Saensouk S *et al.* (2023)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 51, Issue 4, Article number 13190
DOI:10.15835/nbha51413190
Research Article

In vitro regeneration, transplantation and phytochemical profiles of *Kaempferia angustifolia* Roscoe

Surapon SAENSOUK[1,3], Wipa YAOWACHAI[2], Theeraphan CHUMROENPHAT[4], Sukanya NONTHALEE[5], Piyaporn SAENSOUK[2,3]*

[1]*Mahasarakham University, Walai Rukhavej Botanical Research Institute, Mahasarakham 44150, Thailand; surapon.s@msu.ac.th*
[2]*Mahasarakham University, Faculty of Science, Department of Biology, Mahasarakham 44150, Thailand; wipa.y@outlook.com; pcornukaempferia@yahoo.com (*corresponding author)*
[3]*Mahasarakham University, Diversity of Family Zingiberaceae and Vascular Plant for Its Applications Research Unit, Mahasarakham 44150, Thailand*
[4]*Ubon Ratchathani Rajabhat University, Faculty of Thai Traditional and Alternative Medicine, Aesthetic Sciences and Health Program, Ubonratchathani 34000, Thailand; theeraphan.chum@hotmail.com*
[5]*Horticultural Research Institute, Trang Horticultural Research Center, Trang 92150, Thailand; sukanya_014@outlook.com*

Abstract

Kaempferia angustifolia Roscoe is a medicinal plant in the family Zingiberaceae, with wild occurrence decreasing as a result of both natural and human threats. Tissue culture techniques provide an alternative method of propagation for mass production. Microshoots of *K. angustifolia* (1 cm in length) were cultured on MS medium supplemented with various concentrations of cytokinins (BA and Kinetin) and auxins (NAA and IAA) for 8 weeks. Phytochemical profiles were evaluated by total phenolic contents (TPC), total flavonoid contents (TFC), antioxidant activity (2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) assay) and Gas chromatography–mass spectrometry (GC–MS) analysis. Maximum number of shoots was observed in medium supplemented with 2 mg/l BA plus 0.5 mg/l NAA (6.33 shoots/explant) and optimal rooting was induced in MS medium with 2 mg/l Kinetin plus 1 mg/l NAA (35.70 roots/explant). Highest numbers of shoots and roots were obtained when cultured on liquid MS medium supplemented with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA (8.73 shoots/explant and 29.67 roots/explant, respectively). Regenerated plantlets of *K. angustifolia* were transferred to pots containing different types of plant materials under natural conditions for 8 weeks. Optimal survival rate was 100% when transplanting *K. angustifolia* to soil, sand, soil and sand (1:1) or soil and small pieces of rock (1:1) ratio. The methanol extract of leaves *K. angustifolia* from natural plants and *in vitro* derives plants showed a significantly higher amount of TFC and antioxidant activity. GC-MS analysis identified 52 phytochemical compounds in leaves of *K. angustifolia*. This study may be helpful to increase the value of commercial production of *K. angustifolia*, pharmaceutical and medicinal purpose.

Keywords: antioxidant; GC-MS; *Kaempferia angustifolia*; micropropagation; TFC; TPC

Received: 02 May 2023. Received in revised form: 04 Oct 2023. Accepted: 19 Oct 2023. Published online: 15 Nov 2023.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

Kaempferia L. is a genus of monocot herbs containing over 50 species native to east tropical Asia belonging to the family Zingiberaceae (Tang *et al.,* 2014). Many plants in this genus are used for food, as medicinal plants to treat various symptoms and as ornamental leaf displays. This monocot plant is also known as Prab samut (Thai common name) or Toobmoob (I-San local name) and grows wild in the forests of Western and Central Java and in parts of Thailand (Tang *et al.,* 2011). *Kaempferia angustifolia* has a fragrant scent and is often ground and used as traditional medicine to cure fever, cold, stomach pain, cough, dysentery and diarrhea (Yeap *et al.,* 2017). Rhizome extracts of *K. angustifolia* contained terpene, triterpene, cyclohexane derivatives, chalcone, and glycoside showing cytotoxicity against some human cancer cell lines, human promyelocytic leukemia (HL-60), and human breast adenocarcinoma (MCF-7) (Tang *et al.,* 2014).

Plant propagation rate when using the underground rhizome is slow and rhizomes collected from field-grown plants are affected by various pathogens (Saensouk *et al.,* 2016). Overuse by humans and reduction in forest areas have resulted in decrease of *K. angustifolia* plants in the wild. The plant tissue culture technique allows rapid clonal propagation, which is particularly useful when species are threatened and natural regeneration is slow, to quickly provide a large quantity of plant material (Ashmore, 1997). However, limited studies have been investigated total phenolic contents, total flavonoid contents, antioxidant activity and phytochemical profiling (GC-MS analysis) from leaves derived from natural plants and *in vitro* derived plants of *K. angustifolia*. Therefore, the objective of this study was to develop a rapid *in vitro* propagation protocol for *K. angustifolia* using the tissue culture technique, and evaluate total phenolic contents, total flavonoid contents and antioxidant activity of natural plants and *in vitro* derived plants. Gas chromatography-mass spectrometry (GC-MS) was used to analyze the essential oils from leaves of natural plants and *in vitro* derived plants of *K. angustifolia*.

Materials and Methods

In vitro plant regeneration

Surface sterilization procedures and initial culture establishment

Mature seeds of *K. angustifolia* were collected from plants growing in their natural habitat in Udon Thani Province, Thailand. The seeds were washed under running tap water for 30 min and then soaked in 70% (v/v) ethanol for 1 min, followed by disinfecting in 20% and 15% sodium hypochlorite (NaOCl) for 20 min and 15 min, respectively under aseptic conditions. The seeds were rinsed three times for 5 min each in sterilized distilled water, and initially cultured on MS (Murashige and Skoog, 1962) medium supplemented with 2.0 mg/l 6-benzylaminopurine (BA) combined with 0.5 mg/l 1-naphthaleneacetic acid (NAA). After two months of seed culture, excised shoots (1 cm in length) were used as explants for the experiments.

Medium and culture condition

All experiments were cultured on MS medium supplemented with 30 g/l sucrose, 7 g/l bacto agar and various concentrations and combinations of plant growth regulators (PGRs). Microshoots were cultured in solid medium using 120 ml glass culture vessels (25 ml of the media), while for liquid medium, the microshoots were cultured in 250 ml Erlenmeyer flasks (100 ml of the media) for shoot and root induction. In solid culture, single microshoots were inoculated into the culture vessel, with 20 culture vessels used for each treatment. In liquid culture, two microshoots were inoculated into each Erlenmeyer flask, with 10 flasks used for each treatment. All media were adjusted to pH 5.7-5.8 using 1 N NaOH or 1 N HCl and autoclaved at 121 °C for 15 min. The cultures were incubated under 16 hours white fluorescent light with a light level at 27 μmol $s^{-1}m^{-2}$ per day/8 hours darkness at 25 ± 2 °C.

Shoot multiplication

The shoot explants (1 cm in length) were transferred to MS medium supplemented with various concentrations of PGRs as BA (0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) combined with NAA or indole acetic acid (IAA) (0, 0.1, 0.5 and 1.0 mg/l) and Kinetin (0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) combined with NAA (0, 0.1, 0.5 and 1.0 mg/l). Different strengths of solid and liquid MS media (full, half and quarter) were supplemented with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA cultured in a rotary shaker (120 rpm/min) for 8 weeks. Mean shoot number, mean shoot length (cm), mean root number and mean root length (cm) per plantlet were measured.

Acclimatization and transplantation

One-year-old plantlets of *K. angustifolia* (cultured on MS medium without PGRs) were taken out from culture room to room temperature (at 28 ± 5 °C) for 4 weeks during the rainy season to adjust to conditions outside the plant tissue culture room. The plantlets showed well-developed shoots and roots at 9-11 cm in height. Each plantlet was removed from its glass bottle and the roots were washed gently in running tap water to remove culture medium debris before transfer into plastic pots containing soil, sand, soil mixed with sand (1:1 w/w) and soil mixed with small pieces of rock (1:1 w/w) for 8 weeks under greenhouse conditions. Survival percentage, mean shoot number, mean shoot length (cm), mean leaf number, mean leaf length (cm) and mean leaf width (cm) per plantlet were measured. The potted plants were maintained under greenhouse conditions at the Department of Biology, Faculty of Science, Mahasarakham University, Thailand and regularly irrigated with tap water.

Statistical analysis

A completely randomized design (CRD) was applied for all the experiments. Twenty microshoots were used for each treatment and each experiment was repeated three times. Data were analyzed for significance using ANOVA, with differences between treatments assessed using Duncan's multiple range test (DMRT). All statistical analyses were performed at the 5% level using the SPSS program (version 15).

Phytochemical profiling analysis

Plant material

The natural plants of *K. angustifolia* were collected from Amphoe Ban Phue, Udon Thani Province, Thailand in July 2020. The plant sample was identified by Assoc. Prof. Dr. Surapon Saensouk (Walai Rukhavej Botanical Research Institute, Mahasarakham University, Mahasarakham, Thailand). The voucher specimen (SS. Udorn33) was deposited in Mahasarakham University Herbarium. Plant parts used for antioxidant activity consisted of leaves, pseudostem, rhizome and storage root from natural plants and leaves from plants that had been tissue cultured on MS medium and MS medium supplemented with 2 mg/l BA and 0.1 mg/l NAA. Explants from both *in vitro* and natural culture were rinsed with tap water and put in a freezer at -20 °C before freeze-drying for 24 hours. The dried plants were blended into powder and stored at -20 °C for analysis.

Extraction

A dried plant sample (1.00 g) was placed in an Erlenmeyer flask and macerated with 40 ml of methanol at 35 °C for 12 hours in a shaking incubator at 150 rpm. The solvent was then filtered using Whatman filter paper No.1 grade and the extracted about 30 ml kept at -20 °C.

Determination of total phenolic contents (TPC)

The total phenolic contents were investigated following the method described by Thammapat *et al.* (2015). The TPC in the extracts were determined using the Folin-Ciocalteu assay. Extract samples 300 µl (10-fold dilution with ethanol) were added to centrifuge tubes containing 2.25 ml of Folin-Ciocalteu's phenol

reagent, mixed and then incubated in a dark place for 5 min. Next, 2.25 ml of 6% (w/v) sodium carbonate was added, and the mixture was placed in the dark for 90 min. The absorbance was measured by a spectrophotometer at a wavelength of 725 nm. Gallic acid (1.95, 3.90, 7.81, 31.25, 62.50, 250 and 500 mg/l) was used as a standard. TPC were expressed as mg GAE/100 g DW.

