The effect of *Curcuma zedoaria* (Berg) Rosc. plant rhizome extracts on proliferation and viability of myeloid and fibroblasts cancer cells

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**Abstract.** This research aimed to look into the effect of extracts from *Curcuma zedoaria* (Berg) Rosc. plant rhizomes on the proliferation of K-562’s myeloid cancer and WeHi-164’s fibroblast tumor cells. Two types of extracts, comprising water-media and ethanol-media extracts were prepared; and then both tested at varying concentrations (0, 0.1, 1.0, 10.0, and 100.0 g/ml). Proliferation activities were performed on all extract concentrations, either water or ethanol media. Observation focused on morphology-anatomy characteristics of cancer cells. Both extract types (water and ethanol media) inflicted anti-proliferation actions on cancer cells. The lowering/decreasing intensity of the cell proliferation as affected by plant extracts was significant at high extract concentration (100.9 g/ml), with water as well as ethanol media; but less effective at low concentrations (0.1-10.0 g/ml). Anti-proliferation activities by plant extracts at low concentration altered morphology, shapes, and sizes of K-562’s and WeHi-164’s cells, but their cell wall remained intact (unchanged). The use of plant extracts with ethanol media at high concentration altered the shapes/morphology, sizes of K-562’s myeloid cells, fibroblast cells enlarging, cell wall fractured, and the cells fragmented. Plant extracts with ethanol media afforded stronger anti-proliferation activities than those with water media.

1. Introduction

*Curcuma zedoaria* (Berg) Rosc. (temu putih) plants belong to the member of Zingiberaceae family. The origin of *C. zedoaria* plants is not precisely known, but presumably they are indigenous from North West India. The bio-prospects of *C. zedoaria* plants could be as medicine/drug plants and cooking aroma. Currently, those plants have been cultivated under the tree stands for production forest of consecutively teak (*Tectona grandis* Lf), tusam (*Pinus merkusii* Jungh at de Vriese), and mahoni (*Swietenia macrophylla*) species. The rhizomes of *C. zedoaria* plants contain essential compounds for traditional remedies and drug industries [1].

Curcuminoids such as curcumin, di-methoxy-curcumin, and bis-di-methoxy-curcumin typify as bioactive components in *Curcuma* genus, which have been known able to inflict citotoxic effects on OVCAR-3 (human ovarian cancer cells) and have been traditionally used as a remedy for cancers at vagina labium [2]. Curcumin, the phenolic pigment, appears as deep yellow or orange in colour, the substance that exists in the rhizomes of many species of *Curcuma* family. According to [3], the amount of curcumin in rhizomes of *C. zedoaria* by HPTLC analysis is 16.715 mg/g. Curcumene and curcumol are two of these compounds of *C. zedoaria* that have anti-proliferative effects to induce apoptosis in MCF-7 cells by inhibiting cancer cell proliferation [4]. Petroleum ether and chloroform root extracts of plant *C. zedoaria* possess significant anti-inflammatory activity, which was tested in rats [5].

The research results of [6], ermanin and curcumin were the most effective among the isolated compounds from *C. zedoaria* rhizomes, with inhibition of 67.58±3.82 and 95.36±1.58 g/mL, respectively, against collagen induced platelet aggregation at 100 M. Platelet-rich plasma has is its ability to stimulate bone and soft tissue healing. The presence of ermanine and curcumin in the *C. zedoria* rhizome is possible for the treatment/prevention of cardiovascular diseases.
Curcumine typifies as polyphenol compound that could exert biology activities as antioxidant, anti-mutagenic, anticoagulant, antifertility, anti-diabetic, antibacterial, antifungi, antiprotozoa, antivirus, and anti-fibrosis agents [7]. Other researchers reported that curcumine could inhibit the proliferation of carcinoma hepatocellular cells in the human body by inducing (triggering) the stresses on endoplasmic reticulum and dysfunction on mitochondria [6]. In India, China, and South East Asia countries such as Indonesia, people there utilize yellow-colored substances from curcuma plants as additional food, ingredients as well as drugs, which so far do not inflict toxic effects [8].

