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Phyto-Chemical Evaluation and Anti-oxidant potentiality of *Cycas beddomei* Dyer Male cone aqueous Extract

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Abstract:
Cycas beddomei Dyer, an endemic and critically endangered, tropical, dry deciduous, dioecious gymnosperm present in varied region of adjunct areas of Tirumala Hillocks, Seshachalam Biosphere Reserve, Eastern ghats, India. The objective of the study was to investigate antioxidant capacity of aqueous extracts of microsporophylls of male cones of *Cycas beddomei*. This study deals with the quantitative estimation of phytoconstituents viz., Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Flavonols (TF), Total Proanthocyanidins (TPA), Extraction Yield (EY) , Quantitative analysis soluble carbohydrates, Starch, Crude fibre, Proteins, Moisture, Ash and Mineral composition of aqueous extract of male cone of *cycas beddomei* has been carried out. Moreover, the assessment of antioxidant capacity by standard established calorimetric methods viz., DPPH assay, TAC and ABTS assay. Entire data has evaluated statistically. Results depicted High contents of TPC, TFC, TF and TPA and exerted anti-radical aptitude. Significant correlation has been found between quantities of phyto-constituents and anti-oxidant assays. With the results, it is confirmed that the logistics of aqueous cone extract of *Cycas beddomei* as potential anti-oxidant by evaluating the bioavailability of phytoconstituents and provided scientific base as a valuable natural antioxidant and therapeutic agent.

Keywords: *Cycas beddomei, Male cone, Seshachalam Biosphere reserve, Antioxidant activity.*

Introduction

Cycas beddomei Dyer (Cycadaceae) is an endemic and critically endangered gymnosperm confined to Seshachalam Biosphere Reserve, Eastern Ghats, India[1-4]. This plant initially considered as a rare species[5] and vulnerable in Indian Red Data[6]. *Cycas circinalis* var. *beddomei* (Dyer) J. Schust. is a synonym of *Cycas beddomei*[7].

C. beddomei is a xerophytic, palm like dioecious tree (Fig 1). Reproductive structures are cones. Male cones flush during April–June. Male cones are commonly known as "peritha"[8]. These are woody with Oval to pyramidal shaped, pedunculate or shortly stalked, compact, solitary, terminal and are very spectacular with gold coloured tomentum. Each cone has a number of microsporophylls which bear microsporangia on its abaxial (lower) surface in groups and are attached to the central axis perpendicularly in a cross spiral (Fig 2). Each microsporangium has thousands of boat shaped microspores (pollen)[3].

Fig. 1: Habitat showing Coning Stage (Reproductive stage) of *Cycas beddomei* Dyer Male Cone.

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Fig. 2: *Cycas beddomei* Male cone showing Microsporophylls

According to earlier studies and folklore claims from Seshachalam forests, *C. beddomei* has been used for its apparent medicinal value, as a major ingredient in rejuvenating tonics, narcotic agent [6], rheumatoid and muscular pains[7] and to enhance cooling effect in the body when taken with sugarmixture. Natural antioxidants particularly polyphenolics[9], flavonoids[10], flavanols, proanthocyandines exhibit synergetic biological and pharmocological activities. Our previous work revealed its antioxidant and capacity of its n-Hexane, Ethyl acetate and Methanol fractions[11] but not evaluated the aqueous extract. An the previous work did not cover all the phytochemical evaluation such as mineral composition, chemical composition, qualitative analysis of secondary metabolites, flavonol content, anthocyanidins content, etc. The overall phytochemical evalauation is much more meaningful for reporting its anti-oxidative potency. In view of these potential therapeutical aspects, the aim of this present study is to determine the four phytoconstituents possessing antioxidant potency and its radical scavenging capacity. The overall study deals with the analysis of quantification of phytocostituents, Mineral Composition, antioxidative potency and their correlations.

