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IN VITRO INTENSIFICATION OF ANTHRAQUINONES IN CALLUSCULTURES OF *Rubia cordifolia* BY PRECURSOR FEEDING

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*Abstract :*Plants are enriched with various secondary metabolites. It's over exploitation may undermine the existence of plants in this world. So plant tissue culture technology is now emerging as a new frontier in science, as it helps to improve the production rate of metabolites in *in vitro* condition. Callus cultures were developed on different auxin supplemented media and the callus were maintained at the optimum concentration. Precursor feeding experiment were conducted to find the increment of AQ production under the influence of precursors used viz., ketoglutarate (KG) and phenylalanine (PA). Friable calli were produced from nodal segments cultured on MS medium containing 5 µM NAA and estimated 17.76 mg g^{-1} dw alizarin and 3.8 mg g^{-1} dw purpurin. In callus cultures addition 100 µM phenylalanine as precursor has resulted 25 fold higher alizarin production over roots sampled from naturally growing plant. This work is focused to improve the AQ production through callus culture of *R. cordifolia* so that we could maintain the continuous supply of AQ without disturbing the plant intact.

Index Terms **- Alizarin, purpurin, anthraquinone, phenylalanine, ketoglutarate**

1.INTRODUCTION

Demand for plant and plant-based organic products is escalating all over the world due to the realization of its greater biocompatibility and fewer side effects. Each plant is endowed with specific secondary metabolic products, which may undergo various spatial modifications over time by regulating their pathways. The extraction and purification of these metabolites are found to be very difficult as they are generated in a tissue-specific or cell-specific manner at distinct developmental stages (John and Annadurai, 2015) often in trace quantities. Anthraquinone (C14H8O2) is an aromatic dye-stuff group with a basic structure of 9, 10-anthraquinone (IUPAC: 9,10-dioxoanthracene), responsible for the pigmentation of heartwoods and bark of many economically valuable plants. It is the chief coloring agent of natural fibers, food, and hair (Samatha and Vasudevan, 1996). Anthraquinone (AQ) is a medicated compound used to alleviate various ailments. The persuasive antioxidant activity is effectively exploited in the food industry as chemo-preventive agents.

The emerging commercial demand for secondary metabolites has resulted in a new arena to produce these chemicals through plant cell or tissue culture. Under strict and carefully regulated aseptic conditions, predetermined stimulation and process strategies for defined phytochemicals from *in vitro* cultures enhanced the secondary metabolite production. Indeed, the i*n vitro* plant materials are functioning as prospective factories of future secondary metabolites. The efficacy and production of a particular phyto-compound under culture conditions requires the addition of a compound (precursor), which is an intermediate of the beginning of a biosynthesis route (Mulabagal and Tsay, 2004). These compounds exist upstream of target compounds in the pathway and their concentration determines the pace of the reaction and conversion into downstream products (Gaosheng and Jingming, 2012), which either bind to a receptor or activate a particular gene through a signal transduction pathway (Sudha and Ravishankar, 2002) or channels more carbon flux to the biosynthesis of targeted compounds or accumulate upstream compounds in bulk and thus secondary metabolite production is improved.

II.NEED OF THE STUDY

Rubia cordifolia is a highly potent medicinal as well as dye yielding plant, that belongs to the vulnerable category. The roots are chiefly valued for their AQ. In Kerala, the annual consumption of *R. cordifolia* root is increasing many tons/annum because of the huge demand imposed by the pharmaceutical and textile industries. The constant harvesting of roots may lead to complete arrest of the supply of this plant species. So it is time to develop a strategy to improve AQ production by manipulating the available *in vitro* techniques. The main objective of this study is the precursor mediated improvement of AQ production from callus culture.

