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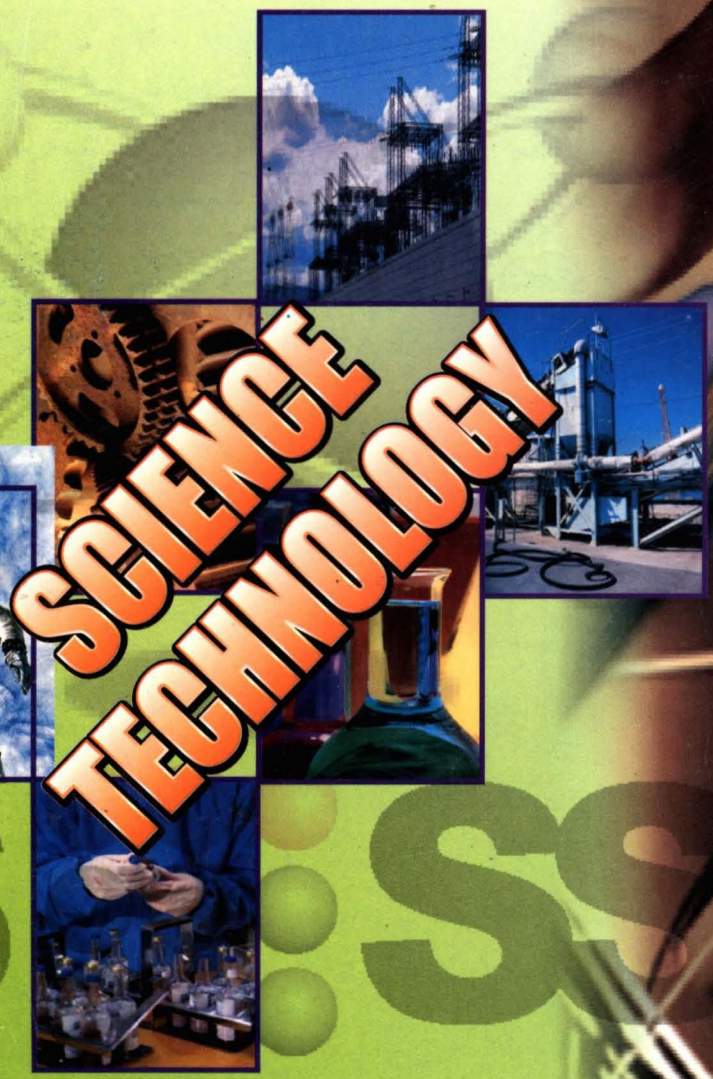

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A Preliminary Investigation of Condensed Tannins from Mangrove

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ABSTRACT

The condensed tannins of mangrove Rhizophora mucronata were extracted using water, methanol or acetone at different concentrations with or without acidification. Among solvents tested, 70% acetone, containing 1% HCl, extracted a maximum amount of condensed tannins from mangrove. Condensed tannins extracted were assayed using a 0.5% vanillin solution in methanol containing 4% HCl (v/v). Fresh leaves of Rhizophora mucronata contained the lowest amounts of condensed tannins (10%) compared to all other parts of the plant. The percentage of the condensed tannins extracted depend on the age of the mangrove tree, the type and percentage of the solvent used, as well as the type and the concentration of the acid used, and the condition of keeping the extracted tannins aliquot.

