Biological Activities of Steamed Miang (*Camellia sinensis* (L.) Kuntze var. *assamica* (J.W. Mast.) Kitam) Juice Extracts

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Abstract: Miang (*Camellia sinensis* (L.) Kuntze var. *assamica* (J.W. Mast.) Kitam.) is an Assam tea which is mainly found in northern Thailand. Fermented Miang is made from young and semi-mature Miang leaves by the local wisdom. Briefly, Miang leaves are harvested and steamed for approximately 2 hours prior to cooling down and subsequently packed into a container with close lid. Fermentation process will take place and run for 1-3 months normally. Steaming step renders a high quantity of steamed Miang juices (SMJ) which will be discarded afterwards. Evaluation of this biological waste will bring about ways of making value-added products. The purpose of this study aims to investigate the effects of steaming time and ages of Miang leaves on biological activities of SMJ obtained. Fresh, young and semi-mature, Miang leaves were collected and steamed for 30 minutes, 1, 2 and 3 hours. Meanwhile, young and old Miang leaves were steamed for 2 hours as compared with the conventional method. All SMJ were further evaporated and lyophilized to obtain SMJ crude extracts (CE). The 3-hour SMJ-CE had the highest antioxidant activity and total phenolic content ($p<0.05$), 548.4±13 μg GAE/ g extract and 460.24±11.8 μg GAE/g extract, respectively. Comparing between SMJ-CE of young and old Miang leaves, the antioxidant activity and total phenolic content of the young leaves SMJ-CE was significantly higher than the old leaves SMJ-CE ($p<0.05$). Moreover, all SMJ-CE had antibacterial activities to inhibit growth of some gastrointestinal pathogenic bacteria including *Bacillus cereus*, *Escherichia coli*, *Escherichia coli* O157:H7, *Salmonella* Typhi, *Shigella dysenteriae*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Vibrio cholerae* with the young leaves SMJ-CE showed the highest activities. The information obtained will be further used as a guideline in food and cosmetic application using SMJ-CE as a raw material.

Key words: Antioxidant, Assam tea, *Camellia sinensis*, Fermented tea, Miang.

1. Introduction

Miang (*Camellia sinensis* (L.) Kuntze var. *assamica* (J.W. Mast.) Kitam.) is an Assam tea which is mainly found in the highlands of northern Thailand [1]. Miang trees usually grow in the shaded area of other forest trees and do not need much care [2].

Miang can refer to a fermented Miang product, which is made and consumed by local people in northern Thailand for centuries. By the conventional method of Miang fermentation process which has been passed down from generation to generation, Miang leaves, young and semi-mature, have been collected and steamed for approximately 2 hours in a steamer. Subsequently, steamed Miang leaves are taken out of the steamer, cooled down to room temperature and further fermented under anaerobic condition in a container lined with banana leaves or a vinyl bag for several days and up to a year [1], [3]. Meanwhile, steamed Miang juice (SMJ) in the steamer is discarded. Fermented Miang has a sour, bitter and astringent taste. It is consumed as a chewing snack and known as a traditional food for religious ceremonies. Nowadays, Miang is less popular to the new generations because of its appearance. Studying about Miang in all aspects will lead to novel product designation, current market evaluation, local wisdom conservation and forest restoration ultimately.

Fresh tea leaves contain high polyphenol content, including (+)-catechins (C), (-)-epicatechin (EC), (-)-gallocatechin (GC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-gallocatechin gallate (GCG) and (-)-epigallocatechin gallate (EGCG). Polyphenols are secondary metabolites, which have potential health properties and benefits for human health, such as strong antioxidants, antibacterial properties, anti-allergic, anti-inflammatory, anticancer and antihypertensive [4].

The SMJ contain various compounds released from steamed Miang leaves [1]. Therefore, the SMJ is an appealing alternative raw material for future applications. This study aims to investigate the effects of steaming time and ages of Miang leaves on some biological activities of SMJ obtained in order to reduce biological waste and to increase the Miang value.

2. Procedure

2.1. Preparation of Steamed Miang Juice Crude Extract (SMJ-CE)

Fresh Miang leaves were collected from a Miang garden in Chiang Mai province. Mixture of young and semi-mature leaves was steamed in a steamer as done by local wisdom except for the time of steaming that was varied in order to evaluate the effect of steaming time on biological activities of wastewater emerged from the whole process of fermented Miang production. Steamed Miang juices (SMJ) were defined as mixture of boiling water at the bottom of the steamer and Miang constituents released from Miang leaves during steaming. SMJs were collected after 30 minutes, 1, 2 and 3 hours of steaming for further experiments.

