Anti-Oxidative Stress Activities of Silibinin on α-Amanitin *In Vitro*

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ABSTRACT

Alpha amanitin (α-amanitin) is the mainly toxic substance in *Amanita* mushrooms in Thailand. Toxicity of α-amanitin is directly on the liver organ leading to body systemic failure. Silibinin is an antioxidant and only one medication that can help reduce the toxicity of α-amanitin, but its mechanism of action is still unclear. The antioxidant activities of silibinin in scavenging free radicals, i.e. superoxide radical, hydroxyl radical, and H_2O_2 as well as the direct trapping between silibinin and α-amanitin were evaluated in vitro. The scavenging free radicals of silibinin in this study were demonstrated in XO-HX, Ribose- and H_2O_2-ABT/HRP systems by comparing to the standard antioxidants; gallic acid, N-acetylcysteine (NAC), and quercetin. Anti-toxicity was studied in human blood from α-amanitin oxidation, then the whole blood glutathione (GSH) and plasma malondialdehyde (MDA) levels were detected with DTNB and TBARs . The results showed that silibinin scavenged superoxide radicals lower than quercetin but higher than gallic acid and NAC. It scavenged hydroxyl radicals better than quercetin, NAC and gallic acid, respectively. However, the scavenging activity on H_2O_2 of silibinin was lowest among the compared antioxidants. Interestingly, silibinin protected the GSH and inhibited MDA production in blood from α-amanitin oxidation in a dose response manner. The study revealed that silibinin had scavenging activity directly on free radicals, especially superoxide, hydroxyl radicals, and H_2O_2. It also protected the GSH and inhibited MDA production in human blood from α-amanitin oxidation.

Keywords: Alpha-amanitin, silibinin, antioxidant activity, scavenging free radical, *Amanita* mushroom

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INTRODUCTION

Bureau of epidemiology, Thailand (2008) reported 1,624 cases with 5 deaths from consuming *Amanita* mushrooms in Chiang Mai and Mae Hong Son. The most poisonous substance of amatoxins is alpha amanitin (α-amanitin). In Thailand, *Amanita verna* (Bull.ex.Fr.) Vitt. and *Amanita virosa* Secr. are the main lethal toadstool.[1] The structure of amatoxins is a bicyclic octapeptides with an indole-(R)-sulphoxide bridge and different amatoxins have been classified in alpha-, beta-, and gamma amanitins. [2,3] α-Amanitin is the most toxic compound (the lethal dose in human adults is 0.1 mg/kg body weight).[4,5] The mechanism of α-amanitin toxicity is due to its strong inhibition on the activity of RNA polymerase II leading to the inability in producing messenger RNA and consequently causing cell death within 24 hour.[4] Other toxic mechanisms have also been proposed, such as, α-amanitin acts synergistically with endogenous cytokine (e.g. tumor necrosis factor)[6] and may cause generation of free radicals within liver cell.[7] Some evidences showed that α-amanitin acted either as an antioxidant in the auto-oxidizing liposome system or as a pro-oxidant in Fenton reaction.[8]

Silibinin is the major compound of the silymarin isolated from seeds of mediterranean milk thistle, *Silybum marianum* (L.) Gaertn. (*Asteraceae*), and clinically used as a hepatoprotectant.[9,10] Silibinin has anti-hepatotoxic activity with anti-inflammatory, anti-allergic and anti-oxidant activities by scavenging various free radicals.[10,11] In animal liver, silibinin is shown to inhibit α-amanitin uptake[12,13] and also inhibit penetration of α-amanitin by competing with α-amanitin at the hepatocyte membrane.[11]

In Thailand, mushroom poisoning especially *Amanita* poisoning was increasingly reported every year. The toxic manifestation of amatoxin poisoning takes a long period of latency. The delay onset of symptoms seems not to be too severe and often lead to misdiagnosis or delayed diagnosis. Several reports claimed the antidote of silibinin to amatoxin is due to antioxidant activity but the actual mechanism of silibinin is still unclear. The aim of this study was to evaluate the mechanism of silibinin on α-amanitin in vitro by observing its scavenging free radicals and activity in human blood.

MATERIALS AND METHODS

Chemicals

N-acetylcystein (NAC), thymol, gallic acid, quercetin, silibinin, hypoxanthine (HPX), xanthine oxidase (XOD) were purchased from Sigma (St. Louis, Mo, USA). 2,2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), nitrotetrazolium blue chloride (NBT), 2-deoxy-D-ribose, 5,5´ dithiobis-(2-nitrobenzoic acid) (DTNB), α-amanitin, ammonium acetate were purchased from Fluka (Buchs, Switzerland). Acetronitrile was purchased from SK chemicals (Korea). Other chemicals were of analytical grade.

