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Deactivation Study of α-Amanitin Toxicity in Poisonous *Amanita* spp. Mushrooms by the Common Substances *In Vitro*

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Abstract

The purpose of this research was to find out the substance which deactivate α-Amanitin Toxicity

The materials and methods used in the study include analysis with high performance liquid chromatography (HPLC) to:

1.Demonstrate the standard α-amanitin at concentrations of 25, 50 and 100 µg/ml

2.Determine the deactivation of α-amanitin with 1) 18% acetic acid 2), calcium hydroxide 40 mg/ml, 3) potassium permanganate 20 mg/ml, 4) sodium bicarbonate 20 mg/ml

3.Report the statistical analysis as the mean ± standard deviation (SD) and paired t-test.

The result revealed that potassium permanganate could eliminate 100 percent of the α-amanitin at 25, 50 and 100 µg/ml. Calcium hydroxide, sodium bicarbonate and acetic acid had lower elimination rates at those concentrations: 68.43 ± 2.58 (-71.4, -67.2, -66.7%), 21.48 ± 10.23 (-29.4, -25.2, -9.9%) and 3.21 ± 0.02% (-3.2, -3.2, +1.1%), respectively. The conclusion of this study was suggested that potassium permanganate could be applied as an absorbent substance during gastric lavage in patients with mushroom poisoning. It also might be effective as a cleansing wash for uncooked mushrooms. Investigation of potassium permanganate's ability to absorb α-amanitin in animal models and humans should be considered. .

Keywords: α-Amanitin; Poisonous *Amanita*; Mushrooms; Acetic acid; Sodium carbonate; Potassium permanganate; Calcium hydroxide

Introduction

The poisonous mushrooms which are very dangerous toxicity are Amanita mushroom species: *A. phalloides*, (death cap) *A. virosa*, (destroying angel), *A. verna*, *A. bisporigera*, *Galerina marginata* and *Conocybe filaris* (Figures 1A and 1B). Alpha-amanitin is the most potent of the toxins occurring in poisonous mushrooms in the genus *Amanita* [1,2]. The lethal oral dose (LD50) of α-amanitin in mice is 0.1-0.3 mg/kg. One cap of *A. phalloides*, *A. virosa* or *A. verna* contains ten amatoxins. Ingestion of *A. phalloides* results in the highest mortality rate of all poisonous mushrooms, ranging from 50 to 90%. Unfortunately, distinguishing poisonous and non-poisonous mushrooms by morphology alone is challenging due to their similar gross appearance. Theoretically, there are ten important amatoxins: [3] -amanitin, β-amanitin, γ-amanitin, ε-amanitin, ð-amanitin, amanullin, amanullinic acid, amaninamide, amanin and proamanullin [4] These differ from the other amatoxins in that they are heat resisitant, alcohol and lipid soluable [5] and indigestible in gastric and small intestinal enzymes. In addition, they can be absorbed rapidly by both gastric and duodenal tissues. One hundred grams of of *A. phalloides* contain eight milligrams of α-amanitin [1] The RNA polymerase II and III inhibitor α-amanitin [2] affects many organs, especially liver cells, resulting in necrotic hepatic cytolysis [3]. The mortality rate of α-amanitin poisoning is very high because of the difficulty diagnosis, the delayed toxicity (average 24 hrs.), the severity of the toxicity and the administration of an ineffective antidote. The cycle peptide chain, α-amanitin, has eight amino acids: asparagine, hydroxyl proline, dihydroxy isoleucine, hydroxyl-mercapto-tryptophan, glycine, isoleucine, glycine and alanine. Hydroxyl proline and dihydroxy isoleucine activate RNA polymerase II and III by binding to the enzyme molecule, enhancing the activating effect by means of glycine and isoleucine binding [6-8]. The immediate cause of death from mushroom poisoning is kidney, liver and respiratory failure which occur within one week of ingestion. Patients with α-amanitin poisoning can develop severe toxic hepatitis, centrilobular necrosis, liver steatosis, and acute tubulointerstitial necrosis leading to hepatorenal syndrome all of which have a high mortality rate. The most effective treatment is emptying the stomach promptly by performing gastric lavage with 1:2,000 tannic acid or 1:10,000 potassium permanganase plus triggering emesis. A warm saline solution of potassium permaganase can be used as an emergency treatment to reduce toxins in the intestines and stomach. Thus, $KMnO_4$, $NaHCO_3$, $Ca(OH)_2$ and acetic acid should be considered as a cleanser for uncooked mushrooms to deactivate α-amanitin toxicity and potentially reduce the mortality rate from liver failure, acute renal failure, respiratory failure and gastro-intestinal haemorrhage[9].

