ARTYKUŁ ORYGINALNY / ORIGINAL PAPER
Otrzymano/Submitted: 24.03.2023 • Zaakceptowano/Accepted: 17.04.2023

Studies on the effect of hormonal variants and the type of explant on callus induction and proliferation of three species of medicinal plants/species with medicinal potential – Eryngium campestre*,* Lychnis flos-cuculi*, and* Boesenbergia pandurata

Anastasia Aliesa Hermosaningtyas[1,2], Barbara Thiem[1], Małgorzata Kikowska[1]

[1] Laboratory of Pharmaceutical Biology and Biotechnology, Department of Practical Cosmetology and Skin Disease Prophylaxis, Poznan University of Medical Sciences, Poland

[2] Doctoral School, Poznan University of Medical Sciences, Poland

Abstract

Background. The totipotency of plant cells widely opens the possibility of studying plant metabolism, understanding the mechanism of cell differentiation, and establishing plant cell cultures for more sustainable production of medicinally-valuable plant metabolites. *Eryngium campestre, Lychnis flos-cuculi, Boesenbergia pandurata* are three medicinally-valuable plants within a broad spectrum of biological and pharmacological activities. ***Aim of the study.*** This study aims to evaluate the influence of various combinations of plant growth regulators and explants to induce and proliferate callus of selected plants– *E. campestre*, *L. flos-cuculi*, and *B. pandurata*. ***Material and methods.*** Donor explants of each plant (hypocotyl, cotyledon, root, rhizome, or leaf) were disinfected and grown in Murashige and Skoog (MS) media with various combinations and concentrations of plant growth regulators. ***Results.*** Callus cultures were successfully induced for *E. campestre* and *L. flos-cuculi* using the combination of auxin 2,4-D (1.0 mg l^{-1}) and cytokinin TDZ (0.1 mg l^{-1}), as well as other auxin 2,4-D (1.0 mg l^{-1}) and cytokinin Kin (0.1 mg l^{-1}), respectively. A high callus proliferation rate for both species was obtained on MS-supplemented 2,4-D (1.0 mg l^{-1}) and Kin (0.1 mg l^{-1}). ***Conclusion.*** Ratio 1:0.1 of auxin and cytokinins are optimal for callus induction and proliferation for our selected species. Meanwhile, the types of explants did not affect the callus induction rate for *E. campestre* and *L. flos-cuculi. (Farm Współ 2023; 16: 72-81) doi: 10.53139/FW.20231612*

Keywords: Field eryngo, Ragged robin, Fingerroot, in vitro culture, callus induction and proliferation, plant growth hormones

Introduction

Eryngium campestre L. (Field eryngo) belongs to the carrot family (Apiaceae), and it is found mainly in southern and central Europe, with its eastern border in Central Russia. It is distributed in Asia Minor, the Middle East, North Africa, and North America [1]. The varied presence of chemical components, i.e., saponins, phenolic compounds, essential oils, polyacetylenes, and others, results in a broad spectrum of biological and pharmacological activities. In folk remedy infusions, extracts, decoctions, liquids, and tinctures of the roots are used as a diuretic, antitussive, stimulant, appetizer, and aphrodisiac [2,3]. Moreover, the herb of *E. campestre* is administered as an extract and in homoeopathic dilutions in the treatment of urinary tract infections and as an adjuvant to treat inflammation [2,4]. In a study conducted by Thiem et al., it was discovered that the leaf and root extracts present strong antifungal and moderate antibacterial activities [5].

Lychnis flos-cuculi L. (Ragged robin) belongs to the carnation (Caryophyllaceae) family. It is a perennial herb native to Europe and Asia, spreading through North America. Wound healing and cures for headaches, stomach discomfort, and, interestingly, malaria are among the therapeutic characteristics of this species. Phytoecdysteroids, triterpenoid saponins, flavono-

ORCID: Anastasia Aliesa Hermosaningtyas 0000-0002-9668-873X, Barbara Thiem 0000-0002-9773-9148, Małgorzata Kikowska 0000-0002-5939-5292

ids, and phenolic acids are found in *L. flos-cuculi* [6-11].

Boesenbergia pandurata (Fingerroot) or *Kaempferia pandurata* belongs to the ginger family (Zingiberaceae) and is mainly found in Southeast Asian countries (Indonesia, Malaysia, Myanmar, and Thailand). This plant is one type in disguise as it has many synonymous botanical names, *Gastrochilus panduratum* RIDL., *Kaempferia pandurata* Roxb., *Curcuma rotunda* L., and *Boesenbergia rotunda* Linn. Mansft [12]. *B. pandurata* has been used as spices and traditional medicine to treat diseases in these countries. This rhizome was previously discovered to be rich in flavonoids, chalcones, and essential oils, showing engaging pharmacological activities [12]. Thorough studies on fingerroot proved that it has antibacterial [13,14], anti-inflammatory [15,16], antifungal [17], and antiviral properties [18-20].

