

# Musa Anticancer

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# Palmitic acid of *Musa Paradisiaca* induces apoptosis through caspase-3 in human oral squamous cell carcinoma

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**Abstract. – OBJECTIVE:** Despite apoptosis processes being conserved, cancer cells have developed mechanisms to inhibit apoptosis by altering anti-apoptotic molecules or inactivating pro-apoptotic. The aim of this study was to determine the palmitic acid of *Musa paradisiaca* var. *sapientum* (L) Kunz (MP) stem extracts against human oral squamous cell carcinoma (hOSCC) through caspase-3.

**MATERIALS AND METHODS:** Ethanol and ethyl acetate extracts of MP stem were analyzed by gas chromatography-mass spectrometry (GC-MS). Computerized models of chemically active compounds were used to predict anticancer activity. Cytotoxicity was evaluated in *Artemia salina* Leach and hOSCC (OM-1) culture at concentrations 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10 µg/mL respectively. The expression level of caspase-3 on hOSCC was measured by enzyme-linked immunoassay (ELISA).

**RESULTS:** We found seven chemically active compounds in the ethanol extract and 15 compounds in the ethyl acetate extract of MP stem. The major component was hexadecanoic acid of palmitic acid derivatives, and this was predicted to have anticancer activities as apoptosis through caspase-3 stimulants. However, cytotoxicity effects against hOSCC culture were assessed by values of the 50% inhibitory concentration (IC<sub>50</sub>) of 15.00 µg/mL for the ethanol extract, and an IC<sub>50</sub>

of 10.61 µg/mL for the ethyl acetate. There was a significant increase of caspase-3 level on treatment groups compared to control.

**CONCLUSIONS:** Hexadecanoic acid of MP stem extracts has anticancer activity by inhibiting cell growth of hOSCC culture through caspase-3 stimulants.

#### Key Words:

Oral squamous cell carcinoma, Anticancer, Herbal medicine, Apoptosis, Caspase-3.

## Introduction

Oral cancer is a common cancer in both developing and developed countries when people undergo lifestyle changes<sup>1</sup>. One of the prominent types of oral cancer is oral squamous cell carcinoma (OSCC), which has reached a high number of cases in Southeast Asia with a prevalence of more than 90% among oral cancers<sup>2</sup>. OSCC occurs place in any region of the oral cavity, pharyngeal region and salivary glands<sup>3</sup>. It was traditionally associated with certain risk factors, such as heavy smoking, consumption of betel nut and alcohol<sup>4</sup>, an impacted

wisdom tooth, poor dental fillings, ill-fitting denture prostheses<sup>5</sup>, unhealthy diet and nutrition<sup>6</sup>, and the human papillomavirus (HPV) infection<sup>7</sup>.

Irritating chemicals and free radicals as stress signals received by intercellular molecules mediate mitochondrial outer membrane permeabilization and released pro-apoptotic molecules into the cytoplasm<sup>8</sup>. The B-cell lymphoma (Bcl-2) family and inhibitors of apoptotic proteins that serve as apoptotic switches, control the permeabilization of mitochondrial membranes<sup>9</sup>. Some suggested that these two pathways may work in synchronization to initiate apoptosis. Despite apoptosis processes being conserved, cancer cells have developed mechanisms to inhibit apoptosis by altering anti-apoptotic molecules or inactivating pro-apoptotic cell death components through decreasing of caspase-3. Cancer cells had adopted several mechanisms to evade apoptosis<sup>10</sup>. These mechanisms can be windows into the development new drugs as cancer therapy.

Chemotherapy and radiotherapy are first-line treatment regimens to manage OSCC. However, these conventional therapies have several severe side effects due to their non-specific actions against highly proliferating normal cells. It is well known that nausea, vomiting, hair loss, Candida infections, and epithelial-ulceration are common manifestations of adverse effects derived from the use of anticancer drugs, such as cisplatin, carboplatin, 5-fluorouracil<sup>11</sup>, docetaxel, paclitaxel, and methotrexate<sup>12</sup>, and radiation-induced xerostomia<sup>13</sup>. Thus, many studies have been conducted to develop non-toxic herbal medicines as alternative to chemical drugs, with the hope of reducing severe side effects in cancer patients.

Plants contain a broad array of phytochemicals with various functional and non-functional activities, and plant extracts might be subjected to particular prescreening prior to detailed investigations. One of methods to screen the active chemical compounds in medicinal plants is through gas chromatography-mass spectrometry (GC-MS)<sup>14</sup>. GC-MS is a key technique to detect and separate volatile compounds, including secondary metabolites in both plant and non-plant species<sup>15,16</sup>. In addition to the intense laboratory-based screening and analytical processes, the emergence of computational screening that utilizes artificial intelligence systems has expedited the development of new candidates through drug discovery. Database applications, such as *Prediction of Activity Spectra for Substances* (PASS SERVER), a web-based application is being used to evaluate the biolog-

ical potentials of organic molecules that can be developed as medicines<sup>17,18</sup>. Prediction results of PASS SERVER are presented as values of Pa (probable activity) and Pi (probable inactivity), with the Pa>Pi ratio indicating a good probability of predicted activity<sup>19</sup>. The robustness of this technique made the screening of new anticancer drugs more feasible, and additional analytical assistance from *in silico* prediction of cell line cytotoxicity (CLC-Pred tools) adds value in predicting the toxicity to cancer cell cultures<sup>20</sup>. Chemically active compounds with potential anticancer activity that are sorted using GC-MS and computational (*in silico*) modeling should be tested for their biological and toxicological activities. The brine shrimp lethality test (BSLT), a bioassay that screens the cytotoxicity of a specific compound or extract, uses shrimp larvae of *Artemia salina* Leach to determine the 50% lethal concentration (LC<sub>50</sub>) through probit analysis<sup>21-23</sup>. A 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) *in vitro* cell proliferation assay is one of the most extensively used methods for assessing preliminary anticancer activities of synthetic derivatives, natural medicines and natural product extracts. This colorimetric-based test is highly reliable and can be used on a wide range of cell lines. This assay indicates whole-cell cytotoxicity; however, additional experiments were required to define particular molecular target<sup>24,25</sup>.

Banana trees are tropical plants that are often used as herbal medicines. The banana tree can be used as an alternative treatment of wounds, fevers, insect bites, digestive disorders, and epilepsy<sup>26</sup>. The sap of the Ambonese banana (*Musa paradisiaca* var. *sapientum* (L) Kunz) (MP) stem has long been used to accelerate wound healing and relieve tooth pain in the village community of Trunyan, Bali, Indonesia<sup>27</sup>. There are significant compounds in banana plants, such as saponins, flavonoids, anthraquinones, and tannins contained in both the fruit and sap, as well as lectin<sup>28</sup>. Sap aspirated from the stem of the MP was shown to have many therapeutic activities, including wound healing<sup>29</sup>. Since high levels of quercetin and lectin are found in banana sap, it was shown to suppress cancer cell proliferation, increase macrophage activity, inhibit matrix metalloproteinases (MMP)-2 and -9 activities<sup>30,31</sup>, increase T cell proliferation<sup>28</sup>, increase fibroblast activity and promote angiogenesis<sup>29</sup>.

