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Investigation of aqueous soluble proanthocyanidins in the inflorescence of *Cocos nucifera* L.

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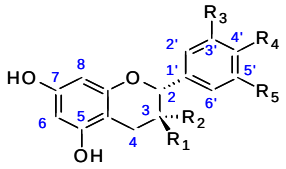
Abstract
Proanthocyanidins belong to a class of polyphenolic compounds called flavonoids and they have been reported to exhibit important pharmacological activities. We have previously reported the extraction, purification and characterization of the ethyl acetate soluble proanthocyanidins (EASPA) of the inflorescence of *Cocos nucifera* L. The aqueous soluble proanthocyanidins (AQSPA) were extracted from the immature inflorescence using acetone/water (7:3) and purified using chromatography on sephadex LH-20. Acid catalyzed cleavage studies and thiolytic studies were carried out to determine the monomeric composition of AQSPA. In addition, AQSPA was subjected to ^{13}C NMR spectroscopy to study the occurrence of proanthocyanidins in this fraction. The AQSPA fraction of an acetone/water (7:3) extract of *Cocos nucifera* L. inflorescence was purified by chromatography on Sephadex LH-20 to yield purified AQSPA as an off white powder in 1.17% on dry weight basis. Acid catalyzed cleavage followed by TLC analysis indicated that AQSPA is composed of (epi)catechin and (epi)afzelechin monomeric units while thiolysis followed by NMR spectroscopy revealed the monomers to be the epi-isomers. ^{13}C NMR studies of AQSPA showed signals characteristic for epicatechin units indicating that AQSPA is composed mainly of epicatechin units with few of epiafzelechin units as previously reported for EASPA. However, AQSPA is comprised of high molecular weight proanthocyanidins and the oligomeric profile is different to that of EASPA.

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

1. Introduction
The coconut palm is botanically known as *Cocos nucifera* L. and is a member of the monocotyledonous family Arecaceae and is the only species of the genus. It is found over the Asian continent, Pacific islands, Africa and in Central and South America [1]. *Cocos nucifera* L. is classified in to three varieties in Sri Lanka: Typica, Nana and Aurantiaca [2]. The orange coloured variety aurantiaca, is intermediate in stature and is popularly known as "thembili" in Sinhala in Sri Lanka. The immature inflorescence of *Cocos nucifera* L. (var. aurantiaca) is used by Ayurvedic and traditional medical practitioners for the treatment of menorrhagia in Sri Lanka. Our previous studies have shown that the inflorescence of *Cocos nucifera* L. predominantly contains proanthocyanidins [3]. In addition, proanthocyanidins in husk fibre of coconut [4], green coconut bark [5] and coconut water [6] have also been reported.
Proanthocyanidins are secondary metabolites that belong to a class of polyphenolic compounds called flavonoids. Apart from lignin, proanthocyanidins represent the most abundant class of natural phenolic compounds and they are the most abundant polyphenols in our diet [7]. Humans take in significant quantities of proanthocyanidins when they consume fruits, vegetables, cereals, legumes and grains, spices such as cinnamon and nutmeg as well as beverages including tea, cocoa, red wine and beer [8, 9]. The occurrence of proanthocyanidins in nature is extremely diverse. That is they exist as dimers, trimers, higher oligomers or polymers consisting of flavan-3-ol units. The most common flavan-3-ol units are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin (Fig. 1) [10]. There are other flavan-3-ols that have been found in plants but rarely such as (+)-afzelechin and (-)-epiafzelechin [10]. These monomers may carry acyl/glycosyl substituents linked to the C-3 or the C-5 position. There are two types of linkages between successive units in proanthocyanidins. When the linkage is between the C-4 of the upper unit and the C-8 and C-6 of the lower unit, proanthocyanidins are named as B-type proanthocyanidins. A-type proanthocyanidins possess an additional ether linkage between C-2 of the upper unit and C-7

of the lower unit and has two hydrogen atoms less compared to the B-type. The proanthocyanidins that exclusively consist of (epi)catechin units are designated as procyanidins, which is the most abundant type of proanthocyanidins in plants. The proanthocyanidins containing (epi)gallocatechin or (epi)afzelechin units are termed as prodelphinidin and propelargonidin respectively.