New sesquiterpene compounds of the so-called furanogermenone that were isolated from the rhizomes of C. zedoaria brought about the effect on increasing the SGOT and SGPT in mice, which were previously inducted (triggered) by CCl₄; and did not cause serious damages to their liver [9]. Ethyl-p- metoxycimamic which was present in their rhizomes of C. zedoaria also revealed its antifungal characteristics [10]. B-1 fraction that was obtained from the purification of B fraction was inherently a single compound with the predicted formula of C₁₃H₁₆O₂ typified as 1(10),5,7,9(11) 8, Guianatetraen-12,8-olide (gweiikurkulakton) compound, which belonged to anti-proliferation bioactive compound further proved efficacious against HeLa cells [11]. Anti-proliferation activities performed by C.zedoaria extracts at high concentration (10%) could effectively inhibit the growth/proliferation of HeLa cells, and reach LC₅₀ value at the extract concentration equal to 60.3 μg/ml [12].

Relevantly in these research activities, it would be examined the rhizome extracts of Curcuma zedoaria plants, which grew under different environments. Further, the rhizome extraction was performed using two different solvent liquids, which comprised water and ethanol (95%) media. Meanwhile, anti-proliferation activities as expected afforded by C. zedoaria rhizome extracts and their cytotoxic effects were tested on two different cancer cells, which covered K-562 (myeloid cancer cells) and WeHI-164 (fibroblast tumor cells).

2. Materials and methods

2.1. Materials
Main research materials were the rhizomes of C. zedoaria plants, collected from the Forestry District Division (BKPH) of Majenang, West Banyumas (Central Java). Further, the rhizomes of the same origins were partly cultivated at the Cibinong Science Centre’s experiment garden in Cibinong, Bogor (West Java). The C. zedoaria rhizomes were allowed for some time to dry naturally under the roof, then milled/ground to pieces, and sieved (using 8-mesh screen) such that the rhizomes in flour shape were obtained. C. zedoaria rhizome flour was then extracted using two kinds of liquid media, i.e. ethanol and water solvent. Extracts from Echinacea purpurea plants were used as a positive control.

In this research, two kinds of cancer cells which were tested, comprised K-562 (myeloid cancer cells) and WeHi 164 (fibroblast tumor cells), simply as a collection of the Animal Husbandry Faculty, Bogor Agricultural University, Bogor-Indonesia.

2.2. Activities of anti-proliferation agent
Anti-proliferation could be defined as the effective action of anti-proliferation agents at their specific concentration (percentage) that could inhibit the growth/proliferation of particular cells as much or greater than 50% (LC₅₀) as their normal growth (proliferation) capacity. The cells which were tested in vitro comprised K-562 (CML myeloid cancer cells) and WeHi-164 (sarcoma-fibroblast tumor cells). Those two kinds of line cells (cancer cells) were each cultured at the concentration equal to 10³ cells/ml in the so-called 24-well dish using the growth media of DMEM (Dulbecco's modified eagle medium) and 10 % FCS/Foetal calf serum [13]. The culture media that contained the two kinds of cultured cancer cells (K-562 and WeHi-164) were then placed into the CO₂ incubator for 24 hours.

The treatment in this regard was the Curcuma zedoaria rhizome extracts with two kinds of solvent liquid (ethanol and water media), which supposedly could act as anti-proliferation agent against those cancer cells. Further, the rhizome extracts (with either ethanol or water media) were made
varying in their concentration (i.e. 0, 0.1, 1.0, 10.0, and 100.0 g/ml; also regarded as the treatment), and then each was put (infected) into the growth media that already contained the cultivated cancer cells (K-562 and WeHi-164). Each of the extract concentrations was replicated three times. The incubation for the infected cultured cancer cells, after the first 24-hour duration, was continued again for another 24 hours. Afterwards, the tested/infected cancer cells could be harvested; and were further counted of their total cells in the dishes using the so-called hemocytometer instrument with the aid of trypan blue’s coloring agent. Results of the cell counting were compared with those (results of the cell counting) for negative control and positive control (Echinacea purpurea plant extracts).

