WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 16, 765-776. Research Article ISSN 2277– 7105

OPTIMIZATION OF CALLUS BIOMASS YIELD AND EXTRACTION OF ACTIVE COMPOUND (PHYLLANTHIN) FROM LEAF AND CALLUS EXTRACT OF PHYLLANTHUS VIRGATUS G. FORST

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Article Received on
04 October 2017,
Revised on 25 October 2017,
Accepted on 15 Nov. 2017
DOI: 10.20959/wjpr201716-10212

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ABSTRACT

The objective of the present study was to evaluate the optimization of callus biomass yield and high-performance liquid chromatography (HPLC) analysis of phyllanthin compound in the leaf and callus culture extract of *Phyllanthus virgatus.* Callus cultures were established from nodal and leaf explants. Leaf explants showed better callus initiation than nodal explants. Maximum callus induction was observed in Murashige and Skoog (MS) medium containing 4.52µM 2,4-D. 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 KN individually and in combinations. Optimum callus biomass of 14.90 ± 0.30 g/L dry weight (163.4 ± 0.20g/L fresh weight) was developed on MS media containing 4.52µM 2, 4-D, 4.65µM KN and 1.11µM BA. The harvested callus biomass was subjected to extraction and purification of phyllanthin compound. In this study, cell biomass extracts were compared with extracts from leaves of mother plants of *Phyllanthus virgatus*. HPLC analysis of these extracts showed that the main components of the active principles namely phyllanthin were present in sufficiently large amounts in the undifferentiated cultured cells.

KEYWORDS: *Phyllanthus virgatus*, Callus biomass, phyllanthin, HPLC analysis.

INTRODUCTION

Medicinal plants are the most exclusive source of life-saving drugs for majority of the world's population. The utilization of plant cells for the production of natural or recombinant compounds of commercial interest has gained increasing attention over past decades.[1] The secondary metabolites are known to play a major role in the adaptation of plants to their environment and also represent an important source of pharmaceuticals.[2]

Plants are the tremendous source for the discovery of new products with medicinal importance in drug development. Today several distinct chemicals derived from plants are important drugs, which are currently used in one or more countries in the world. Secondary metabolites are economically important as drugs, flavor and fragrances, dye and pigments, pesticides, and food additives. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology. Plant cell and tissue culture technologies can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots, and meristems for both the ways for multiplication and extraction of secondary metabolites.[3]

In the last few decades, plants belong to the genus *Phyllanthus* (Euphorbiaceae) came in focus due to their wide distribution, diversity in the genus, broad therapeutic potential and variety in their secondary metabolites. Substantial amount of the genus are used widely in traditional medicine for the treatment of flu, dropsy, diabetes, jaundice, gall bladder calculus, liver disease.[4] *Phyllanthus virgatus* is rich in polyphenols and is known traditionally for its antioxidant.[5] Antimicrobial, antiseptic, anti-inflammatory agent, anticancer activity and antidiabetic properties of various *Phyllanthus* species have been investigated in experimental models.[6]

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 phyllanthin compound from standardised callus culture of *Phyllanthus virgatus*.

MATERIALS AND METHODS

Collection of plant material

The healthy wild *Phyllanthus virgatus* plants (figure 1a) were collected during the middle of October 2015 from Chengalpattu, Tamilnadu, India and were raised in pots containing soil and farm yard manure (1:1) under greenhouse conditions at PG & Research Department of Botany, Presidency College, Chennai and heathy leaves and nodal explants used for further experimental studies.

Explant Preparation

Leaf and nodal explants (nine month old mature plants) were surface sterilized by cleaning thoroughly under running tap water for 20 min, washed with a solution of labolene (2-3 drops in 100 ml of water) for 5 min, and again washed with sterile distilled water. The cleaned explants were then treated with 70% ethanol for 1 min followed by 0.1% mercuric chloride ($HgCl_2$) treatment for 5 min under aseptic conditions and washed six times with sterile distilled water to remove traces of $HgCl_2$.[7]

Initiation of callus

The *in vitro* leaf and nodal explants were cultured on MS basal media containing various concentrations of 2, 4-D (2.26, 4.52, 6.78 and 9.04 µM); NAA (1.342, 2.68, 5.37, and 8.05 µM) and BA (1.10, 2.22, 4.44 and 8.88µM) 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 three weeks. The secondary callus was used for all the experimental studies.

