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# Antiformation and antimutagenic activities of extracts from pericarp and seed of *Zanthoxylum limonella* (Dennst.) Alston

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## ABSTRACT

**Aims:** Extracts from seed and pericarp of Ma-Khwaen (*Zanthoxylum limonella* [Dennst.] Alston) were determined for their direct mutagenic activities with and without nitrite treatment using the Ames test on *Salmonella typhimurium* strains TA98 and TA100. **Results:** Histidine revertants per plate values, measured from mutagenicity testing of seed extracts with and without sodium nitrite treatment on both tested strains, did not meet mutagenicity criteria. Similar results were obtained when the direct mutagenic activity of pericarp extract was tested on *S. typhimurium* strain TA100. Conversely, pericarp extract with sodium nitrite treatment showed mutagenicity on *S. typhimurium* strain TA98. Influence on the formation of the direct mutagen during the interaction between 1-aminopyrene (1-AP) and sodium nitrite in acid solution (pH 3.0–3.5) (antiformation) and influence on mutagenicity of the direct mutagen produced from the same reaction (1-AP-nitrite model) (antimutagen) was also investigated. The mutagenicity modification effect of each sample extract was shown by the percentage of modification (% modification). Positive values of percentage of modification represented a mutagenic decreasing effect while negative values indicated a mutagenic enhancing effect. For antiformation testing, seed extract reduced the formation of the direct mutagen obtained from the 1-AP-nitrite model on *S. typhimurium* strain TA98 (% modification = +69–+72%). When pericarp extract was added at the beginning of the reaction, mutagen formation enhancing effect (% modification = −61–−47%) was observed on strain TA 98. For antimutagen testing, pericarp extract at two higher concentrations (25 and 50 $\mu$l/plate) exhibited decreasing effect on mutagenicity of the standard mutagen on *S. typhimurium* strain TA98 (% modification = +46–+ 84%) and on strain TA100 (% modification = +10–+56%). **Conclusions:** Our investigations indicate that consumption of pericarp from *Z. limonella* with nitrite or nitrite containing food products such as ham and sausage should be avoided to prevent mutagen formation.

**Keywords:** Ames test, antimutagenic activity, *Zanthoxylum limonella* (Dennst.) Alston

## INTRODUCTION

*Zanthoxylum limonella* (Dennst.) Alston or Ma-Khwaen, a spice in the family Rutaceae, is widely distributed in Southeast Asian counties including India, Sri Lanka, Thailand, Myanmar, and the Philippines. In Thailand, *Z. limonella* is an economically important plant in northern areas as Nan, Phayao, and Mae Hong Son Provinces.[1] The fruits of *Z. limonella* can be divided into two parts, the pericarp and the seed. Local people usually use the fruits as a seasoning in the indigenous diet of curry, spicy salad, and fried foods.[2] The pericarp of *Z. limonella* contains more essential oils than the seed and has a pleasant odor.[3] The biological activity of different parts of *Z. limonella* has been previously investigated. Data reported that essential oil extracts of stems, leaves, and fruits from *Z. limonella* have antioxidant, antimicrobial, antiviral, and anticancer activities.[2,4-6] *Z. limonella* also has herbal properties

and is used in traditional medicine. Fruits of *Z. limonella* are sources of essential oils with major components as monoterpenes such as limonene, terpinen-4-ol, and sabinene.[1,2] All activities of *Z. limonella* may result from these phytochemical compounds.

Many foods and dietary components are associated with cancer risk, and increasing or reducing cancer risk depend on dietary constituents.[7] Examples of dietary components with potentially carcinogenic properties include arsenic or heavy metals, polycyclic aromatic hydrocarbons, heterocyclic amines, and nitroso compounds (NOCs),[8] formed by the reaction between nitrosating agents (nitrite or nitrous acid, etc.) and a secondary amino compound from some kinds of food.[9] NOCs are known to act as mutagens and exhibit mutagenicity in microbial systems.[10] Consequently, to prevent cancer, eating healthy food such as vegetables, fruits, and spices that contain nutrients and specific natural phytochemicals may reduce the mutagenic effects of harmful substances.[11] Early determinations of mutagenic and antimutagenic substance activities used bacterial short-term assays. The Ames test, widely used as a short-term mutagenicity assay for detecting chemicals that induce mutations in the DNA of organisms, is based on a reverse mutation in *Salmonella typhimurium*. This assay is rapid, convenient, and simple at relatively low cost.

