Songklanakarin J. Sci. Technol.
45 (3), 331–340, May – Jun. 2023

http://www.sjst.psu.ac.th

Original Article

Callogenesis, growth and bioactive compounds of kaffir lime (*Citrus hystrix* DC.) callus derived from leaf and stem explants

Reva Suzana, Angellia Melliana Pramesthi, and Woro Anindito Sri Tunjung*

Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, 55281 Indonesia

Received: 26 November 2022; Revised: 21 March 2023; Accepted: 23 March 2023

Abstract

Differences in plant organs that are used as sources of explants can cause differences in callus growth and synthesized bioactive compounds. The objective of this study was to induce calli from different sources of explants and analyze their growth and bioactive compounds in the calli from leaf and stem explants of kaffir lime seedlings. Kaffir lime seeds were germinated until they grew into seedlings. On day 35, the leaves and stems of seedlings were harvested and callus was induced. Results showed that callus initiation time of stem explants was 5.66 days faster than that of leaf explants, which required 12.42 days. The colors of these calli were slightly different. Furthermore, callus fresh weight of leaf explants was less than that of stem explants, and the stationary phase of leaf explant-derived callus was earlier than for those from stem explant. Bioactive compounds detected in calli derived from leaf and stem explants were different. The main compounds found in the leaf explant-derived callus were n-decanoic acid and hexanedioic acid, bis (2-ethylhexyl) esters, while stem explant-derived callus had n-hexadecanoid acid. The presence of various bioactive compounds in these calli indicates potential for use as a natural medicine.

Keywords: in vitro seedling, callus, explant, kaffir lime, bioactive compounds

1. Introduction

Kaffir lime (*Citrus hystrix* DC.) has long been used as a traditional medicinal plant because it has biological activities such as antibacterial, antioxidant, antifungal, anti-inflammatory and antiviral (An *et al.*, 2021). Previous studies have shown that kaffir lime leaf extract is cytotoxic against cervical cancer and neuroblastoma cells (Tunjung, Cinatl, Michaelis, & Smales, 2015), and has an inhibitory effect on leukemia K526 cell line (Anuchapreeda *et al.*, 2020). Essential oil from the peel extract of kaffir lime fruit has antibacterial activity against Gram positive and Gram-negative bacteria (Sreepian, Sreepian, Chanthong, Mingkhwancheep, & Prathit, 2019). Kaffir lime fruit extract can inhibit the growth of *Streptococcus mutans* bacteria (Utami *et al.*, 2020). The twigs of kaffir lime can be used as antioxidants because they have as the main component citronellal (Silalahi, 2020; Warsito *et al.*, 2017). Thus, each part of kaffir lime plant has a different bioactive content and biological activities that can be used in traditional medicine.

Several studies have shown that the biosynthesis of secondary metabolites can occur in all plant parts, including roots, leaves, shoots, flowers, fruits, and seeds (Anggraito *et al.*, 2018). Maslakhah, Mutiah, Hakim, Aprinda, and Suryadinata (2018), reported that extracts from the roots, stems, leaves, and seeds of the *Helianthus annuus* L plant had varying amounts of compounds. Accordingly, terpenoid compounds, such as sesquiterpenes, triterpenes, and steroids are found in the flower of *Helianthus annuus* L. extracted using methanol. Leaf extract contains alkaloids, flavonoids, and phenols. Stem extract contains alkaloid compounds, phenols, and flavonoids. Seed extract contains carbohydrates, flavonoids, tannins, alkaloids, saponins, and essential oils. Therefore, it is necessary to produce bioactive compounds from several plant organs.

One of the strategies to produce bioactive compounds from plants on a large scale is to establish callus and cell suspension cultures (Chandana, Nagaveni, Heena, Kolakar, & Lakshmana, 2018). Callus is very important in regenerating plants because each plant cell has the ability to form a new plantlet (Rasud & Bustaman, 2020). Moreover, in

*Corresponding author
Email address: wanindito@ugm.ac.id

vitro seedling is one of the tissue culture techniques that can be used to produce a sterile source of explants. Explants obtained from in vitro seed germination can reduce failure rates in callus cultures due to contamination (Anggraeni, 2016). Each part of the seedling has meristematic properties. Hypocotyl is the longest part of seedling so that this explant is more beneficial for callus production (Setiaji, 2020). Abbas, El-Shabrawi, Soliman, and Selim (2018) successfully induced callus from various explants from in vitro germinated seedlings, namely the stems, roots and leaves of the African locust bean plant *Parkia biglobosa* (Jacq.) Bench.

A previous study by Tunjung *et al.*, (2021) has successfully induced a friable kaffir lime callus using seed explants. The seeds are undifferentiated embryonal tissue which can be induced to be plantlet or callus. Seeds are suitable for use as an explant source because the seed is located inside the fruit, thus it is easy to avoid contamination. However, it takes time to wait for the fruits. Picking ex vitro plant organs directly from the outdoor environment is prone to contamination, thus in vitro seedling is carried out to maintain the availability of explant sources under controlled conditions. The leaves and stems of the kaffir lime plantlet are used as sources of explants for callus induction because they have gone through organogenesis, so the cells and tissues formed are different. Therefore, the objective of this study was to determine the callogenesis, growth and synthesis of bioactive compounds from callus initiated from in vitro seedling leaf and stem explants.

2. Materials and Methods

2.1 Materials

Fruit of kaffir lime were collected from a farm at Pekuten Village, Bayan Purworejo District, Central Java, Indonesia. Only good fresh fruit of diameter approximately 5-6 cm were sampled. The fruit were peeled and seeds at size of ± 0.9-1 cm in length and ± 0.4-0.5 cm in width were excised and used for in vitro germination.

