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Evaluation of Anticancer Bioactive Compounds and Cytotoxicity of *Citrus hystrix* Dc. Callus Extract Post Preservation

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ABSTRACT

Previous studies have shown that kaffir lime leaf extract is toxic to cancer cells. To increase the bioactive compound's production for traditional cancer medicine, we induce kaffir lime callus *in vitro*. One strategy to continuously maintain the production of kaffir lime callus is by using callus preservation. Our preliminary study used two preservation methods of callus stored in 4°C with or without alginate encapsulation. However, low temperatures and sodium alginate can be stress factors for plants, affecting bioactive compounds' production and their anti-cancer ability. Therefore the objective of this study was to determine whether our preservation methods affect the character of the callus by evaluating the bioactive compounds of callus post preservation and their effect on the cytotoxicity against T47D and Vero cells. This study was conducted by inducing kaffir lime seeds to form callus. The generation 1 calluses were divided into control and preserved groups. Callus preservation was performed by stored callus in 4°C with or without alginate encapsulation for 21 days and then recultured for 14 days. The bioactive compounds in the callus extract are detected by GC-MS. Furthermore, cytotoxicity of callus against breast cancer (T47D) and non-cancer cell (Vero) are tested using the MTT method. The results showed that preservation in 4°C with and without encapsulation caused changes in bioactive compounds profile. The terpenoid compounds were detected post preservation are Squalene, Geranyl linalool, and Geranyl acetate. Other anti-cancer bioactive compounds such as Stearic acid, 1-Decanol, Octadecane, 1-Hexcosanol, Hexane, Dodecane, Tetracosane, and 2-Decenoid acid. However, control and post-preservation callus extract are not cytotoxic to both cancer and non-cancer cells. Although there was a slight difference in the type of bioactive compounds, those compounds might be synthesized at a minimal level thus they did not affect cytotoxicity. Our preservation method could well storage the callus thus it can be used to provide continuous supply callus stock for pharmaceutical purposes.

Keywords: Kaffir lime callus, Preservation, Cytotoxicity, Bioactive compounds

INTRODUCTION

Kaffir lime is one of the potential anti-cancer plants and natural products. Our previous study showed kaffir lime leaf extract has cytotoxicity to cervix cancer and neuroblastoma cell (Tunjung *et al*., 2015). Other studies also report that kaffir lime peel and leaves have cytotoxicity effects on leukemia and epidermal cancer cells (Ampasavate *et al*., 2010). However, the direct use of kaffir lime leaf extract has limitations because it required vast samples and was greatly influenced by environmental conditions. Therefore, an effective method is needed to produce anti-cancer compounds in large quantities. The tissue culture technique is one potential method to increase bioactive compounds.

Plant tissue culture is broadly used to produce secondary metabolites. It has many advantages, including season-independent, manageable production systems, more consistent, and not requiring large areas (Kuete *et al*., 2016). Maximizing the synthesize of secondary metabolites, especially anti-cancer compounds by plant cells, elicitation was used to stimulate cells to activate the stress-response mechanism (Neumann *et al*., 2009). A vast and continuous callus supply is needed as raw material to make elicited cell suspension. The callus is usually maintained by subculture on fresh media, which makes them vulnerable. However, The risk of contamination and human error is directly proportional to subculture frequency (Augereau *et al*., 1986). Hence, it is important to store callus using the most stable preservation method so it does not change the callus character.

Callus storage can be done in various ways, such as encapsulation with alginate and low temperature. In this study, we used two callus preservation methods: stored in 4°C with and without alginate encapsulation treatment. These methods are based on a preliminary study which is established in our laboratory.

Although some studies showed that alginate encapsulation and low temperature could preserve plant embryos or callus, however, these treatments may cause stress in plant cells. According to Shabala (2012), cold temperature stress in plants will affect bioactive compounds such as the accumulation of dissolved glucose and secondary metabolite production. Furthermore, according to Augereau *et al*., (1986), there was a difference in the total alkaloid levels in *Catharanthus roseus* G. Son C4 strain after storage at 15°C for 7 weeks. On the other hand, a study in callus of safflower showed that sodium alginate increases the antioxidant compounds under salinity stress, suggesting sodium alginate as a potent elicitor (Golkar *et al*, 2019).

