



## *In vitro* regulation of rotenoid biosynthesis from *lablab purpureus* L.

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### ABSTRACT

Rotenoids are group of natural insecticidal compounds. Six rotenoids (deguelin, dehydrodeguelin, rotenol, rotenone, tephrosin and sumatrol) were isolated and identified from callus cultures of *Lablab purpureus* L using chromatographic and spectroscopic techniques. Growth Index (GI) of *in vitro* grown cultures showed a sigmoid pattern of growth curve with maximum GI at 4 weeks and minimum at 2 weeks old culture after fresh subculturings. Gradual decrease in rotenoid content with age and passage of subculturings of the tissues was observed on maintaining the callus for a period of 12 months. Use of various treatment doses of amino acid precursors (phenylalanine and methionine) and immobilization of cell cultures significantly increased the rotenoid content in callus culture. Immobilization of *in vitro* grown cells released the rotenoids in the medium, which was ideally found useful in maintaining the cell culture and harvesting the insecticides from the nutrient medium.

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### Introduction

*Lablab purpureus* L., belonging to family Fabaceae is a perennial herb alleged to exhibit various pharmacological activities such as antiviral, hypoglycemic, alpha amylase and others (Chau *et al.*, 1967; Kabi *et al.*, 1979; Baslas and Agha, 1986).

Rotenoids are group of bioactive ketonic compounds with potent insecticidal activity, are mostly reported from Fabaceae spp. *Derris*, *Lonchocarpus* and *Tephrosia* spp producing rotenoids have been exploited commercially. Although Rotenoids have also been reported from other members of Fabaceae plants (Sharma and Khanna, 1975; Kamal and Jain, 1978; Kamal and Mehra, 1994; Kamal and Mathur, 2007; Kamal and Mathur, 2008) as well.

Use of biotechnological applications to enhance the yield the metabolite of interest in culture is the need of the hour. The exogenous supply of a biosynthetic precursor to culture medium has been reported to increase the yield of the desired product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases (Moreno *et al.*, 1993; Whitmer *et al.* 1998; Silvestrini *et al.*, 2002). Cell immobilization is another approach, which has led to several commercial processes. Immobilization of microbial and animal cells has been performed for a number of years (Lindsey and Yeoman, 1983). Cells of *Derris indica* were also immobilized for production of rotenoids (Mathur and Kamal, 2001) and harvesting the bioactive metabolites periodically from nutrient medium and replenishing the fresh medium for sustained cell cultures in immobilized beads.

The present study is in continuation of our previous work (Kamal and Mathur, 2010) was undertaken to isolate and identify and evaluate Rotenoids production and regulation in *in vitro* grown culture of *L. purpureus* using precursors and cell immobilization.

### Materials and Methods

#### Tissue culture

Unorganized static cultures of *L. purpureus* were established from its seeds. Seeds were pretreated with 50% sulphuric acid for 5 min, sterilized with 0.1% mercuric chloride, revised and inoculated in MS medium (Murashige and Skoog, 1962). Germination took place in 9–20 days and callus initiated from the radical portion. This callus was maintained for 12 months by periodic subculturings. The callus was harvested at different time intervals of 2, 4, 6, and 8 weeks of subculturings, dried at 60° C and growth index (GI) was calculated, separately. Three replicates of each of the samples were analyzed and mean values were taken. The dried samples were subjected to extraction of Rotenoids.

$$GI = \frac{\text{Final wt. of the tissue} - \text{Initial wt. of the tissue}}{\text{Initial wt. of the tissue}}$$

#### Extraction of Rotenoids

The callus harvested at different time intervals were separately dried and finely powdered. The extraction was carried out by percolating plant material in acetonitrile saturated with n-hexane for 72 h at 22°C, filtered and concentrated to dryness *in vacuo* (Delfel and Tallent, 1969). The concentrated semi-dried extract was dissolved in acetone, and the filtrate was mounted on a column of inert alumina to remove impurities. It was continuously eluted with acetone till the last elutant gave no spot on thin-layer chromatography (TLC). The various eluted fractions were pooled together dried *in vacuo* and weighed separately.