Determination of total flavonoid contents (TFC)

The total flavonoid contents were according to the procedure described by Wanyo *et al.* (2016). A 0.5 ml sample was added to a centrifuge tube and 0.15 ml of 5% (w/v) $NaNO_2$ was added to the mixture and placed in the dark for 6 min. Then, 0.3 ml of 10% (w/v) $AlCl_3$ was added to the mixture and kept in the dark for 5 min before adding 1.0 ml of 1 M NaOH. The absorbance was measured by a spectrophotometer at a wavelength of 510 nm. Rutin (0.97, 1.95, 7.81, 15.62, 31.25, 125, 250 and 500 mg/l) was used as the standard. TFC were expressed as mg RE/100 g DW.

Determination of DPPH radical scavenging assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was determined following the method of Thammapat *et al. (*2015). The DPPH was used to determine the free radical scavenging capacity. The stock solution was prepared by dissolving DPPH with methanol and then stored at 20 °C. The working solution was obtained by mixing 100 µl of sample with 3.0 ml of 0.004% DPPH and incubating in the dark for 30 min. The absorbance was measured by a spectrophotometer at a wavelength of 517 nm. Trolox solution was used as the standard (calibration was performed with seven Trolox standards in the range of 3.90–250 µg/ml), with results expressed as mg Trolox/100 g DW.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing/antioxidant power (FRAP) assay was investigated following the method described by Siriamornpun *et al.* (2016). The FRAP assay is a method of measuring the ability of antioxidants. The samples extract to a 10 dilution with ethanol were added to a centrifuge tube and 180 µl of deionized water (DI) and 1.8 ml of FRAP reagent were added. The mixture was incubated at 37 °C in a water bath for 4 min. The absorbance was measured by a spectrophotometer at a wavelength of 593 nm. $FeSO_4$ solution was used as the standard, seven $FeSO_4$ standards with concentrations between 3.90-250 µg/ml were used for calibration. The results were expressed as mg $FeSO_4$/100 g DW.

Determination of volatile compounds by GC-MS analysis

The plant samples extracts were performed by GC-MS modified from Suphrom *et al.* (2017). One-year-old of *K. angustifolia*, the leaves samples from natural plants and *in vitro* derived plant cultures on MS medium added with 1 mg/l BA plus 0.1 mg/l IAA, MS medium added with 1 mg/l BA plus 0.5 mg/l IAA and MS medium added with 3 mg/l BA plus 1 mg/l IAA were powdered and 0.2 g of each was placed in vials. Vial headspace was maintained at 140 °C for 5 min after coating with a silicone/polytetrafluoroethylene (PTFE) septum. Then, using a heated syringe set to 85 °C, 2,000 µl of headspace was automatically injected. The samples were analyzed using a GC-MS (Shimadzu QP-2010 Plus, Tokyo, Japan) with an Rtx-5Ms fused-silica capillary column (5% diphenyl 95% dimethyl polysiloxane, 30 mm length, 0.25 mm internal diameter, 0.25 µm film thickness: Restek, USA). Helium was used as the carrier gas; 1.0 ml/min of constant column flow rate; injector temperature (250 °C); split ratio (1:5); temperature program, 40 °C for 2 min, with increase at 5 °C/min to 100 °C, 220 °C at 10 °C /min (20 min of holding), and 250 °C at 10 °C /min; ion source temperature (200 °C); transfer line temperature (250 °C) and ionization energy (70 eV). Electron ionization mass spectra were acquired over the mass range 40-550 amu in full scan.

Statistical analysis

Total phenolic contents, total flavonoid contents, and free radical scavenging activity (DPPH and FRAP assays) were measured as three replicates, with results reported as the mean ± standard error (SE). Data were analyzed for significance using ANOVA, with differences contrasted using Duncan's multiple range test (DMRT). All statistical analyses were performed at $p<0.05$ using the SPSS program (version 15).

Results

In vitro plant regeneration

Shoot multiplication

Microshoots of *Kaempferia angustifolia* (1 cm length) were cultured on solid MS medium for 8 weeks with various concentrations of cytokinins (BA and Kinetin) in combination with auxins (NAA and IAA) for shoot and root formation. The explants responded differently to various media. Simultaneous formation of both shoots and root was observed in all the culture media including MS medium without PGRs. When microshoots of *K. angustifolia* were cultured on MS medium added with BA alone, the highest number of shoots was obtained on MS medium supplemented with 2 mg/l BA (4.80±0.55 shoots/explant), while highest average number of roots per explant was observed on MS medium added with 0.5 mg/l BA (20.80±2.40 roots/explant) (Table 1 and Figure 1). Cultured microshoots of *K. angustifolia* on MS medium with various concentrations of auxins (IAA and NAA) showed well-developed shoot and root systems. MS medium supplemented with 2 mg/l BA plus 0.5 mg/l NAA showed the highest number of shoots (6.33±0.25 shoots/explant) (Table 1 and Figure 1), whereas microshoots of *K. angustifolia* cultured on MS medium supplemented with 2 mg/l BA plus 1 mg/l IAA presented the highest number of shoots (4.75±0.94 shoots/explant) (Table 2 and Figure 3). Shoots formed in MS medium with BA plus NAA were green with long leaves, while the roots were short. Maximum shoot length of 4.62±0.31 cm was observed on 5 mg/l Kinetin. Highest average number of roots per shoot was observed in MS medium supplemented with 2 mg/l Kinetin plus 1 mg/l NAA (35.70±2.67 roots/explant) (Table 3 and Figure 4). In the treatment of Kinetin plus NAA, shoot multiplication was significantly lower than the treatment of BA plus NAA and IAA, while in the treatment of Kinetin plus NAA, shoot length was significantly longer than the treatment of BA plus NAA and IAA. *In vitro* flowering was observed on MS medium containing 5 mg/l BA and 5 mg/l BA plus 0.1 mg/l NAA. Flower inflorescence was seen at the tip of the pseudostem, with the calyx, corolla lobes and lateral staminodes white in color (Figure 2). Labellum has been found white with dark purple color at the central. The shape, size and the color of the flower from tissue culture is look like the flower occur in natural condition.

Different strengths of MS solid and liquid media (full, half and quarter) were supplemented with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA for 8 weeks. Solid MS full strength medium showed the highest number of shoots when supplemented with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA (4.13±0.35 shoots/explant), while ½ MS medium showed the same results as full-strength MS medium (4.11±0.26 shoots/explant). The stem of the plant was thin, and the curled leaves were light green with extended white and hairless roots. The ¼ MS medium showed the lowest number of shoots (3.36±0.36 shoots/explant). The liquid full strength MS medium showed the highest number of shoots when supplemented with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA (8.73±0.73 shoots/explant). The liquid ½ MS medium showed the highest number of shoots at 7.73±0.71 shoots/explant and was not significantly different from liquid full-strength MS medium, while the liquid ¼ MS medium gave the lowest number of shoots (4.83±0.42 shoots/explant) (Tables 4-5 and Figure 5).

Acclimatization and transplantation

Plantlets with 3 to 5 leaves and a well-developed root system were removed from the agar medium and transferred into plastic pots containing soil, sand, soil mixed with sand (1:1 w/w) and soil mixed with small

pieces of rock (1:1 w/w) for 8 weeks during the rainy season under greenhouse conditions at the Department of Biology, Faculty of Science, Mahasarakham University, Mahasarakham, Thailand. After 8 weeks, plantlets of *K. angustifolia* transplanted into soil, sand, soil mixed with sand and soil mixed with small pieces of rock produced various sizes of storage roots and new leaves. They adapted well to the external environment and could be used as planting material. Optimal survival rate was 100% when transplanting *K. angustifolia* to soil, sand, soil and sand or soil and small pieces of rock, with maximum number of shoots, average shoot length and number of leaves observed in soil (5.20±0.26 shoots/plant, 8.33±0.34 cm and 10.50±0.37 leaves/plant, respectively). Highest average leaf width and average leaf length were observed in sand mixed with small pieces of rock (1.72±0.05 and 7.02±0.23 cm., respectively) (Table 6 and Figure 6).

Table 1. Effect of combining BA and NAA on shoot and root multiplication of *K. angustifolia*

