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Dioleate and trioleate epoxides from palm oil as anticancer agents through *in vitro* and molecular docking studies

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Abstract: Dioleate and trioleate epoxides were successfully obtained from palm oil through several steps and evaluated as anticancer agent potential via in vitro assays and studies of their binding to the FASN protein. At first, fatty acid methyl esters (FAMEs) were isolated through the transesterification reaction from palm oil. Next, the methyl oleate from FAME was purified through urea inclusion method and then hydrolyzed into oleic acid. The oleic acid was further reacted with 1,2-propanediol and glycerol to produce 1,2-propanediol dioleate (dioleate) and glyceryl-1,2,3-trioleate (trioleate) derivatives, respectively. The dioleate and trioleate compounds were epoxidized with performic acid reagent to yield dioleate and trioleate epoxides, respectively. Trioleate epoxide generated a more potent anticancer activity against T47D and WiDr cancer cells with IC_{50} values of 27.40 and 32.41 mg L^{-1}, respectively. In comparison, the IC_{50} values of dioleate epoxide against T47D and WiDr cancer cells are 35.66 and 38.25 mg L^{-1}. Trioleate epoxide and dioleate epoxide were non-toxic for normal cells with selectivity index values of 9.19–10.87 and 7.60–8.16, respectively. The molecular docking showed that dioleate and trioleate epoxides could inhibit the function of the FASN enzyme with binding affinity values of -27.11 and -27.61 kJ mol^{-1}, respectively.

Keywords: FAME; methyl oleate; epoxidation; selectivity index; FASN.

INTRODUCTION

Cancer has become one of the leading causes of death in the world, whereas the mortality rates of approximately 1 in 9 among men and 1 in 12 among women.[1] Human lifestyles such as smoking, lack of physical activity, and being obese can trigger cancer disease.[2] In general, cancer can be treated by chemotherapy, radiotherapy, anti-endocrine therapy, specialized targeted therapy, and surgery.[3] Prolonged use of chemotherapeutic agents could cause serious side effects due to

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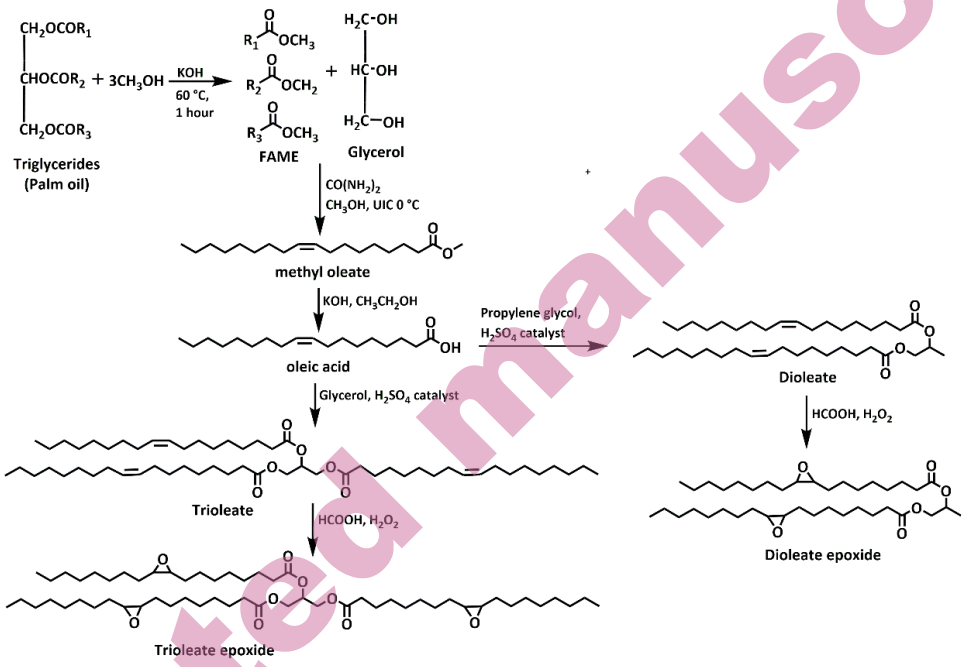
longer exposure to normal cells.[4] In addition, around 70–95% of the population in developing countries use traditional medicine for cancer treatment due to the high cost of modern therapies.[5] Thus, identifying natural compounds that can act as anticancer agents, with low toxicity and side effects, is urgently needed.

Palm oil is a natural product with 99.6% triglycerides, 0.4% free fatty acids, and minor ingredients, such as carotenoids, phytosterols, and vitamin E.[6] The triglycerides consist of saturated and unsaturated fatty acids.[7] Oleic acid, an unsaturated fatty acid with the highest content in palm oil, around 36.4–41.2%,[8] can be isolated via a transesterification reaction and purified by the urea inclusion complex (UIC) method. The principle of the UIC method is the separation of saturated and unsaturated fatty acids based on their size.[9] The oleic acid can be further modified by epoxidation reaction[9], in which the double bond is converted to an oxirane ring,[10] which is a three-membered ring ether cyclic.[11] Fatmayanti *et al*. reported that epoxidized oleic acid exhibited potent anticancer activity against the WiDr and T47D cancer cell lines with an IC_{50} value of less than 20 mg L^{-1} and below 31 mg L^{-1}.[12] This epoxide ring can interact with nucleophilic groups in the proteins and nucleic acids, making them promising candidates for cancer treatment.[13] Therefore, epoxide compounds show good antiproliferative activities such as enzyme inhibition, induction of cell cycle arrest, and apoptosis.[14]

