



Nutritional biotransformation in traditional fermented tea (Miang) from north Thailand and its impact on antioxidant and antimicrobial activities

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Abstract Miang is a traditional fermented tea made from fermentation of Assam tea leaves with mixed microbial culture involving lactic acid bacteria and yeast. Miang has important bioactive benefits such as antioxidant and antimicrobial activity with relevance to health benefits. Miang is categorized into two processes; filamentous fungi growth-based (FFP) and non-filamentous fungi-based (NFP) process, depending on area of production. Further, Miang is also divided into 2 types; astringent Miang and sour Miang, depending on fermentation time. The aim of this research was to determine the important macronutrient biotransformation of Miang diversity under above processes and types and explore the impact on bioactive compounds relevant to antioxidant and antimicrobial activities. During fermentation, pH, total acid, nutritional components, total polyphenols (TP), total tannins (TT), total flavonoids (TF), total catechins (TC), antioxidant activity and antimicrobial activity were evaluated. Miang when fermented for longer sour Miang process compared to shorter time astringent Miang increased crude protein, fiber, and ash contents whereas soluble carbohydrates decreased. Even though TP, TT, TF and TC of sour Miang was lower, the overall antioxidant activity was higher than

astringent Miang. This suggests that in addition to the phenolic compounds, other specific phenolics and substances such as biotransformed protein and fat could contribute to antioxidant properties. Additionally, Miang also contains antimicrobial activities against dental caries pathogenic bacteria *Streptococcus mutans*, gastrointestinal disease causing *Vibrio cholerae* and *Salmonella enterica* serovar Typhimurium through likely effects of organic acids and phenolic compounds.

Keywords Fermented tea · Miang · Nutritional analysis · Antioxidants · Antimicrobials

Introduction

Miang, a traditional fermented food process of north Thailand, is made from Assam tea leaves (*Camellia sinensis* var. *assamica*) through a unique fermentation process (Kawakami et al. 1987; Khanongnuch et al. 2017) and is usually consumed by eating or chewing after meals. It has been part of dietary cultural make-up and socio-economy of Lanna people in north Thailand since several hundred years ago and is commonly served at all ceremonial celebrations (Kanpiengjai et al. 2016). The fermentation processes are different depending on geographical region of the north Thailand. In addition, the diverse ethnicity of Miang producers results in high diversity and variety of Miang producing process among the regions. However, overall process can be categorized into two major processes; (1) filamentous fungi growth-based process (FFP) or two-step fermentation process and (2) non-filamentous fungi-based process (NFP) or single-step fermentation process (Khanongnuch et al. 2017). The generally consumed Miang products are categorized into 2

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main types known as astringent Miang (or *Miang Faad* in Thai) and sour Miang (or *Miang Som* in Thai). Miang fermentation is commonly processed without adding other nutritional substances and the fermentation process is carried out by microbial mixed culture mainly involving lactic acid bacteria and fungi (Kawakami et al. 1987). The nutritional components in steamed tea leaves such as carbohydrates, proteins and lipids are utilized through enzyme-linked catalytic biotransformation process of microorganisms for achieving the necessary metabolites for their growth and functional metabolic activities. These biotransformation processes by mixed culture causes the unique strong aromatic odor and taste of this indigenous fermented food.

Through biotransformation of macronutrients in Miang, some additional beneficial bioactive compounds such as phenolic metabolites are expected to be responsible for health relevant benefits beyond basic nutrition as is well-known in green tea and tea related products, which include catechins and derivatives, gallic acids and tannins (Huang et al. 2016; Kawakami et al. 1987). The functional properties and related health benefits of these bioactive compounds from tea and tea related products have been extensively investigated. Tea polyphenols for example have been suggested to provide protection against all stages of carcinogenesis and is linked to their antioxidant properties through quenching free radicals that otherwise stimulate carcinogenesis (Lee et al. 2013). These compounds are also known for their antimicrobial activity against a wide variety of pathogenic microorganisms and provide additional strategic use in the treatment of infectious diseases (Sanlier et al. 2018). Furthermore, tea polyphenols has been also associated with the reduction of the risk of cardiovascular and neurodegenerative diseases (Lorenzo and Munekata 2016) and diabetes mellitus (Nunes et al. 2015). However, scientific investigations describing the benefits of the health relevant bioactive compounds from fermented tea particularly the traditional fermented tea such as Miang are limited and therefore the focus of this study.

Some factors influence on Miang production process are; tea leaf quality, tea plantation area and most critical fermentation time and processes (Khanongnuch et al. 2017). Miang properties produced from different location are varied both in physical appearances and tastes due to variation in fermentation-based biotransformation of tea leaves. In order to better understand the diverse biotransformation variations linked to fermentation, it critically essential to understand the relevant nutritional biotransformation and associated enhancement of health relevant bioactive properties acquired in Miang products. Therefore this study focused on investigating the basic nutritional biotransformation of Miang from different regions and

processes and evaluated the associated bioactive enriched antioxidant and antimicrobial properties which will provide the foundation for advancing health benefits of Miang and Miang products in contemporary health needs and challenges.

Specifically in this study, the chemical properties, microbial population, nutritional components and the bioactive compounds of Miang products collected from different locations in north Thailand were investigated and properties were compared based on fermentation process and type. Furthermore, the important health relevant properties such as antioxidant and antimicrobial activities of Miang produced with different processes were determined.

Materials and methods

Sample collection

A total of 40 Miang samples, (20 of each astringent Miang and sour Miang) were collected from 20 Miang production areas of Western Lanna (Chiang Mai, Chiang Rai and Lampang) and Eastern Lanna (Phrae, Nan and Phayao) part of north Thailand and maintained in ice box during transportation prior to keeping at 4 °C until use. The raw material as fresh tea leaves of *Camellia sinensis* var. *assamica* to represent unfermented samples were also collected corresponding to area where Miang samples were collected.

Analysis of chemical properties

The pH of Miang products was measured by suspending 10 g of samples in 100 mL of sterile distilled water and mixed well by stomaching for 10 min by Masticator Homogenizer, (Basic/Panoramic, IUL micro, SA). The supernatant was separated by centrifugation at 8000 rpm at 4 °C. The pH value of the supernatant was measured by pH meter and the total acid was determined by NaOH titration method as described by AOAC (2012).

Enumeration of microorganism in Miang

A weight of 50 g of each sample was mixed with 200 mL of sterile 0.85% (w/v) NaCl solution using stomacher (Masticator Homogenizer, IUL, S.A.) for 10 min and 1 mL of each mixed sample was serially diluted. The diluted solutions (100 µL) were spread on nutrient agar (NA), De Man Rogosa and Sharpe (MRS) agar supplemented with 15 ppm bromocresol purple and Sabouraud dextrose agar (SDA) supplemented with 0.1 g/L chloramphenicol for enumeration of total viable bacteria, lactic

acid bacteria (LAB) and yeast, respectively (Jeong et al. 2013). The remaining diluted solutions were incubated in water bath at 80 °C for 12 min then the solutions were spread on NA for counting the viable cell number of endospore forming *Bacillus* sp. (Santana et al. 2008).

