EFFECTS OF SELECTED THAI ANCIENT REMEDIES EXTRACTS ON MUTAGENICITY AND ANTIMUTAGENICITY USING AMES TEST

Preeyakamol Meeyutem[1], Chatubhong Singharachai[2], Chanida Palanuvej[1] Nijsiri Ruangrungsi[1, 3, *]

[1]College of Public Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand
[2]School of Health sciences, Mae Fah Luang University, Muang, Chiang Rai 57100, Thailand
[3]Departments of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: The extracts of selected Thai household ancient remedies namely: Chantaleela, Homtip-osot, Keaw-hom, Prasachandang, Prasamawaeng, Thoraneesantakat, Tree-hom, Ummaluk-kawatee and Wisampayayai were determined for the mutagenic and antimutagenic effects in the absence of metabolic activation using *Salmonella typhimurium* TA98 and TA100. Ethanol was the first solvent used to extract the remedies by soxhlet apparatus till exhaustion. Then the marc was further successively extracted with water. It was found that most ethanolic and water extracts of selected Thai ancient remedies were not directly mutagenic, except that the ethanolic extract of Tree-hom exhibited slightly mutagenicity with the mutagenic index of 3.6 and 2.2 on *S. typhimurium* TA98 and TA100 respectively. However, after treating with nitrite, all extracts showed mutagenic potential on both strains. Antimutagenic assay using 1- aminopyrene-nitrite model revealed that selected Thai ancient remedies could either inhibit or enhance mutagenicity and the concentration-activity relationship was shown. The ethanolic extracts were revealed for more potent activities in mutagenicity and antimutagenicity than water extracts.

Keywords: Mutagenicity, Antimutagenicity, Nitrosation, Ames test

INTRODUCTION

Thai ancient remedies have been widely used in Thailand for a long time until present [1]. Although adverse effects have been less reported during a long historical use of Thai ancient remedies, scientific researches are needed to investigate their safety for consumer protection. Thai ancient remedies, for example, Chantaleela (CL), Homtip-osot (HS), Keaw-hom (KH), Prasachandang (PD), Prasamawaeng (PW), Thoraneesantakat (TK), Tree-hom (TH), Ummaluk-kawatee (UT) and Wisampayayai (WY) have been established as Thai household ancient remedies in the list of Herbal Medicinal Products of the National List of Essential Drugs [2, 3]. These remedies are composed of many herbs, from 7 herbs in PW and UT to 48 herbs in HS. The Ethnomedical uses are for treatment of fever (CL, KH, PD, TH); faint (HS); cough with phlegm (PW, UT); constipation (TK, TH) and flatulence (WY) [3].

In recent years, the interest in the relationship between diet (food/herb) and cancer has increased and there have been numerous studies of the occurrence of mutagens and carcinogens in food. Many types of mutagen are found in foods. Some of them occur naturally, the others can be produced during preparation of foods for consumption [4].

Mutagenic precursors in *dietary ingredient*s may also be important factors causing cancer [5]. Various foods produced in Thailand have been shown to produce a direct-acting on mutagenicity after nitrite treatment [6, 7]. Preserved foods by salt, smoking and pickling processes were associated with an increased risk of gastric cancer due to nitrites and preformed nitroso-compounds [8, 9]. Recently a number of laboratories have reported that fruit and herb extracts contain antimutagenic compounds [10-12] including flavonoids, phenolic, beta-carotene, vitamins C and E, dietary fiber, SH-containing amino acids and peptides. Thai ancient remedies are made up of many herbs in combination and there's been a lack of information about their mutagenic and antimutagenic potential.

The Ames test is a very sensitive and simple procedure. This test uses various strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involving in histidine synthesis, so that they require histidine for growth. Mutagenicity is ability of compound to cause a

[*] *Correspondence to: Nijsiri Ruangrungsi*
E-mail: Nijsiri.R@chula.ac.th

reversion to growth on a histidine-free medium [13-17]. This research assessed the selected Thai ancient remedies extracts for the direct and nitrite–induced mutagenic potential as well as the antimutagenic property against mutagen derived from nitrite treated aminopyrene. Mutagenic expression was investigated by Ames assay using *S. typhimurium* strains TA98 as well as TA100.

