Journal of Pharmacognosy
ISSN: 0976-884X & E-ISSN: 0976-8858, Volume 3, Issue 2, 2012, pp.-67-70.
Available online at http://www.bioinfo.in/contents.php?id=70

DETECTION OF KARANJIN FROM CALLUS CULTURES OF *Pongamia glabra*

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Received: March 21, 2012; Accepted: April 09, 2012

Abstract- Callus cultures derived from the nodal portions of *in vitro* germinated plantlets of *Pongamia glabra* Vent were established. Accumulation of karanjin, a furanoflavanoid was detected and confirmed by thin layer chromatography, ^{1}HNMR and high-pressure liquid chromatography analysis. Hormones and media composition greatly influenced the growth of callus cultures. The accumulation of karanjin was more in the nodal callus grown on Half strength Murashige and Skoog's medium fortified with 1mg/l 2,4-dichlorophenoxyacetic acid and 2mg/l 6-benzylaminopurine than in Full strength MS medium and Woody plant medium. This is the first report on *in vitro* production of a pharmacologically important compound karanjin from callus cultures of *Pongamia glabra*.
Keywords- *Pongamia glabra*, callus cultures, karanjin, furanoflavanoid

Citation: Sreelakshmi L. and Janardhan Reddy K. (2012) Detection of karanjin from callus cultures of *Pongamia glabra*. Journal of Pharmacognosy, ISSN: 0976-884X & E-ISSN: 0976-8858, Volume 3, Issue 2, pp.-67-70.

Introduction

Pongamia glabra Vent is one of the most useful tree, commonly found in all parts of India. It belongs to the family Fabaceae and is known as karanj in Sanskrit and Indian beach in English. It is an evergreen tree, which grows up to 15 m. tall. The whole plant is medicinally important [6]. Seeds contain 27 to 36.4% of bitter fatty oil and traces of an essential oil. Seeds contain karanjin, pongamol and glabrin. Karanjin is reported to be an effective remedy for all skin diseases like scabies, eczema, leprosy and ulcers [16]. Karanjin cures intestinal obstruction, piles, ulcers etc.

Plant cell culture systems are being exploited for the accumulation of a variety of natural products [7,9]. Plants collected as minor forest produce, show a wide disparity in their values, due to lack of information on their life cycles, maturity and regeneration times, all of which change the quality and quantity of active chemical ingredients present.

There are fluctuations in the concentration and quantities of secondary metabolites in field grown plants as the biosynthesis of secondary metabolites, although controlled genetically, is affected strongly by environmental influence [14]. To over come these limitations plant cell culture techniques are used rather than to use whole plants for the extraction of important secondary metabolites of medicinal value.

The present study deals with the induction of callus cultures from nodes and detection of karanjin from callus cultures of *Pongamia glabra*.

Materials and Methods

Seeds of *Pongamia glabra* were obtained from Krishi Vigyan Kendra, Gaddipally, Nalgonda District, Andhra Pradesh, India. The seeds were surface sterilized with 5%Tween-20, thoroughly washed with water, then treated with 70%(v/v) ethanol for 2 min. followed by 0.1%(w/v) Mercuric chloride for 8min.The seeds were rinsed several times with sterilized water and soaked in sterile water to imbibe overnight. The imbibed seeds were germinated *in vitro*.

Establishment of callus cultures

For callus initiation, nodal explants of *in vitro* germinated plantlets were inoculated on half strength MS medium, full strength MS

medium [13] and woody plant medium [12], supplemented with Auxins such as 2,4-D, NAA and cytokinins such as kinetin and BAP in various permutations and combinations. Many explant tissues especially woody tissues produce phenolics, which cause browning of explant material [15]. Antioxidants like polyvinylpyrrolidone (PVP) 200 mg/l, activated charcoal 100 mg/l were used for checking browning problem. Explants were inoculated on to the nutrient media and all the cultures were incubated for 1 week in complete darkness followed by 16 hr. photoperiod (30 mE $m^{-2} S^{-1}$) at 25±2°C. The pH of the medium was adjusted to 5.7 before autoclaving. Induction of callus was observed on 15th day. Callus was sub-cultured every 20 days. Fresh weight of callus was measured by blotting it on a filter paper to remove excess water if any. Dry weight was obtained after drying the callus at 60°C until a constant weight was obtained. Such dried callus samples were used for quantification of karanjin.

