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Research Article
A High Potential of *Kaempferia parviflora* Cell Culture for Phenolics and Flavonoids Production

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Abstract

Background and Objective: Bioactive compound production has been facing many challenges including a long cultivation time, the instability and the impurity of the products from natural plants. This study aimed to produce bioactive compounds from *K. parviflora* by using plant cell culture system and determine their antioxidant potential. **Materials and Methods:** Callus was induced from different organs of the plant including the shoots, rhizomes and roots using Murashige and Skoog (MS) medium supplemented with various Plant Growth Regulators (PGRs). Fast-growing friable calli from all explants were selected and used to establish cell suspension cultures. The biomass production, the accumulation of total phenolics, total flavonoids and the antioxidant activity of the cell culture extracts were determined and compared with those from intact rhizomes of *K. parviflora*. **Results:** All cell suspension cultures of *K. parviflora* exhibited high biomass yields. Furthermore, the productivities of the phenolics and flavonoids from *K. parviflora* cell cultures were also significantly higher than those from the intact rhizomes. The antioxidant capacity of the cell culture extracts was found to be comparable with that detected in the intact rhizomes of this plant. **Conclusion:** Based on the high biomass yield, high bioactive compounds productivity and antioxidant capacity, *K. parviflora* cell culture system displayed great potential for commercial-scale production. Further investigations on the multiple biological properties of the bioactive compounds produced by *K. parviflora* cell suspension cultures as well as the cytotoxicity test will be explored.

Key words: Antioxidant, bioactive compounds, *Kaempferia parviflora*, medicinal plant, plant cell culture

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Kaempferia parviflora Wall. ex Baker or Krachai-dam in Thai, a member of Zingiberaceae is a monocotyledonous indigenous medicinal herb of Thailand which can be found at altitudes between 75 and 500 m[1]. It can also be found in other countries, such as; Laos, Myanmar, India and China. The rhizomes of *K. parviflora* contain several bioactive compounds, e.g., volatile oils, phenolic glycosides and many flavonoids[2]. Traditionally, *K. parviflora* has been used as a Thai medicine for health promotion, stomach discomfort treatment, leucorrhea treatment and oral disease treatment. It can also be used as an antiflatulent agent, an antiplasmodial agent, an antifungal agent and an antimycobacterial agent. It has been reported in Thai traditional medicine that a decoction of *K. parviflora* powder with ethanol is also used to cure allergies, asthma, peptic ulcers and diabetes[3].

The *K. parviflora* is conventionally propagated using rhizomes, but mass propagation by this approach is not sufficiently rapid to meet commercial demand because the best time to harvest rhizomes is approximately 9-10 months after planting. Furthermore, the instability of extracts, the presence of contaminants and plant pathogens make downstream processing more difficult[4]. Plant cell culture can be used to solve these problems. This cultivation strategy offers many advantages over conventional planting methods, such as; a rapid multiplication of plants in a short period[5], provides the genetic identity of pathogen-free and uniform plants[6] and the opportunity to achieve high yield with the high-quality product[7]. There are many research reports on the production of bioactive compounds using plant cell culture. For instance, Yusuf *et al.*[8] reported the production of flavonoids using callus and cell suspension cultures of *Boesenbergia rotunda*. Kumar *et al.*[9] reported the production of phenolics and flavonoids from cell culture of *Heliotropium indicum*. Zhang *et al.*[10] reported the production of flavonoids from the callus culture of *Orostachys cartilaginea*. However, detailed information regarding cell suspension culture establishment and the production of bioactive substances from *K. parviflora* by using plant cell culture has not been documented. Therefore, this research aimed to establish the cell suspension culture of *K. parviflora* for the production of phenolics and flavonoids. The antioxidant activity of these bioactive compounds were evaluated and compared with those from the intact rhizomes of this plant.

MATERIALS AND METHODS

Study area: This study was carried out in the period between October, 2016 and September, 2019 at the Plant Biotechnology Laboratory, Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand.

Plant materials: Rhizomes of a 10-month-old plant collected from Loei province, Northeastern part of Thailand were used in this study. After washing thoroughly with running tap water, they were subjected to surface sterilization using the modified method of Prathanturarug *et al.*[11]. Briefly, the rhizomes were treated with 95 and 70% (v/v) ethanol for 5 and 10 min, respectively. After three rinses with Sterile Distilled Water (SDW), they were cut into 1.0×1.0 cm^2 and treated with a 20 and 10% (v/v) sodium hypochlorite (NaOCl) solution containing 20 L of Tween 80 for 10 min each. After three rinses with SDW, the resulting sterilized explants were placed onto Murashige and Skoog (MS) basal medium[12] containing 30 g L^{-1} sucrose and incubated in a standard culture room at 25±2°C with a light intensity of 2000 Lux and a photoperiod of 16 h. The regenerated *in vitro* plantlets were used as a source of explants for further investigations.

Culture condition and callus induction: The MS basal medium containing 30 g L^{-1} sucrose with a pH of 5.7 was used throughout this study. It was supplemented with various PGRs (2,4-dichlorophenoxyacetic acid (2,4-D), Naphthalene Acetic Acid (NAA) and kinetin) at different concentrations and in different combinations. The culture media were solidified by using 0.8% (w/v) agar and sterilized at 110°C for 28 min.