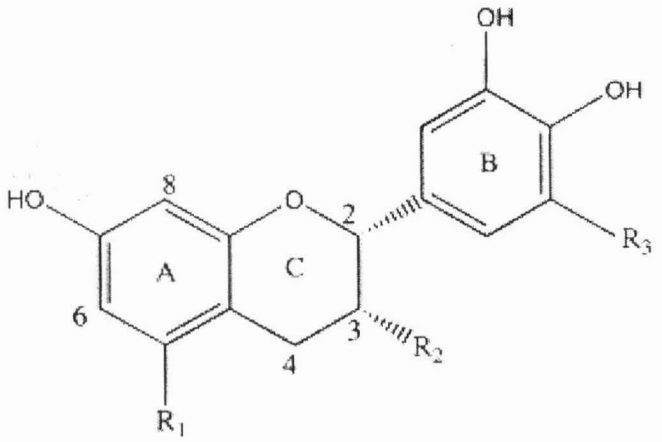
Keywords: *Tannins, Mangrove tannins, Condensed tannins, Extraction*

Introduction

Mangrove trees contain phenolic substances, including tannins (Salunkhe et al. 1990; 1982). Phenolics are secondary compounds widely distributed in the plant kingdom. Tannins are a special group of phenolics, with high molecular weight, that occur only in vascular plants such as flowers, fruits, seeds, leaves, stems, barks, and woods. The chemical diversity of tannins confers many kinds of reactions. Tannins (polyphenols) are produced via condensation of simple phenolics that are secondary metabolites and are widespread in the plant kingdom. Tannins do not constitute a unified chemical group, but have a variety of molecular structures. They are generally divided into hydrolysable (galloyl and hexahydroxydiphenoyl esters and their derivatives) and condensed proanthocyanidins or known as well as condensed tannins, CD (polymers of flavan-3-ols; (Haslam 1989). Hydrolysable tannins are made up of sugar (primarily glucose) and gallic acids, while condensed tannins consists of oligomers and polymers of flavanoids. Tannins are biolog cally active compounds and may have beneficial or adverse nutritional effects and physiological consequences. Endogenous tannins protect unharvested seeds from attack by insects, birds and herbivores, as well as certain diseases and untimely germination (Hulse 1979). Possible harmful effects of certain biological compounds, such as phenolics, trypsin inhibitors and phytates, have received considerable attention (Deshpande & Cheryan 1985), (Deshpande et al. 1984; Reddy et al. 1982). These compounds occur naturally in the plant kingdom, as well as seeds of legumes and cereals and, if present in sufficient quantities, may lower nutritional value and biological availability of dietary proteins and minerals.

Condensed tannins, CD which are also known as proanthocyanidins, PA are polymers of flavan-3-ol unit's i.e.polyhydroxyflavan-3-ol (Fig. 1, Fig. 2, and Fig. 3). The properties of Condensed tannins depend on their structure in terms of monomer units (degree of hydroxylation and 2,3-*cis*- or 2,3-*trans*-stereochemistry), their degree of polymerization (DP) and the linkage-type between flavan-3-ols (4–8 as shown in Fig. 2 and Fig. 3, or 4–6 branched structures as shown in Fig. 3) with a considerable range of structural variation. For the procyanidin, PC- type polymers the constituent flavan-3-ol units are either catechin (*trans*) or epicatechin (*cis*) with R = H (Fig. 1 and Fig. 2), while prodelphinidin, PD- type polymers contain either gallocatechin (*trans*) or epigallocatechin (*cis*) with R = OH (Fig. 1, Fig. 2 and Fig. 3), and many polymers are found to be mixtures of the two classes.

The extractable condensed tannins, CT were estimated to have molecular weight, in the range 5300 –5900 g/mole. The identity of the individual units that make up condensed tannins, CT polymer, and the length of the polymer chain can be estimated by subjecting the polymer to strong acid-catalyzed cleavage in the presence of phloroglucinol or benzyl mercaptan. These reactions result in the release of terminal units as free flavan-3-ols, whereas extender units are distinguished as benzylthioether adducts which are formed by nucleophilic capture of the carbocations generated under the acid conditions of the reaction. The average molecular weights of condensed tannins, CT determined by cleavage with phloroglucinol was in the range of 1900 – 2200 g/mole.



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Fig. 1: The basic repeating unit in condensed tannins. If $R_1 = R_2 = OH$, $R_3 = H$, then the structure is that for (-)-epicatechin. The groups at R_1 and R_3 for other compounds are indicated below the structure. If $R_2 = O$, the structure is galloyl in the catechin gallates.

