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Anticancer Activity of *Curcuma zedoaria* (Berg.) Roscoe Essential Oils Against Myeloma Cells

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ABSTRACT: Cancer is an abnormal and uncontrolled of cell growth. Curcuma zedoaria (Berg.) Roscoe is a plant that can be used for treating various diseases such as cancer. This research aims to identify and to study the bioactivity of the rhizome of Curcuma zedoaria essential oils towards myeloma cells. The rhizome was distilled using steam distillation to obtain its essential oils. Toxicity test towards *Artemia salina* L. was used as a prescreening of anticancer oils. The essential oils were analyzed by GC-MS (Gas Chromatography–Mass Spectroscopy). While In vitro anticancer test was carried out towards myeloma cells. About 20 kg of the rhizome produced 15.30 mL (13.49 g) of yellowish essential oils. The prescreening test towards *Artemia salina* L. showed that the oils were toxic with the LC_{50} of 19.95 ppm. Eight compounds (α-pinene; champhene; camphor; 1-ethenyl-2,4-bis(1-methylethenyl) cyclohexane; 1,8-sineol; furanodiene; germacrone; and velleral) can be identified of the compounds of the oils based on their similarity with the GC-MS database. In vitro test toward myeloma cells showed that oils can inhibit the growth of myeloma cells, but cannot be said to be anticancer since the LC_{50} was greater than 1000 ppm.

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INTRODUCTION

According to the World Health Organization (WHO), cancer patients in the world were increased 6.25 million people each year. In Indonesia there were 100 new cancer patients from 100,000 residents each year. Cancer has become one of the major causes of death of childbearing age. Cancer arise from abnormal growth of some body tissue cells that turn into cancer cells and cancer cells one day could spread throughout the body. Although the cause is still uncertain but there are several factors that can increase the risk of cancer, one of which is carcinogenic.[1]

Cancer treatment can be done medically or traditionally. Medical treatment such as surgery, radiotherapy, and chemotherapy sometimes can cause side effects. Therefore, we need an alternative medicine from natural ingredients that can inhibit or cure cancer selectively, effectively, and no side effects.[2] Indonesia plant diversity provides the potential for the development of a traditional medicine that can inhibit the growth of cancer cells. One plant that is believed to overcome the development of the cancer cells is *Curcuma zedoaria* (Berg.) Roscoe. The rhizome of the plant is usually used for the treatment.

Several studies showed that the rhizomes of *Curcuma zedoaria* have benefits such as having antimicrobial activity.[3–5] Seo *et al.*, (2005) reported that water extracts of the rhizomes played a role in inhibiting the spread of B16 melanoma cells, while Kim *et al.*, (2005) stated that the water extract of the roots of *Curcuma zedoaria* can be used for the treatment of chronic liver disease.[6,7]

Monoterpene contained in Essential oils of the rhizomes of *Curcuma zedoaria* have activity as antineoplastic (anticancer). The essential oils (epikurzerenon and kurzerena) also have antioxidant activity.[8] Sukmana (2006) reported that the curcuma extract given into a male mouse can increase the number of colonic mucosal cells of mice that undergo apoptosis after being exposed 9,10-dimethyl-1,2-benz-(a) anthracene (DMBA), so it can be concluded that the extract can be used to treat colon cancer.[9]

Curcuma zedoaria rhizomes contained 1 to 2.5% of volatile oils with the main composition of sesquiterpene. It also

contained flavonoids, sulfur, gum, resin, starch, and less fat.[10] While the essential oils in the curcuma collected from India contained 1.8-sineol (15.9%) and germacrone (9.0%).[11] Meanwhile in the essential oils *Curcuma zedoaria* rhizome obtained around Bali have antioxidant activity with a reduction of 64.63%% in the first 5 minutes and 73.63% after 60 minutes, and can inhibit the growth of the fungus *Candida albicans*.[12]

Based on preliminary tests, the essential oils contained in the *Curcuma zedoaria* rhizomes was toxic towards the larvae of *Artemia salina* L., with Lethal Concentration (LC_{50}) of 19.96 ppm. Hence the oils are expected to have activity as anticancer agent.