For callus induction, the shoots, roots and rhizomes of *K. parviflora* excised from the *in vitro* plantlets were placed on MS medium containing 30 g L^{-1} sucrose and supplemented with 2,4-D (0.5, 1.0, 2.0, 3.0, 5.0 mg L^{-1}) alone or in combination with NAA (0.5, 1.0, 2.0 mg L^{-1}) or kinetin (1.0, 2.0 mg L^{-1}) at different concentrations. The explants were cultivated in a standard culture room with a light intensity of 2000 Lux, a photoperiod of 16 h and a temperature of 25±2°C. The callus formation (%) was calculated after 90 days of cultivation and its morphology was observed under a light microscope.

Cell line selection: The fast-growing cell lines of friable calli induced from different explants were selected by using the modified method of Tan *et al.*[13]. Briefly, 10% of friable calli were placed into liquid MS medium supplemented with the combination of 2,4-D, NAA and kinetin at a concentration of

1.0 mg L^{-1} each. All cultures were cultivated in a controlled incubator shaker at 110 rpm with a light intensity of 2000 Lux, a photoperiod of 16 h and a temperature of 25±2°C. One milliliter of cell suspension culture was transferred onto solid MS medium supplemented with the same condition of 2,4-D, NAA and kinetin as in the liquid medium and incubated by using the same culture condition as previously mentioned. The fast-growing cell lines from different sources of explants were selected and used for the subsequent experiments.

Callus proliferation and cell suspension cultures: One gram of the selected fast-growing friable calli was transferred into fresh solid MS medium supplemented with PGRs at different concentrations and in different combinations. All cultures were incubated in a standard culture room with a light intensity of 2000 Lux, a photoperiod of 16 h and a temperature of 25±2°C. Fresh Weight (FW) and Dry Weight (DW) of calli were examined at certain time intervals during cultivation.

For cell suspension cultures, 5 g of selected fast-growing friable calli were transferred into 50 mL of liquid MS medium supplemented with 2.0 mg L^{-1} 2,4-D (for calli derived from roots and rhizomes) and with a combination of 2,4-D, NAA and kinetin at a concentration of 1.0 mg L^{-1} each (for calli derived from shoots). After incubation in a controlled incubator shaker at 110 rpm with a light intensity of 2000 Lux, a photoperiod of 16 h and a temperature of 25±2°C, cells were withdrawn and the FW and DW of the suspended cells were measured.

Total phenolic and flavonoid extraction: Total phenolics and flavonoids were extracted from plant cell cultures and the intact rhizomes of *K. parviflora* by using the procedure described by Sudwan *et al.*[14]. Briefly, cell cultures and the intact rhizomes were dried at 50°C and pulverized. Three grams of powdered samples were subjected to Soxhlet extraction by using 95% ethanol. The extracted solution was evaporated at 40°C and the resulting residues were used for the subsequent experiments.

Determination of total phenolics and flavonoids: The determination of total phenolics and flavonoids were carried out by using Folin-Ciocalteu reagent and the aluminum chloride colorimetric method, respectively. All procedures for examination of total phenolics and flavonoids were performed according to the methods described by Chan *et al.*[15] and Chang *et al.*[16], respectively. Gallic acid and quercetin were used as a standard for total phenolics and flavonoids examination, respectively. Total phenolics and flavonoids were expressed as μg g^{-1} gallic acid equivalent and μg g^{-1} quercetin equivalent, respectively.

Determination of antioxidant activity: Antioxidant activity and the free radical scavenging activity of the crude extracts from cell cultures and the intact rhizomes of *K. parviflora* were evaluated by using a Ferric Reducing Antioxidant Power (FRAP) assay and a 1,1-diphenyl-2-picrylhydrazyl (DPPH), respectively. All procedures for examination of antioxidant activity and the free radical scavenging activity were carried out according to the methods described by Benzie and Strain[17] and Chan *et al.*[15], respectively. For calculation of the DPPH radical scavenging activity, the following formula was used:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs control-Abs sample}}{\text{Abs control}} \times 100$$

where, Abs control and Abs sample are the absorbance of the DPPH solution without and with the sample solution, respectively.

Experimental design and data analysis: A Complete Randomized Design (CRD) was used in this study and all experimental data from each treatment were analyzed by using the SPSS version 17.0. The mean difference from each treatment was tested by using the Duncan Multiple Range Test (DMRT) with a p-value of 0.05. All experiments were carried out twice with three replicates and the results were presented as the Mean±Standard Deviation (SD).

RESULTS

Explant preparation: Cultivation of the *K. parviflora* explants without surface sterilization resulted in 100% contamination. By using ethanol and NaOCl solution as the surface sterilizing agents, only 23.3% of contamination was detected. Based on the morphological observation under a microscope, most of the contaminants were bacteria and filamentous fungi. From the preliminary experiments, the survival rate of the explants after 30 days of cultivation was 76.7%. After 90 days of cultivation, the *in vitro* healthy plantlets of *K. parviflora* were regenerated and the result is shown in Fig. 1a.

