

## Antioxidant and Growth Inhibitory Activities on Gastrointestinal Tract Pathogenic Bacteria of Fermented Miang and Miang Leaf Extracts

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### ABSTRACT

Miang (*Camellia sinensis* var. *assamica*) is a tea plant found in mountainous areas of northern Thailand. Miang leaves were collected, cleaned, steamed and fermented with *Lactobacillus acidophilus* TISTR 2365 for 12 months. Meanwhile, Miang leaves were extracted with water and ethanol to obtain crude extracts. The antioxidant activity of fermented Miang juice was increasingly built up from the start, peaked at 3 month of fermentation and gradually decreased against fermentation time. The fermented Miang juices could only inhibit growth of *Staphylococcus aureus* while the crude ethanolic extract of Miang leaves could inhibit growth of *Bacillus cereus*, *Salmonella* Typhi, *Shigella dysenteriae*, *Staphylococcus aureus* and *Vibrio cholerae*. Furthermore, the antioxidant activity was detected in both crude aqueous and ethanolic extracts of Miang leaves.

**Keywords:** *Camellia sinensis* var. *assamica*, Fermented tea, Miang

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## Introduction

Miang, Miang tea or Assam tea (*Camellia sinensis* var. *assamica*) is an indigenous plant in mountainous areas of northern Thailand. Local people have used Miang leaves as foods and food related products for a long period of time. The most popular application of Miang leaves is fermented Miang. Therefore, sometimes the word “Miang” is referred to fermented Miang tea as well. In the past, fermented Miang was usually consumed by local people as a refreshment snack. However, as time goes by, fermented Miang is now far less popular to modernized people or young generation in Thailand due to its unattractive physical appearance, bitter taste and plain product packaging. In order to become a more valuable commodity economy that can be consumed by people of all ages and to conserve local wisdom of fermented Miang, researches on Miang product development and Miang bioactive compounds should be focused. It has been known for decades that tea leaves have high antioxidants, mainly polyphenols especially flavanols. Flavanols in tea include catechins and derivatives of catechins such as (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) (Atomssa and Gholap, 2015). The polyphenols have many benefits such as obesity prevention, reduction of cholesterol levels, prevention of diabetes and heart disease (Jigisha *et al.*, 2012), and growth inhibition of oral bacteria consisting of *Escherichia coli*, *Salmonella* Typhi, *Salmonella* Typhimurium, *Yersinia enterocolitica* (Tiwari *et al.*, 2005), *Bacillus cereus*, *Bacillus subtilis*, *Clostridium perfringens*, *C. sporogenes*, *Micrococcus luteus* (Chan *et al.*, 2011; Keller *et al.*, 2013), methicillin-resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa* (Radji *et al.*, 2013). It was found that EGCG could suppress the expression of *gtf* gene related to biofilm formation of *Streptococcus mutans* resulting in reduction of dental caries (Xu *et al.*, 2012).

## Objectives of the study

To evaluate antioxidant and antibacterial activities against some gastrointestinal tract pathogenic bacteria of fermented Miang juices and Miang crude extracts.

## Methodology

### 1. Miang leaf fermentation

*Lactobacillus acidophilus* TISTR 2365 was cultivated in de Man Rogosa and Sharpe (MRS) broth at 37°C for 48 hours. Fresh Miang leaves were steamed for 2 hours, cooled down and added into fermentation jars prior to inoculation with *L. acidophilus* TISTR 2365, 2% (v/v). The Miang fermentation jars were incubated at room temperature in the dark for 12 months. At intervals of 90 days, the fermented Miang juice samples were collected for investigation of antioxidant and antimicrobial activities.

