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Research Article

OPTIMIZATION OF BIOMASS CULTURE YIELD AND L-DOPA COMPOUND IN THE CALLUS CULTURE FROM COTYLEDONARY LEAVES OF *MUCUNA PRURIENS*

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ABSTRACT

Objective: The objective of the present study was to evaluate the optimization of callus biomass culture yield and high-performance liquid chromatography (HPLC) analysis of L-DOPA compound in the callus culture from cotyledonary leaves of *Mucuna pruriens.*

Methods: *M. pruriens* seed explants were inoculated onto Murashige and Skoog (MS) medium supplemented with different concentrations of 2-isopentenyl adenine (2iP) and Gibberellic acid (GA_3) for germination of plants. The *in vitro* cotyledonary leaf and hypocotyl explants were cultured on MS basal media containing various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), 1-Naphthaleneacetic acid (NAA), 6-Benzylaminopurine (BA), and 2iP for callus induction. A standard approach of Latin square method was followed for screening of media to establish optimum culturing of callus by manipulating the concentration of auxins (2,4-D, Indole-3-acetic acid (IAA) and NAA) and cytokinins (BA and 2iP) alone and in combinations. The harvested callus biomass was screened for a major metabolite namely L-Dopa compound using HPLC.

Results: Cotyledonary leaf explants showed better callus initiation than hypocotyl explants. Maximum callus induction was observed in MS medium containing 4.92 µM 2iP. Further screening of callus culture was carried out on MS medium supplemented with different concentrations and combinations of 2,4-D, NAA, IAA, BA, and 2iP individually and in combinations. Optimum callus biomass of 21.63 g/L dry weight (246.31 g/L fresh weight) was developed on MS media containing 2.26 µM - 2,4-D, 2.22 µM – BA, and 4.92 µM - 2iP. The harvested callus biomass was subjected to extraction and purification of L-Dopa compound.

Conclusion: The present study concludes that HPLC analysis of cell biomass extracts in comparison with extracts from seeds of mother plants of *M. pruriens* showed main component of L-Dopa was present in sufficiently large amounts in the undifferentiated cultured cells.

Keywords: *Mucuna pruriens*, Callus biomass, L-Dopa, High-performance liquid chromatography analysis.

INTRODUCTION

Mucuna pruriens (L.) DC., (Fabaceae) commonly known as velvet bean is a commercially important medicinal plant found in bushes, hedges, at damp places, ravines, and forest of Western and Eastern Ghats of India [1,2]. Traditionally, the seeds of *M. pruriens* are used as a tonic and aphrodisiac for male virility [3]. Seeds contain L-DOPA (3,4-dihydroxyphenyl alanine) content, which is a precursor of the neurotransmitter has found to be used in the treatment of Parkinson's disease and mental disorder [4].

Commercial production of L-Dopa from intact plants is hampered by the herbaceous twining habit and presence of strident trichomes on the pods, which causes a very strong itching sensation creating difficulties in large-scale cultivation of plants and harvesting their pods [5]. Large-scale extraction of L-Dopa compound from wild populations is considered, but the commercial exploitation of this process is hampered because of its limited availability.

Mucuna species possess valuable medicinal properties, and there is a heavy demand of Mucuna seeds containing L-DOPA, in markets across the world. Its demand in international markets has increased many folds in recent years [6,7]. Conventional methods of propagation of this plant are limited to seeds, which pose problems because of allergic properties that cause uncontrolled itching [8,9] and poor viability. *In vitro* cell culture techniques are one of the innovative and effective methods to produce medicinal and aromatic plants for commercial exploitation of valuable plant-derived pharmaceuticals. With the above-mentioned difficulties, callus culture has been an alternative and efficient source for the production of secondary metabolites. Hence, the present investigation was carried out for the production of L-Dopa compound from standardized callus culture of *M. pruriens.*

MATERIALS AND METHODS

Collection of plant material

The healthy wild *M. pruriens* seeds were collected during the middle of February 2013 from Kolli Hills - Namakkal District, Tamil Nadu, India and seeds were cleaned and stored.

