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Original Article

Evaluation of the Antioxidant Activity of Leaves of *Chamaecostus cuspidatus* Plant by *in vitro* Antioxidant Assays

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ABSTRACT

Background: *Chamaecostus cuspidatus*, also known as the insulin plant, has been traditionally used for diabetes management in various medicinal systems. This plant has demonstrated a variety of medicinal properties, including anti-inflammatory, anti-cancer, and anti-diabetic effects. This study aims to evaluate its antioxidant potential, which is crucial for managing oxidative stress, a key factor in the pathogenesis of diabetes and other chronic diseases. **Objectives:** To investigate the antioxidant activity of *Chamaecostus cuspidatus* using a range of *in vitro* assays and to assess its potential as a natural antioxidant source for therapeutic applications. **Materials and Methods:** Plant Material: Fresh leaves of *Chamaecostus cuspidatus* were collected, dried, powdered, and extracted using a Soxhlet method with methanol as the solvent. Antioxidant Assays: The following assays were performed to assess antioxidant activity: DPPH Radical Scavenging Assay, Hydrogen Peroxide (H_2O_2) Scavenging Assay, Phosphomolybdenum Assay, Nitric Oxide Scavenging Activity, Reducing Power Assay, Superoxide Radical Scavenging Assay, Metal Chelating Assay, Hydroxyl Radical Scavenging Assay. **Results:** The extract exhibited significant antioxidant activity across various assays. DPPH Assay: IC_{50} value of 190.4 µg, indicating strong free radical inhibition but less potent than the standard. Hydrogen Peroxide Scavenging Assay: IC_{50} value of 509 µg, indicating superior antioxidant capacity compared to the standard. Phosphomolybdenum Assay: Dose-dependent increase in reducing power. Nitric Oxide Scavenging Activity: IC_{50} value of 2.3 mg, lower efficacy compared to ascorbic acid. Reducing Power Assay: Demonstrated dose-dependent reducing potential, though lower than the standard. Superoxide Radical Scavenging Assay: IC_{50} of 37 µg, better than the standard. Metal Chelating Activity: Effective in binding ferrous ions, similar to the standard. Hydroxyl Radical Scavenging Assay: IC_{50} of 413 µg, moderate scavenging potential. **Conclusion:** *Chamaecostus cuspidatus* demonstrated significant antioxidant activity, especially in scavenging hydrogen peroxide, superoxide, and hydroxyl radicals. Its efficacy in these assays supports its traditional use in managing oxidative stress-related conditions, such as diabetes and neurodegenerative diseases. The plant's antioxidant potential positions it as a valuable candidate for developing natural therapeutic agents for oxidative stress management.

Keywords: *Chamaecostus cuspidatus*, Antioxidant activity, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH), Hydrogen Peroxide (H_2O_2), Nitric Oxide, Phosphomolybdenum Assay.

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INTRODUCTION

Chamaecostus cuspidatus, commonly referred to as the Insulin plant, Spiral flag, or Step ladder, originates from Central and South America. In India, particularly in the southern regions, it is cultivated widely as an ornamental plant and is also found growing in the wild. Belonging to the Costaceae family, this plant is recognized in traditional Siddha medicine for its antidiabetic properties. People often chew its leaves twice daily or consume 0.5 to 1 g of dried leaves in powdered form. Indigenous communities, such as those in Tamil Nadu's Kolli Hills, also use this plant for diabetes management, utilizing its fresh, dried, or powdered leaves. The Costaceae family, formally recognized by Nakai, is distinguished by spirally arranged leaves and rhizomes that lack aromatic essential oils. The family consists of four genera and approximately 200 species, with the genus Costus being the most prominent, encompassing around 150 species. In Indian traditional medicine, the leaves of *Chamaecostus cuspidatus* are commonly consumed to regulate blood glucose

