Indian Journal of Experimental Biology
Vol. 63, November 2025, pp. 949-958
DOI: 10.56042/ijeb.v63i11.16987

Enhanced production of secondary metabolites by *in vitro* cell culture of *Adansonia digitata* (L.) using low-cost options

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Received 06 February 2025; revised 17 May 2025

Adansonia digitata (L.), also known as African baobab, is known for the diverse types of secondary metabolites present in the bark, fruits and leaves. The present study aimed to establish a cost-effective plant cell suspension culture method for the production of secondary metabolites. Cell culture of *A. digitata* (L.) was established by a float culture method using a dried fibrous mesocarp of *Luffa aegyptiaca* Mill. (Luffa sponge), to support the growing cells in a liquid medium, and the possibility of production and enhancement of secondary metabolites was tested. Murashige Skoog medium with 2,4-D (0.5 mg/L) & BAP (0.5 mg/L) and incubation in dark at temperature of 30 ± 2°C was found to be optimum for the induction and growth of the callus. To allow the redifferentiation of callus cells and to optimise the production of secondary metabolites, an organic nitrogen supplement in the form of soybean flour (0.02 g/100mL) and blue-red LED (1350 lux for 16 hours was used as a light source. After 8 weeks of incubation, the redifferentiated callus cells were harvested and the presence of various phytochemicals in the cell extract was determined by qualitative analyses. The cultured cells were found to be capable of producing all the secondary metabolites produced by the leaves and bark of the tree. Quantitative estimation of alkaloids was carried out by HPLC with standard berberine. The alkaloid concentration in the cultured cells sample (with LED light and soy flour) was found to be 18.642 µg/g on a dry weight basis compared to the crude bark extract which contained 4.02 µg/g. The use of Luffa sponge, soybean flour, refined edible table sugar and LED light resulted in an affordable method for the production of plant secondary metabolites, which resulted in the reduction of the cost of 1L medium from ₹ 2170.9/- to ₹154.95/-.

Keywords: *Adansonia digitata,* Alkaloids, Float culture, Luffa sponge, Phytochemical analyses, Plant tissue culture, Secondary metabolites

Adansonia digitata (L.) (Family- Malvaceae) is a very large tree native to the African continent and the southern Arabian Peninsula. All parts of the tree are edible having medicinal and nutritional value. Plants produce a large number of diverse compounds that do not have any direct role in the growth and development of plants; rather, they play an important role in the adaptation of plants to their environment. These compounds, also called secondary metabolites, are an important source of bioactive and antimicrobial pharmaceuticals. Various parts of this tree have been reported to be used for properties such as hydration, antipyretic, antiparasitic, antitussive and for the treatment of diarrhoea in African countries. The GC-MS and LC-MS/QTOF analysis of the fruit pulp, leaves, root and stem bark extract revealed the presence of diverse antimicrobial and bioactive phytochemicals. The extracts of *A. digitata* (L.) have shown a significant cytotoxic effect on the HeLa cell line in a concentration-dependent manner[1,2].

The use of plant cell suspension cultures is emerging as a promising technique for producing bioactive secondary metabolites in controlled and aseptic conditions *in vitro*. Production of secondary metabolites using plant cell culture is a multi-step process that includes the selection of a parent plant, the establishment of cell/callus culture, selection of high-yielding cell lines, the establishment of cell culture, optimization of metabolite production and finally, recovery of the product. There are other concerns, such as the large size of the plant cells with longer doubling time resulting in a slow growth rate. Additionally, the cultured cells show physiological heterogeneity, genetic instability and low metabolite content[3,4]. However, tissue culture-based technology is carried out in highly sophisticated facilities, which include stainless steel surfaces, sterile airflow rooms, expensive autoclaves for sterilization of media and automated bioreactors[5]. The requirements to operate such tissue culture facilities are expensive for developing countries. In a cell culture system, there is a need to develop strategies to lower the cost of production and increase the yield. Many low-cost technology options have been described in the recent past[6,7]. The low-cost media were found to be best for plant vigour and rooting with an increase in the micropropagation rate.

Pure cotton fibre, glass wool, nylon cloth, polystyrene foam, glass beads and filter paper have been successfully used in the past as the supporting

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matrix for the growing cell aggregates or plant propagules in a liquid media[8-10]. In the present work, cells were cultured by the 'float culture' method, wherein a Luffa sponge (fibrous mesocarp of dried sponge guard, *Luffa aegyptiaca* Mill.) was used as a 'raft' to keep the cells floating on the liquid medium ensuring proper aeration. The fibrous nature of the raft enabled the absorption of nutrients through capillary action, for the growth of cells. Using a Luffa sponge for establishing immobilized plant cell cultures has been reported earlier to produce scopadulcic acid B[11].