III.RESEARCH METHODOLOGY

Explant sterilization: Semi- matured leaves were collected from plants growing naturally in Elappara (elevation 1158 m asl; Latitude 9o36',49.89" N, Longitude 77o00', 6.59"E), Kerala, India. A voucher specimen (KUBH 6025) was registered at the Herbarium, Department of Botany, University of Kerala. The pre-sterilization treatment included tap water wash (5 min), carbendazim fungicide treatment (Bavistin, BASF, Mumbai, India) for 5 min followed by repeated rinsing in double-distilled water. The treated explants were subjected to 0.1% (w/v) mercuric chloride (Hi-Media, Mumbai) treatment for 2 min.

Callus initiation and callogenic responses: The leaf segments were trimmed for desired size (1.5 cm2) and were inoculated in agar gelled MS medium supplemented with different NAA (1.0, 2.5, 5.0, and 10.0 µM) concentrations. The standardized NAA concentration served as the basis for further callogenic studies in *R. cordifolia*. To identify the best explants for calli formation, leaf segments, nodal segments, or root segments of *in vitro* plant parts were inoculated on agar gelled MS medium containing different auxins viz., IAA, IBA, NAA, or 2,4-D at 5 µM concentration, and callogenic response (%) of explants, morphology, color, and texture, etc were recorded. After the culture period (30 days) the ethanolic extracts of the dried calli were prepared and were used to quantify alizarin and purpurin (Zenk et al., 1975; Hagendoorn et al., 1994; Borroto et al., 2008) content. The statistical significance of the metabolite content was then duly determined. The calli raised from nodal explants were subcultured on agar gelled MS medium supplemented with 5 µM NAA six times and every 30 days intervals growth stability and AQ production were tested. The data on various parameters *viz.*, FW, DW, GI, alizarin, and purpurin content were recorded. .

Determination of calli growth index (GI): The calli were taken out and wrapped in blotting paper to remove water content and weighed directly to determine the fresh weight and these were oven (Beston Hot air oven, India) dried at 50oC for 2 hours or more till constant weight was obtained. Callus Growth Index (GI) was calculated (Godoy-Hernández and Vazquez-Flota, 2006) as the ratio of the accumulated and the initial biomass.

Anthraquinone determination: About 100 mg of dried tissue was then extracted twice with 10 ml of 80% ethanol for 45 minutes in a water bath at 80°C (REMI water bath) and the extracts were centrifuged at 1500 rpm for 10 minutes. The supernatant was collected and the residue was further extracted till ethanol became colorless. The final volume was made up to 10 ml using the same 80% ethanol and these were used for the determination of AQ using a UV/Vis spectrophotometer (Shimadzu Model No. 1700 series). The standard curve was plotted using alizarin and purpurin standards at 436 nm, and 515 nm absorbance respectively. Based on the standard curve amount of AQ in terms of alizarin and purpurin was determined.

Precursor preparation: The effect of precursors on AQ accumulation was studied by using two different precursor compounds *viz.*, phenylalanine and α–keto glutarate at 50, 100, and 150 µM concentrations. The filter-sterilized (0.22 µm pore size, 25 mm diameter, Hi-Media, Mumbai) precursors were added to the MS medium containing 0.7% agar, 3% sucrose, and 5 µM NAA. After 30 days of culture, developed calli were sampled to determine the fresh weight, and dry weight and to estimate AQ constituents in terms of alizarin and purpurin (Zenk et al., 1975). The cultures were maintained at 25±2oC under continuous white fluorescent light with about 60-65% relative humidity under a controlled environment.

IV. RESULTS AND DISCUSSION

Callus cultures have varying degrees of heterogeneity in terms of size, compactness, and total moisture content and can accumulate a high degree of commercially important metabolites. A unidirectional supply of nutrients and gases and light, chemical, and physical gradients present within callus biomass can be manipulated with the help of intrinsic as well as extrinsic factors. Two-year-old roots were sampled from the Elappara collection showed alizarin (2.19 mg g-1 dw), purpurin (0.71 mg g-1 dw) content and total AQ 2.9 mg g-1 dw (0.29 % DW), which is in accordance to previous reports (Mischenko et al., 1999). For the *in vitro* culture experiments plants sampled from this collection were used and quantification data of both alizarin and purpurin was used to compare the efficiency of *in vitro-based* metabolites production.

