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Note

In vitro evaluation of free flavonoids in *Cassia fistula* Linn. calli of different ages under varying concentrations of plant growth regulators (PGRs)

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Cassia fistula Linn, a paramount medicinal plant consumed by huge population all over the world. Its bioactive metabolites include flavonoids, phenolics, and polyphenols. Major objective of current study is to find right media combination and age of callus for maximum accumulation of free flavonoids in *Cassia fistula* cultures. In this study, the calli were induced in MS + 1.0 mg/L 2,4-D + 0.05 mg/L BAP with 50 mg/L ascorbic acid. These calli were then transferred to basic MS medium with 1.0 mg/L 2,4,5-T and varied BAP concentrations. Four and 8 weeks old calli were used to find out the right concentration of BAP and age for upscaling of free flavonoids. The samples were extracted and HPTLC analyzed for free flavonoids at 260 nm along with the leaf explants. The study revealed that the amount and number of free flavonoids in 4 weeks old calli increased as the amount of cytokinin (0.5 to 1.0 mg/L BAP) increased but decline with further addition of BAP (2.0 mg/L BAP). In aged calli number and amount of free flavonoids exhibit a tremendous increase when compared to the calli in the same medium at 4 weeks. This is inferred that accruing age of callus, reduced quantities of plant growth regulators (optimized for callus growth) accumulate free flavonoids. The best medium for free flavonoid production is MS + 1.0 mg/L 2,4,5-T with 1.0 mg/L BAP may be recommended for producing a high amount of free flavonoids in *C. fistula in vitro*. The results were justified with the results of quantification.

Keywords: Free flavonoids, Golden Shower, Indian Laburnum, Phenolics, Quercetin, Rutin

Cassia fistula Linn. (Caesalpiniaceae), a tropical tree native to South Asia, commonly known as Indian Laburnum, Golden Shower or Amaltas and traditionally referred to as Aragvadha, is used to treat various diseases in the Indian system of medicine[1,2]. Africa, Latin America, Northern Australia and Southeast Asia are home to *C. fistula*[3]. It is cultivated as an ornamental plant in India, Thailand and Pakistan because of its yellow blossoms[4]. *C. fistula* is employed to treat asthma, leprosy, fever, ringworm and heart diseases. Fruit pulp is a good laxative and is employed in the management of constipation. Cases of flu and colds are treated with root extracts, while leaves relieve pain, edema, and skin irritation. Stem, bark and fruits remove toxins and are used as blood purifiers[5]. *C. fistula* seeds are employed for treating diarrhea, gastritis, biliousness and improve appetite. Roots are antibacterial and used for the treatment of tuberculosis, leprosy and syphilis[6]. Fruits are anti-inflammatory and used to manage liver disorders, throat problems, and rheumatism. Different parts of *C. fistula* are used to treat of other complications that are not mentioned[7].

Phenolic substances are the most pronounced secondary metabolites isolated and characterized from parts of *C. fistula.* Significant medicinal properties attributed to this plant are due to its phenolic constituents. Phenolics comprise a variety of compounds, including flavonoids, phenolic acids, polyphenols and colored anthocyanins[8]. Flavonoids are phenolic terpenoid compounds, structurally based on a chromane ring structure[9]. Currently, the number of flavonoids crossed 7000 with beneficial action on human health[10,11]. Active ingredients of *C. fistula* include lupeol, β-sitosterol, hexacosanol (stem bark), fistucacidin, leucoanthocyanidin (heart wood), Kaempferol and proanthocyanidin, rhein, fistulin, alkaloids, triterpenes (flower), chrysophanol, physcion, (-)-epi-afzelechin, (-)-epicatechin, procyanidin B-2, rhein, rhein glucoside, biflavonoids, triflavonoids, sennoside A, sennoside B (leaves), and rhamnetin 3-O-gentibioside (roots). Among vegetative parts young and old leaves showed a maximum amount of total phenolics, flavonoids and proanthocyanidins. The fruit pulp is reported with the highest total phenolics in this species[12]. Flavonoids are significant bioactive secondary metabolites accumulated in free and glycosidic derivatives in several tissues. Above mentioned medicinal properties indicate *C. fistula* is a significant medicinal plant and inspired investigation further from its callus cultures. This is an established fact that stresses and the addition of elicitors or signal molecules are feasible strategies to enhance the

