Original article

Sesquiterpenes from the Rhizomes of *Curcuma zedoaria* and their Cytotoxicity against Leukemic Cell Lines

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Abstract

Phytochemical investigation of the rhizomes of *Curcuma zedoaria* led to the isolation of five sesquiterpenes. Through NMR, IR, UV and GC-MS spectroscopic analysis, they were identified as three guaiane type sesquiterpenes (curcumenol, isoprocurcumenol, and procurcumenol), one caraborane type sesquiterpene (curcumenone), and one germacrane type sesquiterpene (zederone). All the compounds were subjected to cytotoxicity test against mouse myelomonocytic leukemia cells (WEHI-3), promyelocytic human leukemia cells (HL-60) and normal human umbilical vein endothelial cells (HUVEC) cell lines by MTT assay. Procurcumenol showed the strongest inhibition of WEHI-3 and HL-60 cells with IC50 values of 25.6 and 106.8 μM, respectively. However, all these compounds showed cytotoxicity towards HUVEC cells with IC50 values in the range of 16.3 to 50.0 μM, and procurcumenol being the most toxic.

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Introduction

Curcuma zedoaria (Christm.) Rosc. (Family: *Zingiberaceae*) also known as white turmeric, is a perennial rhizomatous herb. It is indigenous to the countries of South East Asian region including Bangladesh, India and Sri Lanka. It is also cultivated in China, Malaysia, Indonesia, Philippines and Papua New Guinea (Habsah *et al.,* 2000). It is known as 'Temu putih' in Malay, 'Ezhu' in Chinese, 'Krachura' in Sanskrit and 'Sutha' in Bengali with its extensive use in traditional medicines including Indian Ayurveda and traditional Chinese medicine. Traditionally the rhizome of the plant is used in the treatment of menstrual disorders, stomach diseases, toothache, leucoderma, tuberculosis, enlargement of spleen and cancer (Saikia and Nath, 2003).

The rhizome of the plant is reported to have antimicrobial (Bugno *et al.,* 2007; Gupta *et al.,* 1976), antiulcer (Watanabe *et al*., 1986), anti-inflammatory (Oh *et al*., 2007; Yoshioka *et al.,* 1998), TNF-α inhibitory (Mi Kyung *et al*., 2001), hepatoprotective activity (Matsuda *et al*., 1998; Rana *et al*., 1992) and cytotoxic activity (Syu *et al*., 1998). The plant is rich in essential oil containing curzerenone, 1, 8-cineole, germacrone, cymene, α-phellandrene and β-eudesmol as the major constituents (Purkayastha *et al.,* 2006; Singh *et al.,* 2002). Several phytochemical investigation of the plant resulted in the isolation of mainly sesquiterpenes including curcumenol, dihydrocurdione (Pamplona *et al*., 2006), furanodiene, furanodienone, zedorone, curzerenone, curzeone, germacrone, 13-hydroxy germacrone, dihydrocurdione, curcumenone and zedoaronediol (De Fátima Navarro *et al.,*

2002), zedoarofuran, and six new guaiane or secoguaiane-type sesquiterpenes, 4-epicurcumenol, neocurcumenol, gajutsulactones A and B and zedoarolides A and B (Hong *et al.,* 2002; Lobo *et al.,* 2009; Makabe *et al*., 2006; Matsuda *et al.,* 2001).

Curcuma zedoaria rhizomes were collected from Tawamangu, Indonesia and a voucher specimen (KL 5764) was deposited at the herbarium of the Chemistry department, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Dried and ground rhizome (1.0 kg) was macerated with n-hexane for three days which resulted in 20.o g of n-hexane extract. The plant material was dried and further macerated with dicholoromethane which yielded 10.0 g of dicholoromethane extract. The extract was subjected to silica gel column chromatography (CC) eluting initially with n-hexane followed by hexane/ethyl acetate gradient. Fractions were then combined according to similarity of the TLC spots to give 21 fractions.

