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Investigation of Abiotic Stress Responses of *Centella asiatica* in Sri Lanka

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Abstract

Plant stress is the term used to describe a plant's inability to grow under certain adverse conditions. This situation can occur only when the stressor exceeds a threshold, which can result in acute growth deficits, permanent damage, and lower agricultural productivity. The aim of this study was to investigate biochemical, anti-oxidant, and nutritional modifications of *Centella asiatica* (Gotu kola) in response to different abiotic stress conditions. *C. asiatica* plants were exposed to different stressors including drought, salinity, heavy metal, nutrient deficiency, and flood. Qualitative tests were performed to identify the presence of bioactive compounds and quantitative tests such as the Folin-Ciocalteu assay for total phenolic count, Aluminium chloride assay for total flavonoid capacity, Lowry assay for total protein content, and Phosphomolybdate assay for total antioxidant capacity were conducted. Additionally, the antibacterial property of *C.asiatica* was examined using antibacterial sensitivity testing for *Escherichia coli*. Based on the qualitative test results, control plants and, salinity, heavy metal, nutrient deficiency, drought, and flood-induced plants showed positive results for tannins, saponins, flavonoids, coumarins, terpenoids, cardiac glycoside, and steroids. However, quantitative test results showed that flood-induced plants had significantly higher values compared to control and other stresses for total phenolic content, total antioxidant capacity, and total protein content. The flood condition sample had higher overall significant values for total phenolic content (14.95 mg $GAEg^{-1}$), total antioxidant capacity (120.5 mg $AAEg^{-1}$), and total protein content (56.55 mg $BSAEg^{-1}$) at 10% error margin. *C. asiatica* showed a negative result for the antibacterial sensitivity test for *Escherichia coli*. This study showed that *C. asiatica* has the potential to be a source of natural antioxidants.

Keywords: *Centella asiatica*, Abiotic Stress, Nutritional modification, Phytochemical, Polyethylene Glycol 6000 (PEG-6000), *Escherichia coli*

1. Introduction

Plant stress refers to the difficulty in growing under certain adverse conditions. This condition occurs when the stressor exceeds a certain tolerance point, which can immediately lead to reduced agricultural production, irreversible damage, and growth deficits.[1]

Major environmental threats that significantly reduce crop yield can be categorized as abiotic and biotic threats. Abiotic stresses include drought, salinity, heavy metals, nutrient deficiencies, flooding, etc. Biotic stresses include various living organisms such as fungi, bacteria, viruses, nematodes and insects.[2] Abiotic environmental conditions significantly impact immobile plants by affecting their biological processes, gene expression, and cellular metabolism.[3]

Drought and high soil salinity are major abiotic stressors that reduce crop yields by causing osmotic stress, ionic toxicity, and impaired growth, metabolism, and cell function.[4] Soil metal concentrations affect plant physiology and seed germination, while flooded soils lead to oxygen loss, impaired respiration, and toxic CO_2 accumulation.[3] *Centella asiatica*, known as Gotu kola in Sri Lanka, shown in Figure 01, is a herbaceous plant in the Mackinlayaceae family, historically used for its therapeutic properties.[2] *C.asiatica* is a tasteless, odorless plant found in rocky terrain, rice fields, and swamps, with small oval fruit, white or light purple-to-pink flowers, and tiny fan-shaped green leaves.[5]

Extreme changes in climate like drought, flood, and changes in soil conditions have an impact on the agricultural aspect, resulting in a decline in crop yield and ultimately the nutritional properties of the crop. The current understanding of plant stress tolerance can be significantly improved through a thorough characterization and its contribution to stress tolerance.[6] Therefore, this study aimed to investigate biochemical, antioxidant, and nutritional modifications of *Centella asiatica* (Gotu kola) in response to different abiotic stress conditions as it is a commonly consumed leafy green vegetable in Sri Lanka.

Figure 1. *Centella asiatica* plant

2. Methodology

2.1 Abiotic stress-induction of Centella asiatica plants. Different abiotic stress conditions were induced according to Table 1.

