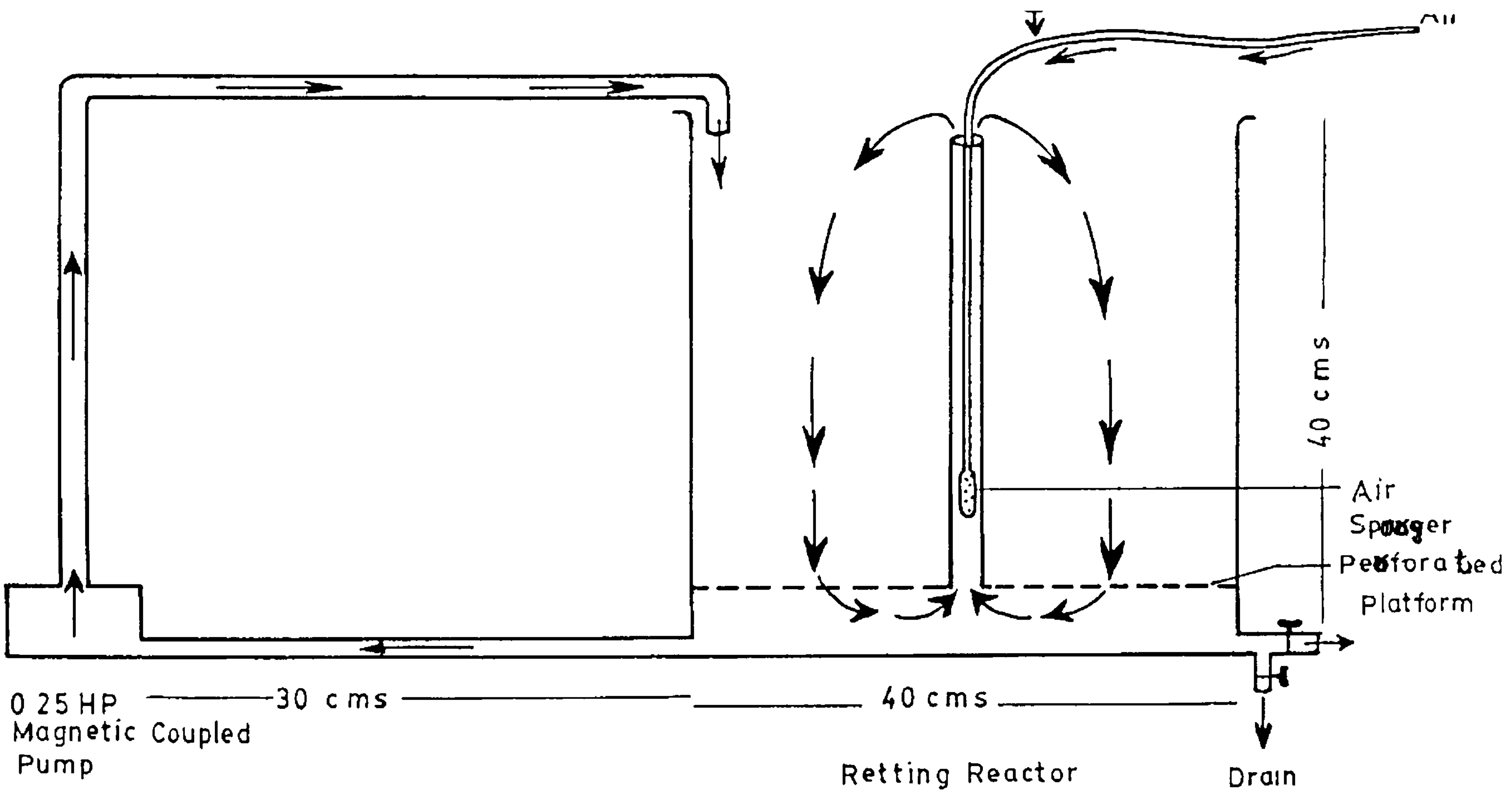


Fig. 2.2 Polyphenol stripping device

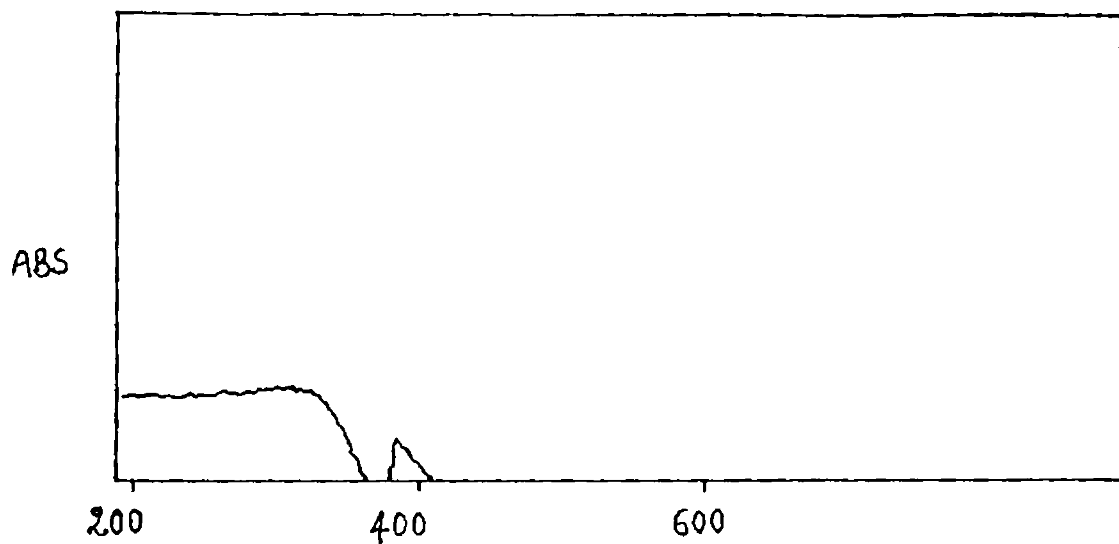


Fig. 2.3 Absorption peaks of polyphenols and the red coloured compound under UV - Visible ranges

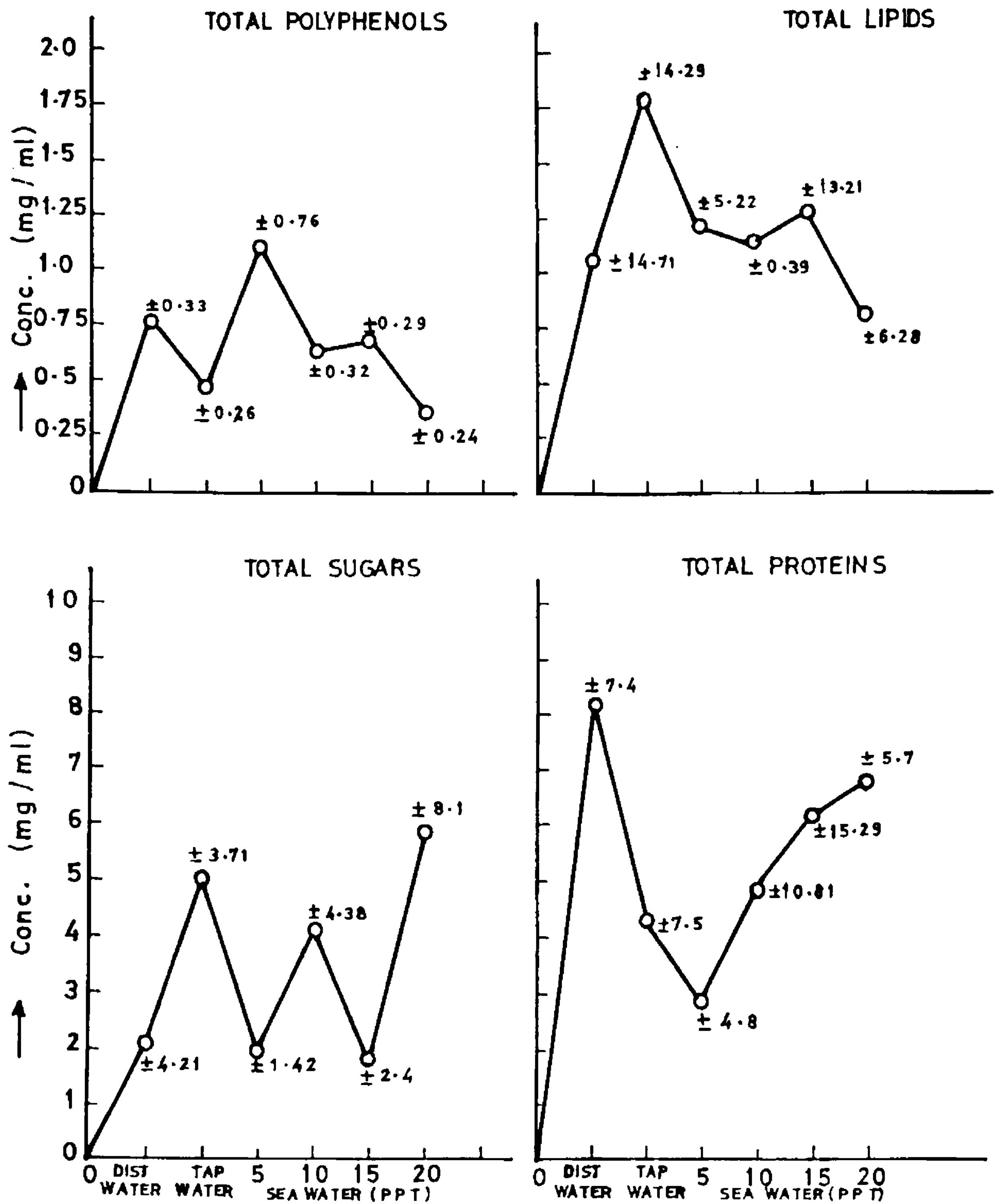


Fig. 2.4 Liberation of total polyphenols, lipids, sugars and proteins into distilled water, tap water and varying salinities.

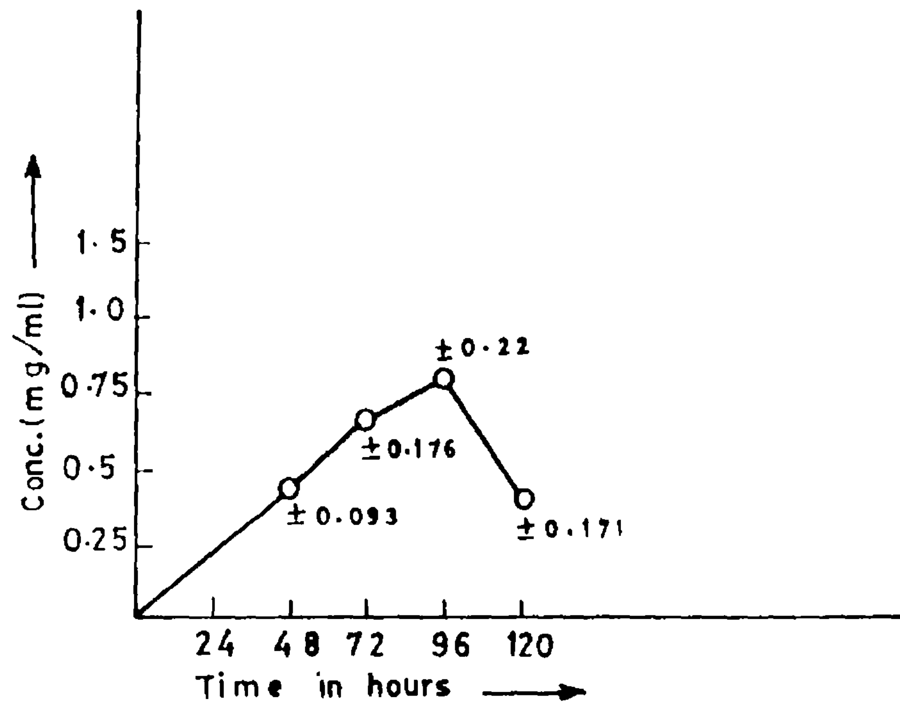


Fig. 2.5 Time required for maximum release of polyphenols at 5PPT sea water.