Effect of PGRs on callus induction: The shoot explants of *K. parviflora* exhibited higher callus formation frequency than the rhizome and root explants. The optimum concentration of PGRs on callus formation varied depending on the sources of

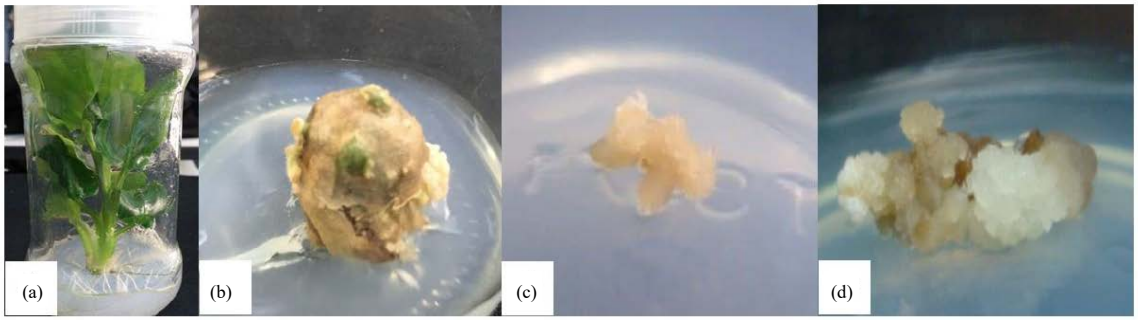

Fig. 1(a-d): Plantlet regeneration and callus formation of *K. parviflora* after culture on MS medium supplemented with various plant growth regulators, (a) *In vitro* plantlet, (b) Callus derived from the rhizome (c) Root and (d) Shoot explants

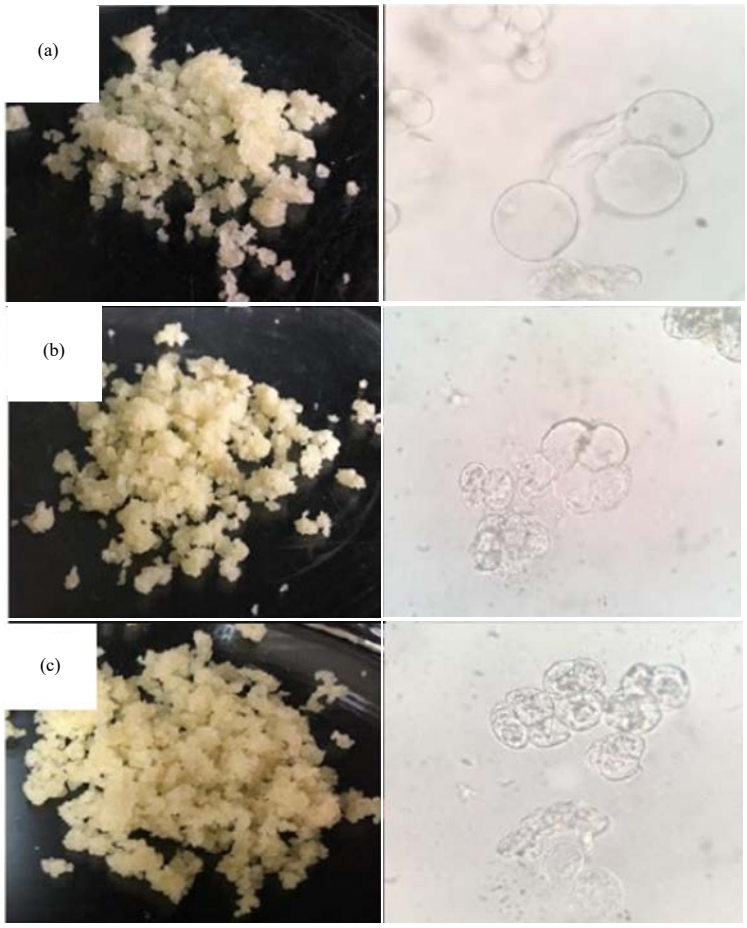

Fig. 2(a-c): Morphology of the selected fast-growing friable calli derived from the (a) Shoots, (b) Roots and (c) Rhizomes of *K. parviflora* under a microscope

the explants. As reported in this study, the combination of 2,4-D, NAA and kinetin at a concentration of 1.0 mg L^{-1} each displayed the highest callus formation (66.7%) from the shoots, while MS medium supplemented with 3 mg L^{-1} 2,4-D exhibited the highest callus formation (53.3%) from the rhizomes. The combination of 2,4-D and NAA at a concentration of 0.5 mg L^{-1} each provided the highest callus formation (40.0%) from the roots of *K. parviflora*. Good callus formation and callus quality in terms of color and texture were obtained from different explants. The morphologies of the

Table 1: Effect of plant growth regulators on callus proliferation of *K. parviflora* after cultivated on MS solid medium for 22 days

