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Original Research Article

Identified Compounds from Ethyl Acetate Phase of Temu Mangga (*Curcuma mangga* Val.) Using LC-MS/MS and Their Potential as Anticancer Against MCF-7 Cells

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ABSTRACT

Curcuma mangga Val. is a well-known species of the Zingiberaceae family. In previous research, acetone extract of this species was highly active in inhibiting MCF-7 cells' proliferation (IC_{50} = 1.45 μg/mL), where crude acetone extract was partitioned consecutively using n-hexane, ethyl acetate (EtOAc), and methanol. The soluble components in the n-hexane phase, identified as zedoarondiol, curcumenol, curcumenone, and 13-hydroxygermacrone, are thought to inhibit MCF-7 cells' proliferation. In contrast, the soluble compounds in the EtOAc phase have not yet been reported. Therefore, this study aims to identify the soluble compounds in the EtOAc phase of acetone extract and examine their anticancer activity toward MCF-7 cells. Fractionation and purification of the EtOAc constituents are achieved by vacuum liquid chromatography, radial chromatography, and preparative thin-layer chromatography, and the chemical structure of the isolated compounds is identified by employing liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Furthermore, the isolated secondary metabolites are examined against the MCF-7 cell line using a 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) assay. By following these protocols, three fractions are obtained. LC-MS/MS spectra show that fraction **1 (α)** contains an unidentified flavanone or chalcone compound, fraction **2 (β)** confines zedoarofuran, and fraction **3 (γ)** is a mixture of zederone, curzerenone, and zedoarofurane. Meanwhile, fractions **2 (β)** and **3 (γ)** are terpenoid compounds. The MTT assay shows that fraction 2 **(β)** contains zedoarofuran (eudesmane-type sesquiterpenoids) and is moderate at inhibiting MCF-7 cells' proliferation (IC50 < 31.25 μg/mL). Fractions 1 and 3 are inactive with IC_{50} < 50 μg/mL.

Keywords: *Curcuma mangga*, Ethyl acetate phase, MCF-7 cells, MTT assay, Zedoarofuran.

Introduction

Temu mangga (*Curcuma mangga* Val.), a member of the *Zingiberaceae* family, spreads widely and thrives in tropical, forested areas. Temu mangga has been used by Indonesian people in spices, supplements, and a variety of traditional medicines. In addition, society and hospitals in Indonesia have used its rhizome to produce traditional medicine, so-called jamu, and to treat many cancers.[1-2] The alternative–complementary therapy combination or the combination of jamu containing various herbs, including temu mangga, and medical cancer therapy can increase the quality of life of cancer patients by up to 79.6%.[2] The use of temu mangga in traditional medicine production (jamu) for cancer therapy is minimal. Indonesians used temu mangga in 5% of jamu varieties, while kunyit putih (*Curcuma zedoaria*) is used in 23% of jamu varieties. Scientific exploration through an *in-vitro* test showed that the essential oil and several organic extracts of temu mangga, which grows in several places in Indonesia, could inhibit numerous cancer cells. Further, temu mangga can inhibit the proliferation of the DU-145, MCF-7, T47D, KB, A549, Ca Ski, HCT 116, and HT-29 cell lines.[3-5]
Such compounds as (*E*)-lambda-8(17),12-diene-15,16-dial, zerumin A, zerumin B, and curcuminoids, isolated from temu mangga, can also inhibit MCF-7 breast cancer cells' proliferation with a half maximal inhibitory concentration (IC_{50}) under 30 μg/mL.[3,6] A previous study on temu mangga grown in the Trop BRC Garden, Bogor, Indonesia, reported the potential of this plant to cure breast cancer. The acetone extract of the rhizomes was active to inhibit the proliferation of MCF-7 cells based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with an IC50 of 1.45 μg/mL.7 The extract was a solid–liquid partitioned using *n*-hexane, ethyl acetate, and methanol, respectively, and the compounds identified in the *n*-hexane phase using *liquid chromatography-mass spectrometry/mass spectrometry* (LC-MS/MS) were zedoarondiol, 13-hydroxygermacrone, curcumenol, and curcumenone.[8] In contrast, the fractionation and purification of the ethyl acetate phase have not been conducted. The ethyl acetate phase of temu mangga had an IC_{50} of 90.80 μg/mL.[9] Hence, this research focused on fractionating and purifying temu mangga's ethyl acetate phase and testing its anticancer activity against MCF-7 cells by *in-vitro* assay.

