Identification of active extracts of *Curcuma zedoaria* and their real-time cytotoxic activities on ovarian cancer cells and HUVEC cells

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**Abstract**

*Curcuma zedoaria* (*C. zedoaria*) is a rare species of *Curcuma*. The rhizomes of *C. zedoaria*, obtained from the central part of Myanmar. To our knowledge, the phytochemical components of *C. zedoaria* from Myanmar and their activities on ovarian cancer cells are not reported in the literature. The objectives of this study are to identify the active extracts of the Myanmar herbal plant, *C. zedoaria*, and to determine their cytotoxic activities on ovarian cancer cells and HUVEC cells. Hexane, chloroform, methanol and water were used for various extractions from the rhizome of *C. zedoaria*. Some of the compounds from the extracts were analysed by GC-MS. The various extracts of *C. zedoaria* were tested with CaOV₃ (epithelial ovarian cancer cells), SKOV₃ (metastatic ovarian cancer cells), HUVEC (Human Umbilical Vein Endothelial Cells) from American Type Culture Collection (ATCC). For cytotoxic activity, the proliferative response and cell death of these cells were determined using Real Time Cell Analyzer (Roche) using Paclitaxel as positive control. Hexane and chloroform extract of *C. zedoaria* were found to have moderately potent cytotoxic activity on SKOV₃ (metastatic ovarian cancer cells) and HUVEC (Human Umbilical Vein Endothelial Cells). Our study reveals cytotoxic activities of various extracts of *C. zedoaria* from Myanmar on metastatic ovarian cancer cells and HUVEC cells. Fifteen compounds identified in Hexane extract by using GC-MS.

**Keywords:** Ovarian cancer, Cytotoxicity, CaOV₃ cells, SKOV₃ cells, HUVEC cells, *C. zedoaria*, Myanmar medicinal plant.

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**Introduction**

Globally, ovarian cancer is a major gynecological problem and one of the leading causes of cancer death in women. It is the seventh most common cancer diagnosed among women in the world. There were estimated 238719 new cases of ovarian cancer worldwide in 2012, accounting for 3.6% of all cancers diagnosed in women [1].

Ovarian cancer cases were highest in the European region, followed by Southeast Asia [2]. According to latest Malaysian National Cancer Registry report, ovarian cancer is fourth most common female cancer, accounting 6.1% of 56,713 new cases of women in 2007-2011 [3]. Sivanesaratnam [4] described as the survival rate is only 41.6% and the elusive nature of the disease results in 70% of the cases being in an advanced stage at presentation. There are also issues of chemoresistance, recurrence even with second line chemotherapy, shorter disease free survival and no adequate screening method for early detection. It affects the quality of life of the patients. In view of these reasons, we aimed to test ovarian cancer cell-lines in order to assess the pharmacological ability of selected medicinal plants to prevent or treat ovarian cancer.

Cordell [5] has highlighted the immense potential of natural products in drug discovery and why it is important to be involved in global sustainable initiatives to improve health care. Plants from the tropical rain forest are biologically and chemically diverse as they synthesize various chemicals as a defense against pests, diseases and predators. They are an excellent reservoir of medicines and chemical leads with which we can design and synthesize new drugs. *Curcuma* is one such popular medicinal plant. *C. zedoaria* is a rare species which is available in some parts of Asia and the world [6].

*Curcuma zedoaria* is one of the native plants of Myanmar [7]. The farms for *Curcuma* are located in a particular area of central Myanmar. Cultivation starts around October and the
land yield good rhizomes. These rhizomes are usually collected in January to February every year. The rhizomes of *C. zedoaria* are yellowish white, smell of camphor, and are known as “Na-nwin Myo”. The rhizomes of *C. zedoaria* are different in appearance, colour and smell from those of *C. longa* / *domestica*.

