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Phytochemical and antioxidant screening of Cocculus hirsutus and Calycopteris floribunda

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Abstract*---Cocculus hirsutus*** and ***Calycopteris floribunda*** are two important Indian medicinal plants that are having multiple health benefits in traditional medicine. The current research aims to investigate the qualitative and quantitative phytochemical screening of ethanolic extracts of the selected plants using standard protocols. The Total phenolic content and total flavonoid content were estimated by Folin–Ciocalteu & Aluminum chloride colorimetric methods respectively. Invitro antioxidant activity by DPPH and NO free radical scavenging assay. Results unveiled that the total phenolic content for the ethanolic extract of *C. hirsutus* and *C. floribunda* are 157.46±1.12 & 113.25±1.03 GAE per gram of sample in dry weight (mg/g) and total flavonoid content was 69±1.05 & 58±1.23 of RE per gram of sample in dry weight (mg/g) respectively. In the DPPH assay, ethanolic extract of *C. hirsutus* is effective to inhibit (71.02%) the DPPH free radicals with IC_{50} values 48.10µg/ml at higher doses that are comparable to standard ascorbic acid (79.26%, IC_{50} =38.39µg/ml) followed by *C. floribunda* (65.24%, IC_{50} =60.49µg/ml). Similarly, in the NO free radical scavenging assay, *C. hirsutus* (75.19%) exhibited the highest free radical scavenging activity with IC_{50} values of 44.43µg/ml followed by *C. floribunda* (71.02%, IC_{50} =52.14µg/ml). Whereas, ascorbic acid is found to inhibit 82.34% with IC_{50} values of 35.84µg/ml. The presence of tannins and flavonoids may directly involve in the observed free radical scavenging property.

***Keywords*---**Cocculus hirsutus, Calycopteris floribunda, antioxidant, total phenolic content, total flavonoid content.

Introduction

The homeostatic imbalance between the oxidative and antioxidant systems will increase cellular stress. The overproduction of the reactive oxygen species that are unable to scavenge by the antioxidant enzymes such as glutathione, catalase, and superoxide dismutase can develop a stressful environment in the tissue and leads to cellular damage. [1] The increased free radical levels are directly involved in the oxidative damage of lipids, proteins, and nucleic acids. [2] The chronic exposure of the cell and cell components to the free radicals causes cellular dysfunction, abnormal immune responses, mutations, and distorted homeostasis. [3] Oxidative stress is one of the prime reasons for metabolic disorders and various pathological conditions such as inflammations and ulcers. Addressing cellular stress through extrinsic factors is an efficient approach to mitigate stress-induced ulcers, inflammations, and other metabolic disorders. [4] Herbs are known to mankind for centuries for treating various chronic and metabolic disorders. [5] The safety profile supported by analytical and pharmacological advancements attracts the globe to shift towards natural medicine. [6]

Cocculus hirsutus, of the Menispermaceae family, is a well-known Indian medicinal plant that is used by the tribals for treating diabetes, skin infections, urinary problems, liver problems, infertility, and bone fractures. [7-10] In South Asia, various parts of *C. hirsutus* are used to relieve fever, joint pains, and vascular disorders. In African folklore medicine, the aerial parts were used as a leafy vegetable and also used to treat constipation. [11-12] *Calycopteris floribunda* Lam of the Combretaceae family is a climber and is widely used in traditional medicine in the treatment of diabetes, liver problems, and infections. [13-15] The decoctions of the herb are used to have anti-diarrhoeal, anti-dysenteric, and anti-malarial properties. all the parts of *C. floribunda* are reported to have several medicinal importance in folklore medicine. Various phytochemicals have been isolated from the plant and need more scientific justification. [16-17] The current research attempts to evaluate total phenolic content, and total flavonoid content and to estimate the antioxidant potential of aerial parts of *Cocculus hirsutus* and *Calycopteris floribunda* using *In vitro* models such as DPPH assay and nitric oxide free radical scavenging assay.

Materials and Methods

Plant material

Cocculus hirsutus aerial parts and *Calycopteris floribunda* leaves were collected in February 2019 from the Osmania University campus, Hyderabad, authenticated by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati.

Reagents and chemicals

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

Extraction Procedure

The shade-dried plant was powdered and subjected to defatting with n-Hexane followed by Soxhlet extraction with ethanol. The crude extract solution was collected by filtration and the solvent residues were removed by evaporation subsequently to get in solid form and the percentage yield was calculated.

