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CYTOTOXICITY ACTIVITY AND DHODH INHIBITION OF ZERUMBONE OF *Zingiber ottensi* Val. RHIZOME AGAINST HELA CERVICAL CANCER CELLS

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ABSTRACT
This study was aimed at determining the cytotoxicity and antimigration activity of zerumbone isolated from *Z. ottensii* Val. rhizomes against HeLa cell lines. Zerumbone was isolated from the hexane fraction of the ethanol extract by a bioassay-guided procedure using column chromatography, and spectroscopic data analysis was used to establish its structure. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure zerumbone's cytotoxic activity. The inhibition of zerumbone against HeLa cell migration was assessed using the scratch/wound healing test. DHODH inhibition activity determined by molecular docking using AutoDock 4.2.6 and in vitro biochemical assay. The results of the MTT assay showed that zerumbone had high cytotoxicity against HeLa cell lines, with the IC50 value of 41.60 µg/mL in the 24-hour treatment. According to the cell migration assay, zerumbone suppressed HeLa cell migration in both the 24 and 48-hour treatments in a concentration-dependent manner. Zerumbone at a concentration of 15 µg/mL significantly inhibited the migration of the HeLa cells compared with the control. Zerumbone has good affinity with the DHODH enzyme and has potential activity as a DHODH inhibitor. This study indicates that zerumbone of the *Z. ottensii* rhizome has potential to be explored further as an anticancer drug candidate.
Keywords: Cytotoxicity, Cell Migration, DHODH, *Zingiber ottensii* Val., Zerumbone
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INTRODUCTION

In Southeast Asia, the Zingiberaceae family is frequently used in traditional medicine. Genus *Zingiber* is the second largest in this family.[1,2] *Z. ottensii* is a species of the Zingiber genus that is native and distributed in Indonesia (Sumatra, Kalimantan, and Java) and Malaysia; it is also found in Thailand, Vietnam, and Myanmar.[1,3,4] *Z. ottensii* is a rhizomatous herb 1-1.9 m tall and has a branched rhizome with a 2.2-3 cm diameter. The internally aromatic rhizome has a dark purple colour and is covered with light brown-yellowish triangular scales; externally, it appears light yellowish brown.[1,3] *Z. ottensii* rhizomes are used in traditional medicine to treat various health issues, including cough, pain, fever, itching, and as a carminative, as well as for postpartum care.[1,3-5] The rhizomes have also been *in vitro* tested for several pharmacological activities. It has potential activity such as antioxidant, tyrosinase inhibition, nitric oxide production inhibition, antimicrobial, antifungal, antibacterial, ACE inhibitor, alpha-glucosidase inhibitor, and also antiproliferative for several human malignant cell lines.[6-12] There is some evidence that the main active compounds of the Zingiber genus, such as 6-gingerol, 6-paradol, 6-shogaol, alpha-terpineol, zingerone, and zerumbone, have effects in various cell lines.[13-20] These pieces of evidence provide drug development of active compounds from Zingiber, especially for the treatment of different cancers. *Z. ottensii* Val. Rhizomes were reported to have terpenoid compounds such as humulene, zerumbone, terpeniol, 1,10,10-trimethylbicyclo[7,4,0]tridecane-3,6-dione, (E)-14-hydroxy-15-norlabda-8(17),12-dien-16-al, (E)-labda-8(17),12,14-trien-15(16)-olide, and (E)-14,15,16-trinorlabda-8(17),11-dien-13-oic acid.[21,22] The metabolite compounds of the *Z. ottensii* rhizomes, such as zingipain protein, a cysteine protease, terpenoids, and diarylheptanoids, as well as its essential oil components such as terpeniol and zerumbone, have potential as anticancer properties.[6,21-23] *Z. ottensii,* as one of the Zingiber genus, has an

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opportunity to be developed as a source of anticancer by a chemotaxonomic approach. Anticancer studies of a drug candidate have been carried out through various approaches, such as viability and cytotoxic assays, and molecular mechanisms. Dihydroorotate dehydrogenase (DHODH) enzyme activity inhibition is one of them. DHODH is a crucial enzyme in the biosynthesis of de novo pyrimidines, which is essential in cancer cells' proliferation.[24-26] Yin *et al.*[27] reported that DHODH activity in HeLa cells was higher than in normal cells. So that the inhibition of the activity of the DHODH enzyme can be related to the inhibition of the growth of these cancer cells. This study aimed at the isolation of zerumbone from *Z. ottensii* and the evaluation of this compound on its cytotoxicity against cervical cancer cell lines and its effect on cell migration and DHODH activity.