Materials and Methods

In this study, experiments were performed on α-amanitin at concentrations of 25, 50 and 100 µg/ml eached mixed with 1) 18% acetic acid 2), calcium hydroxide 40 mg/ml, 3) potassium permanganate 20 mg/ml, 4) sodium bicarbonate 20 mg/ml (results analyzed with high performance liquid chromatography (HPLC) with Luna C18 (150 × 4.6 mm I.D., 5 micron) from Phenomenex®, USA), or 5) mobile phase (a mixture of 0.02 M aqueous ammonium acetate and acetonitrile (88/12, v/v) pH 5.0 at an absorbance of 280 nm). Glacial acetic acid was used to

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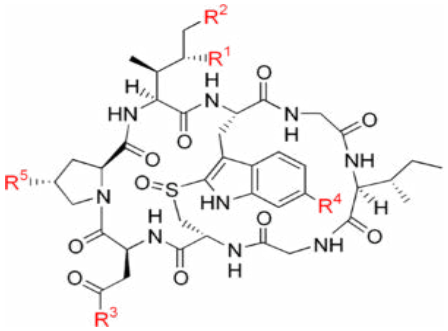

Figure 1: A: *Amanita virosa* **B**: Amanitin structure.

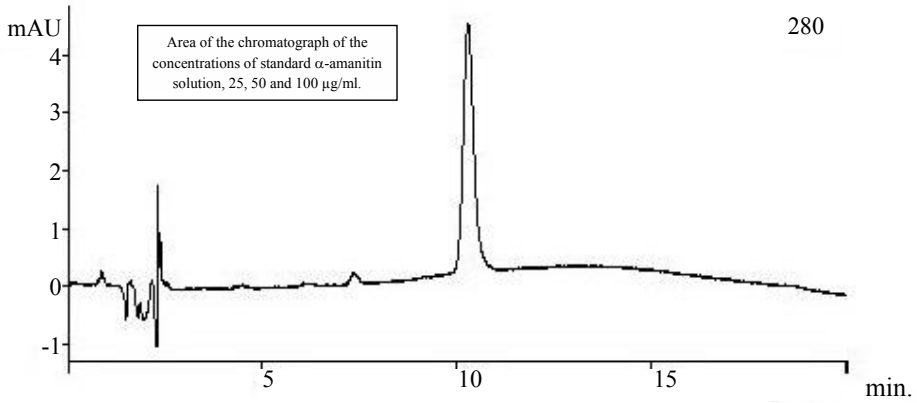

Figure 2: A: Chromatograph of α-amanitin at concentrations at 25, 50 and 100 µg/ml.

α-amanitin (µg/mL)	1st injection	2nd injection	3rd injection	Average ± SD/% Deactivation
25	44748	43498	43956	43727 ± 632.4
50	91448	85370	88120	88312.7 ± 3043.6
100	171481	170648	175971	173309.5 ± 2863.2

Table 1: Area of the chromatograph of the concentrations of standard α-amanitin solution, 25, 50 and 100 µg/mL.

adjust the acid-base condition. Each of the mixtures was injected into the HPLC three separate times [10].

Results of statistical analysis of the α-amanitin concentrations are reported as the mean ± standard deviation (SD) for each group. The levels of α-amanitin concentration inhibited or destroyed by each substance were analyzed by paired t-test.

