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In vitro Hepatotoxicity Study of Barakol Using Human Hepatoma Cell Line HepG2(การศึกษาความเป็นพิษของบาราคอลต่อตับ โดยการทดสอบด้วยเซลล์มะเ...

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นิพนธ์ปฐมภูมิ

การศึกษาความเป็นพิษของบาราคอลต่อตับ โดยการทดสอบด้วยเซลล์มะเร็งเพาะเลี้ยงของตับคน ชนิด เฮพจี2

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บทคัดย่อ

บาราคอล เป็นสารที่สกัดจากใบอ่อนของต้นขี้เหล็ก การศึกษานี้ใช้เซลล์มะเร็งเพาะเลี้ยงของตับคน ชนิดเฮพจี2 ทดสอบความเป็นพิษของบาราคอลต่อตับ โดยใช้บาราคอลในความเข้มข้น 0.25, 0.50, 0.75 และ 1 มิลลิโมลาร์ ทดสอบกับเซลล์เป็นเวลา 24, 48, 72 และ 96 ชั่วโมง แล้วประเมินความเป็นพิษต่อเซลล์โดยใช้ MTT assay รวมทั้งตรวจวัดการทำงานของเอนไซม์แลคเตท ดีไฮโดรจิเนส ที่ถูกปล่อยออกมาอยู่ในสารละลายเพาะเลี้ยงเซลล์ ที่เวลา 24 และ 48 ชั่วโมง การศึกษานี้ได้ใช้ อะเซตามิโนเฟน ซึ่งเป็นสารพิษต่อตับที่รู้จักกันดีในความเข้มข้น 1, 3, 5, 7 และ 10 มิลลิโมลาร์ ทำการศึกษาในทำนองเดียวกันควบคู่ไปด้วย ผลการทดลองเมื่อประเมินความเป็นพิษด้วย MTT assay พบว่า บาราคอลและอะเซตามิโนเฟน มีผลพิษต่อตับที่ขึ้นกับขนาดและเวลาที่ได้รับสาร บาราคอลมีผลพิษต่อเซลล์ตับอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ที่ความเข้มข้นมากกว่าหรือเท่ากับ 0.75 มิลลิโมลาร์ที่เวลา 24 ชั่วโมงของการสัมผัส ส่วนที่เวลา 48, 72 และ 96 ชั่วโมงของการสัมผัส จะพบผลพิษของบาราคอลที่ความเข้มข้นมากกว่าหรือเท่ากับ 0.50 มิลลิโมลาร์ IC_{50} ของบาราคอลต่อเซลล์ที่เวลา 24, 48, 72 และ 96 ชั่วโมงของการสัมผัส มีค่าเท่ากับ 5.70, 0.96, 0.77 และ 0.68 มิลลิโมลาร์ ตามลำดับ เทียบกับ IC_{50}ของอะเซตามิโนเฟนที่เวลาดังกล่าวมีค่าเท่ากับ 12.14, 11.13, 1.39 และ 1.30 มิลลิโมลาร์ ตามลำดับ เมื่อเปรียบเทียบพิษต่อเซลล์ระหว่างบาราคอลและอะเซตามิโนเฟนที่ความเข้มข้น 1 มิลลิ-โมลาร์ พบว่าบาราคอลมีผลพิษต่อเซลล์มากกว่าอะเซตามิโนเฟนอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ที่เวลาต่างๆของการสัมผัส อย่างไรก็ตาม เมื่อวัดการทำงานของเอนไซม์แลคเตท ดีไฮโดรจิเนส ที่เวลา 48 ชั่วโมงของการสัมผัส พบว่าทั้งบาราคอลและอะเซ-ตามิโนเฟนที่ความเข้มข้น 1 มิลลิโมลาร์มีผลทำให้เอนไซม์ดังกล่าวถูกปล่อยออกมาอยู่ในสารละลายเพาะเลี้ยงเซลล์ได้เท่ากัน ทั้งนี้การลดลงของ ระดับรีดิวส์กลูตาไธโอน และอัตราส่วนระหว่างรีดิวส์กลูตาไธโอนและออกซิไดส์กลูตาไธโอน ในเซลล์เมื่อมีการสัมผัสกับบาราคอล อาจช่วยอธิบายกลไกการเกิดพิษต่อตับของบาราคอลได้

