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The protective effects of *Tiliacora triandra* against methomyl induced cytotoxicity in murine macrophage (RAW 264.7) cells

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Abstract

Methomyl, an insecticide belonging to the carbamate types, is an extremely toxic compound that is widely used in agriculture. It exhibits negative effects on human health upon a long-term exposure. Thus, it is necessary to find natural substances that can detoxify this insecticide. Herbs are widely used for therapeutic treatment in local wisdom of Thai people, especially Yanang (*Tiliacora triandra*) which possesses an effective detoxification property. Therefore, this study aimed to investigate the protection property of Yanang water extract on methomyl induced cytotoxicity on murine macrophage cell lines (RAW 264.7 cells). The hemolytic activity assay was used for primary screening of the non-toxic concentration rage of Yanang extract. The obtained result confirmed that Yanang extract at a concentration of 6.25, 12.5 and 25 µg/ml exerted no toxicity towards human red blood cells. Consequently, the dosages of 5, 10 and 20 µg/ml of Yanang extract were evaluated for the protective effects agianst methomyl induced cytotoxicity in RAW 264.7 cells. The results demonstrated that Yanang extract exhibited no toxicity towards RAW 264.7 cells and could promote cell viability of methomyl induced RAW 264.7 cells. In conclusion, the current study suggested that Yanang might contain particular agents with a potential for methomyl detoxification.

Introduction

In recent years, insecticides are widely used in agricultural for controlling various agricultural pests. People are inevitably exposed to insecticides via drinking or using contaminated water as well as breathing contaminated air near application area. There is article which reported that 2 million cases of human were poisoned from insecticide exposure.[1] Carbamate insecticides are widely used, especially methomyl, because it is effective against a wide range of pests and insects. However, the toxicity of methomyl is non-specific. It can cause toxic to many non-target species. Human can be exposed to methomyl by absorption via the skin or eyes as well as oral exposure. Methomyl is well-known to generate the reactive oxygen species (ROS), such as superoxide anion (O^{2-}) and hydroxyl radical ($^{\bullet}OH$), leading to oxidative stress and lipid peroxidation.[2] However, immunotoxicity of methomyl are less studied. Therefore, effects of methomyl toward immune cells are interested.

In order to reduce the toxic of methomyl, several researches have been focused on the natural compounds which are effective to reduce toxicity with relative safety.[3] In this context, *Tiliacora triandra* (Colebr.) or Yanang is one of a medicinal plant that could reduce the human toxicity.[4] It is a popular herb in Thailand. Yanang leaf water extract has been used to reduce

the toxicity of bamboo shoot in Thai cuisine such as Sup Nor Mai (spicy bamboo shoot) and Kaeng No Mai (bamboo shoot soup).[3] Moreover, Yanang is not only used as ingredient of food but also as traditional herbal medicine for detoxication agent, anti-inflammation, anticancer, antibacterial, immune modulator and antioxidant activity.[5] Yanang leaf extract contains antioxidant compounds including vitamin E and phytol.[4] Yanang possess high antioxidant activity which scavenge superoxide anion (O^{2-}) and hydroxyl radicals ($^{\bullet}OH$) resulting in the prevention of oxidative damage. Thus, Yanang shows high potential ability to maintain the balance of oxidation-reduction in cellular level.[6] Although, Yanang extract contains high antioxidant and is found to reduce toxic against many toxic compounds. However, there are a few studies which demonstrate the effect of Yanang water extract on toxic reduction arising from methomyl in immune system. Therefore, this study aimed to evaluate the protective effects of Yanang on methomyl induced murine macrophage cell lines (RAW 264.7 cells).

Methodology

Preparation of plant extract

Tiliacora triandra (Yanang) was purchased from Khon Kaen city market (Khon Kaen, Thailand). Yanang leaves were washed two times with distilled water and air-dried until the water was faded from the leaves. After that, distilled water was added in the ratio of 2 g of Yanang: 1 ml of water and grounded into small pieces. Then, the mixture was filtered through cotton cloth. Next, the filtrate was centrifuged at 8,000 rpm at 4 ºC for 20 min and the supernatant was collected (Yanang water extract). Protein determination was performed using Bradford method.[7]

Hemolytic activity assay

Hemolytic activity assay was performed according to Zohra and Fawzia[9] with minor modifications. Briefly, O type human red blood cells were collected and washed three times with sterile phosphate buffer saline (PBS). The 2% human red blood cell solutions were prepared in sterile PBS. Ten microliters of Yanang extract was mixed with 90 µl of human red blood cell solutions to yield the final concentrations of 6.25, 12.5 and 25 µg/ml. The reaction mixture was placed in water bath for 1 h at 37 ºC. The reaction mixture was subsequently centrifuged at 1,000 x *g* for 5 min. The supernatant was collected and the optical density was measured at 540 nm. Distilled water and 1% Triton X-100 were used as a negative and positive control, respectively. The hemolytic activity was calculated as following equation;

(%) hemolysis = (Absorbance of sample/Absorbance of positive control) x 100

Cell culture

RAW 264.7 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (100 U/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphoteric B). Cells were incubated in a humidified incubator with 5% CO_2 atmosphere at 37 ºC.