Table 1. Induction of abiotic stress conditions

Abiotic Stress condition	Procedure
Heavy Metal	50 mL of 25 mM $Pb(NO_3)_2$ was applied twice a day for two weeks.[7]
Drought	50 mL of 10% Polyethylene Glycol 6000 (PEG-6000) solution was added to the plants once per week and plants were kept under direct sunlight for two weeks without adding water.[8]
Flood	Water was added until the root and the stem were completely submerged and maintained for two weeks.[9]
High Salinity	50 mL of 150 mM NaCl was added every morning and evening for two week.[10]
Nutrient Deficiency	Gotu kola plants were transferred to autoclaved sand and 100 mL of water was added to the plants once a day for two weeks

2.2 Sample collection and preparation of aqueous plant extract of Centella asiatica plant samples. Centella asiatica leaves were plucked and weight was measured. Leaves were then washed with distilled water and placed in the drying oven at 40 ^{0}C for 72 hours. Dried leaves were crushed into a fine powder using a mortar and pestle. Aqueous extract was prepared by adding distilled water and powder in 1:40 ratio. The tubes were kept on the roller mixture for 48 hours. Then the extract was filtrated using a Whatman filter paper through a funnel set-up. The filtrate was used in qualitative and quantitative analysis.

2.3 Determination of moisture content. Using the fresh and dry weight values, of the plant leaves, the percentage of the moisture content was calculated using the following equation

$$\text{Moisture Content \%} = \left(\frac{Fresh\ weight(Wo) - Dry\ weight\ (Wd)}{Fresh\ weight\ (Wo)}\right) \times 100\%$$

2.4 Qualitative analysis of Bioactive compounds in Centella asiatica samples. The presence of bioactive compounds was qualitatively analyzed using the protocols given in Table 2.

Table 2. *Bioactive compounds and respective tests to determine their presence in plant sample*[4]

Bioactive Compound	Tests
Alkaloid	To 1 mL of HCl, 2 mL of an extract was added, followed by 3 – 5 drops of Wagner's reagent
Tannins	A few drops of aq. ferric chloride ($FeCl_3$) were added to 1 mL of the extract

Phenolic Compounds	A few drops of 10% aqueous $FeCl_3$ and 2ml of distilled water were added to 1ml extract
Saponins	To 1 mL of plant extract, 5 m L of water was added and then shaken vigorously
Flavonoids	To 1 mL of extract, 2-3 drops (0.5 mL) of sodium hydroxide (NaOH) were added, followed by a few drops of diluted HCl
Quinone	A few drops of NaOH were added to 1 mL of plant extract
Coumarins	1 mL of NaOH was added to 1 mL of Plant
Terpenoids	2 mL chloroform ($CHCl_3$) and 3 mL of concentrated sulfuric acid (conc. H_2SO_4) was added to 1 mL of plant extract
Cardiac Glycoside	A few drops of $FeCl_3$ solution were added to 2 mL of plant extract, followed by 2 mL of glacial acetic acid with 1 mL of conc. H_2SO_4
Steroids	2 mL $CHCl_3$ was added to 1ml of plant extract, followed by 3 mL of concentrated sulphuric acid (Conc. H_2SO_4)

2.5 Determination of Total Phenolic Content. To measure the total phenolic content, 200 µl of 1:80 Gotu kola extract, 1000 µL of distilled water, and 200 µL of Folin-Ciocalteu reagent were mixed and incubated at room temperature for 6 minutes. Then, 2 mL of 7% sodium carbonate solution and 1000 µL of distilled water were added, and the mixture was incubated in the dark for 90 minutes. Absorbance was measured at 760 nm, and the total phenolic content was calculated as gallic acid equivalents (mg GAE/g) using a gallic acid standard curve.[11]

2.6 Determination of Total Flavonoid Content. To determine the total flavonoid content, 200 µL of a 1:80 Gotu kola extract, 1000 µL of distilled water, and 200 µL of 5% sodium nitrate were mixed and incubated at room temperature for 6 minutes. Then, 300 µL of 10% aluminum chloride and 400 µL of 1 M sodium hydroxide were added. Absorbance was measured at 510 nm using a UV spectrophotometer, and the total flavonoid content was calculated as quercetin equivalents (mg QUE/g) using a standard curve of 0.1-5 mg/mL quercetin.[11]