A standard approach of Latin square method (Collin and Edwards)[8] was followed for screening of media to establish optimum culturing of callus by manipulating the concentration of auxins (2, 4-D, IAA and NAA) and cytokinins (BA and KN) 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.[9] Growth of the callus and its biomass was measured in terms of fresh (FW g/L) and dry weight (DW g/L). Fresh weight of callus was measured after removing the excess moisture and agar adhering to

the callus surface using blotting paper. Dry weight of callus was determined by drying the callus in hot air oven at 60º C for 24 h and was expressed in g/L DW culture.

Age of callus culture

The age of callus of *Phyllanthus virgatus* was determined by standard method.[10] About 0.5gm of secondary callus culture from actively growing callus was inoculated in 250mL Erlenmeyer flask containing 50 ml of MS solid medium supplemented with 24.52µM 2, 4-D, 1.11µM BA and 4.65µM KN. The culture was incubated under 16/8h photoperiod at 25±1ºC. Initial weight of the callus biomass was measured in terms of fresh (FW g/L) and dry weight (DW g/L). Observations were made from the 9th day after incubation up to 36th day with three days intervals and recorded. On the 9th day the first Erlenmeyer flask containing callus was used and it was dried at 50^{o}C in the dark for 24 h. 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 min. Cultures were maintained at 22 ± 1°C under 16/8h photoperiod by cool white fluorescent tubes (50µmol $m^{-2}s^{-2}$) 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.

HPLC analysis of phyllanthin **compound**

The phyllanthin content in *Phyllanthus virgatus* leaf and callus were determined by HPLC analysis. One gram of dried leaf powder and callus of *Phyllanthus virgatus* were used. Ethanolic extracts of the leaf 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 (0.2mm) and 30µl 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, 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 210 nm using a UV detector. The total run time was 20 minutes, but preferably it was extended up to 40 minutes.[11] The results were compared with standard.

RESULT AND DISCUSSION

The growth of callus development varied from leaf and nodal explants. Explants inoculated on MS medium supplemented with individual concentration of 2, 4-D, NAA and BA. Leaf explants inoculated on MS medium containing 452µM 2.4-D was noticed to be significantly higher than nodal explants (Table 1; Fig 1b, c). Callus produced from leaf explants were friable, creamy and light green in colour. The above findings related to the nature of the callus was confirmed with the reports of *Falcaria vulgaris*[12] and *Cornukaempferia aurantiflora*.[13] These findings were seen to be in harmony with those reported in *Stevia rebaudiana*[14] and also, in accordance with the reports of *Cardiospermum halicacabum*[15], 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 was obtained in MS medium supplemented with 2,4-D 4.52µM followed by IAA at 5.71µM, NAA at 2.69µM (Table 2; Fig. 2 a, b, c) whereas in cytokinins 1.11µM BAP was followed by 4.65µM KN(Table 3; Fig. 2 d, e). Callus grown on medium supplemented with 2, 4-D and IAA was pale in colour. Callus grown on medium supplemented with BA and KN was green in colour, hard and granular. The maximum callus growth was found with cytokinins such as KN and BA and auxin (2,4D). Maximum callus growth using auxins and cytokinins has been reported in *G. Sylvestre*[16], *Eurycoma longifolia*[17] and *Rosa bourboniana*.[18]

A total of 58 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 163.4 g/L fresh weight and 14.91 g/L dry weight in MS solid medium supplemented with 4.52 µM2, 4-D, 4.65µM KN and 1.11 µM BA after 21st days of culture (Table.4,5; Fig. 3c). The 24th day callus (Solid medium) extract was used for HPLC analysis. The HPLC analysis of phyllanthin compound from *Phyllanthus virgatus* leaf and callus extract along with the standard phyllanthin has been represented in Fig. 4. Phyllanthin compound eluted through HPLC analysis and based on standard retention time (Rt) 13.67min. The *Phyllanthus virgatus* leaf and callus extract (Chengalpattu accession)

used for HPLC analysis recorded a Rt of 13.73 minutes (leaf) and Rt of 13.79 minutes (Callus) and standard phyllanthin compound recorded a Rt of 13.67 minutes, thus confirming the presence of phyllanthin compound in leaf and callus extract of *Phyllanthus virgatus* (Fig. 4a,b,c). In earlier reports considerable evidences are available on the endogenous accumulation of phyllanthin in cells of *Phyllanthus virgatus* grown *in vitro.*[19-20]

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 synergetic combination of auxins and cytokinins and synthesis of phyllanthin content in callus biomass. The HPLC analysis revealed the identification of active compound namely phyllanthin present in the callus extract of *Phyllanthus virgatus.* Further studies will be directed towards large scale production, testing the efficacy of secondary metabolites through animal cell lines and exploring market potential.