กุญแจคำ

บาราคอล, อะเซตามิโนเฟน, พิษต่อตับ, เซลล์เฮพจี 2

Original Article

In vitro Hepatotoxicity Study of Barakol Using Human Hepatoma Cell Line HepG2

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Abstract

Human hepatoma cell line HepG2 was used to assess the hepatotoxic effects of barakol, isolated from young leaves of *Cassia siamea*. Barakol at concentrations of 0.25, 0.50, 0.75 and 1 mM were added to HepG2 cells for 24, 48, 72 and 96 hours. Cytotoxicity was assessed by a rapid MTT cell viability screening assay. Activities of lactate dehydrogenase enzyme in the culture medium were determined at 24 and 48 hours of barakol incubation. Acetaminophen, a well-known hepatotoxin, at concentrations of 1, 3, 5, 7 and 10 mM were also assessed in parallel in the same manner. Using MTT assay, barakol demonstrated a dose- and time-dependent cytotoxic effect in the same manner as acetaminophen. Barakol exhibited a significant ($p<0.05$) cytotoxic effect at concentrations of $\geq$ 0.75 mM at 24 hours of exposure. At 48, 72 and 96 hours of exposure, cytotoxicities of barakol were shown at concentrations of $\geq$ 0.50 mM. Fifty percent cytotoxicities (IC_{50}) of barakol on HepG2 cells at 24, 48, 72 and 96 hours of exposure were 5.70, 0.96, 0.77 and 0.68 mM, respectively; whereas the corresponding IC_{50} of acetaminophen were 12.14, 11.13, 1.39 and 1.30 mM, respectively. At the same concentration of 1 mM, barakol was significantly ($p<0.05$) more cytotoxic to HepG2 cells than acetaminophen at every time point of compound exposures. However, both barakol and acetaminophen caused lactate dehydrogenase leakage in the same extent at 1 mM concentration and 48 hours exposure. A depleted reduced glutathione as well as a decrease of GSH/GSSG ratio following barakol exposure might explain the mechanism by which barakol induced hepatotoxicity.

Key words

Barakol, Acetaminophen, Hepatotoxicity, HepG2 cells

Introduction

Barakol (3α,4-dihydro-3α,8-dihydroxy-2,5-dimethyl-1,4-dioxaphenalene) is an active constituent extracted from the leaves and flowers of *Cassia siamea* (1,2). Various studies were performed dealing with the physiological and pharmacological effects of either crude extract of *Cassia siamea* leaves (3,6) as well as their purified active constituent, barakol (7-16). Extensive studies have been focused on the

effects of barakol on the central nervous system (7-14). Dopamine agonist-like effects and possible serotoninergic antagonist properties are proposed to explain the anxiolytic effect of barakol (11-14). Regarding the toxicological studies of this compound, only acute toxicities, the median lethal dose (LD_{50}) of either an alcohol-extract of the leaves of *Cassia siamea* or barakol were investigated in rodents (17). Repeated ingestion of leaves of *Cassia siamea* in patients for a traditional purpose of anxiolytic effect resulted in some evidence of chemical induced liver injury (18).

Although animals such as mice or rats have been used for a toxicological evaluation, it is well established that numerous compounds are metabolised quite differently in rodents compared to man *in vivo* and *in vitro* using human hepatocyte cultures (19, 20). Due to an ethical obstacle to study metabolism and toxicity of compound in man *in vivo*, human hepatocyte culture is preferable. However, human hepatocytes are not readily obtainable and levels of drug metabolizing enzymes decrease upon culturing (21). Since human hepatoma cell line HepG2 has been shown by several groups to be useful in studying drug metabolism and toxicity (22-28), we used these cells to assess the hepatotoxicity of barakol *in vitro*. Furthermore, acetaminophen, the well-known hepatotoxin was also evaluated in parallel as a positive control.