Catechin: R_1=OH; R_2=H; R_3=OH; R_4=OH; R_5=H

Epicatechin: R_1=H; R_2=OH; R_3=OH; R_4=OH; R_5=H

Gallocatechin: R_1=OH; R_2=H; R_3=OH; R_4=OH; R_5=OH

Epigallocatechin: R_1=H; R_2=OH; R_3=OH; R_4=OH; R_5=OH

Afzelechin: R_1=OH; R_2=H; R_3=H; R_4=OH; R_5=H

Epiafzelechin: R_1=H; R_2=OH; R_3=H; R_4=OH; R_5=H

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

Proanthocyanidins have recently attracted a considerable amount of attention in the fields of medicine, health and nutrition. They have been reported to exhibit antioxidant [11], anti-inflammatory [11], bacterial anti-adhesion [12], anticancer [13], and cardio protective [14] activities. Today, nutritional supplements containing proanthocyanidins extracts from various plant sources are available, alone or in combination with other nutrients, as herbal extracts, capsules, or tablets [15, 16].

We have previously reported the extraction, purification and characterization of the ethyl acetate soluble proanthocyanidins (EASPA) fraction of the inflorescence of *Cocos nucifera* L. [3]. In this paper, we report the extraction, purification and determination of monomeric composition of the aqueous soluble proanthocyanidin (AQSPA) fraction of the immature inflorescence of *Cocos nucifera* L. (var. aurantiaca).

2. Materials and Methods

2.1 Materials

^{1}H and ^{13}C NMR spectra were recorded in deuterated methanol for purified AQSPA and purified thiolyzed fractions with a Bruker Avance AV-500 NMR spectrometer operating at 600 and 150 MHz, respectively. Preparative HPLC separation was carried out on a LC-908W-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 LH-20 (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, and University of Sri Jayewardenepura, Sri Lanka.

2.3. Extraction of AQSPA

Proanthocyanidins were extracted according to a previously published method with minor modifications [17]. Evenly chopped inflorescence (750 g fresh weight) was placed with 1750 mL of acetone/water (7:3) 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 acetone/water (7:3) containing 0.1% ascorbic acid (200 mL × 3). The resulting acetone layer was evaporated under vacuum at 45 ºC. The viscous residue obtained was then mixed with an equal volume of water (85 mL) and extracted with petroleum ether (40-60 ºC) (175 mL × 3). The aqueous layer was extracted with ethyl acetate (175 mL × 3) to yield crude EASPA. Then the remaining aqueous phase was freeze dried to yield crude AQSPA fraction as a light brown powder (7.81 g).

2.4. Purification of AQSPA

Crude AQSPA was purified by chromatography on sephadex LH-20 according to previously published methods [18]. The AQSPA powder (1.0 g) was dissolved in a minimum volume of methanol/water (3:7) containing 0.1 % ascorbic acid and applied to the column equilibrated with the same solvent. Non-proanthocyanidin fraction was first eluted out with methanol/water (3:7) containing 0.1 % ascorbic acid (1500 mL). Subsequently, proanthocyanidin fraction was eluted with acetone/water (7:3) (500 mL). Collected fractions were tested with Prussian blue and acid catalyzed cleavage tests. Acetone/water fraction, which gave a positive results for both the tests were combined. Acetone in combined fraction was removed under vacuum at 45 °C and remaining aqueous residue was freeze dried to yield 0.25 g of purified AQSPA as an off white powder.

2.5. Acid catalyzed cleavage studies

Purified AQSPA (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 [19].

