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 synthesis 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 Na2SeO3 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 NaCaH₂O₆ (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). CaCl₂H₂O (Merck) solution 0.2M was used as an alginate hardener solution. To prepare 0.2M (w/v) CaCl₂H₂O (MW:147.02), 2.9404g of CaCl₂H₂O 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 CaCl₂H₂O 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 Rtx5MS 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 CO₂ 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 IC₅₀ 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 1a. Profile of bioactive compound of control and treatment callus (Without and With Preservation)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound Name</th>
<th>Peak Area (%)</th>
<th>Group</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lauric acid</td>
<td>4.32</td>
<td>Saturated fatty acid</td>
<td>Antioxidant</td>
<td>Henry et al, 2002</td>
</tr>
<tr>
<td>2</td>
<td>palmitic acid</td>
<td>10.01</td>
<td>Saturated fatty acid</td>
<td>Anti-cancer, anti-inflammatory</td>
<td>Sheela et al, 2019 Lappano et al, 2017</td>
</tr>
<tr>
<td>3</td>
<td>Stearic acid</td>
<td>1.61</td>
<td>Saturated fatty acid</td>
<td>Anticancer</td>
<td>Khan and Ali, 2013</td>
</tr>
<tr>
<td>4</td>
<td>1-Decanol</td>
<td>8.70</td>
<td>Alcohol lipid</td>
<td>Antioxidant &amp; anti-mutagenic</td>
<td>Mohammad et al, 2018</td>
</tr>
<tr>
<td>5</td>
<td>myristic acid</td>
<td>1.22</td>
<td>Saturated fatty acid</td>
<td>Antibacterial</td>
<td>Liang et al, 2020</td>
</tr>
<tr>
<td>6</td>
<td>acrylic acid</td>
<td>46.66</td>
<td>Unsaturated mono carboxylic acid</td>
<td>Antimicrobial</td>
<td>Pfeuffer and Jaudzus, 2016 Gome-Garcia et al, 2019</td>
</tr>
<tr>
<td>7</td>
<td>propionic acid</td>
<td>3.61</td>
<td>Saturated fatty acid</td>
<td>Antimicrobial</td>
<td>Henry et al, 2002</td>
</tr>
<tr>
<td>8</td>
<td>undecylenic acid</td>
<td>1.75</td>
<td>Unsaturated fatty acid</td>
<td>Antifungal, antioxidant</td>
<td>Shi et al, 2016</td>
</tr>
<tr>
<td>9</td>
<td>1 Fluoro-decane</td>
<td>10.27</td>
<td>Haloalkane/alkyl halide</td>
<td>Antimicrobial</td>
<td>(Ujowundu FN 2017)</td>
</tr>
<tr>
<td>10</td>
<td>Oleic acid</td>
<td>1.07</td>
<td>Unsaturated fatty acid</td>
<td>Anticancer</td>
<td>Carrillo et al, 2018</td>
</tr>
<tr>
<td>11</td>
<td>2H-Pyrano-2-one</td>
<td>1.16</td>
<td>Cyclic compound</td>
<td>Antitumor</td>
<td>Dong et al, 2011</td>
</tr>
<tr>
<td>12</td>
<td>ricinoleic acid</td>
<td>1.82</td>
<td>Unsaturated hydroxylated acid</td>
<td>Antibacterial</td>