2.2 Seed germination and growth of seedling

The basal media used was Murashige and Skoog (MS) (Murashige & Skoog, 1962) which contains macronutrients, micronutrients, iron, vitamins, myo-inositol, sucrose, and agar. The pH was adjusted to 5.8. Kaffir lime seeds were sterilized using 5.25% NaClO by shaking for 5 minutes under aseptic conditions. Next, the seeds were rinsed with sterile distilled water for 1 minute 3 times. Then, the sample was sterilized again with 70% alcohol for 5 seconds. The sterilized seeds were inoculated into culture bottles containing solid MS media without growth regulators (MS0). Each bottle contained 4 seeds. The culture was stored and incubated in a culture room under dark conditions at a temperature of 25^{0}C and a humidity of about 50%, until radicle appeared. Then, the culture bottle was placed in a bright illumination until 35-40 days (1000 lux light intensity).

2.3 Callus induction and growth determination

Seedlings at age 35-40 days after germination were used as source of explants for callus induction. The leaf explants were cross-cut to a size of 0.5 x 0,5 cm in length and width, and the stem explants were cut into 0,5 cm length. Both explants were cultured on basal solidified MS medium with plant growth regulator (PGR) 2,4-D: BAP 1:0.5 ppm from a previous study (Tunjung *et al.*, 2021) having the wound side in contact with the culture medium. The culture was incubated in a culture room under dark conditions at a temperature of 25ºC.

Callus induction time was characterized by the appearance of cell masses on the wound surface of explant. Callus biomass in terms of fresh weight and dry weight was measured every 5 days for 50 days in G0 (without subculture). These data were analyzed to obtain the callus growth curve. Callus color was measured using the Royal Horticultural Society color chart whereas the texture of callus was observed by tipping the callus using forceps and subjective scoring.

2.4 Secondary metabolite analysis

Calli derived from both leaf and stem explants were harvested in the stationary phase. The stationary phase is the optimum condition for secondary metabolite analysis because growth has stabilized (Purwaningsih *et al.*, 2016). The calli were then dried at 37^{0}C for 24 hours until constant weight. A dry 100 mg sample of calli was dissolved in 5 ml of ethyl acetate. Maceration was carried out for 24 hours with 3 re-maceration repeats. The extracts were analyzed using gas chromatography with mass spectrometry (GC-MS) on Agilent chromatography 60034. As much as 1μL from the extracted sample was injected into the instrument. Helium was used as the mobile phase at 1 mL/min. The spectrum of components obtained was compared with the National Institute of Standards and Technology (NIST) database 17 GC Method/Retention Index Library. The relative percentage of each component in the compound was calculated by comparing the average peak area with the total area.

3. Results and Discussion

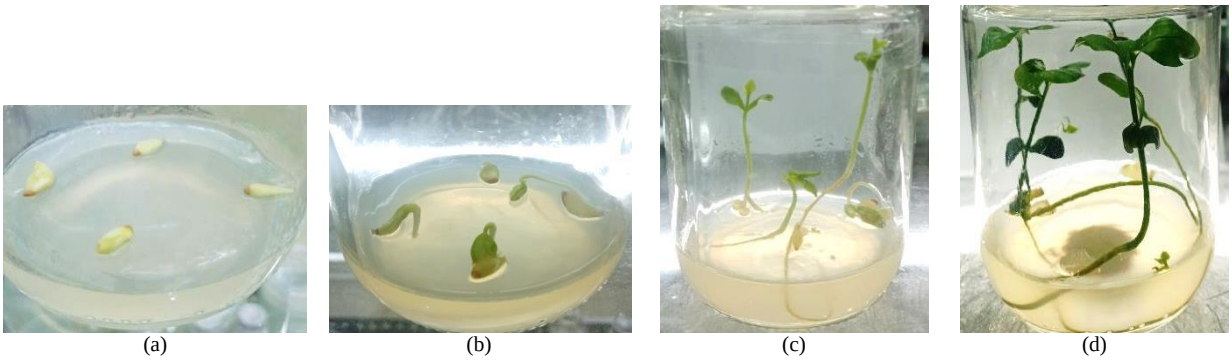
3.1 Results

3.1.1 In vitro germination and growth of seedlings

Germination is characterized by the appearance of radicle from inside the seed. All the kaffir lime seeds were successfully germinated (100%). The radicle continued growing downward and hypocotyl with cotyledons grew later on upward. In this study, the radicle appeared 6 days after sowing the seeds, whereas the cotyledon appeared on day 15. Kaffir lime seedlings at age 35-40 days were producing true leaves of 1 cm length and width, which were used as explants for callus induction. These phenomena can be seen in Figure 1.

3.1.2 Callus induction and growth determination

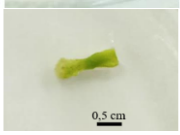
The callus initiation time was recorded when the callus first appeared after the first day of induction (Table 1). The emergence of callus was characterized by the presence of swelling on the explants in the injured area, accompanied by white patches.



(a) (b) (c) (d)

Figure 1. Germination of kaffir lime (a) 0 days, (b) 6 days, (c) 15 days, and (d) 35 days

Table 1. Callus initiation time

Number	Type of explants	Callus initiation time (days)	Picture
1.	Leaves	12,42 ± 0,83[a]	
2.	Stems	5,66 ± 0,29[b]	

Numbers followed by different superscripts are significantly different based on one-way ANOVA analysis.

Table 2. Growth phases of calli

Explant	Growth phases (days)		
	Lag phase	Exponential phase	Stationary phase
Leaves	0-15	15-30	30-50
Stems	0-10	10-35	35-50

Leaf and stem explants were 100% successful in forming calli. This is supported by Tunjung *et al.*, (2021), in that the addition of growth regulators such as 2,4-D and BAP can induce calli from seed explants. However, there was a significant difference in the timing of callus induction between leaf callus and stem callus. Table 1 shows that the initiation times of the leaf and stem explant calli were 12.42 days and 5.66 days, respectively. For comparison, our previous study showed that induced kaffir lime seed explants took 7.78 days to form a callus (Tunjung *et al.*, 2021).

Table 1 shows that the initiation time of leaf explant callus is 12.42 days and for stem explant callus it is 5.66 days. This is in accordance with the research by Naser & Wisnu (2020), showing that the initiation times of the leaf and stem explant calli (*Chrysanthemum morifolium* Ramat cv Dewi ratih) were different. Leaves formed calli on day 13 whereas stems formed calli on day 7. The difference in time required for callus formation might be due to organ differentiation, totipotency, and levels of endogenous auxin hormones (Tarrahi & Rezanejad, 2013).

