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PLANT TISSUE CULTURE

The establishment of cell suspension culture of sabah snake grass (*Clinacanthus nutans* (Burm.F.) Lindau)

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Abstract

Clinacanthus nutans (Burm.F.) Lindau is an herbaceous plant that has long been used for traditional medicinal purposes in Asia. It has recently gained popularity as an alternative treatment for cancer. The aim of this study was to establish cell suspension cultures of *C. nutans* and to identify targeted bioactive compounds in the cultures. Young leaf explants were cultured on Murashige and Skoog medium supplemented with various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin to identify a suitable medium for callus induction and proliferation. Proliferated, friable calluses were cultured in different combinations of plant growth regulators (2,4-D, naphthaleneacetic acid [NAA], picloram, kinetin, and 6-benzylaminopurine) in liquid medium to establish cell suspension cultures. Three cell lines of suspension culture, callus, and intact plant parts were subjected to ethyl acetate extraction followed by thin layer chromatography for identification of selected bioactive compounds. Medium supplemented with 0.25 mg L^{-1} 2,4-D and 0.75 mg L^{-1} kinetin was found to be optimal for callus induction, whereas supplementation with 0.50 mg L^{-1} 2,4-D was efficient for callus proliferation. Liquid medium supplemented with 0.25 mg L^{-1} 2,4-D and 0.50 mg L^{-1} NAA produced the highest growth index (2.52). Quercetin, catechin, and luteolin were present together in the callus and cell suspension cultures of *C. nutans*, but all three compounds were detected separately in young leaves, mature leaves, and stems. This study is the first to report the establishment of cell suspension culture of *C. nutans* with both cell and callus cultures producing quercetin, catechin, and luteolin.

Keywords Callus induction · Callus proliferation · Cell suspension culture · Thin layer chromatography · *Clinacanthus nutans*

Introduction

Clinacanthus nutans (Burm.F.) Lindau, also known as Sabah snake grass, is a well-known medicinal plant belonging to the family Acanthaceae. This plant is used traditionally to treat snake bites, rashes, and inflammation. Several studies reported that flavonoids and glucosides produced by *C. nutans* inhibited the formation of human neutrophil elastase, which triggers the chronic inflammatory response and inhibits healing of injured tissues (Teshima *et al.* 1998; Melzig *et al.* 2001; Wanikiat *et al.* 2008). External application of the leaves of *C. nutans* to snake bite wounds has been used to neutralize snake venom (Makhija and Khamar 2010). In addition, *C. nutans* extracts were found to exhibit significant antiviral activity against herpes simplex disease type 2 through inhibition of virus replication within originally infected cells, thereby preventing continuous spreading (Yoosook *et al.* 1999). Based on Gan *et al.* (2015) and Farooqui *et al.* (2015), *C. nutans* is one of the most commonly used herbs in complementary medicine for treating cancer patients in South East Asia. The Malaysian government has even listed *C. nutans* in the National Key Economic Areas proprietary list (Narayanaswamy and Ismail 2015) indicating its potential in the pharmaceutical and complementary medicine industry.

Various phytochemical compounds have been detected in *C. nutans*, including vitexin, isovitexin, shaftoside, orientin, isoorientin, kaempferol, catechin, quercetin, lupeol, luteolin, isomollupentin-7-O-β-glucopyranoside, 2-cis-entadamide A, clinamides A-C, 1-O-palmitoyl-2-O-linolenoyl-3-O-[α-D-galactopyranosyl-(1′ → 6′)-O-β-D-galactopyranosyl] glycerol, 1,2-O-dilinolenoyl-3-O-β-D-galactopyranosylglycerol,

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132-hydroxyl-(132-R)-pheophytin b, 132-hydroxyl (132–S)-pheophytin a, and 132-hydroxyl-(132-R)-pheophytin (Teshima *et al.* 1997; Sakdarat *et al.* 2009; Vachirayonstien *et al.* 2010; Yang *et al.* 2013; Ghasemzadeh *et al.* 2014; Tu *et al.* 2014). Previous studies concluded that quercetin, catechin, and luteolin are chemopreventive agents that have the ability to inhibit cancer cell growth and to induce cancer cell apoptosis.

Quercetin is a common flavonoid found in plants, mostly in fruits and vegetables, and it has been proven to exert its anticancer properties by inhibiting cancer cell growth and angiogenesis in various types of cancer cells, including pancreatic tumors, prostate cancer, breast cancer, leukemic cells, hepatoma cells, and gastric carcinoma cells (Vidya *et al.* 2010; Gibellini *et al.* 2011). Lee *et al.* (2015) suggested that quercetin could be used also as a chemosensitizer to increase the sensitivity of cancer cells to chemotherapy.

Catechin is a natural antioxidant that modulates the immune system through downregulation of signal transduction pathways involved in metastasis, cell proliferation, inflammation, and transformation, which promotes angiogenesis (Na and Surh 2006; Huang *et al.* 2008; Hu *et al.* 2010). Butt *et al.* (2015) also investigated the efficiency of catechin in suppressing the advancement of various cancer cells, including breast, colon, skin, prostate, and lung cancer cell lines.