<td>Borsotti et al, 2001</td>
</tr>
<tr>
<td>13</td>
<td>Octadecane</td>
<td>1.97</td>
<td>a straight-chain alkane</td>
<td>Antioxidant, anti-microbial &amp; anti-cancer</td>
<td>Gautam et al, 2018</td>
</tr>
<tr>
<td>14</td>
<td>Hexacosane</td>
<td>1.20</td>
<td>Saturated hydrocarbon</td>
<td>Antibacterial</td>
<td>Dayananda, 2016 Kawwari &amp; Darmayasa 2019</td>
</tr>
<tr>
<td>15</td>
<td>Squalene</td>
<td>1.95</td>
<td>Triterpenoid</td>
<td>Antitumor</td>
<td>Yarkoni &amp; Rapp, 1979</td>
</tr>
<tr>
<td>16</td>
<td>1-Hexacosanol</td>
<td>1.59</td>
<td>Primary alcohol fat</td>
<td>Antitumor</td>
<td>Figuereido et al, 2014</td>
</tr>
<tr>
<td>17</td>
<td>Zymosterol</td>
<td>1.30</td>
<td>Sterols</td>
<td>Anticarcinogenic</td>
<td>Shin et al, 2012</td>
</tr>
<tr>
<td>18</td>
<td>Phthalic acid</td>
<td>1.57</td>
<td>Aromatic dicarboxylic acid</td>
<td>Antimicrobial</td>
<td>Zhang, 2016</td>
</tr>
<tr>
<td>19</td>
<td>Hexane</td>
<td>1.31</td>
<td>Alkane</td>
<td>Antioxidant and anti-microbial</td>
<td>(David M. Chambers 2008)</td>
</tr>
<tr>
<td>20</td>
<td>Nonanal</td>
<td>0.74</td>
<td>Saturated fatty aldehyde</td>
<td>Antifungal</td>
<td>Wang et al, 2020</td>
</tr>
<tr>
<td>21</td>
<td>Methane</td>
<td>62.98</td>
<td>Alkane</td>
<td>Anti-inflammatory</td>
<td>Zhang et al, 2017</td>
</tr>
<tr>
<td>22</td>
<td>2-Pentanone</td>
<td>1.71</td>
<td>Lipid aldehyde</td>
<td>Antimicrobial, Antibiotox</td>
<td>(Ivan Ivanov 2017)</td>
</tr>
<tr>
<td>23</td>
<td>Hexadecane</td>
<td>11.53</td>
<td>Alkane</td>
<td>Antibacterial</td>
<td>Widayat et al, 2021</td>
</tr>
<tr>
<td>24</td>
<td>Dodecanone</td>
<td>0.64</td>
<td>Saturated fatty acid</td>
<td>Antibacterial</td>
<td>Nimbeshah et al, 2020</td>
</tr>
<tr>
<td>25</td>
<td>Pentadecane</td>
<td>1.71</td>
<td>Alkane</td>
<td>Antibacterial &amp; anti-inflammatory cytoxicity</td>
<td>Chahal et al, 2018</td>
</tr>
<tr>
<td>26</td>
<td>1-Tetradecane</td>
<td>1.39</td>
<td>Aldehyde</td>
<td>Antifungal</td>
<td>Nimbeshah et al, 2020</td>
</tr>
<tr>
<td>27</td>
<td>Octadecanal</td>
<td>1.86</td>
<td>Fatty Aldehyde</td>
<td>Antimicrobial</td>
<td>Widayat et al, 2021 Rad et al, 2013</td>
</tr>
<tr>
<td>29</td>
<td>Tetracosane Geranyl</td>
<td>7.56</td>
<td>Alkane</td>
<td>Anticancer</td>
<td>Uddin et al, 2012</td>
</tr>
<tr>
<td>30</td>
<td>Linalool Isomer B</td>
<td>5.29</td>
<td>Diterpenoid</td>
<td>Antimicrobial &amp; anti-inflammatory</td>
<td>Beulah et al, 2018</td>
</tr>
<tr>
<td>31</td>
<td>Cyclopentanone-3-carbonic acid</td>
<td>3.20</td>
<td>Cyclic ketone</td>
<td>-</td>
<td>Giry et al, 2018</td>
</tr>
</tbody>
</table>
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).

