

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 $\mu\text{g}/\text{mL}$ 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_{50}) of 15.00 $\mu\text{g}/\text{mL}$ for the ethanol extract, and an IC_{50}

of 10.61 $\mu\text{g}/\text{mL}$ 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 changes¹. 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 cancers². OSCC occurs place in any region of the oral cavity, pharyngeal region and salivary glands³. It was traditionally associated with certain risk factors, such as heavy smoking, consumption of betel nut and alcohol⁴, an impacted

wisdom tooth, poor dental fillings, ill-fitting denture prostheses⁵, unhealthy diet and nutrition⁶, and the human papillomavirus (HPV) infection⁷.

Irritating chemicals and free radicals as stress signals received by intercellular molecules mediate mitochondrial outer membrane permeabilization and released pro-apoptotic molecules into the cytoplasm⁸. The B-cell lymphoma (Bcl-2) family and inhibitors of apoptotic proteins that serve as apoptotic switches, control the permeabilization of mitochondrial membranes⁹. 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 apoptosis¹⁰. 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-fluorouracil¹¹, docetaxel, paclitaxel, and methotrexate¹², and radiation-induced xerostomia¹³. 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)¹⁴. GC-MS is a key technique to detect and separate volatile compounds, including secondary metabolites in both plant and non-plant species^{15,16}. 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^{17,18}. 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 activity¹⁹. 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 cultures²⁰. 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 (LC₅₀) through probit analysis²¹⁻²³. 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^{24,25}.

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 epilepsy²⁶. 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, Indonesia²⁷. There are significant compounds in banana plants, such as saponins, flavonoids, anthraquinones, and tannins contained in both the fruit and sap, as well as lectin²⁸. Sap aspirated from the stem of the MP was shown to have many therapeutic activities, including wound healing²⁹. 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^{30,31}, increase T cell proliferation²⁸, increase fibroblast activity and promote angiogenesis²⁹.

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 light³².

Preparation of Stock Solutions

This study was carried out with 10-100 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/mL³³.

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 1:50¹⁴.

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

The National Centre for Biotechnology Information (NCBI; 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 0.000-1.000 because they are probabilities. Generally, Pa+Pi≠1 since these probabilities were calculated independently¹⁷.

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 area²²; 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 observation²².

Pre-Culture of hOSCC Cells in T75 Flask

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

er (at -80°C). Cells were thawed in sterile DIW at 37°C and moved to Falcon tubes with 5 mL of cell medium. The Falcon tubes were centrifuged at 500 rpm for 5 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°C and 5% 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 cells and culture medium. Then, 25 μL of an extract was added to each well and incubated for 24 h at 37°C , and 5% CO_2 . To each well 10 μL MTT (M2003; Sigma-Aldrich) was added, and then, the mixture was covered with aluminum foil and incubated for 4 h at 37°C . Lastly, 50 μL DMSO was 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 IC_{50} value³⁴.

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 cells culture. The hOSCC cells were initially grown in 6-well plate with a seeding density of $4 \cdot 10^5$ 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°C , 5% 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)³⁵.

Statistical Analysis

Data of larvae mortality were tabulated and assessed to obtain the LC_{50} using a probit analysis²¹. However, the absorbance of the hOSCC culture was measured to obtain the IC_{50} and caspase-3 level. All data were analyzed by GraphPad Prism 8th 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 11.47% 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%