Luteolin is a flavonoid compound present in many plants such as fruits, vegetables, and medicinal herbs. Plants rich in luteolin are traditionally used to treat hypertension, inflammatory disorders, and cancer (Harborne and Williams 2000). This compound was discovered to successfully induce cancer cell apoptosis and inhibit cancer cell proliferation, including the proliferation of multidrug-resistant cancer cells (Cai *et al.* 2012; Rao *et al.* 2012). Recent studies investigated whether *C. nutans* extracts could be effective in suppressing growth of cancer cell lines without exerting cytotoxic effects. They reported that novel biochemical compounds were present in the extracts of *C. nutans* that potentially could be useful as an alternative side treatment for cancer patients (Yong *et al.* 2013; Arullappan *et al.* 2014).

Plant tissue and cell suspension cultures grown under controlled conditions are potential alternatives to chemical synthesis or natural harvesting for the production and extraction of plant secondary metabolites (Dicosmo and Misawa 1995). This approach is not limited by the low production yield from natural harvest or the high cost of complex chemical synthesis (Wilson and Roberts 2012). Methods for the biosynthesis of secondary metabolites by plant tissue cultures have been applied to plants such as *Artemisia annua*, *Panax ginseng*, and *Taxus baccata* to increase the accumulation of novel bioactive compounds (Vanisree *et al.* 2004). Modification of nutrient content using different plant growth regulators and manipulation of *in vitro* culture conditions significantly impacted the production of novel bioactive compounds (Raj *et al.* 2015). This modification included other factors such as explant selection, which played an important role in inducing selected callus morphology with increased biomass (Warghat *et al.* 2011; Andre *et al.* 2015). The establishment of cell suspension culture is an efficient method to scale up the production of novel plant-derived metabolites for industrial applications. In recent years, biotechnology companies incorporated cell suspension culture techniques for the production of pharmaceutical, cosmetic, and food-related secondary products as a cheaper alternative for the extraction of metabolites rather than by chemical synthesis.

Tissue culture attempts on *C. nutans* are not well documented and most studies focused on the identification of the bioactive compounds in the plant. The current study aimed to establish a *C. nutans* cell suspension culture protocol and to evaluate its efficiency for the production and accumulation of quercetin, catechin, and luteolin. This investigation is the first to report on the efficiency of *C. nutans* suspension cultures for producing selected bioactive compounds that can be further studied and applied in the pharmaceutical industry.

Materials and Methods

Callus induction Young leaves from first and second nodes of *C. nutans* plants grown in the herbal garden of Universiti Sains Malaysia were surface sterilized using the previously established protocol of Phua *et al.* (2016). To identify the optimal callus induction medium, the surface-sterilized leaf explants were cultured on solidified Murashige and Skoog (MS, Murashige and Skoog 1962) medium supplemented with 25 combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.25–1.00 mg L^{-1}) (Duchefa, Haarlem, the Netherlands) and kinetin (0.25–1.00 mg L^{-1}) (Duchefa) under a 16-h photoperiod with the light intensity of 32.5 μmol m^{-2} s^{-1} from cool white fluorescence light (Osram, Augsburg, Germany) (Table 1). Plant growth regulators were added before sterilization. The pH of the medium was then adjusted to pH 5.75 using 1 M HCl or 1 M NaOH followed by the addition of 2.75 g L^{-1} Gelrite™ (Duchefa) prior to autoclaving (Tomy ES-315; TOMY Digital Biology, Tokyo, Japan) for 15 min at 121°C. The experiment was carried out using a complete randomized design with ten replicates, with each replicate containing three explants. Callus fresh weight and morphology were recorded after 8 wk.

Callus proliferation Calluses with a weight of 0.65 g were cultured on MS medium supplemented with nine combinations of 2,4-D and kinetin with agarose (First Base, Singapore) as the gelling agent to identify the optimal medium compositions for callus proliferation (Fig. 1). Plant growth regulators were added before sterilization. The pH of the

Table 1. Callus formation and morphology from young leaf explants of *Clinacanthus nutans* cultured on MS basal medium supplemented with different combinations of 2,4-D and kinetin after 8 wk of culture