Materials and Methods

Collection of plant material

C. beddomei Male cone (36 cm long and 13 cm in diameter) was collected on April 20th 2013 at Banglagutta, Talakona reserve forest (GPS Coordinates N 13°42' 41.4", E 79°39' 13.8") at an elevation of 843.6 MSL from the Core zone of Seshachalam biosphere reserve and deposited at Herbarium, Department of Botany, Tirupati with Voucher specimen (No. SVUTY- E/G-1605) and was authenticated by Dr. K. Madhava Chetty, Plant taxonomist, (IAAT No: 357), Department of Botany, Sri Venkateswara University,Tirupati.

Extract preparation

100 gms of decapitated fresh sporophylls used for extract preparation and processed for biological assay[12].

Phytochemical analysis

Qualitative analysis

Qualitative analysis of alkaloids, flavonoids, indoles, leucoanthocyanins, steroids, phenols, proteins, lignins, saponins, terpenoids performed using standard methods[13,14].

Quantitative analysis

Quantitative analysis soluble carbohydrates, Starch, Crude fibre, Proteins, Moisture, Ash and Minerals such as Ca, K, P, Na was performed using standard method employed by Horowitz[15]. Fe were quantified using method suggested AOAC (1975)[16]. Carbohydrates were quantified by the method of Pons *et al.*[17]. Total Fatty composition was determined using anthrone method of Blight and Dyer[18]. The Extraction yield also determined [13].

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Quantitative analysis of antioxidants

Total phenolic content (TPC) was determined by employing Folin–Ciocalteu reagent as per Kim *et al.*[12]. Total flavonoid content (TFC) was measured using standard colorimetric assay[19]. Total flavonols (TF) in the plant extracts were estimated as per the method employed by Kumaran and Karunakaran[20]. Determination of total proanthocyanidins (TPA) was carried by employing standard calorimetric method reported by Sun *et al.*[21].

Invitro Antioxidant assays

The scavenging activity of DPPH was assessed by scavenging of 2, 2-diphenyl-1-picrylhydrazyl radicals by employing the method of Brand-Williams *et al.*,[22]. Reduction of phosphomolybdenum was calculated to determine the total antioxidant capacity (TAC) by adapting the method of Umamaheswari and Chatterjee[23]. ABTS **(Azino-bis (3-ethylbanzthiazoline-6-sulphonic acid)** radicals scavenging activity was evaluated by following the standard Protocol followed by Re R *et al.* [24]

All the chemicals used for assays are of Grade 1 (AR) quality purchased from Bros Scientifics, Tirupati, Andhra Pradesh.

Statistical analysis

The entire work was carried in triplicates. The results graphically represented using ORIGIN 7 software (Software Inc., San Diego, CA, USA). Entire data was statistically analyzed to its standard Ascorbic acid values using paired *t-test* and the correlation between antioxidant assays and TPC, TFC, TF and TPA was calculated using data analysis tool pack in Microsoft excel 2007. Results were considered statistically significant at P <0.05.

Results

Phyto-chemical evaluation

Qualitative analysis

Qualitative analysis of phyto-chemicals indicated the presence of Alkaloids, Flavoinds, Phenols, Carbohydrates, Proteins, Lignins, Steroids and saponins. However, Indoles, Leucoanthocyanins and Terpinoids were absent (Table 1). Table 1. Qualitative analysis of phytochemicals of the extract

Phytochemical test	Occurance (Present (+)/Absent (-))
Alkaloids	+++
Flavonoids	+++
Indoles	-
Leucoanthocyanins	-
Steroids	+
Carbohydrates	++
Phenols	+++
Proteins	++
Lignins	++
Saponins	+
Terpinoids	-

("+" indicates presence; "-" indicates absence; "+"indicates low, "++"indicates moderate; "+++"indicates high)

Quantitative analysis

Quantities of different phyto-constituents such as soluble carbohydrates, Starch, Crude fibre, Proteins, Total Fatty composition, Moisture, Ash are, 439.33±2.52, 245.67±11.50, 7.33±0.21, 93.33±4.04, 18.67±3.06, 904.00±7.21 and 46.33±3.79 g Kg^{-1} respectively. Extraction yield was 23.90±3.77 g (Table 2).