Callus initiation and callogenic response in *R. cordifolia*

To start the callus initiation, leaf explants were cultured on an MS medium supplemented with different concentrations of NAA (1.0, 2.5, 5.0, and 10.0 µM). Cultures incubated in dark for 60 days produced callus. NAA concentration had a significant ($p<0.001$) effect on callus and significantly ($p<0.05$) height % response (73.3%) was at 5 µM NAA (Table 1). In the NAA added medium callus formation occurred within one month of inoculation. Moreover, at the same concentration maximum fresh weight accumulation was noted (1.29 g) and thus 5 µM NAA was selected for further experiments. The calli produced were yellowish-orange in color which later turned yellowish-brown (Figure 1). The induction of callus and its sustained growth was achieved at higher concentrations of auxin with or without cytokinins in the medium.

Callogenic response of different explants to agar gelled MS medium containing 5 µM different auxins (IAA, IBA, NAA, and 2,4D) was compared. The % response of explants on calli induction was statistically significant (Table 2). Different explants placed on auxin-free, basal MS medium did not produce callus. Yellowish orange to dark brown colored calli was produced (Table 2; Figures 2) from different explants of *R. cordifolia*. The maximum (78.3%) calli formation was noticed in nodal explants cultured on a medium supplemented with 5 µM NAA where friable, reddish-orange colored calli along with adventitious roots formation which is in corroboration with the report by Mischenko et al. (1999). The leaf explant also produced friable, orange calli (73.3%).

Table 1: callogenic response of leaf explantsin MS medium containing varying concentrations of NAA

Conc. (µM)	% response	Fresh Weight (g)
1.0	23.3±6.67c	0.96±0.02b
2.5	63.3±6.67b	1.01±0.05b
5.0	73.3±3.33a	1.29±0.30a
10.0	60.0±5.77b	0.88±0.08b
F Value df (n-1)= 3	14.33***	11.12**

Means within a column followed by letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. *** F value highly significant ($p < 0.001$), ** F value significant ($p < 0.01$)

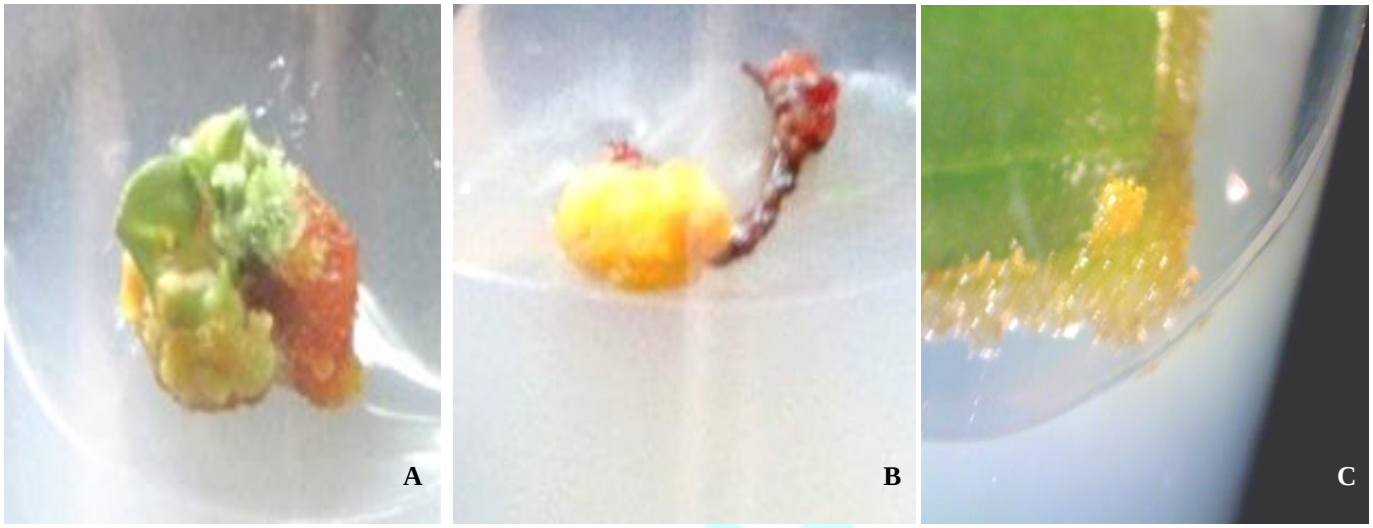
Table 2: Morphology and % response of calli developed in different types of auxin (5 µM) supplemented MS medium