3.1.3 Callus growth curve

The wet and dry weights of the leaf and stem explant calli were determined to create a callus growth curve (Figure 2). The callus growth curves for leaf and stem explants had significant differences in each phase of callus growth (Table 2). Table 2 shows the different of growth phases of calli from leaf and stem explants. The exponential growth phase of stem explant callus was faster than that of leaf explant callus. In addition, the stationary phase of the stem explant callus was slower than that of the leaf explant callus. The growth and development of calli can be seen from the biomass produced. The biomass of stem explant calli was higher than that of leaf explant calli. Our previous study (Tunjung *et al.*, 2021), showed that kaffir lime callus derived from seeds explant enters the exponential phase on day 10 and the stationary phase began on day 35. Therefore, each organ of kaffir lime had a different growth phase. Figure 2 shows that the biomass of stem explant calli was higher than that of leaf explant calli. This is supported by Suhartanto, Astutik, Umami, Suseno, & Haq (2022), where the callus explant stems from the white srikandi corn plant (*Zea mays* L.) produce the highest fresh weight compared to root and leaf explants. The fresh weight of the callus indicates that cell division occurs in the callus, so a high fresh weight indicates the cells are actively dividing.

3.1.4 Callus morphology

Table 3 shows color and texture changes of calli. At day 50 calli from leaf explant had light yellow-green color, while stem explants showed brilliant yellow green color. Callus color indicates the degree of development of the formed callus. Furthermore, both leaf and stem explant calli had a friable texture. The callus's score described changes in texture from the compact seed, into the callus that was getting friable day by day. The friable callus is a good callus for the manufacture of suspension cultures in an effort to multiply cell mass (Yelnititis, 2012). Leaf and stem explant calli induced with ZPT 2,4:BAP (1: 0.5) are of callus type with a friable texture. Induced calli in media with growth regulator 2,4-D: BAP (1: 0.5) have a larger size and are good for long-term storage (Tunjung, 2021).

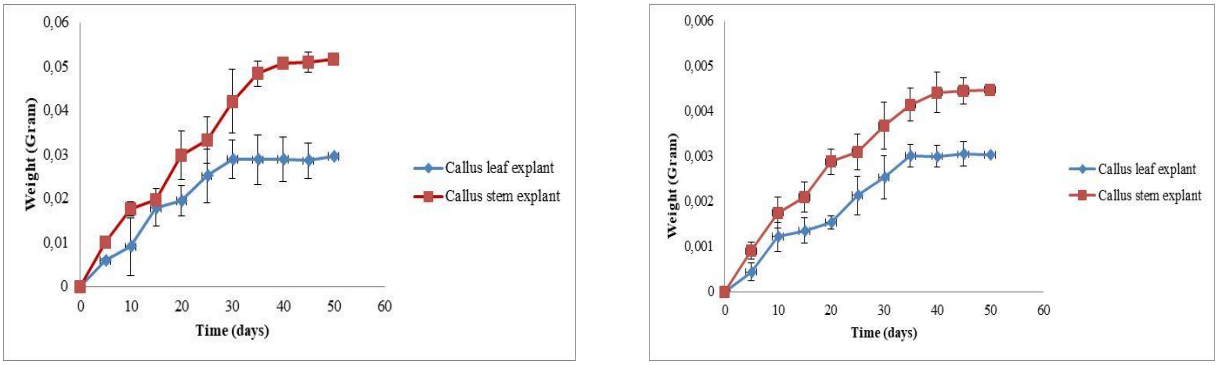
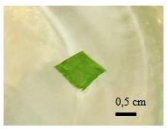
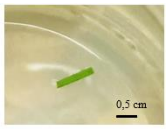
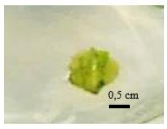
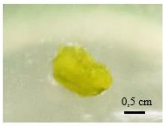
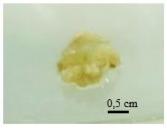
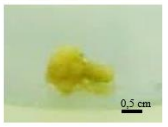
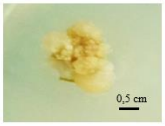
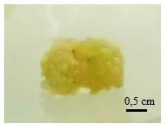


Figure 2. Growth curves of leaf and stem explants. (a) Gross weight, and (b) dry weight

Table 3. The morphology of calli

Day	Type of explant			
	Leaf		Stem	
1		Color : 140 A (Vivid Yellowish Green) Texture : (-)		Color : 141C (Strong Yellowish Green) Texture : (-)
15		Color : 140 B (Briliant Yellow Green) Texture : (+)		Color : N144B (Strong Yellow) Texture : (+)
35		Color : 142 A (Strong Yellowish Green) Texture : (++)		Color : 149 A (Briliant Yellow Green) Texture : (++)
50		Color : 142 C (Light Yellow Green) Texture : (+++)		Color : 150 B (Brilliant Yellow Green) Texture : (+++)

- : no callus has appeared yet
+ : the texture is compact
++ : the callus spreads to all parts and the texture is less friable
+++ : callus mass dominates explant mass and friable texture

Table 3 shows the color and texture of calli from leaf and stem explants. Leaf explant callus was light yellow green in color and stem explant callus was brilliant yellow green in color. Callus color indicates the degree of development of the formed callus. Leaf and stem explant calli induced with ZPT 2,4:BAP (1: 0.5) were of a type with a friable texture. Friable callus is a good callus for the manufacture of suspension cultures in an effort to multiply cell mass (Yelnititis, 2012). Induced callus in media with growth regulator 2,4-D: BAP (1: 0.5) has a larger size and is good for long-term storage.