2.6. Thiolytic studies

Thiolysis reaction for the purified AQSPA was conducted according to a previously reported method [20]. A mixture of AQSPA (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 ethanol/water (9.5:0.5). 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. The 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 epicatechinbenzylthioether and epiafzelechinbenzylthioether 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 3.90 (1H, d, J = 1.8 Hz, H-4), 3.85 (2H, s, -SCH_2-), 4.06 (1H, d, J = 1.8 Hz, H-3), 5.30 (1H, s, H-2), 5.78, 5.85 (each 1H, d, J = 2.4 Hz, H-6,8), 6.71 (1H, dd, J = 1.8, 9.6 Hz, H-6'), 6.79 (1H, d, 9.6 Hz, H-5'), 6.89 (1H, d, J = 1.8 Hz, H-2'), 7.10-7.40 (5H, m, aromatic-H); ^{13}C NMR (CD_3OD, 150 MHz) δ 37.90 (CH_2, -SCH_2-), 43.89 (CH, C-4), 71.51 (CH, C-3), 75.61 (CH, C-2), 95.75 (CH, C-8), 96.76 (CH, C-6), 100.19 (C, C-4a), 115.23 (CH, C-2'), 115.91 (CH, C-5'), 119.15 (CH, C-6'), 127.88 (CH, C-4''), 129.50 (CH, C-3'', C-5''), 130.03 (CH, C-2'', C-6''), 132.02 (C, C-1'), 140.48 (C, C-1''), 145.68 (C, C-3'), 146.01 (C, C-4'), 157.29 (C, C-8a), 158.90 (C, C-5), 159.10 (C, C-7).

Epiafzelechin-4-benzylthioether: 0.005 g; off white amorphous powder; ^{1}H NMR (CD_3OD, 600 MHz) δ 3.80 (1H, d, J = 1.8 Hz, H-4), 3.91 (2H, s, -SCH_2-), 4.01 (1H, d, J = 1.8 Hz, H-3), 5.20 (1H, s, H-2), 5.81, 5.90 (each 1H, d, J = 2.4 Hz, H-6,8), 6.70 (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 38.07 (CH_2, -SCH_2-), 44.10 (CH, C-4), 71.52 (CH, C-3), 75.50 (CH, C-2), 95.00-100.00 (CH, C-6,C-8 and C-2', C-6'), 100.16 (C, C-4a), 115.69 (CH, C-3', C-5'), 127.88 (CH, C-4''), 129.49 (CH, C-3'', C-5''), 130.03 (CH, C-2'', C-6''), 131.31 (C, C-1'), 140.69 (C, C-1''), 157.98 (C, C-4'), 157.29 (C, C-8a), 158.90 (C, C-5), 159.10 (C, C-7).

2.7. Spectroscopic studies

Purified AQSPA was analyzed by ^{13}C NMR spectroscopy.

3. Results and discussion

3.1. Extraction and purification of AQSPA

Extraction of proanthocyanidins in the immature inflorescence of *Cocos nucifera* L. was carried out according to the previously published method upon modification using acetone/water (7:3) containing 0.1% ascorbic acid as the extraction solvent system [17]. During extraction crude proanthocyanidins in the acetone/water extract were partitioned into ethyl acetate and the remaining aqueous layer was freeze dried to yield the crude AQSPA. The yield of crude AQSPA was 1.04 % (by weight) of the fresh inflorescence. Taking into account the moisture in the inflorescence (77.5 %), the proanthocyanidin content by dry weight of the fraction was 4.62 %. In purification of AQSPA, non-proanthocyanidin phenolics were first eluted out with methanol/water (3:7) and adsorbed proanthocyanidins were eluted with acetone/water (7:3). All the fractions eluting from the sephadex LH-20 column were analyzed for the presence of phenolics by the Prussian blue test and proanthocyanidins by the acid catalyzed cleavage to anthocyanidins. Positive results for both the tests were obtained only for the fraction eluting with acetone/water. 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 AQSPA as an off white powder in 0.26 % by weight of the fresh inflorescence. Proanthocyanidin content of the purified AQSPA by dry weight of the inflorescence was 1.17 %. We have previously reported the yield of crude and purified EASPA [3]. When considering both fractions together, AQSPA comprises over 80% of total proanthocyanidins of the inflorescence of *Cocos nucifera* L.