Auxin type	Explant type	Colour of callus	Nature of callus	% Response	Callus induction in scale of intensity
IAA	Leaf	Light orange	Compact with roots	33.3 ± 7.2^{bc}	++
	Node	Creamish brown	Semi compact with roots	37.5 ± 12.5^{bc}	++
	Root	Dark brown	Compact	4.2 ± 7.22^{e}	+
IBA	Leaf	Brownish orange	Compact	29.2 ± 7.22^{cd}	++
	Node	Brownish orange	Friable with roots	29.2 ± 7.22^{cd}	+
	Root	Dark brown	Compact	8.3 ± 7.22^{e}	+
NAA	Leaf	Orange	Friable with roots	73.3 ± 3.33^{a}	+++
	Node	Reddish orange	Friable with root	78.3 ± 7.2^{b}	+++
	Root	Brownish orange	Semi compact	16.7 ± 7.2^{de}	+
2,4-D	Leaf	Brownish orange	Compact	41.7 ± 7.2^{bc}	++
	Node	Yellowish orange	Friable	29.2 ± 7.2^{cd}	++
	Root	Dark brown	Compact	8.33 ± 7.2^{e}	+
F value df (n-1)= 11				12.881***	

Means within a column followed by letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value

Figure 1: Yellowishbrown mature calli of R. cordifolia at 5 µM NAA supplemented MS medium

Figure 2: Calli response of different explants of Rubia cordifolia under culture condition



A: Node derived callli, B: Root derived calli, C: Leaf derived calli

Table 3: Alizarin and purpurin content with different types of auxin at 5 µM concentrations

Auxin type	Alizarin(mg g^{-1} dw)	Purpurin(mg g^{-1} dw)
IAA	12.58±.34^{c}	2.12±.09^{c}
IBA	14.90±1.06^{b}	3.28±.32^{b}
NAA	17.76±.12^{a}	3.80±.02^{a}
2,4-D	8.68±.11^{d}	0.44±.06^{d}
F value df (n-1)= 3	432.729***	4398.443***

Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value

It was noticed that 2, 4-D produced only calli in all explants without any adventitious root formation as reported earlier (Saensouk et al., 2007). The least responding explants were roots which were initially yellowish-orange in color but later turned into dark brown color. This is in contradiction with the findings of Mischenko et al. (1999), where an attempt to develop callus from the root was reported to be completely unsuccessful. This experiment revealed that the nodal segment is the most responding explant for callus induction. The callus developed from nodal explants was reddish-brown and was most responsive and thus used for further experiments. To select the most suitable auxin for AQ production, the nodal segment derived calli raised on MS medium supplemented with 5 µM NAA were further sub-cultured two times on the same media and then cultured on MS medium supplemented with 5 µM IAA, IBA, NAA, or 2, 4-D (Table 3). After 30 days of culture, callus fresh and dry biomass was determined. The maximum accumulation of alizarin was noticed in a 5 µM NAA supplemented medium (17.76 mg g-1 dw) followed by IBA (14.99 mg/g dw). Mischenko et al. (1999) have recorded maximum accumulation of alizarin and purpurin from leaf and stem derived calli. Although there were many reports on the *in vitro* production of AQ in *R. cordifolia*, not much information is available on AQ production concerning explant sources.

