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ANTIOXIDANT ACTIVITY AND ITS CORRELATION OF DIFFERENT SOLVENT EXTRACTS OF MALE CONES OF *Cycas beddomei* Dyer, ENDEMIC TAXA TO SESHACHALAM BIOSPHERE RESERVE

M. MAHENDRA NATH*[1], CH. SANTOSH[2] AND K. MADHAVA CHETTY[1]

[1]Department of Botany, Sri Venkateswara University, Tirupat-517502, Andhra Pradesh, India.
[2]School of Life and Health Sciences, Aadi Kavi Nannayya University, Rajahmundry-533105, Andhra Pradesh, India.

ABSTRACT

Cycas beddomei Dyer. (Cycadaceae), is an endemic and critically endangered, tropical, dry deciduous, dioecious gymnosperm present in varied region of adjunct areas of Tirumala Hills, Seshachalam Biosphere Reserve, Southern Eastern Ghats, India. *In vitro* antioxidant studies by employing different parameters such as DPPH, TAC, ABTS and superoxide radical scavenging activity have been tested with different extracts viz., methanolic, ethyl acetate and n-Hexane. Screening for biochemical availability was done for flavonoids and phenolics and were evaluated statistically which showed positive correlations among biochemical availability and antioxidant activities with all the extracts. This study suggests that all the extractives showed antioxidant potency and laid a scientific proof that *C. beddomei* male cones are new potential source of natural antioxidants.

KEY WORDS: ***Cycas beddomei*** **Dyer.; Seshachalam Biosphere Reserve; DPPH; TAC; ABTS; Antioxidant activity**

M.MAHENDRA NATH
Junior Research Fellow
Department of Botany, Sri Venkateswara University,
Tirupat-517502, Andhra Pradesh, India.

INTRODUCTION

Cycas beddomei Dyer. (Cycadaceae), is an endemic dioecious xerophytic gymnosperm chiefly distributed in Tropical dry deciduous forests of Tirumala hills which is a part of Seshachalam Biosphere reserve in Eastern Ghats portion of Andhra Pradesh in India[1-4](Fig.1). The plant grows on exposed rocky slopes and in the denuded valleys, between elevations of 300-900 m[5]. Male cones are vernacularly known as "Peritha" are reproductive structures which are present at the apex of the stem with peduncle. It has a number of parenchymatous microsporophylls which bear microsporangia on its abaxial (lower) surface in groups (Fig.2). The terminal portion of microsporophylls bends upwards and arranged compactly giving a cony appearance. Each microsporangium has thousands of spores which are boat shaped. Each cone produces pollen grains, microspores in billions[6,7]. Legal Status of *C. beddomei* listed in CITES (Appendix 1), IUCN status as CR[3], Negative List of Export[10]. Local people, Herbal vendors and some ayurvedic medicine manufactures harvest the male cones before pollen shedding for various traditional medicinal purposes like Narcotic, Rheumatoid, Muscle Pain, Cooling effect[7,8,9]. The villagers inhabiting seshachalam forest scour accessible populations for male cones, removing all before their pollen shed [10].

Phyto-polyphenolics such as phenolic acids and flavonoids are proved to be anti-oxidants and are asserted to be a potent natural antioxidants[11-14]. Natural antioxidants are better and safer than synthetic antioxidants[15]. Antioxidants for natural sources are gaining more acceptance due to renewed interest in traditional medicine in providing new potent bioactive compounds which are medicinal and confer antioxidant potency and other medical usage[16,17]. Based on the traditional knowledge of medicinal system, the present study was taken to evaluate the antioxidant efficacy of different microsporophyll extracts. Current research is designed to investigate, evaluate and to screen out naturally occurring antioxidants from juvenile phase of C. *beddomei* male cones.

Fig : 1.Ecological Habitat of *Cycas beddomei* Male plant at Coning Stage

Fig : 2. Juvenile Phase Male Cone of *Cycas beddomei*

MATERIALS AND METHODS

Plant Material

Male cones (18 cm long and 8 cm in diameter) (Fig:2) were collected on April 5th 2013 at Banglagutta, Talakona reserve forest (GPS Coordinates N $13^0 42' 41.4"$, E $79^0 39' 13.8"$) at an elevation of 843.6 sea level in the core zone of Seshachalam biosphere reserve with the prior permission from the Department of Forests, Andhra Pradesh and deposited in the Herbarium, Department of Botany, Tirupati with Voucher specimen (SVUTY-E/G-1605). Authenticated by Dr. K. Madhava Chetty (IAAT No: 357), Plant taxonomist, Department of Botany, Sri Venkateswara University, Tirupati. Fresh and juvenile sporophylls (250 gms) which are the parts of the male cone were used for biological assay.