Meanwhile, young and old Miang leaves were steamed for 2 hours as compared with the conventional method to investigate the effect of Miang leaf ages on biological activities [5].

All SMJ samples obtained above were filtrated by filter papers (Whatman™ No.1), evaporated by a rotary evaporator (Buchi, USA) and freeze dried by a lyophilizer (FTS systems, USA) to obtain crude extracts (CE). All SMJ-CE were kept at room temperature in a desiccator until use. The SMJ-CE was dissolved in sterile distilled water to the desired concentration before using.

2.2. Determination of Antioxidant Activity

Antioxidant activities were determined by the free radical scavenging ability using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [6]. Gallic acid, 0.001-0.01 mg/ml, was used as a standard. Amount of 50 µl of gallic acid or SMJ-CE, 500 mg/ml, was added in triplicate into a 96-well plate. Subsequently, amount of 150 µl of methanolic DPPH solution, 0.1 mM, was added and methanol was used as a control. The solutions were kept in darkness at room temperature for 20 minutes. The absorbance was measured at 517 nm by a microplate reader (Biochrome, UK). The half maximal inhibitory concentration (IC_{50}) and the antioxidant activity were calculated with the equation:

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

$$\text{Antioxidant activity (µg GAE/g extract)} = IC_{50}control/IC_{50}sample$$

where $A_{control}$ is absorbance of methanol, a negative control, and A_{sample} is absorbance of sample.

2.3. Determination of Total Phenolic Content

Total phenolic contents were determined by the Folin-Ciocalteau method [7]. Gallic acid, 0.001-0.01 mg/ml, was used as a standard for a calibration curve. Amount of 20 µl of gallic acid or SMJ-CE and 100 µl of 10% (w/v) Folin-Ciocalteau reagent were added into a 96-well plate. The plate was left in darkness for 5 minutes. Subsequently, amount of 80 µl of 7.5% (w/v) sodium carbonate was added and the mixture was kept in darkness for 1 hour. The absorbance was measured at 725 nm by a microplate reader (Biochrome, UK). The results were expressed as µg of gallic acid equivalent per weight of extract (µg GAE/g extract).

2.4. Preparation of Test Pathogenic Bacteria

A total of 8 test pathogenic bacteria were used in the study including *Bacillus cereus* TISTR 687, *Escherichia coli* ATCC 25922, *Escherichia coli* O157:H7 DMST 12743, *Salmonella* Typhi DMST 22842, *Shigella dysenteriae* DMST 1511, *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* DMST 20625 (MRSA) and *Vibrio cholerae* DMST 2873. All test bacteria were cultivated in Mueller Hinton broth (MHB) (Merck, Germany) 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 [8].

2.5. Antibacterial Activity Evaluation

Antibacterial activity was determined by an agar disc diffusion method [8]. Each test bacterial cell suspension was swabbed on Mueller Hinton agar (MHA) (Merck, Germany). A 6-mm sterile paper disc soaked with 500 mg/ml of SMJ-CE was placed onto the prepared MHA mentioned above. Gentamicin, 0.1 mg/ml, and sterile distilled water were used as positive and negative controls, respectively. The test plates were incubated at 37°C for 24 hours. The diameters of inhibitory clear zone were observed and measured.

2.6. Statistical Analysis

All experiment was performed in triplicate. The data were analyzed using one-way analysis of variance (ANOVA) and the significance was calculated. p values < 0.05 were considered as statistically significant. The statistical analyses were performed using statistical software SPSS 17.0.

3. Results

3.1. Determination of Antioxidant Activity of Steamed Miang Juice Crude Extracts

The antioxidant activity of SMJ-CE was examined by the DPPH radical scavenging assay with gallic acid as a positive control. Using one-way ANOVA analysis, 3-hour SMJ-CE presented the highest antioxidant activity, 548.4±13 µg GAE/ g extract. The antioxidant activities of 30-min, 1-hour and 2-hour SMJ-CE were significantly lower than the 3-hour SMJ-CE ($p<0.05$) with the values of 0.18±0.006, 2.98±0.4 and 0.83±0.1 µg GAE/g extract, respectively (Fig. 1). Furthermore, the antioxidant activity of young leaves SMJ-CE was significantly higher than the old leaves SMJ-CE ($p<0.05$). There antioxidant activities were 1,141.9±68 and 895.2±46 µg GAE/g extract, respectively (Fig. 2).