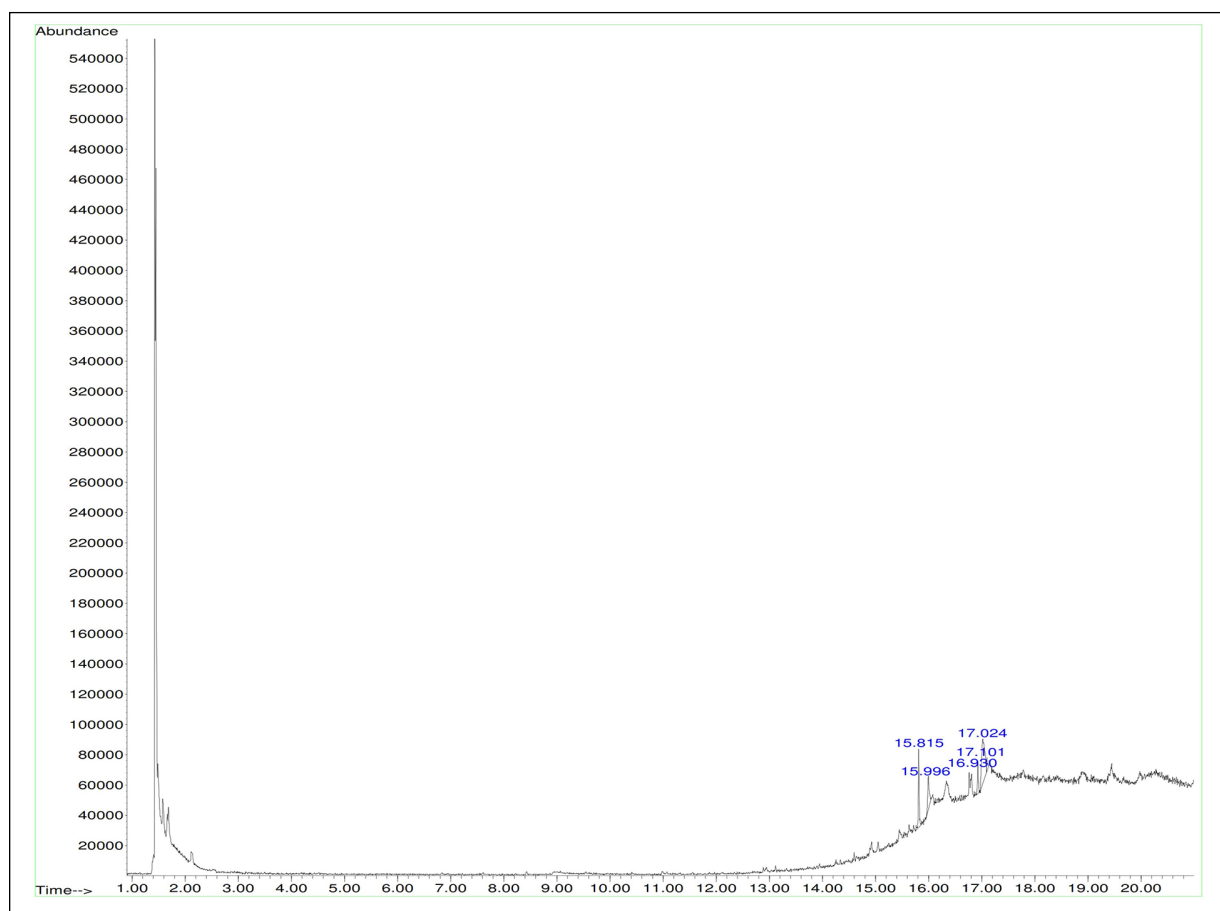


Figure 1. GC-MS chromatogram of MP stem ethanol extract.

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 3'-chlorooxanilic 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% cyclopropaneoctanal, 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 |