Organic supplements are usually provided in the form of yeast extract, casein hydrolysate, malt extract, coconut water or some fruit juices in order to support *in vitro* growth of plant cells. These supplements are easily available sources of many essential amino acids and vitamins. 'Soybean' is a rich protein source containing approximately 40% protein, 30% soluble and insoluble carbohydrates, 21% oil and 14% moisture, ash and other micronutrients such as isoflavones. Soybeanis a rich source of phenylalanine. Soy flour contains 4.0 ± 0.16 g phenylalanine/100 g on a dry weight basis in addition to leucine[12]. The aliphatic and aromatic amino acids (in the cell produced by the Shikimate pathway) are the key ingredients for the synthesis of nitrogen-containing secondary metabolites in plants[13].

Plants require specific regions of visible spectrum or colours for distinct morphogenic responses. LED systems can produce the specific spectra of light required for plants[14]. The emission spectra of red LED, blue LED, green LED and warm white LED are 633 nm, 448 nm, 520 nm and 600 nm, respectively[15]. Blue LED, red LED and a combination of blue and red LED have been reported to increase the production of phenolics, flavonoids, alkaloids and DPPH scavenging activity[16,17]. Blue LED lights are effective in enhancing plant growth by promoting photosynthesis in leaves through an increased stomatal aperture. Blue LED is also reported to play an important role in controlling the distribution of secondary metabolites, such as total phenolics and flavonoids in leaves[18-20]. In the present work, attempts were made to use blue and red LEDs to enhance the production of secondary metabolites.

Material and Methods

Collection and authentication of plant material

Adansonia digitata (L.) tree located on the campus of Dr. Babasaheb Ambedkar Marathwada University, Chhatrapati Sambhajinagar, Maharashtra, India, (19°54'09.9" N, 75°18'37.7" E) was selected for the studies. Mature woody twigs, bark, and fruits were collected manually from the tree in the month of May, in summer. The plant parts were authenticated at the Department of Botany, Government Institute of Science, Chhatrapati Sambhajinagar, India. Voucher samples were prepared and deposited at the same department with code- GISA/BOT/AP-2024-4.

Establishment of Callus culture

Germination of seeds

Seeds from the mature fruits was collected. The round back side of the hard seed coat was scarified with sandpaper until the inside white layer was visible. Seeds (10 in number) were first soaked in water for 24 hours and then sown in a pot containing a mixture of soil and sand (6:4 w/w) with regular watering in the normal environmental conditions.

Surface sterilisation of explants

Juvenile leaves (2-3 cm long) of the plant were collected as explants in the months of April/May, as the juvenile parts exhibit high meristematic activity and are able to grow and differentiate *in vitro*. Leaves were washed under running tap water for 5 minutes. The cut ends of the petiole were sealed with candle wax to prevent entry of harmful chemicals like sodium hypochlorite or ethanol, during the surface sterilisation process. The leaves were soaked in Bavistin (1%, BASF, India) for 20 minutes. The leaves were washed with sterile water and treated with ethanol (95%) for 30 seconds, followed by treatment with sodium hypochlorite (10% v/v, Fischer Scientific, India) and Tween-20 (2-3 drops) for 5 minutes. The explants were finally washed three times with sterile distilled water. After surface sterilisation, the wax-coated petiole ends were removed, the leaves were cut horizontally through the mid-rib, and the explants (1cm × 1cm approx.) were aseptically inoculated on a medium. Murashige Skoog medium (Hi-Media, India, PT O45) was used as the basal medium for all the tissue culture experiments. For callus induction, media with different ratios of auxin, 2,4-dichloro phenoxy acetic acid (2,4-D, Hi-Media) and cytokinin, benzyl aminopurine (6-BAP, Hi-Media) were prepared (MS 1D 1:0, MS 2D 0.8:0.2, MS 3D 0.6:0.4, MS 4D 0.5:0.5 and MS 5D 0.4:0.6 in mg/L). To all the combinations, refined table sugar (3%) and agar-agar powder (0.6%) were added. In a 100mL capacity tissue culture bottle, 20 mL medium was poured and sterilised at 110°C for 30 minutes. For each medium combination, 6 culture bottles were inoculated and incubated in the dark at 30±2°C for 6 weeks.

Optimization of medium and culture conditions for callus growth and secondary metabolite production

The combination ratio of auxin and cytokinin, showing maximum growth in the callus induction step, was selected as the 'callus growth medium'. The callus was subcultured in a liquid medium using the 'float culture method'. For this, the Luffa sponge was cleaned, soaked overnight in dilute hydrochloric acid (1% v/v of commercial grade HCl) and washed thoroughly with water. After drying, it was cut to a suitable size.