In the present study, new chemically active compounds derived from the extract of MP stem were screened using GC-MS methods, *in silico*

prediction activity, the in vitro BSLT and MTT assay, and also apoptotic activity of caspase-3 as potential candidates for anticancer drug.

## Materials and Methods

### Preparation of MP Stem Extract

Stems of MP were cleaned and chopped to a size of 0.5-1 cm. Then, the stems were dried in an oven at a temperature of 50°C for 3 h. The dried stems were blended to become a powder form. Maceration was conducted with 96% ethanol or acetic acid as solvent for 7 days for each solvent. First, 1 kg powder of MP was mixed with 2 L of 96% ethanol and 1 kg powder was mixed with 2 L of 96% ethanol and 20 mL of glacial acetic acid. All process required 7 days and were conducted twice for each solvent. The product was filtered through a No. 41 Whatman membrane (Merck, Kenilworth, New Jersey, USA). The extract and solvent were separated by a rotary evaporator (Heidolph) at a temperature 50°C and a speed of 200 rpm for 2 h until a condensed extract was obtained. This was placed in a covered container to avoid light<sup>32</sup>.

### Preparation of Stock Solutions

This study was carried out with 100 µg/mL as working concentrations. First, a stock solution was made by dissolving 1000 mg of MP stem extract in 1 L of sterile distilled water (1,000 mg/L=1,000 ppm), and then, 1 mL of the stock solution was mixed with 9 mL of sterile distilled water (DIW) to make a concentration of 100 ppm (100 µg/mL). We then mixed 0.9 mL of the stock solution with 9.1 mL of sterile DIW to make 90 ppm (90 µg/mL). The same preparation methods were used to obtain concentrations of 80, 70, 60, 50, 40, 30, 20 and 10 µg/mL<sup>33</sup>.

### Analysis of Chemically Active Compounds by GC-MS

Stem extract of MP was analyzed by GC-MS (Shimadzu QP 5000, Japan). GC-MS spectroscopy was operated using a capillary column with a height of 25 cm, a diameter of 0.25 mm, and a thickness of 0.25 µm. A sample (1 µL) was injected into the mobile phase (helium gas, at a pressure of 12kPa), and it carried the sample mixture to the stationary phase (CP-Sil 5 CB). The capillary column was in an oven that was programmed to gradually increase the temperature (70 to 270°C), at a rate of 10°C/min. Total separation of the mixture components was 30 mL/min, and thus a chromatogram was produced representing an m/z ratio of 1:50<sup>14</sup>.

### Predictions of Chemically Active Compounds of MP Stem Extract on PASS SERVER

The National Centre for Biotechnology Information (NCBI; [www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) was used to obtain chemical structures of selected phytoconstituents of MP stem extracts. Selected structures were downloaded as sdf files for prediction in PASS SERVER software (version 2.0; <http://www.way2drug.com/PASSonline/>), and a compound's predicted activity spectrum was used to calculate the probable activity (Pa) and probable inactivity (Pi). The prediction of this spectrum by PASS SERVER was based on a structure-activity relationship (SAR) analysis of a training set containing over 205,000 compounds with over 3750 different biological activities. Pa and Pi values range 0.00-1.000 because they are probabilities. Generally, Pa+Pi≠1 since these probabilities were calculated independently<sup>17</sup>.

### Preparation of Shrimp Larvae Culture (*A. salina* Leach)

Artificial seawater was prepared. Briefly, shrimp larval eggs were dropped into an aquarium which contained NaCl (15 g) in 1 L of DIW at a neutral pH (8-9). The container placed in the room with light, and the temperature was maintained at around 25-31°C. After 48 h the eggs hatched and the larvae swam to the lighted area<sup>22</sup>; therefore, the larvae could be separated from their shells and then healthy larvae were cultivated and used to test the extracts of MP.

### Toxicity Test by the BSLT

A brine shrimp (*A. salina* Leach) lethality assay is commonly used to check cytotoxic effects of bioactive chemicals. Shrimp eggs (0.25 g) were weighed on an analytical balance, and then grown in 300 mL of artificial seawater for 1-3 days. After 1-3 days, the eggs hatched, and became larvae (nauplii), and we selected healthy larvae for subsequent experiments. Concentrations of 90, 80, 70, 60, 50, 40, 30, 20, and 10 µg/mL of extracts of MP stems in artificial seawater were prepared and placed in test tubes containing 10 nauplii. Nauplii were evaluated, and dead ones were counted after 24 h. A dead larva indicated by a shrimp larva which did not show activity for a few seconds of observation<sup>22</sup>.

### Pre-Culture of hOSCC Cells in T75 Flask

Human oral squamous cell carcinoma (OM-1) cells stored in the vial preserved in a deep freez-

er (at  $-80^{\circ}\text{C}$ ). Cells were thawed in sterile DIW at  $37^{\circ}\text{C}$  and moved to Falcon tubes with 5 mL of cell medium. The Falcon tubes were centrifuged at 500 rpm for 19 min. Cell medium consisted of high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and fetal bovine serum (FBS; Sigma-Aldrich) in a 90:10 ratio. The supernatant was removed, and 5 mL of new medium was carefully added to each Falcon tube. The pellet (containing hOSCC cells) was gently resuspended in the medium and seeded into a flask. The flask was incubated for 2-3 days at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  until a confluent monolayer of cells (80% density) was detected with an inverted microscope at 100x magnification (Nikon, Japan). Cells were detached using TrypLE™ Select Enzyme (Gibco, Rockville, MD, USA), harvested and counted with a cell counter (Scepter; Sigma-Aldrich), and the cells density was  $1.3 \times 10^6/\text{mL}$ .

#### **Anticancer Activity Screening of MP Stem Extract against hOSCC Cells by An MTT Assay**

The microplate containing hOSCC cells was observed under an inverted microscope at a 100x magnification to ensure that cells were confluent. Each group consisted of three replicate wells (triplo). Treatment groups contained the MP stem ethanol extract or ethyl acetate extract at 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10  $\mu\text{g}/\text{mL}$ . Two control groups were control medium that contained culture medium without cells and a control cell group that only contained 35 and culture medium. Then, 25  $\mu\text{L}$  of an extract was added to each well and incubated for 24 h at  $37^{\circ}\text{C}$ , and 5%  $\text{CO}_2$ . To each well 10  $\mu\text{L}$  MTT (M2003; Sigma-Aldrich) was added, and then the mixture was covered with aluminum foil and 36 incubated for 4 h at  $37^{\circ}\text{C}$ . Lastly, 50  $\mu\text{L}$  DMSO was 22 added to each well and shaken vigorously, and then the absorbance level was measured by an Enzyme-Linked Immunosorbent Assay (ELISA) reader at a wavelength of 620 nm to obtain the  $\text{IC}_{50}$  value<sup>34</sup>.

