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# Production of Flavonoids in Callus Culture of *Anthocephalus indicus* A. Rich

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**Abstract:** Manipulation of nutrient medium is the most fundamental approach in enhancing the secondary metabolite production in plant cell culture system. The objective of the experiment was to study the effects of plant growth regulators, L-phenylalanine as precursor and sodium chloride stress on flavonoid production in callus culture of *Anthocephalus indicus*. It was observed that callus cultures grown on WPM medium supplemented with 3 mg $L^{-1}$ benzyladenine, 100 mg $L^{-1}$ phenylalanine and 100 mg $L^{-1}$ sodium chloride produced 77.2±1.92, 104.4±4.39 and 145.31±4.7 mg of flavonoids, respectively per g dry weight. Combined treatment of all the three produced 203±2.82 mg $g^{-1}$ dry weight of flavonoids in callus culture which is highest than produced by single treatment. Qualitative analysis of total flavonoid in all the callus samples and leaf material was analysed by HPTLC fingerprinting. As *Anthocephalus indicus* A. Rich. is a medicinally important plant, the present study offers an alternative method for enhanced production of flavonoid in callus culture.

**Key words:** *Anthocephalus indicus*, callus culture, flavonoid, HPTLC

## INTRODUCTION

*Anthocephalus indicus* A. Rich. (Rubiaceae) commonly known as Kadamba is a herbal remedy that has been mentioned in ancient Indian medical literatures for the treatment of fever, anaemia, uterine complaints, menorrhagia, blood and skin diseases, diarrhoea, colitis, stomatitis, dysentery and as an aphrodisiac (Sandhar *et al*., 2011). *Anthocephalus indicus* leaves have been reported to possess antimicrobial, antioxidant, analgesic, antipyretic, anti-inflammatory and hepatoprotective activity. The plant contains flavonoids as one of its major phytoconsituent. It is mentioned in the Ayurvedic Pharmacopeia of India that the plant has been widely used in the various ayurvedic formulations in the form of churna (nygrodhadikvatha churn) and oil (grahanimihirataila) (Dubey *et al*., 2011).

Phytochemicals present in the plant are used as source of pharmaceuticals, neutraceuticals, colouring agents, flavouring agents, in cosmetics, etc (Balandrin and Klocke, 1998). It is a well-known fact that the modification of the components of the nutrient medium can elevate the secondary metabolite production. In order to enhance the synthesis of secondary metabolites, several organic compounds and plant growth regulators can be added to the culture medium (Ong *et al.*, 2011; Lian *et al*., 2002). There has been no report found on the production of flavonoids in callus culture of *Anthocephalus indicus*. Therefore this study focused on the *in vitro* production of flavonoids in callus culture of *Anthocphalus indicus* using different plant growth regulators, L-phenylalanine as precursor and sodium salt stress.

## MATERIALS AND METHODS

**Establishment of callus culture:** The healthy and young leaves of *Anthocephalus indicus* were obtained from young plantlets growing in locality garden of Kalyan, Mumbai. The plant was identified with the help of "The Flora of Presidency of Bombay" and the voucher specimen was authenticated from Blatter Herbarium, Department of Botany, St. Xavier's College, Mumbai. Leaf explants were washed under running tap water and surface sterilized with 1% bavistin (w/v) for 2 h and 0.2% mercuric chloride solution (w/v) for 7 min. The leaves were then washed several times in sterile distilled water and used as explants. Segments of 1cm leaf explant were inoculated on WPM (woody plant medium) basal medium fortified with 3 mg $L^{-2}$, 4-D and 100 mg $L^{-1}$ activated charcoal. Cultures were incubated at 25±2°C under 8 light/16 h dark photoperiod. After 28 days, the proliferated callus cultures were sub-cultured on WPM medium supplemented with 3 mg $L^{-2}$, 4-D, 100 mg $L^{-1}$ activated charcoal and 20% coconut milk to increase the biomass of calli.