### 2. Miang leaf extraction

Fresh Miang leaves were plucked, cleaned, dried at 60°C and ground. Ground Miang leaves were extracted by two different solvents, water and ethanol, 95%, at a ratio 1:10 of ground Miang leaves and solvent (Dostalova *et al.*, 2014). When using water as a solvent, the mixture was incubated at 45°C in a water bath for 3 hours while the ethanolic extraction was shaking incubated at room temperature for 48 hours. The mixtures were subsequently

filtered by filter papers (Whatman™ No.1), evaporated by a rotary evaporator and freeze dried by a lyophilizer. The crude extracts were kept in a desiccator for further studies. When used, the aqueous extract was dissolved in sterile distilled water while the ethanolic extract was dissolved in dimethyl sulfoxide (DMSO).

### 3. Antioxidant activity measurement by a DPPH radical scavenging assay

Gallic acid, 0.001-0.01 mg/ml, was used as a standard. Amount of 0.5 ml of gallic acid, crude aqueous and ethanolic extracts of Miang leaves or fermented Miang juices was added into 1.5 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 0.1 mM, and mixed well. The mixtures were left in darkness at room temperature for 20 minutes. Afterwards, the absorbance of the sample ( $A_{\text{sample}}$ ) was measured using a spectrophotometer at 517 nm against ethanol blank. Methanol was used as a negative control ( $A_{\text{control}}$ ). The half maximal inhibitory concentration ( $IC_{50}$ ) and the antioxidant activity were calculated (Safdar *et al.*, 2016; Bhadoriya *et al.*, 2012) according to the equations below:

$$\text{DPPH inhibition (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100$$

$$\text{Antioxidant activity} = \frac{IC_{50\text{control}}}{IC_{50\text{sample}}}$$

### 4. Preparation of test pathogenic bacteria

The test pathogenic bacteria including *Bacillus cereus*, *Escherichia coli* O157: H7, *Salmonella* Typhi, *Shigella dysenteriae*, *Staphylococcus aureus* and *Vibrio cholerae* were cultivated in Mueller Hinton Broth (MHB) at 37°C for 24 hours. Each bacterial cell suspension was adjusted equivalent to a No. 0.5 McFarland Standard for using in the antibacterial activity evaluation.

### 5. Antibacterial activity evaluation of crude extracts by an agar disc diffusion method

Each test bacterial suspension was swabbed onto Mueller Hinton Agar (MHA). The concentration of crude extracts was 500 mg/ml. Each sterile paper disc diameter of 6 mm (Macherey - Nagel™) was soaked with aqueous or ethanolic extracts of Miang leaves and placed onto the prepared MHA. The solvents and 0.1 mg/ml of gentamicin were used as negative and positive controls, respectively. All test plates were incubated at 37°C for 24 hours. The inhibitory clear zone was observed and measured using a vernier caliper.

### 6. Antibacterial activity evaluation of fermented Miang juices by an agar well diffusion method

Each test bacterial suspension was swabbed onto MHA. The swabbed agars were punctured by Pasteur pipettes to obtain small wells with diameter of 6 mm each. Afterwards, each fermented Miang juice, 25 µl, was added into each well (Bonev *et al.*, 2008). The solvent and 1 mg/ml of gentamicin were used as negative and positive controls, respectively. All test plates were incubated at 37°C for 24 hours. The inhibitory clear zone was observed and measured using a vernier caliper.

## 7. Statistical analysis

Experiments were performed in triplicates. The data were statistically evaluated using analysis of variance (ANOVA) with SPSS. Duncan's multiple range tests was carried out in order to test any significant difference between the fermented Miang juice and the incubation time. Significance levels were defined using  $p < 0.05$ .