Table 1: Effect of plant growth regulators (2, 4-D, NAA, BA, 2iP) on callus induction from different explants of *Phyllanthus virgatus.*

PGR Concentration (µM)			Leaf explant	Nodal explant
2,4-D	**NAA**	**BA**		
0.45	-	-	29.97 ± 0.15	20.00 ± 0.30
2.26	-	-	40.00 ± 0.10	20.00 ± 0.20
4.52	-	-	**70.00 ± 0.30**	20.00 ± 0.40
9.04	-	-	55.00 ± 0.20	30.07 ± 0.50
-	1.34	-	25.00 ± 0.10	30.00 ± 0.50
-	2.68	-	30.00 ± 0.20	40.00 ± 0.50
-	5.37	-	40.00 ± 0.30	25.00 ± 0.10
-	8.05	-	25.00 ± 0.30	20.00 ± 0.30
-	-	1.11	35.00 ± 0.20	10.00 ± 0.30
-	-	2.22	25.00 ± 0.20	15.00 ± 0.20
-	-	4.44	20.00 ± 0.40	10.00 ± 0.20
-	-	8.88	20.00 ± 0.20	10.00 ± 0.40

Data were recorded after 40 days of culture. Results represent mean ± SD of six replicated experiments.

Table 2: Individual effect of auxins on callus culture of *Phyllanthus virgatus.*

PGR	Concentration (µM)	FW(g/L)	DW(g/L)
2,4-D	0.45	14.16 ± 0.25	1.55 ± 0. 25
	1.13	29.10 ± 0.10	2.54 ± 0. 15
	2.26	39.75 ± 0.25	2.74 ± 0. 25
	4.52	**44.65 ± 0.25**	**3.18 ± 0. 91**
	11.31	31.26 ± 0.25	2.19 ± 0. 30
	22.62	22.55 ± 0.25	1.84 ± 0. 25
	45.25	11.54 ± 0.15	0.90 ± 0. 20
IAA	0.57	24.65 ± 0. 15	1.50 ± 0. 20
	1.43	26.54 ± 0. 25	1.45 ± 0. 15
	2.85	33.25 ± 0. 25	1.51 ± 0. 12
	5.71	**39.60 ± 0. 20**	**2.99 ± 0. 40**
	14.27	22.39 ± 0. 30	2.67 ± 0. 20
	28.54	15.55 ± 0. 15	0.76 ± 0. 15
	57.08	12.85 ± 0. 25	0.75 ± 0. 25
NAA	0.54	15.59 ± 0. 20	2.25 ± 0.15
	1.34	21.15 ± 0. 15	1.35 ± 0.25
	2.69	**35.44 ± 0. 25**	**2.29 ± 0.30**
	5.37	38.20 ± 0. 20	2.95 ± 0.25
	13.43	12.29 ± 0. 20	0.75 ± 0.25
	26.85	10.96 ± 0. 25	0.65 ± 0.15
	53.71	10.35 ± 0. 25	0.64 ± 0.25

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

Table 3: Individual effect of cytokinins on callus culture of *Phyllanthus virgatus.*

PGR	Concentration (µM)	FW(g/L)	DW(g/L)
BA	0.44	11.34 ± 0. 25	2.50 ± 0.10
	1.11	48.55 ± 0. 15	3.59 ± 0.30
	2.22	**32.36 ± 0. 35**	**2.84 ± 0.25**
	4.44	23.35 ± 0. 25	2.50 ± 0.20
	11.1	21.99 ± 0. 30	2.20 ± 0.20
	22.2	13.25 ± 0. 25	1.26 ± 0.15
	44.2	10.29 ± 0. 20	0.85 ± 0.25
KN	0.58	11.50 ± 0.20	0.45 ± 0.15
	1.16	15.70 ± 0.15	0.65 ± 0.15
	2.32	17.40 ± 0.20	1.04 ± 0.15
	4.65	**39.69 ± 0.15**	**2.66 ± 0.25**
	11.62	26.60 ± 0.20	1.30 ± 0.10
	22.00	22.49 ± 0.40	1.99 ± 0.20
	46.5	15.55 ± 0.25	0.80 ± 0.10