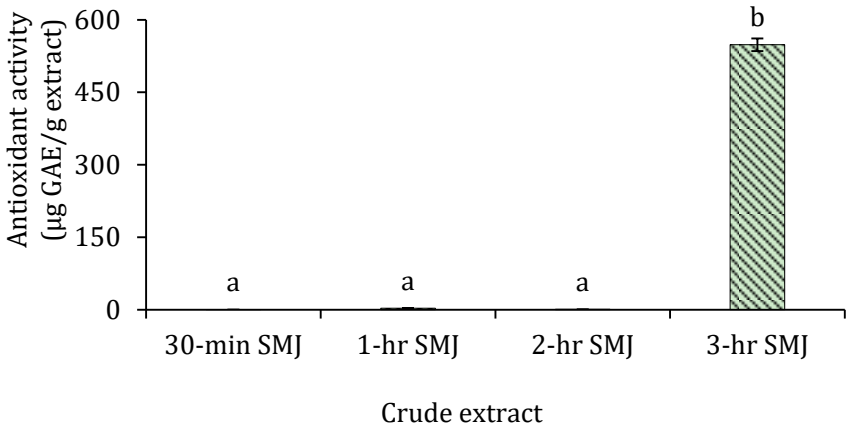

Fig. 1. The antioxidant activity of SMJ-CE of different steamed time. Data were expressed as mean ± standard deviation (n=3) determined by the DPPH radical scavenging assay. The different alphabet on error bar was significantly different (p<0.05) according to the Duncan's multiple range tests.

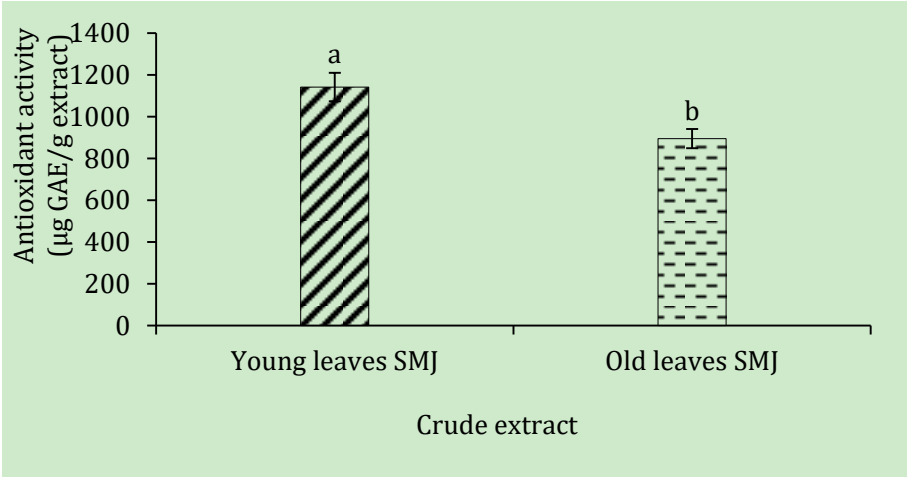

Fig. 2. The antioxidant activity of young and old leaves SMJ-CE. Data were expressed as mean ± standard deviation (n=3) determined by the DPPH radical scavenging assay. The different alphabet on error bar was significantly different (p<0.05) according to the Duncan's multiple range tests.

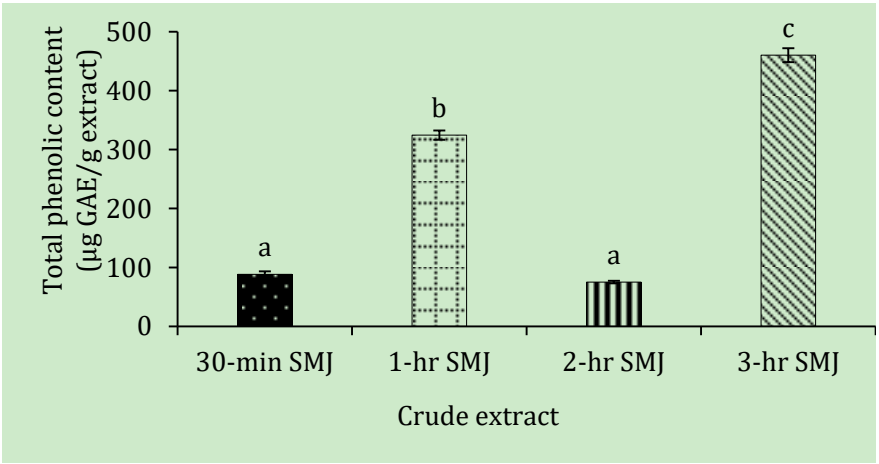

Fig. 3. The total phenolic content of SMJ-CE of different steamed time. Data were expressed as mean ± standard deviation (n=3) determined by the Folin-Ciocalteau method. The different alphabet on error bar was significantly different (p<0.05) according to the Duncan's multiple range tests.