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synthesis of bioactive metabolites both *in vivo* and *in vitro*[13]. The efficient and low cost *in vitro* production of medicinal principles will ensure their availability causing least disturbances to plant natural resources[14]. Continuous and unregulated exploitation of plants for their therapeutic principles makes them endangered and needs conservation efforts. The study proposes an alternate strategy to reduce our direct dependence on plants for medicines[15]. Major benefits of the technology are the synthesis of bioactive secondary metabolites independent of the soil and environmental conditions, cultured calli are free from contamination, cells and tissues of tropical and alpine climates can be multiplied in the laboratory, automation of growth regulation reduces the cost of bioactive metabolite production[16] and low cost downstream processing and quality addition to products further adds to its significance. Therefore, in the current investigation, an attempt has been made to find the impact of plant growth regulator concentrations and age of calli on free flavonoid accumulation. The primary objective of the present study is to screen, identify and quantify important free flavonoids of calli induced from tender leaves of *C. fistula*, on different combinations of PGRs for different time intervals.

Materials and Methods

Murashige & Skoog[17] (MS) basic nutrient medium enriched with sucrose (3% w/v), meso-inositol (100 mg/L) along with a range of PGRs to induce callir callus induction and upscaling of free flavonoid production have been used for this study. The pH was adjusted to 5.6 to 5.8 before autoclaving (at 121°C for 20 min). A 100 mL borosil flasks were used as culture vials. Each flask was dispensed with 30 mL melted medium. The tender leaves of 0.5 to 1.0 cm length were used as explants and were collected from tree growing naturally outside the Kailash Prakash Hostel, CCS University Campus, Meerut (UP) India. The leaf explants collected were first rinsed with tap water and Tween 20 successively. The detergent, Tween 20 was used to remove the dust, microbes and extra substances from the surface. The explants were then taken into the laminar air flow and washed five times each with double distilled water (DDW) and sterile DDW. After that they were treated with 0.1mg/L $HgCl_2$ for 2 min followed by five washing steps with sterile DDW under aseptic conditions[18]. Different concentrations of auxins and cytokinins were combined to induce calli alone or in combination. The cultures were maintained in a culture room with a light of 1400 lux with a 16/8 h light/dark cycle at 25±2°C. The best calli were induced in MS + 1.0 mg/L 2.4-D + 0.05 mg/L BAP with 50 mg/L ascorbic acid. These were then subcultured on different PGR supplementations and left for four and eight weeks. More aged calli showed necrosis and partial death; hence 4 and 8 weeks ages were selected. The accumulation of flavonoids especially free flavonols were observed and compared.

Extraction of free flavonoids

For the comparative study of phenolics and flavonoids, leaf explants, 4 and 8 weeks old calli were used. On the basis of superior physical (growth index, fresh weight, dry weight. and Moisture%) and biochemical attributes calli cultured on 1.0 mg/L 2,4,5-T with 0.5, 1.0 and 2.0 mg/L BAP along with leaf explants were selected for HPTLC analysis. The tissue samples were separately dried powdered and were further used for extraction. Five such replicates were examined and mean values were taken with standard deviation. The samples were analysed at 4 and 8 weeks age for free flavonoids at 260 nm. A 1.0 g of the dried powdered leaf explants and cultured calli were separately soxhlet extracted[19] and then with 80% ethanol (100 mL/gdw) for 24 hours in a water bath. All the extracts were concentrated and extracted again with 40-60°C petroleum ether, ethyl ether, and ethyl acetate in succession and fractions I, II and III collected separately. The process repeated three times to make sure complete extraction in each case. The I lipid rich fraction was discarded whereas II fraction was analysed for free flavonoids in each sample.

