

## Inhibiton Effect of Mahkota Dewa (*Phaleria macrocarpa*) on Benzo(a)pyrene Induced Cytotoxicity in CCRF-CEM Cell Lines

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### Abstract

Mahkota Dewa as a traditional plant has been commonly used as traditional cancer medication. However, the mechanism of usage is not yet clear. The objective of this study was to know the mechanism of the protection effect of Mahkota Dewa on Benzo(a)pyrene (BaP) induced cytotoxicity in CCRF-CEM cell line. The result showed BaP induced cell death with in CCRF-CEM cell line was dose-dependent but not based on time-course. Exposure of this cell for 24 h with variation of dose between 5-20  $\mu\text{M}$  increased the percentage of apoptosis to about 15%. On the other hand, Mahkota Dewa itself has dose-dependently induced cytotoxicity and has no effect in the inhibition of BaP exposure. Phosphorylation of p38 MAPK in both BaP and Mahkota Dewa induced cytotoxicity has been seen but the involvement of oxidative stress is unclear. However, in other cancer cell line SH-SY5Y human neuroblastoma cells, the inhibition effect of Mahkota Dewa in BaP exposure has been seen and no cytotoxicity effect appeared in this cell line. In conclusion, Mahkota Dewa has induced apoptosis in CCRF-CEM cancer cell line but not in SH-SY5Y cell line, so it has a potential anticancer effect; Mahkota Dewa, however, requires more researches on DNA level using other type of cancer to observe the mechanism.

### Abstrak

**Efek Penghambatan Mahkota Dewa (*Phaleria macrocarpa*) pada Sitotoksitas CCRF-CEM Cell Lines yang Terpapar oleh Benzo(a)pyrene.** Mahkota Dewa adalah tumbuhan tradisional yang umumnya digunakan sebagai obat kanker tradisional. Namun belum terdapat kejelasan mengenai mekanisme penggunaannya. Tujuan penelitian ini adalah untuk mengetahui mekanisme efek proteksi Mahkota Dewa pada sitotoksitas CCRF-CEM cell line yang terpapar oleh Benzo(a)pyrene. Hasil penelitian menunjukkan kematian sel dalam CCRF-CEM cell line yang diinduksi oleh BaP terjadi secara dependen terhadap dosis, tetapi bukan didasari oleh jangka waktunya. Paparan sel ini dibiarkan selama 24 jam dengan dosis bervariasi antara 5-20  $\mu\text{M}$  dan mengakibatkan peningkatan persentase apoptosis sampai sekitar 15%. Di lain pihak, Mahkota Dewa itu sendiri telah menginduksi sitotoksitas secara dependen terhadap dosis, dan tidak ditemukan efek terhadap penghambatan paparan BaP. Fosforilasi p38 MAPK baik dalam BaP dan sitotoksitas yang terpapar oleh Mahkota Dewa telah terlihat. Akan tetapi keterlibatan stress oksidatif tidak jelas terlihat. Meskipun demikian, dalam cell line kanker lainnya seperti SH-SY5Y sel neuroblastoma manusia, efek penghambatan Mahkota Dewa dalam paparan BaP telah terlihat dan tidak terdapat adanya efek sitotoksitas yang muncul di cell line ini. Sebagai kesimpulan, Mahkota Dewa telah menginduksi apoptosis pada cell line kanker CCRF-CEM. Namun apoptosis tidak diinduksi pada SH-SY5Y cell line sehingga tumbuhan ini berpotensi memiliki efek antikanker. Meskipun demikian, perlu lebih banyak penelitian mengenai Mahkota Dewa pada level DNA dengan menggunakan jenis kanker lainnya agar mekanismenya dapat diobservasi.

