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

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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 ingredients 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...

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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°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°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°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 D-biotin, mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37°C in darkness for 48 hr, then count number of His⁺ revertant colonies. Each sample was assayed using triplicate plates. 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°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°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]:

\[
\% \text{mutagenic inhibition} = \left[ \frac{(A-B)}{(A-C)} \right] \times 100
\]

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)

<table>
<thead>
<tr>
<th>%</th>
<th>Classification</th>
</tr>
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<tbody>
<tr>
<td>&gt; 60%</td>
<td>strong inhibition</td>
</tr>
<tr>
<td>41 - 60%</td>
<td>moderate inhibition</td>
</tr>
<tr>
<td>21 - 40%</td>
<td>weak inhibition</td>
</tr>
<tr>
<td>0 - 20%</td>
<td>negligible inhibition</td>
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<tr>
<td>&lt; 0%</td>
<td>enhancement</td>
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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 *Salmonella 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 *Drosophila 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.

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