**In Vitro Antioxidant Activities of various extracts of male cone and stem of *cycas circinalis***

**Abstract**

Antioxidants are free radical scavengers that prevent pathological conditions of the human body, mostly, ischemia, anaemia, asthma, arthritis, inflammation, neurodegeneration, and aging process. The present study examines the possible antioxidant activity of *Cycas circinalis*. It was examined by performing various enzymatic and non-enzymatic assays using various extract .Phenolic compounds including flavonoids and phenolic acids are plants secondary metabolites. Due to their ability to act as antioxidant agents, there is growing interest to use those components in traditional medicine for cancer prevention or treatment. The free radical scavenging activity was measured by using diphenyl picrylhydrazyl (DPPH) and Nitric oxide. Methanolic extract of both stem and cone showed better results in Reducing power and in Total antioxidant assay. MECCS showed the lowest IC50 value 3.73 in DPPH assay showing better capacity as compared to other extract. In Nitric oxide AECCS showed the lowest IC50 value of 4.367.

**Keywords:** antioxidants, enzymatic, secondary metabolites, scavenging.

**1. Introduction**

“The importance of reactive oxygen species and free radicals has attracted increasing attention over the past decade. Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals (O2−), hydroxyl radicals (OH·) and non-free-radical species such as H2O2 and singlet oxygen (1O2), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and aging process. ROS are continuously produced during normal physiologic events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. However, they are removed by antioxidant defence mechanisms. There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. Oxidative stress occurs when the production of ROS is beyond the protective capability of the antioxidant defences. Under pathological conditions, ROS are overproduced and result in oxidative stress. ROS are formed when endogenous antioxidant defences are inadequate. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in cellular membrane or intracellular molecules” (Scheng, 2008)

“Antioxidants - Free radicals are being generated at high quantities, particularly at metabolic and contact processes. Tissues and cells are damaged by oxidation as macromolecules (fatty acids, nucleic acids, and proteins) deteriorate. Basically, the reaction that takes place between free radicals and electrons derived from other molecules harms the human body due to its negative effect on the enzyme system. Hence, the complex systems of enzymatic and non enzymatic antioxidants have the capability to address the deteriorating impacts of oxidants and free radicals that may lead to infirmity. An antioxidant is a type of compound that stabilizes, scavenges, and suppresses the generation of oxidants and free radicals. Therefore, consumption of antioxidants based on natural resources (greens, fruits, and herbs) may help to shield one from oxidants and free radicals without side effects” (Zainol haida, 2019)

“*Cycas circinalis* (Cc) belongs to family Cycadaceae, is a native of eastern and southeast asia and is cultivated in many tropical and subtropical areas of ornamental purpose. It usually produces one or more unbranched stems. Plant is dioecious. Male plant is also called the male sago, and its cone has aphrodisiac activity .It is considered to be an invigorating and nutritive tonic for people emaciated  by famine or disease.In Siddha system of medicine, the male cone was used to improve maleness. It is having narcotic, stimulant, and aphrodisiac activity. Male cones are used as stimulant; seeds are used as aphrodisiac as well .Pith in the stem is often harvested because of it’s medicinal value” (Kumar, 2007)

**2. Materials and Methods**

**2.1 Plant extraction**

Male cone and stem of *Cycas circinalis* were collected on 28th July 2022 from the college campus of St. Xavier’s college, Mahapalika Marg, Mumbai, Maharashtra, India. Plant were identified from Blatter Herbarium.

**2.2 Chemical and apparatus required**

* **Chemicals –** Ascorbic acid (vitamin c), Sulphuric acid, sodium phosphate, ammonium molybdate, folin-ciocalteu reagent(FCR), sodium carbonate, sodium chloride(Nacl), potassium chloride(Kcl), di-sodium phosphate(Na2HPo4), monopotassium phosphate(KH2Po4), sulphanilamide, orthophosphoric acid, H3Po4, N-(1-naphthyl) ethylene diamine dihydrochloride, methanol, DPPH(2,2-diphenyl-1-picryl-hydrazyl-hydrate), potassium ferricyanide, Fecl3, gallic acid, Methanol, Aluminium chloride, sodium nitrite, sodium hydrochloride,
* **Apparatus required-** Test tubes, Test tube stand, Holder, Whatman Filter paper, Falcon tubes, Tissue paper, brown bottles, trays, conical flask , Spatula, cotton, Hot air oven , Incubator, Shaker, Spectrophotometer, Centrifuge, Fridge, Weighing machine, Micropipette, Funnel, Weighing machine.