Determination of scavenging activity on H_2O_2

The testing method was followed Okamoto *et al*.[14] Briefly, potassium phosphate buffer (0.1 M, pH 6.0), silibinin, H_2O_2 (0.003%), peroxidase (10 U/ml), and ABTS (0.1%) were thoroughly mixed. Reaction mixture was incubated at 37 °C for 15 min and the absorbance was measured at 415 nm. The concentration of silibinin at 50% inhibitory activity was compared to NAC, gallic acid and quercetin which were used as the standard antioxidants. All tests were repeated 3 times.

Determination of scavenging activity on superoxide anion ($O_2^{\bullet -}$)

The assay was followed Gaulejac *et al*.[15] Superoxide anion radicals were generated in a hypoxanthine/xanthine oxidase (HPX-XOD) system and assayed by NBT reduction. The decrease in absorbance at 560 nm was measured every 15 second for 6 min. The amount of silibinin to reduce the initial $O_2^{\bullet -}$ concentration by 50% was compared to NAC, gallic acid and quercetin. All tests were repeated 3 times.

Determination of scavenging activity on hydroxyl radical (OH^-)

The method of OH^- measurement is by thiobarbituric acid-reactive substances (TBAR) assay as described by Ozyürek *et al*.[16] Oxidative attack of OH^- generated from a Fenton reaction on deoxyribose to produce MDA and measured the formation of MDA-TBA adducts to detect OH^-. The amount of silibinin at 50% inhibition was compared to NAC, gallic acid and quercetin. All tests were repeated 3 times.

Antioxidant activity in human erythrocytes

Four systems were designed: erythrocyte (control group), erythrocyte with α-amanitin (negative control), erythrocyte with α-amanitin plus silibinin, and erythrocyte plus silibinin. Each system was incubated at 37 °C for 2 hr. Whole blood was then evaluated for glutathione and the plasma was separated by centrifugation at 3,000 ×g for 10 min to evaluate MDA. Evaluation of MDA was adapted from Chirico.[17] The measurement of MDA based on MDA-TBA adduct formation and measured at 532 nm. Glutathione assay was applied from Beutler *et al*.[18] Briefly, a whole blood sample was mixed with distilled water and precipitating solution. The mixture was centrifuged at 10,000 xg for 5 min. A supernatant was mixed with 0.3 M Na_2HPO_4 (pH 8.0) and 1 mM DTNB. After incubated at room temperature for 5 min, yellow complex was developed and read absorbance at 412 nm.

Determination of α-amanitin by HPLC

The chromatographic condition was followed the method of Jehl *et al*.[19] The HPLC apparatus was run on the 9012Q pump solvent delivery system with a prostar 310 UV/VIS detector (Varian Chromatography system, USA). α-Amanitin was separated in a Luna C18 (5 mm 125 × 4.6 mm) (Phenomenex®, USA). The mobile phase was a mixture between 0.02 M aqueous ammonium acetate and acetonitrile (88:12, v:v) (pH 5), and α-amanitin was analyzed at 280 nm. The calibration curve was prepared based on the five concentrations of standard α-amanitin (6-100 µg/ml). Determination of α-amanitin was directly trapped with silibinin. α-Amanitin (50 µg/ml) were incubated with various concentration of silibinin (200, 100 and 10 µg) at room temperature for 5 min, and α-amanitin was then analyzed with HPLC.

RESULTS AND DISCUSSION

Free radical scavenging activity

The study showed that gallic acid had the highest scavenging activity (0.34 ± 0.01 µg) on H_2O_2, followed by NAC (0.57 ± 0.01 µg), quercetin (3.78 ± 0.02 µg) and silibinin (4.84 ± 0.01 µg). For the scavenging on $O_2^{\bullet -}$ at 50%, the results showed that quercetin provided the highest scavenging activity (0.07 ± 0.01 µg) followed by silibinin (0.36 ± 0.01 µg), gallic acid (45.42 ± 1.92 µg), and NAC (4,778.34 ± 208.63 µg). Silibinin showed the best scavenging activity (214.67 ± 229.86 µg) on OH^- scavenging compared to quercetin (410.00± 121.66 µg), NAC (666.67 ± 5.77 µg) and gallic acid (713.33 ± 5.77 µg) (Table 1). The study of Mira *et al*[20] has found that silibinin dihemisuccinate (water soluble form of silymarin) was not a good scaven-