Changes in secondary metabolite production may also affect their cytotoxicity against cancer and normal cell. According to study Sae-lee *et al* (2017), Grape (*Vitis vinifera* cv. Pok Dum) cell suspension culture elicited with Na_2SeO_3, showed higher cytotoxicity against BT474 (breast cancer), ChagoK1 (lung cancer), Hep-G2 (liver cancer), KATOIII (stomach cancer) and SW620 (colon cancer cell). In line with this study, Our study showed that elicited kaffir lime suspension cell by *Saccharomyces cerevisiae* had higher cytotoxicity against T47D cell than leaves extract (unpublished data).

Therefore, the study of the effect of preservation on the bioactive compound and its cytotoxicity of kaffir lime callus extract is needed. The objectives of this study were to determine the bioactive compounds of 35th days callus without preservation (control) and callus that preserved for 21 days and recultured for 14 days after preservation. We also examine the cytotoxicity of callus extract to T47D and Vero cells.

MATERIALS AND METHODS

Callus induction

Kaffir lime fruits were taken from Kalijeruk, Candirejo Village, Borobudur, Magelang, Central Java. Seeds from ripe fruit were used as explants for callus induction. Murashige and Skoog (MS) media with two ppm of 2,4-Dichlorophenoxy acetic acid sodium salt monohydrate (Sigma) was used as the induction and subculture medium. This method was based on our previous study (Prabowo *et al*, 2020).

Determination of callus growth curve and treatment

The Callus growth curve was examined to analyze the optimal day for callus preservation. Generation 0 (G0) calluses were subcultured during the exponential phase (21 d), then grown on a new medium (MS media with two ppm of 2,4-D), namely the first generation. The first generation (G1) calluses were grown and their fresh weight was measured every five days until the 50th day. After analyzing the growth curve, the preservation time was set on the exponential phase. There were two groups of callus: the treatment and the control group. As treatment groups, callus was preserved in 4°C with or without alginate encapsulation for 21 days. Then, it subcultured in the new medium for 14 days kept at room temperature. The control group

was callus in the stationary phase (G1 35d) kept at room temperature. Thus treatment and control callus group has a total age of 35 days.

Callus preservation in 4°C without alginate encapsulation

The 21 days of G1 callus was cultured inside the bottle contain MS media with two ppm of 2,4-D then preserved at 4°C for 21 days. This method according to our previous study (Prabowo *et al*, 2020). After the preservation period, callus is being recultured for 14 days at room temperature. Callus was subjected to extraction for further experiment.

Preparation of callus encapsulation

Four grams of $NaC_6H_7O_6$ (Merck) were dissolved into 100mL with ¼ doses of MS medium enriched with 3g sucrose and 2,4-D two ppm with pH range 5.6-5.8. Then the solution was sterilized by using an autoclave. This method according to our previous study (Prabowo *et al*, 2020). $CaCl_2.2H_2O$ (Merck) solution 0.2M was used as an alginate hardener solution. To prepare 0.2M (w/v) $CaCl_2.2H_2O$ (MW:147.02), 2.9404g of $CaCl_2.2H_2O$ dissolved in 100mL aquadest and sterilized by autoclave. This method according to our previous study (Prabowo *et al*, 2020).

Callus preservation in 4°C with alginate encapsulation

G1 21d was covered with alginate solution 4% and dipped in $CaCl_2.2H_2O$ solution 0.2M for 30min. The capsule was preserved inside the petri dish and maintained in 4°C (refrigerator) for 21 days. This method according to our previous study (Prabowo *et al*, 2020). After preservation, callus is recultured in a new medium for 14 days and kept at room temperature. Callus was subjected to extraction for further experiment.

Callus extraction

The harvested callus was dried in an oven (temperature approximately 40°C) until it reached a constant weight. Extraction used the maceration method with ethyl acetate as a solvent. Callus powder in a flacon bottle was soaked with ethyl acetate and was shaken on a shaker for 2 days. The solvent was filtered, then the solution is evaporated until the extract/paste is left at the bottom of the microtube. Maceration was repeated three times. Paste stored in the refrigerator until used. The crude extract was subjected to the GCMS test and MTT assay.