In order to prepare the 'production medium', auxin 2, 4-D (0.5mg/L) in the callus growth medium was replaced with NAA (0.5mg/L, 1-Naphthylacetic acid, Otto Chemie Ltd. India). Soybean flour was used as an organic supplement in the medium. For this, soybean was purchased from the grocery shop, cleaned, ground into a fine powder, and added to the medium at a final concentration of 0.02g/100mL. Liquid media with and without soy flour were prepared. Medium (25mL) was poured in 250 mL capacity bottle with a properly fitting piece of Luffa sponge and the culture bottles were sterilised at 110°C for 30 minutes. The media combinations are given in Table 1. For each combination 6 culture bottles were inoculated. The cultures were incubated at 30±2°C in the dark for 4 weeks to promote the growth of callus. After 4 weeks of dark incubation, 3 culture bottles from each medium combination were shifted under LED lights (blue + red, 90:10 at a total intensity of 1350 Lux (CENTER 337 Lightmeter, Taiwan) in 16 hours day/8 hours night regime for the next 4 weeks. Growth of the callus was measured by taking the fresh weight of the callus after 8 weeks of incubation. The morphological characters of the callus were recorded.

Qualitative phytochemical analyses of leaf bark and cultured cell extract

Qualitative phytochemical analyses of the dried callus, bark and leaf extracts were carried out to study and compare the diversity of secondary metabolites produced. The shade-dried plant and callus powder were subjected to sequential extraction, first with non-polar solvent diethyl ether, then with methanol and finally with highly polar solvent water as follows-

(A) The plant/callus material (10g) was mixed with diethyl ether (100 mL, Loba Chemie Pvt. Ltd., India) and shaken for 24 hours on a rotary shaker. The extract was filtered and used for the identification of lipophilic constituents;(B) After extracting with diethyl ether, the same plant/callus material was dried for 4 hours at room temperature (28°C) and extracted with methanol (100 mL, 80%, Fisher Scientific, India) for 24 hours. The filtered extract was concentrated by evaporation in an oven at 50°C for 2 hours and used to identify methanol-soluble constituents; (C)The plant material, after extraction with ether and methanol, was dried in an oven at 50°C for 16 hours and extracted once again with deionised distilled water (100mL) for 24 hours. The extract was filtered, evaporated at 50°C for 4 hours in an oven, and used for the detection of water-soluble constituents as reported earlier[21-23].

Quantitative analyses of alkaloids in the stem bark and cultured cell extract by reverse-phase HPLC

For comparison, estimation of alkaloids was carried out for bark extract and for 5 different callus samples incubated in different culture conditions. Shade-dried samples were crushed to a fine powder in a mortar and pestle and were suspended in methanol (80%). (MS 4D- 0.09 g Pa 4N(L)- 0.1g/1mL, Pa 4N(D)- 0.1g/1mL, Pe 4N(L)- 0.077g/1mL, Pe 4N(D)- 0.1g/1mL, Bark extract- 0.1g/1mL). The methanol extracts were sonicated in an ultrasonication bath (Kumar Sales Corporation, Mumbai) for 20 minutes

Table 1 — Fresh weight and morphology of callus on different combinations of growth regulators and incubation conditions tested for the production of secondary metabolites in cell cultures of *A. digitata* (L.),incubated at 30±2°C

Medium code	Culture conditions					Weight after 6 weeks (g) on 25 mL medium A.M.± SE	Morphology of callus
	2,4 D 0.5 mg/L	NAA 0.5 mg/L	BAP 0.5 mg/L	Soy flour 0.02%w/v	LED Lights 16 hrs.		
MS 4D	+	-	+	-	-	7.63 (±0.63)	White pink, friable, mucilaginous
Pa 4N (L)	-	+	+	-	+	4.77 (±0.1)	Green, hard
Pa 4N (D)	-	+	+	-	-	2.34 (±1.48)	Brown, friable
Pe 4N (L)	-	+	+	+	+	5.47 (±2.19)	Green, hard
Pe 4N (D)	-	+	+	+	-	3.5 (±2.16)	White, friable

and centrifuged at 10,000 rpm at 10°C. Clear supernatant (100 µL) was mixed with methanol (900 µL, 80 %) and filtered through a 0.22 µm syringe filter. Quantitative estimation of alkaloids in the extracts was carried out on an Agilent Technologies 1260 Infinity HPLC system. Berberine hydrochloride (TCI (India) Pvt. Ltd.) was used as a standard. Standard graph of Berberine was prepared by taking concentrations 5ng, 10ng, 20ng, 30ng, 40ng and 50ng. Water containing 0.02 M o-phosphoric acid [A]: acetonitrile [B] was used as mobile phase for a gradient run with the solvent ratio shift of [A]%- 0 to 2 min:90%, 2 to 10 min: 55%, 10 to 12 min: 55% and 12 to 15 min: 90% with a flow rate of 1 mL/min. An oven temperature of 40°C with a Kromasil column (C-18, 250 ×4.6 mm, 5 µm) was used. The injection volume was 5 µL, and the wavelength of detection was 346 nm.