Myeloma is a cancer that affects B cells, the immune cells responsible for the production of antibodies. Normal B cells develop in bone marrow. Myeloma therefore affects bones. Multiple myeloma arises when the cancer cells travel through the body and form tumors in several different bones. Affected bones may become brittle as the malignant cells proliferate and alter the chemical equilibrium in the marrow.[13]

So far, research on the anticancer activity of the essential oils against murine myeloma cells has never been reported, therefore this study aims to identify compounds contained in the essential oils of *Curcuma zedoaria* rhizome and to examine anticancer activity of the oils against the myeloma cells.

METHODS AND EXPERIMENTAL DETAILS

This study was carried out by exploratory and experimental methods. The research was conducted at the Laboratory of Marine Genetic Resources Development and Genetic Engineering University of Udayana. The anticancer test towards myeloma cells was performed at the Laboratory of Veterinary Virology University of Udayana. Analysis compounds were conducted at the Laboratory of Organic Chemistry Faculty of Gajah Mada University, Yogyakarta.

Materials and Chemicals

Materials used in this study were *Curcuma zedoaria* (Berg.) Roscoe rhizome obtained from Damai Puri Ubud, Bali. Biological material as toxicity tests was *Artemia salina* Leach larvae, while material for anticancer test was a murine myeloma cell type P3UI. Chemicals used in this study were distilled water, dimethylsulfoxide (DMSO), anhydrous calcium chloride ($CaCl_2$), and yeast. Powdered, RPMI 1640 culture medium containing HEPES, murine myeloma cells P3UI type, FBS serum, and Trypan Blue.

Equipments

The equipments used in this study were a set of steam distillation (distillation Stahl), analytical balance, micro-pipettes, pipette volumes, pipette drops, test tubes, multi-well plates (24 well), culture flasks (25 cm), culture flasks (75 cm), pippetes tip (1 mL), pippetes tip (10 mL), filter paper, and Gas Chromatography-Mass Spectroscopy (GC-MS).

Experiments

Isolation of essential oils. Isolation of essential oils was carried out by stem distillation (Stahl distillation). About 20kg of Curcuma zedoaria rhizomes were distilled. By using distilled Stahl, oil can be immediately separated from the water, but the oil needs to be released again from the remnants of the water by adding anhydrous CaCl2 to bind water. Essential oils obtained can be used for toxicity tests on larvae of *Artemia salina* L., anticancer test in vitro against murine myeloma cells, then the compounds were analyzed using GC-MS.

Toxicity test of the essential oils toward *Artemia salina* Leach larvae. Toxicity test used method that had been described in.[14] The test was done by placing a number of seawater in a sealed aquarium in two parts. One part was covered with black paper and the other was left open. Shrimp eggs were laid on the dark part and stored in a place that has adequate lighting for 48 hours, so that the eggs hatched and ready to be used for testing.

In vitro anticancer test against myeloma cells. In vitro anticancer test of the toxic isolate against mice myeloma cells was conducted following the method that has been reported by Hidayat (2002).[15]

Preparation of test solutions. Preparation of test solution was conducted by adding 1mL of 10% DMSO sterile into 50mg of essential oil until dissolved. Then the solution was added 10 mL of distilled water, mixed until homogeneous, and inserted into a sterile tube with a lid in order to obtain the solution with concentrations of 5000 ppm (solution A). Fourscrew-tubes sterile were prepared. 1 mL of solution A was inserted in the tube 1, add 4 mL of distilled water and mixed until homogeneous to obtain a concentration of 1000 ppm (solution B). A 100 ppm solution was obtained by taking 1 mL of solution B in tube ,2 added with 9 mL of distilled water and mixed until homogeneous. The same procedure was done in tubes 3 and 4 in order to obtain a solution concentration of 10 and 1 ppm respectively.

For negative controls, only 1 mL of 10% DMSO and 9 mL of distilled water were added, mixed until homogeneous, and then put into a sterile tube with a lid. It was aimed to

ensure that the cytotoxic effects of this were not caused by the medium.

Preparation of murine myeloma cell culture. P3UI type murine myeloma cells, FBS serum, RPMI medium containing HEPES and NaHCO3 were prepared. The next process was done with aseptic technique under laminar air flow cabinet (LAFC).

Murine myeloma cell thawing process. The myeloma cells in Rosewell Park Memorial Institute media (RPMI) were centrifuged at 1500 rpm for 5 min at 4°C. Supernatant was separated from the residue and then the cells were grown in media using 10% FBS in the culture bottles. Antibiotic ampicillin 100pg/mL was added to prevent contamination, then the culture was stored in 5% CO2 incubator at 37 °C for 24 hours.