Concentration of 2,4-D (mg L^{-1})	Concentration of kinetin (mg L^{-1})	Explants that formed a callus (%)	Color of callus	Texture of callus
0	0	0	No color	No callus
0.25	0	100	Pale white	Friable, watery, sticky
0.50	0	100	Pale yellow	Friable, watery, sticky
0.75	0	100	Pale yellow	Friable, watery, sticky
1.00	0	100	Pale yellow	Friable, watery, sticky
0	0.25	100	Brown	Compact
0.25	0.25	100	Pale white	Friable, watery, sticky
0.50	0.25	63.33	Pale white	Friable, watery, sticky
0.75	0.25	100	Pale white	Friable, watery, sticky
1.00	0.25	100	Light brown	Semi friable
0	0.50	23.33	Brown	Compact
0.25	0.50	100	Pale white	Friable, watery, sticky
0.50	0.50	100	Pale yellow	Semi friable
0.75	0.50	100	Pale white	Friable, watery, sticky
1.00	0.50	80	Gray	Semi friable
0	0.75	80	White	Compact
0.25	0.75	100	Pale white	Friable, watery, sticky
0.50	0.75	100	Pale white	Friable, watery, sticky
0.75	0.75	100	Pale white	Semi friable
1.00	0.75	100	Gray	Semi friable
0	1.00	70	Brown	Compact
0.25	1.00	100	Pale white	Friable, watery, sticky
0.50	1.00	100	Pale white	Friable, watery, sticky
0.75	1.00	100	Pale white	Friable, watery, sticky
1.00	1.00	100	Gray	Semi friable

medium was adjusted to 5.75 using 1 M HCl or 1 M NaOH, then 8 g L^{-1} of agarose was added before autoclaving (Tomy ES-315; TOMY Digital Biology) for 15 min at 121°C. The experiment was carried out with eight replicates, with each replicate containing three explants. Callus fresh weight and morphology were recorded after 21 d.

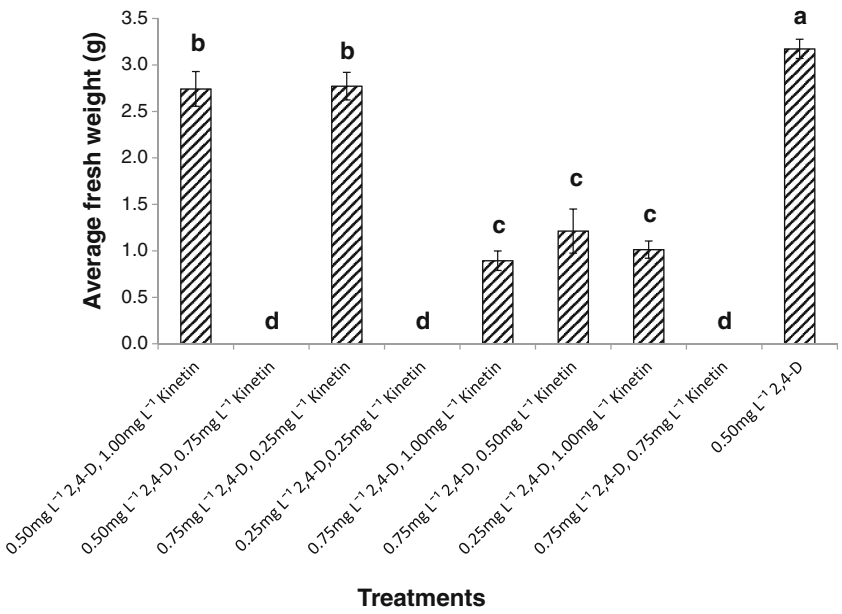

Figure 1. Average callus fresh weight after 3 wk of culture of *Clinacanthus nutans* leaf explants. Mean values followed by the same *letter* were not significantly different ($p \leq 0.05$). MS medium supplemented with 0.50 mg L^{-1} 2,4-D produced a significantly higher callus biomass in comparison to other treatments used. *Error bars* represent mean ± standard error.

Establishment of cell suspension cultures Cell suspension cultures were initiated by transferring 0.5 g of callus obtained from the optimal callus proliferation medium into 150-mL Erlenmeyer flasks containing 25 mL of liquid proliferation medium with aluminum foil (double layer) closure. The pH of the culture medium was adjusted to pH 5.75 1 M HCl or 1 M NaOH and sterilized using the autoclave (Tomy ES-315; TOMY Digital Biology) at 121°C for 15 min. Ten liquid MS proliferation media containing various concentrations and combinations of plant growth regulators (2,4-D, naphthaleneacetic acid [NAA], picloram, kinetin, and 6-benzylaminopurine [BAP]) (Duchefa) were used to establish cell suspension cultures, with six replicates of each treatment (Table 2). The cultures were placed on an orbital shaker with continuous agitation at 100 rotations min^{-1} under continuous, cool white fluorescent lighting with a light intensity of 32.5 μmol m^{-2} s^{-1} (Osram). Cells were harvested after 14 d. The morphology of cells was observed and the growth index of each cell line was calculated, and cell lines were grouped based on the growth index obtained, according the equation:

$$Growth\ index = \frac{Final\ biomass\ (g) - Initial\ biomass\ (g)}{Initial\ biomass\ (g)}$$

Growth patterns of cell suspension cultures Three cell lines were identified that differed in growth index as determined over a period of 30 d: fast growing (CN1), intermediate growing (CN6), and slow growing (CN3). The fresh and dry cell biomass of three replicates was harvested and weighed every 3 d, and the growth of the cell cultures was determined and plotted against the 30-d culture duration.