Phytochemical screening

The preliminary phytochemical investigation of ethanolic extract of *C. hirsutus* and *C. floribunda* was carried out by employing standard protocols. [18]

Estimation of total phenolic content

Folin-Ciocalteu (FC) assay was used to estimate the total phenolic content. 200µl of the extract solution was mixed with 2.5ml of FC reagent (diluted 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 and the values are calculated for triplicates. [19]

Estimation of total flavonoid content

The flavonoid content was determined by a colorimetric method using the Aluminium trichloride method (Zhishen method). A volume of 125µL of the extract is added to 75 µL of a 5% Sodium nitrite ($NaNO_2$) solution. After 6 min, 150 µL of $AlCl_3$ solution (10%) was added followed by the addition of 750 µL of NaOH (1M). The final volume of the solution was made to 2500 µL with distilled water. After 15 min of incubation, the mixture turned pink and the absorbance was measured at 510 nm. The total flavonoid content was expressed as gram equivalence of Rutin per gram dry weight. [20]

In vitro antioxidant assay

DPPH radical scavenging assay

Plant extracts of different concentrations were prepared using DMSO, whereas a solution of 25mg/L DPPH was prepared by using ethanol. Extract solution (5µl) was mixed with 195 µl of DPPH solution 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. [21]

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

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

In the nitric oxide radical scavenging activity assay, 2 mL of sodium nitroprusside (10 mM) and 0.5 mL of phosphate buffer (pH-7.4) were 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. A 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 min. Naphthyl ethylene diamine dihydrochloride (NEDD, 1 mL of 1%) was added and incubated for 30 min at 25 °C. [22] The absorbance was recorded at 540 nm and the percentage of nitric oxide inhibition was calculated as:

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

Where,
A_0 was the absorbance of the control
A_s was the absorbance of the treated sample

Results

Preliminary phytochemical screening

The preliminary phytochemical study of ethanolic extracts of *C. hirsutus* and *C. floribunda* revealed that the extracts are rich in various secondary metabolites such as alkaloids, carbohydrates, flavonoids, phenols, steroids, terpenoids, glycosides, tannins, saponins (Table 1).

Table 1. Phytochemical screening results of ethanolic extracts

Phytochemicals	*C. hirsutus*	*C. floribunda*
Alkaloids	+	+
Glycosides	-	-
Flavonoids	+	+
Steroids	+	+
Tannins	+	+
Proteins	-	-
Carbohydrates	-	+
Amino acids	+	-

+ indicates the presence and – indicates the absence of phytochemicals

Total phenolic content

Phenolic compounds can be directly correlated to their protective effect against cellular stress in the body. Total phenolic contents of ethanolic extracts 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 (Figure 1). The total phenolic content of the extracts was calculated from the regression equation of the calibration curve (Y = 0.0051x

+ 0.0152; $R^2 = 0.9969$) and expressed as mg gallic acid equivalents (GAE). Total phenolic content in the ethanol extract was found to be 157.46±1.12 and 113.25±1.03 GAE per gram of sample in dry weight (mg/g) (Table 2) for *C. hirsutus* and *C. floribunda* respectively.

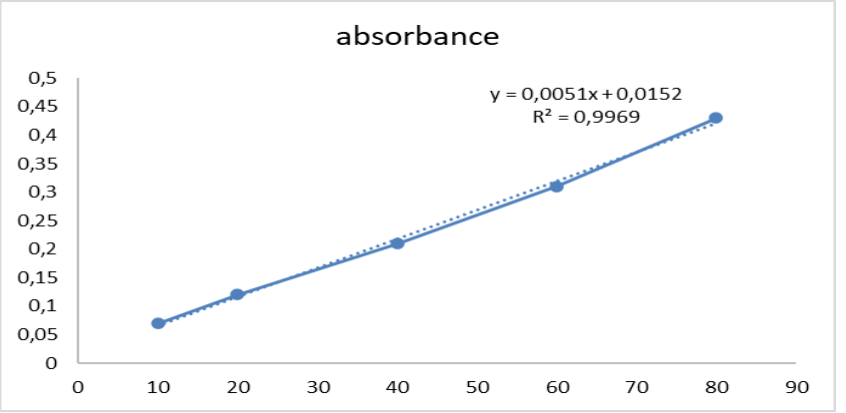

Figure 1. Calibration curve for Gallic acid

Total flavonoid content

Total flavonoid content was determined by Zhishen as gram equivalents of Rutin at 510 nm (Table 2). The calibration curve was made by linear regression and the results represented the average of three determinations for each concentration (Figure 2). The total flavonoid content of the extracts was calculated from the regression equation of the calibration curve ($Y = 3.3914x + 0.0287$; $R^2 = 0.9952$) and expressed as mg Rutin equivalents (RE) per gram of sample in dry weight (mg/g). From the results, it is found that the extracts are rich in flavonoids and measured as 69±1.05 and 58±1.23 of RE per gram of sample in dry weight (mg/g) for *C. hirsutus* and *C. floribunda* respectively.