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levels, with diabetics often taking a leaf per day to help manage their condition.[1] An overaccumulation of free radicals has been linked to various chronic health conditions. These unstable molecules can be neutralized by antioxidants, which help reduce their harmful effects. Oxidative stress, which results from an imbalance between the production of Reactive Oxygen Species (ROS) and the body's antioxidant defenses, contributes to cellular damage over time. This type of damage is implicated in aging and numerous diseases, including cancer, cardiovascular ailments, and neurological disorders. ROS encompass both oxygen-based radicals, such as hydroxyl, superoxide, peroxyl, and alkoxyl radicals-and non-radicals like hydrogen peroxide, hypochlorous acid, singlet oxygen, and ozone. Additionally, nitrogen-based reactive species, like nitric oxide and peroxynitrite, fall under the broader category of Reactive Nitrogen Species (RNS). Despite the body's natural antioxidant defense mechanisms, they may not always suffice to counteract these radicals effectively.[2] A compromised antioxidant system can elevate the frequency of radical-induced cell damage. Excessive free radical activity has been associated with various diseases, including cancer,[3] heart diseases,[4] neurological disorders,[5] Alzheimer's disease,[6] mild cognitive impairment,[7] Parkinson's disease,[8] alcohol-related liver damage,[9] ulcerative colitis,[10] aging,[11] and atherosclerosis.[12] This highlights the urgent need to explore plant-based antioxidants. Free radicals are atoms or molecules with an unpaired electron, rendering them unstable. In an attempt to stabilize, they interact with vital cellular components such as proteins, lipids, and DNA, potentially leading to structural and functional damage.[13] Although oxygen is vital for life, its metabolism in the mitochondria during energy production also gives rise to these reactive species. Lipids, proteins, nucleic acids, and carbohydrates are particularly vulnerable to oxidative attacks. To defend against this, antioxidants play a crucial role by neutralizing free radicals. These compounds donate electrons to unstable molecules, helping to stabilize them and prevent further cellular damage. In doing so, antioxidants convert harmful radicals into excretable byproducts, thereby protecting the body. Scientific research supports that a diet rich in antioxidant-containing fruits and vegetables can lower the risk of diseases associated with oxidative stress.[14] This protective effect is largely attributed to the presence of phytochemicals like polyphenols, carotenoids, and vitamins C and E.[15] Although many endemic plant species are used by local communities for medicinal purposes, there remains a significant gap in scientific data on their antioxidant potential. Yet, phenolic compounds-commonly found in edible and non-edible herbs, grains, fruits, vegetables, spices, and oils—are known for their strong antioxidant properties. Hence, identifying and evaluating these properties in underexplored plant species remains a valuable pursuit, especially in the context of developing functional foods or nutraceuticals.[16] This study, therefore, outlines commonly used methods to assess antioxidant activity in plant materials. The principal assays include:

- DPPH assay (2,2-diphenyl-1-picrylhydrazyl),
- Hydrogen peroxide scavenging assay (H_2O_2),
- Phosphomolybdenum assay,
- Nitric oxide scavenging activity,
- Superoxide radical scavenging assay,
- Metal ion chelation assay,
- Hydroxyl radical scavenging assay,
- These assays are fundamental tools used in antioxidant research to evaluate the free radical-scavenging potential of various plant extracts.

MATERIALS AND METHODS

Plant material

The fresh leaves of *Chamaecostus cuspidatus* were collected from Nadiad, Gujarat. The leaves were dried, powdered, passed through a sieve, and stored in an airtight container.