3.1.5 Bioactive compounds

Figure 3 shows that 37 and 23 peaks of compounds were detected in leaf and stem, respectively. The names of the compounds and their biological activities are presented in Table 4 (calli from leaf explants) and in Table 5 (calli from stem explants). Bioactive compounds in both calli are mostly fatty acids and their derivatives. The n-decanoic acid and hexanedioic acid, bis (2-Ethylhexyl) esters, are the 2 main components in leaf explant callus extract, while the n-hexadecanoic acid was the main compound component of the

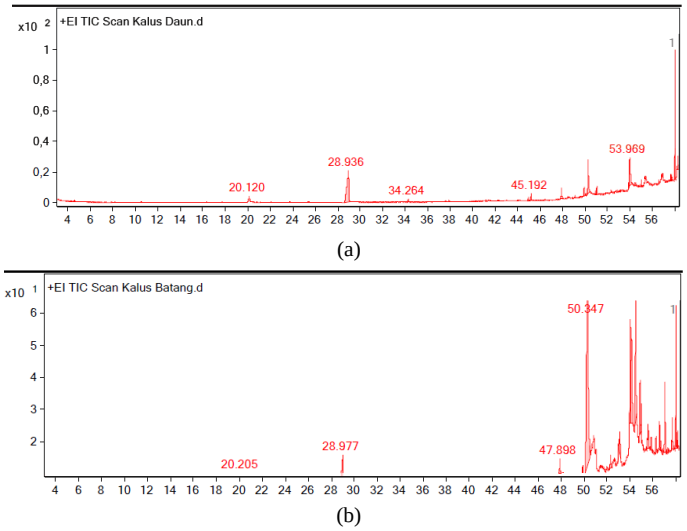


Figure 3. TIC of callus extract. (a) Calli from leaf explants, and (b) calli from stem explants

Table 4. Content of bioactive compounds in callus from leaf explant

No	Compound	Peak area (%)	Retention time (RT)	Group	Biomedical activities
1	2-Hexanol, 3,4-dimethyl	0,26	4,57	Fatty alcohol	-
2	Octanoic acid	1,37	20,12	Saturated fatty acid	Insenticidal, Antimicrobial (Kaczmarek *et al.*,2022)
3	n-Decanoic acid	24,1	28,93	Saturated fatty acid	Antibacterial, Antifungal (Belakhdar *et al.*, 2015)
4	Cyclopentanol	0,35	31,2	Alcohol	-
5	2,4-Di-tert-butylphenol	0,76	34,26	Phenol	-
6	Oxalic acid, allyl pentadecyl ester	0,34	37,59	Dycarboxylic acid	-
7	2-Cyclopropylbutan-2-ol	0,33	37,87	Alcohol	-
8	4-Oxo-.beta.-isodamascol	0,32	44,04	-	-
9	2-Piperidinone, N-[4-bromo-n-butyl]-	0,78; 1,7	44,95; 51,04	Ketone	Antimicrobial activity (Al-Bahadily *et al.*, 2019)
10	Heptadecane, 2,6,10,14-tetramethyl-	1,7	45,19	Alkane hydrocarbon	-
11	Evodinnol	2,97; 0,3	47,89; 49,57	Salicylaldehyde	-
12	7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	0,53	48,47	Cylic ketone	Antineoplastic, antimicrobial and antiviral activities (Tatipamula *et al.*, 2019)
13	Undec-10-ynoic acid, tridec-2-yn-1-yl ester	0,63	49,10	Aliphatic	-
14	1,2-Benzenedicarboxylic acid, butyl octyl ester	2,02	49,90	-	-
15	2,2-Dimethyl-6-methylene-1 [3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol	0,45; 0,27; 0,29; 0,88; 1	50,04; 52,87; 54,89; 56,54; 56,88	Methyl ester	-
16	n-Hexadecanoic acid	11,78	50,26	Long chain fatty acid	Antioxidant, Anti-inflammatory, Antibacterial, Pestiside, cancer preventive (Hameed *et al.*, 2015)
17	Oleic Acid	0,72	50,90	Monounsaturated omega-9 fatty acid	Antioxidant, Antifungal, Antiviral, Anti Bacterial (Dailey *et al.*, 2011), Anticancer activity against MCF-7 and HT-29 cancer cells (Batur *et al.*, 2019)
18	Ethanone, 1-(1,2,3,4,7,7a-hexahydro-1,4,4,5-tetramethyl-1,3a-ethano-3aH-inden-6-yl)-	0,43	52,28	Salicylaldehyde	-

Table 4. Continued.

No	Compound	Peak area (%)	Retention time (RT)	Group	Biomedical activities
19	Limonen-6-ol, pivalate	0,25	53,13	Monoterpene	Antioxidant, Anti-inflammatory, insect repellent activity (Abdulhafiz *et al.*, 2020)
20	2-Pentanone, 4-cyclohexylidene-3,3-diethyl-	1,34	53,84	Acyclic sesquiterpene alcohol	-
21	cis-7-Hexadecenoic acid	11,14	53,96	Monounsaturated Fatty acid	Anti-inflammatory (Astudilo *et al.*, 2018)
22	7-Methyl-Z-tetradecen-1-ol acetate	1,22; 1,15; 0,51; 0,39; 1,61; 0,25	54,45; 54,99; 55,37; 56,78; 57,65;58,16	Acetate ester	Anticancer, Anti-inflammatory, Hepatoprotective (Hameed *et al.*, 2015)
23	Eeicosatetraenoic acid	0,4	55,34	Polyunsaturated Fatty acid	Antimicrobial, Antibacterial (Thien le *et al.*, 2017)
24	9,12,15-Octadecatrienoic acid	1,45	57,62	Polyunsaturated Fatty acid	Anti-inflammatory (Rigoberto *et al.*, 2017)
25	Hexanedioic acid, bis(2-ethylhexyl) ester	22,78	57,98	Long chain fatty acid	Antioxidant, Anti-inflammatory, Antibacterial, Pestiside, cancer preventive (Hameed *et al.*, 2015)
26	Sulfurous acid, cyclohexylmethyl octadecyl ester	3,19	58,25	Long chain fatty acid	Pesticide activity (Ravi *et al.*, 2018)

: compounds that have the highest percent peak area
: compounds found in leaf callus and stem callus

Table 4 shows the bioactive compounds detected in calli from leaf explants. The n-decanoic acid and hexanedioic acid, bis (2-ethylhexyl) esters, are the 2 main components in leaf explant callus extract.

