Effect of Fermentation on Compositional Changes of *Cinnamomum osmophloeum* Kaneh Leaves

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**Abstract.** *Cinnamomum osmophloeum* Kaneh is known as “indigenous cinnamon” with the chemical constituents of its leave’s essential oil are similar to the famous *C. cassia* inner bark oil. Its oil has long been used as a medicinal plant. Fermentation is one of the processes in tea production, which could change the compound’s composition. This research aims to study the compositional changes of *C. osmophloeum* leaves during fermentation compared to unfermented leaves and commercial tea leaves. The main bioactive secondary metabolites in *C. osmophloeum* leaves extract are two flavonol glycosides. Both of this glycosides changed into aglycone during fermentation. By using HPLC and LC-MS analysis the major components and their derivative were identified. The retention time of kaempferol aglycone was 35.21 minute and the concentration showed increased from 0.46 to 46.8 µg. mL⁻¹ after fermentation. There are 3 major groups lactic acid bacteria isolated from fermented *C. osmophloeum* Kaneh leaves, *Bacillus coagulans*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus*, which it plays the key role during the compositional changes of glycosides into aglycone.

1. **Introduction**

*Cinnamomum osmophloeum* Kaneh is native to Taiwan. It is commonly known as “indigenous cinnamon” with Taiwan producing cinnamon tea leaves. *Cinnamomum* species or usually known as cinnamon, belonging to the *Lauraceae* family. Its widely distribute in South and Southeast Asia as spices and traditional medicines. Previous studies reported that *C. osmophloeum* has a bioactivity, such as decreasing the risk of cardiovascular diseases [1], cancer chemoprevention activity [2] and cholesterol lowering effects [3]. Taiwan produced many famous teas, due to the land conditions and the climate, which it very suitable for tea production. However it has been difficult due to the market competition. Since early 2000, Taiwan produced cinnamon tea leaves as a new product variation. Fermentation is process which complex organic compounds, such as glucose, are broken down by the action of enzymes into simpler compounds without the use of oxygen. Fermentation plays an important key to tea quality in terms of appearance, aroma, liquor and infusion [4]. Lactic acid bacteria usually produces secondary metabolites, such as enzymes during fermentation. The β-glucosidase is one of enzyme produced by the lactic acid bacteria in the fermented leaves hydrolyzes the flavonol glycosides to aglycone [5]. This
experiment focused on the effects of fermentation on the compositional changes of *C. osmophloeum* leaves and identified the microorganism involved during fermentation.

2. **Experiment**

2.1 **Plant materials and Fermentation**

The leaves of *C. osmophloeum* Kaneh were collected from Yuli, Hualien County in Eastern Taiwan, dried overnight in room temperature and cut. Fresh leaves were ensiled in an airtight mason jar as spontaneous fermentation for 4-12 weeks of storage at 37°C, and extracted every 2 weeks for analysis. Fresh leaves and fermented, extracted using 100% methanol (1:3), then shaken at 200 rpm for 24 h at 37°C. The solvent was removed then kept at -4°C for short storage.

2.2 **HPLC Analysis**

The analytical HPLC system consisted of a Hitachi (Tokyo, Japan) L-7100 pump, and a Model L-7400 diode array detector (DAD). Separation was carried out with a C18 column (4.6mm×100 mm, 3 μm particle size). The mobile phase consisted of acetonitrile (solvent A) and water containing 0.1% trifluoroacetic acid (solvent B). A linear gradient program was used as follows: 10% A in the first 0 min, linearly gradient to 30% A over 20 min, then hold for 10 min. The mobile phase flow rate was 0.8 mL/min, and the detector was monitored at 265 nm. All the chromatographic operations were carried out at ambient temperature, 25°C [2].

