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Biological Evaluation and Application of Fermented Miang (*Camellia sinensis* Var. *assamica* (J.W.Mast.) Kitam.) for Tea Production

Panee Sirisa-Ard[1,*], Nichakan Peerakam[2], Supadarat Sutheeponhwiroj[1], Tomoko Shimamura[3], Suwalee Kiatkarun[4]

[1]Faculty of pharmacy, Chiang Mai University, Chiang Mai Province, Thailand
[2]Faculty of Pharmaceutical Science, Burapha University, Chonburi Province, Thailand
[3]Faculty of Agriculture, Kochi University, Kochi, Japan
[4]Tea Gallery Group (Thailand) Co. Ltd., Chiang Mai Province, Thailand
*Corresponding author: pmpti008@gmail.com

Abstract Fermented Miang is a traditional masticatory snack of the northern people of Thailand. The aim of this study is to collect data concerning the length of fermentation and maturity of leaves (A01, A02, B01, B02, C) to determine total phenolic content and antioxidant activity. A01 and B02 were selected for drying (A01D and B02D) since it was higher in total phenolic content and antioxidant activity. The results showed that dried young fermented Miang (B02D) possessed higher GAE and TEAC values than A01D. It was developed as a tea product. Then Miang tea product was screened for pharmacological activities. It was found that dried Miang tea product showed a high level of antioxidant activity with TEAC = 216.0 mg/mg DW tea and showed the ability to scavenge O_2^- activity at IC_{50}= 0.038 mg/ml. Furthermore, it showed the capability to inhibit tyrosinase and hyaluronidase including anti-glycation activity.

Keywords: *Camellia sinensis (L.), Total phenolic content, Antioxidant, Tyrosinase, Hyaluronidase, Anti-glycation*

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1. Introduction

Miang is a local name of tea in Thailand. It is of fresh, dry or biological fermented tea leaves. The scientific name of this tea is Camellia sinensis var. assamica (J.W.Mast.) Kitam. Miang occurs in nature as a shrub 1-2 meters tall or a small tree up to 10 meters tall. Camellia sinensis var. assamica (J.W.Mast.) Kitam. are found worldwide in India, Sri Lanka, Pakistan, Indonesia, Vietnam, Russia, Kenya, Argentina and Thailand [1]. Miang appears in nature in green forest on sloped land and grows in groups 600 to 900 meters above sea level. Miang can also be cultivated in many districts of Chiang Mai, Chiang Rai and Lampang provinces. The famous and well-known masticatory snack, Miang-Om is biological fermented Miang (FM) used for masticatory purpose. Fermented Miang appears as a pack or a bundle of wet fermented leaves, produced both in household and as a mass production for business [2]. Currently, fermented Miang is mostly taken by elderly local people after meals or as a snack. The aim of this study is to determine how to manipulate the level of antioxidant in tea production depend on length of fermentation and maturity of the leaves. The tea product will be screened for pharmacological activities and effectiveness for health. The evaluation of fermented Miang for tea production will lead to conservation and sustainability of Miang and to the development of tea products.

2. Material and Methods

All samples were collected from the original of production, from the Pa-Miang village, Lampang Province, Thailand in May, 2014 and 2015, sample codes are showed in Table 1.

Table 1. The description and time of fermentation of samples

Sample codes	Description/ form of use	Years of fermentation
A01	Mature leaves/wet	2
A02	Young leaves/wet	2
B01	Mature leaves/wet	1
B02	Young leaves/wet	1
A01D	Mature leaves/dried	2
B02D	Young leaves/dried	1
C	Mature leaves/fresh	-

2.1. Sample Extraction

2.1.1 Extraction for selection to be developed as tea

All of the fermented Miang (A01, A02, B01 and B02) and dried fermented samples (A01D and B02D) were extracted with 70% ethanol and shaken for 6 hours. Then the extractions were allowed to keep at room temperature (27 ±2°C) for 18 hrs. The mixtures were filtered and evaporated under reduce pressure until crude extracts were obtained.

