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ORIGINAL ARTICLE

Effects of Rotenoids from *Indigofera tinctoria* (L) R. Br. On *Callosobruchus chinensis* and *Trogoderma granarium*

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ABSTRACT
Six rotenoids have isolated from Indigofera tinctoria, characterized by means of their spectral data and/or co-tlc. The total estimation studies revealed that rotenol was major rotenoid in both in vivo and in vitro. Similarly, by elicitation studies, we observed that summatrol synthesis was higher at the concentration of 0.01 mM ergosterol and salicylic acid. Among all sequential fractions, methanol fraction demonstrated modern mortality rate (70%) on 24h against Callosobruchus chinensis and summatrol. the isolated rotenoid, possesses maximum mortality rate (80%) at the concentration of 1.0 and 2.0% after 12h against C. chinensis and 70% mortality rate to Trogoderma granarium.
Keywords: *Insecticidal activity, Rotenoids, elicitors, Callosobruchus chinensis, Trogoderma granarium and Indigofera tinctoria (L.) R. Br.*

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INTRODUCTION

Indigofera tinctoria L. is found nearly throughout India mainly as an escape of cultivation. Asean in origin and widely distributed and cultivated in tropics for the famous dye, "Indigo" neel. It is a shrub, grows widely in Aravali range of Rajasthan and distributed throughout India, China, Malaya Peninsula, Tropical Africa, Madagascar, Ceylon, Burma, Philippines, Siam and Pakistan. The plant is not a palatable to cattle, grown as cover or green manure in coffee plantation and rice fields in South India [1]. The juice of leaves when given with milk is considered to be prophylactic against hydrophobia; used in asthama, whooping cough, palpitation of the heart, in certain lung diseases and kidney disorders, bronchitis, haemorrhoids, hepatitis and scorpion sting [2]. Similarly, the extract of aerial parts demonstrated hypoglycaemic and CNS depressant effects [3] and also showed marked protection against CCl_4 induced liver damage in animals (LD_{50} > 1000 kg bw, mice- i. p.; [4,5]. Likewise antitumour [6-8], larvicidal and insecticidal [9,10], anticonvulsant 11(Rahamathullah *et al*. 1990) and snake venom [12] activities have been reported. Phytochemically, from this plant species, blue dye indigo [13,14], rotenoids, flavonoids and histamine [15,16], galactomannan, indirubin [7] and trigonelline [17] have been reported. But there are no reports in literature on isolation and characterization of rotenoids from *in vivo, in vitro* study and use of elicitors and their insecticidal activities; hence the study was carried out.

MATERIAL AND METHODS

Plant material*:* *Indigofera tinctoria* (L.) R. Br. collected from (Sept-Dec, 1997) Amber Hills, Jaipur and identified by Professor S.K. Mishra and voucher specimens were deposited in the Herbarium, Department of Botany, University of Rajasthan, Jaipur, India were used for present investigation (Sheet no. 2040).

Instruments: Melting points were determined on capillary Toshniwal melting point apparatus and spectral data were recorded on the following instrument : ir, Perkin-Elmer, Model, 283; uv, Perkin-Elmer, Model 200; nmr, JEOL PS 100 at 6.1-6.3 MHz; ms, Hewlett- Packard, HP5930 A; and hplc, Waters Model, 501.

Thin layer chromatography*:* For the separation of rotenoids, following thin layer chromatographic solvent systems were used: $CHCl_3$-Diethyl ether (95: 5.0, v/v), $CHCl_3$-Acetone-Acetic acid (196:3.0:1.0). After development, the tlc plates were sprayed with hydroiodic reagent (HI: 5.0N KI+45% Orthophosphoric acid; 1:30; [18,19], followed by heating in an oven (120°C) for ~ 20 min. Later, different spots were observed and compared with respective reference markers.

Isolation and characterization *:* Shade-dried and powdered aerial parts of plant, extracted with acetonitrile saturated with n-hexane (7.5 ml solvent/gdw) for 72h at room temperature, filtered and concentrated to dryness *in vacuo* [20,21]. Later, the concentrate was dissolved in acetone, filtered and the filtrate was mounted on a column of inert alumina to eliminate the impurities. The column was continuously eluted with acetone till the last elute gave no positive results on various TLC.