BA (mg/l)	NAA (mg/l)	Average no. of shoots/explant mean±SE	Average shoot length (cm) mean±SE	Average no. of roots/explant mean±SE	Average root length (cm) mean±SE
0	0	3.20±0.39^{bcd}	1.77±0.28^{cde}	14.20±3.85^{abc}	1.75±0.52^{a}
0.5	0	3.60±0.45^{bcd}	2.79±0.33^{ab}	20.80±2.40^{a}	1.51±0.36^{abc}
1	0	3.40±0.87^{bcd}	2.09±0.20^{bcde}	10.90±1.99^{bcdef}	1.76±0.23^{a}
2	0	4.80±0.55^{abc}	1.66±0.14^{de}	5.80±1.55^{cdef}	1.06±0.40^{abcdef}
3	0	3.40±0.22^{bcd}	2.25±0.19^{abcd}	6.20±1.40^{cdef}	0.95±0.21^{abcdef}
4	0	3.40±0.37^{bcd}	2.45±0.24^{abc}	5.10±1.30^{def}	0.76±0.16^{bcdef}
5	0	4.20±0.92^{abcd}	2.02±0.17^{cde}	2.50±124^{f}	0.38±0.15^{ef}
0	0.1	3.60±0.56^{bcd}	2.82±0.32^{a}	13.30±1.57^{abcd}	2.20±0.17^{a}
0.5	0.1	3.90±0.38^{bcd}	2.02±0.19^{cde}	15.30±2.55^{ab}	1.70±0.55^{a}
1	0.1	3.70±0.45^{bcd}	2.27±0.21^{abcd}	8.80±2.12^{bcdef}	1.58±0.34^{ab}
2	0.1	4.80±0.66^{abc}	2.28±0.29^{abcd}	6.10±2.15^{cdef}	0.80±0.24^{bcdef}
3	0.1	5.90±1.59^{a}	2.15±0.15^{abcde}	8.40±3.03^{bcdef}	0.85±0.25^{bcdef}
4	0.1	4.10±0.35^{abcd}	2.22±0.20^{abcd}	4.30±2.40^{ef}	0.53±0.33^{def}
5	0.1	5.09±0.53^{ab}	1.83±0.08^{cde}	10.55±2.12^{bcdef}	1.12±0.17^{abcdef}
0	0.5	4.36±0.36^{abcd}	2.16±0.16^{abcde}	16.27±3.33^{ab}	1.07±0.21^{abcdef}
0.5	0.5	3.67±0.33^{bcd}	1.78±0.20^{cde}	12.22±2.53^{bcde}	1.04±0.17^{abcdef}
1	0.5	4.30±0.58^{abcd}	2.12±0.23^{abcde}	14.10±2.25^{abc}	1.16±0.21^{abcde}
2	0.5	6.33±0.25^{a}	2.16±0.18^{abcde}	15.70±2.23^{ab}	1.25±0.15^{abcd}
3	0.5	4.40±0.43^{abcd}	1.69±0.16^{de}	10.00±2.69^{bcdef}	0.94±0.13^{abcdef}
4	0.5	3.10±0.38^{bcd}	2.08±0.26^{bcde}	9.30±2.61^{bcdef}	1.00±0.19^{abcdef}
5	0.5	3.33±0.76^{bcd}	1.78±0.10^{cde}	8.22±2.90^{bcdef}	0.63±0.23^{def}
0	1	2.50±0.40^{d}	2.31±0.34^{abcd}	10.40±3.79^{bcdef}	0.52±0.20^{def}
0.5	1	3.50±0.40^{bcd}	1.44±0.18^{e}	9.60±2.89^{bcdef}	0.70±0.15^{cdef}
1	1	3.20±0.51^{bcd}	1.92±0.16^{cde}	10.80±3.59^{bcdef}	0.72±0.16^{cdef}
2	1	3.13±0.61^{bcd}	1.79±0.20^{cde}	3.63±2.05^{f}	0.31±0.21^{f}
3	1	3.50±0.27^{bcd}	1.76±0.15^{cde}	4.40±1.89^{ef}	0.52±0.18^{def}
4	1	3.20±0.39^{bcd}	1.67±0.13^{de}	3.30±1.45^{f}	0.45±0.15^{def}
5	1	2.88±0.30^{cd}	1.58±0.12^{de}	5.88±0.60^{cdef}	0.84±0.18^{bcdef}

Means followed by the same letters within each column are not significantly different at $p<0.05$ according to DMRT

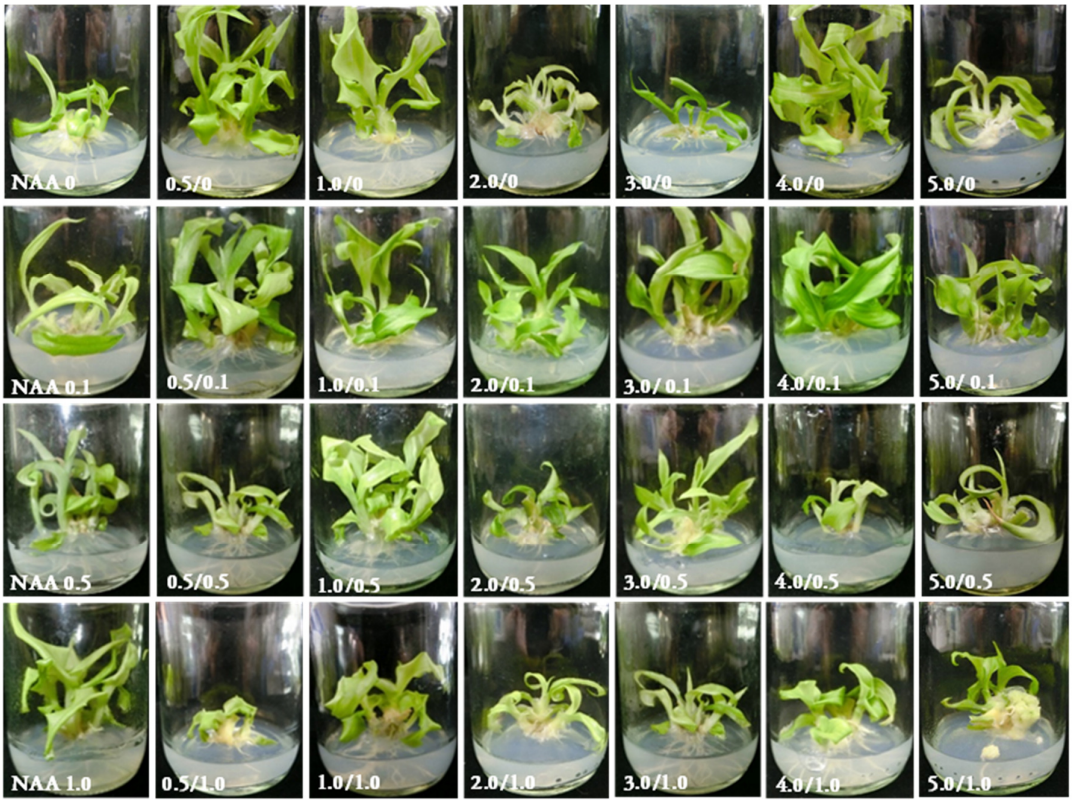

Figure 1. Multiple shoot formation of *K. angustifolia* Roscoe after 8 weeks of cultivation on MS medium supplemented with BA combined with NAA (mg/l)

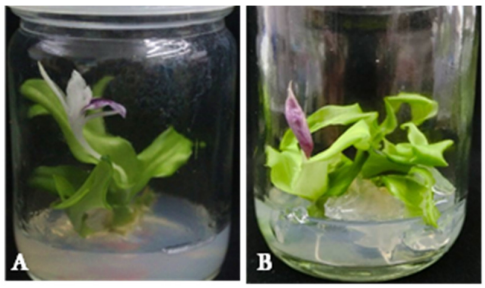

Figure 2. *In vitro* flowering formation of *K. angustifolia* Roscoe after 8 weeks of cultivation on MS medium (A) 5 mg/l BA; (B) 5 mg/l BA plus 0.1 mg/l NAA

Table 2. Effect of combining BA and IAA on shoot and root multiplication of *K. angustifolia*

BA (mg/l)	IAA (mg/l)	Average no. of shoots/explant mean±SE	Average shoot length (cm) mean±SE	Average no. of roots/explant mean±SE	Average root length (cm) mean±SE
0	0	2.70±0.30^{bcd}	3.80±0.27^{b}	13.70±1.31^{abc}	1.45±0.13^{abc}
0.5	0	3.33±0.33^{abcd}	2.58±0.25^{b}	7.78±1.49^{cd}	1.04±0.18^{abc}
1	0	2.78±0.47^{bcd}	2.96±0.32^{b}	9.78±1.96^{bcd}	1.28±0.15^{abc}
2	0	2.67±0.29^{bcd}	2.80±0.38^{b}	8.22±2.05^{cd}	1.09±0.20^{abc}
3	0	2.86±0.46^{bcd}	2.97±0.34^{b}	14.71±1.89^{ab}	1.38±0.06^{abc}
4	0	2.67±0.50^{bcd}	2.53±0.23^{b}	6.89±1.51^{d}	1.03±0.13^{abc}
5	0	3.75±0.45^{abcd}	2.63±0.25^{b}	11.13±2.15^{abcd}	0.93±0.18^{abc}
0	0.1	3.67±0.62^{abcd}	2.37±0.23^{b}	7.22±1.19^{d}	1.12±0.17^{abc}
0.5	0.1	4.14±0.91^{abcd}	3.26±0.22^{a}	16.71±1.11^{a}	1.64±0.13^{ab}
1	0.1	3.78±0.43^{abcd}	2.09±0.17^{b}	12.00±1.72^{abcd}	1.17±0.15^{abc}
2	0.1	3.86±0.14^{abcd}	2.12±0.15^{b}	7.71±1.11^{cd}	1.07±0.20^{abc}
3	0.1	4.00±0.71^{abc}	2.17±0.14^{b}	8.13±1.47^{cd}	1.14±0.22^{abc}
4	0.1	4.29±0.68^{ab}	2.39±0.29^{b}	7.71±1.54^{cd}	1.33±0.20^{abc}
5	0.1	3.00±0.41^{abcd}	2.34±0.23^{b}	9.00±1.78^{bcd}	0.83±0.11^{c}
0	0.5	4.00±0.67^{abc}	2.56±0.33^{b}	9.22±2.13^{bcd}	1.12±0.29^{abc}
0.5	0.5	3.20±0.33^{abcd}	2.73±0.33^{b}	11.60±2.63^{abcd}	1.08±0.17^{abc}
1	0.5	3.50±0.48^{abcd}	2.33±0.28^{b}	6.90±1.95^{d}	1.00±0.22^{abc}
2	0.5	3.80±0.53^{abcd}	2.49±0.21^{b}	7.90±1.30^{cd}	1.32±0.16^{abc}
3	0.5	2.10±0.28^{d}	2.68±0.22^{b}	6.00±1.16^{d}	1.08±0.14^{abc}
4	0.5	3.20±0.33^{abcd}	2.31±0.24^{b}	6.60±1.67^{d}	0.88±0.14^{bc}
5	0.5	2.60±0.37^{bcd}	2.55±0.30^{b}	5.90±1.20^{d}	0.94±0.21^{abc}
0	1	3.50±0.50^{abcd}	2.11±0.29^{b}	8.38±1.48^{cd}	1.67±0.14^{a}
0.5	1	3.33±0.62^{abcd}	1.72±0.18^{b}	8.17±2.73^{cd}	0.83±0.46^{c}
1	1	2.80±0.39^{bcd}	2.14±0.19^{b}	6.80±1.77^{d}	0.84±0.17^{c}
2	1	4.75±0.94^{a}	1.78±0.27^{b}	6.75±2.01^{d}	0.77±0.17^{c}
3	1	4.00±0.45^{abc}	2.85±0.20^{b}	11.70±1.61^{abcd}	1.53±0.85^{abc}
4	1	3.20±0.57^{abcd}	2.52±0.30^{b}	10.00±1.73^{bcd}	1.28±0.17^{abc}
5	1	2.44±0.34^{cd}	2.61±0.34^{b}	9.44±1.79^{bcd}	1.45±0.13^{abc}