Proximate analysis

The moisture, ash, crude fat, crude protein, crude fiber and carbohydrates were determined according to the method of AOAC (2012). Briefly, moisture content was measured using 2 g of sample added in a bottle and dried at 50 °C for 24 h in a material test chamber M720 (Binder GmbH, Tuttlingen, Germany) until constant weight was maintained. After cooling down, the sample was placed in desiccator and weighed for calculation of moisture content. Crude lipid was determined following Soxhlet method as described by the manual of Soxhlet extractor (Model SOXTEC Avanti 2050, Foss, Sweden). The machine was run automatically using the program of 15 min boiling, 45 min rinsing, 10 min recovering and drying for 15 min. Petroleum ether was used for the extraction and then percentage of lipid was obtained. The total nitrogen was determined using combustion automated analyzer (Foss, Denmark). A nitrogen-to-protein conversion factor of 6.25 was used for calculation of protein present in the samples. Ash and crude fiber were analyzed using the method described by AOAC (2012). A 0.3 g of each sample was weighed into crucibles and 100 mL of hot 1.25% (v/v) sulfuric solution was added and the mixture was boiled under reflux for 30 min. The hot solutions were then quickly filtered under suction. The residues were thoroughly washed with hot water until acid free. A volume of 100 mL of hot 1.25% (w/v) sodium hydroxide solution was added and boiled under reflux for 30 min. Each insoluble residue was washed with hot water until it was base free. The crucibles were dried with samples in hot air oven at 105 °C for 2 h or until a constant weight was obtained and placed in a crucible furnace at 550 °C for 2 h and left to cool in desiccator and then weighed. Percentage crude fiber and nitrogen free extract (NFE) were calculated using the following equation:

$$\begin{aligned} \text{Nitrogen free extract (\%)} \\ &= 100 - (\% \text{ of crude fiber} + \text{crude fat} \\ &\quad + \text{crude protein} + \text{ash}) \end{aligned}$$

Extraction of bioactive compounds

Miang sample was dried in a vacuum drier at 50 °C for 24 h and homogenized using blender. A weight of 5 g of the homogenized samples was extracted with 100 mL of

80% (v/v) acetone with 250 rpm on incubator shaker at 30 °C for 1 h as described by Drużyńska et al. (2007). The extracted solution was filtered through Whatman filter paper (No.1) and then the solvent was completely removed by rotary evaporator at 40 °C for 20 min. Dried extract samples were redissolved with 20 mL of 80% (v/v) acetone and the solution obtained was quantified for total phenolic (TP), total flavonoid (TF), total tannin (TT) by the methods described below. The antioxidant activity based on DPPH free radical scavenging activity was also determined.

Total polyphenol content

The total polyphenol content (TP) was determined by the Folin-Ciocalteu assay method as described by Eom et al. (2008). Briefly, 200 µL of sample was added to a test tube containing 200 µL of folic reagent (2 M). The volume was increased by adding 1.8 mL of deionized water. The solution was allowed to stand for 3 min for reaction after vortex. Then, 400 µL of 10% (w/v) sodium carbonate was added and mixed by vortex. The final volume was adjusted to 4 mL by deionized water. Deionized water was used in the control blank. All samples were allowed to stand for 1 h at room temperature and the absorbance was measured at 725 nm.

Total flavonoids content

The total flavonoid content (TF) was determined according to the aluminum chloride colorimetric method. Briefly, 500 µL of sample was mixed with 100 µL of 10% (w/v) aluminum nitrate and 100 µL of 1 M potassium acetate. Then, 3.3 mL of 80% (v/v) methanol was added to adjust the total volume to 4 mL. The mixture was mixed and incubated for 40 min after which the absorbance was measured at 415 nm. Deionized water was used as blank. Quercetin was used as a standard and the value of TF content was represented as mg of quercetin equivalent (QE) per gram of sample (Eom et al. 2008).

Total tannins

The total tannins content (TT) was determined by Folin-Denis method. Briefly, 1 mL of sample was made up to 7.5 mL with deionized water. Then, 0.5 mL of Folin-Denis reagent (2 M) and 1 mL of 10% (w/v) sodium carbonate solution were added. The volume was made up to 10 mL with deionized water. The absorbance was measured at 700 nm. The TT content was represented as mg of tannic acid equivalent (TAE) per gram of sample (Padma et al. 2013).

DPPH free radical scavenging activity

The antioxidant activity of the samples was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described by Braca et al. (2003) with slight modifications. Briefly, 1 mL of each of the extracts at different concentrations (0.5, 1.0, 2.5 and 5 mg/mL) was added to 4 mL (0.15 mM DPPH solution) of DPPH. The mixtures were shaken vigorously and left to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm and the percent inhibition activities of the extracts were calculated against a blank using the following condition.

$$\text{Inhibition (\%)} = (1 - B/A) \times 100,$$

where A is the absorbance of the mixture without extract and B is the absorbance of the mixture containing the extract of the samples.

Preparation of Miang water-extracted (MWE) solution for antimicrobial activity

A 50 g wet weight of Miang samples collected from different locations was determined for moisture content. Each sample of 10 g dried weight calculated based on moisture content was added into bottle and volume was adjusted to 200 mL by sterile water, then it was mixed vigorously for 5 min. The tea leaves were removed by filtration through filter paper Whatman No.1 and the filtrate was centrifuged at 12,000 rpm, 4 °C for 5 min. The supernatants were sterilized by filtering through 0.2 µm sterile microfilter (Whatman™, GE Healthcare UK, Ltd.).

Three samples of Miang water-extracted solutions were randomly selected from astringent Miang and sour Miang and were investigated for their antimicrobial activity against pathogenic bacteria; *Streptococcus mutans*, *Vibrio cholerae*, *Salmonella enterica* serovar Typhimurium TISTR 292 and *E. coli* ATCC 22595 by disc-agar diffusion method (Fehlberg et al. 2016). Each strain was cultured in their selective media for 24 h and was diluted to reach OD₆₀₀ of about 0.1. Each strain was then spread on the plate with 100 µL. Then, paper discs of diameter 6 mm (Macherey–Nagel, GmbH&Co. KG, Germany) were placed on each plate. After that, 50 µL of Miang water-extracted solutions were placed into paper discs. The agar plates were incubated at 37 °C and the diameter of the inhibition zones were measured after 18–24 h.

Results and discussion

pH and total acid

The pH value of Miang water-extracted (MWE) solution obtained from Miang collected from various locations in north Thailand are presented in Fig. 1a. The average pH values of astringent Miang and sour Miang produced by non-filamentous fungi growth-based process (NFP) were 5.1 and 4.2, respectively while those of astringent Miang and sour Miang produced by filamentous fungi growth-based process (FFP) were 5.2 and 4.0. The total acid content of Miang is presented in Fig. 1b. The total acid values of astringent Miang and sour Miang produced by NFP process was in the range of 0.02–0.06 and 0.05–0.19 with the average of 0.05 and 0.11 g/g Miang, respectively while those of astringent Miang and sour Miang produced by FFP process was in the range of 0.02–0.09 and 0.05–0.23 and average of 0.06 and 0.13 g/g Miang. The pH values corresponded to total acid and those of astringent Miang and sour Miang were significantly different in both Miang types produced by NFP and FFP processes. The lower pH value of sour Miang indicated the direct effect from the higher total acid content caused by the prolonged fermentation time. It is evident that some microorganisms converted carbohydrates to organic acids such as lactic acid and acetic acid during their growth (Yadav et al. 2012). It is likely that acid-producing microorganisms in sour Miang could produce more acid than those in astringent Miang.

