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Isolation, Identification, and Biotransformation of Teadenol A from Solid State Fermentation of Pu-erh Tea and *In Vitro* Antioxidant Activity

Xiao-qin Su [1,2,†], Gao-ju Zhang [3,†], Yan Ma [2], Mao Chen [2], Sheng-hu Chen [2], Shuang-mei Duan [2], Jin-qiong Wan [2], Fumio Hashimoto [4], Hai-peng Lv [1], Jia-hua Li [2], Zhi Lin [1,*] and Ming Zhao [1,2,*]

1 Key Laboratory of Tea Biology and Resources Utilization, Ministry of Agriculture, Hangzhou 310008, China; suxiaoqin0520@163.com (X.S.); lvhaipeng@mail.tricaas.com (H.L.)
2 College of Longrun Pu-erh Tea, Yunnan Agricultural University, Kunming, Yunnan 650201, China; mayan202@163.com (Y.M.); yyhscm@126.com (M.C.); aichenhu@163.com (S.C.); duanshuangmei11@163.com (S.D.); wan102@163.com (J.W.); jiahua1124@hotmail.com (J.L.)
3 Yunnan Research Center on Good Agricultural Practice for Dominant Chinese Medicinal Materials, Yunnan Agricultural University, Kunming, Yunnan 650201, China; zhanggaoju2665@163.com
4 Department of Horticultural Science, Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan; k8875711@kadai.jp
* Correspondence: linz@tricaas.com (Z.L.); zhaoming02292002@aliyun.com (M.Z.); Tel.: +86-0571-8665-0617 (Z.L.); Fax: +86-0571-8665-0826 (Z.L.); Tel./Fax: +86-0871-6522-6565 (M.Z.)
† These authors contributed equally to this work.

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Abstract: Post-fermented Pu-erh tea (PFPT) has several health benefits, however, little is known about the bioactive compounds. In this study, a PFPT compound was isolated by column chromatography and identified as Teadenol A by spectroscopic data analyses, including mass spectrometry and 1D and 2D NMR spectroscopy. Teadenol A in tea leaves was biotransformed by *Aspergillus niger* and *A. tamari* at 28 °C for 14 d at concentrations ranging from 9.85 ± 1.17 to 12.93 ± 0.38 mg/g. Additionally, the compound was detected in 22 commercial PFPTs at concentrations ranging from 0.17 ± 0.1 to 8.15 ± 0.1 mg/g. Teadenol A promoted the secretion of adiponectin and inhibited the expression of protein tyrosine phosphatase-1B. Antioxidant assays (e.g., 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, total antioxidant capacity (T-AOC), hydrogen donating ability, and superoxide anion radical scavenging capacity) revealed that Teadenol A has antioxidant properties. Therefore, Teadenol A is an important bio-active component of PFPT.

Keywords: Pu-erh tea; Teadenol A; antioxidant activity; *Aspergillus niger*; solid state fermentation

1. Introduction

Pu-erh tea (PET) is a traditional Chinese tea produced from sun-dried leaves of *Camellia sinensis* (Linn.) var. *assamica* (Masters Kitamura) in Yunnan, China. Based on its processing technology and quality characteristics, PET can be classified into Pu-erh shengcha (non-fermented Pu-erh tea, NFPT) or Pu-erh shucha (post-fermented Pu-erh tea, PFPT). NFPT is prepared by pressing sun-dried green tea leaves into a disk or bowl shape, and PFPT is produced from the solid state fermentation (SSF) of sun-dried green tea leaves at 40–60 °C and high humidity conditions [1,2].

Currently, PFPT has become increasingly popular in Southeast Asia and is beginning to be recognized in Western culture. PFPT has unique sensory characteristics, including a reddish–brownish color, mellow taste, stale flavor, and long shelf life. Additionally, PFPT has anti-obesity [3,4], neuroprotective [5], antioxidant [6], anticancer [7], constipation-relieving [8], anti-allergic, anti-inflammatory [9],

antibacterial [10], and anti-viral (hepatitis B and HIV) properties [11], with retrogressive effects on immunosenescence [12]. Furthermore, clinical studies have shown that the daily consumption of PFPT reduces body weight and prevents obesity [13–15].