MATERIALS AND METHODS

Bacterial tester strain

S. typhimurium tester strains used in this study were histidine dependent strains TA98 and TA100 which carried DNA base frameshift mutation and base-pair substitution mutation respectively. These strains were kindly provided by Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Cultures were stored at -70^{0}C. Each culture was inoculated in Oxoid nutrient broth No.2 overnight at 37°C in a shaking water bath before use.

Preparations of samples

Selected Thai ancient remedies (CL, HS, KH, PD, PW, TK, TH, UT and WY) were exhaustively extracted with ethanol and water respectively. The ethanolic extract was evaporated *in vacuo.* The marc was dried and then extracted with water. The water extract was evaporated in *lyophilizer until* dryness.

Mutagenic activity assay with nitrite treatment

The assay was performed using the pre-incubation method [18]. The ethanolic extracts were dissolved in dimethyl sulfoxide (DMSO) and the water extracts were dissolved in water to make the concentration of 25, 50, 100 and 200 mg/ml. The aliquot of 200 µl was mixed with 550 µl of 0.2 N hydrochloric acid (sufficient to acidify the reaction mixture to pH 3-3.5), then 250 µl of 2M sodium nitrite was added to the reaction mixture. The reaction tube was shaken at 37^{o}C for 4 h and the reaction was stopped by placing the tube in an ice bath for 1 min. Two hundred and fifty microlitres of 2M ammonium sulfamate was added to the tube, mixed well, and allowed to stand for 10 min in an ice bath.

Mixed 100 µl of nitrite treated mixture with 500 µl of 0.5 M phosphate buffer (pH 7.4), added 100 µl of each tester strain (1-2x10^8 cells) and incubated at 37^{o}C in shaking water bath for 20 min. After incubation, added 2 ml of top agar containing 0.5 mM L-histidine and 0.5 mM biotin, mixed well and poured onto a minimal glucose agar plate. The final concentrations of the extracts were 0.4, 0.8, 1.6 and 3.2 mg/plate. The plate was incubated at 37 $^\circ$C in darkness for 48 hr. After that, the number of His$^+$ revertant colonies was counted. Each sample was assayed using triplicate plates. The mutagenic index was calculated as the average number of revertants per plate divided by the average number of the spontaneous revertants per plate. The mutagenic activity of the extract was accepted if the mutagenic index was equal or greater than 2 and also a concentration-response relationship was shown [19].

Mutagenic activity assay without nitrite treatment

The assay was same as described above, but DMSO or water was used instead of sodium nitrite and ammonium sulfamate.

Negative and positive control

DMSO or water was used as a negative control to determine the spontaneous reversion activity. Standard mutagen, nitrite treated 1-aminopyrene was used as a positive control.

Nitrite treated 1-aminopyrene preparation

1-Aminopyrine treated with nitrite in acid solution at the final concentration of 0.06 and 0.12 µg/plate for TA98 and TA100 testing were prepared as aforementioned procedure.

Antimutagenic activity assay

The mixture of nitrite treated 1-Aminopyrine (0.15 mg), the extracts (0, 5, 10 and 15 mg), 500 µl of 0.5 M phosphate buffer (pH 7.4) and 100 µl of each tester strain with the final volume of 700 µl was incubated at 37^{o}C in a shaking water bath for 20 min. After incubation, 2 ml of top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin was added, mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37^{o}C in darkness for 48 hr, then count number of His$^+$ revertant colonies. Each sample was assayed using triplicate plates. Antimutagenic activity was characterized according to the percentage of mutagenic inhibition [20]:

$$\% \text{ mutagenic inhibition} = [(A-B) / (A-C)] \times 100$$

A = a number of histidine revertants induced by nitrite treated standard mutagen (1-AP)

B = a number of histidine revertants induced by nitrite treated standard mutagen (1-AP) in the present of selected Thai ancient remedy extract

C = a number of spontaneous revertants (negative control)

> 60%	=	strong inhibition
41 - 60%	=	moderate inhibition
21 - 40%	=	weak inhibition
0 - 20%	=	negligible inhibition
< 0%	=	enhancement