Materials and Methods

Chemicals

All chemicals, unless otherwise noted, were obtained from Sigma Chemical Co., St Louis, MO. Cell culture materials were purchased from Gibco BRL., NY.

Barakol was extracted from young leaves of *Cassia siamea* Lam. Fresh young leaves of *Cassia siamea* Lam. were obtained from a local Bangkok market and identified by comparing with the herbarium specimens at the Botany Section, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand. The leaves were cut into pieces and boiled twice with 0.5% sulfuric acid for 30 minutes. All fractions of the water extract were filtered, combined, alkalinised with concentrated sodium carbonate solution and extracted with chloroform. The chloroform fraction was concentrated under reduced pressure and shaken with 5% aqueous acetic acid three times. The aqueous acid fraction was neutralised with ammonium hydroxide and cooled. The crude barakol was crystallised as greenish yellow needles which were then dissolved in absolute ethanol. Concentrated hydrochloric acid was added to obtain barakol hydrochloride and recrystallised in distilled water twice to form anhydrobarakol hydrochloride. The compound was characterized by high performance liquid chromatography (Hypersil ODS column and 10% aqueous methanol as a mobile phase) showing a single peak at 254 nm.

Cell cultures

HepG2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in 75 cm2 flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%(v/v) fetal bovine serum, 2 nM L-glutamine, 0.01%(v/v) of the solution containing 5 mg/ml of penicillin, 5 mg/ml of streptomycin and 10 mg/ml of neomycin, and 0.25 μg/ml of insulin. They were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Medium was replenished every 2 or 3 days.

Cell viability assay

Cell viability was determined by the MTT assay as described by Mosman (29) with slight modification. Briefly, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was dissolved in phosphate buffer saline (PBS) at a concentration of 5 mg/ml and filtered. At the time of experimentation, cells were trypsinized from 75 cm2 flask, counted under microscope and 100 μl of dispersed cells of $12x10^3$ cells was placed into each well of 96-well culture plates. Following an overnight incubation at 37°C, 200 μl of new supplemented DMEM containing various concentrations of either barakol (0, 0.25, 0.50, 0.75, 1.00 mM) or acetaminophen (0, 1, 3, 5, 7, 10 mM) were replaced into the wells in triplicate. The culture plates were then incubated at 37°C and assayed for cell viability

by MTT assay at 24, 48, 72 and 96 hours of compound treatments. Except for the 24-hour compound treatment, the culture plates were changed with medium containing the compound everyday till assay for cell viability. At the time of cell viability assay, the medium containing barakol or acetaminophen was removed and 200 µl of new supplemented DMEM as well as 50 µl of MTT solution were added to each well of the assay. After a 2-hour incubation at 37°C, 0.1 ml of the medium was removed from each well and 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate in 50% dimethylformamide) was added. After an overnight incubation at 37°C, the optical densities (OD) at 570 nm were measured using a multiscanner autoreader (Spectra Max Plus, Molecular Devices Corp.) against the blanks. Blanks were prepared by adding 200 µl of supplemented DMEM into each 5 empty wells (without HepG2 cells) in the same plates of the tested compounds. MTT solution and extraction buffer were added to the wells which were incubated in the same manner along with the tested compounds. Percentage of cell viability was determined as follows: % cell viability = (OD_{570} of tested sample/OD_{570} of control sample without the compound) x100.