Cell viability assay

MTT assay was used to evaluate the effects of Yanang extract on the viability of RAW 264.7 cells. According to the previous method described by Phosri et al.[8] with minor modifications, RAW 264.7 cells were seeded at a density of 2.5 x 10^4 cells/well (100 µl) in 96-well plate, and allowed to adhere for 24 h at 37 ºC in 5% CO_2 atmosphere incubator. Then, cells were divided into three groups, first group was incubated with Yanang extract at different concentrations (5, 10, and 20 µg/ml), second group was incubated with methomyl (3,000, 6,000 and 12,000 µM) and the last group was co-incubated between Yanang extract (5, 10, and 20 µg/ml) and methomyl at inhibitory concentration (IC_{50}). After incubation for 24 h, 100 µl of MTT (0.5 mg/ml) was added to each well. The cells were further incubated for 30 min at 37 ºC with 5%

CO_2 atmosphere. After medium was removed, 100 µl of dimethyl sulfoxide (DMSO) was added to each well and the absorbance was measured at wavelength of 570 nm using a microplate reader (Varioskan LUX, USA). The percentage of cell viability was calculated using the following equation;

% Cell viability = (Absorbance of treated cells/Absorbance of control cells) x 100

Statistic analysis

All experiments were performed in triplicate. The results were calculated using statistix 8.0, followed by analysis of variance (ANOVA). Statistical significant was accepted when the *P*-value was less than 0.05 (*$P < 0.05$).

Results and Discussion

Hemolytic activity assay

Hemolytic activity of Yanang extract was investigated against human red blood cells. The result indicated that Yanang extract at concentrations of 6.25, 12.5 and 25 µg/ml exhibited no cytotoxicity effect when compared with a negative control (distilled water treated group) (Figure 1). Therefore, the dose of 5, 10 and 20 µg/ml of Yanang extract were confirmed to have no cytotoxicity when were used in the next experiments.

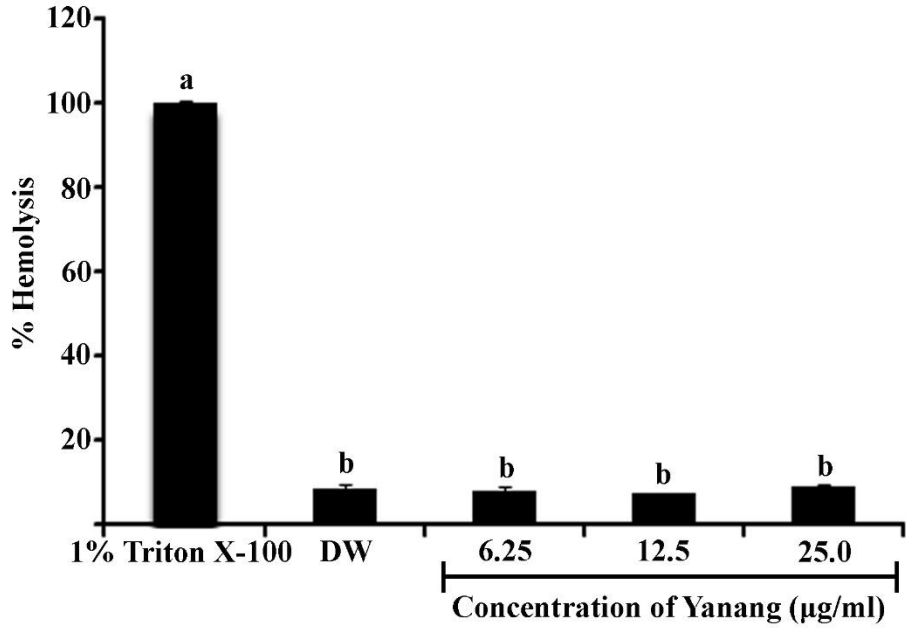

Figure 1. Hemolytic activity of Yanang extract against human red blood cells. 2% human red blood cells were incubated with different concentrations of Yanang extract. A 1% Triton X-100 and distilled water (DW) were used as a positive and negative control, respectively. Each bar was shown as the mean ± SD. The letters on the top of each bar represented statistically significant differences ($p < 0.05$).