Isolation of Karanjin

For isolation of Karanjin 1 gm. of dried callus sample was soxhlet extracted with 80% methanol for 24 hrs on water bath separately. The methanol soluble fractions were filtered dried in vaccum. Each of these fractions was extracted with petroleum ether (Fraction-1), solvent ether (Fraction-2) and ethyl acetate (Fraction-3) thrice. Fraction-1 was discarded due to the presence of fatty substances, where as fraction-2 was concentrated in vaccum and kept for chromatographic analysis. Fraction-3 was hydrolysed with 7% H_2SO_4 for 2 hrs. The ethyl acetate layers were pooled, neutralized and reconstituted in ethanol for TLC [17].

Authentic sample of karanjin was obtained from Natural products laboratory of Chemistry Department, Osmania University, Hyderabad and spotted for TLC. The solvent system was comprised of C_6H_6:CH_3COOH:H_2O (125:75:3). The chromatographic plates were sprayed with 0.1% alcoholic $AlCl_3$, dried in an oven and observed under UV light (254nm) [17]. The crude obtained after extraction was separated by column packed with silica gel and was run with Benzene and Acetic acid in various concentrations. Eluted fractions were concentrated and dissolved in dueteriated chloroform ($CDCl_3$) and subjected for ^{1}HNMR analysis. Spectra and the Rf value of the compound observed from callus sample was compared with that of the authentic sample.

Estimation of Karanjin

The Qualitative and Quantitative estimations of karanjin in the extracts of the callus were carried out following the procedure of Gore, et al. (2000) using karanjin as standard. The HPLC used was an isocratic mode with CH_3OH, H_2O and CH_3COOH as mobile phase (85:13.5:1.5) at a flow rate of 0.5 ml/min on kromasil 100, C18 column (250x46 m) with a particle size of 5m and dual wave length detection at 350 and 300 nm. Authentic sample of karanjin was dissolved in 10ml of methanol and injected into the C18 column with a flow rate of 0.5 ml/min. The peak absorbance was recorded at 300 and 350 nm. Chromatograms of standard Karanjin and extracts of callus samples were obtained and the relative amount of flavanoid was calculated using karanjin as the reference.

Results

Chemical structure of karanjin is given in Fig 1.

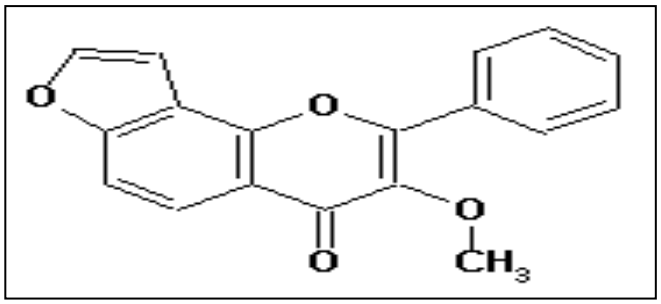

Fig. 1- 7-Methoxy-8-phenylfurano[2,3-h] chromen-6-one

In vitro germinated plantlet nodes were inoculated on different culture media and the percent frequency of callus initiation is represented in Table-1.

Table 1- Effect of auxins and cytokinins on callus induction and proliferation in Pongamia glabra

Hormone treatment (mg/l)	Frequency of callus induction (%)			Growth of callus (g/culture)		
	Full strength M.S	Half strength M.S	Woody plant medium	Full strength M.S	Half strength M.S.	Woody plant medium
1.0 2,4-D+0.5 BAP	Nil	Nil	Nil	Nil	Nil	Nil
0.5 2,4-D+1.0 BAP	20	33	30			
F.W				1.83	2.5	0.92
D.W				0.25	0.72	0.06
2,4-D+2.0 BAP	35	80	40			
F.W				1.9	4.878	1.102
D.W				0.3	1.203	0.081
2,4-D+4.0 BAP	45	72	52			
F.W				1.047	4.012	1.266
D.W				0.049	1.203	0.09
NAA+2.0 BAP	38	68	47			
F.W				0.91	3.52	1.199
D.W				0.07	0.92	0.072
2,4-D+2.0 KN	30	42	20			
F.W				1.75	2.25	0.87
D.W				0.2	0.57	0.01
NAA+4.0 KN	40	58	35			
F.W				2.921	2.65	0.973
D.W				0.82	0.81	0.02

