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# Antiproliferative Effect and Apoptosis Induced in Human Cell Lines by Bruguiera gymnorhiza Barks Methanol Extract

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#### **Abstract**

The Antiproliferative effects of methanol extract from *Bruguiera gymnorhiza* (*B. gymnorhiza*) barks were tested in vitro against three human cell lines: Hela, Raji and Myeloma cells. The extract was found to have antiproliferative effects against Hela, Raji and Myeloma cells with an IC<sub>50</sub> value of 133, 504 and 384 µg/mL, respectively. The antiproliferative test was then performed on Hela, Raji, and Myeloma cells. Cytotoxicity assay of the extract was then determined using MTT method. There were some indications of apoptosis, such DNA fragmentation, as assessed by acrydine orange- ethidium bromide staining. These results indicate that extract from *B. gymnorhiza* barks can induce apoptosis in human cell lines.

Keywords: Antiproliferative, Apoptosis, B. gymnorhiza

# **INTRODUCTION**

Apoptosis is widely observed in various cells of most organisms, from nematodes to mammals, and it is generally accepted to play an important role in the physiological processes during maturation of the immune system, embryogenesis, metamorphosis, endocrinedependent tissue atrophy, and normal tissue turnover (Willye et al, 1980). Furthermore, apoptosis is induced in various cell lines in vitro by incubating the cells with chemicals such as and tumor agent (Tsuruo et al, 2003). Apoptosis is a unique morphological type of cell death that is characterized by chromatin condensation (Cohen, 1993).

Apoptosis plays a crucial role in the regulation of tissue homeostasis. Many studies have indicated that an imbalance between cell death and cell proliferation may result in tumor formation. The killing of tumor cells can be acquired through diverse catatonic approaches, such as anticancer drugs, gamma – irradiation or immunotherapy, which are predominantly mediated through the induction of apoptosis (Pasechnik *et al*, 2000).

Bruguiera gymnorhiza is a family of rhizoporaceae, a mangrove plant. Many Indonesian people use the leaf and stem bark of the plant as antimicrobial, antifungal, antivirus, anticancer, insecticide and antileukemia (Soetarno, 2000). The research on the effect of *B. gymnorhiza's* stem

bark ethanolic extract was reported to be cytotoxic towards myeloma and raji cell lines with IC<sub>50</sub> 301.78 μg/mL and 582 μg/mL (Warsinah, 2005). Stem bark of B. gymnorhiza exhibited antimicrobial activity against Aeromonas sobria, stutzeri Pseudomonas dan parahaemolyticus of udang windu (Chen, et al., 2007). A chemical compound isolated through fermentation of B. gymnorrhiza roots with peniccilium thomi resulted in the finding of 12 compounds: 4',5- dihydroxi -2-3- dimethoxy -4-(hydroxipropyl)-biphenyl (1) and 11 compounds with cytotoxic effect to 3 cancer cells (A549, HepG2 dan HT29). Homhual et al (1996), isolated sulfuric compounds from the flowers of B. namely cyclic 4- hidroxygymnorrhiza, dithiosulfonat (bruguisulfurol), hidroxydithiolane 1-oxida (bruguierol) and isobruguierol.



## **MATERIALS AND METHODS**

#### Chemicals

Medium RPMI (RPMI 1640 (Sigma), sodium bicarbonat (Sigma) dan Hepes (Sigma)), fetal bovin serum (FBS) (Gibco) 10% (v/v), penicillin-steptomycin (Gibco) 1% (v/v), fungizone (Gibco) 0,5% (v/v), sterile aquadest, ethanol 96% v/v, DMSO, Acridine Orange, etidium bromida, ethanol 70% (E Merck), Sodium Dodecyl Sulfat (SDS) 10% in HCl), Silica gel GF 254, MTT (3-(4,5-dimethylthyazole),2,5,diphenyltetrazolium bromide) 5 mg/mL in FBS, Asetic acid glacial (E Merck), Amonia (E Merck), methanol, chloroform, n-hexane, ethyl acetate.

### **Cell line and culture Condition**

The cell lines used in this study were Hela, Raji and Myeloma cell, which were provided by the Parasitology Laboratory of Faculty of Medicine Gadjah Mada University. Hela, Raji and Myeloma cell lines were cultured in RPMI 1640 medium suplemented with 10% fetal bovin serum at 37°C in 5% CO<sub>2</sub>.

