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C. Padumadasa
Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda 10250, Sri Lanka.

D. Dharmadana
Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda 10250, Sri Lanka.

A. M. Abeysekera
Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda 10250, Sri Lanka.

M. G. Thammitiyagoda
Animal Centre, Medical Research Institute, Colombo 8 - 00800, Sri Lanka.

Correspondence:

C. Padumadasa
Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda 10250, Sri Lanka.

Investigation of Ethyl Acetate Soluble Proanthocyanidins in the Inflorescence of *Cocos nucifera* L.

Padumadasa C, Dharmadana D, Abeysekera AM, Thammitiyagoda MG

Abstract
The immature inflorescence of *Cocos nucifera* L. variety aurantiaca is used in Ayurveda for the treatment of menorrhagia in Sri Lanka. Preliminary phytochemical screening revealed that immature inflorescence predominantly contains proanthocyanidins. The ethyl acetate soluble proanthocyanidin (EASPA) fraction obtained from an acetone/water (7:3) extract of the immature inflorescence was effectively purified and separated from other phenolic compounds by chromatography on sephadex LH-20. EASPA has been fully characterized by chromatographic, spectroscopic and spectrometric methods. Acid catalyzed cleavage followed by TLC indicated that EASPA is composed of (epi)catechin and (epi)afzelechin monomeric units while thiolysis followed by ^{1}H and ^{13}C NMR spectroscopy revealed the monomers to be the epi-isomers. ^{13}C NMR studies of EASPA showed signals characteristic for epicatechin units indicating that EASPA is composed mainly of epicatechin units. Confirming these epicatechin oligomers with degrees of polymerization ranging from 2-5 and mixed oligomers, epicatechin-epiafzelechin dimer and epicatechin-epicatechin-epiafzelechin trimer were observed by ESI-MS.

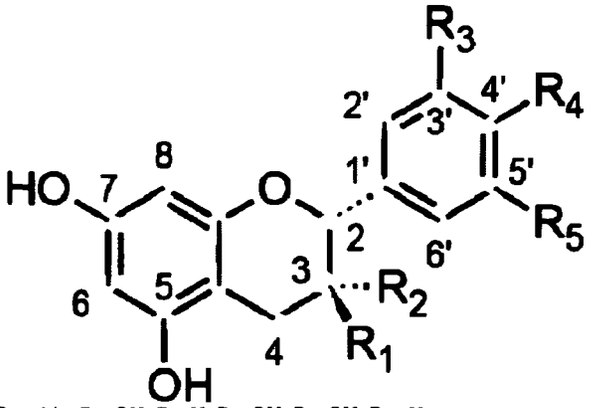
Keywords: *Cocos nucifera* inflorescence, proanthocyanidin, epicatechin, epiafzelechin, thiolysis.

1. Introduction

Cocos nucifera L., the coconut palm is found widely distributed in the Asian continent and in parts of South America and Africa [1]. It is a member of the monocotyledonous family Arecaceae (Palmae) and is the only species of the genus. Coconut palm is a cultivated crop in Sri Lanka, which is a major producer of coconut in the world.

In Sri Lanka, the immature inflorescence of *Cocos nucifera* L. is used by Ayurvedic and traditional medical practioners for the treatment of menorrhagia. *Cocos nucifera* L. is classified in to three varieties in Sri Lanka: Typical, Nana and Aurantiaca [2]. Of this, the orange coloured variety aurantiaca, is used for this purpose. Our preliminary phytochemical screening revealed that the inflorescence of *Cocos nucifera* L. contains high level of proanthocyanidins. Presence of proanthocyanidins in husk fibre of coconut [3], green coconut bark [4] and coconut water [5] have also been reported. Proanthocyanidins have recently attracted a considerable amount of attention in the fields of medicine, health and nutrition. They have been reported to exhibit antioxidant [6], anti-inflammatory [6], bacterial anti-adhesion [7], anticancer [8], and cardioprotective [9] activities. There is evidence also to suggest that proanthocyanidins may play a role in the treatment of menorrhagia [10]. Proanthocyanidins are oligomers or polymers made up of flavan-3-ol monomeric units. The most common flavan-3-ol units are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin, while (+)-afzelechin and (-)-epiafzelechin have been reported to a lesser extent (Fig. 1) [11]. These monomers may carry acyl/glycosyl substituents linked to the C-3 or the C-5 position. The flavan-3-ol units in proanthocyanidins are mainly linked through C-4 to C-8 or sometimes C-4 to C-6 bonds. Proanthocyanidins that contain only these linkages are named as B-type proanthocyanidins. When additional ether linkages are found (usually between C-2 and C-7), the compounds are named as A-type proanthocyanidins. A large variety of different proanthocyanidins have been reported that differ depending on the monomeric unit, substitution pattern of the monomeric unit and the extent of oligomerization.