2.7 Determination of Total Antioxidant Capacity. To measure total antioxidant capacity, 200 µL of a 1:80 Gotu kola extract was mixed with 2 mL of a reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at room temperature for 90 minutes. After cooling, absorbance was measured at 765 nm. Total antioxidant capacity was calculated as ascorbic acid equivalents (mg AAE/g) using a standard curve of 0.01-0.5 mg/mL ascorbic acid.[12]

2.8 Determination of Total Protein Content. To determine total protein content, 200 µL of a 1:80 Gotu kola extract was mixed with 5 mL of reagent C and incubated for 10 minutes at room temperature. Then, 0.5 mL of reagent was added, and the mixture was incubated for 30 minutes in the dark. Absorbance was measured at 660 nm, and total protein content was calculated as BSA equivalents (mg BSAE/g) using a standard curve of 0.2-1.6 mg/mL Bovine Serum Albumin (BSA).[13]

2.9 Antibacterial sensitivity test. The antibacterial activity of *Centella asiatica* was evaluated using the agar well diffusion method against *Escherichia coli* on Muller-Hinton agar (MHA). MHA was prepared and autoclaved, then poured into Petri dishes. A suspension of *E. coli* was evenly spread on the surface of the agar plates. Wells were then created in the agar, and to each well, 100 µL of Gotu kola extract was added. Negative and positive controls were included, with 100 µL of autoclaved distilled water used as the negative control and 100 µL of 0.5 mg/mL gentamicin as the positive control. The plates were incubated at 30°C for 24 hours, after which the zone of inhibition was measured.[14]

2.10 Statistical Analysis. All values are expressed as mean ± Standard Error. Microsoft Excel 2023 was used to calculate the standard error of the mean value.

3. Results

3.1 Morphological changes observed in stress-induced plants. Stress conditions were induced for 14 days and observed morphological changes are detailed in Table 3.

Table 3. Morphological changes observed in stress indued plants after 14 days

Conditions	Morphological Change
Drought	• Reduction in the plant height • Withered leaves • Extremely dry soil
High Salinity	• Decrease in leaf thickness • Withered leaves
Heavy Metal	• Withered leaves • Browning of the leaves
Nutrient Deficiency	• Yellowing of leaves • Stem degradation
Flood	• Plant stem elongation

3.2 Moisture content

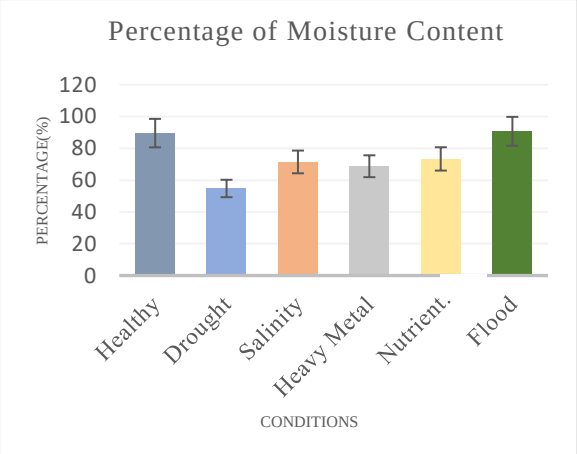

Figure 2. Moisture content percentage of control and stress induced plants. All the values are expressed as the mean ± S.E.

According to Figure 2, moisture content has significantly decreased in drought, salinity, heavy metal and nutrient deficiency induced plants at 10% error margin compared to the control plant

3.3 Bioactive compounds. Table 4 shows the presence and absence of the bioactive compounds of healthy and abiotic stress induced samples. All the samples tested positive for Tannins, Saponins, Flavonoids, Coumarins, Terpenoids, Cardiac glycoside and Steroids. Alkaloids, Phenolic Compound and Quinones were absent in them.

