International Journal of Food Science and Nutrition
ISSN: 2455-4898, Impact Factor: RJIF 5.14
www.foodsciencejournal.com
Volume 1; Issue 3; May 2016; Page No. 30-33

Evaluation of Apoptosis Induction and Necrosis on Leukemia Cell Line in Selected Thai Curry Pastes and Their ready-to-cook Dishes Using *in vitro* Experiments

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Abstract
The combination of fresh herbs and spices used in curry pastes for Thai cooking were one of the uniqueness. Some of curry pastes with or without coconut milk and their ready-to-cook dishes have been scientifically proved for health benefits. Food samples were extracted with water or digested with the simulation of gastrointestinal digestion. The solutions of treated solutions were tested for Apoptosis induction and Necrosis on Leukemia cell line *in vitro*. The results of water extraction from all four curry pastes with coconut milk showed differences in Apoptosis induction and Necrosis of Leukemia cell line. Most of the water extraction from Jasmine rice/Thai spaghetti with curry pastes, coconut milk and chicken were higher potent than that of their curry pastes alone. After simulation of gastrointestinal digestion, Apoptosis induction and Necrosis of Leukemia cell line were higher than the water extraction. The curry pastes without coconut milk namely Gaeng Liang soup was shown to have greatest in Apoptosis induction among the other four curry pastes without coconut milk. Besides, Gaeng Liang soup also had the lowest in %Necrosis among the other curries without coconut milk.

Keywords: Thai **c**urry pastes with coconut milk, Thai curry pastes without coconut milk, their ready-to-cook dishes, Apoptosis induction, Necrosis, Leukemia cell line

1. Introduction
Cancer cell is one of the most concerning health problems in many countries around the world including Thailand. It is well documented that types of food consumed and the eating pattern are largely contributed that types of food consumed and the eating pattern are largely contributed to its cause. Recently, there is an idea of using food in prevention and perhaps threat the disease. Thai food is not only well-known for the delicacy but also known to have physiologically health benefits due to its ingradients; local vegetables, herbs and spices. These ingradients are usually used in a mixed-form called 'Curry Paste'. Although, many health benefits of an individual herb and spice were studied and identified, there is a lacking of scientific evidence supporting the health benefits of Thai foods or Thai food products as commonly consumed. Thus, promotion Thai food consumption was done domestically and internationally. There is a need for the research to determine the health benefits in these products. The Apoptosis induction and Necrosis in Leukemia cell line *in vitro* experiments were briefly studied. Leukemia cell line was incubated with a medium containing test solution, Apoptotic cells will be labeled with protein Annexin V attached with fluorescent dye. The other dye was Propidium iodide which will have red color means the Necrosis cells. The percentage of Apoptotic cells and Necrosis cells occurred could be measured compare to the normal cells without color by using Dual Parameter Flow Cytometry (FACS). The expected outputs were provided the useful information on Apoptosis induction and Necrosis in Leukemia cell line of selected Thai curry pastes with or without coconut milk and their ready-to-cook dishes for manufacturer and general consumers. This information could be used for screening Thai foods/dishes with high potential to further study in human for more supportive evidences.

2. Materials and methods
2.1 Chemicals
All chemicals used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2 Sample preparation for curry pastes with coconut milk
There were 4 kinds of curries pastes with coconut milks such as Red curry, Green curry, Phanang curry and Musaman curry (1). All curry pastes were come from the only one factory. The weight of each curry was one kilogram from triplicating processes at difference time interval and was collected for each testing using nitrogen gas at temperature 4^{o}C until the experiment was done. Each curry with coconut milk was divided for cooking with chicken as the representative meat in the preparation such as Red curry chicken, Green curry chicken, Phanang curry chicken and Musaman curry chicken. Those formulations have been studied and accepted. The detail of composition was shown in recipes for each curry. After that each curry was prepared each curry sample for eating as one plate meal on the following such as rice with Red curry chicken, rice with Green curry chicken, Thai spaghetti with Green curry chicken, rice with Phanang curry chicken and rice with Musaman curry chicken, respectively. Each plate meal had Jasmine rice variety 105 or Thai spaghetti with varies curries according to unit consumption. One unit of consumption was average amount of food which was suitable for eating per person per meal as the following. One unit of consumption rice or spaghetti was 180 grams. One unit of consumption with Red curry chicken or Green curry chicken was 200 grams. One unit of consumption with Phanang curry chicken was 120 grams. Finally, one unit of consumption with Musaman curry chicken was 270 grams. Each one plate meal with each curry including

rice or spaghetti was blended into fine pieces and collected each portion under nitrogen gas at -20ºC until the experiment was started [1].

