NMR Metabolic profiling of green tea (*Camellia sinensis* L.) leaves grown at Kemuning, Indonesia

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Abstract. Green tea (Camellia sinensis L.) has been famous as a beverage and natural medicine. It contains a broad range of primary and secondary metabolites i.e. polyphenols. Nuclear Magnetic Resonance (NMR) has been widely used for metabolic profiling in medicinal plants. It provides a very fast and detailed analysis of the biomolecular composition of crude extracts. Moreover, an NMR spectrum is a physical characteristic of a compound and thus highly reproducible. Therefore, this study aims to profile metabolites of three different varieties of green tea C. Sinensis grown in Kemuning, Middle Java. Three varieties of green tea collected on Kemuning (TR1 2025, Gambung 4/5, and Chiaruan 143) were used in this study. 1H-NMR spectra were recorded at 230C on a 400 MHz Agilent WB (Widebore). The analysis was performed on dried green tea leaves and analyzed by ^{1}H-NMR, 2D-J-resolved and 1H-1H correlated spectroscopy (COSY). MestRenova version 11.0.0 applied to identify metabolites in samples. A 1H-NMR spectrum of tea showed amino acids and organic acids signal at the area δ 0.8-4.0. These were theanine, alanine, threonine, succinic acid, aspartic acid, lactic acid. Anomeric protons of carbohydrate were shown by the region of β-glucose, α-glucose, fructose and sucrose. The phenolic region was depicted at area δ 5.5-8.5. Epigallocatechin derivates and caffeine were detected in the tea leaves. The detail compound identification was observed and discussed in the text.

1. Introduction

Camellia sinensis L. or known as green tea is the popular beverage all over the worlds. It comprises hundreds of both primary and secondary metabolites at different concentrations. The former occurs in tea leaves including sugars, organic acids, amino acids and the latter comprising alkaloids and polyphenols. Those metabolites determine the quality of tea leaves. It is influenced by variety, geography, and climates [1]. Lee [2] reported chemical constituents of tea differed among three varieties naming white tea, green tea and oolong teas. Moreover, the geography of tea leaves grown influences its chemical composition. It revealed that theanine and catechin derivatives were correlated to geographical status, either inter-country or inter-city.

Intermediate and final products of cell metabolism process are noticed as metabolites. Generally, those metabolites are influenced by the current circumstances [2]. Metabolomics as a holistic approach is needed to understand better about primary and secondary metabolites [3]. An advance and powerful

analytical tools such as nuclear magnetic resonance (NMR) has been introduced. Nowadays, NMR is a popular method as a metabolome analysis [4]. Despite its low sensitivity, NMR is a promising feature as its non-destructive method and sample preparation [5].

NMR spectroscopy is favored due to its simple pretreatment. Moreover, it is a non-selective method which covers a wide range of metabolites particularly in the field of agriculture [6]. The use of NMR for metabolites profiling has been reported at different food stuff like coffee [7], wine [8] and tea [9].

The aim of the study was to identify the primary and secondary metabolites of green tea leaves grow at Kemuning, Indonesia using ^{1}H-NMR with some two-dimensional NMR techniques, J-resolved and 1H-1H COSY. It used three varieties available at the tea gardens, TRI 2025, Gambung and Cinruan.

2. Materials and Methods

2.1. Plant Material and Extraction

Green tea (*Camellia sinensis* L.) were freshly collected from Kemuning green tea garden situated at Kemuning, Karanganyar (070 36' 23.6" S, 1110 07' 02.9" E) Middle Java, Indonesia, on May 2016. Kemuning garden is commercially produced tea product for export and local market. There were three varieties such as TRI 2025, Gambung and Cinruan. Three replicates of each sample were used for NMR metabolomics. Fresh green tea samples were plucked and saved in nitrogen liquid. The standard metabolomics protocol of sample preparation and ^{1H}-NMR profiling described by Kim et al. [10]. Samples were mashed under liquid nitrogen and dried in the freeze-drying for 48 hours. Samples of 30 mg plant material were weighed and extracted with 1.5 ml of a mixture of phosphate buffer (pH 6.0) in deuterium oxide containing 0.05% trimethylsilylproprionic acid sodium salt-d4 (TMSP) and methanol-d4 (1:1). Samples were vortexed at room temperature for 1 min, ultrasonicated for 20 min and centrifuged at 13000 rpm for 10 min. An aliquot of 0.6 ml of the supernatant was transferred to 5 mm NMR tubes for ^{1}H-NMR measurement.