**2.3 Extraction Procedure**

Male cone and stem of *Cycas circinalis* collected, were cut down into small pieces and were shade dried for 1 day .After that it was kept in a hot air oven for 4-5 days. It was then grinded into fine powder using a mixer grinder.

For water extraction, a decoction method was selected. 1g of fine powder of stem and male cone was mixed with 50 ml of water and was kept in a water bath. The temperature was set to 45o for 2 hours, 75o for 2 hrs and then to 90o for 1 hrs. Then the extract was filtered using whatman filter paper.

Likewise, In order to determine methanol extraction, 1 g sample of *Cycas circinalis* ground into a fine powder in a mixer grinder and was mixed with 50 ml methanol. The obtained extracts were filtered over Whatman paper and the filtrate was collected. The both extracts of male cone and stem were stored in a bottle. Extracts were named as AECCC (Aqueous extract of *Cycas circinalis* cone) ,MECCC(Methanolic extract of *Cycas circinalis* cone), MECCS(Methanolic extract of *Cycas circinalis* stem), AECCS(Aqueous extract of *Cycas circinalis* stem).

* 1. **Antioxidant assay used**
	+ **Reducing power**

“Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric–ferrous complex .The assay was carried out as described by ‘Glucin, Tel & Kierecci,2008’. 1 ml of different concentrations of sample was added in all test tubes. Sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 mL, 1%) was added. The mixture was incubated at 50°C for 20 min. After cooling (2.5 mL) of trichloroacetic acid (10%) were added to the mixture. The solution is centrifuged at 3000 rpm for 10 minutes. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates an increase of reduction capability” (A. Kumaran, 2008)

* + **Total antioxidant capacity**

This assay is based on the reduction of phosphomolybdate ion in the presence of an antioxidant resulting in the formation of a green phosphate/MoV complex which is measured spectrophotometrically .A method of total antioxidant capacity described by Priya,Rajaram & Suresh Kumar, 2012 was considered. “0.1ml of extract was combined in Test tube with 0.3 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were incubated at 95°C for 90 minutes. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695 nm against blank” (Francis. 2007)

* + **Nitric oxide scavenging activity**

“Nitric oxide reacts with oxygen to produce the stable product nitrates and nitrite through the intermediates NO2 , N2O4 and N3 O4 . It is estimated by using the Griess reagent. In the presence of a test compound, which is a scavenger, the amount of nitrous acid decreases” . Method used as described by ‘Marcocci, Maguire, Droylefaix, and Packer 1994’.0.5ml sample was added to test tubes,  3ml of 10Mm Sodium nitroprusside prepared in PBS(Ph – 7.4). Test tubes were incubated at 25 degree C for 150 mins(2 hrs 30 min),0.5 Gries reagent was added to all test tubes incubated solution.It was re incubated for 30 minutes at room temperature. Absorbance was measured at 546 nm. PBS alone will act as blank. (Avni Patel, 2010)

Nitric oxide scavenging capacity (%) = [(A695 Control−A695 Sample) ∕A695 Control] ×100

* + **DPPH radical scavenging activity**

 “DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in methanol. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colourless methanol solution. Protocol used as described by Alhakmani, Kumar, & Khan, 2013. 0.1mM of ethanolic DPPH is prepared (0.002 g in 50 ml). Methanolic DPPH was covered with aluminium foil and was incubated at room temperature for 30 minutes. 1 ml of sample solution was added in test tubes taken in triplicates, immediately 2 ml DPPH was mixed thoroughly. Test tubes were kept for incubation at 37 degree C for 30 minutes. colour changes from purple to yellow. Absorbance was measured at 515 nm.” (Kumar, 2007)

DPPH scavenging effect (%) = (A Control- A sample)/Acontrol \*100

* + **Total phenolic content**

“A slightly modified version of the method outlined by Singleton and Rossi 1965 was used to determine the content of total phenolics. The total phenolic compounds can be determined using the Folin-Ciocalteu technique. Gallic acid was used as a standard phenolic compound. The principle is the reduction of the Folin–Ciocalteu reagent (FCR) in the presence of phenolics resulting in the production of molybdenum–tungsten blue that is measured spectrophotometrically. In a brief, 0.5 ml sample of different concentration was added in each test tubes. Following the addition of 2.5 mL of Folin-Ciocalteu reagent, the contents of the flask were vigorously mixed.2.5 ml of Na2CO3 (2%) was added after 5 min, and the mixture was then left to stand for 1 h for incubation at room temperature. After cooling to room temperature, absorbance was measured at 725 nm” (Francis., 2007)

