

IN VIVO AND IN VITRO INVESTIGATIONSON ROTENOIDS FROM *INDIGOFERA CORDIFOLIA* AND *I. LINNAEI* AND THEIR BIOLOGICAL ACTIVITY

UPMAN SHIPIKA, *SARIN RENU

Laboratory of Bioactive Natural Products (Lab No. 9), Department of Botany, University of Rajasthan,
Jaipur-302004, INDIA;

*Email: renusarin@sify.com

ABSTRACT.

Various plant parts of *Indigofera cordifolia* and *I. linnaei* were collected separately at different growth stages and analyzed for their rotenoid content. The total rotenoid content decreased with age; among the plant parts, maximum content was in stems and minimum in roots in both the species. The identification of different rotenoids was done by melting point, UV, infrared spectral studies and HPLC. Presence of six rotenoids (deguelin, elliptone, rotenol, rotenone, tephrosin and sumatrol) was confirmed and quantified *in vivo* and *in vitro* in both the species. But in case of *Indigofera cordifolia* one more rotenoid (unknown) was also characterized which could not be identified due to its presence in traces. The static cultures of *Indigofera cordifolia* and *I. linnaei* were established from seeds on MS medium, and maintained for a period of six months by frequent subculturings. All six rotenoids were also present in callus cultures also. The maximum content was found in eight weeks old tissue after fresh subculturings and minimum at 2 weeks old tissue.

The Biological screening of *in vivo* and *in vitro* extract against the insects (*Tribolium castaneum*), showed an immediate paralytic effect 'knock-down' effect. Extracts from callus culture were more effective against the insects than that from plant parts. Knock-down effect was shown more quickly in *Indigofera cordifolia* in comparison to *I. linnaei*.

KEYWORD: Rotenoids; *Indigofera cordifolia*, *I. linnaei*, *Tribolium castaneum*, biological screenings, callus culture.

INTRODUCTION

An important prerequisite for a sustainable ecosystem is the prevention of environmental pollution, arising from the improper and indiscriminate use of hazardous toxicants. Rotenoids, a group of ketonic compounds are naturally occurring insecticides and score over synthetic insecticides due to their extremely low residual toxicity to mammals, non-persistence and their wide spectrum of activity on many insects. As such rotenoids appear ecologically sound and fulfill the criterion of international code of FAO for using them in integrated pest management schedules. The rapid degradation to non-toxic substances and their characteristic

insecticidal activity have created an important place for rotenoids among various insecticidal groups. Nonetheless, the insects have not been able to develop resistance against them [1].

MATERIAL AND METHODS

A regular collection of various plant parts of *I. cordifolia* and *I. linnaei* was made from Aravalli ranges, Amber, Jaipur from May to November.

Tissue culture

Unorganised static cultures of *I. cordifolia* and *I. linnaei* were established from their seeds. Seeds of *I. linnaei* were pretreated with 50% sulphuric acid for 5 min, sterilized with 0.1% mercuric chloride, whereas the seeds of *I. cordifolia* were sterilized with only 0.5%mercuric chloride and both were revised and inoculated in MS medium [2], Germination took place in 28 days in *I. cordifolia* and in 3 months in *I. linnaei*. Callus was subcultured for better growth and maintained for 6 months by periodic subculturings onto fresh media. The callus was harvested at different time intervals of 2, 4, 6, 8 and 10 weeks of subculturings, dried at 60° C and growth indices (GI) were calculated, separately. The dried samples were subjected to extraction of rotenoids.

Extraction

The various plant samples (roots, stem, leaves and callus samples) were separately dried and finely powdered. These plant parts were macerated separately for 72 hrs with methyl cyanide(acetonitrile) saturated with n-hexane at room temperature. Mixture was filtered and residue was dissolved in equal volume of acetone and filtered and dried *in vacuo* [3].

Powered samples were also Soxhlet extracted in hexane for 12 hrs. Mixture was filtered and residue was dissolved in equal volume of acetone and mixture was filtered and dried *in vacuo*.

Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was done on silica gel G coated plates (0.2-0.3mm thick). The plates were air-dried and activated in an oven at 100° C for 30 minutes and cooled. The various extracts along with the standard samples of rotenoids were applied

1cm above the edge of the glass plates. These glass plates were developed in an air-tight chromatographic chamber containing about 200ml of organic solvent mixture of chloroform, acetone and acetic acid (196:3:1; [3]).The developed glass plates were dried at room temperature. The spots were visualized under UV lamp and sprayed with hydroiodic reagent (HI) and heated at 120° C for 20min. to developed the chromatograms [4].

The identity of the rotenoids was further confirmed by melting point (mp), mixed melting point (mmp), gas-liquid chromatography (GLC), UV and IR spectral studies.

PTLC

Glass plates coated (0.4-0.5mm) with silica gel were activated at 100° C for 30 minutes and cooled. The extracts of various tissue samples were applied along with known reference compounds 1cm above the edge of the glass plates and the plates were developed in an organic solvent mixture of chloroform, acetone and acetic acid(196:3:1)a part of the plates were sprayed with hydroiodic acid. The isolated fractions were separately re-extracted in their respective solvent (Acetonitrile saturated with hexane), filtered and the filtrates were dried *in vacuo*. Each of the fractions were re-dissolved in acetone and re-applied on thin layer silica gel coated and activated plates in order to test the purity of the compounds.

Each of the isolated rotenoids was crystallized using carbon tetrachloride and methanol. Crystals were formed after several hours. The crystals of all the isolated rotenoids were subjected to their melting point, GLC, UV and IR spectral studies. The infrared spectral study of each of the isolated compounds were carried out and compared

with that of IR of its respective known reference compounds.