Chemicals and Reagent

All reagents used in this study were of analytical grade and obtained from reputable suppliers. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) was sourced from Natraj Scientific, while methanol, sodium nitroprusside, and potassium ferricyanide were obtained from Rankem. Hydrogen peroxide (H_2O_2), Dimethyl Sulphoxide (DMSO), and Butylated Hydroxytoluene (BHT) were purchased from Finar. Ascorbic acid was procured from Neelam Enterprise. Chemicals such as sulphuric acid, ferrous ammonium sulphate, and ammonium acetate were supplied by Loba Laboratories. Sodium phosphate, Ethylenediaminetetraacetic Acid (EDTA), and glacial acetic acid were obtained from SDFCL, while ammonium molybdate came from Laser Laboratories. Sulphadiazine and phosphoric acid were supplied by Zenith, and Trichloroacetic Acid (TCA) was procured from Himedia. Additional chemicals including ferric chloride, riboflavin, and acetone were purchased from Nilam Chemicals, Ahmedabad. All other chemicals used in the experiments were of analytical grade and acquired from established manufacturers.

Preparation of Plant Extract

The extraction of bioactive compounds from the plant material was carried out using a Soxhlet extraction method. A total of 50 g of powdered leaves were accurately weighed and placed in a filter paper thimble. Methanol was used as the extraction solvent. The Soxhlet apparatus was set up with the solvent heated to a boil, causing it to vaporize, condense in the cooling unit, and drip into the thimble containing the plant powder. This cyclic process was continued until the solvent in the thimble became colorless, indicating that exhaustive extraction had been achieved. The methanolic extract was then concentrated by evaporating the

solvent over a hot plate. The resulting concentrated extract was transferred to a labeled container and stored under appropriate conditions for subsequent analysis.

Antioxidant Assay

DPPH Radical Scavenging Assay

Principle

The foundation of this technique is the antioxidants' ability to reduce the DPPH radical, a stable free radical. When DPPH comes into contact with an antioxidant, it takes up an electron or hydrogen atom, which causes a noticeable change in color from deep purple to yellow.[17]

Procedure

Free radical scavenging was evaluated by mixing 3 mL of plant extract at different doses (50-150 µg/mL) with 1 mL of 0.1 mM DPPH solution made in methanol. After being vortexed, the samples were left to stand at room temperature for half an hour in the dark. The wavelength of absorbance was 517 nm. The baseline was a control sample with all reagents except the extract. An antioxidant reference was vitamin E.

Antioxidant activity was assessed using:

$$\text{Scavenging } \% = [(A0 - A1)/A0] \times 100$$

Hydrogen Peroxide (H_2O_2) Scavenging Assay

Principle

This method evaluates the ability of antioxidants in plant extracts to reduce hydrogen peroxide, monitored through UV absorbance at 230 nm, where H_2O_2 absorbs strongly.

Procedure

Different concentrations (300-900 µg/mL) of the plant extract were prepared. To each test tube, 600 µL of H_2O_2 was added, followed by 400 µL of either the extract or ascorbic acid (standard). Phosphate buffer (pH 7.4) was added to bring the total volume to 2 mL. Samples were incubated at room temperature for 10 min, and absorbance was measured at 230 nm against a blank.

Scavenging efficiency was calculated using the same formula as above.[18]

$$\text{Scavenged percentage} = [(A0 - A1)/A0] \times 100$$

Where, A1= the absorbance for the crude plant extract or standard, and

Ao= the absorbance of the control (without extract).[19]

Phosphomolybdenum Assay

Principle

This assay evaluates the total antioxidant capacity based on the reduction of Mo (VI) to Mo(V) by antioxidants, forming a green-colored phosphate/Mo(V) complex, which is measured spectrophotometrically.

Procedure

A total of 3 mL of reagent (consisting of 1 mL each of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was mixed with 0.3 mL of plant extract (20-100 µg/mL). The mixture was incubated at 95°C for 90 min. After cooling, the absorbance was read at 695 nm against a blank, using BHT as a reference standard.[20]

Nitric Oxide Scavenging Activity

Principle

Nitric Oxide (NO) generated from sodium nitroprusside interacts with oxygen to form nitrite ions, which can be quantified via the Griess reaction. Antioxidants inhibit NO formation by reducing nitrite accumulation.