with 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,

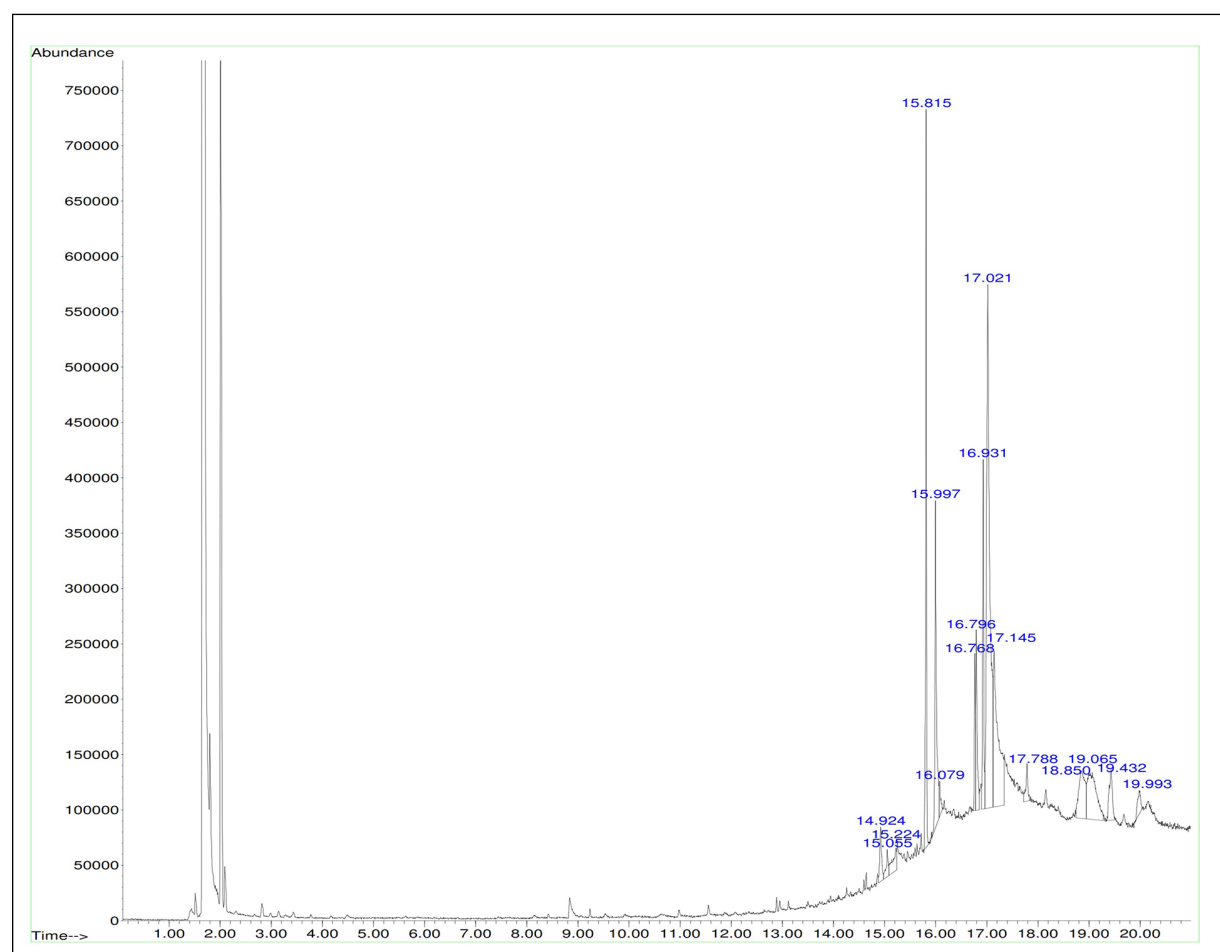
**Figure 2.** GC-MS chromatogram MP stem ethyl acetate extract.

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 | Methyl 10-trans, 12-cis-octadecadienoate 9,12-Octadecadienoic acid (z,z)-, methyl ester 9,15-Octadecadienoic 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 | Cyclopropaneoctanal, 2-octyl- | 90 |
| 12 | 18.850 | 0.81 | 3'-Chlorooxanilic acid N'(3-ethoxy-4-hydroxybenzylidene) hydrazide | 42 |
| 13 | 19.065 | 1.21 | 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 R 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 $500 < LC_{50} < 1,000 \text{ g/ml}$ 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. sapientum (L) Kunz.*