Explant preparation

Seed explants (1-year-old mature plants) were surface sterilized by cleaning thoroughly under running tap water for 20 minutes, washed with a solution of labolene (2-3 drops in 100 ml of water) for 5 minutes, and again washed with sterile distilled water. The cleaned explants were treated with 70% ethanol for 1 minute followed by 0.1% mercuric chloride ($HgCl_2$) treatment for 5 minutes under aseptic conditions and washed six times with sterile distilled water to remove traces of $HgCl_2$.

Germination and plant development

After surface sterilization *M. pruriens* seed explants were inoculated onto Murashige and Skoog (MS) [10] basal medium supplemented with different concentrations of 2-isopentenyl adenine (2iP) (2.46–12.3 µM) and Gibberellic acid (GA_3) (1.44 µM) for germination of plants from the seeds.

Initiation of callus

The *in vitro* cotyledonary leaf and hypocotyl explants were cultured on MS basal media containing various concentrations

of 2,4-dichlorophenoxyacetic acid (2,4-D) (1.13, 2.26, 4.52, 6.78, and 9.04 µM); naphthalene acetic acid (NAA) (1.34, 2.68, 5.37, 8.05, and 10.74 µM), 6-Benzylaminopurine BA (1.11, 2.22, 4.44, 6.66, and 8.88 µM), and 2iP (1.23, 2.46, 4.92, 7.32, and 9.84 µM) for callus induction. Primary callus was established from cotyledonary leaf explants. For secondary callus production, a small portion of primary callus was excised using sterile knife holder and was sub-cultured periodically once in 3 weeks. The secondary callus was used for all the experimental studies.

A standard approach of Latin square method [11] was followed for screening of media to establish optimum culturing of callus by manipulating the concentration of auxins (2,4-D, Indole-3-acetic acid [IAA] and NAA) and cytokinins (BA and 2iP) alone and in combinations. A range of seven concentrations of auxins and cytokinins (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10 mg/L) were used in this study.

Callus growth

The growth measurement of callus was determined by standard method [12]. The growth of the callus and its biomass was measured in terms of fresh (FW g/L) and dry weight (DW g/L). FW of callus was measured after removing the excess moisture and agar adhering to the callus surface using blotting paper. DW of callus was determined by drying the callus in hot air oven at 60°C for 24 hr and was expressed in g/L DW culture.

Age of callus culture

The age of callus of *M. pruriens* was determined by standard method [13]. About 0.5 g of secondary callus culture from actively growing callus was inoculated in 250 mL Erlenmeyer flask containing 50 mL of MS solid medium supplemented with 2.26 µM - 2,4-D, 2.22 µM - BA and 4.92 µM - 2iP. The culture was incubated under 16/8 hr photoperiod at 25±1ºC. Initial weight of the callus biomass was measured in terms of FW g/L and DW g/L. Observations were made from the 9th day after incubation up to 36th day with 3 days intervals and recorded. On the 9th day, the first Erlenmeyer flask containing callus was used, and it was dried at 50ºC in the dark for 24 hr. The same procedure was adopted for the callus harvested at different days of intervals 9th, 12th, 15th, 18th, 21st, 24th, 27th, 30th, 33rd, and 36th days.

Culture medium and conditions

MS basal medium supplemented with 3% (w/v) sucrose was used for all *in vitro* culture studies. The pH of the medium was adjusted to 5.6±0.2 prior to adding 0.9% (w/v) agar and autoclaved at 121°C for 15 minutes. Cultures were maintained at 22±1°C under 16/8 hr photoperiod by cool white fluorescent tubes (50 µmol $m^{-2}s^{-2}$) with 55-60% relative humidity. The plant growth regulators were filter-sterilized using 0.2 µm filter (Minisart®, Sartorius) prior to addition to culture media.