Antimicrobial activity of *A. digitata* (L.) extracts

The antimicrobial sensitivity testing of the aqueous extract of woody twigs (terminal branches along with leaves) was carried out by agar well diffusion method with slight modification from the earlier report[24]. For the antimicrobial activity testing, *Vibrio cholerae* O1(Ogawa) procured from the Government Medical College and Hospital, Chhatrapati Sambhajinagar, *Shigella sonii* procured from M.I.T. Hospital and Research Institute, Chhatrapati Sambhajinagar, *E. coli* (a laboratory isolated strain), *Salmonella typhimurium* (NCIM 2501) and *Staphylococcus aureus* (NCIM 2078) were used. The extract was prepared using sterile distilled water (10g in 100mL) by maceration for 24 hours, filtered through Whatman filter paper no. 1, evaporated in an oven at 60°C for 24 hours and the dried extract was used after resuspending in sterile distilled water (50mg in 1 mL). Muller-Hinton agar/broth (Hi-Media) was used for the antimicrobial susceptibility testing. The turbidity of the actively growing bacterial cultures was adjusted to McFarland standard 0.5 (OD of 0.08 to 0.1 at 600 nm) by adding the culture to the sterile broth. This gives approximately 1×10^8 CFU/mL cell density. Muller-Hinton agar (10 mL, 1.5% agar) was layered on a 100 mm plate. In a test tube, molten Muller-Hinton agar was seeded with 0.1mL bacterial culture, mixed well and poured on the previously plated agar. The plates were allowed to set for 15 minutes in the refrigerator. The plates were taken out and wells were made with a 6 mm sterile stainless-steel borer and filled with samples (20 µL). Sterile distilled water (20 µL) was used as negative control and ampicillin (Hi Media SD002, 10µg) and tetracycline (Hi Media SD0037, 30µg) were used as positive control. The diameters of the clear zones of inhibition were determined on a mm scale[25].

Results and Discussion

Establishment of callus culture

Germination of seeds

Most of the seeds germinated within 15 days. Germination frequency and survival rate were found to be 90%. In one month, plants grew up to 12-15 cm in height.

Initiation of aseptic culture

About 80% of explants survived the surface sterilisation treatment. The use of Bavistin reduced the percentage of fungal contamination from 70% to 30%. After about 10 to 12 days of incubation, masses of white-green cells appeared around the midrib of the explant. Once the cells started proliferation, further growth was very rapid giving rise to friable callus (Fig. 1A). All the auxin: cytokinin combinations of the medium were found to support the induction of callus in the explant, but the growth of the callus was found to be more on MS 4D medium; with 2, 4-D (0.5 mg/L) and BAP (0.5 mg/L), sugar (3.0%) and agar (0.6%). Therefore, MS 4D medium was designated as

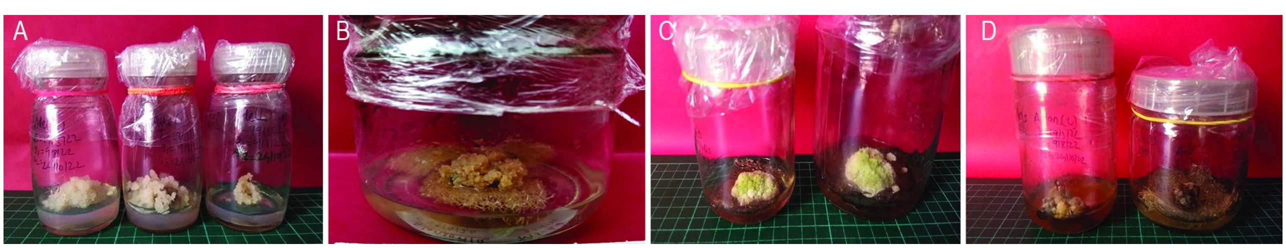

Fig. 1 — Callus induction and growth of *A. digitata* (L.) incubated in shade at 30± 2°C, after 8 weeks on MS 4D medium (A); Growth of *A. digitata* (L.) callus on a liquid medium by 'Float culture method' in the shade at 30±2°C after 8 weeks (B); *A. digitata* (L.) callus cells showing green colouration after incubation in LED at 30± 2°C in Pa 4N(L) and Pe 4N(L) media (C); *A. digitata* (L.)callus turned brown after incubation in dark at 30±2°C in Pa 4N(D) and Pe 4N(D) media(D).

optimized medium for callus induction and growth. The growth was found to be maximum when the temperatures were 30±2°C, as many tropical and subtropical plants show optimum temperatures of 30 to 32°C when cultured *in vitro*[26,27].