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

Table 4: Standardization of plant growth regulators for callus culture of *Phyllanthus virgatus.*

PGR Concentration (µM)									
2,4-D	**NAA**	**BA**	**FW(g/L)**	**DW(g/L)**	**2,4-D**	**KN**	**BA**	**FW(g/L)**	**DW(g/L)**
4.52	2.69	1.11	73.44±0.35	6.35±0.35	**4.52**	**4.65**	**1.11**	**163.4±0.30**	**14.9±0.20**
4.52	2.69	2.22	62.74±0.15	4.74±0.25	4.52	4.65	2.22	132.4±0.10	9.30±0.30
4.52	5.37	1.11	83.23±0.25	5.30±0.30	4.52	11.62	1.11	117.6±0.10	8.30±0.10
4.52	5.37	2.22	93.05±0.25	7.36±0.20	4.52	11.62	2.22	93.4±0.20	7.60±0.20

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

Table 5: Callus biomass in solid medium at different age culture of *Phyllanthus virgatus*

Incubation (Days)	Callus Biomass (Solid medium)	
	FW (g/L)	**DW (g/L)**
9	86.10 ±0.30	5.00 ± 0.20
12	93.40 ±0.20	7.30 ± 0.20
15	129.30 ± 0.30	11.75 ± 0.25
18	145.70 ± 0.10	13.25 ± 0.25
21	**163.40 ± 0.20**	**14.90 ± 0.30**
24	151.07 ± 0.12	12.75 ± 0.35
27	146.90 ± 0.30	11.50 ± 0.40
30	143.10 ± 0.30	10.84 ± 0.35
33	139.50 ± 0.30	9.35 ± 0.25
36	136.70 ± 0.20	9.11 ± 0.20

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

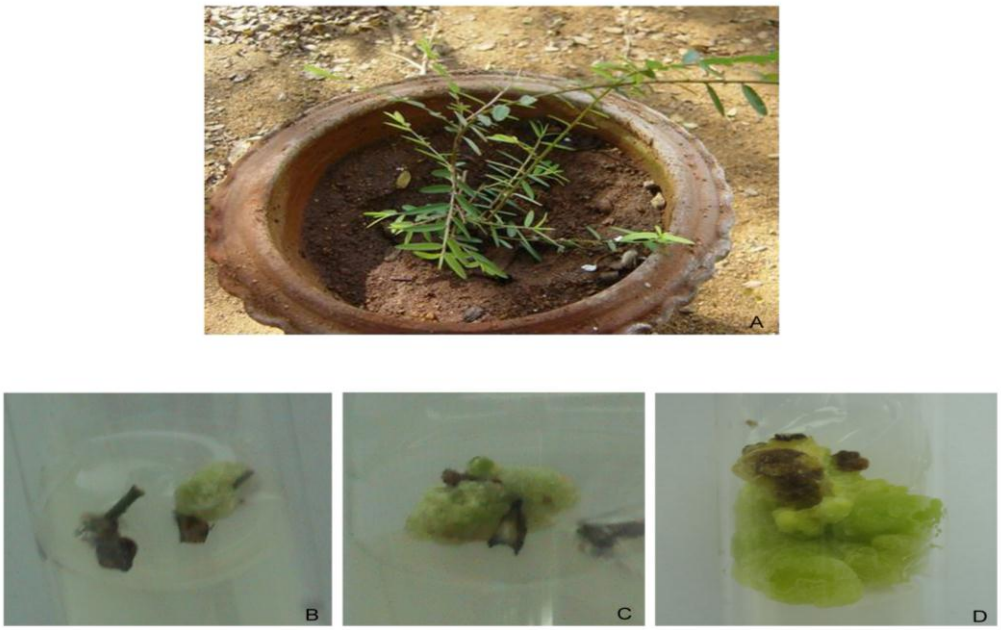

Fig. 1: Mother plant of *Phyllanthus virgatus.*

(a) Mother plant of *Phyllanthus virgatus* collected from Chengalpattu, (b) Callus initiation from nodal explants of *Phyllanthus virgatus*, (c) Callus initiation from leaf explants of *Phyllanthus virgatus*, (d) Secondary callus developed from leaf explants of *Phyllanthus virgatus*.