*Keywords: Mahkota Dewa (Phaleria macrocarpa), Benzo(a)pyrene, apoptosis, CCRF-CEM cell line*

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### Introduction

*Phaleria macrocarpa* (Scheff.) Boerl (Thymelaceae) or *Phaleria papuana* Warb var. *Wichmannii* (Val) Back is a popular herbal medicine in Indonesia known as Mahkota Dewa or MaDe. This plant is a dense evergreen tree

originally found in Irian Jaya, in the eastern part of Indonesia. Its potential as an anticancer agent has been known for generations, and its fruit, seed, and leaf extracts have been widely used by Indonesians, although there are not many researches available on it. In this study, we observed the protection effect of

Mahkota Dewa on Benzo(a)Pyrene (BaP) induced cytotoxicity in Human lymphoblastic leukemia CCRF-CEM cell line. BaP is a carcinogenous Polycyclic Aromatic Hydrocarbon (PAH). PAHs are a large group of diverse environmental organic pollutants formed mainly by incomplete combustion. Many of them are known or suspected carcinogens that have been reported to possess tumor-initiating and/or tumor-promoting properties.<sup>1</sup> There are so many sources of PAH in environment like from car engine, cigarette, smoked food product, etc. Research on cellular level also has proved that Polycyclic aromatic hydrocarbons induce apoptotic result like in Hepa1c1c7 cells.<sup>2</sup> Time- and concentration-dependent changes in gene expression are induced by benzo(a)pyrene in two human cell lines, MCF-7 and HepG2.<sup>3</sup> In this research we use *Human lymphoblastic leukemia cells*, CCRF-CEM and *human neuroblastoma cells*, SH-SY5Y, as a model to observe the inhibition effect of Mahkota Dewa on BaP toxicity and also to observe anti cancer effect of Mahkota Dewa. We study the cell viability, apoptosis and also the involvement of p38 MAPK in the cell death mechanism. p38 is one of MAPKs family Ser/Thr protein kinase which transmit signaling from extracellular to cell nucleus. It has 3 subfamilies: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal Kinase (JNK), and p38 MAPK. All three subfamilies need phosphorylation on one Thr and one Tyr for apoptosis.<sup>4</sup> We can see that the BaP can induce apoptosis by increasing oxidative stress and inducing the p38 MAPK, and Mahkota Dewa is expected to block this mechanism to reduce the apoptosis. This topic is relevant to condition in Indonesia, in which environmental-related pollution has intensified due to increasing industrial activities and transportation, and Indonesia also has many kind of herbal or plant that are interesting to be explored.

## Methods

**Cell culture.** CCRF-CEM human lymphoblastic leukemia cell line was obtained from Health Science Research Resources Bank (HSRRB) Osaka, Japan, and SH-SY5Y human neuroblastoma cell line from American Type Culture Collection (ATCC). Cells were grown in medium RPMI 1640 (GIBCO BRL) + 10% FBS for CCRF-CEM cell line or DMEM/F12 (GIBCO BRL) + 10 FBS for SH-SY5Y cell with penicillin 100 unit/mL (GIBCO BRL), and 100 µg/mL streptomycin (GIBCO BRL, Life Technologies, Inc., Rockville, MD) in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air at 37 °C. For each experiment, exponentially growing cells were plated at  $1 \times 10^5$  cells/well or  $2 \times 10^6$  cells/well in 6-well, 12-well or 96 culture plates, cultured in serum-free medium.

**Treatment with BaP, Mahkota Dewa and antioxidant.** BaP stock solution was made by diluting with DMSO (Wako). Final concentration of BaP solution for

treatment is between 5-20 µM (final concentration of DMSO is 0.2%). Mahkota Dewa (*Phaleria macrocarpa*) was extracted in ethanol solution and added 30-60 minute pretreatment to cells in final concentration 50-100 µg/mL (final concentration of ethanol is 0.01%). In this research, we also use several antioxidant product or radical scavenger like Sodium Formate, Sodium Pyruvate, N-Acetylcysteine (NAC), Curcumin dan Gallic acid (SIGMA) with various doses and 40-60 minute pretreatment before BaP.