Results

1. α-amanitin concentrations as determined by HPLC

The concentrations of standard α-amanitin solution (25, 50 and 100 µg/ml) were injected into the HPLC to measure α-amanitin retention time of 10.2 minutes. The results of the chromatography is shown in Figure 2 and Table 1. The equation of the linear correlation is $Y=1723.8X+1228.6$ ($R^2=0.9999$) (Figure 3).

2. Analysis of the effectiveness of α-amanitin deactivation by 18% acetic acid

The results of deactivation of α-amanitin at concentrations of 25, 50 and 100 µg/ml by 18% acetic acid are shown in the Figure 4-7 and in Table 2. The deactivation levels were not statistically significant.

3. Analysis of the effectiveness of α-amanitin deactivation using sodium bicarbonate

The levels of deactivation of α-amanitin at concentrations of 25, 50 and 100 ug/ml by sodium bicarbonate ($NaHCO_3$) are shown in Figure 8 and Table 3. Deactivation was statistically significant when compared with the control group ($p<0.01$).

4. Analysis of the effectiveness of α-amanitin deactivation by potassium permanganate ($KMnO_4$)

The results of deactivation of α-amanitin at concentrations of 25, 50 and 100 ug/ml by potassium permanganate ($KMnO_4$) are shown in Figures 9-11 and Table 4. The results showed statistically significant deactivation when compared with the control group ($p<0.05$) (Figure 12).

5. Analysis of the effectiveness of α-amanitin deactivation by calcium hydroxide ($Ca(OH)_2$) 40 mg/ml

The effectiveness of deactivation of α-amanitin at concentrations of 25, 50 and 100 ug/ml by calcium hydroxide ($Ca(OH)_2$) is shown in Figure 13-16 and in Table 5. Deactivation was statistically significant when compared with the control group ($p<0.01$).

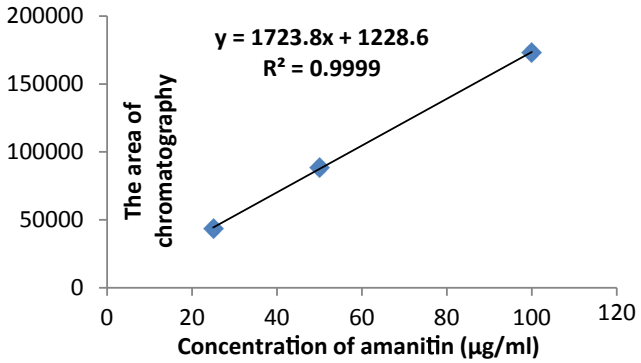

Figure 3: The equation of linear correlation is Y=1723.8X+1228.6 (R^2=0.9999).

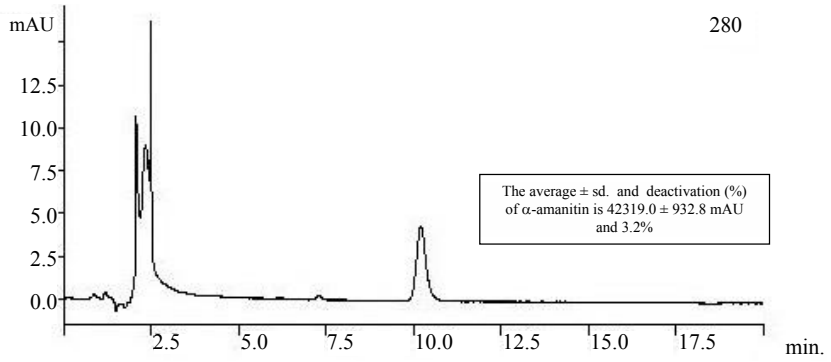

Figure 4: Deactivation of α-amanitin at a concentration of 25 µg/ml by 18% acetic acid.