Microbial population

The microbial population of Miang samples collected from different locations and production processes is presented in Fig. 1c. Even though the raw materials and the fermentation process used in Miang production by NFP and FFP were different, but the average numbers of microbial population detected from Miang samples were not significantly different. The number of total bacterial count was slightly higher but insignificant ($p > 0.05$) when compared to those of lactic acid bacteria and yeast. Furthermore, astringent Miang produced by both NFP and FFP processes showed higher numbers when compared to sour Miang. This means that prolonged fermentation period seems to respond by the slightly decrease in microbial population in all types of Miang samples. However, the number of fungal colony determined from this study was found in a very low number with high fluctuations and in more than half of samples, mold colony were not detected, therefore, it was not presented. It is quite interesting to find number of endospore forming bacteria from Miang in all samples collected with the average number ranging from 40 to 45%

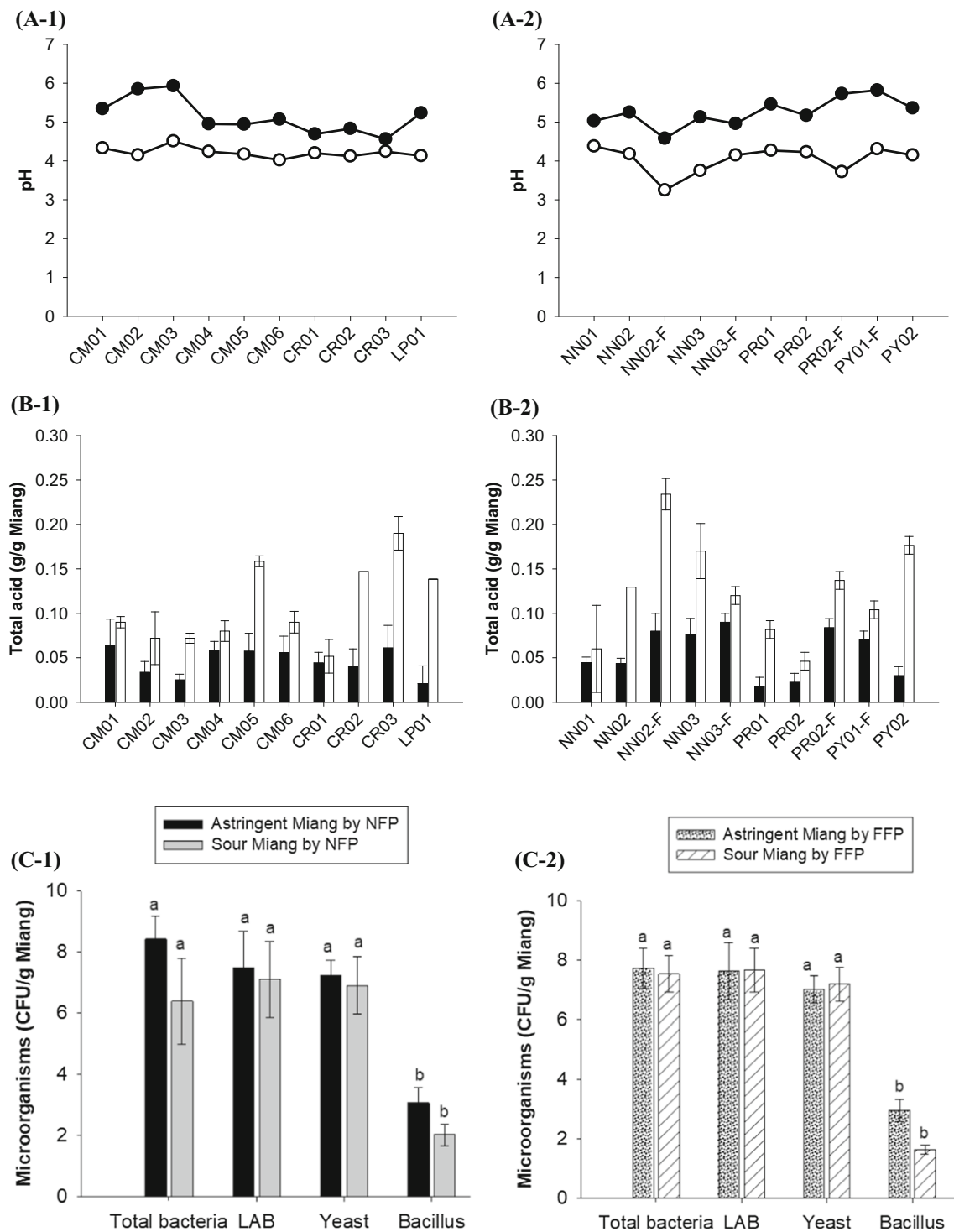


Fig. 1 pH (a), total acid (b), and viable microbial cell (c) of Miang samples; non-filamentous fungi Miang (a-1, b-1, c-1), filamentous fungi Miang (a-2, b-2, c-2), astringent Miang (closed symbol: filled circle, filled square), sour Miang (opened symbol: open circle, open square)

of total bacterial counts in Miang produced by NFP and 35–40% of total bacterial counts in FFP Miang (Fig. 1c), which is a significant high number when compared to the total bacterial counts. It is very likely that this group of bacteria may play some important and specific roles in Miang fermentation.

Nutritional components analysis by proximate analysis

The results in details of proximate analysis of 40 Miang samples collected from 20 producing locations are presented in Table 1. A total of 10 non-filamentous fungi

Table 1 Nutritional components of Miang samples collected from various locations in north Thailand