Cancer cells proliferate faster and therefore have higher metabolic demands than normal cells. Cancer cells obtain lipids from the abnormal activity of the de novo fatty acid synthesis pathway, characterised by increased fatty acid synthesis and the production of palmitoleic acid. The lipid causes cancer cells to experience excessive proliferation, cancer cell metastasis, and triggers death in cancer patients four times faster.[15] Activation of these signalling pathways increases the activity of sterol regulatory element-binding protein-1 (SREBP-1), which in turn induces the expression of fatty acid synthase (FASN). Consequently, FASN has recently been recognised as an oncoprotein that is overexpressed in various cancer types. The administration of FASN inhibitors can prevent the synthesis of long-chain saturated fatty acids to reduce the ability of cancer cells to survive and support apoptosis in cancer cells. Disrupted FASN activity can cause metabolic stress, causing damage to cells (cytotoxicity).[16]

One of the FASN inhibitors that has been developed is cerulenin. The epoxide ring of cerulenin inhibits FASN activity by binding to the enzyme's activity, disturbing the fatty acid synthesis process. This binding involves covalent interactions of epoxide with cysteine residues in the active site β-ketoacyl-ACP synthase (KS) domain of FASN. However, cerulenin has serious side effects, as revealed by the *in vivo* results, especially in the form of anorexia and weight loss.[17] This study aims to synthesize dioleate and trioleate epoxides from oleic acid isolated from palm oil through a series of chemical reactions, as shown in Scheme 1. Then, the *in vitro* anticancer activity of epoxides were investigated against breast

(T47D) and colon (WiDr) cancer cells, as well as against normal cells (Vero). In addition, the interaction of the epoxides in the active site of the FASN was examined by *in silico* molecular docking.



Scheme 1. Synthesis scheme of dioleate epoxide and trioleate epoxide from palm oil

EXPERIMENTAL

Material

Palm oil was purchased from a local market in Yogyakarta, Indonesia. All chemicals and solvents in this work were of pro analysis grade, obtained from Merck (Germany) and used without further purification. The chemicals included: carbamide ($CO(NH_2)_2$), propane-1,2,3-triol, propane-1,2-diol, methanoic acid (HCOOH), hydrogen peroxide (H_2O_2 30%), potassium hydroxide (KOH), sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium hydrogen carbonate ($NaHCO_3$), ethanol, methanol, ethyl ethanoate, *n*-hexane, and anhydrous sodium sulfate (Na_2SO_4). The cell lines used for the cytotoxic evaluation were T47D, WiDr, and Vero. The materials used for the cytotoxic assay included Dulbecco's Modified Eagle Medium (DMEM), medium 199 (M199), Fetal Bovine Serum (FBS), penicillin-streptomycin (Pen-Strep), fungizone, trypsin-EDTA solution, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), mycoexpert mycoplasma, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.01 M HCl, and sodium dodecyl sulfate (SDS).

Instrumentation

The purity and molecular weight of the products were measured by gas chromatography-mass spectrometry (GC–MS) using a Shimadzu-QP 2010S and Agilent GC type 6890-MS type 5973. Liquid chromatography-mass spectrometry (LC–MS) analysis was performed using

ACQUITY UPLC®H-Class System (Waters, USA) equipped with an LC ACQUITY UPLC®HSS C18 (1.8 µm, 2.1 × 100 mm) and a Xevo G2-S QTOF mass spectrometer. The nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-ECZ 500R spectrometer at 500 and 125 MHz, respectively, using CD_3OD as solvent. Fourier-transform infrared (FTIR) spectra were recorded using a Shimadzu Prestige 21 with NaCl plate. The formazan absorbance solution was measured using a Bio-Rad Benchmark ELISA reader. Molecular docking simulations were done using the AutoDock Vina approach implemented in YASARA Structure (version 23.519; license number 645289713). Ligand–receptor interactions was visualized using Discovery Studio Visualizer (version 19.1.0.18287, ©2019).

Synthesis of fatty acid methyl esters (FAME)

Palm oil (50 g, 50 mmol) and KOH (1 g, 18 mmol) were dissolved in methanol (12.15 mL, 30 mmol). The mixture was refluxed and the reaction progress was monitored by GC–MS. The optimum reaction time of FAME was 60 min. After completion, the mixture was extracted with *n*-hexane and washed with distilled water (3 × 15 mL). The organic phase was dried over anhydrous sodium sulfate and the organic solvents were removed by a vacuum evaporator.

Fatty acid methyl esters (FAME)

Bright yellow liquid (41.09 g, 95.69%). FTIR (NaCl, ν_max/cm^{-1}): 3008 (=C–H), 2924, 2854 (C–H), 1743 (C=O), 1658 (C=C), 1458, 1435 (CH_2), 1381 (CH_3), 1165 (C–O), 1118 (=C–H), 725 ((CH_2)_n) (Fig. S-1). GC–MS (EI, 70 eV): methyl myristate (0.42%, R_t = 30.43 min, m/z 242 $[M]^+$), methyl palmitate (36.30%, R_t = 35.02 min, m/z 270 $[M]^+$), methyl linoleate (8.75%, R_t = 38.40 min, m/z 294 $[M]^+$), methyl oleate (51.01%, R_t = 38.62 min, **m/z** 264 $[M - CH_3OH]^+$), methyl stearate (3.27%, R_t = 39.00 min, m/**z** 298 $[M]^+$), and methyl icosanoate (0.25%, R_t = 42.70 min, m/z 326 $[M]^+$) (Fig. S-2).