2.3 **LC-MS Analysis**

The mass spectrometer was run in electrospray ionization mode (ESI). Samples were loaded on to LC symmetry C18 column basic with diameter 150 x 2.1 mm, particle size 5 μm. The mobile phase consisted of 6% acetonitrile + 0.1% formic acid in deionized water (solvent A) and 95% acetonitrile + 0.1% formic acid (solvent B). The mass spectrophotometer operated in positive ion mode using ion trap analyzer and mass range scan of 150 – 1000 m/z with a flow rate of 200 μm/min. The sheath gas flow rate was 50 arb, spray voltage applied for full mass scan was 4 kV and the capillary voltage was 30v with capillary temperature of 300°C. Mass spectral data was acquired using Thermo-Xcalibur™ data acquisition (Thermo Scientific).

A linear gradient program was used as follows for the HPLC gradient: 10% A in the first 0 min, linearly gradient to 30% A over 20 min, then hold for 10 min. The LCQ Fleet ion trap mass spectrometer was operated in positive electrospray mode.

2.4 **Bacterial Isolation**

One gram of *C. osmophloeum* leaves, fresh and fermented, were cut, then diluted with 90 ml sterile saline solution (0.85% sodium chloride), then a serial dilution was conducted. For the last 3 serial dilution and original samples then plated onto de Man-Rogosa-Sharpe (MRS) agar supplemented with CaCO₃, and incubated at 37°C, 48 hours. The lactic acid bacteria produced a clear zone.

The DNA cells were extracted using KIT DNA extraction (FavorPrep Tissue Genomic DNA Extraction Mini Kit #FATGK001, Favorgen Biotech Corp., Taiwan), and confirmed by mean of specific species by PCR. The PCR amplification was then sent to the Genomics Bioscience and Technology Co. Ltd, Taiwan for DNA sequencing.

The PCR analysis was conducted using two primers (partial 16S rRNA gene amplification). 5’-AGAGTTTGATCCTGGCTCAG-3’ as forward primer (P1b 16-F #12041224)) and 5’-GGCTGCTG GCACGTAGTTAG-3’ as reverse primer (P1b 16-R #12041225), were purchased from Genomic Biosd & Tech. The reaction mixture (30 μL) contained 10 μL PCR master mix (Thermo Starr), 6 μL DNA template, and 2 μL for each primer. After an initial denaturation of 4 minutes at 96°C, 30 cycles of 30 sec at 96°C, 30 sec at 56°C, 45 sec at 72°C, and final extension at 72°C for 4 min were performed.
2.5 Measurement of kaempferol aglycone
The quantification of kaempferol aglycone in fermented leaves of C. osmophloeum was performed using calibration with standard solution of kaempferol (Sigma) in the range from 5, 10, 20, 40, 80, and 100 µg.mL⁻¹. The peak area were correlated with the concentrations according to standard. Standard stock solution were made by diluted kaempferol with methanol 100 µg.mL⁻¹. All data are expressed as mean standard deviation of triplicate independent experiment (n=3).

3. Results and Discussion
3.1 Physical Changes during Fermentation
Fresh leaves were ensilaged in an airtight mason jar. A spontaneous fermentation started to occur after 4 weeks of storage at 37°C until 8 weeks, and extracted every 2 weeks for analysis. This experiment focused on fermentation of C. osmophloeum Kaneh, fresh and fermented, then extracted using 100% methanol, and incubated in a shaker for 24 hours. During tea fermentation, tea flavor and color are complex problems since there a number of volatile and nonvolatile compounds formed during this oxidation process on fine granules of tea.

![Figure 1](image)

Figure 1: The color change of C. osmophloeum Kaneh leaves before extraction using 100% methanol from three different samples, (A) fresh leaves (without fermentation), (B) fermented leaves after 8 weeks fermentation, and (C) commercial cinnamon tea (produced by Wenberli Biotechnology Co).

The fresh leaves were green, while the fermented leaves, which have a similarities with the commercial cinnamon tea leaves, were brown. The quality of made tea can be determined by visual inspection of the optimum color during the process as shown at figure 1. The flavor of the fermented leaves also increased significantly compared to the fresh leaves, and it has similar flavor as the commercial one.