2.2. ^{1}H-NMR Measurements

^{1}H-NMR spectra were recorded at 21^{0}C on 400 MHz Agilent spectrometer. Deuterated water was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 128 scans requiring 18 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) of 60^{o} (11.3 μs), and relaxation delay (RD) of 2s. Two-dimensional J-resolved NMR spectra were acquired using eight scans per 128 increments for F1 (chemical shift axis) and eight k for F2 (spin-spin coupling constant axis) using spectral widths of 66 Hz and 5000 Hz respectively. Both dimensions were multiplied by sine-bell functions (SSB = 0) before double complex Fourier transformation. J-resolved spectra were tilted by 450, symmetrized about F1, and then calibrated to TMSP. 1H-1H correlated COSY spectra were acquired with a 1.0 s relaxation delay and 6361 Hz spectral width in both dimensions. The window function for the COSY spectra was Qsine (SSB = 0). MestRenova version 11.0.0 applied to identify metabolites in samples.

3. Result and Discussion

3.1. Qualitative analysis of ^{1}H-NMR Spectra

^{1}H-NMR spectra of three varieties of green teas leaves were analyzed. 2D NMR technique such as J-resolved and COSY (Correlated Spectroscopy) spectra were applied to further metabolites identification. The metabolites identified cover amino acids, organic acids, carbohydrates, alkaloids, flavonoids. All assignments were done by comparing the spectra with other tea leaves reports [2] [1].

A ^{1}H-NMR spectrum is classified into three parts. First, amino acids with few organic peaks placed at the area between δ 0.8 – 4.0 ppm. Secondly, anomeric protons of carbohydrate are found in the region of δ 4.0 – 5.5 ppm and phenolic region placed at the area of δ 5.5 – 8.0 ppm. A comparison of

^{1}H-NMR spectra of three different varieties of green tea, TRI 2025, Gambung and Cinruan depicted at figure 1, figure 2 and figure 3.

3.2. Compound identification

The signals in the amino acids region (δ 0.8 – 4.0 ppm) were useful to identify a number of amino and organic acid signals (Fig. 1). Amino acids like alanine δ 1.48 (d, J = 7.2), threonine (δ 1.32 (d, J = 7.0 Hz); 4.23 (m)) and theanine (δ 1.10 (t, J = 7.4 Hz); 3.2 (q, J = 6.2 Hz). Carbohydrate region exhibited anomeric proton of α-glucose δ 5.17 (d, J = 3.8 Hz) and β-glucose δ 4.59 (d, J = 7.9 Hz). Sucrose was shown by signal at δ 3.74 (t, J = 9.5 Hz); 3.43 (t, J = 9.5 Hz). Signals at δ 3.70 (d, J = 11.6 Hz); 3.52 (d, J = 11.5 Hz) were assigned for fructose (Fig. 2). Phenolic region showed that coffein δ 7.76 (s), and epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) (δ 6.7 (d, J = 8.4); 6.9 (dd, J = 8.4, 2.7); 7.0 (d, J = 2.4)) present in green tea leaves (Fig. 3).

In the current study, we successfully identified the particular amino acids in green tea, theanine. The theanine signals at δ 1.10 (t, J = 7.4 Hz) of H-2 and 3.2 (q, J = 6.2 Hz) of H-1 (Fig. 4) which is correlated in the 1H-1H COSY spectra (Fig. 5). The threonine signals at δ 1.32 (d, J = 7.0 Hz) of H-3 and δ 4.23 (m) of H-4 (Fig. 4) were correlated each other in the 1H-1H COSY spectra (Fig. 5). The epigallocatechin signals at δ 6.7 (d, J = 8.4) of H-5 and δ 6.9 (dd, J = 8.4, 2.7) of H-6 (Fig. 4) were correlated each other in the ^{1}H-^{1}H COSY spectra (Fig. 5). Caffeine, the characteristic alkaloid in green tea, was shown by the singlet at δ 7.8 (s), δ 3.9 (s) and δ 3.5 (s).

Theanine is a characteristic amino acid present in green tea. It is reported to have a beneficial effect in cancer [11], memory ability [12] and blood pressure [13]. Total amino acids such as theanine, alanine, threonine, etc were approximately 1-4% dry weight [14].

