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Research Article

Total Phenolic, Flavonoid Content, and Antioxidant Activity of *Justicia tranquebariensis* LF and *Cycas Beddomei* Dyer. Leaves

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ABSTRACT

Polyphenolics such as tannins and flavonoids are proved as powerful antioxidants to resolve cellular stress, sustain homeostasis in the body and prevent stress-associated disorders. Plant-based medicine gained importance due to its safety margin and multiple benefits. Regular intake of antioxidants is an alternative to avoid serious illness. The current investigation is aimed to measure the phenolic, flavonoid content, the antioxidant activity of *Justicia tranquebariensis L.f.* and *Cycas beddomei* leaves. The selected plants are used by tribes and traditional medicine to cure various diseases. Total phenolic content was estimated using the Folin–Ciocalteu colorimetric method, taking gallic acid as standard. At the same time, total flavonoid content was determined by aluminum chloride colorimetric assay using Rutin as standard. The absorbance was measured at 760 nm and 510 nm, respectively. Antioxidant activity was measured by two *In vitro* methods (DPPH and NO free radical scavenging assay) using standard protocols, where Ascorbic acid served as a reference standard. Results disclosed that the total phenolic content for the methanolic extracts of *C. beddomei* (36.14 ± 1.72) and *J. tranquebariensis* (28.57 ± 0.91) was found to be higher than petroleum ether extracts. Similarly, *C. beddomei* (28.13 ± 2.48) is richer in flavonoids than *J tranquebariensis* (20.32 ± 1.24). In DPPH assay, methanolic extract *C. beddomei* (78.21%) is more effective to inhibit the free radicals than *J. tranquebariensis* (73.05%) with IC_{50} values 41.55µg/ml & 59.52 µg/ml respectively at higher doses that are comparable to standard ascorbic acid (84.34%, IC_{50} =63.27 µg/ml). Similarly, in NO free radical scavenging assay, methanolic extract of *C. beddomei* (72.54%) stood best among all to scavenge the free radicals with IC_{50} values 50.06 µg/ml, whereas ascorbic acid is found to inhibit 83.26% with IC_{50} values 39.75µg/ml. The presence of various secondary metabolites such as alkaloids, carbohydrates, phenols, steroids, terpenoids, glycosides, saponins, especially tannins and flavonoids, either alone or in combination, may be responsible for the observed scavenging property. The quantity of the phenolic compounds and flavonoids can be directly correlated to the exhibited activity.

Introduction

Plants have been the source of medicine for ages to cure several ailments. In virtue of their safety and diverse pharmacological aspects, plant-based medicine is chosen over the synthetics for chronic diseases.[1] Since, plants reserve immeasurable secondary metabolites with multifaceted biological targets, the researchers are fascinated to explore their biological profile towards drug discovery.[2,3]

Justicia tranquebariensis of Acanthaceae family is a small shrub that grows in peninsular India with simple, obovate and opposite leaves. Plant of this genus has well-known traditional applications. They treat pain, inflammations, fever, cold, liver diseases, diarrhea, and diabetes.[4] Few phytochemicals such as lignans, including lariciresinol and cubebin were isolated from the aerial parts.[5]

Cycas beddomei of the Cycadaceae family is used by tribals for rejuvenation purposes.[6,7] Local people use the

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seeds and other parts of the plants for microbial infections, wound healing, pain, fevers, rheumatoid arthritis, muscular spasm, sores, boils, etc. Pharmacologically the plant is proved to have antibacterial, antifungal, analgesic, anti-arthritic, and antiulcer activity.[8-10]

The production of free radicals is a defense mechanism to combat pathogenic entry into the cellular environment. The restoration of this stress load has contended with scavenging enzymes in the body.[11] The disruption of consonance between the production and lavation of these reactive species creates cellular stress and cause chronic and progressive neurodegenerative disorders, for example, diabetes, cardiovascular diseases, cancer, arthritis, etc.[12-14] Antioxidant agents support the scavengers to scrum oxidative stress, improve body health, and delay aging. Naturally derived antioxidants such as flavonoids, tocopherol and tannins are renowned for their potent protective effect and health benefits with minimum risks over the synthetic antioxidants.[15]

In this regard, the current research was planned to explore the total phenolic content and flavonoid content and estimate the antioxidant potential of various extracts of leaves of *Justicia tranquebariensis* and *Cycas beddomei*, using *in vitro* models such as DPPH assay and nitric oxide free radical scavenging assay. The extracts were also subjected to preliminary phytochemical screening to determine the presence of secondary metabolites.