For fresh sample C, mature leaves of Miang were washed and drained. Then the leaves were dried by hot air oven at 50 °C and 70% ethanol was added into the sample and shaken for 6 hrs and allowed to rest for 18 hrs at room temperature. The extract was filtered and dried under reduce pressure by rotary evaporator.

2.1.2. Extraction for Investigation of the Active Ingredients and Biological Activities of Miang Tea

The extracted sample for assessment of the biological activities; 200 g of Miang tea was extracted with boiling water for 10 min and filtered by using a membrane filter (pore size: 0.45 µm) before use (10 mg D.W. eq/mL). For cathechins analysis; Miang tea was soaked with hot water (80°C) for 3 minutes, then cooled with ice and filtered before use.

2.2. Evaluation of Fresh and Fermented Miang for Potential Development as Tea Product

2.2.1. Determination of Total Phenolic Content

Total phenolic content of each sample was examined by Folin-Ciocalteu colorimetric method modification [3]. The stock sample solutions were prepared at 0.20-1 mg/ml by using 70% ethanol as the solvent. Then the sample solutions (250 µL) were mixed with 2.5 mL of the Folin-Ciocalteau reagent (The Folin-Ciocalteau reagent diluted with distilled water in the ratio of 1:10). The sodium carbonate solution (7.5% w/v) was added and incubated in the dark at room temperature. Finally, the absorbance of the test samples were measured at 765 nm by the SHIMADZU® UV-2450 spectrophotometer. The Gallic acid equivalent values (GAE mg/g sample) were calculated and compared with the standard curve of Gallic acid.

2.2.2. Determination of Antioxidant Activity in Fermented Miang

The DPPH radical scavenging assay was used for determination using the method described by Wu *et al*., 2005 [4]. The solution of DPPH radicals was prepared in 95% ethanol and the absorbance (0.8-.0.9 ±0.02) was measured at the wavelength 517 nm before use. The sample solution was diluted at 0.05-0.5 mg/mL of concentration. The triplicates of the test sample solution (20 µL) were transferred onto a 96-well micro titer plate. The DPPH radical solution was added onto each well except negative control (blank) and shaken. It was then left in the dark at room temperature for 30 minutes. The absorbance was measured at the 517 nm wavelength. The calculation of the percentage of inhibition was compared with the dilution curve of trolox standard (the formula is shown underlined). The result of antioxidant activity was interpreted in terms of Trolox equivalence antioxidant capacity value (mg/g sample).

$$\% \text{ Inhibition} = \left[\begin{matrix} (A_{test} - A_{blank}) \\ -(A_{s-test} - A_{s-blank}) / (A_{test} - A_{blank}) \end{matrix} \right] \text{x } 100.$$

Where A_{test} is the absorbance of only DPPH radical solution, A_{blank} is the absorbance of ethanol which replaces DPPH radical solution, $A_{s\text{-}test}$ is the absorbance of sample mixed with DPPH radical solution and $A_{s\text{-}blank}$ is the absorbance of sample mixed with ethanol.

2.3. Assessments of Bioactive Compounds and Biological Activities of Miang Tea

2.3.1. Analysis of Cathechins Quantities

Six chemical standards, Gallocatechin (GC), Epigallocatechin (EGC), Catechin (C), Epicatechin (EC), Epigallocatechin gallate (EGCG) and Epicatechin gallate (ECG), were used to compare the quantities of bioactive compounds in Miang tea (B02D). All of the standards were prepared with deionized water as follows: GC and C were prepared at range of concentrations of 0-2000 ppm, then EC, EGCG and ECG were diluted at 0-1000 ppm and also EGC was used at 0-12 ppm ranges, respectively.