The health benefits of PFPT have been attributed to its components. Flavonoids [16], theabrownin (TB) [17,18], gallic acid (GA) [19], and statin [20] have anti-obesity/hypolipidemic effects and regulate lipid metabolism. Caffeine (CAF) [21] and polysaccharides [22,23] have blood glucose-lowering effects, and 8-C N-ethyl-2-pyrrolidinone substituted flavan-3-ols [24], ethanol-soluble pigment [25], polysaccharides [23,26], and phenolic compounds [27] (including GA, (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate, (-)-epigallocatechin-3-*O*-gallate (-)-epiafzelechin-3-*O*-gallate, kaempferol, and quercetin) have antioxidant properties. However, these components are generally present in other teas (e.g., CAF and catechins), and levels of polyphenols (catechins, theaflavin, thearubigin, myricetin, quercetin, kaempferol, and flavonol glycosides), amino acids, and soluble sugars decrease during SSF of PFPT [2].

Recently, compound **1** was detected during SSF of PFPT, but not in the raw material (Figure 1). To our knowledge, only theabrownin (TB), polysaccharides, α-tocopherol, GA, and CAF increase during SSF of PFPT [2]. In this study, we isolated and identified this novel compound and assessed its antioxidant activity *in vitro*. Furthermore, fungi responsible for the biosynthesis of this compound were identified.

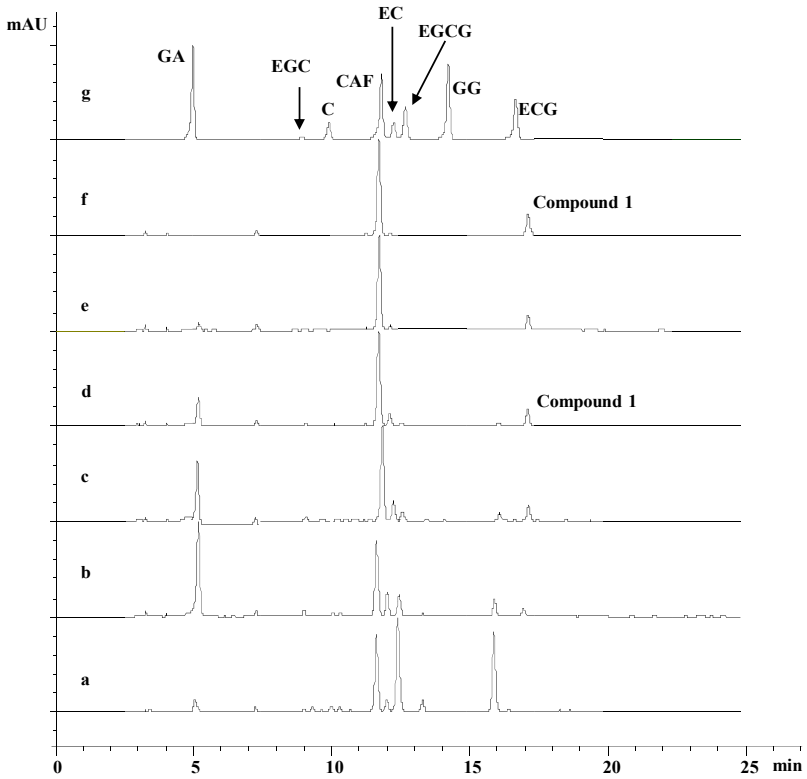


Figure 1. High-Performance Liquid Chromatography (HPLC) chromatogram of caffeine and catechins in raw material (**a**); fermented tea leaves collected on day 7 (**b**), day 14 (**c**), day 21 (**d**), day 28 (**e**), day 35 (**f**); and reference compound (**g**).

2. Experimental Section

2.1. PET Fermentation and Sample Collection

Sun-dried green tea leaves were purchased in Puer City, Yunnan. Green tea leaves (30 kg) were mixed with tap water (15 L) resulting in a solid content of approximately 65% (*w*/*v*). During SSF, the

leaves were mixed to ensure homogeneity, and tap water was added to maintain the solid content at 65%–75%. Samples were collected from the SSF tank every 7 days. Triplicate fermentations were performed. A total of 18 samples were collected and air-dried on days 0, 7, 14, 21, 28, and 35 of SSF.