High-performance liquid chromatography (HPLC) analysis of L-Dopa (3,4-Dihydroxy-L-phenylalanine) compound

The L-Dopa content in *M. pruriens* seed and callus were determined by HPLC analysis [14]. One gram of dried seed powder and callus of *M. pruriens* were used. Methanolic extracts of the seed and callus were prepared by using Soxhlet extraction method. The extracts were incubated at 4ºC for 24 hrs, then filtered and evaporated to dryness vacuum and re-dissolved in a small amount of the same solvent before separation using HPLC. The extract was filtered through Sartorius RC membrane syringe filter (0.2 mm) and 30 µl of the sample used for the HPLC analysis. Chromatography was performed using Shimadzu HPLC (Model SPD-10A UV-VIS Detector) and supelcosil LC-18 column (25 cm × 4.6 mm, 5 µm) 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 20 minutes, but preferably it was extended up to 40 minutes [15]. The results were compared with the standard.

RESULT AND DISCUSSION

The surface sterilized seed explants were inoculated on MS medium containing 2iP (2.46-12.3 µM) with GA_3(1.44 µM). Seed germination in most of the treatments was recorded within 15 days of culture (Fig. 1a and b). The prominent seedling developed on MS basal medium supplemented with 4.92 µM 2iP+1.44 µMGA_3 showed significant growth response of 61.6±2.8% germination with an average shoot length of 5.43±0.05 cm and an average root length of 3.76±0.25 cm (Table 1) and healthy seedlings were developed after 40 days of culture (Fig. 1c). The *in vitro* seedlings were used for further experimental studies.

The growth of callus development varied from cotyledonary leaf and hypocotyl explants. Explants inoculated on MS medium supplemented with an individual concentration of 2,4-D, NAA, BA, and 2iP. Cotyledonary leaf explants inoculated on MS medium containing 4.92 µM 2iP was noticed to be significantly higher than hypocotyl explants (Table 2 and Fig. 1c and d). Callus produced from cotyledonary leaf explants were friable, creamy or light green in color. The above findings related to the nature of the callus were confirmed with the reports of *Ricinus communis* [16] and *Cyclea peltata* [17]. These findings were seen to be in harmony with those reported in *Stevia rebaudiana* [18] and also, in accordance with the reports of *Cardiospermum halicacabum* [19],

Table 1: *In vitro* seedlings developed from seeds of *Mucuna pruriens*

Plant growth regulators (µM)		Germination response (%)	Mean shoot length (cm)±SD	Mean root length (cm)±SD
2iP	GA_3			
2.46	1.44	33.3±5.7	3.56±0.20	2.03±0.15
4.92	1.44	61.6±2.8	5.43±0.05	3.76±0.25
7.38	1.44	43.3±2.8	4.36±0.50	2.43±0.11
9.84	1.44	38.3±5.7	3.33±0.15	2.10±0.52
12.3	1.44	30.0±5.0	2.90±0.10	1.86±0.11

Data were recorded after 40 days of culture. Results represent mean±SD of six replicated experiments. GA_3: Gibberellic acid, 2iP: 2-isopentenyl adenine

Table 2: Effect of plant growth regulators (2,4-D, NAA, BA, 2iP) on callus induction from different explants of *Mucuna pruriens*

PGR concentration (µM)				Cotyledonary leaf response (%)	Hypocotyl response (%)
2,4-D	NAA	BA	2iP		
1.13	-	-	-	20.00±0.00	11.67±0.37
2.26	-	-	-	36.60±0.53	21.67±0.33
4.52	-	-	-	34.33±0.46	18.33±0.88
6.78	-	-	-	19.33±0.85	11.67±0.43
9.04	-	-	-	-	-
-	1.34	-	-	-	-
-	2.68	-	-	20.00±0.00	15.05±0.57
-	5.37	-	-	18.33±0.88	26.67±0.48
-	8.05	-	-	16.67±0.82	18.34±0.96
-	10.74	-	-	-	-
-	-	1.11	-	10.00±0.00	25.00±0.58
-	-	2.22	-	25.00±0.58	15.00±0.58
-	-	4.44	-	-	-
-	-	6.66	-	-	-
-	-	8.88	-	-	-
-	-	-	1.23	26.67±0.78	35.00±0.58
-	-	-	2.46	30.00±1.15	42.33±0.57
-	-	-	4.92	60.00±0.00	38.33±0.51
-	-	-	7.32	23.33±0.64	30.06±0.31
-	-	-	9.84	16.67±0.92	25.00±0.58