We used the Ames test to investigate mutagenicity without metabolic activation (direct mutagenicity) and mutagenicity modification effects of seed and pericarp extracts of *Z. limonella*. Direct mutagenicity assays of the sample extracts were evaluated with and without nitrite treatment on *S. typhimurium* strains TA98 and TA100. The mutagenicity modification assay was determined by measuring the inhibition or enhancement effect of sample extracts on the formation of the standard mutagen during the reaction between 1-aminopyrene (1-AP) and sodium nitrite (anti-formation) and on the mutagenicity of newly formed standard mutagen produced from 1-AP treated with sodium nitrite (anti-mutagen).

## MATERIALS AND METHODS

### Sample Preparation

Dried fruits of *Z. limonella* were purchased from a local market in Nan Province, Thailand. Two samples as pericarp and seed were determined separately because when this fruit matures its pericarp is broken, causing separation from the seed.

The samples were dried at 40°C for 4 h. Each sample (20 g) was extracted with 120 ml of dimethyl sulfoxide with a ratio of 1:6 w/v and transferred to a plastic screw-cap tube. Each sample extract was vortexed vigorously for 15 s using a Vortex-Genie 2 (Scientific Industries Inc., USA) and shaken in a shaking water bath at 30°C for 30 min. The tube was then vortexed again for 5 s and centrifuged at 3000 × g for 30 min at room temperature by a Rotofix 32A centrifuge (Hettich®, Becthai Bangkok Equipment and Chemical Co., Ltd., Thailand). The supernatant of each sample extract was sterilized by filtration through a sterile 0.2 μm filter paper before assay.

### Mutagenicity Activity Assay

*Bacterial tester strain*

*S. typhimurium* strain TA98 (frameshift mutation) and TA100 (base-pair substitution) were used in this study. Both strains were provided by Associate Professor Keaw Kangsadalampai and the manipulation was done as recommended by Maron and Ames.[12] The genotype of each *S. typhimurium* strain was confirmed before utilization, with positive and negative controls routinely included.

*Modified Pre-incubation method*

The mutagenicity assay was evaluated using plate incorporation according to Maron and Ames[12] and the pre-incubation modification method described by Yahagi *et al*.[13] Briefly, 100 μl of reaction mixture was mixed in a test tube with 500 μl of 0.5 M sterile phosphate buffer (pH 7.4) and 100 μl of each overnight culture. The mixture was then incubated at 37°C for 20 min in a shaking water bath (Hotech Instruments Corporation, Taiwan). After pre-incubation, 2 ml of top agar (45°C) containing histidine and biotin was added. Finally, the mixture was vortexed and poured onto a minimal glucose agar plate. The numbers of histidine revertants per plate were counted after the culture plates were incubated at 37°C for 48 h.

*Direct mutagenicity of Z. limonella*

For the nitrite treated procedure, an aliquot of each sample extract (10, 25, or 50 μl) was transferred to a sterilized test tube, and its pH was adjusted to 3.0–3.5 with an appropriate volume of 0.2 N hydrochloric acids. The solution was then mixed with 250 μl of 2 M sodium nitrite with the final volume adjusted to 1000 μl and incubated at 37°C for 4 h in a shaking water bath. After incubation, the reaction tube was placed in an ice bath for 1 min to stop the reaction. After that, 250 μl of 2 M ammonium sulfamate was added into the mixture to decompose the residual nitrite, and the tube was placed again into an ice bath for another 10 min before the pre-incubation modification method.

The procedure of sample extract without nitrite treatment was performed as described above but distilled ultrapure water was added instead of 2 M sodium nitrite and 2 M ammonium sulfamate, respectively.