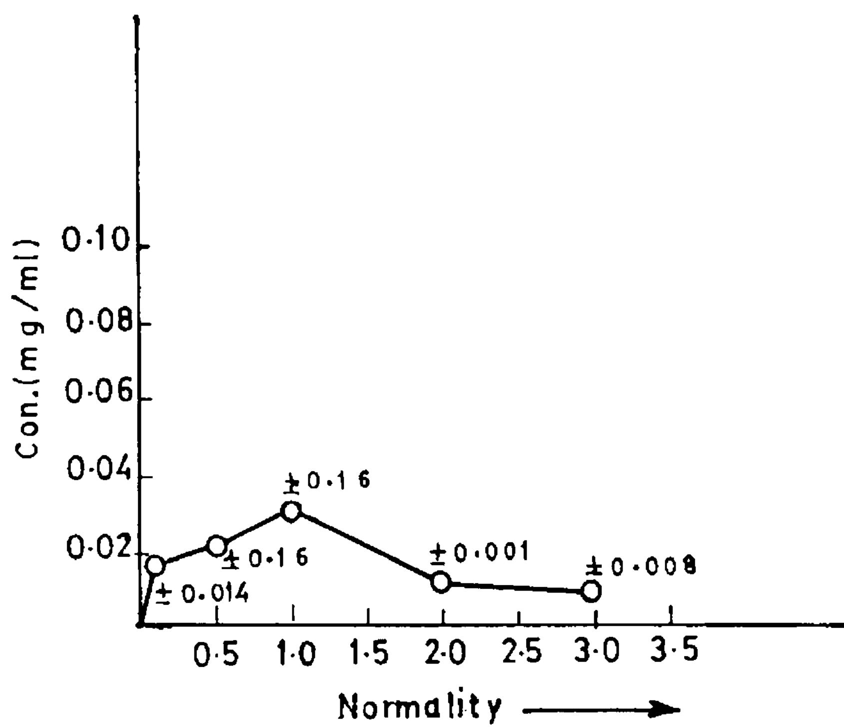


Fig. 2.6 Desorption of polyphenols into various normalities of NaOH

CHAPTER 3

CHARACTERISTICS OF POLYPHENOLS

Chapter 3

CHARACTERISTICS OF POLYPHENOLS

Inroduction

The concentrated ret liquor obtained through reverse osmosis has been found to be rich with polyphenolic compounds. Just like any other natural product, this is also prone to natural degradation which has to be quantified before begining with any sort of investigations either to characterize and identify the individual compounds or to find out their commercial utility. Shelf life of polyphenolics thus forms a crucial component in this study. In another way, shelf life of these natural compounds forms one of the characteristics to be studied.

Separation of polyphenolic compounds in ret liquor and their identification were programmed following the principles of chromatography. For all natural products, thin layer chromatography is an invaluable tool in unravelling the complex nature of the crude extract. The solvent combination used for thin layer chromatography according to Anitha, (1991) is ethyl acetate, benzene and acetic acid and it is stated that the Rf values depend upon the phenolic hydroxyl groups present in the molecule (Roberts, 1956). The chromatogram can be developed on silica coated plates and the spots developed using potassium ferricyanide ferric chloride agent.

Column chromatography is yet another effective technique in separating natural organic substances (Tang et. al, 1995) where a continuous elution with various organic solvents is employed, over a solid support of silica gel powder. Here the column chromatography is clubed to thin layer chromatography for further resolution and identification of the compound.

High performance liquid chrcomatography (HPLC) has been used for separating a wide range of phenolic compounds occuring in plant materials (Elisabeth et.al 1991) and the same has been used here also for the separation of polyphenolic compounds in the concentrated ret liquor. In this study the HPLC used was coupled with UV detector as reported previously by Sdanfelberger and Hosteltmann, (1987) using a reverse phase microbondapak C_{18} column. The polyphenolic compounds were identified by matching the retention time of the sample with those of the standards such as pyrogallol, catechol and tannic acid .

3.2. Materials and methods

3.2.1 Shelf life of concentrated polyphenols

For determining shelf life, polyphenols concentrated by reverse osmosis was filter sterlized initially by passing through a seitz filter under negative pressure. Sterility of the filterate was confirmed by inoculating an aliquot to nutrient broth of the following composition.

Peptone	:	1g
Beef extract		0.5g
NaCl	:	1g
Distilled water		100ml
pH		7.5 ± 0.2

The tubes of 5 ml nutrient broth which received inoculation of 0.5ml were incubated at 28 ± 0.5°C for 7 days. Lack of turbidity confirmed sterility. During this period of incubation, the ret liquor was stored at -20°C.

Shelf life was determined at the temperatures (°C) 0, 4 ± 0.5, 24.5± 0.5, 28 ± 0.5, 37.5 ± 0.5 for 180 days. Aliquots of 5 ml sterile ret liquor were aseptically sealed in sterile vials of 10 each and kept at the above temperatures aseptically, starting from the day of stocking for a period of 180 days one vial each from each set was used for determining the total polyphenol content following APHA, (1985) described earlier. Along with this, sterility of the contents in each vial was ascertained by inoculating 0.5ml in nutrient broth and inculating as mentioned above for 7 days.

3.2.2 Thin layer chromatographic separation of polyphenols and identification.

A slurry of 50% silica gel G meant for TLC (Sisco Research Laboratory, Product No. 194017) was prepared in distilled water and 0.5mm thick coats were prepared on a series of plates of 21cm^2. Then, after drying in oven for two hours at 80°C plates were used for spotting the sample. A spot of 0.05ml concentrated ret liquor was made on the dried silica gel plates along with that of 1% solution of standard reference compounds such as tannic acid, pyrogallol and pyrocatechol side by side maintaining equal distances on a straight line. Care was taken to maintain the spots at a height of 1cm from the base so that they did not get immersed in the solvent system. After allowing the plates for drying for two minutes the plates were kept on a developing chamber which contained a solvent system consisting of ethyl acetate, benzene and acetic acid in the ratio of 50:10:40. This ratio was the one evolved out of a series of experiments with the sample by trial and error method. The solvent was allowed to run over the plate to 17cms and after which the plates were taken out of the developing chamber and air dried. After drying for two hours, spots developed

by spraying with 1% potassium ferricyanide - ferric chloride reagent in water and Rf values were determined for the sample and controls. The experiment was repeated several times for confirmation.

3.2.3 Column chromatographic separation of the polyphenols and identification of the compound.

Column chromatographic separation was performed using micro crystalline silica gel powder in a chromato- graphic glass column of 2.5 cms diameter having a length of 45 cm with a sintered support at the bottom for holding the column of silica gel. The column was packed tightly with silica gel of 100- 200 mesh size (Sisco Research Laboratory, Product No. 194014) and was saturated with methanol with care to have the column completely immersed in methanol. An aliquot of 5 ml concentrated ret liquor which was to be fractionated was applied on top of the column with the help of 5 ml pipette and was eluted with 50ml methanol as solvent A and the fraction was collected. Subsequently, solvents such as acetic acid (solvent B) and water (solvent C) were used in the same quantity for elution and fractionation. These three solvents were the once sorted out by a series of experiments carried out with the sample and thus no other solvents were found to be suitable for elution. All the three fractions collected were concentrated by drying in desicator at room temperature 28±1°C. Each fraction thus recovered was dissolved in distilled water and subjected to thin layer chromatographic analysis along tannic acid, pyrogallol and pyrocatechol as described above.

3.2.4 HPLC analysis of the concentrated polyphenols.

The analysis of concentrated polyphenols was made with reverse phase HPLC system consisting of a HPLC gradient controller, solvent conditioner, and a

dual pump coupled with UV detector. The analytical separation was performed at room temperature on a reverse phase bondapak C_{18} column (Waters) compatible with the system. The separation was achieved using a solvent system of methanol (SRL, HPLC grade) and water (Milli- Q) in the ratio of 4:1.

Aliquot of 0.1 µl concentated ret liquor was injected into the column using a syringe and the solvent was allowed to flow into the column with the help of the pump. The elution got completed after 2 hours and peak areas corresponding to the fractions eluted were detected and recorded using the UV detector at 280 nm. After the elution of the sample, the column was washed with the solvent and the standards such as tannic acid, pyrogallol and catechol prepared as 0.1% aqueous preparations were subjected to HPLC analysis one after the other and the retention time were determined.

Major fraction of the polyphenol in the ret liquor was confirmed by comparing the retention time obtained for the sample and those of the standards. To reconfirm the finding, 0.1% aqueous preparation of the standard tannic acid which was closer to the specific fraction was spiked with the sample and were subjected for the analysis and the retention time was compared with that of the sample.

3.3 Results and Discussion

3.3.1 Shelf life of concentrated polyphenols.

As summerized in Table 3.1, the experiment carried out on the extent of shelf life at various temperatures revealed that at temperatures higher than 4 ± 0.5°C there was a steady decline in the concentration of polyphenols over a period of 180 days resulting in a loss of >90%. On the other hand at 0 and 4 ± 0.5°C,

eventhough there was 10 to 20% decline in the content of the polyphenols during the initial phase of storage, the concentration was found to be stable during the subsequent period. Therefore ret liquor can be stored at 4°C for a period of not less than 180 days and this can be the starting material for research leading to characterization and application of the natural polyphenols.

Polyphenols being secondary metabolites (Haslam, 1986) possess several distinctive molecular characteristics which can be studied only after assessing the degradative nature. The existence of polyphenols in free state invariably depends upon the amount of protein, polysacharides etc in the extract as there can be interaction between polyphenols and proteins, (Bate-Smith, 1981) polyphenols and polysacharides (Martin, et.al, 1986) at temperatures higher than 4°C. The relative abundance of plant polyphenols in nature warrants the study of shelf life to 180 days when compared to synthetic organic substances where shelf lifes are studied for periods greater than 365 days.

3.3.2 Thin layer chromatographic separation of polyphenols and identification.

Results (Rf values) obtained at each trial made to separate the polyphenols of the concentrated ret liquor on silica gel plates along with the standards such as tannic acid, pyrogallol and catechol are summerized in Table 3.2. The Rf values obtained with the concentrated ret liquor closely resembled to the one obtained for tannic acid. Meanwhile the Rf values obtained for the other standards such as pyrogallol and catechol stood very much away from the one obtained for the concentrated ret liquor and tannic acid as well. Interestingly, no other clear spots (separation) could be seen emerging from the concentrated ret liquor on

developing them after resolution with the solvent system. This indicated that the polyphenol in the ret liquor resembled very much to tannic acid in molecular weight and mobility along with a defined solvent system. This compound which can be called as 'Tannin' is the major polyphenol in the ret liquor.

Application of thin layer Chromatography techniques is extremely efficient in the separation of polyphenolic compounds. (Egger, 1969). It can be widely used not only for monitoring the isolation processes but also for preparative purposes invariably aiding the separation of a complex mixture of closely related polyphenols in a simple manner. The Rf values detected in thin layer chromatography depends on the number of hydroxyl groups in the molecule, nature of solvent systems, and the effect of glycosidation. The similarity of Rf values of tannic acid standard and the ret liquor extract is due to the presence of equal number of hydroxyl groups and may be due to the type of glycosidic linkage in the standard and the concentrated ret liquor extract. Since the crude ret liquor extract from coconut husk is not subjected to any heating procedures, which hydrolyse the polyphenols, subjecting this to thin layer chromatography gives more or less a clear cut idea of the major components. The combining ratio of the solvent systems used was carefully selected considering the polar nature of polyphenols. The solvent system contained polar compounds in greater amount when compared to non-polar compounds which greately helps in the separation of polyphenols in the thin layers chromatography giving distinct chromatogram without tailing.

3.3.3 Column chromatographic separation of polyphenols.

The crude polyphenol extract in the concentrated ret liquor was fractionated using three solvents by column chromatography and the fractions were subjected to thin layer chromatographic analysis along with three standards and the results are summarised in Table 3.3. The methanol fractin on TLC plates showed closer Rf values to that of tannic acid, showing complete recovery of tannic acid but differed much from that of pyrogallol and pyrocatechol. However, the fractions in acetic acid and water differed much from the standards as the Rf values were much far apart. The data suggests that as revealed by the thin layer chromatography of the crude polyphenol extract in ret liquor the major fraction is a compound very much closer to tannic acid. Meanwhile, the column chromatographic analysis could reveal the presence of two more fractions which could not be identified as they differed much from the standards used here for comparison.