Preparative TLC: Preparative TLC of rotenoid extracts was carried out on silica gel G coated glass plates along with the standard markers and developed in solvent system ($CHCl_3$-Diethyl ether, 95:5.0). Later, one of the columns in each plate was sprayed with HI reagent and corresponding spots to markers were scrapped from ~ 100 unsprayed plates. Each elute was extracted (19Delfel, 1965) with acetonitrile saturated with n-hexane, concentrated to dryness, crystallized and weighed (%). Each of the isolated compounds was subjected for its mp and other spectral studies along with their respective markers. Later, on the basis of color reactions, TLC behavior and spectral data, the compounds were identified with that of standards.

Tissue culture*:* For seed germination, varied time periods are known in individual plant species of different families. However, in *I. tinctoria*, the seed germination took 7-12 days and the callus tissue initiated after 20-25 days of inoculation from either ends of stem explants. To promote the callus formation, the seedlings grown on Murashige and Skooge [22] medium, were transferred onto MS medium supplemented with 5.0 mg/l 2, 4-dichlorophenoxy acetic acid (2, 4-D) and 2.0 mg/l benzyl amino purine (BAP) in combination. The cultures were incubated at 25±1°C with 60% relative humidity under room light conditions (300 lux). After 25-30 days, the whole explant transformed into the mass of callus. The color of callii, in the initial stages of growth was brown, which later turned reddish-brown with consistency of fragile and irregularly lobed. The callus tissue was transferred onto the fresh medium after 30-35 days interval. The callus tissue was harvested of the age of 2, 4, 6 and 8 weeks and growth index was calculated (GI = Final dry weight of tissue – Initial dry weight of tissue/Initial dry weight of tissue). Fresh tissue samples (600g) were initially dried at 100°C for 15 min to inactivate any enzymatic activity and then at 40°C until the weight became constant. Each of the dried tissue samples was powdered, refluxed in Soxhlet apparatus with 95% ethanol and then evaporated to dryness (yield- 5.38, 9.37, 10.02 and 9.98 %). These extracts (callus tissue) were dried *in vacuo*. The crude ethanolic extract of callus was subjected to fraction with acetonitrile saturated with n-hexane. The n-hexane elute of each samples was used for quantification of rotenoids, by using HPLC, Millipore Waters Model 501, fitted with pump delivery system, injector (Model, 6UK), with solvent delivery system (Millipore Waters, Millford, MA, USA) by using μ Bondapak C_{18} column (30 cm × 3.9 mm, 24±1°C temperature), mobile phase-n-hexane (HPLC grade, E Merck), flow rate – 1.0 ml/min, effluent attenuation at 0.5 AUFS (Lambda Max Model, 481, Spectrophotometer), absorbance adjusted to 254 nm.

Elicitation: Liquid MS medium (50 ml) containing 5.0 g of suspension cells were grown on reciprocal shaker for six days and on seventh day, the medium was supplemented with different concentrations (0.01, 0.025, 0.05 and 0.1 mM) of each ergosterol and salicylic acid as elicitors and the cultures were allowed to grow for next 24 h. Later, these cultures were harvested, dried at 100°C for 20 min to inactivate the enzymatic activity and later at 40°C until constant weight were achieved. The resultant callii were powdered and subjected for rotenoid contents along with the medium separately [23].

Each of the tissue samples was dried, powdered and extracted with acetonitrile saturated with n-hexane (7.5 ml solvent/gdw) for 72 h at room temperature, filtered and concentrated to dryness *in vacuo* [20,21]. Later, each of the concentrate was dissolved in acetone, filtered and filtrate was mounted on a column of inert alumina to eliminate the impurities. The column was continuously eluted with acetone till the last eluate gave no positive results on various TLC. Various fractions were pooled together, dried, weighed and used for TLC and other spectral studies.

Sources of bruchids: The pulse beetle (*Callosobruchus chinensis* L.) and khapra beetle (*Trogoderma granarium* Everts) were obtained from Department of Entomology, Agricultural Research Station, Durgapura, Jaipur and maintained. Both the bruchids were maintained in controlled environment chamber (70% relative humidity, 30±5°C temperature).

Rearing of bruchids: The seeds of green gram (*Vigna radiata* for *C. chinensis*) and wheat (*Triticum vulgare* for *T. granarium*) were cleaned, washed with water, air dried and subjected to temperature for 60°C for 6 h to eliminate infestation. Later, these were air conditioned for 24 h at 55-56% relative humidity and 24±1°C temperature. A culture of pulse beetle (*C. chinensis*) and khapra beetle (*T. granarium*) was developed on green gram and wheat respectively, from a single gravid female. All experiments were carried out at 28±2°C, 50-60% relative humidity, care taken by forceps and camelian hair brushes were invariably.