Tannins are naturally-occurring uncrystallisable colloidal substances with pronounced astringent properties. Their main characteristic is that they bind and precipitate gelatin from solution and form insoluble compounds with gelatin-yielding tissue which is the property which enables them to convert raw hide and skin into leather, consolidating the dermal network of hide into firmer and drier structures of improved thermal stability, durability and water resistance. Because of their protein-binding properties, tannins are of considerable importance in food processing. Tannins have been reported to exert other physiological effects; e.g., they can reduce blood pressure, accelerate blood clotting, decrease the serum lipid level, modulate immune-responses and produce liver necrosis. The dosage and kind of tannins are critical for these effects. Tannins in wine have potent antioxidance against low-density lipoprotein (LDL) of which the oxidized form are a precursor of coronary heart disease. Plant phenols can be considered a nuisance because they can complex proteins by hydrogen bonding.

The objective of this study was to evaluate the effects of various solvent extraction systems on the recovery of mangrove tannins, and to determine the distribution of tannins and phenolic acids in mangrove bark.

Experiment

Mangrove bark

The barks of mangrove 'kurap' or *Rhizophora mucronata* were collected from Port Dickson Beach, Negeri Sembilan, Malaysia in September of 2004. The barks and leaves were separated manually. The total fresh weight and recovery of barks were recorded and samples used for moisture determination immediately after collecting. Samples were dried and then kept at room temperature for further study. The bark was ground using an IKA MF10 WERKE grinder and subsequently sieved with a 60-mesh sieve and used immediately for subsequent analysis.

Reagents

The chemical standards were of the highest purity grade. Tannic acid, Gallic acid, catechin, vanillic acid, caffeic acid, *p*-coumaric acid, protocatechuic acid, ferulic acid, *p*-hydroxy-benzoic acid and *p*-hydroxy-benzaldehyde were all obtained from Sigma–Aldrich Chemie. Folin–Ciocalteau and rhodamine were also obtained from Sigma–Aldrich

Chemie. Methanol, Na_2HPO_4, butanol, glacial acetic acid, ethyl acetate, hydrochloric acid, HCl and sodium sulphate was obtained from Syeterm.