2.2. Determination of Constituents in Tea Samples

Tea leaves were ground in a commercial grinder (Joyoung Co., Jinan, Shandong, China). Ground samples (1 g) were extracted twice with 80% methanol (50 mL) (Tedia Company, Inc., Fairfield, OH, USA) for 1 h, passed through a 0.45-μm Millipore filter (Merck, Darmstadt, Germany), and analyzed by high-performance liquid chromatography (HPLC) (Agilent Technologies, Palo Alto, CA, USA). The contents of GA, CAF, and catechins, including (+)- catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin 3-*O*-gallate (ECG), (−)-epigallocatechin 3-*O*-gallate (EGCG), 1,4,6-tri-*O*-galloyl-β-*D*-glucose (GG), and compound **1** were determined in an Agilent 1200 Series HPLC system equipped with an LC-20AB solvent delivery unit, a SIL-20A auto sampler, a CTO-20A column oven (40 °C), a G1314B Variable Wavelength Detector (VWD) detector (280 nm), and an LC Ver1.23 workstation (Agilent Technologies, Palo Alto, CA, USA). Separation was achieved with a TSK-GEL ODS-80TM column (4.6 mm × 250 mm, Tosoh, Yamaguchi, Japan). The mobile phase consisted of solvents A (0.05 M H_3PO_4-H_2O (Xilong Chemical, Guangzhou, China), 5% CH_3CN (Tedia Company, Inc., Fairfield, OH, USA) and B (0.05 M H_3PO_4-H_2O, 80% CH_3CN). The elution conditions were the following, 0–22 min, 95%–65.5% solvent A; 22.5 min, 34.5%–100% solvent B; 22.5–27.5 min, 100% solvent B; 27.8 min, 0%–95% solvent A; and 28–34 min, 95% solvent A. The flow rate was 1 mL/min, the temperature of the column oven was maintained at 40 °C, and the injection volume was 10 μL. The chemical compounds were identified by comparing their retention times with those of standards. Each sample was extracted three times and analyzed in duplicate.

2.3. Extraction and Isolation of **1**

Ground fermented tea leaves (250 g) were sonicated twice for 1 h in the presence of 1250 mL of aqueous methanol (80%) and filtered. The solvent was concentrated in a rotary evaporator (RE-52AA, Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) at 40 °C. The resulting concentrate (50 mL) was directly added to an MCI-gel CHP 20P column (7.0 × 20 cm; 75–150 μm, Mitsubishi Chemical Industries, Tokyo, Japan) and eluted with 10% ethanol (Fengchuan Chenmical Reagent Technologies, Tianjin, China) in H_2O. Fractions (100 mL) were collected; 300–500 mL aliquots were concentrated in a rotary evaporator, added to a Sephadex^{TM} LH-20 column (5.5 × 20 cm, GE Healthcare, Uppsala, Sweden), and eluted with water (Figure 2a). Fractions (50 mL) were collected; 100–200 mL aliquots were concentrated in a rotary evaporator, added to a Sephadex^{TM} LH-20 column (3.0 × 40 cm, Cherishtech, Beijing, China), and eluted with water (Figure 2b). Fractions (20 mL) were collected, and 40–100 mL aliquots were concentrated in a rotary evaporator (Figure 2c). The concentrate (10 mL) was extracted with acetonitrile (50 mL); the clear upper liquid was concentrated in a rotary evaporator until the acetonitrile was removed. Finally, the concentrate was freeze-dried in an FD5-series freeze-dryer (SIM, Beijing, China), resulting in the isolation of **1**.

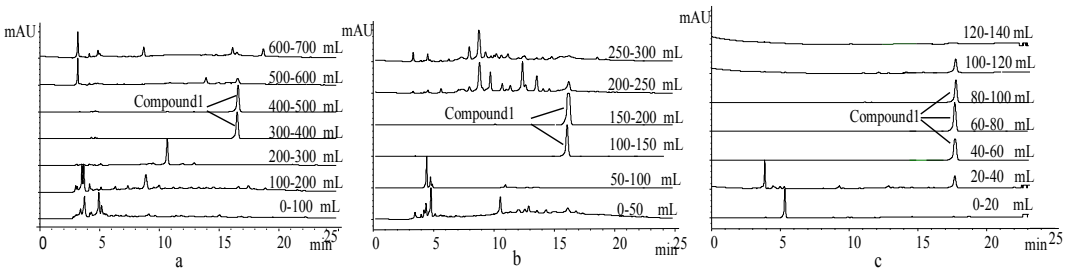


Figure 2. HPLC chromatogram of eluates obtained from column chromatography: (**a**) *d* = 7 cm; (**b**) *d* = 5.5 cm; (**c**) *d* = 3 cm. *d*: diameter.