Sample	Ash		Fiber		NFE	
	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang
<i>Non-filamentous fungi used process (NFP)</i>						
CM01	5.84 ± 0.07 ^{efg}	9.46 ± 0.32 ^{fg}	19.67 ± 0.27 ^{ghi}	22.41 ± 0.43 ^{defg}	56.37 ± 0.17 ^h	43.82 ± 0.14 ^g
CM02	7.43 ± 0.12 ^c	9.87 ± 0.02 ^{fg}	22.05 ± 0.16 ^{bcd}	23.43 ± 0.31 ^{cdef}	34.87 ± 0.08 ^f	32.69 ± 0.12 ⁱ
CM03	4.68 ± 0.19 ^{hij}	5.17 ± 0.01 ^{no}	17.37 ± 0.08 ^k	23.99 ± 0.34 ^{cde}	58.45 ± 0.11 ^e	50.19 ± 0.31 ^d
CM04	4.61 ± 0.12 ^{hijk}	11.62 ± 0.88 ^e	15.28 ± 0.01 ^l	19.19 ± 0.05 ⁱ	60.31 ± 0.45 ^c	41.21 ± 0.32 ^h
CM05	5.45 ± 0.79 ^{fgh}	6.19 ± 0.79 ^{klmn}	19.47 ± 0.84 ^{hi}	20.93 ± 0.27 ^{ghi}	57.68 ± 0.16 ^f	50.23 ± 0.34 ^d
CM06	4.53 ± 0.21 ^{ijk}	6.04 ± 0.31 ^{mno}	18.23 ± 0.79 ^{jk}	19.77 ± 0.49 ^{hi}	62.31 ± 0.02 ^a	57.13 ± 0.28 ^a
CR01	6.35 ± 0.06 ^{de}	7.14 ± 0.30 ^{ijkl}	20.92 ± 0.48 ^{def}	21.22 ± 0.48 ^{fghi}	54.54 ± 0.26 ⁱ	47.22 ± 0.16 ^e
CR02	3.78 ± 0.12 ^k	6.11 ± 0.05 ^{lmn}	24.16 ± 0.21 ^a	26.39 ± 0.62 ^{ab}	43.46 ± 0.08 ^o	38.46 ± 0.54 ^j
CR03	5.99 ± 0.03 ^{efg}	6.29 ± 0.11 ^{klm}	19.52 ± 0.73 ^{ghi}	21.18 ± 1.27 ^{fghi}	42.82 ± 0.22 ^p	32.67 ± 0.13 ⁱ
LP01	4.50 ± 0.23 ^{ijk}	5.00 ± 0.63 ^o	17.35 ± 0.29 ^k	24.75 ± 1.54 ^{bcd}	57.06 ± 0.16 ^g	51.35 ± 0.05 ^c
Average	5.32 ± 1.04	7.29 ± 2.12	19.40 ± 2.42	22.33 ± 2.17	52.79 ± 8.63	44.50 ± 7.79
<i>Filamentous fungi used process (FFP)</i>						
NN01	5.36 ± 0.25 ^{ghi}	19.46 ± 0.77 ^f	19.46 ± 0.77 ^{hi}	10.14 ± 0.19 ^{bcd}	45.12 ± 0.12 ^m	57.77 ± 0.25 ^a
NN02	6.98 ± 0.15 ^{cd}	19.45 ± 0.26 ^c	19.45 ± 0.26 ^{hi}	14.16 ± 0.30 ^{cdefg}	59.32 ± 0.01 ^d	40.11 ± 0.18 ⁱ
NN02-F	8.90 ± 0.01 ^{ab}	21.29 ± 0.38 ^a	21.29 ± 0.38 ^{cde}	19.61 ± 0.04 ^{cdefg}	43.72 ± 0.02 ^o	35.7 ± 0.04 ^k
NN03	9.51 ± 0.04 ^a	20.32 ± 0.23 ^b	20.32 ± 0.23 ^{efgh}	16.51 ± 0.03 ^{cde}	45.13 ± 0.22 ^m	37.69 ± 0.75 ^j
NN03-F	8.52 ± 0.06 ^b	19.98 ± 0.08 ^d	19.98 ± 0.08 ^{fghi}	12.85 ± 0.12 ^{efgh}	47.43 ± 0.10 ^l	35.31 ± 0.27 ^k
PR01	5.65 ± 0.10 ^{efg}	17.64 ± 0.14 ^{ijk}	17.64 ± 0.14 ^k	7.24 ± 0.07 ^{cde}	56.09 ± 0.09 ^h	50.88 ± 0.34 ^{cd}
PR02	6.35 ± 0.02 ^{de}	23.02 ± 0.02 ^{gh}	23.02 ± 0.02 ^{ab}	9.02 ± 0.03 ^{bc}	44.52 ± 0.08 ⁿ	43.72 ± 0.28 ^g
PR02-F	4.12 ± 0.56 ^{jk}	20.73 ± 0.03 ^{ij}	20.73 ± 0.03 ^{efg}	7.27 ± 0.02 ^{cdef}	50.35 ± 0.21 ^k	46.03 ± 0.14 ^f
PY01-F	7.42 ± 0.71 ^c	23.19 ± 0.14 ^f	23.19 ± 0.14 ^{ab}	10.48 ± 0.01 ^a	43.33 ± 0.04 ^{op}	39.96 ± 0.43 ⁱ
PY02	4.05 ± 0.14 ^{jk}	19.02 ± 0.09 ^{mno}	19.02 ± 0.09 ^{ij}	6.03 ± 0.07 ^{ghi}	61.07 ± 0.06 ^b	52.84 ± 0.29 ^b
Average	6.69 ± 1.83	20.41 ± 1.64	20.41 ± 1.64	11.33 ± 4.18	49.61 ± 6.43	44.00 ± 7.31
Sample	Protein		Fat		Moisture	
	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang
<i>Non-filamentous fungi used process (NFP)</i>						
CM01	45.12 ± 0.12 ^{hi}	57.77 ± 0.25 ^f	1.81 ± 0.13 ^{ijk}	3.00 ± 0.19 ^{fghij}	69.07 ± 1.8	80.82 ± 0.33
CM02	59.32 ± 0.01 ^a	40.11 ± 0.18 ^a	3.01 ± 0.16 ^{cde}	4.54 ± 0.23 ^a	78.32 ± 1.20	84.53 ± 0.64
CM03	43.72 ± 0.02 ^{fg}	35.7 ± 0.04 ^{gh}	1.60 ± 0.06 ^{kl}	2.63 ± 0.21 ^{ij}	57.51 ± 0.22	61.53 ± 1.00
CM04	45.13 ± 0.22 ^f	37.69 ± 0.75 ^d	1.59 ± 0.21 ^{kl}	3.94 ± 0.07 ^{abcde}	75.21 ± 1.20	82.14 ± 1.23
CM05	47.43 ± 0.10 ^j	35.31 ± 0.27 ^f	1.54 ± 0.12 ^{kl}	2.34 ± 0.12 ^{jk}	77.36 ± 0.13	84.89 ± 0.53
CM06	56.09 ± 0.09 ^k	50.88 ± 0.34 ^k	1.12 ± 0.09 ^l	1.64 ± 0.10 ^k	76.16 ± 0.11	82.16 ± 1.02
CR01	44.52 ± 0.08 ^{ghi}	43.72 ± 0.28 ^{ef}	1.70 ± 0.22 ^{jk}	2.68 ± 0.05 ^{hij}	78.92 ± 0.52	87.33 ± 1.63
CR02	50.35 ± 0.21 ^d	46.03 ± 0.14 ^c	2.41 ± 0.03 ^{fgh}	3.32 ± 0.20 ^{defghi}	78.36 ± 0.96	88.24 ± 0.21
CR03	43.33 ± 0.04 ^c	39.96 ± 0.43 ^b	2.19 ± 0.26 ^{ghi}	3.48 ± 0.02 ^{cdefgh}	75.99 ± 0.48	81.39 ± 0.67
LP01	61.07 ± 0.06 ^{gh}	52.84 ± 0.29 ^g	1.60 ± 0.23 ^{kl}	2.74 ± 0.03 ^{ghij}	83.36 ± 1.16	86.75 ± 1.05
Average	19.70 ± 6.03	24.29 ± 7.43	1.86 ± 0.51	3.03 ± 0.79	75.03 ± 6.76	81.98 ± 7.25
<i>Filamentous fungi used process (FFP)</i>						
NN01	16.3 ± 0.08 ^{hij}	21.31 ± 0.05 ^{hi}	1.32 ± 0.12 ^{kl}	3.17 ± 0.13 ^{efghij}	70.45 ± 0.14	76.48 ± 0.87
NN02	32.64 ± 0.27 ^l	39.46 ± 0.13 ^g	3.47 ± 0.19 ^{abc}	3.91 ± 0.24 ^{abcde}	67.95 ± 0.12	73.83 ± 0.57
NN02-F	17.41 ± 0.65 ^f	18.50 ± 0.65 ^e	3.53 ± 0.16 ^{ab}	3.96 ± 0.35 ^{abcde}	78.08 ± 1.71	79.77 ± 0.22
NN03	18.21 ± 0.08 ^k	24.03 ± 0.08 ^{gh}	2.54 ± 0.21 ^{efg}	3.33 ± 0.23 ^{defghij}	78.24 ± 0.73	81.59 ± 0.64
NN03-F	15.12 ± 0.10 ^l	21.05 ± 0.06 ^l	3.41 ± 0.13 ^{abc}	4.05 ± 0.21 ^{abcd}	78.52 ± 0.83	81.66 ± 0.17
PR01	13.21 ± 0.20 ^{hij}	15.42 ± 0.33 ^{ij}	2.64 ± 0.02 ^{defg}	3.14 ± 0.22 ^{efghij}	73.64 ± 0.86	74.94 ± 0.95
PR02	16.48 ± 0.38 ^e	21.74 ± 0.11 ^f	3.31 ± 0.13 ^{abc}	3.58 ± 0.18 ^{bcd}	69.05 ± 0.92	79.63 ± 1.36