2.3 Sample preparation for curry pastes without coconut milk

The other curry pastes without coconut milk were 5 curry pastes without coconut milk namely Gaeng Pah, Gaeng Liang, Gaeng Som, Gaeng Leung and Tom Yum. Five curry pastes were made as soup for ready-to-cook product. Each curry pastes without coconut milk 300 g were prepared as triplicate soup without meat and vegetable and filter to separate the residues. Then each soup will be centrifuged at 10,000 rpm at 4ºC. After that each supernatant was kept at -20ºC before further analysis.

2.4 Preparation of samples with coconut milk for study functional health effects

There were two methods for food extraction such as water extraction and simulation of gastrointestinal digestions as the function of human stomach and small intestine. The water extraction of food was started with the weighted each sample 4 grams was put into 50 ml of centrifuge tube and added water 8 grams (triplicate each sample). Then the sample solution was homogenized into homogeneous solution with Homogenizer (ultra turrax T25) at 13,500 rpm for 1 minute. The supernatant was centrifuged (HERMLE Z400 K) at 6,000 rpm for 10 minutes at temperature 4ºC. The filtrate was separated by Whatman no. 541and collected each supernatant at 4ºC for next experiment. The simulation of gastrointestinal digestion as the function of human stomach and small intestine was started with each weighted sample 4 grams into flask (triplicate each sample) and added 120 mM NaCl 60 ml then homogenized into homogeneous solution with Homogenizer (ultra turrax T25) at 13,500 rpm for 30 seconds. The solution was adjusted pH into acidic condition as in human stomach pH 2.1±0.1 with 1N HCl. The enzyme pepsin solution concentration 40 mg/ml in 100 mM HCl was added 4 ml and adjusted the total volume 80 ml with 120 mM NaCl. The stomach digestion was shaked 85 rpm at temperature 37ºC for one hour. Then the solution was adjusted to pH 6.0±0.1 with 1M $NaHCO_3$. The bile extract concentration 40 mg/ml in 100 mM $NaHCO_3$ was added 6 ml and the pancreatine-lipase 10 mg/ml, lipase 5 mg/ml in 100 mM $NaHCO_3$ was added 4 ml. The pH was adjusted to pH 6.9±0.1 with 1 M NaOH and finally added 120 mM NaCl to 100 ml. The solution was digested as in small intestine with shaking 85 rpm at 37ºC for 2 hours. The supernatant was centrifuged at 6,000 rpm, temperature 4ºC. for 30 minutes. The supernatant was filtered on Whatman no.541 and heated to stop the enzyme function in the digestion for 20 minutes then put in ice box immediately. The supernatant solution was cooled at 4ºC for further experiment [1].

2.5 Sample preparation for curry pastes without coconut milk

Each curry pastes were weight 2 g and put into centrifuge tube 50 ml. The water was added 4 ml and homogenized at 13,500 rpm for 1 min. The supernatant was collected after centrifugation at 10,000 rpm for 20 min at 4ºC before the further analysis.

