Volatile compound profiles and cytotoxicity in essential oils from rhizome of *Curcuma aeruginosa* and *Curcuma zanthorrhiza*

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Abstract. *Fitria R, Seno DSH, Priosoeryanto BP, Hartanti, Nurcholis W. 2019. Volatile compound profiles and cytotoxicity in essential oils from rhizome of* Curcuma aeruginosa *and* Curcuma zanthorrhiza. *Biodiversitas 20: 2943-2948.* Cancer becomes one of the uncontagious diseases that occupy the top position in the cause of human death under 70 years in the world. Breast cancer is the most widely experienced cancer by women. The aim of this study was to obtain cytotoxic activity essential oil of *Curcuma aeruginosa* and *Curcuma zanthorrhiza* as antiproliferative substance of cancer cells and to analyze volatile profile of *C. aeruginosa* and *C. zanthorrhiza* essential oils. The essential oils were obtained from rhizomes of *C. aeruginosa* and *C. zanthorrhiza* by using hydrodistillation method. Volatile compositions of essential oils were analyzed using gas chromatography and mass spectrophotometry (GC/MS). The cytotoxic activities of the essential oil samples were evaluated using brine shrimp lethality test and against MCF-7 (Michigan Cancer Foundation-7) and Vero cells. The essential oils of *C. aeruginosa*, and *C. zanthorrhiza* showed cytotoxic activities to brine shrimp and MCF-7. Cytotoxic activity presented in essential oil from *C. aeruginosa* and *C. zanthorrhiza* showed lower activity compared with Doxorubicin. The major compounds of *C. aeruginosa* were tropolone (18.1%), eucalyptol (17.9%), and curcumol (5.7%), whereas *C. zanthorrhiza* were xanthorrhizol (26.8%), β-curcumene (17.0%), ar-curcumene (15.0%), and germacrone (5.4%).

Keywords: Anticancer, *Curcuma aeruginosa, Curcuma zanthorrhiza*, essential oils, GC-MS

INTRODUCTION

Cancer is a non-communicable disease that has been widely spread in the world and leading the main cause of death for under 70 years old human in 91 of 172 countries (WHO 2018). This ailment is caused by a control mechanisms failure of the cell growth and proliferation that can be triggered by a number of factors such as genetic damage, mutations, exposure to carcinogens, chemical and ultraviolet radiation (Lodish 2004). As many as 18.1 million new cases of cancer occurred in 2018 with 9.6 million deaths mortality and 8.6 million of them happened among woman with the most suffered cancer was breast cancer (24.2%) (Bray et al. 2018). Breast cancer comes from excessive ductal proliferation which develops into benign tumors then becomes malignant and can metastasize if continuously exposed to cancer-causing agents (Sonnenschein 2016).

Some attempts that commonly used for cancer treatment therapies are surgery, radiation therapy, and chemotherapy. These methods aim to destroy cancer cells and control the growth of tumors in the breast tissue affected by cancer as well as controlling tumor growth on other tissues that are not affected by cancer (Bruton et al. 2005). Nevertheless, these treatments also have side risk effects that damage normal cell metabolism (Uzuner 2012). Therefore, the herbal materials can be a good option in the treatment of cancer due to the least side effects posed by herbal materials than side effects of chemical drugs (Nageh et al. 2018). In addition, the medicines derived from herbal materials are easily obtained (Nugrahaningtyas et al. 2005).

Pharmaceutical properties of herbal materials are found in essential oils. Essential oil is hydrophobic thus can interact with cell membrane and intracellular components cells or even interfere to damage the cells or even to slay the cells (Cristani et al. 2007). The genus *Curcuma* L (Zingiberaceae) represents a group of perennial rhizoma growing in tropical and subtropical regions such as Asia, Australia and South America (Ravindran et al. 2007). Estimated 93-100 of *Curcuma* species are widely spread. *Curcuma* is commonly used for the flavoring and coloring agent of Asian cuisine, traditional medicine, spices, dyes, perfumes, cosmetics, and ornamental plants (Chuakul et al. 2003). Some species of *Curcuma* have been widely used as medicine to treat bronchial complaints, pneumonia, diarrhea, leucorrhea, dysentery, infectious wounds or abscesses, and insect bites (Basaka et al. 2010). Genus *Curcuma* that widely found in Indonesia is *Curcuma aeruginosa* Roxb and *Curcuma zanthorrhiza* Roxb. *C. aeruginosa* and *C. zanthorrhiza* have a rhizome enriched with essential oil. Some research suggests that essential oils of these herbs contain terpenoids compounds (monoterpenoids and sesquiterpenoids) (Itokawa et al. 2008). *C. aeruginosa* both its extract and essential oil have