## Results

### 1. Antioxidant activity investigation of fermented Miang juices and Miang leaf crude extracts

When using *L. acidophilus* TISTR 2365 as a starter culture of Miang leaf fermentation, the fermented Miang juice obtained after 3 months of fermentation exhibited the highest antioxidant activity, 1979.3±40.5 mg gallic acid/100 ml (Figure 1). Using two-way ANOVA analysis, the antioxidant activity of the fermented Miang juices without addition of a starter culture (control) was significantly lower ( $p < 0.05$ ) than that of the *L. acidophilus* TISTR 2365 fermented Miang juices. Furthermore, the antioxidant activity of *L. acidophilus* TISTR 2365 fermented Miang juices at 3 month fermentation was significantly higher than that of at 12 month fermentation ( $p < 0.05$ ). For the crude aqueous and ethanolic extracts of Miang leaves, their antioxidant activities were 1028.9±2.7 and 1253.4±2.2 mg gallic acid/g extract, respectively (Table 1).

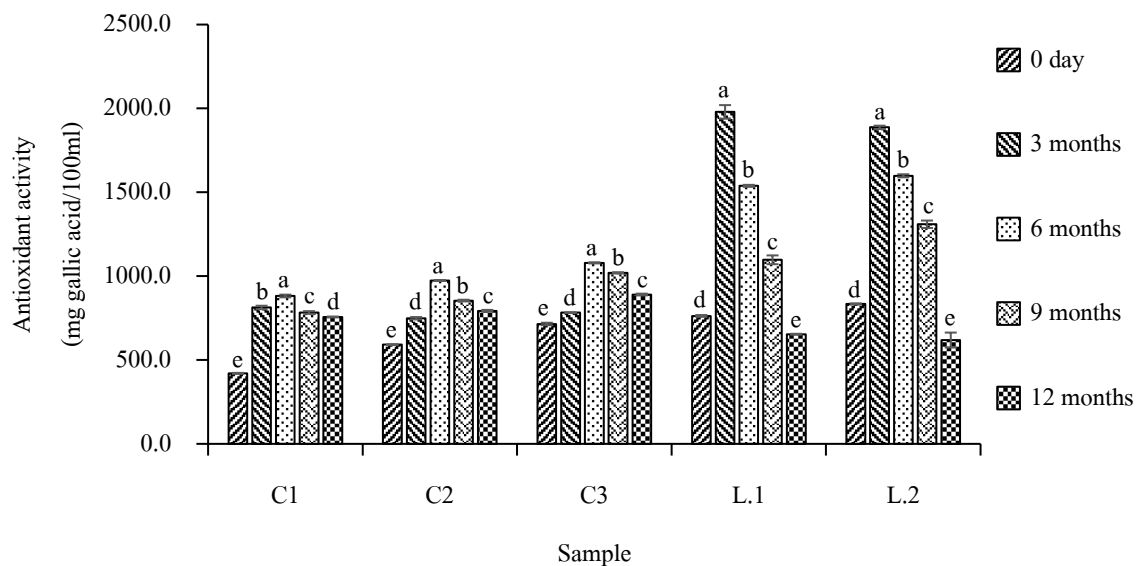


Figure 1. The antioxidant activity of the fermented Miang juices. Data were expressed as mean ± standard deviation (n=3). The symbol "C" referred to fermented Miang juices without addition of a starter culture (control) and "L" referred to fermented Miang juices with *L. acidophilus* TISTR 2365 as a starter culture. The numbers referred to the batch of fermentation. The difference alphabet on error bar was significantly different ( $p < 0.05$ ) according to the Duncan's multiple range tests.

Table 1. Antioxidant activity of Miang crude extracts

Crude extract	Antioxidant activity (mg gallic acid/g extract)
Aqueous extract	1,028.9±2.7 <sup>a</sup>
Ethanollic extract	1,253.4±2.2 <sup>b</sup>

Data were expressed as mean ± standard deviation (n=3). The difference alphabet was significantly different (p < 0.05).

## 2. Antibacterial activity investigation

### 2.1 Fermented Miang juices

Up to 6 months of fermentation time, all fermented Miang juices could only inhibit growth of *S. aureus* with the inhibitory clear zone ranging between 12.3±0.4 -14.0±0 mm (Figure 2). Any fermented Miang juices that had been fermented longer than 6 months were not able to inhibit growth of *S. aureus*.