Data were recorded after 40 days of culture. Results represent mean±SD of six replicated experiments. 2,4-D: 2,4-dichlorophenoxyacetic acid, NAA: 1-Naphthaleneacetic acid, BA: 6-Benzyl adenine, 2iP: 2-isopentenyl adenine, PGR: Plant growth regulators

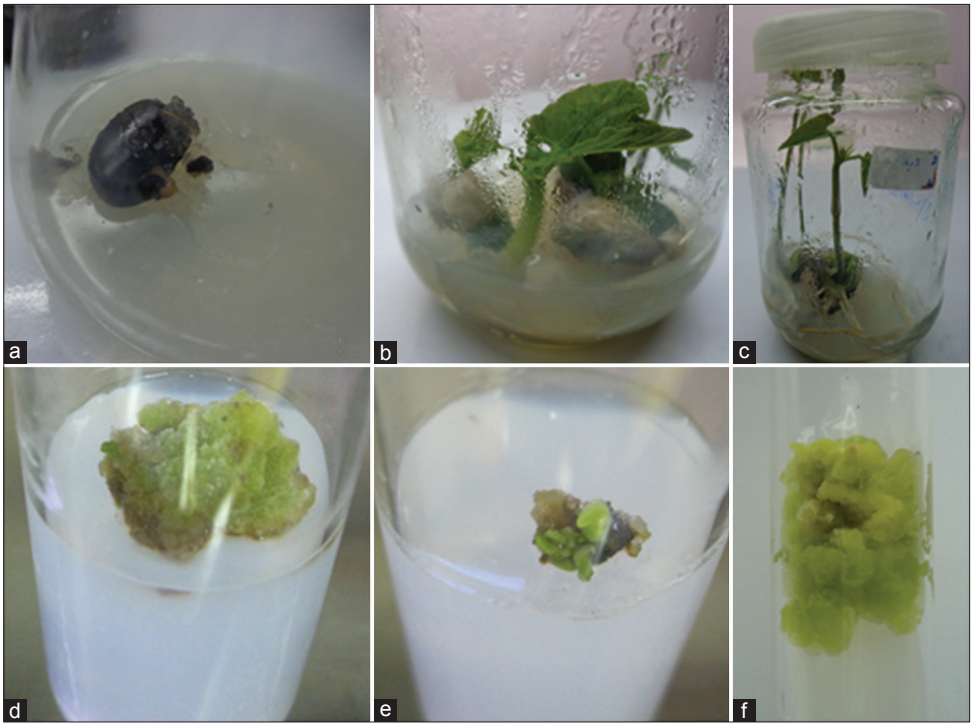

Fig. 1: *In vitro* germinated *s*eedling developed and callus induction from *Mucuna pruriens.* (a) Seeds inoculated on MS medium supplemented with 2-isopentenyl adenine 4.92 µM and Gibberellic acid 1.44 µM. (b) *In vitro* seedlings developed from *Mucuna pruriens* seeds. (c) Cotyledonary leaf and hypocotyl developed from *M. pruriens* seeds. (d) Callus initiation from cotyledonary leaf explants of *M. pruriens*. (e) Callus initiation from hypocotyl explants of *M. pruriens*. (f) Secondary callus developed from cotyledonary leaf explants of *M. pruriens*