#### **Measuring the Caspase-3 Level on hOSCC Cells Treated MP Stem Extract by ELISA**

The apoptotic activity of caspase-3 was measured with ELISA on hOSCC cell 3 culture. The hOSCC cells were initially grown in 6-well plate with a seeding density of  $4.10^5$  cells per well (2 mL volume). When the cells reached 85-90% confluence, cells were treated with 6 different concentrations of MP stem ethanol extract (0, 22.5, 45,

90, 360 ng/ml) and incubated for 0-, 2-, 24-, and 48-hours in  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . The complete DMEM growth medium without penicillin/streptomycin was used during the treatment. Every time points of treatment, a 1 mL of supernatant was collected for ELISA analysis. The sandwich ELISA kit (Bioenzy, Cat. BZ-08124084-EB) was used for a quantitative detection of Human Caspase-3. The collected supernatant was preliminary centrifuged to remove the debris and later transferred to 96-wells ELISA plate. The sample preparation was done by following the assay procedures described in the kit. Each sample was done in triplicate. As controls, untreated cells and serum-free medium were applied. Absorbance was determined at 450 nm using a microplate spectrophotometer (BioTek, Epoch, USA)<sup>35</sup>.

#### **Statistical Analysis**

Data of larvae mortality were tabulated and assessed to obtain the  $\text{LC}_{50}$  using a probit analysis<sup>21</sup>. However, the absorbance of the hOSCC culture was measured to obtain the  $\text{IC}_{50}$  and caspase-3 level. All data were analyzed by GraphPad Prism 8<sup>th</sup> software. The concentration relationship between the ethanol and ethyl acetate extracts was assessed using a linear regression at a 95% confidence interval ( $\alpha=0.05$ ).

## **Results**

#### **Chemically Active Compounds of MP Stem Extract by GC-MS**

The components of the extracts were identified by comparing peak values of mass spectral fragmentation patterns to those of reference compound fragmentation patterns from the Wiley Registry of Mass Spectral Data. Several chemically bioactive compounds were identified in the ethanol extract of MP stems (Figure 1). The main components were 43 47% of 9, 12 octadecadienoic acid, 5.40% of hexadecanoic acid, methyl ester and pentadecanoic acid, 14-methyl which is palmitic acid (a group of saturated fatty acids). Meanwhile, other fatty acid groups with less than 5% concentrations included n-hexadecanoic acid, methyl stearate, heptadecanoic acid, 16-methyl, methyl ester, heptadecanoic acid, 14-methyl, methyl ester, and benzo (h) quinoline, 2, 4-dimethyl (Table I).

There were 15 peak values found by GC-MS in the ethyl acetate extract, but the concentrations were less than 5% (Figure 2). The main constituents were from the fatty acid family, with 4.62%

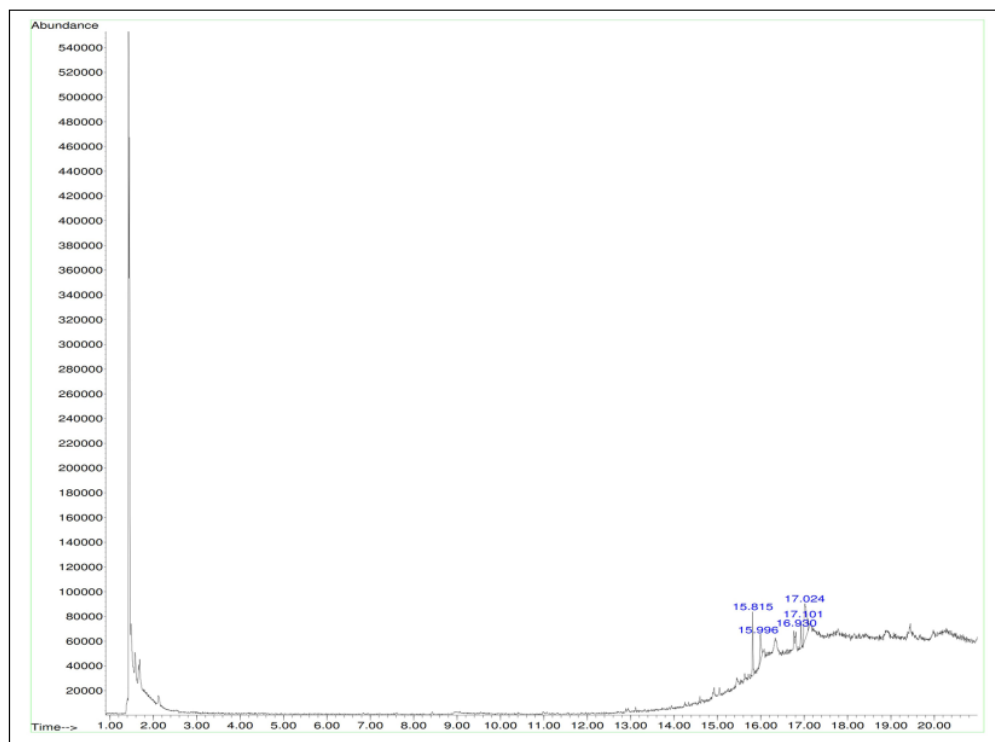


Figure 1. GC-MS chromatogram of MP stem ethanol extract.

oleic acid and octadec-9-enoic acid and, 2.08% 9,12-octadecadienoic acid (z, z). Using ethyl acetate as a solvent increased the extraction of chemically bioactive compounds from MP stem of not only palmitic acid, but also 1.21% of ethanol, 2-bromo, and other compounds with concentration of < 1%, such as 0.81% of chloroanilic acid N'(3-ethoxy-4-hydroxybenzylidene) hydrazide, 0.44% of 2-propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-3, 0.28% of cis-2,4,5-trimethoxy-.beta.-methyl-.beta.-nitrostyrene, 0.25% of 4-fluorobenzoic acid, 2-phenylethyl ester and 0.20% cyclopropanoic acid, 2-octyl- (Table II).

#### **Prediction of Biological Activities of Chemically Bioactive Compounds from MP Stem Extracts Using the Online PASS SERVER**

Some potentially bioactive compounds could be estimated through predictions using the online PASS SERVER. Based on results of chemically bioactive compound screening found using the GC-MS analysis, in general, compounds were members

of the fatty acid group, such as palmitic acid, linoleic acid, oleic acid, and stearic acid. Chemically bioactive compounds with predicted potential were those that had a percentage of similarity to comparison database compounds of ethanol extract (Table I) and ethyl acetate extract (Table II) of MP stem that were greater than 90%. A compound's potential was predicted using a canonical smile obtained from the PubChem online service (Table III).