| No. | Chemically active compounds | Synonym | Canonical SMILE | Pa value | Activity |
|-----|--|---|-------------------------|----------|--------------------------------|
| 1 | Hexadec-anoic acid, methyl ester | Palmitic acid methyl ester Methyl palmitate | CCCCCCCCCCCCCCCC(=O)OC | 0.738 | Superoxide dismutase inhibitor |
| | | | | 0.473 | Apoptosis agonist |
| | | | | 0.609 | Caspase 3 and 8 stimulant |
| 2 | Pentade-canoic acid, 14-methyl-, methyl ester | Pentadecanoic acid Pentadecylic acid 1002-84-2 n-Pentadecanoic acid Pentadecyclic acid | CCCCCCCCCCCCCCCC(=O)O | 0.914 | Superoxide dismutase inhibitor |
| | | | | 0.342 | Apoptosis agonist |
| | | | | 0.562 | Caspase 3 and 8 stimulant |
| 3 | n- Hexadec-anoic acid | Palmitic acid Hexadecanoic acid 57-10-3 Cetylic acid Palmitate | CCCCCCCCCCCCCCCC(=O)O | 0.914 | Superoxide dismutase inhibitor |
| | | | | 0.342 | Apoptosis agonist |
| | | | | 0.562 | Caspase 3 and 8 stimulant |
| 4 | Methyl stearate | Methyl octadecanoate 112-61-8 Octadecanoic acid, methyl ester Stearic acid methyl ester | CCCCCCCCCCCCCCCC(=O)OC | 0.738 | Superoxide dismutase inhibitor |
| | | | | 0.336 | Apoptosis agonist |
| | | | | 0.609 | Caspase 3 and 8 stimulant |
| 5 | Heptade-canoic acid, 16-methyl-, methyl ester | Methyl heptadecanoate 1731-92-6 Methyl margarate Heptadecanoic acid, methyl ester Margaric acid methyl ester | CCCCCCCCCCCCCCCC(=O)OC | 0.738 | Superoxide dismutase inhibitor |
| | | | | 0.473 | Apoptosis agonist |
| | | | | 0.609 | Caspase 3 and 8 stimulant |
| 6 | 9,12-Octadec-adienoic acid (z,z)-, methyl ester Cis-13-Octadecadienoic acid, methyl ester | Linoleic acid 60-33-3 Linolic acid Telfairic acid cis,cis-Linoleic acid | CCCCC=CCC=CCCCCCCC(=O)O | 0.814 | Superoxide dismutase inhibitor |
| | | | | 0.545 | Apoptosis agonist |
| | | | | 0.617 | Caspase 3 and 8 stimulant |
| 7 | Oleic acid Octadec-9-enoic acid | oleic acid 112-80-1 cis-9-Octadecenoic acid (Z)-Octadec-9-enoic acid cis-Oleic acid | CCCCCCCC=CCCCCCCC(=O)O | 0.851 | Superoxide dismutase inhibitor |
| | | | | 0.499 | Apoptosis agonist |
| | | | | 0.592 | Caspase 3 and 8 stimulant |
| 8 | Cyclopr-opaneoctanal, 2-octyl- | 2-Octylcyclopropaneoctanal 8-(2-Octylcyclopropyl)octanal 2-octyl-cyclopropaneoctanal | CCCCCCCC1CC1CCCCCCC=O | 0.653 | Superoxide dismutase inhibitor |
| | | | | 0.386 | Apoptosis agonist |
| | | | | 0.411 | Caspase 3 and 8 stimulant |