Table1: IC_{50} antioxidant scavenging activity of silibinin, NAC, gallic acid, and quercetin

compound	H_2O_2 scavenging activity (µg)	$O_2^{\bullet -}$ scavenging activity (µg)	OH^- scavenging activity (µg)
Silibinin	4.84 ± 0.01	0.36 ± 0.01	214.67 ± 229.86
NAC	0.57 ± 0.01	4,778.34 ± 208.63	666.67 ± 5.77
Gallic acid	0.34 ± 0.01	45.42 ± 1.92	713.33 ± 5.77
Quercetin	3.78 ± 0.02	0.07 ± 0.01	410.00 ± 121.66

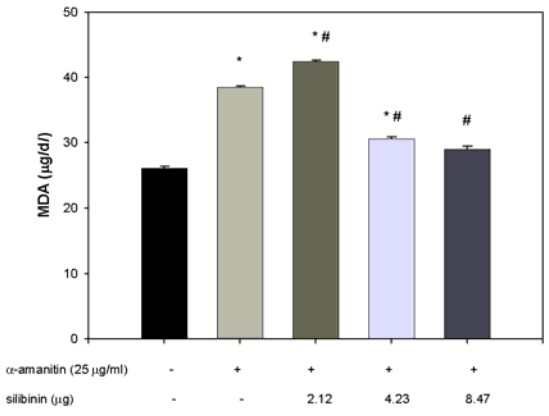

Figure 1 Effect of silibinin at 2.12, 4.23 and 8.47 µg on lipid peroxidation (MDA) level in whole blood from α-amanitin (25 µg/ml) oxidation. Each bar is a mean and standard deviation from triplicates. *, p<0.05 compared to the whole blood without any treatment (first bar); and # , p< 0.05 compared to the whole blood treated with α-amanitin (second bar).

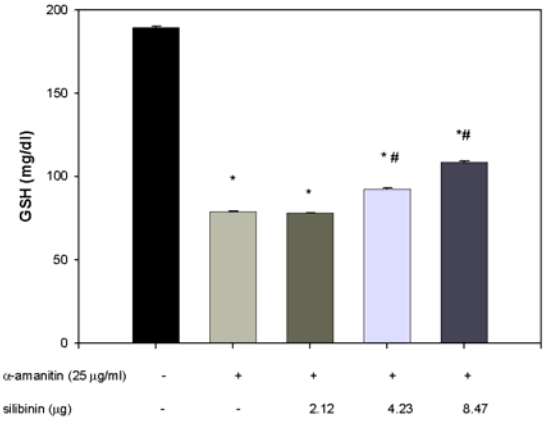

Figure 2 Effect of silibinin at 2.12, 4.23 and 8.47 µg on GSH level in whole blood from α-amanitin (25 µg/ml) oxidation. Each bar is a mean and standard deviation from triplicates. *, p<0.05 compared to the whole blood without any treatment (first bar) and # , p< 0.05 compared to the whole blood treated with α-amanitin (second bar).

ger of $O_2^{\bullet -}$ and gave no reaction with H_2O_2, but could scavenge OH^-. The present study, however, found that silibinin showed antioxidant activity toward H_2O_2, $O_2^{\bullet -}$ and OH^-. Besides the different methods for determine free radical scavenging activity were used, silibinin in this study was a pure compound and insoluble in water. Nevertheless, our results confirmed that silibinin acts as antioxidant.[10,21]

Antioxidant activity in human blood

Figure 1 showed that silibinin inhibited the lipid peroxidation on human erythrocytes from α-amanitin oxidation at 4.23 and 8.47 µg (MDA 30.64 and 28.97 µg/dl) compared to the control (26.11 µg/dl) , which was significantly different (p<0.05). The α-amanitin significantly decreased (p<0 .0 5) the GSH level (78.78 mg/dl) compared to the control (189.51 mg/dl). Silibinin at 4.23 and 8.47 µg significantly increased GSH level (92.34 and 108.30 mg/dl) significantly compared to α-amanitin alone (Figure 2). Silymarin interacts directly to the cell membrane and prevent any abnormalities of lipid fraction responsible for maintaining normal membrane fluidity.[9,21] Thus, it prevented oxidation of membrane lipids resulting in reducing MDA level after treated α-amanitin with silibinin. This confirmed that silibinin helps to maintain and protect the antioxidant system such as GSH and super oxide dismutase.[22,23] Silibinin also protected GSH

from α-amanitin oxidation with a dose response manner.