Bioactive Compounds Profile

The bioactive callus compound was determined using the GC-MS method. This is based on our previous study in which kaffir lime leaves and callus contain many volatile and lipid compounds, which were well detected using GC-MS. We used GC-MS QP Shimadzu 2010S, which is equipped with a non-polar Rtx 5MS column. A sample of 5µL extract was injected into the GC-MS column, which has a 30-meter long glass column, 0.25mm in diameter, and 0.25µm thickness with a stationary phase of EI 70 Ev. The oven temperature was programmed between 70-300°C with a temperature rise rate of 5°C/min and a split ratio of 1: 49 using WILEY and NIST libraries.

Cytotoxic assay (MTT assay)

The MTT assay was conducted to examine the cytotoxicity of kaffir lime callus with and without preservation. We used 2 types of cells: breast cancer T47D cells and Vero cells obtained from the Integrated Research and Testing Laboratory of Gadjah Mada University Yogyakarta.

T47D cells were grown in RPMI (Gibco) culture media while Vero cells in M199 (Gibco) culture media. Both media were added by 10% FBS (Corning), 4% penstrep (Gibco) 0.5% fungizone (BI). The cells were observed by using an inverted microscope until they reached 80% confluency. The serial concentration of callus extracts were 0; 62.5; 125; 250; 500; 1000µg/mL were added to cell lines with triplo replication and then incubated in a CO_2 incubator within 24-48h. Cell viability was determined by adding MTT solution, then incubated between 2-4h. To stop the reaction, add SDS (Merck) 10% (Sodium dodecyl sulfate) overnight and incubated at room temperature. The absorbance measured at λ 550nm using an Elisa plate reader (Bio Rad 680XR). The absorbance data of the treatment was converted into percent viability and used to calculate IC_{50} value.

RESULTS AND DISCUSSIONS

Growth Curve of G1 Callus

The results showed that the lag phase of G1 callus occurs on days 5-10, the exponential phase on days 10-30, the initial stationary phase on days 30-35, and the final stationary phase on days 40-50. According to this data, we decided to choose callus on the exponential phase (21 d) to be preserved.

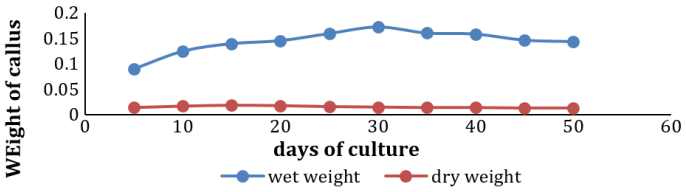

Figure 1. Growth curve of G1 callus

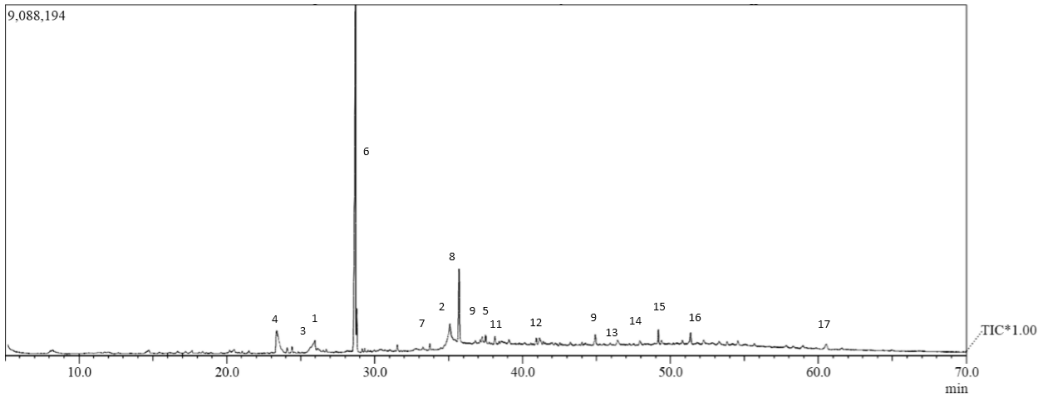

Figure 2. GC-MS Total Ion Chromatogram of control callus.