Few reports were found on their chemical components and biological activities. Lobo et al. [8] compiled 27 biologically active compounds isolated by various researchers. There are some reports on their biological activities, such as antimicrobial, antifungal, anti-amoebic, larvicidal, analgesic, anti-allergic, cytotoxic and anticancer activities. Among these pharmacological activities, cytotoxic and anticancer activities are more interesting for cancer prevention and new anticancer drug discovery. Determination of cytotoxicity is required for pre-clinical screening of potential drugs, including plant extracts [9]. The cytotoxicity and anti-tumour properties of extracts and some compounds of *C. zedoaria* have been demonstrated on various cancer cell lines and animal models [10-16]. Seo and co-workers [17] revealed that the water extract of *C. zedoaria* may play an important role in the inhibition of cancer metastasis.

To date, there is a very limited number of studies in the literature on the activity of *C. zedoaria* on ovarian cancer cells since the earliest work done by Syu et al. in 1998 [10]. Traditionally, the use of *C. zedoaria* is prohibited because of the risk of abortion, as described in the Chinese Pharmacopoeia [18]. Since medicinal herbs and their phytochemicals are potential novel leads for developing anti-angiogenic drugs [19], we postulated that *C. zedoaria* would have anti-angiogenic properties. HUVEC cell lines were used to describe the mechanism of action of a chemotherapeutic agent related to the anti-angiogenic properties as a classic model for anti-angiogenesis [20]. Therefore, we tested CaOV3 (epithelial ovarian cancer cells), SKOV3 cells (metastatic ovarian cancer cells) and HUVEC (human umbilical vein endothelial cells) in our study to investigate the cytotoxicity of various extracts of *C. zedoaria* against these three cell lines.

**Materials and Methods**

**Plant materials**

The rhizomes of *C. zedoaria* were collected from the farm in Kyauk-Se township near Mandalay, central Myanmar. The plant and rhizomes of *C. zedoaria* were authenticated by the taxonomist, University of Traditional Medicine, Mandalay, Myanmar (UTMM). The herbarium number is No.10 of the Herbarium Museum of UTMM.

**Preparation of extracts**

The extracts from rhizomes were prepared as described by Arya [21] at Shimadzu-UMMC Centre for Xenobiotics Study (SUCXes) laboratory, Pharmacology Department, Faculty of Medicine, University of Malaya. Briefly, 500 g of rhizomes were coarsely powdered and placed in a cellulose thimble to extract with 100% n-hexane using hot extraction technique with a Soxhlet extractor for 24 h. Further extraction of the obtained defatted residue was carried out using 100% chloroform, followed by 100% (absolute) methanol and lastly with 100% pure water. The solvents from each crude extract were dried by rotary evaporator under reduced pressure at a maximum temperature of 40ºC. Further to the extraction, all the crude extracts were properly stored in a freezer at -20º C until further use.

**Identification and chemical analysis**

All the crude extracts were subjected to GCMS-MS analysis for the qualitative analysis of major compounds. The potential active compounds from various crude extracts were identified using GCMS-MS as described by Arya et al. [21].

**Chemicals and consumables**

CaOV3 (epithelial ovarian cancer cells), SKOV3 (metastatic ovarian cancer cells) and HUVEC (Human Umbilical Vein Endothelial Cells) and McCoy’s 5A medium (modified) were purchased from ATCC (American Type Culture Collection). Roswell Park Memorial Institute (RPMI) 1640 culture medium, penicillin-streptomycin liquid, and trypsin 2.5% (10X) were purchased from Life Technology, USA (GIBCO). Fetal Bovine Serum, Hank’s Balanced Salt Solution (HBSS) and Dimethyl Sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The culture wares and consumables were procured from Orange Scientific (Braine-l’Alleud, Belgium). Hexane, methanol and chloroform were supplied by Merck, Darmstadt, Germany. E-Plate 96 and E-Plate 16 for Real Time Cell analysis (RTCA) were obtained from Roche Diagnostics, USA. These plates were read to measure the cell proliferation using xCELLigence Real-Time Cell Analysis (RTCA system, Roche Diagnostics GmbH, Germany).