Initiation of murine myeloma cell culture. Three sterile multi welled culture plates were used for the initiation. The myeloma cell of 2 mL from the thawing process was homogenized and poured into each well. Cells are then incubated in an incubator containing 5% CO_2 at 37°C for 24 hours. The number of cells per/mL ≥ 104 cells / mL was calculated.

Inhibition test of the murine myeloma cell growth. If it was considered to have sufficient number of cells, each plate was filled with 0.2mL essential oils and 0.8 mL of myeloma cells, then culture was stored in 5% CO2 incubator at 37 °C for 24 hours. Culture cells were harvested with a cell shed from the walls of wells of each treatment and then examined under a microscope. Each treatment inserted into the vial and 1 mL of cell was taken, added with 1 mL of the dye trypan blue 0.4% in. By using a Thoma counting technique under the microscope, the percentage of cell viability was counted. The percentage of cell viability is the number of live cells divided by the number of total cells (live cells and dead cells) multiplied by 100%. The time between the staining and cell counting was done between 3 and 10 minutes to avoid false positive results.

The number of live cells (not stained) and the number of dead cells (stained) that are visible in the haemocytometer count were counted. Haemocytometer count has 9 squares on each side. There are two rules for calculating the cell, if the cells that touch the left and top of each area are included the count; cells that touch on the right and below the line count are not included in the count.

Media containing myeloma cells were diluted with triptan blue 0.4% staining. Before entering the cell to be counted, haemocytometer was covered with a cover glass and filling the cells have to fill the space provided. Calculations were performed with a magnification of 100×.

Identification of Compounds in Essential Oils by GC-MS

Essential oils obtained were analyzed by GC-MS to determine the constituent of the oil. Mass spectra obtained were compared with standard spectra of known compounds in a database that has been programmed on the GC-MS instrument.

RESULTS AND DISCUSSION

About 20 kg of the Curcuma zedoaria rhizome were distilled and produced 15.30 mL (13.49 g) of yellowish essential oils with a density of 0.88 g/mL. The oil obtained was used for toxicity test on larvae of *Artemia salina* L.

The results of toxicity tests of the rhizome essential oils against *Artemia salina* L. larvae were presented in Table 1. Toxicity test results indicated that the essential oils gave LC_{50} of 19.95 ppm (Figure 1). It can be said that the rhizome essential oils were toxic toward *Artemia salina* L. larvae. LC_{50} is the concentration that causes 50% mortality of larvae. If the LC_{50} was below 1000 ppm, the sample can be said to be toxic,[14] so that the oils can be developed as antitumor agent.

Table 1. Mortality of the Brine Shrimp Larvae after 24 hours of exposure to various concentrations of the Curcuma zedoaria essential oil

Concen-tration	Dead	Alive	Accumulated Dead	Accumulated Alive	Mortality
1000 ppm	10.0	0.0	23.0	0.0	100.0
100 ppm	10.0	0.0	13.0	0.0	100.0
10 ppm	3.0	7.0	3.0	7.0	30.0

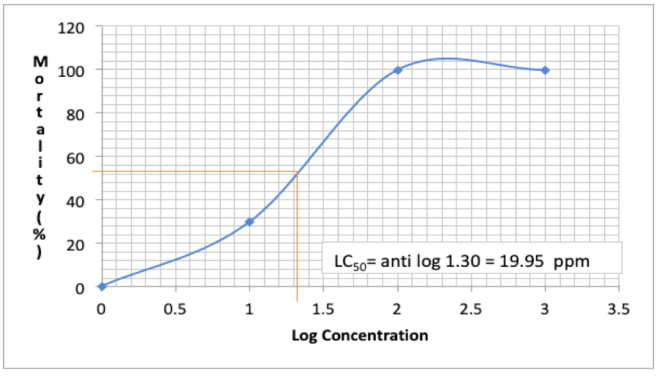

Figure 1. Estimation of LC50 by a plot of percent mortality against log concentration.

Curcuma zedoaria rhizome essential oils obtained from steam distillation process were then analyzed to determine the compound of the oils by using GC-MS. GC-Chromatogram showed the oils contained 8 the main compounds, peaks 1, 2, 3, 5, 8, 10, 14, and 16 (Figure 2).