Materials and Methods

Materials

The sample used in this research was 39.78 g of ethyl acetate phase from the acetone extract of temu mangga. The rhizomes were harvested from Trop BRC Garden, West Java, Indonesia, in January 2019. Chemicals used for fractionation and isolation were a distilled technical-grade solvent, such as methanol (MeOH), ethyl acetate (EtOAc), dichloromethane (DCM), and *n*-hexane (H) (CV Firman).

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Chemicals for the assay were RPMI 1640 media, epirubicin-HCl, MCF-7 cells, and MTT reagent.

Fractionation and Isolation of the Selected Fractions

The ethyl acetate phase of temu mangga was separated into two groups (20.00 g and 19.78 g), both of which were impregnated separately into 60.0 g silica gel 60 (0.2–0.5 mm). The sample was fractionated using vacuum liquid chromatography. Elution started with DCM, a gradient elution from nonpolar to polar using a solvent mixture of DCM:MeOH (9:1–6:4 v/v) and MeOH. Both groups produced four fractions (A1–A4, B1–B4). Fractions A1 (350 mg) and B1 (559.5 mg) were partitioned using radial chromatography with gradient eluent H:DCM (99.5:0.5 v/v) into 25 fractions (A1.1–A1.25, B1.1–B1.25), while fractions A1.22 (36.2 mg) and B (39.8 mg) were fractionated using preparative thin-layer chromatography with eluent H:DCM (3:7 v/v). Three spots that appeared were collected separately, decantated, and then dissolved in DCM. A rotary evaporator concentrated the solutions, and the compound identified in each fraction was analyzed using liquid chromatography-mass spectroscopy-mass spectroscopy (LC-MS/MS). Further, instrument analyses were done at room temperature (25 °C). A sample was filtered by a 0.2 µm filter, and was injected at an injection volume of 5 µL into an LC instrument (ACQUITY UPLC®H-Class, Waters, USA) using an HSS C18 (1.8 µm 2.1×100 mm) column. The column was heated to 50 °C, and the sample elution used gradient elution with a flow rate of 0.2 mL/min. The mobile phases were A (water + 5 mM ammonium format) and B (acetonitrile + 0.05% formic acid). MS analysis was conducted using XEVO G2-S Qtof, Waters, USA with electrospray ionization (ESI). MS detection was done in 50 eV, and all fragment ions were positive. The volume of the injected sample was 250 µL, and the source and desolvation temperatures were 100 °C and 350 °C, respectively. The chromatogram and mass spectrum obtained from the three fractions were analyzed using MassLynx 4.1, and the mass spectrum of the chromatogram peak was compared to the data base such as ChemSpider (http://www.chemspider.com/), HMDB (https://hmdb.ca/), and MassBank (https://massbank.eu/MassBank/) databases.

In-Vitro Anticancer Assay

The anticancer activity of the ethyl acetate phase and its purified fractions was determined by the MTT method on MCF-7 breast cancer cells.[10] The MCF-7 cells grew in a microplate 24 reader at a concentration of 5,000 cells in 100 µL of growth media (D-MEM, RPMI 1640, FBS 5%, penicillin 100 U/mL, and Streptomycin 100 µg/mL). MTT reagent (10 µL, 5 mg/mL) was added to every well on the third day, then incubated for 4 h at 37 °C. The formed formazan crystals were dissolved in EtOH, and its absorbance was measured using a spectrophotometric plate reader at 595 nm. The equation calculated the cells' inhibition percentage:

$$\%\text{Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

The IC_{50} score was calculated by inputting 50 into the curve line equation between the inhibition percentage (y-axis) and sample concentration (x-axis). The cells' morphology was observed under the inverted microscope at 400x magnification.