2.4. Structure Elucidation of **1**

Compound **1** was characterized by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC/MS/MS) (SYNAPT G2 and Xevo G2 QTOF, Waters Corporation, New York, NY, USA), ^{1}H (600 MHz) and ^{13}C Nuclear Magnetic Resonance (NMR) (150 MHz), and Distortionless Enhancement by Polarization Transfer (DEPT) with 2D experiments, e.g., Heteronuclear Singular Quantum Correlation (HSQC), Total Correlation Spectroscopy (TOCSY), and Heteronuclear Multiple Bond Correlation (HMBC) (Bruker BioSpin, Rheinstetten, Germany).

2.5. In Vitro Antioxidant Activity Assays

The antioxidant activity of compound **1** was evaluated by the following assays, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, total antioxidant capacity(T-AOC), hydrogen donating ability, and superoxide anion radical scavenging capacity. DPPH scavenging activity and superoxide anion radical scavenging capacity were determined by the method reported by Zhang [28]. First, DPPH buffer solution (0.1 mmol/mL) (TCI, Shanghai, China) was prepared. Tea extracts (1 mL) of different concentrations were mixed with 3 mL DPPH buffer solution. The mixture was held for 20 min at room temperature. The scavenging effect was determined from ultraviolet (UV) adsorption measurements at 517 nm. The superoxide anion radical scavenging capacity assay consisted of mixing 5 mL Tris–HCl buffer (50 mmol/L, pH = 8.2) (Xilong Chemical, Guangzhou, China) with 0.5 mL of sample, maintaining the solution at 25 °C for 20 min, and adding 0.5 mL 1,2,3-trihydroxybenzene (Sangon Biotech, Shanghai ,China) (30 mmol/L, 25 °C, 4 min). UV absorbance was measured at 325 nm. Total antioxidant capacity and hydrogen donating ability were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Antioxidant activity was expressed as IC50, defined as the concentration of the test compound required to inhibit the formation of radicals by 50%. Vitamin C was used as a positive control.

2.6. Screening of Compound **1** Producing Microbes

Bacteria and fungi were isolated from the fermented tea leaves using the plate dilution method. Tea leaves (25 g) were suspended in 225 mL of sterile 0.9% NaCl (Xilong Chemical, Guangzhou, China), diluted to 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} with sterile water, and spread on Rose Bengal Medium Agar (RBMA; 5 g peptone, 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, and 20 g agar per liter) with 0.1 g/L chloramphenicol and Nutrient Agar (NA; 5 g peptone, 3 g beef extract, 5 g NaCl, and 20 g agar per liter) for fungal and bacterial isolations, respectively. RBMA and NA plates were incubated at 28 °C for 5 d and 37 °C for 2 d, respectively. Bacterial genomic DNA was extracted using the Rapid Bacterial Genomic DNA Isolation Kit (Sangon Inc., Shanghai, China) and used as a template for the Polymerase Chain Reaction (PCR) amplification of bacterial 16S rRNA genes using general bacterial primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGTTACCTTGTTACGACT T-3') [29]. Fungal genomic DNA was extracted using the Rapid Fungal Genomic DNA Isolation Kit (Sangon Inc., Shanghai, China) and used as a template for the PCR amplification of fungal 18S rRNA genes using general primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') [30] and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') [31]. PCR amplification was performed using TaKaRa Ex Taq polymerase (DRR100A, TaKaRa Biotechnology Co., Ltd., Dalian, China). The reaction mixture (50 μL) contained 2 μL of DNA template, 5 μL of Ex PCR buffer, 3 μL of dNTP mixture (25 mM each), and 0.25 μL of TaKaRa Ex Taq polymerase (5 units/μL). The amplification program consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 60 s (extension), and a final extension at 72 °C for 5 min. The PCR fragments were sequenced by Shanghai Sangon Inc. (Shanghai, China) and identified using EzTaxon server [32] and Blastn search.