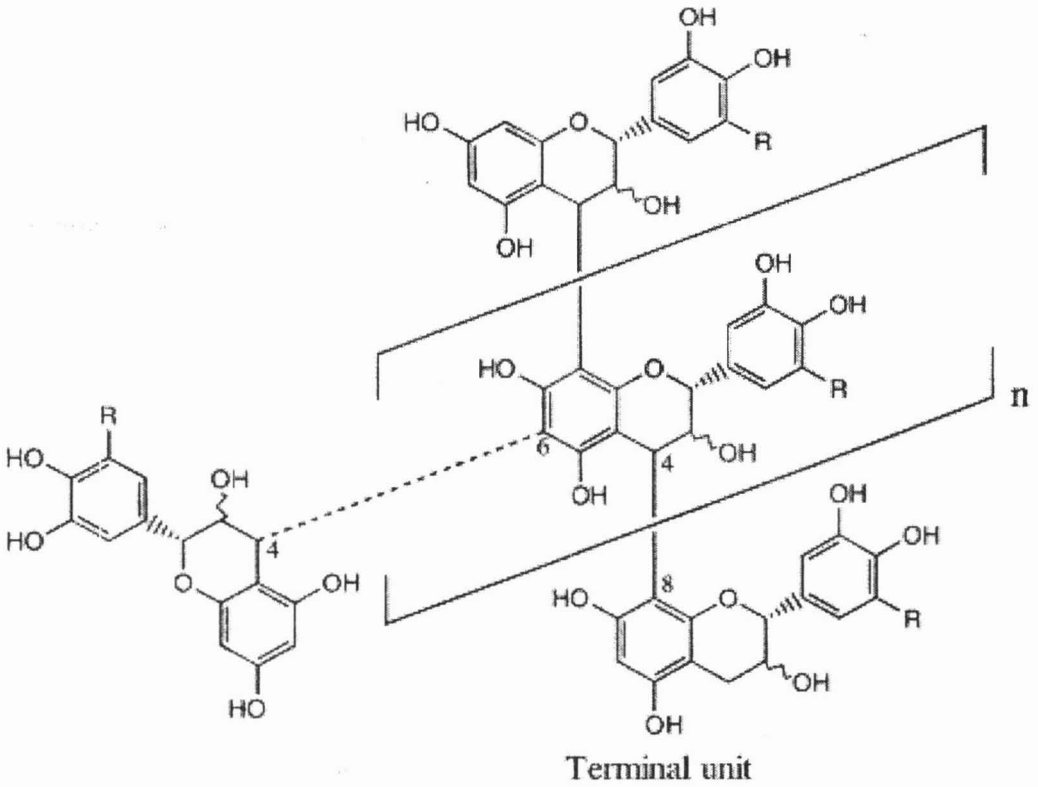


Fig. 2: Model structure for condensed tannin. If R = H or OH then the structure represents a procyanidin or prodelphinidin. The 4→ 6 linkage (dotted line) is an alternative interflavan bond. The terminal unit is at the bottom of such a multi-unit structure.

Extraction of tannins

Mangrove bark chips sample was extracted with different solvents as follows. A 2 g mangrove bark together with 10 ml water (20%, w/v) were mixed and heated in a boiling water bath for 30 min, cooled then centrifuged and the supernatant collected in a clean flask. The procedure was repeated two more times and the combined extracts were evaporated using a rotary evaporator at 40 °C to almost dry and the residue was then dried in the vacuum oven at 40 °C to constant weight.

A 2 g mangrove bark chips together with 10 ml acidified water (1%, v/v, HCl in water) (20%, w/v) were mixed and heated in a boiling water bath for 30 minutes, cooled then and the supernatant collected in a clean flask. This procedure was repeated two more times and the combined extracts were evaporated using a rotary at 40 °C to almost dry and residue was then dried in the vacuum oven at 40°C to constant weight.

A 2 g mangrove bark chips were extracted three times with 10 ml (20%, w/v) absolute methanol, absolute acetone, 90, 80, 70, 60 and 50% methanol or 90, 80, 70, 60 and 50% acetone. In another experiment, 100, 90, 80, 70, 60 and 50% methanol, as well as similar acetone solutions, acidified with 1% concentrated HCl, respectively, were used as extraction solvents. Samples were cooled and then subsequently centrifuged and supernatants collected in a clean flask. This procedure was repeated two more times and combined extracts were evaporated using a rotary evaporator at 40 °C to almost dry and residue was then dried in vacuum oven at 40 °C to constant weight. 2 g of all samples of the dry residue was then dissolved in 25 ml absolute methanol for further analysis.

