

In Vitro Propagation and Determination of Total Phenolic Compounds, Flavonoid Contents and Antioxidative Activity of *Globba globulifera* Gagnep.

Wipa Yaowachai^{1,3}, Surapon Saensouk^{2,3}, Piyaporn Saensouk^{1,3,*}

Wipa Yaowachai^{1,3}, Surapon Saensouk^{2,3}, Piyaporn Saensouk^{1,3,*}

¹Department of Biology, Faculty of Science, Mahasarakham University, Mahasarakham, THAILAND.

²WalaiRukhvej Botanical Research Institute, Mahasarakham University, Mahasarakham, THAILAND.

³Plant and Invertebrate Taxonomic and its Application Research Unit, Mahasarakham University, Mahasarakham, THAILAND.

Correspondence

Piyaporn Saensouk

Department of Biology, Faculty of Science; Plant and Invertebrate Taxonomic and its Application Research Unit, Mahasarakham University, Mahasarakham, THAILAND.

E-mail: pcornukaempferia@yahoo.com

History

- Submission Date: 12-08-2020;
- Review completed: 25-09-2020;
- Accepted Date: 01-10-2020.

DOI : 10.5530/pj.2020.12.236

Article Available online

<http://www.phcogj.com/v12/i6s>

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ABSTRACT

Introduction: Currently, there is a reduction in the number of *Globba globulifera*, which is due to its slow multiplication rate, high susceptibility to pathogenic diseases and overexploitation of the plant from natural sources. *In vitro* culture to study suitable concentrations of plant growth regulators for shoot and root induction of *G. globulifera*. Bioactive compounds were measured by TPC, TFC and FRSA methods for comparison of those from *in vitro* and natural conditions.

Methods: Microshoots were cultured on solid and liquid MS medium supplemented with various concentrations of cytokinins (BA, Kinetin and TDZ) and auxins (NAA and IBA) for eight weeks. Methanol was used as the extraction solvent via the ultrasonic method, TPC and TFC were both measured. DPPH for free radical scavenging activity was investigated. **Results:** The best result for shoot formation was achieved when culturing on MS medium with 3 mg/l and 5 mg/l of BAP or 5 mg/l of BAP plus 1 mg/l of IBA. The plantlets were transplanted to pots in a greenhouse. All the planting material showed a 100% survival rate. The rhizomes of *in vitro*-derived plantlets showed the highest value of TPC (52.28 mg GAE/g crude extract) and FRSA (93.55%) and lowest of IC₅₀ (0.46 mg/ml). **Conclusion:** The *in vitro* culture and antioxidant activity analysis could be the foundation for plant propagation in large quantities and the use of medicine.

Key words: Bioactive compound, DPPH, Micropropagation, TFC, TPC, Zingiberaceae.

INTRODUCTION

The genus *Globba* is the third most diverse of herbaceous plants in the tribe Globbeae, family Zingiberaceae. *Globba* is distributed in tropical regions of Asia, including across all regions of Thailand. The floral structure of the plants in the genus *Globba* is like a dancing woman, so it is commonly known as dancing lady ginger. Many species in the genus *Globba* are used economically, such as *G. schomburgkii* and *G. adhaerens*, which are used as ornamental plants and for export. Some species are used as herbs, such as the roots of *G. leucantha* and *G. patens* that are boiled for use by women after childbirth. Moreover, an extract from the leaves of *G. marantina* is used as eye drops to treat conjunctivitis and eye inflammation. The essential oil of *G. winitii* can inhibit the bacteria *Staphylococcus aureus*, *Proteus mirabilis* and *Micrococcus luteus*.¹

Globba globulifera is a herbaceous plant in the genus *Globba* with a height of 30-50 cm. The inflorescences have dark purple petal-like structures, which are called bracts, with yellow and small flowers. Therefore, it is used as an ornamental plant. The rhizomes and leaves contain essential oils. The flowering occurs from May to September, and dormancy in the winter. *G. globulifera* is propagated by rhizomes, seeds and bulbil, which have a relatively low propagation rate. Only a few research reports in pharmaceutical on *G. globulifera*

have been found. *G. globulifera* is currently threatened by natural and human disasters, deforestation, road making and smuggling for use as ornamental plants and trade. As a result, the number of plants in nature is reducing and are inadequate for exploitation by human beings. Therefore, conservation, preservation of plant genetics and plant breeding are important for the economy. Plant tissue culture techniques are used to propagate *G. globulifera in vitro* to increase the number of plants. Plants are sterile and can propagate throughout the year without restrictions on the season. As a guideline for using *G. globulifera* for medicinal herb purposes, the rhizomes, leaves, stems and root storage parts of *G. globulifera* can be cultured *in vitro* and grown under natural conditions for antioxidant extraction. The total phenolic contents, the total flavonoid contents and the free radical scavenging activity (FRSA) are measured to provide essential details for the therapeutic use of this herb.

MATERIALS AND METHODS

In vitro shoot regeneration

Surface-sterilization procedures and initial culture establishment

The bulbils of *G. globulifera* from plants growing under a natural environment were collected from Udon Thani Province, Thailand. The bulbils were washed for 30 min under running tap water, soaked for 1 min in 70% (v/v) ethanol, followed by

Cite this article: Yaowachai W, Saensouk S, Saensouk P. *In Vitro* Propagation and Determination of Total Phenolic Compounds, Flavonoid Contents and Antioxidative Activity of *Globba globulifera* Gagnep. Pharmacogn J. 2020;12(6)Suppl:1740-7.

disinfection with 20% and 15% sodium hypochlorite (NaOCl) for 20 min and 15 min under aseptic conditions, respectively. Then, rinsed in sterilized distilled water three times for 5 min. Initially, the bulbils were grown on MS medium (Murashige and Skoog)² supplement with a combination of 2.0 mg/l BAP with 1.0 mg/l NAA.