The bacterial and fungal strains isolated from the fermented tea leaves samples were inoculated into sterile sun-dried green tea and fermented for 14 d at 28 °C. The fermented tea leaves were air-dried and subjected to HPLC analysis.

2.7. Determination of Compounds in Commercial PFPT Samples

In this experiment, 22 PFPT samples were purchased from local markets (Table 1). GA, CAF, catechins, and compound **1** were measured by HPLC.

Table 1. Concentrations of gallic acid, catechins, caffeine, and Teadenol A in commercial post-fermented Pu-erh tea (PFPTs).

No.	PFPT Name	GA	EGC	C	CA	EC	EGCG	GG	ECG	Teaden-ol A
		Mean Content (mg/g) (n = 6)								
1	Jijin ziyin	39.16 ± 1.1	10.85 ± 0.3	8.46 ± 0.5	39.35 ± 0.1	1.80 ± 0.0	22.47 ± 0.0	10.39 ± 0.0	9.52 ± 0.0	0.25 ± 0.0
2	Zichun	53.56 ± 2.1	9.02 ± 0.1	5.35 ± 0.1	46.06 ± 0.9	1.37 ± 0.1	18.48 ± 0.3	9.09 ± 0.3	4.57 ± 0.1	0.20 ± 0.0
3	Shunde Jijin	121.81 ± 1.6	25.06 ± 0.8	15.9 ± 0.5	52.87 ± 0.6	5.02 ± 0.1	43.39 ± 1.4	13.91 ± 0.4	1.90 ± 0.1	1.15 ± 0.0
4	Gongting pu-er	13.23 ± 0.1	1.52 ± 0.0	0.59 ± 0.2	34.88 ± 0.4	0.35 ± 0.1	0.32 ± 0.0	0.20 ± 0.0	0.67 ± 0.1	0.17 ± 0.0
5	Nanye repaocha	11.62 ± 0.8	4.60 ± 0.1	1.85 ± 0.1	30.29 ± 0.8	2.34 ± 0.2	1.68 ± 0.2	0.46 ± 0.1	1.25 ± 0.1	0.40 ± 0.0
6	Mabang	1.55 ± 0.1	3.67 ± 0.3	1.51 ± 0.1	12.17 ± 0.2	1.01 ± 0.3	0.72 ± 0.0	0.47 ± 0.1	0.60 ± 0.1	1.00 ± 0.2
7	Huilong shengtaiqizibing	124.50 ± 1.1	12.24 ± 0.0	11.23 ± 0.0	55.70 ± 0.0	2.74 ± 0.0	4.29 ± 0.1	1.23 ± 0.0	7.87 ± 0.0	1.43 ± 0.1
8	Pinzang bing	6.33 ± 0.2	3.80 ± 0.5	1.18 ± 0.2	36.78 ± 0.5	1.18 ± 0.4	0.98 ± 0.5	0.26 ± 0.1	0.19 ± 0.0	1.18 ± 0.2
9	Menghai qizibing	8.09 ± 0.1	6.93 ± 0.0	2.14 ± 0.0	32.24 ± 0.1	2.23 ± 0.0	0.91 ± 0.0	0.54 ± 0.0	1.37 ± 0.2	1.17 ± 0.2
10	Lizhi hong	22.80 ± 0.5	54.64 ± 1.0	26.16 ± 0.6	55.83 ± 0.6	1.60 ± 0.0	78.07 ± 0.3	20.12 ± 1.0	10.78 ± 0.5	1.28 ± 0.0
11	Nannuo yihao	4.30 ± 0.5	1.50 ± 0.2	0.61 ± 0.1	30.31 ± 0.5	0.34 ± 0.1	0.25 ± 0.0	0.69 ± 0.1	0.47 ± 0.0	5.03 ± 0.2
12	Nanfangjiamu paka	21.62 ± 1.2	11.18 ± 1.1	2.41 ± 0.1	40.45 ± 0.9	2.91 ± 0.2	1.74 ± 0.1	0.59 ± 0.1	1.86 ± 0.1	1.25 ± 0.2
13	Puxiuqizibing	23.96 ± 1.6	9.98 ± 0.1	7.65 ± 0.2	34.12 ± 0.1	1.15 ± 0.1	17.34 ± 0.1	8.46 ± 0.1	1.61 ± 0.1	3.15 ± 0.3
14	Menghaitie bing	18.11 ± 0.4	3.59 ± 0.1	2.15 ± 0.2	36.04 ± 0.1	0.47 ± 0.0	8.34 ± 0.1	3.76 ± 0.0	1.75 ± 0.1	6.20 ± 0.3
15	Xiangyupu-er	60.02 ± 49.5	3.27 ± 0.0	3.99 ± 0.2	44.66 ± 0.1	0.54 ± 0.0	12.30 ± 0.0	6.23 ± 0.1	3.16 ± 0.1	4.10 ± 0.0
16	Nannuoyihao (shucha)	33.78 ± 0.4	4.82 ± 0.2	3.50 ± 0.4	50.99 ± 1.5	1.18 ± 0.1	15.81 ± 0.6	6.99 ± 0.1	3.72 ± 0.5	8.15 ± 0.1
17	Qingyungongma-o	44.34 ± 1.6	6.94 ± 0.6	2.91 ± 0.1	37.88 ± 1.9	1.04 ± 0.1	13.34 ± 0.9	6.69 ± 0.1	2.13 ± 0.3	4.38 ± 0.1
18	Chunqiaomuch	32.90 ± 0.4	9.92 ± 0.5	7.55 ± 0.1	43.67 ± 1.2	1.73 ± 0.0	17.83 ± 0.4	7.20 ± 0.1	4.31 ± 0.4	1.31 ± 0.0
19	Banzhangwang	22.80 ± 0.5	6.86 ± 0.1	4.58 ± 0.4	40.77 ± 0.6	1.60 ± 0.0	18.82 ± 0.5	9.65 ± 0.6	5.96 ± 0.2	7.44 ± 0.3
20	Daixiangyubing	36.82 ± 1.6	9.00 ± 0.2	5.13 ± 0.1	52.79 ± 0.5	1.73 ± 0.1	24.49 ± 0.9	13.53 ± 0.2	5.00 ± 0.3	1.28 ± 0.1
21	Lanxiangguiqi	20.67 ± 0.6	9.49 ± 0.6	6.83 ± 0.6	53.14 ± 1.2	1.18 ± 0.0	21.65 ± 0.5	10.52 ± 1.6	2.05 ± 0.1	5.93 ± 0.0
22	Pu-er sancha	11.75 ± 0.2	1.90 ± 0.0	0.42 ± 0.0	32.52 ± 0.0	0.62 ± 0.0	0.33 ± 0.0	0.15 ± 0.0	1.25 ± 0.9	6.98 ± 0.4