The amount of cathechin and cathechin derivatives were analyzed by using high-performance liquid chromatography (HPLC, SHIMADZU Scientific Instrument, Japan) [5]. The systems were as follows, SCL-10A controller, GT-154 degasser, FCV-10AL Mixer, LC-10AD liquid chromatography pump, SPD-10A UV-VIS detector, CTO-10Avp column oven and CBM-10A communications BUS modules. The use column chromatography was C_{18} reversed-phase (4.6 x 250 mm, Water, Ireland). Mobile phases consisted of the mixture of an eluents A (86% v/v phosphoric acid (0.2% v/v) in 12% acetonitrile and 1.5% v/v tetrahydrofuran) and B (73.5% v/v phosphoric acid (0.2% v/v) in 25% acetonitrile and 1.5% v/v tetrahydrofuran). The linear gradient of elution was followed by 0-100% of mobile phase A (30 min) and gradually increased volume of mobile phase B from 0-100% (10 min, hold 20 min) with the flow rate 1 mL/min, then slowly decreased mobile phase B into an initial condition of 100% solvent A (10 min, hold 20 min) for next analysis. The wavelengths of the detector were set at 280 nm (detector wavelength 1) and 210 nm (detector wavelength 2) with column temperature at 25-30° C. The results were compared with peak area of standards (Gallocatechin (GC), Epigallocatechin (EGC), Catechin (C), Epicatechin (EC), Epigallocatechin gallate (EGCG) and Epicatechin gallate (ECG)).

2.3.2. Determination of Antioxidant Activity in Miang Tea

DPPH radical scavenger assay was used to evaluate Miang tea which followed the procedure of Shimamura *et al.*, 2014 [6]. The mixture of 200 µL sample solution and 800 µL of 0.1 M Tris-HCL buffer (pH 7.4) were

transferred into test tube, 1 mL of the DPPH solution was added and shaken by mixer for 10 s. The test sample solution was left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm by spectrophotometer. The mixture of 1.2 mL of ethanol and 800 µL of Tris-HCL buffer were used as blank. The percentage of inhibition was calculated and compared with trolox standard using the formula as before.

2.3.3. Determination of O_2^- Scavenging Activity

The estimation of O_2^- scavenging activity was examined using SOD Assay Kit-WST of Dojino Molecular Technologies [7]. The sample solution (20 µL) was added to a 96-well micro titer plate. Then, 200 µL WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfo-phenyl)-2H-tetrazolium, monosodium salt) working solution and 20 µL of buffer were mixed and incubated at 37°C for 20 minutes. Thereafter, the absorbance was monitored at the 450 nm wavelength by using micro plate reader. All of the volume used in the procedure of blanks (1-3) and test sample were shown in Table 2. The percentage of inhibition was calculated using the formula which is shown underlined, and the ability of anti O_2^- scavenging activity was reported in terms of IC_{50} (mg/mL).

Table 2. The volume of each solution used to evaluate O_2^- scavenging activity

List	Sample	Blank1	Blank2	Blank3
Sample solution	20 µL	-	20 µL	-
ddH_2O	-	20 µL	-	20 µL
WST working solution	200 µL	200 µL	200 µL	200 µL
Enzyme working solution	20 µL	20 µL	-	-
Dilution buffer	-	-	20 µL	20 µL

$$\% \text{ Inhibition} = \left[\begin{matrix} \left(A_{blank1} - A_{blank3}\right) \\ -\left(A_{sample} - A_{blank2}\right) / \left(A_{blank1} - A_{blank3}\right) \end{matrix} \right] \text{ x } 100.$$

Where A_{blank1} is the absorbance of the mixture of double distilled water (ddH_2O) with WST working solution and enzyme working solution, A_{blank3} is the solution of ddH_2O mixed with WST working solution and dilution buffer, A_{sample} is the solution of sample mixed with WST working solution and enzyme working solution, A_{blank2} is a similar mixture of A_{blank3} but instead used sample solution ddH_2O.