Procedure

2 mL of 10 mM sodium nitroprusside was mixed with 0.5 mL phosphate buffer saline (pH 7.4) and 0.5 mL of extract at different concentrations. The mixture was incubated for 150 min at 25°C. Then, 1 mL of the incubated sample was treated with 3 mL of Griess reagent (1% sulfanilamide, 2% phosphoric acid, 0.1% NED dihydrochloride). After 30 min at room temperature, absorbance was recorded at 540 nm. Ascorbic acid was used as the standard.

$$\text{Scavenged } \% = [(A0 - A1)/A0] \times 100$$

Where, A1= the absorbance for the crude plant extract or standard, and

Figure 1: *Chamaecostus cuspidatus* plant.

Ao= the absorbance of the control (without extract).[21]

Reducing Power Assay

Principle

This test assesses an antioxidant's ability to reduce Fe^{3+} to Fe^{2+}. In an acidic medium (pH 3.6), the increased absorbance at 700 nm indicates higher reducing capacity due to electron transfer activity.[22]

Procedure

2.5 mL of 0.2 M phosphate buffer (pH 6.6), 2.5 mL of 1% potassium ferricyanide, and 1 mL of extract were mixed and incubated at 50°C for 20 min. After adding 2.5 mL of 10% TCA, the mixture was centrifuged. The supernatant (2.5 mL) was combined with 0.5 mL of 0.1% $FeCl_3$ and 2.5 mL distilled water. Absorbance was measured at 700 nm.[23]

Superoxide Radical Scavenging Assay

Principle

Riboflavin in the presence of light initiates the generation of superoxide radicals, which reduce NBT to formazan. Antioxidants inhibit this reaction by scavenging the superoxide radicals.

Procedure

A reaction mixture (1 mL) containing 50 mM phosphate buffer (pH 7.4), 10 µM riboflavin, 56 µM NBT, 12 µM EDTA, and different concentrations of the extract (10-200 µg) was prepared. The mixture was exposed to light for 2 min, and absorbance was measured at 590 nm at 30-second intervals. Vitamin E was used for comparison.[24]

Metal Chelating Assay

Principle

Ferrozine forms a red complex with Fe^{2+} ions. When a chelating agent is present, it binds Fe^{2+}, reducing complex formation and absorbance at 562 nm.

Procedure

0.5 mL of 0.2 mM $FeCl_2$ was added to 0.1 mL of extract, followed by 0.2 mL of 5 mM ferrozine. The mixture was incubated for 10 min at room temperature. Absorbance was measured at 562 nm. Citric acid or EDTA served as positive controls.[25]

Hydroxyl Radical Scavenging Assay

Principle

Hydroxyl radicals generated by the reaction between Fe^{2+} and H_2O_2 react with DMSO to form formaldehyde. The amount of formaldehyde, detected by Nash reagent, reflects hydroxyl radical levels.

Procedure

Various extract concentrations (100-500 µg) were combined with 1 mL of Fe-EDTA, 0.5 mL EDTA (0.018%), and 1 mL DMSO (0.85% in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of 0.22% ascorbic acid and incubated at 80-90°C for 15 min. After cooling, 1 mL of cold 17.5% TCA and 3 mL of Nash reagent were added. After 15 min at room temperature, absorbance was recorded at 412 nm.

Each assay used the same formula to calculate radical scavenging or inhibitory activity:

$$\text{Inhibition percentage} = [(A0 - A1)/A0] \times 100$$

Where, A1= the absorbance for the crude plant extract or standard, and

Ao= the absorbance of the control (without extract).[26]

RESULTS AND DISCUSSION

The antioxidant efficacy of the plant extract was thoroughly assessed using a comprehensive set of well-established *in vitro* assays. The extract exhibited notable free radical scavenging and antioxidant properties in the following assays: DPPH (2,2-Diphenyl-1-picrylhydrazyl), hydrogen peroxide scavenging, phosphomolybdenum reduction, nitric oxide scavenging,

Table 1: Observation Table of DPPH, H_2O_2, Nitric oxide Assay.