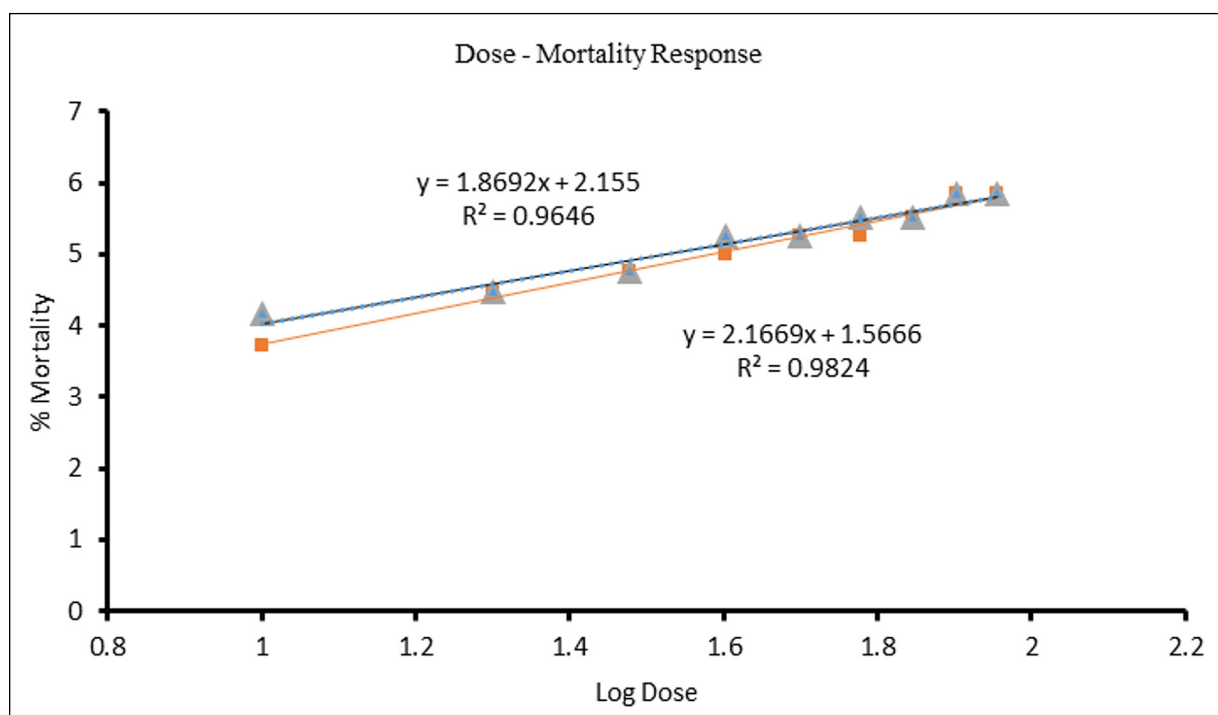


Figure 3. Correlation between extract concentration and Brine Shrimp Lethality Test. The orange line represented MP stem ethanol extract, and the blue line represented MP stem ethyl acetate extract.

length of 650 nm. The concentration inhibition of the ethanol extract of MP stem on hOSCC cells was 15.00 µg/mL ($IC_{50}=15.00\mu\text{g/mL}$). 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 (IC_{50}) of ethyl acetate extract was 10.61 µg/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 | DMS-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 |

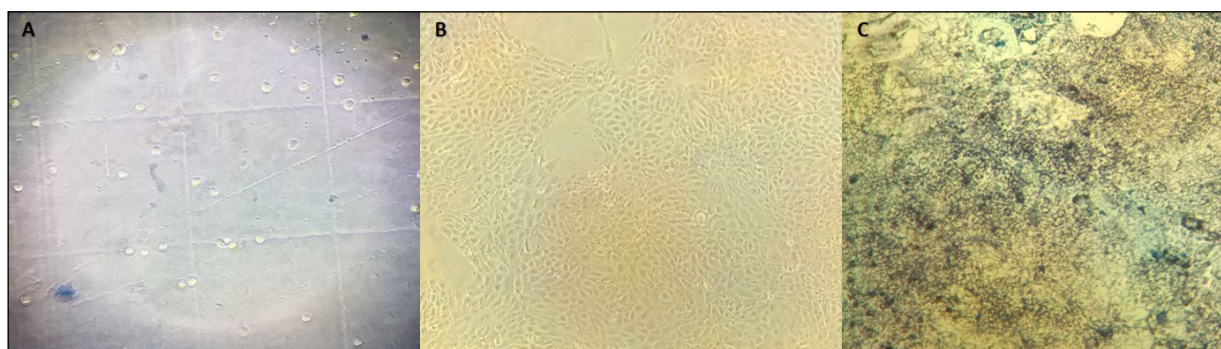


Figure 4. Cytotoxicity assay of hOSCC culture by MTT method and observed with inverted microscope at 100x magnification. **A**, Seeding cell. **B**, Harvesting cell. **C**, Formazan formation.