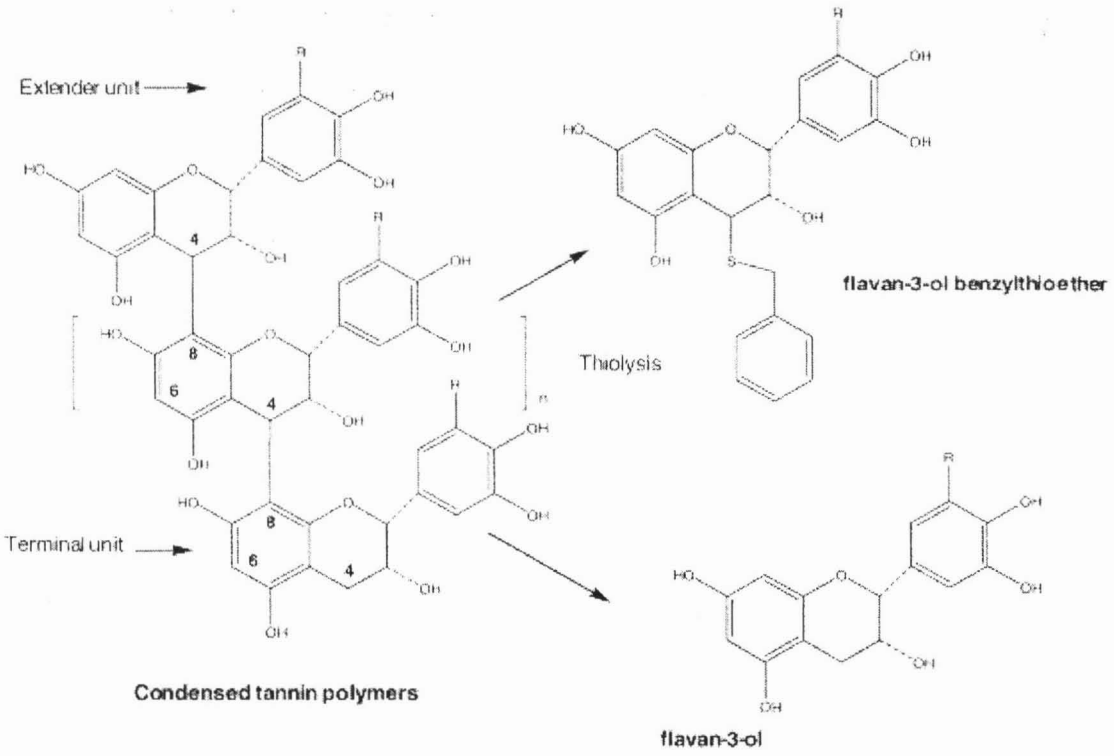


Fig. 3: Thiolysis reaction of condensed tannin polymers with procyanidin (R = H) and/or prodelphinidin (R = OH) units. The *trans*-stereochemistry is associated with catechin and gallocatechin (not shown), while *cis*-stereochemistry is associated with epicatechin and epigallocatechin. All the terminal units in the polymer were released as flavan-3-ols, and the extender units as flavan-3-ol benzylthioethers.

Extraction of polyphenols

Ground mangrove bark samples (2 g) were placed in a flask of 10 ml capacity on ice. Methanol–water, 1:1 v/v (10 ml), was added and the flasks were suspended on a shaker for a minute. The flasks were then centrifuged for 10 min. The supernatants were collected, filtered and stored in a deep freezer. The pigments were removed using diethyl ether in 1% acetic acid (Makkar et al. 1993).

Extraction of phenolic acids

The samples (2 g) of ground mangrove bark were extracted twice each with ethyl acetate (10 ml). The ethyl acetate was dried over sodium sulphate and then the sample was concentrated by rotary evaporation at 30 °C. The concentrates were transferred into small sample bottles and stored in a freezer for analysis.

Determination of condensed tannins (vanillin's assay)

The condensed tannins were assayed colorimetrically by the method of Price, Van Scoyoc and Butler (Makkar et al. 1995). To 0.2 – 1 ml of methanolic solution of condensed tannins, 5 ml of 0.5% vanillin reagent were added; a 5 ml volume of 4% concentrated HCl in methanol was used as a blank. The absorbances of samples and blank were read at 500 nm after standing for 20 min at room temperature. Catechin (3.5 moles of water per mole of catechin) was used as a standard in these experiments. The content of condensed tannins in the mangrove bark was expressed as mg or g catechin equivalents per 100 g sample.

Quantification of the polyphenol

In methanol solution, the total phenolics were quantified using the Folin–Ciocalteau method, gallotannins by the rhodamine assay and flavanols by the vanillin assay. All the methods were adopted from Makkar et al. (1993).

