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Research Article

Isolation of Sesquiterpenes Lactone from *Curcuma aeruginosa* Rhizome and the Cytotoxic Activity Against Human Cancer Cell Lines

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ABSTRACT
The objectives of this research were to isolate bioactive compounds from *Curcuma aeruginosa* Roxb. and to study the cytotoxic activity against human cancer cell lines. The *in vitro* cytotoxicity test was done on human cancer cell lines such as *Breast carcinoma MCF-7* and *T-47D; Cervical carcinoma Ca Ski* and *Hela S3* by MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cytotoxicity test was also conducted on *Vero* cells (normal cells). The isolation of bioactive compounds from this extract of *C. aeruginosa* rhizome was carried out by chromatographic method and the structure elucidation was performed by interpretation of spectroscopic data, including UV, IR, ^{1}H and ^{13}C NMR 1D and 2D. The study showed that *n*-hexane and chloroform fraction from *C aeroginosa* had low cytotoxic activity against *MCF-7* and *Ca-ski* (IC_{50}<100µg/mL), but not toxic against *Hela S3*, *T-47D*, and *Vero* cell lines (IC_{50}>500µg/mL). From the chloroform fraction of *C. aeroginose* we isolated a new sesquiterpene lacton aeruginon (1) and a known compound curcumenon (2). It can be concluded that according to the present study, *C. aeruginosa* can be used as a potent source of natural bioactive compounds that is rich in sesquiterpene compounds.

Keywords: *Curcuma aeroginosa; cytotocix effects; aeruginon; curcumenon*

INTRODUCTION

Nature is still a rich source of active compounds against cancer cells. Identification and development of natural compounds and their derivatives have greatly contributed to this progress, and many of these compounds are now used in clinical practice resulting in increased effectiveness of the therapy. The discovery of drugs to fight against cancer is mostly done by experts mainly from traditional medicinal plants. Nonetheless, a number of naturally derivative agents has been included in clinical trials and is terminated due to lack of efficacy or unacceptable toxicity[1,2]. Zingiberaceae family constitutes a vital group of rhizomatous medicinal and aromatic plants characterised by the presence of volatile oils and oleoresins of export value. Generally, the rhizomes and fruits are aromatic, tonic, stimulant, and occasionally they are nutritive. Some are used as food as they contain starch in large quantities while others yield an astringent and diaphoretic juice. The important genera coming under Zingiberaceae are *Curcuma, Kaempferia, Hedychium, Amomum, Zingiber, Alpinia, Elettaria* and *Costus*[2,3]. *Curcuma* is widely distributed in tropical and subtropical regions of Asia, especially Indonesia, Thailand, and Malaysia. One of *Curcuma* species that is found in Indonesia is *C. aeruginosa*. The plant is known by local name,"temuireng". The rhizome of *C. aeruginosa* has been used as disinfectant, expectorant, anthelmentic, antifungal, febrifuge, anti-inflammatory and tonic[2,3]. Thaina[4] reported that the extracts of *C. aeruginosa* in methanol and chloroform might be useful as tocolytic agents for the prevention of preterm labor. The *C. aeruginosa* rhizome contains various chemical classes of compounds including terpenoids, sterols, organic acids, fatty acids and sugars[5,6]. Suphrom[7] reported six classes, sesquiterpenes germacrone, zederone, dehydrocurdione, curcumenol, zedoarondiol, and isocurcumenol from *C. aeruginosa* rhizome. The compounds showed inhibitory activity against the conversion of testosterone to dihydrotestosterone[7]. This paper reports the cytotoxicities against the *Breast carcinoma MCF-7* and *T-47D*, *Cervical carcinoma Ca Ski* and *Hela S3* cell lines through 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays for the extract of *C. aeruginosa* rhizome; the cytotoxicity against normal cells using a *Vero* cell lines, and characterization of the two sesquiterpenes isolated from *C. aeruginosa* rhizome.