HPTLC analysis

The samples prepared in such a way were subjected to HPTLC with standards. The fraction II extract was dissolved in methyl acohol (3×10 mL) at 25±2°C. This extract was mixed, sieved and dried by evaporation in the water bath. A 10 mg dried extract was again mixed with 1.0 mL methanol and then 15 µL of this was implicated with CAMAG automatic TLC sampler III on a HPTLC percolated Silica Gel Merck 60 F254 test plate with consistent coating of 0.2 mm[20]. In CAMAG twin trough chamber the plate immersed in solvent system Ethyl acetate: Acetic acid: Water (100:1:10) to develop it and screened at 260 nm[21]. The scanner settings were plate size (width X height = 20X20), application position Y (10 mm), position of solvent front (80 mm), scan start position

Y (10 mm), scan end position Y (90 mm), scan start position X (27.9 mm), distance between tracks X (13.9 mm), number of tracks (12), lamps (Duterium/tungsten/ mercury), monochromator bandwidth (20 nm), wave-length (260 nm) and slit dimension (6.0×0.45 mm).

Results and Discussion

Callus induction from tender leaf explants of *C. fistula* requires an adsorbant or antioxidant in the medium owing to the high percentage of phenolics exuded in the medium inhibiting growth. The best calli were induced in MS+1.0 mg/L 2.4-D+0.05 mg/L BAP with an antioxidant 50 mg/L ascorbic acid. These were then subcultured on different PGR supplementations and left for four and eight weeks to obtain the required amount with upscaled flavonoid production. Four and eight weeks old calli were used to optimize the concentration of BAP and age of subculture for flavonoid upscaling (Fig. 1).

Free flavonoids have been analysed in the present investigation during varying ages of calli to pinpoint the specific changes in flavonoid accumulation patterns. The leaf explants were screened for free flavonoids at 260 nm before subjecting to callus induction. Four free flavonoids were recorded, each ranging between 0.08 to 0.83 Rf in the tender leaves. Interestingly, in callus cultures grown on 1.0 mg/L 2,4,5-T with increasing concentration of BAP, the number of free flavonoids in callus rose from two to five; however, the Rf of flavonoids varied as well, ranging between 0.17 and 0.92 Rf. Another interesting feature was decline in flavonoids with the highest BAP concentration used (2.0mg/L). Besides, in all the concentrations of BAP at 4 weeks stage of *callus*, a compound with 0.92 Rf appeared stably (Fig. 2A).

At Eight weeks the subcultured calli exhibited seven, nine and four free flavonoids, respectively with 0.5, 1.0 and 2.0 mg/L BAP, with Rf values ranging between 0.08 to 0.92. Interestingly, the lower values of Rf were retained upto 1.0 mg/L BAP. Further increasing BAP concentration up to 2.0 mg/L led to a decline in free flavonoids with a lower Rf value (Fig. 2B). The quantification of identified flavonoids led to a doubling of quercetin on 2.0 BAP and 1.0 BAP, respectively, through 4 to 8 weeks. On the other hand, rutin increased by 1.5 times upon reduction of BAP from 1.0 to 0.5 mg/L through 4 to 8 weeks, indicating ageing of subcultures to be a cause of free flavonoid accumulation at lower expense BAP. However, HPTLC could not identify these important flavonoids in tender leaves (explants) (Table 1). Similar studies have been reported exhibiting a quantitative increase total phenolics content and antioxidant activity in *Clinacanthus nutans* (Burm. f.) Lindau the [22] or by modification of growth regulator concentrations in *Artemisia annua*[23], or by using elicitors in *C. tora*[24] and *Pueraria candollei*[25]. In *C. fistula* presence of rutin and its accumulation with age by reducing the concentration of BAP is a unique finding, besides the accumulation of quercetin too with age of callus and reduction of BAP. HPTLC analysis revealed a high accumulation of free flavonoids besides quercetin and rutin compared to 4 weeks old subcultures on all the media tested. The explant was recorded on 1.0 mg/L 2,4,5-T + 1.0 mg/L BAP, 8 weeks old *C. fistula* calli were recorded too.