Table 5. Content of bioactive compounds in callus from stem explant

No	Compound	Peak area (%)	Retention time (RT)	Group	Biomedical activities
1	1,8-Nonadien-3-ol	1,71	3,11	Fatty alcohol	Antioxidant potential, Antibacterial (Muthumperumal *et al.*, 2016)
2	Octanoic acid	1,13	20,20	Saturated fatty acid	Insenticidal activity, Antimicrobial (Kaczmarek *et al.*, 2022)
3	n-Decanoic acid	3,74	28,97	Saturated fatty acid	Antibacterial, Antifungal (Belakhdar *et al.*, 2015)
4	Tetradecane, 1-chloro	0,85	47,89	Ester	Antioxidant and anti-inflammatory (Palariya *et al.*, 2019)
5	n-Hexadecanoic acid	41,35	50,34	Long chain fatty acid	Antioxidant, nematicide, pesticide, Hypocholesterolamic (Siswadi *et al.*, 2021)
6	2,2-Dimethyl-6-methylene-1-[3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol)	0,85; 2,69; 2,21; 0,62, 1,1; 1,36; 4,09; 1,98; 0,61; 0,68	52,29; 54,86; 55,56; 55,84; 56,25; 56,54; 57,03; 57,67; 58,08; 58,15	Methyl ester	-
7	Z-8-Methyl-9-tetradecenoic acid	0,48	52,96	Fatty acid	Antibacterial activity (Kadhim *et al.*, 2016)
8	Tetradecane, 2,6,10-trimethyl	1,77	53,1	Fatty acid	Antimicrobial (Sheoran *et al.*, 2015)
9	cis-Vaccenic acid	8,83; 7,12	54,03; 54,12	Fatty acid	Antibacterial activity and hypolipidemic effect in rat (Semwal *et al.*, 2018)
10	Octadecanoic acid	8,67	54,48	Stearic acid	-
11	9,12,15-Octadecatrienoic acid	1,11	54,97	Polyunsaturated Fatty acid	Anti-inflammatory (Rigoberto *et al.*, 2017)
12	Limonen-6-ol, pivalate	1,11	56,62	Monoterpene	Antioxidant, Anti-inflammatory (Abdulhafiz *et al.*, 2020), Insect repellent activity (Mohiuddin *et al.*, 2018)

Table 5. Continued.

No	Compound	Peak area (%)	Retention time (RT)	Group	Biomedical activities
13	Adipic acid	5,93	57,98	Long chain fatty acid	Antioxidant, nematicide, pesticide, Hypocholesterolamic (Siswadi *et al.*, 2021)

: compounds that have the highest percent peak area
: compounds found in leaf callus and stem callus

Table 5 shows the bioactive compounds present in callus from stem explants. The n-hexadecanoic acid is the main compound component from the stem explant callus.

stem callus explant. Furthermore 2,2-dimethyl-6-methylene-1 [3,5-dihydroxy-1-pentenyl]cyclohexan-1-perhydrol and limonen-6-ol, pivalate were the common compounds detected in both stem and leaf derived calli.

3.2 Discussion

The objective of this study was to induce kaffir lime calli from several plant organs. Previously we successfully induced friable calli from seed explants. In this study, we used leaves and stems produced by in vitro seedling as the explant sources. To our knowledge, this is the first study using kaffir lime plantlets for callus induction. Plantlet as a source of explants has advantages. This is supported by Umami, Respati, Rahman, Umpuch, & Gondo (2022), which state that each part of the plantlet can be induced into a callus because it has meristematic properties that the cells are still actively dividing. Study by Nursadi *et al.*, (2003) in Purba, Yuswanti, & Astawa (2017), used explants or meristematic planting material to avoid browning in callus. Differentiation leads to the formation of permanent tissues of meristematic tissue that have a certain structure and function. Furthermore, this study revealed that the use of plantlets as a source of explants will produce more biomass because each part of the plantlet can be used for callus induction.

Callus initiation is characterized by the presence of swelling of the explants on the injured area, accompanied by white patches (Khaniyah & Habibah, 2012). Swelling or thickening of the explant occurs due to the interaction between the explant and the planting medium, hormones, and the appropriate environment so that the explant increases in size (Rasud & Bustaman, 2020). The initiation time of the stem explant was 5.66 days, while the leaf explant took 12.42 days. Accordingly, stem explants were faster in forming calli than leaf explants. This is in accordance with the study by Naser and Wisnu (2020), showing that the initiation times of the leaf and stem explant calli (*Chrysanthemum morifolium* Ramat cv Dewi ratih) were different. The leaf explant callus is formed on day 13 and the stem explant callus on day 7. Differences between explants indicate different types of organ differentiation, totipotency, and levels of endogenous auxin hormones (Tarrahi & Rezanejad, 2013).

Callus growth is characterized by an irreversible increase in biomass (I'anatushshoimah, Nurchayati, Prihastanti, & Hastuti, 2020). Callus biomass also depends on the morphology of the callus, the speed of cell division, and the enlargement of the callus so that the role of growth regulators is very important for callus growth (Shinta, Minarno, & Rofiqoh, 2020). In this study, the biomass of the stem explant callus was higher than that of the leaf callus, where on the 50th day the weight of the stem callus reaches 0.05 grams while the leaf callus is only 0.03 grams. These data are supported by Kartikasari, Hidayat, and Ratnasari (2013), as the growth of callus in one plant species can differ depending on the growth conditions and the location of the explant part used from the plant. Furthermore Mastuti, Widoretno, and Harijati (2020), also say that different types of explants give different responses in speed of growth and development of the calli.