Table 2. Growth index grouping of the 10 cell lines of *Clinacanthus nutans* cell suspension culture after 14 d of culture

Grouping	Line[z]	Average growth index ± S.E
High (growth index > 2.0)	CN1	2.52 ± 0.17
Intermediate (growth index of 1.0–2.0)	CN10	1.40 ± 0.19
	CN6	1.39 ± 0.15
	CN9	1.25 ± 0.07
Low (growth index < 1.0)	CN3	0.94 ± 0.23
	CN4	0.05 ± 0.10
Not viable (growth index < 0)	CN5	− 0.07 ± 0.09
	CN8	− 0.18 ± 0.12
	CN2	− 0.20 ± 0.05
	CN7	− 0.31 ± 0.05

Cell lines were grouped into high, intermediate, low, and not viable growth index groups

[z] CN1 0.25 mg L^{-1} 2,4-D + 0.50 mg L^{-1} NAA; CN2 0.50 mg L^{-1} picloram; CN3 0.50 mg L^{-1} 2,4-D; CN4 0.50 mg L^{-1} NAA + 0.50 mg L^{-1} kinetin; CN5 0.50 mg L^{-1} 2,4-D + 1.00 mg L^{-1} kinetin; CN6 1.00 mg L^{-1} 2,4-D + 0.10 mg L^{-1} kinetin; CN7 2.00 mg L^{-1} picloram; CN8 3.00 mg L^{-1} NAA + 0.60 mg L^{-1} BAP; CN9 4.50 mg L^{-1} NAA; CN10 0.25 mg L^{-1} 2,4-D

Thin layer chromatography analysis Cells from the three cell lines (CN1, CN6, and CN3) were harvested 1 d before the stationary phase based on the growth curve data (CN 1, 23 d; CN3, 17 d; CN6, 14 d). Calluses from the sixth subculture cycle (3-wk-old) were used as the initial source for cell suspension cultures and were also harvested from solid proliferation medium supplemented with 0.50 mg L^{-1} 2,4-D. The 3-wk-old young leaves, mature leaves, and the stem from *C. nutans* plant grown at the School of Biological Sciences, Universiti Sains Malaysia were collected and air-dried for 5 d before the extraction. The samples were ground into powder using a mortar and pestle and extracted with ethyl acetate at the ratio of 1:20 (*w*/*v*). The mixture was vortexed for 60 s before being sonicated in an ultrasonicator water bath (Ultrasonic Cleaner Set) (Daihan Scientific, Gangwon-do, Korea) for 30 min. The mixture then was centrifuged at 11,200×*g* for 5 min, and the supernatant was filtered through Whatman filter paper No. 1 (90 mm diameter) (Whatman, Maidstone, UK). The filtrate was analyzed by thin layer chromatography (TLC). Each crude extract was analyzed qualitatively using TLC aluminum plates (TLC Silica gel 60 F_{254}) (Merck, Kenilworth, NJ) based on comparisons to the standards (quercetin, kaempferol, and catechin) (ChromaDex Inc., Irvine, CA). The mobile phase was toluene/ethyl acetate/formic acid (2.2:2.2:0.6 *v*/*v*/*v*). All the solvents used were of analytical grade (Sigma-Aldrich®, St. Louis, MO). The spots were detected under UV irradiation (254-nm) using a Spectroline® ENF-240C/FBE (Spectronics Corporation, Westbury, NY). The retention factor (R_f) of each spot was then calculated. R_f is equal to the distance traveled by the standard compound over the total distance covered by the solvent.

Statistical analysis All data were analyzed using one-way analysis of variance (ANOVA) followed by comparison of means using Duncan's multiple range test at $p < 0.05$.

Results and Discussion

Callus induction Callus induction was the initial step in the identification of callus suitable for cell suspension culture. Control treatment explants (MS0) showed no sign of callus formation and remained green after 8 wk of culture (Fig. 2*a*).

Table 1 lists the callus formation rate and morphology of calluses for all treatments of young leaf explants. The leaf explants placed on MS medium supplemented with kinetin alone produced the lowest callus fresh weight, with a callus morphology that was compact in texture (Fig. 2*b*). Explants

cultured on medium with 0.50 mg L^{-1} kinetin had the lowest average fresh weight (0.007 g). Cells turned brown in certain media because of unsuitable plant growth regulators. The callus turned brown and was not viable when growth factors were insufficient for the culture media to support cell growth. Fernando and Gamage (2000) also reported that medium composition was an important factor in increasing callus induction whereby different plant growth regulators produced different responses in different media formulations. Previous studies reported that the presence of abscisic acid increased oxidase activity and induced anti-oxidative activities in cells, which facilitated the excretion of phenols into the medium (Fernando and Gamage 2000; Fazelienasab *et al.* 2004). Toxic metabolites secreted by callus accumulate in the medium and cause phytotoxicity (Razdan 2003; George *et al.* 2008; Appleton *et al.* 2012).