2.3.4. Determination of Tyrosinase Inhibitory Activity

The evaluation of anti tyrosinase activity was prepared using mushroom tyrosinase dissolved in 50 mM potassium phosphate buffer (pH 6.5) solution to give 71.5 units/mL, which was stored at -20°C until use. In a 96-well micro titer plate, 140 µL of 0.1 M phosphate buffer (pH 6.8), 4 µL of sample, and 60 µL of the tyrosinase solution were added, followed by 5 min of pre-incubation at 22°C on shaker. Then 60 µL of 5 mM L-DOPA was added to each well. The mixture was incubated for exactly 5 min at 22°C on a shaker and measured at the absorbance of 475 nm [8]. The inhibition rate was calculated using the following equation:

$$\text{Inhibition rate } (\%) = \left[1 - \frac{\left(A_{sample} - A_{sample-blank}\right)}{\left(A_{control} - A_{control-blank}\right)} \right] \text{ x } 100.$$

Where A_{sample} is the absorbance of sample with tyrosinase and $A_{sample\text{-}blank}$ is the absorbance of sample without tyrosinase, $A_{control}$ is the absorbance of control with tyrosinase and $A_{control\text{-}blank}$ is the absorbance of control without tyrosinase. The incubation temperature during the assay was set at 22° C, unless otherwise specified.

2.3.5. Determination of Hyaluronidase inhibitory Activity

Before the assay, 3200 units/mL of hyaluronidase stock solution was prepared with water and stored -20°C until use. Hyaluronidase solution was defrosted and diluted 1:2 with 0.1 M acetate buffer (pH 4.0) before use. The activating solution was prepared by adding 1 mg of compound 48/80 and 7.5 mg of calcium chloride dihydrate into 10 mL of 0.1 M acetate buffer (pH 4.0). Potassium borate solution was prepared by adding 2.24 g potassium hydroxide to 1 L of 0.8 M boric acid. *p*-Dimethylaminobenzaldehyde (DMAB) solution was prepared dissolving 100 mg of DMAB in 1 mL of the mixed solution containing 6 mL of 10 N HCl and 44 mL of acetic acid. DMAB solution was diluted 1:10 with acetic acid before use.

In a sample tube, 30 µL of sample and 15 µL of 1600 unit/mL hyaluronidase solution were added and incubated for 20 min at 37°C, followed by addition of 30 µL of the activating solution. The sample mixture was further incubated for 20 min at 37°C. After incubation, 75 µL of 0.32 mg/ml of potassium hyaluronate was added to start the reaction. The sample mixture was incubated for exactly 60 min at 37°C, and 30 µL of 0.4 N NaOH was added to stop the reaction. In accordance with Morgan-Elson method modification [9]. The sample mixture, to which 30 µL of the potassium borate solution was added, was heated at 100°C for exactly 3 min. The sample was immediately cooled in a cold water bath. In a 96-well micro plate, 84 µL of the sample mixture and 180 µL of DMAB solution were added and incubated at 37°C for exactly 20 min. The sample solution was measured for absorbance 585 nm. The following calculation was used to determine the inhibitory activity of the sample:

$$\text{Inhibition rate } (\%) = \left[\begin{matrix} \left(A_{control} - A_{control-blank}\right) \\ -\dfrac{\left(A_{sample} - A_{sample-blank}\right)}{\left(A_{control} - A_{control-blank}\right)} \end{matrix} \right] \text{ x } 100$$

Where A_{Sample} is the absorbance of sample with hyaluronidase and $A_{Sample\text{-}blank}$ is the absorbance of sample without hyaluronidase. $A_{Control}$ is the absorbance of control with hyaluronidase and $A_{Control\text{-}blank}$ is the absorbance of control without hyaluronidase.