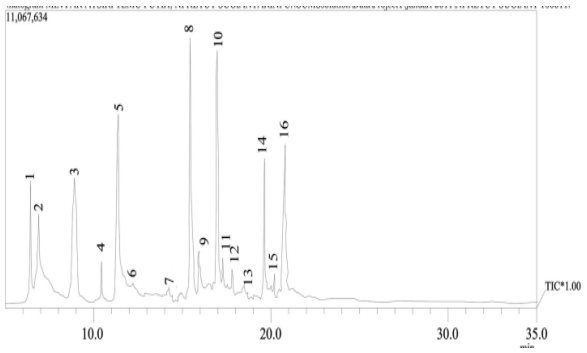

Figure 2. Chromatogram of Curcuma zedoaria essential oils.

Each peak was identified further by a mass spectrometer, wherein each compound has a specific mass fragmentation pattern. Identification of the compounds were done by comparing the mass spectra of the compounds in GC-MS database, so it can be presumed the mayor compounds of the *Curcuma azedoaria* essential oil. The names of the compounds were shown in Table 2, while the structures of the compounds were presented on Figure 3.

Table 2. Major compounds of essential oil of *Curcuma zedoaria*

Peak	M$^+$	Retention time (minutes)	Compounds
1	136	6.442	α-pinene
2	136	6.884	Camphen
3	145	8.906	1,8-sineol
5	152	11.403	Camphor
8	189	15.460	β-elemene
10	216	16.988	Furanodiene
14	218	19.624	Germakron
16	232	20.776	Velleral

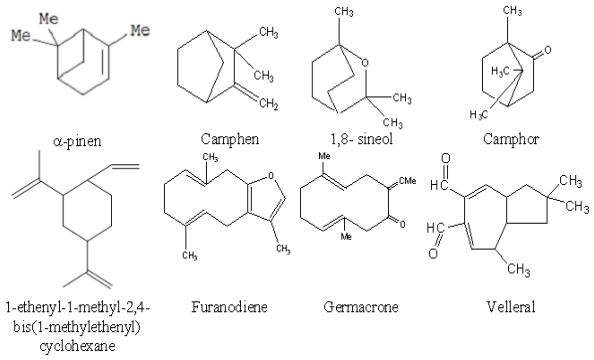

Figure 3. Structure of the compounds contained in the essential oil of *Curcuma zedoaria.*

There were 8 mayor compounds in *Curcuma zedoaria* essential oils, those were α-pinene, Camphen, 1,8-sineol, Camphor, β-elemene, Furanodiene, Germakron, and Velleral. Several scientists reported that β-elemene and furanodiene have responsibility to the oil anticancer activity.

Sun *et al.*[16] reported that furanodiene was able to inhibit the growth of Hela, Hep-2, PC3, HL-60, SGC-7901 and HT-1080 cells with IC_{50} between 0.6-4.8 μg/mL. In vivo, furanodiene was also found to exhibit inhibitory effects on the growth of uterine cervical (U14) and sarcoma 180 (Sl80) tumors in mice. Furanodiene combined with tamoxifen (TAM) could enhance the growth inhibitory of breast cancer cells, without contributions to the cytotoxicity of TAM.[17]

Yao *et al.* (2008) reported that beta-Elemene extracted from *Curcuma wenyujin* has been used as an anti tumour drug for different tumours, including glioblastoma. The growth of glioblastoma cell-transplanted tumours in nude mice was inhibited by intra peritoneal injection of beta-elemene.[18] While Zheng *et al.* (2009) stated that β-elemene combined with aclaburin has ability to enhance the effects of apoptosis of HL-60 cells and can be used in the treatment of anti leukemia.[19] The beta-elemene obviously inhibited the proliferation of RPMI-8226 cells in both time- and dose-dependent manners.[20] Li *et al.* (2010) reported that beta-elemene exerted broad-spectrum anti tumour activity against many types of solid carcinoma and supported a proposal of beta-elemene as a new potentially therapeutic drug for castration-resistant prostate cancer and other solid tumours.[21] Chen *et al.* (2011) reported that β-elemene inhibited melanoma growth and metastasis through suppressing VEGF-mediated angiogenesis. It is a natural potential anti angiogenic agent.[22]

Determination of anticancer activity against murine myeloma cells was done using the cell viability. From the observations, the number of cells was as much as 5 × 10^5 cells/mL. This amount is considered to have been sufficient to test the anticancer of the essential oils against myeloma cells. The living and dead cells can be observed under the microscope. Myeloma cells, before and after being given the oils under the microscope, are shown in Figure 4.