Materials and Methods

Plant Material

Justicia tranquebariensis and *Cycas beddomei* leaves were collected from rural Nalgonda, Telangana, and authenticated by Dr. K. Venkata Ratnam, Assistant Professor, Department of Botany Rayalaseema University, Kurnool; leaf specimens have been submitted (RU/BD/VSN-135&136) for future reference.

Reagents and Chemicals

All the chemicals and reagents were procured from Sigma Aldrich (laboratory grade).

Preparation of Extracts

The leaves of *Justicia tranquebariensis* and *Cycas beddomei* were collected and dried under shade. The dried leaves were powdered and subjected to exhaustive extraction with petroleum ether and methanol, respectively, using the Soxhlet apparatus. The solvent was evaporated to dryness to get the solid extract, and percentage yield was calculated.[16]

Phytochemical Screening

The preliminary phytochemical investigation of petroleum ether and methanolic leaf extract of *Justicia tranquebariensis* and *Cycas beddomei* was carried out by employing standard protocols.[17]

Estimation of Total Phenolic Content

Folin-Ciocalteu (FC) assay was used to estimate the total phenolic content of extracts of *J. tranquebariensis*, and *C. beddomei* leaves. 200 µl of the extract solutions were mixed with 2.5ml of FC reagent (diluted to 10 times) and 2 ml of Na_2CO_3 solution (7.5% w/v) followed by proper mixing and incubation at 30°C for 90 minutes. Absorbance for all the samples was recorded at 765 nm and expressed in terms of mg equivalents of gallic acid. The calibration curve was constructed by plotting the absorbance against concentration from the equation $y = 6.1047x + 0.1334$, $R^2 = 0.9904$. The values are calculated for triplicates.[18]

Estimation of Total Flavonoid Content

The flavonoid content of the leaf extracts was determined by a colorimetric method using aluminum trichloride method (Zhishen method). A volume of 125µL of the extracts is added to 75 µL of a 5% Sodium nitrite ($NaNO_2$) solution. After 6 minutes, 150 µL of $AlCl_3$ solution (10%) was added, followed by 750 µL of NaOH (1-M). The final volume of the solution was made to 2500 µL with distilled water. After 15 minutes of incubation, the mixture turned pink, and the absorbance was measured at 510 nm. The total flavonoids content was expressed as gram equivalence of Rutin per gram dry weight.[19]

In vitro Antioxidant Assay

DPPH Radical Scavenging Assay

The free radical scavenging activity of *J. tranquebariensis* and *C. beddomei* leaf extracts was estimated. Plant extracts of different concentrations were prepared using DMSO, whereas a solution of 25 mg/L DPPH was prepared by using methanol. In 96-well plate, 5µl of the extract solution followed by 195 µl of DPPH solution was added and incubated for 20 minutes at room temperature. The absorbance was measured at 515 nm for individual extracts, and the free radical scavenging activity was recorded by comparing the absorbance values with the blank. The above procedures were repeated using ascorbic acid as positive controls in triplicates. The antioxidant activity was calculated using the formula given below.[20]

$$\% \text{ Free radical scavenging activity} = [(A_0 - A_s)/A_0] \times 100$$

Where,

A_0 is the absorbance of blank (DPPH solution alone)

A_0 is the absorbance of extracts (DPPH + sample)