Results and Discussion

Temu mangga is a plant from *the Curcuma* genus and the *Zingiberaceae* family, primarily found in tropical countries, such as Indonesia, Malaysia, Thailand, and India. The phytochemical qualitative assay showed that the acetone extract, *n*-hexane fraction, ethyl acetate fraction, and methanol fraction contained secondary metabolites, including alkaloid, phenolic, flavonoid, and terpenoid.[8] The first stage of *the in-vitro* assay was the fractionation and isolation of a select fraction in the ethyl acetate fraction. Three fractions were successfully isolated from the ethyl acetate phase of the acetone extract. The weight and Rf in eluent H:DCM (3:7) of fractions 1 (**α**), 2 (**β**), and 3 (**γ**) were 6.1 mg and 0.44, 6.1 mg and 0.33, and 10.8 mg and 0.26, respectively. The position of the isolated fractions in the TLC plate is shown in Figure 1. The LC-MS/MS analysis showed that the fraction 1 (α) chromatogram displays several weak peaks and one prominent peak, with a retention time (t_R) of 11.03 min and an area of 49.70%. Peak 11.03 min was suspected as $C_{15}H_{12}O_3$, with a similarity of 91.78% at base peak *m/z* $[M + H]^+$ 241.0877. The mass spectrum information showed that the molecule formed fragment ions at *m/z* 191, 152, 141, 128, and 115. The suspected compound was a chalcone-type or flavanone-type flavonoid-suspected compounds contained in fraction 1 (Table 1**).**

Table 1: Predicted compounds identified in fraction 1 at 11.03-min time retention

Compound	Molecular Structure
3',4'-Dihydroxychalcone	
2',5'-Dihydroxychalcone	
2',4-Dihydroxychalcone	
o-Hydroxydibenzoylmethane	
6-Hydroxyflavanone	
2'-Hydroxyflavanone	
Lettucenin A	

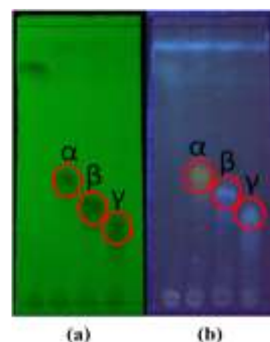

Figure 1: TLC plate of the three isolated fractions in eluent H:DCM (3:7) under UV lamp. (a): 254 nm; (b): 366 nm.

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The fraction 2(**β**) chromatogram indicated several weak peaks, the main peak being at a retention time of 10.90 min and an area of 49.43%. The peak with a retention time of 10.90 min had a base ion at *m/z* $[M + H]^+$ 247.1345. The compound was suspected as the zedoarofuran ($C_{15}H_{20}O_4$) terpenoid with a similarity of 99.95%. Zedoarofuran has a molecular weight of 246 g/mol and fragment ions at *m/z* 229, 201, 173, 121, 105, and 91. The fraction 3 chromatogram (**γ**) showed several weak peaks and three main peaks at retention times and areas of 9.87 min and 19.96%, 10.35 min and 14.61%, and 10.93 min and 19.82%, respectively. Peak 1 showed *m/z* $[M + H]^+$ 247.1345. The molecular formula of peak **1,** based on MassLynx 4.1., is $C_{15}H_{18}O_3$, with a similarity of 89.64%. An analysis of the formed fragment ions was conducted using bank data, and the result showed that $C_{15}H_{18}O_3$ was suspected as zederone, which has several fragment ions at *m/z* 229, 217, and 175. Peak **2** had a base peak at *m/z* $[M + H]^+$ 231.1396, and the MassLynx analysis predicted that peak **2** is $C_{15}H_{18}O_2$ with a similarity of 87.97%. The similarity comparison between the mass spectrum and bank data indicated that the identified compound was terpenoid curzerenone, and there were some similar peaks at *m/z* 213, 203, 191, and 105. The retention time of peak **3** from fraction **3** was similar to that of the peak in fraction 2, which was 10.93 min. The identified compound in peak **3's** mass spectrum was suspected as zedoarofuran ($C_{15}H_{20}O_4$), with a similarity of 74.65%. The chemical structures of zederone **(a)**, curzerenone (b), and zedoarofuran (c) are shown in Figure 2, and the proposed fragmentation scheme of zederone and curzerenone are illustrated in Figure 3. The LC-MS/MS analysis of the isolated fraction from temu mangga's ethyl acetate fraction presented flavonoid chalcone or flavanone and terpenoids, such as zedoarofuran, zederone, and curzerenone. Several flavonoid chalcones and flavanones that have been identified in the Zingiberaceae family, such as *Curcuma longa*, *Kaempferia parviflora*, and *Boesenbergia pandurata, are displayed* in Figure 4.[11-13] Zederone is commonly found in the essential oils and rhizomes of *C. aromatica*, *C. caesia*, *C. xanthorrhiza*, *C. longa*, *C. zedoaria*, *C. phaeocaulis*, and *C. wenyujin*.[14-17] Meanwhile, curzerenone has been identified in the essential oils and rhizomes of *C. caesia*, *C. zedoaria*, *C. angustifolia*, *C. aromatica*, *C. xanthorrhiza*, *C. phaeocaulis*, *C. longa*, and *C. Aeruginosa*.[14,16,18-21-22] Further, curzerenone was the dominant compound in the essential oils of *C. zedoaria* (96.82%) and *C. angustifolia* (95.24%), while curzerenone was found in only a small quantity (2.40%) in the essential oils of *C. amada*.[18] Zedoarofuran has now been identified in *C. wenyujin* and *C. zedoaria*.[17,22-23]