Table 2: Chemicals composition of the extract

Chemical Composition	SC	Starch	Crude Fibre	Proteins	Total Fatty composition	Moisture	Ash	E. Y (g)
Quantity (g kg^{-1})	439.33 ± 2.52	245.67 ± 11.50	7.33 ± 0.21	93.33 ± 4.04	18.67 ± 3.06	904.00 ± 7.21	46.33 ± 3.79	23.90 ± 3.77

(All the values are the means of replicates (n=3); values after "±" indicates the Standard Deviation between replicates (SD),SC – Soluble Carbohydrates, EY – Extraction Yield)

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The mineral composition of the extracts showed Ca, K, P, Na and Fe in the quantity of 5.63±0.39, 1.89±0.10, 1.44±0.06, 0.40±0.06 and 0.13±0.03 g/kg respectively (Table 3).

Table 3: Mineral composition of the extract

Mineral	Ca	K	P	Na	Fe
Quantity (g/kg)	5.63 ± 0.39	1.89 ± 0.10	1.44 ± 0.06	0.40 ± 0.06	0.13 ± 0.03

(All the values are the means of replicates (n=3); values after "±" indicates the Standard Deviation between replicates (SD))

Quantitative analysis of antioxidants

The quantities of anti-oxidants of extract are as follows: Total Phenolic Content (TPC, Gallic Acid Equivalent) 135.69±1.53 mg/g; Total Flavonoid Content (TFC 311.39±6.09 mg/g, Quercetin Equivalent); Total Flavanols 145.58±9.75 mg /g (TF, Catechin Equivalent); Total Proanthocyanidines 48.66±1.80 mg/g (TPA, Catechin Equivalent) and Extraction yield of the aqueous extract of *C. beddomei* 15.60±1.58 g (Table 4).

Table 4. Quantitative estimation of Phyto-constituents of Aqueous extracts of *C. beddomei* Male cone.

Assay	Quantity
TPC	135.69±1.53
TFC	311.39±6.09
TF	145.58±9.75
TPA	48.66±1.80
EY	15.60±1.58

Values are the means ± SD (n=3); values are expressed to their respective standards. TPC- mg of GA/g of dry extract; TFC- mg of R/g of dry extract; TF- mg of QU/g of dry extract; TPA- mg of CA/g of dry extract; EY- g.

In-vitro antioxidant assays:

The DPPH free radical scavenging activity of the aqueous extract of *C. beddomei* male cone was in a concentration dependent manner, the activity was increased with increase in the concentration of the extract. The lowest DPPH activity has exerted at 25µg/ml concentration (13.00±1.00) and the highest activity has exerted at 250µg/ml (86.00±2.00). The TAC also increased with increase in the extract concentration. The lowest TAC observed at 25µg/ml concentration (12.00±1.00) and the highest TAC has observed at 250µg/ml (81.67±1.53) (Figure 3). ABTS scavenging activity also had the concentration dependency as DPPH increase in the concentration increased the radical scavenging capacity. The lowest ABTS activity has exerted at 25µg/ml concentration (16.67±0.58) and the highest activity has exerted at 250µg/ml (42.00±2.65).

The radical scavenging pattern observed by the ascorbic acid is as same as the aqueous extract of *C. beddomei* male cone. The activity was increased with the increase in the concentration of the ascorbic acid. The lowest DPPH activity has exerted at 25µg/ml concentration (21.00±1.00) and the highest activity was exerted at 250µg/ml (98.67±0.58). The lowest TAC observed at 25µg/ml concentration (16.67±0.58) and the highest TAC was observed at 250µg/ml (92.67±0.58) (Figure 4). The values at all the concentration showed significant variation ($p \leq 0.01$) when compared with the standard ascorbic acid except for TAC at 25µ/mg concentration. The lowest ABTS activity was exerted at 25µg/ml concentration (18.33±0.58) and the highest activity was exerted at 250µg/ml (91.33±1.15).