Analysis: Melting point:

Each of the isolated compounds was crystallized by Tetrachloride and methanol method except deguelin which could not be crystallized. Melting points (Toshniwal, melting point apparatus) of the isolated compounds coincided with those of their respective reference standard compounds.

HPLC:

The High Pressure Liquid Chromatography was performed using wave length-254nm; 60% Chloroform+40% Hexane; 0.5cm/min; column-Microporasil.

Biological screening

The isolated rotenoids mixture was dissolved in kerosine oil and tested for insecticidal property by spraying on insects (*Tribolium castaneum*). On spraying the mixture the insect showed an immediate knock-down effect and within half an hour all the insects died.

RESULTS

The callus of *Indigofera cordifolia* was formed after 28 days of inoculation of seeds on MS medium, whereas the callus of *Indigofera linnaei* was formed after 3 months of inoculation of seeds on MS medium. The callus showed better growth on MS medium after subsequent subculturing. The unorganised callus mass of *I. cordifolia* and *I. linnaei* were creamish white in colour and fragile and turned brownish after 8 weeks of subculturing. The growth index of *I.*

cordifolia was found to be maximum (4.2; based on wet weight and 3.75; based on dry weight) in eighth week old cultures. However the growth index of *I. linnaei* was found to be maximum (3.98; based on wet weight and 3.51; based on dry weight) at the transfer age of 6 weeks..

The TLC of the rotenoids extracted from different plant parts at various growth stages of plant revealed six spots. The spots coincided in Rf values and characteristic colour with reference rotenoids on spraying with HI reagent. The TLC analysis of tissue culture samples showed only six rotenoids (sumatrol, tephrosin, rotenone, degulin, rotenol and elliptone) coinciding with authentic markers of rotenone, rotenol, tephrosin and elliptone. The melting points of the crystals of tephrosin, rotenone and sumatrol coincided with that of the standard. The mixed melting point was undepressed. The IR spectral studies of each of the isolated compounds showed characteristic superimposable absorption peak corresponding to that of reference markers. The UV maximum absorption (E max) of isolate gave maximum absorption at 223 nm of rotenone and others ranging between 222 to 225 nm comparable to standard reference rotenoids. The rotenoids were further confirmed by HPLC when the peaks (min) were compared with those of their respective standard samples. The rotenoids content decreased progressively with age of the plant. The occurrence of all six rotenoids was not universal in different plant parts. Among the six rotenoids in *Indigofera cordifolia* elliptone was absent in roots whereas in *Indigofera linnaei* elliptone was very much less in roots. Maximum amount of rotenoids was observed in the stems followed by leaves and roots.

Only four rotenoids were found in tissue culture samples harvested at different time intervals. Sumatrol and tephrosin were absent. However, callus cultures gave better rotenoid recovery than plant parts . The maximum rotenoid content (0.72%) was in 8 week old tissue and minimum (0.25%) in 2 week old tissue in *Indigofera cordifolia* and (0.70%) was in 8 week old tissue and (0.20%) in 2 week old tissue in *Indigofera linnaei*. There was a progressive increase in rotenoids content from 2 to 8 week of callus growth; however the rotenoids content decreased subsequently in 10 weeks old cultures.

The GI was minimum at 2 weeks and maximum at 8 weeks. The total amount of rotenoids was found to be higher in all the samples extracted with acetonitrile, as compared to other solvent. It indicates that acetonitrile is a good solvent for recovery of rotenoids in this plant species. Isolated rotenoids mixture when dissolved in kerosene oil and sprayed on insect (*Tribolium castaneum*) showed an immediate knock-down effect. The result was found to be more effective in *Indigofera cordifolia* than *Indigofera linnaei*.

DISCUSSION

The present findings identifies *I .cordifolia* and *I .linnaei*, as a source of rotenoids. The variation in the rotenoids content at different stages of growth was observed. The data suggests that early harvest at young stage gives better rotenoid recovery. Fukui *et al* [5] have reported biosynthesis of rotenoids from isoflavonoids. Barnes and Freyre [6] showed loss of rotenoid content with the decrease in moisture content of the plant tissue [7]. Delfel *et al* [8] reported the influence of environmental conditions on levels of rotenoids in plants. The above facts supports

the idea that plants at young stage show a higher concentration of rotenoids then older plants, which may be due to reduction in moisture retaining capacity. Greater rotenoid recovery from callus culture may be due to the maintenance of moisture content through fresh subculturings. The present study revealed that callus culture has more rotenoid biosynthetic potential than plant parts which can be an important source of rotenoid. Fukami *et al* [9] reported that insecticidal activity of rotenone, deguelin and dehydrorotenone is much higher than that of other rotenoids . So the higher bioefficacy of callus extract as compared to plant part extract against may be due to various combination of ratios of active rotenoids and higher recovery from tissue cultures. The presence of six rotenoids (elliptone, sumatrol, deguelin, toxicarol, rotenone and tephrosin) from *in vivo* and *in vitro* tissue of *Crotalaria burhia* has been reported by Khanna and Bansal [10] and Aminuddin and Khanna [11] respectively. Isolation of rotenoids (rotenone, deguelin, dehydrodegulin, sumatrol and tephrosin) have also been demonstrated from plant parts of *Indigofera tinctoria* [12]. In the present investigation presence of six rotenoids (sumatrol, tephrosin, rotenone, degulin, rotenol and elliptone) in roots, stem, seeds, leaves and in callus tissue of *Indigofera cordifolia* and *I .linnaei* has been reported for the first time. The amount found in both the plants are encouraging. The total amount of rotenoids was found to be higher in all the samples extracted with acetonitrile as compared to other solvent. It indicates that acetonitrile is a good solvent for recovery of rotenoids in these plant species.

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