Lactate dehydrogenase (LDH) assay

LDH determination used in this assay is based on the kinetic spectrophotometric method of Wroblewski and LaDue (30). Briefly, 1 ml of dispersed cells of 2.5×10^5 cells was placed into each well of 24-well culture plates. Following an overnight incubation at 37°C, 1 ml of supplemented DMEM containing various concentrations of either barakol (0, 0.50, 1 mM) or acetaminophen (0, 1, 5, 10 mM) were replaced into the wells in triplicate. The culture plates were incubated at 37°C for 24 and 48 hours and the medium were then removed and used for the LDH assay. The activities of LDH was measured by monitoring the rate of which the substrate, pyruvate, was reduced to lactate. The reduction was coupled with the oxidation of reduced nicotinamide adenine dinucleotide (NADH), which was followed spectrophotometrically in term of reduced absorbance at 340 nm. The extinction coefficient of 6.22 mM^{-1} cm^{-1} of NADH was used in the calculation.

Determination of glutathione

Total glutathione and oxidized glutathione (GSSG) were determined according to the method of Griffith (31). Reduced glutathione (GSH) was calculated from a substraction of GSSG from total glutathione. Briefly, 1×10^6 cells in 25 ml of supplemented DMEM were grown in each 150 mm culture plate till established adherent monolayer cells were approximately 80% confluency. The medium was then replaced with 25 ml supplemented DMEM containing various concentrations of barakol (0, 0.50 and 1 mM) and further incubated at 37°C. Following a 24-hour incubation, the medium was aspirated and cells were washed twice with cold PBS before harvested with 50 mM phosphate buffer pH 7.8 containing 1.34 mM of diethylenetriamine pentaacetic acid. Cells were homogenized and the homogenates were used for the assay of glutathione. Protein concentrations of the cell homogenates were determined by using Bio-Rad protein assay kit (Bio-Rad Lab, U.S.A.).

Statistics

The data were shown as mean and standard deviation. Statistical analysis was performed by analysis of variance (ANOVA), followed by Student Newman-keuls test. Comparing between two treatments was performed by Student t-test. Probability of $p<0.05$ was accepted as statistically significant.

For estimation of IC_{50}, the percents of inhibition were transformed to probit units. The linear regression method was used to fit a curve between probit unit and log dose using Cricket graph program. The IC_{50} was calculated from the log dose probit line.

Results

Using MTT cell viability assay, barakol and acetaminophen demonstrated a similar profile of cytotoxicity on HepG2 cells. Their effects on HepG2 cells were concentration- and time-dependent (Figure 1). At 24-hour exposure, barakol exhibited significant cytotoxic effects at concentrations of 0.75

mM and above, whereas acetamionphen began to show a significant cytotoxicity at the concentration of 5 mM, comparing to the corresponding controls. At 48, 72 and 96 hours of exposure, barakol at concentrations of 0.50 mM and above demonstrated significant cytotoxic effects on HepG2 cells and the effects were increased as the concentration and the time of exposure increased. The 50% cytotoxicity (IC_{50}) of barakol on HepG2 cells at 24, 48, 72 and 96 hours of exposures were 5.70, 0.96, 0.77 and 0.68 mM, respectively, whereas the corresponding IC_{50} of acetaminophen were 12.14, 11.13, 1.39 and 1.30 mM, respectively (Figure 1). At the same concentration of 1 mM, barakol was significantly more cytotoxic to HepG2 cells than acetaminophen at every time point of compound exposures (Figure 2).