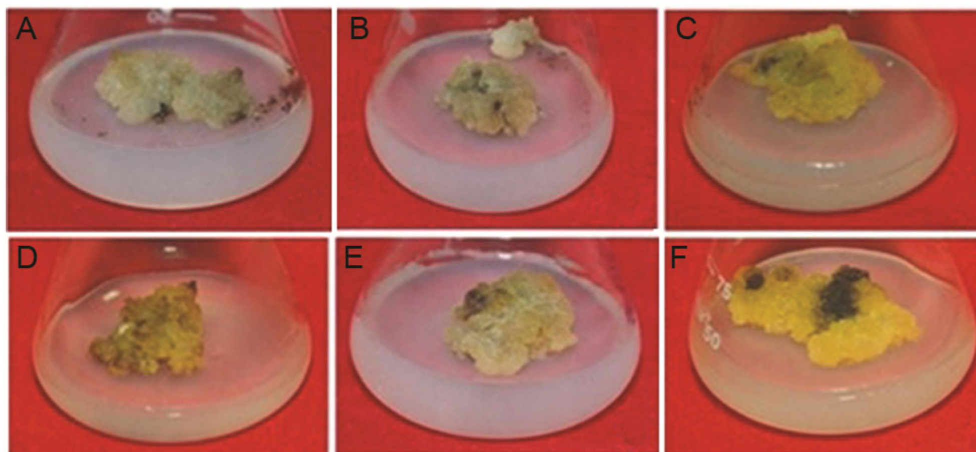

Fig. 1 — Cultured *Cassia fistula* calli on MS medium supplemented with 1.0 mg/L 2,4,5-T and (A-C) 0.5, 1 and 2 mg/L BAP for 4 wk, respectively; and (D-F) the same as in A-C for 8 wk