As demonstrated by Bate-Smith, (1981) the plant polyphenols are associated with proteins and polysacharides the two fractions obtained in acetic acid and water which did not resemble the used standards in Rf values suggest that they are non polyphenolic compounds. This assumption is based on the fact that the solvents used for extraction varied with polarity and solvation. Thus the methanol fraction could solvate polyphenols and acetic acid and water could solvate proteins and sugars. As suggested by Craig et. al, (1951) the column chromatography is a better technique in isolating polar compounds found in plants. The fractions obtained after column chromatography can be further confirmed by HPLC, thus improvising the technique as a feasible method for structural elucidation in combination with spectroscopy

3.3.4 HPLC analysis of the concentrated polyphenols

The chromatograms generated out of the HPLC analysis of the concentrated ret liquor , tannic acid as standard, and a mixture of ret liquor and 0.1% aqueous tannic acid in 1:1 ratio is presented as Fig. 3.1. From the concentrated ret liquor 7 peaks could be generated with only one major peak which corresponded to a fraction having retention time of 5.05. This is very much comparable with the peak obtained with the standard tannic acid retention time of 5.11. This suggest that the major fraction of polyphenol in the ret liquor is a compound very much similar to tannic acid. This inference was confirmed when the concentrated ret liquor was spiked with tannic acid (aqueous) and subjected to HPLC analysis. The major peak obtained with retention time of 5.09 resembled the ones described earlier. Meanwhile, the retention time of the peaks corresponding to catechol and pyrogallol did not show much similarity to concentrated ret liquor (Fig. 3.2). As revealed in the chromatogram, the concentrated ret liquor contained several other fractions which are minor and as such not investigated.

High performance liquid chromatography is a versatile and potent technique in the separation of non volatile and heat sensitive polyphenols (Lyne et. al, 1976.) Though, there was only a single major peak in the chromatogram of the ret liquor extractive, the smaller peaks speak of some more associated compounds in the extract. This fact is observed in all the chromatographic techniques and it is already proven experimentally by spectrophotometric methods. The use of reverse phase HPLC gave complete resolution of the extract as compared with the normal phase HPLC. The HPLC of other standards like catechol, pyrogallol that were used for TLC was also performed in addition to tannic acid standard

and the retention time clearly indicated the presence of tannic acid as the major component in the concentrated liquor in comparison to pyrogallol and catechol. In all three cases, the elution was in the order of decreasing polarity. The separation identification and quantification of plant phenolics always present challenges. However, it is suggested that some improvements are to be made, particularly for extraction by use of different chromatographic techniques such as with sephadex LH-20, Phenyl-Sepharose and C_{18} (Sep-pak).

Conclusion

The ret liquor concentrated in a reverse osmosis module has a shelf life of 180 days at 4±0.5°C during which the polyphenol content remained almost steady. The thin layer chromatographic analysis of the concentrated ret liquor suggested tannic acid as the major polyphenol. This fact was further confirmed by fractionating it in a chromatographic column. Meanwhile, the retention time (RT) of the peaks obtained in the chromatogram by subjecting the concentrated ret liquor to high performance liquid chromatography analysis confirms Tannic Acid (Tannin) as the major polyphenol in ret liquor.

Table 3.1 SHELF LIFE OF CONCENTRATED POLYPHENOLS

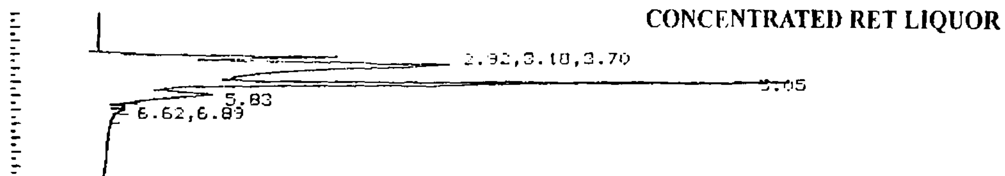
Time period	TEMPERATURES														
	0°C			4°C ± 0.5			24.4°C ± 0.5			Room Temperature			35 - 37°C		
	Abs.	Conc. Mg/ml	Sterility	Abs.	Conc. Mg/ml	Sterility	Abs.	Conc. Mg/ml	Sterility	Abs.	Conc. Mg/ml	Sterility	Abs.	Conc. Mg/ml	Sterility
0 Hour	0.072	0.886	Sterile	0.072	0.886	Sterile	0.072	0.886	Sterile	0.072	0.886	Sterile	0.072	0.886	Sterile
After 7 days	0.063	0.775	Sterile	0.042	0.516	Sterile	0.072	0.886	Sterile	0.02	0.246	Sterile	0.054	0.664	Sterile
After 14 days	0.052	0.64	Sterile	0.042	0.516	Sterile	0.031	0.381	Sterile	0.021	0.258	Sterile	0.018	0.221	Sterile
After 30 days	0.047	0.578	Sterile	0.041	0.504	Sterile	0.014	0.172	Sterile	0.01	0.123	Sterile	0.01	0.123	Sterile
After 60 days	0.051	0.603	Sterile	0.047	0.578	Sterile	0.014	0.172	Sterile	0.016	0.196	Sterile	0.01	0.123	Sterile
After 90 days	0.047	0.578	Sterile	0.048	0.59	Sterile	0.013	0.283	Sterile	0.014	0.172	Sterile	0.01	0.123	Sterile
After 120 days	0.048	0.59	Sterile	0.047	0.578	Sterile	0.02	0.27	Sterile	0.014	0.172	Sterile	0.01	0.123	Sterile
After 180 days	0.046	0.566	Sterile	0.046	0.566	Sterile	0.014	0.172	Sterile	0.01	0.123	Sterile	0.01	0.123	Sterile

Table 3.2 THIN LAYER CHROMATOGRAPHIC ANALYSIS OF CONCENTRATED RET LIQUOR

Rf values on using the solvent system (Ethyl acetate, acetic acid and benzene)				
Sl.No.	**Concentrated Sample**	**Tannic Acid standard**	**Pyrogallol standard**	**Catechol standard**
1.	0.860	0.853	0.924	0.934
2.	0.906	0.915	0.875	0.896
3.	0.885	0.891	0.908	0.921
4.	0.870	0.883	0.903	0.935
5.	0.914	0.921	0.853	0.843

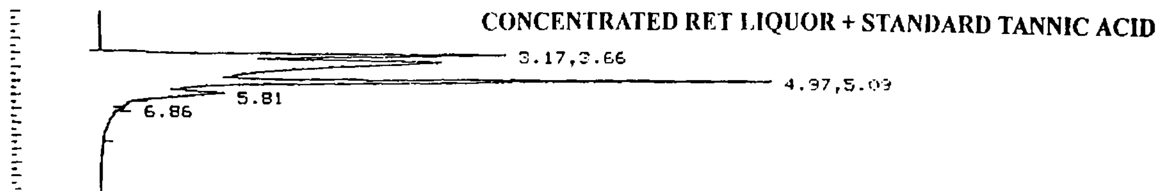
Table 3.3 THIN LAYER CHROMOGRAPHY OF MELTHANOL, ACETIC ACID & WATERFRACTION AFTER COLUMN CHROMATOGRAPHY.

Sl. No.	fraction	Rf values of fractions	Rf values of standards		
			Tannic Acid	**Pyrogallol**	**Catechol**
1.	Methanol	0.872	0.863	0.928	0.943
2.	Acetic Acid	0.793	0.858	0.908	0.934
3.	Water	0.904	0.842	0.954	0.968



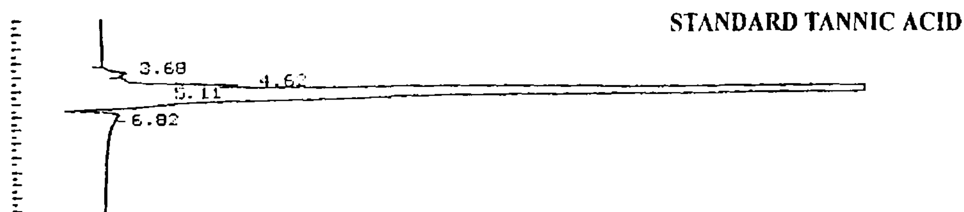
Date Nov 07 1997 Data file : sat01_06.dat id :
Time 14:38:46 Method file : capsacin.mtd % on Area

SRN	R.TIME	HEIGHT	AREA	HT%	AREA%	TYPE	AR/HT
1	2.92	1135	213099	2.5184	0.7925	BV	0.128
2	3.18	7353	2727853	16.3153	10.1444	VV	0.253
3	3.70	10762	12737345	23.8795	47.3679	VV	0.808
4	5.05	23407	9852272	51.9371	36.6388	VB	0.287
5	5.83	1999	1238892	4.4355	4.6072	TTT	0.423
6	6.62	172	44601	0.3816	0.1659	TTV	0.177
7	6.89	240	76180	0.5325	0.2833	TVT	0.217



e Nov 07 1997 Data file : sat01_07.dat id :
e 14:53:46 Method file : capsacin.mtd % on Area

R.TIME	HEIGHT	AREA	HT%	AREA%	TYPE	AR/HT
3.17	12484	5109221	24.2445	16.6062	BV	0.279
3.66	10445	12334057	20.2847	40.0888	VV	0.806
4.98	23163	11981004	44.9837	38.9413	VB	0.353
5.09	3377	300084	6.5583	0.9754	TTT	0.061
5.81	1885	1007355	3.6608	3.2742	TTT	0.365
6.86	138	35147	0.2680	0.1142	TTT	0.174

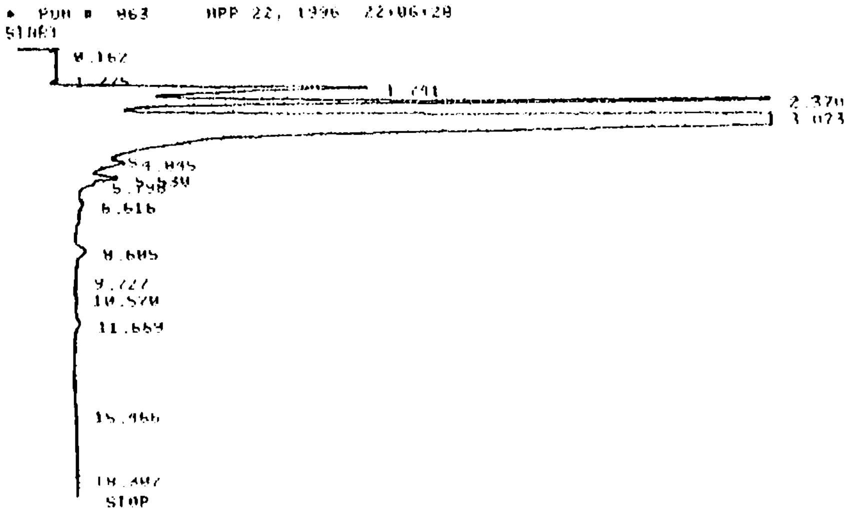


Date Nov 07 1997 Data file : sat01_02.dat id :
Time 13:35:38 Method file : capsacin.mtd % on Area

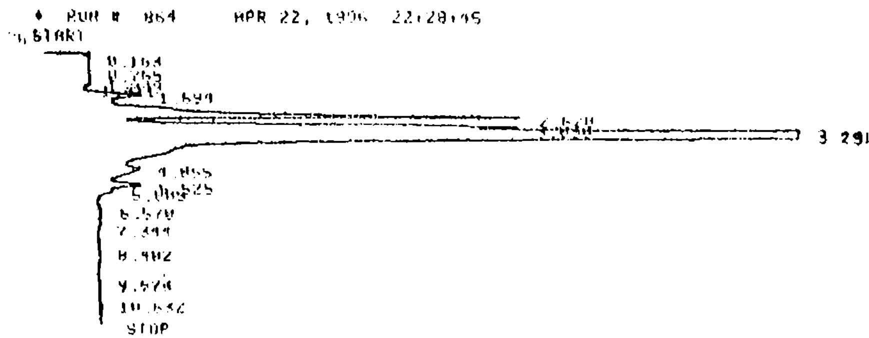
SRN	R.TIME	HEIGHT	AREA	HT%	AREA%	TYPE	AR/HT
1	3.68	822	435047	1.3116	1.1635	BV	0.411
2	4.62	4552	2222736	7.2634	5.2241	VV	0.333
3	5.11	56639	39218072	90.3766	92.1750	VP	0.473
4	6.82	657	611583	1.0483	1.4374	PB	0.635

Fig. 3.1 Chromatograms of concentrated ret liquor, standard tannic acid and concentrated ret liquor spiked with tannic acid as given by HPLC.