Table 4. Qualitative analysis of bioactive compounds

Tests	Healthy	Drought	High salinity	Heavy metal	Nutrient Deficiency	Flood
Alkaloid	Ab	Ab	Ab	Ab	Ab	Ab
Tannins	P	P	P	P	P	P
Phenolic Compound	Ab	Ab	Ab	Ab	Ab	Ab
Saponins	P	P	P	P	P	P
Flavonoid	P	P	P	P	P	P
Quinones	Ab	Ab	Ab	Ab	Ab	Ab
Coumarins	P	P	P	P	P	P
Terpenoids	P	P	P	P	P	P
Cardiac Glycoside	P	P	P	P	P	P
Steroids	P	P	P	P	P	P

P- Present, Ab – Absent

3.4 Total Phenolic Content

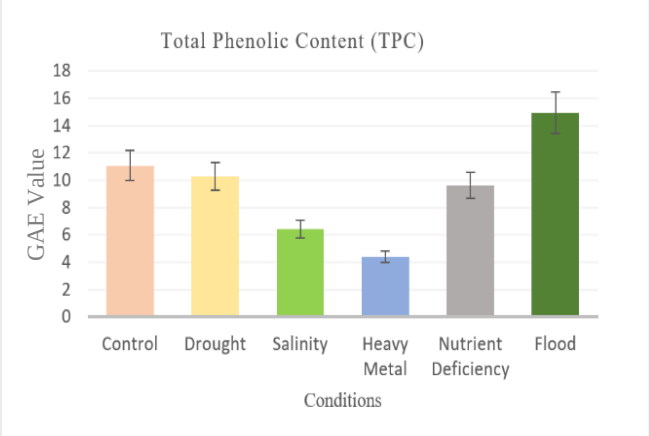

Figure 3. Total phenolic content of control and stress induced *Centella asiatica* plants.

All the values are expressed as the mean ± S.E.

The flood-induced plants had the highest total phenolic content, while the heavy metal-contaminated plants had the lowest. At a 10% error margin, the non-overlapping error bars for salinity, heavy metal, and flood-induced plants indicate statistically significant changes in phenolic content compared to control plants in Figure 3.

3.5 Total Flavonoid Content

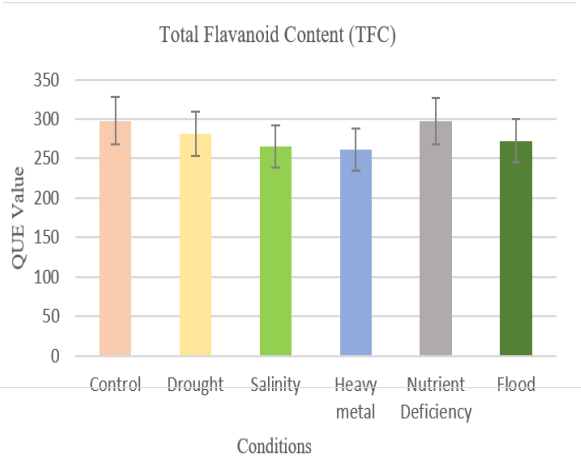

Figure 4. Total Flavonoid Content of control and stress-induced *Centella asiatica* plants. All the values are expressed as the mean ± S.E.

The control plants had the highest total flavonoid content, while the heavy metal-contaminated plants had the lowest. However, at a 10% error margin, the overlapping error bars for drought, salinity, heavy metal, nutrient deficiency, and flood-induced plants indicate that the changes in flavonoid content are not statistically significant compared to control plants in Figure 4.

3.6 Total Antioxidant Capacity.

The flood-induced plants showed the highest total antioxidant capacity, while the salinity-induced plants had the lowest. At a 10% error margin, the non-overlapping error bars for salinity and flood-induced plants suggest statistically significant changes in antioxidant capacity compared to control plants in Figure 5.