The callus is a massive growth of unorganized cells with totipotency characteristics [21]. Callus can be induced by applying exogenous auxin and cytokinins within *in vitro* culture media. The ratio between both growth regulators could determine the order of tissue morphogenesis [21,22]. The totipotency of plant cells widely opens the possibility of studying plant metabolism, understanding the mechanism of cell differentiation, and establishing plant cell cultures for more sustainable production of medicinally-valuable plant metabolites. In the biotechnology industry, suspension cultures obtained from callus are used to produce chemical compounds of pharmaceutical, food, and industrial importance on an industrial scale, which are difficult or unprofitable to obtain from natural sources or chemical synthesis.

This study aims to evaluate the influence of various combinations of plant growth regulators and explants to induce and proliferate callus of selected plants with medicinal values – *E. campestre, L. flos-cuculi*, and *B. pandurata*.

Materials and methods

Donor plant materials

The fruits of *Eryngium campestre* were collected from the steppe reserve Owczary (Poland) in August 2009. The voucher specimen (No. 6731) is deposited in the Herbarium of the Laboratory of Pharmaceutical Biology and Biotechnology, Poznan University of Medical Sciences. The fruits were washed under running tap water for 30 min to remove any adherent particles, then in sterilized double-distilled water for 5 min and dipped in 70% (v/v) ethanol for 30 s followed by rising in 60% (v/v) commercial bleach with two drops of Tween 80, for 10 min. They were rinsed in sterilized double-distilled water to obliterate the chemicals. All surface disinfection procedures were performed under aseptic conditions in a laminar air cabin. The disinfected fruits were placed in culture tubes containing 15 ml of Murashige and Skoog (MS) medium [23] supplemented with 1.0 mg l^{-1} gibberellic acid (GA_3) to provide aseptic seedlings. 20-day-old seedlings were aseptically cut into explants and used for callus induction on the following solidified media: MS with 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.1 or 1.0 mg l^{-1} kinetin (Kin), MS with 1.0 mg l^{-1} 2,4-D + 0.1 or 1.0 mg l^{-1} N6-benzylaminoprine (BAP), MS with 1.0 mg l^{-1} 2,4-D + 0.1 or 1.0 mg l^{-1} thidiazuron (TDZ) (table I). The following hormonal variants were selected for callus proliferation: MS with 2,4-D (1.0 mg l^{-1}) + Kin (0.1 mg l^{-1}), MS with 2,4-D (1.0 mg l^{-1}) + BAP (0.1 mg l^{-1}), and MS with 2,4-D (1.0 mg l^{-1}) and NAA (0.1 mg l^{-1}) (table IV).

The leaves of *Boesenbergia pandurata* underwent surface sterilization using the same protocol as *E. campestre*. Meanwhile, we established a new protocol to disinfect the rhizome. The machine for ultrasonication with a heating feature (POLSONIC, Polsonic Palczynski Sp. J., type SONIC-3, PL) was cleaned with 70% ethanol, put into the laminar airflow cabinet, and sterilized under UV light. In the mean, the rhizomes were washed and brushed lightly under running water for 10 minutes, then 5 minutes with a few drops of Tween-20 (Sigma). After washing with detergent, the rhizomes were rinsed once in distilled water and brought into a sterile laminar airflow cabinet for further processing.

Inside the cabinet, the explants were immersed in ethanol 70% for 30 seconds and then rinsed with distilled water before thermosonication. The POLSONIC ultrasonication machine was running on 40 kHz frequency, 2x160 Watts power, and on selected temperatures (30°C, 40°C, and 50°C) for 5 minutes or 10 minutes using hydrogen peroxide (H_2O_2) as a sterilant. The fingerroot rhizomes were then fragmented and placed in solidified MS medium; ten explants were obtained for each treatment. The initial explants were then cultured on a callus induction medium of solidified MS basal medium. The supplemented plant growth regulators are 2,4-D, 3,6-dichloro-2-methoxy benzoic acid (dicamba, Dic), BAP, Picloram, and 1-phe-

Table I. Callus induction of *Eryngium campestre* on several combinations of plant growth hormones

Plant growth regulator (mg l^{-1})		Hypocotyl		Cotyledon		Root		Morphology
		[%]	P	[%]	P	[%]	P	
2,4-D	**Kin**							
1.0	0.1	100	++	0	-	100	+++	Light, yellow, watery
1.0	1.0	100	+	0	-	80	++	Light, yellow, watery
2,4-D	**BAP**							
1.0	0.1	100	+++	33	++	100	+++	Yellow-green, watery, with organogenic structures
1.0	1.0	100	+++	80	++	100	+++	Yellow-green, watery
2,4-D	**TDZ**							
1.0	0.1	100	+++	80	+++	100	+++	Yellow-green, compact, fragile
1.0	1.0	100	+++	80	+	100	+++	Green compact and organogenic

*P – Proliferation: - not visible; + very weak; ++ weak; +++ good; ++++ very good