*Mutagenicity modification effect of Z. limonella extract*

Effect of extracts on the formation of the standard mutagen

The modification effect of each sample on mutagen formation during the reaction between 1-AP and sodium nitrite was investigated. Briefly, 0.075 mg/ml of 1-AP was mixed with an appropriate volume of 0.2 N hydrochloric acids to obtain a pH of 3.0–3.5. Then, an aliquot of each sample extract (10, 25, or 50 μl) was added and treated with 250 μl of 2 N sodium nitrite in an acidic condition. The remaining steps followed the procedure described in 2.2.3.

Effect of extracts on the mutagenicity of the standard mutagen

The procedure was performed as described above except an aliquot of each sample extract was added after the reaction mixture was mixed with 250 μl of 2 M ammonium sulfamate and placed into an ice bath for 10 min. This was done to determine the effect of each sample on the newly formed mutagen.

## Data Evaluation

Data were reported as means with standard deviations of six plates from two different determinations. A sample was classified as a mutagen if the results satisfied three criteria.[14,15] First, sample concentration is dose-responsive with a number of revertants per plate. Second, at least two concentrations of the sample have a higher number of histidine revertants per plate than spontaneous revertants. Finally, at least one sample concentration has a higher number of histidine revertants per plate than two-folds of spontaneous revertants.

The mutagenicity modification effect of *Z. limonella* extract was evaluated by calculating the percentage of modification using the following equation, as suggested by Calomme *et al*.[16]

$$\% \text{ Modification} = (A-B/A-C) \times 100$$

Where A is the number of histidine revertants per plate induced by sodium nitrite treated 1-AP (standard mutagen), B is the number of histidine revertants per plate induced by the mutagen in the presence of *Z. limonella* extract, and C is the number of spontaneous revertants per plate (negative control).

Percentage of modification was qualitatively ranked following Bunkova *et al*.[17] The inhibition effect (+) and enhancement effect (−) were classified into four levels as strong or potent activity (> ± 60%), effective or moderate activity (± 40–60%), weak activity (± 20–40%), and no effect (< ± 20%).

# RESULTS

## Direct Mutagenicity of *Z. Limonella* Extract

Table 1 shows direct mutagenicity of the *Z. limonella* pericarp extract and the *Z. limonella* seed extracts with and without nitrite treatment on *S. typhimurium* strains TA98 and TA100. Extracts of either *Z. limonella* pericarp or *Z. limonella* seed without sodium nitrite treatment did not meet the criteria of mutagenicity. Both samples were not mutagenic on either strain of *S. typhimurium*. After treatment with nitrite, the seed extract still showed no mutagenic activity. Conversely, 25 and 50 µl/plate of the pericarp extract induced 29 ± 6 and 66 ± 7 histidine revertants of *S. typhimurium* strain TA98 per plate, respectively. These data showed that the sample was mutagenic in accordance with criteria of the Ames test.

## The Modification Effect of *Z. Limonella* Extract on Mutagenicity of Direct Mutagen

Each sample was added at the beginning of the reaction between 1-AP and sodium nitrite to determine whether the sample interfered with mutagen formation in the pH range of gastric fluid. Results are shown in Table 2. Extract from pericarp had an enhancing effect on the formation of direct mutagen (% modification = −61–−47%) on *S. typhimurium* strain TA98 while the seed extract decreased the formation of mutagen (% modification = +69–+72%) on the same strain of bacteria. When performing the same test using *S. typhimurium* strain TA100, the pericarp extract showed no effect on the formation of mutagen (% modification = −19–−8%) while the seed extract slightly decreased the formation of mutagen (% modification = +20–+29%).