Most of the leaf explants turned brown or white and did not produce callus (Fig. 2*c*). Leaf explants cultured on media supplemented with combinations of 2,4-D and kinetin showed better results than when 2,4-D or kinetin were used alone. All of the calluses produced were pale white in color and were friable, watery, and sticky in texture (Fig. 2*d*). Overall, young leaves cultured on MS medium supplemented with 0.25 mg L^{-1} 2,4-D and 0.75 mg L^{-1} kinetin produced the highest average callus fresh weight (0.896 g), which was significantly higher than that of the other 24 combinations tested (Fig. 3). This formulation was identified to be suitable for the establishment of cell suspension culture due to the friable texture of callus induced.

An optimal combination of auxin and cytokinin was also found to speed up cell division and callogenesis in *Brassica napus* (Afshari *et al.* 2011) and *Solanum lycopersicum* (Shah *et al.* 2015). Gorst *et al.* (1991) discovered that auxin alone was able to induce expression of the cdc2 class of cyclin-dependent kinases. Moreover, the catalytic activity of the kinases increased when explants were placed on medium supplemented with a combination of auxin and cytokinin, which allowed the cells to enter mitosis. This observation was further supported by Hemerley *et al.* (1993), who reported that treatment with both hormones led to higher expression of the gene encoding β-glucuronidase under the control of the cdc2 promoter in tobacco leaf protoplasts than treatment with either auxin or cytokinin alone. This indicated that auxin and cytokinin synergistically influence cdc2a-At kinase transcription. These phenomena showed that auxin renders cells with the competency to divide, but cytokinin is still needed for cells to enter the division cycle (Hemerley *et al.* 1993).

Callus proliferation A suitable callus proliferation medium was required to obtain healthy and regenerable calluses. In some cases, the optimal callus induction medium can differ from the optimal callus proliferation medium, possibly because of the effects of phytohormone types and concentrations on cell division and enlargement. Different genotypes or plants differ significantly in callus induction and regeneration even when cultured using the same plant growth regulators. The degree of cell dispersion is also particularly influenced by the concentration of growth regulators in the culture medium. Ling *et al.* (2009) reported that the callus induction medium for *Mirabilis jalapa* leaf-derived calluses differed from the callus proliferation medium; half-strength MS medium supplemented with 20 μM picloram induced the highest biomass of calluses, whereas half-strength MS medium with the addition of 10 μM picloram was best for maintenance of callus growth. Hu (2012) also reported the use of different plant growth regulators for the callus induction and proliferation medium in *Arctium lappa L.* In that report, it was evident that leaf explants were best induced from MS medium supplemented with 1 mg L^{-1} BAP and 2 mg L^{-1} 2,4-D, whereas medium supplemented with 1.5 mg L^{-1} NAA and 1 mg L^{-1} 2,4-D was found to be more efficient as the callus proliferation medium.

In the present study, MS basal medium supplemented with 0.50 mg L^{-1} 2,4-D was optimal for callus growth and proliferation (3.174 g) (Fig. 1). In addition, the morphology of the callus was friable, indicating that this callus proliferation

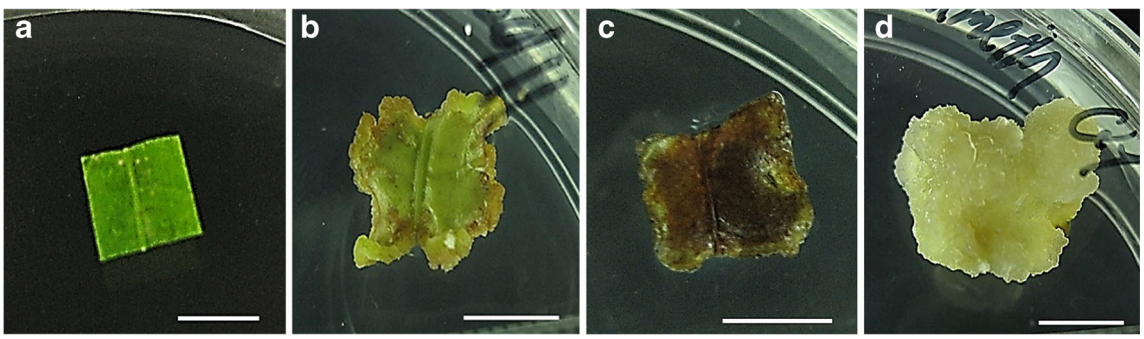

Fig. 2 Callus initiation from *Clinacanthus nutans* leaf explants placed in a combination of 2,4-D and kinetin after 8 wk of culture. (*a*) Control (MS0). (*b*) Compact callus formed on MS medium supplemented with 0.25 mg L^{-1} kinetin (callus formed after 14 d of culture). (*c*) Explant that turned brown on which no callus formed on MS medium supplemented with 0.50 mg L^{-1} kinetin. (*d*) watery, sticky, and friable callus formed on medium supplemented with 0.25 mg L^{-1} 2,4-D and 0.75 mg L^{-1} kinetin (callus formed after 12 d of culture). *Bars* = 0.5 cm.