Optimization of medium and culture conditions for callus growth and secondary metabolites production

Callus cells were sub-cultured in a liquid medium, both with and without the Luffa sponge raft. It was observed that the growth was fast, reaching to maximum size in 6 weeks on the Luffa sponge raft (Fig. 1B**)**. The results pointed to the efficient aeration and nutrient transfer to the cells through the raft.

MS 4D, a callus induction medium containing auxin in the form of 2,4-dichloro phenoxy acetic acid, was found to be the best for biomass production as seen in fresh weight measurement (Table 1). The growth regulator 2,4-D, although it supports the dedifferentiation of cells and growth of undifferentiated callus cells, has been known to inhibit the production of secondary metabolites. Therefore, in the production medium 2,4-D was replaced by NAA[28-30.] Among the different production media combinations, incubation in LED stimulated maximum callus growth in both the media with and without soybean flour [Pa4N(L) & Pe4N(L)]. The addition of soybean flour alone was not beneficial, as evident from fresh weight measurements (Table 1**)**. A friable callus, a mass of undifferentiated and proliferating cells, was used to start a cell suspension culture. The undifferentiated cells are thin-walled, non-uniform in size and shape and unable to produce secondary metabolites. It has been reported that cells that have thick cell walls and more differentiated structures, or large cell aggregates, which develop vascular elements, can produce secondary metabolites[27]. In the presence of light, the callus mass turned green (chlorophyll synthesis) and became hard, indicating the formation of vascular tissue, a process known as ‘cytodifferentiation’. This transformation is known to enhance the production of secondary metabolites by the cells. On the other hand, cells kept in the dark did not undergo any redifferentiation and remained as an undifferentiated mass of cells (Fig. 1C & D). Earlier, there are many reports of developing micropropagation protocols for *A. digitata* (L.)[31,32]. This is the first report of the establishment of a cell culture in a liquid medium for the production of secondary metabolites *in vitro*.

Qualitative phytochemical analyses of shoot, bark and cultured cell extract

When extracted with methanol (80%), 10g of dried woody twigs powder of *Adansonia digitata* (L.) yielded 1.5g of dried extract, which was dark brown and crystalline, and 10g of dried powder of bark yielded 1.2g of dried extract, which was green and sticky. It could be seen (Table 2) that the cultured

Table 2 — Phytochemical analyses of shoot, bark, and cultured cells extract

	Name of the secondary metabolite tested	Name of the test	Shoot extract	Bark extract	Cultured cells extract
Ether extract	Essential oils	Pleasant smell	Nil	Nil	Nil
	Lipids & Fats	Spot test	Nil	Nil	Nil
	Steroids & Triterpenes	Salkowski reaction	Nil	Nil	Nil
	Carotenoids	Sulfuric acid test	Nil	Nil	Nil
Alcohol extract	Cardiac glycosides	Trichloroacetic acid test	+	Nil	+
	Saponins	Lead acetate test	Nil	+	Nil
	Phenolic Glycosides	Potassium ferrocyanide test	+	+	+
	Phloroglucides	Conc. nitric acid test	Nil	+	+
	Anthrocenocides	Sodium hydroxide test	Nil	Nil	Nil
	Flavonoids	Alkaline reagent test	+	Nil	+
	Quinones	Alcoholic KOH test	Nil	Nil	Nil
	Tannins	Ferric chloride test	Nil	Nil	Nil
Aqueous extract	Alkaloids	Mayer’s reagent test	+	+	+
	Glucides	Molisch’s reagent test	+	+	+
	Polyphenols	Potassium ferrocyanide test	+	+	+
	Tannins	Ferric chloride test	Nil	Nil	Nil
	Polyuronides (Pectin, gum mucilage)	Alcohol test	+	+	+