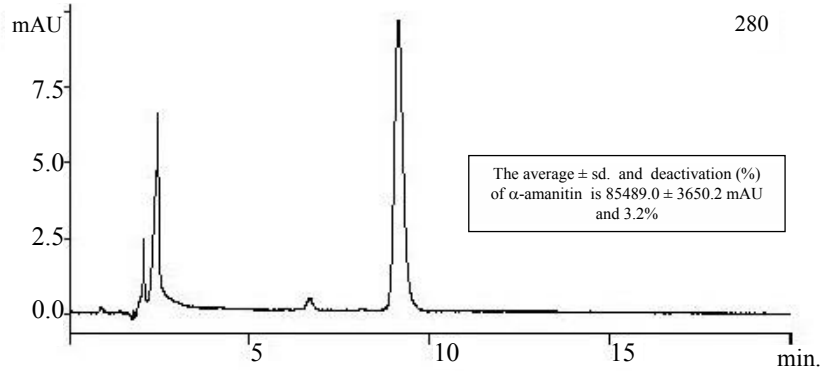

Figure 5: Deactivation of -amanitin at a concentration of 50 µg/ml by 18% acetic acid.

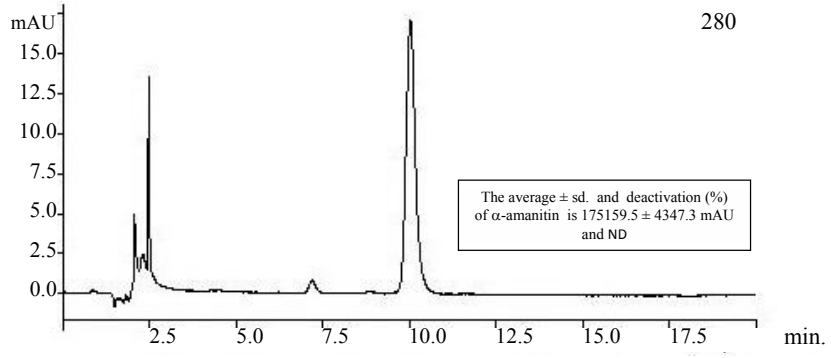

Figure 6: Effective de-activation of α-amanitin at a concentration of 100 µg/ml by 18% acetic acid.

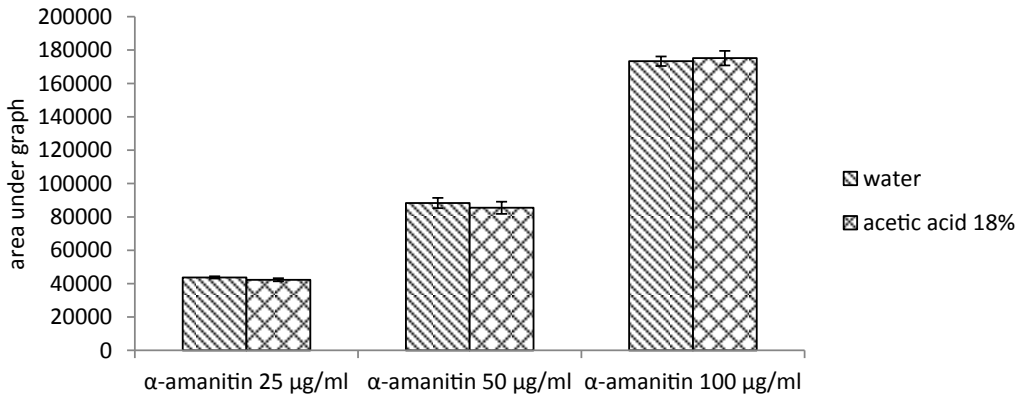

Figure 7: Comparison of Area under the Curve for concentrations of α-amanitin before and after 18% acetic acid.

Testing	Area of Chromatograph			
	1st injection	2nd injection	3rd injection	Average ± SD (% Deactivation)
α-amanitin 25 µg/mL + water	44748	43498	43956	43727.0 ± 632.4
α-amanitin 50 µg/mL + water	91448	85370	88120	88312.7 ± 3043.6
α-amanitin 100 µg/mL + water	171481	170648	175971	173309.5 ± 2863.2
α-amanitin 25 µg/mL + acetic acid 18%	43108	41505	43133	42319.0 ± 932.8 (**3.2%**)
α-amanitin 50 µg/mL + acetic acid 18%	86701	88379	81387	85489.0 ± 3650.2 (**3.2%**)
α-amanitin 100 g/mL + acetic acid 18%	168497	177185	173134	175159.5 ± 4347.3 (ND)

Table 2: Area of chromatograph showing deactivation of α-amanitin at concentrations of 25, 50 and 100 µg/mL by 18% acetic acid.