Shoot multiplication

The shoot explants were transferred to solid MS medium supplemented with various concentrations of plant growth regulators. The plant growth regulators were BAP (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l), Kinetin (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l), BAP (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with NAA (0.5 and 1 mg/l), BAP (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with IBA (0.5 and 1 mg/l), Kinetin (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with NAA (0.5 and 1 mg/l) and Kinetin (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with IBA (0.5 and 1 mg/l). The MS liquid medium supplemented with 3.0 mg/l of Kinetin in combination with 0.5 mg/l of NAA (Parida *et al.*)³, 2.0 mg/l of BAP (Chuengpanya *et al.*)⁴ and 2.0 mg/l of TDZ (Saensouk *et al.*)⁵ was used for the culture media. The explants were cultured on liquid and solid (7 g/l of bacto agar) MS media to study the effect of the type of media. For all media, the pH was modified to 5.7–5.8 and the media was autoclaved at 121 °C for 15 min, with 10 replications for each procedure. Mean number of shoots, mean length of shoot (cm), mean number of roots and mean length of root (cm) per explant were measured. Cultures were incubated with fluorescent lamps at 25 ± 2 °C under a 16 h photoperiod for eight weeks.

Acclimatization

The plantlet was kept at room temperature for four weeks and then after culturing in the MS medium for another eight weeks. The plantlets had shoots and roots that were well established, and they were 6–8 cm in height. Then the plantlets were removed from the glass bottle. The roots were gently washed in running tap water to remove the crop media debris and transferred to the greenhouse for eight weeks in plastic pots containing soil, sand and soil mixed with sand (1:1 w/w). The survival rate, mean number of shoots, mean length of shoot (cm), mean number of leaves, mean length of leaf (cm) and mean width of leaf (cm) per plantlet were measured for eight weeks.

Bioactive Compounds

Plant material

In vitro-derived plantlets for one year and those derived from natural conditions were used as the plant material. The parts of *G. globulifera* used in the study were the rhizomes, root storage, leaves and pseudostems. Healthy plants were selected, wash thoroughly with running tap water and cut into small pieces. The pieces of *G. globulifera* were first frozen for 12 hours at -80 °C, and then quickly put into a freeze dryer. Ground thoroughly with a herb grinder, and then the specimens were stored in a desiccant jar.

Antioxidant extraction

Antioxidant extraction was according to the procedure described by An *et al.*⁶ with modifications. For this, 5 g of *G. globulifera* explant powder was precisely weighed, then added to 30 ml 80% methanol. Each different explant was extracted ultrasonically for 30 min three times and filtered. The methanol was removed in a freeze dryer through drying. The extraction procedure was repeated three times. The sample solution was prepared from the crude extract. For this, 1 mg of the crude extract was added into the microcentrifuge tube, and then 1 ml of 80% methanol was added and mixed with a vortex (1 mg/ml stock solution).

Determination of total phenolic contents (TPC)

The total phenolic contents in the extracts was determined using the Folin–Ciocalteu assay as describe by Lister *et al.*⁷ with modification. Samples (100 µl) were added to a microcentrifuge tube that contained 500 µl of 10% (v/v) Folin–Ciocalteu phenol reagents. They were mixed and incubated in a dark place for 1 min. Then a solution of 400 µl of 7.5% (w/v) sodium carbonate was added to the mixture, and then placed in the dark for 30 min. Then 80% (v/v) methanol was used as a blank. The absorbance was measured at 765 nm using a spectrophotometer. Gallic acid (3–100 mg/l) was used as a standard. The total phenolic content was expressed as mg gallic acid equivalent per gram of crude extract (mg GAE/g crude extract).

Determination of total flavonoid contents (TFC)

TFC in the extract was determined using the Aluminium trichloride method as describe by Zhishen *et al.*⁸ with modification. Initially, 50 µl of each sample was added to a microcentrifuge tube. Then 30 µl of 5% (w/v) NaNO₂ was added to the mixture, and after 6 min it was placed in the dark, 60 µl of 10% (w/v) AlCl₃ was added. After 6 min in the dark, and 300 µl of 1 M NaOH was added. Following this, 560 µl of deionized water was added to the reaction and mixed well. Then 80% (v/v) methanol was used as a blank. The absorbance was measured at 510 nm using a spectrophotometer. Catechin (3–200 mg/l) was used as the standard. TFC was expressed as mg catechin equivalent per gram of crude extract (mg CE/g crude extract).

Determination of DPPH radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), which is stable, was used to determine the free radical scavenging capacity as described by Yen and Hsieh⁹ with modification. For this, 335 µl of a range of dilutions of the extracts (0.05–1 mg/ml) was added to 665 µl of 0.2 mM DPPH solution in methanol and mixed well, and after this it was incubated at room temperature in the dark for 30 min. The absorbance was measured at 510 nm using a spectrophotometer. The absorbance was used to calculate the free radical scavenging activity (FRSA, %) with the equation: %FRSA = [(A_{Blank} - A_{Sample})/A_{Blank}] x 100. The half maximal inhibitory concentration (IC₅₀) was determined from the plotted graph of %FRSA and scavenging activity against the concentration of the sample extracts. The scavenging ability of the antioxidants by the IC₅₀ value of each sample was compared with the IC₅₀ values of ascorbic acid.

Statistical analysis

The experiments were conducted using a completely randomized design (CRD). Data were analyzed for significance using ANOVA and differences were contrasted using a Duncan's multiple range test ($p < 0.05$) with the SPSS program version 15.