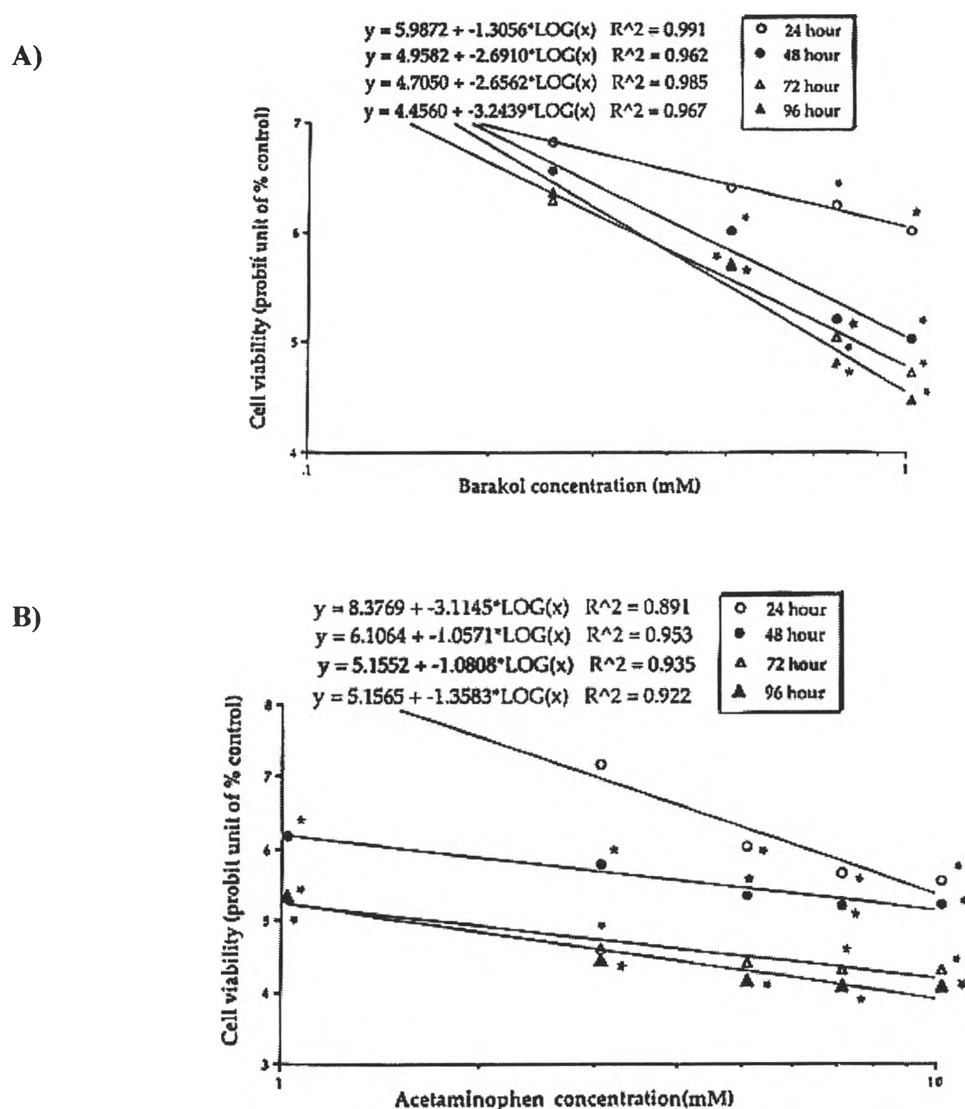

Figure 1. Cytotoxicity of barakol (A) and acetaminophen (B) on human hepatoma HepG2 cells at various concentrations and times of exposure. The figures shown were mean of 3 replicated wells per point. * denotes significant differences ($p < 0.05$) between the compound treatment and the corresponding control (0 mM). IC_{50} of barakol at 24, 48, 72 and 96 hours of exposure were 5.70, 0.96, 0.77 and 0.68 mM, respectively (A); whereas the corresponding IC_{50} of acetaminophen were 12.14, 11.13, 1.39 and 1.30 mM, respectively (B).