Furthermore, growth regulators such as auxins and cytokinins with balanced concentrations are able to initiate cell division and optimize cell growth (Prashariska, Pitoyo, & Solichatun, 2021). The auxin hormone 2,4-D is used in callus culture because of its strong activity to push ahead the process of cell differentiation, suppress organogenesis, and maintain callus growth (Indah & Ermavitalini, 2013). Moreover, 2,4-D stimulates the optimal proliferation and growth of embryogenic callus (Carsono *et al.*, 2021). BAP plays a role in spurring explant growth, cell division, and bud formation (Nadeak, Anna, & Siregar, 2012). It can stimulate cell division, increase protein synthesis and influence callus growth and the production of secondary metabolites. Moreover, the addition of BAP to MS medium produces higher callus biomass than without the addition of BAP (Tunjung *et al.*, 2021). Callus induced in a medium with a combination of auxin and cytokinin hormones has a good effect on the formation and growth of callus, and also affects the production of secondary metabolites (Bienaimé *et al.*, 2015).

The color of both calli changed day by day until it became brownish-yellow. The callus of the leaf explants was light yellow-green while the stem explant callus was brilliant yellow-green. Callus color was influenced by various factors, such as variations in the type of organ differentiation, the level of activity of endogenous hormones, pigmentation, and the type of explants used (Garcia, Pacheco, Falcão, Borges, & Mansur, 2011). The difference in callus color indicates the degree of callus development (Royani *et al.*, 2020). A green callus indicates that the callus cells are still actively dividing and contain chlorophyll (Sinaulan, Lenkong, & Tulung 2019). Green callus has a high content of bioactive compounds and has the potential to serve as an antioxidant (Ashokhan, Othman, Abd Rahim, Karsani, & Yaacob, 2020). A callus is brown due to an increase in the accumulation of phenolics such as lignin, and a decrease in peroxidase activity towards the end of the culture period. The cells on the callus continue to turn brown and die (Chaudhary & Dantu, 2015).

On the other hand, calli from leaf and stem explants had different types of bioactive compounds. This is supported by Lahsin *et al.*, (2016), where calli from seeds, leaves and

groundcherry fruit (*Physalis peruviana*) explants contained different bioactive compounds. Some secondary metabolites are unevenly distributed in the plant organs. Moreover, the expression of the secondary metabolite compounds synthesis depends on the stage of development of the organ (Anggraito *et al.*, 2018). Young leaves on tobacco plants (*Nicotiana tabacum* L.) produce the most salicylic acid compared to adult leaves and stems and it is not found in the roots (Nugroho, 2014).

According to Koperuncholan *et al.*, (2015), bioactive compounds are generally produced on certain synthesis pathways that can differ between types of compounds and plant species. The presence of differences in the amount and type of bioactive compounds in kaffir lime leaf and stem explant calli revealed that the bioactive compounds were distributed unevenly in various organs. The bioactive compounds detected in both types of calli had several biological activities (Tables 4 and 5) such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, anticancer etc. Therefore, the calli in the recent study can be used as valuable sources of natural medicines because the different compounds in each plantlet organ can complement each other. In addition, the results of this study showed that leaf and stem explants from in vitro germination were able to produce callus with good growth. Therefore, further studies need to be conducted on the addition of elicitors or cofactors to enhance the growth and synthesis of secondary metabolite compounds in callus.

4. Conclusions

Stem explants had a shorter callus initiation time than leaf explants. The callus color and texture differed by the type of explants used. The stem derived-callus biomass was higher than the leaf derived-callus but the identified bioactive compound content was less. The compounds detected in the leaf and stem explant callus extracts were mostly fatty acid groups and their derivatives, namely n-decanoic acid, hexanedioic acid, bis (2-ethylhexyl) ester, and n-hexadecanoid acid, which have various biological activities.

References

Abbas, M. S., El-Shabrawi, H. M., Soliman, A. Sh., & Selim, M. A. (2018). Optimization of germination, callus induction, and cell suspension culture of African locust beans *Parkia biglobosa* (Jacq.) Benth. *Journal of Genetic Engineering and Biotechnology*, *16*(1), 191–201. Retrieved from https://doi.org/10.1016/j.jgeb.2017.10.012

Anggraeni, A. (2016). Sterilization Techniques and Growth Regulator Hormone to Germination of Kenikir Seed (*Cosmos caudatus*). *Biology Journal*. 5(5). Retrieved from https://journal.student.uny.ac.id/ojs/index.php/kingdom/article/view/5876/5615

Anggraito, Y. U., Susanti, R., Iswari, R. S., Yuniastuti, A., Lisdiana, Nugrahaningsih, . . . Bintari, S. H. (2018). *Metabolit Sekunder dari Tanaman: Aplikasi dan Produksi*. Universitas Negeri Malang. Retrieved from http://lib.unnes.ac.id/31113/

An, T. N. T., Ngan, T. T. K., Van, C. K., Anh, H. L. T., Minh, L. V., & Ay, N. V. (2021). The major and minor components of Kaffir Lime (*Citrus hystrix* DC.) essential oil in the steam distillation process. *IOP Conference Series: Materials Science and Engineering*, *1092*(1), 012082. Retrieved from https://doi.org/10.1088/1757-899x/1092/1/012082

Anuchapreeda, S., Chueahongthong, F., Viriyaadhammaa, N., Panyajai, P., Anzawa, R., Tima, S., & Okonogi, S. (2020). Antileukemic Cell Proliferation of Active Compounds from Kaffir Lime (*Citrus hystrix*) Leaves. *Molecules*, *25*(6), 1300. Retrieved from https://doi.org/10.3390/molecules25061300

Ashokhan, S., Othman, R., Abd Rahim, M. H., Karsani, S. A., & Yaacob, J. S. (2020). Effect of plant growth regulators on coloured callus formation and accumulation of Azadirachtin, an essential biopesticide in *Azadirachta indica*. *Plants*, *9*(3), 352. Retrieved from https://doi.org/10.3390/plants9030352

Bienaimé, C., Melin, A., Bensaddek, L., Attoumbré, J., Nava-Saucedo, E., & Baltora-Rosset, S. (2015). Effects of plant growth regulators on cell growth and alkaloids production by cell cultures of *Lycopodiella inundata*. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *123*(3), 523–533. Retrieved from https://doi.org/10.1007/s11240-015-0856-6