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**Manipulation of medium for flavonoid production:** The proliferated callus culture was sub-cultured on WPM medium fortified with different plant growth regulators (2, 4-D, NAA, IAA, IBA, BAP and Kinetin) at 1.0, 3.0 and 5.0 mg $L^{-1}$ of concentration. Similarly the study of effect of precursors and sodium salt stress was initiated by sub-culturing the calli on WPM medium fortified with L- phenylalanine and sodium chloride at 100, 300 and 500 mg $L^{-1}$ of concentration. WPM medium supplemented with 3 mg $L^{-1}$ 2, 4-D and 100 mg $L^{-1}$ activated charcoal was used as the control. Cultures were incubated at 25±2°C under 8 light/ 16 h dark photoperiod for the period of 28 days. After 28 days cultures were harvested, dried at 60°C in an oven and used for the estimation of total flavonoid content.

**Estimation of flavonoids:** All the dried samples were ground and extracted with ethanol for 3 days. Subsequently the extract was filtered and air dried. The crude extract obtained was dissolved in ethanol at the concentration of 1 mg $mL^{-1}$ and estimation of total flavonoid content was carried out by aluminium chloride colorimetric method (Patel *et al*., 2010).

**HPTLC fingerprinting of flavonoid:** Ethanolic extract of callus samples was analyzed by TLC for the presence of alkaloid and flavonoid. Samples were prepared according to the method of Wanger and Bladt (1996). Silica gel G 60 F254 TLC precoated plate (Merck) was used as adsorbent. The plate was developed using ethyl acetate: glacial acetic acid: formic acid: water (100: 11: 11: 26) as mobile phase. The number of bands present in the samples was detected by spraying the plate with 5% ethanolic aluminium chloride solution for flavonoids (Kumar *et al*., 2011; Wanger and Bladt, 1996).

**Statistical analysis:** All the experiments were performed with minimum of five replicates per treatment. Significance of treatment effects was determined by one way analysis of variance (ANOVA) using SPSS 20.0 software and *post hoc* Duncan test (p = 0.05) to determine significant differences among treatment means.

## RESULTS AND DISCUSSION

**Effect of plant growth regulators:** Plant growth regulators, regulates the metabolism and in plant cell cultures they affect both growth and secondary metabolites production (Lian *et al*., 2002). Among all the regulators used to increase the flavonoid content in callus culture 3 mg $L^{-1}$ benzyladenine (BAP) concentration resulted in the highest production of flavonoid in callus which is about 3.6% highest as compared to control (Table 1, Fig. 1). BAP increases the flavonoid content

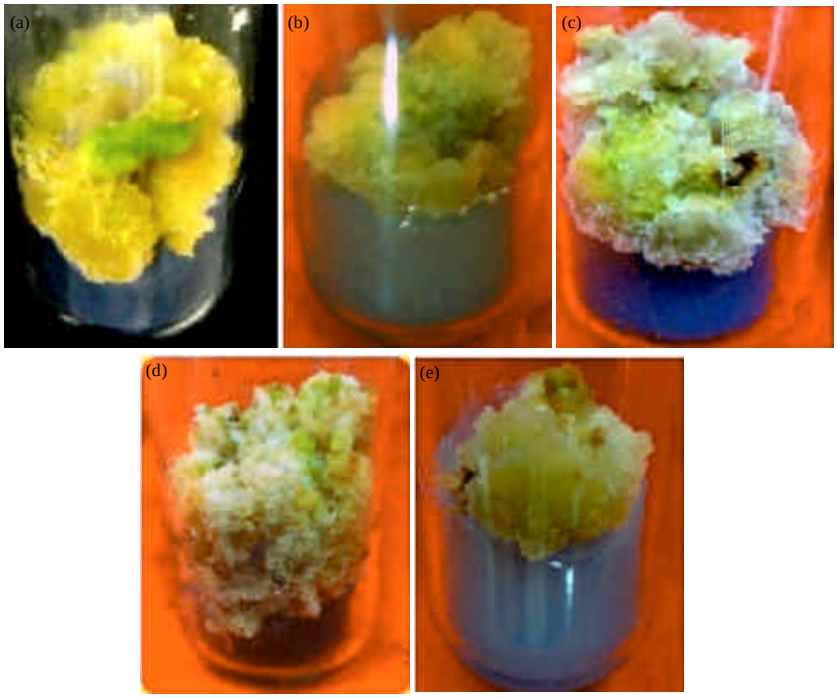


Fig. 1(a-e): (a) Callus treated with BAP, (b) Callus treated with L-phenylalanine, (c) Callus treated with sodium chloride (d) Callus treated in combination of BAP, L-phenylalanine and sodium chloride and (e) Control callus