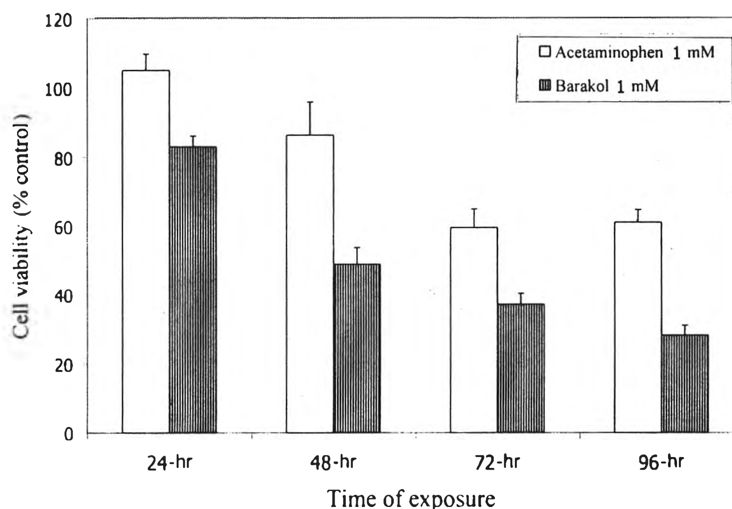

Figure 2. Cytotoxicity of barakol and acetaminophen at the same concentration of 1 mM in culture medium of HepG2 cells at various times of exposure. The figures shown were mean ± SD of 3 replicated wells per point. * denotes significant differences ($p< 0.05$) between the barakol-treated and the acetaminophen-treated cells.

Table 1 shows LDH activities in the culture medium following 24 and 48 hours of barakol and acetaminophen exposures. Both barakol (0.50 and 1 mM) and acetaminophen (1, 5, 10 mM) did not cause LDH leakage from the cells at 24-hour exposure. However, following 48-hour exposure, 1 mM of either barakol or acetaminophen caused significant increase of LDH activities in the culture medium comparing to their corresponding controls. In contrast with the MTT assay, the extent of LDH leakage by these two compounds at the same concentration of 1 mM was not significantly different.

Preliminary data of the effect of barakol on hepatic glutathione was shown in Figure 3. Barakol decreased hepatic total glutathione, GSH and the glutathione status (GSH/GSSG ratio) in a concentration-dependent manner. Barakol, at a concentration of 0.50 mM, depleted total glutathione and GSH to about 40% of the nontreated levels. Much more depletions of total glutathione and GSH to about 5-10% of the corresponding nontreated levels were shown when 1 mM of barakol was in the culture medium.

Table 1. LDH activities in the culture medium of HepG2 cells at 24 and 48 hours of barakol and acetaminophen exposures.

Compound	Concentration in medium (mM)	24-hr exposure[a, b]	48-hr exposure[a, b]
Acetaminophen	0	24.68 ± 0.34	25.19 ± 1.46
	1	25.88 ± 1.05	34.92 ± 2.83*
	5	28.35 ± 0.09	55.52 ± 1.86*
	10	28.51 ± 2.37	66.08 ± 9.11*
Barakol	0	21.94 ± 0.57	23.15 ± 1.54
	0.5	22.19 ± 0.74	22.35 ± 0.58
	1.0	20.85 ± 2.46	30.83 ± 5.14*

