BIOACTIVE COMPOUNDS OF INDONESIAN RED BETEL (PIPER CROCATUM) EXTRACT AND ITS INHIBITORY ACTIVITY IN MCF-7 CELL LINE

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ABSTRACT

Piper crocatum, known as red betel, is a potential medicinal plant that grows in the tropics as an anti-cancer. Several previous studies of red betel showed the ability to inhibit various types of cancer cells. The research aims to analyze the bioactive compounds in red betel leaf extract origins from Indonesia on ethanol by GC-MS method, identify toxicity with MTT assay. Further, the morphology of MCF-7 breast cancer cells was determined to lead the ethanol extract of P. crocatum. The bioactive compounds of P. crocatum leaves have a function in cytotoxicity to cancer cells, such as phenol (1.36%), phytol (5.72%), and vitamin E (1.76%). Red betel leaves extract against MCF-7 cells has an IC₅₀ = 6.68 μg mL⁻¹. The results showed that morphological changes occurred in MCF-7 breast cancer cells. Cell morphological changes were indicated by loss of contact with surrounding cells and decreased the number of cells after 24 h exposed to ethanol extract of P. crocatum. The ethanol extract of P. crocatum can inhibit MCF-7 breast cancer cells' growth and potential as an anti-cancer agent. However, further research is needed to confirm the mechanism of inhibition and expression of apoptotic genes.

Keywords: Cytotoxicity, Piper crocatum, Breast Cancer Cell, Cell Morphology, MTT Assay

INTRODUCTION

Red betel (Piper crocatum) is a plant originally from the Southeast Asian region which belongs to the Piperaceae. Red betel plants are generally cultivated by vegetative methods such as cutting, grafting, and budding.¹ Propagation of red betel plants with vegetative techniques has weaknesses, such as impractical propagation stages, storage of planting material is short, and planting material is small and susceptible to viral infections.² Sianipar et al.³ have succeeded in reproducing red betel through in vitro culture. It can be optimally utilized as a raw material for medicine.

Red betel is traditionally often used to treat various diseases. Based on pharmacological activity, red betel has been used as an anti-bacterial⁴,⁵ anti-hyperglycemic⁶, anti-cancer breast⁷, and anti-inflammatory.⁸ Red betel plants contain bioactive compounds, including alkaloids, flavonoids, tannins, polyphenols, saponins, terpenoids, and essential oils.⁴ Red betel also contains several bioactive compounds such as carotene, essential oils, and ascorbic acid.⁹ Various genus of Piper such as P. argyrophyllum, P. betel, P. chaba, P. sarmentosum, and P. longum were identified as anti-inflammatory activity.¹⁰ Fitriyani et al.¹¹ have proven the carcinogenic induction method in rats exposed with red betel extract concentration of 50 mg/kg.

Antiproliferation in red betel was detected from ethyl acetate extract, which can inhibit MCF-7 breast cancer cells in vitro with IC₅₀ of 64 μg mL⁻¹ incubated for 48 h.¹¹ Red betels has been reported to have cytotoxicity against various types of cancer cells, such as 4T1 cells (breast cancer) with IC₅₀ values of 120 μg mL⁻¹ ¹² and WiDr cells (colon cancer) with IC₅₀ values of 100 μg mL⁻¹.¹³ Cancer is one of the diseases that causes 9.6 million deaths in the world, with 18.1 million cases in 2018.¹⁴ Gas Chromatography-Mass Spectrometry (GC-MS) is one method to identify the bioactive compounds in plant extracts.¹⁵ This method can analyze the number of compounds quantitatively and find out the molecular structure. Furthermore, cytotoxicity tests of extracts on a cancer cell can use Microtetrazolium...
(MTT) assay method. This method has advantages such as rapid testing, sensitive, non-radioactive isotopes, and can measure large samples at one time. The aim of this study was conducted to analyze the bioactive compounds in red betel leaves extract using the GC-MS and determine the anti-cancer activity and morphology changes of red betel leaves extract against MCF-7 cells. Data have obtained the name of compounds, and the IC$_{50}$ values of red betel leaf extracts on MCF-7 breast cancer cells.