* + **Total flavonoid content**

“Total flavonoids amount in the both extracts was determined by method of Zhishen, Mengcheng & Jianming, 1999 as follows: 1 ml of sample was added to 4 ml of distilled water in each test tube. 0.3 ml 5% sodium nitrate was added, after 5 minutes 0.3ml Aluminium chloride was added and 2 ml of 1M NaOH was added to them, immediately all test tubes were vortexed. After 40 min incubation at room temperature, the absorbance was determined spectrophotometrically at 510 nm. Total flavonoids concentration was calculated using catechin as standard.” (Zainol haida, 2019)

**3. Results and Discussion**

**3.1  Reducing power**

In this assay, the antioxidants present in sample would result in the reduction of Fe3+ to Fe2+ by giving one electron. The yellow colour solutions present in the test tubes changes to various shades of blue and green colour depending on the reducing power of sample. Which can be monitored by measuring formation of Pearl’s Prussian blue at 700 nm.

Graph 1 shows the reducing power of AECCC, MECCC, MECCS, AECCS (A and Standard (Ascorbic acid). The reducing power of AECCC, MECCC, AECCS, MECCS and standard compound exhibited the following order:- standard> MECCS > MECCC > AECCS> AECCC

[Insert graph 1]

**3.2 Total antioxidant capacity**

Antioxidant activity of the extracts of varying concentrations ranging form 10- 10000 µg/ml was evaluated by various in vitro models. Graph 2 shows the Total antioxidant capacity of AECCC, MECCC, MECCS, AECCS and Standard(Ascorbic acid). The Total antioxidant capacity of AECCC, MECCC,AECCS, MECCS and standard compound exhibited the following order:- standard> MECCS> AECCS> AECCC> MECCC

[Insert Graph 2]

[Insert table 1]

**3.3 DPPH radical scavenging activity**

“For screening of antioxidant activity of plant extracts DPPH assay is one of the most widely used methods. DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. It was reduced to a yellow coloured product, diphenyl picryl hydrazine, with the addition of all fractions in a concentration-dependent manner. Lower the IC50 value or better is the scavenging ability of the sample. There was no significant difference found between MECCC and MECCS.IC50 value of AECCC and MECCC was 11.14 µg/ml and 3.8 µg/ml. And of AECCS and MECCS was 9.89 µg/ml and 3.73 µg/m” (Kumar, 2007)

**3.4 Nitric oxide radical scavenging activity**

“Nitric oxide radical inhibition in vitro is used to test the antioxidant effectiveness of plant-based medicines.

The production of nitric oxide from sodium nitroprusside in buffered saline, which combines with oxygen to form nitrite ions that can be detected using Griess reagent, is the basis for the scavenging of nitric oxide radicals.

Due to the presence of antioxidant components in the extract, EEHS reduced the amount of nitrite produced during the in vitro breakdown of sodium nitroprusside.

As extract concentration increases, so does the percentage scavenging activity. The IC50 value of AECCC and MECCC was 5.517µg/ml and 35.57 µg/ml . And of AECCS and MECCS was 4.367µg/ml and 57.078µg/ml”(Avni Patel, 2010)

[Insert table 2]

[Insert graph 3]

[Insert graph 4]

**3.5 Total phenolic and flavonoid content**

Total phenolic compounds are reported as pyrocatechol equivalents. The total phenolic content of 1 gm of WECC and MECC  of cone were 0.0127 mg and 0.00165 mg and total phenolic content of phenolics content of WECC and MECC  of stem were 0.0127 mg and  0.00065 mg of standard gallic acid/gram of sample. In both extract (cone and stem) of *Cycas circinalis* WECC possessed the highest phenolic compounds.

Flavonoids are one of important group of natural compounds, which helps in preventing coronary heart disease and have antioxidant properties.The content of total flavonoids of 1 gm of WECC and MECC  of cone were 0.115 mg and 0.03 mg quercetin equivalent /g of sample.The content of total flavonoids of 1 gm of WECC and MECC  of stem were 0.12665 mg and  0.07mg quercetin equivalent /g of sample.

[Insert table 3]

**4. Conclusion**

According to the findings of the current investigation, AECCC, MECCC, AECCS and MECCS were found to be effective antioxidant in different in vitro assay including DPPH, Nitric oxide scavenging activity, reducing power, Total antioxidant capacity when it is compared with standard antioxidant compound such as ascorbic acid. Methanolic extract showed better results in Reducing power and in Total antioxidant assay. In DPPH scavenging activity methanolic extract gave better results. In Nitric oxide Aqueous extract showed better results. A methanolic extract of *Cycas circinalis* cone and stem demonstrates strong antioxidant and free radical scavenging properties. Additionally, it possesses reducing power and chelates iron. These in vitro tests show that this plant extract is a considerable natural antioxidant source, which may be useful in halting the progression of various oxidative stressors. Therefore, additional research must be done to isolate and characterize the antioxidant chemicals found in the plant extract.