Purification of FAME by urea inclusion complex

The urea inclusion complex was carried out to produce a high purity of methyl oleate from FAME. Urea solution in methanol (12.5 g/50 mL) was heated at 50 ºC for 15 min. Immediately, FAME (6.25 g) were added to the urea solution and stirred for 10–15 min. The solution was allowed to reach room temperature and kept in the freezer for 24 h. The formed crystals were filtered and rinsed with *n*-hexane (3 × 15 mL). The filtrate was acidified with distilled water and 6 M HCl to pH 1. The organic phase was separated and evaporated to obtain enriched-methyl oleate product.

Purified FAME

Pale yellow liquid (5.56 g, 88.96%). FTIR (NaCl, ν_max/cm^{-1}): 3008 (=C–H), 2924, 2854 (C–H), 1743 (C=O), 1658 (C=C), 1458, 1435 (CH_2), 1381 (CH_3), 1165 (C–O), 1118 (=C–H), 725 ((CH_2)_n) (Fig. S-3). GC–MS (EI, 70 eV): methyl palmitate (3.26%, R_t=29.24 min, m/z 270 $[M]^+$), methyl linoleate (9.84%, R_t=32.47 min, m/z 294 $[M]^+$), and methyl oleate (89.90%, R_t=32.67 min, m/z 296 $[M]^+$) (Fig. S-4).

Hydrolysis of oleic acid

Methyl oleate (2.95 g, 10 mmol) and KOH (1.68 g, 30 mmol) were dissolved in ethanol (10 mL). The mixture was refluxed for 120 min. The mixture was acidified with H_2SO_4 to pH 1–3. Afterward, the mixture was extracted by using *n*-hexane and washed by distilled water (3 × 20 mL) to remove excess H_2SO_4 and K_2SO_4. The organic solvents were removed with the help of a vacuum evaporator.

Oleic acid

Light yellow liquid (2.59 g, 92%). FTIR (NaCl, ν_max/cm^{-1}): 3100 (O–H), 2924, 2854 (C–H), 1712 (C=O), 1458 (CH_2), 1373 (CH_3), 1280 (C–O), 725 ((CH_2)$_n$) (Fig. S-5). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.85 (t, J = 7.0 Hz, 3H), 1.20–1.32 (m, 20H), 1.56–1.63 (m, 2H), 1.95 (q, J = 7.0 Hz, 4H), 2.35 (t, J = 8.0 Hz, 2H), 5.30–5.35 (m, 2H) (Fig. S-6). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 14.18, 22.77, 24.74, 27.23, 27.29, 29.12, 29.15, 29.24, 29.42, 29.62, 29.76, 29.85, 32.00, 34.15, 34.20, 127.79, 130.08, 180.53 (Fig. S-7).

Synthesis of 1,2-propanediol dioleate (dioleate)

Oleic acid (6.21 g, 22 mmol) was reacted with 1,2-propanediol (0.76 g, 10 mmol) with sulfuric acid catalyst (200 µL) at 90–100 °C. After reaction for 4 h, the mixture was extracted with *n*-hexane and washed with distilled water (3 × 20 mL) to remove impurities. The organic phase was dried over anhydrous sodium sulphate and then removed in vacuum to produce the title compound.

1,2-Propanediol dioleate

Yellow liquid (6.01 g, 82.8%). FTIR (NaCl, ν_max/cm^{-1}): 3008 (=C–H), 2924, 2854 (C–H), 1736, 1712 (C=O), 1658 (C=C), 1458 (CH_2), 1381 (CH_3), 1180 (C–O), 725 ((CH_2)$_n$) (Fig. S-8). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.81–0.84 (m, 6H), 1.14–1.32 (m, 43H), 1.54–1.61 (m, 4H), 1.92 (q, J = 6.8 Hz, 8H), 2.25–2.30 (m, 4H), 3.95–4.05 (m, 2H), 5.08–5.12 (m, 1H), 5.28–5.32 (m, 4H) (Fig. S-9). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 14.17, 16.55, 22.76, 24.80, 27.23, 27.28, 29.02, 29.11, 29.18, 29.34, 29.42, 29.47, 29.58, 29.68, 31.98, 34.19, 65.98, 68.07, 129.78, 130.06, 173.64 (Fig. S-10).

Synthesis of glyceryl-1,2,3-trioleate (trioleate)

Oleic acid (9.04 g, 32 mmol) was reacted with glycerol (0.92 g, 10 mmol) in the presence of sulfuric acid catalyst (300 µL) at 90–100 °C for 6 h. Then, the mixture was extracted with *n*-hexane and washed with distilled water (3 × 15 mL) to remove undesired compounds. The organic phase was dried over anhydrous sodium sulphate and then removed by a vacuum evaporator to produce the title compound.

Glyceryl trioleate

Yellow liquid (7.08 g, 80%). FTIR (NaCl, ν_max/cm^{-1}): 3008 (=C–H), 2924, 2854 (C–H), 1736 (C=O), 1658 (C=C), 1458, 1435 (CH_2), 1357 (CH_3), 1172 (C–O), 725 ((CH_2)$_n$) (Fig. S-11). ^{1}H NMR (500 MHz, $CDCl_3$, δ): 0.85 (t, J = 7.1 Hz, 9H), 1.18–1.30 (m, 60H), 1.55–1.63 (m, 6H), 1.98 (q, J = 7.2 Hz, 12H), 2.28 (t, J = 8.4 Hz, 6H), 4.09–4.16 (m, 4H), 4.73–4.77 (m, 1H), 5.28–5.33 (m, 6H) (Fig. S-12). ^{13}C NMR (125 MHz, $CDCl_3$, δ): 14.18, 24.81, 24.94, 27.24, 27.29, 29.17, 29.25, 29.41, 29.61, 29.77, 29.85, 31.68, 31.99, 34.16, 34.26, 65.09, 70.34, 129.87, 130.08, 180.03 (Fig. S-13).