3.2 Monomeric composition of AQSPA

Proanthocyanidins are compounds that yield anthocyanidin pigments upon acid hydrolysis. (Epi)catechin based proanthocyanidins produce cyanidin, whereas (epi)gallocatechin based proanthocyanidins yield delphinidin. The rare mono substituted (epi)afzelechin based proanthocyanidins yield pelargonidin. TLC analysis of the acid hydrolyzate of purified AQSPA against anthocyanidin standards showed the presence of cyanidin and pelargonidin indicating that (epi)catechin and (epi)afzelechin as the monomer units. To confirm that the proanthocyanidins are composed of (epi)catechin and (epi)afzelechin monomers, depolymerization through thiolysis reaction was carried out according to a method previously reported using benzyl mercaptan [20]. In this reaction, the flavanyl extension units of the proanthocyanidins are captured by benzyl mercaptan to form the benzylthioether derivatives and only the terminal units are released as its native form. Two benzylthioether fractions were obtained after preparative HPLC. ^{1}H and ^{13}C NMR spectroscopic data of the two fractions revealed them to be benzyl thioethers of epicatechin and epiafzelechin same as in EASPA. ^{1}H NMR spectra of the two benzyl thioether fractions are shown in Fig. 2 and 3. The two compounds are easily differentiated from each other by the pattern obtained for the flavanyl B-ring in ^{1}H and ^{13}C NMR spectra. The stereochemistry (2, 3-*cis*/2, 3-*trans*) of the flavanyl C-ring is indicated by the chemical shift of C-2 in the ^{13}C NMR spectra. According to published data, the C-2 chemical shift of δ 75.61 for the benzylthioether of (epi)catechin clearly indicates a 2, 3-*cis* stereochemistry [21]. Although published NMR spectral data is not available for the benzylthioether of (epi)afzelechin to the best of our knowledge, data is available for afzelechin and epiafzelechin units of polymeric proanthocyanidins [22]. The C-2 chemical shift for the benzylthioether of (epi)afzelechin is δ 75.50. We provisionally assigned the 2, 3-*cis* stereochemistry to the epiafzelechin unit by 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 [22]. Thus, the AQSPA is also composed of epicatechin and epiafzelechin units as EASPA.

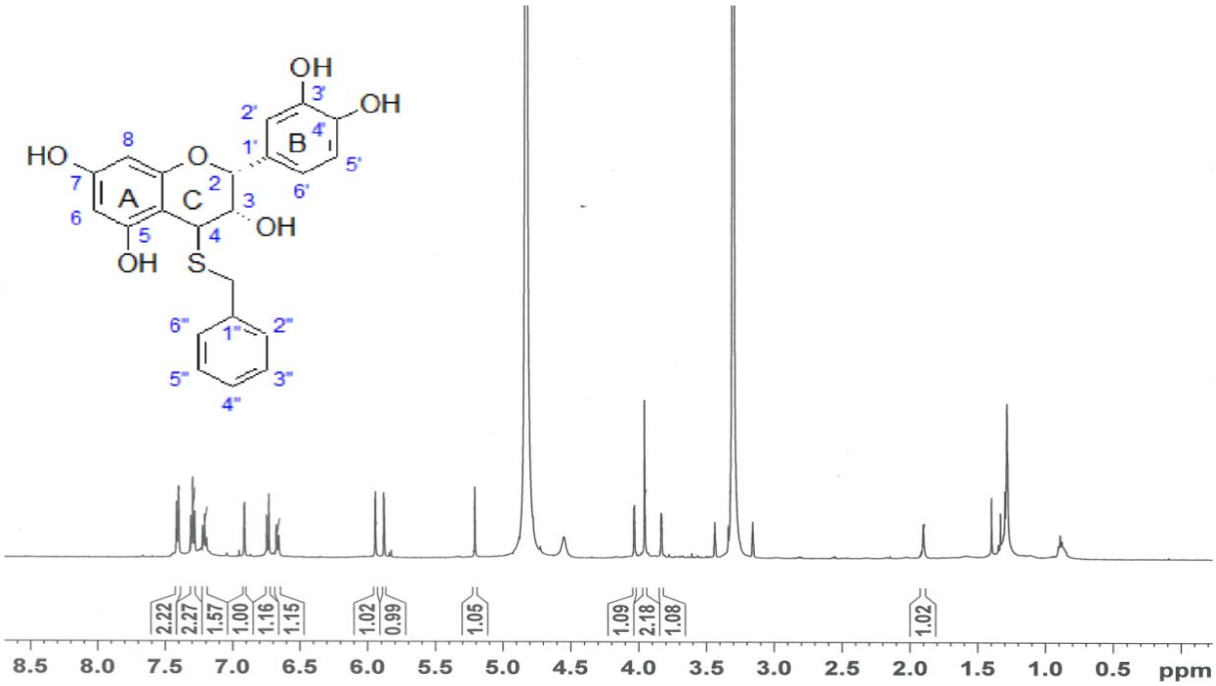


Fig 2. [1]HNMR spectrum of epicatechin-4-benzylthioether.

