



Effect of Zamzam Water Extract of *Curcuma zedoaria* Treatment on Apoptosis, p53 and Bcl2 Protein Expression of T47D Cell Culture

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Abstract

Introduction: People worldwide drink Zamzam water either medicinally or religiously. However, the effect of zamzam water extract of CZ in breast cancer cells remains unclear. **Objective:** To evaluate the zamzam water extract of CZ effect on cell apoptosis, p53 and Bcl2 protein expression in T47 D cells line culture. **Methods:** In the control group design, 16 Wells which was filled with T47 D cell line culture in the density of $5 \times 10^4/100 \mu\text{l}$, were assigned into 4 groups. Control group (C-G), were only filled with T47D cell line culture; Zamzam 50 (ZZ-50); Zamzam 100 (ZZ-100), and Zamzam 500 (ZZ-500) groups, were filled with T47D cell line cultures and 50, 100, and 500 $\mu\text{g/ml}$ zamzam water extract of CZ respectively, and then incubated for 48 hours. The number of apoptotic cell was counted by flow cytometry, while p53 and Bcl2 measured by immunohistochemistry staining method. **Results:** Mann Whitney analysis indicated that the percentage of apoptotic cell and p53 protein expression in ZZ-100 was significant higher compared to that of C-G ($p < 0.05$). In contrary the percentage number of Bcl2 protein expression in ZZ100-G was significantly lower when compared to that of C-G ($p < 0.05$). **Conclusion:** Treatment of zamzam water extract of CZ on T47D cell line cultured was capable of increasing the number of apoptotic cells, p53 protein expression, but decreased Bcl2 protein expression in T47D cell line culture. However the numbers of apoptotic cells were less than 10%.

Keywords: Apoptosis, Breast Cancer, Chemoprevention, Flavonoids, p53mutan

1. Introduction

Cancer is still considered as the most detrimental for survival of humans amongst different diseases¹. In women, the second leading cause of cancer death across the world is breast cancer^{2,3}. Fortunately, since two decades ago, incidence rate of breast cancer was decreased around 3.5% per year³. In that period, the mortality rate of breast cancer had also reduced to 24% particularly, on women with Estrogen-Progesterone Receptor positive cancer disease⁴. The decrease in breast cancer mortality rate was attributable to the combination of early detection with screening programs and the efficacious adjuvant

systemic therapy⁵. Flavonoids from plants are the most prominence chemical constituents that were widely used as adjuvant systemic therapy. Various studies have pointed out those flavonoids or flavonoid derivatives having pivotal roles in cancer chemoprevention and chemotherapy⁶. Some epidemiological studies indicate that high flavonoids intake may be associated with declining of cancer risk, and therefore can be proposed as a chemoprotective treatment against cancer⁶. *In vitro* studies also provide evidence that flavonoids may be associated with cell proliferation inhibition, adhesion, and invasion, inducing cell differentiation, cell cycle arrest, and apoptosis⁷.

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It is well known that *Curcuma zedoaria* (CZ) (Zingiberaceae) also known as white turmeric possess several active chemical components such as terpenoids, flavonoids, phenylpropanoids and sesquiterpenes. The medicinal properties of CZ depend upon the presence of these active chemical component⁸. The prominence medicinal properties of CZ includes cytotoxic, anti-mutagenic^{8,9}, antitumor, antioxidant, antimicrobial, anti-inflammation, and antiangiogenesis activities⁹. In Indonesia *Curcuma zedoaria* (CZ) has been traditionally proven capable of inhibiting breast cancer cells progression *in vivo*³. There are study reports that CZ containing isocurcumenol from ethanol extract is capable of inhibiting the proliferation, increased apoptosis and expression of caspase-3 in cancer cells¹⁰. In addition, curzerenon and alismol from hexane fraction of CZ were able to inhibit the MCF7 proliferation and possess a cytotoxic effect in MCF7 cell culture¹¹. Another study has also reported that treatment with CZ ethanol extraction at the dose of 300 mg/kg and 750 mg/kg body weight (BW) has been proven to inhibit expression of p53 and H-Ras in mutant mammary gland cells. Moreover, treatment of CZ extract is also capable of reducing the mammary gland tumor incidence in rats¹². Another study reported by Papademetrio DL, et al., (2013) indicated that flavonoid contained in CZ was able to induce apoptosis through decrease in Bcl2 expression and otherwise increase in expression¹³.