In this paper, we report the extraction, purification and characterization of the ethyl acetate soluble proanthocyanidin (EASPA) fraction of the immature *Cocos nucifera* L. inflorescence.



Catechin: R_1=OH; R_2=H; R_3=OH; R_4=OH; R_5= H
Epicatechin: R1=H; R2=OH; R3=OH; R4=OH; R5= H
Gallocatechin: R1=OH; R2=H; R3=OH; R4=OH; R5= OH
Epigallocatechin: R1=H; R2=OH; R3=OH; R4=OH; R5= OH
Afzelechin: R_1=OH; R_2=H; R_3=H; R_4=OH; R_5= H
Epiafzelechin: R1=H; R2=OH; R3=H; R4=OH; R5=H

Fig 1: Chemical structures of flavan-3-ol units found in proanthocyanidins.

2. Materials and Methods

2.1. Materials

^{1}H and ^{13}C NMR spectra were recorded in deuterated methanol for purified EASPA and purified thiolyzed fractions with a Bruker Avance AV-500 NMR spectrometer operating at 600 and 150 MHz, respectively. The electrospray ionization mass spectrum (ESI-MS) of purified EASPA was obtained from an Applied Biosystems QSTAR XL quadrupole time of flight mass spectrometer in positive ion mode. Sample was dissolved in methanol and the solution was introduced into the ion spray source with a 100 µL syringe. Preparative HPLC separation was carried out on a LC908W-C60 Recycling Preparative HPLC (Japan Analytical Industry Co., Ltd.), equipped with UV Detector 310 and RI Detector RI-5 using a 20 mm x 270 mm JAIGEL-ODS-L-80 C18 column with 2 mL/min flow rate. Column chromatography was performed using Sephadex LH20 (25-100 µm, Bio-Science, Uppsala). Thin layer chromatography (TLC) was performed on Cellulose F_{254} (0.2 mm, Merck) and developed with forestal solvent system (conc. HCl-glacial acetic acid-water, 3:30:10) and precoated Kieselgel 60 F_{254} plates (0.2 mm, Merck KGaA) with ethyl acetate: dichloromethane: methanol: formic acid (5.8:3.8:0.2:0.2) as the solvent system and visualized by spraying with Vanillin-HCl reagent upon heating. All chemicals and solvents were of AR or HPLC grade and purchased from Sigma-Aldrich, Germany. Water when used was distilled using GFL distillation apparatus. HPLC grade solvents and water purified by a Milli-Q system were used for preparative HPLC work.

2.2. Plant material

Inflorescences were collected from healthy adult *Cocos nucifera* L. (var. aurantiaca) palms situated in the University of Sri Jayewardenepura premises, Sri Lanka from May 2012 to April 2014. Immature inflorescence (the inflorescence which was situated just above the freshly opened inflorescence in the palm) was plucked and the spathe was removed. The inflorescence was botanically authenticated by Mr. I. U. Kariyawasam of the Department of Botany and voucher specimen (Assess. No. A3 S13, 001) was deposited in the herbarium of the Department of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka.

2.3. Extraction of proanthocyanidins

Proanthocyanidins were extracted according to a previously published method with minor modifications [12]. Evenly chopped inflorescence (750 g fresh weight) was placed with 1750 mL of 70% aqueous acetone containing 0.1% ascorbic acid and refluxed for 2 hours. After cooling to room temperature and filtration, the extract was saturated with sodium chloride. The acetone layer that salted out was removed and washed with the aqueous layer of sodium chloride saturated 70% aqueous acetone containing 0.1% ascorbic acid (200 mL × 3). The resulting acetone layer was evaporated under vacuum at 45 °C. The viscous residue obtained was then mixed with an equal volume of water (85 mL) and extracted with petroleum ether (40-60 °C) (175 mL × 3). The aqueous layer was then extracted with ethyl acetate (175 mL × 3). The combined ethyl acetate extracts were dried over anhydrous sodium sulphate and evaporated under vacuum at 45 °C to produce the crude ethyl acetate soluble proanthocyanidin (EASPA) fraction as a light brown powder (1.55 g).