EXPERIMENTAL

Plant Materials

The rhizomes of *Z. ottensii* were gathered from a nearby garden in Bandung, Indonesia. The species was recognised and verified at the herbarium of the Department of Biology, Padjadjaran University, Indonesia. The rhizomes were cleaned, cut and dried in an oven at 40 °C. Rhizomes that had dried were ground and sifted through a 20-mesh sieve.

Cell Culture and Condition

The HeLa cell lines (human cervical cancer) were used to evaluate the cytotoxic and cell migration inhibitory activity of ethanol extract, fractions, and an isolate from *Z. ottensii* rhizome. The medium that was used to culture HeLa cell lines was RPMI medium (pH 7.4) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL streptomycin and 100 U/mL penicillin). Cells were incubated at 37 °C under 5% CO_2 for 12-24 hours.

Extraction and fractionation of *Z. ottensii* Val. rhizomes

The powders of the *Z. ottensii* rhizomes (1500 g) were extracted with 70% ethanol (3 times, each time for 24 h) at room temperature using the maceration method. The extract was evaporated using a rotary evaporator at 50-60 °C (Heidolph Hei-Vap ML Precision Rotary Evaporator) to obtain a concentrated extract (231.6 g; yield of extract 15.44%). The extract (145 g) was fractionated by liquid-liquid extraction using the mixture of hexane and water (1:1) to yield a hexane fraction (6.8 g) and a water layer. The water layer was extracted with ethyl acetate (1:1) to yield an ethyl acetate fraction (20.9 g) and a water layer. The water layer was further extracted with butanol (1:1) to give a butanol fraction (23.5 g) and a water fraction (88.5 g).

Isolation of Zerumbone

The hexane fraction (5.56 g) was column chromatographed using silica gel 60 (0.063-0.200 mm/70-230 mesh ASTM; Merck, Germany) as a stationary phase and a mixture of hexane-chloroform as a mobile phase with gradient polarity to give subfractions H1-11. Crystalised zerumbone in the subfraction H5 was purified by recrystallisation using a mixture of chloroform and methanol. The process of recrystallisation was done by dissolving the subfraction H5 in warm methanol, then chloroform was added until saturated and crystallised overnight to produce a crystalline solid (zerumbone). The molecular structure of zerumbone was determined using a UV-Vis spectrophotometer (Shimadzu UV-1800 series) and FT-IR Spectrometer (Agilent® Cary 630) and recorded using a OneSource PerkinElmer instrument, NMR Agilent® series DD2 spectrophotometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR).

Cytotoxicity Test

The cytotoxicity test was done to evaluate cell viability after treatment with *Z. ottensii* Val. extract and fractions, and the cytotoxic effect of the extract, fractions, and Zerumbone. The cytotoxicity was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[28] HeLa cell lines ($2x10^{-4}$ cells per well) were seeded on a 98-well plate and incubated for 24 hours at 37 °C under 5% CO_2. After 24 h incubation, the medium was removed and replaced with 200 µL fresh growth medium containing different doses of the test sample as treatment and incubated for 24 h-72 h at 37 °C under 5% CO_2. When incubation finished, the medium was removed, and a 20µL MTT solution (10%) per well was added and incubated for 4 hours. The supernatant fluid was removed, and the reaction was stopped by 100

µL DMSO per well. Samples were read at 550 nm with a plate reader (Thermo Scientific® Multiscan EX, Singapore). The absorbance value is directly proportional to the number of living cells. The cell viability was determined by the measured percentage of living cells in treated cells compared to untreated cells. The cell viability was examined at 24 h, 48 h, and 72 h of treatment. The cytotoxic effect was determined by measuring the percentage of dead cells of treated cells to untreated cells after 24 h of treatment, and determining the 50% inhibitory concentration (IC50). IC50 values were analysed using four parametric logistic models by Sigmaplot ver.12.