MATERIAL AND METHOD

Apparatus and reagent

UV and IR spectra were recorded by Varian Cary 100 Conc and Shimadzu 8300 FTIR. ^{1}H and ^{13}C NMR spectra were recorded with Jeol JNM A-5000 spectrometer, operating at 500.0 MHz (^{1}H) and 125.0 MHz (^{13}C) using residual and deuterated solvent peaks as internal standards. Vacuum liquid chromatography (VLC) was carried out using Si gel Merck 60 GF_{254} (230-400 mesh), column

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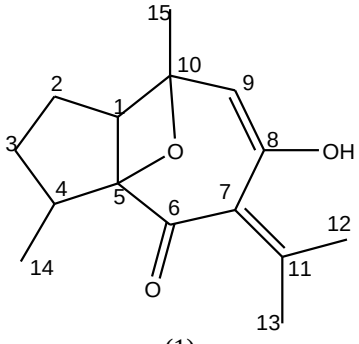
Table 1: Activity test of methanol extract and fraction of *C. aeroginosa* against cancer cell lines

S No.	Extract/ fraction	LC_{50} (µg/mL)				
		MCF-7	Ca Ski	Hela S3	T-47D	Vero
1	Methanol extract of *C. aeroginosa*	>100	95.73 ±3.06	> 500	> 500	> 500
2	*n*-Hexane *fraction of C. aeroginosa*	69.47± 2.16	66.02 ±0.45	> 500	> 500	> 500
3	Chloroform *fraction of C. aeroginosa*	92.60 ± 4.10	94.87±1.94	> 500	> 500	> 500

Table 2: ^{1}H and ^{13}C NMR data of compounds (1 and 2)* in chloroform

No carbon	Aeruginon (1)		Curcumenon (2)	
	δ C ppm	δ H (∑ H; m; *J* Hz)	δ C ppm	δ H (∑ H; m; *J* Hz)
1	60.7	1.93 (1H; br s)	24.2	0.62 (1H; m)
2	27.3	2.46 (2H; dd; 15.3; 10,7)	23.5	2.07 (2H; m)
3	37.3	1.93 (1H, br s); 1,95 (1H, br s)	43.9	2.40 (2H; m)
4	37.0	1.62 (1H, s)	208.9	-
5	83.2	-	24.2	1.63 (1H; m)
6	194.8	-	28.1	2.77 (2H; d;
7	133.0	-	128,2	-
8	152.3	-	201.8	-
9	127.9	5.6 (1H, s)	49.0	2.40 (2H, br s)
10	86.2	-	20.2	-
11	144.0	-	147.6	-
12	22.3	1.72 (3H, s)	23.6	1.76 (3H, s)
13	22.4	1.87 (3H,s)	23.5	0.63 (3H,s)
14	23.1	1.91 (3H, s)	19.1	1.09 (3H, s)
15	23.9	1.23 (3H,s)	30.1	2.12 (3H,s)

* measured with chloroform ($CDCl_3$) 500.0 MHz (^{1}H) and 125.0 MHz (^{13}C)



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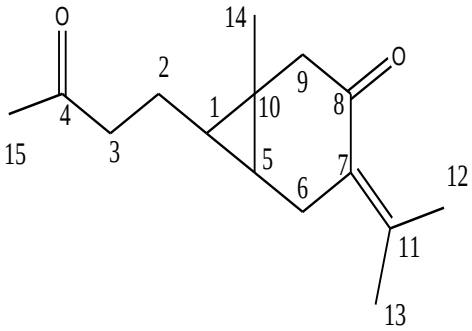

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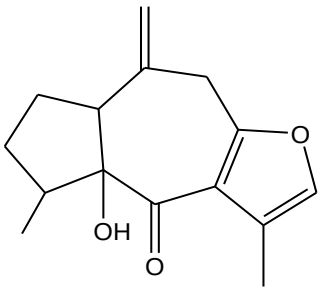
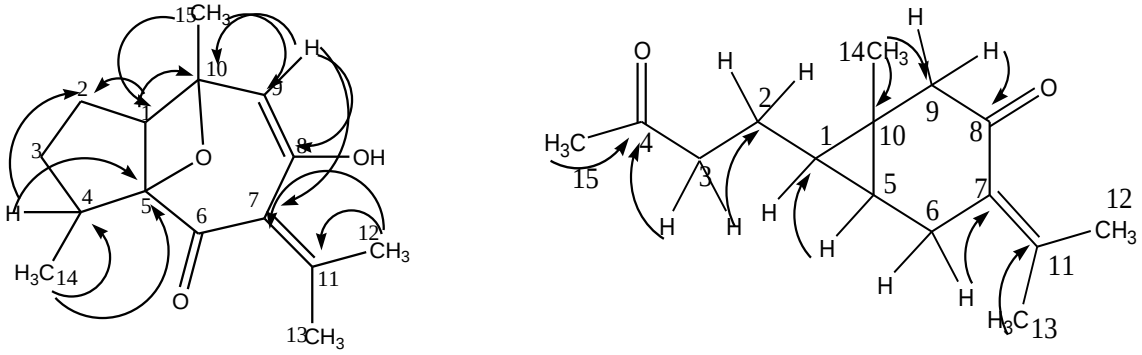