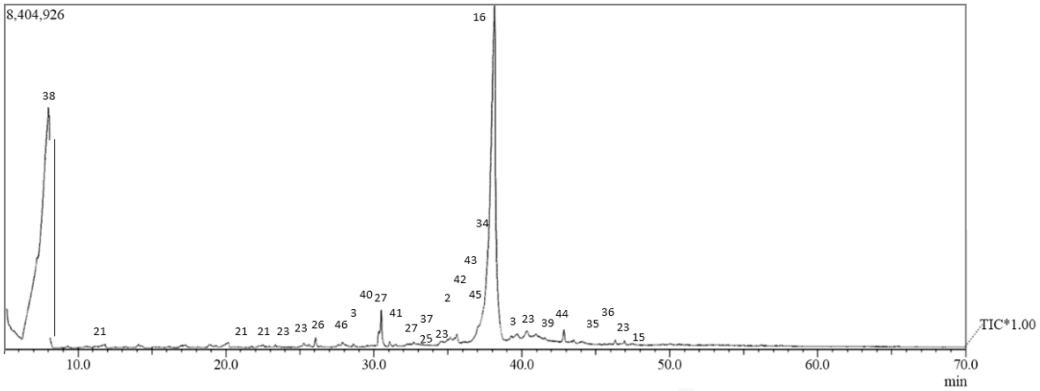

Figure 3. GC-MS Total Ion Chromatogram of callus maintained in 4°C without alginate encapsulation

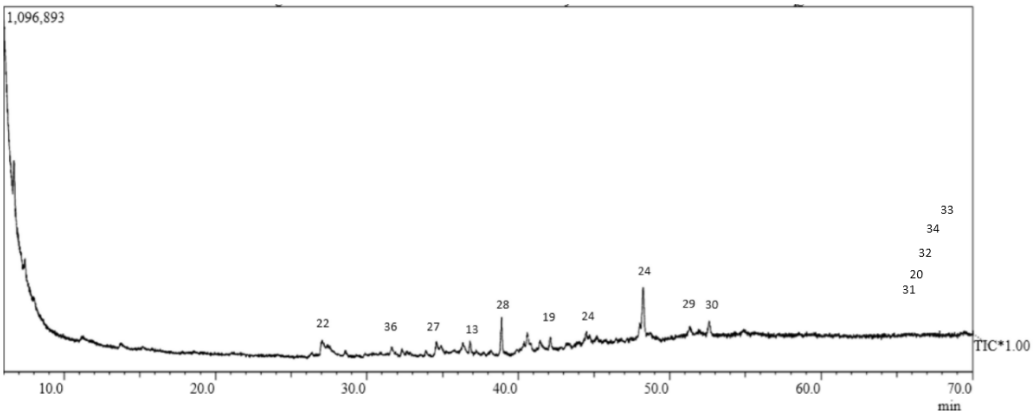

Figure 4. GC-MS Total Ion Chromatogram of callus maintained in 4°C with alginate encapsulation

Table Ia. Profile of bioactive compound of control and treatment callus (Without and With Preservation)