Chacko [15] reported catechin derivatives particularly EGCG are important to metabolic syndrome. It occurred in higher amount of EGCG particularly in green tea than other varieties [15]. Catechins and caffeine in green tea act synergistically to increase energy expenditure and fat oxidation [14]. Polyphenols and caffeine content in green tea leaves were reported approximately as 45-90% and 0.4-10%, respectively [14]. It varies due to varieties, plant origin and growing condition [16].

4. Conclusions

The ^{1}H-NMR-based metabolomics approach allowed for simultaneous qualitative analysis in green tea leaves. Amino-organic acids and phenolic metabolites could be detected in the spectra. Green tea (*Camellia sinensis* L.) leaves located in Kemuning, Karanganyar, Indonesia contained characteristic amino acid in green tea, theanine, and characteristic phenolic, epicatechine derivates. The quantitative measurement for each metabolite is further analysis to be done to compare the effect of varieties on green tea.

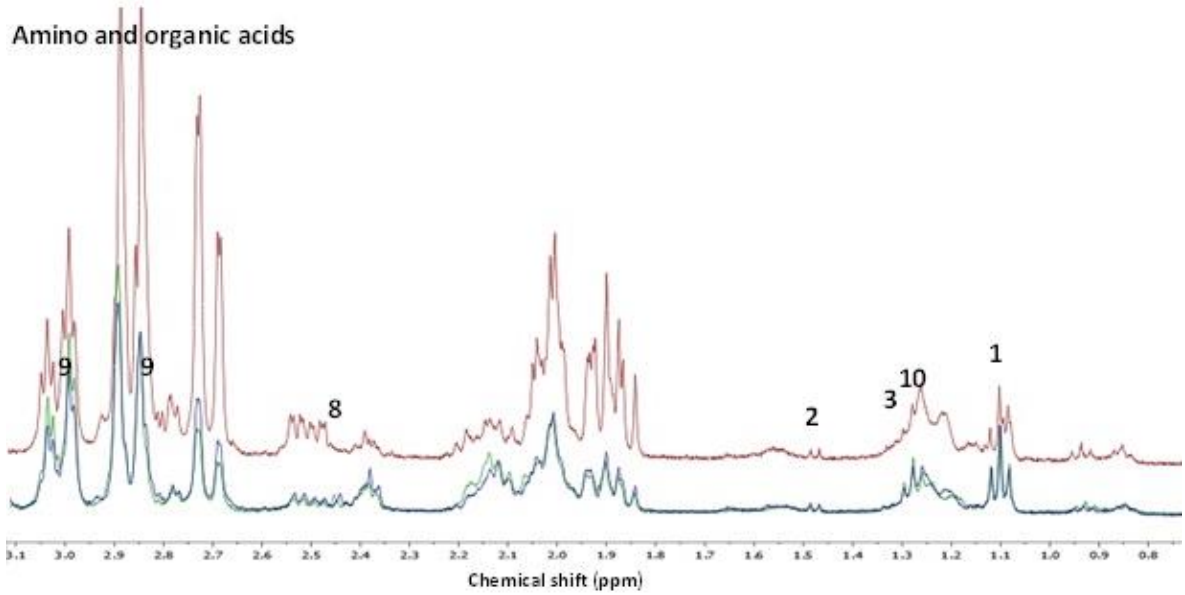

Figure 1. [1]H-NMR spectra of Gambung (red), TRI 2025 (blue) and Chinruan (green) at the area between δ 0.8 – 3.0 ppm collected on May 2016. 1: theanine, 2: alanine, 3: threonine, 4: α-glucose, 5: β-glucose, 6: sucrose, 7: fructose, 8: succinic acid, 9: aspartic acid, 10: lactic acid, 11: coffein, 12: EGC/EGCG, 13: gallic acid