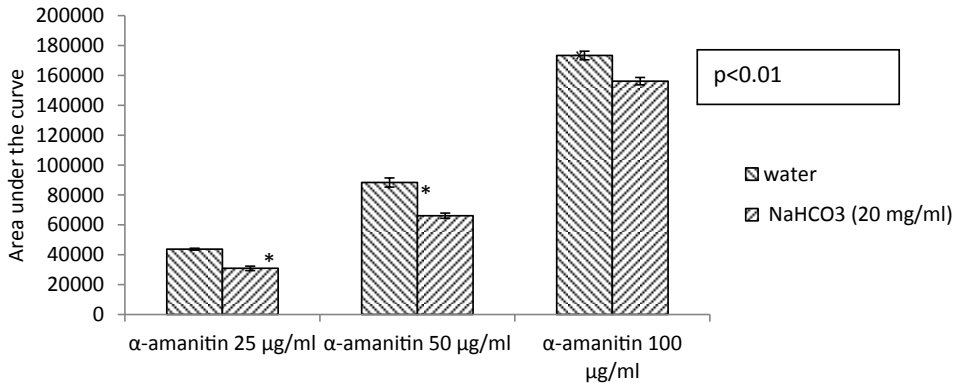

Figure 8: Area under the curve comparing concentrations of α-amanitin before and after sodium bicarbonate 20 mg/ml. Deactivation was statistically significant when compared with the control group.

Testing	Area of Chromatograph				
	1st injection	2nd injection	3rd injection	Average ± SD/% Deactivation	P
α-amanitin 25 µg/mL + water	44748	43498	43956	43727.0 ± 632.4	
α-amanitin 50 µg/mL + water	91448	85370	88120	88312.7 ± 3043.6	
α-amanitin 100 µg/mL + water	171481	170648	175971	173309.5 ± 2863.2	
α-amanitin 25 µg/mL + $NaHCO_3$ 20 mg/mL	43108	41505	43133	30879.7 ± 1487.5 **29.4%**	<0.01
α-amanitin 50 µg/mL + $NaHCO_3$ 20 mg/mL	86701	88379	81387	66092 ± 1748.9 **25.2%**	<0.01
α-amanitin 100 µg/mL + $NaHCO_3$ 20 mg/mL	168497	177185	173134	156153.3 ± 2482.1 **9.9%**	<0.01

Table 3: Area of chromatograph showing deactivation of α-amanitin at concentrations of 25, 50 and 100 µg/mL by sodium bicarbonate 20 mg/mL .

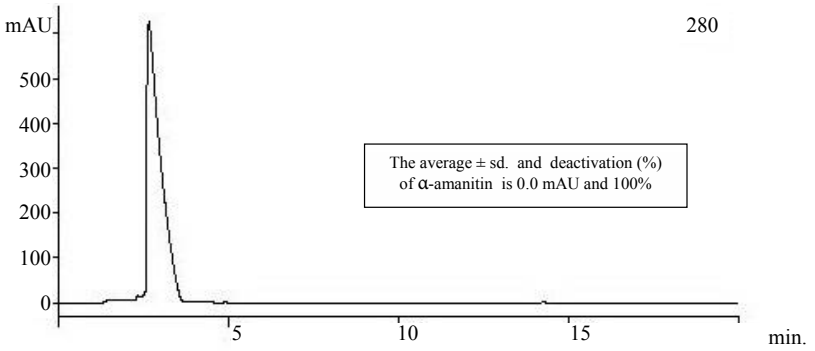

Figure 9: Deactivation of -amanitin at a concentration of 25 µg/ml by 20 ug/ml of potassium permanganate ($KMnO_4$).