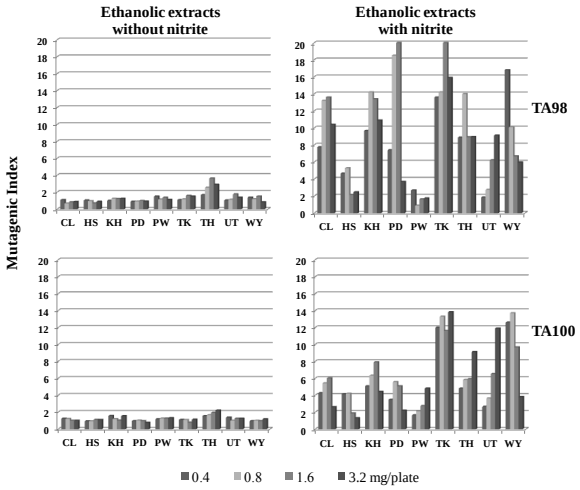


Figure 1 Mutagenic activity among ethanolic extracts of selected Thai ancient remedies on *Salmonella typhimurium* strains TA98 and TA100. CL: Chantaleela, HS: Hoptip-osot, KH: Keaw-hom, PD: Prasachandang, PW: Prasamawaeng, TK: Thoraneesantakat, TH: Tree-hom, UT: Ummalukkawatee, WY: Wisampayayai.

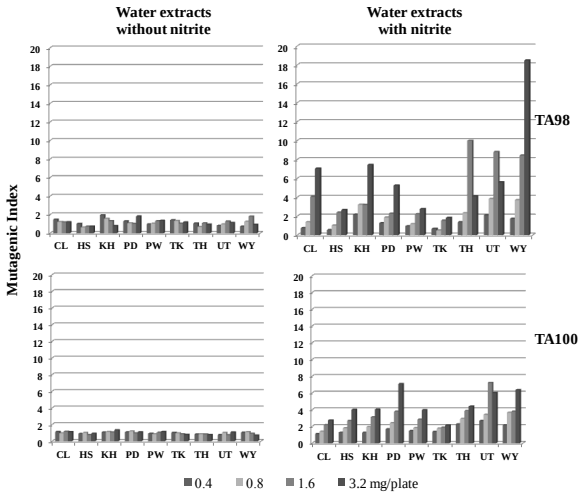


Figure 2 Mutagenic activity among water extracts of selected Thai ancient remedies on *Salmonella typhimurium* strains TA98 and TA100. CL: Chantaleela, HS: Hoptip-osot, KH: Keaw- hom, PD: Prasachandang, PW: Prasamawaeng, TK: Thoraneesantakat, TH: Tree-hom, UT: Ummalukkawatee, WY: Wisampayayai.

RESULTS

Mutagenicity of Thai ancient remedies extracts

Most of the extracts tested without nitrite treatment were not directly mutagenic except the ethanolic extract of TH which exhibited slightly mutagenicity (Figure 1 and 2). The mutagenic index of ethanolic TH extracts was 3.64 and 2.21 on TA98 and TA100 respectively.

With nitrite treatment, all extracts showed the mutagenicity against both strains (Figure 1 and 2). Ethanolic extracts had stronger mutagenic activity than water extracts.

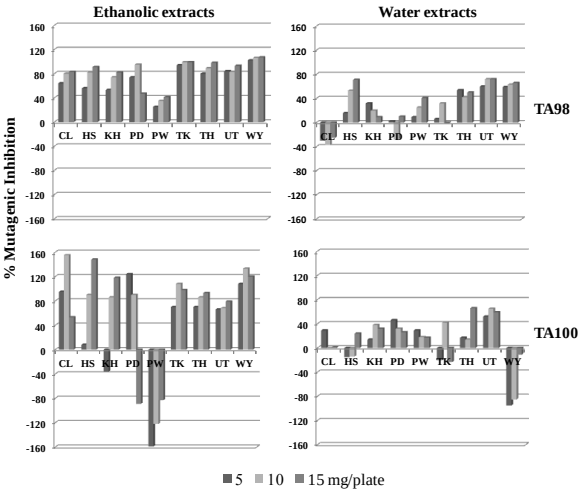


Figure 3 Antimutagenic activity among ethanolic and water extracts of selected Thai ancient remedies on *Salmonella typhimurium* strains TA98 and TA100. CL: Chantaleela, HS: Hoptip-osot, KH: Keaw-hom, PD: Prasachandang, PW: Prasamawaeng, TK: Thoraneesantakat, TH: Tree-hom, UT: Ummalukkawatee, WY: Wisampayayai.