Table II. Callus induction of *Lychnis flos-cuculi* on several combinations of plant growth hormones

Plant growth regulator (mg l^{-1})		Hypocotyl		Cotyledon		Root		Morphology
		[%]	P	[%]	P	[%]	P	
2,4-D	**Kin**							
1.0	0.1	100	++++	100	+++	100	+++	Light and fragile
1.0	1.0	67	+	50	++	67	+	Dark and compact; organogenic
2,4-D	**BAP**							
1.0	0.1	100	++	100	++	80	++	Yellow, compact
1.0	1.0	80	+	80	+	80	+	Dark and compact; organogenic
2,4-D	**TDZ**							
1.0	0.1	100	+++	100	++	100	++	Light, yellow, and compact
1.0	1.0	60	++	80	++	80	+	Dark and compact

*P – Proliferation: - not visible; + very weak; ++ weak; +++ good; ++++ very good

Table III. Callus induction of *Boesenbergia pandurata* on several combinations of plant growth hormones

Plant growth regulator (mg l^{-1})		Rhizome		Leaves		Morphology
		[%]	P	[%]	P	
Dic	**TDZ**					
1.0	0.1	0	-	80	++	White, watery, friable
2.0	0.2	0	-	80	++	White, watery, friable
3.0	0.3	0	-	100	+++	White, watery, friable
4.0	0.4	0	-	100	+++	White, watery, friable
2,4-D	**BAP**					
2.0	0.2	0	-	75	++	Translucent and colourless, watery, friable
3.0	0.3	0	-	75	++	Translucent and colourless, watery, friable
2,4-D	**TDZ**					
2.0	0.2	0	-	55	+	Translucent and colourless, watery, friable
3.0	0.3	0	-	35	+	Translucent and colourless, watery, friable
4.0	0.4	0	-	100	++	Translucent and colourless, watery, friable

Table IV. Callus proliferation of *Eryngium campestre* on several combinations of plant growth hormones

Plant growth regulator (mg l^{-1})	Passage 3	Passage 4	Passage 5	Growth index [%] (mean + SE)
2,4-D 1.0 Kin 0.1	845.6	891.7	905.7	881 + 18.16[a]
2,4-D 1.0 BAP 0.1	686.3	663.2	721.2	690.2 + 16.86[b]
2,4-D 1.0 NAA 0.1	451.7	506.8	521.4	493.3 + 21.22[c]

Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan's Multiple Range Test)

Table V. Callus proliferation of *Lychnis flos-cuculi* on several combinations of plant growth hormones

Plant growth regulator (mg l^{-1})	Passage 4	Passage 5	Passage 6	Growth index [%] (mean ± SE)
2,4-D 1.0 Kin 0.1	564.7	501.9	589.9	552.17 ± 18.46[a]
2,4-D 1.0 NAA 0.1	361.5	343.5	328.3	344.46 ± 28.43[c]
Pic 1.0 TDZ 0.1	416.7	388.1	433.9	412.79 ± 13.41[b]
Dic 1.0 TDZ 0.1	423.6	445.2	464.9	440.88 ± 14.59[b]

Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan's Multiple Range Test)

nyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron, TDZ), at the various combination of concentrations.

The seeds of *Lychnis flos-cuculi* were garthered in June 2016 from plants growing enar Kuźnica Trzecińska, Wielkopolskie Voivodeship, Poland (51°09'21" N 18°03'24" E). The voucher specimen (No. CP-LFc-2016-0601) is deposited in the Herbarium of Laboratory of Pharmaceutical Biology and Biotechnology, Poznan University of Medical Sciences. The seeds were washed under running tap water for 30 min. The surface was sterilized with 70% ethanol (v/v) and washed for 30 minutes in 30% commercial bleach solution with a drop of Tween-80. The seeds were then rinsed five times with sterile distilled water. The disinfected fruits were placed in culture tubes containing 15 ml of Murashige and Skoog (MS) medium [23] supplemented with 1.0 mg l^{-1} gibberellic acid (GA_3). The studied callus was induced from hypocotyl, cotyledons, and the root of the axenic seedling. The following solidified media were used for callus induction: MS with 1.0 mg l^{-1} 2,4-dichlorophenoxycetic acid (2,4-D) + 0.1 or 1.0 mg l^{-1} kinetin (KIN), MS with 1.0 mg l^{-1} 2,4-D + 0.1 or 1.0 mg l^{-1} N6-benzylaminoprine (BAP), MS with 1.0 mg l^{-1} 2,4-D + 0.1 or 1.0 mg l^{-1} thidiazuron (TDZ) (table II). The following hormonal variants were selected for callus proliferation: MS with 2,4-D (1.0 mg l^{-1}) + Kin (0.1 mg l^{-1}), MS with 2,4-D (1.0 mg l^{-1}) + NAA (0.1 mg l^{-1}), MS with Pic (1.0 mg l^{-1}) + TDZ (0.1 mg l^{-1}), and MS with Dic (1.0 mg l^{-1}) + TDZ (0.1 mg l^{-1}) (table V).