Plant growth regulator (mg L^{-1})			Shoots*		Rhizomes*		Roots*	
2,4-D	NAA	Kinetin	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
0.5	-	-	4.09±0.38^{e}	0.17±0.02^{b}	2.74±0.35^{c}	0.06±0.01^{g}	2.46±0.44^{c}	0.17±0.07^{a}
1.0	-	-	0.55±0.13^{j}	0.09±0.05^{c}	3.59±0.09^{a}	0.13±0.01^{b}	0.56±0.13^{f}	0.05±0.01^{f}
2.0	-	-	1.08±0.08^{j}	0.04±0.01^{c}	3.60±0.12^{a}	0.14±0.02^{a}	4.56±0.28^{a}	0.17±0.02^{a}
3.0	-	-	0.76±0.15^{j}	0.10±0.04^{b}	2.03±0.07^{g}	0.08±0.04^{e}	2.05±0.30^{d}	0.08±0.03^{f}
5.0	-	-	0.36±0.07^{j}	0.04±0.01^{b}	1.00±0.06^{i}	0.07±0.03^{f}	1.92±0.52^{d}	0.13±0.01^{d}
0.5	0.5	-	3.23±0.03^{k}	0.16±0.02^{b}	0.95±0.19^{i}	0.10±0.06^{c}	1.88±0.01^{d}	0.13±0.01^{d}
	1.0	-	3.47±0.66^{d}	0.15±0.01^{b}	0.34±0.08^{j}	0.03±0.01^{j}	1.34±0.19^{e}	0.06±0.01^{f}
	2.0	-	4.36±0.14^{a}	0.16±0.01^{b}	0.33±0.02^{j}	0.04±0.01^{j}	4.04±0.38^{a}	0.15±0.02^{b}
1.0	0.5	-	2.78±0.19^{j}	0.14±0.03^{b}	2.46±0.01^{e}	0.17±0.04^{a}	2.90±0.46^{c}	0.18±0.02^{a}
	1.0	-	2.44±0.56^{j}	0.16±0.06^{b}	3.12±0.04^{a}	0.11±0.01^{b}	2.22±0.08^{c}	0.13±0.05^{d}
	2.0	-	3.11±0.19^{j}	0.16±0.01^{b}	0.24±0.02^{j}	0.03±0.01^{j}	0.30±0.03^{f}	0.04±0.00^{f}
2.0	0.5	-	1.26±0.18^{j}	0.09±0.01^{b}	1.47±0.90^{h}	0.09±0.04^{e}	3.06±0.41^{b}	0.16±0.01^{b}
	1.0	-	1.44±0.16^{j}	0.12±0.03^{b}	2.57±0.27^{d}	0.06±0.01^{g}	3.24±0.05^{b}	0.14±0.02^{c}
	2.0	-	2.95±0.20^{f}	0.16±0.01^{b}	1.55±0.49^{h}	0.06±0.02^{h}	3.97±0.36^{b}	0.15±0.03^{bc}
1.0	-	1.0	2.63±0.21^{f}	0.14±0.05^{b}	2.87±0.57^{c}	0.08±0.02^{e}	2.12±0.46^{c}	0.12±0.05^{e}
	-	2.0	3.64±0.41^{d}	0.18±0.01^{b}	2.57±0.23^{d}	0.06±0.02^{g}	2.01±0.06^{d}	0.12±0.02^{e}
2.0	-	1.0	2.58±0.33^{k}	0.13±0.04^{b}	2.35±0.14^{f}	0.03±0.01^{j}	0.42±0.38^{f}	0.07±0.04^{f}
	-	2.0	2.20±0.32^{j}	0.11±0.04^{b}	1.82±0.09^{g}	0.12±0.03^{b}	1.30±0.65^{e}	0.11±0.02^{e}
1.0	1.0	1.0	4.55±0.27^{a}	0.24±0.02^{a}	2.98±0.01^{b}	0.10±0.01^{c}	3.11±0.33^{b}	0.11±0.01^{ae}
	2.0	1.0	3.59±0.33^{a}	0.17±0.01^{b}	2.15±0.19^{g}	0.05±0.00^{i}	0.55±0.25^{f}	0.05±0.01^{f}
2.0	1.0	1.0	3.07±0.27^{j}	0.15±0.02^{b}	1.07±0.06^{i}	0.09±0.01^{d}	0.91±0.21^{jf}	0.07±0.01^{f}
	2.0	1.0	3.97±0.56^{c}	0.17±0.01^{b}	2.70±0.23^{c}	0.07±0.01^{f}	2.19±0.63^{c}	0.15±0.02^{c}

*Mean±SD with different letters in the same column are significantly different at $p< 0.05$ based on DMRT analysis, NAA: Naphthalene acetic acid

generated calli are shown in Fig. 1. Most of the calli originating from rhizomes were compact (Fig. 1b), while those originating from roots (Fig. 1c) and shoots (Fig. 1d) were in a friable type. After repeated subculturing onto the MS medium supplemented with 3 mg L^{-1} 2,4-D, friable calli originating from rhizomes were obtained. All friable calli originating from different sources of the explants were chosen for further experiments.

Cell line selection: After ten successive sub-cultures by using MS liquid and solid media, the fast-growing friable calli or cell lines with consistent growth performance from all explants were successfully identified and selected. These selected fast-growing cell lines revealed a similar cell morphology, i.e., nearly a spherical form under a microscope (Fig. 2). However, their morphologies in terms of color and texture were different. The cell lines originating from the shoots (Fig. 2a) and rhizomes (Fig. 2b) were creamy with loosely aggregated cells, while those from the roots were pale yellow with tightly aggregated cells (Fig. 2c). The distinct growth performances of these selected fast-growing cell lines were also observed. The selected fast-growing cell lines originating from the shoots displayed higher growth as determined by the FW and DW than the other selected fast-growing cell lines. The maximum growth of the selected fast-growing cell lines from the shoots (4.55 g FW and 0.24 g DW), roots (3.11 g FW and 0.11 g DW) and rhizomes (2.98 g FW and 0.10 g DW) were obtained after 21 days of cultivation by using MS solid medium.