Carsono, N., Juwendah, E., Liberty, L., Sari, S., Damayanti, F., & Rachmadi, M. (2021). Optimize 2,4-D concentration and callus induction time enhance callus proliferation and plant regeneration of three rice genotypes. *Biodiversitas Journal of Biological Diversity*, *22*(7). Retrieved from https://doi.org/10.13057/biodiv/d220702

Chandana, B. C., Nagaveni, H. C. K., Heena, M. S., Kolakar, S. S., & Lakshmana, D. (2018). Role of plant tissue culture in micropropagation, secondary metabolite production and conservation of some endangered medicinal crops. *J Pharmacogn Phyitochem*, *SP3*, 244-251. Retrieved from https://www.phytojournal.com/archives/2018/vol7issue3S/PartF/SP-7-3-77-449.pdf

Chaudhary, G. & Dantu, P. K. (2015). Evaluation of callus browning and develop a stratrgically callus culturing of *Boerhaavia diffusa* L. J. *Plant Development*, *22*, 47-58. Retrieved from https://plant-journal.uaic.ro/docs/2015/6.pdf

Garcia, R., Pacheco, G., Falcão, E., Borges, G., & Mansur, E. (2011). Influence of type of explant, plant growth regulators, salt composition of basal medium, and light on callogenesis and regeneration in *Passiflora suberosa* L. (Passifloraceae). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *106*(1), 47–54. Retrieved from https://doi.org/10.1007/s11240-010-9892-4

I'anatushshoimah, Nurchayati, Y, Prihastanti, E., & Hastuti, R. H. (2020). Effects of light for callus induction of mangrove plant (*Rhizophora Apiculata* Bi) by *In Vitro*. *Life Science*, *9*(2), 139-147. Retrieved from https://doi.org/10.15294/lifesci.v9i2.47157

Indah, P. N., & Ermavitalini, D. (2013). Induksi Kalus Daun Nyamplung (*Calophyllum inophyllum* Linn.) pada Beberapa Kombinasi Konsentrasi 6-Benzyl aminopurine (BAP) dan 2,4- Dichlorophenoxyacetic Acid (2,4-D). *Jurnal Sains dan Seni Pomits*, *2*(1), 1-

6. Retrieved from http://dx.doi.org/10.12962/j23373520.v2i1.2571

Kartikasari, P., Hidayat, M.T., Ratnasari, E. (2013). Pengaruh Zat Pengatur Tumbuh 2,4-D (2,4- dichlorophenoxy acetic acid) dan Kinetin (6- furfurylaminopurine) untuk Pertumbuhan Tunas Eksplan Pucuk Tanaman Jabon (Anthocephalus cadamba Miq. ex Roxb.) secara in Vitro. *Lentera Bio*, 2(1), 75–80. Retrieved from https://jurnalmahasiswa.unesa.ac.id/index.php/lenterabio/article/view/1391/1036

Khaniyah, S., & Habibah, N. A. (2012). Pertumbuhan kalus daun dewa (*Gynura prochumbens*) dengan kombinasi 2,4-Dichlorophenoxyacetic acid dan kinetin secara in vitro. *Biosantifika:Journal of Biology and Biology Education*, *2*(4), 98–105. Retrieved from https://doi.org/10.15294/biosantifika.v4i2.2274

Koperuncholan, M., & Manogaran, M. (2015). Edible plant mediated biosynthesis of silver and gold nanoflakes against human pathogens. *World Journal of Pharmaceutical Research*, *4*(1), 1757-1775. Retrieved from https://www.researchgate.net/publication/277812975_EDIBLE_PLANT_MEDIATED_BIOSYNTHESIS_OF_SILVER_AND_GOLD_NANOFLAKES_AGAINST_HUMAN_PATHOGENS

Lahsin, I. I., & Elhaw, M. H. (2016). Evaluation of secondary metabolite in callus and tissue of *Physalis peruviana*. *International Journal of Modern Botany*, *6*(1), 10-17. Retrieved from http://doi:10.5923/j.ijmb.20160601.03

Maslakhah, F. N., Mutiah, R., Hakim, A., Aprinda, R., & Suryadinata, A. (2018). Metabolite Profiling Bagian Akar, Batang, Daun, dan Biji *Helianthus annuus* L. Menggunakan Instrumen UPLC-MS. *MPI (Media Pharmaceutica Indonesiana)*, *2*(2), 64–81. Retrieved from https://doi.org/10.24123/mpi.v2i2.1361

Mastuti, R., Widoretno, W., & Harijati, N. (2020). Kultur Kalus Tanaman Obat *Physalis angulata* L. (Ciplukan). *Biotropika: Journal of Tropical Biology*, *8*(1), 26–35. Retrieved from https://doi.org/10.21776/ub.biotropika.2020.008.01.05

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*(3), 473–497. Retrieved from https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nadeak, R., Anna, N., & Siregar, E. B. M. (2012). Effect of plant growth regulator NAA and IBA on seed explants agarwood (*Aquilaria malaccencis* Lamk.) *In Vitro*. *Peronema Forestry Science Journal*, *1*(1). Retrieved from Retrieved from https://media.neliti.com/media/publications/156169-ID-respon-eskplan-biji-gaharu-aquilaria-mal.pdf

Naser, A. S., & Wisnu, M. (2021). Callus induction of leaves and stems in Krisan (*Chrysanthemum morifolium* Ramat cv Dewi ratih) with alternative foliar fertilizers media. *Biology, Medicine, and Natural Product Chemistry*, *9*(2), 109–115. Retrieved from https://doi.org/10.14421/biomedich.2020.92.109-115

Nugroho, L. H. (2014). *Peran Anatomi dalam Studi Biosintesis dan Akumulasi Metaboli Sekunder pada Tumbuhan*. Universitas Gadjah Mada. Retrieved from https://repository.ugm.ac.id/136700/1/2015_2014_l_hartanto_nugroho_pt_aap.pdf

Purba, R. V., Yuswanti, H., & Astawa, I. N. G. (2017). Induction can Callus Eksplan the Vine (*Vitis vinivera* L.) with the Application 2,4-D in *In Vitro*. *E-Jurnal Agroekoteknologi Tropika*, *6*(2), 218-228. Retrieved from http://ojs.unud.ac.id/index.php/JAT