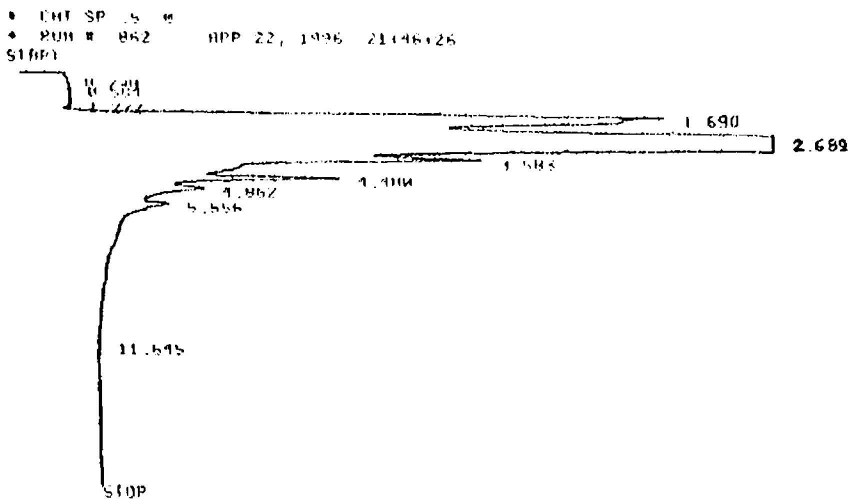
STANDARD PYROGALLOL



STANDARD CATECHOL



STANDARD TANNIC ACID



CONCENTRATED RET LIQUOR

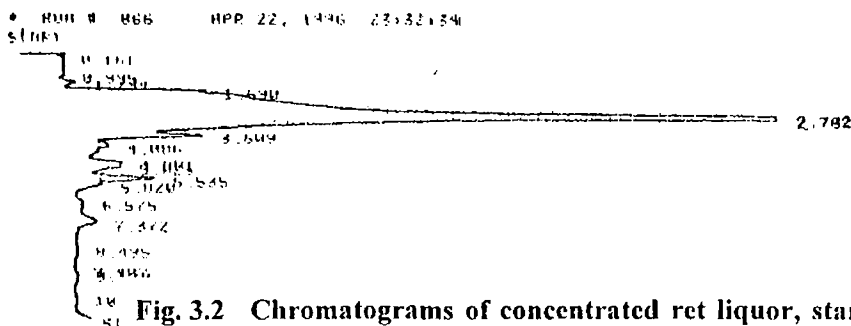


Fig. 3.2 Chromatograms of concentrated ret liquor, standard pyrogallol, standard catechol and standard tannic acid as given by HPLC.

CHAPTER 4

POSSIBLE APPLICATIONS

Chapter 4

POSSIBLE APPLICATIONS

4.1 Introduction

Tannin obtained from plants can find applications as natural mordants for dyeing fabrics/fibres such as cotton, wool, silk, jute, hemp etc especially using natural dyes, which is due to their affinity to bind to natural polymers, (Haslam, 1996). The function of the mordant is to fix the dye on to the material or fibre, so that the rate of exhaustion of the dye is greater. There are may natural mordants that are used in combination either with synthetic dyes or natural dyes. The dyeing industry these days pose severe environmental problems due to accumulation of the synthetic dyes and mordants. Majority of these synthetic dyes and mordants are not bio-degradable and consequently most of them are being banned. At this juncture, the use of natural colouring material as mordants and dyes play a very important role and now-a-days the industry is trying out on natural forms to avoid environmental hazards. Hence the importance of testing the ret liquor containing tannins as the major polyphenol for its mordanting property. The mordantingproperty depends upon temperature, and, a certain moderate temperature is needed for each fibre to absorb the organics. In the present study the possibility of using tannnin from concentrated ret liquor in mordanting cotton fabric with dyes such as 135 H, Eco-rust blue and Eco-rust red has been evaluated.

The astringent organic compounds obtained from plant kingdom capable to convert raw hides and skins into leather are called Tannins. Tannins of different plants are different in composition, structural properties and the leather obtained from them

also varies in their properties. Tannins obtained from different parts of a plant are always associated with plant pigments, acids salts, carbohydrates and many other known and unknown compounds called non-tannins. Though these non-tannins have no tanning power, their presence in the tan liquor is important and essential to control the rate and to impart many qualities to the finished leathers. The distribution of tannin throughout the thickness of the leather and also the rate of tanning are largely controlled by the non tannin content of the tan liquor (Edwards, 1949). If the non-tannin content of a tanning material is more than its tannin content, the tanstuff is considered unsuitable for tanning (Shuttleworth, 1941). The tan/non-tan ratio of a tanstuff for satisfactory tannage should therefore be always greater than one. The tan/non-tan ratio varies from one sample to another, mainly based on the age of the tree from which it is obtained, season of the year in which it is collected, the locality or region where the tree grew, the girth of the stem or branch from which the bark is obtained and many such other factors, while tanning is also controlled by an optimum pH (5.5 to 6.5) and temperature. (Edwards, 1949).

The active substance responsible for tannage are phenolic substances (Theodore White, 1956) having molecular weight greater than 500. The major polyphenolic substance in the concentrated ret liquor was found to be tannic acid (tannin) which was tested for its tanning property.

Natural products from plants have provided immense application in biological and pharmacological activity. Over the past two decades researchers have turned into many of the traditional folk medicines to uncover the scientific basis of their medicinal effects. Natural polyphenols from plant are found to have such biological activities. (Haslam and Cai, 1994). The concentrated ret liquor obtained from coconut

husk is rich in polyphenols with tannic acid (tannin) as the major polyphenol. It is also found that the tannin containing extracts of plants inhibit the growth of pathogenic bacteria. (Sotoby *et al* 1995). Tannins in general are a new family of bioactive natural organic compound (Okuda, 1995) which form a substitute for the artificially synthesised bio-active organic compounds, and are more environment friendly.In vitro testing of natural polyphenols has revealed their potentiality over several biological and pharmacological activities (Haslam, 1996). The physical and chemical properties of polyphenols associated with the possession of a concatenation of phenolic nuclei within the molecule are taken into account in testing the activity of polyphenols on fungal cultures like *Myrothecium roridum*, *Cylindrocladium quinqueseptatum*, *Corticium salmonicolor*, *Alternaria alternata*, *Rhizoctania solani* and *Cytospora sp* supplied by Kerala Forest research institute, Peechi , Trichur, Kerala and also on fungal cultures like *Colletrichumm falcatum*, *Pythium*, *Phytopthora*, *Rhizoctonia solani* and *Pestalotia* supplied by Dr. M.C. Nair, Kerala Agricultural University. The activity of the concentrated polyphenols were also tested on pathogenic bacteria like *Staphylococcus aureus*, *Photobacterium*, *Vibrio Sp.* (ANM 607), *Aeromonas* (680 a), *Vibro Cholerae*, *Non-Cogaulase Staphylococcus sp.*, *Aeromonas* (AAC 1102 a), *Enterobacter aglomerans*, *Salmonella typhi*, *E. coli*, *Shigella dysentereae*, *Pseudomonas aerogenosa*, *Klebsiella pneumoniae and Citrobacter frueindi* and on 47 bacterial cultures from prawn hatchery system.

Bacterial biofilms are part of aquatic environment and account for much of the biomass and microbial activity in the system (Liu et.al, 1994), and they attract the macrofoulants to settle on substrata. Over the years much efforts have been put in to develop antifouling compounds which would either prevent the development of biofilms or/and

the settlement of macrofoulers. For experimenting with a compound it has always been much easier to deal with macrofoulers than with biofilms. Amongst macrofouling organisms, barnacles are the major target organism for developing antifouling technology. While many barnacles such as *Balanus balamoides* reproduce only once per year (Crisp and Spencer, 1958), other barnacles, such as *Balanus amphitrite*, reproduce frequently, once per week (Holm, 1990) and are therefore used in the larval swimming behaviour in toxicity assays. This study therefore inventigates the possibility of using natural polyphenols from coconut husk for the development of non-toxic, non polluting, natural anti-fouling additive that can be incorporated in practical coatings. Nauplii of the barnacle *(Balanus amphitrite)* are used for such studies as the larvae swim continuously till they perish making the observations easier.

In the present study mordanting, tanning, antifungal, anti bacterial and antifouling properties of polyphenols in ret liquor have been investigated.

4.2 Materials and Methods

4.2.1 Mordanting Property

The method known as 'Test Tube' mordanting was adopted for evaluating the mordanting property of the polyphenols in ret liquor. Cotton fabric uniformly weighting 0.25 gm each were mordanted with ret liquor containing 4,2,1,0.5 and 0.25 mg/ml polyphenol in a boiling water bath for 20 minutes. For dyeing the fabric three types of natural dyes such as 135 H (SITRA, Coimbatore) Eco-Rust Red and Eco-Indo blue. (National Hand Loom Corporation, Lucknow) prepared by dissolving 0.1 gm in 100 ml distilled water were used. The cotton fabric samples squeezed of the mordant were impregnated with the dye preparation in a

test tube and heated in a water bath at 60°Cfor 10-20 minutes, and subsequently they were boiled over a bunsen flame for 5 minutes for complete adhesion of the dye on to the fabric. The fabric samples were washed with distilled water till no more dye came off and subsequently dried in an oven at 60°C for 30 minutes and observed for the colour that was imparted on to the cotton fabric. Beside negative controls a positive control with $FeSO_4$ as the mordant was also run.

4.2.2 Tanning property

Since the tanning property of a plant extract is judged based on the tan/non-tan ratio of the tan stuff the ret liquor which supposed to contain the tanning property was subjected for the analysis of total solids, total solubles, total insolubles, and when the non-tannin content of a tanning material becomes more than its tannin content the tan stuff is considered unsuitable for tanning and as a rule tan/non tan ratio of the transtuff should be around 1.

Total Solids

25 ml concentrated ret liquor was pipetted out into a tared basin weighed and evaporated on a water bath and dried in an oven at 102 ± 2°C for 3 hours, cooled in desciator weighed and the percentage of the dry matter determined and expressed as total solids in the ret liquor.

Total Solubles

For the determinatin of total solubles the following procedure was adopted. In a beaker, 100ml ret liquor was mixed with 1 gm Kaolin and filtered through whatman filter paper No. 2. The filtrate collected is returned to the filter paper along with the left over kaolin repeatedly in such a way that the entire

kaolin was returned to the filter paper. The filter paper was again filled with the same ret liquor and the process was repeated till it became optically clear. From the filtrate 25 ml clear fluid was pipetted out in a porcelain basin, weighed and dried at 102 ± 2°C for about 3 hours cooled in a desicator and weighed again. The process of drying and weighing was repeated until constant weight was attained and the total solubles calculated.

Non-tannins

For the determinatin of non-tannins the following procedure was followed. Hide powder (CLRI, Madras) was weighed out (6.2 g) and soaked over night in 62.5 ml water containing 6.2 ml of 3% chrome alum. Weight of the hide powder was brought down to 26.2g by squeezing it and expelling the water (weight was further adjusted by the addition of water). To the hide powder 100ml ret liquor was added and stirred for 10 minutes. Subsequently it was filtered through Whatman no. 2 filter paper and to the filtrate 2gm Kaolin was added stirred and filtered through the same filter paper. Filteration of the same fluid through the same filter paper was continued several times (4 to 5 times) until the fluid was clear. From the clear filtrate 25 ml of clear non-tan solution was pipetted out in a tared basin, evaporated, dried and weighed and the weight represented the weight of non-tannins as the tannin components had already been absored to the hide powder.