### Bioactive compound profile of callus

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound Name</th>
<th>Peak Area (%)</th>
<th>Group</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>Bis(trimethylsilyl) derivative of 5-Pregnene-3-beta</td>
<td>2.65</td>
<td>-</td>
<td>Anxyolytic agent</td>
<td>[Raymond A. Dombroski 1997]</td>
</tr>
<tr>
<td>33</td>
<td>2-Decenoic acid</td>
<td>2.52</td>
<td>unsaturated fatty acid</td>
<td>Antitumor, antibiotic, collagen production promoting activity</td>
<td>[Hattori et al, 2007]</td>
</tr>
<tr>
<td>34</td>
<td>1-Nonene</td>
<td>2.29</td>
<td>Unsaturated aliphatic hydrocarbon</td>
<td>Antibacterial</td>
<td>[Gueret et al, 1991]</td>
</tr>
<tr>
<td>35</td>
<td>Nonacosane</td>
<td>2.07</td>
<td>Alkane</td>
<td>Antibacterial</td>
<td>[Widayat et al, 2021]</td>
</tr>
<tr>
<td>36</td>
<td>Di-n-octyl-phtalate</td>
<td>1.57</td>
<td>Phthalate ester</td>
<td>Antivenom</td>
<td>[Di Bella et al, 1999]</td>
</tr>
<tr>
<td>37</td>
<td>2-Hexen-1-ol</td>
<td>1.31</td>
<td>Unsaturated alcohol</td>
<td>Antifungal</td>
<td>[Guha et al, 2014]</td>
</tr>
<tr>
<td>38</td>
<td>Hexanal</td>
<td>4.57</td>
<td>Saturated fatty aldehyde</td>
<td>Antimicrobial</td>
<td>[Panseri et al, 2011]</td>
</tr>
<tr>
<td>39</td>
<td>1H-Indene</td>
<td>1.03</td>
<td>Polycyclic aromatic hydrocarbon</td>
<td>Antimicrobial</td>
<td>[Wanibuchi et al, 2021]</td>
</tr>
<tr>
<td>40</td>
<td>1-Hexanedecanethiol</td>
<td>0.79</td>
<td>Alkanethiol</td>
<td>-</td>
<td>[Node et al, 2001]</td>
</tr>
<tr>
<td>41</td>
<td>Nonane</td>
<td>0.74</td>
<td>Alkane</td>
<td>Antimicrobial</td>
<td>[Genny et al, 2011]</td>
</tr>
<tr>
<td>42</td>
<td>3-Dodecene</td>
<td>0.64</td>
<td>Alkane</td>
<td>Antioxidant</td>
<td>[Cibulka and Alexiou, 2010]</td>
</tr>
<tr>
<td>43</td>
<td>1,3-Dioxocane</td>
<td>0.61</td>
<td>Ether</td>
<td>-</td>
<td>[Cibulka and Alexiou, 2010]</td>
</tr>
<tr>
<td>44</td>
<td>Imidazole-1,4,5-b-3</td>
<td>0.5</td>
<td>Heterocyclic Aromatic</td>
<td>Antioxidant</td>
<td>[Noriega-Iribe et al, 2020]</td>
</tr>
<tr>
<td>45</td>
<td>6-Tridecanone</td>
<td>0.34</td>
<td>Ketone derivative</td>
<td>Antibacterial</td>
<td>[Zhao et al, 2012]</td>
</tr>
<tr>
<td>46</td>
<td>Undecanal</td>
<td>0.32</td>
<td>Saturated fatty aldehyde</td>
<td>Antimicrobial</td>
<td>[Al-Shanqietat et al, 2015]</td>
</tr>
<tr>
<td>47</td>
<td>Butanal</td>
<td>1.72</td>
<td>Aldehyde derivative</td>
<td>-</td>
<td>[Silva, 2006]</td>
</tr>
<tr>
<td>48</td>
<td>Geranylacetat</td>
<td>2.81</td>
<td>Monoterpenoid</td>
<td>Antibacterial</td>
<td>[Ganjewala and Luthra, 2008]</td>
</tr>
<tr>
<td>49</td>
<td>1,2-Benzenedicarboxylic acid</td>
<td>23.88</td>
<td>Aromatic dicarboxylic acid</td>
<td>-</td>
<td>[Klepper and Nilsen, 2010]</td>
</tr>
<tr>
<td>50</td>
<td>Cyclopentanone-3-carboxylic acid</td>
<td>3.20</td>
<td>Cyclic ketone</td>
<td>-</td>
<td>[Giry et al, 2018]</td>
</tr>
</tbody>
</table>

Information: A = without preservation (G1 35d); B1 = with preservation in 4°C; B2 = with preservation in 4°C and alginate encapsulation.
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](image1.png)
**Figure 5. Viability of T47D cells after treated by kaffir lime callus extracts**

![Figure 6](image2.png)
**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>
<thead>
<tr>
<th>Treatment</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; value range</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (G1 35d)</td>
<td>2000 - &gt;100.000 µg/mL</td>
<td>100.000 µg/mL</td>
</tr>
<tr>
<td>Preservation in 4°C</td>
<td>&gt;100.000 µg/mL</td>
<td>&gt;100.000 µg/mL</td>
</tr>
<tr>
<td>Preservation in 4°C with alginate encapsulation</td>
<td>&gt;100.000 µg/mL</td>
<td>&gt;100.000 µg/mL</td>
</tr>
</tbody>
</table>

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

![Figure 7](image3.png)
**Figure 7. Morphology T47D Cell. A. before treatment, B. after treatment (200x magnification using an inverted microscope)**

![Figure 8](image4.png)
**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 CaCl₂·2H₂O 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\textsubscript{50} values of all callus extracts were above 1000\mu g/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\textsubscript{50} value < 20\mu g/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\textsubscript{2}(SO\textsubscript{4})\textsubscript{3}, KH\textsubscript{2}PO\textsubscript{4}, KNO\textsubscript{3}, Na\textsubscript{2}SeO\textsubscript{3}, and chitosan in various concentrations. The results showed that type and dose of elicitor have a different effect. Na\textsubscript{2}SeO\textsubscript{3} (50mg/L and 100mg/L), and Al\textsubscript{2}(SO\textsubscript{4})\textsubscript{3} (50mg/L) enhanced phenolic content 1.7, 1.4, and 1.0-fold – higher than control, respectively. Furthermore, Al\textsubscript{2}(SO\textsubscript{4})\textsubscript{3} (50mg/L) and Na\textsubscript{2}SeO\textsubscript{3} (100mg/L) showed higher accumulation of resveratrol and higher antioxidant activity compared with cells treated with KNO\textsubscript{3} and chitosan. However, only Na\textsubscript{2}SeO\textsubscript{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-Hexacosanol, 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 store the callus thus it can be used to provide continuous supply callus stock as raw material for pharmaceutical purposes.

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