Predictions were based on results of online computing according to the PASS SERVER, and four fatty acid compounds had potential that could be used as a starting point of this study for anti-cancer agents. These compounds were palmitic acid, linoleic acid, enolic acid, and stearate. They were identified by examining Pa value in the activity of superoxide dismutase (SOD) inhibitors, apoptosis agonists, and caspase-3/caspase-8 stimulants. The data revealed that the bioactive compounds of hexadecanoic acid (palmitic acid) and pentadecanoic acid, 14-methyl, methyl ester had the highest Pa values against the activity of SOD inhibitors at Pa=0.914. The bioactive compounds

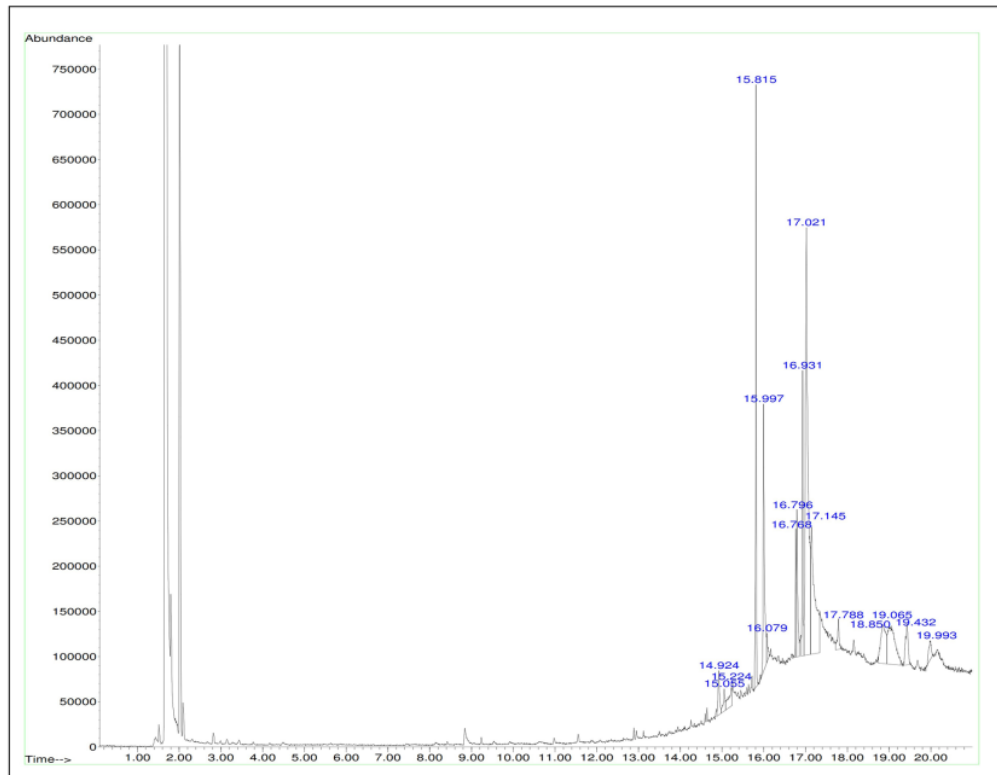
**Table I.** GC-MS analysis of chemically active compounds in *Musa paradisiaca* var. *sapientum* (L) Kunz ethanol extract.

Peak	Retention time	Peak area (%)	Chemically active compounds	Qual
1	15.815	5.40	Hexadecanoic acid, methyl ester	98
			Pentadecanoic acid, 14-methyl-, methyl ester	97
2	15.996	3.85	n-Hexadecanoic acid	95
			Methyl stearate	91
3	16.930	2.29	Heptadecanoic acid, 16-methyl-, methyl ester	91
			Heptadecanoic acid, 14-methyl-, methyl ester	90
4	17.024	11.47	9,12-Octadecadienoic acid	46
5	17.101	0.96	Benzo (h) quinoline, 2,4-dimethyl	38

The highest apoptosis agonist activity were 9,12-octadecadienoic acid (z,z), methyl ester and cis-13-octadecadienoic acid, methyl ester (linoleic acid) with Pa=0.545. Furthermore, the bioactive compound with the highest activity as a caspase-3/caspase-8 stimulant was linoleic acid (Pa=0.617) (Table III).

The predicted result of bioactive compounds obtained Pa>0.7, indicating that the test com-

pound's activity was the same as the drugs, and there were bioactive compounds with values of 0.5<Pa<0.7, indicating that the test compounds had lower biological activities than the drug. Table IV shows predictions of hexadecanoic acid (palmitic acid) compounds with toxic activity against cancer cell cultures (lung carcinoma, metastatic melanoma, melanoma, and stage 3 non-small cell lung cancer cells). In our results,



**Figure 2.** GC-MS chromatogram MP stem ethyl acetate extract.

**Table II.** GC-MS analysis of chemically active compounds in *Musa paradisiaca* var. *sapientum* (L) Kunz ethyl acetate extract.

Peak	Retention time	Peak area (%)	Chemically active compounds	Qual
1	14.924	0.28	Cis-2,4,5-Trimethoxy-beta-methyl-beta-nitrostyrene	38
2	15.055	0.14	Triamterene	41
3	15.224	0.25	4-Fluorobenzoic acid, 2-phenylethyl ester	27
4	15.815	1.73	Pentadecanoic acid, 14-methyl-, methyl ester Hexadecanoic acid, methyl ester	99
5	15.997	1.33	n-Hexadecanoic acid	99
6	16.768	0.39	10-methyl 10-trans, 12-cis-octadecadienoate 9,12-Octadecadienoic acid (z,z)-, methyl ester 9,15-tetradecadienoic acid (z,z)-, methyl ester	99 99 99
7	16.796	0.68	9-Octadecadienoic acid (z)-, methyl ester Cis-13-Octadecadienoic acid, methyl ester	99 99
8	16.931	0.98	Methyl stearate	98
9	17.021	4.62	Oleic acid Octadec-9-enoic acid	96 96
10	17.145	2.08	9,12-Octadecadienoic acid (z,z)-, Oleic acid	95
11	17.788	0.20	20-Cyclopropanoic acid, 2-octyl-	90
12	18.850	0.81	3'-Chlorooxanilic acid N'(3-ethoxy-4-hydroxybenzylidene) hydrazide	42
13	19.065	1.21	11-Ethanol, 2-bromo-	35
14	19.432	0.44	2-propen-1-one, 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-3	64
15	19.993	0.19	Oleic acid	47

predictions of toxic compounds from MP stem extract using CLC-Pred tools showed  $P_a > 0.5$ .