No.	Compound Name	Peak Area (%)			Group	Function	Reference
		A	B				
			B1	B2			
1	Lauric acid	4.32			Saturated fatty acid	Antioxidant	Henry *et al*, 2002
2	palmitic acid	10.01			Saturated fatty acid	Anti-cancer, anti-inflammatory	Sheela *et al*, 2019 Lappano *et al*, 2017
3	Stearic acid	1.61	0.32		Saturated fatty acid	Anticancer	Khan and Ali, 2013
4	1-Decanol	8.70			Alcohol lipid	antioxidant, & antimutagenic	Gautam *et al*, 2018
5	myristic acid	1.22			Saturated Fatty acid	Antibacterial	Mohammad *et al,* 2018
6	acrylic acid	46.66			Unsaturated mono carboxylic acid	Antimicrobial	Liang *et al*, 2020 Gratzl *et al*, 2014
7	propionic acid	3.61			Saturated fatty acid	Antimicrobial	Pfeuffer and Jaudszus, 2016 Gomez-Garcia *et al*, 2019
8	undecylenic acid	1.75			Unsaturated Fatty acid	Antifungal, antioxidant	Henry *et al*, 2002 Shi *et al*, 2016
9	1 Fluoro-decane	10.27			Haloalkane/alkyl halide	Antimicrobial	(Ujowundu FN 2017) (Eze 2015) Chinonye *et al*, 2019
10	Oleic acid	1.07			Unsaturated Fatty acid	Anticancer	Carrillo *et al*, 2018
11	2H-Pyran-2-one	1.16			Cyclic compound	Antitumor	Dong *et al*, 2011
12	ricinoleic acid	1.82			Unsaturated hydroxylated acid	Antibacterial Antimycobacterial	Borsotti *et al*, 2001 Kuppala *et al*, 2016
13	Octadecane	1.97	1.86	2.95	a straight-chain alkane	antioxidant, antimicrobial & anticancer	Gautam *et al*, 2018; Alkahalf *et al*, 2018
14	Hexacosane	1.20			Saturated hydrocarbon	Antibacterial	Dayananda, 2016 Kawuri & Darmayasa 2019
15	Squalene	1.95	1.64		Triterpenoid	Antitumor	Yarkoni & Rapp, 1979
16	1-Hexcosanol	1.59	5.56		Primary alcohol fat	Antitumor	Figueiredo *et al.,* 2014
17	Zymosterol	1.30			Sterols	Anticarcinogenic	Shin *et al*, 2012
18	Phthalic acid		1.57		Aromatic dicarboxylic acid	Antimicrobial	Zhang, 2016 Al-Bari *et al*, 2006
19	Hexane		1.31	3.69	Alkane	Antioxidant and antimicrobial	(David M. Chambers 2008) (Ivan Ivanov 2017)
20	Nonanal		0.74	2.85	Saturated fatty aldehyde	Antifungal Antimicrobial	Wang *et al*, 2020 Zhang *et al*, 2017
21	Methane		62.98		Alkane	Antiinflammatory Antioxidant Antiapoptosis	Boros *et al* 2012 Jia at al 2018
22	2-Pentanone		1.71	16.57	Lipid aldehyde	Antimicrobial Antibiotic	Esterbauer and Zolnner 1989 Al-Andal *et al*, 2019
23	Hexadecane		11.53		Alkane	antibacterial	Widayat *et al*, 2021 Nimvbeshaho *et al* 2020
24	Dodecane		0.64	2.77	Saturated fatty acid	Antioxidant	Henry *et al*, 2002
25	Pentadecane		1.71		Alkane	Antibacterial and anti-inflammatory cytotoxicty	Chuah *et al*, 2018 Nimbeshaho *et al* 2020
26	1-Tetradecane		1.39		Aldehyde	Antifungal Antibacterial	Nimbeshaho *et al* 2020
27	Octadecanal		1.86	3.95	Fatty Aldehyde	Antimicrobial	Widayat *et al*, 2021 Rad *et al*, 2013
28	palmitic aldehyde			14.49	Fatty aldehyde	-	Ford *et al*, 20166
29	Tetracosane			7.56	Alkane	Anticancer	Uddin *et al*, 2012
30	Geranyl Linalool Isomer B			5.29	Diterpenoid	Antimicrobial & anti-inflammatory	Beulah *et al*, 2018
31	Cyclopentanon-3-carbonic acid			3.20	Cyclic ketone	-	Giry *et al*, 2018

Table Ib. Profile of bioactive compound of control and treatment Callus (Without and With Preservation)