2.4. Purification of proanthocyanidins

The EASPA was purified by chromatography on sephadex LH-20, according to reported methods [13]. The crude EASPA (1.00 g) was dissolved in a minimum volume of 95% aqueous ethanol and applied to the column equilibrated with the same solvent. The non-proanthocyanidin phenolics were eluted first with 95% aqueous ethanol (1600 mL). Proanthocyanidin phenolics were then eluted with 70% aqueous acetone (500 mL). Collected fractions were examined with Prussian blue and acid catalyzed cleavage tests. Fractions that gave a positive result for both the tests were combined. The acetone was removed under vacuum at 45 °C and the resulting aqueous residue was freeze dried to yield 0.15 g of purified EASPA as an off white powder.

2.5. Acid catalyzed cleavage studies

Purified EASPA (0.025 g) was refluxed with 2M HCl and the anthocyanidin fraction was extracted using amyl alcohol. The resulting amyl alcohol solution was co-chromatographed alongside anthocyanidin standards on cellulose TLC plates using forestal as the solvent system [14].

2.6. Thiolytic Studies

Thiolysis reaction for the purified EASPA was conducted according to a previously reported method [15]. A mixture of EASPA (0.025 g), benzyl mercaptan (1 mL) and acetic acid (2 mL) in ethanol (5 mL) was refluxed for 4.5 hours. The reaction mixture after cooling to room temperature was concentrated under vacuum at 50 °C. The resulting residue was dissolved in ethanol and chromatographed over sephadex LH-20 using 95% aqueous ethanol. The column fractions were monitored by thin layer chromatography on silica TLC plates using ethyl acetate: dichloromethane: methanol: formic acid, 5.8:3.8:0.2:0.2 as the solvent system and visualized by spraying with vanillin-HCl reagent upon heating. The fractions containing the benzylthioethers were combined and subjected to recycling preparative HPLC. Mobile phase consisted of 60% aqueous acetonitrile containing 0.5% acetic acid. The flow rate was 2 mL/min. The eluate was monitored at 280 nm. Two fractions corresponding to epicatechin benzylthioether and epiafzelechin benzylthioether were obtained. Each benzylthioether fraction was concentrated under vacuum at 45 °C, freeze dried and subjected to ^{1}H and ^{13}C NMR spectroscopic analyses.

Epicatechin-4-benzylthioether: 0.008 g; off white amorphous powder; ^{1}H NMR (CD_3OD, 600 MHz) δ 3.83 (1H, d, J = 1.8 Hz, H-4), 3.95 (2H, s, -SCH_2-), 4.03 (1H, d, J = 1.8 Hz, H-3), 5.20 (1H, s, H-2), 5.88, 5.94 (each 1H, d, J = 2.4 Hz, H-6, 8), 6.66 (1H, dd, J = 1.8, 9.6 Hz, H-6'), 6.74 (1H, d, 9.6 Hz, H-5'), 6.91 (1H, d, J = 1.8 Hz, H-2'), 7.10-7.40 (5H, m, aromatic-H); ^{13}C NMR (CD_3OD, 150 MHz) δ 37.95 (CH_2, -SCH_2-), 43.96 (CH, C-4), 71.56 (CH, C-3), 75.59 (CH, C-2), 95.71 (CH, C-8), 96.77 (CH, C-6), 100.16 (C, C-4a), 115.26 (CH, C-2'), 115.89 (CH, C-5'), 119.18 (CH, C-6'), 127.93 (CH, C-4''), 129.52 (CH, C-3'', C-5''), 130.06 (CH, C-2'', C-6''), 132.09 (C, C-1'), 140.50 (C, C-1''), 145.78 (C, C-3'), 146.00 (C, C-4'), 157.31 (C, C-8a), 158.95 (C, C-5), 159.13 (C, C-7).