Migration Assay

The scratch/wound healing assay in HeLa cells was conducted to determine the anti-cell migration of Zerumbone from Z. ottensii Val. According to Liang *et al.*'s procedure.[29] HeLa cells were grown to 70% confluence in 24-well plates. Gaps were created by scraping the monolayer using a P10 pipette tip. After scraping, cells were washed three times to eliminate debris using PBS. Cells were treated with Zerumbone at various concentrations in complete medium and then incubated in an incubator. Untreated cells were used as a control. The 0, 3, 24 and 48 hours of treatment were photographed under a microscope, which was connected to a computer and Toupview Software (version x64, 3.7.7892). The gap area was measured using ImageJ software. The percentage of gap area closure was determined by comparing the width of the scratch area at a specific time point (t) to the width of the scratch area at zero time. Data were presented as mean and SD from triplicate data. *$p < 0.05$; **$p < 0.01$ to control.

Molecular Docking Simulation

The target protein in this study was the human dihydroorotate dehydrogenase (DHODH) enzyme. The 3D structure of the target protein used was downloaded from the Protein Data Bank (PDB) with the code PDB 3G0X. The docking method that is considered valid is the docking method that gives an RMSD value of less than 2 Å. Validation is carried out by redocking the natural ligands on the target protein. For molecular docking simulation of Zerumbone against human DHODH, we are using the molecular docking program AutoDock 4.2.6. The grid spacing was set at 0.375 Å. The Lamarckian Genetic Algorithm was implemented with several GA runs, which was 10.

In Vitro DHODH Inhibition Assay

The zerumbone effect on *Hs*DHODH was examined using the enzymatic biochemical method in vitro. The zerumbone solution was prepared at a concentration of 10 mg/mL. Two µL of the solution was placed into the well plate, and thus the concentration of zerumbone in the system was 20 µg/mL. After the addition of 2 µL of samples in a 96-well plate, 2 µL of water was added as a positive and negative control. A quantity of 190 µL *assay mixture* (contains 100 mM HEPES pH 8, 10% (v/v) glycerol, 50 mM NaCl, 0.05% (w/v) Triton X-100, 120 µM DCIP, and 18 µM decylubiquinone) was added into all wells, and homogenised by shaking at 500-700 rpm for 30 seconds. The absorbance was read in kinetic mode by a Multiskan GO multiplate reader (Thermo) at 600 nm, 25°C for 1 minute and recorded as background. Eight µL of 5 mM L-DHO was then added to each well except the positive control and homogenised by shaking at 500-700 rpm for 30 seconds. The absorbance at 600 nm was read. The inhibition activity was calculated by the following equation:

$$\%\,inhibition = 100 - [(\frac{A.Sample - A.positive\ control}{A.negative\ control}) \times 100\%]$$

A.sample is the decrease of absorbance of the sample; *A. positive control* is the average decrease of absorbance of the well of the positive control, and *A. negative control* is the average decrease of absorbance of the well of the negative control.

RESULTS AND DISCUSSION

Cytotoxicity of Ethanol Extract and Fractions of the *Z. ottensii* Rhizomes

The extract and fractions of the *Z ottensii rhizomes* were evaluated for their effects on HeLa cancer cell lines using the MTT bioassay over treatment periods of 24, 48, and 72 hours. The cell viability of HeLa cell lines treated with the ethanol extract (100 µg/mL), *n*-hexane fraction (75 µg/mL), ethyl acetate fraction (100 µg/mL), *n*-butanol fraction (100 µg/mL), and water fraction (100 µg/mL) showed a decrease

following the 48- and 72-hour treatment (Fig.-1). The cytotoxic effect was indicated by the percentage of cell death, which increases with rising concentration and was quantified as the IC50 value. The IC50 values for all samples are presented in Table-1. The hexane fraction exhibited an IC50 value of 40.01 ± 3.686 µg/mL, the lowest among the tested samples. The significant reduction in HeLa cell viability caused by the hexane fraction at lower concentrations compared to other samples suggests that the hexane fraction possesses cytotoxic potential. Consequently, the focus of the isolation process for the cytotoxic compound was directed toward the hexane fraction, which ultimately led to the identification of zerumbone.