**EXPERIMENTAL**

**Preparation of Extract from Red Betel Plant**
The plant materials for ethanol extraction were exerted by red betel leaves (Piper crocatum) from West Java, Indonesia. The red betel leaves were dried and macerated in ethanol 96% for three days stirred periodically. The solvents were filtered and evaporated using the rotary vacuum evaporator (Rotavapor® R-300, Buchi) at 50 °C. Ethanol extracts were obtained and kept for further analysis.

**GC-MS Analysis of the Extract**
The bioactive compounds in red betel leave ethanol extract were analyzed using gas chromatography-mass spectrometry (GC-MS) instrumentation. The extracts were injected a 5µl with a 5:1 split ratio into a gas chromatography column at 250 °C. The carrier gas of Helium was applied with a velocity of 0.8 µl per min. The following temperature reached at 100 °C, stable for 1 min and it will be followed by heating at 300 °C for 20 min. The ionization energy was used 70 eV voltage for indices the mass spectrometers. Identifying the bioactive compounds was based on comparing the retention time and mass spectra with data published in the National Institute Standard Technique (NIST) database.

**Determination of Cytotoxic Activity by MTT Assay**
Red betel (Piper crocatum) leaves extract were used for cytotoxicity test toward MCF-7 cells. The MCF-7 breast cancer cells cultured was provided with a collection from the Division of Biology Activities at the Central Laboratory of Padjadjaran University, Indonesia. The cell proliferation inhibition (CPI) was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MCF-7 cultured suspension cells (100 µL) were obtained and seeded into a 96-well plate. The MCF-7 cells were incubated at 37°C for 24 h. The test solutions contained 100 µL of medium red betel leaves extract at a concentration of 1000, 500, 250, 125, 62.5 µg mL$^{-1}$. The serial concentration of test solution was added to each well plate and incubated at 37 °C for 24 h. The cultured medium was removed and washed by using 100 µL phosphate saline buffer (PBS). The culture medium with MTT was added to each well plate and subsequently re-incubated at 37 °C for 4 h. The MCF-7 cells were reacted with MTT to form purple formazan. After the incubation period of 4 h, the stopper reagent was added to dissolve formazan crystals. The disk plate was allowed for shaker at 10 min, followed incubated to stand overnight in the dark place and room temperature. The sample absorbances were measured using the ELISA reader at wavelength 595 nm. The IC$_{50}$ values were assessed through the logarithm of the test solution of serial concentration and the percentages of MCF-7.

**Morphological Changes of MCF-7 breast cancer cells**
The morphological changes of MCF-7 cells were treated with the IC$_{50}$ value of ethanol extract P. crocatum. The MCF-7 cells were incubated at 0 h, 12 h, and 24 h. The cells that have been attached to the glass cover were washed with PBS, then added with Propidium Iodide and Hoechst as coloring reagents. The morphological changes in MCF-7 cells were examined using a fluorescent microscope (Carl Zeiss, Germany) using magnification 1000 times. The living cells will show in blue color, and dead cells appear red color.

**RESULTS AND DISCUSSION**
Based on GC-MS analysis, it was revealed that ethanol extract of P. crocatum leaves extract contains several bioactive compounds including Phytol (2.32%), C- (1- (4-methoxyphenyl -2-phenylethene (1.91%), Phenol, p- 1-indanyl (1.36%), Naphthalene-2,6-bis (1.22%), 24s-3.beta., 29-dihydroxy-5A-stigmast-7-ene (1.28%), Euclein (2.65%), 4, 4-diaminostilbene (26.78%), 6,8-decadient-2-ol, 1-nitro (26.75%), 2-amino-
5- (1-propenyl) -3-thioamidopyrazine-1-oxide (4.02%), Phenol, p-1-indanyl (3.40%) and 1H-Phenalen-1-one, hydrazone (2.96%), Dibenzo (A, C) Cyclooctene-3,8-diol, 5,6,7,8-tetrahydro -2,4,10,11,12-pentamethoxy-6,7-dimethyl-, stereoisomer (2.83%), vitamin E (1.76%), as shown in Table 1.