Tannins

The difference between the weight of solubles and the non-tannin component was taken as the tannin component.

4.2.3. Antifungal Property

The concentrated ret liquor was filter sterlized by passing through seitz filter under negative pressure. Sterility of the fluid was tested by inoculating 1ml aliquot of the ret liquor into 10ml nutrient broth and incubating at 28 ± 0.4°C for 7 days. Lack of turbidity in the tubes confirmed sterility of the ret liquor. The sterile ret liquor was preserved aseptically in screw caped bottles at 4°C till used. At the time of use the ret liquor was diluted with a diluent having the following composition. Composition of the diluent was arrived at from the analysis made with the concentrated ret liquor for Na, K, Ca using flame photo meter, for SO_4, PO_4 and Fe colourimetrically and for Mg titrimetrically. The quantity of salts required for supplying the above elements and radicals to match with the ret liquor was estimated and reconstituted appropriately.

Composition of the diluent.

Calcium carbonate		4.8 mg
Magnesium carbonate		0.5 mg
Sodium chloride		147.4 mg
Potassium chloride	:	1148.01 mg
Ferrous ammonium sulphate		0.052 mg.
Potassium phosphate		3900 mg
Sodium sulphate	:	1158 mg
Distilled water		1000 ml.

For testing the fungicidal or fungistatic property the ret liquor was diluted to get a final concentration of 1000ppm polyphenol.

The fungal cultures used were the following.

Myrothecium roridum

Cylindrocladium quinquiseptatum.

Cortcium salmonicolor

Alternaria alternata

Rhizoctonia solani

These organisms were grown in saboraud dextrose agar slants for 10 days so that sporulation took place. The spores thus produced were harvested in 0.5% sterile saline. Standard well diffusion method was followed to test the antifungal property of the polyphenolics. For this 5 mm diameter wells were cut and the bottom sealed with sterile 2% molten agar aseptically. Surface of the plate was seeded with the fungal spores by swabing from the spore suspension prepared in saline. Subsequently the wells were filled with ret liquor and plates incubated straight at 28 ± 0.4°C for 4 to 5 days. Daily observations were made for the growth of fungi on plates and for the inhibition zone around the wells.

Alternatively, a series of dilutions of polyphenols were made with the diluent mentioned above ranging from 100 to 1000 ppm, (as 100, 250, 500, 750 and 1000 ppm.) and tested the sensitivity of the fungal spores to the compound. For this the fungal species such as *Colletrichium, Pythium, Rhizoctonia solani, Phytophthora* and *Pestalotia* were grown in sabourad dextore agar slants till they

produced sufficient quantity of spores. The spores were harvested in 5ml aliquots diluent and observed microscopically to ascertain their presence. From each such preparation one loopful each of spores were introduced into 1ml aliquot of the polyphenol preparation as mentioned above in such a way that for every fungal species uniformity in inoculum size between different concentration of polyphenols was maintained. The preparation was maintained at 28 ± 4°C for 24 hours and at the end of which a loopful of the preparation was transfered to 10ml saboraud dextrose broth and incubated at 28 ± 4°C for 7 day and observed daily for germination of spores, development of hyphae and mycelium.

4.2.4 Antibacterial Property.

Antibacterial property was tested against *Staphylococcus aureus, Photobacterium, Vibrio Sp.*(ANM 607), *Aeromonas* (680a), *Vibrio Cholerae, Non-coagulase Staphylococcus sp, Aeromonas* (AAC 1102a), *Enterobacter aglomerans, Salmonella typhi, E.coli, Shigella dysenterae, Pseudomonas aerogenosa, Klebsiella pneumoniae,* and *Citrobacter fruendi.* Along with them 47 bacterial isolates representing the family Vibrionacecae associated with the larvae of *Macrobrachium rosenbergi* (Bhat and Singh, 1998) were also used.

The above cultures were grown in Nutrient agar slants of the following composition:

Peptone : 0.5 g

Beef extract : 0.1 g

NaCl : 1 g

Agar : 2 g

pH : 7.5 ± 0.2

Distilled water 100 ml

The cultures after 24 hour growth were harvested in the diluent mentioned above (section 4.2.3) and O.D adjusted to 0.5 absorbancy at 600 nm. The ret liquor was filter sterilized as described under section 3 and the sterility tested. It was then diluted using the diluent described above to a final concentration of 100, 250, 500, 750, and 1000 ppm aseptically and transferred as one ml aliquots into sterile test tubes. For testing the anti bacterial property of polyphenols a loopful of the bacterial suspension prepared was inoculated to the ret liquor preparations and incubated for 24 hr. Starting from 0 hr to 24 hr at 12 hourly intervals a loopful of broth from each of the preparations was transferred to 10ml nutrient broth having the composition mentioned above. This was incubated at 28 ± 0.4°C for 7 days and growth manifested as turbidity was examined.

4.2.5. Antifouling property

Swimming behaviour of nauplii of barnacles is considered for toxicity assays of polyphenols as antifouling agents. Precisely the inability of the larvae to stay in the water column in the presence of polyphenols is scored as the toxic response. Barnacles were bred in laboratory and the nauplii were collected from the culture system. The experimental set up consisted of 100ml seawater in 250ml beakers with added ret liquor to obtain the final concentration of 75, 100, 250, 500, 750 and 1000 ppm polyphenol. The larvae were attracted by light to a point of light source and collected in minimum volume of seawater. For the assay 60 to 80

nauplii were added to each beaker, and incubated for 1 hour at 28°C. The beakers were held up to light and were counted for the larvae which were attracted towards light and the percentage of larvae indifferent to light was expressed as the measure of toxicity of polyphenols as the macrofouling agents.

4.3 Results and Discussions

4.3.1. Mordanting property

The mordanting property of polyphenols in ret liquor in terms of the exhaustion of natural dyes into cotton fabric in the presence of varying concentration of polyphenols is summarised in Table 4.1. For all the concentration of polyphenols ranging from 0.25 to 4 mg/ml the percentage exhaustion of the dyes 135 H and Eco-Rust Red was 20 and for Eco-Indo blue it was only 10. This indicated that an increase in concentration of polyphenols from 0.25 to 4mg/ml did not enhance the dyeing property of the natural dyes. If a compound has to function as a mordant it should be effective in giving an exhaustion of >80% dye into the fabric. But the highest exhaustion obtained in the present study is only 20% which may be due to the lower amount of tannic acid (4%) in the ret liquor used. However, with this concentration also it has shown a little bit of mordanting property and having more concentration of polyphenols being used there could be an enhancement in the dyeing property. It has to be remembered that the polyphenols in ret liquor used in the present study is a by-product of retting cocount husk in a biorector and according to the present method employed for the extraction there is a limitation in obtaining polyphenols beyond certain limits from the husk. Probably if the method of extraction is modified, to get the polyphenols fully, to have higher concentration for application the mordanting property also could be

enhanced.

Tannins that are derived naturally from the plants like oak, walnut etc and are known for their astringency and are therefore used as natural mordants and as substitutes for the artificial ones such as $CuSO_4$, $Al_2(SO_4)_3$, $FeSO_4$ etc. The type of mordanting that has been found with tannic acid (tannins) present in the concentrated ret liquor is the premordanting type because mordanting was done before dyeing.

The possibility of using tannins in ret liquor as the natural mordant along with other synthetic dyes other than the natural dyes used here also has to be looked into. Moreover experimentation of utilizing ret liquor as a mordant has to be extended to other fabrics such as silk, wool, jute etc before coming into a conclusion about its full potential.

4.3.2. Tanning property

The characteristics of ret liquor in terms of the tanning property is summarised in Table 4.2. As is well evident that bulk of the ret liquor is moisture and the content of total polyphenols, total solids, total solubles, non tannins and tannin are very negligible. Moreover the tan/non- tann ratio is very low. This indicated that ret liquor in the persent form does not have the tanning property.

The function of tannin in the tanning material appears to be to displace the cross links of the natural protein fibre with tannin links. The natural cross links are susceptible to acid, alkali and salt solutions, whereas the tannin links are resistant. (Jordan and Lloyd, 1935).

From the tanners point of view, high tannin content is a very desirable factor, and a certain minimum non-tannin content is essential. The function of non-tans is to aid in separating the collagen molecules, which consists of long chains oriented in parallel by virtue of their polar groups. This separation permits penetration of tannin molecules (Philips, 1932). As non-tannis are essential to obtain good distribution of tannin throughout the thickness, it will be seen that their function is an important factor, and is a necessity to the action of tannin. The ratio of tannins to non-tannins also influence the rate of fixation (astringency) of the tanning material. Therefore, it is essential that the tannin non-tannin ration is to be more than one. The colour of the tanning material is also a very important factor in tanning leather.

The ratio of tannin to non-tannin content in the concentrated ret liquor is less than one and thus is found unsuitable for tanning. Since the percentage of non-tannins (1.27%) is higher than tannins (0.01%), there may be higher penetration of tannins into the hide but due to insufficient amount of tannins, the process of tanning does not take place. Therefore the amount of tannin is the most important criteria in tanning when compared to non-tans, astringenacy etc.

The lower quantity of Tannins (0.01%) in the concentrated ret liquor may be due to the mode of extraction of the polyphenols from the husk, The binding of polyphenols with other molecules and the fibres in the cocount husk is so strong that simple mechanical extraction using a stripping device may not be sufficient enough to extract the total amount of polyphenols (tannins) from the husk, Therefore it is suggested that alternate extraction processes are to be adopted for drawing out the maximum tannins from coconut husk, so that the tannin/non

tannin ratio is brought above one. Thus, if the extraction method is altered then there can be a possibility of using the tannins fom coconut husk in tanning industry also.

4.3.3. Antifungal Property

Two sets of experiments were conducted to test the antifungal property of the polyphenols in ret liquor. In the well diffusion method tried under the first category with 1000 ppm polyphenol on *Myrothecium roridum, Cylindrocladium quinqueseptatum, Corticium salmonicolour, Alternaria alternata, Rhizoctonia solani* no substantial inhibition zone could be seen on incubating for 7 days (Table 4.3). Eventhough there was a lag in the germination of spores (Table 4.4), in the second category of experiments for examining the germination of spores in saboraud dextrose broth in the presence of varying concentrations of polyphenols the germination was found to be delayed temporarily, but after 10 days of incubation myeelia were found to grow in the broth. This indicated that there is a level of antifungal property with the polyphenols in ret liquor but yet not sufficient to kill the organism.

4.3.4. Antibacterial property

The antibacterial property of the polyphenolics in ret liquor is summarised in Table 4.5.Out of ten strains of bacteria pathogenic to man and aquatic animals tested *Photobacterium, Vibrio Sp* (ANM, 607), *Aeromonas* (ANM 680a) *Vibrio cholerae* lost viability even at 0 hour at the lowest polyphenol concentration of 100 ppm tested. At the same time *Non-coagulase staphylococcus sp.* and *Aeromonas* (AAC 1102a) were killed during the exposure of first 12 hours. In the case of *Shigella dysentereae* loss of viability was observed at 500 ppm

polyphenol during an incubation of 12 hours. At the same time for *Staphyloccus aureus* and *Enterobacter aglomerans* 750 ppm polyphenol and an exposure period of around 24 hours were required for the loss of viabiity. However, *Salmonella typhi* was killed at 1000 ppm only that too during an exposing for 24 hours.

The cidal effect of polyphenol on the opportunistic pathogens under the family Vibrionaceae associated with the larvae of *M. rosenbergii* is summarised in Table 4.6. Out of 47 cultures tested 37 were found to loose viability within 24 hour exposure to polyphenol at all concentrations tested ranging from 100 to 1000 ppm indicating an inhibition of 72% of the members of the family.

The overwhelming action of the polyphenols on human pathogenic bacterial cultures and the cultures from the prawn hatchery system reveals the possibility of using natural polyphenols from coconut husk as the source of potential drungs against bacteria.