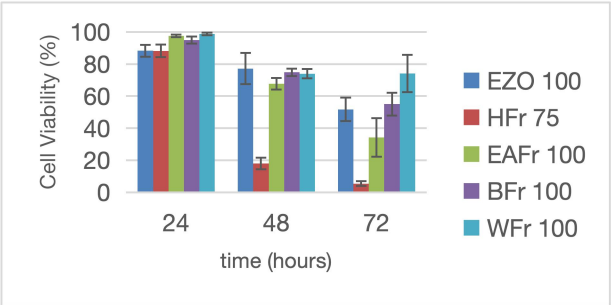

Fig.-1: The HeLa Cells Viability after Exposure with 100 µg/mL Extract (EZO 100), 75 µg/mL Hexane Fraction (HFr 75), 100 µg/mL Ethyl Acetate (EAFr 100), 100 µg/mL Butanol Fraction (BFr 100), and 100 µg/mL Water Fraction (WFr 100) of the *Z. ottensii* rhizomes. Determinations Were Made in Triplicate. Data represents the Mean of Three Measurements, SD

Table-1: The Cytotoxic Effect of Extract and Fractions of *Z. ottensii* Rhizome against HeLa cell line*)

Sample	IC50 (µg/mL)
Extract (EZO)	295.91±0.196
Hexane Fraction (HFr)	40.01±3.686
Ethyl Acetate (EAFr)	358.41±4.201
Butanol Fraction (BFr)	250.66±10.001
Water Fraction (WFr)	251.58±1.194

*Determinations were made in triplicate. Data represent the mean of three measurements ± SD. Data were analysed using four parametric logistic model by Sigmaplot ver.12.

Structure Determination of Zerumbone

Further work on the *n*-hexane fraction, which exhibited the highest cytotoxicity against HeLa cell lines, led to the identification of an active compound, zerumbone. The compound appeared as white crystals. The spectroscopic data were analysed to determine the molecular structure of the compound. A molecular ion peak was observed at 219 m/z in the EI-Mass. The UV spectrum showed an absorption peak at a wavelength of 323 nm, and the IR spectrum showed the presence of functional groups of C-H stretch (3000-2855 cm^{-1}, alkyl), C=O stretching (1655-1615 cm^{-1},α,β-unsaturated keton), C-H bend (1470-1385 cm^{-1}, CH_2/CH_3), and C-H bend, out of plane (825-760 cm^{-1}, CH_2/CH_3). In the ^{1}H-NMR spectrum, four singlets were observed at δ 1.06 (3H), 1.19 (3H), 1.53 (3H), and 1.81 (3H), which were derived from the methyl hydrogens at carbons C-14, C-15, C-12, and C-13, respectively. Three multiplets appeared at δ 1.9 (1H), 2.2 (2H), and 2.3 (2H), which were due to methylene hydrogens attached to C1, C-5, and C-4, respectively. In addition, there was a broad doublet at δ 5.25 (1H) attributed to the hydrogen at C-2 and two doublets at δ 5.85 (1H) and at δ 5.95 (1H) derived from the α- and β-hydrogens of a carbonyl group, respectively, and one double doublet at δ 6.01 (1H) of the other β- hydrogen of the carbonyl group. The ^{13}C-NMR spectrum had a total of 15 carbon signals, consisting of one carbonyl carbon signal appearing at δ 204.49 (C=O), 4 primary carbon signals at δ 11.93 (CH_3, C-13), 15.36 (CH_3, C-12), 24.33 (CH_3, C-15), and 29.57 (CH_3, C-14), 3 seqondary carbon signals at δ 24.54 (CH_2, C-5), 39.59 (CH_2, C-4), and 42.55 (CH_2, C-1), 4 tertiary carbon signals at δ 125.12 (CH, C-2), 127.29 (CH, C-6), 148.96 (CH, C-9), and 160.88 (CH, C-10), and 3 quaternary carbon signals at δ 38.00 (C, C-11), 136.41 (C, C-3), and 138.08 (C, C-7). These ^{13}C-NMR and ^{1}H-NMR data supported the assumption that this compound was zerumbone.