RESULTS

In vitro shoot regeneration

Microshoots of *G. globulifera* (1 cm length) were cultured on MS solid medium for eight weeks with various concentrations of a plant growth regulator for shoot and root formation. The highest number of shoots was obtained on the medium supplemented with 3 mg/l and 5 mg/l of BAP (4.40 shoots/explant). The best shoot length was observed on 3 mg/l of BAP (2.67 cm). The highest average number of roots per shoot and the best root length were observed in 5 mg/l and 4 mg/l of BAP, respectively (24.20 roots/explant and 1.67 cm) (Table 1 and Figure 1). The MS medium containing 5 mg/l of Kinetin showed the highest number of shoots (4.80 shoots/explant), shoot length (2.42 cm), average number of roots (23.20 roots/explant) and root length (2.26 cm) (Table 2 and Figure 1).

Table 1: Effect of BAP on shoot and root multiplication of *G. globulifera* for eight weeks.

BAP (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	2.60 ± 0.22 ^c	1.07 ± 0.06 ^c	11.90 ± 1.14 ^d	0.69 ± 0.04 ^d
1	3.10 ± 0.23 ^{bc}	1.36 ± 0.03 ^d	13.20 ± 0.55 ^d	1.20 ± 0.04 ^c
2	3.50 ± 0.27 ^b	1.52 ± 0.05 ^d	20.00 ± 0.49 ^c	1.21 ± 0.02 ^c
3	4.40 ± 0.22 ^a	2.67 ± 0.10 ^a	21.20 ± 0.79 ^{bc}	1.33 ± 0.05 ^c
4	4.10 ± 0.28 ^{ab}	2.10 ± 0.06 ^c	22.80 ± 0.59 ^{ab}	1.67 ± 0.03 ^a
5	4.40 ± 0.31 ^a	2.38 ± 0.09 ^b	24.20 ± 0.53 ^a	1.50 ± 0.07 ^b

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

Table 2: Effect of Kinetin on shoot and root multiplication of *G. globulifera* for eight weeks.

Kinetin (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	2.60 ± 0.22 ^c	1.07 ± 0.06 ^c	11.90 ± 1.14 ^c	0.69 ± 0.04 ^d
1	2.80 ± 0.25 ^c	2.11 ± 0.15 ^c	19.60 ± 0.50 ^b	1.52 ± 0.11 ^c
2	3.80 ± 0.25 ^b	1.96 ± 0.09 ^c	18.70 ± 0.67 ^b	1.77 ± 0.12 ^{bc}
3	3.90 ± 0.28 ^b	1.95 ± 0.05 ^c	21.30 ± 0.73 ^{ab}	1.42 ± 0.06 ^c
4	3.80 ± 0.29 ^b	2.23 ± 0.13 ^{ab}	22.70 ± 0.67 ^a	1.95 ± 0.07 ^b
5	4.80 ± 0.29 ^a	2.42 ± 0.09 ^a	23.20 ± 0.79 ^a	2.26 ± 0.09 ^a

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

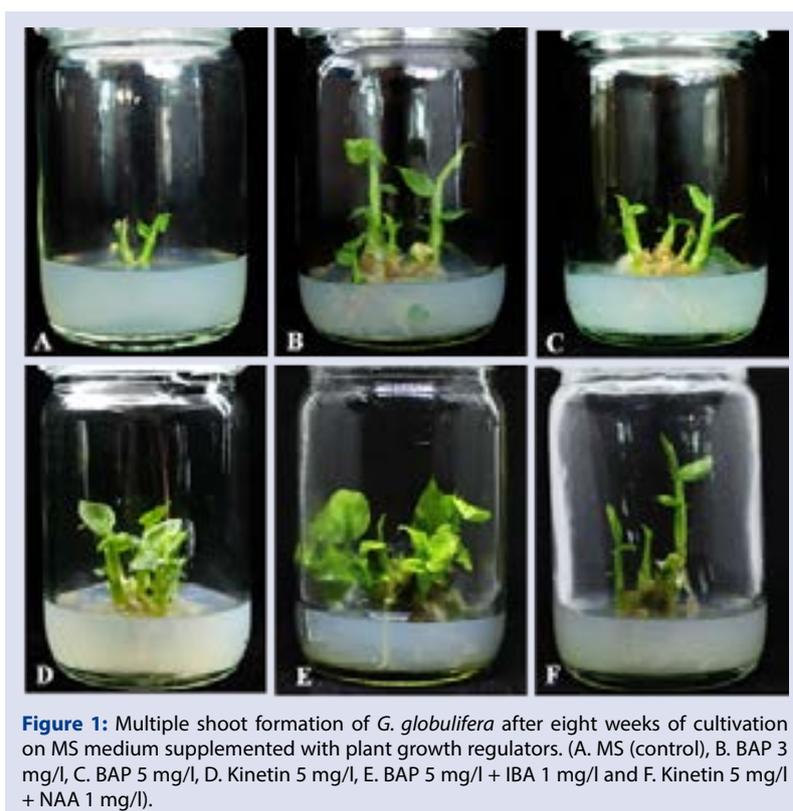


Figure 1: Multiple shoot formation of *G. globulifera* after eight weeks of cultivation on MS medium supplemented with plant growth regulators. (A. MS (control), B. BAP 3 mg/l, C. BAP 5 mg/l, D. Kinetin 5 mg/l, E. BAP 5 mg/l + IBA 1 mg/l and F. Kinetin 5 mg/l + NAA 1 mg/l).

