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Distribution of progestogenic proanthocyanidins within the inflorescence of *Cocos nucifera* L.

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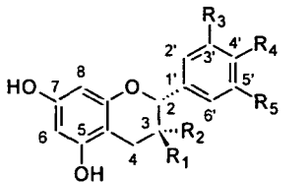
Abstract
In Sri Lanka, the immature inflorescence of *Cocos nucifera* L. is used by Ayurvedic and traditional medical practitioners for the treatment of menorrhagia. We have previously reported that the inflorescence of *Cocos nucifera* L. predominantly contains proanthocyanidins. In addition, the progestogenic activity of ethyl acetate soluble proanthocyanidins of *Cocos nucifera* L. inflorescence has also been reported in relation to its ethnomedical usage. *Cocos nucifera* L. inflorescences at different maturity stages were used to determine the variation of the yield of ethyl acetate soluble proanthocyanidins (EASPA) and aqueous soluble proanthocyanidins (AQSPA) with maturity. In addition, variation of proanthocyanidin content of the inflorescence along the length of the inflorescence at different maturity stages and proanthocyanidin content of different floral parts of the inflorescence were determined by the acid butanol assay. The percentage yield of crude EASPA decreases, whereas percentage yield of crude AQSPA increases, with increasing maturity of the inflorescence. In all stages of development, the middle section of the inflorescence contained the highest level of proanthocyanidins. The female flower has higher proanthocyanidin content than any other floral part of the inflorescence. The current Ayurvedic and traditional medical practice of using the total immature inflorescence just prior to opening appears to optimize the procedure for obtaining an adequate concentration of EASPA (which has been shown to contain progestogenic activity) for therapeutic purposes. Thus, our studies do not indicate the need to change the current Ayurvedic and traditional medical practice of the use of total immature inflorescence just prior to opening for the treatment of menorrhagia.

Keywords: Proanthocyanidin, *Cocos nucifera* inflorescence, acid butanol assay.

1. Introduction
Cocos nucifera L., the coconut palm is found widely distributed in the Asian continent, 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 one of the major producers of coconut in the world.
Ethnomedical usages and biological activities of different parts of the *Cocos nucifera* L. have been reported. In Nigeria, coconut dispersions are used to counteract the effects of over dosage of drugs, poisoning and untoward drug reaction [2]. Traditional healers in Cameroon use coconut shells and roots for the treatment of oral diseases [3]. Various coconut products are used as antihemorrhagic, anti bronchitis, febrifugal and antigingivitic agents in Indian folk medicine [4]. Different extracts of husk fibre have been shown to possess antimicrobial [5], radical scavenging [6], analgesic [6, 7], anti-inflammatory [7], anthelmintic activity [8] and antiproliferative activities [9]. Recent studies have revealed that different extracts from mesocarp exhibit antimalarial [10], vasorelaxant [11] and antihypertensive activities [11]. Several studies found that virgin coconut oil has numerous medicinal properties, including cardioprotective [12], antithrombotic [13] and hypolipidemic activities [13]. Coconut water exhibit antioxidant [14] and hypolipidemic [15] activities. The results obtained from a recent study suggested that the coconut inflorescence has cytoprotective and antihyperglycemic properties [16]. Protective and curative effects of *Cocos nucifera* L. inflorescence on alloxan-induced pancreatic cytotoxicity in rats have also been reported [17].
In Sri Lanka, *Cocos nucifera* L. immature inflorescence, which is just about to open is used by Ayurvedic and traditional medical practitioners for the treatment of menorrhagia [18, 19]. *Cocos nucifera* L. is classified in to three varieties in Sri Lanka: Typica, Nana and Aurantiaca [20]. Of this, the orange coloured variety aurantiaca, which is popularly known as "thembili" in Sinhala is used for this purpose.