Epoxidation of dioleate and trioleate.

The synthesis of dioleate epoxide was done with a mole ratio of dioleate (0.076 g), formic acid (0.276 g), and 30% hydrogen peroxide (0.714 g) of 1:6:21. Meanwhile, the synthesis of trioleate epoxide was carried out with a mole ratio of trioleate (0.89 g), formic acid (0.41 g), and 30% hydrogen peroxide (1.05 g) was 1:9:31. Formic acid (1.08 g, 24 mmol) and hydrogen peroxide 30 wt% (2.86 g, 84 mmol) were firstly mixed in a closed Erlenmeyer at 28 ºC for 1 h to generate performic acid. Afterwards, dioleate and trioleate compounds were separately added and the mixture was stirred at 28 ºC for 24 h. The product was extracted with ethyl acetate (3 × 20 mL) and then the organic phase was washed with sodium bicarbonate 5 wt% and sodium

chloride 5 wt%. The organic phase was dried over anhydrous sodium sulphate, filtered, and evaporated to yield epoxide product.

Dioleate diepoxide

White solid (0.60 g, 94.49%). FTIR (NaCl, ν_max/cm^{-1}): 2924, 2854 (C–H), 1735 (C=O), 1465 (CH_2), 1381 (CH_3), 1172 (C–O), 840 (oxirane C–O), 725 ($(CH_2)^n$) (Fig. S-14). LC–MS (ESI, negative ion mode): purity 86.49%, R_t=12.28 min, m/z 722 ([M + CH_3CN + HCOO]$^-$) (Fig. S-15). ^{1}H NMR (500 MHz, CD_3OD, δ/ppm): 0.80 (t, *J* = 7.0 Hz, 6H), 1.23–1.27 (m, 43H), 1.40 (q, *J* = 5.5 Hz, 4H), 1.55 (q, *J* = 8.0 Hz, 8H), 2.25 (t, *J* = 7.2 Hz, 4H), 2.85–3.32 (m, 4H), 4.04–4.07 (m, 2H), 4.84–4.90 (m, 1H) (Fig. S-16). ^{13}C NMR (125 MHz, CD_3OD, δ/ppm): 14.18, 24.81, 24.94, 27.24, 27.29, 29.17, 29.25, 29.41, 29.61, 29.73, 29.77, 29.85, 30.53, 31.88, 33.50, 34.01, 57.41, 57.50, 65.95, 68.07, 178.13 (Fig. S-17).

Trioleate triepoxide

White solid (0.81 g, 92.04%). FTIR (NaCl, ν_max/cm^{-1}): 2924, 2854 (C–H), 1728 (C=O), 1465 (CH_2), 1373 (CH_3), 1242 (C–O), 840 (oxirane C–O), 725 ($(CH_2)_n$) (Fig. S-18). LC–MS (ESI, negative ion mode): purity 93.51%, R_t=12.10 min, m/z 985 ([M+ NH_4Cl]$^-$) (Fig. S-19). ^{1}H NMR (500 MHz, CD_3OD, δ/ppm): 0.81 (t, *J* = 7.0 Hz, 9H), 1.21–1.25 (m, 60H), 1.40–1.44 (m, 6H), 1.56 (q, *J* = 8.3 Hz, 12H), 2.26 (t, *J* = 7.2 Hz, 6H), 2.87–3.34 (m, 6H), 4.06–4.08 (m, 4H), 4.81–4.87 (m, 1H) (Fig. S-20). ^{13}C NMR (125 MHz, CD_3OD, δ/ppm): 14.14, 21.06, 22.70, 24.72, 26.60, 27.79, 28.99, 29.05, 29.25, 29.35, 29.56, 29.73, 31.89, 33.49, 34.03, 57.43, 57.48, 60.53, 72.24, 171.44 (Fig. S-21).

Cytotoxicity evaluation

The cytotoxic evaluation was conducted in the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada. The ethical clearance approval letter is shown in Fig. S-22. The MTT assay was performed to examine the cytotoxic activity and selectivity of all synthesized epoxides. The T47D, WiDr and Vero cell line were separately cultured in a 10% FBS medium at 37 ºC with a flow of 5% CO_2. Each cell line (100 µL/well) was incubated at 37 ºC with 5% CO_2 flow for 24 h. Doxorubicin and 5-fluorouracil were chosen as positive controls. The evaluated compounds were examined at two-fold serial dilutions in DMSO. After exposing the cell with the tested compounds for 24 h, the MTT solution (100 µL) was added to each well and placed in an incubator for 4 h. Then, the SDS reagent stopper solution (100 µL) was added and the mixture was stored in a darkroom overnight. The absorbance was measured by using an ELISA reader at 595 nm. Each sample concentration was assayed in triplicate. The cell viability percentage was evaluated and expressed in half-maximal inhibitory concentration (IC_{50}). The IC_{50} value represents the effective concentration required to inhibit 50% of cell viability. The selectivity of tested compounds was determined by calculating selectivity index (SI) value. The SI represents the safety of epoxidized oleate in cancer and normal cells.