2.3. Toxicity test and cell morphology
Sediment of the infected cultured cancer cells that occurred due to the treatment (infection) by supposedly anti-proliferation agent (C. zedoaria rhizome extracts) was immersed in glutaraldehyde (2%) liquid for 24 hours, then added with buffer chucodylate, and allowed to stand in the immersion, until the sediment dissolved to form a solution. The solution was further centrifuged vigorously, such that new sediment was formed; and afterwards the supernatant (clear liquid part) was separated (discarded). The remaining sediment was then added with osmium tetraoxyde (1%) liquid until it was immersed completely and then kept there for one hour. Afterwards, the sediment was separated from the immersing liquid and, then dried, and further immersed in alcohol (80%) for 20 minutes. After the immersion, the sediment was separated and then suspended by adding butanol liquid; and the sediment suspension was placed on the cover slip, which had already been stuck to the aluminum stub. In this way, the suspension would dry gradually, leaving behind the dried sediment. The dry sediment on the cover slip further underwent the vacuuming for 20 minutes, and then meticulously observed using Scanning Electron Microscope (SEM 5000) device for examining and scrutinizing the morphology of the two tested cancer cells (K-562 cells, and WeHi-164 cells). Results of the SEM’s morphology scrutiny on those two kinds of cells were compared with results of the SEM scrutiny on the Hella cells, already treated with anti-proliferation agent (C. zedoaria plant extracts) obtained from the previous research [12].

3. Results and discussion
Viability (i.e. living cells which are still able to proliferate for their growth) of the tested cancer cells in the initial testing averaged about 95.3% of their normal capable growth (pointed-out in the negative control) (Table 1).

Table 1. Average inhibition of the tested cancer cells/IC50 of C. zedoaria rhizome extracts, at varying extract concentrations; and with two types of extracts.

| Treatment | Average cell viability (%) / IC50 | K - | Low | High |
|-----------|---------------------------------|-----|-----|------|
| Extract concentrations | | 95.3a | 72.4a | 39.7b | 24.6b |
| Types of extracts (solvent media) | | 95.3a | Water | Etanol |
| | | 43.4ab | solvent | Solvent |
| | | 25.5b | | 24.6b |

Remarks: Average figures followed horizontally by the same letters are not significantly different (based on Duncan’s multiple range test at 5% level; a > b > c); cell inhibition (%) = 100% - cell viability (%); Low = 0.1-10 g/ml; High = 100 g/ml; K - = negative control; K + = positive control (extracts from Echinacea purpurea plants).

Testing results on anti-proliferation activities revealed that the treatment by suspected anti-proliferation agent (C. zedoaria rhizome extracts) at their low concentration (0.1-10 μg/ml) did not inflict significant effect on the proliferation capability of K-52’s as well as WEHI-164’s cancer cells, either using water solvent or using ethanol solvent as the extraction media (Table 1). Increasing the
concentration of *C. zedoaria* rhizome extracts (to 100 µg/ml) brought about more intensive inhibition effects or inflicted more active anti-proliferation actions significantly on the tested cancer cells (K-52 and WeHi-164), compared to the situation (inhibition effects/anti-proliferation actions) in the negative control. It was indicated that activities of anti-proliferation by rhizome extracts could strongly relate to the chemical content of their extractives.

Cell viability at low concentration of *C. zedoaria* rhizome extracts (0.1-10 µg/ml) reached 40.3-92.0% (in range) for either of the two kinds of cancer cells (K-562 and WeHi-164), or 72.4% as average. That viability was regarded as still high, indicating that the anti-proliferation activities by low concentration *C. zedoaria* extracts were less active in inhibiting the cancer cell proliferation (less inhibition effect). However, high concentration of *C. zedoaria* extracts (100 µg/ml) apparently exhibited the most actively inhibiting the proliferation of the tested cancer cells (K-562 and WeHi-164), as indicated by their low cell viability (22.0–47.3% in range or 39.7% in average).

Such cancer cell viability (39.7% in average) at high concentration of *C. zedoaria* extracts (100 µg/ml) was significantly lower than the viability (72.4% in average) at low extract concentration (0.1-10 µg/ml); and also lower than the viability of the negative control (95.3% in average) (Table 1), whereas the difference in viability between low concentration *C. zedoaria* extracts (72.4% in average) and the negative control (95.3% in average) was not significant. Concurrently, the difference in viability between high concentration *C. zedoaria* extracts (39.7% in average) and the positive control (24.6% in average) was not significant either (Table 1). Judging from those quantitative results, accordingly, high concentration of *C. zedoaria* rhizome extracts (100 µg/ml) associated with the average cancer-cell viability (39.7%) could be regarded as their reliable LC$_{50}$ value. This is because the cell inhibition achieved by those extracts could reach ≥50% (inhibition: 100%–39.7% = 60.3%); and that IC$_{50}$ value applied to both K-562 and WeHi-164 cancer cells.