**Data represent an average of 10 replicates.*

When explants were inoculated on full strength MS, half strength MS and Mc Cown & Lyod medium supplemented with various concentrations of Auxins and Cytokinins, callus formation was observed on 15th day. Out of the three media tried callus initiation frequency (CIF) was 80% in half strength MS medium containing 1mg/l 2,4-D and 2 mg/l BAP along with 200 mg/l glutamine and 500mg/l casein hydrolysate. The frequency of response was low on Mc Cown and Lyod medium and full strength MS medium (35%

and 40% respectively)The maximum fresh weight of callus from half strength medium, full strength MS medium and Mc Cown and Lyod medium were 4.878 gm, 1.047 gm and 1.266 gms and corresponding dry weights were 1.539 gm, 0.049 mg and 0.099 mg respectively. (Table- 1)(Fig. 2).

Fig. 2- Callus from nodes of *Pongamia glabra*

When an authentic sample of karanjin spotted on TLC plates was run in a solvent system, the Rf value was found as 0.48.The coloured spots from the callus samples corresponding to the Rf value of the authentic sample were eluted from the TLC plates and subjected to ^{1}HNMR analysis. ^{1}HNMR spectrum of the extracted karanjin was obtained from dueteriated chloroform ($CDCl_3$) at 200MHz.The Rf value of the TLC analysis and ^{1}HNMR studies revealed that the extracts of calli confirm that the compound under investigation is karanjin. (Fig-3)

^{1}HNMR results are as follows:

H-9-7.18, d, J=2.2Hz{furon proton}
H-8-7.76, d, J=2.2Hz{[furon proton}
H-5-8.2, d, J=8.5Hz
H-2'&H-6'-8.15,m, 2Hz
H-6, H-3', H-4', H-5'-7.55,m, 4Hz
3-OCH3-3.93, s.

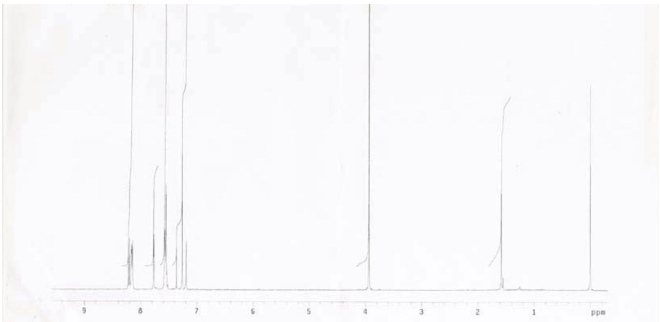

Fig. 3- ^{1}HNMR spectrum of the extracted karanjin

The retention time for standard karanjin was observed as 8.801 at 300nm (97.63%) and 8.800 at 350nm (95.43% pure) (Fig. 4 and Fig. 5).

Comparison with HPLC of the standard karanjin revealed that fraction-3 showed the presence of karanjin at 8.928 (300nm) with a percentage of 0.03 while fraction-2 showed 0.02% at 8.873(300 nm) (Table- 2) (Fig. 6 and Fig. 7). Karanjin was earlier isolated from the seeds of *Pongamia glabra* [1,8,10]. But this is the first report on detection of furanoflavanoid from callus cultures. Since the content of karanjin was less in callus cultures when compared to the seeds, cell line selection, elicitation and immobilization of cells is highly needed to enhance the content.

Table 2- HPLC analysis of karanjin obtained from nodal callus.