RESULTS AND DISCUSSION

FAME isolation and purification

Fatty acid methyl esters (FAMEs) were successfully isolated from palm oil through the transesterification reaction. Base catalyst (KOH) was selected due to its higher reaction rate and lower operating temperature compared to acid-catalyzed processes.[18] The used molar ratio of palm oil and alcohol was 1:6 to accelerate the reaction rate to form the ester product, as reported earlier.[19] The methoxide ion attacks the carbonyl carbon of triglyceride, forming a tetrahedral

intermediate. This intermediate then undergoes a nucleophilic substitution reaction, where one of the acyl chains on the triglyceride is released, producing methyl ester (FAME) and diacylglyceride (DAG) as intermediate products. This reaction continues until the entire acyl chain on the triglyceride is converted to methyl ester, with glycerol as a by-product.[20] Based on the results of the GC-MS analysis, six methyl ester peaks were detected, comprised four saturated fatty acids (myristate, palmitate, stearate, and eicosanoate) and two unsaturated fatty acids (oleate and linoleate).

The unsaturated fatty acid fraction was further purified using the UIC method. Urea has a tetragonal lattice with a diameter of 5.67 Å. The large size of the urea crystal can accommodate the aliphatic chain of fatty acid esters with more than six carbon atoms to form a hexagonal complex through van der Waals interactions. In contrast, urea crystal canals cannot accommodate unsaturated fatty acid esters due to their non-linear form.[21] In this work, methyl oleate was purified using the UIC method with a mol ratio of FAME:urea:methanol of 1:2:8 at 0 °C for 24 h. Based on GC–MS results, the purity of methyl oleate was significantly increased from 51.0% to 89.9%. A slight increase was detected also for methyl linoleate, from 8.8% to 9.8%. The concentration of methyl palmitate decreased from 36.3% to 3.3%, while methyl myristate, methyl stearate, and methyl icosanoate were no longer detected (see Table I).

Table I. Compositions of FAME before and after purification by UIC method

Entry	Methyl ester	Percentage (%)	
		Before purification	After Purification
1	Myristate	0.4	0.0
2	Palmitate	36.3	3.3
3	Linoleate	8.8	9.8
4	Oleate	51.0	89.9
5	Stearate	3.3	0.0
6	Icosanoate	0.2	0.0

The FTIR spectra of FAME before and after purification showed no significant changes (Fig. 1), except for an increase in the intensity of the =C–H sp^2 stretching band at 3008 cm^{-1}. The FAME sample after purification had a sharper intensity with a typical peak of C=O and C–O ester vibrations appearing at 1743 and 1165 cm^{-1}, respectively.

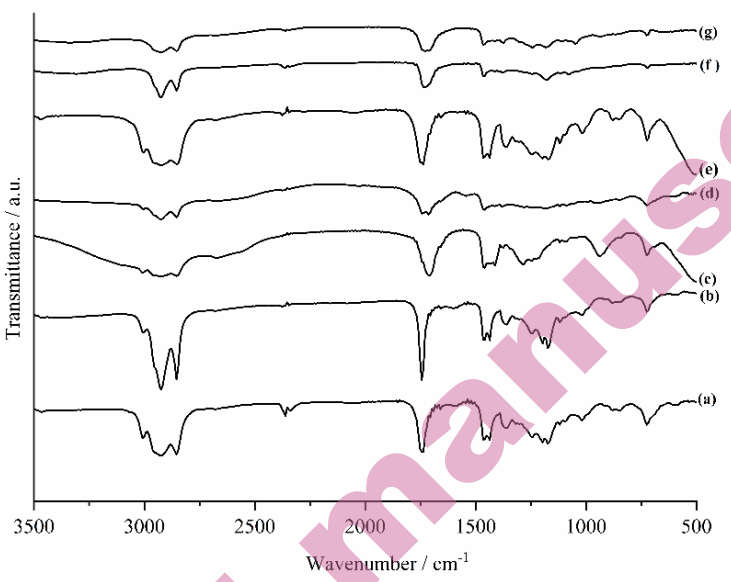


Fig. 1. FTIR spectra of (a) FAME, (b) purified FAME, (c) oleic acid, (d) dioleate, (e) trioleate, (f) dioleate epoxide, and (g) trioleate epoxide

Hydrolysis of methyl oleate

The refined methyl oleate was hydrolyzed at a molar ratio of methyl oleate to KOH of 1:3, respectively. The FTIR spectrum (Fig. 1) confirmed the formation of oleic acid by the appearance of a broad O–H stretching band of the carboxylic group at 3100 cm^{-1}. In addition, a strong absorption at 1712 cm^{-1} (shifted from 1743 cm^{-1} in methyl oleate) corresponds to the C=O of the carboxylic group. In the ^{1}H NMR spectrum (Fig. 2), the double bond of oleic acid appeared as a multiplet at δ 5.32 ppm (J = 6.3 Hz) indicating a cis configuration. The hydroxyl (-OH) proton peak on oleic acid does not appear in the ^{1}H-NMR spectrum because this proton easily exchanges with protons from the solvent or water present in the sample, causing the signal to broaden or disappear.[22] The α-methylene protons (H_{α}) appeared as a triplet at 2.35 ppm, while the β-methylene protons (H_{β}) were observed as a multiplet at δ 1.59 ppm.

The ^{13}C-NMR spectrum confirmed the presence of 18 carbon atoms in oleic acid. The carbonyl carbon appeared at δ 180 ppm because it binds to a hydroxy group, making it the least protected shift. The olefinic carbons were observed at δ 129 and 130 ppm, while fourteen other carbon peaks are found at a chemical shift range of 20–30 ppm.