3.2. Determination of Total Phenolic Content

The total phenolic content of SMJ-CE was examined by the Folin-Ciocalteau method with gallic acid as a positive control. 3-hour SMJ-CE presented the highest total phenolic content, 460.24±11.8 µg GAE/g extract. The total phenolic content of 1-hour SMJ-CE was significantly higher than 30-min and 2-hour SMJ-CE (p<0.05) with the values of 324.58±7.9, 88.36±4.9 and 75.17±2.1 µg GAE/g extract, respectively (Fig. 3). Moreover, the total phenolic content of young leaves SMJ-CE was significantly higher than old leaves SMJ-CE (p<0.05). There antioxidant activities were 600.57±10.2 and 559.1±1.9 µg GAE/g extract, respectively (Fig. 4).

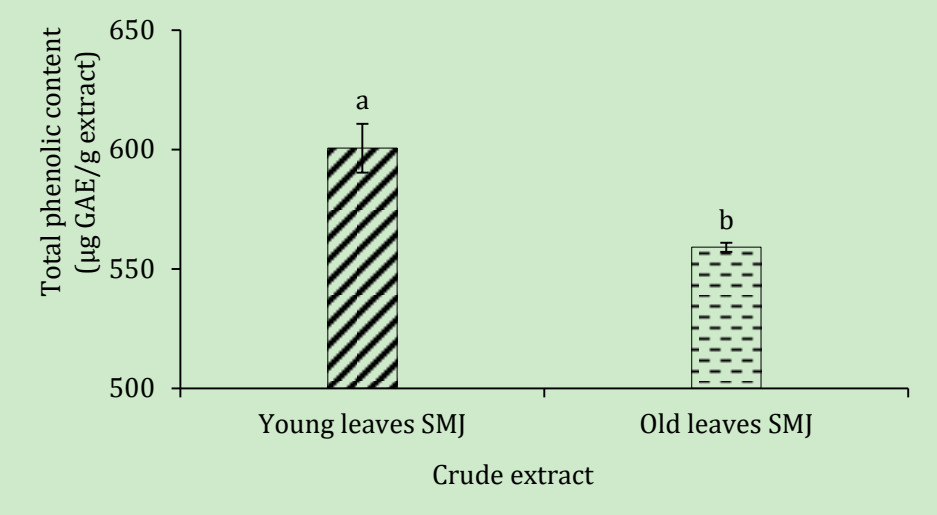

Fig. 4. The total phenolic content of young and old leaves SMJ-CE. Data were expressed as mean ± standard deviation (n=3) determined by the Folin-Ciocalteau method. The different alphabet on error bar was significantly different (p<0.05) according to the Duncan's multiple range tests.

3.3. Antibacterial Activity Evaluation

Using the agar disc diffusion method, 3-hour SMJ-CE could inhibit growth of *Shi. dysenteriae*, *S. aureus* and MRSA with the inhibitory clear zone of 9.9±0.5, 16.1±1.3 and 18.2±0.3 mm, respectively. However, 30-min, 1-hour and 2-hour SMJ-CE were not able to inhibit growth of any test pathogenic bacteria (Table 1). Furthermore, young leaves SMJ-CE could inhibit growth of all test pathogenic bacteria with the inhibitory clear zone ranging between 6.9±0.3-26±0.4 mm. Meanwhile, old leaves SMJ-CE could only inhibit growth of *B. cereus*, *Sal*. Typhi, *Shi. dysenteriae*, *S. aureus* and MRSA with the inhibitory clear zone of 7.4±0.5, 9.9±0.3, 11.2±0.6, 18.25±0.3 and 21.2±0.3 mm, respectively (Table 2).