Table 3 reveals the effect of each sample extract on the direct mutagenicity of the newly formed standard mutagen. This information was compared with the former experiment to assess whether the extracts had an inhibitory effect on the formation of the substance (anti-information) or affected the mutagenic properties of the newly formed reactant (antimutagen). Results presented that seed extract showed no effect on direct mutagenicity of newly formed mutagens for both tested strains. Conversely, pericarp extract at the lowest amount exhibited an enhancing effect (% modification = −52%) on

**Table 1:** The direct mutagenic activity of the extracts from different part (seed and pericarp) of *Z. limonella* on *S. typhimurium* strain TA98 and TA100

| Sample | Amount of the extract (µl/plate) | Number of His$^+$ revertants of *S. typhimurium*/plate[a] | | | |
|---|---|---|---|---|---|
| | | TA98 | | TA100 | |
| | | w/o nitrite | w/nitrite | w/o nitrite | w/nitrite |
| Pericarp | Spontaneous[b] | 15±3 | 15±3 | 136±8 | 136±8 |
| | 0[c] | 10±3 | 9±4 | 128±9 | 117±15 |
| | 10[d] | 10±2 | 20±2 | 122±9 | 157±11 |
| | 25[e] | 10±2 | 29±6 | 128±8 | 208±16 |
| | 50[f] | 12±4 | 66±7 | 77±7 | 220±34 |
| Seed | Spontaneous[b] | 26±1 | 26±1 | 92±3 | 92±3 |
| | 0[c] | 28±2 | 27±3 | 86±10 | 88±9 |
| | 10[d] | 33±2 | 32±5 | 81±7 | 92±10 |
| | 25[e] | 32±4 | 30±2 | 92±7 | 89±11 |
| | 50[f] | 34±3 | 31±4 | 96±9 | 91±11 |

[a]Reported as mean±standard deviation of six plates from two different determinations, [b]Spontaneous reversion, [c]Negative (solvent) control; DMSO, dimethyl sulfoxide, [d]equivalent to 2.5 mg dry sample/plate, [e]Equivalent to 6.5 mg dry sample/plate, [f]Equivalent to 12.5 mg dry sample/plate, Values in bold indicate that the induced number of His$^+$revertants per plate was >2 times of the spontaneous mutation, His$^+$ revertants/plate of S. typhimurium strain TA98 induced by the positive mutagen was 1943±111, His$^+$revertants/plate of *S. typhimurium* strain TA100 induced by the positive mutagen was 821±19. *Z. limonella: Zanthoxylum limonella, Salmonella typhimurium: S. typhimurium*

**Table 2:** The effects of the extracts from different parts (seed and pericarp) of *Z. limonella* on the formation of the direct mutagen during the interaction between 1-AP and sodium nitrite (anti-information)

| Sample | Amount of the extract (µl/plate) | Sample presented at the beginning of the reaction | | | |
|---|---|---|---|---|---|
| | | TA98 | | TA100 | |
| | | Number of $His^+$ revertants/plate[a] | % Modification[b] | Number of $His^+$ revertants/plate[a] | % Modification[b] |
| Pericarp | 0[c] | 1966±133 | | 1154±73 | |
| | 10[d] | 3162±158 | -61 | 1234±101 | -8 |
| | 25[e] | 2980±235 | -52 | 1288±54 | -13 |
| | 50[f] | 2885±207 | -47 | 1351±98 | -19 |
| Seed | 0[c] | 1641±152 | | 1270±121 | |
| | 10[d] | 510±63 | +69 | 1043±88 | +20 |
| | 25[e] | 595±60 | +64 | 1015±41 | +22 |
| | 50[f] | 465±117 | +72 | 938±80 | +29 |

[a]Reported as mean±standard deviation of six plates of two different experiments, [b]+or - represents inhibiting or enhancing effects of *Z. limonella* extracts, respectively, [c]Standard mutagen: 1-aminopyrene interacted with nitrite, [d]Equivalent to 2.5 mg dry sample/plate, [e]Equivalent to 6.5 mg dry sample/plate, [f]Equivalent to 12.5 mg dry sample/plate, number of spontaneous revertants/plate of *S. typhimurium* strain TA98 was 13±2, number of spontaneous revertants/plate of *S. typhimurium* strain TA100 was 131±20. *Z. limonella: Zanthoxylum limonella, S. typhimurium: Salmonella typhimurium,* 1-AP: 1-aminopyrene