We have previously reported that the inflorescence of *Cocos nucifera* L. predominantly contains proanthocyanidins [21]. In addition, the progestogenic activity of ethyl acetate soluble proanthocyanidins of *Cocos nucifera* L. inflorescence has also been reported in relation to its ethnomedical usage [22]. A broad spectrum of medications is used to treat menorrhagia of which synthetic progestogens make up 55% in total of prescriptions [23]. Progestogens act by opposing the action of oestrogens by minimizing the effects of estrogen on target cells, thereby maintaining the endometrium in a state of down-regulation. The end result is suppression of endometrial glandular growth, stromal decidualization, leukocytic infiltration, glandular atrophy and stromal focal necrosis, thereby leading to a reduction in menstrual blood loss [24]. The increase in progesterone levels in female rats administered with EASPA suggests a possible mode of action, which explains the use of coconut inflorescence in controlling menorrhagia in Ayurveda and traditional medicine in Sri Lanka.

Proanthocyanidins, also known as condensed tannins, are oligomers or polymers made up of flavan-3-ol monomeric units. Apart from lignin, they represent the most abundant class of natural phenolic compounds [25]. The most commonly reported flavan-3-ols are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin while (+)-afzelechin and (-)-epiafzelechin have been reported to a lesser extent [25] (Fig. 1). Flavan-3-ol subunits may carry acyl or glycosyl substituents at C3 or C5 positions [25]. There are two types of linkages between successive units in proanthocyanidins. When the linkage is between C4 of the one unit and C8 or C6 of the other unit, proanthocyanidins are named as B-type proanthocyanidins. A-type proanthocyanidins mainly possess an additional ether linkage between C2 of the one unit and C7 of the other unit, this subclass has two hydrogen atoms less compared to the B-type [25]. Thus, the occurrence of proanthocyanidins in nature is extremely diverse.



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 [26], anti-inflammatory [26], bacterial anti-adhesion [27], anticancer [28], and cardioprotective [97] activities. There is evidence also to suggest that proanthocyanidins may play a role in the treatment of menorrhagia [30].

Since proanthocyanidins are one of the major compound groups present in *Cocos nucifera* L. inflorescence and it may be responsible for the ethnomedical usage of *Cocos nucifera* L. inflorescence in menorrhagia, we carried out a quantitative study of the proanthocyanidin distribution within the inflorescence as well as its variation with the maturity of the inflorescence. The results will be useful to verify the use of the total immature inflorescence of *Cocos nucifera* L. just prior to opening for the treatment of menorrhagia in Ayurveda and traditional medicine in Sri Lanka.

2. Materials and Methods

2.1. Materials

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.

2.2. Plant materials

Inflorescences were collected from healthy adult king coconut palms situated in the University of Sri Jayewardenepura premises. 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. Inflorescence was plucked and the spathe was removed. Maturity stages of the inflorescences used (Fig. 2) are described according to literature [29]. Inflorescences at stage 0, 1, 2 and 3 were used to determine the variation of the yield of ethyl acetate soluble proanthocyanidins (EASPA) and aqueous soluble proanthocyanidins (AQSPA) with maturity. Further, inflorescences at stage 1, 2, 3 were used in quantification studies.

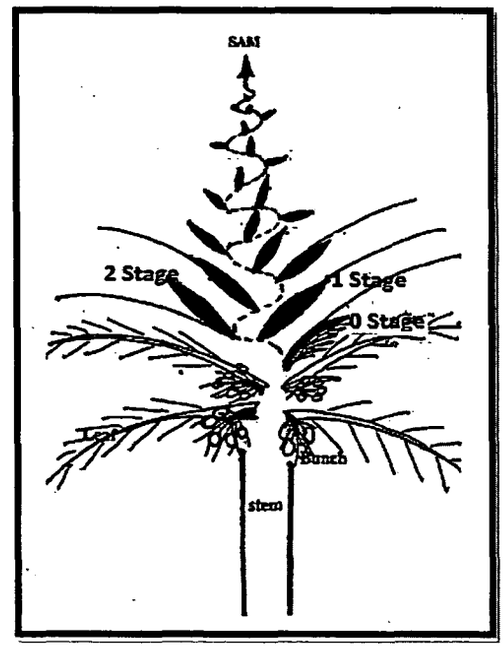


Fig 2: Schematic diagram of different maturity stages of inflorescences within the coconut palm. *SAM=Shoot Apical Meristem.*

Stage 0 - The freshly open (youngest mature) inflorescence in the palm.