Growth Index of *R. cordifolia* callus culture

To determine stability in growth and AQ production of callus raised on agar containing (7 gL-1) MS medium supplemented with 5 µM NAA were sampled after 30 days. FW, DW, and GI of callus were determined. . Growth index reflects the growth characteristic of the callus. When the growth rate was analyzed, there was a progressive increase in biomass in terms of FW, DW, and GI was noticed up to the III subculture (Table 4). Thereafter, fresh weight of callus showed stable growth, with non-significant ($p< 0.05$) variation among the mean fresh weight of IV to VI subcultures. Dry weight records also achieved stability in V and VI subcultures. Maximum dry weight accumulation was noted on the V subculture (76.65 mg). Thereafter dry weights were stable (Figure 3). Growth index records indicate that at the initial stages of subcultures, I and II, steady progress showed, and later on in the V and VI subculture non significant ($p < 0.05$) variation in growth index (0.33 and 0.39 respectively) was recorded. GI index data also support that when callus is continuously subcultured in the same media formulation growth becomes stable and a consistent yield of metabolites thus can be expected.

Alizarin and purpurin production through callus subculture

Alizarin and purpurin production showed a gradual increase and V subculture (17.79 mg g-1 dw, 4.86 mg g-1 dw respectively) onwards production became stable. Previous reports on *in vitro* production of AQ suggest increased AQ accumulation using 0.2 mg Kin and 2.0 mg NAA (Suzuki et al., 1984) in *R. cordifolia*. Zenk et al. (1975) reported that NAA and IAA accelerated the production of AQ in *M. citrifolia* whereas 2, 4-D, in the medium inhibited AQ production. In *R. Cordifolia*, lower concentration of NAA (0.4 mgL-1) enhanced AQ accumulation. (Suzuki et al., 1984). In *Aloe barbadensis* 2, 4-D and Kin combination showed improved growth and AQ accumulation than NAA and IAA (Supe, 2013). In *R. akane*, modified MS without NH4NO3 and NAA, Kin (5 mg L-1 each) was found to be the best production media for AQ (Mizutani et al., 1997).

Table 4: Continuous subculture and growth stability study of callus

Subculture	Fresh Weight (mg)	Dry Weight (mg)	Growth Rate	Alizarin (mg g^{-1} dw)	Purpurin (mg g^{-1} dw)
I	528.60±1.61^{f}	58.21±0.22^{c}	0.06±0.00^{f}	10.12±0.21^{e}	3.59±0.11^{e}
II	539.97±1.46^{e}	67.55±0.23^{b}	0.08±0.00^{e}	12.88±0.32^{d}	3.93±0.07^{d}
III	577.07±1.14^{d}	69.45±0.95^{b}	0.16±0.01^{d}	15.21±0.23^{c}	4.15±0.10^{cd}
IV	629.47±6.14^{c}	68.84±4.43^{b}	0.26±0.01^{c}	17.04±0.19^{b}	4.34±0.14^{bc}
V	665.63±4.19^{b}	76.65±2.93^{a}	0.33±0.01^{b}	17.79±0.13^{a}	4.86±0.22^{a}
VI	694.40±4.75^{a}	66.65±3.41^{b}	0.39±0.01^{a}	17.64±0.06^{a}	4.52±0.07^{b}
F value df (n-1)= 5	991.79***	15.48***	1245.90***	665.77***	36.47***