**Cell viability assay.** We determined cell viability using the WST-8 assay. Cell Count Reagent SF (10 µL; Nacalai Tesque, Kyoto, Japan), which consists of 5 mM WST-8 (4-{3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio}-1,3-benzene disulfonate, sodium salt), 0.2 mM 1-methoxy-5-methylphenazinium methosulfate, and 150 mM NaCl were added to each well of 96-well culture plates. After incubation for 1 hr at 37 °C, the absorbance of each well was measured at 450 nm with a reference wavelength at 655 nm.<sup>5</sup>

**Flow cytometric analysis for apoptosis measurement.** CCRF\_CEM cells ( $3 \times 10^6$ ) incubated BaP for period of time, washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol on ice for 30 min. The fixed cells were washed twice with PBS, incubated with 250 µg/mL Rnase at 37 °C for 1 hr, washed again twice, and then stained with 50 µg/mL propidium iodide solution. The DNA content of cells ( $1 \times 10^4$  for each sample) was determined with a Coulter EPICS XL (Coulter Electronics, Miami, FL, USA). Apoptotic cells were estimated by the percentage of cells in the sub-G<sub>1</sub> peak as described previously.<sup>6</sup>

**Western blots for detecting of p38 phosphorylation.** After incubation, cells were washed with PBS and lysed with SDS-polyacrylamide gel Laemmli sample buffer. Cell lysates were collected, sonicated, and boiled for 5 min. Aliquots equivalent to  $4 \times 10^5$  cells were subjected to SDS-PAGE on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Buckinghamshire, England). The membrane was blocked with 5% nonfat milk or 1% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 for 1 hr at room temperature. We used the following antibodies: phospho-p38 antibody and phosphorylation state-independent p38 MAPK antibody (Cell Signaling Technology, Inc., Beverly, MA, USA). The membrane was incubated overnight at 4 °C with the primary antibody diluted 1:1000 in 5% bovine serum albumin in 1:10,000. Tris-buffered saline containing 0.1% Tween-20. It detected protein using the Phototope-HRP Western blot detection kit (Cell Signaling Technology, Inc.). For densitometric analysis, the films were scanned by an Epson GT-9800F scanner and quantified using Scion Image Software (Scion, Frederick, MD, USA).<sup>7</sup>