2.3.6. Determination of Anti-glycation Activity

The anti-glycation activity was examined by using Collagen Glycation Assay Kit [10]. The collagen solution

(stored on ice <10°C before testing) was added to a 96-well black plate and incubated at 37° C for 18-24 hrs with high humidity to avoid the well drying up. The sample solution was filtered with a 0.22 µm filter and transferred to each well (10 µL). Then, 50 µL of sample dilution buffer and 200 mM glucose were added to the mixture. The intensity of the test samples were recorded by a fluorescent micro plate reader at 37°C with an excitation wavelength of 370 nm and an emission wavelength of 440 nm (0 week: A). After that, the test samples were incubated for 1, 2, 3 and 4 weeks at 37°C in high humidity conditions to avoid the wells drying up, the fluorescent intensity was measured after incubations (1, 2, 3 and 4 weeks: B). The inhibition effects of samples on glycation were calculated per the underlined equation, and reported in term of the percentage inhibition rate.

Inhibition effect of glycation

= Fluorescent intensity B – Fluorescent intensity A

Where fluorescent intensity A is the intensity of the test samples before incubation (at 0 week) and fluorescent intensity B is the intensity of the tests sample at 1, 2, 3 and 4 weeks after incubation

2.4. Statistical Analyses

Each experiment was performed in triplicate. The results were expressed as mean ± SD using Microsoft Excel software.

3. Result and Discussion

The results indicated that all of the samples showed quantities of phenolic compounds and anti-radicals capacities. It was clearly shown that the total phenolic content from the two year fermentation samples were higher than the one year fermentation samples. The GAE of the samples were 438.51 (A01), 428.31 (A02), 416.55 (B02) and 377.33 (B01) mg/g sample, respectively. Whereas the antioxidant activity of the young fermented Miang leaves B02 (TEAC=15,025.85 mg/g sample) exhibited the highest level of antioxidant capacity, next A01 (TEAC=14,430.32 mg/g sample) and B01 (TEAC=11,283.50 mg/g sample), respectively as the results shown in Table 3. The total phenolic content corresponds to the chemical compounds that contain the phenol group in the molecule which resulted in the potential antioxidant properties [4,11,12,13]. The phenolic compounds found in plants are important in biological systems. They can act as radical scavengers and have been used to prevent various diseases [14].

The A01 and B02 were selected for continual evaluation for development of dry fermented Miang tea (A01D and B02D). The result showed that dried young fermented Miang B02D presented higher total phenolic content (GAE=422.04 mg/g sample) and antioxidant activity (TEAC=18,315.21 mg/g sample) than A01D. It was selected to be as fermented Miang tea product for other investigations. Therefore, the results can be summarized that one year fermentation of young Miang leaves is suitable to use for the application of Miang tea. Nevertheless, in the region of northern Thailand, fermented Miang leaves are commonly eaten therefore, two years of fermentation might be an appropriate suggestion.

The biological investigation: Miang tea showed ability of inhibition in all assays. The potential of Miang tea when extracted similar to normal tea still revealed a capability of antioxidant activities. The results showed that Miang tea acts as a radical scavenger on O_2^-, which is danger free radical in the body, and it also showed capability of scavenging DPPH radicals. Miang tea showed a great capability to scavenge O_2^- which was the IC_{50}= 0.038 mg/mL and displayed the ability of anti DPPH radicals equivalent to trolox standard which was TEAC= 216.0 mg/mg D.W. tea. Moreover, this product exhibited anti-tyrosinase, anti-hyaluronidase and anti-glycation activities. The percentage of inhibitions showed activity of tyrosinase inhibitor (67.2%) which was similar to anti-glycation (69.2%) including exhibited hyaluronidase inhibitor (48.6%). All of the inhibitions were shown in Table 4. The activities of Miang tea may result from bioactive compounds such as phenolic and flavonoid groups that usually are found in various tea products. Well known polyphenols, especially catechin groups such as epigallocatechingallate, epigallocatechin, epicatechin-gallate, epicathechin and cathecin are the main compounds in teas which have shown the strongest anti-oxidant activity. They can generate α-tocopherol which protects the LDL fraction against oxidation [15,16,17,18]. Various investigations reported that tea polyphenol has shown chemoprotective effects in animal models (skin, lung, esophageal and gastrointestinal cancers) [19], anti-mutagenic, anti-carcinogenic, anti-proliferative and anti-neoplastic activities [20,21,22,23] and also shows several mechanisms to scavenge the reactive molecules, hydroxyl radical, and against peroxyl radicals in a liposomal and aqueous system [24,25].