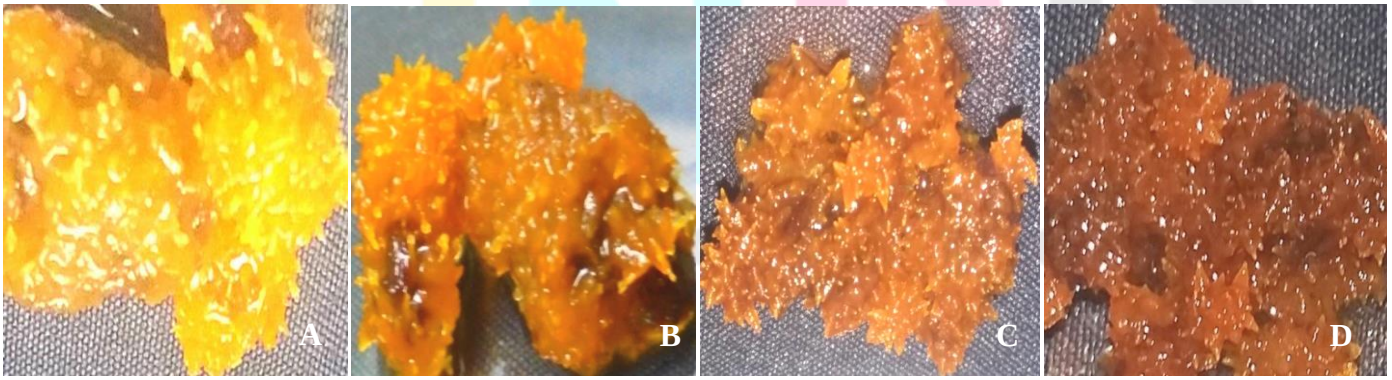
Means within a column followed by letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. ***highly significant ($p < 0.001$) F value

Effect of different precursors on AQ production in callus cultures

The biosynthetic pathway of anthraquinones revealed that the first aromatic intermediate (o-succinyl benzoate) is formed from isochorismate and ∝–ketoglutarate (AK) in presence of thiamine pyrophosphate (Simantiras et al., 1991) and links with shikimate-mevalonate pathway. Thus ∝-ketoglutarate was selected as one of the precursors.

Phenylalanine (PA) is an aromatic amino acid involved as a precursor in a wide variety of secondary metabolites, which functions as a substrate for phenylalanine ammonia-lyase that converts L-phenylalanine into trans-cinnamic acid, the first step of the biosynthesis of phenols in many plant species.. The addition of precursors of AQ in the culture medium is an important strategy in cell culture technique to improve the production of specialized metabolites in medicinal plants. Fresh weight accumulation was influenced by the media composition in which the calli grow. Statistical analysis revealed a highly significant ($p < 0.001$) effect of precursor addition in the culture medium for the production of AQ. In the present study, AK accumulated maximum fresh weight in 50 µM (909.83 mg) followed by 50 µM PA (864.03 mg), while higher concentrations of both precursors failed to enhance FW (Table 5). Dry weight data recorded maximum (93.33 mg) at 50 µM PA and least at 100 µM AK (54.68 mg). The growth rate conformed with these results. The precursors when applied to the medium, they function as substrates for enzymes involved in the biosynthetic pathway. PA supported the accumulation of increasing intracellular alizarin (Table 5). The highest accumulation of alizarin (54.55 mg g-1 dw) was noticed in 100 µM PA, with about 3 fold increase over control and 25-fold higher than roots of naturally growing plant (2.19 mg g-1 dw). Purpurin accumulation was highest in AK at 150 µM concentration (16.84 mg g-1 dw), which was about 4 times greater than the control and 24 fold higher than the root sample of naturally growing plant. According to Zaprometov (1989), precursor feeding is effective only if they are capable of penetrating the cell and if their formation in the cultured cell is limited.

Figure 3: Calli grown in MS supplemented with 2.5 µM NAA and various precursors *A:* at 100 µM Phenyl alanine before addition of precursor;*B:* at 100 µM Phenyl alanine after 30th day of growth; *C:* at 150 µM α-ketoglutarate on 20th day of culture; *D*: at 150 µM α-ketoglutarate on after precursor feeding



In the present investigation, precursor addition has a positive influence on the accumulation of AQ in *R. cordifolia* callus cultures. Previous reports suggest that AK can enhance the accumulation of anthraquinone e.g. *Morinda citrifolia* (Sreeranjini and Siril, 2013) and *Oldenlandia umbellata* (Krishnan and Siril, 2016). According to Shin and Chi (1989) α-ketoglutarate (100 mg L-1) and shikimic acid (500 mg L-1) enhanced the bioaccumulation of pigments in *R. cordifolia*. However, in *R. akane* (Jin et al., 1999) there was no significant increase in AQ by the addition of these precursors in the medium. *In vitro* production of radio-labeled taxol revealed PA as the best precursor for taxol production (Strobel et al., 1992) and colchicines in callus and roots of *Gloriosa superba* (Sivakumar et al., 2004).

