Pak. J. Bot., 55(5): 1623-1631, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-5(17)

INFLUENCE OF PLANT GROWTH REGULATORS ON THE PHENOLIC COMPOSITION OF *ELAEOCARPUS GRANDIFLORUS* J.E. SMITH (ELAEOCARPACEAE) CELL CULTURE

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Abstract

Elaeocarpus grandiflorus (Elaeocarpaceae) has the potential to be developed as an antidiabetic agent and as a source of antioxidants. However, massive exploration of this species is very risky and likely to be extinct because it is rare and difficult to cultivate. Cell culturing of *E. grandiflorus* is an effective alternative technique for producing secondary metabolites, especially phenolic acid. Picloram and 2,4-Dichlorophenoxyacetic acid (2,4-D) are plant growth regulators in the form of synthetic auxins which are widely used to increase the production of secondary metabolites in the cell culturing process. Therefore, this study aimed to analyze the effect of the type and concentration of picloram and 2,4-D on the composition of phenolic acids. The organ samples for the cell suspension culture were petioles of mature leaves obtained from two-year-old *E. grandiflorus* plants. Petiole was prepared aseptically and inoculated on a woody plant medium and induced using 2,4-D at a dose of 1.5 ppm, 2.5 ppm, and 3.5 ppm and picloram at a dose of 2.5 ppm, 5.0 ppm, and 7.5 ppm. Cultures were maintained for 35 days and then analyzed using liquid chromatography-mass spectrometry to measure the phenolic content. The results showed that picloram and 2,4-D with various concentrations had the same effect in inducing the formation of phenolic compounds in *E. grandiflorus* cell cultures. However, cell suspension cultures derived from the petiole of young leaves produced various types of phenolic acid compounds. The dominant compound found was kaempferol, which accounted for more than 16% of the total identified secondary metabolites. Further research is needed to determine the mechanism of action of picloram and 2,4-D in influencing the productivity of phenolic acid compounds.

Key words: Kaempferol**,** Picloram**,** Flavonoid, 2,4-D.

Introduction

The water extract of the leaves and fruits of the *Elaeocarpus* plant can scientifically be used for antidiabetic treatment (Prasannan *et al*., 2020; Rahayu *et al*., 2018). In addition, *Elaeocarpus grandiflorus* has been proven to contain various secondary metabolites, especially flavonoids, which reach up to more than 50% and have potential as antioxidants (Habibah *et al*., 2021a; 2021b). However, massive and explorative use of *E. grandiflorus* is very difficult because this plant is rarely found and also difficult to cultivate (Rahayu *et al*., 2018). Therefore, one of the technologies that can be used is the cell culture technique for *E. grandiflorus*.

Several studies have shown that cell cultures of *E. grandiflorus* are capable of producing various secondary metabolites, especially phenolic compounds (Anggraito *et al*., 2020; Habibah *et al*., 2019a, 2020). Moreover, more than 100 secondary metabolites were also identified from the methanol extract of *E. grandiflorus* cell culture (Habibah *et al*., 2021b). Cell culturing provides opportunities to produce secondary metabolites in high quantity with minimal resources and can be continuously increased by adding plant growth regulators (PGRs).

Furthermore, the use of PGRs has been shown to increase secondary metabolic synthesis (Habibah *et al*., 2019b; Jamwal *et al*., 2018; Radić *et al*., 2016) and to increase the antioxidant activity (Kousalya & Bai, 2016). In general, the PGRs used in cell culturing are auxin phytohormones (Bedetti *et al*., 2017), such as 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Melo *et al*., 2019). Picloram and 2,4-D are systemic herbicides used for the control of broadleaf weeds and grasses (Dayan *et al*., 2010; Mwakalesi & Potter, 2020; Yang *et al*., 2021). However, the presence of picloram and 2,4-D in low concentrations can be used as a callus growth inducer in cell culture (Moura *et al*., 2017). Several studies have shown that the distribution of picloram in low concentrations can trigger the production of metabolites, especially phenolic acid (Zaman *et al*., 2020), while at high concentrations, it does not show an increase (Habibah *et al*., 2021a). Furthermore, high concentrations of picloram and 2,4-D were shown to reduce the root growth length in *Thalictrum foliolosum* (Mishra *et al*., 2020), the callus formation in *Physalis angulata* (Mastuti *et al*., 2017), and the production of phenolic acid in *Aconitum violaceum* culture (Rawat *et al*., 2013).

Phenolic compounds are widely distributed in plant tissues and are often considered secondary metabolites of plant metabolism that contribute to the physiological or ecological functions of the plant (Liu *et al*., 2017). These phenolic substances, or polyphenols, contain numerous varieties of compounds: simple flavonoids, phenolic acids, complex flavonoids, and colored anthocyanins. Plant phenolic compounds can act as antioxidants, structural polymers (lignin), attractants (flavonoids and carotenoids), UV screens (flavonoids), signal compounds (salicylic acid and flavonoids), and defense response chemicals (tannins and phytoalexins) (Muszyńska *et al*., 2021; Singh *et al*., 2018).

Based on the above explanation, the administration of PGRs still needs to be evaluated to establish a clear justification for the use of synthetic auxins, in this study, picloram and 2,4-D in the cell culture of *E. grandiflorus*. Therefore, this study aimed to analyze the effect of the type and concentration of PGRs on the composition of phenolic acids. This is to obtain information on the best medium for production of phenolic compounds in *E. grandiflorus* cell culture.

Method

The sample of *E. grandiflorus* cell suspension culture was a two-year-old plant seed obtained from the Bali Botanical Garden, Indonesian Institute of Sciences, Bali, Indonesia. The plants were then stored in the Plant Culture Laboratory, Universitas Negeri Semarang, Central Java, Indonesia for six months for acclimatization and adaptation. The inoculum was obtained from mature petioles of leaves or the third to fifth leaves of the shoot.

Cell culture induction: Woody plant medium (WPM) was used as the callus induction medium and cell culture medium. Callus induction was carried out using picloram and 2,4-D (Sigma Aldrich, Darmstadt, Germany) as PGRs at doses of 1.5 ppm, 2.5 ppm, and 3.5 ppm for 2,4-D and 2.5 ppm, 5.0 ppm, and 7.5 ppm for picloram. Cell suspension cultures were prepared by inserting 1 g of callus aged 5 months into 20 ml of liquid woody plant medium with the same PGRs concentration as the callus induction medium by following the procedure of Habibah *et al*., (2021b). The culture was shaken using a shaker at a speed of 120 rpm and maintained for 35 days in the dark. The harvested cells were dried in an oven at 60°C for extraction. A total of ± 0.2 g dried culture cell was obtained from the drying process.