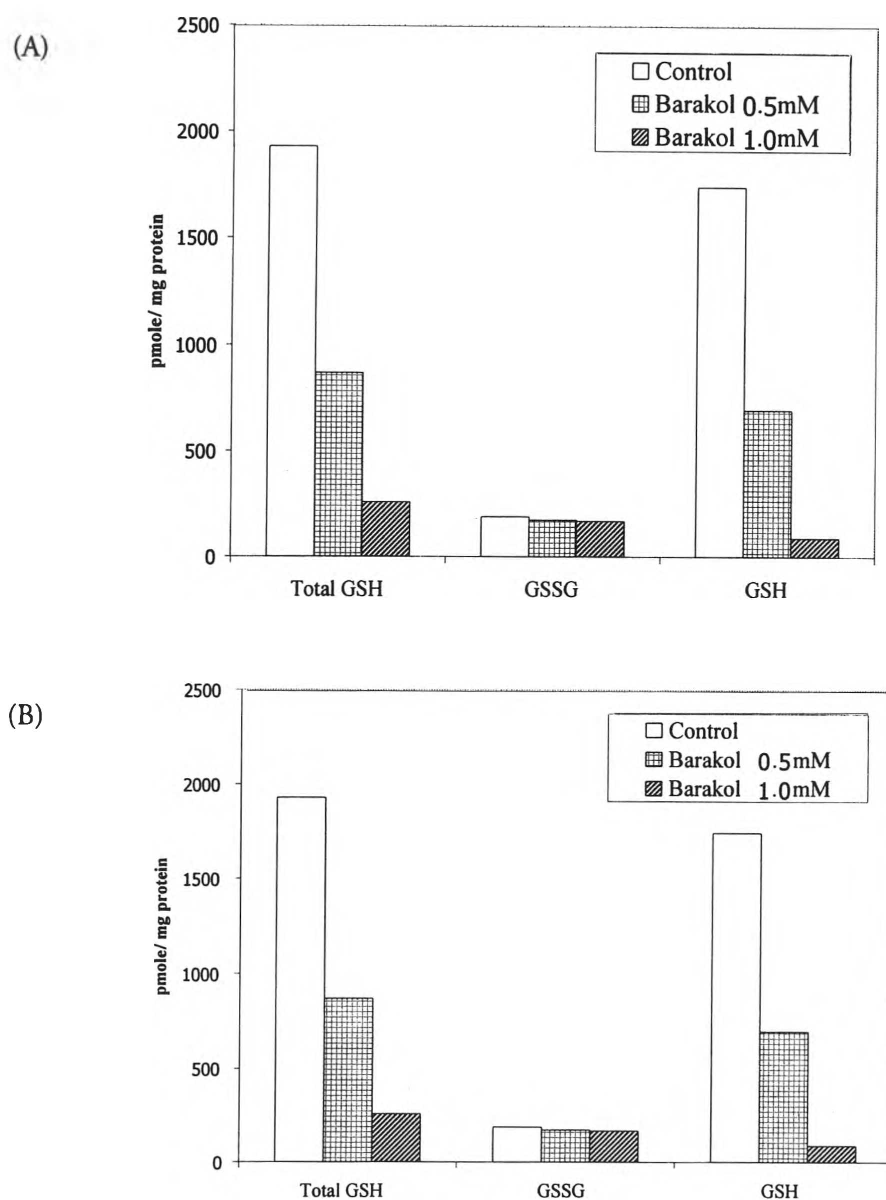

Figure 3. Effect of barakol on total glutathione, GSSG, GSH (A) and GSH/GSSG ratio (B) at 24 hour exposure.

Discussion and Conclusion

The results from this study showed that barakol exhibited a concentration- and time-dependent hepatotoxicity *in vitro* using human hepatoma cell line HepG2 as a model study. Using MTT cell viability assay, the cytotoxic effects of barakol were found at the lower concentrations and earlier than the appearance of LDH leakage. This could be explained by the different principal of detection between the assays. MTT assay is based on the tetrazolium salt MTT of which the tetrazolium ring is cleaved by active mitochondria containing various active dehydrogenase enzymes or the method measures the reducing capacity of mitochondrial dehydrogenase enzymes and mitochondrial integrity. The reaction occurs only in living cells or the method measures only living cells (29). Thus, MTT assay can detect cell toxicity early at the beginning of cell injury. In contrast, an increase of LDH activity in the culture medium indicates cell leakage, the final event of cell damage/necrosis. Moreover, MTT assay might be a more sensitive method than a detection of LDH leakage in this study. Due to its simplicity, rapidity, high degree of precision, strong correlation with cell viability and capacity of measuring large number of

samples (29), MTT assay has been used by several groups for a preliminary screening of cell viability. Nevertheless, another cytotoxicity measurements are necessarily confirmed.

In this study, we used human hepatoma HepG2 cells to investigate an *in vitro* hepatotoxicity of barakol instead of using animal cell lines or conventional *in vitro* methods using animal liver preparations such as rat primary hepatocyte cell cultures, rat liver homogenates, etc. Species differences in compound metabolism and toxicity limit an extrapolation of animal data to human (19, 20, 32). Even though primary human hepatocyte cultures eliminate these drawbacks of animal model, human hepatocytes are not readily obtainable and levels of drug-metabolizing enzymes decrease upon culturing (21). HepG2 cells are human hepatoma cell lines which have been found to retain many drug-metabolizing enzymes (25) and have been shown by several groups to be useful in studying drug metabolism and toxicity (22-28).