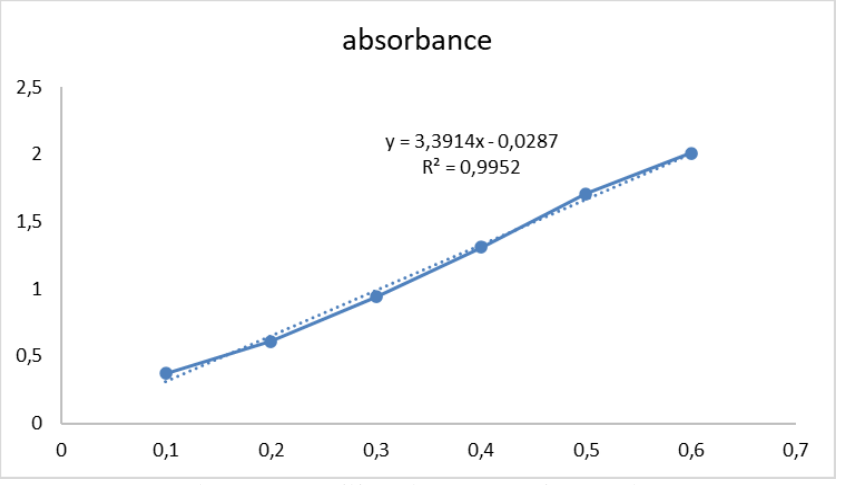

Figure 2. Calibration curve for Rutin

Table 2. The total phenolic and flavonoid content

Extract	*C. hirsutus*	*C. floribunda*
Total phenolic content	157.46±1.12 GAE	113.25±1.03 GAE
Total flavonoid content	69±1.05 RE	58±1.23 RE

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

DPPH radical scavenging assay

In the present study, when compared to the standard Ascorbic acid, *C. hirsutus* has exhibited significant free radical scavenging activity than *C. floribunda* in a dose-dependent manner. A standard curve was plotted using various concentrations of ascorbic acid (Figure 3). At a higher concentration (100 µg/mL), the *C. hirsutus* ethanol extract exhibited 71.02% of inhibition next to ascorbic acid (79.26%) (Table 3). Whereas *C. floribunda* exhibited the next higher inhibition 65.24%. The IC_{50} value for ascorbic acid, *C. hirsutus,* and *C. floribunda* were found to be 38.39 µg/mL, 48.10 µg/mL, and 60.49µg/mL.

Table 3. Percentage inhibition and IC_{50} values of ascorbic acid, *C. hirsutus,* and *C. floribunda* at different concentrations in the DPPH assay

Concentration µg/mL	Ascorbic acid	*C. hirsutus*	*C. floribunda*
100	79.26	71.02	65.24
75	76.25	65.68	50.51
50	73.37	63.22	57.13
25	61.37	55.86	49.52
IC_{50} µg/ml	38.39	48.1	60.49

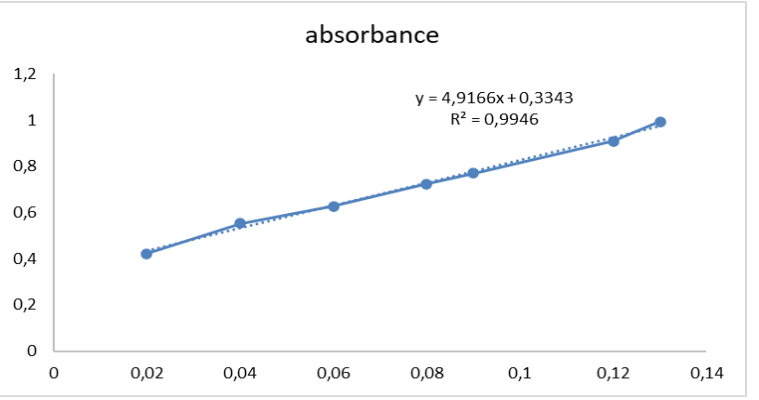

Figure 3. Calibration curve for Ascorbic acid

Nitric oxide radical scavenging activity

C. hirsutus ethanol extract exhibited better NO free radical scavenging activity than *C. floribunda* in a dose-dependent manner compared to standard ascorbic acid (Table 4). At a higher concentration of 100 µg/mL, *C. hirsutus* is showing 75.19% of inhibition with IC_{50} values of 44.43 µg/ml and *C. floribunda* exhibited

71.02% of inhibition with IC_{50} values of 52.14 µg/ml. Whereas for ascorbic acid it is found to be 82.34% with IC_{50} values of 35.84µg/ml.

Table 4. Percentage inhibition and IC_{50} values at different concentrations in NO free radical scavenging assay

Concentration µg/mL	Ascorbic acid	*C. hirsutus*	*C. floribunda*
100	82.34	75.19	71.02
75	79.55	69.02	65.88
50	76.34	65.78	61.81
25	63.05	57.97	44.25
IC_{50} µg/ml	35.84	44.43	52.14

Discussions

Secondary metabolites such as tannins, flavonoids, and a few aromatic alkaloids are produced through the shikimic acid pathway that are having a good correlation with the antioxidant mechanism. [23] These antioxidant phytochemicals directly scavenge the free radicals and diminish the oxidative damage to the biomolecules. Herbs that are rich in polyphenols can be a source of antioxidants that combat the reactive oxygen species and can benefit the therapeutic outcome of chronic inflammatory and metabolic disorders. [24-25]

In the present study, the ethanol extracts of *C. hirsutus,* and *C. floribunda* were studied for qualitative and quantitative phytochemicals. From the results, it is apparent that both plants were wealthy in diversified plant metabolites. Especially, ethanol extract of *C. hirsutus* is rich in phenolic compounds, including flavonoids. The ethanol extracts also exhibited significant antioxidant activity in the DPPH assay and NO free radical scavenging assay. Further investigation is in progress to evaluate the complete phytochemical and pharmacological profile of the plant to justify its traditional applications and the reported antioxidant activity.

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