Preparation and purification of methanolic extract: Extraction was carried out by following the method of Hao *et al*., (2009). A total of 5 mL methanol containing 1% HCl (v/v) was used to macerate 0.2 g dried culture cell of *E. grandiflorus*, followed by the addition of 2 N HCl and was incubated at 90°C for 1 hour. The extract was then dried and resuspended in methanol.

Liquid chromatography–mass spectrometry (LC-MS) analysis: The extract obtained was dissolved with a methanol solvent to a concentration below 100 ppm to obtain a homogeneous solution. Then it was centrifuged at 8000 rpm for 10 minutes to separate the solids. The supernatant obtained was used for the protein precipitation step. Two ml of the extract supernatant was put into a centrifuge tube, added 3 ml of acetonitrile acidified with 0.2% formic acid, and centrifuged at 8000 rpm for 30 seconds. The supernatant was used for the purification process by the solid phase extraction method. The solution was filtered using a 0.45 m cellulose acetate filter membrane, and degassing was carried out. The solution was ready to be used for injection into the LC-MS apparatus. The LC-MS apparatus used was Shimadzu LC-MS-8040 LC-MS with the column Shimadzu Shim-pack FC-ODS (2 mm × 150 mm, 3 m). The conditions used were as follows: injection volume 1 l, capillary voltage 3.0 kV, column temperature 35°C, isocratic mobile phase mode, flow rate 0.5 ml/min, mobile phase methanol 90% with water, MS focused ion mode Io type [M]+, collision energy 5.0 V, desolvation gas flow 60 ml/hr, desolvation temperature 350°C, fragmentation method low energy collision-induced dissociation, ionization electrospray ionization, scanning 0.6 sec/scan (Mz: 10–1000), source temperature 100°C, and run time 80 minutes.

Results and Discussion

The LC-MS result showed, around 87-90 various secondary metabolites were identified from cultured cell of *E. grandiflorus* induced with picloram or 2,4-D (Fig. 1). This condition revealed high potential of *E. grandiflorus* cell culture for producing bioactive compounds through *In vitro* technique.

Identification and quantification of approximately 39 phenolic compounds form cultured cell of *E. grandiflorus*, enhanced with picloram or 2,4-D was done. This study has determined the optimal conditions related to the callus culture induction process and *E. grandiflorus* cell suspension to produce maximum secondary metabolites. However, the synthesis of secondary metabolites is more dominantly influenced by environmental stress factors or plant defense systems in the face of pathogens (Caser *et al*., 2019; Kiokias *et al*., 2020). In addition, the *In vitro* production of secondary compounds may also influence by the variations of growth regulator type and concentration in the medium (Ali *et al*., 2017; Castro *et al*., 2021). Regarding to this study, the cell culture of *E. grandiflorus* induced with picloram and 2,4-D produces various secondary metabolites, which is dominated by polyphenolic compounds up to more than 60% and monophenolic compounds were up to 6% from the total compounds (Table 1). Other compounds identified also included alkaloids, grandiflorus acids, terpenoids, and vitamins (Fig. 2). Phenolic acid compounds are aromatic compounds containing a phenolic ring and an organic carboxylic acid function (C_6–C_1) (Liu *et al*., 2017).

Several studies have shown that PGR substances regulate the synthesis of secondary metabolites, both as suppressors and enhancers. Specifically, the administration of picloram at a dose of 1 mg/L was able to increase the production of azadirachtin compounds in *Azadirachta indica* (Farjaminezhad & Garoosi, 2019). Moreover, picloram and 2,4-D effectively increased the total antioxidant activity of *Polyalthia bullata* (Zaman *et al*., 2020). This report differs from the results of the LC-MS analysis of secondary metabolites of *E. grandiflorus* cell culture induced by picloram and 2,4-D in this study. Induction of picloram and 2,4-D resulted in secondary metabolite profiles that were not significantly different. The concentrations of picloram used up to 7.5 ppm and 2,4-D up to 3.5 ppm also did not show significant differences in the production of secondary metabolites with lower inducer concentrations. In other words, both picloram and 2,4-D gave the same effect on the production of secondary metabolites, especially flavonoids and phenolic acid.