2.8. Statistical Analyses

Data were analyzed by one-way ANOVA and least-significant difference (LSD) for paired data. Data were expressed as mean ± SD (Standard Deviation). Statistical analyses were performed with SPSS 19.0 software packages (v19.0, SPSS Inc., Chicago, IL, USA, 2010); $p < 0.05$ was considered to be statistically significant.

2.9. Nucleotide Sequence Accession Numbers

The bacterial 16S rRNA and fungal 18S rRNA gene sequences were deposited in GenBank under accession numbers KR149596-KR149624 and KR149625-KR149644, respectively.

3. Results and Discussion

3.1. Isolation and Identification of Compound **1**

Ground samples were extracted twice with 80% methanol. Compound **1** was isolated from the extract using column chromatography followed by purity analysis by HPLC. The compound had one peak, and the peak area of the purified compound was 99.94%. Compound **1** consisted of a white powder with a molecular ion peak at *m/z* 276.0638, which corresponded to the molecular formula $C_{14}H_{12}O_6$ (Figure 3). The ^{1}H-NMR spectrum of **1** (Table 2) showed one methylene ($^{\delta}$H 2.85, m), two methine ($^{\delta}$H 4.39, m; and δ 4.52, s), *meta*-coupled ($^{\delta}$H 5.78, J = 2.4 Hz; and δ 5.91, J = 2.4 Hz) aromatic, and three olefinic ($^{\delta}$H 6.51, J = 0.9 Hz; δ 5.19, br s; and δ 5.27, br s) proton signals. The ^{13}C-NMR spectrum (Table 2) of **1** revealed the presence of one phloroglucinol-type aromatic ($^{\delta}$C 95.9, 96.7, 99.3, 156.6, 157.8 and 157.9), one methylene ($^{\delta}$C 25.4), two methine ($^{\delta}$C 72.9 and 73.5), and four olefinic ($^{\delta}$C 109.8, 116.6, 139.3, and 148.1) carbon signals. From these ^{1}H- and ^{13}C-NMR spectral data, we predicted that **1** had ring moieties that resembled the A- and C-ring structures in flavan-3-ol (catechins). The HMBC and HSQC spectra of **1** (Table 2, Figure 4) showed an olefinic carbon sequence (from C-12 to C-15) in the correlation peaks from H-13 to C-12 and C-14, and from H-15 to C-14. Additionally, the correlation peaks from H-15 to C-2 and from H-13 to C-16 in the HMBC spectrum showed the presence