It is suggested that the biological and pharmacological activity of the polyphenols may be by virtue of three distinctive general chacteristics which all natural polyphenols possess, namely (i) their complexation with metal ions (iron, manganese, vanadium, copper, aluminium, calcium etc) (ii) their antioxidant and radicle scavenging activities and (iii) their ability to complex with other molecules including macromolecules such as proteins and polysaccharides.

The ability to complex with macromolecules like proteins and enzymes produced by the various bacterial cultures may be the reason for the bacterial inhibition by the polyphenols extracted from coconut husk. These protein complexation can occur by hydrophobic effects and hydrogen bonding (Haslam, 1996).

Polyphenols derived from various parts of the plant exhibit antibacterial properties. In this study the polyphenols derived from the coconut husk in the form of concentrated ret liquor was examined in the crude form and therfore the antimicrobial activity pertains to a group of polyphenols like tannins, pyrogallol, catechol etc. Hence experiments are to be done after isolating the pure individual components from the concentrated ret liquor for developing potent natural drugs, as substitutes for the synthetic ones for avoiding major side effects including the development of drug resistance.

4.3.5. Antifouling property.

Antifouling property of the polyphenols in ret liquor is expressed in terms of the inability of the barnacle larvae to stay in the water column in the presence of polyphenols (Table 4.7) On starting from 75 ppm and going upto 1000ppm the larvae were found to become motionless from 100 ppm onwards while at 75 ppm all larvae were active. *Balanus amphitrite* is an excellent model organism for studies concerned with antifouling compounds because of its rapid larval development, the ease of raising synchronous mass culture, and the predictable settlement (Branscomb and Rittschof, 1984). The mechanism of action of several natural antifouling compounds is not known (Rittschof et.al, 1992). However, toxicity of the polyphenols at 100 ppm level to the barnacle larvae is a clear indication of its usefulness as an antifouling compound. As mentioned in the previous section the polyphenols have considerable antibacterial property also on natural isolates and this indicates that the compounds can take part substantially in the prevention of primary film formation on a surface. Based on this, purification of the compounds and their incorporation in surface coatings can be

tried with the objective to develop an environment friendly antifouling agent commercially.

4.4 Conclusion

Five possible applications of polyphenols in ret liquor were examined. The mordanting property was judged based on the exhaustion of dye to fabrics and it reveled that the preparation in the present form was not satisfactory as a natural mordant. At the same time if the extraction procedures are modified to yield more concentration of polyphenol in ret liquor it is likely to exhibit more such properties. The tannin content in ret liquor is comparitively lower compared to non tan content and hence the ret liquor as such has poor tanning property. Here also if the extraction procedures are modified to release more polyphenols from husk and the tan: non tan ratio is brought near to one the tanning potential of the preparation may be enhanced On experimenting with several species of fungi it was revealed that the ret liquor can arrest the germination of spores of fungi temporarily and at the same time cannot lead to death of the organism. Meanwhile it exhibited profound antibacterial property even though it varied from organism to organism. The bactericidal property was very much pronounced in *Photobacterium, Vibrio cholerae* and *Aeromonas sp.* even at 100 ppm, the lowest concentration tested at zero hour exposure. In the same way ret liquor exhibited antifouling property also by inhibiting the larvae of barnacles to settle.

Thus, among the five properties tested the antibacterial and antifouling properties were worth to be studied further with the ret liquor in the present form which can be used as the raw material for the extraction of the active compounds.Other properties have to be looked into after modifying the extraction procedures.

Table 4.1 RATE OF EXHAUSTION OF NATURAL DYES INTO COTTON FABRIC

Conc. (mg/ml)	% exhaustion by using Conc. ret liquor as mordant.			% exhaustion by using $FeSO_4$ as mordant			% exhaustion by using no mordants.		
	135 H.	Eco Rust Red	Eco-Indo Blue	135 H	Eco Rust Red	Eco-indo blue	135 H	Eco Rust Red	Eco-Indo blue
0.25	20	20	10	90	85	80	0	0	0
0.5	20	20	10	90	85	80	0	0	0
1	20	20	10	90	85	80	0	0	0
2	20	20	10	90	85	80	0	0	0
4	20	20	10	90	85	80	0	0	0

Table 4.2 CHARACTERISTICS OF THE RET LIQUOR IN TERMS OF TANNING PROPERTY.

Sl.No.	Total polyphenol %	Total Solids %	Total Solubles %	Non Tannin %	Tannin %	Tannin/Nontannin ratio
1	0.4	1.39	1.28	1.27	0.01	0.007

Table 4.3 ACTIVITY OF CONCENTRATED POLYPHENOLS OVER FUNGAL CULTURES FROM FOREST ECOSYSTEM

Sl. No.	Cultures	Inhibition zone formed after			
		0 hr incubation	48 hrs incubation	96 hrs incubation	192 hrs incubation
1.	*Myrothecium rodium*	-	-	-	-
2.	*Cylindrocladium quinqueseptatum*	-	-	-	-
3.	*Corticium salmonicolor*	-	-	-	-
4.	*Alternaria alternats*	-	-	-	-
5.	*Rhizoctonia solani*	-	-	-	-
6.	*Cytospora sp.*	-	-	-	-

'-' Indicates no activity

Table 4.4 EFFECT OF POLYPHENOLS ON THE GERMINATION OF FUNGUS

Fungal cultures	Response of fungal spores to different concentration of polyphenols on exposure for 0 hour																			
	24 hour incubation in saboraud dextrose broth after the exposure					48 hour incubation in saboraud dextrose broth after the exposure					96 hour incubation in saboraud dextrose broth after the exposure					168 hour incubation in saboraud dextrose broth after the exposure				
	Concentration of polyphenols (ppm)					Concentration of polyphenols (ppm)					Concentration of polyphenols (ppm)					Concentration of polyphenols (ppm)				
	100	250	500	750	1000	100	250	500	750	1000	100	250	500	750	1000	100	250	500	750	1000
Colletrichum fulcatum	-	-	-	-	-	+	+	+	+	+	++	++	++	++	++	+++	+++	+++	+++	+++
Pythium	-	-	-	-	-	+	+	+	+	+	++	++	++	++	++	+++	+++	+++	+++	+++
Rhizoctonia solani	-	-	-	-	-	+	+	+	+	+	++	++	++	++	++	+++	+++	+++	+++	+++
Phytopthora	-	-	-	-	-	+	+	+	+	+	++	++	++	++	++	+++	+++	+++	+++	+++
Pestalotia	-	-	-	-	-	+	+	+	+	+	++	++	++	++	++	+++	+++	+++	+++	+++

'-' indicates No growth
'+' indicates growth

Table 4.5 EFFECT OF CONCENTRATED POLYPHENOLS FROM RET LIQUOR ON PATHOGENIC BACTERIAL CULTURES

Bacteria	Time Intervals	Effect of polyphenols					
		100 ppm	250 ppm	500 ppm	750 ppm	1000 ppm	Control
Staphylococcus aureus	0hr	+	+	+	+	+	+
	12 hr	+	+	+	+	+	+
	24 hr	+	+	+	-	-	+
Photo bacterium	0 hr	-	-	-	-	-	+
	12 hr	-	-	-	-	-	+
	24 hr	-	-	-	-	-	+
Vibrio Species ANM 607	0 hr	-	-	-	-	-	+
	12 hr	-	-	-	-	-	+
	24 hr	-	-	-	-	-	+
Aeromonas ANM 680 a	0 hr	-	-	-	-	-	+
	12 hr	-	-	-	-	-	+
	24 hr	-	-	-	-	-	+
Vibrio cholerae	0 hr	-	-	-	-	-	+
	12 hr	-	-	-	-	-	+
	24 hr	-	-	-	-	-	+
Noncoagulase staphylococcus sp.	0 hr	+	+	+	+	+	+
	12 hr	-	-	-	-	-	+
	24 hr	-	-	-	-	-	+
Aeromonas AAC 1102 a	0 hr	+	+	+	+	+	+
	12 hr	-	-	-	-	-	+
	24 hr	-	-	-	-	-	+
Shigella dysentereae	0 hr	+	+	+	+	+	+
	12 hr	-	-	-	-	-	+
	24 hr	-	-	-	-	-	+
Salmonella Typhi	0 hr	+	+	+	+	+	+
	12 hr	+	+	+	+	+	+
	24 hr	+	+	+	+	-	+
Enterobacter aglomerans	0 hr	+	+	+	+	+	+
	12 hr	+	+	+	+	+	+
	24 hr	+	+	+	-	-	+

'-' indicates no growth '+' indicates growth

Table 4.6 ACTION OF CONCENTRATED POLYPHENOLS ON BACTERIAL CULTURES FROM PRAWN HATCHERY SYSTEM

Sl.No.	Culture Number	Species to which affiliation is seen	Effect of 24 hour action of Polyphenols (100ppm-100ppm)
1.	AAC 629 b	*V. aestuarianus, V. tubiashi, V. ichthyoenterii, P. leiognathi*	-
2.	ANM 594 b	*V. aestuarianus, V. tubiashi, V. ichthyoenterii, P. leiognathi*	-
3.	ANM 597	*V. aestuarianus, V. tubiashi, V. ichthyoenterii, P. leiognathi*	-
4.	ANM 708	*V. fischeri, V. logei, V. marinus P. angustum*	-
5.	ANM 610 a	Group E-3	-
6.	ANM 1020	*V. fischeri, V. logei, V. marinus P. angustum*	-
7.	ANM 594 a	*V. tubiashi, V. anguillarum, V. ichthyoenterii, V. ordalli*	+
8.	ANM 1003	Group E-3	+
9.	AAC 1104 a	Group E-3	-
10.	AAC 1142 b	*V. fischeri*	+
11.	AAC 1101 b	*P. angustum, P. phosphoreum, V. logei, V. marinus, V. fischeri*	-
12.	API 684 a	*V. costicola, V. furnissi, V. fluvialis, V. hollisae, V. proteolyticus*	-
13.	AAC 1114 b	*P. angustum, P. phosphoreum, V. logei, V. marinus, V. fischeri*	-
14.	ANM 718 aa	*V. anguillarum, V. mestschnik-ovii, V. ordalli, V. tubiashi, V. ichthyoenterii*	+
15.	AAC 1109 b	*V. fischeri*	-
16.	AAC 1128 a_2	*V. campbelli, V. cholerae, V. harveyi, V. vulnificus*	-

Sl.No.	Culture Number	Species to which affiliation is seen	Effect of 24 hour action of Polyphenols (100ppm-100ppm)
17.	AAC 1101 a	*P. angustum, V. logei, V. marinus, V. fischeri*	-
18.	AAC 672 b_1	*Aeromonas sobria, A. hydrophila*	-
19.	AAC 669 B	Out of range for Vibrionaceae	-
20.	AAC 880 c	*Aeromonas salmonicida*	-
21.	AAC 1108 d	13 species of Vibrios	-
22.	API 644 b	*A. hydrophila, A. sobria*	-
23.	AAC 654 b	*A. caviae-like*	-
24.	ANM 598 C_2	*V. anguillarum, V. metschnik-ovii, V. ordalli, V. tubiashi, V. ichthyoenterii*	-
25.	AAC 639 b_1	Group E-3	-
26.	AAC 536	*V. cholerae*	-
27.	ANM 1008 a	*A. salmonicida*	-
28.	ANM 625	*P. angustum, V. logei, V. marinus, V. fischeri*	+
29.	AAC 701	*V. cholerae, V. harveyi, V. campbelli, V. vulnificus*	-
30.	ANM 712	*V. anguillarum, V. metschnikovii V. mytili, V. navarrensis, V. ordalli, V. orientalis, V. pelagius* bv. I and II, *V. splemdidus* bv. I and II, *V. alginolyticus*	+
31.	ANM 719	*V. fischeri*-like	-
32.	API 1555	*V. proteolyticus, V. hollisae, V. costicola, V. furnissi*	-
33.	API 768	*A. hydrophila, A. sobria, A. caviae*	-