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 cells³⁶. Natural drugs can be an alternative to chemo- and radiotherapy³⁷. The phytochemicals often exert their effects through modulating molecular pathways associated with the development and progression of can-

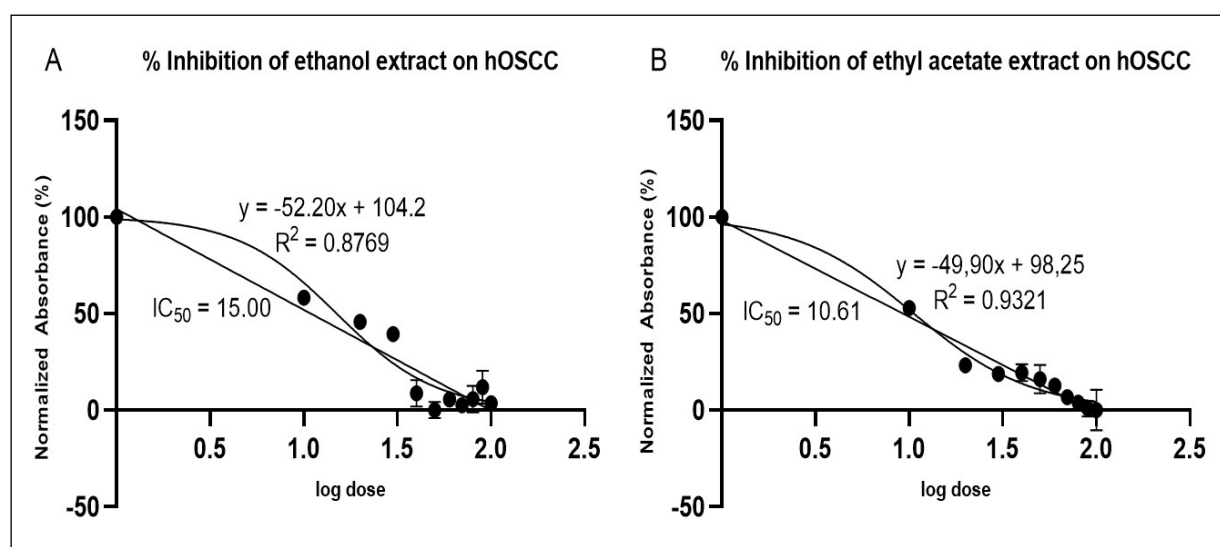


Figure 5. Inhibition concentration of MP stem extract to hOSCC culture. **A**, IC_{50} of ethanol extract and **B**, IC_{50} of ethyl acetate extract.

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 ₅₀ (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 system³⁸.

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 damaged³⁹. 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 points⁴⁰. Excessive cell stressors are captured by Toll-like receptors (TLRs), which then phosphorylate nuclear factor (NF)-κB and initiate the *p53* gene to perform apoptosis⁴¹. If stress cannot be eliminated by a cell, the *p53* gene mutates, and there is a failure of the process of apoptosis³⁵. Failure of apoptosis allows damaged cells to continue to replicate and eventually form cancer⁴². 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 malignancies⁴³.

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, 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 cancer⁴⁴.

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 G₂/M arrest and apoptosis in MCF-7 cells via upregulation of *p53* and the Bax/Bcl-2 ratio^{45,46}, cytotoxicity against human leukemic cells, and inhibition phagocytic activity and nitric oxide production of certain cells⁴⁷, and could lower levels of TNF-α, prostaglandin E (PGE)-2, and interleukin (IL)-10 without affecting ATP levels^{48,49}. Con-

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

| Concentration | Log concentration | Life | Mortality | Probit | LC ₅₀ (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 | |