Results and Discussion

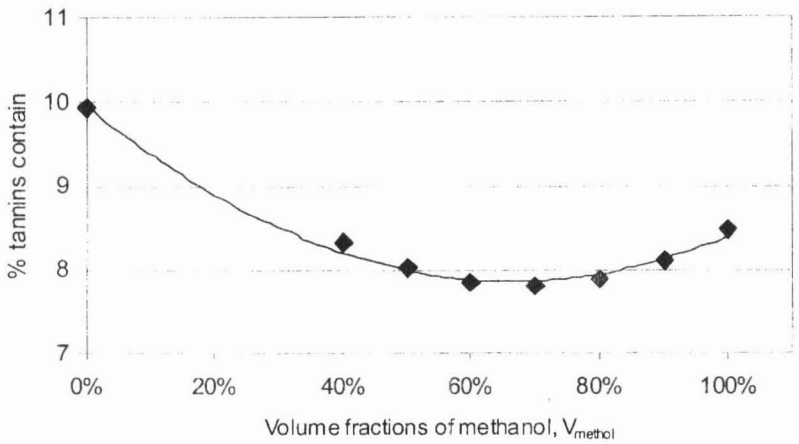


Fig. 4: Variation of volume fractions of methanol-water mixture against tannins contain.

In previous studies they have demonstrated that most of plant kingdoms served as an excellent source of condensed tannins, CT (Makkar et al. 1993; Shahidi et al. 2001). In the literature, different solvent systems have been used for extraction of condensed tannin, CT from plant materials as the extraction efficacy of condensed tannin, CT depends on their chemical nature, solvent system used (Table 1, and Fig. 4) and extraction conditions employed (Shahidi & Naczk 1995). The crude extracts of condensed tannin, CT contain low-molecular-weight phenolics as well as condensed tannin, CT. The low- molecular-weight phenolics may include phenolic acids, as well as several subclasses of flavonoids (Merken & Beecher 2000). In addition the extracted condensed tannin, CT consist of a series of oligomeric and polymeric compounds (Salunkhe et al. 1990) that differ in their sensitivity toward the reagents used for their determination (Shahidi & Naczk 1995). This makes the selection of appropriate methods for quantization of phenolics a difficult task. Merken & Beecher (2000) reviewed High Pressure Liquid Chromatography, HPLC methodologies for measurement of food flavonoids. According to these authors the existing HPLC methods can only separate a limited number of flavonoids and other phenolics and a method for simultaneous determinations of all prominent flavonoids is still needed. The molecular composition of crude mangrove bark condensed tannin, CT extracts is still unknown and therefore it is difficult, based on available data, to select both appropriate standards and HPLC methodologies for separation and quantization of phenolics involved. Since the aim of this study was to evaluate the effectiveness of solvent systems for extraction of condensed tannin, CT from mangrove bark, we selected the vanillin assay for quantification of condensed tannin, CT crude extract. This method is commonly used for quantification of condensed tannin, CT due to its specificity toward flavanols and dihydrochalcones (Shahidi & Naczk 1995). Moreover, according to Oszmainski & Bourzeix (1966), the vanillin method provides the most accurate estimate of the content of condensed tannin; CT. Methanol is usually used for carrying out the vanillin assay because, in methanol, the vanillin reaction is more sensitive toward polymeric condensed tannin, CT than monomeric flavanols (Price et al. 1978). However, it may still lead to over estimation of condensed tannin, CT content of crude extracts that are rich in monomeric components.