Extract preparation

Decapitated part of the cone was air dried at room temperature under shade for 3 weeks and grinded to 60 mm mesh size by using Willy Mill. Powder of 100 g was soaked in 200 mL of 95% methanol (3 times) and filtered the extract with Whatman No.1 filter paper. Filtrate was dried under vacuum by using rotary evaporator. The extract was suspended in methanol, ethyl acetate and n-Hexane and residual was used as methanolic, ethyl acetate and n-hexane extracts respectively. Extracts were dried by using rotary evaporator and preserved at 4°C[18].

Quantitative analysis of antioxidants

Quantification of Total Phenolic Content (TPC)

Total phenolic content (TPC) was determined by employing Folin–Ciocalteu reagent as per Kim *et al* (2003)[19]. Folin–Ciocalteu reagent of 400µl was mixed with 200µl of extracts/standard (1.0 mg/ml) in a volumetric flask. The solution was placed at 25^0C for 5–10 min and mixed with 0.2 ml of 7% Na_2CO_3 solution, and finally diluted the mixture to 10.0 ml of volumetric flask with deionised distilled water. The mixture was vortexed for 20 minutes and placed for 2 h at 25^0C and absorbance was than measured at 725 nm using UV-VIS Spectrophotometer (Shimadzu, USA). Ascorbic acid was used for plotting calibration curve. Total content of phenols was expressed in terms of Gallic acid equivalent, GAE, mg of GA/g of dry extract.

Quantification of Total Flavonoid Content (TFC)

Total flavonoid content was measured using standard colorimetric assay [20]. In a volumetric flask of 10.0 ml, an aliquot of 0.1 ml of extracts/standard (1.0 mg/ml), distilled water (4.0 ml), 5% $NaNO_2$ (0.3 ml) and 10% $AlCl_3$.6H2O (0.3 ml) was mixed and placed for 6 min. 2 ml of NaOH (1 M) was added and diluted the solution with 2.4 ml of distilled water. Absorbance of pink color solution was recorded at 510 nm against a blank (containing all reagents except extract/standard). Rutin was used for plotting calibration curve. Total content of phenols was expressed in terms of rutin equivalent, RE, mg of R/g of dry extract.

Antioxidant assays

DPPH, Superoxide, ABTS radical scavenging and Total antioxidant Capacity assays were evaluated. IC_{50} values obtained as to determine the 50% inhibition radicals. Ascorbic acid was used as standard.

Scavenging activity of DPPH radicals

The scavenging activity of DPPH was assessed by scavenging of 2, 2-diphenyl-1-picrylhydrazyl radicals by employing the method of Brand-Williams (1995)[21]. 2.4 mg DPPH was dissolved in 100 ml of methanol to prepare the stock solution of DPPH. The DPPH solution was diluted with methanol to achieve an absorbance of 0.980±0.02 at 517 nm. DPPH solution of 500µl was added to 500µl of the dried fractions at varying concentrations (25–250 µg/ml) and vibrated vigorously. After the incubation of 15 min in the dark, absorbance was recorded at 517 nm. The DPPH scavenging activity of various extracts was calculated by the following equation:

Percentage inhibition (%) = [(Absorbance of control-Absorbance of sample)/(Absorbance of control)]×100

Scavenging activity of Superoxide anion radicals

Superoxide radical scavenging activity was assessed by following the calorimetric method followed by Bushra Ahmad (2013)[22]. Reaction solution was prepared by mixing 0.5ml of phosphate buffer (50 mM, pH 7.6), 0.3ml of riboflavin (50 mM), 0.25 ml of PMS (20 mM) and 1 ml of NBT (50 mM). Sample (100 µl) was mixed with the 1 ml of reaction solution and kept under fluorescent lamp for 20 min. Absorbance was measured at 560 nm. The Superoxide scavenging activity of various extracts was calculated by the following equation:

Scavenging activity (%) = (1-absorbance of sample/absorbance of control)×100

Scavenging activity of ABTS (Azino-bis3-ethylbanzthiazoline-6-sulphonicacid)radicals

ABTS scavenging activity was evaluated by following the standard Protocol[23]. To prepare a dark colored ABTS working solution, ABTS solution (7 mM) was mixed with potassium oxidopersulphate (2.45 mM) solution and was placed in the dark for 12–16 h. The solution was diluted with 50% methanol and absorbance was adjusted at 0.7 (±0.02) at 734 nm. Extract (100 µl) was mixed with 1 ml of ABTS working solution and decrease in absorbance was read 1 min after adding the extract and then up to 6 min. The ABTS scavenging activity of various extracts calculated by the following equation:

Inhibition(%) = [(Absorbance of control– Absorbance of sample)/ Absorbance of control] ×100

Total antioxidant capacity (TAC)

Reduction of phosphomolybdenum was calculated to determine the total antioxidant capacity by adapting the method of Umamaheswari and Chatterjee[24]. 100 µl of extract was mixed with 1.0 ml of the reagent solution consisting of phosphate buffer, 0.6 M H_2SO_4, 28 mM sodium molybdate and 4 mM ammonium molybdate. The mixture was incubated in a water bath at 95^0C for 90 min. The absorbance was measured at 765 nm, after cooling the mixture at room temperature. A standard of ascorbic acid was employed. Total antioxidant scavenging capacity of various extracts was calculated using the following equation:

Total antioxidant capacity (%) = [(Absorbance of control-Absorbance of sample)/(Absorbance of control)]×100

Statistical analysis

The entire work was carried out in triplicates (n=3). Experimental results were calculated as Mean±SD. For three parallel measurements and the graphs were plotted. Samples data was compared to their respective standards using paired *t-test*. Correlation analysis between quantities of antioxidants and the anti-oxidative properties was done for Linear regression (Y) and the determination coefficient (R^2) using statistical analysis tool SPSS 20.0 (SPSS Inc, Chicago, IL). Differences were considered significant at p≤0.05.

RESULTS

Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Extraction Yield (EY) of the Extracts:

The TPC values ranged from 9.33±0.21 to 116.49±5.22 mg Gallic acid equivalents per g of dry weight. TFC values ranged from 122.08±7.33 to 315.92±1.98 mg of Catechin equivalents per g of dry weight. The EY ranged from 11.33±0.94 g to 32.21±1.78 g. The order of the different extracts based on their TPC, TFC and EY values is:

n-Hexane<Ethyl Acetate<Methanol (Table 1).

Table 1
Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Extraction Yield (EY) of the Extracts

Plant Extract	TPC (mg of gallic acid equivalents /g of dry weight)	TFC (mg of Catechin equivalents / g of dry weight)	EY (g)
n-Hexane	9.33±0.21	122.08±7.33	11.33±0.94
Ethyl Acetate	75.22±2.77	224.72±4.87	22.77±0.90
Methanol	116.49±5.22	315.92±1.98	32.21±1.78

Values are the means of Triplicates±SD.

In-vitro antioxidant assays

The percentage of DPPH, Superoxide and ABTS scavenging activity was increased with increase in the concentration of the extract (Graph 1,2,4). The highest DPPH scavenging activity was exerted by methanolic extract following ethyl acetate and n-hexane having the IC_{50} values 325.04±4.61, 185.07±5.06 and 67.02±5.04 respectively. The higher Superoxide scavenging capacity also exerted by methanolic extract following ethyl acetate and n-hexane having the IC_{50} values 257.96±3.51, 271.56±0.34 and 64.64±0.30 respectively. The high ABTS scavenging activity was also exerted by methanolic extract following ethyl acetate and n-hexane having the IC_{50} values 318.20±5.35, 231.51±10.06 and 133.61±0.36 respectively (Graph 5). All the extracts exerted noticeable TAC. It also increased with the increase in the concentration (Graph 3). The highest TAC exerted by methanolic extract following ethyl acetate and n-hexane and their IC_{50} values are 578.79±0.30, 90.49±0.42 and 71.87±0.76 respectively. All the IC_{50} values were compared to its standard ascorbic acid and found significant variation ($P \leq 0.05$). The antioxidant efficacy of standard ascorbic acid increased with increase in the concentration. The IC_{50} values of ascorbic acid for DPPH, Superoxide, TAC and ABTS are 26.64±0.27, 24.45±0.40, 37.86±0.39 and 77.55±0.30 respectively.