Antimutagenicity of Thai ancient remedies extracts

Inhibition or enhancing effect of the extracts on the mutagenicity induced by 1-aminopyrene treated with nitrite was shown (Figure 3). Most of the ethanolic extracts of selected Thai ancient remedies exhibited moderate to strong inhibition (41 to more than 60%) on *S. typhimurium* TA98 and TA100. However, it was found that the ethanolic extracts of KH, PD and PW expressed mutagenic enhancement on TA100. PW ethanolic extract weakly inhibited mutagenicity on TA98 and enhanced mutagenicity on TA100. The mutagenic enhancing activity of PW ethanolic extract decreased reciprocally as the concentration increased. Mutagenic enhancing activity of KH ethanolic extract on TA100 was diminished and the anti mutagenic activity was dominated by increasing concentration. On the contrary, PD ethanolic extract expressed the mutagenic enhancing activity at high concentration. According to three concentrations tested, the ethanolic extract of WY remedy exhibited highest potential in antimutagenicity in the absence of metabolic activation on both strains of *S. typhimurium*. The water extracts showed less antimutagenic potency than the ethanolic extracts. The water extract of WY remedy enhanced mutagenicity on TA100 with reciprocal proportion to the concentration.

DISCUSSION

Mutagenicity of Thai ancient remedies extracts

Most ethanolic and water extracts of selected Thai ancient remedies in this study were not mutagenic on both *S. typhimurium* TA98 and TA100.

However, when the extracts were treated with nitrite, they became mutagenic on both strains. The results were in accordance with the previous experiments on medicinal plants that the plant extracts exhibited the mutagenicity after nitrosation [21, 22]. Tongyonk also reported that ethanol and water extracts of Ya-ris-si-duang-mahakal remedy had mutagenic activity when treated with nitrite [15]. The finding that selected Thai ancient remedies showed genotoxicity by Ames test after treating with nitrite implied that some chemical component in the remedies could react with nitrite under acidic condition to form *N-nitroso* typed mutagenic compounds. The results in this study were in accordance with the experiment of Kangsadalampai that the ethanolic and hexane extracts of CL, PW, KH and TH treated with sodium nitrite, exhibited the mutagenicity [23]. Therefore co-administration of the remedies with nitrite-containing food should be avoided. Nevertheless the mutagenic assay in this study was performed in the absence of metabolic activation. Hepatic metabolizing enzymes could modulate biotransformation of chemicals and affect on increasing or decreasing of chemical toxicity *in vivo*. In the condition with rat liver enzyme (S9 mix) in Ames system, the mutagenicity of medicinal herbs and remedies might to either diminished or enhanced [23, 24].

Antimutagenicity of Thai ancient remedies extracts using 1- aminopyrene-nitrite model

The reaction between nitrite and dietary amines and amides under the stomach could lead to the formation of nitrosated products which possible to develop pH gastric cancer in human [25]. It is well known that ingredients in diet including herbs, fruits and seeds may exert anticarcinogenic and antimutagenic activities [26-28]. This study found that selected Thai ancient remedies could either inhibit or enhance mutagenicity and the concentration-activity relationship was shown. In addition, the effect on different strains of *S. typhimurium* could be diverse.

Wongwattanasatheun [17] and Botting et al. [29] reported that the extract derived from low polar solvent caused higher inhibition of mutagenicity than the extract derived from high polar solvent. In this study, ethanol was the first solvent used to extract the remedies by soxhlet apparatus till exhaustion. Then the marc was further successively extracted with water. The ethanolic extracts were revealed for more potent activities in mutagenicity and antimutagenicity than water extracts. However, it should be concerned that the effect might be altered if metabolic activation was accounted.