Means followed by the same letters within each column are not significantly different at $p<0.05$ according to DMRT

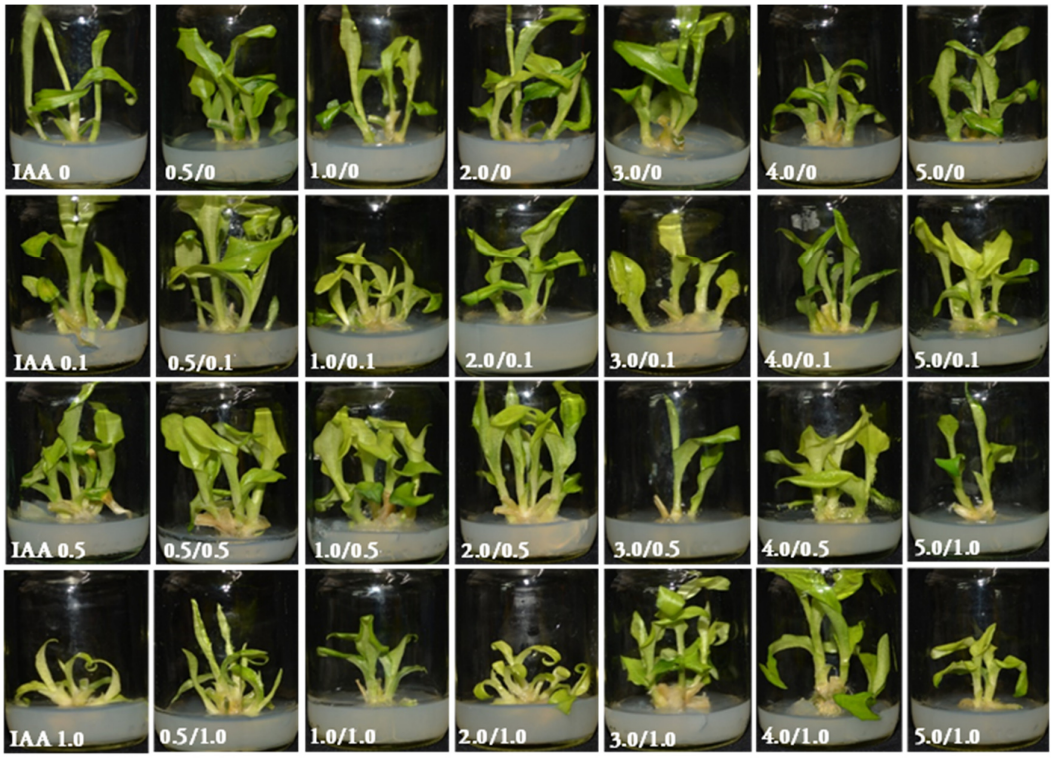

Figure 3. Multiple shoot formation of *K. angustifolia* Roscoe after 8 weeks of cultivation on MS medium supplemented with BA combination with IAA (mg/l)

Table 3. Effect of combining Kinetin and NAA on shoot and root multiplication of *K. angustifolia*

Kinetin (mg/l)	NAA (mg/l)	Average no. of shoots/explant mean±SE	Average shoot length (cm) mean±SE	Average no. of roots/explant mean±SE	Average root length (cm) mean±SE
0	0	3.80±0.20^{abc}	3.93±0.28^{abcd}	20.70±2.93^{bcdef}	1.90±0.15^{abcd}
0.5	0	2.67±0.24^{cdef}	3.45±0.17^{bcde}	20.80±0.94^{bcdefg}	2.03±0.05^{ab}
1	0	3.40±0.50^{abcde}	4.20±0.30^{abc}	16.30±1.84^{bcdefgh}	2.04±0.04^{ab}
2	0	3.10±0.41^{abcdef}	4.59±0.37^{a}	14.30±1.94^{cdefgh}	2.06±0.13^{ab}
3	0	2.89±0.51^{abcdef}	4.38±0.45^{ab}	12.00±1.94^{defgh}	1.68±0.10^{abcdefg}
4	0	3.10±0.46^{abcdef}	3.98±0.23^{abcd}	11.30±1.82^{gh}	1.90±0.17^{abcd}
5	0	2.70±0.42^{cdef}	4.62±0.31^{a}	11.70±1.58^{fgh}	1.94±0.12^{abc}
0	0.1	2.60±0.31^{def}	4.19±0.41^{abc}	16.50±3.05^{bcdefgh}	1.71±0.20^{abcdef}
0.5	0.1	2.80±0.25^{bcdef}	3.08±0.27^{cde}	20.80±0.94^{bcdef}	1.76±0.13^{abcde}
1	0.1	2.10±0.18^{f}	3.31±0.17^{bcde}	8.90±1.02^{h}	2.22±0.22^{a}
2	0.1	3.20±0.29^{abcdef}	3.18±0.25^{cde}	16.80±2.22^{bcdefgh}	1.12±0.14^{ghijk}
3	0.1	3.10±0.38^{abcdef}	4.31±0.27^{ab}	19.80±2.78^{bcdefgh}	1.58±0.21^{bcdefg}
4	0.1	3.22±0.36^{abcdef}	4.05±0.33^{abcd}	16.89±2.47^{bcdefgh}	1.21±0.15^{efghij}
5	0.1	3.00±0.30^{abcdef}	4.17±0.26^{abc}	22.40±1.87^{bc}	1.53±0.12^{bcdefgh}
0	0.5	2.90±0.31^{abcdef}	2.94±0.27^{de}	11.80±2.50^{efg}	0.72±0.15^{jk}
0.5	0.5	2.89±0.31^{abcdef}	3.10±0.30^{cde}	19.78±1.26^{bcdefg}	1.21±0.08^{efghij}
1	0.5	3.30±0.33^{abcde}	3.42±0.32^{bcde}	21.10±3.06^{bcd}	0.99±0.14^{hijk}
2	0.5	2.44±0.34^{ef}	3.87±0.49^{abcde}	20.89±2.68^{bcde}	1.67±0.21^{bcdefg}
3	0.5	2.70±0.21^{cdef}	3.81±0.41^{abcde}	17.20±2.32^{bcdefgh}	1.36±0.22^{defghi}
4	0.5	2.80±0.25^{bcdef}	3.68±0.25^{abcde}	20.80±2.34^{bcdef}	1.34±0.19^{efghi}

5	0.5	4.00±0.30^{a}	3.41±0.17^{bcde}	23.30±1.74^{bc}	1.48±0.12^{cdefgh}
0	1	3.30±0.33^{abcde}	3.79±0.42^{abcde}	25.10±2.63^{b}	1.12±0.21^{ghijk}
0.5	1	3.20±0.20^{abcdef}	2.98±0.22^{de}	21.90±1.15^{bc}	1.48±0.12^{fghijk}
1	1	2.70±0.40^{cdef}	4.18±0.59^{abc}	18.10±3.64^{bcdefg}	0.84±0.19^{ijk}
2	1	3.90±0.35^{ab}	4.32±0.25^{ab}	35.70±2.67^{a}	1.19±0.14^{fghij}
3	1	3.22±0.36^{abcdef}	3.70±0.47^{abcde}	22.00±4.95^{bc}	1.23±0.34^{efghij}
4	1	3.56±0.18^{abcde}	2.79±0.29^{e}	15.44±3.04^{cdefgh}	0.65±0.13^{k}
5	1	3.67±0.24^{abcd}	2.97±0.23^{de}	18.00±2.07^{bcdefg}	0.92±0.13^{ijk}

Means followed by the same letters within each column are not significantly different at $p<0.05$ according to DMRT

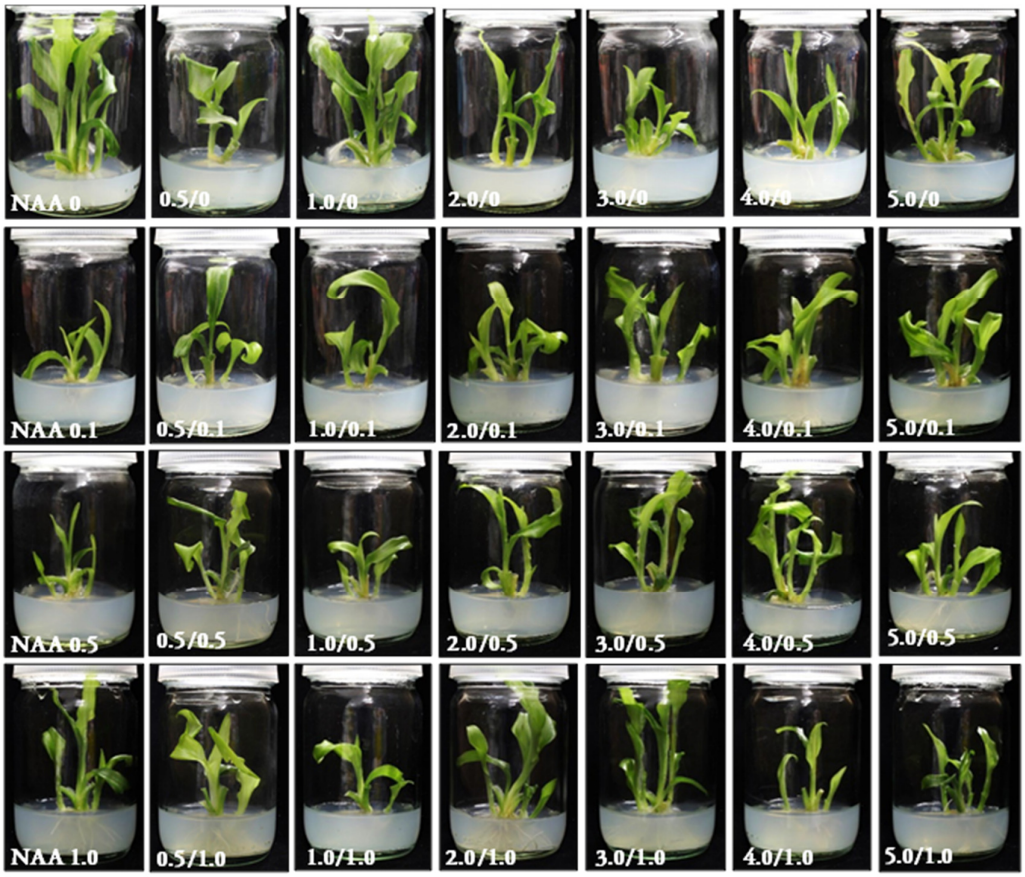

Figure 4. Multiple shoot formation of *K. angustifolia* Roscoe after 8 weeks of cultivation on MS medium supplemented with Kinetin combined with NAA (mg/l)