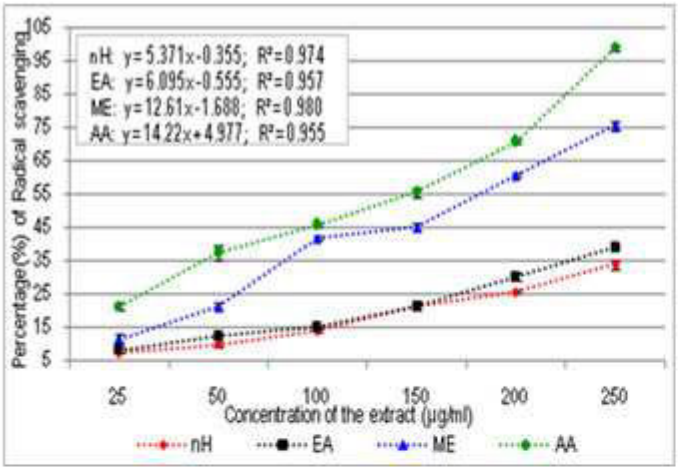
Correlations between anti-oxidant assays and phenolic, flavonoid contents of different solvent extracts

IC_{50} values of DPPH, Superoxide and ABTS scavenging capacity correlated to TPC and TFC of the extracts (Table 2). A strong positive correlation was found for TPC and radical scavenging assays with significant R^2 values (correlation coefficient) (0.964, 0.918) for IC_{50} values of DPPH, Superoxide and a positive correlation was found (0.760, 0.833) for IC_{50} values of TAC and ABTS of n-Hexane extract. Ethyl acetate extracts given a strong positive correlation for TPC and radical scavenging assays with significant R^2 values (0.948, 0.930, 0.980, 0.914) for its IC_{50} values of DPPH, Superoxide, TAC and ABTS. Methanol extract had a strong positive correlation with TPC and radical scavenging assays with significant R^2 values (0.995, 0.957, 0.976) for IC_{50} values of DPPH, TAC and ABTS where as IC_{50} values of Superoxide had a positive correlation with TPC (0.847). A strong positive correlation was found for TFC and radical scavenging assays with significant R^2 values (0.928, 0.998, 0.998) for IC_{50} values of DPPH, TAC and ABTS of n-Hexane extract where as the superoxide had a weak positive correlation (R^2 = 0.491). Ethyl acetate extracts had a strong positive correlation with significant R^2 values (0.977, 0.987, 0.737, 0.993) for IC_{50} values of DPPH, Superoxide, TAC and ABTS and TFC whereas, TAC showed a positive correlation. Methanol extract had a strong positive correlation (0.981) for IC_{50} values of DPPH, Superoxide, where as the other anti-oxidant assays DPPH, TAC and ABTS had a positive correlation with TFC (0.791, 0.896, 0.860). The paired t test showed significant variation at different significant levels ($p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$) in between anti-oxidant assays and the TPC and TFC (Table 2). However, the TFC of ethyl acetate extract remained insignificant with the Ethyl acetate ABTS scavenging capacity.

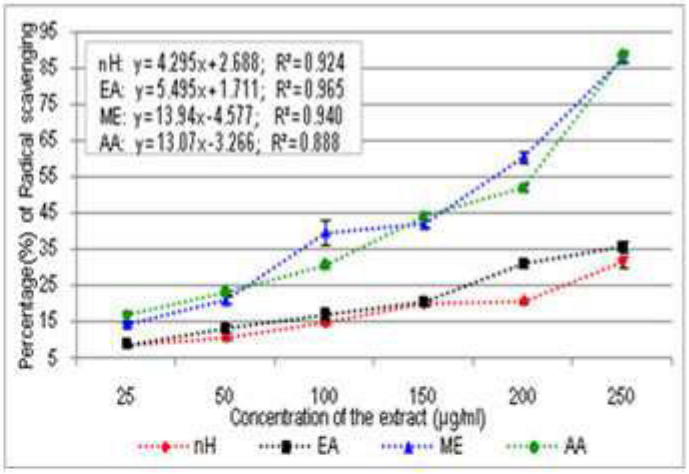
Table 2
Correlation between IC_{50} values and TPC, TFC of Plant Extracts