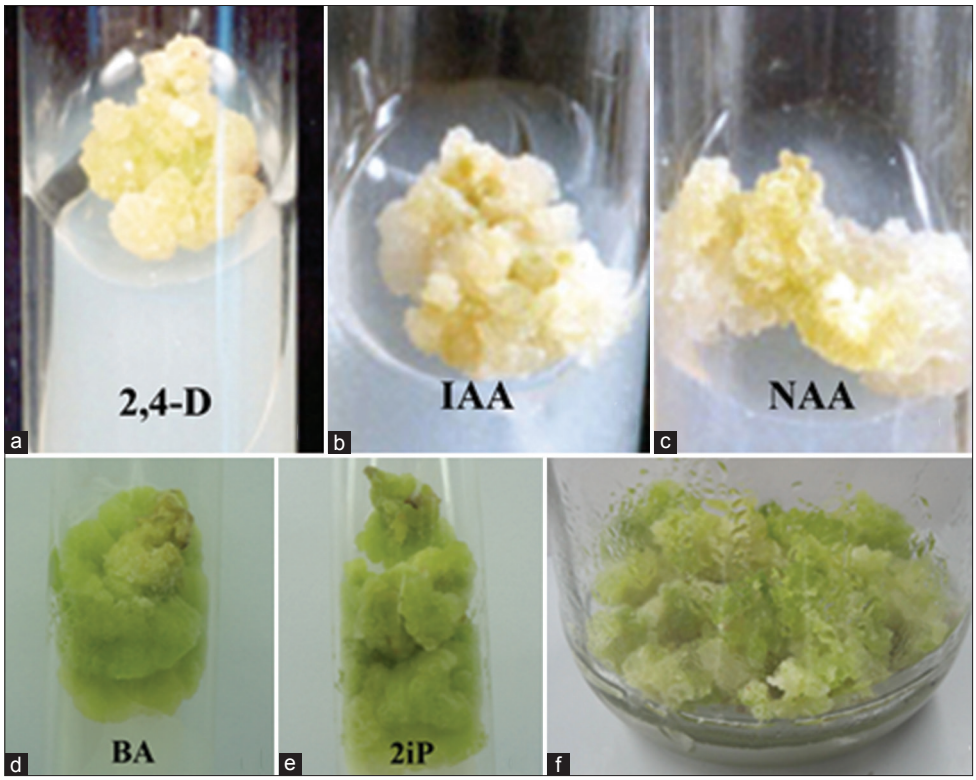

Fig. 2: Individual effect of auxins and cytokinin on callus culture of *Mucuna pruriens*. (a) Murashige and Skoog (MS) basal medium with 2,4-dichlorophenoxyacetic acid (2,4-D) (2.26 µM). (b) MS basal medium with Indole-3-acetic acid (5.71 µM). (c) MS basal medium with 1-Naphthaleneacetic acid (2.69 µM). (d) MS basal medium with 6-Benzylaminopurine (BA) (2.22 µM). (e) MS basal medium with 2-isopentenyl adenine (2iP) (4.92 µM). (f) Optimum callus biomass developed on MS medium 2,4-D (2.26 µM), BA (2.22 µM) and 2iP (4.92 µM)

in which callus induction was highest (90%) in the leaf explants of *C. halicacabum* on MS medium supplemented with 2,4-D. Calli were transferred to MS medium supplemented with different concentrations and combination of auxins and cytokinins. In auxins maximum growth

of callus were obtained in MS medium supplemented with 2,4-D 2.26 µM followed by IAA at 5.71 µM, NAA at 2.69 µM (Table 3) whereas in cytokinins 4.92 µM 2iP was followed by 2.22 µM BA (Table 4 and Fig. 2 a-e). Callus grown on medium supplemented with 2,4-D and IAA was pale, yellowish. Callus grown on medium supplemented with BA and 2iP was green in color, more compact, hard, and granular. The maximum callus growth was found with cytokinins such as 2iP and BA and auxin (2,4D). Maximum callus growth using auxins and cytokinins has been reported in *G. Sylvestre* [20,21], *Eurycoma longifolia* [22], and *Rosa bourboniana* [13].

A total of 79 combinations of auxin and cytokinins were tried for optimum callus biomass production. The hormone combination for optimum callus biomass production was standardized, and the callus biomass was 246.31 g/L FW and 21.63 g/L DW in MS solid medium supplemented with 2.26 µM - 2,4-D, 2.22 µM – BA, and 4.92 µM - 2iP after 24 days of culture (Tables 5 and 6; Fig. 2f). The 24th day callus (solid medium) extract was used for HPLC analysis. The HPLC analysis