2.6 Methods for Testing Apoptosis Induction and Necrosis on functional health effects

The Leukemia Jurkat cells clone E6-1 (Catalog No. TIB -152, ATCC) was cultured with the food sample. The comparison of Apoptosis and the normal white blood cells were tested with the positive control which had Etoposide to kill the cancer cells. The detection of Apoptosis or Necrosis cells was done by using Annexin V staining assay and Dual Parameter Flow Cytometer (FACS) for separation the normal cell, Apoptosis or Necrosis cell. The cells were cultured with RPMI 1640 which were composed of 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin-streptomycin in humidity at 5% CO_2 and temperature 37ºC in culture cell flask 25 cm^3. The cells were grown optimum and subcultured every 2-3 days. The cells had density $3x10^5$ cell/ml in RPMI medium 1640 with the volume of 950 µl in each well of 24-well plate. The food solution was pipetted 50 µl into each well with had the cells by using as negative control with the normal cells and positive control with 10 µg/ml of Etoposide instead of the food sample. The cells were incubated for 24 hours at temperature 37ºC with humidity 97% and 5% CO_2. After that the cells were collected from each well at 13,400 rpm at temperature 25ºC for 1 minute. The supernatant was discarded. The cell pellets were resuspended with buffer solution 1X assay buffer 100 µl for the reaction and transferred to Flow Cytometry tube. Propidium iodide (PI) 0.05 µg/ml 1µl was added and Annexin V-FITC concentration 0.02 µg/ml 0.5 µl. The reaction was incubated in the dark for 15 minutes and 1X assay buffer was added with 400 µl.

The Apoptosis cells were checked immediately with Dual Parameter Flow Cytometry (FACS). The evaluation of Apoptosis cells and Necrosis were done. When Apoptosis occurred and resulted to form phosphatidylserine on the plasma membrane with inside out. The detection was monitored by using annexin-V with fluorescence attached such as Annexin V-FTIC was shown red color whereas the Necrosis cells was not shown. Propidium iodide (PI) was colored on double helix of living thing. The proportion of fluorescence color of Annexin and PI was determined to classify each cell as the following. Annexin V $FITC^-/PI^-$ showed the living cells and Annexin V-$FITC^+/PI^-$ showed the beginning of Apoptosis cells. Annexin V-$FITC^+/PI^+$ showed the last stage of Apoptosis cells or tertiary stage of Necrosis and PI^+ showed Necrosis cells. The symbol"+" was mean the fluorescence color and "-" no fluorescence color. Finally, the report of surviving cell and dead cells by Apoptosis or Necrosis was in percentage of total cells [1].

3. Results and Discussion

3.1Apoptosis Induction and Necrosis of Curry Pastes with Coconut Milk, Chicken and Their Ready-to-Cook Dishes (Jasmine Rice or Thai Spaghetti)

The study of Apoptosis induction and Necrosis in Leukemia cell lines was cultured Jurkat Leukemia cells clone E6-1. The cell line was tested by using the solution after digestion only (Table 1). The result was expressed in % of Apoptosis cells when cultured with each food sample solutions after simulation of gastrointestinal digestion. Each food sample had mixture of enzymes with heat as the blank. The natural induction occurred in natural and then compared with the change in induction (Table1). From Table 1, the result was shown that Apoptosis induction was found at the level between 20-40%. All food samples after simulation of gastrointestinal digestion were increased on Apoptosis induction very significance figure

($p<0.5$) when compared to the control. The order of Apoptosis induction were the highest to the lowest as the following; Jasmine rice with Musaman curry and chicken, Jasmine rice with Red curry and chicken, Jasmine rice with Phanang curry and chicken, Jasmine rice with Green curry and chicken and Thai spaghetti with Green curry and chicken, respectively. In addition, Jasmine rice alone was increased Apoptosis induction too but the increased was less than the dishes. Finally, Thai spaghetti was not induced Apoptosis at all [1].

The ingradients in each curry, coconut milk, chicken was typical formula and showed difference in % Apoptosis induction clearly. Garlic was composed of Allicin [2] and derivative was Ajoene [3] had affected in inhibition of various cancer cells include Leukemia. Therefore the mixture of enzymes in simulation of gastrointestinal digestion was involved in Apoptosis process in Leukemia cell line. Some chemical compounds such as 1-acetoxychavicol acetate in *Alpinia nigra* (Gaertn.) B.L. Burtt [4], capsainoids in chili [5] and polyphenol in various vegetable and herbs could induce Apoptosis in cancer cells.