DPPH Assay				H_2O_2 Assay				Nitric oxide assay			
Sample		Standard		Sample		Standard		Sample		Standard	
Conc. (µg)	%Scavenging	Conc. (µg)	%Scavenging	Conc. (µg)	%Scavenging	Conc. (µg)	% Scavenging	Conc. (µg)	% Scavenging	Conc. (µg)	% Scavenging
100	20.43	5	1.2	200	24.67	100	26.26	100	5.7	100	15.19
200	52.52	10	4.6	300	40.74	200	29.33	300	7.9	200	23.86
300	62.74	20	12.74	600	58.9	300	33.61	500	10.2	300	28.11
400	71.03	25	21.63	700	68.72	400	35.36	700	14.7	400	31.37
500	72.83	30	31.73	800	78.85	500	40.95	900	24.6	500	44.58
				900	97.90			1500	35.7		
								2000	42.66		

reducing power, superoxide radical scavenging, metal chelation, and hydroxyl radical scavenging. Figures 1-3 represent picture of *Chamaecostus cuspidatus* plant, Graphs of DPPH and Reducing power assay and Graph of Hydrogen Peroxide radical, ferrous iron chelating, phosphomolybdenum. Hydroxyl radical, Nitric oxide, superoxide radical scavenging assay respectively. While Tables 1-3 represent Observation of DPPH, H_2O_2, Nitric oxide Assay, Observation of Superoxide, Metal chelating, Hydroxyl radical Assay and Comparison of IC_{50} between sample and standard extract respectively.

DPPH Radical Scavenging Assay: The extract showed effective free radical inhibition in the DPPH assay. It exhibited an IC_{50} value of 190.4 µg, which, while indicative of strong antioxidant potential, was less potent than the standard (47.3 µg). The comparatively higher IC_{50} value suggests that a greater quantity of the extract is needed to achieve similar radical scavenging effects.

Hydrogen Peroxide Scavenging Assay: The extract demonstrated superior antioxidant capacity with an IC_{50} of 509 µg, outperforming the standard (610 µg). These findings highlight the potential of the extract as a natural alternative to synthetic antioxidants.

Phosphomolybdenum Assay: This assay, which assesses the total antioxidant capacity through molybdenum reduction, revealed that increasing the concentration of the extract correspondingly increased absorbance at 695 nm, indicating a dose-dependent rise in reducing power.

Nitric Oxide Scavenging Activity: The extract showed nitric oxide scavenging activity with an IC_{50} of 2.3 mg. However, its efficacy was lower compared to the reference standard, ascorbic acid (560 µg), suggesting a relatively moderate capacity in quenching nitric oxide radicals.

Reducing Power Assay: The extract displayed dose-dependent reducing potential, albeit lower than that of the standard

Table 2: **Observation table of Superoxide, Metal chelating, Hydroxyl radical Assay.**

Superoxide Assay			Metal chelating assay			Hydroxyl radical assay		
Conc. (µg)	%Scavenging (Sample)	%Scavenging (Standard)	Conc. (µg)	%Scavenging (Sample)	%Scavenging (Standard)	Conc. (µg)	%Scavenging (Sample)	%Scavenging (Standard)
10	6	14.28	10	21.9	31.8	100	22.22	28.8
25	23.57	43.57	20	35	44.5	200	33.95	36.6
50	67.5	54.64	30	38.8	49.6	300	38.68	43.2
100	74.64	72.5	40	42.1	54.6	400	48.35	54.52
200	75.71	77.85	50	50.9	60	500	55.76	59.87