Bioassays: The flour, with or without added test extracts and isolated compounds, was packed into Davcap no. 2 (7×20cm) hard gelatin capsule each holding 500 mg of diet, mated and newly emerged.

Female beetles (age 12-24 h) were allowed to lay 20 eggs on each capsule. The capsules were then incubated for two days, monitoring larval development and adult emergence. Five capsules were made for each treatment of the test extracts, isolated compounds and the control and mean values were recorded [24].

RESULTS AND DISCUSSION

Rotenoids are known to be harmless to warm blooded animals while toxic to variety of insects. In present study six rotenoids have been isolated and characterized. All the rotenoids isolated from n-hexane elute by using co-TLC and HPLC. All these compounds were identified on the basis of spectral data and direct comparison with reference markers viz., tephrosin (1, $R_f \sim 0.30$, mp 198°C, $UV_{(nm)}$ λ_{max} 222-225, yield- 423 mg), rotenone (2, $R_f \sim 0.56$, mp 163°C, $UV_{(nm)}$ λ_{max} 223, yield- 230 mg), deguelin (3, $R_f \sim 0.71$, mp 171°C, $UV_{(nm)}$ λ_{max} 222-224, yield- 324 mg), rotenol (4, $R_f \sim 0.72$, mp 119°C, $UV_{(nm)}$ λ_{max} 223-224, yield- 242 mg), dehydrodeguelin (5, $R_f \sim 0.74$, mp 223°C, $UV_{(nm)}$ λ_{max} 223-224, yield- 290 mg) and summatrol (6, $R_f \sim 0.78$, mp 200°C, $UV_{(nm)}$ λ_{max} 223-225, yield- 560 mg).

Isolated compounds	% of isolated compounds				
	In vivo	*In vitro**			
		2	4	6	8
Tephrosin	0.42	0.009	0.032	0.054	0.053
Rotenone	0.59	0.018	0.045	0.066	0.062
Deguelin	0.52	0.016	0.048	0.073	0.070
Rotenol	0.63	0.027	0.067	0.098	0.089
Dehydrodeguelin	0.45	0.022	0.054	0.078	0.074
Summatrol	0.56	0.024	0.063	0.076	0.069

Table 1. Estimation of rotenoids from *I. tinctoria* (L.) R. Br. both *in vivo* and *in vitro*.
*** Age of callus in weeks**

Accumulations of secondary metabolites in cell cultures have been reported by various workers. The isolated rotenoids from *in vitro* cell cultures are presented in Table 1. The observed results revealed that the growth index (GI = 0.95, 2 weeks, < 2.43, 4 weeks, < 5.82, 6 weeks > 5.23, 8 weeks) was higher at six weeks old callus and later starts decrease in growth [25, 26]. In these results, rotenol (0.63%) was found to be higher in quantity in intact plant while lower in cell cultures. This is evidenced from results that cell culture study showed lower biosynthetic potentiality in comparison to intact plant species. The growth cycle of tissue cultures generally follow a normal sigmoid growth curve. Microscopical observations suggest that such of increase in cell dry weight at 6 weeks old callus is due to accumulation of starch grains which subsequently disappear later in the growth cycle [27]. The rotenoid values increases to reach sharp peak at 6 weeks then gradually diminish to the initial inoculum value. As a result characteristic fluctuations in cell composition develop during the initial induction from a non-dividing to a dividing condition and over all cell metabolism is obtained cell growth becomes retarded due to nutrient limitation [28], presumably cell division has stopped, there is small loss a break-down of rotenoids from the cells, it seems likely that cessation of rotenoids synthesis is related to the depletion of major nutrients, particularly sucrose from the medium or synthesis may be related to the period of substrate availability [29]. Although, there was relatively greater uptake of nitrate between 3-5 weeks of culture period, ammonium was depleted by 15 days [30], while carrot suspension cells utilized ammonium more rapidly in preference to nitrate. In this respect the pattern of rotenoid biosynthesis by *I. tinctoria* is similar to the examples of secondary metabolite production of cultured plant cells, for example, pyrrolizidine alkaloids in *H. marifolium* [26], tropane alkaloids in *Datura scopolia* cultures [31,32]. These observed results supported the view that the callus growth and quantity of rotenoids interrelated to biosynthetic potentiality of plant.