Further, Table 1 revealed that *C. zedoaria* rhizome extracts at low concentrations (0.1–10 µg/ml) as described before still revealed their anti-proliferation activities, whereby unfortunately the viability or proliferation rate of the tested cancer cell was still greater than or > 50% (72.4%). In this situation, therefore, the capability of *C. zedoaria* extracts at 0.1–10 µg/ml concentrations to inhibit the cancer cell proliferation was still less than or < 50% (inhibition: 100%–72.4% = 27.6%). On the other hand, *C. zedoaria* rhizome extracts at high concentrations/dosages (100 µg/ml) also as described before could reduce the cancer-cell viability to 37.9% (viability < 50%); or concurrently could inhibit the cancer cell proliferation as much as >50% (inhibition: 100%–39.7% = 60.3%). Accordingly, again such high dosages (100 µg/ml concentrations) was confirmed as the reliable LC$_{50}$ value for *C. zedoaria* rhizome extracts.

The main compounds in *C. zedoaria* extracts, such as particularly arylheptanoid (curcuminoid), volatile (essential) oils with their varying monoterpenes and sesquiterpenes, and polysaccharides could exhibit remarkable anticancer actions; and therefore were immensely effective to press down the growth/proliferation of K-562’s myeloid cells and WeHi-164’s tumor cells; or were so to other cells that might exhibit cancer behavior [11,14]. The increase in cell proliferation could occur due to the activation of several oncogenes involved in the mitogenic signals such as specific race, whereas the inhibition action on biology processes which were deadly to the cancer cells was brought about by the so-called bcl2’s over-expression [14].

Use of different solvent liquids (water and ethanol media) for the extraction of *C. zedoaria* rhizome apparently brought about prominently different extract performances as antiproliferation agents against the cancer cells (Table 1). It revealed that *C. zedoaria* extracts with water media at particular concentrations brought specific inhibition effect on the K-562’s cancer cells (viability/proliferation: 41.0–90.3%) and WeHi’s cancer cells (viability/proliferation: 42.0–92.0%). However, the extracts with ethanol media, at similar concentrations (as water media) exerted stronger inhibition effects on the cancer cells (indicated by lower cell viability/proliferation rate) consecutively for K-562’s cancer cells (viability/proliferation: 22.0–77.6%) and for WeHi’s cancer cells (viability/proliferation: 22.6–85.3%). Results of Duncan’s multiple range test confirmed that allegation (Table 1), whereby the average cell
viability/proliferation rate was 43.4% (inhibition: 100 – 43.4% = 56.6%) due to the use of *C. zedoaria* extracts with water media. On the other hand, due to the use of *C. zedoaria* extracts with ethanol media, the average cell viability/proliferation rate of cancer cells was significantly lower (cell viability lower [25.5%]; or greater inhibition [100% - 25.5% = 74.5%]).

Further still related, in inhibiting the cancer cells the performance of *C. zedoaria* rhizomes’ ethanol extracts (cell viability 25.5% or cell inhibition 74.5%) was statistically comparable with that of positive control/K+ (cell viability 24.6% or cell inhibition 75.4.5%) (Table 1). Meanwhile, in doing so the performance of *C. zedoaria* rhizomes’ water extracts (cell viability 43.4% or cell inhibition 56.6%) as stated before was statistically lower than that of *C. zedoaria* rhizomes’ ethanol extracts, but still better than that of negative control/ K- (cell viability 95.3% or cell inhibition 4.7%). Results of the previous research which used *C. zedoaria* extracts (with ethanol media as well) could inhibit the proliferation of the cervix cancer cells in the range of 32.0-87.6% (inhibition); or cell viability range of 68.0–12.4% [12]. In that research, the average cell viability reached 95.3% (under normal condition or in negative control), whereas concurrently the related LC50 value for *Echinacea* sp. extracts was achieved at 24.5% cell-viability/proliferation (or cell inhibition: 100%–24.5% = 75.5% [>50%]). Compared to the LC50 value in this research results (cell viability = 39.7%; or cell inhibition = 60.3%), then in inhibiting the cancer cells, the performance of *C. zedoaria* rhizome extracts was slightly lower than that of *Echinacea* sp. extracts.