Effect of PGRs on callus proliferation: The response of calli originating from different sources of explants varied depending on the type and concentration of PGRs. The results are summarized in Table 1. Among the different concentrations of auxins and cytokinin tested, 2,4-D at a concentration of 1 and 2 mg L^{-1}, NAA at 2 mg L^{-1} and kinetin at 1 mg L^{-1} seemed to be the best concentration in promoting callus proliferation of *K. parvoflora* compared with the other concentrations. Besides, the combination of PGRs also enhanced the proliferation of callus. In this study, MS medium supplemented with 2,4-D, NAA and kinetin at a concentration of 1.0 mg L^{-1} each promoted the biomass production of calli originating from the shoots, with the highest biomass production of 4.55 g FW and 0.24 g DW. On the other hand, supplementation of 2.0 mg L^{-1} of NAA into the MS medium also gave relatively high levels of biomass production (4.36 g FW and 0.16 g DW) of calli originating from the shoots. For biomass production of calli originating from the rhizomes and roots of *K. parviflora*, MS medium supplemented with 2.0 mg L^{-1} 2,4-D proved to be the best condition for biomass production, yielding the maximum growth of 3.60 g FW and 0.14 g DW for rhizome-derived calli and 4.56 g FW and 0.17 g DW for root-derived calli.

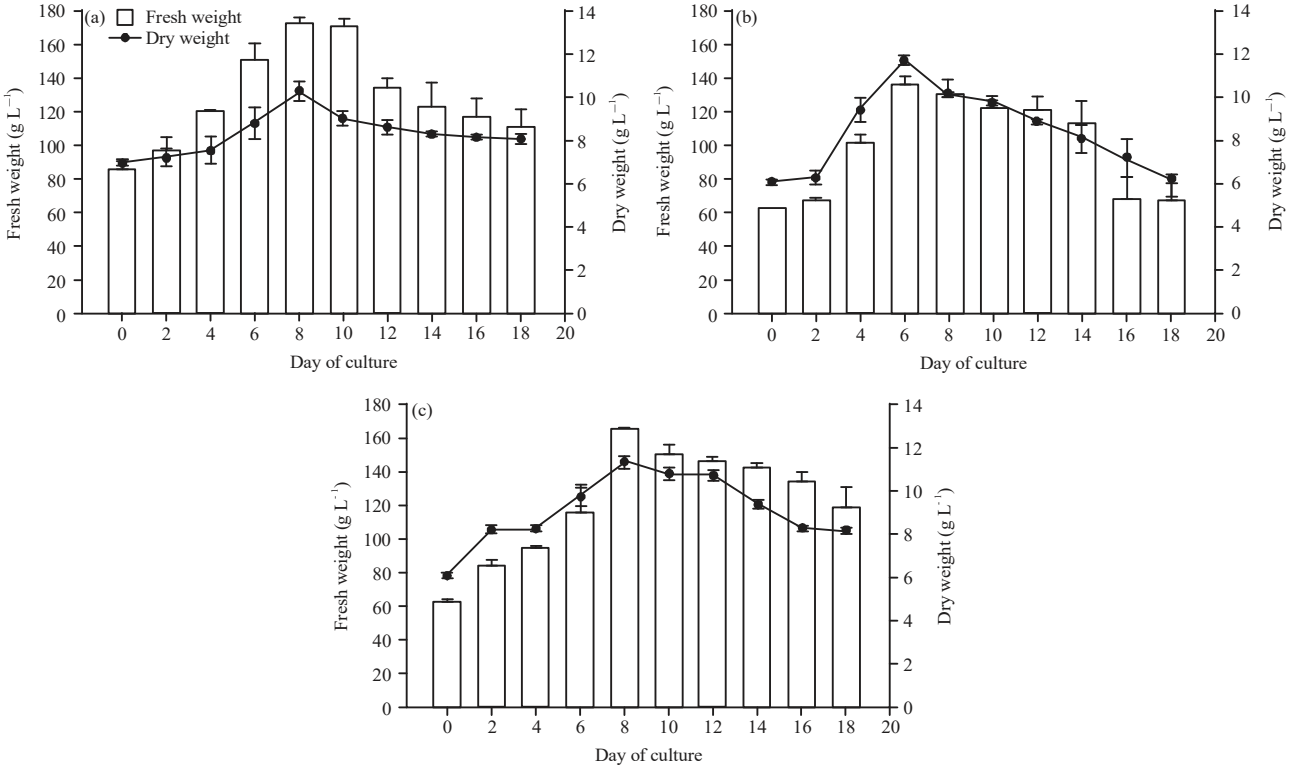

Fig. 3(a-c): Time courses of the growth of cell suspension cultures by using fast-growing friable calli originating from the (a) Shoot, (b) rhizome and (c) Root explants of *K. parviflora* in MS medium