Table 3: Individual effect of auxins on callus culture of *Mucuna pruriens*

PGR	Concentration (µM)	FW (g/L)	DW (g/L)
2,4-D	0.45	34.17±0.96	2.56±1.27
	1.13	39.11±1.63	3.53±0.79
	2.26	59.74±0.90	4.73±0.88
	4.52	54.66±1.00	4.61±0.73
	11.31	51.28±1.33	3.18±1.06
	22.62	42.56±1.53	2.82±0.99
	45.25	31.51±1.11	2.31±0.90
IAA	0.57	34.64±1.06	2.51±0.74
	1.43	36.52±0.89	3.45±0.73
	2.85	43.26±1.22	2.63±0.76
	5.71	53.59±2.23	3.68±1.10
	14.27	42.37±1.47	3.62±0.73
	28.54	35.55±0.86	2.79±1.10
	57.08	32.86±1.59	2.26±0.89
NAA	0.54	25.57±0.96	2.24±0.92
	1.34	31.15±0.85	3.37±0.84
	2.69	45.43±1.29	4.28±1.04
	5.37	35.20±0.95	2.25±0.99
	13.43	22.28±0.76	2.26±0.79
	26.85	20.27±0.89	2.53±0.60
	53.71	19.34±0.76	1.83±0.86

Data were recorded after 40 days of culture. Results represent mean±SD of six replicated experiments. FW: Fresh weight, DW: Dry weight, PGR: Plant growth regulators, 2,4-D: 2,4-dichlorophenoxyacetic acid, NAA: 1-Naphthaleneacetic acid, IAA: Indole-3-acetic acid

Table 4: Individual effect of cytokinins on callus culture of *Mucuna pruriens*

PGR	Concentration (µM)	FW (g/L)	DW (g/L)
BA	0.44	21.33±1.13	2.50±1.37
	1.11	38.54±1.09	2.58±0.89
	2.22	62.37±0.67	4.83±0.60
	4.44	43.35±1.52	3.50±0.64
	11.1	41.97±1.11	2.18±1.02
	22.2	33.24±0.93	2.27±0.80
	44.2	30.28±1.20	1.85±1.02
2iP	0.615	21.51±1.21	4.24±1.15
	1.23	35.78±1.08	5.44±0.70
	2.46	37.41±0.84	4.43±1.39
	4.92	68.62±1.23	5.87±1.15
	12.3	46.60±1.40	3.31±0.81
	24.6	42.47±2.16	2.67±1.23
	49.2	35.54±1.06	2.81±1.06

Data were recorded after 40 days of culture. Results represent mean±SD of six replicated experiments. FW: Fresh weight, DW: Dry weight, BA: 6-Benzyl adenine, 2iP: 2-isopentenyl adenine, PGR: Plant growth regulators

of L-Dopa compound from *M. pruriens* seed and callus extract along with the standard L-Dopa has been represented in Fig. 3. L-Dopa compound eluted through HPLC analysis and based on standard retention time (Rt) 3.27 minutes. The *M. pruriens* seed and callus extract (Kolli Hills accession) used for HPLC analysis recorded a Rt of 3.31 minutes (leaf), and Rt of 3.36 minutes (Callus), and standard L-Dopa compound recorded a Rt of 3.27 minutes, thus confirming the presence of L-Dopa compound in seed and callus extract of *M. Pruriens* (Fig. 3a-c). In earlier, reports considerable evidences are available on the endogenous accumulation of L-Dopa in cells of *M. pruriens* grown *in vitro* [23-25].

CONCLUSION

In conclusion, a cell culture methodology for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds. The increased use of plant cell culture systems in recent years is perhaps due to an improved understanding of the secondary metabolite pathway in economically important plants. The present study developed an efficient and optimum callus biomass using a synergetic combination of auxins and cytokinins and synthesis of L-DOPA content in callus biomass. The HPLC analysis revealed the identification of active compound namely L-DOPA present in the callus extract of *M. pruriens.* Further studies