Table 1: Effect of different plant growth regulators on flavonoid production in calli derived from leaf explant of *Anthocephalus indicus* A. Rich

| Concentration PGR (mg $L^{-1}$) | 1.0 | 3.0 | 5.0 |
|---|---|---|---|
| 2, 4-D | 17.2±1.92$^{k}$ | 20.98±0.62$^{j}$ | 17.4±2.30$^{k}$ |
| IAA | 25.11±1.9$^{ef}$ | 23±1.22$^{j}$ | 17.2±1.92$^{k}$ |
| IBA | 27.6±2.07$^{i}$ | 38.2±1.09$^{fg}$ | 17.8±1.30$^{k}$ |
| NAA | 35.6±1.94$^{gh}$ | 59.4±1.94$^{b}$ | 48±2.12$^{d}$ |
| BAP | 57.6±1.81$^{b}$ | 77.2±1.92$^{a}$ | 42±2.91$^{e}$ |
| Kinetin | 51.4±1.34$^{c}$ | 27.90±1.67$^{ef}$ | 34.6±1.14$^{h}$ |

The values represent the mean±SD of five samples. Results are significant at $p \leq 0.05$ as per one way ANOVA statistical analysis. Same superscript indicates no significant difference between the values whereas variation in the superscript letters indicates significant difference at $p \leq 0.05$

Table 2: Effect of L-phenylalanine (precursor) and sodium chloride (elicitor) on flavonoid production in calli derived from leaf explant of *Anthocephalus indicus* A. Rich

| Concentration of PGRs (mg $L^{1}$) | 100 | 300 | 500 | Control |
|---|---|---|---|---|
| L-Phenylalanine | 104.4±4.39$^{b}$ | 73.6±2.30$^{d}$ | 46±1.58$^{e}$ | 20.98±0.62$^{g}$ |
| Sodium chloride | 145.31±4.7$^{a}$ | 79.26±2.27$^{c}$ | 37.696±3.59$^{f}$ | |

The values represent the mean±SD of five samples. Results are significant at $p \leq 0.05$ as per one way ANOVA statistical analysis. Same superscript indicates no significant difference between the values whereas variation in the superscript letters indicates significant difference at $p \leq 0.05$

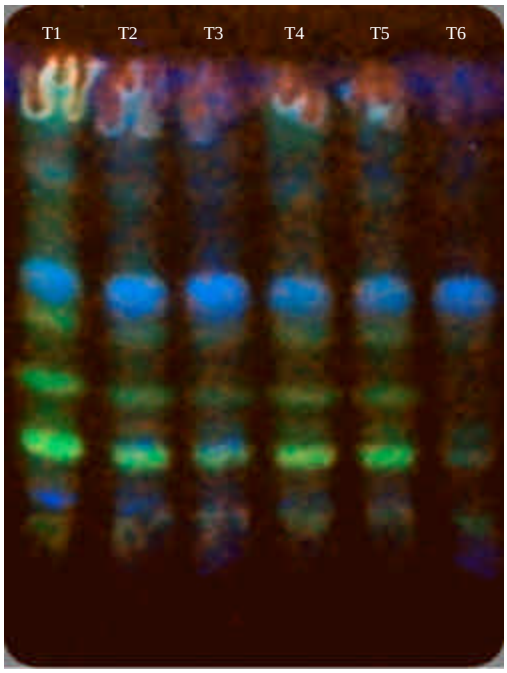


Fig. 2 (T1-T6): HPTLC chromatogram; (T1)- leaf material, (T2): Callus treated with BAP, (T3): Callus treated with L-phenylalanine, (T4): Callus treated with sodium chloride, (T5): Callus treated in combination treatment and (T6): Control callus

in callus culture. For example, when kinetin was replaced with 1 mg $L^{-1}$ of BAP, it increased the anthocyanin production upto 3.65 fold as compared to control in stem callus culture of *Crataegus sinaica* (Maharik *et al*., 2009).