Four sets of experiments were planned to evaluate rotenoid biosynthesis capacity of callus tissue with age and passage of subculturings. Callus tissues were maintained for 3, 6, 9 and 12 months (Set I, II, III, and IV, respectively) by periodic subculturings, separately. In each set, the calli were harvested at the age of 2, 4, 6 and 8 weeks after fresh subculturings. The various samples were dried at 100° C for 20 min to inactivate the enzymes and then at 60° C till constant dry weight was achieved. These were then subjected to qualitative and quantitative

analysis of rotenoids, separately.

#### TLC

TLC was done on silica gel G (BDH-250  $\mu\text{m}$  wet thickness for qualitative and 500  $\mu\text{m}$  wet thickness for preparative TLC) coated plates, air dried, activated and developed two-dimensionally for better resolution of the spots using the chloroform/ether (95:5) in first direction solvent system and chloroform/acetone/acetic acid (196:3:1) in the second. The spots were visualized by spraying developed chromatograms with hydroiodic reagent (HI) and heated at 120° C for 20min (Delfel,1966). The identity of the compound was further confirmed by melting point (mp), mixed melting point (mmp), gas-liquid chromatography (GLC), UV and IR spectral studies.

#### GLC

GLC of isolated rotenoids along with the standard was performed on a Perkin- Elmer FII gas Chromatograph equipped with a dual column, hydrogen flame ionization detectors, steel columns (3 to 6 ft  $\times$  0.125 in), o.d. (0.035 in, wall) packed with 3% JXR gas Chrom Q at 380° C for 16 h, helium flow rate 20 ml/min and inject temperature programmed at 4°C/min from 230-320° C. GLC curves were obtained after reduction of rotenoids with sodium borohydride. About 100  $\mu\text{g}$  of 100% concentrated extract was injected. Retention times were calculated separately and compared with those of reference compounds. The peak areas were measured and computed with the triangulation method for quantification of individual rotenoids (Fig 1).

#### Effect of Feeding Amino Acids as Precursors on Growth and Rotenoid Production in Tissue Cultures of *L. purpureus*.

Three treatment doses of amino acids, phenylalanine and methionine in the ratio of 0.025mM, 0.05mM and 0.1mM each were premixed with MS media, separately. Six months old established calli were transferred to the MS media supplemented with above three treatment doses of amino acids, separately. The MS media without amino acids served as control. The tissues grown in different media were harvested at regular time intervals of 2, 4, 6 and 8 week after fresh subculturing. The growth indices of the tissues harvested at various intervals of time in different precursor treatment doses were calculated, separately. Various tissue samples thus obtained were dried at 100°C for 20 min and later at 60°C till constant weights were achieved. The calli were powdered separately and subjected to qualitative and quantitative estimation for rotenoids.

#### Immobilization

The suspension cultures of *L. purpureus* were subjected to immobilization as per the procedure of Brodelius *et al* (1979). The cultures were sieved through nylon net and cells obtained were added approximately 1:1 (W/V), to a 2.5 % solution of sodium alginate (1.25 g in 50 mL of medium). Shaking above contents formed a homogenous mixture. Immobilized tissues were obtained by pouring drop wise the alginate- tissue mixture in to 50mM calcium chloride solution (1.036g in 150 mL of medium) using a cut end pipette. Beads formed were allowed to harden for 30-40 min, washed thoroughly with MS medium for 3-4 times and transferred in the flask having liquid MS medium. The flasks were incubated on shaker (125rpm) and harvested periodically after 2 week time intervals. Contents of the flasks were sieved. The mediums as well as beads were analyzed for the rotenoid content, separately.

