IRJSE Int. Res. J. of Science & Engineering, Special Issue A8, July, 2020 SJIF Impact Factor 6.70
ISSN: 2322-0015
RESEARCH ARTICLE OPEN ACCESS

In vitro Callogenesis and L-Dopa Evaluation in *Mucuna Pruriens* L. by High Performance Liquid Chromatography.

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Manuscript Details

Available online on https://www.irjse.in
ISSN: 2322-0015

Editor: Dr. Arvind Chavhan

Cite this article as:
Simran Kotwal and Nathar VN. *In vitro* Callogenesis and L-Dopa Evaluation in *Mucuna Pruriens* L. by High Performance Liquid Chromatography, *Int. Res. Journal of Science & Engineering,* 2020, Special Issue A8, pages: 115-120.

Article published in Special issue of International e-Conference on "Sustainable Development : A Biological and Socioeconomical Perspective" organized by Government Vidarbha Institute of Science and Humanities Amravati, Maharashtra, India date, 26-27 January 2020.

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Abstract

Mucuna pruriens L. commonly called as velvet bean, cowitch, cowhage, alkushi belongs to family Fabaceae. In the current study an efficient protocol and optimum callus biomass was developed using a synergetic combination of auxins and cytokinins 2, 4-D + NAA (1.5+1.5, 1.0+1.5, 2+1 mg/l), BAP + KIN (1.5 + 1 and 2.5 + 1.5 mg/l) and synthesis of L-DOPA content in callus biomass was evaluated with the help of HPLC analysis. By the HPLC analysis, revealed the identification of active compound, L-DOPA present in the callus extract of *M. pruriens.* The quantitative amount of L-dopa in present callogenesis study was 1000ug/l in 1000 ppm of sample extract.

Keywords: *Mucuna pruriens,* Callogenesis, L-Dopa, High-Performance Liquid Chromatography analysis.

Introduction

In the discover for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites [1]. The 3-(3, 4-dihydroxyphenyl)-L-alanine (L-DOPA) is a neurotransmitter precursor. It has found a wide use for the relief of Parkinson's disease.

The genus *Mucuna* belongs to the family Fabaceae [2]. Currently the genus accounts for 137 species, subspecies or varieties [3] of climbing vines and shrubs. The name of the genus is derived from the word mucuna [4].

The genus is found all over the world in the woodlands of tropical areas especially in tropical Africa, India and the Caribbean. In India it is found in the foothills of the Himalayas, the plains of West Bengal, Madhya Pradesh, Karnataka, Kerala and Andhra Pradesh [5].

Material and Methods

Standard and reagents:
Standard L-DOPA was purchased from Himedia laboratories private limited Mumbai India. HPLC-grade Methanol and water were purchased from SD FINE Chemical Industries Ltd. (Mumbai, India).

Standard solution:
A stock standard solution (1000 g/mL) was prepared by dissolving 20 mg of standard L-DOPA in 20 ml methanol. By diluting the stock solution with methanol working standard solutions were prepared in the concentration range of 0.5–100 g/mL.

Sample:
In the present investigation the healthy wild *M. pruriens* seeds were collected during the month of January 2016 from Amravati region and were inoculated under aseptic and controlled conditions.

Plant development and germination:
After the surface sterilization of *M. pruriens* seed explants were inoculated on to Murashige and Skoog (MS) basal medium supplemented with different concentrations of 2, 4-D (0.5-3mg/l), NAA(0.5-3mg/l), BAP(0.5-3mg/l), IAA(0.5-3mg/l), IBA(0.5-3mg/l), and Kinetin (0.5-3mg/l), for germination of plants from the seeds.

Result and Discussion

Callus induction:
The aseptic plants prepared first, from where the explants were taken for callogenesis. After the date of inoculation (12-16) days shooting and rooting were observed from *in-vitro* inoculated seeds in combination of BAP + KIN (2.5 + 1.5 mg/l). The length varies from size 15 to 16cm touches the cotton plug of test tube in fact it comes out, when cotton plug was removed and root length varies from 4 to 5cm. The average shoot length varies from size 14 cm to 15 cm and root length varies from 3 to 5 cm.