Comparison of its spectral data with those of zerumbone in the literature[30] confirmed the assumption. The molecular structure was shown in the Fig.-2.

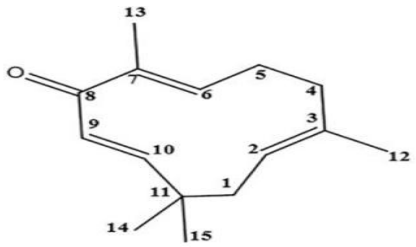

Fig.-2: Molecular Structure of Zerumbone

Cytotoxicity of Zerumbone

Zerumbone from *Z. ottensii* rhizomes showed a cytotoxic effect on HeLa cell lines in a dose-dependent manner, as shown in Fig.-3.

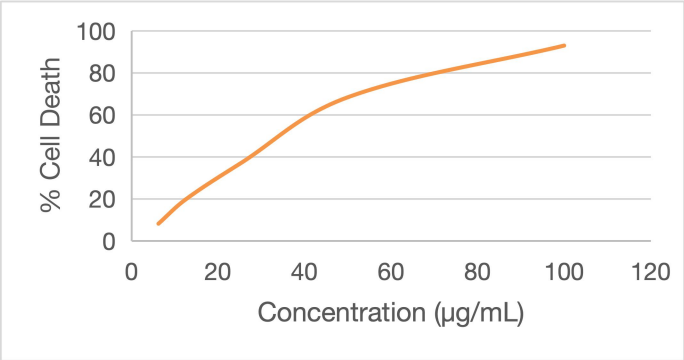

Fig.-3: The Cytotoxicity Effect of Zerumbone of *Z. ottensii* Rhizome against HeLa Cell Lines in 24 hours

The IC50 value was analysed using four parametric logistic models by SigmaPlot ver.12, showing that the IC50 value of zerumbone against HeLa cell lines in the 24-hour treatment was 41.60 µg/mL. In this study, zerumbone was proven to have strong cytotoxic activity against HeLa cell lines in the 24-hour treatment with the IC_{50} of 41.60 μgmL^{-1}. This result was in line with those reported previously concerning the effect of zerumbone on the HeLa cells and other cancer cells. Zerumbone exhibits a cytotoxic effect against HeLa cells by increasing the levels of cellular caspase-3 and producing distinct morphological features characteristic of apoptotic cell death.[30] Abdelwahab *et al*.[31] reported that zerumbone of *Z. zerumbet* inhibits cancer cell growth through arresting the cycle at G2/M phase, induces apoptosis, and inhibits IL-6 secretion in HeLa cells, whereas Ashraf *et al*.[32] revealed zerumbone to inhibit HeLa cell proliferation and induce mitotic block through tubulin binding.[31,32] The effect of zerumbone on other cancer cell lines was also demonstrated in some reports.[17, 33,34]