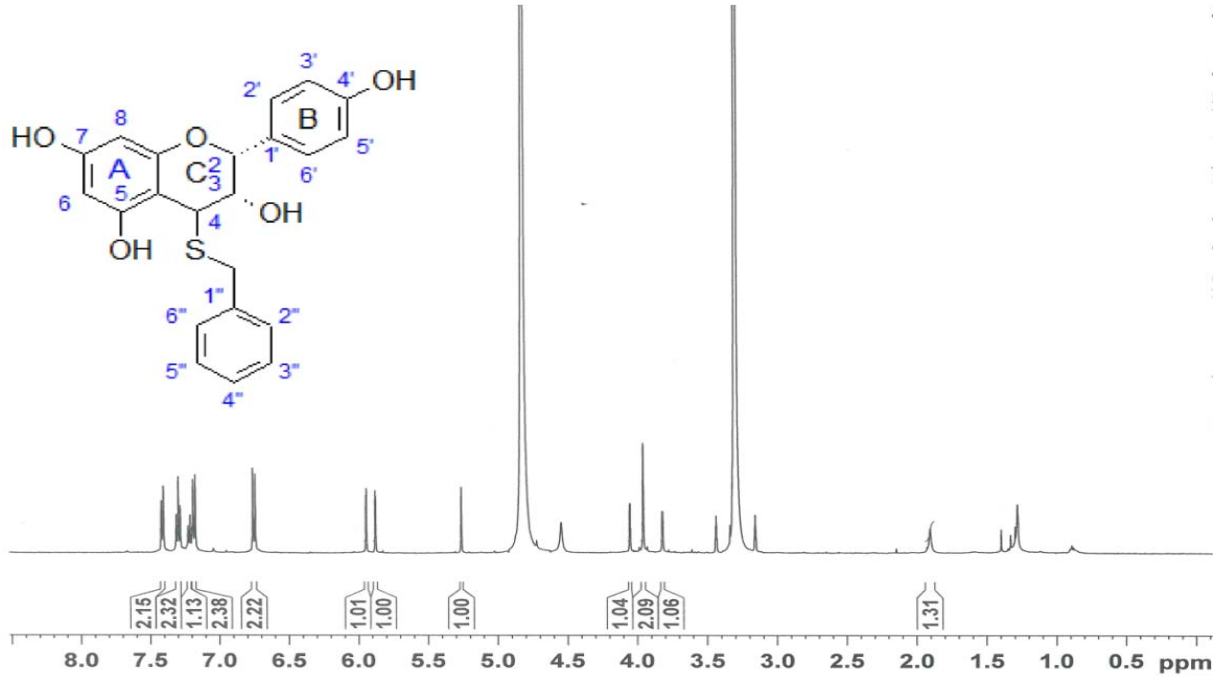


Fig 3. [1]H NMR spectrum of epiafzelechin-4-benzylthioether.

With the confirmation of the monomers of the proanthocyanidin fractions it was necessary to get an idea about their occurrence in the AQSPA fraction. Thus, the purified AQSPA fraction was subjected to ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of the purified AQSPA is shown in Fig. 4. The signals were assigned by comparison with published data for other proanthocyanidins [23]. The ^{13}C NMR spectral data of purified AQSPA was similar to that of EASPA. All the signals in the ^{13}C NMR spectrum except for the signal at 128.5 ppm, which is due to benzene, which occurs as an impurity were assigned to epicatechin and there were no detectable signals for epiafzelechin indicating that it occurs in the AQSPA fraction in low extent as in EASPA. The aromatic carbons of the A-ring appeared in the range δ 160-90 localized towards the two ends. Comparison of the C-8 chemical shift of the extension unit with that published suggests the probable inter flavanoid 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 rest of the signals in the spectrum starting from δ 90 are due to the aliphatic carbons of the C-ring. 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-flavanyl extension units and terminating with both 2,3-*cis* and 2,3-flavanyl units have been reported previously [24]. The very weak signals for C-2 terminal carbon relative to the corresponding signals for extender carbon suggest that