Cell viability assay

The results showed that Yanang extract at concentrations of 5, 10, and 20 µg/ml did not exhibit cytotoxicity to RAW 264.7 cells which revealed the cell viability as 102.62%, 98.77% and 101.06%, respectively (Figure 2). Regarding to methomyl, statistical test indicated that 3,000, 6,000 and 12,000 µM methomyl significantly induced cytotoxicity in RAW 264.7 cells, resulting in cell viability was found approximately 73.76%, 54.74% and 39.95%, respectively (Figure 3). The IC_{50} of methomyl at a concentration of 12,000 µM was selected for further evaluation of the protective effect of Yanang on methomyl stimulated RAW 264.7 cells. The result showed that methomyl could induce toxicity on RAW 264.7 cells by decreasing cell

viability approximately 50%. However, supplementation with Yanang extract at concentrations of 5, 10, and 20 μg/ml significantly increased the cell viability, resulting in cell viability was found approximately of 59.77%, 67.32% and 71.31%, respectively, when compared with methomyl treated group (Figure 4). Previously, Jang et al.[10] mentioned that insecticides commonly generated reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and superoxide anion (O^{2-}), leading to the generation of oxidative stress and lipid peroxidation that promoted macrophage cells death. Whereas, antioxidant activity in Yanang water extract have previously been documented.[6] This information suggested that Yanang is possible to reduce toxic in methomyl stimulated damage in RAW 264.7 cells because of their ability to inhibit a free radical arising from methomyl. Our reports demonstrated that Yanang extract has potential to decline the death of methomyl induced macrophages.

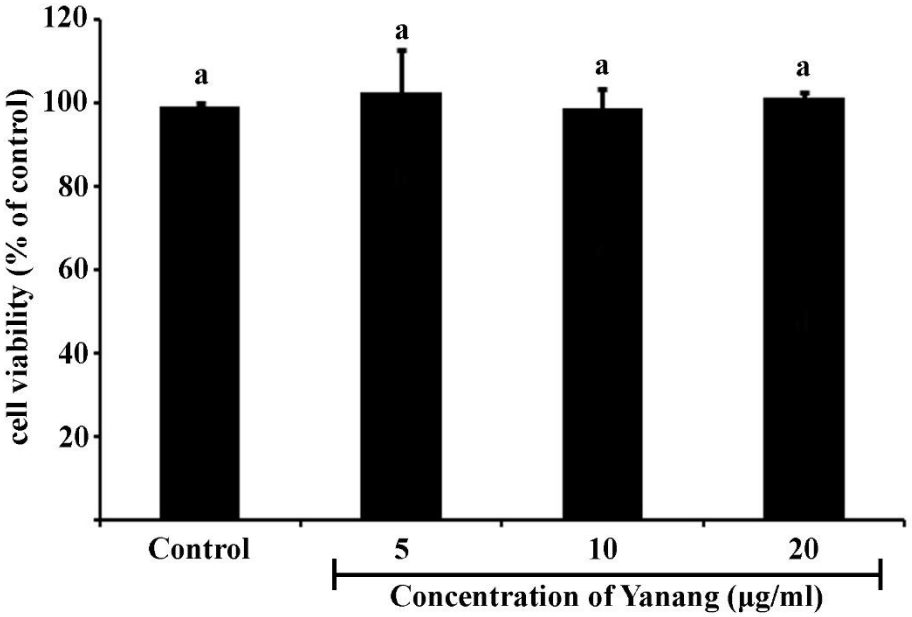

Figure 2. Effect of Yanang extract on cell viability by MTT assay. RAW 264.7 cells were incubated with Yanang extract at different concentrations of 5, 10, and 20 μg/ml for 24 h. The RAW 264.7 cells cultured in RPMI 1640 only was used as an untreated control. Each bar is shown as the mean ± SD. The letters on the top of each bar represented statistically significant differences ($p < 0.05$).