Detection of L-Dopa in crude callus extracts of *Mucuna pruriens* by High Performance Liquid Chromatography.
The callus extracts were obtained from different samples like leaf, petiole, nodal, intermodal calli of *Mucuna,* that grown in MS solid medium containing different combinations of plant growth regulators. The L-Dopa content in *M. pruriens* callus was determined by HPLC analysis[3]. One gram of dried callus powder of M. pruriens was used. Methanolic extract of the callus was prepared by using Soxhlet extraction method. The extract was filtered through Sartorius RC membrane syringe filter (0.2 µm) and 20 µl of the sample was used for the HPLC analysis.

Table 1.1: Response of *in vitro* inoculated seeds to different combinations and concentrations of growth regulators.

Sr. No	Plant Growth Hormones	Concentrations mg/lit	Time taken to obtain shooting (days)	Time taken to obtain rooting (days)	Shoot/ root length in cm (average)
1	BAP + KIN	2.5 + 1.5	12 - 18	16 – 20	14/ 5
2	BAP + KIN	0.5 + 1	5 - 10	10 – 13	13/ 4
3	BAP +NAA	2 + 1	18 - 22	12 – 18	13/ 4
4	IBA	2.5	14 - 18	10 – 15	15/ 5

Table 1.2: Response of leaf explants for callus induction at different combinations and concentrations of growth regulators.

Sr. No.	Explant	PGR	Concentration mg/L	Callus (Days)	Result	Callus Morphology	
1	Leaf	2, 4-D	2.5	11 - 15	+	Colour	Texture
2	Leaf	2, 4-D + NAA	1.5 + 1.5	15 - 23	+++	Yellowish	Fragile
			1.0 + 1.5	20 - 23	++	Yellowish	Friable
			2 + 1	12 - 18	++	Yellowish	Friable
3	Leaf	BAP	1.5	14 - 18	++	Yellowish	Fragile
4	Leaf	BAP+KIN	1.5 + 1	22 - 26	++	Yellowish	Friable
			2.5 + 1.5	16 - 22	++	Yellowish	Soft

Table 1.3: Response of petiole explants for callus formation to different combinations of growth regulators used at different concentrations

Sr. No	Explant Used	Growth regulators	Concentration mg/lit	Time taken by explants To form callus (days)	Result	Colour and Morphology	
						colour	Texture
1	Nodal	2, 4-D and NAA	1.5 + 1.0	20 - 27	+	White-yellow	Soft
2	Petiole	BAP +NAA	2.0 + 1.0	20 - 25	++	greenish	Hard
3	Internodal	2, 4-D and NAA	2.5 + 1.5mg	23 - 26	++	White-yellow	Hard

(-) No callus; (+) Little amount of callus (++); Average amount of callus; (+++) Large Callus.