**Cell culture**

The tests were performed in the CENAR (Centre for Natural Product Research and Drug Discovery), Department of Pharmacology and Department of Pharmacy, Faculty of Medicine, University Malaya. CaOV3 cells (adenocarcinoma ovary, epithelial ovarian cancer cell line), SKOV3 cells (metastatic ovarian cancer cell-line, which were derived from ascities of chemo-resistant adenocarcinoma ovary), and HUVEC cells (human umbilical vein endothelial cell-line) were obtained from American Type Culture Collection (ATCC). The frozen cells in cryo vials were thawed and subcultured in a sterile manner as per protocol described by ATCC. Initially, McCoy medium was used for CaOV3 cells and SKOV3 cell culture. Later, these cells were gradually familiarized with RPMI 1640. Endothelial Growth Medium (EMG) was used to maintain HUVEC cells. The cells were kept at 37ºC with 5% CO2 incubator. The cells in culture flasks were observed under the microscope for growth, and were used to seed when 70-80% confluency was achieved.
Cytotoxicity and real-time cell growth assay

In order to measure the cell viability and cell growth in a continuous manner at multiple time points, we performed Cytotoxicity and Real-Time Cell Growth Assay using xCELLigence Real-Time Cell Analysis (RTCA) system, as described by Looi et al. [22]. In brief, SKOV3 cells in exponential growth phase were seeded in E-96 plates with electrodes after taking background measurement of 50 μl of culture medium. The optimal cell numbers in each well were determined before the actual experiment. After seeding the cells at a density of $3 \times 10^3$, the plate was placed back in the RTCA system and kept with 5% CO$_2$ at 37°C for 24 h to grow to the log phase. Then, the cells were treated with different concentrations of various extracts of C. zedoaria (200, 100, 50, 25, 12.5, 6.25 μg/ml) dissolved in medium. Paclitaxel (taxol) 1 mmol/ml (0.85 μg/ml) was used as a positive control. The cells were monitored continuously for 72 h.

Similar procedures were performed to culture and treat the CaOV3 and HUVEC cells.

Results

Cytotoxicity and cell growth

We observed the different responses of cell growth in different types of cells to each extract of C. zedoaria in real-time cell growth inhibition for 3 consecutive days using RTCA. These inhibitions were in time- and dose-dependent manner (SKOV3 cell-lines in Figures 1a-1d), CaOV3 Cell-line in Figures 2a-2d and HUVEC cell-line in Figures 3a-3d.

Identification of chemical components from C. zedoaria

Compounds identified in hexane fraction of C. zedoaria using GC-MS were shown in Table 2.

Table 1. Real time cytotoxic activity of various extracts of CZ against selected human cancer cell lines determined by Real Time Cell Analyzer (RTCA) (IC$_{50}$ in μg/ml).

<table>
<thead>
<tr>
<th></th>
<th>SKOV3</th>
<th>CaOV3</th>
<th>HUVEC</th>
</tr>
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<tbody>
<tr>
<td>Hexane</td>
<td>60.48</td>
<td>93.87</td>
<td>13.2</td>
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<tr>
<td>Chloroform</td>
<td>61.05</td>
<td>164.57</td>
<td>14.9</td>
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Table 2. Compounds tentatively identified in hexane fraction of C. zedoaria using GC-MS.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>RT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mol. weight</th>
<th>Mol. formula</th>
<th>Similarity index</th>
<th>Compound&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>1</td>
<td>9.653</td>
<td>152</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;O</td>
<td>96</td>
<td>L. Camphor</td>
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<tr>
<td>2</td>
<td>10.212</td>
<td>154</td>
<td>C&lt;sub&gt;10&lt;/sub&gt;H&lt;sub&gt;16&lt;/sub&gt;O</td>
<td>96</td>
<td>Isoborneol</td>
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<tr>
<td>3</td>
<td>10.545</td>
<td>154</td>
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<td>96</td>
<td>Borneol</td>
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<tr>
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<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;24&lt;/sub&gt;</td>
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<td>88</td>
<td>Germacrene-D</td>
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<td>27.8</td>
<td>250</td>
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<td>65</td>
<td>Hydroxy valereric acid</td>
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</table>

RT<sup>a</sup>: Retention Time in Minute; Compound<sup>b</sup>: Compounds listed in order of their relative area percentage.