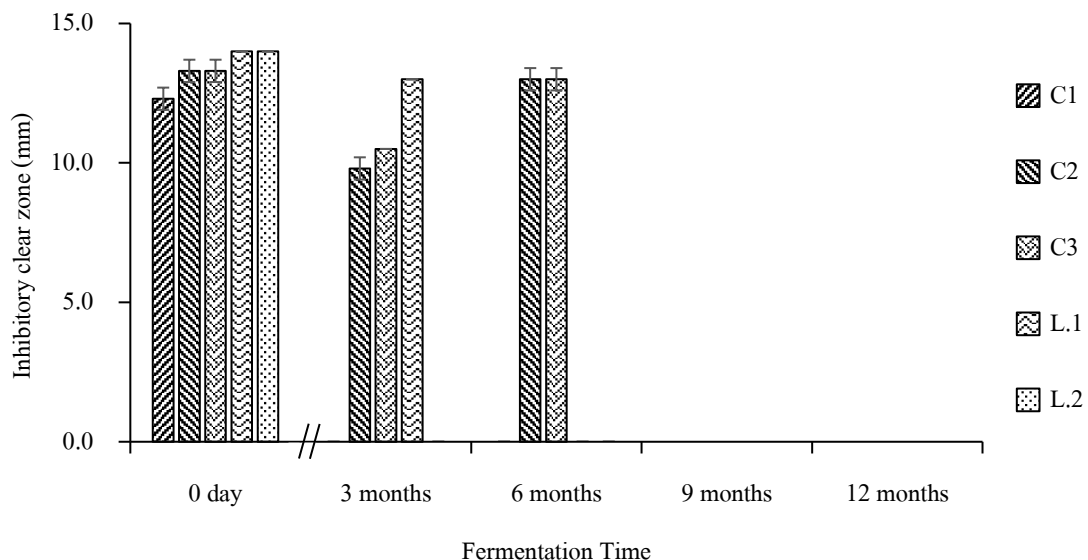


Figure 2. Antibacterial activity of the fermented Miang juices was evaluated on *S. aureus* by an agar well diffusion method. Data were expressed as mean ± standard deviation (n=3); The symbol “C” referred to fermented Miang juices without addition of a starter culture (control) and “L” referred to fermented Miang juices with *L. acidophilus* TISTR 2365 as a starter culture.

### 2.2. Miang leaf crude extracts

Using a paper disc diffusion method, the aqueous extract could inhibit growth of *B. cereus*, *Sal. Typhi* and *S. aureus* with the inhibitory clear zones of 7.8±0.3, 7.0±0.0 and 7.0±0.0 mm, respectively, while the ethanolic extract could inhibit growth of *B. cereus*, *Sal. Typhi*, *Shi. dysenteriae*, *S. aureus* and *V. cholerae* with the inhibitory clear zones of 10.3±0.3, 10.5±0.0, 8.8±0.3, 17.3±0.3 and 9.3±0.3 mm, respectively (Figure 3). The antibacterial activity of the aqueous extract was significantly lower than that of the ethanolic extract (p < 0.05) using one sample t-test

analysis. Interestingly, *Escherichia coli* O157:H7 was resistant to both crude aqueous and ethanolic extracts of Miang leaves.