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**7. Graphs and Tables**



P< 0.05(AECCC,AECCS,MECCC,MECCS)

Graph 1: Total reductive potential of AECCC, MECCC, AECCS and MECCS



P<0.05(AECCC,AECCS) P>0.05(MECCC,MECCS)

Graph 2: Total Antioxidant capacity of AECCC, MECCC, AECCS and MECCS

|  |  |  |
| --- | --- | --- |
|  | **REDUCING POWER** | **TOTAL ANTIOXIDANT CAPACITY** |
|  concentration( µg/m) | AECCC | MECCC | AECCS | MECCS | AECCC | MECCC | AECCS | MECCS |
| 0.005 | 0.094±0.005 | 0.089±0.16 | 0.101±0.005 | 0.099±0.016 | 0.014±0.004 | 0.06±0.03 | 0.011±0.004 | 0.063±0.009 |
| 0.01 | 0.096±0.01 | 0.081±0.25 | 0.107±0.01 | 0.147±0.025 | 0.02±0.003 | 0.061±0.002 | 0.026±0.005 | 0.065±0.005 |
| 0.015 | 0.098±0.015 | 0.2±0.39 | 0.108±0.015 | 0.183±0.039 | 0.04±0.002 | 0.063±0.001 | 0.041±0.003 | 0.066±0.004 |
| 0.02 | 0.099±0.02 | 0.15±0.49 | 0.111±0.02 | 0.238±0.04 | 0.05±0.005 | 0.065±0.001 | 0.054±0.009 | 0.072±0.013 |
| 0.025 | 0.1±0.025 | 0.15±0.050 | 0.112±0.025 | 0.265±0.05 | 0.07±0.001 | 0.066±0.004 | 0.080±0.007 | 0.077±0.001 |

 Table 1: Reducing power and Total antioxidant capacity

|  |  |
| --- | --- |
|  | **% SCAVENGING ACTIVITY OF DPPH AND NITRIC OXIDE** |
| **DPPH** | **NITRIC** |
| concentration( µg/m) | AECCC | MECCC | AECCS | MECCS | AECCC | MECCC | AECCS | MECCS |
| 0.01 | 24.74±0.009 | 29.03±0.02 | 24.66±0.0.005 | 29.03±0.042 | 10.84±0.017 | 75.62±0.01 | 1.25±0.005 | 79.65±0.05 |
| 0.025 | 25.96±0.03 | 34.39±0.02 | 25.41±0.003 | 35.14±0.038 | 16.3±0.018 | 76.34±0.02 | 14.15±0.016 | 80.37±0.03 |
| 0.05 | 27.11±0.0.017 | 41.25±0.005 | 26.69±0.0015 | 41.25±0.004 | 19.98±0.001 | 76.79±0.01 | 22.75±0.029 | 81±0.01 |
| 0.075 | 33.33±0.02 | 47.28±0.001 | 34.24±0.002 | 48.94±0.009 | 29.21±0.009 | 77.59±0.02 | 29.74±0.018 | 81.36±0.02 |
| 0.1 | 33.93±0.01 | 64.4±0.003 | 35.29±0.008 | 64.4±0.027 | 48.38±0.01 | 78.49±0.02 | 42.38±0.008 | 81.72±0.03 |

Table 2: Percentage scavenging activity of DPPH and Nitric oxide

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P<0.05(AECCS,MECCC) P>0.05(AECCC,MECCS)

Graph 3: % Scavenging activity DPPH  of AECCC, MECCC, AECCS and MECCS

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P<0.05(AECCC,AECCS, MECCC,MECCS)

Graph 4: % Scavenging activity Nitric oxide  of AECCC, MECCC, AECCS and MECCS

|  |  |  |
| --- | --- | --- |
|   | Total phenolics(mg/ml) | Total flavanoids(mg/ml) |
| AECCC | 0.0127±0.006 | 0.115±0.02 |
| MECCC | 0.00165±0.013 | 0.0006±0.006 |
| AECCS | 0.0127±0.018 | 0.126±0.003 |
| MECCS | 0.00065±0.004 | 0.07±0.006 |

 Table 3: Total phenolic and flavonoid content in mg/ml