Stage 1 - The inflorescence, which was situated just above the freshly open inflorescence in the palm (the inflorescence which is to open next)

Stage 2 - The inflorescence, which was situated just above the stage 1 inflorescence

Stage 3 - The smallest (the most immature) inflorescence in the palm

2.3. Extraction of proanthocyanidins from Cocos nucifera L. inflorescences at different maturity stages

Inflorescences of four different maturity stages (stage 0, 1, 2 and 3) were collected from the same plant. Extraction was carried out as previously reported for each inflorescence [21, 32, 33]. Evenly chopped inflorescence was placed with 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. 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). The aqueous layer was extracted with ethyl acetate to yield crude EASPA. Then the remaining aqueous phase was freeze dried to yield crude AQSPA.

2.4. Quantification of proanthocyanidins by acid butanol assay

The variation of proanthocyanidin content of the inflorescence along the length of the inflorescence with maturity was determined. In addition, proanthocyanidin content of different floral parts of stage 1 inflorescence (the inflorescence used in Ayurveda and traditional medicine for the treatment of menorrhagia) was studied as well.

2.4.1 Preparation of calibration curve

AQSPA was purified according to a previously published method and was used as the standard to prepare the calibration curve [34]. Stock solution of AQSPA was prepared by dissolving 5.0 mg of AQSPA in 25.00 mL of 70% aqueous acetone containing 0.1% ascorbic acid. Stock solution was used to prepare following concentrations of AQSPA solutions: 0.02 mg/mL, 0.04 mg/mL, 0.06 mg/mL, 0.08 mg/mL, 0.10 mg/mL, 0.17 mg/mL and 0.20 mg/mL. Acid butanol assay was carried out in triplicate for each concentration. Mean absorbance of the each concentration was calculated. Calibration curve was drawn using six concentrations against absorbance.

2.4.2 Preparation of sample solutions

Each inflorescence was divided into three equal parts according to the length: bottom, middle and top sections (Fig. 3). Rachis, rachilla, male flower and female flower were used as different floral parts (Fig 4). Each section or floral part was chopped and approximately 1.00 g of sample was refluxed with 300.00 mL of 70% aqueous acetone containing 0.1% ascorbic acid for 4 hours. After cooling to room temperature, the acid butanol assay was performed. Experiment was carried out in triplicate for each section or floral part.

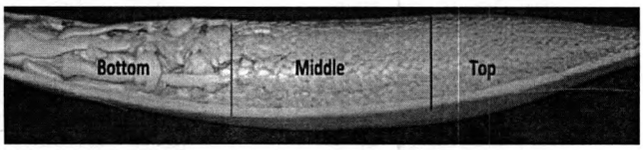


Fig. 3: Bottom, middle and top sections of *Cocos nucifera* L. inflorescence.

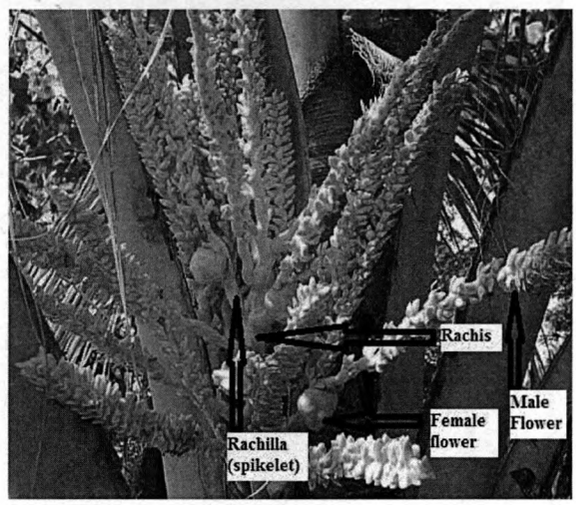


Fig 4: Different floral parts of the inflorescence of *Cocos nucifera* L.