Sl.No.	Culture Number	Species to which affiliation is seen	Effect of 24 hour exposure of Polyphenols (100ppm-1000ppm)
34.	ANM 721	*A. vulnificus, V. campbelli, V. cholerae, V. harveyi, V. tubiashi,* marine luminous bacteria	-
35.	ANM 702	*Aeromonas*	-
36.	API 1519	*V. proteolyticus, V.hollisae, V. costicola, V. furnissi, V. fluvialis*	-
37.	AAC 730 a	*V. gazogenes, V. cholerae, V. campbelli, V. penaecida, V. diazotrophicus, V. nereis, V. parahaemolyticus, V. pelagius* bv. I and II, V. *vulnificus* bv I, *V. nigripul-chritudo, V. harveyi, V. natreig-enes, V. navarrensis, V. alginolyticus*	+
38.	ANM 932	*V. fluviallis, V. costicola, V. furnissi*	-
39.	API 1561	Group E-3	-
40.	API 1558	*A. hydrophila, A. caviae, A. sobria*	+
41.	AAC 727	*V. fischerii*	-
42.	AAC 740	*V. fluvialis, V. costicola, V. furnissi, V. proteolyticus, V. hollisae*	+
43.	ANM 723	Group E-3	+
44.	API 1546	*V. fischerii*	-
45.	API 810	Out of range of Vibrionaceae	+
46.	AAC 717	Out of range of Vibrionaceae	+
47.	API 781	Out of range of Vibrionaceae	+

'+' indicates growth
'-' indicates No growth

Table 4.7 ACTIVITY OF POLYPHENOLS ON BARNACLE NAUPLII

Organism	Concentration of polyphenols (ppm)					
	75	100	250	500	750	1000
Barnacle Nauplii	+	-	-	-	-	-

'+' mobility of larvae
'-' no mobility of larvae

CHAPTER 5

CONCLUSION

Chapter 5

CONCLUSION

For combating pollution caused by the natural retting process, a bio-reactor has been developed where the ret liquor generated is treated by microbial cultures and recirculated. This controlled retting process included a pre-retting operation for the removal of polyphenols prior to retting which would speeed up the microbial processes making the retting faster. The polyphenols liberated during this process have to be effectively separated by various chemical and physical means so that they can be utilized for various bio-medical and industrial applications. The study focuses attention on developing a viable technology for the extraction of polyphenols, their characterization and evaluation of the potential to use for tanning, in textile industry, as novel drugs for controling bacterial and fungal pathogens and as antifouling compounds. The results obtained and the conclusions made are listed below:

* Experiments were conducted for determining the optimum salinity for the liberation of polyphenols from husk and the time required for their maximum liberation at this salinity. Accordingly 5PPT was found to be the optimum salinity for the polyphenol stripping and a period of 96 hours was required for their liberation by gentle swirling which was comparable to the natural movement of water in the environment.

* For separating the polyphenols from ret liquor, various organic solvents like diethyl ether, benzene, hexane, chloroform, carbontetrachloride and petroleum ether were tried and among them the highest percent extraction obtained was only 2.9 ± 1.8 with diethyl ether.

* Activated charcoal was found to be useful in separating the polyphenols from ret liquor by adsorption and the maximum was 96% at the normal pH of the ret liquor (6.5 ± 0.2)

* As the polyphenols are adsorbed onto activated charcoal, they have to be desorbed for their effective utilization. For desorption from activated charcoal NaOH at various normalities such as 0.1. 0.5, 1, 2 and 3 N were tried and the highest desorption obtained was just 0.34% at 1N NaOH.

* For desorption of polyphenols from activated charcoal, various organic solvents like ethyl alcohol, acetone, methanol, hexane, carbontetrachloride and petroleum ether were also tried and the maximum desorption obtained was 0.44 ± 0.13% in ethyl alcohol.

* Meanwhile polyphenols could be effectively separated using a flat reverse osmosis module consisting of a cellulose acetate -14 polymer membrane in a metallic seitz filter holder with a meshed support. A known quantity of the ret liquor was poured into the module which was connected to a pressure control device, whereby a pressure of 100 psi was applied with stirring at 550 rpm; the pressure applied forced the clear permeate through the membrane while the higher molecular weight polyphenols were rejected to a tune of 95% with a flux of 0.5ml/min.

* The ret liquor concentrated by the reverse osmosis employing the cellulose acetate polymer membrane could be lyophilized using a bench scale lyophilizer and the total polyphenol content in the case of ret liquor prepared in distilled water was 499 ± 38 mg/g and in case of the ret liquor prepared in 5 PPT sea water it was 302 ± 94.5 mg/g

* For the effective removal of polyphenols from the coconut husk and to enhance the retting process a polyphenol stripping device was fabricted as the basic unit of coir-retting bio-reactor. The stripping device consisted of a tank of $32cm^2$ base and 40cm height. A perforated platform is positioned 10cms above the base to support the coconut husk. From the bottom of the tank an outlet pipe with 2cm diametre connects with a 0.25 Hp monoblock electric pump which facilitates drawing water forcefully from the bottom and to discharge at the top, thereby a strong circulation of water to an amount of 40 litres per minute can be generated. Through another outlet pipe fixed at the opposite side ret liquor can be drained off for concentration of polyphenols. The tank is made with fibre glass to avoid corrosion. The coconut husk after crushing with hammer are arranged inverted in the tank fitted with 5 PPT sea water and the stripping device operated.

* Using the polyphenol stripping device, the maximum liberation of polyphenols (1.7668mg/ml) took place within 24 hours and this was found to be the right time for drawing off the ret liquor from the reactor as the concentration of the polyphenols declined subsequently. This formed the first stage of stripping of polyphenols in the stripping device of the bioreactor.

* The ret liquor after 24 hours stripping is drawn out and the reactor is filled with fresh 5 PPT sea water and the process repeated. This is the second stage of stripping, during which, the polyphenol concentration get declined and interestingly a red coloured compound having different absorption peak entirely different from that of the polyphenols is found to get liberated.

* Thus during the first stage of stripping, polyphenols could be extracted and during the second stage the polyphenols got decreased with the liberation of the red coloured compound.

* The red coloured compound liberated in the stripping device during the second stage could be effectively separated by flocculation by maintaining the pH at 7.8 and salinity at 6 PPT. By this technique 88% of the red coloured compound could be separated.

* The polyphenols separated and concentrated from the ret liquor by reverse osmosis for their effective utilization was filter sterilized by passing through a seitz filter , tested for sterility and the shelf life determined at temperatures of 0, 4 ± 0.5, 24 ± 0.5, 28 ± 0.5 and 37.5 ± 0.5 °C for 180 days by keeping the sterile concentrated ret liquor asceptically in vials and in determining the total polyphenol content at each period of time.

* From the studies on shelf life it was found that the concentrated polyphenols were stable at or below 4 ± 0.5°C for a period not less than 180 days.

* The concentrated ret liquor was further characterized for their identity following various chromatographic techniques like thin layer chromatography (TLC), column chromatography and high performance liquid chromatography(HPLC).

* The thin layer chromatographic resolution of the concentrtated ret liquor on comparing with standards like tanninc acid, pyrogallol and catechol revealed tannic acid (tannin) as the major polyphenol in the concentrated ret liquor.

* Fractionation of the ret liquor by column chromatography using various solvents gave three fractions soluble in Methanol, Acetic acid and water respectively.

These fractions were further characterised by thin layer chromatography individually, which also revealed tannic acid (tannin) as the major component.

* HPLC, being the versatile technique for fractionating natural compound, was applied here for ascertaining the major polyphenol in the concentrated ret liquor. On elution by reverse phase employing a C_{18} μ bondapak column with methanol and water at the ratio of 4:1 as the solvent system also revealed tannic acid (tannin) as the major fraction, as confirmed by the retention time (RT) obtained from the chromatogram.

* Tannins are generally used as mordants in dyeing industry. From the chromatographic studies, tannins were said to be the major polyphenol in ret liquor and were tried for mordanting cotton fabrics. On mordanting with concentrated ret liquor and subsequent dyeing using three natural dyes 135H, Eco-rust red, Eco-indo blue, the percentage exhaustion was 20,20,10 for the three dyes resepectively which were very low when compared to $FeSO_4$ as the mordant with the same dyes. This showed a limited extent of mordanting property with the content of polyphenol in the ret liquor prepared.

* As another industrial application, tannins were tried in tanning hide for the preparation of leather. The ability of the tanning material depended on the tannin-nontannin ratio which should be 50:50 or 1 at the minimum. On studying the tannin - nontannin ratio of the concentrated ret liquor it was found to be less than one and therefore it was inferred that the concentrated ret liquor did not possess the required tanning property in the present form.

* The concentrated ret liquor containging polyphenols were studied for their antifungal property on cultures of *Myrothecium roridum, Cylindrocladium quinquispetatum, Corticium salmonicolor, Alternaria alternata and Rhizoctonia solani* obtained from KFRI, Peechi following well method and on *Colletrichum, Pythium, Rhizoctonica solani, Phytopthora* and *Pestalotia* obtained from Kerala Agricultural University for testing the sensitivity of the spores in broth suspensions. In the first set of fungal cultures there were no substantial inhibitory activity and in the second set of cultures, there was a lag in the germination of spores on exposing to polyphenols. This indicated a level of antifungal property but not sufficient to kill the organism.

* The concentrated polyphenols were again tested for their antibacterial property by serial dilution technique on bacteria pathogenic to man and aquatic animals like *Staphylococcus aureus, Photobacterium, Vibrio Sp.* (ANM 607), *Aeromonas* (ANM 680a), *Vibrio cholerae, Non coagulase staphylococcus. sp., Aeromonas* (AAC 1102a), *Shigella dysentereae, Salmonella typhi* and *Enterobacter aglomerans* and 47 cultures from prawn hatchery system. Out of these strains *Photobacterium, Vibrio sp.* (ANM 607), *Aeromonas* (ANM 680a), *Vibrio cholerae* lost viability at the 0 hour exposure to a polyphenol concentration of 100 ppm. At the same time *Aeromonas* (AAC 1102a) and *Non-coagulase Staphylococcus sp.* were killed during 12 hour exposure at a concentration of 100 ppm. *Shigella dysentereae* lost viability at 500 ppm at 12 hours. At the same time *Staphylococcus aureus* and *Enterobacter aglomerans* lost viability at 750 ppm concentration over an exposure period of 24 hours. However *Salmonella typhi* was killed at 1000 ppm after exposing for 24 hours.

Out of the 47 cultures from prawn hatchery system, 37 lost viability indicating 72% inhibition at concentrations ranging from 100 to 1000 ppm. These indicated a tremendous bactericidal activity of concentrated polyphenols on pathogenic bacteria.

* Barnacle nauplii are natural macrofouling agents in the aquatic system. Swimming behaviour of this larvae is considered for toxic assays. In this study the possibility of inhibition of barnacle nauplii by concentrated polyphenols were tested using various concentrations such as 75, 100, 250, 500, 750 and 1000 ppm polyphenols. It was found that the larvae stopped swimming at concentrations above 100 ppm. These natural polyphenols thus after purification can be incorporated in surface coatings which can act as antifouling agents.

The present study brings out a viable technology for the extraction of polyphenols from coconut husk through a process known as pre-retting operation in a polyphenol stripping device which is an integral part of the bioreactor developed for coir retting. The ret liquor generated by the pre-retting operation is further concentrated by reverse osmosis and the polyphenols separated for commercial application. This compound having a shelf life of more than 180 days below 4°C has profound antibacterial and antifouling potential. Obviously new drugs can be thought of from this compound and it can form an additive in paints used for preventing biofouling. The major polyphenol in this preparation has been proved to be a compound very much closer to tannic acid (tannin). The experiments carried out revealed that modifications in the extraction procedures of polyphenols from husk may result in the extraction of higher concentration of polyphenols from husk in such levels suitable for using them in textile industry as mordants and also in leather industry for tan-

ning because a limited extent of these two properties were also exhibited by the polyphenols present in the ret liquor.

It has to be emphasised at this juncture that the study opens up immense potential to utilize a toxic waste generated during coir retting for bio-medical and industrial applications at present is being lost in open waters polluting and impairing the aquatic system.

REFERENCES

REFERENCES

*Acker, T.E., Breneisen, P.E and Thambenbaum, S.W., 1966. J.Am. Chem. Soc, **88:** 834.