Table 4. Effect of solid MS media (MS, ½ MS and ¼ MS) supplemented with 2 mg/l BA plus 2 mg/l kinetin and 1 mg/l NAA on shoot and root multiplication of *K. angustifolia*

Solid MS media	Average no. of shoots/explant mean±SE	Average shoot length (cm) mean±SE	Average no. of roots/explant mean±SE	Average root length (cm) mean±SE
MS	4.13±0.35^{a}	2.65±0.07^{a}	24.63±1.30^{a}	0.95±0.05^{b}
½ MS	4.11±0.26^{a}	2.30±0.17^{a}	24.33±1.72^{a}	1.11±0.08^{ab}
¼ MS	3.36±0.36^{a}	1.92±0.10^{b}	18.00±1.40^{b}	1.28±0.10^{b}

Means followed by the same letters within each column are not significantly different at $p<0.05$ according to DMRT

Table 5. Effect of liquid MS media (MS, ½ MS and ¼ MS) supplemented with 2 mg/l BA plus 2 mg/l Kinetin and 1 mg/l NAA on shoot and root multiplication of *K. angustifolia*

Liquid MS media	Average no. of shoots/explant mean±SE	Average shoot length (cm) mean±SE	Average no. of roots/explant mean±SE	Average root length (cm) mean±SE
MS	8.73±0.73[a]	4.96±0.21[a]	29.67±1.56[a]	2.74±0.14[a]
½ MS	7.73±0.71[a]	3.92±0.17[b]	28.73±2.03[a]	2.84±0.25[a]
¼ MS	4.83±0.42[b]	3.32±0.21[c]	27.58±3.16[a]	1.99±0.26[b]

Means followed by the same letters within each column are not significantly different at $p<0.05$ according to DMRT

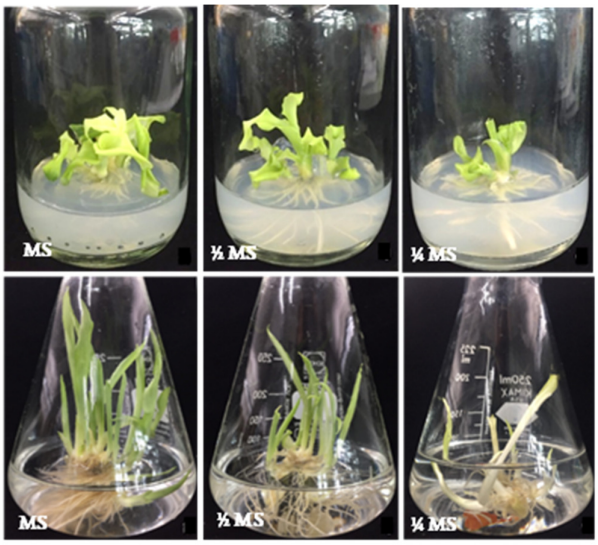

Figure 5. Effect of solid and liquid MS media (MS, ½ MS and ¼ MS) supplemented with 2 mg/l BA plus 2 mg/l Kinetin and 1 mg/l NAA on shoot and root multiplication of *K. angustifolia*

Table 6. Effect of potting media on plantlet performance of *K. angustifolia* after 8 weeks of acclimatization

Plant material	Percentage of surviving plantlets (%)	Average no. of shoots/explant mean±SE	Average shoot length (cm) mean±SE	Average no. of leaves/explant mean±SE	Average width of leaves/explant mean±SE	Average length of leaves/explant mean±SE
Soil	100	5.20±0.26[a]	8.33±0.34[a]	10.50±0.37[a]	1.69±0.07[a]	6.79±0.24[a]
Sand	100	2.95±0.25[bc]	5.10±0.38[c]	5.26±0.25[b]	1.35±0.05[b]	5.08±0.19[c]
Soil:sand	100	3.65±0.27[b]	6.30±0.50[b]	5.46±0.26[b]	1.50±0.05[b]	5.82±0.24[b]
Soil:small pieces of rock	100	2.90±0.22[c]	6.30±0.32[b]	5.67±0.12[b]	1.72±0.05[a]	7.02±0.23[a]

Means followed by the same letters within each column are not significantly different at $p<0.05$ according to DMRT

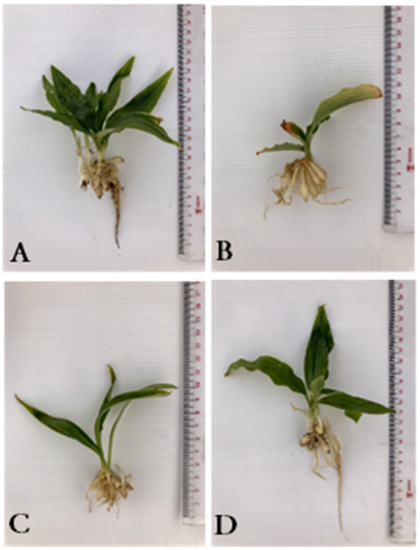

Figure 6. Acclimatization of *K. angustifolia* plantlets 8 weeks after transfer to pots in a greenhouse (A) soil; (B) sand; (C) soil: sand (1:1); (D) soil: small pieces of rock (1:1)

Phytochemical profiling analysis

Total phenolic contents and total flavonoid contents

Total phenolic contents (TPC) and total flavonoid contents (TFC) were determined in both natural and *in vitro* derived plant parts with high variation. The rhizome and storage root of *ex vitro* plantlets exhibited highest TPC (139.70 mg GAE/100 g DW), followed by pseudostem of natural plants (74.41 mg GAE/100 g DW). The leaves of micropropagated plants presented the lowest TPC of 22.46 mg GAE/100 g DW. There were significant variations in TFC for different plant organs, ranging from 23.08 mg RE/100 g DW to 343.08 mg RE/100 g DW. Highest TFC were recorded in the leaves of natural plants (343.08 mg RE/100 g DW), followed by the leaves of *in vitro* propagated plants cultured on MS medium supplemented with 2 mg/l BA and 0.1 mg/l NAA (323.59 mg RE/100 g DW), and the leaves of *in vitro* propagated plants cultured on MS medium without plant growth regulators (207.69 mg RE/100 g DW). Lowest TPC were found in pseudostem of natural plants (23.08 mg RE/100 g DW) (Table 7).

Antioxidant activity

Results showed that 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric reducing antioxidant power (FRAP) of different plant parts of *K. angustifolia* were significantly different at $p < 0.05$ between natural plants and *in vitro* derived plants. Different types of propagation (natural or micropropagation) and different plant parts significantly affected the DPPH scavenging activity and FRAP values. Highest DPPH scavenging activity (323.46 mg Trolox/100 g DW) and FRAP (166.00 mg $FeSO_4$/100 g DW) were found in leaves of *in vitro* regenerated plants cultured on MS medium added with 2 mg/l BA and 0.1 mg/l NAA, followed by leaves of natural plants 295.96 mg Trolox/100 g DW and 155.96 mg $FeSO_4$/100 g DW, respectively. Lowest DPPH scavenging activity (98.46 mg Trolox/100 g DW) and FRAP (2.00 mg $FeSO_4$/100 g DW) were recorded in pseudostem of natural plants (Table 7).

Table 7. TPC, TFC, DPPH and FRAP in methanolic extract of *K. angustifolia* by maceration method from different explant parts

Explant	Condition	TPC (mg GAE/100 g DW) mean±SE	TFC (mg RE/ 100 g DW) mean±SE	DPPH (mg Trolox/100 g DW) mean±SE	FRAP (mg $FeSO_4$/100 g DW) mean±SE
Leaf	MS	22.46±0.23^{c}	207.69±1.78^{c}	252.21±1.50^{c}	60.00±1.15^{c}
Leaf	MS+BA 2 mg/l+ NAA 0.1 mg/l	39.70±1.61^{c}	323.59±5.71^{b}	323.46±1.50^{a}	166.00±1.15^{a}
Leaf	Natural	28.90±1.19^{d}	343.08±4.70^{a}	295.96±5.61^{b}	156.00±5.03^{b}
Pseudostem	Natural	74.41±1.44^{b}	23.08±1.78^{d}	98.46±2.73^{c}	2.00±0.00^{d}
Rhizome and storage root	Natural	139.70±2.65^{a}	26.15±1.78^{d}	155.96±4.64^{d}	7.33±0.67^{d}

Means followed by the same letters within each column are not significantly different at $p \leq 0.05$ according to DMRT

Determination of volatile compounds by GC-MS analysis

Comparisons were made between the leaves of *K. angustifolia* natural plants and leaves of *in vitro* regenerated plants when cultured under different concentrations of PGRs by GC-MS analysis to identify the phytochemical constituents. Fifty-two volatile compounds were detected, as listed in Table 8, with 34 found in natural plants, 19 in MS medium without PGRs, 13 in MS medium supplemented with 1 mg/l BA and 0.5 mg/l IAA, 5 in MS medium supplemented with 1 mg/l BA and 0.1 mg/l IAA, and 4 in MS medium supplemented with 3 mg/l BA and 1 mg/l IAA.