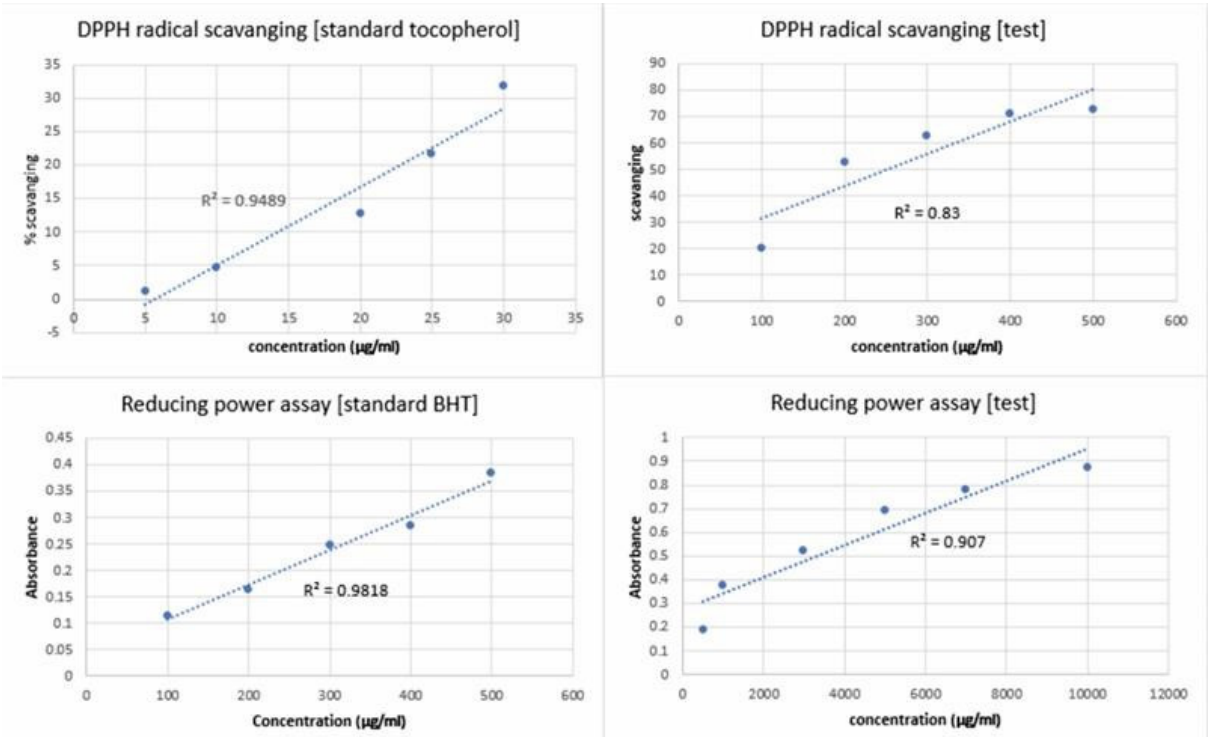

Figure 2: Graph of DPPH and Reducing power assay.