Anti-oxidant property	n-Hexane		Ethyl Acetate		Methanol	
	TPC	TFC	TPC	TFC	TPC	TFC
DPPH	0.964***	0.928***	0.948***	0.977***	0.995***	0.791***
Superoxide	0.918***	0.491***	0.930***	0.987**	0.847**	0.981***
TAC	0.760***	0.998***	0.980**	0.737***	0.957**	0.896***
ABTS	0.833***	0.998***	0.914***	0.993^{NS}	0.976*	0.860***

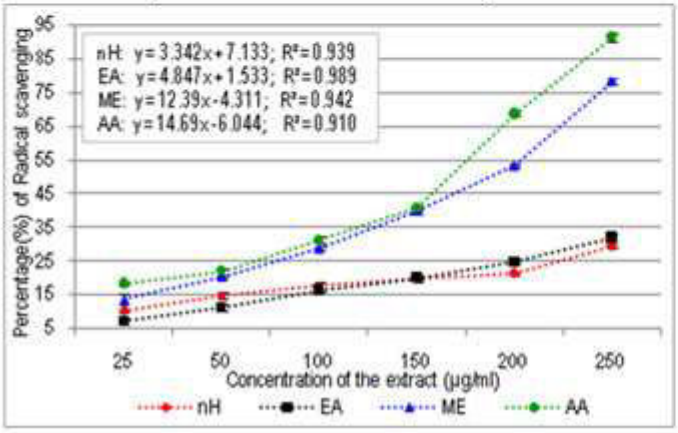
** Represents significant variation levels. *p≤0.05, **p≤0.01, ***p≤0.001, NS - not significant. (R^2 :correlation coefficient)*



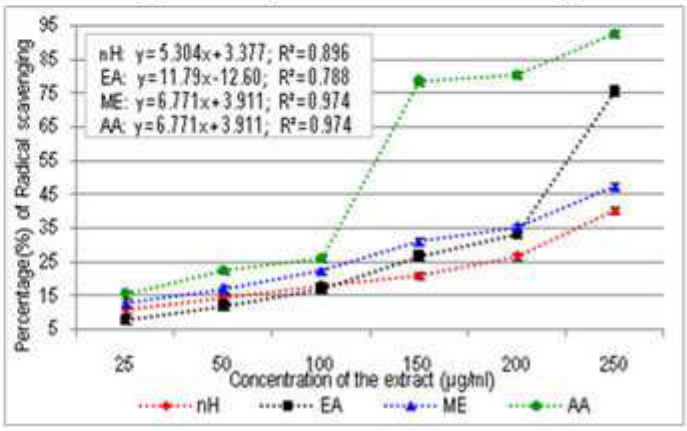
Graph 1: DPPH radical assay



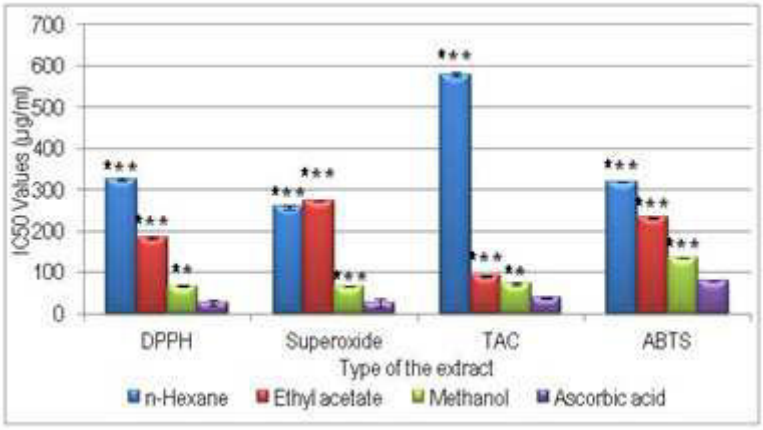
Graph 2: Superoxide radical assay



Graph 3:TAC of different extracts



Graph 4: ABTS radical asssay



Graph 5
IC_{50} Values of different extracts.