Isolated compounds	Callus (Suspension cells) Elicitor concentration (mM)					Liquid medium (Leachate) Elicitor concentration (mM)				
	Control	0.01	0.025	0.05	0.10	Control	0.01	0.025	0.05	0.10
Tephrosin	0.053*	0.173	0.126	0.160	0.168	-	-	-	-	-
Rotenone	0.085	0.216	0.189	0.184	0.175	0.007	0.026	0.008	0.006	0.005
Deguelin	0.045	0.196	0.185	0.190	0.165	0.004	0.024	0.008	-	-
Rotenol	0.059	0.124	0.118	0.198	0.110	-	-	-	-	-
Dehydrodeguelin	0.056	0.135	0.142	0.145	0.161	0.012	0.005	-	-	-
Summatrol	0.099	0.286	0.256	0.245	0.220	0.010	0.009	0.004	-	-

Table 2. Effect of ergosterol as biotic elicitor on rotenoids level of *I. tinctoria* (L.) R. Br.
***Yield in percentage**

Accumulation of secondary metabolites in cell cultures by the use of elicitors has attributed the attention of the workers in recent years [33, 34, 35]. In the present study, effect of ergosterol (a biotic elicitor) on rotenoids biosynthesis has been evaluated using suspension cultures treated at various concentrations (0.01, 0.025, 0.05 and 0.1 mM). From the results, it is evidenced that different treatment doses (yield – 0.286% at 0.01 mM concentration: summatrol > yield – 0.216% at 0.01 mM concentration: rotenone). It was interesting to note that liquid medium subsided the biosynthesis of rotenoids, tephrosin and rotenol, which were totally absent in nutrient medium but, rotenone and deguelin was maximum at 0.1 mM treatment dose. Rotenone was present in both suspension cells as well as medium (Table 2). The above findings showed that elicitor concentration is a factor that strongly affects the biosynthetic yield of the individual rotenoid, which respond differently. Likewise, salicylic acid (abiotic elicitor) also exhibited strong effect on the rotenoids yield in cell suspension cultures. Tephrosin and summatrol were maximum in quantity at 0.01 mM dose in cells than the liquid medium at 0.01 mM dose subsequently, but decrease or eliminated with increase treatment doses. It is noteworthy that deguelin was totally absent in the liquid medium (Table 3). Elicitors in a cell cultures offer a novel approach for rapid accumulation of certain secondary metabolites which could improve the efficacy of product via induction of various biosynthetic pathways [36]. Elicitors have been used to increase *de novo* synthesis of secondary metabolites in cell cultures [23].

Isolated compounds	Callus (Suspension cells) Elicitor concentration (mM)					Liquid medium (Leachate) Elicitor concentration (mM)				
	Control	0.01	0.025	0.05	0.10	Control	0.01	0.025	0.05	0.10
Tephrosin	0.082*	0.333	0.093	0.086	0.084	0.006	0.010	0.032	0.029	-
Rotenone	0.065	0.219	0.245	0.268	0.289	0.002	-	-	-	-
Deguelin	0.053	0.116	0.114	0.098	0.085	-	-	-	-	-
Rotenol	0.023	0.099	0.086	0.066	0.070	0.003	-	-	-	-
Dehydrodeguelin	0.039	0.118	0.109	0.128	0.213	0.001	-	-	-	-
Summatrol	0.126	0.438	0.395	0.390	0.380	0.001	-	-	-	-

Table 3. Effect of salicylic acid as abiotic elicitor on rotenoids level of *I. tinctoria* (L.) R. Br.

***Yield in percentage**

Salicylic acid, which is an abiotic elicitor, it is basic compound of salicylate, an important group of pharmaceutical agent while ergosterol is a fungal sterol is used as a biotic elicitor to enhance the yield of useful metabolites. It has been observed that application of exogenous biotic and abiotic elicitors induce expression of defense related genes and provide partial protection against pathogens.

Nature of test sample	Concentration (%) used	Bruchids used							
		Callosobruchus chinensis				*Trogoderma granarium*			
		K.D.E.* after (min)	Mean mortality rate			K.D.E*after (min)	Mean mortality rate		
			6	12	24		6	12	24
Petroleum ether	0.5	40-50	-	-	-	60-70	-	-	-
	1.0	40-50	10	10	20	50-60	-	-	-
	2.0	30-40	20	10	20	50-60	-	10	10
n-hexane	0.5	40-60	-	-	-10	40-50	-	-	10
	1.0	40-50	10	10	30	40-50	-	20	20
	2.0	30-40	10	20		40-50	10	20	30
Chloroform	0.5	50-60	-	-	-	50-60	-	-	-
	1.0	40-50	-	10	10	40-50	-	10	10
	2.0	40-50	-	10	20	40-50	-	10	10
Methanol	0.5	30-40	10	10	20	40-50	10	10	10
	1.0	30-40	10	20	30	40-50	20	30	30
	2.0	30-40	20	40	70	40-50	30	50	60
Ethanol	0.5	60-70	-	-	-	70-80	-	-	-
	1.0	30-40	20	20	30	70-80	10	10	10
	2.0	20-30	20	20	40	60-70	10	20	30

Table 4. Insecticidal properties of crude extracts and fractions of *I. tinctoria* (L.) R. Br.