Culture conditions

All callus cultures were maintained in an environmentally controlled room at 22°C ± 1°C with 60-70% relative humidity and under 16h/8h per day photoperiod. Callus cultures were maintained by repeated sub-culturing at four weeks intervals.

Callus induction and proliferation

Initiated calluses were stabilized, and subcultures were performed at four weeks intervals. Callus initiation and development of homogenous callus lines were first observed, and then the growth callus index was calculated for each species. The percentage of callus formation was calculated using the following formula:

$$\% \text{ callus formation} = \frac{number\ of\ responsing\ explants}{total\ inoculated\ explants} \times 100$$

For callus growth, fresh weight (FW) and dry weight (DW) were taken into account to calculate the growth index [GI] during three consecutive subcultures from the following:

$$\text{Growth index} = \frac{(Fresh\ final\ callus\ weight - Fresh\ initial\ callus\ weight)}{Fresh\ initial\ callus\ weight} \times 100$$

Phytochemical analysis by thin-layer chromatography

The callus biomass was oven-dried at 40°C for 24 h. Dried callus biomass was extracted with methanol (*Lychnis flos-cuculi* callus) or ethanol (*Eryngium campestre* callus) 70% v/v under reflux at 80°C (three times for an hour) at the boiling point temperature under reflux. The combined extract and filter were concentrated under a vacuum on a rotary evaporator. For TLC analysis, exactly 0.1000 (± 0.0001) g of the extract (*E. campestre*) or the butanol fraction (*L. flos-cuculi*) was dissolved in 1 ml of methanol.

TLC analysis used ready-made plates coated with silica gel or self-made plates with polyamide-6 as the adsorbent. After the sample was applied, the plates were placed in a chromatographic chamber filled with one of the solvent systems and developed. Reactions characteristic for a given group of secondary metabolites were applied using the selected reagents (details below).

For phenolic acids and flavonoids detection, each extract was applied as 1.0 cm streaks to the cellulose and HPTLC silica gel plates (Merck, Germany). The plates were developed with ethyl acetate – acetic acid – water (8:1:1 v/v/v) mixture, dried, and viewed under $UV_{366\ nm}$ or daylight before and after spraying with the following reagents: (i) 2-diphenylboranyloxy ethanamine (NA; Roth) 0.1% solution in ethanol for detection of phenolic acids (blue bands under UV) and flavonoids (yellow bands under UV), (ii) $AlCl_3$ 1% solution in ethanol for detection of flavonoids.

For saponins detection, each extract was applied as 1.0 cm streaks to the HPTLC silica gel plates (Merck, Germany). The plates were developed with 1-buthanol-acetic acid-water 4:1:5 (v/v/v) mixture and viewed under daylight after spraying with the vanillin-sulphuric acid reagent. The spots with violet-pink colour in daylight were considered as those of saponins.

Standards were as follows: phenolic acids – rosmarinic acid (Sigma-Aldrich), chlorogenic acid (EDQM), caffeic acid (Fluka), ferulic acid (Sigma-Aldrich), flavonoids –hyperoside (Sigma-Aldrich), rutoside (Sigma-Aldrich), quercetin (Sigma-Aldrich), kaempferol (Sigma-Aldrich), apigenin (Sigma-Aldrich), phytoecdysteroids – polypodine B (isolated from the herb of *L. flos-cuculi*), 20-hydroxyecdysone (isolated from the herb of *L. flos-cuculi*), saponins – easculin (aescin, Teva).

Compounds in callus extracts of the studied species were identified based on the colour and Rf value of the spots on TLC plates before and after detection with the specific reagents.

Statistical analysis

The statistical analyses were carried out using Statistica 11 (StatSoft) software. A one-way analysis of variance (ANOVA) and Duncan's post hoc test were performed on the collected data. A two-sided P value of 0.05 was used to calculate statistical significance.

Result

The calluses from *Eryngium campestre* and *Lychnis flos-cuculi* were initiated from the hypocotyl, cotyle-

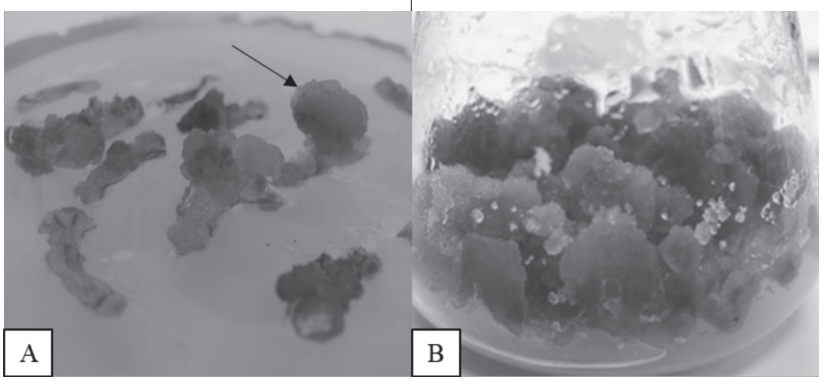

Figure 1. Callus induced on the explant (A) and homogenous callus (B) of *E. campestre*