To date ethanol is an important organic solvent and substrate which is used extensively to extract flavonoids and polyphenol from plants¹⁴. There are growing evidences that CZ extraction with ethanol have capability killing cancer cells⁷. Unfortunately, ethanol has also been proved capable of inducing hemorrhagic gastric lesions, at least in part by increasing oxidative stress¹⁵. Therefore, natural water should be considered as an appropriate alternative solvent for this purpose. Aside from plain tap water, Zamzam water is a type of natural water obtained from well located in holy mosque in Mecca, Saudi Arabia have been consumed by millions of Muslims all over the world^{16,17}. Based on Muslim religious beliefs zamzam water is holy and miraculous water therefore to be scientifically superior to plain tap water. Accordingly to many Muslims worldwide including people in Indonesia drink Zamzam water either medicinally or religiously¹⁷.

Based on phytochemistry analysis, zamzam water extract of CZ contained higher flavonoids, phenolic, and saponin content compared to that of plain tap water¹⁸. However, the effect of zamzam water extract of CZ remains unclear. In this study zamzam water was used to replace ethanol as a solvent for extraction of CZ and to measure the extracts effect on p53 and Bcl2 expression, and apoptosis cells on T47D cell line cultured. T47D cell lines were selected because of an appropriate and ideal experimental model to elucidate the estrogen and progesterone-specific effects of a luminal A subtype of breast cancer^{19,20}. In addition, some previous studies have also pointed out that T47D cell line express p53 mutation, therefore unable to bind DNA and loss its capacity to regulates cell cycle, inhibit apoptosis, and hence serve as pro-survival in breast cancer²¹.

2. Methods

In experimental study, post test only control group design, 12 wells which was filled with culture of T47 D cell line in the density of $5 \times 10^4/100 \mu\text{l}$ were assigned into 4 groups, three wells of each consist of: Control group (C-G), was only filled with T47D cell line culture; Zamzam 50 (ZZ50-G), Zamzam 100 (ZZ100-G), and Zamzam 500 (ZZ500-G) groups, were filled with T47D cell line culture and treated with 50, 100, and 500 $\mu\text{g/ml}$ CZ diluted in Zamzam water respectively. All replications were then incubated at 37°C temperature in CO₂ incubator during 48 hours. The number of cell apoptosis was analyzed by flow cytometry, while mutant p53 and Bcl2 expression were measured by immunohistochemistry method. The expression of p53 and Bcl2 were assessed under the light microscope with 400x magnification by the hot spot method. This study was approved by Ethics Committee of the Faculty of Medicine, Sultan Agung Islamic University Semarang.

2.1 *Curcuma zedoaria* Extract

Curcuma zedoaria (CZ) obtained from market was washed, sliced, air dried, and powdered. 200g of dry powder of CZ was extracted by Soxhlet method with zamzam water as a solvent. Zamzam water boiling point is similar to other plain water (100°C), however in this study zamzam waters boiling point was set at 80°C in order to extract all flavonoid of CZ. The extract was

concentrated using a rotary evaporator. The extraction process was carried out in the Chemical laboratory of Sultan Agung Islamic University Medical Faculty, Semarang, Central Java Indonesia.

2.2 Immunohistochemistry Stained Slides Preparation for p53 and Bcl2 Protein Expression

The immunohistochemical stained slides were prepared by deparaffining process for totally 110 minutes, then the slides were immersed in Mayer Hematoxylin stain for 6 minutes. It was washed in running water, dehydrated, cleared, and mounted.

2.3 Analysis of Apoptotic Cells by Flow Cytometry

Apoptosis T47D cells following the treatment with *Curcuma zedoaria* was dissolved in zamzam water was analyzed by flow cytometry FITC apoptosis detection kit with Propidium Iodide (Sigma-Aldrich). In this flow cytometry Annexin V was used to detect apoptosis cells by its ability to bind with phosphatidylserine located in the outer leaflet of the plasma membrane. In normal cell phosphatidylserine is hidden within plasma membrane, however during apoptosis phosphatidylserine is translocated from the cytoplasmic face of the plasma membrane to the cell surface as an endpoint indicator of early and late apoptosis.

2.4 Statistical Analysis

The total number of p53 and Bcl2 protein expression and cell apoptosis were presented as the mean \pm SD. Differences between groups were analyzed for statistical

significance, using Anova and followed by Post Hoc LSD test with the significance level of 95%.