cells were able to produce all the metabolites produced by the natural plant cells, except for saponins. In addition, cultured cells also revealed the presence of flavonoids that were absent in the bark extract. The presence of various phytochemicals such as alkaloids, flavonoids, steroids, tannins and terpenoids in stem bark, leaves and fruit pulp is reported earlier by GC-NS and HPLC analysis. The results also revealed the presence of strong radical scavenging activity in the leaves extract with IC50 of 0.23 ±0.01 mg/ml[33-35]. In this study, all three studied aqueous extracts (woody twigs, bark and callus) show the presence of mucilage gum. The friable callus was very sticky and highly viscous. There is a report of investigation of physicochemical properties and applications of the mucilage in *A. digitata* (L.), and it was found to have the potential for many pharmaceutical applications like binding, coating, gelling, and matrix forming[36]. The results point to the possibility of using plant tissue culture for the production of secondary metabolites under controlled and aseptic conditions on a large scale in order to overcome the limitation of space for growing plants and the long time required for their maturation. Moreover, cell culture systems are independent of environmental factors like season, climate change, pests or any microbial disease.

Quantitative analyses of alkaloid in the bark and cultured cell extract by reverse-phase HPLC

Quantitative analysis of alkaloids was carried out by reverse-phase HPLC, as all three extracts showed the presence of alkaloids in a qualitative test (Mayer′s reagent test).4-hydroxybenzyl alcohol is commonly found in most of the alkaloids, and therefore, berberine was used as a standard for the HPLC. It is a standard practice to use berberine as a standard for the detection and quantitative estimation of alkaloids. Since maximum absorption of berberine is at 346nm, this wavelength was selected for quantitative estimation of alkaloids. Solvent/buffers used for the sample dissolution did not show absorbance at 346 nm.The retention time for standard berberine was found to be 12.177 min at the injection volume of 5 µL, at 346 nm (Fig. 2). The amount of alkaloid

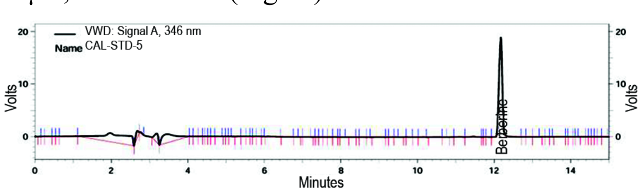

Fig. 2 — Chromatogram for estimation of standard berberine.

synthesised by the cultured cell samples, incubated in different cultural conditions was calculated from Fig. 3A-F in µg/ gram dry weight (Table 3). It was evident that the amount of alkaloid synthesised in all the cultured cell samples was higher than in the bark extract (Fig. 4). The amount of alkaloid was found to be highest (18.642 µg/gram dry weight) in the cell extract, cultured on medium Pe 4N(L), grown in 16h LED (1350 lux) and supported with the soybean flour (0.02g/100mL). Thus, there was a 5-fold increase in the production compared to the amount in the bark extract (4.02 µg/gram dry weight). It was also revealed that MS 4D, a callus induction medium although supported cell growth, did not enhance alkaloid synthesis. Other samples, Pa 4N(L) and Pe 4N(D), which received either LED light or soybean flour could not reach the alkaloid level as in Pe

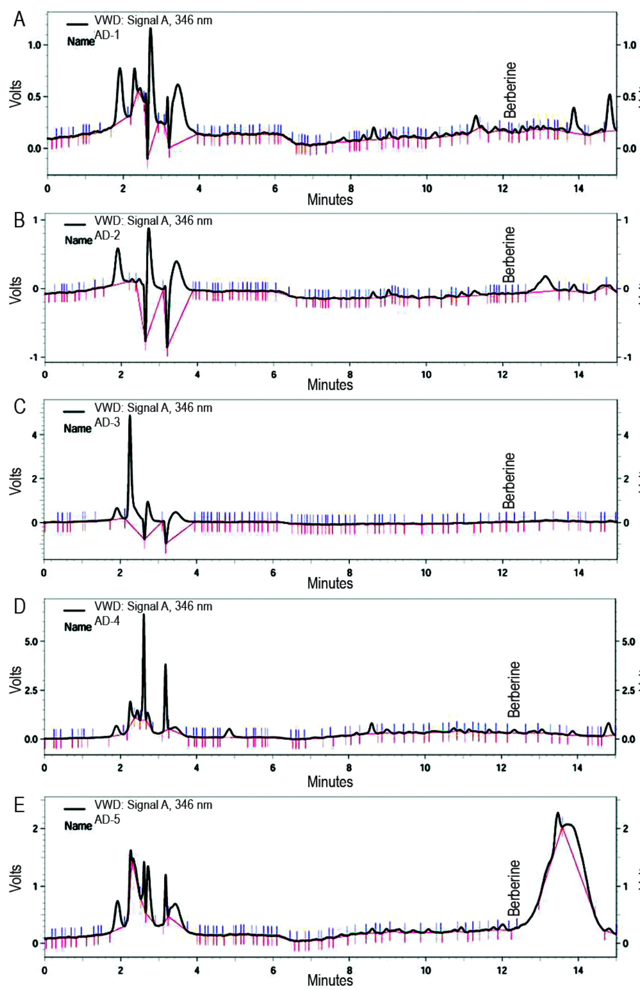

Fig. 3 — Chromatogram for (A) sample AD-1 (MS 4D); (B) sample AD-2 (Pa 4N L); (C) sample AD-3 (Pa 4N D); (D) sample AD-4 (Pe 4N L); (E) sample AD-5 (Pe 4N D) and (F) sample AD-6 (Bark extract).