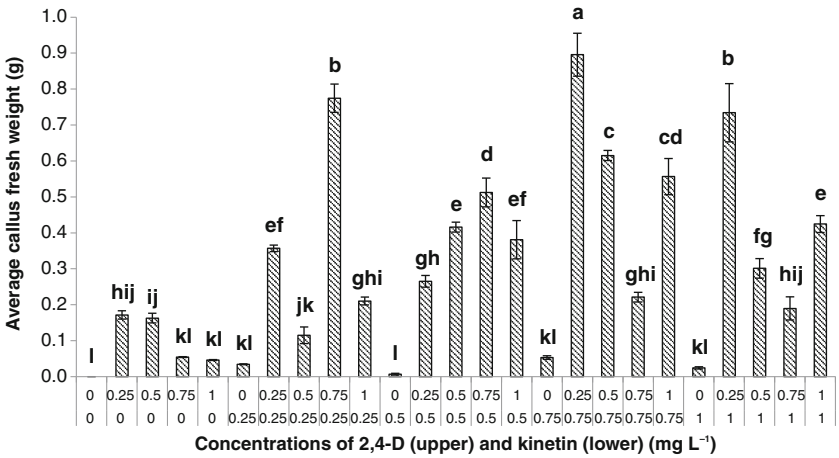

Figure 3. Average of callus fresh weight induced from leaf explants of *Clinacanthus nutans* supplemented with different combinations and various concentrations of 2,4-D and kinetin after 8 wk of culture. Mean values followed by the same letter were not significantly differently ($p \leq 0.05$). Error bars represent mean ± standard error.

medium was optimal for establishing a cell suspension culture. The growth characteristics were probably due to the role of 2,4-D in determining the growth and development of the plant cells. Previous reports stated the importance of 2,4-D in stimulating callus formation in many medicinal plants such as *Falcaria vulgaris* (Hamideh *et al.* 2012), *Achyranthes aspera* (Sen *et al.* 2014), *Ricinus communis* (Elaleem *et al.* 2015), and *Glinus lotoides* (Teshome and Feyissa 2015). These results were consistent with the present study where healthy calluses proliferated was observed on medium with addition of 2,4-D. Endogenous and exogenous plant growth regulators play essential roles in the growth and proliferation of calluses, as optimal levels of these hormones induce expression of the cdc2 class of cyclin-dependent kinases, which allow the cells to enter the division cycle, leading to callus growth (Gorst *et al.* 1991; Hemerley *et al.* 1993).

Establishment of cell suspension cultures Proliferated, friable calluses were cultured in liquid media to enable dispersal of cell clumps and single-cell proliferation for the establishment of cell suspension cultures. MS medium supplemented with 0.25 mg L^{-1} 2,4-D and 0.50 mg L^{-1} NAA resulted in the highest growth index (2.52) (Table 2). This group was categorized as the fast-growing cell lines. MS medium supplemented with 0.25 mg L^{-1} 2,4-D, 4.50 mg L^{-1} NAA, and the combination of 1.00 mg L^{-1} 2,4-D and 0.10 mg L^{-1} kinetin achieved an intermediate average growth index, with the values of 1.40, 1.25, and 1.39, respectively, and were categorized as intermediate-growing cell lines (Table 2). MS medium supplemented with 0.50 mg L^{-1} 2,4-D and the combination of 0.50 mg L^{-1} NAA and 0.50 mg L^{-1} kinetin was grouped as slow-growing cell lines due to low average growth index produced with the values of 0.94 and 0.05, respectively (Table 2). The four remaining cell lines were categorized as not viable due to their average growth index of below 0.

MS medium supplemented with 0.25 mg L^{-1} 2,4-D and 0.50 mg L^{-1} NAA was the most suitable for cell growth and resulted in small aggregates with high biomass. Sharma (2012) reported higher biomass and the production of a large quantity of andrographolide from *Andrographis paniculata* cultured in medium supplemented with 2,4-D and NAA compared to medium supplemented with 2,4-D, NAA, and BAP or 2,4-D and kinetin. This could be due to the crucial role of these hormones in plant cell division and proliferation. Auxin causes DNA to be more methylated, which promotes cell division (George *et al.* 2008). According to George and Sherrington (1984), auxin causes the secretion of hydrogen ions through the cell wall, which increases cell wall extensibility. Potassium ions are taken into the cells to counteract the electrogenic export of hydrogen ion resulting in water molecules moving into the cells and cell expansion due to the low water potential in the cell.