Table 8. Volatile compounds in leaves of *K. angustifolia* determined by GC-MS analysis

No.	Volatile compound	RI	Molecular formula	Molecular weight	Peak area (%)				
					Natural	MS	BA 1 mg/l +IAA 0.1 mg/l	BA 1 mg/l +IAA 0.5 mg/l	BA 3 mg/l +IAA 1 mg/l
1	l-Gala-l-ido-octose	2221	$C_8H_{16}O_8$	240					8.15
2	Octadecane, 1-chloro-	2036	$C_{18}H_{37}Cl$	288	0.82				
3	Humulene	1579	$C_{15}H_{24}$	204	0.76	0.51			
4	Caryophyllene oxide	1507	$C_{15}H_{24}O$	220	0.38				
5	Caryophyllene	1494	$C_{15}H_{24}$	204	13.43	9.49	4.46	8.67	11.02
6	Naphthalene	1481	$C_{15}H_{24}$	204	0.29	1.02			
7	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-	1474	$C_{15}H_{24}$		2.30				
8	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	1469	$C_{15}H_{24}$	204	0.43				
9	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4aα,7α,8aβ)]-	1469	$C_{15}H_{24}$	204	0.80				
10	Alpha-farnesene	1458	$C_{15}H_{24}$	204				1.49	
11	Isoledene	1419	$C_{15}H_{24}$	204		0.62			
12	Alpha-cubebene	1344	$C_{15}H_{24}$	204	0.05	0.24			
13	Alpha-copaene	1221	$C_{15}H_{24}$	204	0.07	0.23			
14	Beta-copaene	1216	$C_{15}H_{24}$	204		1.34			
15	2-Decanone	1151	$C_{10}H_{20}O$	156	0.41				

16	Terpinen-4-ol	1137	$C_{10}H_{18}O$	154	0.15				
17	3-Fluorophenylacetone	1103	C_9H_9FO	152				2.74	
18	l-Alanine ethylamide	1097	$C_5H_{12}N_2O$	116		12.63	80.79	54.10	73.80
19	Linalool	1082	$C_{10}H_{18}O$	154	0.77	0.47			
20	Eucalyptol	1059	$C_{10}H_{18}O$	154		4.11			
21	D-Limonene	1018	$C_{10}H_{16}$	136	1.37	2.84			
22	Gamma.-terpinene	998	$C_{10}H_{16}$	136	0.10				
23	3-Hexen-1-ol,	989	$C_7H_{12}O_2$	128	1.96				
24	Beta-myrcene	985	$C_{10}H_{16}$	136	14.77	26.74			
25	3-Carene	984	$C_{10}H_{16}$	136	0.23				
26	Formic acid, hexyl ester	981	$C_7H_{14}O_2$	130	0.27				
27	Beta-ocimene	976	$C_{10}H_{16}$	136	0.75	0.83			
28	Trans-beta-ocimene	976	$C_{10}H_{16}$	136	0.56				
29	(1R)-2,6,6-Trimethylbicyclo [3.1.1]hept-2-ene	948	$C_{10}H_{16}$	136	3.59	8.33			
30	2-Carene	948	$C_{10}H_{16}$	136	0.10				
31	Camphene	943	$C_{10}H_{16}$	136	0.18	0.48			
32	β-Pinene	943	$C_{10}H_{16}$	136	20.59	28.03			0.98
33	Carene	919	$C_{10}H_{16}$	136	0.08	0.17			
34	(+)-Sabinene	897	$C_{10}H_{16}$	136	5.67	1.08			
35	Pentane, 1-(2-propenyloxy)-	882	$C_8H_{16}O$	128				3.24	
36	Trans-2,3-epoxyoctane	869	$C_8H_{16}O$	128				2.19	
37	2-Hexenal, (E)-	814	$C_6H_{10}O$	98	3.24				
38	Hexanal	806	$C_6H_{12}O$	100	1.68				
39	Furan, 2-ethyl-	742	C_6H_8O	96	1.12				
40	Pentanal	707	$C_5H_{10}O$	86	0.15			1.57	
41	Propane, 1-nitro-	701	$C_3H_7NO_2$	89		0.65			
42	1-Penten-3-ol	671	$C_5H_{10}O$	86	0.27				
43	Cyclobutanol	668	C_4H_8O	72				2.60	
44	3-Butyn-1-ol	659	C_4H_6O	70	21.87				
45	Oxiranemethanol, (S)-	653	$C_3H_6O_2$	74					6.05
46	Butanal, 2-methyl-	643	$C_5H_{10}O$	86				0.65	
47	2-Propen-1-ol, 3,3-difluoro-, acetate	616	$C_5H_6F_2O_2$	136				5.39	
48	2-Butanone, 3-methyl-	590	$C_5H_{10}O$	86	0.20				
49	Propanal, 2-methyl-	543	C_4H_8O	72			0.50	2.88	
50	2,3-Epoxybutane	471	C_4H_8O	72				3.14	
51	Trimethylsilyl trifluoroacetate	430	$C_5H_9F_3O_2Si$	186			4.82		
52	Acetaldehyde	408	C_2H_4O	44			9.43	11.33	
Total					34	19	5	13	4

Discussion

Suitable concentrations of auxin and cytokinin in the culture medium are important factors for shoot and root formation. This study exhibited shoot induction of 6.33±0.25 shoots/explant with MS medium supplemented with 2 mg/l BA plus 0.5 mg/l NAA within 8 weeks. BA is a cytokinin hormone commonly used to induce shoot and root multiplication of plants in the genus *Kaempferia* (Zingiberaceae) such as *Kaempferia galanga* (Shirin *et al*., 2000; Rahman *et al*., 2005; Kalpana and Anbazhagan, 2009; Parida *et al*., 2010; Mohanty *et al*., 2011; Ibemhal *et al*., 2012; Kochuthressia *et al*., 2012; Bhattacharya and Sen, 2013; Sahoo *et al*., 2014; Anbazhagan *et al*., 2015), *Kaempferia marginata* (Saensouk *et al*., 2016), *Kaempferia parviflora* (Park *et al*., 2021) and *Kaempferia siamensis* (Nonthalee *et al*., 2022). Our results differed from Rahman *et al.* (2022) who reported that young shoots of *K. angustifolia* cultured on MS medium with a combination of 5 mg/l BAP and 0.5 mg/l NAA resulted in 5.80±0.83 shoots/explant after 60 days of culture. The culture medium supplemented with cytokinins (BA and Kinetin) displayed a strong effect on the proliferation rate of micropropagation. BA and Kinetin hormones are most popularly used as PGRs for micropropagation, while cytokinins mediate the responses to irrelevant factors such as light conditions in the shoot and accessibility of nutrients and water in the root (Werner and Schmulling, 2009). BA increases the level of cytokinins in plants, protects pith tissue, prolongs active photosynthetic duration, and improves photosynthetic efficiency factors (Ren *et al.*, 2016), while Kinetin maintains the stability of chlorophyll and enhances the activities of antioxidant enzymes (Wang *et al.*, 2014). These results differed from Kalpana and Anbazhagen (2009) who reported that medium supplemented with 2 mg/l BA plus 0.2 mg/l NAA gave optimal shoot induction (19.40±0.42 shoots/explant) in *Kaempferia galanga*. In our study, using PGRs in combination or individually induced shoot and root formation. NAA and IAA are PGRs in the auxin group and are often applied in plant tissue culture. They are also rooting agents and are used for vegetative propagation of plants from stem and leaf explants (Khandaker *et al.*, 2017). The number of shoots induced per explant depended on the ratio of cytokinin and auxin combinations used during shoot induction propagation. Cytokinins regulate plant growth by exerting differential influence on the number or duration of cell division cycles in the root and shoot meristems (Werner *et al.*, 2001), while auxins play an important role in the growth of root, apical dominance and plant senescence, and are associated with cell expansion, cell division and cell differentiation (Vadassery *et al.*, 2008). In this study, high concentrations of BA induced flowering of *K. angustifolia*. The application of cytokinins induces molecular changes associated with the floral transition growth regulator. Cytokinins and auxins induce different results in different species and also induce the same response in a wide range of species.

The effect of different strengths (full, half, quarter) of solid and liquid MS medium added with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA were investigated. Results showed that in solid MS medium or ½ MS medium supplemented with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA produced the number of shoots of *K. angustifolia* at 4.13 and 4.11 shoots/explant, respectively but less than when cultured on liquid medium. In liquid MS medium or ½ MS medium supplemented with the same concentration of BA, Kinetin and NAA produced the highest number of shoots of *K. angustifolia* at 8.73 and 7.73 shoots/explant, respectively but with no significant statistical difference. Therefore, ½ MS medium can be used instead of full-strength MS medium for shoot and root induction to decrease the cost of plant tissue culture. The efficiency of the MS medium in producing shoots and root formation was the same for both half and full-strength MS media in *K. angustifolia*. These results differed from Haque and Ghosh (2018) who reported that rhizome bud of *K. angustifolia* showed the highest number of shoots (6.60 shoots/explant) when cultured on solid MS medium supplemented with 2 mg/l BA, 2 mg/l Kinetin and 1 mg/l NAA and 2.0 mM spermidine. One disadvantage of culturing explants in solid media is that they are only exposed to one side of the media and do not receive the same level of illumination. Explants on the surface of the media are exposed to more light than explants submerged in the media. As a result, not all cultured explants grew at the same rate. By contrast, culture in liquid media ensures that all parts of the explant are in contact with the media and absorb nutrients, with the tissues developing

uniformly (Mehrotra *et al.,* 2007). In the present study, it was found that when cultured the plantlet of *K. angustifolia* in liquid medium showing higher average number of shoots, shoot length, average number of roots and root length than a solid medium. These results concurred with Stanly and Keng (2007) in *Curcuma zedoaria* and *Zingiber zerumbet*; Chong *et al.* (2012) in *C. zedoaria*; Alizah *et al.* (2019) in *Curcuma aeruginosa* and Yaowachai *et al.* (2020) in *Globba globulifera*. Therefore, explants cultured in liquid medium produce more shoots and grow faster than those cultured in solid medium.

In vitro plantlets of *K. angustifolia* derived using the plant tissue culture technique produced new shoots, roots and leaves. In this study, *in vitro* plantlets produced healthy plants, morphologically similar to the natural plants after transfer to the field. In the wild, *K. angustifolia* grows in areas of soil mixed with rocks. The *in vitro* plantlets transplanted into soil mixed with small pieces of rock (1:1) had flatter leaves than plants in other planting materials. Small pieces of rock mixed with the soil enhance drainage, ventilation, and plant material density. These findings varied from Parida *et al.* (2010) who reported that 95% of *K. galanga* plantlets transplanted to pots containing a 1:1:1 mixture of soil, cow dung and sand survived and developed to maturity in field conditions.

This is the first report on the phytochemical profile of leaves of *K. angustifolia*. The rhizome and storage root of natural plants of *K. angustifolia* showed highest the TPC. Highest TFC was recorded in the leaves of natural plants, followed by the leaves of *in vitro* propagated plants (cultured on MS medium added with 2 mg/l BA and 0.1 mg/l NAA), and the leaves of *in vitro* propagated plants (cultured on MS medium without PGRs). Yeap *et al.* (2017) found that chloroform and methanolic extracts of the rhizomes of *K. angustifolia* showed strong free radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH), with values of 616±17 and 616±13 mg Trolox/g extract, respectively. The strengths of DPPH free radical scavenging extract activities were chloroform and methanolic extracts > ethyl acetate extracts > hexane extracts, with ethyl acetate extracts showing the strongest reducing power in the FRAP assay of 342±41 mg Trolox/g extract. In this study, antioxidant activities by DPPH and FRAP assays showed better results in leaves of *in vitro* derived plants and natural plants. The leaves of *in vitro* regenerated plant extracts (cultured on MS medium added with 2 mg/l BA and 0.1 mg/l NAA) exhibited strong antioxidant properties in both DPPH and FRAP assays, further supporting wide applicability for medicinal purposes. The leaf extract from *in vitro* culture may be an alternative to the plant extract.