Table 1 continued

Sample	Protein		Fat		Moisture	
	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang
PR02-F	21.76 ± 0.21 ^k	25.71 ± 0.47 ^j	3.73 ± 0.24 ^a	4.28 ± 0.10 ^{abc}	78.13 ± 0.34	82.39 ± 1.43
PY01-F	29.17 ± 0.45 ^{hij}	36.68 ± 0.03 ⁱ	2.78 ± 0.16 ^{def}	4.08 ± 0.11 ^{abcd}	74.78 ± 0.74	85.03 ± 0.10
PY02	16.67 ± 0.11 ^{ij}	18.99 ± 0.53 ^{jk}	3.09 ± 0.08 ^{bcd}	4.34 ± 0.16 ^{ab}	75.66 ± 0.70	80.74 ± 0.81
Average	14.80 ± 3.07	18.30 ± 2.45	2.98 ± 0.67	3.78 ± 0.42	74.45 ± 3.84	79.61 ± 3.34

process (NFP) were from Western Lanna including Chiang Mai (6 samples) Chiang Rai (3 samples) and 1 sample from Lampang while 10 Miang samples produced by filamentous fungi process (FFP) were collected from Eastern Lanna including Nan (5 samples), Phrae (3 samples) and Phayao (2 samples). To understand the transformational change during Miang fermentation, the nutritional components of young and mature fresh tea leaves Western Lanna and Eastern Lanna areas (as the representatives of NFP and FFP, respectively) were also analyzed in comparison between young and mature leaves. There is no significant difference between the young and mature tea leaves except the fat content which was found in higher quantity in mature tea leaves (Fig. 2b-1 and b-2). The

overall changes of nutritional components after short fermentation (astringent Miang) and longer fermenting period (sour Miang) are also presented in Fig. 2b and Table 1. Ash content of Miang produced by FFP was found to be higher responding to the longer fermentation time (sour Miang > astringent Miang and mature fresh leaves) while those values of Miang produced by NFP were similar for both Miang types. This can be simply explained by the extent of maturity of tea leaves where the mature leaves contain more structural components such as lignin or cellulose complex that potentially leads to the higher ash content and therefore higher ash content in sour Miang was observed in comparison to astringent Miang. The increased ash content may be the result from improved substrate

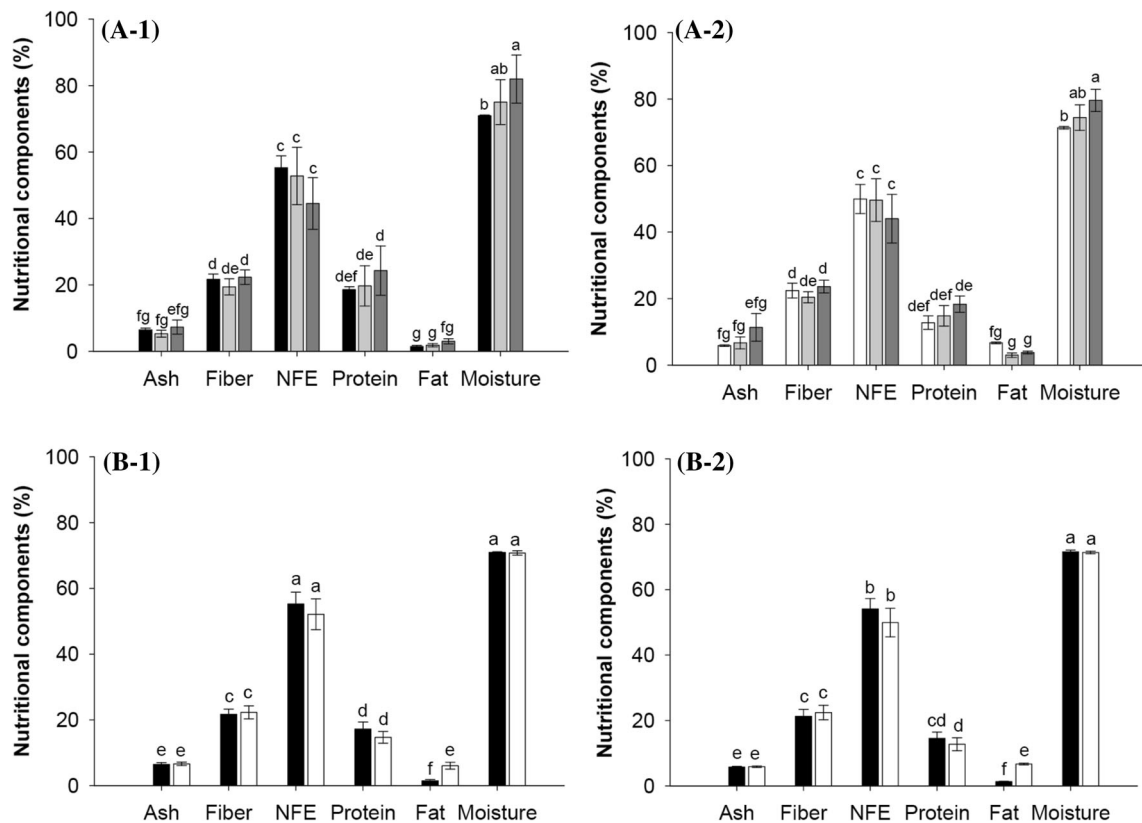


Fig. 2 Nutritional components of Miang and fresh tea leaves; Miang of western area; NFP (a-1), Miang of eastern area, FFP (a-2), fresh tea leaves of western area; (b-1), fresh tea leaves of eastern area (b-2),

raw material; Young tea leaves, (filled square), Mature tea leaves (open square), astringent Miang (light shaded square), sour Miang (thick shaded square)

compositions which is effectively changed from microbial process (Ogodo et al. 2017). On the other hand, the decrease in the soluble carbohydrate of sour Miang produced by both NFP and FFP process was noticeably observed, however the statistical analysis indicates all values are in the same group ($p > 0.05$). The soluble carbohydrate is easier to metabolize by fermenting microorganisms to give the simple sugars which are important substrates as energy source for microbial growth (Kaboré et al. 2011). The simple sugars were likely converted from complex carbohydrate (cellulosic material of tea leaf) by microbial cellulolytic enzyme in fermented tea (Murugesan et al. 2002) and leads to increase of the available nutrients for the fermentation process (Nnam and Obiakor 2003).