**Table 3:** The effects of the extracts from different parts (seed and pericarp) of *Z. limonella* on the mutagenicity of direct mutagen produced from the reaction between 1-AP and sodium nitrite (antimutagen)

| Sample | Amount of the extract (µl/plate) | Sample added after the formation of direct mutagen | | | |
|---|---|---|---|---|---|
| | | TA98 | | TA100 | |
| | | Number of $His^+$ revertants/plate[a] | % Modification[b] | Number of $His^+$ revertants/plate[a] | % Modification[b] |
| Pericar | 0[c] | 1951±192 | | 1149±90 | +10 |
| | 10[d] | 2949±207 | -52 | 1045±128 | +53 |
| | 25[e] | 1069±89 | +46 | 614±77 | +56 |
| | 50[f] | 320±44 | +84 | 578±49 | |
| Seed | 0[c] | 1590±88 | | 1266±102 | -8 |
| | 10[d] | 1745±160 | -10 | 1353±131 | -10 |
| | 25[e] | 1863±163 | -17 | 1385±83 | -7 |
| | 50[f] | 1778±128 | -12 | 1343±74 | |

[a]Reported as mean±standard deviation of six plates of two different experiments, [b]+or - represents inhibiting or enhancing effects of *Z. limonella* extracts, respectively, [c]Standard mutagen: 1-aminopyrene interacted with sodium nitrite, [d]Equivalent to 2.5 mg dry sample/plate, [e]Equivalent to 6.5 mg dry sample/plate, [f]Equivalent to 12.5 mg dry sample/plate, number of spontaneous revertants/plate of *S. typhimurium* strain TA98 was 13±2, number of spontaneous revertants/plate of *S. typhimurium* strain TA100 was 131±20. *Z. limonella: Zanthoxylum limonella, S. typhimurium: Salmonella typhimurium*, 1-AP: 1-aminopyrene

*S. typhimurium* strain TA98 but showed an inhibiting effect with the other two higher amounts on *S. typhimurium* strain TA98 (% modification = +46–+84%) and on *S. typhimurium* strain TA100 (% modification = +53–+56%).

## DISCUSSION

Fruits of *Z. limonella* are widely used as a flavoring agent for food in Northern Thailand. Wongsrisom *et al*.[3] reported that pericarp extracts of *Z. limonella* had higher amounts of essential oil than seed extracts. Most plants that contain essential oils are known to have a wide range of biological activities.[18] Some studies showed that essential oils of fruit from *Z. limonella* exhibited antioxidant, antimicrobial, and anticancer activities.[4,6,19] However, no previous studies have been conducted to compare the antimutagenic activity of *Z. limonella* pericarp extract and *Z. limonella* seed extract.

Here, *Z. limonella* seed extract, either with or without nitrite treatment, had no direct mutagenicity on both *S. typhimurium* strains. This indicated that *Z. limonella* seed extract did not contain compounds which could produce a direct mutagen after treatment with nitrite. Conversely, the pericarp extract after nitrite treatment exhibited direct mutagenicity only on *S. typhimurium* TA98, a strain that detected frameshift mutation. Namiki *et al*.[20] also reported a similar result and they found that many spices including chili, laurel, nutmeg, and pepper exhibited mutagenic activities on *S. typhimurium* strains TA98 and TA100 after treatment with nitrite. Phytochemical constituents of essential oils of *Z. limonella* fruit are monoterpene hydrocarbons, oxygenated monoterpene, and phenolic compounds[1] and *Z. limonella* pericarp contains higher amounts of essential oils than *Z. limonella* seed.[3] Therefore, it is possible that certain substances such as phenolic compounds, which are components

of *Z. limonella* essential oils, may react with nitrite and become a frameshift mutagen. This hypothesis is supported by Ohshima *et al*.[21] who found that simple phenolic compounds (including phenol, 3-methoxycatechol, catechol, and vanillin) had direct mutagenicity on *S. typhimurium* strains TA98 and TA100 after being treated with sodium nitrite. Accordingly, consumption of *Z. limonella* pericarp with nitrite or food products that contain nitrites such as sausages, ham, and bacon should be avoided to prevent the formation of mutagens.