No.	Compound Name	Peak Area (%)			Group	Function	Reference
		A	B				
			B1	B2			
32	Bis(trimethylsilyl) derivative of 5-Pregnene-3.beta			2.65	-	Anxyolytic agent	(Raymond A. Dombroski 1997)
33	2-Decenoic acid			2.52	unsaturated fatty acid	Antitumor, antibiotic, collagen production promoting activity	Hattori *et al*, 2007
34	1-Nonene		2.29		Unsaturated aliphatic hydrocarbon	Antimicrobial	Boughendjioua, 2019 Gueret *et al*, 1991
35	Nonacosane		2.07		Alkane	Antibacterial	Widayat *et al*, 2021 Ryu *et al*, 2020
36	Di-n-octyl-phtalate		1.57		Phthalate ester	Antivenom	Di Bella *et al*, 1999 Ibrahim *et al*, 2012
37	2-Hexen-1-ol		1.31		Unsaturated alcohol	Antifungal	Guha *et al*, 2014 Velivelli *et al* 2014
38	Hexanal		4.57		Saturated fatty aldehyde	Antimicrobial	Panseri *et al*, 2011 Song *et al*, 1996
39	1H-Indene		1.03		Polycyclic aromatic hydrocarbon	Antimicrobial	Yuan *et al*, 2010 Wanibuchi *et al*, 2021 Chiarini *et al* 1980
40	1-Hexanedecanethiol		0.79		Alkanethiol	-	Node *et al*, 2001
41	Nonane		0.74		Alkane	Antimicrobial	Genny *et al*, 2011 Kawuri & Darmayasa 2019
42	3-Dodecene		0.64		Alkane	Antimicrobial Antiinflammatory Antioxidant Anticancer Cytotoxicity	Sumithra & Purushothaman, 2017
43	1,3-Dioxocane		0.61		Ether	-	Cibulka and alexiou, 2010
44	Imidazole-1,4,5-D-3		0.5		Heterocyclic Aromatic	Antioxidant	Noriega-Iribe *et al*, 2020
45	6-Tridecanone		0.34		Ketone derivative	Antibacterial	Zhao *et al*, 2012 Lopez-Lara *et al*, 2018
46	Undecanal		0.32		Saturated fatty aldehyde	Antimicrobial	Caldwell *et al*, 2003 Al-Shuneigat *et al*, 2015
47	Butanal		1.72		Aldehyde derivative	-	(Silva, 2006)
48	Geranyl acetat			2.81	Monoterpenoid	Antibacterial	Ganjewala and Luthra, 2008. Mandal and Mandal, 2015
49	1,2 Benzenedicarboxylic acid			23.88	Aromatic dicarboxylic acid	-	Klepper and Nilsen 2010
50	Cyclopentanon-3-carbonic acid			3.20	Cyclic ketone	-	Giry *et al*, 2018

Information: A = without preservation (G1 35d); B1 = with preservation in 4°C; B2 = with preservation in 4 0C and alginate encapsulation

Bioactive compound profile of callus

We determined the bioactive compounds of control and treatment callus by GC-MS (Figure 2, 3 and 4).

The control callus (G1 35d) callus preserve in 4°C and callus group preserves in 4°C with alginate encapsulation consists of 17, 30 and 15 compounds respectively (Figure 2,3 and 4). compounds included in the group of fatty alcohols, saturated and unsaturated fatty acids, carboxylic acids, and alkanes. Callus preserve in 4°C consists of 23 compounds in saturated and unsaturated fatty acids, fatty aldehydes, lipid aldehyde, and alkanes. Callus group preserves in 4°C with alginate encapsulation consist of 15 compounds. There are similar bioactive compounds between control and

preserved callus such as stearic acid, octadecane, squalene, hexosanol, hexane, nonanal, and pentanone. The compounds detected in all groups were octadecane, and those classified as a straight-chain alkane. However, terpenoid as the main anti-cancer compound was present in G1 35d (control group) and in callus preserve in 4°C without alginate encapsulation, namely, squalene geranyl acetate and Geranyl linaloxol in 4°C with encapsulation. Some compounds act as anti-cancer in preserved callus such as Lauric acid, palmitic acid, stearic acid, 1-Decanol, undecylecil acid, oleic acid, 2H pyran-2-one, octadecane, 1-Hexcosanol, Hexane, Methane, dodecane, tetracosane, 2-Decenoid acid, and 3-dodecane (**Table I**).

Cytotoxicity of Control and Treatment Callus

Low-temperature treatment and alginate encapsulation possibly act as elicitors. Since we detected some terpenoid and other anti-cancer compounds in the callus, therefore we subjected the callus to the cytotoxicity assay on cancer and non-cancer cell. The viability of cancer and non-cancer cells after treatment with kaffir lime callus extract (**Figures 5 and 6**).