The average protein content of Miang from different fermentation process was also slightly different (Table 1). Crude protein of NFP Miang (astringent Miang; 19.70 ± 6.03 , sour Miang; 24.29 ± 7.43) was noticeably increased when compared to the fresh tea leaves before fermentation and was higher than those values of FFP Miang (astringent Miang; 14.80 ± 3.10 , sour Miang; 18.30 ± 2.50). However, the protein content of all Miang samples was statistically indicated to be the non-different group (Fig. 2b-1 and b-2). The slightly higher crude protein of Miang produced by NFP may be due to the different in raw material used as NFP Miang was made from young tea leaves that its protein content was higher than the mature tea leaves which is normally used for FFP Miang production. This was in agreement with the report in poplar (*Populus alba* × *Populus tremula*) leaves by Barbehenn et al. (2015), where protein content of young leaves was higher than mature leaves. In addition, we also found an increase of crude protein content during fermentation in sour Miang compared to astringent Miang both in NFP and FFP processes, this is probably due to the increase in microbial population which leads to the increase of both microbial biomass and their extracellular enzymes (Çabuk et al. 2018).

In case of fat content, even though the data from Table 1 showed that the average fat value of FFP Miang (astringent Miang; 2.98 ± 0.70 , sour Miang; 3.78 ± 0.40) were slightly higher than the value of NFP Miang (astringent Miang; 1.86 ± 0.51 , sour Miang; 3.03 ± 0.79) but these was not significantly different ($p > 0.05$) (Fig. 2a). Mature tea leaves used for FFP Miang production contains higher fat than the young tea leaves, and fat degradation products in tea leaves are believed to be responsible for generating flavor and aroma compounds (Liu et al. 2017). Long-time fermented sour Miang showed higher fat accumulation than short-time fermented astringent Miang. This means the longer fermentation process resulted in fat accumulation of Miang product. However, an overall decrease in the fat content of raw material of FFP Miang was observed.

During fermentation, fat breakdown compounds such as fatty acid and glycerol are likely generated by lipase activity of microorganisms particularly yeast (Odufa 1983).

Bioactive compounds and antioxidant activity

The quantitative analysis of bioactive compounds in Miang samples was analyzed and the results were presented in Table 2. The difference of TP content between Miang produced by both NFP and FFP was observed. Total polyphenols (TP) content of astringent Miang-NFP and astringent Miang-FFP were found in the range of 59–205 and 69–201 mg/g dw with the average value of 117 and 120 mg/g dw, respectively while those of sour Miang-NFP and sour Miang-FFP were found only in range of 41–155 and 48–109 mg/g dw and average 77 and 82 mg/g dw, respectively. However, the longer fermentation time in sour Miang in both NFP and FFP fermentation processes significantly affected the decrease in TP. This suggested that microorganisms in Miang fermentation process metabolized or transformed the phenolic compounds leading to the changes of metabolic structure of tea leaves that could not be detected by the methods used in this experiment.

In similar pattern to TP, total tannins (TT) of NFP and FFP astringent Miang was average of 142 and 127 mg/g dw, respectively, while those of NFP and FFP sour Miang was 113 and 99 mg/g dw, respectively. Like the TP content, TT of NFP and FFP Miang was not significantly different, but the prolonged fermentation time significantly resulted in the decrease of total tannins in both fermentation types.

Total flavonoids (TF) content of NFP and FFP astringent Miang was in the range of 0.58–6.69 and 0.53–1.17 mg/g dw and average of 1.53 and 0.91 mg/g dw, respectively while TF content of NFP and FFP sour Miang was in the range of 0.23–2.29 and 0.27–0.56 mg/g dw and average of 0.62 and 0.38 mg/g dw, respectively. TF content of NFP and FFP Miang was not significantly different. It was observed that when Miang had prolonged fermentation time as in sour Miang compared to astringent Miang, TP content of Miang decreased from 1.53 and 0.91 mg/g dw to 0.62 and 0.38 mg/g dw, respectively. Total catechin (TC) content of NFP and FFP astringent Miang was 45 and 50 mg/g dw, respectively while those of NFP and FFP sour Miang was 32 and 23 mg/g dw, respectively. It is clear that prolonged fermentation time of sour Miang compared to astringent Miang clearly showed a decrease of TC content, except CM06 sample.

The antioxidant activity was presented in terms of IC_{50} value, which reflects the quantity of dry Miang in $\mu\text{g/mL}$ required for 50% inhibition. A large fluctuation in the

Table 2 Bioactive compounds of Miang samples collected from various locations in north Thailand