Table 5: Effect of different precursors on AQ production in callus cultures of *R. cordifolia* raised on agar gelled MS medium containing 5 µM NAA and varying conc. of precursors (α keto glutaric acid and phenylalanine)

Precursors	Concentration (µM)	Fresh Weight (mg)	Dry Weight (mg)	Alizarin (mg g^{-1} dw)	Purpurin (mg g^{-1} dw)
α keto glutaric acid (AK)	50	909.83±1.65^{a}	75.20±.15^{c}	26.40±.35^{d}	3.51±.01^{e}
	100	772.17±2.29^{e}	54.68±.17^{g}	26.71±.16^{d}	4.77±.07^{e}
	150	854.77±1.5^{c}	81.48±.13^{b}	45.64±.66^{bc}	16.84±.4^{a}
Phenyl alanine (PA)	50	864.03±1.69^{b}	93.33±.19^{a}	43.11±.51^{c}	7.65±.49^{cd}
	100	782.17±1.68^{d}	69.17±.30^{d}	54.55±.37^{a}	8.58±.15^{c}
	150	600.30±2.6^{f}	67.73±.49^{e}	45.68±.49^{bc}	11.26±.1^{b}
Control	0.0	647.20±1.28^{g}	64.90±.45^{f}	17.60±.13^{f}	4.42±.22^{e}
F value df (n-1)= 6		5879***	2771***	1741***	319.7***

Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's multiple range test. *** F value highly significant ($p \leq 0.001$)

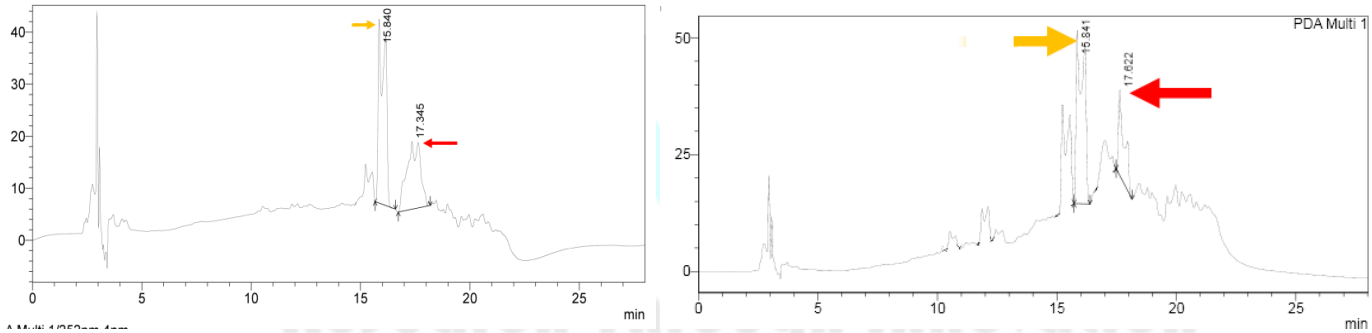
Estimation of alizarin and purpurin through HPLC analysis

Samples from the callus culture, which is found to have the highest AQ production was selected for HPLC analysis. About 100 mg (dw) samples were extracted with HPLC grade methanol (Merck India Ltd., Mumbai) and the extracts were filtered through AXIVA, Syringe filter (2.5mm diameter, 0.2 µm pore size) with cellulose acetate membrane. For HPLC analysis, the chromatographic condition was fixed as follows; C18 column (250 × 4.6 mm); alizarin and purpurin were isocratically eluted with a solvent system composed of methanol: water (80:20, v/v), pH 3.5 (adjusted with formic acid), at a flow rate of 1 mL/min, and UV detection at 254 nm using a photodiode array detector as suggested by Ajum et al. (2014). About 20 µL of the sample was injected and the analysis of alizarin (Sigma) and purpurin (Sigma) was done in Agilent 1100 system with a DAD UV detector.