2.4.3 Acid butanol assay

Acid butanol assay was performed according to the method described in literature [37].The Butanol-HCl reagent (95:5 v/v) was prepared by mixing 950 mL of *n*-butanol with 50 mL of concentrated HCl acid (37%). Ferric reagent (2% ferric ammonium sulphate in 2M HCl) was prepared by dissolving 2.00 g of ferric ammonium sulphate in 2 M HCl acid and stored in dark bottle. To a test tube, 1.00 mL of sample solution, 6.00 mL of butanol-HCl reagent and 0.20 mL of ferric reagent were added. Tubes were shaken using a vortex. Then the tubes were loosely capped and placed in a boiling water bath for 50 minutes. After cooling the tubes to room temperature, absorbance was recorded at 550 nm. Blank contained 70% aqueous acetone containing 0.1% ascorbic acid instead of sample solution.

2.5. Statistical analysis

The results are represented as the mean ± SEM. Every statistical analysis was performed with one-way ANOVA, followed by student T test using Minitab 17.0 software. Differences were accepted as statistically significant at $P \leq 0.05$.

3. Results and Discussion

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 [21, 32, 33]. During extraction crude proanthocyanidins in the acetone/water extract were partitioned into ethyl acetate to obtain the crude EASPA and the remaining aqueous layer was freeze dried to yield the crude AQSPA. The variation of the crude EASPA and AQSPA yields with respect to the stage of maturity of the inflorescence was determined and the results are given in Table 1.

Table 1: The yield of EASPA and AQSPA respect to the maturity stage of the inflorescence of *Cocos nucifera* L (on fresh weight basis).

Maturity stage of Inflorescence	Weight of Inflorescence (g)	Weight of EASPA (g)	Yield of EASPA (%)	Weight of AQSPA (g)	Yield of AQSPA (%)	Total Yield (%)
Stage 0	1320	1.95	0.15	18.51	1.40	1.55
Stage 1	850	1.75	0.21	9.38	1.10	1.31
Stage 2	500	1.13	0.23	5.10	1.02	1.25
Stage 3	50	0.17	0.34	0.36	0.72	1.06

Maturity of the inflorescences decreases from stage 0 to stage 3.

According to the results the proanthocyanidin content in the inflorescence increases with maturity. In addition, percentage yield of crude EASPA decreases whereas percentage yield of crude AQSPA increases with maturity. Hence, the freshly open inflorescence (stage 0), which is the most mature inflorescence used for the study has the highest percentage of AQSPA and lowest percentage of EASPA. Despite the fact that the total proanthocyanidin content increase with maturity, the most immature flower has the highest percentage of EASPA. EASPA is comprised of low molecular weight proanthocyanidins, whereas AQSPA is composed of higher molecular weight proanthocyanidins [33]. Therefore, it can be suggested that proanthocyanidins are undergoing further polymerization during inflorescence development, which leads to a high percentage yield of AQSPA in the mature inflorescence. The higher molecular weight polymeric proanthocyanidins are poorly absorbed by the body leading to low bioavailability compared to low molecular weight proanthocyanidins [33, 37]. Further, our previous results show that the progestogenic activity lies in the EASPA and not in AQSPA. After the administration of EASPA and AQSPA for 28 consecutive days, EASPA administered rats showed highly significant increase of progesterone levels in their serum compared to control and AQSPA administered groups [22]. The low weight of the smallest inflorescence (stage 3) does not make it practical to use it as a source for EASPA. Thus, these results provide a rationalization for the use of the immature inflorescence, which is just about to open (stage 1) in Ayurveda and traditional medicine for the treatment of menorrhagia.

Next the proanthocyanidin content of the inflorescence was quantified along the length of the inflorescence with respect to maturity stage and with respect to different floral parts (rachis, rachilla, male flower and female flower) with the aim of standardizing the current pharmaceutical preparations used in ethnomedicine by using a specific portion of the inflorescence instead of using the whole inflorescence.