Nitric Oxide Radical Scavenging Assay

Leaf extracts of *J. tranquebariensis* and *C. beddomei* were also screened for nitric oxide radical scavenging activity using sodium nitroprusside. In this experiment, 2 mL of sodium nitroprusside (10 mM) and 0.5 mL of phosphate buffer (pH-7.4) will be mixed with 0.5 mL of the test solution and incubated for 150 min at 25°C. Ascorbic acid solution and DMSO served as standard and control, respectively. Sulfanilic acid reagent (1mL 0.33% of

sulfanilic acid in 2% glacial acetic acid) was added to 0.5 mL of nitrite and kept for 5 minutes. Naphthyl ethylene diamine dihydrochloride (NEDD, 1-mL of 1%) was added and incubated for 30 minutes at 25 °C.[21] The absorbance was recorded at 540 nm, and the percentage of nitric oxide inhibition was calculated as:

Percentage of nitric oxide radical scavenging assay = $[(A_0-A_s)/A_0] \times 100$

Where,

A_0 was the absorbance of control

A_s was the absorbance of the treated sample

Results

Preliminary Phytochemical Screening

The preliminary phytochemical study of petroleum ether and methanolic extracts of *J. tranquebariensis* and *C. beddomei* exposed that the extracts are instituted with various secondary metabolites such as alkaloids and carbohydrates flavonoids, phenols, steroids, terpenoids, glycosides, tannins, saponins (Table 1).

Total Phenolic Content

Phenolic compounds can be directly correlated to their protective effect against the cellular stress in the body. Total phenolic contents of petroleum ether and methanolic extracts of *J. tranquebariensis* and *C. beddomei* leaves were evaluated by Folin–Ciocalteu method taking gallic acid as the reference standard. A calibration curve was plotted against the absorbance values versus different concentrations of gallic acid (Fig. 1). The total phenolic content of the extracts was calculated from the regression equation of calibration curve ($Y = 0.0051x + 0.0162$; $R^2 = 0.998$) and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g) (Table 2). Total phenolic content values were observed high in methanolic extract of *C. beddomei* (36.14 ± 1.72) than *J. tranquebariensis* (28.57 ± 0.91).

The Flavonoid Content

The polyphenolic compounds such as flavonoids are important antistress agents that help the body to adapt to innumerable harsh environment. These defensive agents also improve human health from chronic ailments. The flavonoid content of the leaf extracts of *J. tranquebariensis* and *C. beddomei* was determined by a colorimetric method using the Zhishen technique and is found to be 20.32 ± 1.24, and 28.13 ± 2.48 of gram equivalence of Rutin at 510 nm (Table 3 and Fig. 2). The calibration curve was made by linear regression, and the results represented the average of three determinations to each concentration. The total phenolic content of the extracts was calculated from the regression equation of the calibration curve ($Y = 3.54x + 0.0673$; $R^2 = 0.9973$) and expressed as mg Rutin equivalents (RE) per gram of sample in dry weight (mg/g).

The results show that methanolic extracts of both plants are rich in flavonoids compared to the petroleum ether extracts. The polarity of the methanol is sufficient to

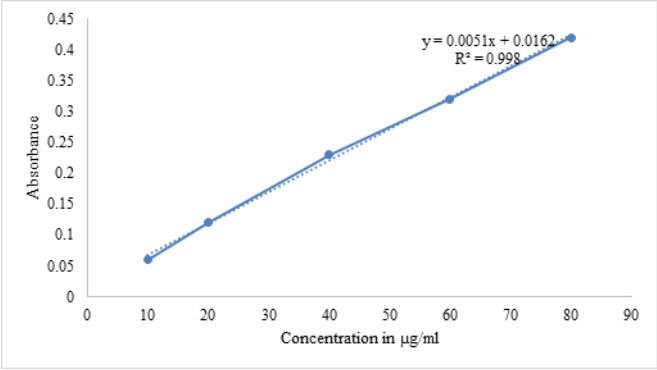

Fig. 1: Calibration curve for Gallic acid

Table 1: Phytochemical screening of *J. tranquebariensis* and *C. beddomei*

	J. tranquebariensis		*C. beddomei*	
Phytochemicals	*Petroleum ether extract*	*Methanolic extract*	*Petroleum ether extract*	*Methanolic extract*
Alkaloids	+	+	+	+
Glycosides	-	+	-	+
Flavonoids	-	+	+	+
Terpenoids	+	+	+	+
Steroids	+	+	+	-
Tannins	-	+	-	+
Proteins	-	+	-	+
Carbohydrates	-	+	-	+
Amino acids	-	-	-	+
Saponins	-	+	-	+