pharmacological activity. 70% ethanol extract of this plant had secondary metabolites compound that might inhibit the proliferation of cancer cells such as eucalyptol, β-elemene, camphor and isocurcumenol (Nurcholis et al. 2017). Essential oil of *C. aeruginosa* had antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* (Kamazeri 2012) and potent antibacterial activity against *Enterococcus faecalis* (MIC, Minimum Inhibitory Concentration = 6.25 µg/mL) and *Streptococcus mutans* (MIC = 15.63 µg/mL) (Theanphong et al. 2015; Wahyuni et al. 2017). The oil also showed strong radical-scavenging power evaluated by DPPH scavenging assay (EC_{50}, Efficiency Concentration = 24.32 µg/mL) due to the presence of germacrone and curzerenone (Theanphong et al. 2015). Other pharmacological activities of this oil were as antiandrogenic, antinociceptive, anti-inflammatory and antifungal (*Candida albicans*, MIC = 250 µg/mL) (Srivilai et al. 2018; Reanmongkol et al. 2006; Akarchariya et al. 2017). *C. zanthorrhiza* commonly known as *Temulawak* contains peculiar metabolite volatile compound Xanthorrhizol. Both extract and essential oil also showed pharmacological activity. Ethyl acetate extract of *C. zanthorrhiza* was able to inhibit the proliferation of MCF-7 cancer cells with IC_{50} (Inhibitory Concentration) value 1.17 µg/mL (Ngawi accession) and 6.83 µg/mL (Sukabumi accession) (Nurcholis et al. 2018). Biological activity of *C. zanthorrhiza* including as hepatoprotective and antinociceptive activities (Hartiwi et al. 2014). This oil effectively inhibited copper-mediated oxidation of LDL in thiobarbituric acid reactive substances assay (IC_{50} = 2.2 ± 0.1 µg/mL) (Jantan et al. 2012) and also showed some pharmacological activities such as antibacterial against *S.aureus* (ZOI, Zone of Influence = 11.53 ± 0.27 µg/mL), antifungal against *Candida albicans* (ZOI = 7.29 ± 0.17 µg/mL) (Akarchariya et al. 2017), antiproliferative, anti-inflammatory, antidiuretic, antioxidant, antihyperlipidemic, antiobesogenic and larvicidal (Ozaki 1990; Yasni et al. 1994; Sukari et al. 2007; Schmidt et al. 2015; Salleh et al. 2016).

To the best of our knowledge, cytotoxic potential and antiproliferative activity essential oil of *C. aeruginosa* and *C. zanthorrhiza* against larva shrimp and MCF-7 breast cancer cells in vitro have not been investigated previously. Therefore, the present study was focussed on understanding *C. aeruginosa* and *C. zanthorrhiza* essential oil as antiproliferative substance and to figure out its volatile compound.

MATERIALS AND METHODS

Plants materials
Samples of *C. aeruginosa* and *C. zanthorrhiza* (rhizome) were collected from Biofarmaka Conservation Units, Biofarmaka Tropika Research Center with latitude (S) 6˚34'37.95", longitude 106˚47'20.37" and altitude (m) 238, Bogor, Indonesia on July 2018.

Distillation of essential oil
Extraction of *C. aeruginosa* and *C. zanthorrhiza* essential oils were carried out using steam distillation. Total of 3000 g of the sliced samples was inserted into the distillation container and added water by ratio of 1:5 (sample: water) then heated to a temperature 100-105 ˚C for 4 hours. The essential oil produced then stored on 4 ˚C. Percent yield of essential oil obtained was calculated according to the following equation:

$$\% \ Yield = \frac{oil \ weight}{dry \ sample \ weight} \times 100\%$$

Essential oil cytotoxicity test with Brine Shrimp Lethality Test (BSLT)
The larvae egg of *Artemia salina* Leach were hatched on beaker glass contained seawater and aerator for 24 hours in case to alter it into larva. Essential oil solutions were made by dissolving it using seawater and tween 80 into concentration 1000, 500, 100, 10 µg/mL and 0 µg/mL (control). After 24 hours, a set of test plate containing 10 shrimp larvae, 1 mL seawater and 0.5 mL essential oil solution with each concentration was incubated for 24 hours then the number of dead larvae was calculated. The control was treated similarly except without the granting of essential oils. LC_{50} (Lethality Concentration) values were determined by using probit analysis SPSS 16.0.