Graphs 1, 2, 4: Antioxidant assays of DPPH, Super Oxide, ABTS
Graph 3: TAC of Different solvent extracts
All the values are the means of replicates (n=3). Error bars indicate SD (Standard Deviation).
Y= Fitted equation/solvent extract;
R^2 values: correlation coefficient.
*indicates the level of significant variation between extract and standard ascorbic acid value; ***p≤0.05**; ****p≤0.01**; *****p≤0.001.**

DISCUSSION

Radical scavenging activities are very important to prevent the deleterious role of free radicals in different diseases. Various plants were identified to possess antioxidative property, due to their phenolic and flavonoid content[25,26]. *C. beddomei* male cone extracts has found to contain various phenolic and flavonoid components. The recent study reported the presence of various phyto-chemicals such as alkaloids, flavonoids, terpenoids, tannins, glycosides and bifloavonoids[27,28]. The presence of such components turned the view on its antioxidative property. Different parameters such as DPPH, TAC, ABTS and superoxide radical scavenging activity assays have been proved to be reliable for determining Antioxidant activity which is tested in the present work. DPPH free radical scavenging is an accepted mechanism by which antioxidants act to inhibit lipid peroxidation[29]. The results of DPPH scavenging activity had increased along with increase in the concentration. The results coincide with the recent study[25]. The IC_{50} values indicated the anti-radical capacity of the extracts. The methanolic extracts have the highest DPPH scavenging capacity whereas n-Hexane extracts exerted the lowest capacity. The superoxide radical produced by various biological reactions is very lethal free radicals. The present work showed the scavenging of the free superoxide radical by the application of the extract. The effect was concentration and solvent dependent. The methanolic extract found to possess high superoxide radical scavenging capacity following ethyl acetate and n-Hexane extracts. IC_{50} values also indicated its

superoxide radical scavenging capacity. All the extracts represented noticeable TAC. Both the TAC and ABTS increased with increase in the concentration.

Based on the results of the DPPH, superoxide, TAC and ABTS, it was found that the extracts of *C. beddomei* are natural antioxidants, it possess free radical scavenging efficacy. The activity depended on solvent and concentration of the extract. Highest TPC was found in methanol whereas n-Hexane extract reflected small quantities of TPC. The TFC values varied among all three of the extracts and they showed the order as in TPC *i.e*. Hexane<Ethyl Acetate<Methanol. Another main objective of the work was to relate the phenolics and flavonoids contents to its antioxidant capacity. The correlation results between TPC, TFC and Antioxidant assays strongly correlates the antioxidative properties of the extracts are as their phenolic and flavonoid contents. Kalt et al[30] had found a strong correlation between antioxidant capacity and total phenolics. We confirmed that, antioxidant capacity was higher in the methanolic extracts following the Ethyl acetate and n-Hexane. The reason of high antioxidant activity of methanolic extract than other solvent extracts could be its phenolics and flavonoid contents. The extraction yield results also support it. The Extraction Yield was higher in methanolic extract. The maximum yield with methanolic extract represented the highest extractable compounds in the methanolic extract than other extracts. So, the order of the extractable compounds and the anti-oxidative capacity of the extracts is Hexane<Ethyl Acetate<Methanol. To our knowledge this investigation has not been carried out earlier. Synergism between the evaluated antioxidant compounds in different solvent extracts showing antioxidant activity not only dependent on the concentration, but also on the other phytoconstituents and their interaction with antioxidant constituents. Our results indicated and laid scientific proof that *Cycas beddomei* Dyer male cone extracts are a promising potential natural antioxidants.

CONCLUSION

The main objective of this work is to evaluate *C. beddomei* male cone as antioxidant using essential antioxidant and radical scavenging assays such as DPPH, Superoxide, TAC and ABTS. The assays results indicated that it possess an antioxidant efficacy and can eliminate free radicals. The results also indicated that the antioxidant capacity is solvent and concentration dependent. The methanolic extracts found to be more effective among all. There was a positive correlation in between phenolic, flavonoid and radical scavenging assays, which strongly explains the reason of antioxidant capacity is as its phenolic and flavonoid contents. With this work, our observations demonstrate that the different solvent extracts of *C. beddomei* male cone are antioxidative in nature. The plant male cone is a good source of phenolics and flavonoids which acts as anti-oxidants. There is a need for conservation of Male populations at its coning stage and necessary protection should be taken up for its natural regeneration *in vitro* and *in vivo* against further loss.

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