Breast cancer is the most common type of cancer across the world. The World Health Organization (WHO) reported breast cancer as a top cancer in 109 of 193 countries in 2020.[24] MCF-7 cells were the leading cause of breast cancer cells and the most common cell used in cancer research since its identification in 1973.[25] The anticancer activity of the ethyl acetate fraction and isolated fractions aganist MCF-7 cells was tested using the MTT assay. The working principle of the MTT assay is to measure the color intensity of the final product, named blue formazan crystals, which form from a reduction in yellow-colored MTT reagent by the dehydrogenase succinate mitochondrial enzyme in living cancer cells.[26] The sample could inhibit the proliferation of cancer cells in several categories, such as highly active (IC_{50} < 5 ppm), active (IC_{50} = 5–10 ppm), and intermediate (IC_{50} = 11–30 ppm).[27] The result of breast cancer growth inhibition is shown in Table 2.

Fraction 2 (**β**) had an IC_{50} <31.25 µg/mL, a moderate level to inhibit the proliferation of MCF-7 cells, followed by the ethyl acetate phase, fraction 3, and fraction 1. The previous research reported that the IC_{50} of zederone isolated from the *C. zedoaria* and *C. caesia* rhizomes, having an inhibition capacity with IC_{50} >100 µg/mL and 98.4 µg/mL, respectively, fall into the inactive category. In contrast, curzerenone from both rhizomes was more active, with IC_{50} values of 40.0±0.5 µg/mL and 52.8±0.4 µg/mL, respectively.[28-29] There is no report on zedoarofuran bioactivity as an anticancer agent for MCF-7 cells based on *in-vitro* assay. Therefore, an approach based on the compounds' similar skeleton structure was adopted to predict the bioactivity of zedoarofuran (Table 3).

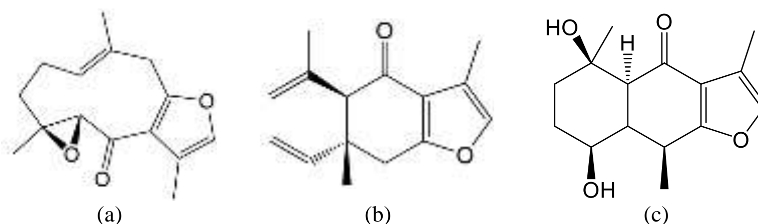

Figure 2: Identified terpenoids in the ethyl acetate phase of temu mangga. (a): zederone; (b): curzerenone; (c): zedoarofuran

