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A new method to prepare and redefine black tea thearubigins

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ABSTRACT

Thearubigins (TRs) are the major components of black tea, which are formed during the fermentation reactions. Although anti-inflammatory and anti-cancer activities of TRs have been reported, the prepared TRs according to the literature methods still contain many floating peaks. It is puzzling whether the observed activities are from TRs or these floating peaks. Thus, it is urgent to develop a method to prepare pure TRs and redefine them. In the present study, we developed a new method, the combination of caffeine precipitation and Sephadex LH-20 column chromatography, to prepare pure TRs. The floating peaks on the hump of the crude TRs were removed, and pure TRs were prepared. The chemical profile of the floating peaks was established using LC/MS, and the major compounds in this fraction were identified as apigenin glycosides, quercetin glycosides, kaempferol glycosides, theaflavins, theasinensin, and galloylglucoses based on the analysis of their tandem mass spectra and in comparison with literature data. This study will pave the way to further study the chemistry and biological activities of TRs and the health effects of black tea consumption.

1. Introduction

Black tea is one of the most popular beverages in the world, produced from young green shoots of the tea plant (*Camellia sinensis*) by fermentation [1]. Consumption of black tea has been associated with many health benefits including the prevention of cardiovascular disease [2], cancer [3,4], and obesity [5]. These effects are attributed to the polyphenol compounds in black tea [6,7], which include catechins, phenolic acids, theaflavins (TFs) and thearubigins (TRs) [8–11].

Aside from TRs, other polyphenols in black tea have been well characterized structurally. TRs were thought to be the major components of black tea which are believed to account for up to 60% of the solids in a black tea infusion [12,13]. However, the content of TRs was overestimated because of a lack in an effective method to prepare pure TRs. On reverse-phase high performance liquid chromatography (HPLC), the crude TRs appeared as a large Gaussian-shape hump that is overlaid by a number of well-resolved sharp peaks [1,14]. In the 1960s, Roberts reported the crude TRs as three fractions, SI, SII, and SIa, which showed a broad streak on two-dimensional paper chromatography [15,16]. In the 1990s, Bailey reinterpreted the issue with division of the crude TRs into groups I, II, and III with the development of HPLC techniques [17,18]. At that time, Powell began to investigate the TRs via the caffeine precipitation method [19]. During the past sixty years, many scientists have been working on the isolation, purification, and characterization of TRs [1,18,20–23]. Most of the reports on the separation of TRs were based on and modified from the Roberts' method and the caffeine precipitation method. However, Roberts' method involves many steps and cannot produce pure TRs, and the TRs from the caffeine precipitation method still showed a hump with many floating peaks [20]. Ulf W Stodt prepared the more clean TRs via Amberlite XAD-7 resin and high-speed counter-current chromatography but with low yields [20].

Numerous studies have reported the biological activities of TRs. Murad and colleagues reported that TRs protected against acetaminophen-induced hepatotoxicity and nephrotoxicity in mice [24], and improved the sildenafil-induced delayed gut motility in mice [25]. Halder et al. found that TRs have significant antimutagenic and anticlastogenic effects in Salmonella assay *in vitro* and *in vivo* in bone marrow cells of mice [26]. TRs have also been reported to protect against the neuromuscular blocking action of botulinum neurotoxin types A, B, and E by binding with the toxins [27,28]. However, the TRs used in these studies were prepared based on the Roberts' method and the caffeine precipitation method, which are crude TRs and contain many floating peaks. It

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is puzzling whether the observed activities are from TRs or these floating peaks.

In the present study, we developed a new method by combination of caffeine precipitation and the Sephadex LH-20 column chromatography to remove the floating peaks and prepare pure TRs. We also analyzed the chemical profile of these floating peaks and elucidated their structures based on the analysis of their tandem mass spectra and in comparison with literature data.

2. Materials and methods

2.1. Chemicals and reagents

Black tea was purchased from Yunnan Province of China in 2016 (Baoshan Changninghong Tea Industry Group Co., LTD, Yunnan, China). Caffeine and Sephadex LH-20 were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). ACS-grade ethanol, acetone, HPLC grade methanol, LC/MS-grade solvents and other reagents were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of black tea extract

Black tea was soaked with 70% methanol at room temperature for 4 days and repeated for four times. The extracts were then concentrated by a rotary evaporator under vacuum at 35 °C to remove the methanol, and then freeze-dried to obtain the black tea extract (yield: 33%).