Loh et al. performed Ames test for antimutagenic assay of aqueous and methanol extracts from *Euphorbia hirta* and found that the extracts exhibited strong antimutagenic activity only in the presence of S9 mix. The antimutagenic property of the extracts was related to the ability to modulate the metabolising enzymes, either by preventing the metabolic activation of the mutagen or by altering the enzymatic activity in the detoxification pathway of the mutagen leading to induce the disposal of the mutagen. In addition, it was also possible that antimutagenic metabolites were generated *via* the extracts biotransformation by the metabolizing enzymes [30].

In conclusion, likewise other modern medicines, Thai ancient remedies should be concerned for nitrosation induced mutagenicity. Further studies were required to develop clearer understanding of the mutagenic activity regarding to Thai ancient remedies especially in the presence of metabolic activation. The studies should also be performed using eukaryotic system, for example the Somatic Mutation and Recombination test (SMART) on *Drosophilia Melanogaster* [31]. SMART is non-mammalian *in vivo* model representing metabolism similar to that found in mammalian cell and has been used in genotoxicity and antigenotoxicity detection of chemical substances as well as herbal extracts [32]. Ames in combination with SMART tests can provide more reliable evidences on the mutagenic and antimutagenic potential of Thai ancient remedies.

ACKNOWLEDGEMENTS

The authors are thankful to the Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 Years Chulalongkorn University Fund. The authors are also grateful to College of Public Health Sciences, Chulalongkorn University and all the staff members for necessary assistance and instrumental support. This publication with partial support provided by the funds made available under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (No.AS1148A).

REFERENCES

1. World Health Organization [WHO]. WHO monographs on selected medicinal plants. Geneva: WHO; 1999.
2. Thai National Drug Committee. List of Herbal Medicinal Products A.D. 2008. Bangkok: National Health Security Office [NHSO]; 2008.
3. Ministry of Public Health Notification on Traditional Household Remedies, 26 June 1999, In: Thai Royal