The situation is further complicated by the act that the flavor is unstable and its odor is changing with time as and when chemical composition of the tea changes. These changes are contribute towards the color and flavor of made-tea [6], that a series of chemical reactions take place during this process due to the severe damage of the leaf cells, which is mostly influenced by oxidized flavonol formed during fermentation. Proteins get degraded, the chlorophyll are transformed into pheophytins and some volatile compounds are generated due to transformations of certain aroma precursors present in leaves. Sharma and Bhattacharya (2013) reported that at the time of fermentation grassy smell continues change to the floral smell due to some complex chain of biochemical reaction inside the tea leaf and greenish color changes into the coppery brown.

3.2 Chemical Changes during Fermentation
Every samples from fermentation time of C. osmophloeum leaves were extracted, following the procedure above. The volume of 20 µL of the filtered solution was injected directly and separated under the optimum condition mentioned earlier. Each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing the retention times and on-line UV spectra with those of the standards. Retention time for compounds 1 and 2 were 23.4 and 25.1 minute, and compound 3 were identified as kaempferol aglycone has retention time around 35.21 minute, respectively (see figure 2).

Figure 2. Representative HPLC profiles of methanol extract of C. osmophloeum Kaneh leaves. These figures compared among the extraction of 3 different samples. A) Fresh leaves, B) 8 weeks fermented leaves, C) commercial tea. Compound 1 identified as kaempferol 3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside, and compound 2 is kaempferitrin (in accordance with previous research [2]), compound 3 is kaempferol (aglycone) which identified by compared with kaempferol standard (Sigma) then continued with LC-MS identification.

Fresh C. osmophloeum Kaneh leaves showed two major glycosides after analysis using high performance liquid chromatographic (HPLC) as shown in figure 2. Compound 1 and 2 were identified as kaempferol 3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside and kaempferitrin as identified in the previous research, by using HPLC and NMR analysis [2]. Both compounds are glycosides.

These results are in accordance with the previous research [2] about determination of kaempferol glycosides in C. osmophloeum leaves by HPLC. Glycosides are water soluble and less reactive and volatile than aglycone, possible explaining why plant (fresh leaves) store a great number of compounds in glycosides form [7]. The determination were initially by isolation and characterization of compounds with explained method [2].

Figure 2. Shows the similar pattern, peaks 1 and 2 were found in three different samples, but peak 3 only showed in fermented leaves and commercial tea. Commercial tea is also already fermented, which means that compound 3 is produced after fermentation. The methanol extract of fermented C. osmophloeum leaves was then analyzed using LC-MS, as shown in figure 3, selection ion chromatogram in m/z 287. Kaempferol has molecular weight 286 and the m/z 287 for positive ionization [8]. Kaempferol found in the retention time of 8.07 minutes. This means that kaempferol was present in the methanol extract of fermented C. osmophloeum Kaneh leaves.
Figure 3. Mass spectrum (LC-MS) of kaempferol from crude extract of fermented leaves of *C. osmophloeum* Kaneh found in the retention time of 8.07 minutes, with selection ion chromatogram in m/z 287.

With longer fermentation the amount of kaempferol aglycone in the leaves increased significantly. Measurement was done by comparing the peak area from HPLC analysis, which by week 8 of fermentation the amount of kaempferol aglycone increased significantly compared with 0 week fermentation, from 0.46 to 46.81 µg.mL⁻¹. It is increased about more than 100 fold.

Figure 4 showed that length of fermentation effects the composition of the *C. osmophloeum* Kaneh leaves. These results explained the similar HPLC pattern between fermented leaves and commercial tea leaves, that the activity of β-glucosidase produced by lactic acid bacteria during fermentation of *C. osmophloeum* Kaneh leaves converted two kaempferol glycosides stored at a fresh leaves become kaempferol aglycone. Yang, *et al.* reported that the concentration of aglycone in meju was increased to reach to approximately 40% of total isoflavones in 90 day meju fermentation. Some amino acids increased and others remained almost constant with increased fermentation time [9].