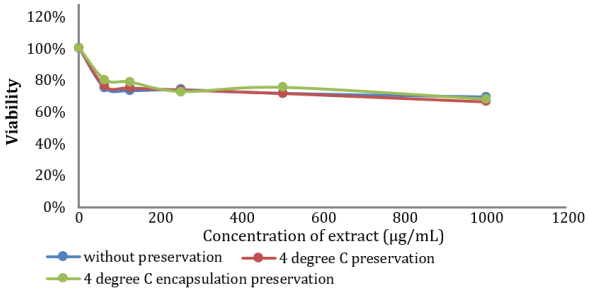

Figure 5. Viability of T47D cells after treated by kaffir lime callus extracts

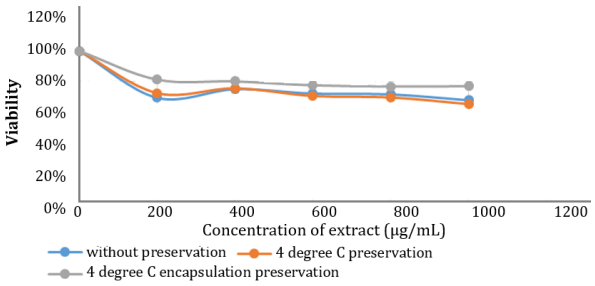

Figure 6. Viability of Vero cells after treated by kaffir lime callus extracts

The data showed that the higher concentration of kaffir lime callus extract causes decreased T47D and Vero cells' viability. However, at the highest concentration of extract, the viability percentage of T47D and Vero cells were 68.77% and 69.57%, respectively, which means more than half of the cells were still alive. Hence, the extract of kaffir lime callus was not cytotoxic to the cell.

Table 2. IC_{50} Values of kaffir lime callus extracts

Treatment	IC_{50} value range		Information
	Sel T47 D	Sel Vero	
Control group (G1 35d)	2000 - >100.000 µg/mL	> 100.000 µg/mL	Not toxic
Preservation in 4°C	>100.000 µg/mL	> 100.000 µg/mL	Not toxic
Preservation in 4°C with alginate encapsulation	>100.000 µg/mL	> 100.000 µg/mL	Not toxic

The morphology of cancer and non-cancer cells after treatment with kaffir lime callus extract (**Figures 7 and 8**).

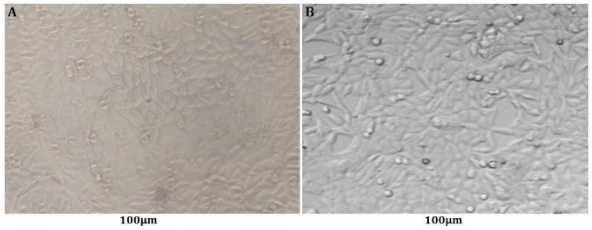

Figure 7. Morphology T47D Cell. A. before treatment, B. after treatment (200x magnification using an inverted microscope)

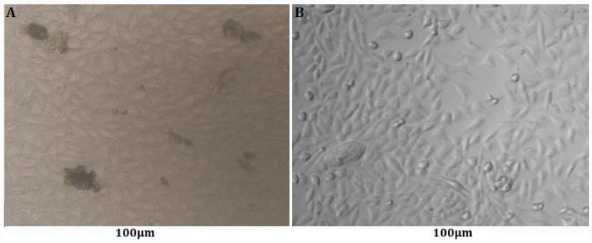

Figure 8. Morphology Vero Cell. A. before treatment, B. after treatment (200x magnification using an inverted microscope)

In this study we used 4% sodium alginate dipped in $CaCl_2.2H_2O$ to encapsulate the callus then storage at low temperature (4°C) for 21 days. After that, the preserved callus was recultured for 14 days on a fresh medium before subjected to the next

experiment. Meanwhile, the control group was callus generation 1 cultured on MS medium for 35 days. Sodium alginate has the potential as an elicitor to increase plant tolerance in stressful environments such as drought and salinity. (Salachna *et al*. 2018 in Golkar *et al*, 2019). According to a study by Golkar *et al* (2019), safflower callus induced by the addition of sodium alginate 0.075% and 0.15% inside the culture medium under salinity stress produced more secondary metabolites (total phenolic content (TPC), total flavonoids (TFD), total flavonols (TFL), anthocyanin (Ant)) and had antioxidant activity (total antioxidant capacity (TAC), phenylalanine ammonia-lyase (PAL), catalase (CAT)) higher than non-salinity and non-elicitation.

On the other hand, a study by Shabala (2012) showed cold temperature stress in plants will affect bioactive compounds such as the accumulation of dissolved glucose and secondary metabolite production. Furthermore, according to Augereau *et al*., (1986), there was a difference in the total alkaloid levels in *Catharanthus roseus* G. Son C4 strain after storage at 15°C for 7 weeks. Moreover, a study in Brassica napus callus tissue showed that low temperature (2°C) caused the changes in PAL activity and phenolic compounds content (Hura *et al* 2015).