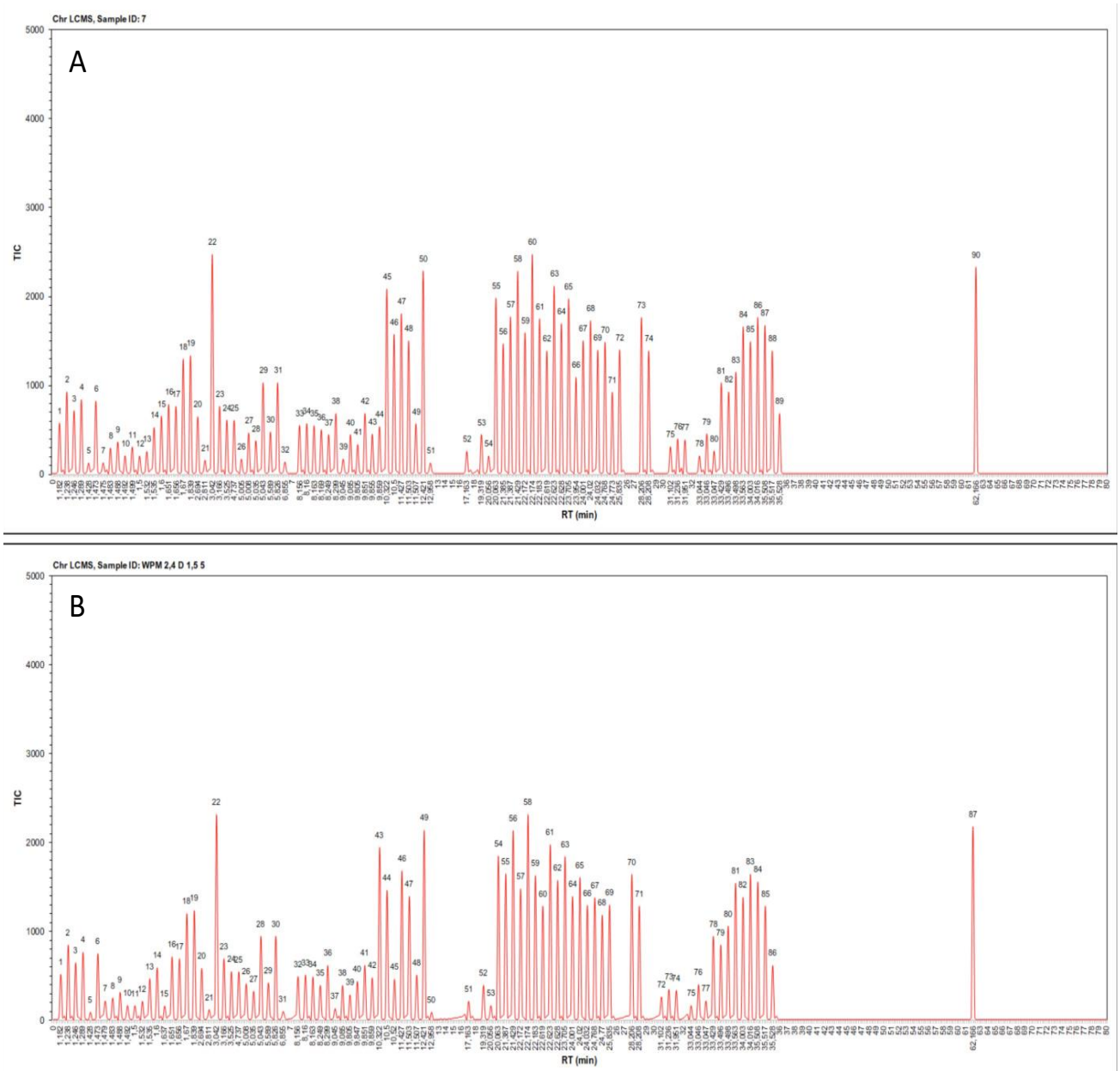

Fig. 1. LC-MS chromatographs represent *E. grandiflorus* culture cell extract. Approximately, more than 95 – see text compounds were identified from the picloram induced-culture cell (A); and 87 compounds from the 2,4-D inducement (B).

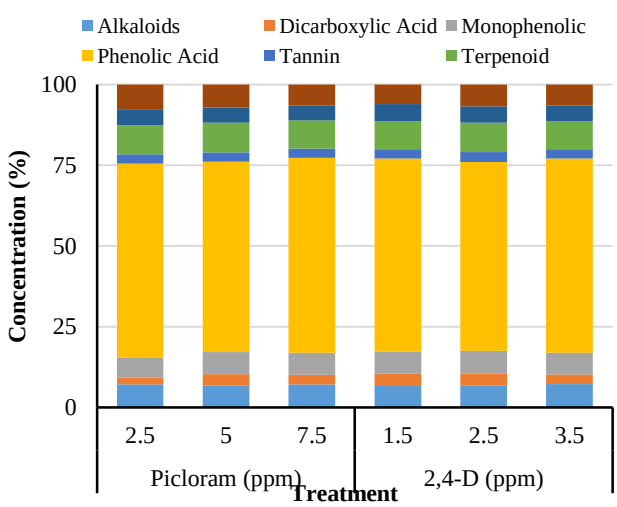

Fig. 2. Types of secondary metabolites of *E. grandiflorus* cell culture on various PGR variations.

In detail, there were at least 19 flavonoid polyphenol compounds that had been identified using the LC-MS method, with the dominant compound being kaempferol, which consisted of 13 derived compounds. The total concentration of kaempferol compounds produced from the cell culture of *E. grandiflorus* was more than 25% of the total components per each induced callus (Table 1). Furthermore, the induction of picloram and 2,4-D at various concentrations in the cell culture of *E. grandifloras* also showed no significant difference in monophenolic production. Induction of picloram and 2,4-D at various concentrations also produced four monophenolic compounds, which were dominated by gallic acid with a concentration of more than 2.00% of the total secondary metabolites.

The results of LC-MS showed that four compounds from the polyphenol group were not found in *E. grandiflorus* cell cultures, including isovitexin,

kaempferol 3-(4″-acetylrhamnoside), and kaempferol 3-glucuronide, which were not detected in the 1.5 ppm 2,4-D treatment. In addition, the kaempferol compound 3-(3″-acetylrhamnoside) was also not detected in cell cultures of *E. grandiflorus* maintained in the medium with the addition of 7.5 ppm picloram. This indicates a possible link between the presence of certain compounds and the levels of synthetic auxin PGR administration. Therefore, further research is needed to identify the mechanism of this relationship.

Furthermore, there was no difference in the production of phenolic compounds due to the influence of picloram and 2,4-D, possibly because the two types of growth regulators were synthetic auxins with similar mechanisms (Wang *et al*., 2018). Meanwhile, there was no difference in the production of secondary metabolites in the *E. grandiflorus* cell culture, possibly because the presence of PGR, used by *E. grandiflorus* cells for growth. Picloram and 2,4-D undergo cellular metabolism like natural indole-3-acetic-acid (IAA) involving in growth through regulation of auxin-influx carrier or auxin resistant 1 or aux1 (AUX1/LAX) protein (Chandler, 2016). Further research needs to be carried out to analyze the effect of the type and dose of PGR on the concentration of phenolic compounds to explain the best cell culture technique for *E. grandiflorus*.

Other studies have also shown that there is an inverse correlation between the growth and production of secondary metabolites in PGR-induced plant cultures. Research on differentiated *In vitro* cell lines of *Azadirachta indica* showed that the production of phenolic compounds had an inverse relationship with cell growth, which was determined by the callus weight (Ashokhan *et al*., 2020). The relationship between cell growth and accumulation of secondary metabolites has been reported (Ho, *et al*., 2020) although it is still not well understood. Decreased production of secondary metabolites may be related to the energy use of sucrose in culture media for growth. Several studies have shown that auxin contributes to the biosynthesis of flavonoid compounds by inhibiting the production of anthocyanins (Liu *et al*., 2021; Wang *et al*., 2018), increasing the synthesis of epicatechin, and having no effect on the concentration of catechins, as in *Camellia sinensis* (Zhao *et al*., 2020).