A number of methods have been developed for the quantification of proanthocyanidins. The methods can be divided into three main categories: methods based on biological effects, colorimetric methods and chromatographic methods. Of these methods, colorimetric and chromatographic methods are frequently used in quantifying proanthocyanidins. Colorimetric methods include the Folin-Ciocalteu method, acid-butanol assay, vanillin assay and 4-dimethylamino-cinnamaldehyde (DMAC) assay [38, 39]. The acid butanol assay is the currently available most reliable method to quantify proanthocyanidins and uses the characteristic reaction that gave the proanthocyanidins their name. Proanthocyanidins are cleaved into the carbocation units of corresponding extension units and the flavan-3-ol unit (terminal units) by diluted mineral acids. Subsequently, the carbocations are rapidly converted into anthocyanidins by autoxidation [37]. The formation of reaction products of acid butanol assay is shown in Figure 5.

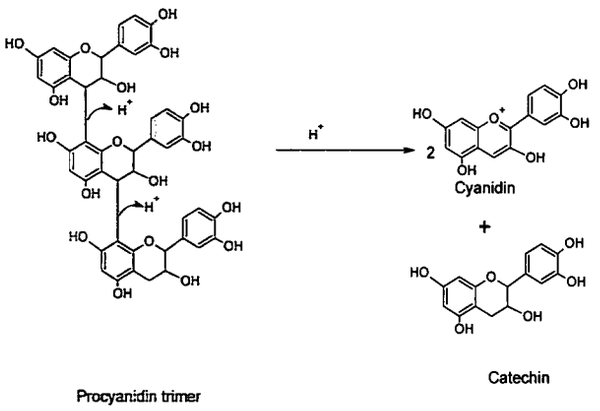


Fig 5: The formation of reaction products of the acid butanol assay.

According to literature, purified proanthocyanidins are used as a standard to quantify the proanthocyanidin content in plant materials [40]. While it is possible to use commercially available proanthocyanidins as standards, it is most suitable to use purified proanthocyanidin fraction for the material under study as the standard. This is because the anthocyanidins formed in the acid butanol reaction depends on the building blocks of the proanthocyanidin chain. Equally important is the fact that the anthocyanidin yield depends on the nature of the linkage and molecular weight of the proanthocyanidin as well. The AQSPA comprises over 80% of the total proanthocyanidin content in the *Cocos nucifera* L. inflorescence [34]. Therefore, purified AQSPA was used as the standard for the preparation of the calibration curve for the acid butanol assay. The calibration curve was drawn using six concentrations ranging from 0.02 mg/mL to 0.20 mg/mL. Calibration curve is shown in Fig. 6. A linear calibration curve ($y = 5.153x + 0.013$) with $R^2 = 0.997$, was obtained, going through zero.

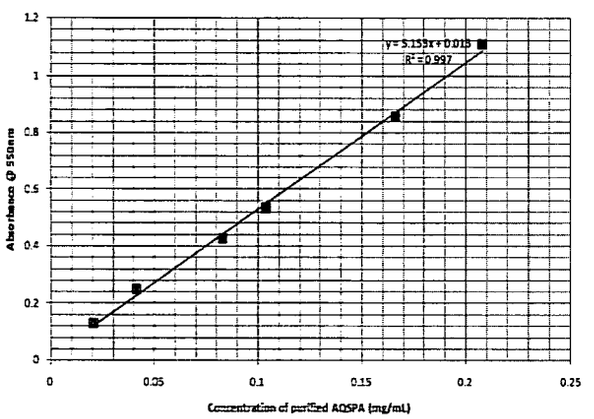


Fig 6: The calibration plot of proanthocyanidins from the inflorescence of *Cocos nucifera* L.