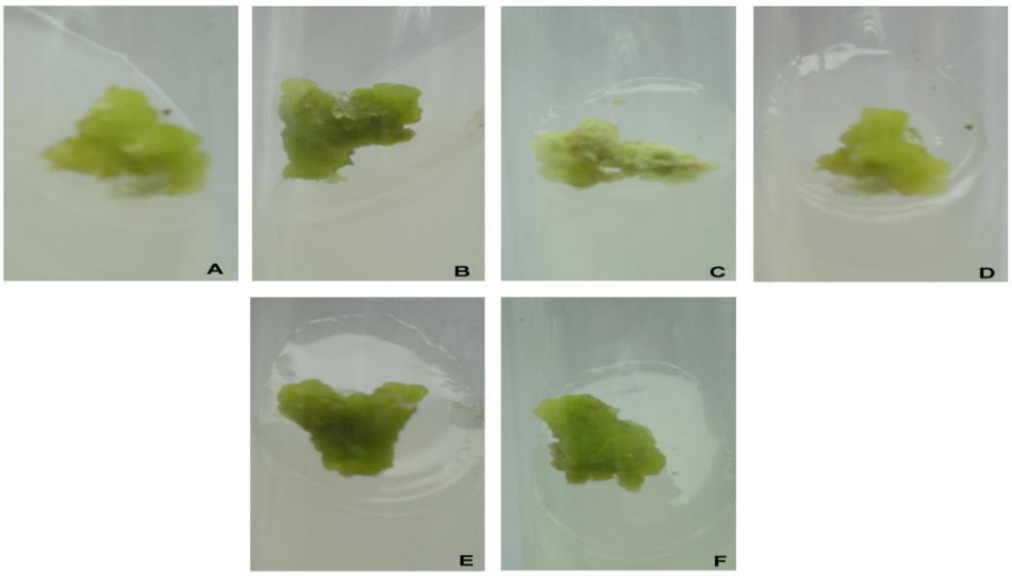

Fig. 2: Individual effect of auxins and cytokinin on callus culture of *Phyllanthus virgatus*.

(a) Murashige and Skoog (MS) basal medium with 2,4-dichlorophenoxyacetic acid (2,4-D) (4.52 µM). (b) MS basal medium with Indole-3-acetic acid (IAA) (5.71 µM). (c) MS basal medium with 1-Naphthaleneacetic acid (NAA) (2.69 µM). (d) MS basal medium with 6-Benzyladenine (BA) (2.22 µM). (e) MS basal medium with Kinetin (KN) (4.65 µM).

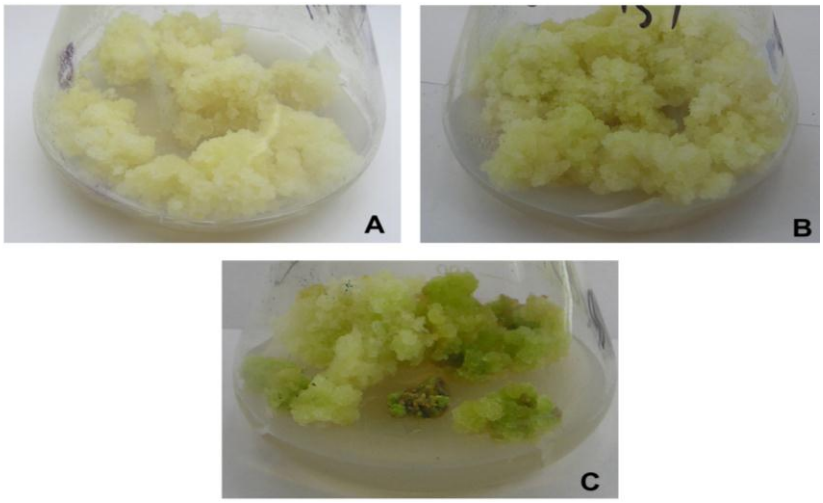

Fig. 3: Standardization of plant growth regulators for callus culture of *Phyllanthus virgatus*.

(a) Optimum callus biomass developed on MS medium 2,4-D (4.52 µM), KN (11.62 µM) and BA (1.11 µM), (b) Optimum callus biomass developed on MS medium 2,4-D (4.52 µM), KN (4.65 µM) and BA (2.22 µM), (c) Optimum callus biomass developed on MS medium 2,4-D (4.52 µM), KN (4.65 µM) and BA (1.11 µM).