3. Results

After 48 hours incubation at 37°C temperature in CO₂ incubator, the total number of p53 and Bcl2 protein expression were assessed under the light microscope with 400x magnification by the hot spot method (Figure 1) whereas the apoptotic cells were analyzed with flow cytometry (Figure 2). The mean of total number of protein p53 and Bcl2 expression, apoptosis and necrotic cells are presented in Table 1.

The expression of p53 protein was highest in ZZ100-G, followed by ZZ50-G, ZZ500-G, and the lowest was in C-G and ZZ500-G. In contrary, the highest expression of Bcl2 protein was in C-G. Followed by ZZ50-G, ZZ500-G, and the lowest was in ZZ100-G. Since the data of p53 percentage was abnormal, nonparametric test Kurskall Wallis was used. On the other hand considering the data in number of Bcl2 protein expression percentage was normal distribution, therefore data was analyzed used Anova. Both of Kurskall Wallis and Anova analysis indicated that there were significant differences among groups, $p < 0.05$ (Figure 1).

Apoptotic cells were measured by flow cytometry which showed that the highest apoptotis was in ZZ500-G, followed by ZZ100-G, ZZ50-G, and the lowest was in C-G. Kurskalwallis statistical analysis on the percentage of apoptotic cells indicated that there were significant differences among groups, $p < 0.05$ (Figure 2).

In order to identify which one of the p53, Bcl2 protein expression and apoptosis cell group possessed significant difference between two groups were studied further.

Table 1. Total Number of p53 and Bcl2 Protein expression, and apoptosis Cells

Variables	Groups			
	C-G (n=5) χ (\pm)	ZZ50-G (n=5) χ (\pm)	ZZ100-G (n=5) χ (\pm)	ZZ500-G (n=5) χ (\pm)
P53 Expression (%)	10(\pm 0.70)	12(\pm 0.70)	14(\pm 0.70)	10(\pm 0.70)
Bcl2 Expression (%)	40(\pm 0.70)	39(\pm 0.70)	37(\pm 0.70)	38(\pm 0.70)
Apoptotic Cells (%)	4.01(\pm 0.12)	3.67(\pm 0,06)	5.16(\pm 0,12)	8.87(\pm 0,23)
Survival Cells (%)	94.67 (+0.29)	95.55 (\pm 0.05)	95.33(\pm 0.09)	90.91(\pm 0.20)