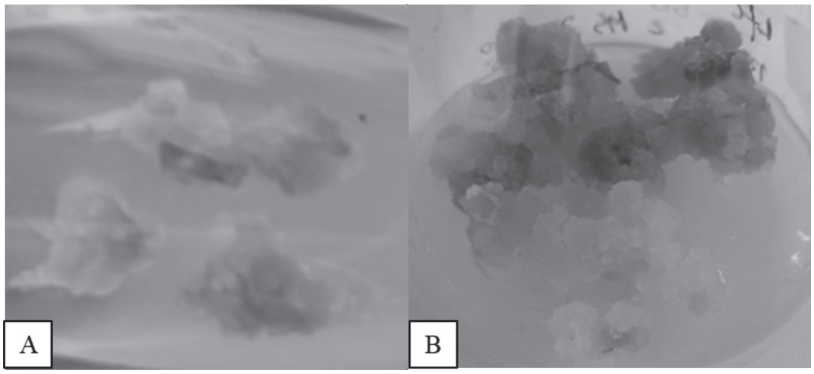

Figure 2. Callus induced on the explant (A) and homogenous callus (B) of *L. flos-cuculi*

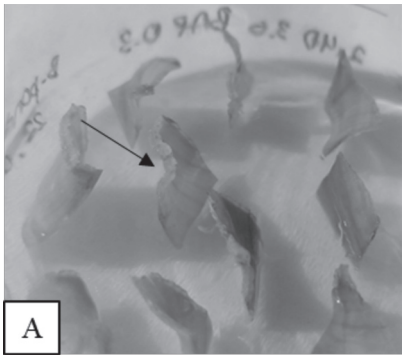

Figure 3. Callus induced on the explant (A) of *B. pandurata*

don, and root fragmets isolated from *in vitro*-derived seedlings (figure 1 and 2). Meanwhile, callus induction of *Boesenbergia pandurata* was completed using rhizome and leaves as initial fragments (figure 3). This experiment aims to obtain a callus line with appropriate morphological parameters: homogenous, fragile, easy to crush, hydrated, and light colour, and with rapid growth. Therefore, we used the combinations of auxin 2,4-D with one of cytokinins Kin, BAP, or TDZ for all donor explants along with the combination of Dic and TDZ to induce callus of *B. pandurata*. The different responses of callus frequency and proliferation suggest that plant cells have different needs for auxins and cytokinins at each induction or proliferation stage, and the level of auxins and cytokinins depends not only on their uptake from extracellular sources but also on their metabolism and endogenous interaction [24].

The hypocotyl and root fragments of *E. campestre* responded positively to all combinations of plant growth regulators (PGRs) ranging from 80-100% callus induction, as seen in table I. On the other hand, callus culture from cotyledon is only optimal in media supplemented with 2,4-D (1.0 mg l^{-1}) and TDZ (0.1 mg l^{-1}) with an 80% induction rate. There is no significant difference in the concentration of cytokinins on callus induction in all donor explant types. However, the best callus induction and proliferation rate was observed from callus grown in 2,4-D (1.0 mg l^{-1}) and TDZ (0.1 mg l^{-1}). This combination of growth regulators produced a massive amount of yellow-green calli with compact and fragile features, which may regenerate the organs. Therefore, this hormonal option was not selected for further callus proliferation.

A massive amount of light yellow and watery callus of *E. campestre* was grown on MS medium supplemented with 2,4-D with Kin and BAP. Therefore, these hormonal options as well as 2,4-D with NAA, were selected for further callus proliferation. The calluses were transferred into three different media compositions, as seen in table IV. The growth index of callus cultured on selected media in passages 3-5 was determined to select the callus line with the highest biomass increase. Callus in all types of media grew significantly. Nevertheless, the best growth index of *E. campestre* was obtained from callus-line grown on MS medium supplemented with 2,4-D 1.0 mg l^{-1} and Kin 0.1 mg l^{-1}, with 881% growth indexes on average from all three passages. Followed by 2,4-D 1.0 mg l^{-1} + BAP 0.1 mg l^{-1}, and the least effective for *E. campestre* callus proliferation was 2,4-D 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} (table IV). The morphological and anatomical observation indicated the formation of a yellow-green callus with a light structure with a dominant group of meristematic cells forming the centers.

L. flos-cuculi callus development was observed to varied degrees on all PGRs treatments and explants. The ratio of 1:0.1 of auxin and cytokinins was the optimal combination for callus induction. This ratio of auxin and cytokinin induced massively the non-morphogenic callus from all types of explants, with the most optimal supplementation being 2,4-D (1.0 mg l^{-1}) with Kin (0.1 mg l^{-1}). Callus induced on this media supplementation had light and fragile characteristics, fulfilling the criterion to proceed to the proliferation phase. Meanwhile, increasing the concentration of cytokinins impact negatively the induction rate, regardless of the explant type or PGRs type. Moreover, it is also observed that the callus cultures tended to be darker and organogenic when the auxin and cytokinin were in the same ratio (table II).