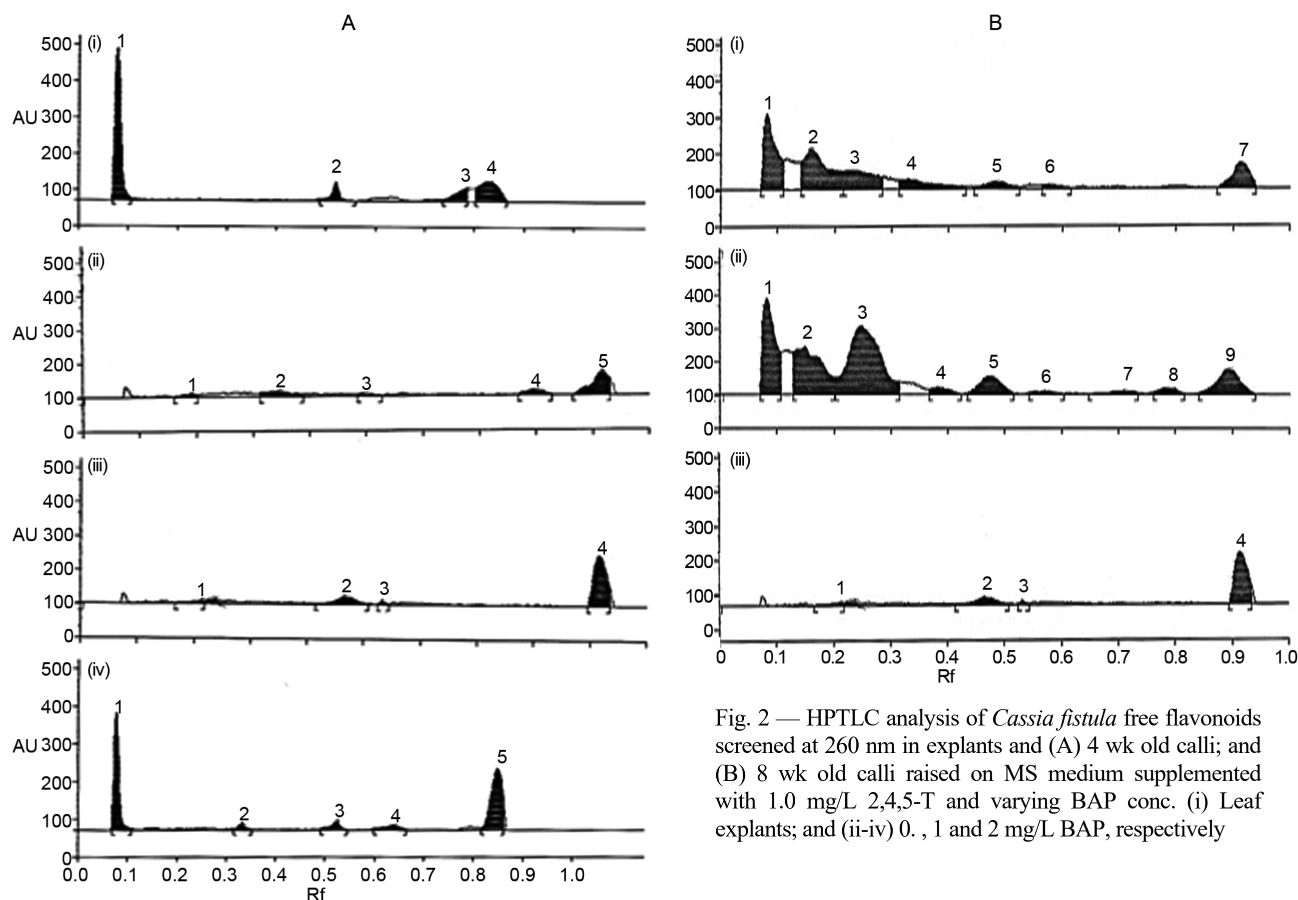

Fig. 2 — HPTLC analysis of *Cassia fistula* free flavonoids screened at 260 nm in explants and (A) 4 wk old calli; and (B) 8 wk old calli raised on MS medium supplemented with 1.0 mg/L 2,4,5-T and varying BAP conc. (i) Leaf explants; and (ii-iv) 0. , 1 and 2 mg/L BAP, respectively

Table 1 — Quantification of free flavonoids through HPTLC in *Cassia fistula* call

Age/ sub-culture	Growth Regulators with MS	Free Flavonoids		
		Quer-cetin (%w/w)	Quercetin like Flavo-noids (%w/w)	Rutin (%w/w)
	Leaf Explant	-	-	-
4 wk old	1 mg/L 2,4,5-T+0.5 mg/LBAP	-	-	-
	1 mg/L 2,4,5-T+1.0 mg/LBAP	-	-	0.049
	1 mg/L 2,4,5-T+2.0 mg/LBAP	0.031	-	-
8 wk old	1 mg/L 2,4,5-T+0.5 mg/LBAP	-	-	0.64
	1 mg/L 2,4,5-T+1.0 mg/LBAP	0.065	-	-
	1 mg/L 2,4,5-T+2.0 mg/LBAP	-	-	-

Conclusion

The analysis of experimental data leads to an inference that with accruing age of callus, reduced quantities of plant growth regulators (optimized for callus growth) accumulate free flavonoids. The best medium for free flavonoid production is MS + 1.0 mg/L 2,4,5-T with 1.0 mg/L BAP may be recommended for producing a high amount of free flavonoids in *Cassia fistula in vitro*.

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Conflict of Interest

Authors declare no conflict of interests.

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