Phenolic compounds synthesized by plants via the shikimate pathway that requires molecular precursors or aromatic compounds include the amino acids phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr) (Barros & Dixon, 2020). The main aromatic phenolic compounds synthesized from Phe and Tyr are flavonoids such as anthocyanins (Qu *et al*., 2011) and anthraquinone (Anjusha & Gangaprasad, 2017; Demirci *et al*., 2021) and monophenolic acid (Szewczyk *et al*., 2021). Trp is a precursor of alkaloids in secondary metabolism, including various hydroxybenzoic acids and aromatic aldehydes (Sharma *et al*., 2018). Flavonoids are synthesized from the amino acid phenylalanine through the phenylpropanoid metabolism pathway. The process produces 4-coumaroyl-CoA and reacts with malonyl-CoA to form the main structure of the flavonoid chain, called chalcone, which contains two phenyl rings (Ferreyra *et al*., 2012). The ring closure of the chalcone conjugate produces a three-ring structure of the flavone (Fig. 3) and only one ring in monophenolic compounds (Fig. 4). The metabolic pathway continues to produce flavanones followed by dihydroflavonols to produce anthocyanins or other flavonoid compounds through a series of enzymatic modifications (Metsämuuronen & Sirén, 2019). This biosynthesis sequence produces intermediates and derivatives, including naringenin, flavan-3-ols, flavonols, proanthocyanidins (tannins), and several other polyphenols.

Meanwhile, monophenolics are produced from the main precursor of Phe through two main pathways, namely the formation of 3-dehydroshikimate by the shikimate dehydrogenase enzyme until the final product gallic acid is obtained (Al-Jitan *et al*., 2018). In the second pathway, Phe undergoes hydrolysis to form an intermediate compound p-coumaric acid, which is then produced by ferulic acid and eugenol. In general, the structural similarity of the four compounds is found in the structure of the main phenyl ring with the end of the side chain attached to the C_1 and C_4 atoms. Meanwhile, in further reactions, p-coumaric acid also becomes an intermediate compound for the biosynthesis of flavonoid and lignin compounds.

In this study, the use of resources in the medium is prioritized for growth rather than the need for the biosynthesis of phenolic compounds. However, several studies have shown the effect of administering picloram and 2,4-D on the production of phenolic compounds followed by callus or cell growth significantly. This is probably because auxin signaling regulates flavonoid biosynthesis at the nuclear receptor scale. The presence of auxin in low concentration triggers the activation of the auxin response factor (ARF), which binds to the Aux/IAA complex and suppresses the expression of the auxin-induced gene (Luo *et al*., 2018; Yamauchi *et al*., 2019). This has an impact on increasing the rate of cell division and increasing the number of cells.

Meanwhile, in high concentrations, auxin acts as an initiator of Aux/IAA binding to the F-box protein-transport inhibitory response 1 (TIR1) factor and triggers Aux/IAA degradation (Ljung, 2013). This process reduces the amount of Aux/IAA protein in the cytoplasm, which increases the formation of ARF homodimers to bind to auxin response elements (AuxRE), thereby triggering the expression of growth-related genes (Fendrych *et al*., 2018). In addition, the Aux/IAA–ARF complex is associated with the MdARF receptor complex, which is responsible for gene expression in the biosynthesis of various types of polyphenols, such as flavones and anthocyanins (Wang *et al*., 2018). However, the explanation of the auxin effect, in this study picloram and 2,4-D, on flavonoid production is still unclear. We suspect that the absence of stress on cells leads to priority use of resources for cell growth, thereby reducing the availability of Phe precursors. This is because phosphoenolpyruvate as a precursor of the shikimate pathway tends to be used to produce ATP rather than producing secondary metabolites (Zabalza *et al*., 2017).

Table 1. Composition of phenolic compounds in the cell culture of *E. grandiflorus* on various PGR variations.

Comp. name	Chem. Structure	Accession No.	Composition (%)					
			Picloram (ppm)			2,4-D (ppm)		
			2.5	5	7.5	1.5	2.5	3.5
Polyphenolic								
Vitexin	$C_{21}H_{20}O_{10}$	5280441	2.08	2.05	2.06	2.14	2.04	2.05
Cryptoflavin	$C_{40}H_{56}O_2$	5376350	0.21	0.24	0.21	0.22	0.21	0.21
Epicatechin	$C_{15}H_{14}O_6$	72276	1.85	1.83	1.83	1.90	1.81	1.82
Epicatechin gallate	$C_{22}H_{18}O_{10}$	107905	2.06	2.03	2.03	2.11	2.01	2.03
Epigallocatechin-3-*O*-gallate	$C_{22}H_{18}O_{22}$	71316355	2.33	2.29	2.30	2.39	2.28	2.30
Epigallocatechin	$C_{15}H_{14}O_7$	72277	1.76	1.74	1.74	1.81	1.73	1.74
Hyperoside	$C_{21}H_{20}O_{12}$	5281643	2.03	2.00	2.01	2.09	1.99	2.00
Isoorientin	$C_{21}H_{20}O_{11}$	114776	1.62	1.61	1.60	1.67	1.59	1.60
Isoquercetin	$C_{21}H_{20}O_{12}$	5280804	1.64	1.62	1.61	1.68	1.60	1.61
Isovitexin	$C_{21}H_{20}O_{10}$	162350	1.72	1.70	1.70	n.d.	1.69	1.70
Kaempferol	$C_{15}H_{10}O_6$	5280863	2.46	2.42	2.43	2.53	2.41	2.43
Kaempferol 3-(2″,4″-diacetylrhamnoside)	$C_{25}H_{24}O_{12}$	44258958	2.08	2.05	2.05	2.13	2.03	2.05
Kaempferol 3-(2″-acetylrhamnoside)	$C_{23}H_{22}O_{11}$	44258953	1.74	1.72	1.72	1.79	1.71	1.72
Kaempferol 3-(3″,4″-diacetylrhamnoside)	$C_{25}H_{24}O_{12}$	44258959	1.63	1.61	1.60	1.67	1.59	1.60
Kaempferol 3-(3″-acetylrhamnoside)	$C_{23}H_{22}O_{11}$	-	1.50	1.48	n.d.	1.54	1.47	1.48
Kaempferol 3-(4″-acetylrhamnoside)	$C_{23}H_{22}O_{11}$	44258955	1.07	1.07	1.06	n.d.	1.05	1.06
Kaempferol 3-glucuronide	$C_{21}H_{18}O_{12}$	5318759	1.27	1.27	1.25	n.d.	1.24	1.25
Kaempferol-3-(5″-feruloylapioside)	$C_{30}H_{26}O_{13}$	44259012	2.08	2.05	2.05	2.13	2.03	2.05
Kaempferol-3-(6″-caffeoylglucoside)	$C_{30}H_{26}O_{14}$	44259011	1.97	1.94	1.94	2.02	1.93	1.94
Kaempferol-3-*O*-D-glucoside	$C_{21}H_{20}O_{11}$	25203515	2.50	2.46	2.46	2.57	2.45	2.46
Kaempferol-3-*O*-rhamnoside	$C_{21}H_{20}O_{10}$	5835713	2.70	2.65	2.66	2.77	2.64	2.66
Kaempferol-4′-rhamnoside	$C_{21}H_{20}O_{10}$	44258925	2.34	2.30	2.31	2.40	2.29	2.31
Kaempferol-7-rhamnoside-4′-glucoside	$C_{27}H_{30}O_{15}$	74978093	1.75	1.73	1.73	1.80	1.71	1.73
Luteolin-7-glucoside	$C_{21}H_{20}O_{11}$	5280637	1.99	1.97	1.97	2.05	1.95	1.96
Naringin	$C_{27}H_{32}O_{14}$	442428	1.95	1.93	1.92	2.00	1.91	1.92
Orientin	$C_{21}H_{20}O_{11}$	5281675	1.87	1.85	1.85	1.92	1.83	1.84
Petunidin 3-sambubioside	$C_{27}H_{31}O_{16}$	44256968	0.78	0.79	0.77	0.80	0.77	0.77
Procyanidin A1	$C_{30}H_{24}O_{12}$	9872976	1.20	1.19	1.18	1.23	1.17	1.18
Procyanidin B1	$C_{30}H_{26}O_{12}$	11250133	1.07	1.08	1.06	1.10	1.05	1.06
Procyanidin B2	$C_{30}H_{26}O_{12}$	122738	1.34	1.33	1.32	1.38	1.31	1.32
Quercetin	$C_{15}H_{10}O_7$	5280343	2.13	2.09	2.10	2.18	2.08	2.10
Quercetin 3-glucoside	$C_{21}H_{19}O_{12}$	5280804	1.76	1.74	1.74	1.81	1.73	1.74
Quercetin-3-*O*-rhamnoside	$C_{21}H_{20}O_{11}$	5280459	2.93	2.87	2.89	3.01	2.87	2.89
Querciturone	$C_{21}H_{18}O_{13}$	5274585	1.64	1.63	1.62	1.69	1.61	1.62
Rutin	$C_{27}H_{30}O_{16}$	5280805	1.63	1.61	1.60	1.67	1.59	1.60
Monophenolic								
Eugenol	$C_{10}H_{12}O_2$	3314	0.74	0.75	0.73	0.76	0.72	0.73
Ferulic acid	$C_{10}H_{10}O_4$	445858	1.20	1.19	1.18	1.23	1.17	1.18
p-Coumaric acid	$C_9H_8O_3$	1549106	1.56	1.55	1.54	1.61	1.53	1.45
Gallic acid	$C_7H_6O_5$	370	2.93	2.87	2.89	3.01	2.87	2.89
Total phenolic acid			69.11	68.3	66.71	66.81	67.66	68.05
Total other compounds			30.89	31.7	33.29	33.19	32.34	31.95