Epiafzelechin-4-benzylthioether: 0.005 g; off white amorphous powder; ^{1}H NMR (CD_3OD, 600 MHz) δ 3.82 (1H, d, J = 1.8 Hz, H-4), 3.95 (2H, s, -SCH_2-), 4.05 (1H, d, J = 1.8 Hz, H-3), 5.26 (1H, s, H-2), 5.88, 5.94 (each 1H, d, J = 2.4 Hz, H-6, 8), 6.75 (2H, d, J = 10.2 Hz, H-2', 6'), 7.10-7.50 (7H, m, H-3', 5', aromatic-H). ^{13}C NMR (CD_3OD, 125 MHz) δ 38.02 (CH_2, -SCH_2-), 44.14 (CH, C-4), 71.57 (CH, C-3), 75.57 (CH, C-2), 95.00-100.00 (CH, C-6, C-8 and C-2', C-6'), 100.16 (C, C-4a), 115.74 (CH, C-3', C-5'), 127.90 (CH, C-4''), 129.53 (CH, C-3'', C-5''), 130.07 (CH, C-2'', C-6''), 131.36 (C, C-1'), 140.72 (C, C-1''), 157.93 (C, C-4'), 157.33 (C, C8a), 158.95 (C, C-5), 159.16 (C, C-7).

2.7. Spectroscopic and spectrometric studies

Purified EASPA was analyzed by ^{13}C NMR spectroscopy and electrospray ionization-mass spectrometry in positive ion detection mode.

3. Results and Discussion

Extraction and purification of proanthocyanidins in the immature inflorescence of *Cocos nucifera* L. was carried out according to previously published methods with minor modifications [12, 13]. Proanthocyanidins in the ethyl acetate soluble fraction of an acetone/water (7:3) extract of *Cocos nucifera* L. inflorescence was purified on Sephadex LH-20 [13]. The proanthocyanidin fraction was obtained by eluting the Sephadex LH-20 column with acetone/water (7:3) after first eluting the non-proanthocyanidin phenolics with ethanol/water (9.5:0.5). All the fractions eluting from the sephadex LH-20 column were analyzed for the presence of phenolics by the Prussian blue test and the proanthocyanidins by the acid catalyzed cleavage to anthocyanidins. Positive results for both the tests were obtained only for the fraction eluting with acetone/water (7:3). This confirmed the presence of proanthocyanidins in that fraction and an effective separation from non-proanthocyanidin phenolics. This fraction upon freeze drying yielded the purified EASPA as an off white powder in 0.2% by weight of the fresh inflorescence. The moisture content of the inflorescence was determined to be 77.49%.

Acid catalyzed cleavage followed by thin layer chromatographic studies of the EASPA showed the presence of two anthocyanidins, cyanidin and pelargonidin, indicating that EASPA is composed of (epi)catechin and (epi)afzelechin monomeric units. This was confirmed by thiolysis studies of the EASPA carried out according to published procedures. In addition, thiolysis studies revealed the monomers to be the epi-isomers. In the thiolysis reaction, the flavanyl extension units of the proanthocyanidins are captured by benzylmercaptan to form the corresponding benzylthioether derivatives while the terminal units are released in the native form. Purification on Sephadex LH-20 followed by recycling preparative HPLC yielded two benzylthioether fractions which were identified as epicatechin-4-benzylthioether and epiafzelechin-4-benzylthioether by ^{1}H and ^{13}C NMR spectroscopy (Fig. 2). The two compounds are easily distinguished from each other from the pattern obtained for the flavanyl B-ring in ^{1}H and ^{13}C NMR spectra. The chemical shift for the C-2 in the ^{13}C NMR spectra is diagnostic for the relative stereochemistry (2,3-*cis*/2, 3-*trans*) of the flavanyl C-ring. The C-2 chemical shift of δ 75.59 for the benzylthioether of (epi)catechin clearly indicates a 2,3-*cis* stereochemistry in accordance with published data [16]. To the best of our knowledge published NMR spectral data is not available for the benzylthioether of (epi)afzelechin. However, data is available for afzelechin and epiafzelechin units of polymeric proanthocyanidins [17]. The C-2 chemical shift for the benzylthioether of (epi)afzelechin is δ 75.57. Taking in account the fact that there is a slight lowering in the chemical shift for the C-2 signal going from the polymeric proanthocyanidin to benzylthioether derivative, we provisionally assigned the 2,3-*cis* stereochemistry to the (epi)afzelechin unit as well [17]. Thus, the EASPA is composed of epicatechin and epiafzelechin units.