Antiproliferative test of essential oils against MCF-7 cancer cells
Complete growing mediums containing 850 µL Dulbecco's Modified Eagle's Medium (DMEM), 10 µL gentamicin (antibiotic), 10 µL fungizone and 30 µL Fetal Calf Serum (FCS) were made for each MCF-7 and Vero cells line. Essential oil solutions were made with concentrations 30, 65, 100, 135 and 170 µg/mL and 0 µg/mL (controls) using DMSO 10% as solvent. Cell cultures then were made on 24 microplates which contained complete growing medium and essential oil solutions with 5 different concentrations. Positive control using doxorubicin and without the granting of essential oils as negative controls. 50 µL cell suspensions were inserted into each well triple. The cells culture then incubated inside the incubator at a temperature of 37 ˚C (5% CO_2).

After confluence about 3 days, the cells then harvested and counted. The calculation was conducted using a microscope with 100 times magnification. A total of 100 µL of cells suspension was homogenized in a microplate that had contained 5 µL trypan blue and 10 µL of homogenized cells suspension were placed in the hemocytometer and calculated the total number of cells. The percentage of percent inhibition activity was determined as follows:

$$\% \ Inhibition \ activity = 100\% - \left(\frac{treatment \ cell \ number}{negative \ cells \ number} \times 100\%\right)$$

GC-MS analysis

Analysis of volatile compounds contained in essential oil of each plant by using Agilent Technologies GC-MS machine with mass selective detector. 20 μL of essential oils were dissolved into 2000 μL dichloromethane (1:100 v/v). The samples were separated using capillary column HP INNOWAX (30 m x 0.25 mm, 0.25 μm thickness) with helium as carrier gas (0.6 mL/minute). Initial temperature for analysis was 60 °C raised to 150 °C and with the rise of 2 °C /min for about 1 minute then increased to 210 °C with the rate increase 20 °C /minute and maintained constant for 10 minutes. Separation rate ratios were 250:1. The temperature when the injection was 150 °C for 1 μL essential oil.

Data analysis

Statistical analysis using SPSS 16.0 probit analysis to measure the value of LC_{50}. Counted the IC_{50} value of cells inhibition by using linear regression analysis and Duncan test to find out the significant difference between samples.

RESULTS AND DISCUSSION

The yield of essential oils produced from distillation process was not plenty. This was due to several factors such as the condition of the material and the distillation time. Wither material will produce more yield of essential oil compared to fresh material because moisture content in the gland of the material had been reduced and the extraction was easier to do. Longer distillation times will result in more essential oil yields since the volatile compounds that are hard to evaporate will require longer heating time to evaporate, in addition, growing land and soil conditions also affect the yield of essential oils (Cassel and Vargas 2013). Distillation process obtained the volume of essential oils produced by each plant that presented in Table 1.

Preliminary test (BSLT) was conducted to find out the cytotoxicity of essential oils and declared as LC_{50}, the minimum concentration required to kill 50% of the population. An extract allegedly to have cytotoxic potential if it LC_{50} value is less than 1000 μg/mL (Meyer et al. 1982). All essential oils from tested plants had a toxic potential because it had a LC_{50} value below 1000 μg/mL (78,2 and 83.6 μg/mL for *C. aeruginosa* and *C. zanthorrhiza* respectively). Essential oils cytotoxicity was defined in LC_{50} (Table 2). The BSLT test results showed that all plants had toxic potentials thus also had potential as antiproliferative substance against MCF-7 cells.

Essential oil antiproliferative test against MCF-7 cancer cells are shown in Table 3. *C. zanthorrhiza* essential oil showed the most excellent inhibitory against MCF-7 cells (IC_{50}, 139.8 μg/mL) followed by *C. aeruginosa*. Antiproliferation activity of essential oils tested against MCF-7 cancer cells by using the total cells method according to Priosoeryanto et al. (1994) with the principle of negative control in the microplate containing cancer cells and growth media is considered to proliferate by 100% thereby the total treatment cells (given essential oils) as well as positive control (doxorubicin) will be compared to the total cells on the negative control and percent proliferation as well as percent inhibition can be obtained. Cells were incubated in incubator for 3 to 4 days because it takes 3-4 days for cells to grow and proliferate optimally. Less than 3 days the cells have not grown optimally and more than 4 days the cells will suffer death due to the lack of growth nutrients. The total cells count was performed with the hemocytometer under the microscope with magnification 100 times with trypan blue to clarify the display of cells under the microscope.