This study showed the compounds that dominate in the control and preserved callus were fatty acids. The fatty acid compounds in the control callus were palmitic and stearic acids. Palmitic and stearic acids are de novo-synthesized compounds from acetyl precursors resulting from a discharge of carbon sources (photosynthates and stored carbohydrates). According to Harwood (2019), Palmitic and stearic acids can further to 3 processes. Firstly, elongation to very long-chain fatty acids with carbon chains of more than 18. Secondly, desaturation to unsaturated fatty acids and the third has been modified into compounds such as oxy-, epoxy-, hydroxy-, and cyclic compound. Therefore the differences of bioactive compounds in control and preserved callus indicate the difference in growth phases rather than the stress caused by preservation methods. The compounds detected in the control group, mainly as precursor compounds. While in the preservation group, the compounds that appear were intermediate or final.

IC_{50} values of all callus extracts were above 1000µg/mL in both T47D breast cancer cells and Vero cells. According to the U.S. NCI plant screening program, a crude extract is generally considered to have in vitro cytotoxic activity with an IC_{50} value < 20µg/mL (Graidist *et al*, 2015). This value indicates that the extract of kaffir lime (preserved or control) calluses was not toxic to the cancer cell (**table 2**). Our previous study showed that kaffir lime leaves extract cytotoxic to Hela and neuroblastoma cancer cells (Tunjung, 2015). We analyze the type of bioactive compounds, but we did not measure their level. Thus, it is possible the content of anti-cancer compounds in callus has a minimal level compare to leaf so that it may not have a cytotoxic effect on T47D cells and Vero cells. These results are in line with Jafarian *et al* (2014), where leaf extract of Moringa oleifera was more potent than callus extract on Hela cells, where the content of phenolic compounds of leaf extract was higher than callus extract.

Furthermore, not all stress can increase the cytotoxicity of cell culture. In a study by Sae-Lee *et al* (2017), Grape (*Vitis vinifera* cv. Pok Dum) cell suspension culture was elicited by $Al_2(SO_4)_3$, KH_2PO_4, KNO_3, Na_2SeO_3, and chitosan in various concentrations. The results showed that type and dose of elicitor have a different effect. Na_2SeO_3 (50mg/L and 100mg/L), and $Al_2(SO_4)_3$ (50mg/L) enhanced phenolic content 1.7, 1.4, and 1.0-fold – higher than control, respectively. Furthermore, $Al_2(SO_4)_3$ (50mg/L) and Na_2SeO_3 (100mg/L) showed higher accumulation of resveratrol and higher antioxidant activity compared with cells treated with KNO_3 and chitosan. However, only Na_2SeO_3 (100mg/L) showed significantly higher cytotoxicity against BT474 (breast cancer), ChagoK1 (lung cancer), Hep-G2 (liver cancer), KATOIII (stomach cancer), and SW620 (colon cancer cell) than the control group. These results revealed that elicitors possibly increase the targeted bioactive compounds in callus or cell culture, but it did not always enhance their cytotoxicity against cell cancer. In line with this study, our data showed that there were a lot of bioactive compounds which act as anticancer detected in both control and preserved groups. We did not measure the concentration of those compounds, they might be synthesized at a minimal level which could not enhance cytotoxicity. Since our preservation method using sodium alginate and low temperature did not affect cytotoxicity, hence this preservation method suitable to produce continuous callus supply as raw material for pharmaceutical purposes. Future study about type and dose of elicitor in kaffir lime callus or cell suspension that can produce high efficacy cancer medicine is important to elucidate.

CONCLUSIONS

There were differences in the profiles of bioactive compounds in control and preserved kaffir lime callus. The terpenoid compounds detected after preservation are Squalene, Geranyl linalool, and Geranyl acetate. Other anti-cancer bioactive compounds such as stearic acid, 1-Decanol, octadecane, 1-Hexcosanol, Hexane, Dodecane, tetracosane, and 2-Decenoid acid. However, the preservation has no effect on the cytotoxicity of kaffir lime callus on breast cancer cells (T47D) and Vero cells. Although there was a slight difference in the type of bioactive compounds, those compounds might be synthesized at a minimal level thus they did not affect cytotoxicity. Our preservation method could well storage the callus thus it can be used to provide continuous supply callus stock as raw material for pharmaceutical purposes.

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