The effect of a combination of cytokinins (BA and Kinetin) and auxins (NAA and IBA) showed well-developed shoot and root formation. MS medium supplemented with BAP combination with NAA or IBA had suitable shoot and root multiplication. The highest number of shoots was observed on the medium containing 5 mg/l of BAP plus 1 mg/l of IBA (6.40 shoots/explant) and the best shoot length was found on medium supplemented with 4 mg/l of BAP plus 0.5 mg/l of NAA (2.68 cm). The highest average number of roots and the best root length was observed in 2 mg/l of BAP plus 0.5 mg/l of IBA (34.50 roots/explant)

and 5 mg/l of BAP plus 0.5 mg/l of NAA (1.99 cm), respectively (Table 3). The MS medium supplement with 5 mg/l of Kinetin in combination with 1 mg/l of NAA showed the highest number of shoots (6.50 shoots/explant), shoot length (3.20 cm) and average number of roots (46.50 roots/explant) (Table 4). There were statistically significant differences when comparing the different plant growth regulators on liquid media. The liquid MS medium supplemented with 2.00 mg/l of TDZ show the highest number of shoots (12.25 shoots/explant) (Table 5 and Figure 2).

Table 3: Effect of combination with BAP plus NAA and BAP plus IBA on shoot and root multiplication of *G. globulifera* for eight weeks.

BAP (mg/l)	NAA (mg/l)	IBA (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	0	2.60 ± 0.22 ^e	1.07 ± 0.06 ^f	11.90 ± 1.14 ^e	0.69 ± 0.04 ^g
1	0.5	0	4.60 ± 0.27 ^{bcd}	2.09 ± 0.11 ^{bc}	22.60 ± 1.61 ^{bcd}	1.87 ± 0.17 ^{ab}
2	0.5	0	4.00 ± 0.42 ^{cd}	2.12 ± 0.15 ^{bc}	21.50 ± 2.83 ^{bcd}	1.68 ± 0.13 ^{abcd}
3	0.5	0	4.70 ± 0.40 ^{bcd}	1.96 ± 0.12 ^{bcd}	20.70 ± 1.96 ^{bcd}	1.72 ± 0.12 ^{abcd}
4	0.5	0	5.00 ± 0.26 ^{abcd}	2.68 ± 0.21 ^a	24.00 ± 2.17 ^{bcd}	1.92 ± 0.11 ^a
5	0.5	0	4.40 ± 0.27 ^{bcd}	2.25 ± 0.17 ^{ab}	23.00 ± 1.53 ^{bcd}	1.99 ± 0.13 ^a
1	1	0	5.90 ± 0.31 ^{ab}	2.38 ± 0.22 ^{ab}	27.70 ± 1.69 ^{ab}	1.97 ± 0.15 ^a
2	1	0	4.80 ± 0.47 ^{bcd}	2.34 ± 0.23 ^{ab}	29.20 ± 1.87 ^{ab}	1.82 ± 0.16 ^{abc}
3	1	0	4.10 ± 0.31 ^{cd}	2.28 ± 0.25 ^{ab}	24.90 ± 2.48 ^{bcd}	1.92 ± 0.25 ^a
4	1	0	4.70 ± 0.15 ^{bcd}	1.84 ± 0.11 ^{bcd}	23.50 ± 2.21 ^{bcd}	1.40 ± 0.09 ^{bcd}
5	1	0	4.40 ± 0.43 ^{bcd}	1.67 ± 0.10 ^{cde}	17.60 ± 3.19 ^{de}	1.38 ± 0.21 ^{bcd}
1	0	0.5	4.30 ± 0.42 ^{cd}	1.68 ± 0.14 ^{cde}	29.50 ± 2.80 ^{ab}	1.10 ± 0.05 ^{fg}
2	0	0.5	4.60 ± 0.50 ^{bcd}	1.87 ± 0.15 ^{bcd}	34.50 ± 2.65 ^a	1.53 ± 0.18 ^{ab}
3	0	0.5	4.80 ± 0.73 ^{bcd}	2.04 ± 0.24 ^{bcd}	33.70 ± 6.65 ^a	1.56 ± 0.20 ^{ab}
4	0	0.5	4.50 ± 0.34 ^{bcd}	1.70 ± 0.13 ^{cde}	18.50 ± 1.98 ^{cde}	1.35 ± 0.12 ^{cdef}
5	0	0.5	5.00 ± 0.21 ^{abcd}	1.69 ± 0.09 ^{cde}	22.90 ± 0.86 ^{bcd}	1.37 ± 0.04 ^{cdef}
1	0	1	3.80 ± 0.49 ^{cde}	1.60 ± 0.11 ^{cde}	26.80 ± 2.57 ^{abc}	1.62 ± 0.27 ^{abcde}
2	0	1	3.60 ± 0.37 ^{de}	1.51 ± 0.17 ^{def}	21.90 ± 2.01 ^{bcd}	1.17 ± 0.09 ^{ef}
3	0	1	5.20 ± 0.77 ^{abc}	1.43 ± 0.11 ^{ef}	21.40 ± 3.39 ^{bcd}	1.22 ± 0.07 ^{def}
4	0	1	5.90 ± 0.64 ^{ab}	1.60 ± 0.13 ^{cde}	25.10 ± 2.76 ^{bcd}	1.33 ± 0.18 ^{cdef}
5	0	1	6.40 ± 0.70 ^a	2.05 ± 0.26 ^{bcd}	26.00 ± 2.45 ^{abcd}	1.35 ± 0.09 ^{cdef}

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

Table 4: Effect of combination with Kinetin plus NAA and Kinetin plus IBA on shoot and root multiplication of *G. globulifera* for eight weeks.