Determination of α-amanitin by HPLC

The retention time of α-amanitin was 11.78 ± 0.47 min. The limit of detection (LOD) and the lower limit of quantitation (LOQ) of alpha amanitin were 4.3 and 5.73 µg/ml, respectively. The peak area responded to the dose concentration at 6-100 µg/ml with a good correlated coefficience (r = 0.999). The method showed the high accuracy with % recovery of the measurement of α-amanitin more than 95% (95-98%), including good intra-day coefficient of variation (3.53 to 7.73% in range) from 10 repetitive tests. The direct scavenging effect of silibinin on α-amanitin was done by varying concentrations of silibinin (10, 100, or 200 µg) with 50 µg/ml of α-amanitin. The results showed no different change compared to the untreated α-amanitin (Figure 3). This finding suggested that silibinin did not affect directly on α-amanitin, but the inhibitory action of silibinin is by binding to cell membranes; and therefore inhibit α-amanitin penetration of the cell membrane. Silibinin also competed with α-amanitin for the specific bile salt transport systems of hepatocyte membrane.[24]

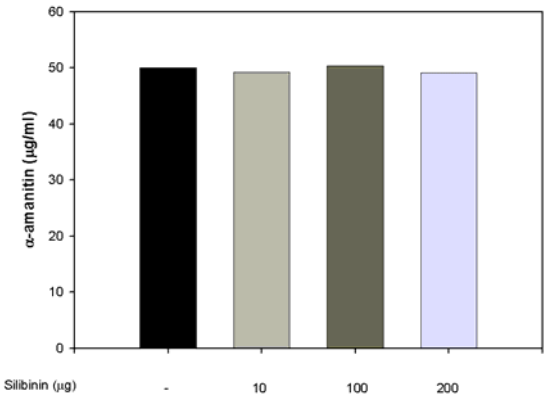

Figure 3 Effect of Silibinin at 10, 100, and 200 µg on α-amanitin at 50 µg/ml.

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การต้านอนุมูลอิสระของซิลิบินินต่อความเป็นพิษของแอลฟา-อะมานิติน ในหลอดทดลอง

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บทคัดย่อ

แอลฟา-อะมานิติน (Alpha amanitin) มีความเป็นพิษที่รุนแรงที่สุดที่พบในเห็ดสกุล *Amanita* ในประเทศไทย โดยมีความเป็นพิษต่อตับโดยตรงและทำให้ระบบการทำงานต่างๆ ของร่างกายล้มเหลวได้ ส่วน ซิลิบินิน (Silibinin) เป็นสารต้านอนุมูลอิสระที่นำมาใช้รักษาความเป็นพิษที่เกิดจากแอลฟา-อะมานิติน แต่กลไกการทำงานของซิลิบินินนั้นยังไม่แน่ชัด ดังนั้นวัตถุประสงค์ในครั้งนี้ เพื่อศึกษาฤทธิ์ในการต้านอนุมูลอิสระของซิลิบินินในการกำจัดสารอนุมูลอิสระชนิดต่างๆ ได้แก่ superoxide anion, hydroxyl radical และ hydrogen peroxide โดยอาศัยวิธี XO-HX, Ribose- และ H_2O_2-ABT/HRP รวมทั้งความสามารถของซิลิบินินในการจับกับแอลฟา-อะมานิตินได้โดยตรงหรือไม่ โดยเปรียบเทียบกับสารต้านอนุมูลอิสระชนิดอื่น ได้แก่ gallic acid, N-acetylcysteine (NAC) และ quercetin นอกจากนี้ได้ศึกษาการต้านความเป็นพิษจากแอลฟา-อะมานิตินในเลือดคน โดยการวัดปริมาณ glutathione (GSH) ในเลือด และ malondiladehyde (MDA) ในพลาสมา โดยอาศัยวิธี DTNB และ TBARs ตามลำดับ ผลการศึกษาพบว่า ซิลิบินินสามารถกำจัดอนุมูลอิสระ superoxide anion ได้น้อยกว่า quercetin แต่มากกว่า NAC และ gallic acid ส่วนการกำจัดอนุมูลอิสระชนิด hydroxyl radical ได้มากกว่า quercetin, NAC และ gallic acid ตามลำดับ และการกำจัด hydrogen peroxide ได้น้อยกว่าสารต้านอนุมูลอิสระชนิดอื่น นอกจากนี้ ซิลิบินินสามารถป้องกัน GSH และลดปริมาณ MDA ได้ตามความเข้มข้น และยังพบว่าซิลิบินินไม่สามารถจับกับแอลฟา-อะมานิตินได้โดยตรง โดยสรุปซิลิบินินสามารถกำจัดสารอนุมูลอิสระชนิดต่างๆ โดยเฉพาะ superoxide anion, hydroxyl radical และ hydrogen peroxide ได้ และยังสามารถป้องกัน GSH และลดการเกิด MDA จากแอลฟา-อะมานิติน

คำสำคัญ : แอลฟา-อะมานิติน, ซิลิบินิน, การต้านอนุมูลอิสระ, เห็ด *Amanita*

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