Discussion

Ovarian cancer is known to be a most lethal gynaecological cancer even with optimal therapy. Therefore, the discovery of newer and more effective chemotherapeutic agents is inevitably required to improve the clinical outcome, survival and quality of life. In this innovative research, we were able to evaluate the anti-proliferative effect of four different extracts from C. zedoaria from Myanmar. We found that these extracts contained potential active compounds according to polarity and there were more compounds detected in hexane extract of C. zedoaria rhizomes.

Previously, some compounds were isolated from CZ from various countries such as China, Japan, Korea, India, Bangladesh, Thailand, Indonesia and Brazil [10,23-31]. Because of the typical smell of CZ rhizomes, we postulated that CZ might contain more chemical constituents in hexane extract. Hexane extract contains essential oil with volatile non-polar compounds which give rise to the aroma of a particular plant or its rhizomes. We found a majority of these compounds in hexane extract.

The phytochemical, biological, pharmacological and ethnomedical properties of CZ were extensively reviewed by Lobo [8]. For cytotoxic and anti-proliferative activities, some cancer cell lines such as human lung carcinoma cell line and prostate cancer cell lines [12], lung, nasopharyngeal cancer cells, leukaemia and murine lymphoma cells, human promyelocytic leukemia HL-60 cells [28], liver cancer cells, melanoma cells and HUVEC cell lines [32] were tested in-vitro using extracts and compounds from CZ. Regarding the ovarian cancer of our interest, the cytotoxic activity of CZ was demonstrated against human ovarian cancer OVCAR-3 cells [10]. Recently, the compounds isolated from CZ rhizomes collected from Indonesia were reported to exhibit strong anti-proliferative activity and also found to induce apoptotic cell death on MCF-7 breast cancer cell line [15] and on MDA-MB-231 human breast cancer cells by petroleum extract from CZ from China [33].

In this study, we investigated the cytotoxic activity of four crude extracts against two ovarian cancer cell lines and primary endothelial cell lines. To the best of our knowledge, this is the first report on the cytotoxic activity of CZ rhizomes cultivated in Myanmar.

There are various spectrophotometric or fluorometric assays for determination of cytotoxic activities as described by Cordier and Steenkamp [9]. We chose RTCA as this technology enables us to measure cytotoxic effect of CZ extracts in real-time. In addition, the results can be monitored up to 72 h with a simultaneous comparison with a standard chemotherapeutic drug [34]. Our findings are similar with cytotoxic activity of CZ obtained from Indonesia [14]. However, cytotoxic activities were shown against cervical, breast, colorectal and lung cancer cell lines in their study.
Identification of active extracts of Curcuma zedoaria and their real-time cytotoxic activities on ovarian cancer cells and HUVEC cells

We proved that our extracts, particularly hexane extract, had bioactivity on SKOV3 cells and HUVEC cells, rather than the CAOV3 cell line. Even though water extract from CZ was found to be highly toxic to SKOV3 cells and HUVEC cells, the compounds in water extract may contain some fatty material in micelle forms which may contribute to the toxicity. Therefore, we focused on hexane extract to identify compounds by using GCMS.

Conclusion

Our study showed that HE-CZ from Myanmar showed a more cytotoxic effect on Metastatic Ovarian Cancer cells and HUVEC cells than primary ovarian cancer CaOV3 cells. This finding demonstrates that HE-CZ from Myanmar may have potential anti-cancer activity in metastatic ovarian cancer.

Acknowledgement

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Conflicts of Interest

The authors declare no conflict of interest.

References

23. Shiobara Y, Kodama M, Yasuda K, Takemoto T. Curcumenone, curcuminolide A and curcuminolide B,


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