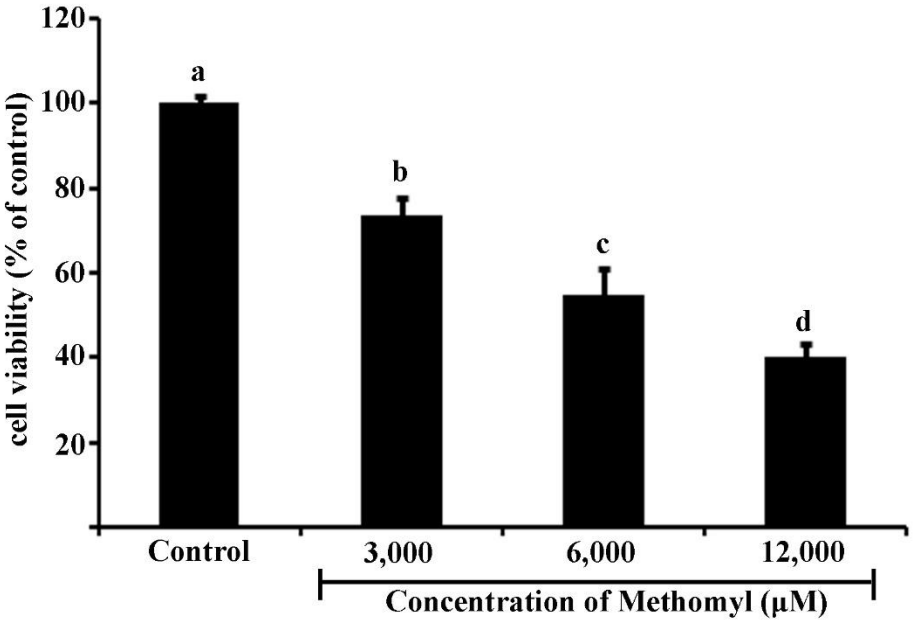

Figure 3. Effect of methomyl on cell viability by MTT assay. Murine macrophage cell lines were incubated with methomyl at different concentrations of 3,000, 6,000, and 12,000 μM for 24 h. The RAW 264.7 cells cultured in RPMI 1640 only was used as an untreated control. Each bar is shown as the mean ± SD. The letters on the top of each bar represented statistically significant differences ($p < 0.05$).

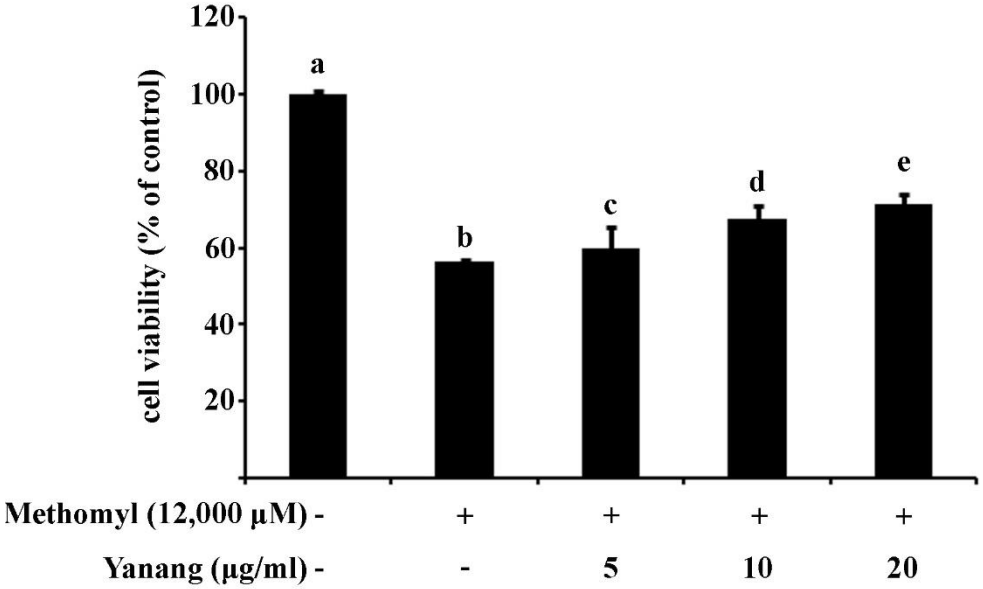

Figure 4. Effect of Yanang extract on cell viability by MTT assay. Murine macrophage cell lines were treated with methomyl concentration at 12,000 μM and co-incubated with Yanang extract with different concentrations at 5, 10, and 20 μg/ml for 24 h. Each bar is shown as the mean ± SD. The letters on the top of each bar represented statistically significant differences ($p < 0.05$).

Conclusion

The study of efficient protection of Yanang water extract against methomyl induced RAW 264.7 cells was investigated. The results showed that the Yanang extract at concentrations of 6.25, 12.5 and 25 μg/ml had no toxic to RAW 264.7 cells and human red blood cells. The methomyl was strong toxic to RAW 264.7 cells by decreasing cell viability. However, the damage of cells could decline by incubation with Yanang water extract. Therefore, our results suggested that certain natural components in Yanang extract might provide the potential ability to neutralize methomyl induced toxicity in RAW 264.7 cells. Hence, Yanang may afford as a representative of natural derived therapeutic agents and a healthy supplementary foods in the future.

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