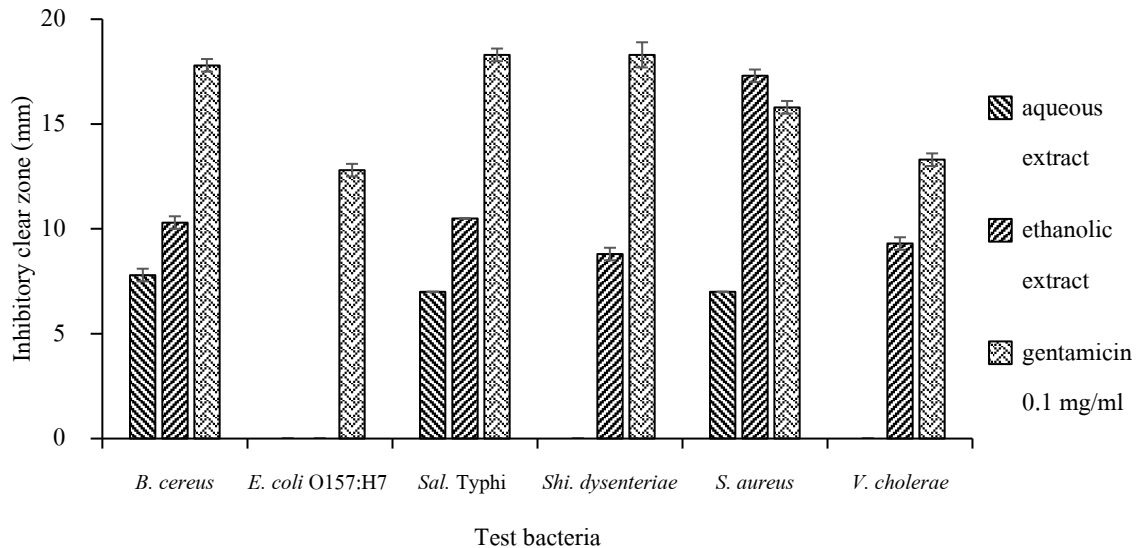


Figure 3. Antibacterial activity of Miang crude extracts by an agar disc diffusion method. Data are expressed as mean  $\pm$  standard deviation (n=4).

### Discussion

Deb *et al.* (2016) found that green tea had antioxidant activity particular in catechins and derivatives of catechins such as (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC). When antioxidant activity was evaluated by DPPH radical scavenging assay, it was found that the 3 month fermentation of *L. acidophilus* TISTR 2365 fermented Miang juice had the highest antioxidant activity. It was anticipated that the initial phase of fermentation, Miang leaves released the antioxidants. Over time, the antioxidants were destroyed by the acid condition. Moreover, the rising temperatures during fermentation process resulting from bacterial metabolism could reduce the amount of antioxidants. Settharaksa *et al.* (2012) found that when pH decreased and temperature increased, the levels of antioxidants could be reduced.

The 3 month fermentation of *L. acidophilus* TISTR 2365 fermented Miang juice had the highest activity to inhibit growth of *S. aureus*. This was consistent with the antioxidant test that the 3 month fermentation of *L. acidophilus* TISTR 2365 fermented Miang juice had the highest antioxidant activity as well. The crude ethanolic extract of Miang leaves could inhibit growth of *B. cereus*, *Sal. Typhi*, *Shi. dysenteriae*, *S. aureus* and *V. cholerae*, but not *Escherichia coli* O157:H7 possibly due to its antioxidant activity and other substances which would be further studied in the future. Reygaert (2014) studied the antibacterial activity of green tea and found that ECG, EGC and EGCG could inhibit growth of pathogenic bacteria. In addition, green tea could use for treatment and prevention of microbial infection. Catechin and its derivatives could destroy cell membranes and inhibit bacterial fatty acid synthesis and enzyme activity. Kumar *et al.* (2012) reported that the antibacterial activity of green tea extract could

inhibit growth of *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Escherichia coli*, *Proteus* and *Bacillus*. Furthermore, the antibacterial effect was a result of EGCG, which would react with dissolved oxygen to produce hydrogen peroxide which caused DNA damage and mutation (Steinmann *et al.*, 2013).

### Conclusions

The *L. acidophilus* TISTR 2365 fermented Miang juice has high antioxidant activity and can inhibit growth of *S. aureus*. Additionally, both crude aqueous and ethanolic extracts of Miang leaves have high antioxidant and antibacterial activities which will be suitable for further product development in the future.

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