Table 1. Antibacterial Activity of SMJ-CE of Different Steamed Time

Pathogenic bacteria	Inhibitory clear zone (mm)				
	30-min SMJ	1-hr SMJ	2-hr SMJ	3-hr SMJ	Gentamicin (0.1 mg/ml)
B. cereus	0	0	0	0	16.8±1.3
E. coli	0	0	0	0	13.0±1.2
E. coli O157:H7	0	0	0	0	13.2±0.5
Sal. Typhi	0	0	0	0	18.2±0.8
Shi. dysenteriae	0	0	0	9.9±0.5	12.8±0.8
S. aureus	0	0	0	16.1±1.3	15.0±0.3
MRSA	0	0	0	18.2±0.3	11.7±0.7*
V. cholerae	0	0	0	0	16.3±0.6

Data were expressed as mean ± standard deviation (n=3), *Gentamicin 50 mg/ml

Table 2. Antibacterial Activity of Young and Old Leaves SMJ-CE

Pathogenic bacteria	Inhibitory clear zone (mm)		
	Young leaves SMJ	Old leaves SMJ	Gentamicin (0.1 mg/ml)
B. cereus	10.9±0.25	7.4±0.5	16.8±1.3
E. coli	6.9±0.3	0	13.0±1.2
E. coli O157:H7	7.1±0.3	0	13.2±0.5
Sal. Typhi	11.9±0.6	9.9±0.3	18.2±0.8
Shi. dysenteriae	16.4±0.3	11.2±0.6	12.8±0.8
S. aureus	21.6±0.5	18.25±0.3	15.0±0.3
MRSA	26±0.4	21.2±0.3	11.7±0.7*
V. cholerae	7.0±0.0	0	16.3±0.6

Data were expressed as mean ± standard deviation (n=3), *Gentamicin 50 mg/ml

4. Discussion

Miang or tea contains polyphenols known as catechins. The catechins of green tea leaves are GC, EGC, ECG, C, EC, EGCG and GCG [9]. When antioxidant activity was evaluated by the DPPH radical scavenging assay, it was found that the 3-hour SMJ-CE had the highest antioxidant activity. It was anticipated that the hot steam was able to soften and damage Miang leaves to release some antioxidant compounds. Releasing of antioxidants in Miang leaves depends on steaming time as well as age of Miang leaves. The antioxidant activity of young leaves SMJ-CE was higher than old leaves SMJ-CE. Similarly, it had been found that between tea leaves of different ages, young leaves had total phenolic content and antioxidant activity higher than mature leaves [10]. However, the SMJ-CE had antioxidant activity lower than Miang leaves CE when compared with the previous study [11]. This is because the anti-oxidative compounds are remained to the large extent in Miang leaves, only the lesser is released during steaming.

1-hour SMJ-CE had higher total phenolic content than the 30-min SMJ-CE whereas its total phenolic content was lower than 2-hour SMJ-CE. It was estimated that Miang leaves were able to release some phenolic compounds at 2-hour steaming and it was degraded when spending more time. Tea polyphenols are susceptible to deterioration by heating temperature [12]. However, 3-hour SMJ-CE had the highest total phenolic content. Possibly, the longer time of steaming encouraged Miang leaf cell breakage to release more phenolic compounds. Additionally, young leaves SMJ-CE had higher amount of total phenolic content than old leaves SMJ-CE which was consistent with antioxidant activity presented.

3-hour SMJ-CE could inhibit growth of *Shi. dysenteriae*, *S. aureus* and MRSA. Moreover, young leaves SMJ-CE could inhibit growth of all test pathogenic bacteria whereas old leaves SMJ-CE could only inhibit growth of *B. cereus*, *Sal*. Typhi, *Shi. dysenteriae*, *S. aureus* and MRSA. Polyphenols have the antibacterial properties and catechins, which can inhibit bacterial growth by interact with bacterial lipid bilayer causing damage to cell membrane [13]. Furthermore, it has been reported that the ethanolic extract of Miang leaves have antibacterial activity against *B. cereus*, *Sal*. Typhi, *Shi*. *dysenteriae*, *S. aureus* and *V. cholerae* [11].

5. Conclusion

3-hour SMJ-CE had the highest antioxidant activity and total phenolic content. Meanwhile, the young leaves SMJ-CE had higher antioxidant activity and total phenolic content than old leaves SMJ-CE. Additionally, the SMJ-CE had antibacterial activities to inhibit growth of some gastrointestinal tract pathogenic bacteria. Consequently, the SMJ, which is considered as biological waste from Miang fermentation process and usually discarded, will be further studied its potential ability to be a raw material

for food, therapeutic and cosmetic applications.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

VC and NT designed the experiments and analyzed the data. VC prepared the steamed Miang juice extracts and evaluated their biological activities. VC and NT wrote and edited the manuscript.

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