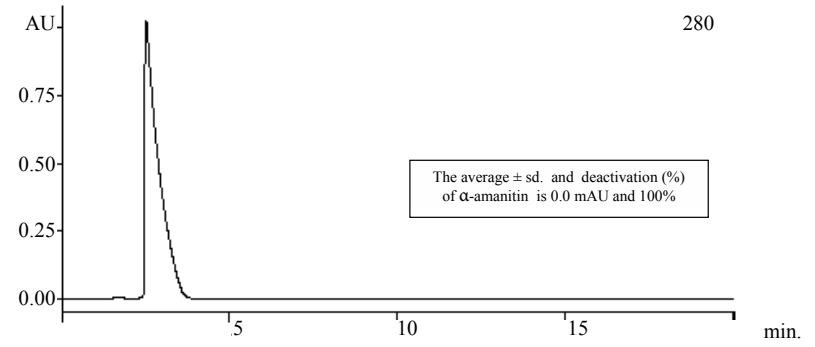

Figure 10: Deactivation of -amanitin at 50 µg/ml by 20 ug/ml potassium permanganate ($KMnO_4$).

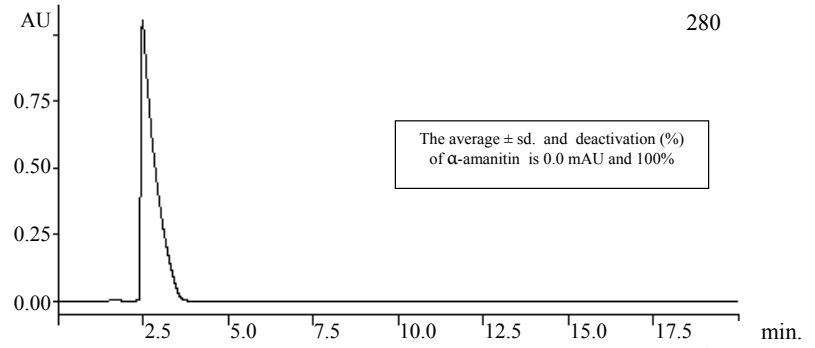

Figure 11: Deactivation of -amanitin at a concentration of 100 µg/ml by 20 ug/ml of potassium permanganate ($KMnO_4$) ($p<0.05$).

Testing	Area of Chromatograph				
	1st injection	1st injection	1st injection	Average ± SD/% Deactivation	p
α-amanitin 25 µg/mL + water	44748	43498	43956	43727.0 ± 632.4	
α-amanitin 50 µg/mL + water	91448	85370	88120	88312.7 ± 3043.6	
α-amanitin 100 µg/mL + water	171481	170648	175971	173309.5 ± 2863.2	
α-amanitin 25 µg/mL + $KMnO_4$ (20 µg/mL)	43108	41505	43133	0/-**100%**	<0.05
α-amanitin 50 µg/mL + KMnO4 (20 µg/mL)	86701	88379	81387	0/-**100%**	<0.05
α-amanitin 100 µg/mL + KMnO4 (20 µg/mL)	168497	177185	173134	0/-**100%**	<0.05

Table 4: Area of chromatograph showing deactivation of α-amanitin at concentrations of 25,50 and 100 µg/mL by 20 ug/mL of Potassium permanganate ($KMnO_4$).

Discussion and Conclusions

Four common substances, Potassium permanganate ($KMnO_4$), calcium hydroxide ($Ca(OH)_2$), sodium carbonate ($NaHCO_3$) and acetic acid are regularly used for vegetable detoxification. As mushrooms are one of the most popular ingredients in Thai food, especially in rural areas, routine detoxification of potential toxins in uncooked mushrooms is one solution that should be considered to avoid mushroom poisoning, particularly α-amanitin toxicity.