#### BSLT Results of MP Stem Extracts

Brine shrimp lethality test (BSLT) is an acute toxicity test that applies a probit analysis to determine toxic concentration within 24 h. At each concentration, the death of shrimp larvae was measured, and a concentration-response relationship was determined using a regression analysis. The following equation was used to calculate the toxicity of the ethanol extract of MP stem:

$$y = 2.1669x + 1.5666 \quad (1)$$

Regression equation (1) achieved a value  $R^2 = 0.9824$ , so that the R calculated value was 0.9912. Based on the table value of R person, i.e., ( $R = 0.666$ ), which means the calculated R value was greater than the value from the R table. This indicated a significant correlation or relationship between the concentration and the percentage of shrimp larval death when the ethanol extract of MP stem was administered (Figure 3). There was a significant difference in the number of larval deaths between groups ( $p = 0.027$ ). In the probit analysis, the ethanol extract was found to be toxic at  $LC_{50} = 38.41 \mu\text{g/mL}$  (Table V). The following regression equation was obtained to calculate the toxicity of the ethyl acetate extract of MP stem:

$$y = 1.8692x + 2.155 \quad (2)$$

In regression equation (2),  $R^2 = 0.9646$  and  $R$  count = 0.9821 were obtained. The R-value of the R person table was ( $R = 0.666$ ), indicating that counts were greater than those of the R table. There was a significant correlation or relationship between the concentration of the ethyl acetate extract of MP stem and the percentage of shrimp larval death (Figure 3). There was a significant difference in the number of larval deaths between groups ( $p = 0.010$ ). The probit analysis revealed that ethyl acetate extract was toxic at  $LC_{50} = 33.27 \mu\text{g/mL}$  (Table VI).

Whenever the results were compared to toxicity level criteria, the crude extract at  $LC_{50} < 100 \mu\text{g/mL}$  was in the strong toxicity category, and crude extracts in the range of  $LC_{50}$  values of  $100 < LC_{50} < 500 \mu\text{g/mL}$  were classified as having moderate toxicity. The crude extracts with the range of  $LC_{50}$  values for  $500 < LC_{50} < 1,000 \mu\text{g/mL}$  were considered to have low toxicity activity, while those with  $LC_{50}$  values greater than 1,000  $\mu\text{g/mL}$  were considered non-toxic.

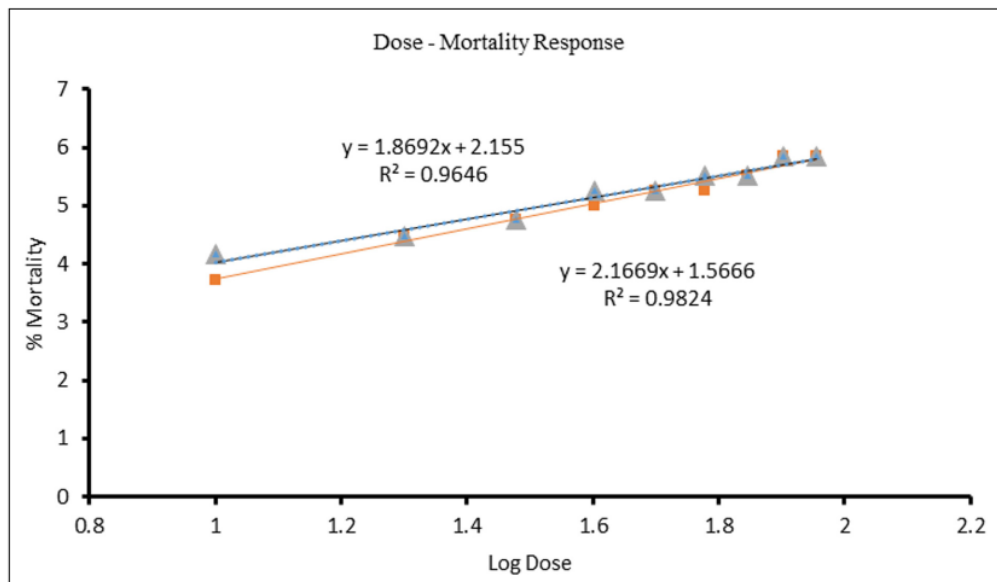
#### Cytotoxicity Assay of MP Stem Extracts to hOSCC Cells

The cytotoxicity test results of MP stem sap deposited in a hOSCC (OM-1) cell culture revealed differences in the formazan formed in each group. Purplish crystal formation was observed in the reaction of MTT with the succinic dehydrogenase enzyme found in mitochondria (Figure 4). Formazan formed in each well was observed using an ELISA reader at a wave-



**Table III.** Canonical smile of fatty acid compounds group has anticancer potential in *Musa paradisiaca* var. *sapientium* (L) Kinz.

No.	Chemically active compounds	Synonym	Canonical SMILE	Pa value	Activity
1	Hexadecanoic acid, methyl ester	Palmitic acid methyl ester Methyl palmitate	CCCCCCCCCCCCCCCC(=O)OC	0.738 0.473 0.609	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant
2	Pentadecanoic acid, 14-methyl-, methyl ester	Pentadecanoic acid 1002-84-2 n-Pentadecanoic acid Pe <sub>12</sub> cyclic acid Palmitic acid	CCCCCCCCCCCCCCCC(=O)O	0.914 0.342 0.562	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant
3	n- Hexadecanoic acid	Hexadecanoic acid 57-10-3 Cetyllic acid Palmitate	CCCCCCCCCCCCCCCC(=O)O	0.914 0.342 0.562	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant
4	Methyl stearate	Methyl octadecanoate 8 112-61-8 Octadecanoic acid, methyl ester	CCCCCCCCCCCCCCCC(=O)OC	0.738 0.336 0.609	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant
5	Heptadecanoic acid, 16-methyl-, methyl ester	Methyl margarate 44 1731-92-6 Heptadecanoic acid, methyl ester	CCCCCCCCCCCCCCCC(=O)OC	0.738 0.473 0.609	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant
6	9,12-Octadecadienoic acid (z,z)-, methyl ester	Margaric acid methyl ester 5 Linoleic acid 60-33-3 Linolic acid	CCCC=CCC=CCCCCCCC(=O)O	0.814 0.545 0.617	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant
7	Oleic acid Octadec-9-enoic acid	9 Telfairic acid cis,cis <sub>5</sub> linoleic acid oleic acid 112-80-1 cis-9-Octadecenoic acid (Z)-Octadec-9-enoic acid cis-Oleic acid	CCCCCCCC=CCCCCCCC(=O)O	0.851 0.499 0.592	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant
8	Cyclopropanoic acid, 2-octyl-	2-Octylcyclopropanoic acid 8-(2-Octylcyclopropyl)octanal 2-octyl-cyclopropanoic acid	CCCCCCCC(CCC1CCCCCCCC=O	0.653 0.386 0.411	Superoxide dismutase inhibitor Apoptosis agonist Caspase 3 and 8 stimulant



**Figure 3.** Correlation between extract concentration and Brine Shrimp Lethality Test. The orange line represented MP stem ethanol extract, and the blue line represented MP stem ethyl acetate extract.

length of 650 nm. The concentration inhibition of the ethanol extract of MP stem on hOSCC cells was 15.00  $\mu\text{g/mL}$  ( $\text{IC}_{50}=15.00\mu\text{g/mL}$ ). The inhibition of hOSCC cells was measured, and a concentration-response relationship was determined using a regression analysis. The following equation was used to calculate the concentration inhibition of the ethanol extract of MP stem against hOSCC culture:

$$y = -52.20x + 104.2 \quad (3)$$

Regression equation (3) obtained the value  $R^2=0.8769$ , so that the calculated value was  $R=0.9364$ . Based on the table value of R person, i.e., ( $R=0.344$ ), this means that the calculated R value was greater than the value from the R table.