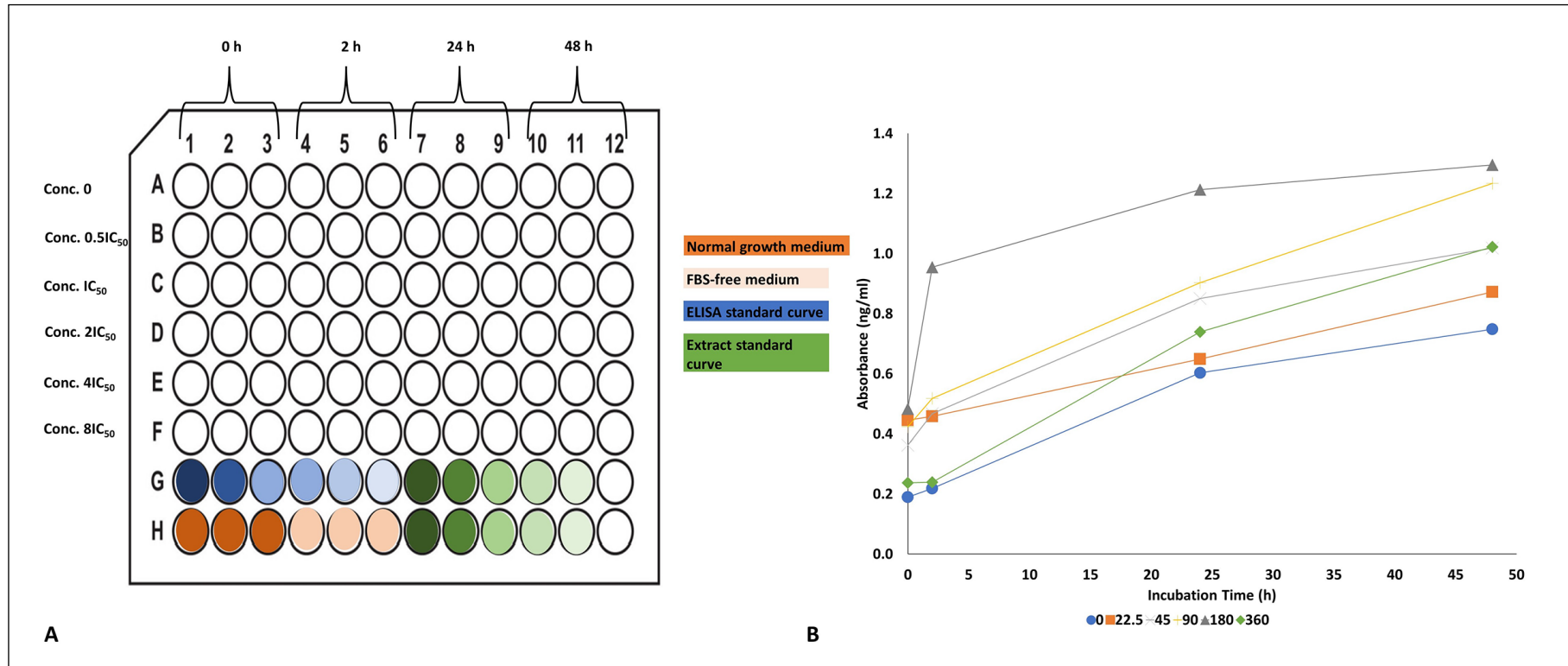


Figure 6. Caspase-3 level on hOSCC culture treated MP stem ethanol extract. A. 96 well plate scheme, B. Concentration of caspase-3 in different doses and incubation time. Conc.: concentration.

strast, octadec-9-enoic acid and oleic acid had the potential to reduce inflammation, aid digestion, and promote probiotics and pro-omega 3⁵⁰. 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- κ B activation all contribute to a wide range of diseases, including cancer⁵¹.

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^{17,19}. 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)⁵¹. 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 p53⁵². 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 drugs⁵³. The overexpression of SOD was associated with an increased incidence of tumor metastasis^{54,55}. 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 LC₅₀ value of the ethanol extract was 38.41 μ g/mL, and that of the ethyl acetate extract was 33.27 μ g/mL, indicating that the ethyl acetate extract was more toxic than the ethanol extract. The LC₅₀ values for both extracts were less than 100 μ 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 $0.5IC_{50}$, IC_{50} , $2IC_{50}$ and $4IC_{50}$ to increase apoptotic activity by measuring caspase-3. However, there was a decrease in apoptotic activity with a hefty dose of $8IC_{50}$ (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 curation, 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.

Ethical 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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