The production of *Lychnis flos-cuculi* callus biomass on several media types was studied (table V). In line with *E. campestre*, the media MS + 2,4-D 1.0 mg l^{-1} + Kin 0.1 mg l^{-1} yielded the most significant growth index for *L. flos-cuculi*, with a mean growth index of 552.17%. Therefore, this plant growth hormone variation is optimal for the induction and proliferation of *L. flos-cuculi* callus biomass. The combination of dicamba and thidiazuron in a 1:0.1 ratio also yielded a satisfactory result with a 440.88 growth index on average from subsequent passaging 4-6, the second-best callus line of *L. flos-cuculi*.

The induction of callus from *B. pandurata* from rhizome fragments was unsuccessful, and induction from leaf fragments gave intermediate results. The combination of Dic and TDZ induced callus formation with a range between 80-100% (table III). As both growth regulators' concentration increases, the induced-callus amount also increases. The induced calli were homogenous, white, translucent, had watery features, and were friable. Unfortunately, we could not obtain sufficient callus for the growth index calculation. After the second week of the induction phase, rapid oxidation occurred, resulting in severe callus browning. Eventually, the calli deteriorated and became unusable to proceed to the proliferation phase.

Callus biomass of *E. campestre* was checked for the most important groups of secondary metabolites present in the *Eryngium* genus. Callus cells synthesized rosmarinic acid, chlorogenic and caffeic acids from the group of phenolic acids; flavonoids were not detected, while the presence of several compounds from the group of triterpene saponins was shown (table VI).

Callus biomass of *L. flos-cuculi* was not able to produce phytoecdysteroids, characteristic of the herb and roots of this species, but contained phenolic acids and flavonoids as well as unknown triterpene saponins (table VII).

Table VI. Secondary metabolites of *Eryngium campestre* callus detected by TLC method

Secondary metabolites	Callus	Rf
Phenolic acids		
rosmarinic acid	+	0.92
chlorogenic acid	+	0.54
caffeic acid	+	0.96
Flavonoids		
hyperoside	Not detected	
rutoside	Not detected	
quercetin	Not detected	
kaempferol	Not detected	
Triterpenes		
triterpenoid saponins	+	0.43; 0.51; 0.57

Table VII. Secondary metabolites of *Lychnis flos-cuculi* callus detected by TLC method

Secondary metabolites	Callus	Rf
Phytoecdysteroids		
polypodine B	Not detected	
20-hydroxyecdysone	Not detected	
Phenolic acids		
ferulic acid	+	0.68
caffeic acid	+	0.83
chlorogenic acid	+	0.25
Flavonoids		
apigenin	+	0.22
Triterpenes		
triterpenoid saponins	+	0.17; 0.27; 0.33; 0.55; 0.64; 0.81; 0.96

Discussion

The influence of explant type and PGRs on callus induction and subsequent growth was studied to determine the best donor explant for establishing a callus culture to create critical therapeutic compounds of three selected species, *E. campestre, L. flos-cuculi*, and *B. pandurata*. In this study, the hypocotyl and root explants of

E. campestre were highly responsive, and we obtained watery and fast-growing callus, regardless of the type of PGRs used. Callus induction of the *Eryngium* species has also been reported from the mature leaf blade, petiole, and axenic seedling [25-27]. These findings enhanced the versatility of Eryngo species to develop calluses and, further, cell suspension culture to produce valuable secondary metabolites on a larger scale.

Callus was also successfully induced from all types of explants of the ragged robin. The types of explants did not affect the induction rate; instead, the combination of growth hormones that were used in the experiments did it. Effective callus induction using hypocotyl and cotyledon was also reported in *Nigella damascene* L. [28]. A few related studies on *Lychnis wilfordii* (Regel) Maxim, *L. cognata* (Maxim), and *L. fulgens* Fisch. Ex Spreng showed that callus formation of this genus varied significantly depending on the types of explants and hormones used [29-30]. Leaf explants induced callus formation relatively faster than using stem and root explants, with eight weeks and ten weeks of culture, respectively. Ogita et al. (2009) also observed the callus formation of *L. senno* Siebold et Zucc. using leaves as donor explants within eight weeks of cultures [31]. Employing Pic and TDZ in various concentrations, they discovered that higher concentrations of TDZ led to colour characteristics and proliferation rate, similar to the results we obtained in this study.

The callus culture of *B. pandurata* has been reported to be induced by newly emerging buds [32]. In this study, we attempted to induce callus formation from the rhizome and mature leaf explants. However, the proceeding is complicated as monocotyledons have low regenerative capacity. Johnson (2000) successfully initiated the embryonic callus of three monocot plants (*Aniozanthos* 'Harmony', *Blandfordia grandiflora*, and *Thysanotus juncifolius*) from the leaf segments. The basal sections of the leaf blades were more responsive than the tip or middle sections [33]. As studied in maize, the basal sections generally contain meristematic tissues with better regeneration properties [34].