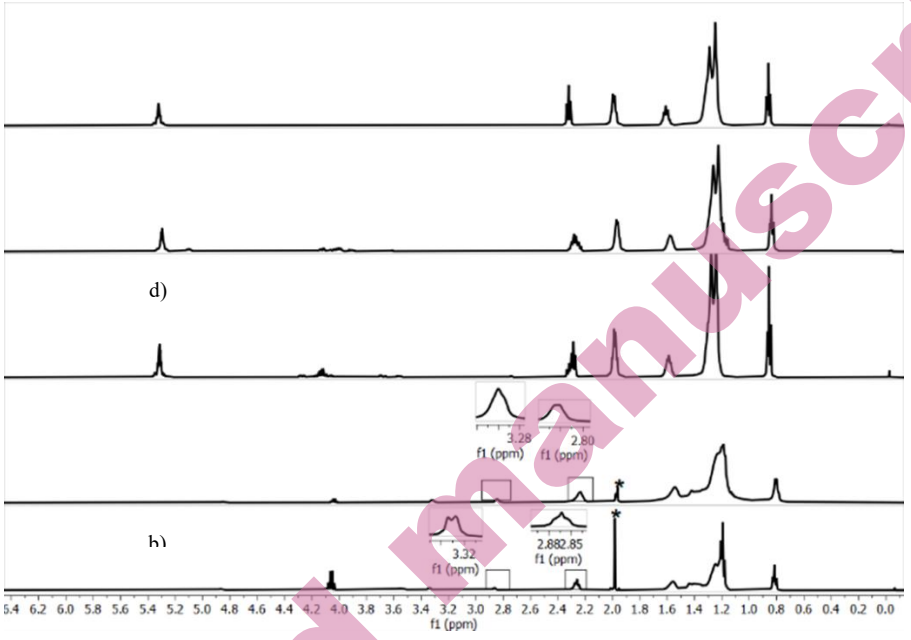


Fig. 2. ^{1}H-NMR spectra of (a) trioleate epoxide, (b) dioleate epoxide, (c) trioleate, (d) dioleate, and (e) oleic acid

Synthesis of dioleate and trioleate

Dioleate was synthesized by reacting an excess amount of oleic acid with 1,2-propanediol at a molar ratio of 2.3:1.0 in the presence of sulfuric acid as a catalyst at 100 °C for 4 h. Oleic acid was added in excess to reduce the possibility of the monoester formation as the side product. The FTIR spectrum confirmed the formation of dioleate by the disappearance of the O–H stretching band at 3100 cm^{-1} from oleic acid and propanediol. The formation of dioleate product is proven by the absence of OH protons of oleic acid and 1,2-propanediol in the ^{1}H-NMR profile, which appear at δ 10–13 ppm. The alkene proton (-CH=) appeared at δ 5.30 ppm. On the other hand, the ^{13}C-NMR analysis showed that the carbon in the unsaturated double bond remains at δ 129 and 130 ppm while C=O carbon appears at δ 173 ppm.

Trioleate was also synthesized by esterification of oleic acid and glycerol at a molar ratio of oleic acid and glycerol of 3.3:1.0, respectively, at 100 °C for 6 h. In the ^{1}H-NMR spectrum, trioleate showed 8 proton signals. The formation of trioleate was confirmed by the absence of hydroxyl proton signals from oleic acid and glycerol, which appear at a chemical shift of δ 10–13 ppm. The FTIR data also support this evidence by showing no signal at 3100 cm^{-1} regions, which are found only for oleic acid and glycerol. Moreover, the characteristic ester absorptions at 1713 cm^{-1} (C=O) and 1226 cm^{-1} (C–O) are observed. The alkene protons (–CH=)

appeared at δ 5.32 ppm, while the alkene and carbonyl carbons appeared at δ 129–130 and 180 ppm in the ^{13}C-NMR spectrum. Although similar compound has been prepared by Radojčić *et al*. in 2022, no further epoxidation reaction is performed.[23] Furthermore, the previous work did not provide sufficient structural elucidation for the synthesized target compound.

Synthesis of dioleate and trioleate epoxides

At first, formic acid and hydrogen peroxide were reacted *in situ* for 1 h to produce performic acid (Fig. S-23). Performic acid was then reacted with dioleate and trioleate compounds to yield targeted epoxide product (Fig. S-24 and S-25). The molar ratio of dioleate, formic acid, and hydrogen peroxide (30 wt%) was 1:6:21 mmol, while the molar ratio of trioleate, formic acid, and hydrogen peroxide (30 wt%) was 1:9:31 mmol. Due to high molecular weights of dioleate and trioleate (636.53 and 932.77 g mol^{-1}), respectively, LC-MS analysis was empolyed to identify the purity of the products. The purities of dioleate epoxide and trioleate epoxide were 94.5% and 92.0%, respectively. The LC chromatogram showed dioleate epoxide as the major component at a retention time of 12.28 min with an m/z value of 722.49, corresponding to the $[M+CH_3CN+HCOO]^-$ species (Fig S-14). Trioleate epoxide was detected at 12.10 min with an m/z value of 985.74, assigned to the $[M+NH_4Cl]^-$ species (Fig S-18). In the ^{1}H NMR spectra, the epoxide ring protons of the dioleate epoxide appeared as a multiplets at δ 2.85–3.55 ppm (J=7 Hz), in agreement with another report.[31] The alkene proton at 5.32 ppm no longer appears in the ^{1}H-NMR spectra of both epoxides. Additionally, the alkene carbon peaks disappeared after the epoxidation reaction. Instead, the epoxide carbon signals appeared at δ 57.41–57.50 ppm. This is in accordance with Mai *et al*., stating that the ^{13}C-NMR peaks of the epoxide ring of vegetable oil is observed at δ 54–58 ppm.[24] The characteristic =C-H and C=C bands at 3008 and 1658 cm^{-1} were also absent in the FTIR spectra. Meanwhile, a new peak showing the C–O–C oxirane appeared at 840 cm^{-1}, in which agree with previous work.[24] The disappearance of alkene groups and the appearance of oxirane groups indicate the presence of epoxide moieties. These results revealed that both dioleate and trioleate epoxides have been successfully obtained.