Sample	Retention time	Area percentage	U.V absorbption
Authentic	8.801	97.63	300nm
Authentic	8.8	95.43	350nm
Callus:			
Fraction-2	8.873	0.56	300nm
Fraction-3	8..928	5.5	300nm

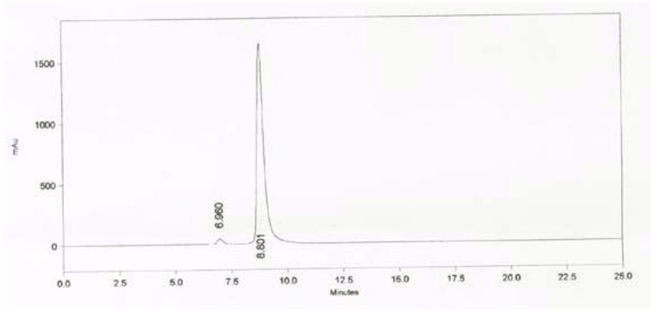

Fig. 4- HPLC analysis of authentic karanjin at 300nm

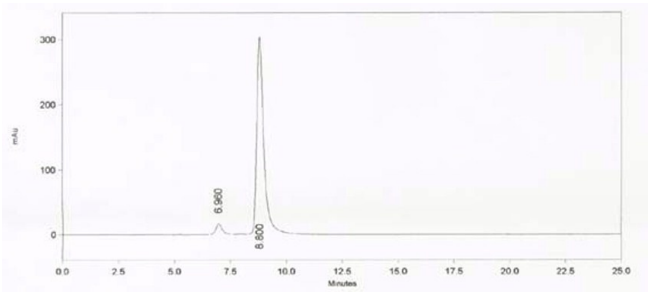

Fig. 5- HPLC analysis of authentic karanjin at 350nm

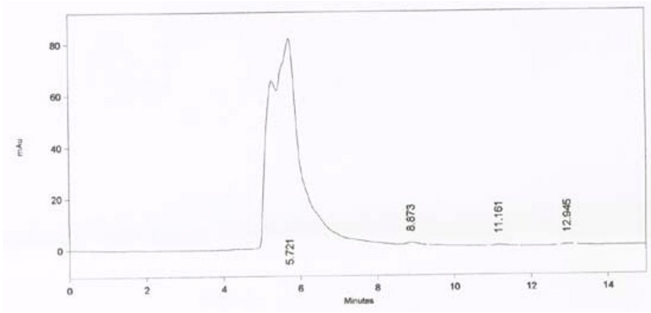

Fig. 6- HPLC analysis of extracted karanjin from Fraction- II at 300nm

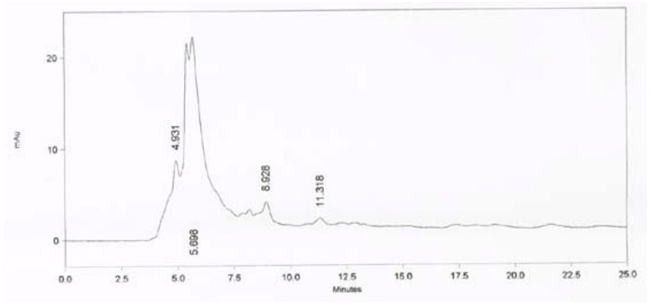

Fig. 7- HPLC analysis of extracted karanjin from Fraction III at 300nm.

Discussion

The composition of nutrient medium is an important factor for successful initiation and establishment of tissue cultures [18]. Half strength MS, Full strength MS and McCown and Lyod media employed in the present study are widely used for initiation of callus in other woody plants earlier [3,7]. Study of available literature on callus cultures of other woody plants indicated that higher concentrations of cytokinins induce callus formation in woody plants [5, 11,18]. The present experiment demonstrates that medium with lower mineral concentration supplemented with glutamine (200 mg/l) and casein hydrolysate (500 mg/l) has increased the callus induction. It is concluded that *in vitro* accumulation of karanjin an important secondary metabolite and its detection from callus cultures of *Pongamia glabra* are possible. However further investigations to enhance the content of karanjin and also screening for new compounds in callus and suspension cultures are necessary by employing specialized techniques such as elicitation, immobilization and permeabilization.

Acknowledgement

The authors are thankful to Prof. G. Srimannarayana and Prof. David Krupadanam for their help in isolation of karanjin and Head Dept. of Botany for providing the laboratory facilities.

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