(3)

Figure 1: Structure of aeruginon (1), curcumenon (2), and zedoarol (3)

chromatography using Si-gel Merck 60 (200-400 mesh), and TLC analysis on pre-coated Si gel plates Si-gel Merck Kieselgel 60 F_{254} 0.25 mm, 20 x 20 cm. Many solvents such as chloroform, *n*-hexane, ethyl acetate, acetone, ethanol, and methanol were used for isolating the compound. *T-47D* and *Hela S3* cell culture were obtained from Parasytology laboratory, Gadjah Mada University, Indonesia, and grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with fetal bovine serum 10% (FBS; Gibco), and1% Penicillin-Streptomycin



(1) (2)

Figure 2: Significant HMBC (H→C) correlations of aeruginon (1) and curcumenon (2)

(Gibco) at 37^{0}C and with a flow of CO_2 5% (Heraeus). *MCF-7* and *Caski* cell culture were obtained from laboratory of Faculty Biosain, University of Malaya, Malaysia. MTT ([3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] (Sigma), DMSO (Dimethyl sulfoxide), SDS (Sodium dodecyl sulphate) 10%, and 0.01 N chloride acid were purchased and used without any treatment.

Plant Material

Samples of the rhizome of *C. aeruginosa* were collected from the market of Yogyakarta, Indonesia. The plant was identified by the staff at the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia, and a voucher specimen had been deposited at the Herbarium.

Extraction

The milled dried rhizome of *C. aeruginosa* (3 Kg) was extracted exhaustively in methanol. Each of methanolic extract from the dried rhizomes of *C. aeruginosa* was partitioned in *n*-hexane, chloroform, and ethyl acetate. The extract and fraction were dried by vacuum rotavapor.

Measurement of cytotoxic activity through MTT ([3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] assay

Each extract and fraction were measured the cytotoxic activity test against human cancer cell lines, that were *Breast carcinoma MCF-7*and *T-47D* and *Cervical carcinoma Hela S3 and Ca Ski*, and *Vero* cell lines. The in vitro cytotoxicity test was investigated using plate with 96 wells, with cell density 2x10^4 cells per ml. Into each well was added 100 µl cells in culture medium (87,5% RPMI 10,4 g/L; 2% penstrep; and 10% FBS) which was then incubated in CO_2 incubator for 12-24 hours at 37 ^{0}C. Each sample was dissolved in culture medium containing 0,05% DMSO, and 100 µL of each sample in different concentrations was added into each well in triplicate and was then incubated in CO_2 incubator for 12-24 hours at 37 ^{o}C. MTT solution (10 µL per 100 µL medium) was added to all wells of an assay, and plates were incubated for 4 hours at 37 ^{o}C in CO_2 incubator. About100µl of formazon (10% SDS and 0, 01 N hydrochloric acid) was added into each well and mixed on a shaker for 5 minutes. The wells were incubated in the dark room for 12-24 hours at room temperature. The absorbance was recorded using multi-well scanning spectrophotometers (ELISA reader) at 595 nm. The absorbance is directly proportional to the number of living cells. So the dead cell could be calculated to determine IC_{50}. Activity test was also measured its cytotoxic activity on *Vero* cell lines as normal cell control comparison. The cytotoxic activity of the samples against cancer cell line measured as IC_{50} was shown in Table 1.