Inhibition of Zerumbone on HeLa Cell Migration

The capacity of the zerumbone of *Z. ottensii* to prevent HeLa cell migration was evaluated by a cell migration assay. The HeLa cell lines were treated with zerumbone at concentrations of 5, 10, and 15 μgmL^{-1}. The results are shown in Fig.-4A (cell migration photograph) and Fig.-4B (quantification of gap enclosure). The Fig.-4 indicates that zerumbone at a concentration of 10 µg/mL had an open gap until the 24-hr treatment, whereas the concentration of 15 µg/mL showed an open gap in the 24 and 48 hr treatment. This means that zerumbone had inhibitory activity on the HeLa cell migration. The migration and invasion of cancer cells to different tissues is an important initial step in metastasis. These processes can be a target of an anti-metastatic agent to inhibit cancer cell aggression. Our study indicated that zerumbone of *Z.ottensii* at the concentrations of 10 and 15 µg/mL was effective in preventing HeLa cell migration in a dose-dependent manner in the 24 and 48 hr treatment. Sung *et al.*[35] reported that zerumbone isolated from *Z. zerumbet* suppressed CXCR4 expression on HER2-overexpressing breast cancer cells and had a potential to suppress cancer metastasis.[35]

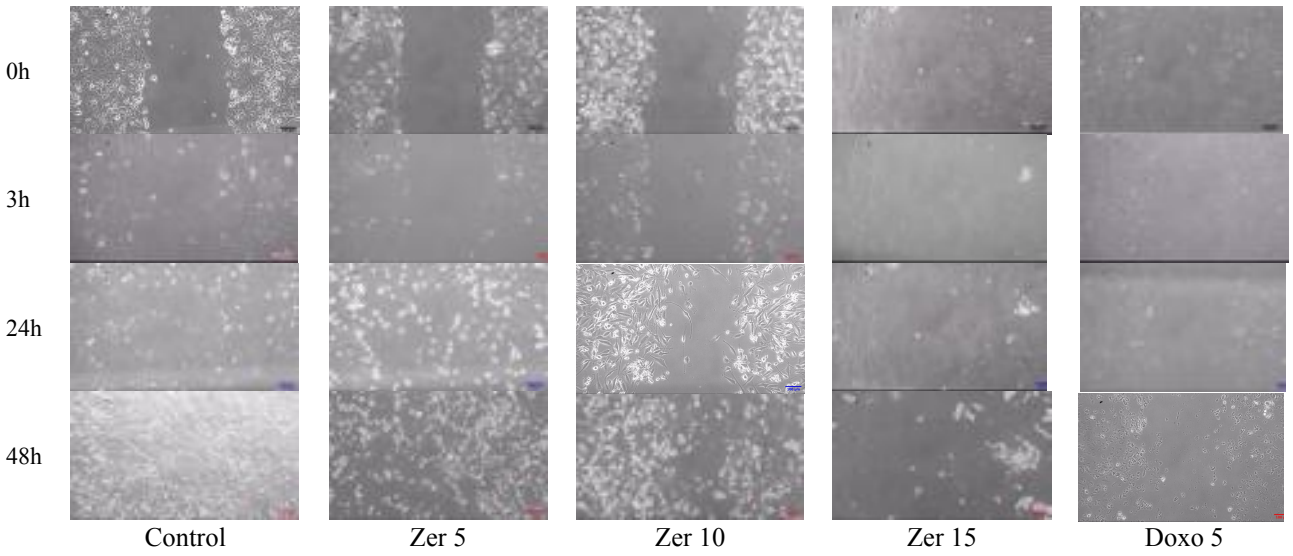

Fig.-4A: HeLa Cell Migration Photograph of the Cell Lines Treated with Zerumbone and Doxorubicin

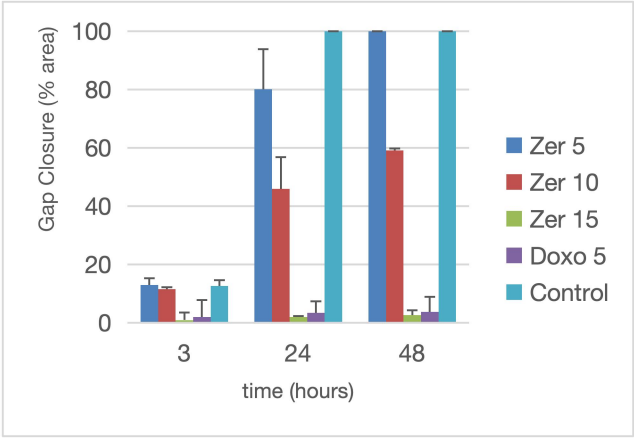

Fig.-4B: Quantification of Gap Enclosers of HeLa Cell Lines Treated with Zerumbone and Doxorubicin