#### Results

**Rotenoids-** TLC of isolated rotenoids from callus cultures of *L.*

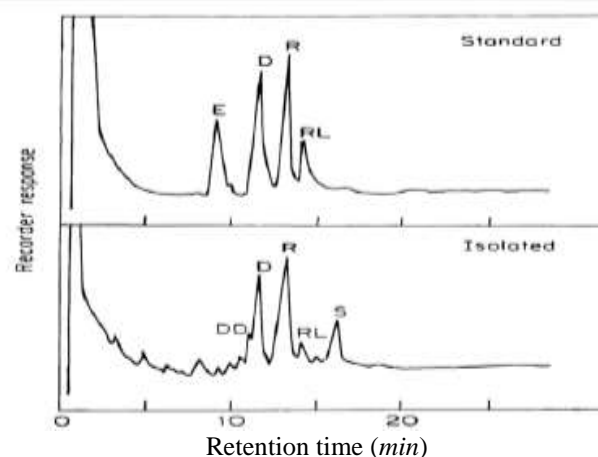
*purpureus* gave six spots in two different solvent systems, which were distinctly coinciding with their standard reference compounds (deguelin, dehydrodeguelin, rotenol, rotenone, tephrosin and sumatrol). In two-dimensional TLC the isolated rotenoids were following the hyperbola pattern giving characteristic colours on spraying with HI reagent spots (Table-1). Isolated individual rotenoids were crystallized and were subjected to melting point determination. The melting point of different rotenoids was rotenone (163°C) and elliptone (179°C) coincided to their respective standard compounds. However, deguelin, dehydrodeguelin, rotenol and sumatrol could not be crystallized. The UV absorption curves at  $\lambda$  max 223 nm of rotenone and other between 222-225 nm were also comparable with that of standard rotenoids (Table1). The IR studies of each of the isolated compound showed characteristic super imposable absorption peaks corresponding to their respective reference marker.

#### GLC

The GLC analysis also showed characteristic retention times of individual rotenoids similar to that of the standard. The GLC analysis of plant part samples showed that rotenone was predominant followed by deguelin, elliptone and other (Fig 1)

**Table 1: Chromatographic behavior and chemical characteristics of isolated rotenoids**

Rotenoids	R <sub>f</sub> Value		Color After Spray		M.P. (°C)	UV absorption (in EtOH) $\lambda$ max (nm)
	S <sub>1</sub>	S <sub>2</sub>	Before heating	After heating		
Sumatrol	0.78	0.27	Grayish blue	Bluish gray	200	223-225
Tephrosin	0.30	0.30	None	Pink	198	222-225
Rotenone	0.56	0.58	Blue	Blue	163	223
Deguelin	0.70	0.72	None	Pink	-	222-224
Rotenol	0.72	0.74	None	Blue	-	-
Elliptone	0.85	0.87	Reddish-violet	Purplish blue	179	225



**Fig 1** GLC analysis of isolated and standard rotenoid. E- Elliptone; DD- dehydrodeguelin; D- deguelin; R- rotenone; RL- rotenol; S- sumatrol

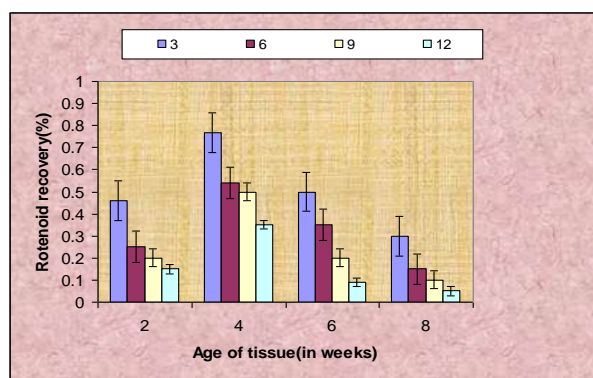


Fig 2. Rotenoid recovery (%) at different stages of callus cultures of *L.purpureus*

Rotenoid content was calculated individually in the four sets (I to IV) of experiments 3, 6, 9 and 12 months old maintained tissues, respectively at the time interval of 2, 4, 6 and 8 weeks of fresh subculturings, which also showed a close parallelism with sigmoid pattern of GI. A gradual decrease of rotenoid content was recorded with the passage of subculturings of the tissue in all the four experimental sets. The maximum amount was observed in set I and lowest in set IV. The maximum rotenoid content (0.77%) was observed in callus harvested after 4 weeks of subculturings whereas in set IV lowest content (0.05%) was observed in callus harvested after 8 weeks of subculturings.

Table 2- Two way interaction of treatment doses of amino acids (phenylalanine and methionine) and time treatment doses (week) on growth index of *L. purpureus* cultures.