Isolation of bioactive compounds

The isolation of bioactive compounds from chloroform fraction of *C. aeroginosa* was done by using the chromatographic method. A portion (50 g) of the total chloroform fraction was fractionated by vacuum liquid chromatography (VLC) and purified by repeated column chromatography on silica gel eluted with various solvent systems. From this method we obtained two sesquiterpen compounds, a sesquiterpen lacton, aeruginon (1) (60 mg) and curcumenon (2) (120 mg). The structures of these compounds were established on the basis of their spectral data, including UV, IR, and NMR spectra.

RESULTS

Cytotoxic activity against human cancer cell lines

The cytotoxic activity of the methanol extract and this fraction of *C. aeroginosa* against cancer cell lines measured as LC_{50} were shown in Table 1. In this study we observed activity cytotoxic with some cancer cell lines, i.e *Breast carcinoma (MCF-7* and *T-47D), Cervical carcinoma (Hela S-3* and *Ca Ski),* and *Vero* cell lines as control normal cell. The data IC_{50} showed that the extract and fraction have low activity as anticancer with IC_{50} > 100 µg/mL. Table 1 indicates that the methanol extract, *n*-hexane, and chloroform fraction of *C aeroginosa* exhibits cytotoxic activity againt *MCF-7* and *Ca-ski* (IC_{50} > 100µg/mL), but not toxic against *Hela S3* and *T47-D* (IC_{50} >500 µg/mL. The extract and fraction from *C aeroginosa* are not toxic against *Vero* cell. Several studies have reported that the role of curcuminoids from some *Curcuma* is known as chemotherapeutic compounds, and they do not cause any damage to the normal cells[4,9-11].

Isolation and identification of bioctive compounds

From the chloroform fraction of *C. aeroginose*, after separated and repeated purification by extensive chromatography, we were produced two compounds, aeruginon (1) and curcumenon (2). Aeruginon (1) was obtained as brown oil, 60 mg, UV (MeOH) $\lambda_{max.}$ 229 and 250 nm, IR (KBr) $\upsilon_{max.}$: 3322; 2933; 1713;1650; 1598; 1441; 1373; 1313; and 1019 cm^{-1}.^{1}H and ^{13}C NMR

(Me_2CO-d_6, 500.0 and 125 MHz) as shown in Table 2. Curcumenon (2) was obtained as brown oil, 90 mg, UV (MeOH) $\lambda_{max.}$: 214 and 238 nm, IR (KBr) $\upsilon_{max.}$: 2922; 2870; 1713; 1678; 1600; 1453; 1369; and 1270 cm^{-1}, ^1H and ^{13}C NMR (Me_2CO-d_6, 500.0 and 125 MHz) as shown in Table 2.