Identifying the bioactive compounds of red betel leaves using GC-MS analysis showed similar data to Setiawan et al. The research has identified several bioactive compounds such as naphthalene, phenol, β-phellandrene, sesquiterpene, and monoterpene. Furthermore, Li et al. reported that red betel leaves extract consists of essential oils and phenolic compounds. This shows that most of the bioactive compounds obtained from red betel leaves are generally phenolic compounds.

Table-1: The Bioactive Compounds in P. crocatum Leaves Extract on Ethanol using GC-MS Analysis (RT = Retention Time)

<table>
<thead>
<tr>
<th>RT</th>
<th>Quality</th>
<th>Name of the Compound</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28.948</td>
<td>91</td>
<td>Phytol</td>
<td>2.32</td>
</tr>
<tr>
<td>31.602</td>
<td>42</td>
<td>C-(1-(4-methoxyphenyl -2-phenylethene</td>
<td>1.91</td>
</tr>
<tr>
<td>31.644</td>
<td>46</td>
<td>Phenol, p-1-indanyl</td>
<td>1.36</td>
</tr>
<tr>
<td>31.706</td>
<td>49</td>
<td>Naphthalene-2,6-bis</td>
<td>1.22</td>
</tr>
<tr>
<td>31.761</td>
<td>72</td>
<td>24s-3.beta.,29-dihydroxy-5A-stigmast-7-ene</td>
<td>1.28</td>
</tr>
<tr>
<td>32.044</td>
<td>38</td>
<td>Euclien</td>
<td>2.65</td>
</tr>
<tr>
<td>32.175</td>
<td>38</td>
<td>2-amino-5-(1-propenyl)-3-thioamidopyrazine-1-oxide</td>
<td>4.02</td>
</tr>
<tr>
<td>32.285</td>
<td>45</td>
<td>Acethydrazide,2(2-isopropyl-5-methylphenoxy)-n2- (4-carboxybenzyldeno</td>
<td>2.00</td>
</tr>
<tr>
<td>32.519</td>
<td>62</td>
<td>4,4′-diaminostilbene</td>
<td>6.79</td>
</tr>
<tr>
<td>32.568</td>
<td>70</td>
<td>Phenol, p-1-indanyl</td>
<td>3.40</td>
</tr>
<tr>
<td>32.630</td>
<td>30</td>
<td>1H-Phenalen-1-one, hydrazone</td>
<td>2.96</td>
</tr>
<tr>
<td>32.961</td>
<td>38</td>
<td>6,8-decadien-2-ol, 1-nitro-</td>
<td>26.75</td>
</tr>
<tr>
<td>33.016</td>
<td>90</td>
<td>Methyl (25R)-5-oxo-anor-3,5-secospirostan-3oate</td>
<td>2.76</td>
</tr>
<tr>
<td>33.223</td>
<td>43</td>
<td>4,4′-diaminostilbene</td>
<td>26.78</td>
</tr>
<tr>
<td>33.285</td>
<td>90</td>
<td>Dibenzo (A,C) Cyclooctene-3,8-diol,5,6,7,8-tetrahydro-2,4,10,11,12-pentamethoxy-6,7-dimethyl-, stereoisomer</td>
<td>2.83</td>
</tr>
<tr>
<td>33.285</td>
<td>99</td>
<td>Vitamin E</td>
<td>1.76</td>
</tr>
</tbody>
</table>

Several bioactive compounds produced from GC-MS of ethanol extract of red betel leaves have potential biological activity. Some compounds such as phytol (2.32%), methoxyphenyl (1.91%), phenol (5.72%), acethydrazide (2.00%), and vitamin E (1.76%) are used as antioxidants and anti-cancer, as shown in Table-2.