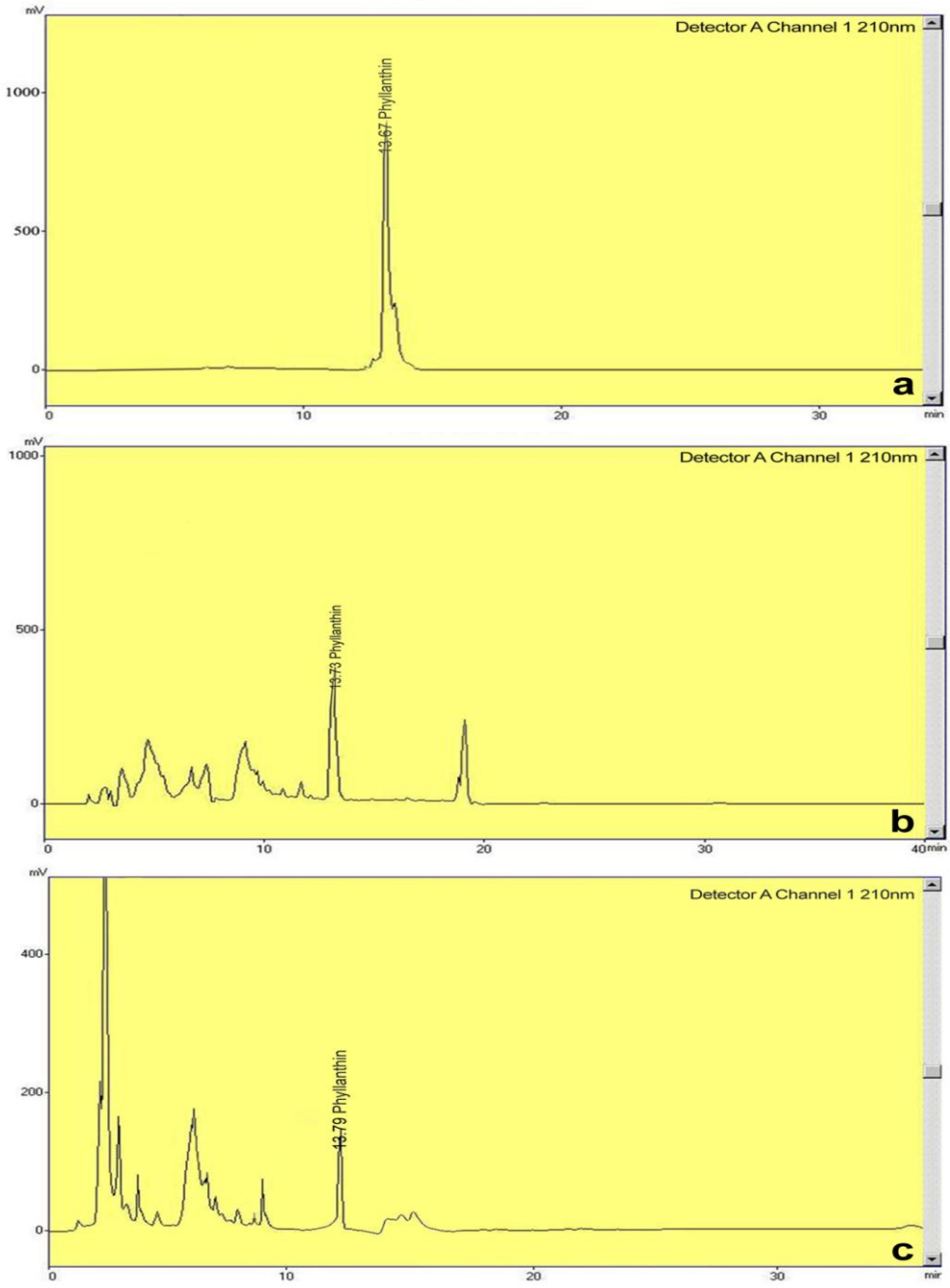

Fig. 4: High-performance liquid chromatography analysis of Phyllanthin compound from *Phyllanthus virgatus* leaf and callus extract.

(a) Phyllanthin standard (1 mg/1 mL) - Sigma-Aldrich. (b) The leaf extract of *Phyllanthus virgatus*. (c) Callus extract of *Phyllanthus virgatus.*

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