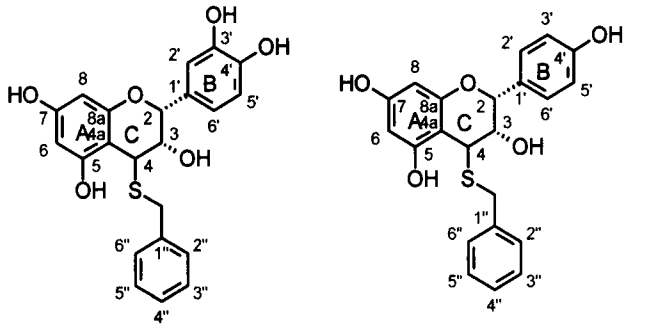


Fig 2: Thiolysis products of EASPA from the inflorescence of *Cocos nucifera* L.

The ^{13}C NMR spectrum of the purified EASPA is shown in Fig. 3. The signals were assigned by comparison with published data for other proanthocyanidins [18]. The spectrum shows characteristic ^{13}C signals consistent with proanthocyanidins predominantly composed of epicatechin units. The aromatic carbons of the A-ring appeared in the range δ 160-90 localized towards the two ends. The signals clustered between δ 160-155 correspond to C-5, C-7 and C-8a. The signal due to C-8 of the extension unit fall between δ 110-105, whereas that of the terminal unit gives a much sharper signal at δ 97 close to C-6, which is slightly more deshielded. The small lines between δ 100 and 105 are due to C-4a. Comparison of the C-8 chemical shift of the extension unit with that published suggests the probable interflavanoid linkage to be B-type where the flavanyl units are singly linked between C-4 and C-8. The aromatic carbons of the B-ring appear in a narrow range between δ 150-110. The signal at δ 145.5 is assigned to C-3' and C-4' and signals at δ 132.3 and 119.0 are assigned to C-1' and C-6', respectively. The fairly sharp signal at δ 115.8 is attributed to C-2' and C-5'. The rest of the signals in the spectrum starting from δ 90 are due to the aliphatic carbons of the C-ring. The C-2, C-3 and C-4 of the extension unit give signals at δ 77.0, 73.0 and 37.5,

respectively. The region between δ 90 – 70 is sensitive to the stereochemistry of the C-ring. The sharp signal at δ 77.0 of the C-2 extension unit is consistent with 2,3-*cis* stereochemistry, whereas a small signal at δ 84.0 and even smaller signal at δ 80.0 correspond to C-2 of the terminal unit in 2,3-*trans* and 2,3-*cis* stereochemistry, respectively. Proanthocyanidins consisting of 2,3-*cis* or 2,3-*trans* flavanyl extension units and terminating with both 2,3-*cis* and 2,3-*trans* flavanyl units have been reported previously [19]. The very weak signals for C-2 terminal carbon relative to the corresponding signals for extender carbon suggest that EASPA is predominantly composed of epicatechin units. The C-3 and C-4 of the terminal unit gives rise to small signals at δ 70 and 30, respectively. The signal at δ 128.5 is due to benzene, which occurs as an impurity. Thus, all the signals in the ^{13}C NMR spectrum could be assigned to epicatechin units. There were no detectable signals for epiafzelechin units although its presence was shown by acid catalyzed cleavage and thiolysis studies. This indicates that EASPA is comprised mainly of epicatechin units and only a few of epiafzelechin units. Analysis of ^{13}C DEPT spectra of the EASPA allowed identification of the tertiary and quaternary carbons of epicatechin units, which further confirmed the above assignments.