**Effect of L-phenyalanine:** Flavonoid originated from phenylalanine, an upstream metabolic precursor through phenylpropanoid pathway. Considering this phenylalanine supplementation which is expected to increase the metabolic flux through phenylpropanoid biosynthetic pathway and elevate the level of targeted compound. Phenylalanine supplementation has been reported to enhance secondary metabolite production in plant cell cultures (Masoumian *et al*., 2011). The data revealed that the most suitable concentration for increased production of flavonoid was 100 mg $L^{-1}$ phenylalanine with flavonoid content 4% increased than control (Table 2, Fig. 2). The findings are in agreement with the Masoumian *et al*. (2011) reported that higher concentration of phenylalanine seemed to be unsuitable for flavonoid production.

**Effect of sodium chloride:** The application of salt stress enhances the flavonoid content in callus (Table 2, Fig. 2) which is 5% highest than control in the presence of 100 mg $L^{-1}$ sodium chloride. It may be due to the inductions in enzymatic activity occurring under salinity condition, thereby favouring the production of different flavonoid compounds (Haghighi *et al*., 2012). Sodium chloride at low concentration significantly increased the flavonoid content. Accumulation of compatible solutes, such as proline, glycine, betaine, polyols, sugar alcohols and soluble sugars occurs at low sodium chloride concentration. These solutes appear to behave as osmoprotectant and enhance the synthesis of secondary metabolites (Haghighi *et al*., 2012). As the concentration of salt increases it will decreases the metabolite production. High sodium chloride stress disrupts homeostasis in water potential and ion distribution in cells. This disruption of homeostasis occurs at both the cellular levels. Drastic changes in ion and water homeostasis lead to molecular damage, growth arrest and even death (Zhu, 2001). The combination treatment of 3 mg $L^{-1}$ BAP, 100 mg $L^{-1}$ phenylalanine and 100 mg $L^{-1}$ sodium chloride increases the total flavonoid content up to 203±2.82 mg $g^{-1}$ dry weight of callus.

**HPTLC Fingerprinting:** Development of yellow and blue colour bands (Fig. 2) after spraying the TLC plates with particular spraying reagents indicates the presence of flavonoids and alkaloids in the callus samples (Kumar *et al*., 2011, Wanger and Bladt, 1996). Bands with common Rf values at 0.38, 0.63 and 0.75 was observed in all the samples with 0.01-0.03 deviation (Table 3, Fig. 3). HPTLC fingerprinting helps in rapid identification of the unknown compounds in the samples and assigns them into particular group of phytoconstituents depending on its reaction with spraying reagents and separation at specific Rf value (Patel *et al*., 2010).

The WPM medium with 3 mg BAP $L^{-1}$, 100 mg phenylalanine $L^{-1}$ and 100 mg sodium chloride $L^{-1}$ produced the highest concentration of flavonoid in callus culture of *Anthocephalus indicus*. However further modification in the medium, elicitors and complex addenda