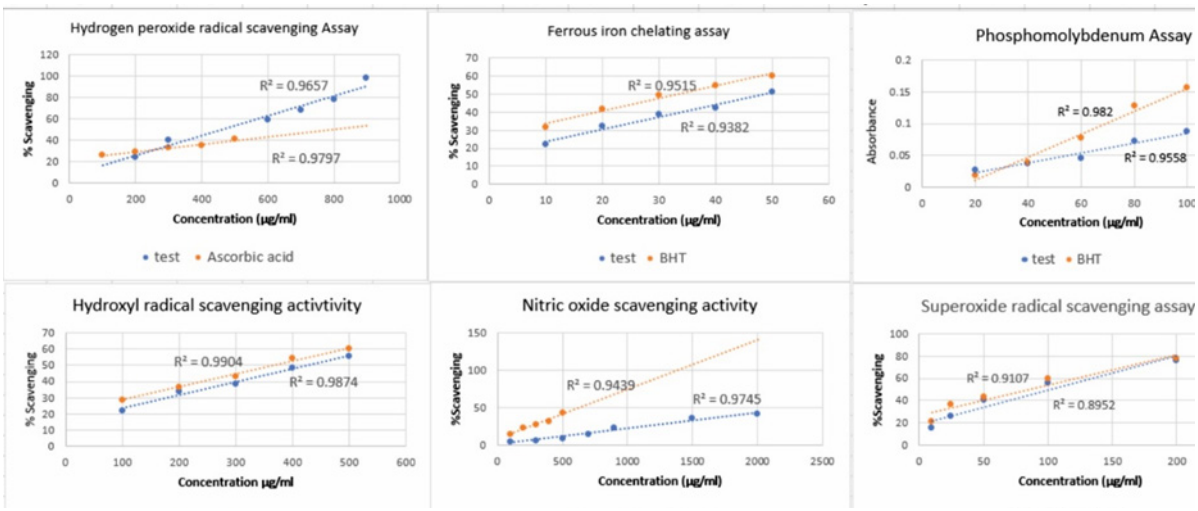

Figure 3: Graph of Hydrogen Peroxide radical, ferrous iron chelating, phosphomolybdenum. Hydroxyl radical, Nitric oxide, superoxide radical scavenging assay.

Table 3: Comparison of IC_{50} between sample and standard extract.

Assay	Sample extract IC_{50} (µg)	Standard IC_{50} (µg)
DPPH radical scavenging assay	190.4	47.3 (Vitamin E)
H_2O_2 radical scavenging assay	509.3	610.5 (Ascorbic acid)
Nitric oxide scavenging assay	2300	560 (Ascorbic acid)
Superoxide radical scavenging assay	37	45.83 (Ascorbic acid)
Metal chelating assay	50	30 (BHT)
Hydroxyl radical scavenging assay	413	366 (BHT)

antioxidant, BHT. The observed reducing activity is likely due to the complex mixture of phytochemicals present in the crude extract, each contributing differently to the antioxidant mechanism.

Superoxide Radical Scavenging Assay: The extract exhibited stronger activity than the standard, ascorbic acid, with an IC_{50} of 37 µg compared to 45.83 µg. This indicates that the extract has potent superoxide scavenging ability, likely owing to its intrinsic antioxidant components.

Metal Chelating Activity: The extract (50 µg) demonstrated noteworthy metal ion chelating ability, closely comparable to that of the standard (30 µg). This suggests that the extract can effectively bind ferrous ions and reduce metal-catalyzed oxidative reactions.

Hydroxyl Radical Scavenging Assay: The hydroxyl radical scavenging capability of the extract was moderate, with an IC_{50} of 413 µg, which was slightly less effective than that of the standard antioxidant BHT (366 µg). Despite being less potent, the extract still showed significant radical scavenging potential.

CONCLUSION

The current investigation highlights *Chamaecostus cuspidatus* as a potent natural source of antioxidants, exhibiting significant efficacy in neutralizing hydrogen peroxide, superoxide, and hydroxyl radicals. Notably, the extract outperformed standard antioxidants in hydrogen peroxide and superoxide scavenging assays, suggesting the presence of phytochemicals with strong radical-quenching properties that can effectively reduce oxidative stress. These findings support the traditional medicinal uses of *C. cuspidatus* and point to its potential application in the development of pharmaceutical, nutraceutical, and functional food products aimed at preventing or managing oxidative stress-related conditions. The plant has demonstrated therapeutic promise in the management of diabetes, protection of pancreatic cells from age-related damage, and in mitigating neurodegenerative disorders such as Alzheimer's and Parkinson's disease, as well as inflammatory diseases like rheumatoid arthritis. Its effectiveness, coupled with a favorable safety profile, positions *C. cuspidatus* as a valuable candidate for the development of plant-based oral antidiabetic formulations.

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ABBREVIATIONS

g: Gram; **min:** Minutes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

This study provides a solid foundation for future research focused on isolating and characterizing its active constituents, thereby advancing the development of evidence-based herbal therapeutics for managing chronic diseases.

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