IC_{50} values of DPPH, TAC and ABTS of the aqueous extract of *C. beddomei* male cone are 66.06±26.64 µg/ml, 87.46±37.86 µg/ml and 82.13±77.55 µg/ml respectively. The IC_{50} values of DPPH, TAC and ABTS of extract were significantly varied ($p \leq 0.01$) when compared with the standard ascorbic acid (Figure 5).

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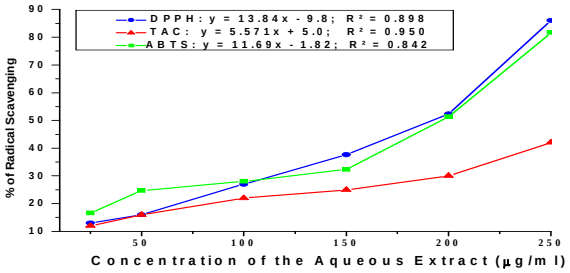

Figure 3: Antioxidant activity exerted by Aqueous extract of *C. beddomei* Male cone

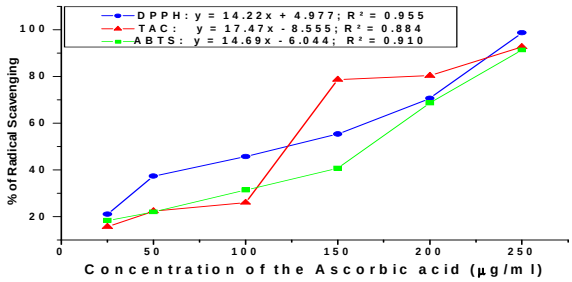

Figure 4: Antioxidant activity exerted by Ascorbic Acid

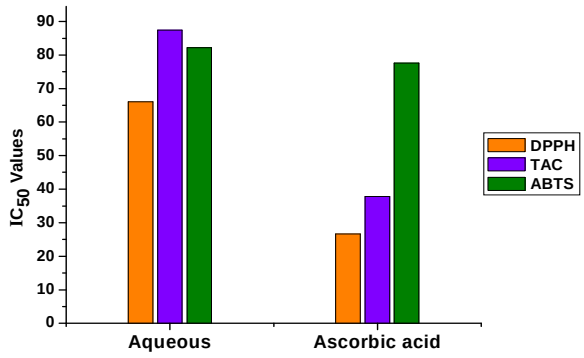

Fig 5: Anti-oxidant effect (IC_{50}) on DPPH, TAC and ABTS of Aqueous extract of *C. beddomei* Male cone.

Correlation studies in between antioxidants of the aqeous extract and the antioxidant capacity of the extract

The radical scavenging activities (IC_{50} values) of the aqueous extract of *C. beddomei* male cone and TFC, TPC, TF and TPA values has been correlated. DPPH showed strong positive correlation with its TPC, TFC, TF and TPA with significant R^2 values (0.98, 0.98 and 0.99) ($p \leq 0.01$, 0.001) whereas it has a weak positive correlation with TF (R^2=0.30) ($p \leq 0.01$). TAC showed strong positive correlation with its TPC, TFC, TF and TPA with significant R^2 values (0.99, 0.91 and 0.99) ($p \leq 0.001$) whereas it has a weak positive correlation with TF (R^2=0.53) ($p \leq 0.01$). ABTS showed strong positive correlation with its TPC, TFC, TF and TPA with significant R^2 values (0.94, 0.99 and 0.96) ($p \leq 0.001$) whereas it has a weak positive correlation with TF (R^2=0.15) ($p \leq 0.01$). Y= Fitted equation/solvent extract; R^2 values are represented as correlation coefficient (Table 5).

Table 5: Correlation between IC 50 Values of Antioxidant Activities and Phytoconstituents of *C. beddomei* Male cone.