AQSPA is predominantly composed of epicatechin units. The ratio of the peak heights of the signals for C2, C3 and C4 of the extension unit with that of terminal unit indicates the extent of oligomerization of the sample [23]. This ratio is higher in the case of AQSPA compared to that of EASPA, suggesting that the extent of oligomerization is higher in AQSPA than EASPA. Thus, AQSPA is comprised of high molecular weight proanthocyanidins and the oligomeric profile is different to that of EASPA although both fractions are made up of the same monomeric units. This observation is in accordance with published reports, which state that during extraction low molecular weight proanthocyanidins are partitioned into ethyl acetate layer, whereas high molecular weight proanthocyanidins remain in the aqueous layer [18].

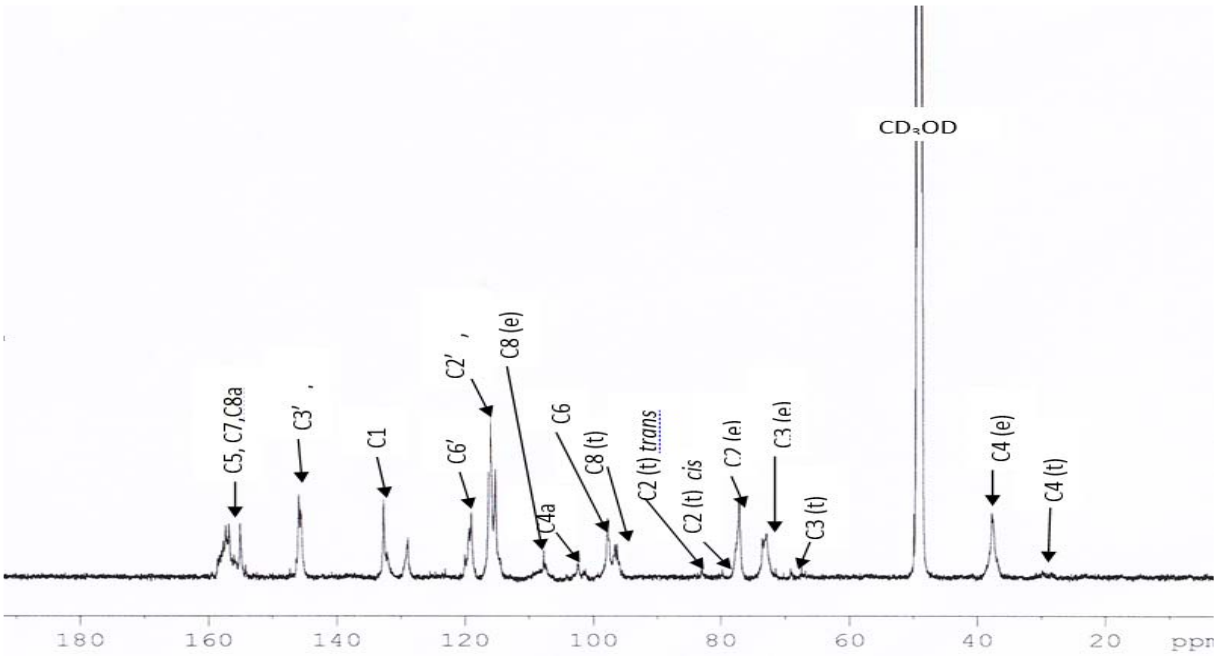


Fig 4. ^{13}C-NMR spectrum of AQSPA from the inflorescence of *Cocos nucifera* L. (solvent CD_3OD).

4. Conclusion

Proanthocyanidin fraction, AQSPA obtained from the acetone/water extract of inflorescence of *Cocos nucifera* L. has been effectively purified and separated from other phenolic compounds by chromatography on sephadex LH-20. It is composed of epicatechin and epiafzelechin monomeric units with epicatechin being the predominant monomeric unit as previously reported for EASPA. However, AQSPA is comprised of high molecular weight proanthocyanidins and the oligomeric profile is different to that of EASPA.

5. Acknowledgement

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