Growth pattern of cell suspension culture The composition of liquid medium affects the cell biomass and morphology of cultured cells. In the present study, cell color and degree of cell aggregation differed when different concentrations and types of plant growth regulators were used. Dziadczyk *et al.* (2013) reported that the medium composition was the main factor required to provide appropriate culture conditions to disperse friable callus tissue as individual cells in the liquid medium. In the present study, the growth patterns of a fast-growing (CN1), intermediate-growing (CN6), and slow-growing (CN3) were studied to further evaluate cell growth throughout the 30-d culture period. The fresh weights and dry weights of all cell lines showed a sigmoid curve pattern, which consists of lag, exponential, stationary, and death phases (Fig. 4). The cells displayed a prolonged lag phase, as long as 9 d, before the cells actively divided and proliferated. Cell lines reached maximum biomass at 24, 15, and 18 d for CN1, CN6, and CN3, respectively. After the exponential phase, all cell lines underwent a decline phase during which the cell growth rate began to decrease (Fig. 4). The different cell lines reached different growth phases at different time intervals, likely because the duration needed to establish cell suspension culture depends greatly on the liquid medium composition (Joshi 2006; Rahman and Bari 2012).

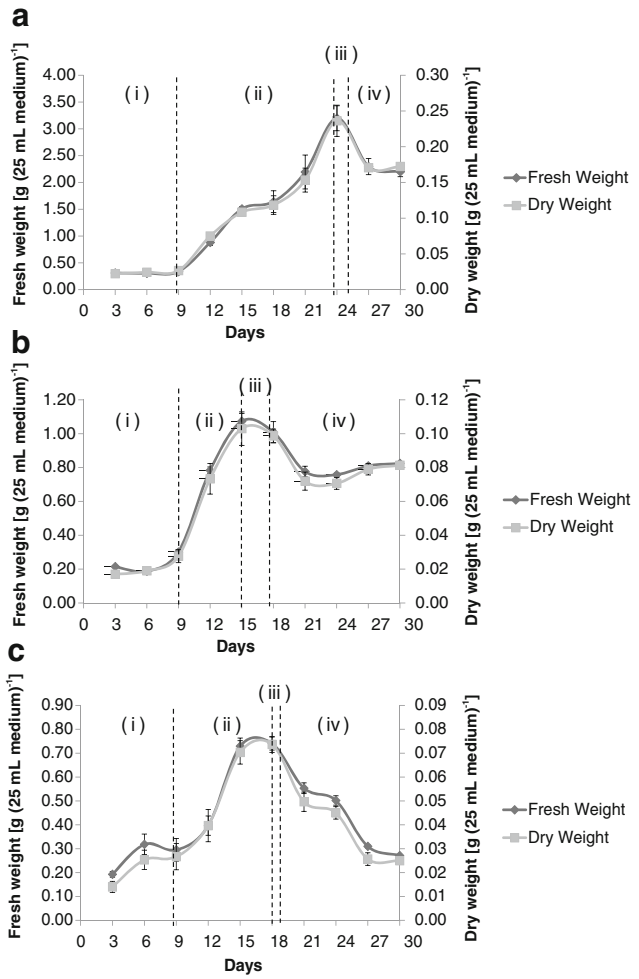

Figure 4. Growth curve of *Clinacanthus nutans* suspension cultures indicating the average fresh weight and dry weight of cells of different cell lines over 30 d of culture. (*a*) CN1. (*b*) CN6. (*c*) CN3. *Error bars* represent mean ± standard error. (*i*), (*ii*), (*iii*), and (*iv*) = lag, log, stationary, and death phases, respectively.

The growth rate in liquid medium decreases as the cell mass increases for several reasons such as the depletion of nutrients and oxygen and the accumulation of toxic waste (Neumann *et al.* 2009). In addition, cell proliferation and primary metabolism greatly decreased, and the primary metabolites were converted into secondary metabolites. This indicated that secondary metabolites accumulated in the stationary phase. Thus, cells should be harvested at this stage before the degradation of secondary metabolites occurs.

The establishment of single-cell cultures provides an excellent opportunity to investigate the properties and potentials of plant cells. Such systems contribute to our understanding of the inter-relationships and complementary influences of cells in multicellular organisms. The dynamic movement of the cells in relation to nutrient medium facilitates higher gaseous exchange, removes any polarity of the cells due to gravity, and eliminates the nutrient gradients within the medium and at the surface of the cells.

Thin layer chromatography analysis TLC was performed on the cell, callus, and plant samples to quickly identify the targeted biochemical compounds that were present. The callus culture and all three cell suspension cell lines (CN1, CN6, and CN3) produced six spots on the TLC plate (Fig. 5) with R_f values of 0.86, 0.68, 0.60, 0.44, 0.28, and 0.08 (Table 3). Of these spots, some had R_f values similar to those of quercetin, catechin, and luteolin. This could be a result of the callus used to initiate cell suspension cultures being from the same source (MS medium supplemented with 2,4-D). Hengel *et al.* (1992) reported that *Camptotheca acuminate* suspension culture cell lines derived from the same solid medium produced similar amounts of camptothecin. Gami *et al.* (2010) reported that *Mimusops elengi* calluses in the same medium composition contained similar chemical constituents and exhibited similar banding patterns on TLC plate.