Anitha Das Ravindranath., 1991. Preliminary studies on the polyphenols in coconut husk, Coir, XXXV, No.1.

Anon., 1998. Coir Retting: Process Upgradation and Pollution abatement through Environmental Biotechnology, Project Completion report, Sponsored by Coir Board, Govt. of India, Implemented at School of Enviornmental Studies, CUSAT, Cochin- 16, 62.

APHA., 1989. Standard methods for the examination of water and waste water- 17th Edn., Washington D.C.

Bal, A.S and Lutade, S.L., 1979. Evaluation of NEERI made Cellulose Acetate tubular reverse osmosis membrane. Indian Journal of Environmental Health, **21**(2): 173-181.

Bal, A.S., 1992. Evaluation of operating pressure on reverse osmosis membrane properties. Indian Journal of Environmental Health, **34**(3): 200-208.

Barnes, H and Blackstock, J., 1973. Estimation of lipids in marine and animal tissues. J. Exp. Mar. Biol. Ecol, **12**: 103-108.

*Bate-Smith., 1973. Phytochemistry, **12**: 703.

Bhat, J.V., 1969. Souvenir, Third International Congress on the Global impacts of Applied Microbiology, Bombay, 39.

Bhat, J.V and Nambudiri, A.M.D., 1971. The Uniquity of Coir Retting. Journal of Scientific and Industrial Research, **30**(12): 17-28.

Bhatt, B.J., Bhargava, D.S and Panesar, P.S., 1983. Effect of pH on phenol removal in moving media reactors. Indian Jounral of Enviornmental Health, **25**: 261-267.

Bhat, S. G and Singh, I. S. B., 1998. Vibrionaceae associated with the larvae and larval rearing system of *Macrobrachium rosenbergii* : Systematics and Pathogenecity. Advances in shrimp Biotechnology, Flegel, T. W (Ed.), National Centre for Biotechnology, 225.

Billek, G and Shimpl, A., 1966. Biosynthesis of Aromatic Compounds, Billek, G (Ed.), Pergamon, Oxford, 37.

Blat, W. F., Dravid, A., Michael, A.S and Nelsen, L ., 1970. Solute polarisation and cake formation in membrane ultrafiltration. Membrane Science and Technology, Flynn, J.E (Ed.), Plenum press, New York.

Branscomb, E.S and Rittchof, D., 1984. An investigation of low frequency sound waves as a means of inhibiting barnacle settlement. J. Exp. Mar. Biol. Ecol, **79** (149) : 54

Brown, S. A., 1966. Biosynthesis of Aromatic compounds, Billek. G (Ed.), Pergamon, Oxford, 15.

Cadman, C. H., 1960. Phenolics in plants in Health and Disease, Pridham, J.B (Ed.), Pergamon press, Oxford, 101-105

*Cheek, P. J and Lilley, T. H., 1988. J. Chem. Soc. Faraday Trans 1, **84**: 1927-1940.

Craig, L. C., Craig, D., Scheibel, E. G., 1951. Techniques of Organic Chemistry, Weisberger (Ed.), Interscience, New York, **3**:1

Crisp, D.J and Spencer, C.P., 1958. The control of the hatching process in barnacles. Proc. R. Soc. London, B **149**: 278-299.

*Dewick, P.M and Haslam, E., 1968. Chem. Commun, 673.

Edwards, P.M., 1949. Notes on the Species of South Indian Wattle, Indian For. Leaflet (71): 9.

Egger, K., 1969. Thin layer chromatography. A laboratory handbook, E.Stahl (Ed.), Springer, Berlin, 686.

Elisabeth, D., Bohuon, G., Baron, A and Drilleau, J. F., 1991. High performance liquid chromatography of the phenolic compounds in the juice of some French Cider apple varieties. Journal of Chromatography, **555**: 125-136.

*Farkas, P and Pallos, L., 1967. Fortschr. Chem. Org. Naturstoffe, **25**:150.

Gornall, A.G., Bradawill, C.G and David, M.M., 1949. Determination of total serum protein by means of Biuret reaction. J. Biol. Chem, **177**:751-766.

Grisebach, H., 1965. Chemistry and biochemistry of plant pigments, Goodwin,T.W (Ed.), Academic press, London, 271.

Grisebach, H., 1975. Recent advances in Phytochemistry, Runeckles, V.C (Ed.), Plenum press, New York, Vol.9.

Gupta, P and Lewis, J. R., 1971. J. Chem Soc (c), 629.

*Halliwell, B., 1995. Biochemist, **17**:3-6.

Hanson, K.R and Havir, E. A., 1972. Recent advances in Phytochemistry, Runeckles, V.C., Watkans, J.F (Eds.), Appleton- Century - Crofts, New York, **2**: 48.

Haslam, E., 1974. The Shikimate pathway, Butterworth, London.

* Haslam, E., 1986. Chem. Soc. Nat. Prod. Rep, 217.

Haslam, E., 1989. Plant polyphenols: Vegetable tannins Re-visited, Cambridge University press, Cambridge.

*Haslam, E and Cai, Y., 1994. Nat. Prod. Rep, **11**:41-66.

Haslam, E., 1996. Natural polyphenols (Vegetable tannins) as drugs: Possibel modes of action. J. Nat.Prod, **59**:205-215.

*Hathway, D. E., 1959. Biochem. J, **71**:533.

Hillis, W.E and Carle, A., 1960. Biochem. J, **74**:607.

Hillis, W.E and Swain, T., 1959. J. Sci. Food. Agric, **10**:135

Hillis, W.E and Inoue, T., 1968. The Polyphenols formed in *Pinus radiata* after *Sirex* attack. Phytochemistry, **7**:13-22.

Holm, P.R., 1990. Effect of density- dependant mortality on the relationship between recruitment and larval settlement. Mar. Ecol. Progr. Ser, **60** (141): 146.

Jayashankar, N. P., 1966. Studies on the anaerobic microflora associated with the retting of coconut husks. Ph.D. thesis, Indian Institute of Science.

Jordan Lloyd, D., 1935. Theory of the tanning process with special reference to vegetable tanning and chrome tanning. J. Intern. Soc. Leather Trades Chem, **19**:336-344.

*Kashiwada, K., Nonaka, G., Nishioka, I., Chang, J and Lee, K.H., 1992. J. Nat. Prod, **55**: 1033-1043.

*Konishi, E and Hotta, S., 1979. Microbiol. Immunol, **23**:659-667.

Kopecek, J and Sourirajan, S., 1969. Performance of porous Cellulose Acetate membranes in some reverse osmosis studies. Indian Engg. Chem. Prod. Res. Develop, **8**:274.

Lea, A.G.H., 1982. J. Chromatogram, **238**: 253

Liu, D., Lan, Y. L., Chan, Y. K.,Pacepavicius, G., 1994. Simple technique for estimation of bio-film accumulation. Bulletin - Envron - Contam -Toxiocol, **53**(6) : 913-918.

*Loomis, W. D., 1974. Methods Enzymol, **31**: 528-544.

Lyne, R. L Mulheim, L. J and Leworthy, D. P., 1976. J. Chem. Soc. Chem. Commun, 497.

Malhotra, S. K., 1979. Kinetics and adsorption mechanism for phenol removal on flyash in a batch reactor. M. E. thesis, University of Roorkee.

*Martin., Gaffney., Lilley., Magnolato and Haslam., 1986. J. Chem. Soc. Chem. Commun, 107.

*Mc Credie, R. S., Ritchie, E. R., Taylor, W.C., 1969. J. Chem. **22**:1011.

*Menon, S. R. K., 1935. Biochem Journal, **29** :282.

Menon, K. P. V and Pandalai, K. M., 1958. Coconut palm - A monograph (Indian Central Coconut Committee, Ernakulam).

Neish, A., 1965. Plant Biochemistry, Bonner, J and Varner, V. E (Ed.), Academic press, New York, 581.

Nord, F. F and Schubert, W.J., 1967. Biogenesis of Natural compounds, Bernfeld, P(Ed.), 2nd Ed., Pergamon, Oxford.

Okuda, T., 1993. Polyphenolic phenomena, Scalbert, A (Ed.), INRA publications, Paris, 221-235.

Okuda, T., 1995. Tannins, a new family of bio-active natural organic compounds, Yakugaku-Zashi(Japan), **115**(2):81-100

*Pandalai, K. M., Nair, W. K and Menon, K. P. V., 1957. Coir I, 30.

Philips, H., 1932. Nontans - their nature and function. J. Intern. Soc. Leather Trades Chem, **16**:324-328.

*Pridham, J. V., 1965. Ann. Rev. Plant Physiol, **16**:13

Remani, K. N., Nirmala, E and Nair, S. R., 1989. Pollution due to Coir Retting and its effects on estuarine flora and fauna. Intern. J. Environ. Studies, **32**:285-295.

Ritschoff, D., Clare, A. S., Gerhart, D. J., Sister Avelin Mary and Bonaventura, J., 1992. Barnacle *invitro* Assays for Biologically Acvtive Substances: Toxicity and settlement inhibition assays using mass cultured Balanus amphitrite amphtrite Darwin. Biofouling, **6**:115-122.

Roberts, E. A. H., 1956. Paper chromatography as an aid to the elucidation of the structure of polyphenols occuring in tea. Paper presented at a symposium held in the university of Cambridge on April 12th-13th under the auspices of the society of leather trades chemists, 87-97.

Roe, J. H., 1955. Determination of sugar in blood and spinal fluid. J Biol. Chem. **212**: 335-343.

*Scalanfelberger, D and Hosteltmann, K., 1987. J. Chromatogram, **389**:450

Seguin, A., 1796. Ann. Chim (Paris), **20**:15.

Seshadri,G and Shimpl, A., 1966. Bio-synthesis of Aromatic compounds, Billek, G (Ed.), Pergamon,Oxford, 37.

*Sherry, S.P., 1952. Wattle Research Institute, Univ.Natal, S.Africa, Report No. 4 - 5, 64.

Shuttleworth, S. G., 1941. Story of Wattle. Union of South Africa, Dep. Agr and For. Sci. Bull., **168**:28.

Sotoby, S. A., Muller, W and Ismail, A. A., 1995. "Invitro" effect of Egyptian tannin containing plants and their extracts on the survival of pathogenic bacteria. DTW Dt-Sch Tierarztl Wochenschr (Germany), **102**(9):334-338.

Sourirajan, S., 1970. 'Reverse Osmosis', Logos press Ltd, New York.

Swain, T., 1965. Plant Biochemistry, Bonnes, J and Varner, V. E (Eds.), Academic press, New York, 552.

Tang, H. R., Hancock, R. A and Convington, A. D., 1995. Studies of vegetable tannins, Characterisation of commercial Chestnut tannin extract. Journal of the Society of Leather Technologists and Chemists, **66**:15-24.

Theodore White., 1956. The scope of vegetable tannin Chemistry, Paper presented at a symposium held in the University of Cambridge on April 12th - 13th under the auspices of the Society of Leather Trades Chemists, 7-29.

Turner, W. B., 1971. Fungal metabolites, Academic press, London.

Vaden Berghe, D. A., Vleietinck, A. J and Van Hoof, L., 1985. Advances in medicinal plant Research, Vleietinck, A. J and Dommisse, R. A (Eds.), Wissenschaftliche Verlagsgesellschafte mbh, Stuggart, 47-54.

*Varrier, N. S and Moudgill, K. L., 1947. Journal of Scientific and Industrial Research, **6**:114.

Vogel, W., 1931. Handbuch der Gerbereichemie und Lederfabrikation, Bergmann, M (Ed.), Springer, Vienna, Vol. II, Part 1.

*Waterhouse, A. L., 1995. Chem. Ind, 338-341.

*Weinges, K., Bahar, W., Ebert, W., Goritz, K and Marx, H. D., 1964. Fortschr. Chem. Org. Naturstoffe, **22**:115.

Williams, C. O., 1930. Bull. South African. Dep. Agric, No. 74.

Zogaski, J. C and Fouri, S. D., 1977. Removing Phenols via Activated Carbons. Chemical Engg. Progress, **235** : 65.

**As available from the source material*