#### Extraction of stem bark

Five hundred grams of stem bark powder was macerated with 3 L of methanol for 3x24 hrs. The filtrate was evaporated with rotary evaporator, residue of the methanol extract obtained was 53.6 gram.

# **Preparation of Test Solution**

Methanol extracts of 20 mg was weighed and dissolved in 100 mL DMSO. Then a series of extract concentration in RPMI medium 1640 was made into the treatment stock solution. A total of 20 mL of stock solution dissolved in RPMI medium to obtain concentrations of 1000  $\mu$ g/mL, further diluted to concentrations of 500, 250, 125 and 62.5  $\mu$ g/mL. Preparation of test solution was performed in a laminar air flow cabinet aseptically.

# Antiproliferative effect with MTT methods

Three cell lines, Hela, Raji and Myeloma cell were used for cytotoxicity and antiproliferative assay. Near-confluent cells were collected and diluted to a concentration of 2 x 10<sup>4</sup> cells/mL with culture medium. One hundred microliter aliquot of cell suspension were dispensed into each well 0f 96- well plate. The residue from extract stem bark *B. gymnorhiza* was redisolved in DMSO. The treatment concentrations were 1000, 500, 250,125 and 62,5 µg/. After 24 hrs of incubation at 37°C in 5% CO<sub>2</sub>, each well was treated with doxorubicin at

concentrations of 0.2, 0.1, 0.05, 0.025 and 0.0125 mg/mL and control of 100  $\mu$ l suspension cell in medium. It was then incubated at 37°C of 24 hrs. In each well 10  $\mu$ l of MTT 5mg/mL in RPMI was added followed by 4 hrs of incubation at 7°C. The reaction was then stopped with SDS. After incubation for 24 hrs the absorbance of each well was measured 595 nm using *Elisa reader*.

# **Detection of apoptosis Induction**

Apoptosis of the cells were detected by ethidium bromide-acrydine orange. Near-confluent cells were collected and diluted to a concentration 3 x  $10^4/100$  cells/mL. One hundred microliter aliquots of cell suspension were distributed on cover slips in a 24-well plate. The extract was dissolved in DMSO and was diluted to 30, 15 and 7.5  $\mu L$ . The extract was then used to treat the cells. Then the plate was incubated for 24 hours. The cells were then observed by flourescence microscope after adding ethidium bromide-acridine orange to the cover slips .

#### **RESULTS AND DISCUSSION**

Initially, we examined the antiproliferative effects of the extract obtained from the stem bark of B. gymnorhiza in human cancer cell lines. The extract showed antiproliferative effects towards HeLa, Raji and Myeloma cell in this study, with an IC<sub>50</sub> value of 133, 504 and 384  $\mu g/mL$ , respectively.

To investigate its antiproliferative activity further, the methanol extract from *B. gymnorhiza* was subjected to cell antiproliferation assay using HeLa, Raji and Myeloma cells. HeLa, Raji and Myeloma cells were treated with the stem bark *B. gymnorhiza* extract (75 μg/mL), and cell proliferation was examined at intervals by MTT assay. As shown in Fig. 1. The proliferation of the cells increased in time–dependent manner. Similar to the control.

The difference in cell number between the control and treated with tested extract metanol at any time were analyzed by LSD test with an error rate of 5%. The results of the analysis presented in Table I. Morphological changes in the cell caused by the extract methanol of stem bark *B. gymnorhiza* were observed by microscopy, as shown in Fig. 2. After 1-hrs incubation with the stem bark extract, the HeLa cells had swelled in comparison to the control cells (Fig.2, Extract 1 hour and control 1 hour). After 24 hours, dead cells were observed among the treated cells.

Next, the Hela cells were exposed to 133 µg/mL of extract for 24 hrs and the occurence of



apoptosis was observed. This result comfirm that induce apoptosis of HeLa cells (Fig. 3) methanol extract of stem bark *B.gymnorhiza* can

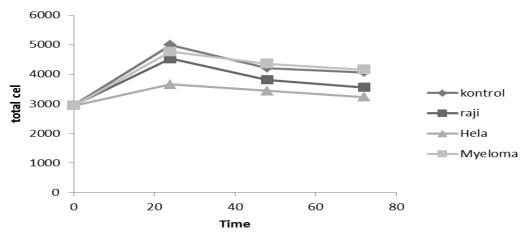


Figure 1. Cell proliferation (absorbance) by the extract methanol from stem bark of B. gymnorhiza.