	DPPH	TAC	ABTS
TPC	0.98***	0.99***	0.94***
TFC	0.98***	0.91***	0.99***
TF	0.30**	0.53**	0.15**
TPA	0.99**	0.99***	0.96***

*Indicates the level of significance when compared using paired t-test. *= ($p \leq 0.05$); **= ($p \leq 0.01$); ***= ($p \leq 0.001$)

The results of the IC_{50} indicated that the aqueous extract of *C. beddomei* male cone exert anti-oxidant capacity, it is highly anti-oxidative in nature (Figure 3). The significant correlation between antioxidant activities and phyto-constituents strongly indicates that the reason behind the antioxidant activities exerted by the aqueous extract of *C. beddomei* male cone is the presence phyto-constituents (Table 4).

Discussion

Cycas beddomei have been strictly governed by the CITES and is legally by schedule 6 of Indian wildlife protection Act 1972. Rao *et al.*[4] made assessment and commented on conservation status done and analyzed *C. beddomei* to be

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Endangered (not critically endangered) in IUCN Criterion B.

Phytochemicals protect against oxidative stress, which in term helps in maintaining the balance between oxidants and anti-oxidants. Other organic extracts of *C. beddomei* male cones proved to be rich in phenols and flavonoids[11]. Previous works reported its phenolic studies, chemical analysis of biflavonoid[25], Reproductive ecology[3], AFLP studies[26], GC-MS studies[27], Chetty and Rao[28] claimed that Hill habitation ecosystem might be associated with high contents and greater number of phenolic compounds[29].

These flavonoids, Polyphenols, flavonols and Proanthocyanadins proved to be antioxidant in nature[9,10,30]. The present investigation proves presence of various phytochemicals and quantified its mineral, chemical compositions. The present results also proved the presence of all antioxidative phytochemicals (Flavonoids, Polyphenols, flavonols and Proanthocyanadins) in the aqueous extracts of the male cones which indicates its antioxidative potentiality. As the safety limits of natural antioxidants are mostly not known, but they are hardly safer than synthetic antioxidants[31]. Radical scavenging activities are very important to prevent the deleterious role of free radical. So, here we had made an attempt to test its in-vitro anti-oxidative potentiality through radical scavenging assays and found the positive results i.e., the aqueous extract is highly anti-oxidative and acting as radical scavenger. Radical scavenging capacity was increased with the increase in the concentration of the extract for all the anti-radical tests. The IC_{50} values of the assays also proved its radical scavenging capacity. The radical scavenging capacity of the aqueous extract is more than the inorganic extracts of the same male cone[11].

This is a first approach to employ all the compounds together with appropriate statistical technique to differentiate between Antioxidants. In our previous study we investigated the antioxidant potency of different extracts of male cone. In this investigation, we tested the aqueous extracts which showed the potent antioxidant activities than the other solvent systems. The anti radical potency of the aqueous extract can be compared to the anti radical potency of standard Ascorbic acid. Here, we correlated both the antioxidants with their radical scavenging capacity and found that the presence of Phenols, flavonoids, flavonols and anthocyanidines are the reason behind its radical scavenging potency.

Conclusion

This study is a preliminary investigation considering anti radical potentiality of the male cone aqueous extract of *Cycas beddomei*. And the work also covers all the possible phytochemical investigation of the aqueous extracts. Antioxidants such as Phenols, flavanoids, flavonols and anthocyanidines were quantified. Together with the support of statistical data evaluation, the results predicted promising constituents which embrace a considerable range of antioxidant potency. With this study we conclude that the aqueous extract of the *C. beddomie* male cones as the bio-source of the antioxidants and acts as highly potent radical scavenger.

High bioavailability of phenolics, flavonoids, flavonols, proanthocyanidins which confer antioxidant potentiality have a great need to conserve and multiply the population for the

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multiple benefits from these constituents of the coning episode of *cycas beddomei*.

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Ethical issues and competing interests

None to be declared.

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