Fraction 8 was separated by PTLC using petroleum ether (40-60°C) (PE) and EtOAc in a ratio of 85:15 for the first run and 82:18 for the second run to get curcumenol (3, 15.5 mg). Isoprocurcumenol (5, 10.2 mg) was isolated from fraction 9 by successive development on PTLC using PE:EtOAc in the ratio of 90:10 and 85:15, respectively. Curcumenone (6, 16.4 mg) was purified from fraction 12 by PTLC using three times run with PE:EtOAc:MeOH in a ratio of 85:14:1. Procurcumenol (7, 8.9 mg) was isolated from fraction 15 as a colourless oil by PTLC (PE:EtOAc:formic acid 85:14.5:0.5). The DCM extract (10 g) was fractionated on a silica gel column (Hex:EtOAc95:5-0:100) to give 23 fractions. Fraction 2 was subjected to micro silica gel column (0.043-0.063 mm) with a gradient elution system of Hex and EtOAc (100:0-0:100) followed by RP-HPLC (H2O:MeOH 40:60, run time 80 min, flow rate 2.5 ml/min) which afforded zederone (9, 24.4 mg) at the retention time of 15.26 min. The spectral data of the isolated compounds were in agreement with the literature (Firman *et al.,* 1988, Giang and Son, 2000 and Kuroyanagi *et al.*, 1990).

To test for cytotoxicity, MTT based test was performed against mouse myelomonocytic leukemia cells (WEHI-3), promyelocytic human leukemia cells (HL-60) and normal human umbilical vein endothelial cells (HUVEC) cell lines. The result was expressed as half maximal inhibitory concentration (IC50). All the experiments were done in triplicate.

In brief, the cells were maintained in an atmosphere saturated with 5% CO_2 at 37°C. Briefly, 4×103 cells/well of HL- 60, WEHI-3 cells were seeded on 96-well microtitre plates. Different concentrations (0-100 µg/ml) of isolated compounds were added to the cells, then incubated for 24, 48 and 72 h at 37°C , 20 µl of , 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution which react with mitochondrial enzymes of living cells to form purple formazan was added per well followed by 4 hrs incubation at 37°C, to dissolved purple formazan, 100 µl of dimethylsulfoxide DMSO was added into each well . Optical density was measured at wavelength 570- 590 nm. The percentage of cell viability was determined as follows:

% Viability = OD sample × 100%. (Raskin *et al.,* 2002)
(where OD control)

Among the sesquiterpenes isolated from the *Curcuma* rihzomes, a guaiane skeleton type, procurcumenol is the most active.

Cancer is a disease manifested by uncontrolled cell growth that presents over 100 distinct clinical pathologies .The insidious nature of the disease, as well as the challenges associated with its effective treatment, has made this disease a leading cause of death in many countries (Kufe *et al.,* 2003).

Alternative approaches in cancer treatment are important due to the emergence of new cases and some limited effectiveness of present treatments. Much intervention in cancer cases involves surgery and chemotherapy that aims to eliminate cancer tissues. In addition, there is an increase in the number of cancer drugs that have been found to exhibit a relatively short clinical life span and then to become ineffective. In addition, the very potent drugs frequently exhibit serious side effects. Hence, there is an urgent need to search and develop new anticancer agents that are safe as well as being effective. Thus, possible remedies have been sought from unconventional sources, such as marine environments, and from the use of traditional information. This is particularly apparent in Asian countries

where there has been a long-standing tradition for the use of natural products in health management and in cancer treatment (Wijesinghe *et al.,* 2013).