Cell suspension culture of *K. parviflora*: The biomass production of the cell suspension cultures by using the selected fast-growing friable calli originating from different sources of explants is illustrated in Fig. 3. The growth profiles of all *K. parviflora* cell suspension cultures were almost similar, i.e., the biomass production slightly increased in the first stage and reached the maximum at day 6 or 8 of cultivation depending on the explant source. Thereafter, the biomass declined. The cell suspension culture by using the selected fast-growing friable calli originating from the rhizomes exhibited faster growth than those of the selected fast-growing friable calli originating from the shoots and roots. The maximum biomasses of the cell suspension cultures cultivated by using the selected fast-growing friable calli originating from the shoots (172.30 and 10.27 g L^{-1} FW and DW, respectively) (Fig. 3a), rhizomes (141.60 and 11.73 g L^{-1} FW and DW, respectively) (Fig. 3b) and roots (165.34 and 11.33 g L^{-1} FW and DW, respectively) (Fig. 3c) were obtained in this study.

Total phenolic and flavonoid contents: The cell suspension culture by using the selected fast-growing friable calli originating from roots produced higher total phenolics and flavonoids than those by using the selected fast-growing friable calli originating from shoots and rhizomes. The production profiles of total phenolics and flavonoids from cell suspension cultures of *K. parviflora* are presented in Fig. 4. The suspension cells gradually accumulated the total phenolics and flavonoids in the first stage of growth (during the first 2-4 days) and reached the highest level at day 8 of cultivation. Thereafter, the accumulation of these bioactive compounds gradually declined. The maximum concentrations of total phenolic compounds produced by cell suspension cultures by using the selected fast-growing friable calli originating from roots, shoots and rhizomes were 343.78, 301.78 and 142.00 µg GAE g^{-1} DW, respectively (Fig. 4a). The total flavonoid content followed the same pattern as total phenolic content with the highest total flavonoid content in cell suspension culture of root-derived calli (292.67 µg QE g^{-1} DW), followed by those of shoot-derived (269.33 µg QE g^{-1} DW) and rhizome-derived calli (150.44 µg QE g^{-1} DW) (Fig. 4b). The accumulation of total phenolics and flavonoids in the intact rhizomes of a 10-month-old plant was also determined and the results are presented in Table 2. Although, the concentrations of total phenolics and flavonoids from the cell suspension cultures of *K. parviflora* were remarkably lower

Table 2: Production of total phenolics and flavonoids from *K. parviflora* cell cultures and the intact rhizomes of a 10-month-old plant

Sources	Total phenolics		Total flavonoids	
	Concentration (μg GAE g^{-1} DW)	Productivity (μg GAE d^{-1})	Concentration (μg QE g^{-1} DW)	Productivity (μg GAE d^{-1})
Cell culture by using calli originating from roots	343.78±2.04	42.97	292.67±3.02	36.58
Cell culture by using calli originating from shoots	301.78±1.89	37.72	269.33±2.64	33.67
Cell culture by using calli originating from rhizomes	142.00±1.60	17.75	150.44±1.86	18.81
Rhizome from 10-month-old plant	1,207.00±5.68	4.02	1,077.00±4.45	3.59

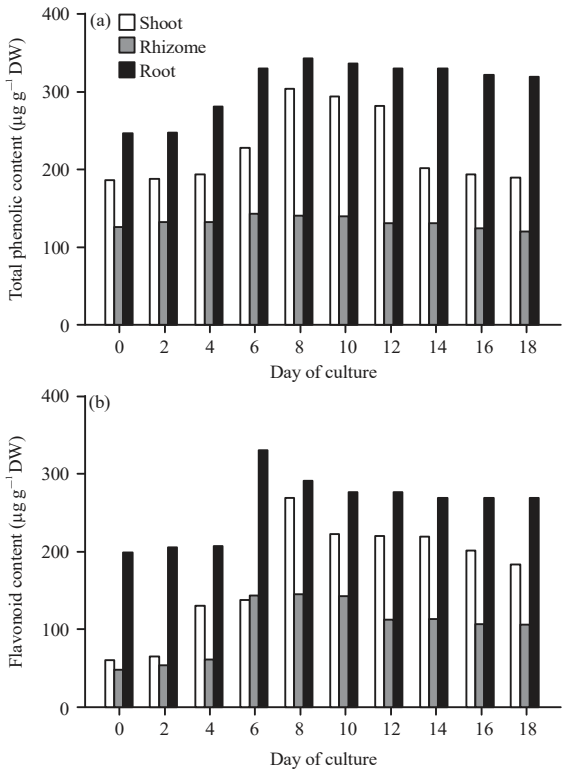

Fig. 4(a-b): (a)Total phenolic and (b) Flavonoid contents from cell suspension cultures using fast-growing friable calli originating from the shoot, rhizome and root explants of *K. parviflora*