Molecular Docking Complex of Zerumbone to DHODH

In this study, we also examined the inhibition of zerumbone on DHODH activity. In the fourth and rate-limiting step of the de novo pyrimidine synthesis pathway, DHODH, an important mitochondrial enzyme, catalyses the conversion of dihydroorotate (DHO) to orotate.[36] DHODH is linked to the mitochondrial respiratory chain (MRC) and is crucial to de novo pyrimidine production. The MRC inhibition causes an increase in the tumour suppressor p53 level and its activity, and activates programmed human cancer cell death.[37] Furthermore, the suppression of DHODH activity induces apoptosis in human colon cancer cells upon MRC complex inhibition.[37] Preclinical studies have demonstrated that inhibiting DHODH stops the development of numerous cancer types.[26,36] So, one of the efforts of discovering new effective anticancer drugs could be targeted at the DHODH activity inhibitors.

The molecular docking of the molecule Zerumbone revealed that the value of the bond-free energy (ΔG) was -8.10 kcal/mol, with an inhibition constant (Ki) of 1.15 µM. Meanwhile, the interactions that occur between zerumbone and the target protein are 2 hydrogen bonds, 10 alkyl-alkyl and pi-alkyl interactions, and 6 van der Waals interactions (Fig.-5). Hydrogen bonding occurs between the zerumbon and the amino acid residues ARG136 and GLN47. All zerumbone interactions with the target occurred at the binding site. Based on these results of molecular docking, zerumbone has a good affinity with the enzyme and has activity as a DHODH inhibitor.

Inhibition of DHODH Enzyme Activity

The in vitro DHODH inhibition assay showed that zerumbone at the concentration of 20 µg/mL had potential inhibition against DHODH activity, with its percentage inhibition of 24.2 ± 2.8 %. IC_{50} prediction was 41.8 ± 4.9 µg/mL. In our study, zerumbone provided good inhibitory activity against DHODH activity with an IC_{50} value of 41.8±4.9 µg/mL.

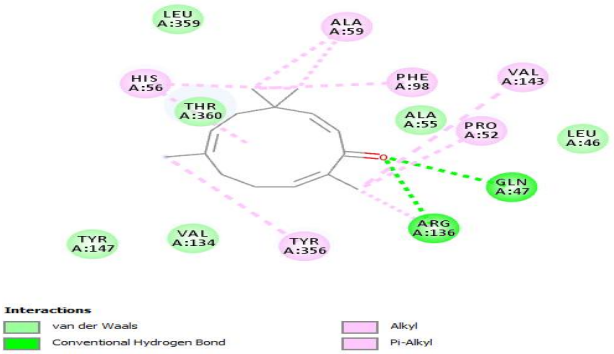

Fig.-5: Interaction between Zerumbone and Human DHODH

This result was supported by the molecular docking study, which showed that zerumbone had good affinity with the DHODH enzyme, and all interactions occurred at an enzyme binding site. The value ΔG (-8.10 kcal/mol) and Ki (1.15 µM) indicated that zerumbone had the potential to inhibit enzyme activity by blocking enzyme binding with its natural ligands. Although the molecular mechanism of zerumbone on HeLa cells was not determined in this study, the overall data above revealed the potential of zerumbone as a new anticancer candidate. The overall data support the previous findings that zerumbone has potential as a new anticancer candidate.

CONCLUSION

This study provided evidence that zerumbone of *Z. ottensii* had cytotoxicity on the HeLa cell lines, inhibited the cell migration, and suppressed the DHODH enzyme activity. These results indicated that zerumbone has potential as a candidate anticancer agent through some pathways, which should be continuously studied in order to be applicable for cancer treatment, including cervical cancer treatment.

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AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3*: *Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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