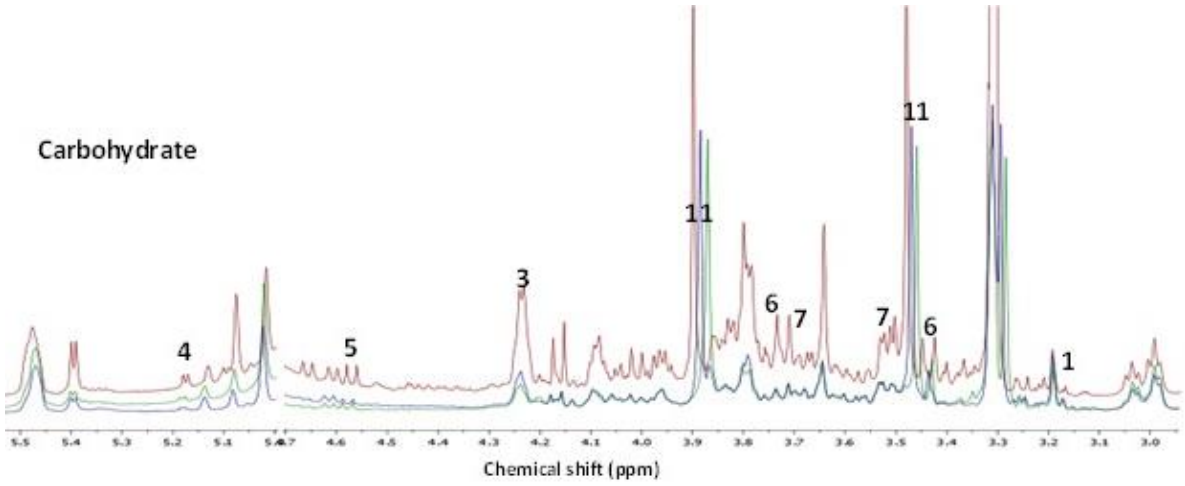

Figure 2. [1]H-NMR spectra of Gambung (red), TRI 2025 (blue) and Chinruan (green) at the area between δ 3.0 – 5.5 ppm collected on May 2016. 1: theanine, 2: alanine, 3: threonine, 4: α-glucose, 5: β-glucose, 6: sucrose, 7: fructose, 8: succinic acid, 9: aspartic acid, 10: lactic acid, 11: caffeine, 12: EGC/EGCG, 13: gallic acid

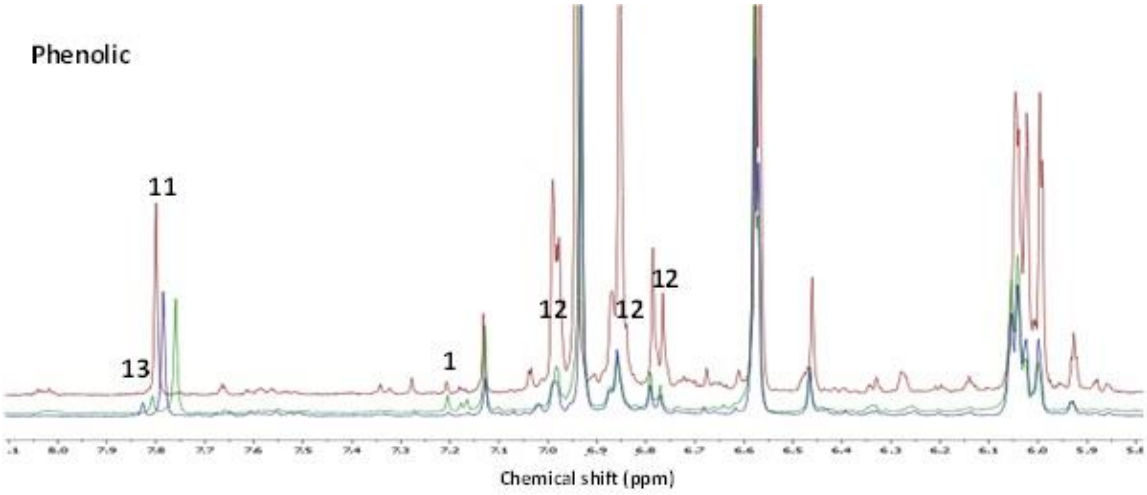

Figure 3. [1]H-NMR spectra of Gambung (red), TRI 2025 (blue) and Chinruan (green) at the area between δ 5.5 – 8.0 ppm collected on May 2016. 1: theanine, 2: alanine, 3: threonine, 4: α-glucose, 5: β-glucose, 6: sucrose, 7: fructose, 8: succinic acid, 9: aspartic acid, 10: lactic acid, 11: caffeine, 12: EGC/EGCG, 13: gallic acid