Results showed that cancer cells proliferation decrease as the concentration of the extract given increase. The biggest concentration in each essential oil of *C. aeruginosa* and *C. zanthorrhiza* were capable of inhibiting 50.2% and 59.1% of MCF-7 cancer cells in vitro respectively (Figure 1). N-hexane fraction of *C. aeruginosa* had potential as an antiproliferation against the MCF-7 cancer cells with IC_{50} value 69.5 μg/mL (Sriatun et al. 2016), thus proved that *C. aeruginosa* as volatile metabolite compounds as well as extracts can be functioned as antiproliferative cancer cells compound. Then *C. zanthorrhiza* essential oil was able to inhibit the growth of YBM-1 cancer cells with IC_{50} value 2.9 μg/mL (Udin 2013).

Test against Vero normal cells was also conducted to determine the effect of essential oils granting of these plants to normal cells proliferation. Figure 2 shows that essential oils of *C. aeruginosa* and *C. zanthorrhiza* do not interfere with normal cell proliferation with percent inhibition of 7.5% and 7.7% respectively. Inhibition of essential oils against MCF-7 cancer cells and Vero normal cells also compared to the administration of doxorubicin (drug used for cancer treatment) on both cells. Figures 1 and 2 indicate that doxorubicin at concentrations of 100 μg/mL not only inhibited MCF-7 cells by 65.1% but also damage the proliferation of Vero normal cells by 35.2%, while *C. aeruginosa* and *C. zanthorrhiza* at equal concentrations only inhibited the proliferation of Vero normal cells by 4.8% and 7.29% respectively and thus indicated that essential oils of *C. aeruginosa* and *C. zanthorrhiza* were safer against normal cells compared to doxorubicin.

Table 1. Yield and volume of essential oils distillation

Plant	Volume (mL)	Yield (%)
C. aeruginosa	1	0.03
C. zanthorrhiza	2	0.06

Table 2. Plant essential oil cytotoxicity against *A. salina*

Plant	LC_{50} (μg/mL)
C. aeruginosa	78.2 ± 7.3
C. zanthorrhiza	83.6 ± 12.1

Table 3. IC_{50} essential oils of each plant against MCF-7 cell

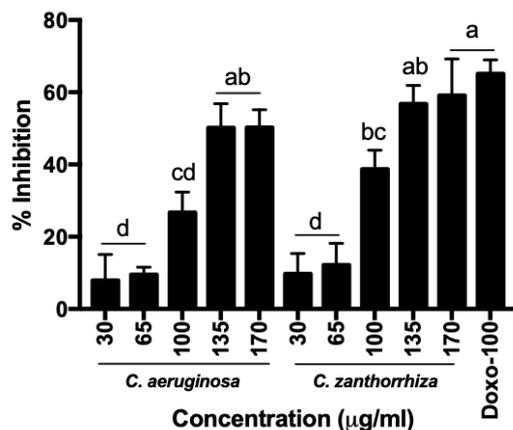| Plant	IC_{50} (μg/mL)
C. aeruginosa	161.0
C. zanthorrhiza	139.8

Figure 1. Inhibition graph of *C. aeruginosa* and *C. zanthorrhiza* essential oil against proliferation of MCF-7 cells line. Value followed by the same letters indicates they are not significantly different by Duncan test at p ≤ 0.05. Doxo = doxorubicin.
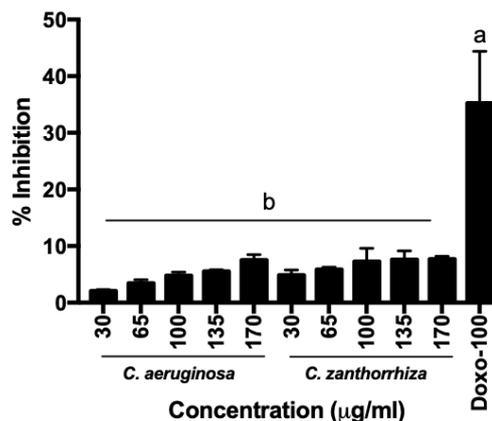

Figure 2. Inhibition graph of *C. aeruginosa* and *C. zanthorrhiza*'s essential oil against proliferation of Vero normal cells line. Value followed by the same letters indicates they are not significantly different by Duncan test at p ≤ 0.05. Doxo = doxorubicin.