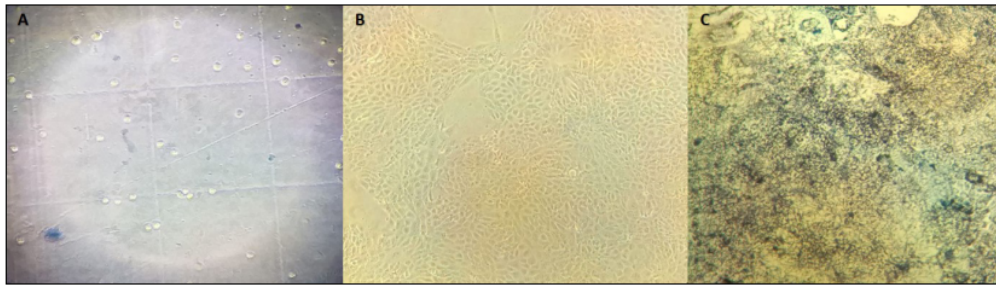
This indicates a significant correlation or relationship between the concentrations of the ethanol extract on inhibition of cancer cell (hOSCC) proliferation (Figure 5A). There was a significant difference in the inhibition of hOSCC cells between groups ( $p=0.000$ ). However, the 50% inhibitory concentration ( $\text{IC}_{50}$ ) of ethyl acetate extract was 10.61  $\mu\text{g/mL}$ . The regression equation of the ethyl acetate extract was as follows:

$$y = -49.90x + 98.25 \quad (4)$$

Regression equation (4) obtained an  $R^2$  value of 0.9321, so that the calculated R value was 0.9654. Based on the table value of R person i.e., ( $R=0.344$ ), this means that the calculated R value was greater than the value from the R table. This

**Table IV.** Toxicity of hexadecanoic acid on cell cancer culture used the CLC- Pred tools

Pa	Pi	Cell-line	Cell-line full name	Tissue	Tumor type
0.558	0.017	MS-114	Lung carcinoma	Lung	Carcinoma
0.545	0.017	SK-MEL-1	Metastatic melanoma	Skin	Melanoma
0.521	0.009	A2058	Melanoma	Skin	Melanoma
0.537	0.033	NCI-H838	Non-small cell lung cancer (stage 3)	Lung	Carcinoma



**Figure 4.** Cytotoxicity assay of hOSCC culture by MTT method and observed with inverted microscope at 100x magnification. **A,** Seeding cell. **B,** Harvesting cell. **C,** Formazan formation.

indicates a significant correlation or relationship between the concentrations of the ethyl acetate extract on inhibition of cancer cell (hOSCC) proliferation (Figure 5B). There was a significant difference in the inhibition of hOSCC cells between groups ( $p=0.000$ )

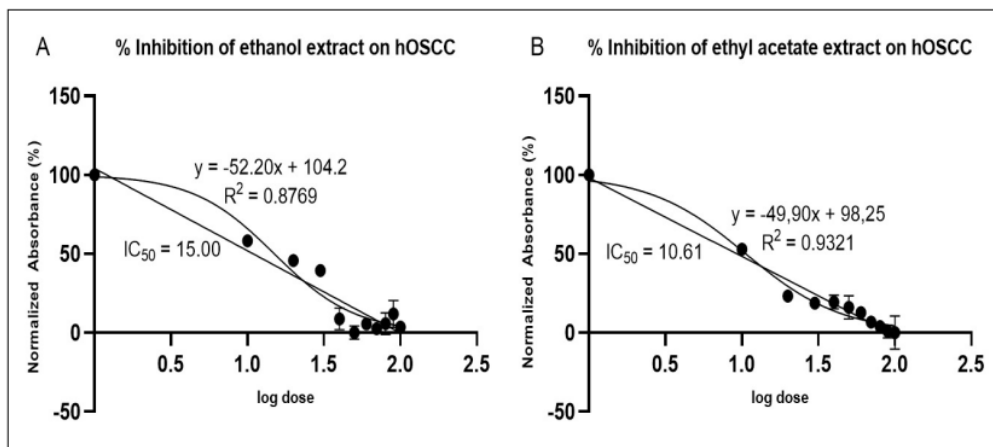
#### Apoptotic Activity of Caspase-3 on hOSCC Culture

The time of incubation and concentration of MP stem ethanol extract had an influence on the concentration of caspase-3 in hOSCC culture. Whenever the MP stem ethanol extract was administered, the concentration of caspase-3 steadily increased after 2-, 24-, and 48-hours, as determined by ELISA. The Figure 6 revealed that administering an ethanol extract of MP stem at a dose of 180 ng/ml significantly increased caspase-3 levels

compared to doses of 0, 22.5, 45, 90, 180, 360 ng/ml. However, after 2-, 24-, and 48-hours incubation, the quantity of caspase-3 was lower at the 0, 22.5, 45, 360 ng/ml than the 90 and 180 ng/ml. There was a significant difference for caspase-3 level in 2-hours ( $p=0.000$ ), 24-hours ( $p=0.000$ ), and 48-hours ( $p=0.000$ ).

#### Discussions

Recent anticancer drug research focused on the genetic side with a fundamental goal in mind while avoiding damaging healthy cells<sup>36</sup>. Natural drugs can be an alternative to chemo- and radiotherapy<sup>37</sup>. The phytochemicals often exert their effects through modulating molecular pathways associated with the development and progression of can-



**Figure 5.** Inhibition concentration of MP stem extract to hOSCC culture. **A.**  $IC_{50}$  of ethanol extract and **B.**  $IC_{50}$  of ethyl acetate extract.

**Table V.** Probit analysis of Brine Shrimp Lethality Test on *Musa paradisiaca* var. *sapientum* (L) Kunz ethanol extract.

Concentration	Log concentration	Life	Mortality	Probit	LC <sub>50</sub> (µg/mL)
10	1	9	10%	3.72	38.41276
20	1.30103	7	30%	4.48	
30	1.477121	6	40%	4.75	
40	1.60206	5	50%	5.00	
50	1.69897	4	60%	5.25	
60	1.778151	4	60%	5.25	
70	1.845098	3	70%	5.52	
80	1.90309	2	80%	5.84	
90	1.954243	2	80%	5.84	

cer. Specific processes include boosting antioxidant status, inactivation of carcinogens, inhibition of cell growth, promotion of cell cycle arrest and apoptosis, and control of the immune system<sup>38</sup>.