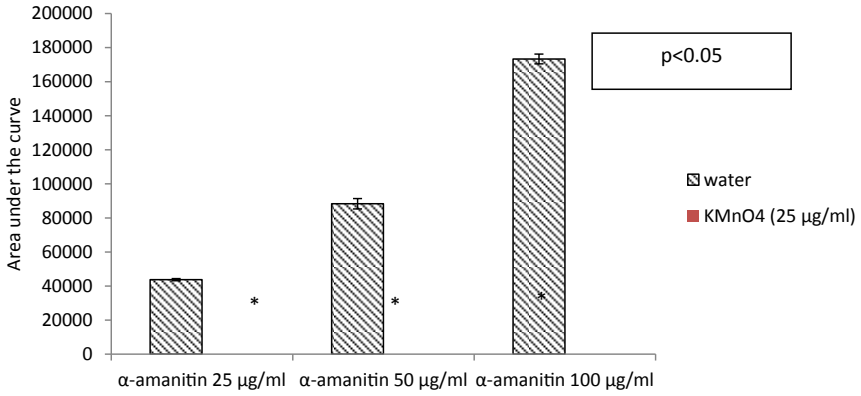

Figure 12: Area under the Curve with three concentrations of α-amanitin before and after potassium permanganate ($KMnO_4$) 20 ug/ml showing statistically significant deactivation.

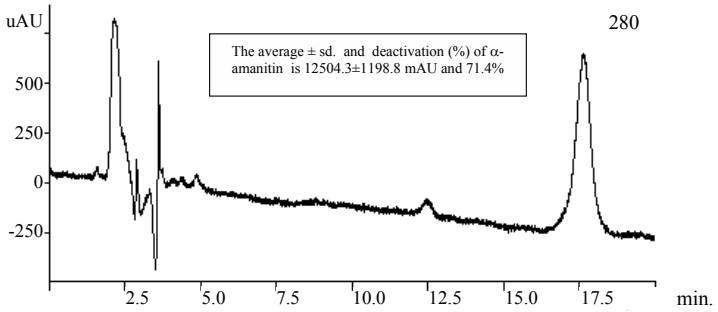

Figure 13: Deactivation of -amanitin at 25 µg/ml by 40 mg/ml of Calcium hydroxide ($Ca(OH)_2$).

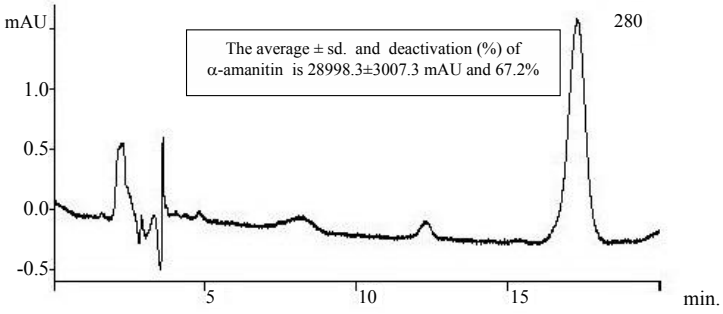

Figure 14: Deactivation of -amanitin at 50 µg/ml by 40 mg/ml of calcium hydroxide ($Ca(OH)_2$).

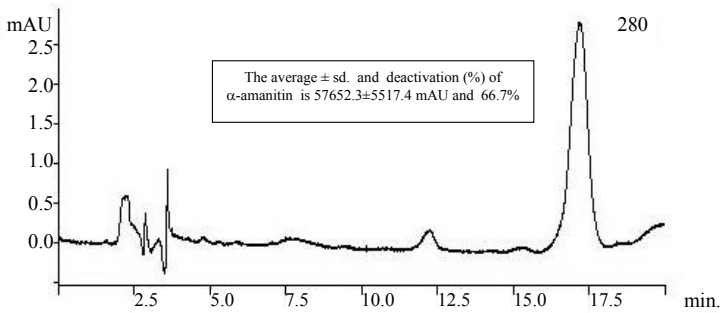

Figure 15: Deactivation of -amanitin at 100 mg/ml by 40 mg/ml of calcium hydroxide ($Ca(OH)_2$).