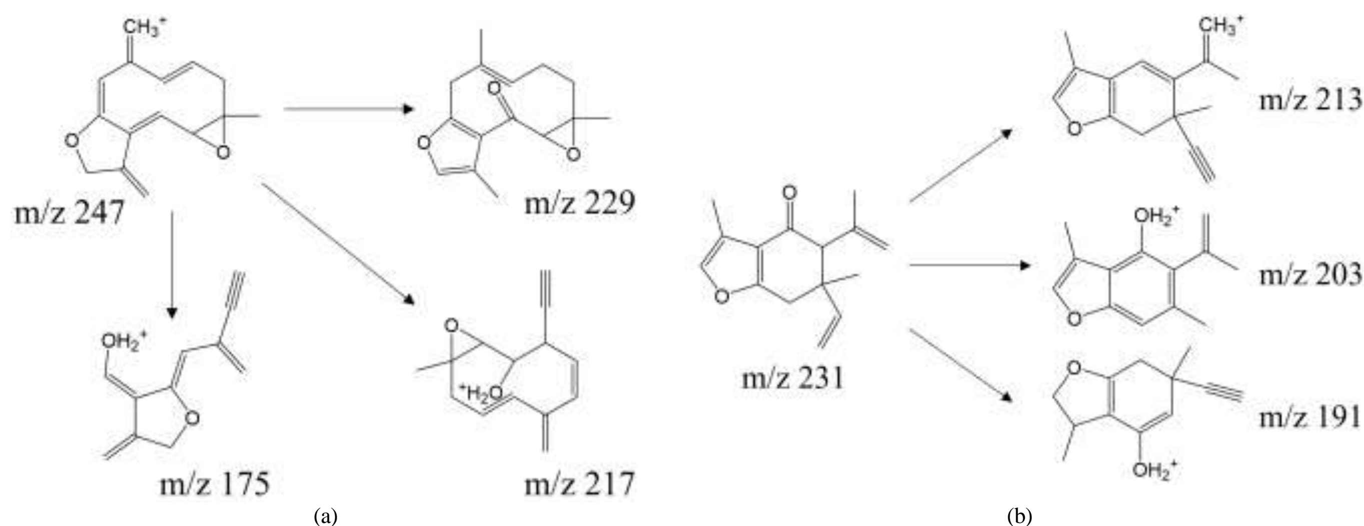

Figure 3: Fragmentation scheme terpenoids in the ethyl acetate phase. (a): zederone (*m/z* 247) at peak 1 (**γ1**); (b): curzerenone (*m/z* 231) at peak 2 (**γ2**)

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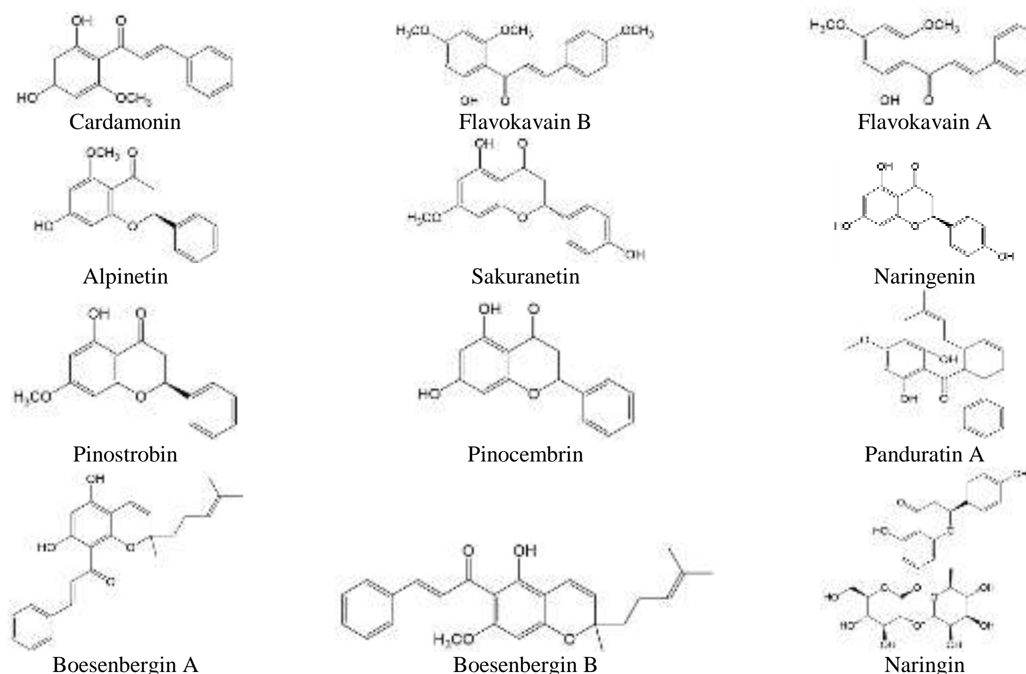

Figure 4: Chalcone and flavanone compounds from the Zingiberaceae family.[11-13]

Table 2: IC_{50} values of the ethyl acetate fraction and the three selected fractions against MCF-7 cells' proliferation

Sample	IC_{50} (µg/mL)
Ethyl acetate phase	38.63
Fraction 1 (α)	128.4
Fraction 2 (β)	< 31.25
Fraction 3 (γ)	58.94

Zedoarofuran has an eudesmane skeleton, and eudesmane and several of its derivatives isolated from numerous plants give various activities. Eudesmane isolated from terrestrial plant *Juglans sinensis* and *Kaunia lasiophthalma* was highly active in inhibiting the proliferation of MCF-7 cells, while the compounds from red algae *Laurencia obtusa* had minimal anticancer activities. Based on the growth zone, zedoarofuran in temu mangga tended to be active as an anticancer against MCF-7 cells. Figure 5 shows the morphology of MCF-7 cells in the negative control, positive control (Epirubicin-HCl), and fraction 2 (β) with a concentration of 31.25 ppm, respectively.