Time Treatment (weeks)	0.5:0.5 PA:M	0.5:1.5 PA:M	0.5:2.5 PA:M	1.5:0.5 PA:M	1.5:1.5 PA:M	1.5:2.5 PA:M	2.5:0.5 PA:M	2.5:1.5 PA:M	2.5:2.5 PA:M	Control
2	0.69	0.32	0.42	0.38	0.39	0.44	0.14	0.13	0.32	0.32
4	1.88	1.20	0.81	1.16	1.54	0.92	0.75	0.18	1.18	0.65
6	0.93	0.92	0.67	0.53	0.80	0.78	0.47	0.16	0.60	0.50
8	0.60	0.35	0.40	0.24	0.57	0.45	0.18	0.14	0.50	0.35

PA: M = Phenylalanine: Methionine (mg%) SEM = 0.398, CD 5% = 0.780

The growth index and rotenoid recovery data of 6 month old callus cultures (control) of *L. purpureus* and callus sample obtained from nine treatment doses of two amino acids phenylalanine and methionine in the ratio of 0.5:0.5, 0.5:1.5, 0.5:2.5, 1.5:0.5, 1.5:1.5, 1.5:2.5, 2.5:0.5, 2.5:1.5, 2.5:2.5 mg per 100 mL of MS medium were subjected to statistical analysis. Taking different concentrations of two amino acids along with the control, the marginal mean GI was highly significant. The maximum GI (1.025) was observed in the callus with the treatment dose of 0.5mg% each of phenylalanine and methionine and the minimum (0.152) in the callus with the treatment dose of 2.5mg of phenylalanine and 1.5 mg of methionine in 100 mL of medium. Taking time factor as a parameter, marginal mean GI in all the four time segments gradually increased from 0.355 in 2<sup>nd</sup> week to 1.027 in 4<sup>th</sup> week of subculturing and thereafter decreased from 6<sup>th</sup> week (0.636) to 8<sup>th</sup> week (0.378). The interaction between different treatment doses of amino acids and the time treatments showed the maximum GI (1.88) in 4week callus obtained from treatment dose 0.5mg% each of phenylalanine and methionine, and minimum (0.13) in 2 week callus with treatment dose 2.5 mg of phenylalanine in

combination with 1.5mg of methionine in 100 ml of medium. Taking different concentration of amino acids along with the control, the marginal mean of rotenoid content was highly significant. The maximum content (0.535%) was in callus with treatment dose of 1.5mg% each of phenylalanine and methionine, and minimum (0.137) in the callus with treatment dose of 2.5 mg each of phenylalanine and methionine in 100 mL of medium. Taking time treatment individually, maximum rotenoid content recovery was estimated in 4-week callus (0.471%), which was significantly superior to rest of the time treatments. The interaction between different treatment doses of amino acids and time treatments showed a maximum recovery (0.92%) in 4 week callus obtained from 1.5 mg each of phenylalanine and methionine in 100 mL of medium, and minimum (0.05%) in 8 weeks old callus obtained from 3 different combinations, 2.5 mg methionine with 0.5, 1.5 and 2.5 mg in 100 mL medium, separately.

The 6 month old callus cultures of *L.purpureus* grown on static medium were transferred to liquid MS medium. It formed a uniform suspension when kept on shaker, composed of free cells and cellular aggregates ranging from a few to several hundred cells as observed under microscope. The cultures were harvested after 2, 4, 6 and 8 week time interval. The rotenoid content was estimated from cells obtained from sieving the cultures through nylon net, on fresh weight basis. The maximum amount of rotenoid (0.50%) was found in the cells harvested after 4 week and minimum (0.09%) in 8 week which was more than 12 months old static culture.

The suspension cells were subjected to immobilization using calcium alginate as substratum. The cells embedded calcium alginate beads thus formed were transferred to fresh liquid medium (MS) and were harvested after 2, 4, 6 and 8 weeks of transfer. The beads as well as the medium were analyzed, separately for their rotenoid contents. The rotenoid content was found to be maximum (0.60%) in the medium of 4 week old immobilized cultures, where as minimum (0.12%) was observed in 8 week old cultures, which was found to be more than the suspension cultures.



Fig. 3 *Lablab purpureus* immobilized culture

The rotenoid content was maximum (0.04%) in beads harvested from 4-week-old medium of immobilized cultures and traces (0.001%) were found in the beads from 8 week old cultures.