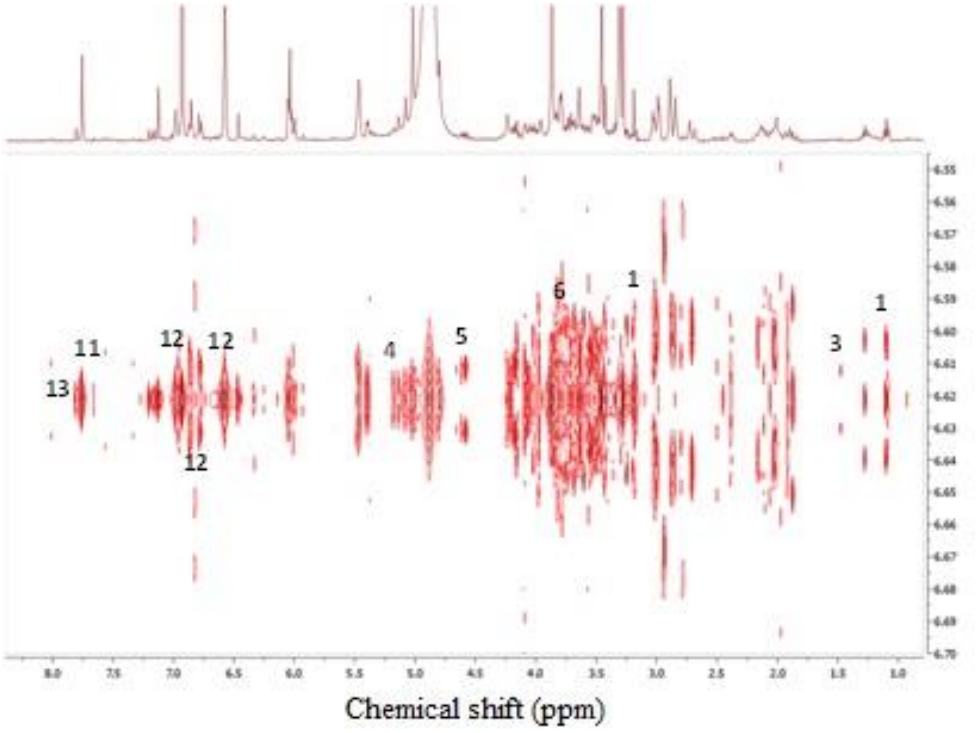

Figure 4. Two dimensional [1]H-[1]H *J*-resolved spectra of Chinruan shows 1: H-2 of theanine, 3: H-4 of threonine, 4: H anomeric of α-glucose, 5: H anomeric of β –glucose.

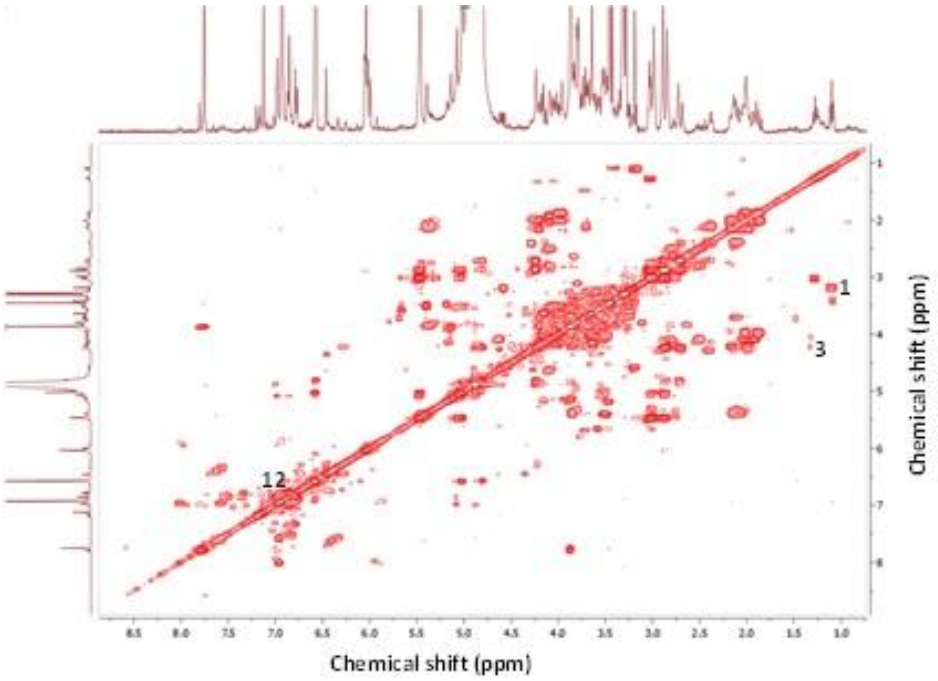

Figure 5. Two dimensional ^{1}H-^{1}H COSY spectra of Chinruan shows correlation between 1: H-1 and H-2 of theanine, 3: H-3 and H-4 of threonine, 12: H-5 and H-6 of epigallocatechin

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