In vitro cytotoxic evaluation

Cytotoxicity tests of epoxides were evaluated against T47D, WiDr, and Vero cells using the *in vitro* MTT assay (Tables S-1–S-4). The principle of the MTT method is based on color measurement.[25] The IC_{50} values of both epoxides (Table II) fall into the moderate category in killing cancer cells, according to *National Cancer Institute of the United States*.[26]

Table II. IC_{50} value of tested compounds against cancer and normal cell lines

Compound	IC_{50} value (µg/mL)			Index selectivity	
	T47D	WiDr	Vero	T47D	WiDr
Dioleate epoxide	35.66	38.25	291.04	8.16	7.60
Trioleate epoxide	27.40	32.41	297.93	10.87	9.19
Doxorubicin	12.58	-	-	-	-
5-fluorouracil	-	1.58	-	-	-
Epoxidized methyl oleate[12]	12.63	1.56	226.53	17.94	145.13
Epoxidized ethyl oleate[12]	30.13	10.05	244.10	8.10	24.28

Trioleate epoxide exhibited a lower IC_{50} value than dioleate epoxide against both T47D and WiDr cells. This difference in cytotoxic activity is thought to be related to the differences in the molecular structures of both compounds. Trioleate epoxide has a greater number of oxirane rings than dioleate epoxide, thereby providing more reactive centers capable of interacting with intracellular biomolecules, thus disruption of lipid metabolism in cancer cells. WiDr and T47D cancer cells are known to undergo metabolic reprogramming, characterised by increased de novo fatty acid synthesis to meet high proliferation demands. This increased demand for lipids is primarily mediated by the overexpression of FASN, which functions to produce palmitate as a precursor for various structural lipids and signalling molecules.[15] Structurally, dioleate epoxide and trioleate epoxide retain the lipophilic characteristics of the long oleate chain, thereby enabling both molecules to interact with the hydrophobic environment of FASN. These interactions may disrupt FASN activity, leading to a reduction in palmitate production and endogenous lipid synthesis, thereby impairing the formation of membrane phospholipids and lipid rafts required to maintain the activation of pro-survival signalling pathways, such as PI3K/Akt/mTOR and MAPK/ERK.[16]

Compared with the methyl ester and ethyl ester derivatives previously reported - namely epoxidized methyl oleate and epoxidized ethyl oleate[12] - both dioleate epoxide and trioleate epoxide exhibited lower cytotoxic activity against both T47D and WiDr cells. This finding indicates that structural modification of the monoester compound into glycerol-based diester and triester derivatives does not enhance cytotoxic potential due to bulky three-dimensional structures. Larger molecular size also has the potential to reduce the efficiency of penetration into the cell or limit the access of the epoxide group to intracellular biological targets, resulting in lower cytotoxic activity compared to monoester compounds. The results of cytotoxicity tests on dioleate and trioleate epoxides against normal Vero cells showed the IC_{50} values of 291.04 and 297.93 mg L^{-1} respectively. This indicates that dioleate and trioleate epoxide compounds are non-toxic for normal cells. Furthermore, all test compounds exhibited a SI > 6 against T47D and WiDr cancer cells. These data indicate that dioleate and trioleate epoxide compounds are

selective anti-cancer agents, as they are capable of inhibiting the growth of cancer cells without significantly inhibit the growth of normal cells.

Molecular docking study

The cytotoxic activities of dioleate and trioleate epoxides were further supported by molecular docking studies against the thioesterase domain of FASN. FASN has a co-crystallized ligand, orlistat, which is a lipid-derived molecule possessing a long aliphatic hydrocarbon chain and a highly lipophilic character. Orlistat was previously known as a weight-loss drug and is now recognised as a promising candidate for an anti-cancer agent due to its ability to irreversibly inhibit FASN by forming a covalent bond with the thioesterase domain.[27] Orlistat bears a structural resemblance to the epoxide compounds, both possess long-chain alkyl groups that are lipophilic, and are therefore thought to be capable of interacting with the hydrophobic pockets of FASN, which naturally accommodate long-chain fatty acid substrates. In this work, the redocking of native ligands against the FASN enzyme was performed within a cube-shaped grid box with a distance of 5 Å from the vicinity of the selected ligand. The root-mean square deviation (RMSD) value obtained from the redocking of native ligand (i.e., orlistat) was 1.40 Å. When the RMSD value is smaller than 2 Å, it indicates that the pose of orlistat after redocking has no significant difference from the experimental pose from the crystallographic data. The re-docking results show that the orlistat interacts with various amino acid residues in the FASN enzyme, generating a binding affinity value of -22.87 kJ mol^{-1}.

After confirming the redocking of orlistat, the next stage is the molecular docking of dioleate and trioleate epoxides against FASN. Docking of dioleate epoxide and trioleate epoxide in the active site of FASN resulted in a binding affinity value of -27.11 and -27.61 kJ mol^{-1}. The dioleate epoxide, trioleate epoxide, and native ligand interacted with the identical amino acid residues, namely Leu2222, Ile2250, Ser2308, Tyr2309, Ala2367, Phe2370, Phe2371, Gln2374, Phe2375, Phe2423, Lys2426, Leu2427, His2481, and Arg2482. The oxygen atom on the oxirane ring of both epoxides interacts with Ser2308 residue through a hydrophobic interaction. Based on the results, trioleate epoxide has a stronger bond affinity value because trioleate epoxide has 2 hydrogen bonds and 2 carbon-hydrogen bonds, while epoxide dioleate only has 2 carbon-hydrogen bonds. Hydrogen and carbon-hydrogen bonds have the most important role in stabilizing enzyme-ligand bonds, whereas the energy of hydrogen bonds and carbon-hydrogen bonds is 10–63 and 4 kJ mol^{-1}.[28] Trioleate epoxide is more stable for FASN binding through Ser2308 residue compared to dioleate epoxide. This computational finding agrees with the experimental *in vitro* result, in which trioleate epoxide exhibits a stronger anticancer activity than dioleate epoxide one. The interactions in the docking results are listed in Table III.