Auxins and cytokinins are two plant growth regulators that have been extensively used [21]. 2,4-D has been acknowledged to induce callus formation in many plant species effectively. The results from this study revealed that the presence of 2,4-D in the culture media was essentially required to induce callus formation in both *E. campestre* and *L. flos-cuculi,* especially when it is combined with cytokinins with a proportional ratio. Therefore, the addition of cytokinins further exerts the physiological effect. Combining auxins and cytokinins was essential to producing calli with the desirable morphology in our selected species. Similar results were also presented from the study of *E. planum* and *L. senno* [26,31].

E. campestre callus is rich in triterpene saponins and phenolic acids, as is the callus of the related species *E. planum* [26,35]. The saponins present in the callus of *E. campestre* are most likely those previously identified for the species [3,36]. On the other, the ecdysteroids accumulated in the herb and roots of *L. flos-cuculi* [9-11] were absent in the callus biomass.

Conclusion

The callus culture of three species with medicinal value, *Eryngium campestre, Lychnis flos-cuculi,* and *Boesenbergia pandurata* were successfully initiated using Murashige and Skoog media with supplementation of auxin and cytokinin in 1:0.1 ratio. These protocols could initially start large-scale production of valuable phytochemicals from both species.

Acknowledgement
Anastasia Hermosaningtyas participates in the Poznan University of Medical Science STER Internationalization of Doctoral Schools Programs of the NAWA Polish National Agency for Academic Exchange No. PPI/STE/2020/1/0014/DEC/02.

Conflict of interest
None

Correspondence address
Anastasia A. Hermosaningtyas
Laboratory of Pharmaceutical Biology and Biotechnology, Department and Division of Practical Cosmetology and Skin Disease Prophylaxis, Poznan University of Medical Sciences
Collegium Pharmaceuticum
3 Rokietnicka St., 60-806, Poznań
☎ (+48 61) 641 85 22
anastasia.hermosaningtyas@student.ump.edu.pl