A characteristic abnormality of some types of leukemia such as ALL, AML and APL is that they are blocked at an early stage of their developments and fail to differentiate into functional mature cells (Nouri *et al.,* 2011). Recently, several scientific achievements have emphasized on using differentiation therapy as an elegant alternative to chemotherapy with potent cytotoxic agents. This approach could theoretically limit the patient exposure to unwanted side effects of cytotoxic drugs and, more importantly, it would modulate the remission and the cure rates (Wijesinghe *et al*., 2013). So recently, few studies on the rhizomes of *Curcuma zedoaria* showed the essential oil could inhibit the proliferation human promyelocytic leukemia HL-60 cells (Lai *et al.,* 2004). Antitumour compound isolated from *Curcuma zedoaria*, a isocurcumenol has shown inhibition the cell cell proliferation in human leukemia (K562) have attracted a great deal attention (Lakshmi *et al*., 2011) . Nevertheless, there are few reports on the biological activity of the pure compounds isolated from *C. zedoaria* as anti-leukemic plant. Therefore, this study had been conducted to determine which isolated compounds might have anti-leukemic activities by evaluating their cytotoxicities on HL60 (human leukemic cell line) and WEHI-3 (mouse cell line). Human promyelocytic leukemia cells, derived from a patient with acute promyelocytic leukemia (APL), following treatment with respective inducers could be differentiated either along granulocytic or along monocytic lineage, and thus this cell line provide a good *in vitro* model for research and differentiation studies

Materials and Methods

Chemicals and Reagents

RPMI 1460 media , fetal bovine serum (FBS) and penicillin-streptomycin were obtained from from Biowest Ltd. Phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were from Santa Cruz Biotechnology (Santa Cruz, CA USA).

Experimental

NMR spectra were obtained using JEOL JNM-FX100 (400 MHz). Deuterated chloroform (CDCl3) was used as NMR solvent. Coupling constant were presented in Hz. Chemical shifts were reported in ppm. Infra-red spectra were recorded using Perkin Elmer FT-IR Spectrometer RX1, with chloroform as solvent. The UV spectra were measured by Shimadzu UV-1650 PC UV-VIS Spectrophotometer with methanol (hexadecyl-(E)-ferulate) and dichloromethane (stigmasterol and β-sitosterol) as solvent. Mass spectra were recorded using Agilent Technologies 6530 Accurate –Mass Q-TOF LC/MS. Industrial grade solvents (hexane, dichloromethane and methanol) were used for bulk extraction. These solvents were distilled before use. For chromatographic separation of compounds, silica gel 60 of size (0.043-0.063mm) and (0.063-0.200 mm) were used, while aluminium - and glass-supported silica gel 60 GF254 plates were used for TLC and PTLC, respectively. TLC and PTLC spots were revealed and marked under UV light (254 and 365 nm) followed by spraying with vanillin reagent. Vanillin reagent was prepared by dissolving 1.0 g of vanillin (commercially available) in a mixture of 25 ml of ethanol and 25 ml of concentrated sulphuric acid.

Results and Discussion

A total of five sesquiterpenes, namely curcumenol, isoprocurcumenol, procurcumenol, curcumenone, and zederone, were isolated from the dichloromethane extract of the rhizomes of *Curcuma zedoaria* (Figure 1). The structures of the purified sequiterpenoids were confirmed by comparing with the spectral vlaues. However, none of these compounds were previously tested for its cytotoxicity against the leukemia cell lines used in this study. This is the first time cytotoxic activity evaluation of these sesquiterpenes against one mouse leukemic myelocytic (WEHI-3) cells and one human promyelocytic leukemia (HL-60) cell lines.