Sample	Total polyphenols			Total flavonoids			Total tannins			Total catechins		
	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang	Astringent Miang	Sour Miang
<i>Non-filamentous fungi used process (NFP)</i>												
CM01	96.47 ± 0.42 ^j	86.30 ± 0.91 ^f	6.69 ± 0.22 ^a	2.29 ± 0.19 ^a	125.38 ± 0.21 ^{jk}	110.19 ± 0.32 ^g	50.54 ± 3.00 ^{cd}	20.57 ± 1.81 ^{ghi}				
CM02	86.30 ± 0.91 ^m	53.35 ± 0.84 ^l	1.04 ± 0.13 ^{bcd}	0.68 ± 0.05 ^b	96.24 ± 1.56 ^p	86.63 ± 1.52 ^j	67.65 ± 4.54 ^b	52.63 ± 3.01 ^c				
CM03	68.71 ± 0.45 ⁿ	41.18 ± 1.32 ⁿ	0.74 ± 0.04 ^{cde}	0.29 ± 0.02 ^{ef}	130.32 ± 1.06 ^{hi}	102.38 ± 1.67 ^h	56.97 ± 2.63 ^{bc}	13.27 ± 1.60 ^{ijk}				
CM04	59.51 ± 1.11 ^p	52.60 ± 0.86 ^l	1.39 ± 0.07 ^{bc}	0.60 ± 0.05 ^{bcd}	129.03 ± 1.88 ^{ij}	78.83 ± 0.83 ^k	36.69 ± 3.94 ^{fghi}	19.04 ± 1.59 ^{ghi}				
CM05	181.25 ± 0.67 ^d	86.99 ± 1.4 ^f	0.66 ± 0.15 ^{de}	0.23 ± 0.02 ^f	185.57 ± 1.72 ^b	149.73 ± 1.96 ^{cb}	40.93 ± 2.34 ^{defgh}	14.95 ± 1.54 ^{bijk}				
CM06	63.63 ± 0.62 ^o	57.21 ± 0.91 ^k	0.58 ± 0.17 ^e	0.26 ± 0.02 ^f	126.71 ± 0.42 ^{ij}	96.32 ± 0.95 ⁱ	38.07 ± 1.64 ^{efghi}	74.54 ± 1.12 ^a				
CR01	126.62 ± 0.43 ^h	44.13 ± 1.02 ⁿ	0.99 ± 0.08 ^{bcd}	0.56 ± 0.18 ^{bcd}	183.57 ± 1.70 ^b	154.92 ± 0.16 ^b	49.73 ± 2.68 ^{cd}	11.32 ± 1.70 ^{jk}				
CR02	197.34 ± 0.31 ^c	129.39 ± 0.48 ^b	1.64 ± 0.09 ^b	0.62 ± 0.09 ^{bc}	165.72 ± 1.02 ^c	127.17 ± 1.47 ^e	30.56 ± 3.32 ^{hi}	37.57 ± 2.41 ^{de}				
CR03	91.67 ± 1.92 ^k	70.79 ± 0.19 ^{hi}	0.90 ± 0.08 ^{cde}	0.33 ± 0.01 ^{def}	92.42 ± 1.12 ^q	70.91 ± 0.56 ^m	28.79 ± 3.48 ⁱ	37.27 ± 2.19 ^e				
LP01	205.26 ± 0.73 ^a	155.97 ± 1.95 ^a	0.65 ± 0.10 ^e	0.38 ± 0.08 ^{cdef}	193.67 ± 1.13 ^a	162.05 ± 1.17 ^a	51.23 ± 2.74 ^{cd}	45.05 ± 1.60 ^d				
Average	117.68 ± 53.81	77.79 ± 36.21	1.53 ± 1.75	0.62 ± 0.58	142.86 ± 34.95	113.91 ± 31.21	45.12 ± 11.63	32.62 ± 19.57				
<i>Filamentous fungi used process (FFP)</i>												
NN01	126.81 ± 0.15 ^h	96.47 ± 0.35 ^e	0.77 ± 0.07 ^{cde}	0.29 ± 0.09 ^{ef}	135.47 ± 1.81 ^{fg}	109.99 ± 1.08 ^g	35.19 ± 3.17 ^{ghi}	22.22 ± 1.32 ^{gh}				
NN02	90.24 ± 0.99 ^{kl}	71.85 ± 1.08 ^h	1.17 ± 0.10 ^{bcd}	0.45 ± 0.07 ^{bcd}	103.28 ± 0.89 ^o	75.13 ± 1.12 ^l	44.04 ± 3.91 ^{defg}	26.47 ± 3.47 ^{fg}				
NN02-F	115.43 ± 1.07 ⁱ	82.03 ± 0.44 ^g	0.95 ± 0.13 ^{bcd}	0.30 ± 0.02 ^{ef}	121.72 ± 0.91 ^{kl}	84.36 ± 1.11 ^j	43.44 ± 3.96 ^{defg}	22.48 ± 3.53 ^{gh}				
NN03	85.46 ± 1.13 ^m	68.79 ± 0.63 ⁱ	0.53 ± 0.06 ^e	0.27 ± 0.05 ^{cdef}	108.41 ± 1.13 ⁿ	75.74 ± 0.79 ^{kl}	47.52 ± 3.33 ^{cdef}	18.20 ± 2.54 ^{bij}				
NN03-F	150.29 ± 0.96 ^f	97.63 ± 0.49 ^e	0.98 ± 0.07 ^{bcd}	0.37 ± 0.04 ^f	134.12 ± 0.94 ^{gh}	95.61 ± 1.21 ⁱ	44.40 ± 4.05 ^{defg}	19.18 ± 3.41 ^{bij}				
PR01	69.38 ± 0.56 ⁿ	51.68 ± 0.51 ^l	1.04 ± 0.17 ^{bcd}	0.56 ± 0.15 ^{bcd}	115.23 ± 0.73 ^m	104.04 ± 1.56 ^h	48.76 ± 3.14 ^{cde}	30.48 ± 2.64 ^{ef}				
PR02	130.54 ± 0.12 ^g	96.70 ± 0.65 ^e	0.86 ± 0.16 ^{cde}	0.44 ± 0.02 ^{bcd}	155.26 ± 0.89 ^d	128.88 ± 0.95 ^e	88.17 ± 3.58 ^a	32.09 ± 3.31 ^{ef}				
PR02-F	201.52 ± 0.52 ^b	100.67 ± 0.83 ^d	0.97 ± 0.24 ^{bcd}	0.39 ± 0.05 ^{cdef}	150.03 ± 1.04 ^e	134.23 ± 1.44 ^d	66.05 ± 4.28 ^b	31.58 ± 3.73 ^{ef}				
PY01-F	69.71 ± 0.48 ⁿ	48.10 ± 0.41 ^m	0.86 ± 0.16 ^{cde}	0.37 ± 0.16 ^{cdef}	119.37 ± 0.93 ^l	86.78 ± 0.44 ^j	49.58 ± 4.08 ^{cd}	9.49 ± 2.78 ^k				
PY02	169.43 ± 0.76 ^e	109.78 ± 1.86 ^e	0.92 ± 0.86 ^{cde}	0.40 ± 0.02 ^{cdef}	132.97 ± 0.78 ^{gh}	95.74 ± 1.19 ⁱ	42.51 ± 4.34 ^{defg}	18.88 ± 1.39 ^{bij}				
Average	120.88 ± 41.65	82.37 ± 20.33	0.91 ± 0.16	0.38 ± 0.08	127.59 ± 16.18	99.05 ± 19.43	50.97 ± 14.49	23.11 ± 6.80				

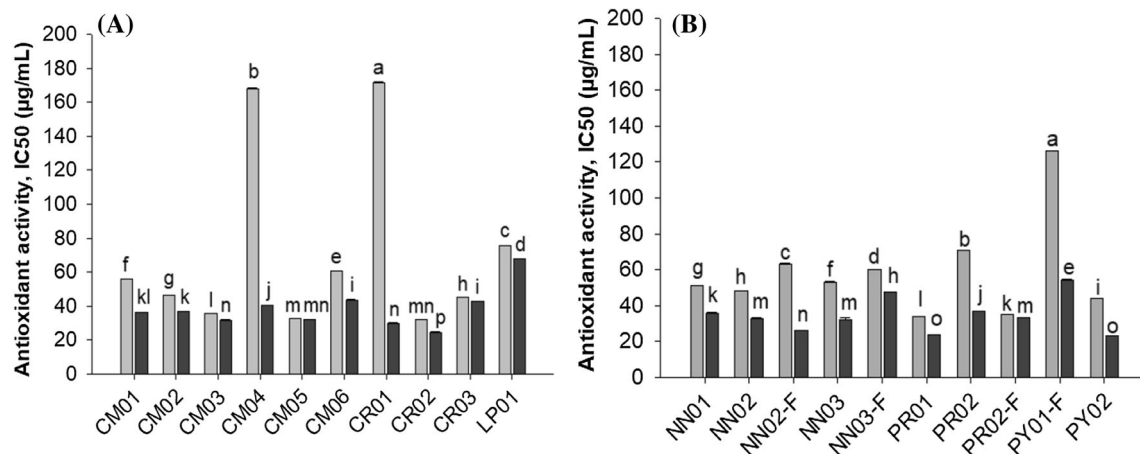


Fig. 3 Antioxidant activity, IC₅₀ (µg/mL) of Miang samples; non-filamentous fungi Miang, NFP (a), filamentous fungi Miang, FFP (b), astringent Miang (light shaded square), sour Miang (thick shaded square)

antioxidant properties was found in all types of Miang samples (Fig. 3). The IC₅₀ of astringent Miang produced by NFP and FFP processes was in the range of 32–171 and 34–126 µg/mL with the average of 45 and 50 µg/mL, respectively while those NFP and FFP sour Miang was in the range of 24–67 and 23–54 µg/mL with the average of 38 and 34 µg/mL, respectively. IC₅₀ of Miang produced by different fermentation processes was not significantly different, but with prolonged fermentation time, IC₅₀ of Miang decreased from 72 (NFP) and 58 (FFP) µg/mL in astringent Miang to 38 (NFP) and 34 (FFP) µg/mL in sour Miang, respectively. The decrease in IC₅₀ value indicated that the increase in antioxidant activity with prolonged fermentation of sour Miang as less material was needed for IC₅₀ value. This result is very interesting as it suggested that in sour Miang when other bioactive compounds such as polyphenols, tannins, flavonoids and catechins overall decreased along with the long fermentation time, at the same time the antioxidant activity increased indicating likely role of other bioactives that are biotransformed products not measured in this study.