Table III. Molecular docking results of the native ligand and epoxide compounds

Compound	Binding affinity (kJ mol^{-1})	Interaction			
		Hydrogen	Carbon hydrogen	Hydrophobic	Electro-static
Native ligand (orlistat)	-22.87	Ile2250, Ser2308, His2481	-	Leu2222, Pro2249, Val2256, Tyr2307, Tyr2309, Tyr2343, Ala2367, Phe2370, Phe2371, Gln2374, Phe2375, Ala2419, Ser2422, Phe2423,Lys2426, Leu2427, Arg2482	Glu2251
Dioleate epoxide	-27.11	-	Thr2342, Ala2363	Leu2222, Ile2250, Glu2251, Ser2308, Tyr2309, Asp2338, Gly2339, Ser2340, Val2343, Leu2344, Ala2345, Tyr2350, Ala2363, Glu2364, Glu2366, Ala2367, Phe2370, Phe2371, Gln2374, Phe2375, Phe2423, Tyr2424, Lys2426, Leu2427, Arg2428, Ala2430, Glu2431, Ala2452, Tyr2453, Gly2454, Asp2456, Tyr2462, His2481,Arg2482.	-
Trioleate epoxide	-27.40	Ser2308, Arg2351	Thr2342, Ala2367	Leu 2222, Ile2250, Glu2251, Tyr2309, Val2343, Ala2345, Tyr2350, Glu2366, Phe2370, Phe2371, Gln2374, Phe2375, Ala2419, Phe2423, Lys2426, Leu2427, Arg2428, Ala2430, Glu2431, Ala2452, Tyr2453, Tyr2462, His2481, Arg2482	-

Compared with previously reported oleate epoxide derivatives—namely epoxidized methyl oleate (-30.54 kJ mol^{-1}) and epoxidized ethyl oleate (-33.89 kJ mol^{-1})[12] - the two compounds synthesised in this study exhibited weaker binding affinity for FASN. This strengthen that structural modification of monoester compounds into glycerol-based diester and triester derivatives does not enhance the strength of interaction with the target protein due to steric effect, which is in agreement with the cytotoxic results.

CONCLUSIONS

Dioleate and trioleate epoxides have been successfully prepared from palm oil through five consecutive steps with a purity of 86.49% and 93.51%, respectively. Based on the *in vitro* and *in silico* tests, trioleate epoxide generated more potent *in vitro* anticancer activity in killing T47D and WiDr cancer cells than dioleate epoxide. Meanwhile, the cytotoxicity results revealed that SI values of dioleate and trioleate epoxides were found in a range of 7.60–10.87 demonstrating their non-

toxic category towards Vero normal cells. The docking results revealed that both epoxides were able to interact with key amino acid residues, i.e., Ser2308, Thr2342, Arg2351, Ala2363, and Ala2367, in the FASN's active site. In future, *in vivo* tests of these oleate epoxides need to be carried out for further development as new anticancer agents.

SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/13896, or from the corresponding author on request.

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И З В О Д

ЕПОКСИДИ ИЗВЕДЕНИ ИЗ ДИОЛЕАТА И ТРИОЛЕАТА ПАЛМИНОГ УЉА КАО АНТИТУМОРСКИ АГЕНСИ ИСПИТИВАЊЕМ *IN VITRO* АКТИВНОСТИ И МОЛЕКУЛСКОГ ДОКИНГА

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Епоксиди изведени из диолеата и триолеата палминог уља добијени су у неколико корака, и испитан им је антитуморски потенцијал у *in vitro* тестовима, а молекулским докингом је испитано могуће везивање за FASN протеин. Прво, поступком трансестерификације палминог уља добијени су метил-естри масних киселина FAMEs (**f**atty **a**cid **m**ethyl **e**sters). Затим су метил-олеати из FAME пречишћени инклузионом методом помоћу урее и затим је пречишћен метил-олеат хидролизован до олеинске киселине. Олеинска киселина је у поступку естерификације са 1,2-пропандиолом и глицеролом преведена у 1,2-пропандиол-диолеат (диолеат) и глицерил-1,2,3-триолеат (триолеат), редом. Диолеат и триолеат су помоћу пермрављe киселине преведени у одговарајуће епоксиде. Триолеат-епоксид показује бољу антитуморску активност према T47D и WiDr ћелијама рака са IC_{50} вредностима 27,40 и 32,41 mg L^{-1}, редом. У поређењу са њим, диолеат-епоксид према T47D и WiDr ћелијама ракаа има активност 35,66 и 38,25 mg L^{-1}, редом. Триолеат- и диолеат-епоксиди нису токсични према нормалним ћелијама са вредностима индекса селективности 9,19–10,87 и 7,60–8,16, редом. Молекулским докингом је показано да би диолеат- и триолеат-епоксиди могли да инхибирају функционисање FASN ензима са вредностима афинитета везивања -27,11 и -27,61 kJ mol^{-1}, редом.

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