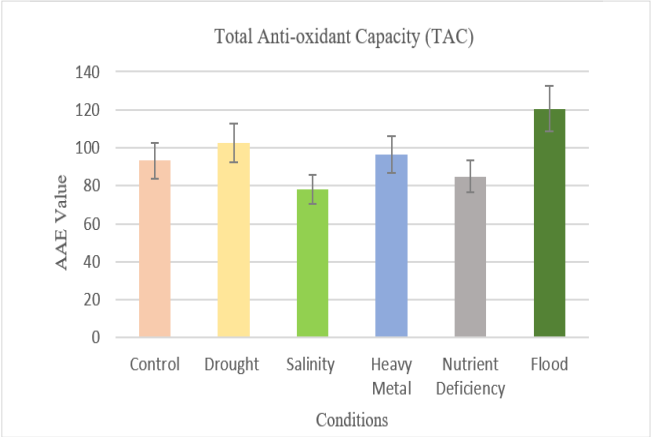

Figure 5. Total Antioxidant capacity of control and stress induced *Centella asiatica* plants. All the values are expressed as the mean ± S.E.

3.7 Total Protein Content

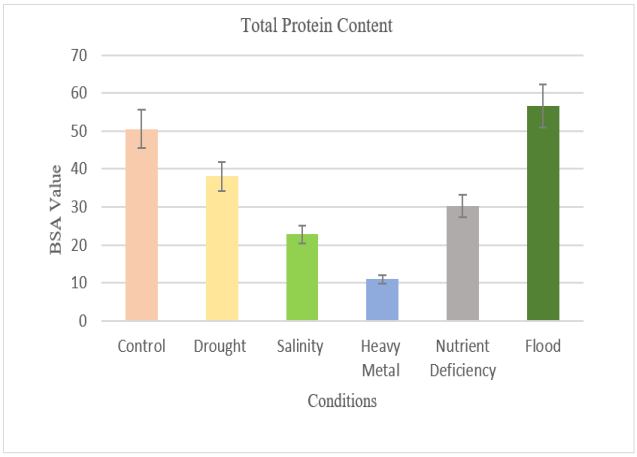

Figure 6. Total Protein Content of control and stress induced *Centella asiatica* plants. All the values are expressed as the mean ± S.E.

The flood-induced plants had the highest total protein content, while the heavy metal-affected plants had the lowest. At a 10% error margin, the non-overlapping error bars for drought, salinity, heavy metal, and nutrient-deficient plants indicate that the changes in total protein content under these conditions are statistically significant compared to control plants in Figure 6.

3.8 Anti-bacterial sensitivity test. Using the well diffusion method, only the positive control (0.5 mg/mL Gentamicin) showed an average zone of inhibition of 23 mm. Neither the negative control nor *Centella asiatica*

produced any zone of inhibition. Gentamicin effectively inhibits the growth of *E. coli*, confirming its activity and the absence of a zone of inhibition suggests that *Centella asiatica* has no antimicrobial activity against the *E. coli* bacteria at this plant extract concentration.

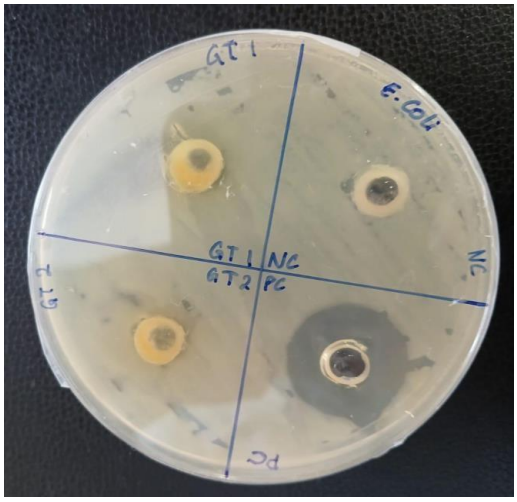

Figure 07. Anti-bacterial sensitivity test of *Centella asiatica* against *E. Coli*

4. Discussion

Centella asiatica, a plant used in traditional medicine, contains bioactive components that are influenced by various abiotic stress conditions, affecting its biochemical, antioxidant, and nutritional properties.[15] Quantitative analysis of *C. asiatica* under stress conditions revealed unique nutritional properties, including bioactive compounds with additional benefits beyond essential nutritional value.[16]