Table 3. Total phenolic content and antioxidant activity of fresh and fermented Miang

Sample	GAE mg/g sample	TEAC Value (mg/g sample)
A01	438.51±0.018	14,430.32±0.022
A02	428.31±0.014	9,728.68±0.032
B01	377.33±0.023	11,283.50±0.012
B02	416.55±0.027	15,025.85±0.013
A01D	379.69±0.022	8,616.37±0.015
B02D	422.04±0.016	18,315.21±0.022
C	147.48±0.006	5,578.34±0.019

Table 4. The biological activities of fermented Miang tea (B02D)

Sample	Antioxidant activity		% Inhibition		
	DPPH (TEAC mg/mg D.W. tea)	O_2^- IC_{50} (mg/mL)	Tyro- sinase	Hyaluro- nidase	Anti-glycation
Miang tea	216.0	0.038	67.2	48.6	69.2

Table 5. Quantities of bioactive compounds in Miang tea (B02D)

Compounds	Active in gradients (mg/g dry sample)
Gallocatechin (GC)	9.65±0.34
Epigallocatechin (EGC)	0.84±0.05
Catechin (C)	16.13±1.66
Epicatechin (EC)	61.60±0.13
Epigallocatechin gallate (EGCG)	6.46±0.38
Epicatechin gallate (ECG)	1.93±0.15

The bioactive compounds analysis: Miang tea showed high quantity of epicathechin (EC) (61.60 mg/g dry sample) next catechin (C) (16.13 mg/g dry sample), then gallocatechin (GC) (9.65 mg/g dry sample) and epigallocathecin gallate (EGCG) (6.46 mg/g dry sample), while epicatechin gallate (ECG) was similar to amounts of epigallocatechin (EGC), as the results showed in Table 5. As catechins are the main chemicals compounds found in green tea, they are flavan derivatives which are members of the flavonoids group [26]. The level amount of catechins depend on many factors such as drying conditions, degree of fermentation, preparation of the infusion and decaffeination [27]. From this study the quantities of catechins may result from fermentation and proper temperature of extraction as an identical report of Khokhar and Magnusdottir (2002) reported that the highest quantity of catechins was extracted at a temperature of 77-80°C [28]. Other investigations have reported that catechins showed a high level of antioxidant activities, EC exhibited the strongest anti radical activity among catechins [29] while EGC showed a high capability of anti-oxidative activity as well [30]. All of the biological activities shown in Table 4 resulted from two major compounds, EC and C. The results proved that Miang tea showed beneficial activities for health.

4. Conclusion

Traditionally the people of the northern region of Thailand consume the fermented Miang leaves after meals. Besides providing caffeine stimulation there is a great indirect health care benefit. The results of this study provide the explanations that the fermented Miang leaves (one and two year fermentations) revealed a higher total phenolic content and antioxidant activity than fresh mature leaves. The fermented Miang leaves when developed to be Miang tea, still showed the properties of antioxidant, anti-tyrosinase, anti-hyaluronidase and anti-glycation activities which are an indirect beneficial health to skin in term of anti-aging activity. Moreover, this product contains large amounts of the bioactive compounds, which are EC, C, GC, EGCG, ECG and EGC. The consumption of Miang tea is good for consumers because Miang tea plays an important role as a natural anti-oxidative agent in the body. Moreover, it also shows the potential to be developed into other products such as cosmetics with the activities of whitening and anti-aging etc. It would be an excellent alternative for many health conscious people and could also add to the value of the product as well.

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