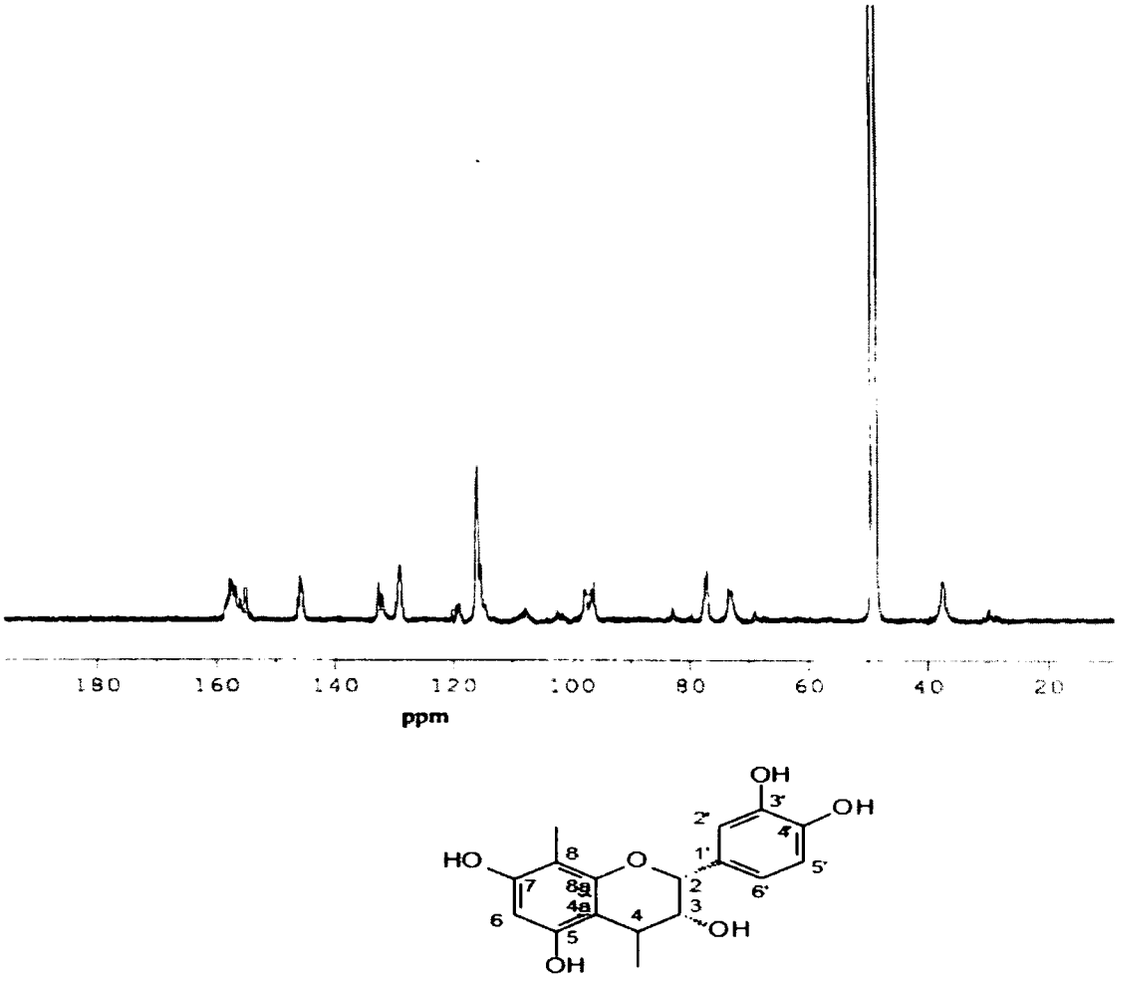


Fig 3: ^{13}C NMR spectrum of EASPA from the inflorescence of *Cocos nucifera* L. (solvent CD_3OD).

The ESI-MS data performed in the positive ion mode provided further evidence in support of ^{13}C NMR spectral data of the EASPA being composed mainly of epicatechin units. In addition, it provided information about the oligomeric profile of the EASPA. According to the mass spectrum, the highest oligomer detected was a pentamer. The peaks at *m/z* 579, 867, 1155 and 1443 correspond to molecular masses $(M+H)^+$ of epicatechin units with degrees of polymerization ranging between 2-5. In addition, mixed oligomers of epicatechin and epiafzelechin were detected in the mass spectrum with the peaks at *m/z* 563 and 851 being assigned to epicatechin-epiafzelechin dimer and epicatechin-epicatechin-epiafzelechin trimer, respectively.

4. Conclusion

Proanthocyanidin fraction, EASPA obtained from the inflorescence of *Cocos nucifera* L. has been effectively purified and separated from other phenolic compounds by chromatography on sephadex LH-20. This fraction has been fully characterized. EASPA is composed of epicatechin and epiafzelechin monomeric units and contains epicatechin oligomers ranging from dimer to pentamer, epicatechin-epiafzelechin dimer and epicatechin-epicatechin-epiafzelechin trimer.

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