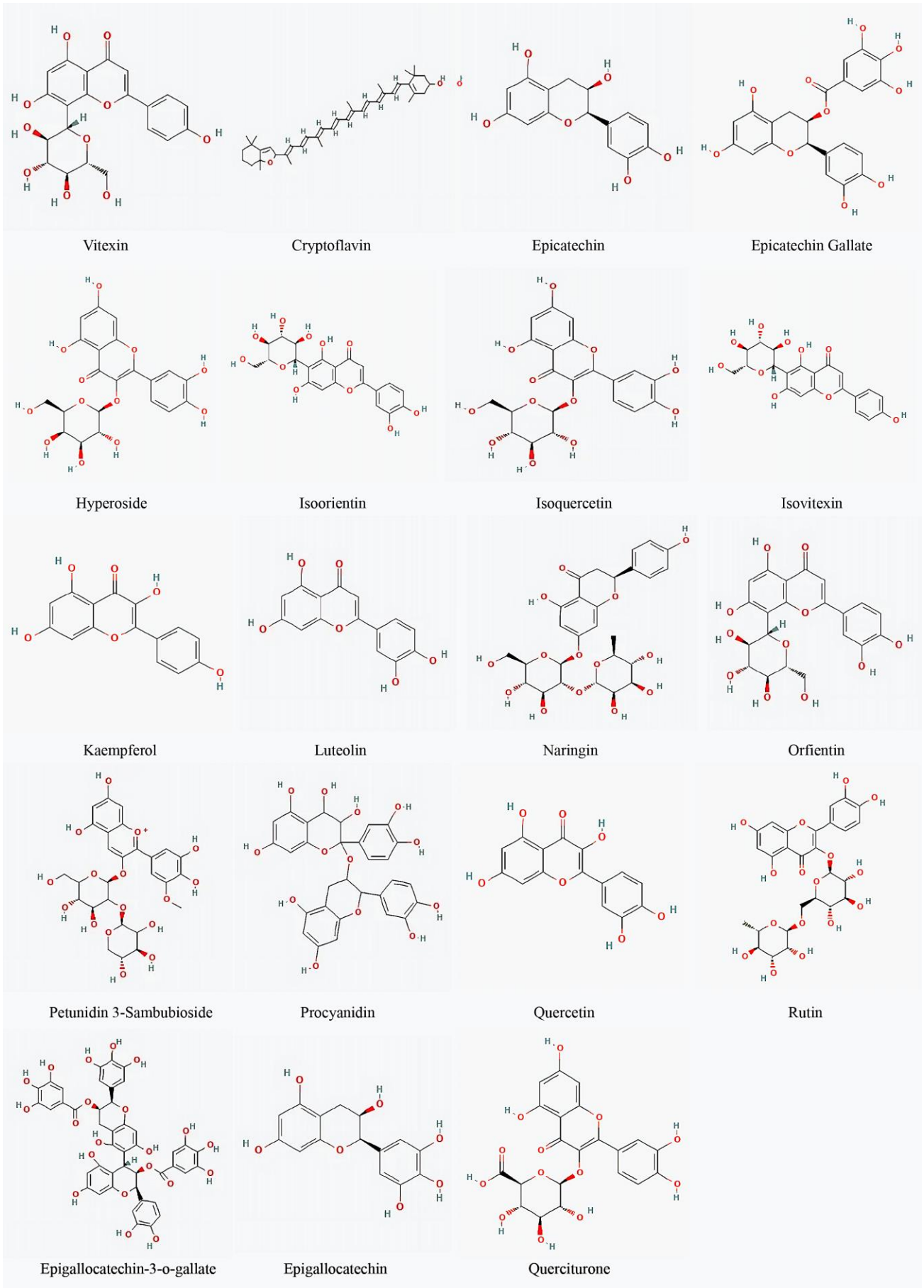

Fig. 3. Similarity in the structure of polyphenol compounds, especially flavonoids, having the main structure of the chalcone chain containing two phenyl rings and compounds that have undergone ring closure of the chalcone conjugate to produce three phenyl rings.