Table 3— Alkaloid yield of *A. digitata* (L.)cell cultures grown in various media and incubation conditions from HPLC analyses. Shaded cells indicate experimental conditions resulting in the highest yield

Sample code	Medium Code	Incubation and culture conditions	Amount of alkaloid, µg/gram dry wt	Projected yield for 1L culture medium	
				Biomass dry wt. g/L	Alkaloid µg/L of medium
AD1	MS 4D	Callus induction medium, incubation in dark, 30±2°C	4.2851 µg	29.44	126.15
AD2	Pa 4N(L)	Blue LED 1350 lux at cap level, without organic N, 30±2°C	4.3600 µg	15.08	65.740
AD3	Pa 4N(D)	Incubation in dark, Without organic N, 30±2°C	4.3400 µg	9.36	40.622
AD4	Pe 4N(L)	Blue LED 1350 lux at cap level, with organic N, 30±2°C	18.662 µg	22.90	427.35
AD5	Pe 4N(D)	Incubation in dark, With organic N, 30±2°C	4.1600 µg	14.00	58.240
AD6	Bark extract	-	4.0200 µg	-	-

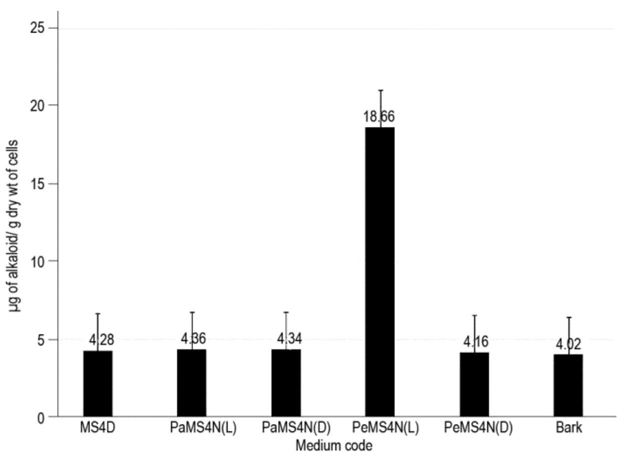

Fig. 4 — Estimated amount of alkaloids in µg/g dry weight in different samples by HPLC.

4N(L). The result indicated that LED light and organic nitrogen supplement had a collective effect on enhancing alkaloid production.

Antimicrobial activity of *Adansonia digitata* (L.):

The experimental results showed that the aqueous woody twigs extract of *A. digitata* (L.) exhibited intermediate activity against *E. coli, Salmonella typhimurium* (NCIM 2501) and *Staphylococcus aureus* NCIM 2078(Table 4). Moreover, the extract showed significant level of bioactivity against a clinical isolate *Shigella sonii* that was resistant to ampicillin[37]. However, no inhibition zone was observed for *Vibrio cholerae* (Ogawa). There are reports of the presence of antimicrobial activity in the stem bark and fruit pulp of *A. digitata* (L.).Stem bark of the tree is traditionally used for treatment of several diseases such as typhoid fever, malaria and UTI (urinary tract infection). The fruit pulp is used as medicine like antipyretic (febrifuge), antidysenteric, diaphoretic, immunostimulant, analgesic, anti-inflammatory and probiotic, and also used to treat diarrhoea in children[38].The antimicrobial activity could not be detected in the callus extract, possibly due to insufficient volume of callus extract available for the experiment.