Gas chromatography profiles of volatile oils from natural plants and *in vitro* regenerated plants of *K. angustifolia* were different, especially when cultured on MS medium supplemented with different types and concentrations of PGRs. GC-MS analysis of the leaf extracts in natural plants and *in vitro* regenerated plant extracts (MS, MS+BA 1 mg/l+IAA 0.1 mg/l, MS+BA 1 mg/l+IAA 0.5 mg/l, MS+BA 3 mg/l+IAA 1 mg/l) of *K. angustifolia* found only l-alanine ethylamide from leaf extracts of *in vitro* regenerated plants but not in leaves of natural plants. Caryophyllene was found in the leaves of natural plants and *in vitro* regenerated plants of *K. angustifolia*. The 14 phytochemical compounds identified in leaves from *in vitro* regenerated plants (cultured on MS medium) were identical to those found in leaves of natural plants. GC-MS analysis of leaf extracts of natural plants presented more volatile compounds than *in vitro* regenerated plants. The medicinal activities of these compounds as anti-inflammatory, antibacterial, antifungal, anticancer, antioxidant, antiparasitic, antitumor and antidiabetic are reported in Table 9. Our results were contrary to Yeap *et al.* (2017) who reported boesenboxide, crotepoxide, 2´-hydroxy-4,4´,6´-trimethoxychalcone, kaempfolienol and zeylenol in the rhizome of *K. angustifolia* from Java, Indonesia using liquid chromatography-mass spectrometry (LC-MS), while Tang *et al.* (2014) also reported 8 phytochemical compounds; kaempfolienol, crotepoxide, boesenboxide, 2'-hydroxy-4,4',6'-trimethoxychalcone, zeylenol, 6-methylzeylenol, (24S)-24-methyl-5α-lanosta-9(11), 25-dien-3β-ol and sucrose, β-sitosterol, and its glycoside in the rhizome of *K. angustifolia* from Java, Indonesia using mass spectrometry (MS). Nonthalee *et al.* (2023) used comparative phytochemical

profiling (GC-MS and HPLC) to evaluate the antioxidant activities of wild, *in vitro* cultured, and greenhouse plants of *Kaempferia grandifolia* and *Kaempferia siamensis* from Thailand. Their GC-MS results revealed 38 compounds in *K. grandifolia* and 19 compounds in *K. siamensis*. Major constituents in the leaf and rhizome extracts were 9-octadecenamide, (Z)-, hexadecanamide, and octadecanamide. They also found that TPC and TFC of leaf extracts decreased from wild to *in vitro* cultured and greenhouse plants, respectively whereas rhizome extract contents increased from wild to greenhouse plants. Antioxidant activities of rhizome extracts showed higher activity than leaf extracts in DPPH and ABTS assays. Different extraction solvents, plant organs, and plant conditions are important factors that influence the contained volatile compounds. Leaves of *in vitro* regenerated plants showed potential as suitable for industrial pharmacy use as an alternative material to natural plants.

Table 9. Phytochemical analysis of leaves of *K. angustifolia* and their medicinal activity in methanolic extract

Compound name	Medicinal activity
Caryophyllene oxide	Analgesic, anti-inflammatory (Chavan *et al*., 2010)
Caryophyllene	Antitumor, analgesic, antibacterial, anti-inflammatory, sedative, fungicide (Gopalakrishnan and Vadivel, 2011)
Alpha-farnesene	Antioxidant (Devi *et al*., 2018)
Alpha-copaene	Antibacterial (Shareef *et al*., 2016)
Beta-copaene	Antibacterial (Al-Rekaby and Atiyah, 2020)
Terpinen-4-ol	Antifungal (Mondello *et al*., 2006)
Linalool	Anti-inflammatory, anticancer, antihyperlipidemic, antimicrobial, antinociceptive, analgesic, anxiolytic, antidepressant and neuroprotective properties (Pereira *et al*., 2018)
Eucalyptol	Against respiratory disorders (Mao *et al*., 2019)
D-Limonene	Antioxidant, anticancer (Ajayi *et al*., 2019)
Gamma-terpinene	Antioxidant (Babri *et al*., 2015)
3-Carene	Antimicrobial, antioxidant, anticancer (Kang *et al*., 2019)
Camphene	Antibacterial, antifungal, anticancer, antioxidant, antiparasitic, antidiabetic, anti-inflammatory, hypolipidemic activity (Hachlafi *et al*., 2021)
3-Butyn-1-ol	Antibacterial, anticancer, antifibrinolytic, antimalarial (Alabi *et al*., 2019)

Conclusions

This is the first report describing the micropropagation of *K. angustifolia* Roscoe from shoot explants using the tissue culture technique. *In vitro* micropropagation of *K. angustifolia* from shoot explants in MS medium supplemented with 2 mg/l BA plus 0.5 mg/l NAA regenerated multiple shoots (6.33±0.25 shoots/explant) after 8 weeks. MS medium supplemented with 2 mg/l Kinetin plus 1 mg/l NAA was recommended for rooting (35.70±2.67 roots/explant). Soil was chosen as the most suitable potting material for the acclimatization of *K. angustifolia*. *In vitro* plantlets derived from the tissue culture technique produced healthy plants that were morphologically similar to the natural plants after transfer to the field. TFC and antioxidant activities (DPPH and FRAP assays) of *K. angustifolia* leaf extract from natural plants and *in vitro* derived plants were higher than rhizome (with storage root) and pseudostem for medicinal purposes. GC-MS analysis on the methanolic extract identified 34 compounds from leaf samples of natural plants, more than found in *in vitro* cultured plants. Only one compound (caryophyllene) was found in both natural and *in vitro* regenerated plants. Results identified the potential of using leaves of *K. angustifolia* from natural and *in vitro* cultured plants as a suitable substitute in the pharmaceutical industry.

Authors' Contributions

Conceptualization PS and SS; Data curation PS, WY, TC, SN and SS; Formal analysis PS, SS, WY, TC and SN; Funding acquisition PS; Investigation PS, WY, TC, SN and SS; Methodology PS, WY and TC; Project administration PS and SS; Resources SS and PS; Software PS, WY, TC and SN; Supervision PS and SS; Validation PS, SS and TC; Visualization PS and WY; Writing - original draft PS and WY; Writing - review and editing PS, SS, WY, TC and SN. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

We would like to thank the Department of Biology, Faculty of Science, Mahasarakham University for use of facilities. This research project was financially supported by Thailand Science Research and Innovation (TSRI). We would like to thank Mr. Peter Charge for language editing, with thanks also to Tippawan Srithet, Alisa Suthimoon and Thitima Boonchu for assistance with plant tissue culture.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Ajayi GO, Akinsanya MA, Agbabiaka AT, Oyebanjo KS, Hungbo TD, Olagunju JA (2019). D-Limonene: a major bioactive constituent in *Allium fistulosum* identified by GC-MS analysis. The Journal of Phytopharmacology 8(5):257-59. *https://doi.org/10.31254/phyto.2019.8509*

Alabi OS, Koleoso OB, Abiala AM (2019). Antimicrobial screening and GC-MS analysis of bioactive compounds from strains of *Pseudomonas aeruginosa* isolated from poultry fecal littered soil in Ibadan, Nigeria. Journal of Pure and Applied Science 32(1):3347-57. *https://doi.org/10.6084/m9.figshare.12278864*

Alizah Z, Nurulaishah Y, Adilah A (2019). *In vitro* propagation of *Curcuma aeruginosa* Roxb in liquid culture. South Asian Research Journal of Biology and Applied Biosciences 1(3):87-89. *https://doi.org/10.36346/SARJBAB.2019.v01i03.006*

Al-Rekaby LS, Atiyah KM (2020). Antibacterial activities for root extracts of (goldenrods) *Solidago canadensis* L. treated by nano and bio fertilizer. Al-Qadisiyah Journal of Pure Science 25(4):1-14. *https://doi.org/10.29350/qjps.2020.25.4.1201*

Anbazhagan M, Balachandran B, Arumugam K, Nagar A (2015). *In vitro* propagation of *Kaempferia galanga* (L.)-an endangered medicinal plant. Journal of Phytology 15:63-69.

Ashmore SE (1997). Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. International Plant Genetic Resources Institute, Rome.

Babri RA, Khokhar I, Mahmud S (2015). Antioxidant potential and iron chelating activity of some bioactive compounds. Journal of the Chemical Society of Pakistan 37(3):514-519.

Bhattacharya M, Sen A (2013). *In vitro* regeneration of pathogen free *Kaempferia galanga* L. -a rare medicinal plant. Research in Plant Biology 3(3):24-30.

Chavan MJ, Wakte PS, Shinde DB (2010). Analgesic and anti-inflammatory activity of caryophyllene oxide from *Annona squamosa* L. Bark. Phytomedicine 17(2):149-51. *https://doi.org/10.1016/j.phymed. 2009.05.016*

Chong YH, Khalafalla MM, Bhatt A, Chan LK (2012). The effects of culture systems and explant incision on *in vitro* propagation of *Curcuma zedoaria* Rosc. Pertanika Tropical Agricultural Science 35(4):863-874.

Devi RB, Barkath TN, Vijayaraghavan P, Rejiniemon TS (2018). GC-MS analysis of phytochemical from *Psidium guajava* Linn. leaf extract and their *in vitro* antimicrobial activities. International Journal of Pharmacy and Biological Sciences 8(1):583-589.

Gopalakrishnan S, Vadivel E (2011). GC-MS analysis of some bioactive constituents of *Mussaenda frondosa* Linn. International Journal of Pharma and Bio Sciences 2(1):313-320.