Kinetin (mg/l)	NAA (mg/l)	IBA (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	0	2.60 ± 0.22 ^h	1.07 ± 0.06 ^g	11.90 ± 1.14 ^j	0.69 ± 0.04 ^j
1	0.5	0	5.40 ± 0.52 ^{abcd}	1.95 ± 0.14 ^{def}	28.40 ± 1.19 ^{defgh}	1.88 ± 0.11 ^{cde}
2	0.5	0	4.70 ± 0.37 ^{bcd}	2.60 ± 0.21 ^{abcd}	31.90 ± 4.10 ^{bcd}	2.13 ± 0.15 ^{bc}
3	0.5	0	4.60 ± 0.31 ^{bcd}	2.73 ± 0.38 ^{abc}	32.20 ± 3.30 ^{bcd}	2.32 ± 0.19 ^{ab}
4	0.5	0	5.20 ± 0.65 ^{abcde}	3.01 ± 0.26 ^{ab}	36.80 ± 3.64 ^{bcd}	2.50 ± 0.14 ^a
5	0.5	0	5.70 ± 0.60 ^{ab}	2.98 ± 0.27 ^{ab}	38.60 ± 3.59 ^{abc}	2.49 ± 0.19 ^a
1	1	0	4.00 ± 0.30 ^{defg}	2.66 ± 0.27 ^{abcd}	32.70 ± 4.35 ^{bcd}	1.96 ± 0.18 ^{cd}
2	1	0	4.50 ± 0.22 ^{bcd}	3.00 ± 0.35 ^{ab}	40.20 ± 3.22 ^{ab}	1.97 ± 0.11 ^{cd}
3	1	0	4.30 ± 0.21 ^{bcd}	2.56 ± 0.21 ^{abcd}	33.00 ± 2.82 ^{bcd}	1.83 ± 0.06 ^{cdef}
4	1	0	5.50 ± 0.37 ^{abc}	2.83 ± 0.31 ^{abc}	37.90 ± 5.10 ^{abcd}	1.75 ± 0.13 ^{defg}
5	1	0	6.50 ± 0.50 ^a	3.20 ± 0.32 ^a	46.50 ± 3.43 ^a	1.96 ± 0.13 ^{cd}
1	0	0.5	3.70 ± 0.26 ^{gh}	1.50 ± 0.11 ^{fg}	17.80 ± 1.54 ^{ij}	1.19 ± 0.06 ⁱ
2	0	0.5	4.60 ± 0.27 ^{bcd}	1.97 ± 0.13 ^{def}	26.00 ± 2.34 ^{fghi}	1.52 ± 0.04 ^{fghi}
3	0	0.5	4.70 ± 0.33 ^{bcd}	1.74 ± 0.14 ^{efg}	25.70 ± 2.82 ^{fghi}	1.43 ± 0.06 ^{ghi}
4	0	0.5	5.50 ± 0.56 ^{abc}	2.14 ± 0.20 ^{cdef}	28.30 ± 3.09 ^{defgh}	1.45 ± 0.11 ^{ghi}
5	0	0.5	4.60 ± 0.76 ^{bcd}	2.45 ± 0.30 ^{abcde}	29.90 ± 4.09 ^{cdefg}	1.48 ± 0.06 ^{fghi}
1	0	1	3.50 ± 0.17 ^{gh}	1.63 ± 0.04 ^{fg}	20.10 ± 1.50 ^{ghij}	1.39 ± 0.02 ^{ghi}
2	0	1	4.10 ± 0.46 ^{cdefg}	2.14 ± 0.12 ^{cdef}	27.10 ± 1.73 ^{efghi}	1.55 ± 0.08 ^{efghi}
3	0	1	3.90 ± 0.38 ^{efg}	1.59 ± 0.13 ^{fg}	18.70 ± 2.13 ^{hij}	1.27 ± 0.04 ^{hi}
4	0	1	5.10 ± 0.43 ^{bcd}	2.19 ± 0.20 ^{cdef}	28.80 ± 2.40 ^{cdefg}	1.62 ± 0.07 ^{defgh}
5	0	1	4.10 ± 0.48 ^{cdefg}	2.38 ± 0.27 ^{bcd}	23.10 ± 2.00 ^{fghi}	1.65 ± 0.06 ^{defg}

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

Table 5: Effect of liquid medium supplemented with Kinetin, NAA, BAP and TDZ on shoot and root multiplication of *G. globulifera* for eight weeks.

Kinetin (mg/l)	NAA (mg/l)	BAP (mg/l)	TDZ (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	0	0	3.67 ± 0.19 ^b	2.25 ± 0.19 ^b	12.08 ± 1.09 ^b	1.10 ± 0.07 ^b
3	0.5	0	0	3.75 ± 0.28 ^b	1.97 ± 0.14 ^b	8.58 ± 1.14 ^b	1.27 ± 0.08 ^b
0	0	2	0	4.92 ± 0.45 ^b	2.84 ± 0.18 ^a	20.92 ± 1.54 ^a	1.51 ± 0.05 ^a
0	0	0	2	12.25 ± 0.88 ^a	2.00 ± 0.16 ^b	4.08 ± 0.58 ^d	0.76 ± 0.11 ^c

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

Plantlets (6-8 cm in height) with well-developed shoots and roots were transferred for eight weeks during the rainy season into a greenhouse at the Department of Biology, Faculty of Science, Mahasarakham University, Mahasarakham, Thailand. After eight weeks, plantlets of *G. globulifera* transplanted into the soil, sand and soil mixed with sand produced various sizes of storage roots and new leaves. They are well adapted to the external environment and can be used as planting material. All the planting material showed 100% survival rate. The maximum number of shoots (4.35 shoots/plant), average shoot length (14.03 cm) and number of leaves (31.55 leaves/plant) were observed in soil (Table 6 and Figure 3).