Purwaningsih, W., Febri S., & Kusdianti. 2016. Formation flavonoid secondarymetabolites in callus culture of *Chrysanthemum cinerariefolium* as alternatif provision medicine. *Proceedings of International Seminaron Mathematics, Science, and Computer Science Education*. Retrieved from https://doi.org/10.1063/1.4941150

Prashariska, K., Pitoyo, A., & Solichatun, S. (2021). Pengaruh Indole-3-Acetic Acid (IAA) Dan Benzyl Amino Purine (BAP) Terhadap Induksi Dan Deteksi Alkaloid Kalus Kamilen (*Matricaria chamomilla* L.). *Innofarm: Jurnal Inovasi Pertanian*, *23*(2). Retrieved from https://doi.org/10.33061/innofarm.v23i2.5916

Rasud, Y., & Bustaman, B. (2020). In vitro callus induction from clove (*Syzigium aromaticum* L.) leaves on medium containing various auxin concentrations. *Jurnal Ilmu Pertanian Indonesia*, *25*(1), 67–72. Retrieved from https://doi.org/10.18343/jipi.25.1.67

Royani, J. I., Tanjung, A., Angga, I. W., Surachman, M., Rachmawati, A., Susiyanti, & Rohmawati, I. (2020). In vitro callus initiation from *Indigofera* (*Indigofera zollingeriana* Miq.). *IOP Conference Series: Earth and Environmental Science*, *457*, 012066. Retrieved from https://doi.org/10.1088/1755-1315/457/1/012066

Setiaji, A. (2020). Effect of different strenght of medium on germination and seedling growth of tomato and sucrose effect on biomass of tomato callus. *Jurnal Biota*, *6*(1), 12-18. Retrieved from https://doi.org/10.19109/Biota.v6i1.4184

Shinta, S., Minarno, E. B., & Rofiqoh, I. (2020). In vitro embryogenic callus induction of *Carica pubescens* Lenne and K.Koch using 2,4-D (2,4-Dichloro phenoxy Acetic Acid) and BAP (6-Benzyl aminopurin). *Berkala Penelitian Hayati*, *25*(2), 38-44. Retrieved from https://doi.org/10.23869/49

Silalahi, M. (2020). Pemanfaatan *Citrus hystrix* DC. oleh Pedagang Tumbuhan Obat di Pasar Tradisional Kabanjahe Kabupaten Karo. *Bioscientist: Jurnal Ilmiah Biologi*, *8*(2), 317. Retrieved from https://doi.org/10.33394/bjib.v8i2.3154

Sinaulan, J. F., Lenkong, E. D., & Tulung, S. (2019). The respon of the formation of the plant embryonic callus of Krisan Kulo (*Chrysanthemum morifolium*) to the subtance a growth of regulator of Cytockines, *1*(1). Retrieved from https://doi.org/10.35791/cocos.v1i1.22203

Sreepian, A., Sreepian, P. M., Chanthong, C., Mingkhwancheep, T., & Prathit, P. (2019). Antibacterial activity of essential oil extracted from *Citrus hystrix* (Kaffir Lime) peels: An in vitro study.

Tropical biomedicine, *36*(2), 531–541. Retrieved from https://pubmed.ncbi.nlm.nih.gov/33597415/

Suhartanto, B., Astutik, M., Umami, N., Suseno, N., & Haq, M. S. (2022). The effect of explants and light conditions on callus induction of srikandi putih maize (*Zea mays* L.). *IOP Conference Series: Earth and Environmental Science*, *1001*(1), 012006. Retrieved from https://doi.org/10.1088/1755-1315/1001/1/012006

Tarrahi, R. & Rezanejad, F. (2013). Callogenesis and production of anthocyanin and chlorophyll in callus cultures of vegetative and floral explants in *Rosa gallica* and *Rosa hybrida* (Rosaceae). *Turkish Journal of Botany*, *37*(6), 1145-1154. Retrieved from http://dx.doi.org/10.3906/bot-1205-42

Tunjung, W. A. S., Cinatl, J., Michaelis, M., & Smales, C. M. (2015). Anti-cancer effect of kaffir lime (*Citrus Hystrix* DC.) leaf extract in cervical cancer and Neuroblastoma cell lines. *Procedia Chemistry*, *14*, 465–468. Retrieved from https://doi.org/10.1016/j.proche.2015.03.062

Tunjung, W. A. S., Widyasari, A. F., Iskandar, A., Nurulita, A. J., Sasongko, A. B., Indrianto, A., & Maryani. (2021). Effect of 2,4-D and BAP on morphological characters and genetic stability of kaffir lime (*Citrus hystrix* DC.) callus cultures among generations. *Chiang Mai University Journal of Natural Sciences*, *20*(3). Retrieved from https://doi.org/10.12982/cmujns.2021.067

Umami, N., Respati, A. N., Rahman, M. M., Umpuch, K., & Gondo, T. (2022). Somatic embryogenesis and plant regeneration from the apical meristem of Wrukwona Napiergrass (*Pennisetum purpureum*) treated with Thidiozuron and Cupric sulfate. *Tropical Animal Science Journal*, *45*(2), 220–226. Retrieved from https://doi.org/10.5398/tasj.2022.45.2.220

Utami, S., Larasati, R., Hidayati, S., & Sugito, B. H. (2020). The effectiveness of involvement test of kaffir lime (*Citrus hystrix*) fruit extract on the growth of streptococcus mutans bacteria (in vitro). *The 2nd International Conference of Dental and Oral Health (ICoDOH)*. Retrieved from https://www.icodohp.poltekkesdepkessby.ac.id/index.php/icodoh/article/download/21/20

Yelnititis, Y. (2012). Pembentukan Kalus Remah Dari Eksplan Daun Ramin (*Gonystylus bancanus* (Miq) Kurz.). *Jurnal Pemuliaan Tanaman Hutan*, *6*(3), 181–194. Retrieved from https://doi.org/10.20886/jpth.2012.6.3.181-194