![Kaempferol Mw = 286](image)

**Figure 4.** The effects of fermentation on the compositional changes of *C. osmophloeum* Kaneh leaves shows that the longer fermentation done the kaempferol aglycone (green graphic) increased significantly. Both glycosides (blue and red graphic) were decreased after fermentation.
3.3 Microbiological Analysis

Lactic acid bacteria plays an important role in the fermentation process, producing lactic acid, ethyl alcohol, carbon dioxide and aromatic compounds, which contributed to changes of color, flavor, and odor during fermentation. Ten strains were isolated from 4 weeks fermented C. osmophloeum Kaneh leaves. The DNA of each strain extracted using Tissue Genomic DNA Extraction Mini KIT (FavorPrep, FATGK001), then continued by PCR analysis.

![Figure 5](image)

**Figure 5.** Lane A-J shown the results of PCR amplification products (500 bp) of LAB isolated from fermented tea leaves. Lane C and D didn’t showed an any bend in 500 bp

In this study, PCR amplification products was 500 base pair fragment (figure 5). After PCR amplified, continued to sequences the 16S rRNA gene from bacteria, then use BLAST (Basic Local Alignment search Tools, Program BLASTN 2.2.28+) on the complete NCBI genomic database to identify the species that most closely matches their sequence results for the genes. This experiment showed that there are three groups of lactic acid isolated from the fermented leaves, B. coagulans, L. plantarum, and P. pentosaceus (table 1). The activity of β-glucosidase produced by those lactic acid bacteria during fermentation of C. osmophloeum Kaneh leaves converted two kaempferol glycosides stored at a fresh leaves become aglycone form, kaempferol.

| Strain Code | Species Identified* | Max. Identified (%) |
|-------------|---------------------|---------------------|
| A           | Bacillus coagulans   | 99                  |
| B           | Bacillus coagulans   | 99                  |
| E           | Lactobacillus plantarum | 98            |
| F           | Pediococcus pentosaceus | 97           |
| G           | Pediococcus pentosaceus | 97           |
| H           | Lactobacillus plantarum | 97            |
| I           | Lactobacillus plantarum | 98            |
| J           | Pediococcus pentosaceus | 97           |

Table 1. Sequencing DNA then compared to the world gen bank data by BLAST (Basic Local Alignment search Tools, Program BLASTN 2.2.28+)

Naturally, the lactic acid bacteria involved in food fermentation process produced enzymes as secondary metabolites. β-glucosidase activity was found in wine making by L. plantarum [10]. β-glucosidase also produced by lactic acid bacteria from local fermented food of Indonesia [11]. Its
enzymes generated isoflavone aglycones during fermentation by enzymatic hydrolysis of glycosides [5]. Similar results also reported by [12] that Bacillus strain enhanced the aglycone production during soybean fermentation.

The molecule of aglycone is smaller than glycosides which made aglycone easily incorporated into target cells through simple diffusion mechanism because of its high hydrophobicity. The isoflavone aglycones were absorbed faster and greater amounts than their glycosides in human, which means it may be more effective than glycoside products in preventing chronic disease [13]. These results demonstrated that consumption of fermented tea leaves of C. osmophloeum Kaneh are way better than the fresh leaves.

4. Conclusion
Fermentation could change the phsyco-chemical and organoleptic properties of C. osmophloeum Kaneh leaves by 3 major groups of lactic acid bacteria activity, B. coagulans, L. plantarum and P. pentosaceus. The HPLC analysis showed that the chemical composition changed with the length of fermentation, compared to fresh leaves. The presence of kaempferol aglycone produced during fermentation was confirmed by LC-MS. However, its need further research to understand the stability of kaempferol aglycone during fermentation and the possibility that it will use by other bacteria.

Acknowledgment
This experiment was supported by National Pingtung University of Science and Technology, Taiwan and Universitas Brawijaya, Indonesia from Beasiswa Unggulan DITKTI for Double Degree Program.

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