Larvicidal activity and in vitro regulation of rotenoids from *Cassia tora* L.

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Received: 7 April 2017 / Accepted: 5 December 2017 / Published online: 11 December 2017
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Abstract

The search for new insecticidal natural products is very important viewing the global incidence of malaria. In the present study, rotenoids viz., sumatrol, rotenone, tephrosin, rotenol, deguelin, and elliptone were identified from the plant parts and callus culture of *Cassia tora* L. Maximum content of rotenoids was observed in roots ($1.96\% \pm 0.03$) and minimum in the stem ($0.52\% \pm 0.02$). Rotenoid showed larvicidal activity against *Anopheles stephensi* larvae (LC_{50} -120.61 ppm; $P < 0.05$). To enhance the production of rotenoids in vitro, the callus culture of *C. tora* was supplemented with different concentrations of precursors (phenylalanine and methionine). More than onefold increase in the rotenoid content was observed as compared to the control. The present study highlights the insecticidal potential of rotenoids from *C. tora*. Moreover, the enhanced production of rotenoids using precursors can be exploited commercially.

Keywords *Cassia tora* · Rotenoids · Larvicidal activity · Precursors

Introduction

Cassia tora L. (commonly known as chakod and sickle senna) is a member of the family Fabaceae. The plant has been used in Chinese medicine to improve visual acuity and as a health drink tea (Saravanakumar et al. 2015). *Cassia tora* has been explored for a wide range of pharmacological activities viz., antiallergic, antimicrobial, antihepatotoxic, antimutagenic. The herb contains many active substances, including alaternin, cassiaside, β -sitosterol, chrysophanol, emodin, rhein, anthraquinones, etc. (Kirtikar and Basu 1999). However, there are no reports on the presence of rotenoids in this plant.

Malaria, a life-threatening disease, is caused by a parasite (*Plasmodium*), which is transmitted from one individual to another through disease vector *A. stephensi*. As per the report of World Health Organization (WHO), 207 million people were found to be affected from malaria, which led to approximately 627,000 deaths. Africa and South East Asia still happen to be the most affected areas

with almost 3.4 billion people at risk of this disease (WHO 2013). Synthetic insecticides have been effectively used as a measure to control mosquitoes. However, their reckless use has adversely affected the environment and also led to the occurrence of resistant vectors. Plant based products are an effective alternative to overcome the aforesaid problem, thus, exploring bioactive compounds for mosquito larvae control activity forms an interesting perspective among researchers. There are scanty reports on the insecticidal activity of extracts of *C. fistula* (Govindarajan et al. 2008), *C. obtusifolia* (Rajkumar and Jebanesan 2009) and *C. roxburghii* (Muthukumaran et al. 2015). However, it is always important and necessary to identify and characterize compounds having insecticidal activity from the crude extract for commercial reasons. Rotenoids, isoflavone derivatives, commonly occur in the family Fabaceae. These compounds have low mammalian toxicity and thus a favored bioinsecticide (Lim et al. 2015). They are used in the integrated pest management and meet the requirements of the WHO and Food and Agriculture Organization (FAO).

Ever-rising demand for phytocompounds has adversely affected plant biodiversity globally. Thus, manipulating the components of the biosynthetic pathway, using plant tissue culture techniques, to regulate the metabolite production has always attracted researchers. One of the common applications in this regard is the use of precursors and elicitors in

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the tissue culture media to achieve an enhanced yield of the metabolite of interest (reviewed by Rao and Ravishankar 2002).

The present investigation was undertaken to isolate and identify rotenoids in various plant parts and cell culture of *C. tora* together with its larvicidal efficacy against *A. stephensi* larvae. Various treatment doses of precursors were also used to enhance the production of the rotenoids in the culture system. The present study on *C. tora* has been reported for the first time.

Materials and methods

Plant material

Plant parts viz., stem, roots, leaves, and seeds of *C. tora* were collected from University of Rajasthan, Jaipur and authenticated from Department of Botany, University of Rajasthan (Rajasthan University Botanical Library-RUBL No. 20,384). Plant parts were washed under running tap water and oven dried at 60 °C and then finely powdered for biochemical analysis.