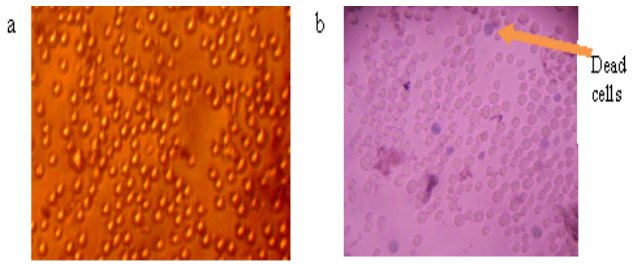

Figure 4. Myeloma cellsunder the microscope (100x): a) the health cells; b) after given the *Curcuma zedoria* essential oils.

Staining cells with trypan blue solution aims to distinguish the living and dead cells. The dead cells will look blue because of lysis, so that the plasma protein will bind to the trypan blue, the cells become blue. In addition,

cells that die will look darker in color and shape is not round anymore or shrinks as the contents of the cell (cytoplasm) come out. This does not occur in living cells because no damage on the cell membrane, so that living cells are still looking round, brighter, and clearer.[15]

Table 3. Murine myeloma cell viability after being given the essential oils

Concentration (ppm)	Repetition	The number of cells (10^4) Alive	Dead	Total	Percent of Viability	Percent of Mortality
0	1	38.50	0.50	39.00	98.72	1.56[a*]
	2	36.25	0.50	36.75	98.64	
	3	35.75	0.75	36.50	97.95	
1	1	34.00	1.50	35.50	95.77	4.00[b]
	2	34.75	1.25	36.00	96.53	
	3	28.50	1.25	29.75	95.80	
10	1	26.50	2.25	28.75	92.17	8.00[c]
	2	20.75	2.00	22.75	91.21	
	3	22.00	1.75	23.75	92.63	
100	1	27.25	4.50	31.75	85.83	15.33[d]
	2	14.25	3.00	17.25	82.61	
	3	20.75	3.50	24.25	85.57	
100	1	7.75	3.00	10.75	72.09	26.78[e]
	2	11.25	4.25	15.50	72.58	
	3	15.00	5.00	20.00	75.00	

*Values followed by the same letter in the same column are not significantly different according to the Duncan's Multiple Range Test at P < 5%.

Observation and calculation of percent viability of myeloma cells after a given number of essential oils are presented in Table 3. The table showed that the mortality percentage of myeloma cells increased significantly (P<0.05) with the increase of the oil concentration. Based on the table, it can be concluded that oils can inhibit the growth of myeloma cells, because the increase of the oil concentration was followed by the higher of percent mortality cells. However, it cannot be said to be anticancer agent since the LC_{50} was greater than 1000 ppm. It can also be presented in Figure 5.

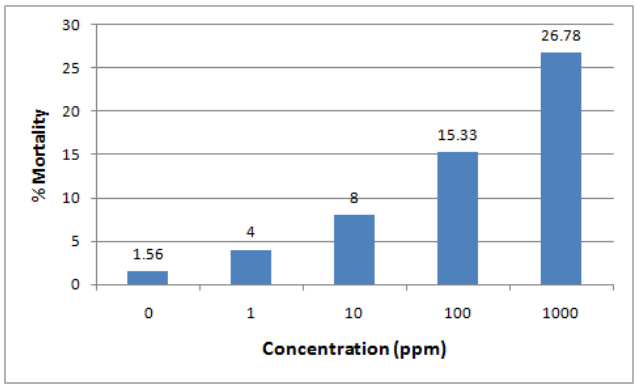

Figure 5. The graphs of comparisons between the mortality of the concentration.

CONCLUSION

Based on these results, it can be concluded that Curcuma zedoaria rhizome essential oils contained 8 mayor compounds, i.e. α-pinen, camphen, 1,8- sineol, camphor, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane, furanodiene, Germacrone, and velleral. The oils can inhibit the growth of murine myeloma cells, but cannot be recommended to be anticancer agent for the cells.

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