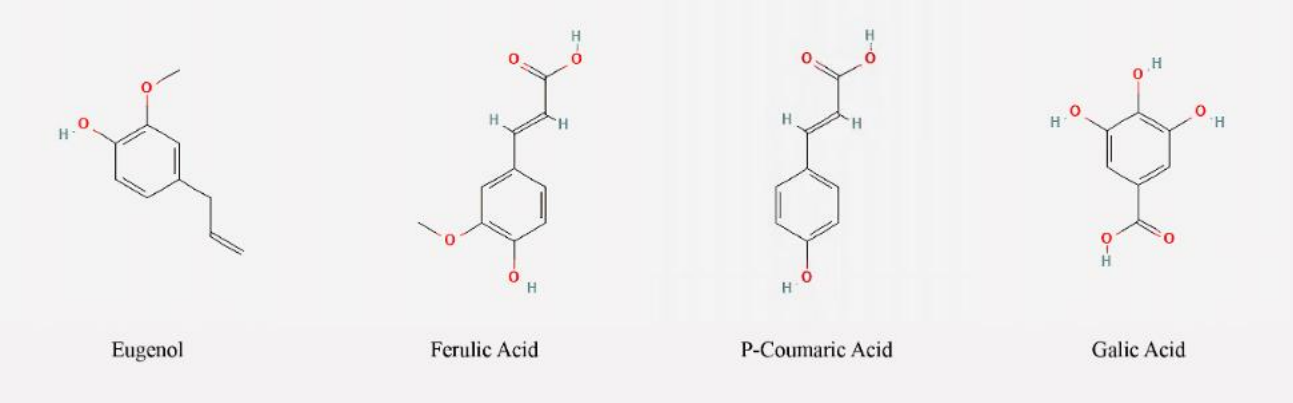

Fig. 4. Monophenolic compounds having only one phenyl aromatic ring with the side chain end of a hydroxyl or carboxyl group attached to C_1 or C_4.

The increase in the content of phenolic compounds was probably due to an increase in the quantity of cell growth during culture. The secondary metabolite profile may be influenced by metabolic activity at the cellular level as a result of the process of differentiation, specification, and organogenesis (Park *et al*., 2020). In other words, it is necessary to have a tissue differentiation process to increase the biosynthesis of secondary metabolites in cell culture. This is in line with the studies of Wojtania *et al*., (2020) who suggest that tissue-specific differentiation is a prerequisite for secondary metabolite production. Furthermore, the current study further explains that polyphenol compounds show an activity that inhibits auxin transport and accumulation in cells (Sharma *et al*., 2020). Rutin, scularein, and naringenin induce the distribution of auxin from shoots to roots by altering the formation of PIN proteins and triggering efflux via a polar auxin transport mechanism (Zhang *et al*., 2021).

Conclusion

Picloram and 2,4-D are PGRs in the form of synthetic auxins, which have the same effect in inducing the formation of secondary metabolites in *E. grandiflorus* cell cultures. In addition, cell culture induction treatment using picloram and 2,4-D can be used to produce phenolic compounds. Based on this study, four main monophenolic compounds were identified namely eugenol, ferulic acid, p-coumaric acid, and gallic acid. Them, kaempferols is the main phenolic compounds identified in all treatment, which is composed by 12 derived compounds. However, this study proved that cell suspension cultures for callus derived from petiole young leaves of the plant *E. grandiflorus* are capable of producing various types of phenolic acid compounds. The dominant compound found was kaempferol, which accounted for more than 16% of the total identified secondary metabolites.

Acknowledgment

The authors would like to thank the Directorate of Research and Community Service, Directorate General of Research and Development Strengthening Ministry of Research, Technology and Higher Education of Indonesian Republic, for funding this research with contract number 44.25.3/UN37/PPK.6.8/2021.

References

Al-Jitan, S., S.A. Alkhoori and L.F. Yousef. 2018. Phenolic acids from plants: extraction and application to human health. In Rahman, A. *Studies in Natural Products Chemistry* (1st ed., Vol. 58). Elsevier B.V, Switzerland, pp. 389-417.

Ali, M., B.H. Abbasi, N. Ahmad, H. Khan and G.S. Ali. 2017. Strategies to enhance biologically active-secondary metabolites in cell cultures of Artemisia–current trends. *Crit. Rev. Biotechnol.,* 37(7): 833-851.

Anggraito, Y.U., W.H. Nugrahaningsih, F. Musafa, K. Mukhtar, W. Wijawati, Y. Rostriana, S. Safitri and N.A. Habibah. 2020. Secondary metabolites in elaeocarpus grandiflorus cell culture in WPM medium with various concentrations of PGR. *J. Phys. Conf. Ser.*, 1524(1): 2-7.

Anjusha, S and A. Gangaprasad. 2017. Callus culture and *In vitro* production of anthraquinone in *Gynochthodes umbellata* (L.) Razafim. & B. Bremer (Rubiaceae). *Ind. Crops. Prod.*, 95: 608-614.

Ashokhan, S., R. Othman, M.H.A. Rahim, S.A. Karsani and J.S. Yacob. 2020. Effect of plant growth regulators on coloured callus formation and accumulation of azadirachtin, an essential biopesticide in *Azadirachta indica*. *Plants*, 9(352): 1-17.

Barros, J. and R.A. Dixon. 2020. Plant phenylalanine/tyrosine ammonia-lyases. *Trends Plant Sci.*, 25(1): 66-79.

Bedetti, C.S. G.P. Bragança and R.M.D.S. Isaias. 2017. Influence of auxin and phenolic accumulation on the patterns of cell differentiation in distinct gall morphotypes on *Piptadenia gonoacantha* (Fabaceae). *Aust. J. Bot.*, 65(5): 411-420.

Caser, M., W. Chitarra, F. D'Angiolillo, I. Perrone, S. Demasi, C. Lovisolo, L. Pistelli, L. Pistelli and V. Scariot. 2019. Drought stress adaptation modulates plant secondary metabolite production in *Salvia dolomitica* Codd. *Ind. Crops. Prod.*, 129(6): 85-96.