Callus culture

Mercuric chloride (0.1%) was used to treat the seeds of *C. tora* initially for 4 min, followed by 70% ethanol for 5 min and then washed with sterile water thrice. Surface sterilized seeds were inoculated in the basal MS medium. The cotyledonary leaves from the germinated seedling were used as an explant for the callus culture on MS medium supplemented with 2.5 ppm 2,4-dichlorophenoxyacetic acid (2,4-D): 2.5 ppm naphthaleneacetic acid (NAA): 0.02 ppm kinetin (KN) as this combination gave the best results as reported in our earlier study (Vats and Kamal 2014b). Culture flasks were incubated at 25 ± 1 °C and 1200 Lux light intensity for 16 h.

Rotenoids

Rotenoids were identified according to the method of Vats and Kamal (2014a). Briefly, samples were extracted with acetonitrile saturated with *n*-hexane. The filtered and concentrated extracts were dissolved in acetone, mounted on a column of inert alumina to eliminate impurities using acetone as eluent. The fractions were pooled together, dried and weighed for rotenoid content (expressed as mean % rotenoid content $\pm$ SD).

Thin layer chromatography (TLC)

TLC was performed on silica gel G (BDH 250- μ m wet thickness for qualitative and 500- μ m wet thickness for

preparative TLC) coated plates using 2-D chromatography. Chloroform:ether (95:5) solvent system was used initially followed by chloroform:acetone:acetic acid (196:3:1). The TLC plates were sprayed with hydroiodic reagent (HI) and heated at 120 °C for 20 min (Delfel 1973). The compounds were also confirmed by mixed melting point (mmp), melting point (mp), UV, gas–liquid chromatography, and IR spectral studies.

Gas liquid chromatography (GLC)

The isolated rotenoids with the standards was subjected to GLC on a Perkin–Elmer FII gas chromatograph equipped with a dual column, hydrogen flame ionization detectors, steel columns (3–6 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 injected temperature programmed at 4 °C/min from 230 to 320 °C. Rotenoids were reduced with sodium borohydride to obtain the GLC curves. About 100 μ g of concentrated extract (100%) was injected. Retention times were compared with standard compounds (Vats and Kamal 2014a).

Larvicidal assay

The larval susceptibility test was performed according to WHO on late third/early fourth instar larvae of *A. stephensi*. Four replicates having 25 larvae/replicates were used. The assay was monitored for 24 h and larval deaths were expressed as percent mortality. Positive control consisted of pure water and acetone and pure water alone served as the control. Where needed, the mortality in the test replicate was correlated with control mortality using Abbott's formulae. Probit analysis was used to calculate LC₅₀ with a 95% confidence limit (upper–lower) (WHO 1981).

Precursor feeding

Phenylalanine (P) and methionine (M) were used as precursors premixed with MS at an equal concentration of 0.025, 0.05 and 0.1 mM each, separately. Callus maintained for 6 months were transferred to the MS media supplemented with the above mentioned concentrations of amino acids. The MS media without amino acids served as control. The callus, thus, obtained was analyzed for the Growth index ($GI = \frac{\text{Final weight of tissue} - \text{initial weight of tissue}}{\text{Initial weight of tissue}}$) and rotenoid content after 2, 4, 6 and 8 weeks of treatment. The callus culture experiments were repeated thrice with 20 flasks each time. The results were analyzed by one-way ANOVA and the difference between the means of the sample was analyzed by Duncan's multiple range test (DMRT) at *P* 0.05.