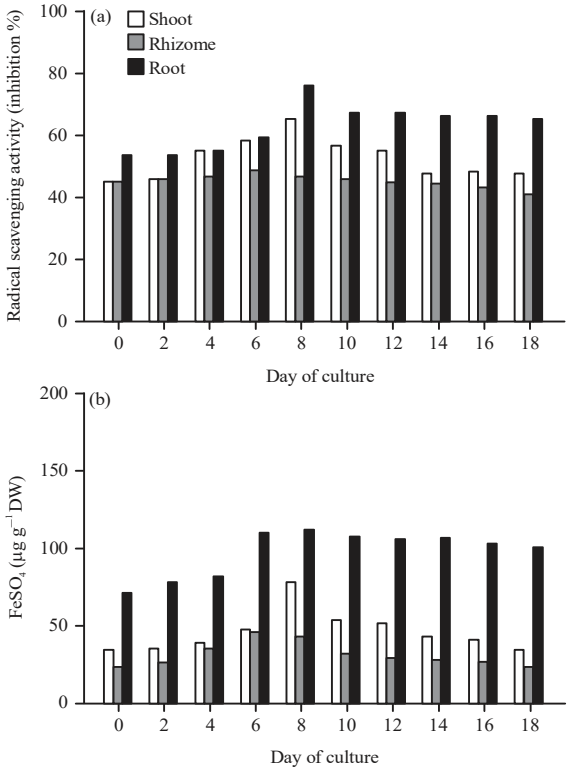

Fig. 5(a-b): (A) DPPH free radical scavenging activity and (b) Antioxidant activity of the crude extracts from cell suspension cultures using fast-growing friable calli originating from the shoot, rhizome and root explants of *K. parviflora*

than those from the intact rhizomes, the production rates or productivities of these bioactive compounds were significantly greater than those from the intact rhizomes. The productivities of total phenolic compounds produced by cell suspension cultures using the selected fast-growing friable calli originating from roots, shoots and rhizomes were 42.97, 37.72 and 17.75 μg GAE d^{-1}, respectively, whereas that from the intact rhizomes was 4.02 μg GAE d^{-1}. Likewise, the productivities of the flavonoids produced by cell suspension cultures using the selected fast-growing friable calli originating from roots, shoots and rhizomes were 36.58, 33.67 and 18.81 μg QE d^{-1}, respectively, while that from the intact rhizomes was 3.59 μg QE d^{-1}. Therefore, these findings strongly suggested that the cell suspension cultures of *K. parviflora* displayed high potential for the production of total phenolic compounds and flavonoids on a commercial scale.

Antioxidant activity: The DPPH free radical scavenging and antioxidant activities of the cell culture extracts from the selected fast-growing friable calli originating from different explant sources are shown in Fig. 5. The cell culture extracts from the calli originating from roots exhibited greater DPPH

free radical scavenging and antioxidant activities than those detected in the other calli. The highest percentage of DPPH free radical scavenging activity of the cell culture extract from the calli originating from roots was 76.34%, which was slightly higher than that of the intact rhizomes (75.53%), while those of the cell culture extracts from the calli originating from shoots and rhizomes were 65.06 and 48.89%, respectively (Fig. 5a). Similarly, the highest antioxidant activity determined by the FRAP assay of the cell culture extract from the calli originating from roots was 113.00 μg Fe^{2+} g^{-1} DW, which was comparable to that of the intact rhizomes (125.70 μg Fe^{2+} g^{-1} DW), while those of the cell culture extracts from the calli originating from shoots and rhizomes were 77.83 and 46.33 μg Fe^{2+} g^{-1} DW, respectively (Fig. 5b).

DISCUSSION

Natural plants are usually contaminated with various pathogens, such as; bacteria and fungi, thus, surface sterilization is needed to eliminate all contaminants of the explants. Several techniques have been used for surface sterilization, including physical (ultrasonication, heat treatment) and chemical treatments (NaOCl, ethanol, antifungals or antibiotics)[18]. Among the chemical treatments, ethanol and NaOCl have been widely used for surface sterilization of several plant species in a member of Zingiberaceae[19]. In the present study, ethanol and NaOCl exhibited a great potential in eliminating contaminants on the surface of *K. parviflora* explants. One possible explanation is that ethanol, a wetting agent, acts as a surfactant to effectively remove surface contaminants. It should be noted that ethanol is not only considered as a powerful sterilizing agent, but also extremely toxic to the plant cells. A high concentration of ethanol may cause cell death[20]. In this study, a relatively high survival rate of the *K. parviflora* explants (76.7%) was obtained after ethanol and NaOCl treatments, which was consistent with that reported by Zuraida[21]. Apart from ethanol and NaOCl, other chemicals for surface sterilization have also been reported. For instance, Alveera *et al.*[22] reported the application of 0.1% (w/v) $HgCl_2$ for the treatment of *Alternanthera sessilis* and they found that only 5.5% contamination was detected. When the concentration of $HgCl_2$ increased from 0.1 to 0.5% (w/v), 83% of the cells died suggested that this chemical was also toxic to the plant cells.