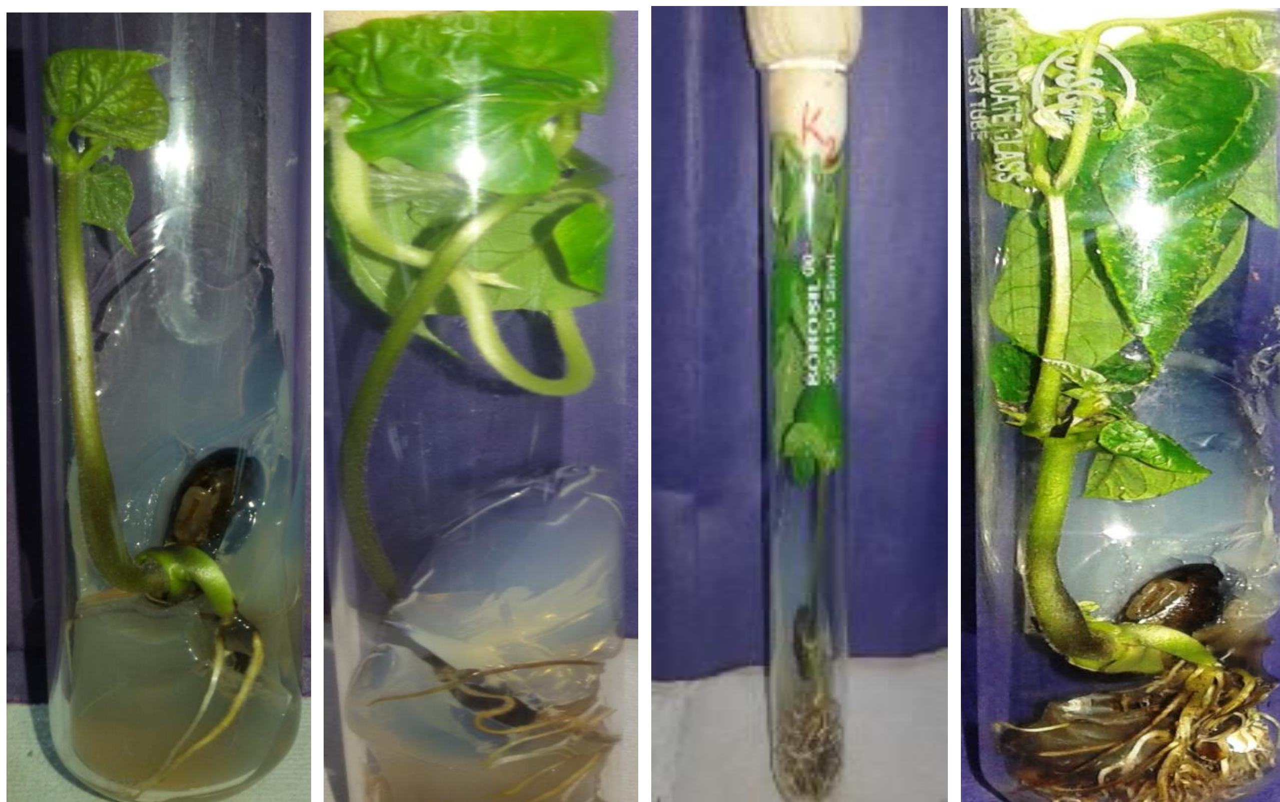

Figure 1: Initiation of shoot and root from in vitro inoculated seeds of *M. pruriens* in combination of (a) BAP + KIN (2.5 + 1.5 mg/l), (b) BAP + KIN (0.5 + 1 mg/l), (c) BAP +NAA (2 +1 mg/l), (d) IBA (2.5 mg/l).

Fig. 2. Callus obtained from Leaf explants of ***M. pruriens*** **(a) 2, 4-D (2.5 mg/lit) (b) 2, 4-D and NAA (1.5 + 1.5mg/lit.), (1.0 + 1.5mg/lit) (c) BAP (1.5mg/lit) (d) BAP+KIN, 1.5 + 1, 2.5 + 1.5.**

Fig.3. Callus obtained from Nodal plant of *M. pruriens* (a) 2, 4-D and NAA (1.5 + 1.0mg/lit) (b) Petiole BAP and NAA (2.0 + 1.0mg/lit), (c) Internodal 2, 4-D and NAA (2.5 + 1.5mg/lit).

Chromatography was performed using Shimadzu HPLC (Model SPD-10A UV-VIS Detector) and supelcosil LC-18 column (25 cm × 4.6 mm, 5µm) with mobile phase, linear gradient elution profile started with methanol: water (75:25), and ended with methanol : water (50:50). Flow rate was maintained at 1.0 mL/minute with a back pressure of 250 psi, and the compounds were read at 254 nm using a UV detector. The total run time was 10 minutes, but preferably it was extended up to 20 minutes [6]. The results were compared with the standard.