One of the mechanisms used in the development of anticancer drugs is the *p53* gene pathway. The *p53* gene is a tumor-suppressor gene that accumulates when DNA is damaged<sup>39</sup>. This gene serves as a controller of damaged cells dividing by inhibiting the cell cycle in the interphase phase/G1 and G2/M phase correction points<sup>40</sup>. Excessive cell stressors are captured by Toll-like receptors (TLRs), which then phosphorylate nuclear factor (NF)-κB and initiate the *p53* gene to perform apoptosis<sup>41</sup>. If stress cannot be eliminated by a cell, the *p53* gene mutates, and there is a failure of the process of apoptosis<sup>35</sup>. Failure of apoptosis allows damaged cells to continue to replicate and eventually form cancer<sup>42</sup>. Caspases are a type of proapoptotic protease that cleaves key sites for apoptosis execution. Caspase-3 is an important member of this family that causes apoptosis by inducing nuclear alterations. Many forms of cancer, such as cervical and breast cancer, have reduced caspase-3 levels, whereas elevated caspase-3 levels are a good predictive factor for malignancies<sup>43</sup>.

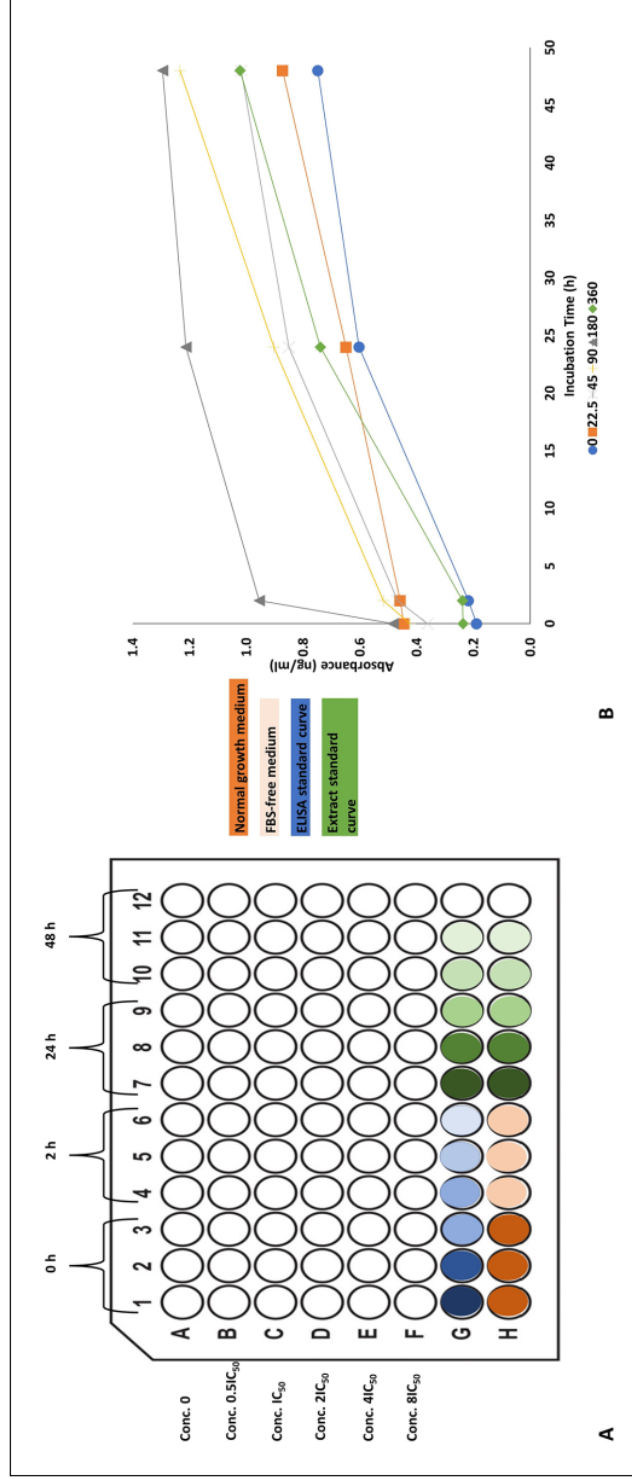
Several chemically bioactive compounds were found in the ethanol extract, including hexade-

canoic acid, methyl ester; n-hexadecanoic acid; pentadecanoic acid, 14-methyl, methyl ester; heptadecanoic acid, 16-methyl, methyl ester; and 9,12-octadecadienoic acid, which were palmitic acid and stearate acid group compounds (Table I). Palmitic acid, stearate acid, oleic acid and linoleic acid group compounds were also found in the ethyl acetate extract, although at lower concentrations (Table II). In a previous study, 12 members of the palmitic acid group contained a saturated long-chain fatty acid with a 16-carbon backbone that was shown to play a role in cancer suppression, particularly in colon cancer<sup>44</sup>.

The concentration of hexadecanoic acid methyl ester had the highest percentage (5.40%) in the ethanol extract, while 9.12-octadecadienoic acid was 11.47% according to the results of the GC-MS analysis of the ethanol extract and ethyl acetate extract of MP stem. A previous study found that hexadecanoic acid compounds were a potential source of anti-inflammatory agents that selectively induced G<sub>2</sub>/M arrest and apoptosis in MCF-7 cells via upregulation of *p53* and the Bax/Bcl-2 ratio<sup>45,46</sup>, cytotoxicity against human leukemic cells, and inhibition of phagocytic activity and nitric oxide production of certain cells<sup>47</sup>, and could lower levels of TNF-α, prostaglandin E (PGE)-2, and interleukin (IL)-10 without affecting ATP levels<sup>48,49</sup>. Con-

**Table VI.** Probit analysis of *Musa paradisiaca* var. *sapientum* (L) Kunz on ethyl acetate extract.

Concentration	Log concentration	Life	Mortality	Probit	LC <sub>50</sub> (µg/mL)
10	1	8	20%	4.16	33.26914
20	1.30103	7	30%	4.48	
30	1.477121	6	40%	4.75	
40	1.60206	4	60%	5.25	
50	1.69897	4	60%	5.25	
60	1.778151	3	70%	5.52	
70	1.845098	3	70%	5.52	
80	1.90309	2	80%	5.84	
90	1.954243	2	80%	5.84	



**Figure 6.** Caspase-3 level on hOSCC culture treated MP stem ethanol extract. A. 96 well plate scheme, B. Concentration of caspase-3 in different doses and incubation time. Conc.: concentration.

strast, octadec-9-enoic acid and oleic acid had the potential to reduce inflammation, aid digestion, and promote probiotics and pro-omega 3<sup>50</sup>. It is possible that either of these identified compounds or other unknown compounds, or a synergistic effect of all these compounds combined contributed to the excellent anticancer and anti-inflammatory activity. The role of inflammation in cancer initiation and progression is well understood, and the underlying molecular mechanisms have been extensively studied. As a result, it has become feasible to target inflammatory pathways for cancer prevention and therapy in recent decades. DNA-repair proteins, caspases, lipid peroxidation, mutation, and NF- $\kappa$ B activation all contribute to a wide range of diseases, including cancer<sup>51</sup>.