References

1. Strzelecka H, Kowalski J, editors. Encyclopedia of herbalism and herbal medicine (in Polish). Naukowe PWN; 2000.
2. Fleming T (eds). PDR for Herbal Medicines. Medical Economics, Montvale New Jersey, 2000.
3. Thiem B, Wiatrowska I. Eryngium campestre L. (Field Eryngo) and other species of Eryngium L. – Little known medicinal plants. Herba Pol. 2007;53(1):93-102.
4. Küpeli E, Kartal M, Aslan S, et al. Comparative evaluation of the anti-inflammatory and antinociceptive activity of Turkish Eryngium species. J Ethnopharmacol. 2006;107:32-37.
5. Thiem B, Goslinska O, Kikowska M, et al. Antimicrobial activity of three Eryngium L. species (Apiaceae). Herba Pol. 2010;56(4).
6. Bucharow WG, Chirva VJ, Bucharowa IL. Triterpene glycosides from Coronaria flos-cuculi. Pharmazie. 1974;29:540.
7. Báthori M, Lafont R, Girault JP, et al. Structural diversity of ecdysteroids of Lychnis flos-cuculi. Acta Pharm Hung. 2001;71:157-67.
8. Tomczyk M. Preliminary phytochemical investigation of Lychnis flos-cuculi herbs. J Nat Med (Tokyo). 2008;62:473-5.
9. Maliński MP, Michalska AD, Tomczykowa M, et al. Ragged Robin (Lychnis flos-cuculi)—a plant with potential medicinal value. Rev Bras Farmacogn. 2014;24:722-30.
10. Maliński MP, Kikowska M, Kruszka D. et al. Various in vitro systems of Ragged Robin (Lychnis flos-cuculi L.): a new potential source of phytoecdysteroids? Plant Cell Tiss Organ Cult. 2019;139:39-52.
11. Maliński MP, Kikowska MA, Soluch A, et al. Phytochemical screening, phenolic compounds and antioxidant activity of biomass from Lychnis flos-cuculi L. in vitro cultures and intact plants. Plants. 2021;10(2):206.
12. Chahyadi A, Hartati R, Wirasutisna KR, et al. Boesenbergia pandurata Roxb., An Indonesian medicinal plant: phytochemistry, biological activity, plant biotechnology. Procedia Chem. 2014;13:13-37.
13. Rukayadi Y, Lee K, Han S, et al. In vitro activities of panduratin A against clinical Staphylococcus strains. Antimicrob Agent Chemother. 2009;53(10):4529-32.
14. Thongson C, Davidson PM, Mahakarnchanakul W, et al. Antimicrobial effect of thai spices against Listeria monocytogenes and Salmonella Typhimurium DT104. J Food Prot. 2005;68(10):2054-8.
15. Morikawa T, Funakoshi K, Ninomiya K, et al. Medicinal foodstuffs. XXXIV. Structures of new prenylchalcones and prenylflavanones with TNF-α and aminopeptidase N inhibitory activities from Boesenbergia rotunda. Chem Pharm Bull. 2008;56(7):956-62.
16. Tewtrakul S, Subhadhirasakul S, Karalai C, et al. Anti-inflammatory effects of compounds from Kaempferia parviflora and Boesenbergia pandurata. Food Chem. 2009;115(2):534-8.
17. Phongpaichit S, Subhadhirasakul S, Wattanapiromsakul C. Antifungal activities of extracts from Thai medicinal plants against opportunistic fungal pathogens associated with AIDS patients. Mycoses. 2005;48:333-8.
18. Cheenpracha S, Karalai C, Ponglimanont C, et al. Anti-HIV-1 protease activity of compounds from Boesenbergia pandurata. Bioorg Med Chem 2006;14:1710-4.
19. Wu N, Kong Y, Zu Y, et al. Activity investigation of pinostrobin towards herpes simplex virus-1 as determined by atomic force microscopy. Phytomed. 2011;18:110-8.
20. Kanjanasirirat P, Suksatu A, Manopwisedjaroen S, et al. High-content screening of Thai medicinal plants reveals Boesenbergia rotunda extract and its component Panduratin A as anti-SARS-CoV-2 agents. Sci Rep. 2020;10:19963.
21. Ikeuchi M, Sugimoto K, Iwase A. Plant callus: mechanisms of induction and repression. Plant cell. 2013;25(9):3159-73.
22. Yücesan B. Thidiazuron (TDZ): A callus minimizer for in vitro plant production. In: Ahmad N, Faisal M. (eds) Thidiazuron: from urea derivative to plant growth regulator. Springer, Singapore, 2018.
23. Murashige T, Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant. 1962;15:473-97.
24. Jiménez VM, Bangerth F. Endogenous hormone levels in explants and in embryogenic and non-embryogenic cultures of carrot. Physiol Plant. 2001;111(3):389-95.
25. Ignacimuthu S, Arockiasamy S, Antonysamy M, et al. Plant regeneration through somatic embryogenesis from mature leaf explants of Eryngium foetidum, a condiment. Plant Cell Tissue Organ Cult. 1999;56:131-7.
26. Kikowska M, Kowalczyk M, Stochmal A, et al. Enhanced accumulation of triterpenoid saponins in in vitro plantlets and dedifferentiated cultures of Eryngium planum L.: a medicinal plant. Hortic Environ Biotechnol. 2019;60:147-54.
27. Kikowska M, Thiem B, Szopa A, et al. Accumulation of valuable secondary metabolites: phenolic acids and flavonoids in different in vitro systems of shoot cultures of the endangered plant species—Eryngium alpinum L. Plant Cell Tissue Organ Cult. 2020; 141:381-91.
28. Klimek-Chodacka M, Kadluczka D, Lukasiewicz A, et al. Effective callus induction and plant regeneration in callus and protoplast cultures of Nigella damascena L. Plant Cell Tissue Organ Cult. 2020;143(3):693-707.
29. Bae KH, Lee MH, Choi YE, et al. Callus induction and plant regeneration of Lychnis wilfordii (Regel) Maxim a critically endangered plant in Korea. J Plant Biotechnol. 2014;41(1):33-7.
30. Bae KH, Yoon ES. Seed germination and in vitro plant regeneration through callus culture of two Lychnis species. Plant Tissue Cult Biotechnol. 2015;25(1):1-12.
31. Ogita S, Miyazaki J, Godo T, Kato Y. Possibility for selective accumulation of polyphenolics in tissue cultures of senno (Lychnis senno Siebold et Zucc.). Nat Prod Comm. 2009;4(3):1934578X0900400312.

32. Tan SK, Pippen R, Yusof R, et al. Simple one-medium formulation regeneration of fingerroot [Boesenbergia rotunda (L.) Mansf. Kulturpfl.] via somatic embryogenesis. In Vitro Cell Dev Biol-Plant. 2005;41:757-61.
33. Johnson KA. Development of non-zygotic embryos from callus in three Australians monocots. In: Wilson KL, Morrison DA (eds) Monocots: Systematic and Evolution. CSIRO, Melbourne, 2000.
34. Ahmadabadi M, Ruf S, Bock R. A leaf-based regeneration and transformation system for maize (Zea mays L.). Transgenic Res. 2007;16: 437-48.
35. Kikowska M, Budzianowski J, Krawczyk A, et al. Accumulation of rosmarinic, chlorogenic and caffeic acids in in vitro cultures of Eryngium planum L. Acta Physiol Plant 2012;34(6):2425-33.
36. Kartal M, Mitaine-Offer AC, Abu-Asaker M, et al. Two new triterpene saponins from Eryngium campestre. Chem Pharm Bull 53(10): 1318-20.