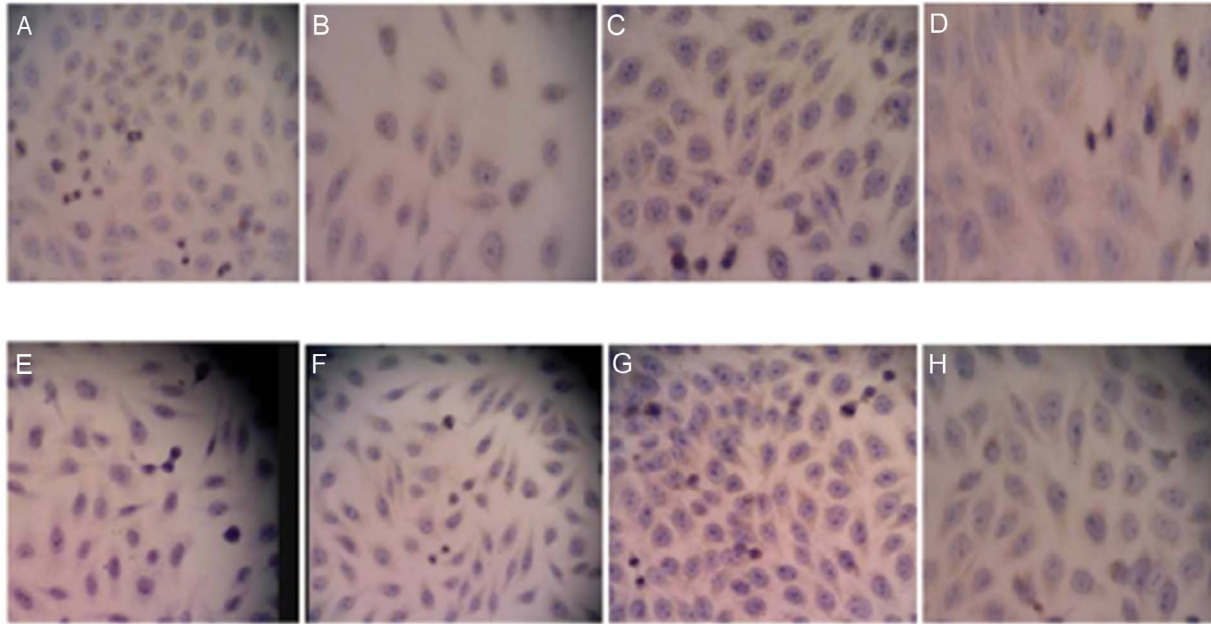


Figure 1. P53 Protein Expression: A (C-G), B (ZZ50-G), C (ZZ100-G), D (ZZ500-G); Bcl2 Protein Expression: E (C-G), F (ZZ50-G), G (ZZ100-G), H (ZZ500-G).

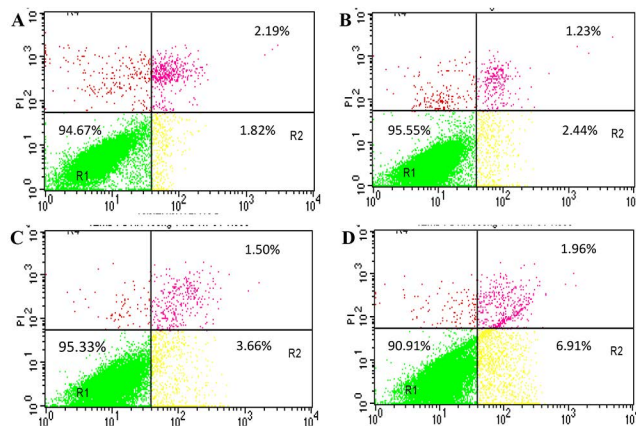


Figure 2. The Results of Annexin V FLUOS test by Flow cytometry: Control group (A); ZZ50-G (B); ZZ100-G (C); ZZ500-G (D)

3.1 Apoptotic Cells

Mann Whitney analysis indicated that number of apoptotic cells in ZZ100-G and ZZ500-G were significant compared to that of C-G ($p < 0.05$). Similar result also occurred in ZZ100-G when compared to that of ZZ500-G ($p < 0.05$) (Figure 3).

3.2 P53 Protein Expression

Mann Whitney analysis indicated that p53 protein expression in ZZ50-G and ZZ100-G were significant compared to that of C-G ($p < 0.05$). However, p53 protein expression in ZZ500-G compared to that of C-G was not significant ($p > 0.05$) (Figure 3).

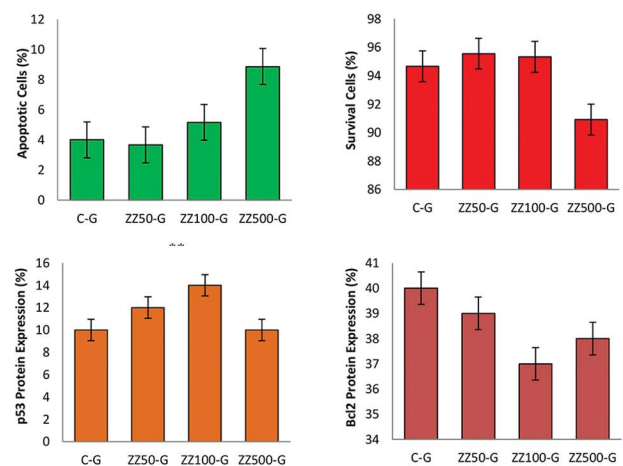


Figure 3. Mann Withney analysis on apoptotic cells and p53 Protein expression. Anova analysis on Bcl2 Protein expression: * $p < 0.05$; ** $p > 0.05$.

3.3 Bcl2 Protein Expression

Post Hoc LSD analysis showed that Bcl2 protein expression in ZZ50-G, ZZ100-G, and ZZ500-G was significant when compared to that of C-G ($p < 0.05$). Similarly, Bcl2 protein expression in ZZ500-G was significant when compared to ZZ100-G and ZZ50-G ($p < 0.05$) (Figure 3).

4 Discussion

The result of the present study indicated that zamzam water extract of CZ treatment on T47D cell culture was able to induce apoptosis particularly in the dose of 100 and 500 $\mu\text{g/ml}$. However the apoptosis of T47D cells were very limited, less than 10%, in contrary, the survival cells were majority, more than 90% (Figure 3). This result showed that the treatment of zamzam water extract of CZ on T47D cell line was not inducing apoptosis effectively. This result implied that zamzam water extract of CZ treatment by the dose of 100 and 500 μl on T47D cell line was unable to trigger DNA damage largely and subsequently apoptosis.