Results and discussions

Tissue culture

Seed germination in *C. tora* was observed after 10–12 days of inoculation. Initially the calli was off-white in color. During the first 4 weeks the proliferation of callus was slow, which increased during the 6th week. Later (8th week) the media as well as the callus started to turn brown. The callus tissue subcultured after the 8th week showed negligible proliferation. However, on subculturing the callus tissue at 5–6th week the proliferation of cells in callus was more. The synergistic effect of 2,4-D along with NAA and kinetin was observed, which resulted in maximum callusing. This was in confirmation with the study done on *C. occidentalis* (Vats and Kamal 2014c). Growth, differentiation and dedifferentiation of explants in vitro depend on the optimum use of phytohormones either alone or in combinations together with culture conditions (Vats and Kamal 2013, 2014b, 2016). Browning of callus might be due to the leaching out of phenols and accumulation of phenolics in the medium as well as in culture. It happens mainly due to decompartmentalisation through wounding or during senescence with the initiation of oxidation process (Hanumanaika and Krishna 2008).

Negligible growth of callus on subculturing after the 8th week may be due to the formation of quinones, product of oxidation of phenolic compound, which are highly reactive and toxic to plant tissue (Hanumanaika and Krishna 2008). Quinones have a distinct property of binding to other biological molecules and arresting the growth of plant cells. Frequent subculturing of calli to fresh medium was used as an alternative to overcome the problem of browning of callus (Sundram et al. 2012). This maintains the meristematic nature of calli and prevents accumulation of phenols and their subsequent oxidation.

Rotenoids

The 2-D TLC showed the presence of six rotenoids (sumatrol, rotenone, tephrosin, rotenol, deguelin, and elliptone) in plant parts and callus, which matched with the standard rotenoids with respect to R_f , mp, UV absorption max, IR and GLC spectra and compared with our previous data (Vats and Kamal 2014a). Rotenol and deguelin could not be crystallized. Roots showed the maximum content of rotenoids ($1.96\% \pm 0.03$) followed by seeds ($1.0\% \pm 0.01$), stem ($0.52\% \pm 0.02$) and leaves ($0.85\% \pm 0.02$). This observation seconds the fact that synthesis of some secondary metabolites is dependent on cell/tissue/organ types (Sharma et al. 2013).

Larvicidal assay

The LC_{50} value of standard rotenone and rotenoid extract against larvae of *A. stephensi* was found to be 3.20 (in confirmation with our previous study Vats and Kamal 2014a) and 120.61 ppm, respectively ($P < 0.05$) (Fig. 1). Larvicidal activity of the rotenoid extract from roots of *C. tora* was evaluated to be 42.78 times less toxic than the standard rotenone. Rotenoids inhibit the mitochondrial electron transport chain at the specific site causing death of the organism (Fukami and Nakajima 1971). The efficacy was found to be more than *Trigonella polycerata* and *T. foenum-graecum* and *Lablab purpureus* (Kamal and Mathur 2010), which may be attributed to the different combinations and the amount of the rotenoids in the extract and the part of plants used. The results are a step ahead in exploring natural insecticidal plants/metabolites which can be a part of biopesticides minimizing the huge loss of life due to malaria all over the globe particularly in developing and underdeveloped countries.

Precursor feeding

As the demand for herbal medicine is ever-increasing, search for alternative approaches to harvest plant metabolites has been a major concern to conserve biodiversity (Mathur and Shekhawat 2013). It is established that the cultures are heterogeneous collection of cells and their composition changes with successive cultures. In vitro biosynthesis capacity may be lost due to unavailability of precursors with the passage of subculturing and accumulation of degradative enzymes. Moreover, it is generally accepted, not all the cells in the cultures are active in product synthesis. This leads to a differential yield of secondary metabolites in different time intervals (Khan et al. 2016). In the present study, P and M supplemented in equal concentration of 0.05 mM showed the maximum mean GI (1.22 ± 0.10) in 6 weeks old cultures. Minimum GI (0.08 ± 0.02) was observed in the 2 weeks old callus treated with 0.01 mM dose of both P and M. In the control flask, maximum GI was found to be 0.98 ± 0.03 from 6 weeks old callus and minimum (0.16 ± 0.02) in 2 weeks old tissue. Growth of callus showed a sigmoid pattern in all the sets of experiments (Table 1). P and M at equal dose of 0.05 mM showed the highest content of rotenoid ($1.58\% \pm 0.05$) in the 6 week old callus tissue (Table 2). Least content ($0.71\% \pm 0.03$) was evaluated in the 2-week-old callus culture supplemented with 0.1 mM of each of the amino acids.