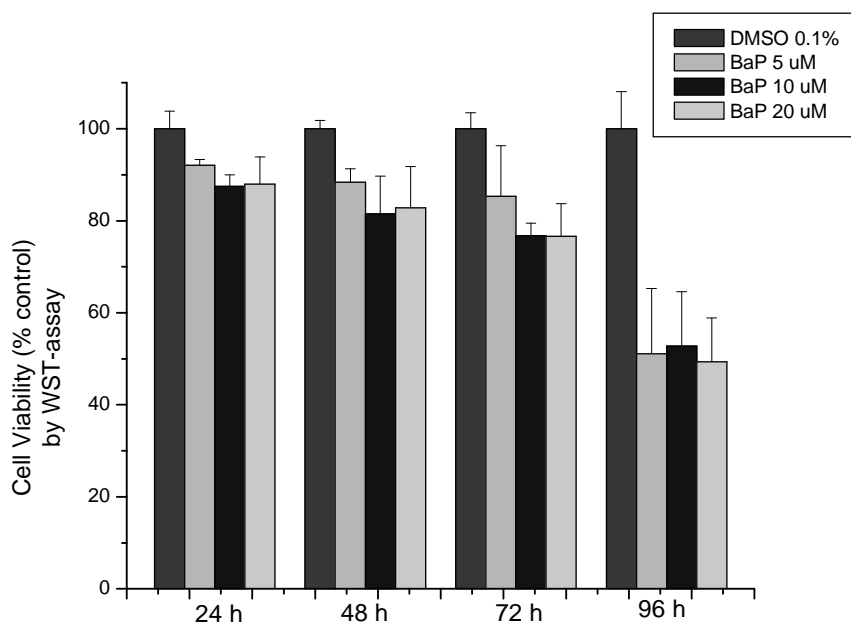
**Results and Discussion**

Our results showed that BaP induced cell toxicity in CCRF-CEM with dose-dependent result (5-20  $\mu$ M) but not time-course result (24-96 jam), as shown in Figure 1. The cell viability decreased to 80% at 24 h exposure and about 50% in 96 h exposure. However, Mahkota Dewa did not show inhibitory effect in BaP induced toxicity and decreased cell viability in the cells (Figure 1).

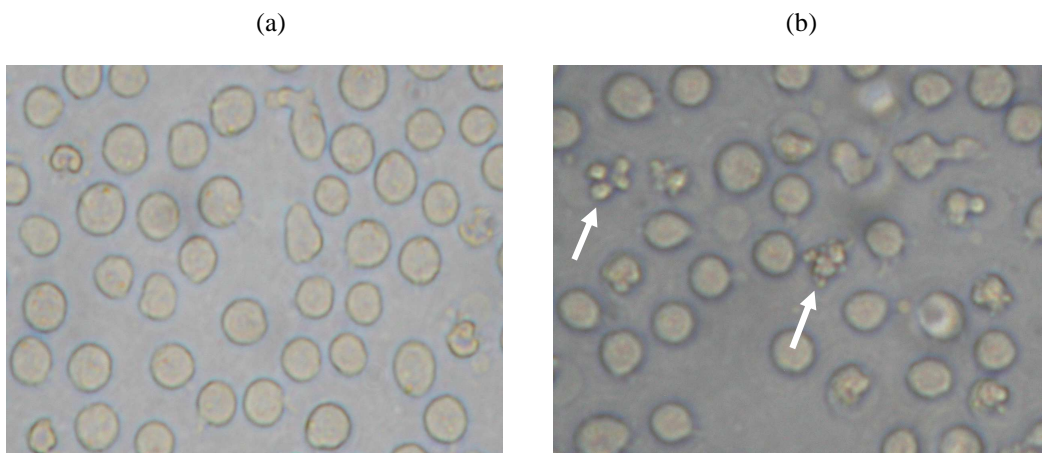
The characteristic of cell-death in BaP induced toxicity in CCRF-CEM is apoptotic under light microscopic

observation as shown in Figure 2A. The percentage of apoptotic cells was about 25% in BaP or Mahkota Dewa and increased to about 40% when the cells were treated in combination of Mahkota Dewa and BaP (Figure 2B). It means that Mahkota Dewa increased the apoptotic feature and could not protect BaP induced apoptosis in CCRF-CEM cell line.

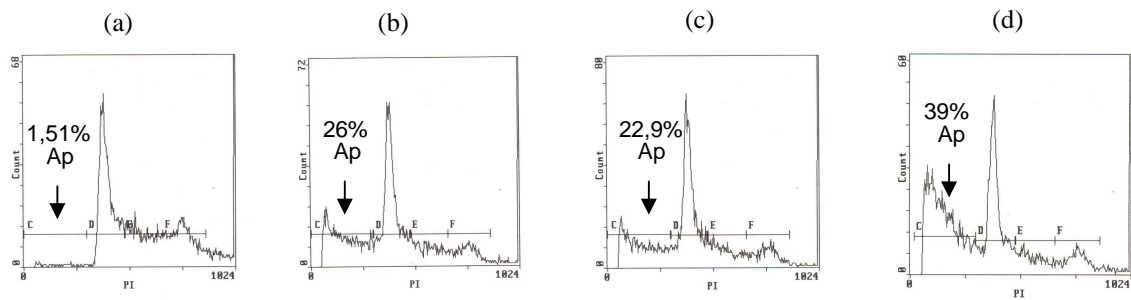
Our results also showed that in the early time (1 h exposure), BaP and Mahkota Dewa induced phosphorylation of p38 MAP in CCRF-CEM cell line, as shown in Figure 4.