Castro, A.H.F., M.C. Coimbra, C.R.N. Crema, R.M.O Thomasi and V.R. Cardos. 2021. Exploring plant cells for the production of compounds of interest. In: (Ed.): Malik, S. *Exploring plant cells for the production of compounds of interest* (Issue April). Springer International Publishing, Switzerland. pp. 195-226.

Chandler, J.W. 2016. Auxin response factors. *Plant Cell Environ.*, 39(5): 1014-1028.

Dayan, F.E., S.O. Duke and K. Grossmann. 2010. Herbicides as probes in plant biology. *Weed Sci.*, 58(3): 340-350.

Demirci, T., A.O. Ascı and N.G. Baydar. 2021. Influence of salicylic acid and L-phenylalanine on the accumulation of anthraquinone and phenolic compounds in adventitious root cultures of madder (*Rubia tinctorum* L.). *Plant Cell, Tissue Organ Cult.*, 144(2): 313-324.

Farjaminezhad, R. and G.A. Garoosi. 2019. New biological trends on cell and callus growth and azadirachtin production in *Azadirachta indica.* 3 *Biotech*, 9(8): 1-17.

Fendrych, M., M. Akhmanova, J. Merrin, M. Glanc, S. Hagihara, K. Takahashi, N. Uchida, K.U. Torii and J. Friml. 2018. Rapid and reversible root growth inhibition by TIR1 auxin signalling. *Nat. Plants*, 4(7): 453-459.

Ferreyra, M.L.F., S.P. Rius and P. Casati. 2012. Flavonoids: Biosynthesis, biological functions, and biotechnological applications. *Front. Plant Sci.*, 3(9): 1-15.

Habibah, NA., W.H. Nugrahaningsih. Y. U. Anggraito, K. Mukhtar, N. Wijayanti, F. Mustafa and Y. Rostriana. 2019a. Effect of growth regulators on cell growth and flavonoid production in cell culture of *Elaecarpus grandiflorus. IOP Conf. Ser.: Earth Environ. Sci.*, 391(1): 012061

Habibah, N.A., T. Widiatningrum, Y.U. Anggraito, E.S. Rahayu, K. Mukhtar, Wijayanti, N and F. Mustafa, F. 2019b. Growth of *Elaeocarpus grandiflorus* callus cultures in MS medium with various concentrations of growth regulators. *J. Phys. Conf. Ser.,* 1321(3): 2-7.

Habibah, N.A., W.H. Nugrahaningsih, F. Musafa, Y. Rostriana, K. Mukhtar. N. Wijawati and Y.U. Anggraito. 2020. Bioactive compounds from callus culture of *Elaeocarpus grandiflorus*. *J. Phys. Conf. Ser.*, 1567(3): 032055

Habibah, N.A., Y.U. Anggraito. N. Nugrahaningsih, S. Safitri, F. Musafa and N. Wijayati. 2021a. LC-MS based secondary metabolites profile of *Elaeocarpus grandiflorus* J.E. Smith. cell suspension culture using picloram and 2,4-dichlorophenoxyacetic acid. *Trop. J. Nat. Prod. Res.*, 5(8): 528-533.

Habibah, N.A., N. Nugrahaningsih, F. Musafa and N. Wijawati. 2021b. Profile of flavonoid and antioxidant activity in cell suspension culture of *Elaeocarpus grandiflorus*. *Biosaintifika.*, 13(3): 328-335.

Hao, G., X. Du, F. Zhao, R. Shi and J. Wang. 2009. Role of nitric oxide in UV-B-induced activation of PAL and stimulation of flavonoid biosynthesis in *Ginkgo biloba* callus. *Plant Cell Tissue Organ Cult.*, 97(2): 175-185.

Ho, T.T., H.N. Murthy and S.Y. Park. 2020. Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. *Int. J. Mol. Sci*., 21(3): 716.

Jamwal, K., S. Bhattacharya and S. Puri. 2018. Plant growth regulator mediated consequences of secondary metabolites in medicinal plants. *J. Appl. Res. Med. Aromat. Plants*, 9(6): 26-38.

Kiokias, S., C. Proestos and V. Oreopoulou. 2020. Phenolic acids of plant origin-a review on their antioxidant activity *In vitro* (O/W emulsion systems) along with their *In vivo* health biochemical properties. *Foods*, 9(4): 1-22.

Kousalya, L and V.N. Bai. 2016. Effect of growth regulators on rapid micropropagation and antioxidant activity of *Canscora decussata* (Roxb.) Roem. & Schult. A threatened medicinal plant. *Asian Pac. J. Reprod.*, 5(2): 161-170.

Landi, M., M. Zivcak, O. Sytar, M. Brestic and S.I. Allakhverdiev. 2020. Plasticity of photosynthetic processes and the accumulation of secondary metabolites in plants in response to monochromatic light environments: A review. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1861(2): 148131.

Liu, H., Z. Liu, Y Wu, L. Zheng and G. Zhang. 2021. Regulatory mechanisms of anthocyanin biosynthesis in apple and pear. *Int. J. Mol. Sci.*, 22(8441): 1-13.

Liu, J., H. Pu, S. Liu, J. Kan and C. Jin. 2017. Synthesis, characterization, bioactivity and potential application of phenolic acid grafted chitosan: A review. *Carbohydr. Polym.* 174: 999-1017.

Ljung, K. 2013. Auxin metabolism and homeostasis during plant development. *Development* (*Cambridge*), 140(5): 943-950.

Luo, J., J.J. Zhou and J.Z. Zhang. 2018. Aux/IAA gene family in plants: Molecular structure, regulation, and function. *Int. J. Mol. Sci.*, 19(1): 1-17.

Mastuti, R., A. Munawarti and E.R. Firdiana. 2017. The combination effect of auxin and cytokinin on *In vitro* callus formation of *Physalis angulata* L. - A medicinal plant. *AIP Conf. Proc.*, 1908(9): 040007.

Melo, P.B.D.S., A.C.P.P. de Carvalho, C.H.C. Bertini, C.R.M. Muniz and A.G. Rossetti, 2019. Induction of somatic embryogenesis in two cultivars of anthurium analysed by scanning electron microscopy. *Revista Agroambiente*, 13(0): 1-13.

Metsämuuronen, S. and H. Sirén. 2019. Bioactive phenolic compounds, metabolism and properties: a review on valuable chemical compounds in Scots pine and Norway spruce. *Phytochem. Rev.*, 18(3): 623-664.