DISCUSSION

C. aeruginosa is a rhizome that contains essential oil, and consists of over 20 different types of terpenoids. Sesquiterpene compounds as have been reported are isolated curzerenone, zedoarol, furanodienone, and furanogermenone, zedoalactone A, zedoalactone B, and zedoarondiol[12,13]. Based on chromatographic techniques using a variety of solvents in this study were obtained two sesquiterpene compounds aeruginon (1) and curcumenon (2). Aeruginon (1) was obtained as brown oil. Its UV spectrum showed absorption maximum at 229 and 250 nm suggesting the presence of double bond conjugated chromophore. The IR spectrum exhibited hydroxyl group (3322 cm^{-1}), C-H aliphatic (2933cm^{-1}), and C=O (1713; 1650 cm^{-1}).^{13}C NMR spectrum showed fifteen signals of carbons suggesting a sesquiterpen compound. This carbon showed four signals for aliphatic carbon methyl at δ 22.34 (C-12), 22.42 (C-13), 23.10 (C-14), and 23.92 (C-15) ppm, two oxyalkyl carbon at δ 86,25 (C-5), and 83,19 (C-10) ppm spesific for carbon lacton, two methylene carbon δ 27.32 (C-2) and 37.35 (C-3) ppm, one carbon carbonyl δ 194.87 (C-6) ppm), two quarterner carbon at δ133.0 (C-7) and 144.0 (C-11) ppm, alkene alcohol carbon at δ 152.0 (C-8) ppm, alkene carbon atδ 127.5 (C-9), and one carbon methin at δ 60.0 (C-1) ppm. The ^1H NMR spectrum of 1 in $CDCl_3$ exhibited signals for four sets of methyl group at δ 1.87 (3H, *s*), 1.84 (3H, s), 1.27 (3H, s), and 1.77 (3H, s) ppm. The ^1H NMR spectrum also showed four proton signals from methylene group at δ 1.93 (1H, br s) and 1.93 (1H, br s), and 2.46 (2 H, dd) and one proton signal from methyne group at δ 1.93 (1 H, m) ppm. These spectral data indicated that compound 1 has a sesquiterpen with lacton group at C-5 with C-10, and two cyclic rings cyclopentane and cycloheptane. The connection between protons and their corresponding carbons was established by HMQC (Heteronuclear Multiple Quantum Coherence). Further support for the structure 1 was obtained from HMBC (Heteronuclear Multiple Bond Connectivity) measurement (Figure 2). The HMBC spectrum of 1 showed long-range correlations between H-1/C-2, H-15/ C-1, H-15/C-9, and H-9/C-10, H-9/ C-8. Long-range correlations were also observed for the methylene proton between H-4/C-5, H-4/C-2, and Long-range correlations for the methyl group H-14/C-4 and H-14/C-5. These data conclude that compound isolated 1 is a sesquiterpen with lacton group, hydroxyl group, two alkene groups, and two cyclic rings. The sesquiterpene lactone in this study is different from that reported by Suphrom[8], Takano[12], and Sirat[13]. However, it has a structural biogenetic relationship with zedoarol (3) as previous reported[8,13]. Curcumenon (2) was obtained as brown oil, with of absorption maxima observed at 214 and 238 nm in the UV spectrum attributable to double bond cromophor. The IR spectrum exhibited C-H aliphatic (2922 cm^{-1}), carbonyl group (1713 cm^{-1}), and C=C aliphatic (1678 cm^{-1}). The ^1H NMR (Table 2) showed four sets proton of methyl groups at δ 1.76 (3H, s), 0.63 (3H, s), 1.09 (3H, s), and 2.12 (3H, s). The ^1H NMR spectrum also showed four proton signals from methylene group at δ 2.07 (2H, m), 2.40 (2H, m), 2.77, and (2.40 (2H, br s) ppm, and two proton signals of methyne group at δ1.63 (1H, m) and 0.62 (1H, m) ppm. The connection between protons and their corresponding carbons was established by HMQC. The ^{13}C NMR spectrum showed two carbonyl signals at δ 201.16 (C-8) and 208.95 (C-4), four carbon signals of methyl group at δ 23.56 (C-12), 23.51 (C-13), 19.14 (C-14), and 30.13 (C-15) ppm. The ^{13}C NMR spectrum also showed four carbons of methylene group at δ 23.48 (C-2), 43.99 (C-3), 28.09 (C-6), and 49.01 (C-9), and two proton signals from methyne carbon at δ 24.23 (C-1) and 24.19 (C-5), and five quarterner carbon signals δ 128.16 (C-7), 20.19 (C-10), and 147.56 (C-11) ppm. The data obtained from the spectrum of the compound 2 is characteristic for sesquiterpene having two carbonyl groups. Further support for the structure 2 was obtained from HMBC measurement (Figure 2). The HMBC spectrum of 2 showed long-range correlations between H-15 with C-4 and H-3 with C-4 (δ 208.95 ppm), confirming that position of carbonyl group. Long-range correlations were also observed for the methylene proton between H-6/C-7, H-9/C-8 and correlation for the methyl proton between methyl group H-13/ C-11, and H-14/ C-10 indicated the position of methyl group. This structure of isolated compounds 2 has similarities with curcumenon that was first isolated from *Curcuma zedoria*[10]. Researchers have previously reported crude extract and some sesquiterpene compounds of the plant *Curcuma* rhizomes which showed cytotoxicity properties against the CEM-SS cells[13]. In this research we concluded that the *n*-hexane and chloroform fraction of *C. aeroginosa* exhibited cytotoxic activity against *MCF-7* and *Ca-ski*, but not toxic against *Hela S3* and *T-47D*. This research showed that the extract and fraction of *C. aeroginosa* are not toxic (LC_{50}> 500 µg/mL) against *Vero* cell lines. From the chloroform fraction of *C. aeroginose* we isolated a new sesquiterpene lacton aeruginon (1) and a known compound curcumenon (2).

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Conflict of interest statement

We declare that we have no conflict of interest

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