Gazette. 1999; 116 (Part 67d, 24 August).
4. Paris MW, Felton JS, Aeschbacher HU, Sato S. Mutagen and carcinogen in the diet. New York: Wiley-Liss; 1990.
5. Doll R, Peto R. The cause of cancer: quantitative estimates of avoidable risk of cancer in US today. J Natl Cancer Inst. 1981 Jun; 66(6): 1191-308.
6. Kangsadalampai K, Butryee C, Manoophol K. Direct mutagenic of polycyclic aromatic hydrocarbon containing fraction of smoked and charcoal – broiled foods treated with nitrate in acid solution. Food Chem Toxicol. 1997 Feb; 35(2): 213-8.
7. Jitwiriyatham P. Antimutagenicity against urethane of durian products, mangosteen and their mixtures in somatic mutation and recombination test. [Master's thesis, Program in Food and Nutritional Toxicology]. Nakhonpathom: Institute of Nutrition, Mahidol University; 2009.
8. Kaewngam N. Mutagenicity and the modulating effects on the mutagenicity of urethane and the reaction products of sodium nitrite and methylurea of four salted foods. [Master's thesis, Program in Food and Nutritional Toxicology]. Nakhonpathom: Institute of Nutrition, Mahidol University; 2009.
9. Barthilomew BA, Hill MJ. The pharmacology of dietary nitrate and the origin of urinary nitrate. Food Chem Toxicol. 1984 Oct; 22(10): 789-95.
10. Chavoenrat P. Nutrition value of Thai fruit juice and fruit drink and effect of storage time on vitamin C content in orange juice. [Master's thesis]. Nakhompathom: Faculty of Graduate Studies, Mahidol University; 1993.
11. Franke K, Ckless JD. Study of antioxidant and mutagenic activity of different orange juices. Food Chem. 2004; 88: 45-55.
12. Ikken Y, Cambero I, Maria L. Effect of fruit and vegetable aqueous extracts against *N*-Nitrosamines evaluated by the Ames test. J Agric Food Chem. 1998; 46: 5194-200.
13. Ames BN, Lee FD, Duston WE. An improved bacteria test system for the detection and classification of mutagens and carsinogens. Proc Natl Acad Sci USA. 1973 Mar; 70(3): 782-6.
14. Moretelmans K, Zeiger E. The Ames *salmonella* microsome mutagenicity assay. Mutat Res. 2000 Nov 20; 455(1-2): 29-60.
15. Tongyonk L, Tunsaringkarn T, Palanuvej C, Rungsiyothin A, Issaravanich S, Chuthaputti A, et al. Mutagenicity and antimutagenicity of Thai traditional medicine: Ya-ris-si-duang-mahakal. Thai J Health Res. 2006; 20(2): 155-68.
16. Kruawan K, Kangsadalumpai K, Tongsmith B, Sriyapai T. Effect of Manascus color on the mutagenicity of nitrite-treated 1-Aminopyrene using Ames test. Thai J Pharm Sci. 2005; 29: 29-41.
17. Wongwattanasatheun A. Antimutagen and anti oxidative activities of some flowers. [Doctoral dissertation]. Bangkok: Faculty of Pharmaceutical Sciences, Chulalongkorn University; 2008.
18. Singharachai C, Palanuvej C, Kiyohara H, Yamada H, Ruangrungsi N. Safety evaluation of Thai traditional medicine remedy: Ben-Cha-Lo-Ka-Wi-Chian. J Health Res. 2011; 25 (2): 83-90.
19. Calomme M, Pieters L, Vlietinck A, Vanden Berghe D. Inhibition of bacterial mutagenesis by Citrus flavonoids. Planta Med. 1996 Jun; 62(3): 222-6.
20. De Serres FD, Shelby MD. Recommendations on data production and analysis using the *Salmonella*/ microsome mutagenicity assay. Mutant Res. 1979; 64: 159-65.
21. Horn RC, Vargas VMF. Mutagenicity and antimutagenicity of teas used in popular medicine in the *Salmonella*/microsome assay. Toxicol In Vitro. 2008 Jun; 22(4):1043-9.
22. Higashimoto M, Purintrapiban J, Kataoka K, Kinouchi T, Vinitketkumnuen U, Akimoto S, et al. Mutagenicity and antimutagenicity of extracts of three species and a medicinal plant in Thailand. Mutat Res. 1993 Nov; 303(3): 135-42.
23. Kangsadalampai K, Roajchanapho W. Mutagenic potential of Thai folklore medicines formulated under the Provision of Ministry of Public Health and some singer medicinal plants by using ames salmonella mutagenicity test. Proceeeding in Thai traditional medicine and research in the future, Institute of Thai traditional medicine; 2000. p. 236-7.
24. Youvraj R, Sohni MMT, Kale PG. Bacterial mutagenicity of eight medicinal herbs from Zimbabwe. Mutat Res. 1994; 322: 133-40.
25. Mirvish SS. The etiology of gastric cancer: intragastric nitrosamide formation and other theories. J Natl Cancer Inst. 1983; 71: 629-47.
26. Reid KA, Maes J, Maes A, van Staden J, De Kimpe N, Mulholland DA, et al. Evaluation of the mutagenic and antimutagenic effect of South African plants. J Ethnopharmacol. 2006; 106: 44-50.
27. Hartman PE, Shankel DM. Antimutagens and anticarcinogens: a survey of putative interception molecules. Environ Mol Mutagen. 1990; 15: 145-82.
28. Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr. 2003 Sep; 78(3 Suppl): 517S-20S.
29. Botting KJ, Young MM, Pearson AE, Harris PJ, Ferguson LR. Antimutagens in food plants eaten by Polynesians: micronutrients, phytochemicals and protection against bacterial mutagenicity of the heterocyclic amine 2-amino-3-methylimidazo [4-5-*f]* quinoline. Food Chem Toxicol. 1999 Feb-Mar; 37(2-3): 95-103.
30. Loh DSY, Er HM, Chen YS. Mutagenic and antimutagenic activities of aqueous and methanol extracts of *Euphorbia hirta.* J Ethnopharmacol. 2009; 126: 406-14.
31. Hallstrom I, Blanck A, Atuma S. Genetic variation in cytochorm P 450 and xenobiotic metabolism in *Drosophila melanogaster*. Biochem Pharmacol. 1984; 33: 13-20.
32. Sroysa-ard H. Genotoxicity testing of emmena-gogues with and without nitrite using the wing somatic mutation and recombination test (SMART) in *Drosophila melanogaster*. [Master's thesis]. Bangkok: Faculty of Science (Nutrition), Mahidol University; 1997.