+ indicates the presence and – indicates the absence of phytochemicals

Table 2: The total phenolic content of leaf extracts of *J. tranquebariensis* and *C. beddomei*

Extract	*Total phenolic content mg/ml*
JTPE (*J. tranquebariensis* petroleum ether extract)	2.26 ± 0.83
JTME (*J. tranquebariensis* methanol extract)	28.57 ± 0.91
CBPE (*C. beddomei* petroleum ether extract)	5.18 ± 0.14
CBME (*C. beddomei* methanol extract)	36.14 ± 1.72

*All values are expressed as mean±SD for three determinations

Table 3: The total flavonoid content of leaf extracts of *J. tranquebariensis* and *C. beddomei*

Extract	*Total flavonoid content mg/ml*
J	UNE 2 ± 0.64
JTME	29 ± 1.32
CBPE	27.54 ± 1.15
CBME	107 ± 0.96

*All values are expressed as mean±SD for three determinations

solubilize the majority of the flavonoids, while petroleum ether failed to do maybe a reason to get higher values for these extracts. Moreover, methanolic leaf extracts of *C. beddomei* (107±0.96) have more flavonoid content than *J. tranquebariensis* (29 ± 1.32). Petroleum ether extract of *J. tranquebariensis* (2 ± 0.64) has a very low concentration of flavonoids.

DPPH Radical Scavenging Assay

In the present study, when compared to the standard Ascorbic acid, *C. beddomei* has exhibited significant free radical scavenging activity in a dose-dependent manner than *J. tranquebariensis* leaf. A standard curve was plotted (Fig. 3) using various concentrations of ascorbic acid. At a higher concentration (100 µg/mL), the *C. beddomei* extract with 78.21% of inhibition stood high among the extracts next to ascorbic acid (84.34%) (Table 4 and Fig. 4).