For the anti-information assay, results showed that *Z. limonella* seed extract decreased mutagen formation. The direct or standard mutagen model used in this study was a mutagenic nitro compound (1-nitropyrene),[22] obtained by the reaction between 1-AP and sodium nitrite in an acidic condition. This indicated that some compounds in the seed extract inhibited this reaction model. Supabphol and Tangjitjareonkun[1] reported the presence of oil and unsaturated fatty acid accumulated in seeds of *Z. limonella*. Fatty acids reacted with nitrite under the acidic condition to produce nitro-fatty acids[23] and inhibited the nitrosation reaction by scavenging nitrite.[24] Therefore, the mechanism to reduce the formation of standard mutagens of *Z. limonella* seed extract may involve the scavenging activity of fatty acids contained in the seeds. However, limited information exists on the phytochemical composition of *Z. limonella* seed; therefore, further experiments to determine the active compounds of the seed are required.

Conversely, pericarp extract of *Z. limonella* increased mutagen formation during the reaction between 1-AP and sodium nitrite on *S. typhimurium* strain TA98. This result may be caused by compounds contained in the *Z. limonella* pericarp extract which reacted with nitrites, leading to the formation of a new mutagen.[21] This explanation was supported by the result of the direct mutagenicity test of pericarp extract which showed that after being treated with nitrite this pericarp extract exhibited mutagenic effects on *S. typhimurium* strain TA98. In the same system, a standard mutagen was generated from the reaction between 1-AP and nitrite. Therefore, the pericarp extract increased the mutagenicity of the standard mutagen by a combination of mutagenic activities of the new mutagen and the standard mutagen. However, consumption of *Z. limonella* fruits may not be harmful to customers since most spices are used in small quantities.

For the antimutation assay, *Z. limonella* pericarp extract reduced the mutagenicity of the newly formed standard mutagen on both strains of bacteria. Normally, the standard mutagen from 1-AP treated with sodium nitrite requires bacterial biotransformation to activate their mutagenicity on bacterial DNA.[22] The nitro group is reduced to a hydroxylamine intermediate which may bind to DNA by the enzymes of bacteria, nitroreductase, and O-transferase.[25] Thus, the antimutagenicity of the pericarp extract may be involved with an inhibitory effect on such enzymes. This hypothesis was suggested by findings of a previous study that the antimutagenicity of terpene (linalool and β-caryophyllene) against the mutagenicity of 2-nitrofluorene on *S. typhimurium* strains TA98 and TA100 resulted from inhibition on bacterial nitroreductase and/or O-transferase.[26] However, pericarp extract showed the effect with a biphasic dose response only on *S. typhimurium* strain TA98. Pericarp extract increased the mutagenicity of the standard mutagen at the lowest amount, but a decreasing effect was found with the other two higher amounts of extract. Similarly, Stavric *et al*.[27] reported that extract of green tea increased the mutagenicity of some heterocyclic aromatic amines (PhIP, Trp-P-1, and Trp-P-2) at a lower dose but this decreased at higher doses. They suggested that there may be sufficient quantities of enhancer to increase the mutagenic activity of mutagens in diluted tea extracts. On the other hand, higher concentrations may contain more inhibitory factors that hindered promotional activity. However, *Z. limonella* pericarp extract at different concentrations may need further investigation to confirm this hypothesis.

## CONCLUSION

*Z. limonella* seed extract showed no direct mutagenicity either with or without nitrite treatment on *S. typhimurium* strains TA98 and TA100. On the other hand, pericarp extract exhibited direct mutagenicity after treatment with nitrite on *S. typhimurium* strains TA98. The seed extract decreased the formation of direct mutagen in the reaction between 1-AP and sodium nitrite in an acid condition. In addition, the pericarp extract reduced mutagenicity of the standard mutagen produced from 1-AP reaction with sodium nitrite in both tested strains. However, antimutagenicity mechanisms of phytochemical constituents from *Z. limonella* pericarp and that of *Z. limonella* seed require further studies. In addition, since consumers use the whole fruit of *Z. limonella* as a spice for cooking, further detailed exploration should be carried out using an extract from the whole fruit (seed mixed with pericarp).

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