Mishra, M.K., S. Pandey, P. Misra and A. Niranjan. 2020. *In vitro* propagation, genetic stability and alkaloids analysis of acclimatized plantlets of Thalictrum foliolosum. *Plant Cell Tissue Organ Cult.*, 142(2): 441-446.

Moura, L., A.C. Xavier, A.C.F. Cruz, R. da, Gallo, C. Gatti, N.A. Miranda and W.C. Otoni. 2017. Effects of explant type, culture media and picloram and dicamba growth regulators on induction and proliferation of somatic embryos in *Eucalyptus grandis x E. Urophylla. Rev. Arvore.,* 41(5): e410502

Muszyńska, E., K,M. Tokarz, M. Dziurka, M. Labudda, K. Dziurka and B. Tokarz. 2021. Photosynthetic apparatus efficiency, phenolic acid profiling and pattern of chosen phytohormones in pseudometallophyte *Alyssum montanum. Sci. Rep.,* 11(1): 1-15.

Mwakalesi, A.J. and I.D. Potter. 2020. Removal of picloram herbicide from an aqueous environment using polymer inclusion membranes. *J. Environ. Chem. Eng.*, 8(5): 103936.

Park, J.S., Z.K. Seong, M.S. Kim, J.H. Ha, K.B. Moon, H.J. Lee, H.K. Lee, J.H. Jeon, S.U. Park and H.S. Kim. 2020. Production of flavonoids in callus cultures of Sophora flavescens aiton. *Plants*, 9(6): 1-13.

Prasannan, P., Y. Jeyaram, A. Pandian, R. Raju and S. Sekar. 2020. A review on taxonomy, phytochemistry, pharmacology, threats and conservation of *Elaeocarpus* L. (Elaeocarpaceae). *Bot. Rev.*, 86(3-4): 298-328.

Qu, J., W. Zhang and X. Yu. 2011. A combination of elicitation and precursor feeding leads to increased anthocyanin synthesis in cell suspension cultures of Vitis vinifera. *Plant Cell Tissue Organ Cult.*, 107(2): 261-269.

Radić, S., V. Vujčić, M. Glogoški and M. Radić-Stojković. 2016. Influence of ph and plant growth regulators on secondary metabolite production and antioxidant activity of Stevia rebaudiana (Bert). *Period. Biol.*, 118(1): 9-19.

Rahayu, E.S., N.K. Dewi and F.P.M.H. Bodijantoro. 2018. Profile of *Elaeocarpus grandiflorus* and *Ziziphus mauritiana* as identity plants of Salatiga and Tegal towns, Central Java Province, Indonesia. *J. Phys. Conf. Ser.*, 983(1): 012195

Rawat, J.M., B. Rawat, R.K. Agnihotri, A. Chandra and S. Nautiyal. 2013. *In vitro* propagation, genetic and secondary metabolite analysis of Aconitum violaceum Jacq.: A threatened medicinal herb. *Acta Physiol. Plant*, 35(8): 2589-2599.

Sharma, A., P. Verma, A. Mathur and A.K. Mathur. 2018. Overexpression of tryptophan decarboxylase and strictosidine synthase enhanced terpenoid indole alkaloid pathway activity and antineoplastic vinblastine biosynthesis in Catharanthus roseus. *Protoplasma*, 255(5): 1281-1294.

Sharma, P., V. Kumar, R. Khosla and P. Guleria. 2020. Exogenous naringenin improved digestible protein accumulation and altered morphology via VrPIN and auxin redistribution in Vigna radiata. 3 *Biotech*, 10(10): 1-14.

Singh, B., J.P. Singh, A. Kaur and N. Singh. 2018. Phenolic compounds as beneficial phytochemicals in pomegranate (*Punica granatum* L.) peel: A review. *Food Chem.*, 261(4): 75-86.

Szewczyk, A., I. Kwiecień, M. Grabowski, K. Rajek, E. Cavò, M.F. Taviano and N. Miceli. 2021. Phenylalanine increases the production of antioxidant phenolic acids in *Ginkgo biloba* cell cultures. *Molecules*, 26(4965): 1-16

Wang, Y.C., N. Wang, H. Xu, S. Jiang, H.C. Fang, M.Y. Su, Z.Y. Zhang, T.L. Zhang and X.S. Chen. 2018. Auxin regulates anthocyanin biosynthesis through the Aux/IAA-ARF signaling pathway in apple. *Hortic. Res.*, 5(1): 1-11.

Wojtania, A., M. Dziurka and E. Skrzypek. 2020. *In vitro* rooting response of yellow-flowered magnolia in relation to the phenolic acids content. *Agronomy*, 10(12): 1-12.

Yamauchi, T., A. Tanaka, H. Inahashi, N.K. Nishizawa, N. Tsutsumi, Y. Inukai and M. Nakazono. 2019. Fine control of aerenchyma and lateral root development through AUX/IAA- And ARF-dependent auxin signaling. *Proc. Natl. Acad. Sci. U.S.A.*, 116(41): 20770-20775.

Yang, X., X. Cao, L. Zhang, Y. Wu, L. Zhou, G. Xiu, C. Ferronato and J.M. Chovelon. 2021. Sulfate radical-based oxidation of the aminopyralid and picloram herbicides: The role of amino group on pyridine ring. *J. Hazard. Mater.*, 405(9): 124181.

Zabalza, A., L. Orcaray, M. Fernández-Escalada, A. Zulet-González and M. Royuela, 2017. The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots. *Pestic. Biochem. Physiol.*, 141: 96-102.

Zaman, M.A.K., A.M. Azzeme, I.K. Ramle, N. Normanshah, S.N. Ramli, N.A. Shaharuddin, S. Ahmad and S.N.A. Abdullah. 2020. Induction, multiplication, and evaluation of antioxidant activity of Polyalthia bullata callus, a woody medicinal plant. *Plants*, 9(12): 1-21.

Zhang, X., X. Huang, Y. Li, F. Tao, Q. Zhao and W. Li. 2021. Polar auxin transport May Be responsive to specific features of flavonoid structure. *Phytochemistry*, 185(6): 112702.

Zhao, S., X. Mi, R. Guo, X. Xia, L. Liu, Y. An, X. Yan, S. Wang, L. Guo and C. Wei. 2020. The biosynthesis of main taste compounds is coordinately regulated by miRNAs and phytohormones in tea plant (*Camellia sinensis*). *J. Agric. Food Chem.*, 68(22): 6221-6236.

(Received for publication 28 December 2021)