Twofold increase in the rotenoid content in callus culture of *T. polycerata* was observed on feeding phenylalanine and methionine at concentrations of 0.1, 0.3 and 0.5 mM, each (Kamal et al. 1997). In the present investigation 1.18 times increase in the rotenoid content was observed in 6-week-old culture as compared to the control. On the other hand,

Fig. 1 Effect of different doses of rotenoids from *C. tora* on *A. stepensi* larvae

Table 1 Effect of precursors (P-phenylalanine and M-methionine) on growth index (GI) at different time intervals of *C. tora* callus culture

Time treatment (weeks)	Growth index Treatment dose of each amino acid			Control
	0.025 mM	0.05 mM	0.1 mM	
2	0.15 ± 0.03 ^a	0.22 ± 0.03 ^a	0.08 ± 0.02 ^a	0.16 ± 0.02 ^a
4	0.29 ± 0.05 ^b	0.44 ± 0.01 ^b	0.21 ± 0.03 ^b	0.38 ± 0.02 ^b
6	0.95 ± 0.01 ^d	1.22 ± 0.1 ^d	0.76 ± 0.04 ^d	0.98 ± 0.03 ^d
8	0.36 ± 0.04 ^c	0.96 ± 0.02 ^c	0.38 ± 0.05 ^c	0.86 ± 0.01 ^c

Mean values ± standard deviation represented by the same superscript letters within the same column are not significantly different at $p < 0.05$

Table 2 Effect of precursors (P-phenylalanine and M-methionine) on rotenoid content (%) at different time intervals of *C. tora* callus culture

Time treatment (weeks)	Rotenoid content (%) Treatment dose of each amino acid			Control
	0.025 mM	0.05 mM	0.1 mM	
2	0.77 ± 0.03 ^a	0.82 ± 0.01 ^a	0.71 ± 0.03 ^a	0.78 ± 0.03 ^a
4	1.16 ± 0.05 ^b	1.26 ± 0.05 ^b	1.08 ± 0.11 ^b	0.94 ± 0.03 ^b
6	1.36 ± 0.02 ^d	1.58 ± 0.05 ^d	1.29 ± 0.02 ^c	1.33 ± 0.09 ^d
8	1.29 ± 0.02 ^c	1.48 ± 0.02 ^c	1.24 ± 0.05 ^c	1.14 ± 0.03 ^c

Mean values ± standard deviation represented by the same superscript letters within the same column are not significantly different at $p < 0.05$

the increase was more than one-fold in all the other time intervals. This increment in the production can be exploited commercially. It is a well known fact that the primary

metabolites act as precursors for the synthesis of secondary metabolites (Hrazdina and Jensen 1992). The increase in the metabolite content may be due to the incorporation of P and M in the ring structure of rotenoids thereby replenishing the loss of availability of the precursors (Butcher 1997). A direct biosynthetic link between amino acids and rotenoid formation has been established when labeled phenylalanine and methionine were fed as precursors to *D. elliptica* and *Amorpha fruticosa*, which were incorporated in different rings of the chromanochromanone structure of rotenoids. An enhancement in rotenoid content was also reported in these plants (Crombie and Whiting 1998).

Conclusions

Rotenoids from *C. tora* showed promising larvicidal activity against *A. stephensi* (vector of malaria). Viewing the problems of plant biodiversity associated with excessive use of plant/plant products for various purposes, tissue culture of the plant for rotenoids recovery was attempted with success. Moreover, in vitro regulation of rotenoids in the culture system using precursors helped in enhanced recovery of the metabolite of interest. The results can be taken further to have mass production of rotenoids either through suspension culture or bioreactor.

Acknowledgements The author is grateful to Late Prof. Raka Kamal, Department of Botany, University of Rajasthan for her supervision and support during the study.

Compliance with ethical standards

Conflict of interest The author declares that there is no competing interests.

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