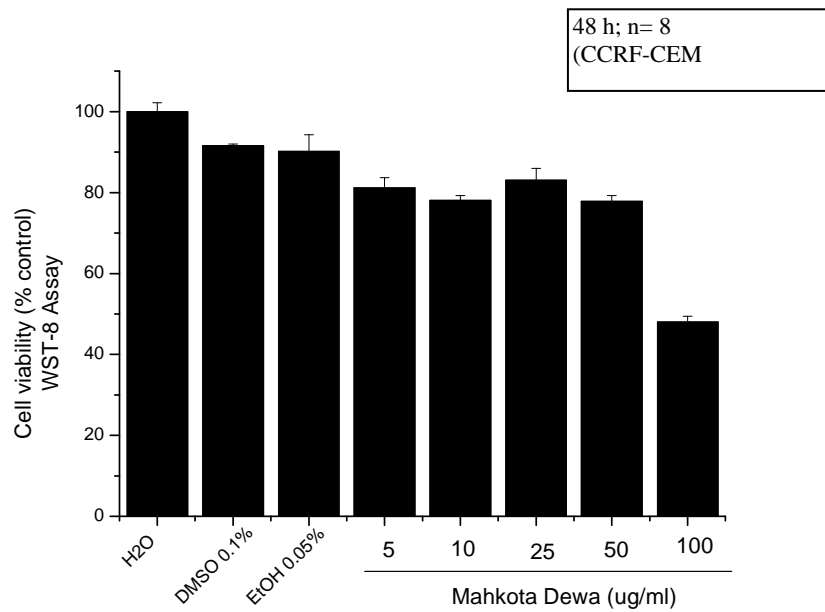
**Figure 1. BaP Induced Cell Toxicity in CCRF-CEM Cell Line**



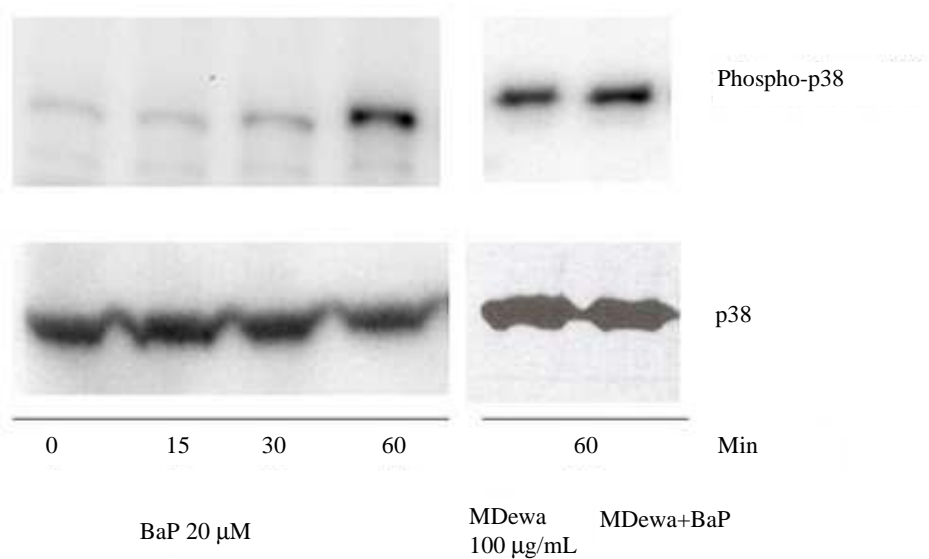
**Figure 2A. Apoptosis Feature of CCRF-CEM Cell Line (a) Normal, (b) Apoptosis**



**Figure 2B. Flowcytometry-percentage of Apoptosis in CCRF-CEM Cell Line After 24 h Exposure (a) DMSO 0,1%, (b) BaP 20 μM, (c) Mahkota Dewa 100 μg/mL, (d) BaP+Mahkota Dewa**