The PGRs 2,4-D, NAA and kinetin are widely used to stimulate callus from several plant species. However, the callus formation frequency depends on the type of explants and the concentrations and combinations of PGRs. As shown in this study, the shoot explants of *K. parviflora* exhibited higher callus formation frequency than the roots and rhizomes. It could be attributed that the shoot explants possessed a higher number of meristematic cells than the other explants. Concerning the PGRs, 2,4-D at a concentration of 3 mg L^{-1} or the combinations of 2,4-D and NAA or kinetin at a low concentration (0.5-1.0 mg L^{-1}) displayed a great potential in promoting the callus formation of *K. parviflora*. These results were in good agreement with those reported for *Cornukaempferia aurantiflora*[23], *Hedychium coronarium*[24], *Curcuma attenuata*[25], *K. parviflora*[26] and *Zingiber officinale*[27]. The same phenomenon was also observed in the callus proliferation of *K. parviflora*, i.e., callus formation and response might vary depending on the sources of explants as well as the types and concentrations of PGRs. The highest biomass production was detected in the callus origination from the roots followed by the shoots and rhizomes. Based on the biomass production observed in this study, 2,4-D at a low concentration (1-2 mg L^{-1}) seemed to be a promising PGR for the callus proliferation of *K. parviflora*, which was similar to previous reports of *B. rotunda*[8], *C. aurantiflora*[23], *Curcuma caesia*[26] and *Z. officinale*[27,28]. It should be noted in the present study that a decline in biomass production in the later stage of cultivation (Fig. 3) was due to cell lysis, resulting in the loss of macro and micro-elements from the plant cells. The results were in good agreement with the results reported for *B. rotunda*[8], *C. asiatica*[13] and *K. parviflora*[26].

The screening and selection of high potential cell lines are one of the most important steps for biomass and bioactive compound production using plant cell culture systems. In this study, the fast-growing cell lines with relatively high biomass production from different sources of *K. parviflora* explants were successfully selected and their morphologies in terms of color and texture were different (Fig. 2). There are several research reports on screening and selection of high potential cell lines from many plants, e.g., Zhao *et al.*[29] reported the selection of cell lines from calli originating from the leaves of *Saussurea medusa* using MS medium. Based on the growth performance and color of the calli, 3 different calli, yellow, red and white were selected. Among these selected cell lines, the red cell line exhibited the highest growth and jaceosidin accumulation. Tan *et al.*[13] reported the selection of cell lines from calli originating from the leaves of *Centella asiatica* based on biomass and flavonoid accumulation. One of the potential elite cell lines, designated as UPM03, displayed the highest biomass and flavonoid accumulation. The selected cell line exhibited the highest FW (0.67 g), DW (0.041 g) and flavonoid concentration (10.75 mg g^{-1} DW) after 12 days of cultivation using MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 1.0 mg L^{-1} kinetin. Jin and Keng[30] reported the

selection of cell lines from calli originating from the leaves of *Artemisia annua* by using MS medium containing 0.5 g L^{-1} casein hydrolysate and supplemented with the combination of NAA and BA at a concentration of 0.5 mg L^{-1} each. Based on the Growth Index (GI) and morphology of the calli, 34 cell lines with consistent GIs and sustainable biomass production were selected and categorized into 3 groups, i.e., fast-(GI>20), intermediate-(GI 15-20) and slow-growing (GI<15) groups.

Most research on the production of phenolic compounds and flavonoids from *K. parviflora* used intact rhizomes[2] and the production of these bioactive compounds by using a plant cell culture system has not yet been reported. As shown in this study, all of the suspension cultures by using the selected fast-growing friable calli originating from different explant sources synthesized and accumulated total phenolics and flavonoids similar to those of the intact rhizomes. Although, the concentrations of these bioactive compounds from the suspension cultures were relatively low compared to those from the intact rhizomes, the productivities of total phenolics and flavonoids were approximately 4.4 to 10.7-fold and 5.2 to 10.2-fold greater than those from the intact rhizomes of a 10-month-old plant. Phenolic and flavonoid compounds are ubiquitous plant secondary metabolites that have been reported to possess multiple biological effects, e.g., antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory and anticancer effects[2,31-33]. As shown in the present study, all of the cell culture extracts from different explant sources displayed relatively high DPPH free radical scavenging activity and antioxidant activity similar to those found in the intact rhizomes suggested that the plant cell culture system did not affect the antioxidant properties of these bioactive compounds derived from cell suspension cultures of *K. parviflora*. The DPPH free radical scavenging and antioxidant activities of the cell culture extracts were correlated with the amounts of total phenolics and flavonoids produced by this cell type. Apart from the antioxidant properties, total phenolics and flavonoids from *K. parviflora* have also been reported to exhibit other biological effects, e.g., antiplasmodial, antifungal, antimycobacterial and cytotoxicity effects[2]. To clarify the multiple biological properties and the toxicity test of these bioactive substances in the cell suspension cultures of *K. parviflora*, further investigation is needed.

CONCLUSION

The cell suspension cultures for the production of biomass, total phenolics and flavonoids from the medicinal herb, *K. parviflora* were successfully established. Based on the biomass, total phenolic and flavonoid production and the antioxidant capacity of the cell culture extracts, the plant cell suspension culture by using the selected fast-growing friable calli originating from the roots represents a potential source for the continuous production of valuable bioactive compounds from this plant on a commercial scale.

SIGNIFICANCE STATEMENT

The present study revealed that a well-established *K. parviflora* plant cell culture system provides a reliable, predictable and sustainable platform for the production of high-value plant secondary metabolites at a high efficiency within a short period of time when compared with the conventional method. By using the best cell line of *K. parviflora*, a commercial-scale production of plant-derived natural products from this medicinal herb will be economically feasible with potential applications in medical, pharmaceutical and nutraceutical industries.

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