Bioactive Compounds

Antioxidant activity is an excellent example of how plant extracts can provide a practical gain.¹⁰ From the results of TPC, TFC and free radical scavenging activity (FRSA) of methanol extracts of *ex vitro* plantlets and *in vitro*-derived plantlets, the results specified that there is a significant

difference between the extracts of different parts of the plantlets. The TPC demonstrated in gallic acid equivalents showed the highest value of 52.28 mg GAE/g crude extract in the rhizome of *in vitro*-derived plantlets, whereas the rhizome of *ex vitro* plantlets showed 50.56 mg GAE/g crude extract (Table 7). Likewise, the rhizome of the *in vitro*-derived plantlets had the highest FRSA (93.55%) and lowest IC₅₀ (0.46 mg/ml). TFC was expressed in catechin equivalents and showed the highest value of 33.62 mg CE/g crude extract in the rhizome of *ex vitro* plantlets. The DPPH scavenging ability had higher correlations with TPC (r = 0.913), and less correlation with TFC (r = 0.894).

DISCUSSION

When the microshoots of *G. globulifera* were cultured on the MS medium with various concentrations of BAP or Kinetin alone, BAP plus NAA or IBA, Kinetin plus NAA or IBA added, the shoot and root formation were different. In the same genus, the BAP treatments are consistent

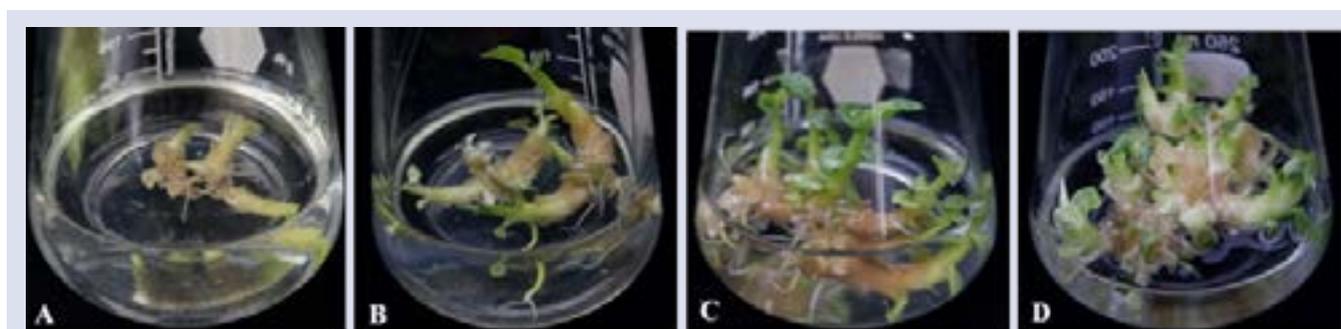


Figure 2: Multiple shoot formation of *G. globulifera* after eight weeks of cultivation on liquid MS medium supplemented with plant growth regulators. (A. MS (control), B. Kinetin 3 mg/l + NAA 0.5 mg/l, C. BAP 2 mg/l and D. TDZ 2 mg/l).

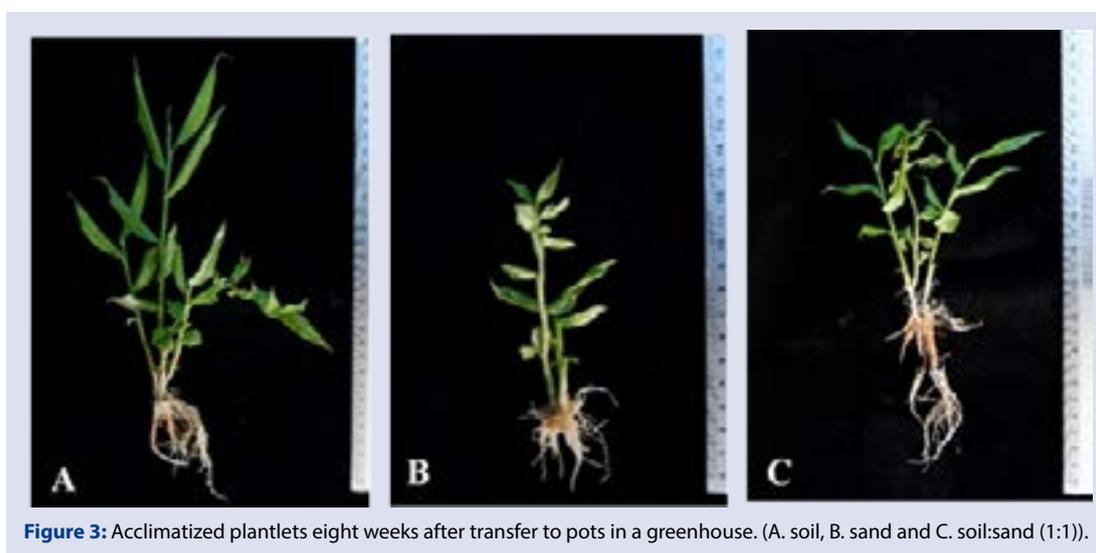


Figure 3: Acclimatized plantlets eight weeks after transfer to pots in a greenhouse. (A. soil, B. sand and C. soil:sand (1:1)).

Table 6: Effect of potting media on plantlet performance of *G. globulifera* after eight weeks of acclimatization.

Potting medium	Average no. of shoots/plant mean ± SE	Average shoot length (cm) mean ± SE	Average no. of leaves/plant Mean ± SE	Survival rate (%)
Soil	4.35±0.42 ^a	14.03±0.51 ^a	31.55 ± 2.19 ^a	100
Sand	2.90 ± 0.2 ^b	6.27 ± 0.37 ^b	14.80 ± 1.11 ^c	100
Soil mixed with sand	3.55 ± 0.27 ^{ab}	7.23 ± 0.58 ^b	19.90 ± 1.46 ^b	100

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

Table 7: TPC, TFC, FRSA and IC₅₀ in 80% (v/v) methanol extract of *G. globulifera* by ultrasonically method from different parts of explant.