Hachlafi NEL, Aanniz T, Menyiy NEl, Baaboua AEl, Omari NEl, Balahbib A, ... Bouyahya A. (2021). *In vitro* and *in vivo* biological investigations of camphene and its mechanism insights: a review. Food Reviews International 2-28. *https://doi.org/10.1080/87559129.2021.1936007*

Haque SM, Ghosh B (2018). Micropropagation of *Kaempferia angustifolia* Roscoe-an aromatic, essential oil yielding, underutilized medicinal plant of Zingiberaceae family. Journal of Crop Science and Biotechnology 21(2):147-53. *https://doi.org/10.1007/s12892-017-0051-0*

Ibemhal A, Laishram JM, Dhananjoy C, Naorem B, Toijam R (2012). *In vitro* induction of multiple shoot and root from the rhizome of *Kaempferia galanga* L. An International Journal of Environment and Biodiversity 3(3):46-50.

Kalpana M, Anbazhagan M (2009). *In vitro* production of *Kaempferia galanga* L.-an endangered medicinal plant. Journal of Phytology 1(1):56-61.

Kang GQ, Duan WG, Lin GS, Yu YP, Wang XY, Lu SZ (2019). Synthesis of bioactive compounds from 3-Carene (II): synthesis, antifungal activity and 3D-QSAR study of (Z)- and (E)-3-caren-5-one oxime sulfonates. Molecules 24(3):1-14. *https://doi.org/10.3390/molecules24030477*

Khandaker MM, Rasdi MZMD, Naeimah NN, Mat N (2017). Effects of naphthalene acetic acid (NAA) on the plant growth and sugars effects on the cut flowers *Mokara chark kuan* orchid. Bioscience Journal 33(1):19-30. *https://doi.org/10.14393/BJ-v33n1a2017-34908*

Kochuthressia KP, Britto SJ, Jaseentha MO (2012). *In vitro* multiplication of *Kaempferia galanga* L. an endangered species. International Research Journal of Biotechnology 3(2):27-31.

Mao QQ, Xu XY, Cao SY, Gan RY, Corke H, Beta T, Li HB (2019). Bioactive compounds and bioactivities of ginger (*Zingiber officinale* Roscoe). Foods 8(185):1-21. *https://doi.org/10.3390%2Ffoods8060185*

Mehrotra, S, Goel MK, Kukreja AK and Mishra BN (2007). Efficiency of liquid culture systems over conventional micropropagation: a progress towards commercialization. African Journal of Biotechnology 6(13):1484-1492.

Mohanty S, Panda M, Sahoo S, Nayak S (2011). Micropropagation of *Zingiber rubens* and assessment of genetic stability through RAPD and ISSR markers. Biologia Plantarum 55(1):16-20. *https://doi.org/10.1007/s10535-011-0002-1*

Mondello F, Bernardis FD, Girolamo A, Cassone A, Salvatore G (2006). *In vivo* activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and-resistant human pathogenic *Candida* species. BMC Infectious Diseases 6(158):1-8. *https://doi.org/10.1186/1471-2334-6-158*

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay tobacco tissue culture. Physiologia Plantarum 15:473-497. *https://doi.org/10.1111/j.1399-3054.1962.tb08052.x*

Nonthalee S, Maneechai S, Saensouk S, Saensouk P (2022). *In vitro* propagation, microrhizome induction, and evaluation of genetic variation by RAPD markers of *Kaempferia siamensis* Sirirugsa. Propagation of Ornamental Plants 22(1):11-22.

Nonthalee S, Maneechai S, Saensouk S, Saensouk P (2023). Comparative phytochemical profiling (GC-MS and HPLC) and evaluation of antioxidant activities of wild, *in vitro* cultured and greenhouse plants of *Kaempferia grandifolia* Saensouk and Jenjitt and *Kaempferia siamensis* Sirirugsa; rare plant species in Thailand. Pharmacognosy Magazine 19(1):1-12. *https://doi.org/10.1177/09731296221145066*

Parida R, Mohanty S, Kuanar A, Nayak S (2010). Rapid multiplication and *in vitro* production of leaf biomass in *Kaempferia galanga* through tissue culture. Electronic Journal of Biotechnology 13(4):1-8. *https://doi.org/10.2225/vol13-issue4-fulltext-12*

Park HY, Kim K, Ak G, Zengin G, Cziáky Z, Jekő J, Adaikalam K, Song K, Kim DH, Sivanesan I (2021). Establishment of a rapid micropropagation system for *Kaempferia parviflora* Wall. Ex Baker: phytochemical analysis of leaf extracts and evaluation of biological activities. Plants, 10(698):2-28. *https://doi.org/10.3390/plants10040698*

Pereira I, Severino P, Santos AC, Silva AM, Souto EB (2018). Linalool bioactive properties and potential applicability in drug delivery systems. Colloids and Surfaces B: Biointerfaces 171(1):566-578. *https://doi.org/10.1016/j.colsurfb.2018.08.001*

Rahman MM, Amin MN, Ahamed T, Ahmad S (2005). *In vitro* rapid propagation of black thorn (*Kaempferia galanga* L.): a rare medicinal and aromatic plant of Bangladesh. Journal of Biological Sciences 5(3):300-304. *https://doi.org/10.3923/jbs.2005.300.304*

Rahman ZA, Othman1 AN, Ghazalli MN, Adlan NAS (2022). Micropropagation of *Kaempferia angustifolia* Roscoe via direct regeneration. American Journal of Plant Sciences 13:734-43. *https://doi.org/10.4236/ajps.2022.136049*

Ren B, Zhu Y, Zhang J, Dong S, Liu P, Zhao B (2016). Effects of spraying exogenous hormone 6-benzyladenine (6-BA) after water logging on grain yield and growth of summer maize. Field Crops Research 188:96-04. *https://doi.org/10.1016/j.fcr.2015.10.016*

Saensouk P, Muangsan N, Saensouk S, Sirinajun P (2016). *In vitro* propagation of *Kaempferia marginata* Carey ex Roscoe, a native plant species to Thailand. The Journal of Animal and Plant Sciences 26(5):1405-1410.

Sahoo S, Parida R, Singh S, Padhy RN, Nayak S (2014). Evaluation of yield, quality and antioxidant activity of essential oil of *in vitro* propagated *Kaempferia galanga* Linn. Journal of Acute Disease 3(2):124-130. *https://doi.org/10.1016/S2221-6189(14)60028-7*

Shareef HK, Muhammed HJ, Hussein HM, Hameed IH (2016). Antibacterial effect of ginger (*Zingiber officinale)* Roscoe and bioactive chemical analysis using gas chromatography mass spectrum. Oriental Journal of Chemistry 32(2):817-37. *https://doi.org/10.13005/ojc/320207*

Shirin F, Kumar S, Mishra Y (2000). *In vitro* plantlet production system for *Kaempferia galanga* L., a rare Indian medicinal herb. Plant Cell, Tissue and Organ Culture 63:193-97. *https://doi.org/10.1023/A:1010635920518*

Siriamornpun S, Tangkhawanit E, Kaewseejan N (2016). Reducing retrogradation and lipid oxidation of normal and glutinous rice flours by adding mango peel powder. Food Chemistry 201:160-67. *https://doi.org/10.1016/j.foodchem.2016.01.094*

Stanly C, Keng CL (2007). Micropropagation of *Curcuma zedoaria* Roscoe and *Zingiber zerumbet* Smith. Biotechnology 6(4):555-560. *https://doi.org/10.3923/biotech.2007.555.560*

Suphrom N, Sonyota W, Insumronga K, Sawangsupa, P, Sutamuanga, P, Ingkaninanb, K (2017). GC-MS analysis and *in vitro* anti-androgenic activity of *Kaempferia rotunda* Linn. extract. Naresuan University Journal: Science and Technology 4(25):34-43.

Tang SW, Sukari MA, Neoh BK, Yeap YS, Abdul AB, Kifli N (2014). Phytochemicals from *Kaempferia angustifolia* Rosc. and their cytotoxic and antimicrobial activities. BioMed Research International 417674:1-6. *https://doi.org/10.1155/2014/417674*

Tang SW, Sukari MA, Rahmani M, Lajis NH, Ali AM (2011). A new abietene diterpene and other constituents from *Kaempferia angustifolia* Rosc. Molecules 16(4):3018-3028. *https://doi.org/10.3390/molecules16043018*

Thammapat P, Meeso N, Siriamornpun S (2015). Effects of NaCl and soaking temperature on the phenolic compounds, α-tocopherol, γ-oryzanol and fatty acids of glutinous rice. Food Chemistry 175:218-224. *https://doi.org/10.1016/j.foodchem.2014.11.146*

Vadassery J, Ritter C, Venus Y, Camehl I, Varma A, Shahollari B (2008). The role of auxins and cytokinins in the mutualistic interaction between *Arabidopsis* and *Piriformospora indica*. Molecular Plant-Microbe Interactions 21(10):1371-1383. *https://doi.org/10.1094/MPMI-21-10-1371*

Wang Y, Wang J, Shi B, Yu T, Qi J, Meyerowitz EM (2014). The stem cell niche in leaf axils is established by auxin and cytokinin in *Arabidopsis*. The Plant Cell 26(5):2055-2067. *https://doi.org/10.1105/tpc.114.123083*

Wanyo P, Kaewseejan N, Meeso N, Siriamornpun S (2016). Bioactive compounds and antioxidant properties of different solvent extracts derived from Thai rice by products. Applied Biological Chemistry 59:373-384. *https://doi.org/10.1007/s13765-016-0173-8*

Werner T, Motyka V, Strnad M, Schmulling T (2001). Regulation of plant growth by cytokinin. Proceedings of the National Academy of Sciences of the United States of America 98(18):10487104-92. *https://doi.org/10.1073/pnas.171304098*

Werner T, Schmulling T (2009). Cytokinin action in plant development. Current Opinion in Plant Biology 12(5):527-38. *https://doi.org/10.1016/j.pbi.2009.07.002*

Yaowachai W, Saensouk S, Saensouk P (2020). *In vitro* propagation and determination of total phenolic compounds, flavonoid contents and antioxidative activity of *Globba globulifera* Gagnep. Pharmacognosy Journal 12(6):1-8. *https://doi.org/10.5530/pj.2020.12.236*

Yeap YSY, Kassim NK, Ng RC, Ee GCL, Yazan LS, Musa KH (2017). Antioxidant properties of ginger (*Kaempferia angustifolia* Rosc.) and its chemical markers. International Journal of Food Properties 20:1158-1172. *https://doi.org/10.1080/10942912.2017.1286508*