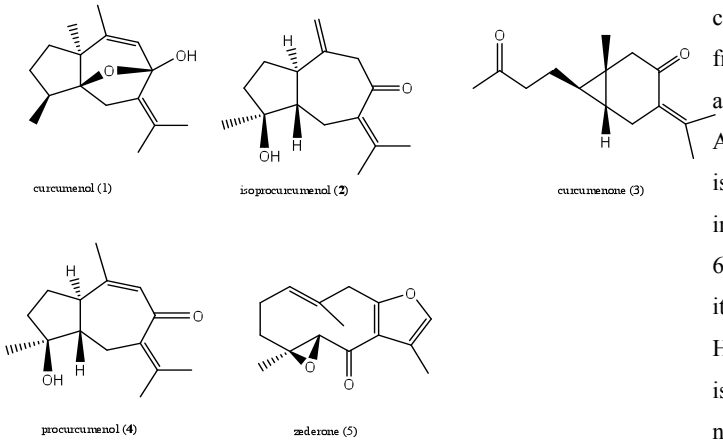

Fig. (1). Structures of the isolated compounds

Human promyelocytic leukemia cells, derived from a patient with acute promyelocytic leukemia (APL), is a well known cell line which provide a good in vitro model for anti-leukemic effect of compounds. Among the sesquiterpenes tested, curcumenone compound showed the most potent cytotoxic effect against WEHI-3 and HL-60 cells with a IC50values 25.6 and 106.8 µM, for WEHI-3 and HL-60 cell line respectively (Table 1). curcumenone Compound also showed cytotoxicity against the normal human umbilical vein endothelial (HUVEC) cells with a IC50vlaue 69.6 µM. All other compounds showed mild to no toxicity against all the tested cell lines (IC50: >100 µM) (Table 1).

Table 1. Anti-proliferative activity [IC50 values (µg/ml) and (µM)] of isolated compounds against WEHI-3 , HL60 and human umbilical vein endothelial cells (HUVEC)

Compound name	WEHI-3 in ug/ml (µM)	HL60 in ug/ml (µM)	HUVEC in ug/ml (µM)
Procurcumenol	6 ± (25.6)	25 ± (106.8)	16.3±1.0 (69.6±4.2)
Curcumenol	66 ± (282.0)	39 ± (166.6)	25.9±1.4 (110.6±5.9)
Isoprocurcumenol	81 ± (246.1)	>100	45.1±3.0 (192.7±12.8)
Curcumenone	35 ± (149.5)	99 ± (423.0)	50.0±8.6 (213.6±35.4)
Zederone	> 100	> 100	42.1±2.7 (171.1±10.9)

Curcuma is one of the well-known species of Zingiberaceae family and a number of antineoplastic compoundshave been isolated from this species. Curcuminoids, epicurzerenone, curdione and a polysaccharide fraction have been reported from *C. zedoaria* rhizomes were demonstrated to be cytotoxic against different human cancer cell lines (Lakshmi *et al.,* 2011). Among these epicurzerenone and curdione has been reported, isolated as an essential oil from *C. zedoaria*, to possess inhibitory effects against human promyelocytic leukemia HL-60 cells. However, none of the above sesquiterpenes reported its cytotoxic activity againstthecell lines used in this study. However, recent study reports that compound (isocurcumenol) isolated from *Curcuma zedoaria* possess to have significant nontoxic nature and antitumour effects on the human lung, nasopharyngeal, and leukemic cells as well as the murine lymphoma cells (Lakshmi *et al*., 2011). Interestingly, the results of our study showed that compound curcumenone possesses cytotoxic activity against the normal human umbilical vein endothelial (HUVEC) cells which support the previous literature (Lai *et al*., 2004).

*Cytotoxicity screening models provide important preliminary data to help to select medicinal plant or isolated compounds with potential anticancer properties. Thus, the finding of the present study supports the use of *C. zedoaria* in cancer-related diseases in traditional medicine.

Conclusions

*As the rhizomes are also widely consumed as salad in food without any known undesirable side effect, it can be assumed that.

*Among the isolated terpenoids from *C. zedoaria*, three compounds showed some cytoxcity activity, IC-50 values ranging from 60 to 89.6 (µM) which were calcaratarin A, a labdane Type diterpenoid, Dehydrocudione a germacrane Type sesquiterpene , and zerumbone epoxide a humulane Type sesquiterpene. This is the first report of the three pure active compounds against lung cancer cells.

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