With reference to Kala (2014), *in vitro* culture can produce higher levels of plant secondary metabolites than the intact plant. Hakkim *et al.* (2007) reported that the accumulation of rosmarinic acid in callus cultures was 10-fold higher than in the intact plant. In the current study, the intensity of compounds (spots) detected (qualitatively) on the TLC plate were darker in the cell lines and callus culture compared to the intact plant parts, indicating that the compounds were either elicited or synthesized during the culture process. Previous studies reported that this observation could be due to stress stimuli, such as light and plant growth regulators in the medium, which trigger the expression of genes (*e.g.*, *Phenylalanine ammonia-lyase* and *Cinnamate-4-hydroxylase*) involved in plant secondary metabolism (Zhao *et al.* 2005; Jiao *et al.* 2015). Pasquali *et al.* (1992) reported that plant secondary metabolism is not restricted to certain specific tissues but

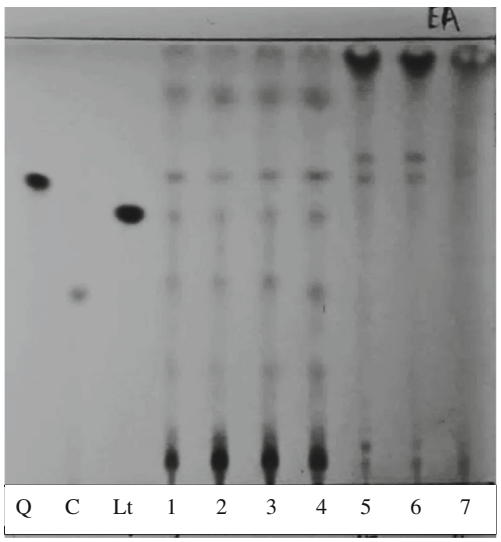

Figure 5. Thin layer chromatography plate indicating separation of the ethyl acetate extract of intact plants and *in vitro* cultured cells: *Q* quercetin, *C* catechin, *Lt* luteolin, *1* cell line 1, *2* cell line 6, *3* cell line 3, *4* callus, *5* young leaves, *6* mature leaves, and *7* stem.

Table 3. The retention factor (R_f) value of intact plants and *in vitro* culture cells of *Clinacanthus nutans* on a thin layer chromatography plate

Source	R_f value
Quercetin standard	0.68
Luteolin standard	0.60
Catechin standard	0.44
Cell line 1	0.86
	0.68
	0.60
	0.44
	0.28
	0.08
Cell line 6	0.86
	0.68
	0.60
	0.44
	0.28
	0.08
Cell line 3	0.86
	0.68
	0.60
	0.44
	0.28
	0.08
Callus	0.86
	0.68
	0.60
	0.44
	0.28
	0.08
Young leaves	0.72
	0.68
	0.10
	0.06
Mature leaves	0.72
	0.68
	0.10
	0.06
Stem	0.68
	0.06

The presence of the standard compounds was identified through comparison of the R_f values of the standard to the R_f values of the plant materials

instead is influenced by environmental and developmental factors. Several factors, such as composition of the culture medium, photoperiod, and environmental conditions, can affect the accumulation and synthesis of a specific compound *in vitro*. Wink (1989) and Sesterhenn *et al.* (2007) found that some important genes involved in plant secondary metabolism may be missing in the differentiated plant. This further indicates that *in vitro* cultures have the potential to produce compounds that are not found in the differentiated plant.

Among the plant parts tested, the stem extracts had only two spots, with R_f values of 0.68 and 0.06, and one of those was similar to the R_f value of quercetin. Four spots with R_f values of 0.72, 0.68, 0.10, and 0.06 were detected on the TLC plate for the young leaf and mature leaf extracts, with one spot having the same R_f value as quercetin. Sarega *et al.* (2016) also reported that the leaf extract of *C. nutans* contained more total phenolic compounds than the stem extract. The extract of *C. nutans* leaves can significantly inhibit cell proliferation of cancer cell lines such as SNU-1, LS-174T, IMR32, HepG2, NCL-H23, HeLa, K562, A549, MCF-7, and Raji (Yong *et al.* 2013; Arullappan *et al.* 2014; Ghani *et al.* 2015; Sulaiman *et al.* 2015). Furthermore, *C. nutans* leaves are one of the herbs most commonly consumed by cancer patients. Therefore, more bioactive compounds may be present in the leaves compared to other parts of the *C. nutans* plant.

The results of this study indicated that the medium composition significantly affected callus induction and callus proliferation of *C. nutans*. MS medium with agarose as a gelling agent supplemented with the combination of 0.25 mg L^{-1} 2,4-D and 0.75 mg L^{-1} kinetin was best for callus induction, and MS medium with agarose as a gelling agent containing 0.50 mg L^{-1} 2,4-D was best for callus proliferation. Calluses were successfully established in cell suspension culture, and quercetin, catechin, and luteolin were potentially detected in the callus and cell suspension cell lines using TLC analysis. This shows the potential of cell suspension culture and *in vitro* callus culture for the production of essential secondary metabolites linked to cancer prevention and treatment.

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