Table 4. Major volatile compound of each plant identified using GC-MS analysis

Retention time (Min)	Library identification	Peak area (%)	
		C. aerruginosa	*C. zanthorrhiza*
3.84	Toluene	4.78	-
7.31	**Eucalyptol**	**17.90**	**0.35**
20.33	**Camphor**	**5.31**	**9.06**
49.92	**Tropolone**	**18.07**	**-**
51,27	**Curcumol**	**5.69**	**-**
51.35	(Z, Z)- 3,6-Nonadienal	3.86	-
21.97	**alpha-Curcumene**	**-**	**15.05**
22.46	**Curzerene**	**-**	**3.90**
23.11	**beta-Curcumene**	**-**	**17.04**
24.64	Germacrene B	-	2.08
26.10	Epicurzerenone	-	5.97
27.61	**Germacrone**	**-**	**5.43**
28.61	**Xanthorrhizol**	**-**	**26.77**

Note: Bold faceted compounds allegedly play a role as antiproliferative compound

Volatile compounds from GC-MS results of the plants acquired a total of 38 compounds identified. Major compounds in *C. aeruginosa* were 5 compounds: eucalyptol (17.90%), camphor (5.31%), tropolone (18.07%), curcumol (5.69%) and (Z, Z)-3.6-nonadienal (3.86%) while major compounds found in *C. zanthorrhiza* were 8 compounds: camphor (9.06%), alpha-curcumene (15.05%), curzerene (3.90%), beta-curcumene (17.04%), germacrene B (2.08%), epicurzerenone (5.97%), germacrone (5.43%), and xanthorrhizol (26.77%). GC-MS chromatogram showed that mostly terpenoid (monoterpenoid and sesquiterpenoid) group were the major volatile compound contained in essential oil of each plant (Table 4).

GC-MS chromatogram analysis showed that major compounds of the plants were terpenoid group (monoterpenoid and sesquiterpenoid). Terpenoids are one of the secondary metabolites compounds which are widely produced by plants, especially in its vacuoles. Terpenoids are contained in the essential oil of plants. Terpenoids are compounds with a large structure constructed from units of isoprene (C5). Isoprene is derived from metabolism of acetic acid through the mevalonic acid (MVA) line (Sukadan 2008). Total of volatile compounds identified in GC-MS analysis were 38 compounds, but only 13 major compounds identified with peak area greater than 2%. Among those 13 major compounds, 9 compounds were allegedly played role as antiproliferative compound (Table 4).

Tropolone was a compound contained in *C. aeruginosa* that was suspected to inhibit the proliferation of cancer cells. Tropolone and its derivatives were great to inhibit several cancer cells such as HCT-116 (colon cancer), BXPC-3 (pancreatic cancer), Jurkat (leukemia) and HuT-78 (T-lymphocytes) with Growth Inhibitory value (GI_{50}) ranges between 5 and 30 μM. It had a good selectivity for normal cell hDf (Human Dermal Fibroblasts cell) with GI_{50} value 95 μM (Ononye 2013). Tropolone was suspected to inhibit proliferation of cancer cells by inhibiting Histon Deacetylase (HDAC), an enzyme that plays a role in several biochemical processes such as transcription regulation, tubulin regulation and cytoskeletal function, controlling cardiac growth, thymocyte development regulations and facilitating DNA repair (Huangfu et al. 2008; Granger et al. 2008; Ouaissi et al. 2011; Estiu et al. 2010; Somoza et al. 2004). Some epigenetic pathways related to cancer, one of them is acetylated gene controlled by Histon Acetyltransferases and Histon Deacetylase which regulates gene expression and chromatin structure. Histone deacetylase inhibitors become a new target in anticancer therapy by reactivating tumor suppressor gene (Goswami et al. 2018). By inhibiting HDAC it will inhibit cell growth and induce cell differentiation (Lemoine and Younes