**Figure 3. Mahkota Dewa Induced Cell Toxicity in CCRF-CEM Cell Line**



**Figure 4. BaP and Mahkota Dewa Induced Phosphorylation of p38 MAPK in CCRF-CEM Cell Line**

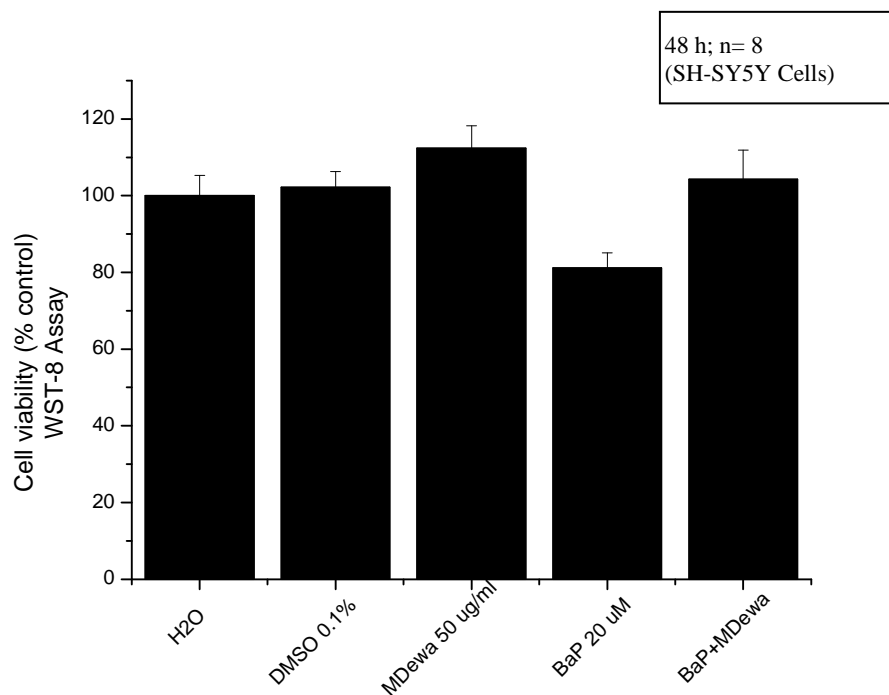


Figure 5. BaP Induced Cell Toxicity and Inhibition Effect of Mahkota Dewa in SH-SY5Y Cells

On the other hand, in another cancer cell line, SH-SY5Y, the effect of Mahkota Dewa showed different result. In these cells, Mahkota Dewa increased the cell viability with no increase in apoptosis. Mahkota Dewa also inhibited BaP induced cell toxicity (Figure 5). We also want to know whether oxidative stress play a role in the mechanism of apoptosis. We used oxidative scavenger like sodium pyruvate and sodium formate and also antioxidant N-Acetylcysteine (NAC) that has capacity in protecting of cadmium in LLC-PK1 cell line.<sup>8</sup> We also used Gallic Acid, a component in Mahkota Dewa which has anti cancer effect on human cancer cell line.<sup>9</sup> The result shown that no protecting effect of sodium pyruvate, sodium format and NAC, which means that the mechanism BaP induced toxicity in CCRF-CEM cell line is not through the formation of oxidative stress (data not shown). On the other hand, Gallic Acid has induced toxicity in CCRF-CEM cell line same as the Mahkota Dewa itself.

## Conclusions

In conclusion, there is different effect of Mahkota Dewa in capacity of inducing apoptosis in two different cancer cell line CCRF-CEM and SH-SY5Y. Further research should be done to know the mechanism of Mahkota Dewa induced apoptosis in human cancer cell line especially in DNA or gene expression level. Observation of Mahkota Dewa inhibiting BaP toxicity should also be done in different cell lines to know whether the inhibition effect is cell-type different.

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