The PASS SERVER is commonly used to examine a compound's biological activity prior to chemical synthesis and biological testing. This method predicts the highest values of bioactive compounds based on a comparison of the Pa (probable active): Pi (probable inactive) ratio and the accuracy of the prediction was 95% based on a leave-one-out cross-validation (LOO CV) assessment<sup>17,19</sup>. This study found that hexadecanoic acid had a Pa=0.914 and Pi=0.003 values in the pathway via SOD inhibitors. In normal cells that experience oxidative stress, antioxidants in the body, such as SOD, dampen reactive oxygen species (ROS)<sup>51</sup>. High ROS and low SOD activities allow cell damage to continue, which can trigger DNA damage. Cells with damaged DNA can activate repair mechanisms through the apoptosis pathway by p53<sup>52</sup>. If cells with DNA damage are not immediately repaired, those cells may develop into abnormal cells (cancer). Nevertheless, antioxidant activity should be suppressed from another perspective. Some anticancer treatments, such as xenobiotics and radiation, kill tumor cells by releasing ROS. Cells with high amounts of such antioxidant enzymes are resistant to anticancer drugs<sup>53</sup>. The overexpression of SOD was associated with an increased incidence of tumor metastasis<sup>54,55</sup>. That was in accordance with this study in which potential compounds with the highest activity as agonists against apoptosis were linoleic acid (Pa=0.545) and a caspase-3/caspase-8 stimulant (Pa=0.617). Thus, the value of the Pa>Pi ratio indicated that it had the potential to be a good anticancer compound, because analysis of it using the CLC-Pred tools for in silico predictions of cell line cytotoxicity revealed that hexadecanoic acid exhibited toxicity to some cancer cell cultures such as melanomas. Therefore, MP stem extract might well inhibit the development of oral cancer

(OSCC) due to its ability to induce apoptotic pathways.

Relying on the absorption computing system, distribution, metabolism, excretion, and toxicity (ADMET) screening, hexadecanoic acid or palmitic acid compounds could be well absorbed in the human intestinal tract (Pa=0.8417) because palmitic acid is a fatty acid that is highly fat-soluble, easily penetrates cell membranes, and was found in the subcellular milieu in mitochondria. This indicates that the ethanol and ethyl acetate extracts of banana stems could be orally administered.

*In vitro* testing should be used to validate the GC-MS data and computational predictions made with the PASS SERVER. They were calculated using the BSLT and Finney's probit analysis tool, and results showed that the LC<sub>50</sub> value of the ethanol extract was 38.41  $\mu$ g/mL, and that of the ethyl acetate extract was 33.27  $\mu$ g/mL, indicating that the ethyl acetate extract was more toxic than the ethanol extract. The LC<sub>50</sub> values for both extracts were less than 100  $\mu$ g/m, and thus may be classified as highly toxic.

The use of ethanol and ethyl acetate extracts of MP stem in the culture method suppressed the growth of oral cancer (hOSCC) cells. The concentration of ethyl acetate extract that inhibited the proliferation of oral cells was lower than that of the ethanol extract. This was consistent with the wide range of chemicals discovered using the GC-MS technique on the ethyl acetate extract. As a result, when utilizing the ethanol extract to prevent cancer cell proliferation, a larger concentration will be necessary than when using the ethyl acetate extract. In the ethyl acetate extract, we found 15 chemically active compounds, and we found seven in the ethanol extract. The active compound of octadec-9-enoic acid and oleic acid which had potential as pro-omega 3. This would contribute to be a good anticancer.

In this study, apoptotic activity was observed by measuring the concentration of caspase-3 in hOSCC cell cultures treated with ethanol extract. The screening results through the GC-MS method showed that the concentration of palmitic acid derivative compounds, especially hexadecanoic acid, was higher in ethanol extracts. Compounds of hexadecanoic acid are predicted to have anticancer activity in several types of cancer, one of which is through the stimulation of caspase-3 activity. An increase in the concentration of caspase-3 in the application of ethanol extract of MP stem showed an increase in the apoptotic activity of cancer cells. The content of

hexadecanoic acid can inhibit the growth of oral cavity cancer cells (hOSCC) according to the dose. There is a dose relationship with the administration of ethanol extract of MP stem at doses of 0.5IC<sub>50</sub>, IC<sub>50</sub>, 2IC<sub>50</sub>, and 4IC<sub>50</sub> to increase apoptotic activity by measuring caspase-3. However, there was a decrease in apoptotic activity with a hefty dose of 8IC<sub>50</sub> (360mg/ml). A high-dose administration of MP stem ethanol extract in hOSCC cell cultures could inhibit apoptotic activity, which was indicated by low concentrations of caspase-3. As a result, the dose- and time-dependence of drug-induced apoptosis should be thoroughly assessed for both experimental and conventional anticancer agents.

Although *Musa paradisiaca* var. *sapientum* (L) Kunz extract has been used to treat various forms of cancer, its effect as an oral anticancer agent is still unknown. One of the limitations is that in silico studies (target and receptor) were not included in the computational stage of oral cavity anticancer screening, which was the focus of this current study. Caspases have a vital role in regulating apoptosis (programmed cell death) (apoptosis). Caspase-3 is a commonly activated death protease that cleaves numerous essential cellular proteins. However, this study can serve as a springboard for further development of an oral anticancer drug.

## Conclusions

We concluded that the chemically active compounds such as hexadecanoic acid obtained from the *Musa paradisiaca* var. *sapientum* (L) Kunz (MP) stem could be developed as candidates for new anticancer drugs. Hexadecanoic acid is palmitic acid derivatives of MP stem extract has apoptotic activity by inhibiting cancer cell growth of hOSCC culture through caspase-3 stimulants.

## Conflict of Interest

We have no competing interests.

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## Authors' Contributions

Conceptualization, H.S.B. and Y.K.S.; methodology, H.S.B. and Y.K.S.; investigation and data collection, N.M.U. and M.A.S.; data analysis, R.R.; resources, H.S.B.; writing-original draft preparation, H.S.B. and S.A.; writing-review and editing, supervision, H.S.B. Y.K.S. and C.Z.W. All authors contributed to the final version and approved the manuscript.

## Ethics Statement

Ethics approval was not required for this study.

## Data Availability Statement

The data used to support the finding of this study are included within the article.

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