In this study of *C. asiatica* plants, qualitative analysis revealed the presence of tannins, saponins, flavonoids, coumarins, terpenoids, cardiac glycosides, and steroids, while alkaloids, phenolic compounds, and quinones were absent. Similar findings were reported by Roy M. and Krishnan in 2018, where tannins, flavonoids, terpenoids, saponins, and steroids were present, but alkaloids were absent. The results of this study align with their findings for the qualitative analysis of *C. asiatica* (Table 4).[17]

The moisture content of Gotu kola was measured, revealing that flood-induced plants had the highest moisture content at 90.72%, while drought-stressed plants had the lowest at 54.75%. These differences highlight the significant impact of water availability on the plant's hydration (Figure 02).

The Folin-Ciocalteu (FC) method, which measures total phenolic content by oxidizing phenolic compounds to produce a blue-colored reagent, was used to analyze the phenolic content in Gotu kola under various conditions. In healthy plants, the total phenolic content was 11.08 mg $GAEg^{-1}$. This aligns with Zainol *et al.* (2003), who discovered *Centella asiatica* phenolic content ranging from 3.23 to 11.7 g/100g in aqueous extracts.[18] Under abiotic stress, heavy metal exposure reduced the phenolic content to 4.417 mg $GAEg^{-1}$, and salinity stress to 6.452 mg $GAEg^{-1}$. However, flood-induced stress significantly increased the phenolic content to 14.95 mg $GAEg^{-1}$ (Figure 03). These results demonstrate the varying impact of different abiotic stresses on phenolic content, with flooding notably enhancing phenolic production.

The total flavonoid content in Gotu kola was measured using the aluminum chloride complex formation assay, with quercetin as the standard. In this study, the flavonoid content showed no significant variation between control (297.81 mg $QUEg^{-1}$) and abiotic stress conditions, including salinity (264.83 mg $QUEg^{-1}$), heavy metal (261.12 mg $QUEg^{-1}$), drought (281.55 mg $QUEg^{-1}$), nutrient deficiency (297.13 mg $QUEg^{-1}$), and flood (272.18 mg $QUEg^{-1}$). This finding contrasts with a study by Minarti *et al*. (2021), which reported a much lower flavonoid content of 9.33 mg $QUEg^{-1}$ [19,20] (Figure 4). The differences may be due to varying environmental conditions and growth factors, suggesting that Gotu kola may have an inherent resistance to abiotic stress, maintaining its flavonoid production as an adaptive response to challenging environments.

In this study, the total antioxidant capacity of Gotu kola was 97.16 mg $AAEg^{-1}$ in control plants. Under abiotic stress, flood conditions increased the antioxidant capacity to 120.59 mg $AAEg^{-1}$, while salinity slightly reduced it to 78.09 mg $AAEg^{-1}$ (Figure 05). These results align with a similar study by Rashid *et al.* (2023), which found 102.32 mg $AAEg^{-1}$ in *C. asiatica*. Despite the variations, both stress conditions produced antioxidant levels close to those of control plants,

indicating the plant's resilience and adaptive mechanisms under stress.[20]

In this study, the total protein content in healthy Gotu kola plants was 50.45 mg $BSAEg^{-1}$. Under abiotic stress, protein levels significantly varied with salinity (22.79 mg $BSAEg^{-1}$), drought (38.12 mg $BSAEg^{-1}$), nutrient deficiency (30.26 mg $BSAEg^{-1}$), and heavy metal stress (11.05 mg $BSAEg^{-1}$) induced plants, all showing notable reductions compared to control plants (Figure 6).

Infectious diseases drive global morbidity and mortality, and medicinal plants provide a promising, cost-effective alternative to antibiotics, with fewer side effects. In the antibacterial sensitivity test, *Centella asiatica* showed no antibacterial activity against *Escherichia coli*, likely due to insufficient active compounds in the extract. Gentamicin, used as the positive control, demonstrated effective antibacterial properties against aerobic gram-negative bacteria (Figure 7).

5. Conclusion

Centella asiatica under flood conditions showed higher values for total phenolic content, total antioxidant capacity, and total protein content compared to healthy samples, indicating its potential as a source of natural antioxidants.

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