2010). Another most prevalent volatile metabolites found in *C. aeruginosa* was eucalyptol. 1,8-cineole, which is another name of eucalyptol, was able to inhibit the growth of HL-60 cancer cells (human leukemia) by inducing apoptosis process (Moteki et al. 2002). Eucalyptol can activate survivin (an apoptosis protein inhibitor that lowers the apoptosis process) and Akt pathway/phosphatidylinositol-3-kinase (PI3K) (cell signaling pathway that promotes cell growth and will be the target of apoptosis) as well as activating the P38 MAP kinase (part of the MAP kinase family that is activated by cellular stress and causes apoptosis). Eucalyptol also induced the cutting of Poly ADP ribose polymerase (PARP) and caspase-3 thereby causing apoptosis (Murata et al. 2013). Eucalyptol/1, 8-cineole was also supposed to have a role in suppressing the proliferation of MCF-7 cancer cells due to its abundance in *C. aeruginosa*. Curcumol had been widely reported to have potential as antiproliferation of cancer cell. Granting of curcumol in MDA-MB cancer cells (breast cancer) was able to inhibit the proliferation of cells with IC_{50} value 100.2 μg/mL by arresting cell in G1 phase and triggering apoptosis through mitochondrial pathway (Huang et al. 2017). Other compounds that were also identified in *C. aeruginosa* were camphor. According to Itani et al. (2008), camphor was able to inhibit cell cycles and induced apoptosis via mitochondrial pathway in HCT-116 cells in in vitro by 30% then stopped the cell proliferation. Choudhury et al. (2018) explain that cancer cells were able to avoid apoptosis process by over-activation of anti-apoptosis genes and deregulation pro-apoptosis gene. B-cell lymphoma 2 (BCL-2) is an important member in regulating death and survival of cells. BCL-Xl is an anti-apoptosis protein. P53 which is a Tumor suppressor protein confers control over cell cycle and apoptosis. Camphor allegedly led to up-regulation of p53 thereby lowering the anti-apoptosis protein (BCL-Xl) levels and raising pro-apoptosis protein (Bax) levels.

Peculiar volatile metabolites contained in *C. zanthorrhiza* is xanthorrhizol which had the potential as antiproliferation substance of cancer cells. Xanthorrhizol was able to inhibit the proliferation of MDA-MB (breast cancer) cells with GI_{50} value 8.67 μg/mL by triggering apoptosis through mitochondrial pathways (Cheah et al. 2008) as well as inhibiting the proliferation of MCF-7 cancer cells with Effectivity Concentration value (EC_{50}) 1.71 μg/mL by apoptosis induction through modulation of BCL-2, p53 and PARP-1 protein (Cheah et al. 2006). Xanthorrhizol was also able to decrease the proliferation of HeLa cells (cervical cancer) with EC_{50} value 6.16 μg/mL through up-regulation of tumor suppressor proteins p53 and Bax apoptosis pro proteins. Beta-curcumene and Ar-curcumene contained in *C. zanthorrhiza* also reported being an anti-proliferation compound for cancer cells. Curcumene causing apoptosis in cancer cells through mitochondrial pathway that secreted the cytochrome C and activates the caspase resulting in cell apoptosis (Shin and Lee 2013). Germacrone was a potential anti-proliferation compound of cancer cells. The provision of germacrone in MDA-MB cells lowered the viability of the cell through

apoptosis mitochondrial pathway and arrested the cell cycle in the G0/G1 and G2/M phases by decreasing retinoblastoma protein suppressor tumor (Rb) which plays a role in regulation of cell proliferation, lowering cyclin-dependent kinases (CDK-2) thus preventing cells from entering the mitosis phase as well as lowering total phosphorylation of CDK-2 (Zhong et al. 2011). Another volatile metabolite compound on *C. zanthorrhiza* potentially as antiproliferation substance was curzerene. According to Wang et al. (2016), curzerene was able to inhibit the proliferation of SPC-A1 (lung cancer) cells with an IC_{50} value 47 μM by arresting cell in the G2/M phase and triggering cell apoptosis. Curzerene was able to lower the expression of GSTA1 proteins and mRNA expressions in SPC-A1 cells.

The abundance of volatile compounds contained in *C. aeruginosa* and *C.zanthorrhiza* essential oil that is supposedly potential to inhibit the proliferation of cancer cells, resulting in its necessity to explore further the potential of these plants.

ACKNOWLEDGEMENTS

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