Explant		TPC (mg GAE/g crude extract) mean ± SE	TFC (mg CE/g crude extract) mean ± SE	FRSA (%) mean ± SE	IC ₅₀ (mg/ml) mean ± SE
Nature	Rhizome	50.56 ± 0.54 ^b	33.62 ± 0.32 ^a	92.01 ± 0.07 ^a	0.47 ± 0.002 ^f
	Storage root	33.63 ± 0.37 ^f	15.31 ± 0.90 ^d	73.47 ± 0.41 ^c	0.65 ± 0.004 ^d
	Leaf	45.81 ± 0.71 ^c	19.53 ± 0.22 ^b	70.25 ± 0.26 ^d	0.67 ± 0.001 ^d
	Pseudostems	20.75 ± 0.26 ^g	5.61 ± 0.59 ^e	37.59 ± 0.42 ^f	1.31 ± 0.002 ^a
<i>In vitro</i> -derived plantlets	Rhizome	52.28 ± 0.41 ^a	32.20 ± 0.77 ^a	93.55 ± 0.18 ^a	0.46 ± 0.002 ^f
	Storage root	39.91 ± 0.60 ^d	17.39 ± 0.52 ^c	87.48 ± 0.16 ^b	0.52 ± 0.003 ^e
	Leaf	36.56 ± 0.14 ^e	15.53 ± 0.13 ^d	59.32 ± 1.72 ^e	0.81 ± 0.015 ^c
	Pseudostems	21.93 ± 0.17 ^g	6.87 ± 0.26 ^e	38.42 ± 0.43 ^f	1.28 ± 0.007 ^b

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

with Kho *et al.*¹¹ who reported the medium supplement with 3 mg/l of BAP showed the highest number of shoots (6.60 shoots/explant) when culturing *G. brachyanthera*. Nevertheless, these results differed from those of Chuengpanya *et al.*⁴ who reported that the maximum number of shoots was 24.1 shoots/explant and 180.4 roots/explant when culturing *G. siamensis* on MS medium containing 2.0 mg/l of BAP. Saensouk *et al.*⁵ reported different results, the MS medium supplemented with 3.0 mg/l of BAP show the number of shoots was 3.80 shoots/explant of *G. schomburgkii*. Then, 5 mg/l of Kinetin showed the best response on shoot and root induction. These results are different from Parida *et al.*¹² who reported the maximum number of shoots was 5.8 shoots/explant when culturing *G. marantina* on a medium that contained 3 mg/l Kinetin. The result of comparing different plant growth regulators on liquid media showed the differences from Saensouk *et al.*⁵ who reported *in vitro* propagation of *G. schomburgkii* on MS medium supplemented with 2.00 mg/l of TDZ (9.10 shoots/explant).

A culture medium was supplemented with cytokinins (BAP, Kinetin and TDZ) displayed a strong effect on the proliferation rate of the micropropagation process. In general, BAP, Kinetin and TDZ hormones have been the most commonly used media and plant growth regulators in plant micropropagation. Cytokinin also mediates the responses to irrelevant factors, such as light conditions in the shoot and accessibility of nutrients and water in the root.¹³ BAP could increase the level of cytokinin in plants, protects the pith tissue and prolongs the active photosynthetic duration and improves the photosynthetic efficiency factors.¹⁴ Kinetin could maintain the stability of chlorophyll and enhances the activities of antioxidant enzymes.¹⁵ TDZ could be an effective bioregulator in plant tissue cultures in a wide array of plant species.¹⁶

In the present study, it was found that using a plant growth regulator combination (BAP plus NAA or IBA and Kinetin plus NAA or IBA) could induce shoot formation in similar amounts. The results show the best shoot and root induction occurred on the medium supplement with BAP plus NAA or IBA. These results are different from Parida *et al.*¹² who studied the effect of combinations of plant grow regulators. MS medium supplement with 3 mg/l of BAP plus 0.5 mg/l of NAA showed the best number of shoots (6.5 shoots/explant). The effect of Kinetin in combination with NAA or IBA demonstrated an increase in the number of plantlets as well. In the same family, these results are consistent with Nguyen *et al.*¹⁷ who reported a medium supplement with 1 mg/l of Kinetin plus 0.5 mg/l of NAA have the highest number of shoots (5.10 shoots/explant) and best shoot length (6.43 cm). However, root induction was effective when using a combination of cytokinin and auxin. NAA is a plant growth regulator in the auxin group and is used for plant tissue culture. It is also a rooting agent and used for vegetative propagation of plants from stem and leaf explants.¹⁸ The number of

shoots per explant induced depended on the ratio of cytokinin and auxin used for shoot multiplication. Cytokinin is responsible for the regulation of plant growth through an influence on the number or duration of cell division cycles in the root and shoot meristems.¹⁹ Auxins play a major role in root development, apical dominance and plant senescence, and is associated with cell expansion, cell division and cell differentiation.²⁰

In the present study, *in vitro* plantlets derived using the tissue culture technique produced new shoots, roots and leaves. The plantlet was healthy and morphologically like the mother plants after being transferred to the field. These results differ from those of Saensouk *et al.*⁵ who reported that when the plantlets of *G. schomburgkii* were transferred to pots containing sand they showed the highest percentage of surviving plantlets (80%), average number of leaves per shoot (21.62) and average shoot length (15.00 cm). In this study, *in vitro* plantlets derived from tissue culture techniques produced healthy and plants morphologically similar to the mother after being transferred to the field.