The decrease of total tannin content during fermentation process may be due to polyphenolic compounds was hydrolyzed to simpler metabolites by polyphenol oxidase or the breakdown of the tannin complexes such as tannin-protein, tannic acid–starch and tannin-iron complexes to make free nutrients. Furthermore, decrease of total polyphenols, total flavonoids and total catechins during fermentation process was also observed and this may cause from the transformation of catechins to other phenolic metabolites by the action of polyphenol oxidase (Tang et al. 2018). The TP, TT, TF and TC contents in astringent Miang were high and the antioxidant capacity was also high because antioxidant activity in terms of reducing power was likely linked to the total phenolic content

(Paixão et al. 2007). On the other hand, in sour Miang, it was found that even as the TP, TT, TF and TC contents decreased than those of astringent Miang the antioxidant ability was maintained at higher levels. This result indicated that the higher total phenolic content may not always be responsible for the greater antioxidant capacity, because the difference of phenolic profiles in different samples can provide different responses (Nunes et al. 2015). The phenolic compounds might be further transformed through the polymerization, epimerization and degradation by extracellular enzymes of microorganisms, such as tannase which play important role in catalyzing chemical reactions and promoting the transformation of the key chemical components during the fermentation (Lv et al. 2013). Moreover, some other compounds such as degraded proteins and lipids could have contributed to the antioxidant activity as the crude protein and fat content of sour Miang was higher than astringent Miang. This was in agreement with result of Ismail et al. (2010), where fatty acids were responsible for antioxidant activity. Proteins and peptides can also inhibit lipid oxidation in food by contributing to the endogenous antioxidant capacity of food with biologically designed mechanisms such as antioxidant enzymes and iron-binding proteins or nonspecific mechanisms through multiple pathways including inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, reduction of hydroperoxides, and alteration of the physical properties of food systems (Elias et al. 2008).

Antimicrobial activities on foodborne pathogens and oral cavity pathogenic bacteria

Samples of astringent Miang and sour Miang collected from different fermentation process from different regions were investigated for their antimicrobial activity against

pathogenic bacteria including dental caries linked *Streptococcus mutans*, as well as food borne pathogens *Salmonella enterica* serovar Typhimurium, *Vibrio cholerae* and *Escherichia coli* ATCC 22595 by agar-disc diffusion method. The overall results (Fig. 4) indicated the capability of Miang extracts to inhibit these targeted pathogenic bacteria and both with non-neutralized pH (pH about 4.0) and neutralized pH (pH about 7.0). However, the pattern of inhibition was different among the pathogens. All of Miang extracts showed strong inhibitory effect against *V. cholerae* and *S. mutans* (Fig. 4a, b) both in with the unneutralized extracts (pH 4.0) and neutralized extracts (pH 7.0). Different pattern was found with the inhibitory effect against

S. enterica (Fig. 4c) and *E. coli* ATCC 22595 (Fig. 4d) in which the acidic or unneutralized sample (pH 3.9–4.0) showed higher inhibition effect compared to the neutralized samples (pH 7.0). The unneutralized solutions could likely inhibit bacterial growth due to the major effect of organic acids content from fermentation process (Talawat et al. 2006). Organic acids can slowly diffuse through a bacterium's cell wall and dissociate into anions and protons in the cell cytoplasm and lead to change of pH in the cell from neutral to acidic pH, which can then disrupt proton motive force and inhibit substrate transport mechanisms (Raftari et al. 2009).

However, the inhibitory effect on all pathogenic bacteria was found in neutralized samples which indicated that beside the acidic condition there are other bioactive compounds which are capable of antimicrobial activity in neutral condition. This phenomenon might be the result of antimicrobial activity of phenolic compounds in tea such as tannins, polyphenols, flavonoids and catechins, and protein hydrolyzed products (Fazal and Rauf 2015; Hendra et al. 2011; Talawat et al. 2006). In this situation the antimicrobial action of phenolic compounds was likely responsible for the disruptive action on the outer membrane leading to cellular enzymes inactivation, which depend on the rate of penetration of the phenolic metabolites into the cell leading to membrane permeability changes that cause disruption of plasma membrane leading to cell death (Pupponen-Pimiä et al. 2001).

Conclusion

It is clear from this study that during fermentation, there is nutritional biotransformation of indigenous Miang of north Thailand under NFP and FFP process in astringent Miang and sour Miang types. The most exciting result from this study is that while macronutrients such as carbohydrates, proteins and lipids of tea are used by fermenting microorganisms, the structural modification of tea phenolic polymers towards bioactive polyphenols, tannins, flavonoids, and catechins as well as possible further degraded metabolites contribute to higher value antioxidant and antimicrobial activity. This is of significant relevance to show that this traditional fermented product has benefits beyond basic nutritional benefits. Therefore the understanding of nutritional biotransformation of Miang, an important fermented and culturally relevant dietary source of north Thailand region will advance the value added benefits of increased antioxidant and antimicrobial activity with traditional fermentation process and is relevant to current health challenges.

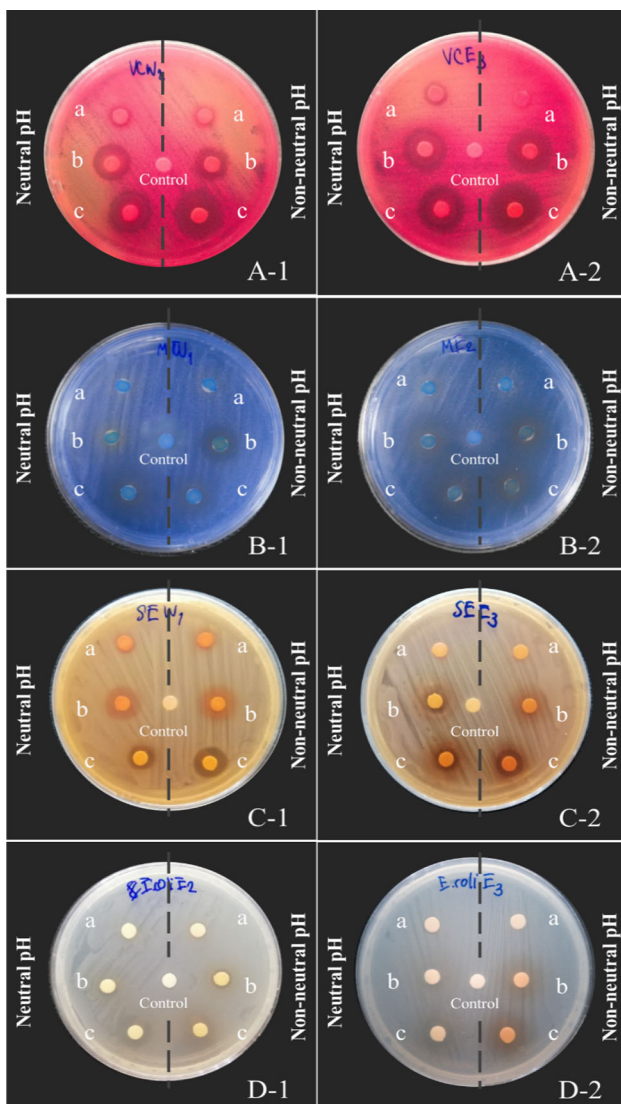


Fig. 4 Antimicrobial activity of Miang water extracted solution against *Vibrio cholerae* (a); *Streptococcus mutans* (b); *Salmonella enterica* (c) and *Escherichia coli* (d); NFP (a-1, b-1, c-1, d-1); FFP (a-2, b-2, c-2, d-2); a control (steamed tea leaves), b astringent Miang (short time fermentation), c sour Miang (long time fermentation)

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