In the biological system, apoptosis is used to delineate the intrinsic cell suicide program, and morphologically is characterized by global cell shrinkage, cell blabbing, and chromatin condensation accompanied by nuclear and DNA fragmentation into specific fragment sizes form DNA ladder pattern²². Initiation of apoptosis by the cell is determined by gene activities, leading to cellular self-destruction through either intrinsic or the extrinsic or both pathways²³. DNA damage is initiators of apoptosis throughout process, by which wild type p53 in nucleus and cytoplasm may be activated and stabilized, which in turn serve as tumor suppressor function²¹. Consequently, activated p53 is able to drive intrinsic and extrinsic apoptotic pathway through amplifying the apoptotic signal consisting of apaf 1, cell death receptor Fas (CD95), PUMA, Bax, Bak, and caspases²³. On the other hand p53 mutant is missense mutation within the core domain, leading to the expression of a full-length mutant p53 protein, subsequently abrogate the tumor suppression function of wild type p53, and even gain new oncogenic activities to promote tumourigenesis²¹. When mutation at sequence of DNA specific binding domain containing serine residue occur result in p53 unable

to bind mdm2. Moreover, mdm2 that serve as a protein anti p53 will be freed and activated leading to inhibition of p53 activity. Decrease in p53 activity is associated with the disappearance of the function of checkpoint control potency. Subsequently, the cell cycle does not stop in G1 phase (G1 arrest) in order to perform apoptosis, albeit p53 protein expression is increasing²³. Consequently, mutated p53 is unable to bind DNA, induce p21 inactivity, and subsequently loss its capacity to regulates cell cycle and inhibit apoptosis cells²¹.

In the present study the T47D cells that underwent apoptosis were very limited, suggesting that treatment with zamzam water extract of CZ in 50, 100, and 500 μl dosing were incapable of inducing DNA damage, activate and stabilizes p53 to trigger apoptosis. It was plausible, since T47D cells were used in this study constitute p53 mutant, accordingly lost its capacity to suppress tumor proliferation as reported²¹. In the present study, p53 expression in zamzam water extract of CZ treatment in 50, 100, and 100 μl dosing were increasing significantly, indicating that treatment with CZ zamzam extract was able to induce p53 protein expression. However, due to its mutation the increase in p53 expression was not capable of inducing T47D cells apoptosis due to incapability of binding with DNA. The increase in p53 protein expression following CZ treatment, also suggested that CZ stimulate p53 protein expression at DNA level since controlling of p53 by mdm2 but not at DNA level, may be in protein level²³. In contrary, Bcl2 protein expression following zamzam water extract of CZ treatment in the dose of 50, 100, and 500 μl were decreased significantly, suggesting that Bcl2 protein could be down regulated. This result was similar to the study reported by Chen et al., (2013) that treatment with CZ essential oil decreased the levels of Bcl-2 and Bcl-xL and increased the ratio of Bax/Bcl-2²⁴.

According to phytochemical analysis, zamzam water extract of CZ contained flavonoids, phenol, and saponin and their concentration were lower compared to that of ethanolic extract of CZ¹⁸. The calculated IC_{50} value of CZ zamzam water extract was 28.24 $\mu\text{g/ml}$ or 20 $\mu\text{g/ml}$ slightly higher compared to that of ethanol extract of CZ 13.71 $\mu\text{g/ml}$ or less than 20 $\mu\text{g/ml}$. This study point out the IC_{50} value in zamzam water extract of CZ is defined as fairly active category, whereas the ethanol extract of CZ is defined as very active

category²⁵. Similarly, the study reported by Khaing et al., (2017) indicated that plain water extract of CZ posses strong inhibition with an IC₅₀ value of 23.50 µg/ml in the cell growth of cells metastatic ovarian cancer cells (SKOV3) in a time and dose-dependent manner²⁶. Taken together, the effect of zamzam water extract of CZ on T47D apoptosis cell culture in order to be more effective need more doses of CZ.

5. Conclusion

Treatment of zamzam water extract of CZ with dose of 100µl on T47D cell line culture was capable of increasing the percentage number of apoptotic cells and p53 protein expression, but decreasing Bcl2 protein expression in T47D cell line culture. However the apoptotic cells were very limited less than 10%.

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7. Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

8. References

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