The inflorescence of *Cocos nucifera* L. takes over two years to complete the entire floral development process. Each inflorescence along the length was divided into three sections:

bottom, middle and top (Fig. 3). The proanthocyanidin content of the inflorescence along the length of the inflorescence with respect to different maturity stages (stage 1, 2 and 3) were determined and results are given in Fig. 7. In Ayurveda and traditional medicine the immature inflorescence, which is just about to open (stage 1) is used to treat menorrhagia. Thus, the freshly open inflorescence (stage 0) was not included in the study. According to the results there is a gradual increase in proanthocyanidin content in all parts of the inflorescence with increasing maturity. At all stages of inflorescence maturity the middle part contained the highest level of proanthocyanidins. This may be due to the fact that middle section of the inflorescence contained more floral parts such as male flowers, rachis and rachilla than other two sections. The proanthocyanidin content in top and bottom sections of the inflorescence is approximately the same and is not low enough to allow the middle section of the inflorescence only to be taken as a source for EASPA.

Cocos nucifera L. inflorescence has a main axis (rachis) and appendages (rachillae), which are attached to the main axis [31]. Each rachilla carries a few female flowers at the base and several hundreds of male flowers above it [31]. The different floral parts of the inflorescence of *Cocos nucifera* L. are shown in Fig. 4. The results for proanthocyanidin content of different floral parts of the immature inflorescence, which is just about to open (stage 1) are shown in Table 2. The rachilla has higher proanthocyanidin content than the rachis and the male flower has a higher proanthocyanidin content than the rachilla. Female flower has the highest proanthocyanidin content of all the floral parts of the inflorescence, however, the weight percentage per flower is low. The current Ayurvedic and traditional medical practice of using the total immature inflorescence just prior to opening appears to optimize the procedure for obtaining an adequate concentration of EASPA (which has been shown to contain progestogenic activity) for therapeutic purposes.

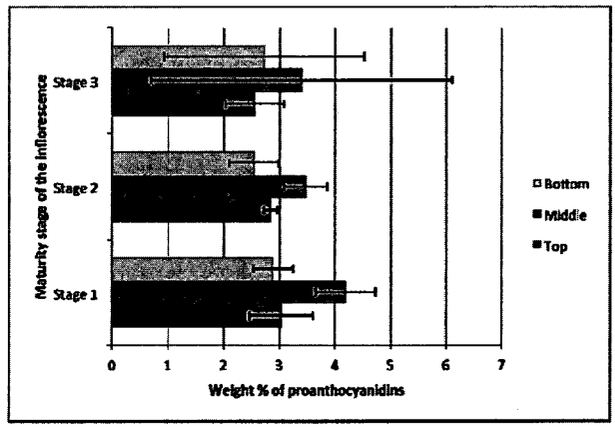


Fig 7: Proanthocyanidin content along the length of the *Cocos nucifera* L. inflorescence at different stages of maturity.

Table 2: Proanthocyanidin content of floral parts of the *Cocos nucifera* L. inflorescence at stage1 maturity.

Maturity stage of inflorescence	Whole rachis only	Whole rachilla only	Male flower	Female flower
Stage 1	1.84 ± 0.12	3.08 ± 1.79	3.95 ± 0.48	6.26 ± 1.26

All weight percentages are expressed on fresh weight basis and presented as mean ± SEM (n = 3).

4. Conclusion

To best of our knowledge this is the first report on quantification of proanthocyanidins in the inflorescence of *Cocos nucifera* L. Although the proanthocyanidin content increases, the percentage yield of EASPA decreases with increasing maturity of the inflorescence. The low weight of the smallest inflorescence (stage 3) does not make it practical to use it as a source for EASPA. While the middle section of the inflorescence contains a higher percentage of total proanthocyanidin, the difference between the middle and the other two other sections is not large enough to allow the middle section of the inflorescence only to be taken as a source for EASPA as well. According to the results the female flower has the highest content of proanthocyanidins among the floral parts, although the weight percentage per flower is low. The current Ayurvedic and traditional medical practice of using the total immature inflorescence just prior to opening appears to optimize the procedure for obtaining an adequate concentration of EASPA (which has been shown to contain progestogenic activity) for therapeutic purposes. Thus, our studies do not indicate the need to change the current Ayurvedic and traditional medical practice of the use of total immature inflorescence just prior to opening for the treatment of menorrhagia.

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