Table 6: **HPLC profile of *R. cordifolia* in various experimental systems**

Expt Systems	Area	Height	Ret Time	Area	Height	Ret Time	mg/g HPLC	mg/g HPLC
	Alizarin Res Factor= 28721			Purpurin Res Factor = 14641			Alizarin	Purpurin
	28721689	1366750	15.875	14641589	592540	17.688		
In vitro root	798770	37185	15.841	297394	18219	17.622	27.81	20.31
Calli at 5µM NAA	254143	13998	15.848	1188587	34897	17.625	8.85	8.11
Calli grown in PA (100 µM)	744457	35287	15.840	560603	13047	17.345	25.92	3.89
Mature root	86932	3018	15.813	1247636	30875	17.611	3.03	0.852

Figure 4: HPLC profile- A: *In vitro* grown root after 3 months; B: Calli grown at PA (100 µM)



The root samples collected from the wild (about 2 years of growth) were selected as control, which was compared with the methanol extracts of three different *in vitro* systems where maximum yield was obtained by the spectrometric method. Callus developed on 5 µM NAA after the 5th subculture, was found to accumulate 3 fold increased accumulation of alizarin (8.85 mg g-1 dw) and sustained comparable level of purpurin (8.11 gm g-1 dw) production. Similarly, the callus grew in presence of PA also sampled (Table 6), and it was found that a significant increase in alizarin (25.92 mg g-1 dw) production occurred by the addition of 100 µM PA but conc. of purpurin was; less than control. In the case of phenylalanine, the alizarin accumulation was about 8.6 fold greater than that of control (3.03 mg g-1 dw). HPLC data conforms with the spectrophotometric value where maximum alizarin was recorded in PA treated callus. When an overall comparison was made, it was found that the *in vitro* grown roots after achieving 3 months of growth (Figure 4), produced the highest content of alizarin similar to that of calli grown in culture (Figure 4) for 30 days under the influence of PA (25.925 and 3.89 mg/g dw respectively).

V.SUMMARY AND CONCLUSION

Madder (*R. cordifolia*) root is a well-recognized source of AQ. An intensive collection of roots from the naturally growing plants leads to decreasing resource and impose a threat to the plant in the region. Therefore efforts to *in vitro* production of metabolites received due attention. By suitable modifications of media and culture conditions, improved production of AQ was achieved in

the present study through callus culture. Since the current natural sources of AQ could not meet the mounting demand, a continuous production system must be developed by applying plant cell culture technology.

Callus culture is capable to produce an enhanced level of alizarin and purpurin over control. Friable calli were produced from nodal segments cultured on MS medium containing 5 µM NAA and an estimated 17.76 mg g-1 dw alizarin and 3.8 mg g-1 dw purpurin. In callus cultures, the addition of 100 µM phenylalanine as a precursor showed about` 25-fold higher alizarin production over roots sampled from the naturally growing plant. The HPLC analysis revealed that the *in vitro* produced alizarin and purpurin were similar to that of commercially available purpurin sample.

In conclusion, the present study is an initial step toward enhanced AQ production in *R. cordifolia* to meet the market demand for this particular dye in the pharmaceutical, textile, and cosmetic industries. Further research is needed to achieve 100-fold or more enhancements in AQ production. Over expression of key genes in the AQ synthesis pathway, isolation, cloning, and expression of key genes of the AQ pathway of *R. cordifolia* in the prokaryotic system, etc. through the involvement of genetic engineering tools will be the right direction for future research programs in *R. cordifolia*.

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