Table 3: IC_{50} values of eudesmane-type sesquiterpenoid toward MCF-7 cells

Compound	Plant	IC_{50} (µg/mL)	Reference
Eudesma-4(15),7-diene-5,11-diol	*L. obtusa*	39.5	30
Teuhetenone A	*L. obtusa*	22.8	30
Chabrolidione B	*L. obtusa*	63.6	30
11-Hydroxy-2,4-cycloeudesman-8-one	*J. sinensis*	>2.1	31
(4S,5S,7R,8R,14R)-8,11-Dihydroxy-2,4-cyclo-eudesmane	*J. sinensis*	>2.4	31
(4S,5S,7R,8S,14R)-8,11-Dihydroxy-2,4-cyclo-eudesmane	*J. sinensis*	0.4	31
15-Hydroxy-α-eudesmol-11-O-β-D-glucopyranoside	*J. sinensis*	>4	31
Acetyl 1-epireynosine	*K. lasiophthalma*	7.0	32
4-epi-1α-Acetoxyarbusculin A	*K. lasiophthalma*	11.1	32
Santamarine	*K. lasiophthalma*	4.2	32
11,13-Didehydrovulgarin	*K. lasiophthalma*	6.3	32

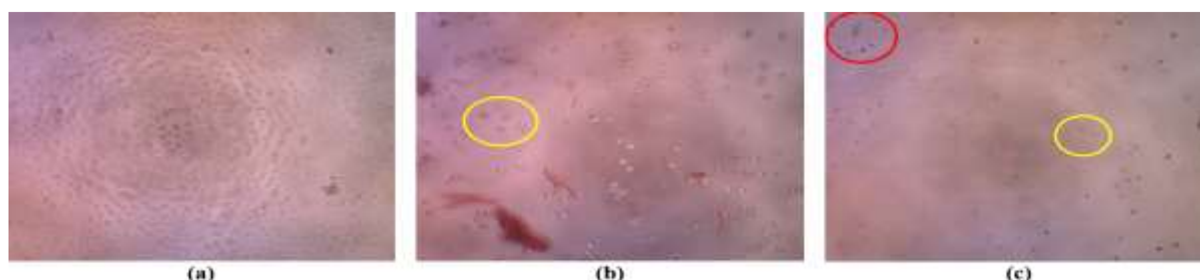

Figure 5: Morphological visualization of MCF-7 cells under 400x magnification. (a): Cells without adding test sample (negative control). (b): adding 31.25 ppm epirubicin HCl (positive control). (c): Adding 31.25 ppm fraction 2 (**β**). The cells' form was round and shrunk (red circle). The dead cells became dark and formed an aggregate suspension (yellow ring)

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Cell morphology in the negative control (Fig. 5a) showed cells growing over the plate surface (confluency close to 100%), polygonal and rigid. Meanwhile, after the addition of epirubicin HCl and fraction **2 (β)** at a concentration of 31.25 μg/mL, the morphology of MCF-7 cells changed to a round shape and shrunk, leading to a dead cell, which is indicated with a dark suspension (Figure 5b and 5c).[33-34]

Conclusion

The ethyl acetate phase from the acetone extract of *Temu mangga* was separated through several chromatographic techniques. Its fractionation and purification afforded three fractions with different factor retentions. Based on LC-MS/MS analysis, compounds suspected in them were secondary metabolites, flavonoids of the chalcone/flavanone group, and terpenoids. Further, fraction 2 (**β**) (IC_{50} < 31.25 μg/mL) was suspected as zedoarofuran (eudesmane-type sesquiterpenoid) with moderate activity, whereas **1** (**α**), and **3** (**γ**) were inactive, with IC_{50} > 50 μg/mL to inhibit the proliferation of the MCF-7 breast cancer cells.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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