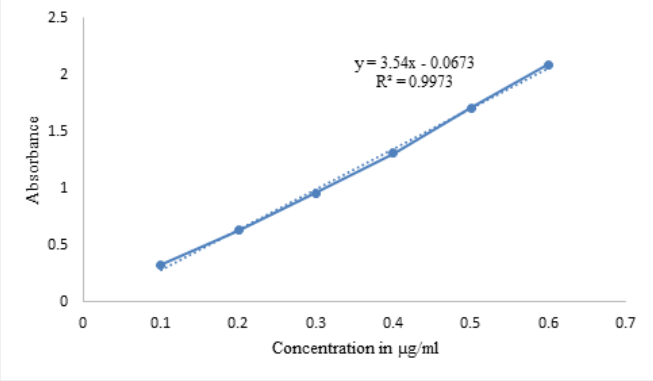

Fig. 2: Calibration curve for Rutin

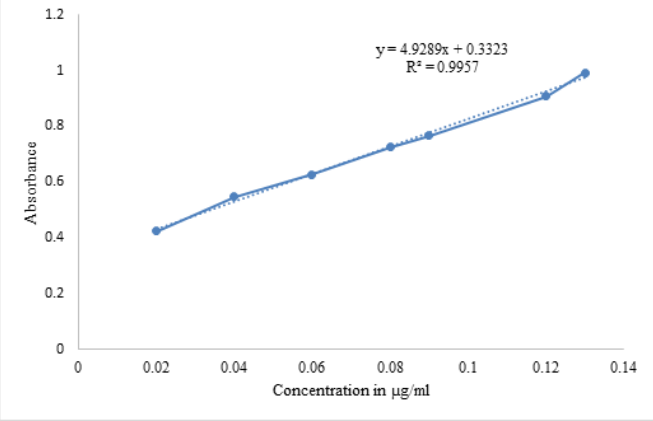

Fig. 3: Calibration curve for Ascorbic acid

Methanolic extracts of *C. beddomei* (IC_{50} = 41.55) and *J. tranquebariensis* (IC_{50} = 59.52) were inferred to be potent antioxidants than the petroleum ether extracts (Fig. 5). The ability of these extracts to scavenge DPPH could also reflect its ability to impede stress and, subsequently, the protective effects in the body.

Nitric Oxide Radical Scavenging Activity

When compared to the standard Ascorbic acid, *C. beddomei* leaf extract exhibited high NO free radical scavenging activity in a dose-dependent manner than *J. tranquebariensis* leaf extract (Table 5 and Fig. 6). At a higher concentration of 100 µg/mL, the methanolic *C. beddomei* extract shows 72.54% of inhibition with IC_{50} values 50.06 µg/ml followed by petroleum ether extract 71.34% with IC_{50} values 61.36µg/ml. Whereas for ascorbic acid, it is found to be 83.26% with IC_{50} values 39.75µg/ml

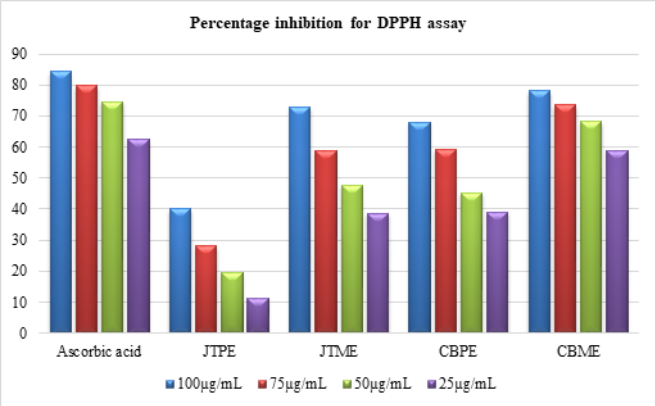

Fig. 4: Percentage inhibition of extracts at different concentrations for DPPH assay

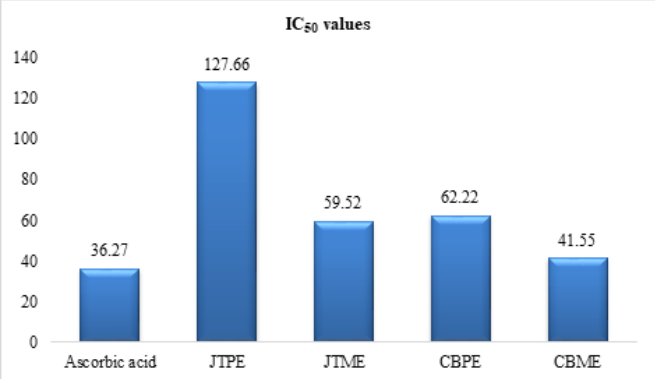

Fig. 5: IC_{50} values of extracts for DPPH assay

Table 4: Percentage inhibition and IC_{50} values of extracts and ascorbic acid at different concentrations in DPPH assay

		J. tranquebariensis		*C. beddomei*	
Concentration µg/mL	*Ascorbic acid*	*Petroleum ether extract*	*Methanolic extract*	*Petroleum ether extract*	*Methanolic extract*
100	84.34	40.19	73.05	68.07	78.21
75	79.87	28.07	58.84	59.37	73.64
50	74.53	19.48	47.82	45.24	68.27
25	62.36	11.14	38.63	39.06	58.81
IC_{50} µg/mL	36.27	127.66	59.52	62.22	41.55

Table 5: Percentage inhibition and IC_{50} values of extracts and ascorbic acid at different concentrations in NO free radical scavenging assay