Table I. Antiproliferative test results on the cells Raji, Hela, Meyloma and control of methanol extract at different times.

Time	Control	Raji	Hela	Meyloma
0	2953.3 ± 72.3a	2950.0 ± 69.3a	2940.0 ± 62.a	2940.0 ± 62.5a
24	4991.3 ± 121.1a	4529.7 ± 113.5a	3655.3 ± 60.3a	4759.3 ± 150.4ab
48	4209.7 ± 29.0a	3807.0 ± 67.09a	3441.7 ± 60.3a	4357.0 ± 47.6a
72	4064.7 ± 105.1a	3559.3 ± 103.4a	3233.3 ± 130.5ab	4157.7 ± 90.9a

Description: Different superscript in the same column indicate significant differences (P<0.05)

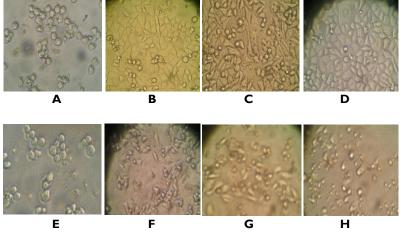
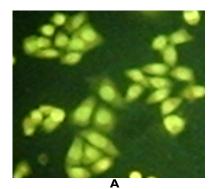


Figure 2. Morphological change in HeLa cells incubated with extract methanol (A) Control 0 hours (B) Control 24 Hours (C) Control 42 hours (D) Control 72 hour (E) Extract 0 hours (F) Extract 24 hours (G) Extract 48 hours (H) Extract 72 hours.





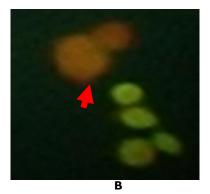


Figure 3. The result of apoptosis induction Cells by ethidium bromide-acridine orange exhibited the occurance of apoptotic cells, indicated by red ( ) arrows. (A) Control cell (B) Cell and extract

Previously we found that the ethanolic extract of B. gymnorhiza were cytotoxic towards Myeloma and raji cells line with IC<sub>50</sub> of 301,78  $\mu$ g/mL and 582  $\mu$ g/mL (Warsinah, 2005). Mimaki et al (1996) have shown that the saponins isolated from C. Comusum have inhibitory activity of Hela Cells as primary screening test to identification on new antitumor promoter compounds. B gymnorhiza has been traditionally used as a folk medicine for cough in china (Jiang, 1977).

In the present study, we have shown that methanol extract from the stem barks of B gymnorhiza has antiproliferative activity on HeLa, Raji and Myeloma cells in MTT assay. The IC<sub>50</sub> value of the methanol extract in HeLa, Raji and Myeloma cells were between 150  $\mu$ g/mL and did not vary much among the cell lines.

To confirm these effects by biochemical analysis, the effects of the methanolic extract was evaluated using HeLa cells. The antiproliferative activity of the methanol extract toward HeLa cells increased in a time dependent manner, as did the activity of the negative control cells. Examination of the morphology changes in HeLa cells treated with the extract showed that the cells swelled into circular form 1 hrs, and then died without fragmentation after 72 hrs, which differed from the effect of the negative control (Fig 2). These results suggest that the methanol extract of stem barks of *B. gymnorhiza* contains antiproliferative materials that kill HeLa cells.

To invesigate the relationship between the antiproliferative activity and apoptosis inducing activity of the methanol extract analysis by double staining (etidium bromide-acridine orange). The presence of DNA fragments indicates that the HeLa cells had undergone apoptotis. Detection of apoptotis using microscope flourescense futher indicate that methanol extract also induced apoptosis in Hella cells. Thus, these results show

that the antiproliferative activity of the extract was due to induction of apoptosis.

In summary, the methanolic extract from stem barks of *B. gymnorhiza* induces difinite apoptosis in various human cells lines.

#### **CONCLUSION**

The ethanolic of *B. Gymnorhiza* stem bark is cytotoxic towards Hela, Raji, and Myeloma cells but the molecular mechanism of action must be studied futher to provide a more conclusive evidence.

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