*** K.D.E. = Knock Down Effect**

In the present study, two months old callus of *I. tinctoria* when fed with different doses of ergosterol and salicylic acid in the medium, the recovery of the rotenoids was recorded to be highly significant as compared to the control. Such an increase in rotenoid content in conformity with studies of Kamal *et al*. [37], that salicylic acid might act as a signal compound in the induction of plant defense mechanism thereby increasing the production of secondary metabolites, specifically rotenoids as demonstrated in the present investigation on *I. tinctoria*.

Isolated compounds	Concentration (%) used	Bruchids used							
		Callosobruchus chinensis					*Trogoderma granarium*		
		K.D.E.* after (min)	Mean mortality rate				K.D.E.* after (min)	Mean mortality rate	
			6	12	24		6	12	24
Tephrosin	0.5	30-40	10	10	20	50-60	-	10	10
	1.0	40-50	20	30	30	50-60	-	10	10
	2.0	50-60	10	20	20	40-50	-	-	-
Rotenone	0.5	40-60	20	30	30	30-40	10	10	10
	1.0	50-60	10	20	20	40-50	10	20	20
	2.0	30-40	20	30	30	40-50	20	30	30
Deguelin	0.5	30-40	20	20	20	30-40	20	20	20
	1.0	40-50	10	20	20	40-50	20	30	30
	2.0	30-40	20	20	20	50-60	20	30	40
Rotenol	0.5	50-60	20	20	20	30-40	30	40	40
	1.0	30-40	20	30	30	40-50	20	30	30
	2.0	50-60	20	30	30	40-50	-	-	-
Dehydrodeguelin	0.5	30-40	30	40	40	40-50	30	40	50
	1.0	50-60	30	30	30	50-60	40	50	50
	2.0	40-50	20	30	30	50-60	30	40	40
Summatrol	0.5	20-30	50	50	60	30-40	40	50	50
	1.0	30-40	60	70	70	20-30	50	50	50
	2.0	40-50	60	70	80	20-30	50	60	70

Table 5. Insecticidal properties of isolated rotenoids from *I. tinctoria* (L.) R. Br.
*** K.D.E. = Knock Down Effect**

The petroleum ether, n-hexane, chloroform and methanol fractions of ethanolic extract and isolated rotenoids have been screened for insecticidal activity and found that among all fractions, methanol fraction demonstrated maximum mortality rate (60%) against *T. granarium* at the 0.5% concentration, similarly this fraction also showed higher mortality rate (70%) against *C. chinensis* at 2.0% concentration after 24 h (Table 4). Among all the isolated rotenoids, the summatrol possesses the maximum mortality rate (80%) at the concentration of 2.0 against *C. chinensis* while 70% mortality rate against *T. granarium* at 2.0% concentration after 24 h (Table 5). The wide and indiscriminate use of conventional insecticides have posed the products are the only group of compounds against which the resistance development in vectors especially mosquitoes have not been reported so far. Earlier, Khanna [38] observed that black pepper in combination with mustard oil used in eradication of various vectors and insect-pests and the isolated bioactive products also intervene the evolution of insects. Interesting results on the bioefficacy tests of rotenoids from *I. tinctoria* against two vector cyclops (Guinea worm), *Anopheles stephensi* (malaria) and pulse beetle (*C. chinensis*) have been reported [10]. In the present investigation sequential fractions of *I. tinctoria* were found to be moderate toxic than the standard rotenoids. However, maximum mortality was observed in methanol fraction followed by n-hexane fraction while minimum in petroleum ether fraction against *C. chinensis* and *T. granarium*. The higher mortality rate in methanol fraction may be due to certain combinations and complementing effect of various phytoceuticals present that may be effective against the insect- pests while in n-hexane may be due to partial extractabilities of rotenoids. The present study focus on the isolation of pure compounds (rotenoids) from *in vivo* and *in vitro,* similarly effect of elicitors on quantity of compounds and insecticidal activities. The future investigation of changes in cell physiology associated with specific change in the environment may lead to a better understand of the regulation of secondary metabolites in plant cells.

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