		J. tranquebariensis		*C. beddomei*	
Concentration µg/mL	*Ascorbic acid*	*Petroleum ether extract*	*Methanolic extract*	*Petroleum ether extract*	*Methanolic extract*
100	83.26	39.37	69.39	71.34	72.54
75	72.64	24.19	54.51	58.11	69.82
50	68.21	14.21	46.17	45.27	58.25
25	62.14	10.24	29.54	38.35	49.17
IC_{50} µg/ml	39.75	132.31	65.38	61.36	50.06

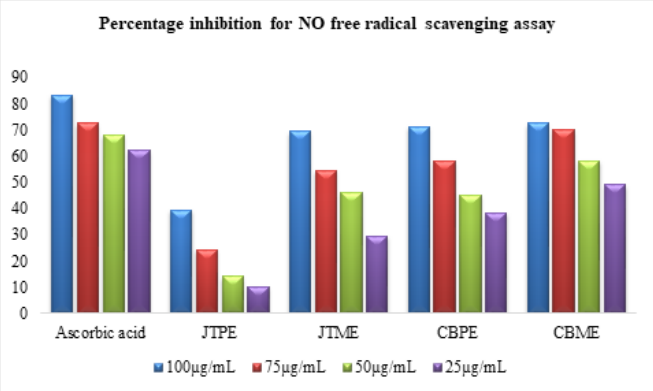

Fig. 6: Percentage inhibition of extracts at different concentrations for NO free radical scavenging assay

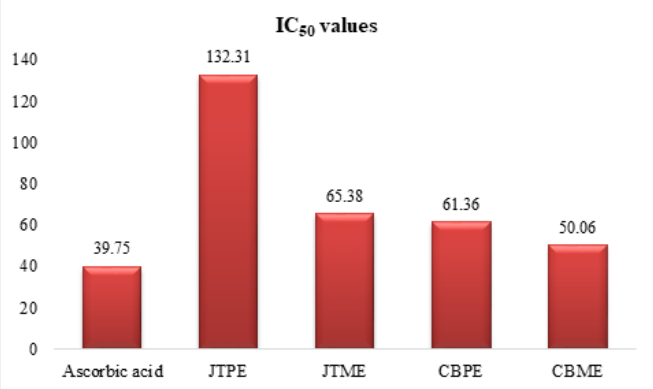

Fig. 7: IC_{50} values of extracts for NO free radical scavenging assay

(Fig. 7). The polyphenolic compounds such as tannins and flavonoids present in the *C. beddomei* may neutralize the free radicals liberated by the nitroprusside in the given procedure and may offer protection in the animal body related to cellular stress.

Discussions

Flavonoids and tannins are the major polyphenolic compounds produced as metabolic end products of plants that help to the detoxification process. They are considered powerful agents to neutralize the free radicals due to their ability to react with them and are regarded as antioxidants, supporting the scavenging mechanism of the human body. Ample literature is available to support the protective effect of these polyphenolics to address various chronic and lifestyle disorders associated with stress. Since synthetic agents are associated with fewer side effects, naturally occurring antioxidants are preferred in nutraceuticals for promoting health.[22]

It is important to determine the antioxidant potential of the plants to understand their efficiency in protecting against cellular stress. Numerous procedures were adopted to screen the antioxidant (*in vitro and in vivo*), such as DPPH assay, nitric oxide free radical assay, etc.[23]

Total phenolic, total flavonoid content for petroleum ether and methanol extracts of *J. tranquebariensis* and *C. beddomei* were evaluated using standard protocols. *All extracts performed in vitro* antioxidant screening in DPPH and NO free radical assay. To curb the results, it is apparent that *C. beddomei* is rich in phenols and flavonoids and can be correlated to the high antioxidant activity observed in both methods. Whereas, *J. tranquebariensis,* although rich in phytochemicals, the lower concentrations of the protective agents may be a reason to exhibit the lesser scavenging potential. Further, the investigation is in progress to evaluate the plant's complete phytochemical and pharmacological profile to justify its traditional applications and the reported antioxidant activity.

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