Scrutiny using the scanning electron microscopy (SEM) could provide clear illustration associated with the effect of *C. zedoaria* rhizome extracts (with ethanol as well as water media) at high concentration (100 g/ml) on the visual appearance of the tested cancer cells (K-562 and WeHi-184) (Figure 1). Toxicity of *C. zedoaria* rhizome extracts with water media affected the shape (morphology) of K-562’s cancer cells which became enlarged, while the shape/morphology of WeHi-164’s cancer cells was unchanged; both kinds of cells were compared to their corresponding cells in the control. Meanwhile, the toxicity of *C. zedoaria* rhizome extracts with ethanol media revealed that the line cells tended to get bigger, cell wall destroyed, cell fragmentation (necrosis) occurred. Therefore, this visual illustration confirmed that *C. zedoaria* rhizomes’ ethanol extracts inflicted stronger antiproliferation effects on the tested cancer cells than *C. zedoaria* rhizomes’ water extracts, both at high concentration (100 µg/ml). At high extract concentration, the ethanol fraction was able to explore bioactive components in *C. zedoaria* rhizomes more perfectly than the water fraction. Ethanol extracts afforded better polarity than water extracts; and consequently, chemical compounds in *C. zedoaria* rhizome extracts such as flavonoids in free form as well as in their glycoside forms could be extracted more intensively.

According to [2], curcuminoide compounds in *Curcuma* genus were known to have cytotoxic effects on OVCAR-3 (human ovarian cancer cells); and traditionally already used a remedy for cancers at vagina labia. *C. zedoaria* extracts contained as much 0.1% as curcumine compounds [15]. Use of SEM could provide visual illustration about morphology changes (Figure 1) in the two kinds of tested cancer cells (K-562 and WeHi-164) at high concentration of *C. zedoaria* rhizome extracts (100 µg/ml).

From the viability data (Table 1), then it could be formulated that *C. zedoaria* rhizomes’ ethanol extracts inflicted stronger antiproliferation actions on the cancer cells than *C. zedoaria* rhizomes’ water extracts. Results of the SEM’s scrutiny (Figure 1) provided stronger and clearer illustration regarding the effect of treatment (*C. zedoaria* rhizomes extracts with ethanol as well as water media) on the line cells (tested cancer cells). It appeared as visually disclosed before that the use of *C. zedoaria* rhizomes’ water extracts induced K-562’s cells to grow bigger, whereas WeHi-164’s cells remained unchanged, compared to the control. On the other hand, the effects of using *C. zedoaria* rhizomes’ ethanol extracts revealed that all line cells tended to grow bigger, cell wall destroyed to pieces (fractured), and all cells fragmented. Further, ethanol extracts at high concentration brought about stronger inhibition activities or enhanced more active anti-proliferation effects on the two kinds of tested cancer cells.
Figure 1. Scanning electron micrograph visually featuring the effect of *C. zedoaria* rhizomes extracts using water and ethanol media on the morphology of tested cancer cells (using SEM under 15,000 times magnification): A. Control; B. Water-media extracts; C. ethanol-media extracts.

4. Conclusion and suggestions

The use of *C. zedoaria* rhizome extracts (with ethanol as well as water media) at low concentrations (0.1-10 g/ml), as allegedly antiproliferation agent, seemed less effective in inhibiting the growth (proliferation) of the tested cancer cells (K-562 and WeHi-164). However, at high extract concentration (100 g/ml), there occurred notable changes in the cell morphology and sizes which became enlarged, cell wall fractured, and all cells fragmented.

At high extract concentration, antiproliferation activities became more pronounced; and the extract effect on inhibiting the proliferation of cancer cells tended to be more severe or brought about the cell viability less than 50% (39.7%) or inhibition of cell growth greater than 50% (100%–39.7% = 60.3%). Therefore, extract concentration at 100 g/ml at which the cell viability was 39.7% (or cell growth/proliferation inhibition 60.3%) was regarded as the reliable LC50 value for the *C. zedoaria* extracts (as the alleged antiproliferation agent).

*C. zedoaria* extracts, either with ethanol or water media, exhibit potentiality as antiproliferation agent against K-562’s and WeHi-164’s cancer cells. However, *C. zedoaria* extracts with ethanol media inflicted stronger antiproliferation actions compared to the extract with water media. At high concentration (particularly with ethanol media) strongly deserves thoroughly further research continuation.
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