The study of Necrosis was very surprising on the simulation of gastrointestinal digestion on Thai spaghetti with Green curry, coconut milk, chicken. %Necrosis of the digestion was highest among the other curry pastes under simulation of gastrointestinal digestion (Table 1).

Table1: The results of Apoptosis Induction and Necrosis on Leukemia cell line with the solution after simulation of gastrointestinal digestion of Jasmine rice, Thai spaghetti and Jasmine rice/ Thai spaghetti with various curries, coconut milk and chicken.

Food samples	%Normal cell		%Apoptosis cell		%Necrosis cell	
	Control	Digestion	Control	Digestion[b]	Control	Digestion
Jasmine rice	62.9	47.7	23.7	47.5	13.4	4.8
Thai spaghetti	59.6	55.1±2.9	36.0	39.4±4.8	4.4	5.5±2.0
Jasmine rice with Red curry, coconut milk and chicken	55.9	3.3±3.7	35.1	88.1±0.5	9.0	8.6±3.6
Jasmine rice with Green curry, coconut milk and chicken	56.0	18.4±1.6	29.1	66.9±3.0	14.9	14.7±1.6
Thai spaghetti with Green curry, coconut milk and chicken	56.5	2.7±0.7	30.9	45.9±3.4	12.7	51.4±3.3
Jasmine rice with Phanang curry, coconut milk and chicken	49.3	0.5±0.1	38.4	8.70±8.4	12.4	12.5±8.3
Jasmine rice with Musaman curry, coconut milk and chicken	58.9	0.5±0.1	28.0	98.2±0.5	13.3	1.3±0.5

Control was enzyme solution digested food samples and heat to denature enzymes activity

[b]mean±SD, n=3, except Jasmine rice n=2

3.2 Apoptosis induction and Necrosis of curry pastes without coconut milk and their ready-to-cook soup

The Apoptosis induction in curry pastes without coconut milk such as Gaeng Pah, Gang Liang, Gaeng Som, Gaeng Lerng, Tom Yum. All curry pastes were in the soup without vegetables and meats were used to culture Leukemia cell line Jurkat cells and analysis %Apoptosis induction and %Necrosis compare to the control with C-RPMI. The result was shown that Etoposide [6] can induce to Apoptosis at the highest 70.8±20.3% as the reference, Gaeng Liang 47.0±6.9%, Gaeng Pah 45.0±4.2%, Gaeng Som 43.6±2.2%, Tom Yum 33.5±1.0%, and Gaeng Leung 24.2±2.6%, respectively.

In addition, %Necrosis of each curry pastes without coconut milk was the highest in Gaeng Leung 45.1±13.7% and the lowest in Gaeng Liang 3.0±1.3%. Finally, the ratio of Apoptosis induction to Necrosis 15 time was the important to make decision to choose Gaeng Liang soup as the best for consumption curry soup in Leukemia cell line (Table2).

Table 2: The Apoptosis induction and Necrosis of Leukemia cell line on curry soup without coconut milk

Sample	%Survival	%Apoptosis	%Necrosis
C-RPMI	93.1±2.0	5.7±2.6	1.0±0.5
Etoposide	14.0±12.6	70.8±20.3	11.4±6.0
Gaeng Pah	44.6±12.0	45.0±4.2	10.4±7.8
Gaeng Liang	50.0±6.4	47.0±6.9	3.0±1.3
Gaeng Som	29.0±5.5	43.6±2.2	27.3±6.5
Gaeng Leung	30.7±15.6	24.2±2.6	45.1±13.7
Tom Yum	46.6±5.5	33.5±1.0	19.0±3.4

mean±SD

4. Conclusion

The selected curry pastes with coconut milk for the best of Apoptosis induction on Leukemia cell line (>90%) was Jasmine rice with Musaman curry, coconut milk and chicken. Gaeng Liang soup was the best curry pastes without coconut milk for the highest %Apoptosis induction and lowest %Necrosis in Leukemia cell line.

5. Acknowledgement

The laboratory work was performed by Tada Sueblinwong, M.D.'s Laboratory. The financial support came from Thailand research Fund and Numprik Nittaya Company Limitted.

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