Since acetaminophen is a well-documented hepatotoxin and several cytotoxicity studies were performed using HepG2 cells (26-28), we investigated cytotoxicity of barakol in parallel with this drug. We used concentrations of acetaminophen as 1, 3, 5, 7 and 10 mM in this study in the same manner as a study by Hall et al (27). In that study, they found that acetaminophen produced 50% hepatotoxicity in acute exposure (24 hours) studies at concentration of 7 mM. In addition, in chronic exposure (up to 10 days) studies, acetaminophen produced significant morphological changes of HepG2 cells at 1 mM, the concentration which had no significant effects in the acute studies (27). Consistently, in our study, 1 mM of acetaminophen did not produce significant cytotoxicity on HepG2 cells at 24 hours of exposure. However, the IC_{50} of acetaminophen at 24-hour exposure in our study (12.14 mM) was different from the result reported (7 mM) by Hall et al (27) probably due to the different methods used for cell viability assay. In this study, barakol could not be used at concentrations above 1 mM due to its limited solubility in culture medium as well as its strong alkali property. Therefore, we could compare the toxic effects of these two compounds only at 1 mM concentration. Barakol demonstrated more toxic effects on HepG2 cells than acetaminophen using MTT assay. However, the cytotoxicity of these two compounds determined by LDH leakage was not significantly different. At 48-hour exposure, 1 mM concentration of either barakol or acetaminophen caused LDH leakage into the culture medium significantly higher than their corresponding controls, but the activities were not significantly different between these two compounds at this concentration.

While the mechanism by which barakol-induced hepatotoxicity needs to be further clarified, we preliminary explored whether the toxicity was associated with oxidative stress. Glutathione is presented in high concentrations as reduced glutathione (GSH) in most mammalian cells, with minor fraction being glutathione disulfide (GSSG) and other cellular thiol (33). Glutathione redox cycle is a major endogenous protective system of mammalian cells in minimizing injurious events that result from the oxidative stress produced by toxic chemicals or their reactive metabolites, as well as normal oxidative products of cellular metabolism (34, 35). Thus, the glutathione status (GSH/GSSG ratio) is a good indicator of oxidative stress (36). Glutathione depletion to about 20-30% of total glutathione level can impair cell's defense mechanism and may lead to cell injury and death (37-38). In this study, barakol depleted total glutathione, GSH as well as decreased GSH/GSSG ratio in the concentration-dependent manner at 24 hours of exposure. The depletion effects of barakol on glutatione correlated with the cytotoxic effects of this compound at 24-hour exposure determined by MTT method. This is a preliminary information showing that oxidative stress is possibly associated with barakol-induced hepatotoxicity. However, metabolic pathway of barakol need to be further explored whether barakol undergoes metabolic bioactivation and oxidative stress is resulted from the reactive metabolites.

In conclusion, barakol demonstrated hepato-toxicity *in vitro* using human hepatoma HepG2 cells as a model study. Using MTT cell viability assay , barakol exhibited a significant toxic effect at concen-

trations of ≥ 0.75 mM at 24-hour exposure. At 48, 72 and 96 hours of exposure, cytotoxicities were shown at concentrations of ≥ 0.50 mM of barakol. IC_{50} of barakol on HepG2 cells at 24, 48, 72 and 96 hours of exposures were 5.70, 0.96, 0.77 and 0.68 mM, respectively whereas the corresponding IC_{50} of acetaminophen were 12.14, 11.13, 1.39 and 1.30 mM, respectively. At the same concentration of 1 mM, barakol was significantly more cytotoxic to HepG2 cells than acetaminophen at every time point of compound exposures. However, similar extent of LDH leakage was shown when HepG2 cells were exposed to barakol or acetaminophen at a concentration of 1 mM for 48 hours. A depletion of GSH as well as a decrease of GSH/GSSG ratio following barakol exposure might explain the mechanism by which barakol induced hepatotoxicity.

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