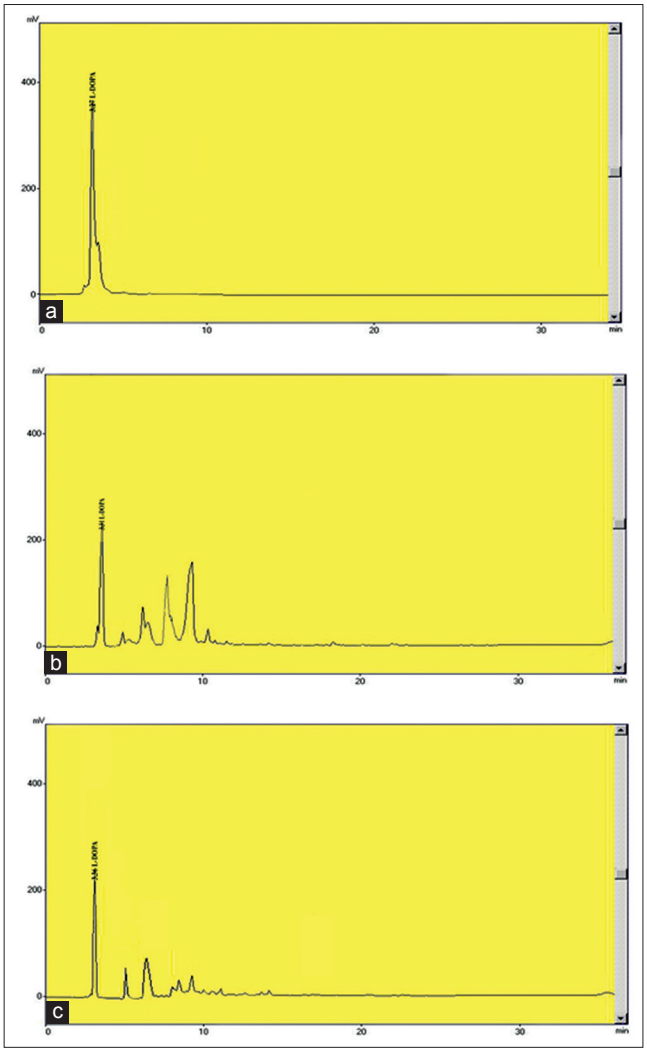

Fig. 3: High-performance liquid chromatography analysis of L-DOPA compound from *Mucuna pruriens* seed and callus extract (a) L-DOPA standard (1 mg/1 mL) - Sigma-Aldrich. (b) The seed extract of *M. pruriens.* (c) Callus extract of *M. pruriens*

Table 5: Standardization of plant growth regulators for callus culture of *Mucuna pruriens*

PGR concentration (µM)									
2,4-D	**2iP**	**BA**	**FW (g/L)**	**DW (g/L)**	**2,4-D**	**2iP**	**BA**	**FW (g/L)**	**DW (g/L)**
2.26	4.92	2.22	246.31±2.0	21.63±1.4	4.52	4.92	2.22	162.42±2.0	13.45±0.8
2.26	4.92	4.44	173.28±1.3	16.59±0.7	4.52	4.92	4.44	176.33±1.5	14.50±0.8
2.26	12.3	2.22	161.67±1.5	13.62±0.7	4.52	12.3	2.22	184.00±1.0	15.53±1.1
2.26	12.3	4.44	154.67±1.5	15.30±1.3	4.52	12.3	4.44	156.33±2.5	14.50±1.0

Data were recorded after 40 days of culture. Results represent mean±SD of six replicated experiments. FW: Fresh weight, DW: Dry weight, BA: 6-Benzyl adenine, 2iP: 2-isopentenyl adenine, PGR: Plant growth regulators, 2,4-D: 2,4-dichlorophenoxyacetic acid

Table 6: Callus biomass in solid medium at different age culture of *Mucuna pruriens*

Incubation (days)	Callus biomass (solid medium)	
	FW (g/L)	**DW (g/L)**
9	40.3±1.5	3.0±0.1
12	51.7±1.2	3.2±0.1
15	90.0±1.0	8.1±0.7
18	161.3±3.2	14.4±0.2
21	170.0±2.0	15.82±0.3
24	246.3±2.0	21.63±1.4
27	166.7±3.1	16.2±0.2
30	161.3±1.5	13.8±0.5
33	148.4±2.5	13.2±0.2
36	146.2±5.1	12.4±0.2

Data were recorded after 40 days of culture. Results represent mean±SD of six replicated experiments. FW: Fresh weight, DW: Dry weight

will be directed toward large scale production, testing the efficacy of secondary metabolites through animal cell lines and exploring market potential.

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