Table-2: The Evaluation of Total Bioactive Compounds in P. crocatum Leaves Extracts on Ethanol and its Biological Activities Potential

<table>
<thead>
<tr>
<th>Name of the Compound</th>
<th>Compound Nature</th>
<th>Concentration (%)</th>
<th>Biological Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytol</td>
<td>Phytol</td>
<td>2.32</td>
<td>Antioxidant activity&lt;sup&gt;19&lt;/sup&gt;,&lt;sup&gt;20&lt;/sup&gt; and anticancer&lt;sup&gt;21&lt;/sup&gt;,&lt;sup&gt;22&lt;/sup&gt;</td>
</tr>
<tr>
<td>C-(1-(4-methoxyphenyl -2-phenylethene</td>
<td>Methoxyphenyl</td>
<td>1.91</td>
<td>Anesthetic for relieving pain&lt;sup&gt;23&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phenol, p-1-indanyl</td>
<td>Phenol</td>
<td>5.72</td>
<td>As an anti-cancer agent and antioxidant potential&lt;sup&gt;24&lt;/sup&gt;,&lt;sup&gt;25&lt;/sup&gt;,&lt;sup&gt;26&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acethydrazide,2(2-isopropyl-5-methylphenoxy)-n2-(4-carboxybenzyldeno</td>
<td>Acetic acid hydrazide</td>
<td>2.00</td>
<td>An antibiotic for the treatment of &lt;i&gt;Mycobacterium tuberculosis&lt;/i&gt; &lt;sup&gt;27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Vitamin E</td>
<td>1.76</td>
<td>Antioxidant activity&lt;sup&gt;28&lt;/sup&gt; and anticancer&lt;sup&gt;29&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
The GC-MS spectrum confirms various bioactive compounds with different retention times, as illustrated in Fig.-1. The mass of each compound eluted at other times produces the properties and structure of the compound. Fragments of large compounds into small compounds give rise to peak appearance at different m/z ratios.

Interaction between the concentration of red betel leaves extract and inhibition of breast cancer cell (MCF-7) polymerase is shown in Fig.-2. Morphological visualization showed that the increased red betel leaves extract concentration was inversely proportional to the percentage of breast cancer cell inhibition. The concentration of red betel extract at 100 μg mL\(^{-1}\) has a percentage of cell polymerase inhibition of 52%. Based on the linear equation \(y = -14.9\ln(x) + 103.48\), the IC\(_{50}\) value of red betel leaves extract on ethanol is 6.68 μg mL\(^{-1}\).

This value confirms that the red betel extract's effectiveness with a concentration of 6.68 μg mL\(^{-1}\) can kill 50% of the multiplication of MCF-7 breast cancer cells. The American National Cancer Institute groups cytotoxic compounds into four categories. The very toxic category with IC\(_{50}\) values ≤ 20 μg mL\(^{-1}\). The moderate category if IC\(_{50}\) values range from 21-200 μg mL\(^{-1}\), a weak category with IC\(_{50}\) values between 201-500 μg mL\(^{-1}\), and not toxic with an IC\(_{50}\) value of ≥ 500 μg mL\(^{-1}\). Based on this group, ethanol extracts of red betel leaf in this study is the very toxic category.