### Discussion

Rotenoids inhibit mitochondrial electron transport system (ETS) at a characteristic site of insects (Fukami and Nakajima, 1971). It has low mammalian toxicity (Haag, 1937) and high biodegradability thus fulfilling the criterion of international code of FAO for using them in integrated pest management schedule. Rotenoids have been reported from various species of family Fabaceae. The review article of Roark (1932) proved a stepping stone in search of naturally occurring insecticides and resulted in

the exploitation of fabaceous plants for the rotenoids. Among them *Derris* sps has gain the maximum attention on commercial scale production of the rotenoids (Tattersfield and Roach, 1923; Haller et al. 1942; Bose et al. 1976; Yamakawa et al, 1987; Mathur and Kamal, 2001). Various sps of *Derris* such as *D. elliptica* (Tattersfield and Roach, 1923; Yamakawa et al, 1987), *D. malaccensis* (Tattersfield and Martin, 1935), *D. ulginosa* (Bose et al, 1976), *D. indica* (Mathur and Kamal, 2001) etc have been worked out. Rotenoids namely tephrosin, sumatrol, rotenone, ellipton, deguelin, toxicarol and dehydrorotenone have been identified from these species. Four rotenoids (tephrosin, rotenone, deguelin and 6a, 2a dehydrodeguelin) have been reported from *T. vogelii* (Castagne, 1935; Clark, 1930, 1931; Rangaswami and Ramashastri, 1956; Delfel and Tallent, 1969). Whereas rotenone, deguelin and tephrosin has been reported from *Lonchocarpus nicou* (Clark, 1930; Jones, 1939; Delfel and Tallent, 1969). Different rotenoid derivatives (Yenesew et al, 2006) and dehydrodeguelin, dehydrorotenone, 12a-hydroxy rotenone, tephrosin have been reported from *D. trifoliata* (Xu et al.2007) and elliptone, deguelin, dehydrodeguelin, tephrosin, rotenol and rotenone have been reported from *Lablab purpureus* plant parts (Kamal and Mathur, 2010). Besides these various rotenoids have also been reported from *in vivo* and *in vitro* cultures of *I. tinctoria*, *Medicago sativa*, *Parkinsonia aculeata*, *T. foenum-graecum*, *T. polycerata*, *T. corniculata* and *Vigna sinensis* (Kamal and Mathur, 1994; Kamal and Mehra, 1994; Kamal and Mathur, 2007)

Evaluation of metabolite content in culture at different interval of time is an important parameter to assess its biosynthetic capacity. Sharma and Khanna (1975) reported an increase in rotenoid content in 4 week old static and suspension cultures of *Tephrosia purpurea* and *T. vogelii*. Later, Uddin and Khanna (1979) reported sustained biosynthetic potentialities of static cultures raised from seeds and maintained for 12 months. Whereas, Kodama et al (1980) have reported unstable rotenoid biosynthesis ability in leaflet callus mass, wherein a decrease in rotenoid content in unorganized callus mass maintained for 14 months was observed by them with passage of subculturings, which was finally lost in *D.elliptica*. A significant decrease in rotenoid content from 2 to 10 months old tissues have also been observed in *I. tinctoria* by Kamal and Mangla (1993).

In the present study callus tissue showed a gradual decrease in rotenoid contents with age and passage of subculturings. However, the content in individuals set was directly proportional to the growth index. The present findings are in agreement with Kodama et al (1980), Kamal and Mangla (1993) that the tissues with age and passage of subculturings in static culture loose the rotenoid biosynthetic potentialities. It has been known for long time that cultures are heterogeneous collection of cells and their composition changes with successive cultures. The synthesis of many secondary metabolites is associated either with specialized differentiated cell types or organized tissue system. Loss of such rotenoid producing cell lines and or specialized cells may be responsible for the loss of rotenoid biosynthesis capacity.

In order to meet the ever increasing demand for phyto-products there should be a culture system that gives enhanced yield of metabolite of interest. Rotenoid recoveries in fed tissue was highly significant in comparison to control which may be due to incorporation of both amino acids acting as the precursors in biosynthesis of rotenoids, and recoup the loss of availability of the precursors, which is supported by the observations of Crombie et al (1973) and Butcher (1977). The maximum GI and

rotenoid recovery was in the callus with lower treatment doses of 0.5mg% and 1.5mg%, respectively. In immobilization aggregated or partially organized cells where growth can be limited, might be particularly suited to the production of high yields of secondary compounds (Lindsey and Yeoman, 1983) due to induced stress. In the present investigation enhanced level of rotenoid production in immobilized cells was recorded. This enhancement in the content compared to free suspension cultures may be attributed to the fact that the growth rate of immobilized cells is restricted and less than that of freely suspended cells.

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