2.3. Preparation of crude TRs

In our study, considering the effects of 1) concentrations of caffeine to precipitate tea polyphenols, 2) the order of decaffeination by chloroform and the partition against ethyl acetate, and 3) the pre-extraction of ethyl acetate before caffeine precipitation on the purity and amount of the TRs, six methods (methods 1, 2, 3, 1′, 2′, and 3′) were used to prepare the crude TRs. Their schematic depictions of the procedure are shown in Fig. 1. The only difference between methods 1, 2, and 3 and methods 1′, 2′, and 3′, respectively, is the concentration of caffeine. We used 20 mM caffeine in methods 1, 2, and 3, and 40 mM caffeine in methods 1′, 2′, and 3′. For method 1, TRs were prepared according to the method of caffeine precipitation with modification [1,23]. Briefly, black tea extract (1 g) was added to boiling water (60 mL) with 20 mM of caffeine, stirred to ensure dissolution (Fig. 1). The solution was allowed to stand at 4 °C for 2 h, and then centrifuged at 8819 rpm for 10 min to form the precipitate. The precipitate was suspended in boiling water again and extracted by ethyl acetate (100 mL/time, 6 times), which produced the aqueous fraction and the ethyl acetate fraction. The aqueous phase was partitioned against chloroform (100 mL/time, 4 times) to remove caffeine, then the decaffeinated aqueous phase was dried by a rotary evaporator under vacuum at 35 °C, to get the crude TRs fraction. Similarly, in method 2, the caffeine precipitate was suspended in boiling water and extracted by chloroform first, and then ethyl acetate instead of ethyl acetate first and then chloroform in method 1. Then, the aqueous phase was dried by a rotary evaporator to get the crude TRs fraction. While, in method 3, black tea extract was extracted by ethyl acetate, then the aqueous phase was precipitated by caffeine. The crude TRs were obtained by decaffeination of the precipitate.

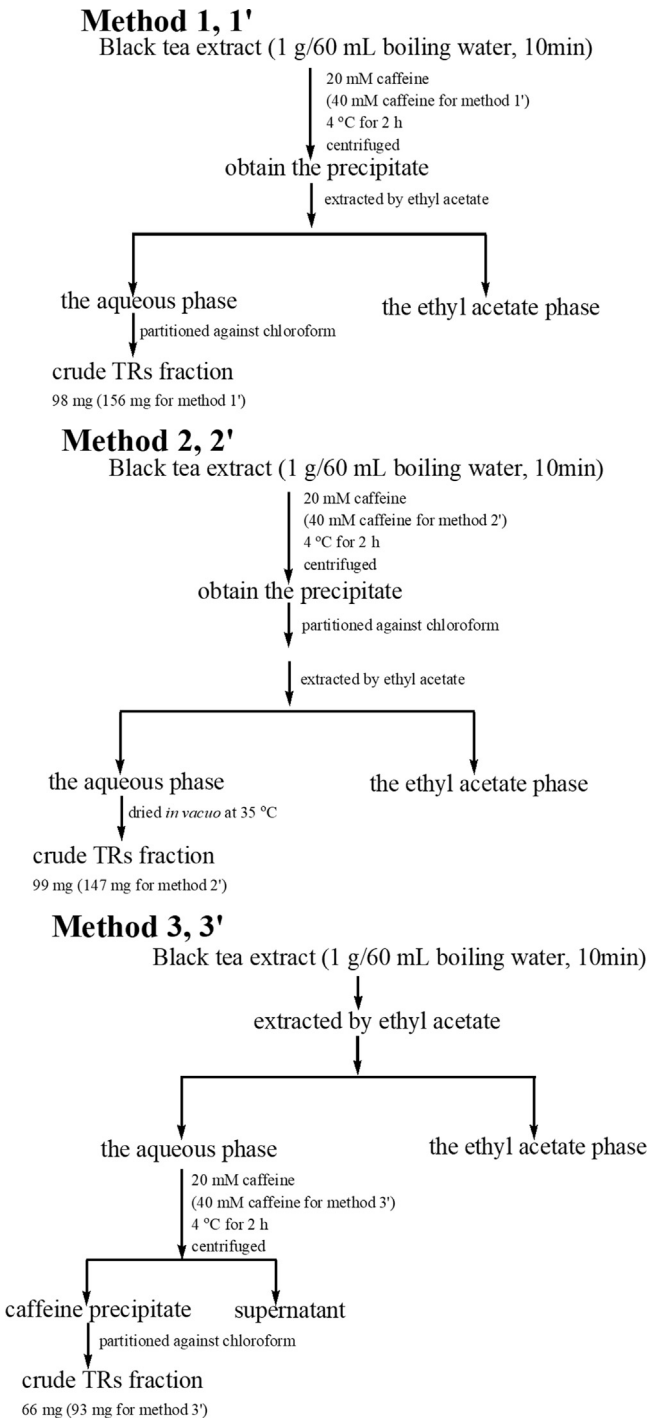

Fig 1. Schematic depictions of the procedure of methods 1, 1′, 2, 2′, 3, and 3′ for preparation of the crude thearubigins (TRs) fraction from black tea extract.

2.4. Separation of the floating peaks from the crude TRs and preparation of pure TRs fractions

The crude TRs fraction prepared based on method 1 was further chromatographed over Sephadex LH-20 column (38 × 3.2 cm) with ethanol and 50% aqueous acetone as eluents. HPLC-DAD was used to monitor the separation. All fractions containing caffeine were combined and named as the caffeine fraction (F1). Fractions with just the floating peaks were combined as fraction F2. Fractions with a hump with some floating peaks were combined as fraction F3. F3 was partitioned against ethyl acetate to further remove the floating peaks to get the ethyl acetate phase and the aqueous phase. The ethyl acetate phase was combined with F2 to obtain the floating peaks fraction. The aqueous phase was named as the TRs-a1 fraction. Fractions eluted by 50% acetone showed a clean hump and were combined as the TRs-a2 fraction. The detailed procedure

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