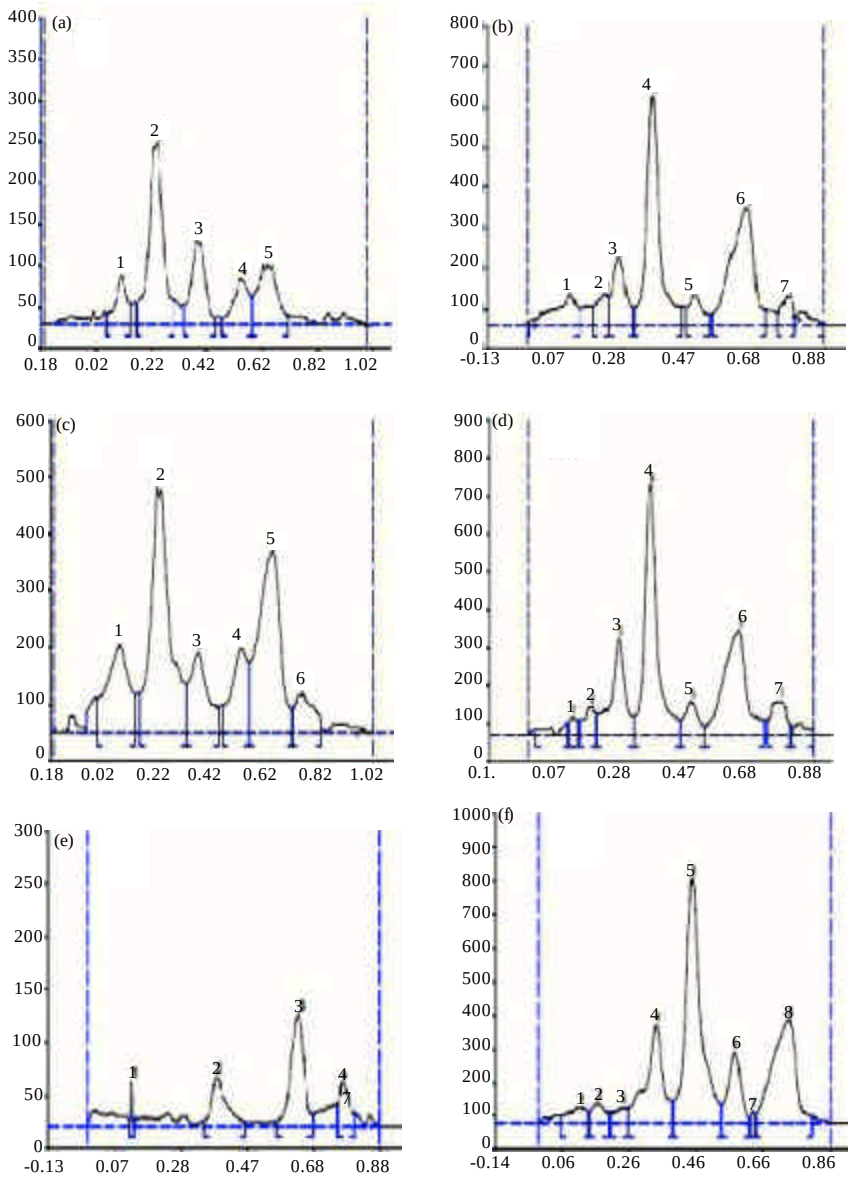


Fig. 3 (a-f): HPTLC fingerprinting; (a) Callus treated with BAP, (b) Callus treated with L- phenylalanine, (c) Callus treated with sodium chloride, (d) Callus treated in combination treatment, (e) Control callus, and (f) leaf material

Table 3: HPTLC fingerprinting of callus samples and leaf material of *Anthocephalus indicus*