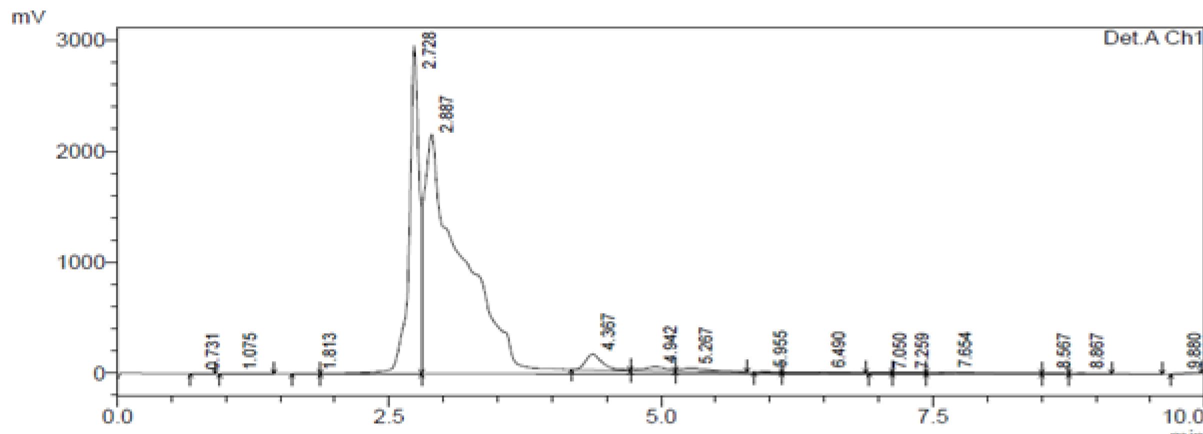

Fig: 4 HPLC chromatogram of L-Dopa in standard of *Mucuna pruriens.*

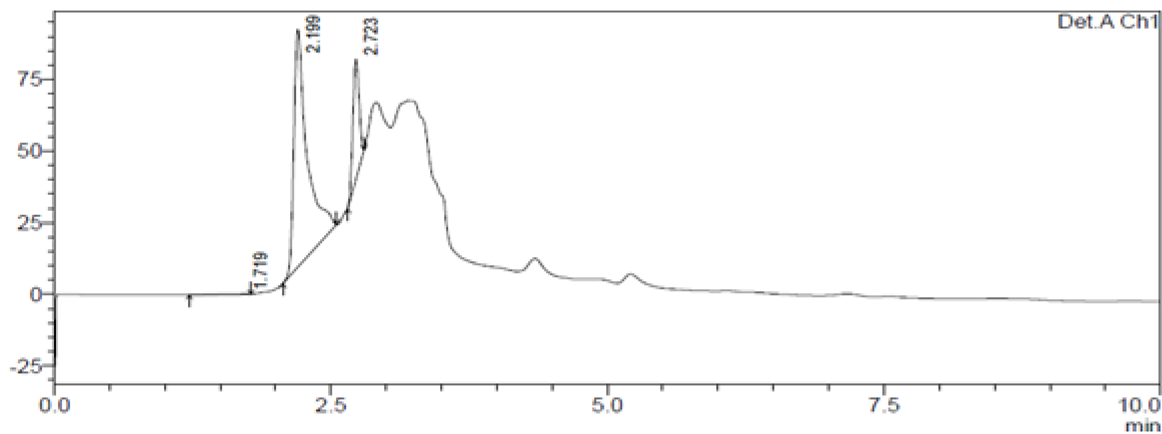

Fig: 5 HPLC chromatogram of L-Dopa *Mucuna pruriens* callus.

HPLC is a versatile and widely used technique for the isolation of natural products. It is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture [7-8].

The standard L-dopa graph shows peak at RT = 2.72. The HPLC analysis of L-Dopa compound from *M. pruriens* callus extract along with the standard L-Dopa has been represented in Fig. 4. L-Dopa compound eluted through HPLC analysis and based on standard retention time (Rt) 7 minutes. The *M. pruriens* callus extract used for HPLC analysis recorded at Rt =2.72 minutes (Callus), and standard L-Dopa compound recorded at Rt = 2.72 of 10 minutes, thus confirming the presence of L-Dopa compound in callus extract of *M. Pruriens*. In earlier, reports considerable evidences are available on the endogenous accumulation of L-Dopa in cells of *M. pruriens* grown in vitro [9-11].

Chromatographic Conditions

The following chromatographic conditions were used to quantify the L-dopa

Stationary phase: Eurosphare, C18, 254 × 4.0 mm

Column oven temperature: 30ºC

Mobile phase: methanol: 0.5% v/v.

Different concentration of the standard solution of L-Dopa of 10 – 80 µg were prepared from stock solution and used for HPLC analysis.

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The content of L- dopa in different dilution is given as:
10 ppm = 38.8ug/ml
100 ppm = 163.33ug/ml
1000 ppm = 1000ug/l

This study concludes that the quantitative amount of L-dopa in callogenesis is 1000ug/l in 1000ppm of sample extract that is three times higher than the ex-vitro plants.

Conflicts of interest: The authors stated that no conflicts of interest.

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