Table 1: Effect of different solvents on extraction of condensed tannins from mangrove barks

Solvent	*Rhizophora mucronata* (%)
Water	8.31
Water + HCl	9.92
100% Methanol	6
90% Methanol	5.34
80% Methanol	5.14
70% Methanol	5.4
60% Methanol	6.12
50% Methanol	7.3
40% Methanol	8.94
100% acidified Methanol	8.47
90% acidified Methanol	8.12
80% acidified Methanol	7.9
70% acidified Methanol	7.81
60% acidified Methanol	7.85
50% acidified Methanol	8.02
40% acidified Methanol	8.32
100% acetone	4.7
90% acetone	6.7
80% acetone	8.1
70% acetone	8.8
60% acetone	9.0
50% acetone	8.5
40% acetone	7.4
100% acidified acetone	6.3
90% acidified acetone	8.3
80% acidified acetone	9.6
70% acidified acetone	10.1
60% acidified acetone	10.0
50% acidified acetone	9.1
40% acidified acetone	7.5

The content of condensed tannins of mangrove barks in *Rhizophora mucronata* were around 4 to 12% depending on what solvent being used as extractor (Table 1). The content of condensed tannins in different plant parts of mangrove was significantly different (2 to 17%) (Table 2); the highest amount was present in fresh brownish bark (14%), followed by dark brown barks (12%), branches plus stems (11%), fresh green leaves (10%), and dried brown leaves (12%). The synthesis of tannins in different plant parts may depend on the metabolic rate of tannin synthesis at a particular site. Another reason may be higher polymerization of existing polyphenolic compounds in the bark to high-molecular-weight compounds during maturation. These results indicate that, as the maturity progressed, delocalization of condensed tannins from leaves to stems to branch to bark occurred and this was followed by their po-

lymerization into high-molecular-weight compounds (Price et al. 1980). Several factors, such as plant type, cultivar, age of the plant or plant parts, stage of development, and environmental conditions, govern the tannin content in plants (Table 2. and Fig. 5). The changes observed during development or maturation was mostly due to metabolism of polyphenolic compounds or polymerization of existing phenolic compounds.

The condensed tannins in different plant parts of mangrove tree were extracted into 70% acidified acetone; results are presented in Table 2 and Fig. 5. Mature bark had a higher tannin content than physiologically immature bark (light in weight, light green colour, relatively smaller size tree). Thus, as the maturity stage progressed, the concentration of condensed tannins increased. Fresh green leaves (10%), and dried brown leaves (12%) had different content of tannins than all other plant parts. This increase in tannin content may be due to a higher polymerization of existing polyphenolic compounds in the bark to high-molecular-weight compounds during maturation. Similar results and conditions have been reported by earlier workers for beans (Dashpande et al. 1984).

Table 2: Condensed tannins in different plant parts of mangrove

Plant part	Condensed tannins (%)
Fresh green leaves	10.4
1 day dried leaves	10.1
2 days dried leaves	10.2
3 days dried leaves	10.7
4 days dried leaves	11.4
Fresh branches	13.7
Dried branches	14.3

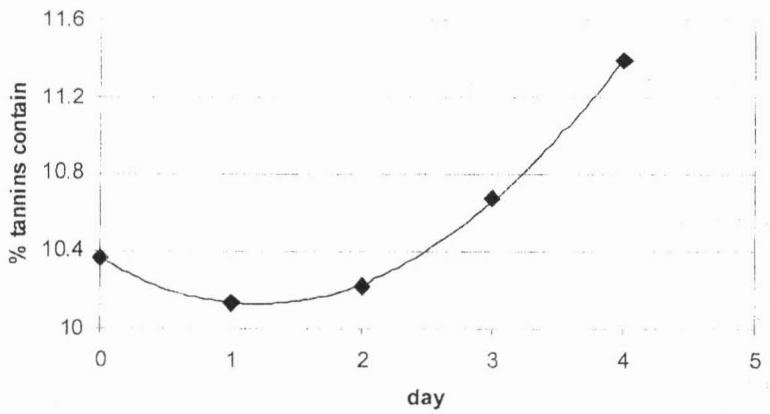


Fig. 5: Variations of tannins contain against days they kept

Conclusion

In conclusion, fresh dark brown mature bark contained higher amounts of tannins than their immature counterparts. Acidified acetone-water served as an efficient system for recovery of a maximum amount of condensed tannins from mangrove tree.

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