| Peak | Start Rf | Start height | Max Rf | Max height | Max (%) | End Rf | End height | Area |
|---|---|---|---|---|---|---|---|---|
| **Leaf control callus** | | | | | | | | |
| 1 | 0.12 | 6.7 | 0.13 | 43.4 | 17.09 | 0.14 | 9.6 | 245.8 |
| 2 | 0.34 | 6.4 | 0.38 | 47.7 | 18.80 | 0.47 | 4.6 | 2070.5 |
| 3 | 0.56 | 5.3 | 0.63 | 107.1 | 42.16 | 0.67 | 12.4 | 4016.7 |
| 4 | 0.75 | 20.9 | 0.75 | 21.2 | 8.35 | 0.80 | 14.6 | 760.2 |
| **Leaf** | | | | | | | | |
| 1 | 0.05 | 24.5 | 0.11 | 46.8 | 2.68 | 0.14 | 35.5 | 2185.8 |
| 2 | 0.14 | 35.6 | 0.16 | 59.2 | 3.39 | 0.20 | 33.3 | 1896.9 |
| 3 | 0.20 | 33.5 | 0.24 | 45.7 | 2.62 | 0.25 | 44.6 | 1484.1 |
| 4 | 0.34 | 44.7 | 0.38 | 293.7 | 16.83 | 0.39 | 66.8 | 11976.7 |
| 5 | 0.39 | 66.8 | 0.45 | 729.6 | 41.81 | 0.53 | 55.7 | 27430.8 |
| 6 | 0.54 | 55.7 | 0.52 | 210.1 | 12.04 | 0.62 | 20.0 | 6588.9 |
| 7 | 0.62 | 20.3 | 0.63 | 35.9 | 2.06 | 0.63 | 25.2 | 300.2 |
| 8 | 0.64 | 25.8 | 0.74 | 305.9 | 17.53 | 0.81 | 19.2 | 17662.7 |
| **Leaf callus treated in combination with precursor, PGR and elicitor (phenylalanine at 100 mg $L^{-1}$, BAP at 3.0 mg $L^{-1}$ and sodium chloride at 100 mg $L^{-1}$)** | | | | | | | | |
| 1 | 0.12 | 27.7 | 0.13 | 45.8 | 3.00 | 0.15 | 37.0 | 1029.3 |
| 2 | 0.15 | 37.1 | 0.19 | 48.8 | 3.20 | 0.20 | 47.4 | 1799.6 |
| 3 | 0.20 | 47.6 | 0.27 | 253.8 | 16.63 | 0.32 | 49.8 | 9953.5 |
| 4 | 0.32 | 49.9 | 0.37 | 667.3 | 43.73 | 0.46 | 38.2 | 24147.0 |
| 5 | 0.46 | 38.3 | 0.50 | 87.1 | 5.71 | 0.54 | 21.3 | 3362.7 |
| 6 | 0.54 | 21.8 | 0.64 | 277.9 | 18.21 | 0.72 | 37.7 | 18360.5 |
| 7 | 0.73 | 37.5 | 0.75 | 42.4 | 2.78 | 0.80 | 19.9 | 1916.1 |
| **Leaf callus treated with precursor phenylalanine at 100 mg $L^{-1}$** | | | | | | | | |
| 1 | 0.08 | 27.0 | 0.12 | 57.5 | 4.75 | 0.15 | 22.3 | 2100.1 |
| 2 | 0.19 | 48.7 | 0.22 | 73.8 | 5.44 | 0.24 | 68.6 | 2496.1 |
| 3 | 0.24 | 68.9 | 0.27 | 170.8 | 12.59 | 0.31 | 43.6 | 5959.6 |
| 4 | 0.31 | 43.7 | 0.36 | 571.2 | 42.10 | 0.45 | 45.4 | 19819.3 |
| 5 | 0.47 | 42.3 | 0.49 | 71.8 | 5.29 | 0.53 | 25.5 | 2822.9 |
| 6 | 0.54 | 29.2 | 0.64 | 292.6 | 21.56 | 0.70 | 38.1 | 38.1 |
| 7 | 0.74 | 34.2 | 0.74 | 34.3 | 2.53 | 0.79 | 17.8 | 1190.9 |
| **Leaf callus treated with sodium chloride at 100 mg $L^{-1}$** | | | | | | | | |
| 1 | 0.05 | 24.5 | 0.11 | 46.8 | 2.68 | 0.14 | 35.5 | 2185.8 |
| 2 | 0.15 | 69.9 | 0.21 | 435.8 | 32.58 | 0.32 | 84.9 | 18904.4 |
| 3 | 0.32 | 85.2 | 0.36 | 140.6 | 10.51 | 0.43 | 44.6 | 5776.9 |
| 4 | 0.45 | 46.1 | 0.52 | 147.2 | 11.01 | 0.55 | 123.0 | 5425.5 |
| 5 | 0.55 | 123.2 | 0.63 | 318.2 | 23.79 | 0.71 | 40.9 | 16320.7 |
| 6 | 0.71 | 41.1 | 0.72 | 43.0 | 3.21 | 0.81 | 18.7 | 1873.8 |
| **Leaf callus treated with BAP at 3.0 mg $L^{-1}$** | | | | | | | | |
| 1 | 0.06 | 13.0 | 0.11 | 59.7 | 10.99 | 0.14 | 25.9 | 1539.7 |
| 2 | 0.16 | 25.5 | 0.24 | 219.1 | 40.35 | 0.30 | 29.1 | 7329.9 |
| 3 | 0.34 | 22.8 | 0.39 | 101.4 | 18.67 | 0.45 | 7.6 | 3262.1 |
| 4 | 0.47 | 7.6 | 0.54 | 55.5 | 10.21 | 0.58 | 32.6 | 1937.7 |
| 5 | 0.59 | 32.7 | 0.64 | 72.7 | 13.39 | 0.71 | 10.6 | 3277.0 |

should be considered amongst other factors to optimize the flavonoid production in cell culture system of *Anthocephaus indicus*.

## CONCLUSION

As *Anthocephalus indicus* is a medicinally important plant, the present study offers possibility of enhanced production of flavonoid in callus culture using commonly available source of additives. This biotechnological approach of production of secondary metabolites provides an alternative mean for large scale production of flavonoids in cell culture system of *Anthocephalus indicus* A. Rich. This can also help in preventing the plant from becoming endangered due to routine use of conventional methods of collection of plant material and extracting bioactive compounds. Further the scale up in production of specific type of flavonoid in cell culture system of *Anthocephalus indicus* can be carried out using some additional additives.

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