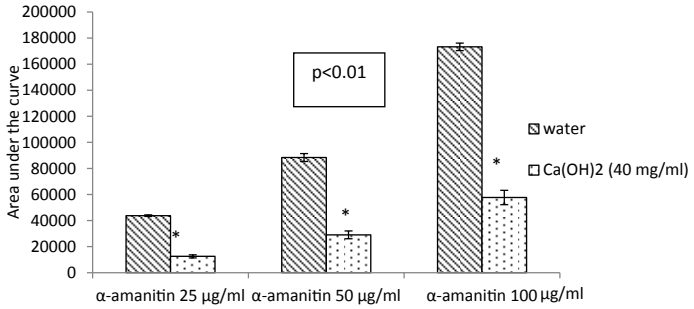

Figure 16: Area under the curve comparing three concentrations of α-amanitin before and after calcium hydroxide 40 mg/ml. Deactivation was statistically significant compared with the control group.

Testing	Area of Chromatograph				
	1st injection	2nd injection	3rd injection	Average ± SD/% Deactivation	P
α –amanitin 25 µg/mL + water	44748	43498	43956	43727.0 ± 632.4	
α –amanitin 50 µg/mL + water	91448	85370	88120	88312.7 ± 3043.6	
α –amanitin 100 µg/mL + water	171481	170648	175971	173309.5 ± 2863.2	
α –amanitin 25 µg/mL + $Ca(OH)_2$ (40 mg/mL)	43108	41505	43133	12504.3 ± 1198.8/-71.4%	<0.01
α –amanitin 50 µg/mL + $Ca(OH)_2$ (40 mg/mL)	86701	88379	81387	28998.3 ± 3007.3/-67.2%	<0.01
α –amanitin 100 µg/mL + $Ca(OH)_2$ (40 mg/mL)	168497	177185	173134	57652.3 ± 5517.4/-66.7%	<0.01

Table 5: Area of chromatograph showing deactivation of α-amanitin at 25,50 and 100µg/mL by 40 mg/mL of Calcium hydroxide.

Potassium permanganate ($KMnO_4$) is an antiseptic, alkali, and highly soluble detoxification substance. It contains potassium (K^+) and permanganate ions which are strong oxidizing and organic substance destroying agents. Manganese oxide (MnO_2) destroys the cell wall and cell membrain of bacteria, protozoa, parasites and algae. This study revealed that potassium permanganate ($KMnO_4$) 20 mg/ml completely destroyed α-amanitin at concentrations of 25, 50 and 100 µg/ml. We found that potassium permanganate ($KMnO_4$) is the strongest of the oxidizing agents tested and that it has the ability to destroy or deactivate peptides of α-amanitin molecules *in vitro* [11,12]. Potassium permanganate ($KMnO_4$) could potentially be used for gastric lavage in patients with mushroom poisoning. Additional experiments in animal models should be conducted to evaluate the effectiveness of these four substances.

Calcium hydroxide ($Ca(OH)_2$) and sodium carbonate ($NaHCO_3$) have an alkaline property which can significantly deactivate alkaline hydrolysis [13,14] caused by α-amanitin. Acetic acid has an antiseptic acid property but it cannot destroy the cell membranes or cell walls of any vegetable including α-amanitin because it has a low pKa value. α-amanitin in poisonous mushrooms cannot be destroyed by acids or by gastric or duodenal enzymes [15].

Potassium permanganate ($KMnO_4$) is the most effective deactivation substance for α-amanitin. It completely inhibits α-amanitin activation. Both calcium hydroxide ($Ca(OH)_2$) and sodium carbonate ($NaHCO_3$) (to a lesser degree) inhibit α-amanitin, but they do not do so completely. Acetic acid only very weakly inhibits α-amanitin. Based on these findings, washing uncooked poisonous *Amanita* spp. mushrooms with $KMnO_4$ to detoxify or deactivate α-amanitin before ingestion would definitely be beneficial to health, especially in rural areas where many people gather their own mushrooms from forested areas rather than purchasing them at a market.

Ethical Approval

This research did not involve any human participants or animals.

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