Plants contain a wide range of bioactive compounds with essential health and food applications. Among these compounds, a strong correlation was observed between antioxidant activity and the total plant phenolic content, indicating that phenolic compounds may be the main contributor to their antioxidant ability.²¹ The characterization of the phytochemical contents of different tissues of *G. globulifera* is critical to establishing its medicinal importance. The total phenol and flavonoid contents of *in vitro*-derived plantlets for one year and natural conditions are the rhizomes, storage root, leaves and pseudostems. In this study, it was demonstrated that *in vitro*-derived plantlets had higher levels of phenolic and antioxidant activities than those in natural plantlets. This is consistent with Shohael *et al.*²² who reported that *in vitro* conditions enhanced the secondary production of metabolites through the modulation of primary plant metabolism. The rate of synthesis and gene expression of secondary metabolites may be significantly affected by the nutritional content and plant hormones used in the *in vitro* culture.²³

CONCLUSION

In conclusion, a wide-ranging investigation into *in vitro* propagation by shoot proliferation, measurement of the total phenolic contents, total flavonoid contents and the free radical scavenging activity (FRSA) analysis, was conducted. Consequently, the present research will contribute to commercial production and pharmaceutically significant conservation. The determination of the free radical scavenging potential of methanol extracts from *G. globulifera* can be used for therapeutic benefits. This study presents the first report of shoot multiplication, acclimatization, total phenolic contents, total flavonoid contents and

antioxidant activity of *G. globulifera*. This significant study could be useful for propagating *G. globulifera* at a commercial scale, as well as for genetic improvement of this valuable plant in the future.

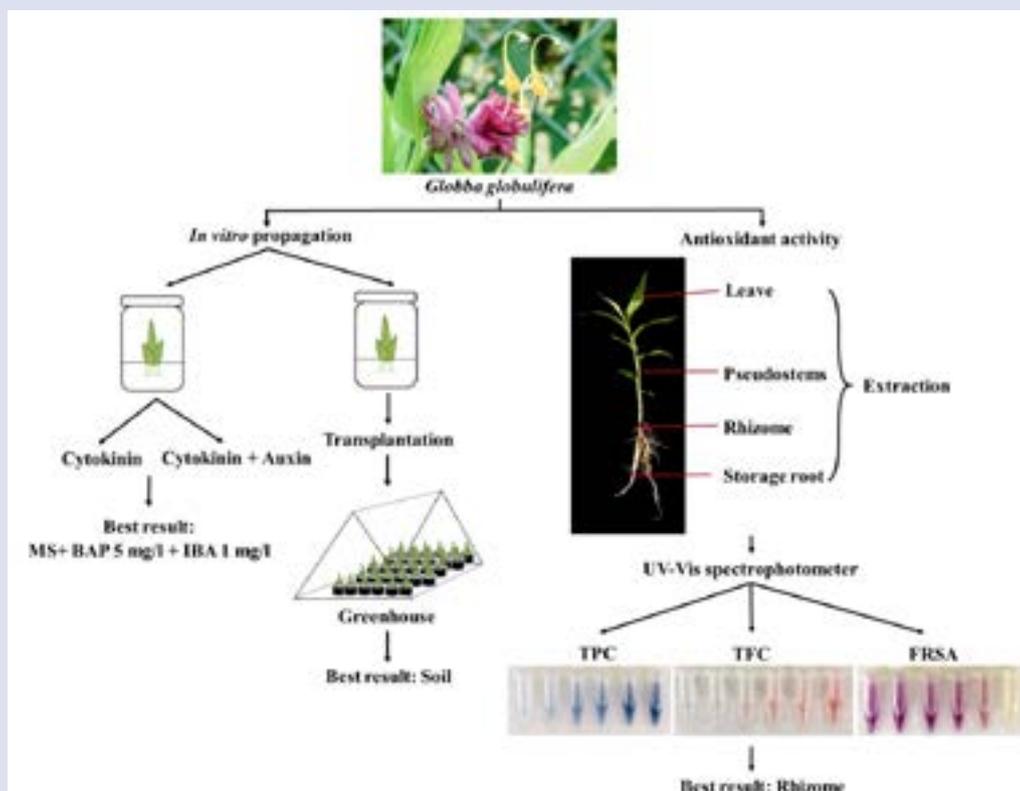
ACKNOWLEDGEMENTS

The authors would like to thank the Department of Biology, Faculty of Science, Mahasarakham University for their facilities during the research. Also, thanks to Dr. Jolyon Dodgson for language editing and suggestions to improve the manuscript. This research was financially supported by the Faculty of Science, Mahasarakham University (Grant year 2020).

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GRAPHICAL ABSTRACT



ABOUT AUTHORS



Wipa Yaowachai is a student studying for a M.Sc. degree in Biology at Department of Biology, Faculty of Science, Mahasarakham University, Thailand. Her research projects focus on Plant Tissue Culture, Plant extraction and Bioactive compounds.



Surapon Saensouk is an Associate Professor and lecturer at Walairukhavej Botanical Research Institute, Mahasarakham University, Thailand. His research projects focus on Plant Taxonomy, Biodiversity, Chromosome, Pollen, Plant Anatomy, Plant Geography and Ethnobotany.



Piyaporn Saensouk is an Assistant Professor and lecturer at Department of Biology, Faculty of Science, Mahasarakham University, Thailand. Her research projects focus on Plant Tissue Culture, Plant Anatomy, Palynology and Plant Chromosome.

Cite this article: Yaowachai W, Saensouk S, Saensouk P. *In Vitro* Propagation and Determination of Total Phenolic Compounds, Flavonoid Contents and Antioxidative Activity of *Globba globulifera* Gagnep. Pharmacogn J. 2020;12(6)Suppl:1740-7.