![Fig.-1: GC-MS Chromatogram of P. crocatum Leaves Extracts on Ethanol](image1.png)

![Fig.-2: The Percent Cell Proliferation Viability and the IC\(_{50}\) Value of P. crocatum Leaves Extracts on Ethanol against Breast Cancer Cell Line MCF-7](image2.png)

The cytotoxicity of a compound is influenced by two main factors: the type of plant extract and the type of target cancer cell. The difference in IC\(_{50}\) values is due to the kind of betel and plant sources used as samples. Cytotoxic test of the red betel extract on MCF-7 breast cancer cells has been carried out by several researchers and showed various IC\(_{50}\) values of 65 μg mL\(^{-1}\), 8.33 μg mL\(^{-1}\), and 114 μg mL\(^{-1}\).
The observation of MCF-7 cell morphology at the beginning of time (0 h) showed that some cells were already indicated death. Cell death is visualized in red while living cells are blue. This is seen in Fig.-3A shown by the arrow. The cell staining results in Fig.-3B shown MCF-7 cells have died after being exposed to red betel leaves extract for 12 h. The extracts treatment is caused cell death with morphological changes of MCF-7 cells. It was seen that some cell walls had lysis and decreased cell count after 24 h, which was indicated as pre-apoptosis (Fig.-3C). The potential of red betel leaves extract to stimulate cell apoptosis is feasible due to the presence of phytol (2.32%), phenol (5.72%), and vitamin E (1.76%) compounds, in which some of these compounds have antioxidant and anti-cancer activities.

Fig.-3: The effect of P. crocatum leaves extracts on ethanol against breast cancer cell line MCF-7 on morphological changes. The magnification using 1000 times. The arrows indicate differences in the morphology of cells that experience cell death

Sakhtivel et al. demonstrated that phytol compounds could inhibit cell growth and induce apoptosis in A549 lung cancer cells with IC_{50} values of 70.81 ± 0.32 μM at 24 h and 60.7 ± 0.47 μM at 48 h. The formation of shrinking cells is characterized by damage to cell membranes and cell wall lysis. Pejin et al. have reported that MCF-7 (breast cancer) and HeLa (cervical cancer) cells were susceptible to phytol compounds, so they were inhibitory on cancer cells' growth. Phytol compounds purified from marine algae (Gracilaria edulis) have been shown to have antiproliferation activity and significantly inhibit MCF-7 breast cancer cells' growth.

Owen et al. reported that phenol compounds have potential as antioxidants and anti-cancer contained in olive oil. Phenolic compounds can occur as potential reactive oxygen inhibitors involved in neoplasms' etiology, such as breast and colorectal cancer. Phenol compounds have been identified as having active mechanisms as anti-carcinogenic in vivo. Polyphenol compounds will react in cellular mechanisms under oxidative stress through redox modulation and intracellular signal transduction associated with cell proliferation, differentiation, apoptosis, inflammation, angiogenesis, and metastasis. Tragopogon porrifolius plant has a high phenol compound content, so it has anti-cancer activity against two types of osteosarcoma cancer cells.

Some types of vitamin E have been shown to have anti-cancer and anti-inflammatory activity. Vitamin E has a vital role in the prevention and inhibition of cancer cells. Ronco et al. have shown a reduction in breast cancer risk by regularly administering vitamin E on the food menu. Vitamin E has been informed as an effective anti-cancer agent, and its utilization in cancer treatment has a significant clinical trial subject. The process of metabolizing vitamin E on cancer cells affects the bioactivity of cancer cells. This metabolism is influenced by two factors, the concentration of compounds and groups of genes involved.

CONCLUSION

The results obtained from the present study indicate that the extract of P. crocatum leaves extracts contains phytol (2.32%), phenol (5.72%), and vitamin E (1.76%), which may contribute to anti-cancer. The study results concluded that the extract of red betel leaf has anti-cancer activity with an IC_{50} value of 6.68 μg mL^{-1}. Morphological differences appear that cell death occurs, and cell numbers decreased after exposure to red betel leaves extract for 24 h. This study indicates that the cell has pre-apoptosis. However, further studies are required to prove the activity of the mechanism and expression of genes responsible for the process causes of cytotoxicity and apoptosis of MCF-7 breast cancer cells in vitro.
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