Assessment of low-cost strategy:

It is worth mentioning that dry sponge guards are available at the rate of ₹80/- kg. A total of 10 pieces of sponge are required for 1L medium (10 culture bottles) amounting to ₹120/. The cultures could be maintained for 8 weeks without changing the medium and for about 4-5 months by replacing the medium intermittently without disturbing the cell mass. This novel technique also saved electricity to the tune of about ₹2000/- by eliminating the need for a rotary shaker that consumes about 4 units per day. A typical plant cell culture experiment requires sub-culturing the growing cells in a fresh medium every 10 to 12 days. For a 6-week culture, about 3L medium is consumed. The use of Luffa sponge raft eliminated the need for frequent sub-culturing, and thus resulting in more cost savings. These factors point to the usefulness of the Luffa sponge raft for growing plant cell cultures. Sucrose is the most widely used carbon source for the heterotrophic plant cell cultures which contributes to 83% cost of the medium. There are early reports of using a commercial grade sugar successfully to reduce cost of production. The ajmalicine production by *C. roseus* suspension cultures in pilot plant bioreactor was found to be efficient with reduction in cost by 36%[39]. In the present study refined table sugar was used to reduce

Table 4 — Antimicrobial activity of aqueous woody twigs extracts of *A. digitata* (L.)

Name	Zone of Inhibition in mm, arithmetic Mean ± Standard error				
	E. coli	*S. typhimurium*	*S. sonii*	*V. cholerae*	*S.aureus*
A. digitata aq. extract (1000 µg)	13.65 (±1.24)	12.88 (±1.0)	10.26 (±0.64)	0.00 (±0.00)	12.83 (±1.0)
Ampicillin (10 µg)	28.00 (±0.00)	34.66 (±1.5)	0.00 (±0.0)	30.00 (±0.0)	11.33 (±0.5)
Tetracycline (30 µg)	12.66 (±0.5)	33.33 (±1.54)	13.22 (±1.5)	13.66 (±0.5)	23.00 (±1.0)
*Sterile distilled water	-	-	-	-	-

[*No zone of inhibition found for sterile distilled water]

Table 5 — Comparative account of requirements for conventional plant cell culture medium and low-cost options using Luffa sponge

Components of medium	Running cost for 1 kg or 1 unit/day (₹)	Requirement for 1L medium	Conventional protocol 1L medium (₹)	Low-cost options 1L medium (₹)
MS Basal medium (PT 045 Hi-Media)	614/5L pack	4.9 g	122.80	122.80
Luffa sponge	10/unit	3 Units	-	30.0
Electricity	44/day	4 units/day	2000	-
Sucrose	770/Kg	30 g	23.10	-
Refined table sugar	45/Kg	30 g	-	1.35
Coconut water	25/unit	100 mL	25.0	-
Soy flour	40/Kg	200 mg	-	0.80
Total cost/1L medium	-		2170.9	154.95

the cost of production. In order to enhance the productivity of the cultures, organic supplements are provided in the form of pure amino acids, yeast extract, casamino acids, peptone and malt extract. Coconut water is also used as a well-known organic supplement usually added at a concentration of 10 % v/v[27]. Table 5 provides a comparative costing for preparation of 1L plant cell culture medium by conventional protocols and the low-cost options as described in the present work.

A combination of these approaches offers multiple advantages such as simplicity, ease of setup and maintenance and the upscaling of plant cell cultures to a suitable level for enhanced production of bioactive metabolites. Large pieces of the Luffa sponge can be made available for large-scale production in batch and immobilised culture modes. Cultured cells could be protected from shear damage, a problem usually occurring in rotary shaking conditions. By virtue of the stability and proper aeration, cells grow into large clumps that undergo redifferentiation which is necessary for the production of secondary metabolites. It has been reported that adansonine, an alkaloid from the bark of *Adansoniadigitata* has antimalarial and antidepressant properties[40]. There is evidence of beneficial effects of bioactive compounds of *A. digitata* on diabetes through different mechanisms of action, namely, decreasing digestive enzyme activities, translocation of glucose transporter 4 into cells, and activation of insulin and the AMPK-signalling pathway[41]. This study is, therefore, a step forward for the production of pharmaceutically important plant metabolites on a large scale by using a cost-effective and simple plant tissue culture technique.

Conclusion

This is the first report of the establishment of an *in vitro* cell suspension culture of *A. digitata* (L.) by a cost-effective 'float culture' method using a Luffa sponge, for the synthesis and enhancement in the production of secondary metabolites. Easy availability of 'Luffa sponge' was pivotal for establishing float culture method that eliminates the need for any aeration device and significantly reduces the dependence on electricity. For the optimization of the alkaloid production, organic supplement was provided through 'soybean flour'. This is the first report of using soybean flour as a cheaper organic source for *in vitro* growth of plant cells. Other cost-effective options used were LED light source and refined table sugar as a source of sucrose. In the present work the cost of 1 L medium was successfully reduced from ₹2170.9/- to ₹154.95/-. This approach offered multiple advantages such as simplicity, ease of setup and maintenance. The production of plant secondary metabolites can be up-scaled for large-scale production of therapeutic molecules in batch and continuous culture modes by applying these strategies.

Conflict of interest

The authors declare that there is no conflict of interest.

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