of C-2–C-14 and C-12–C-16 bonds, respectively. Furthermore, NOE spectral data (Table 2, Figure 4) supported the structural correlations observed in the HMBC spectrum. The small *J* value (2.4 Hz) of H-2 observed in the ^{1}H-NMR spectrum of compound **1** (Table 2) indicated a *cis* conformation between the C-2 and C-3 positions. The ^{1}H-NMR and ^{13}C-NMR spectral data of **1** (Table 2) were similar to those of Teadenol A [33,34]. Therefore, compound **1** was identified as Teadenol A (Figure 5).

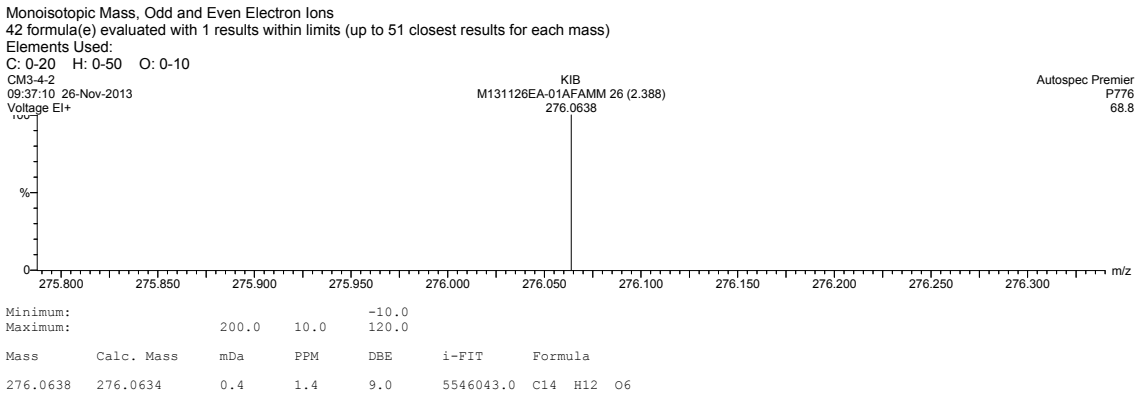
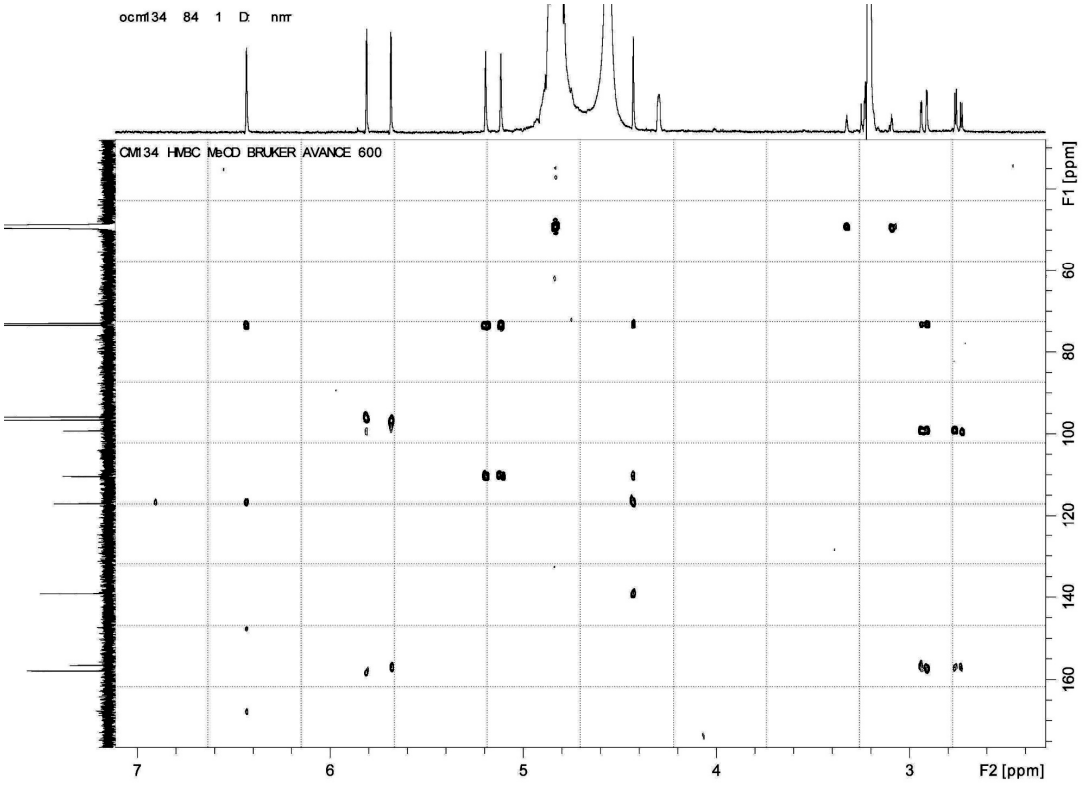
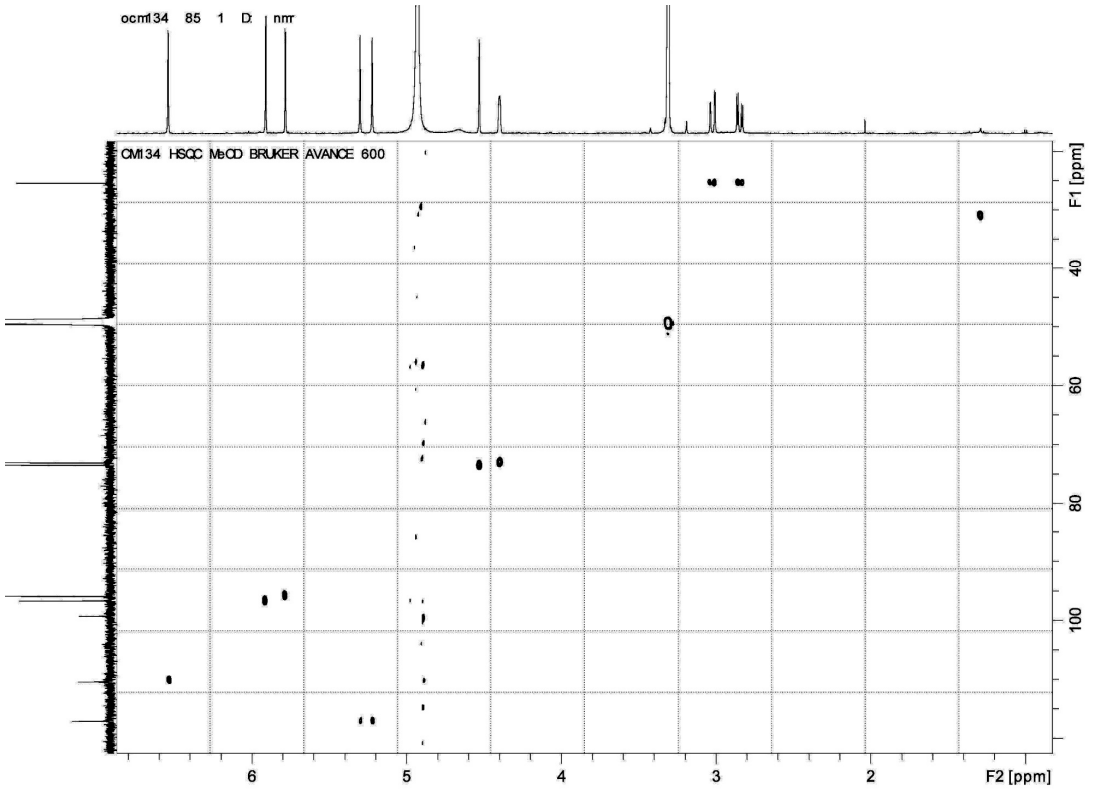


Figure 3. High resolution mass spectrometry assay of compound **1**.

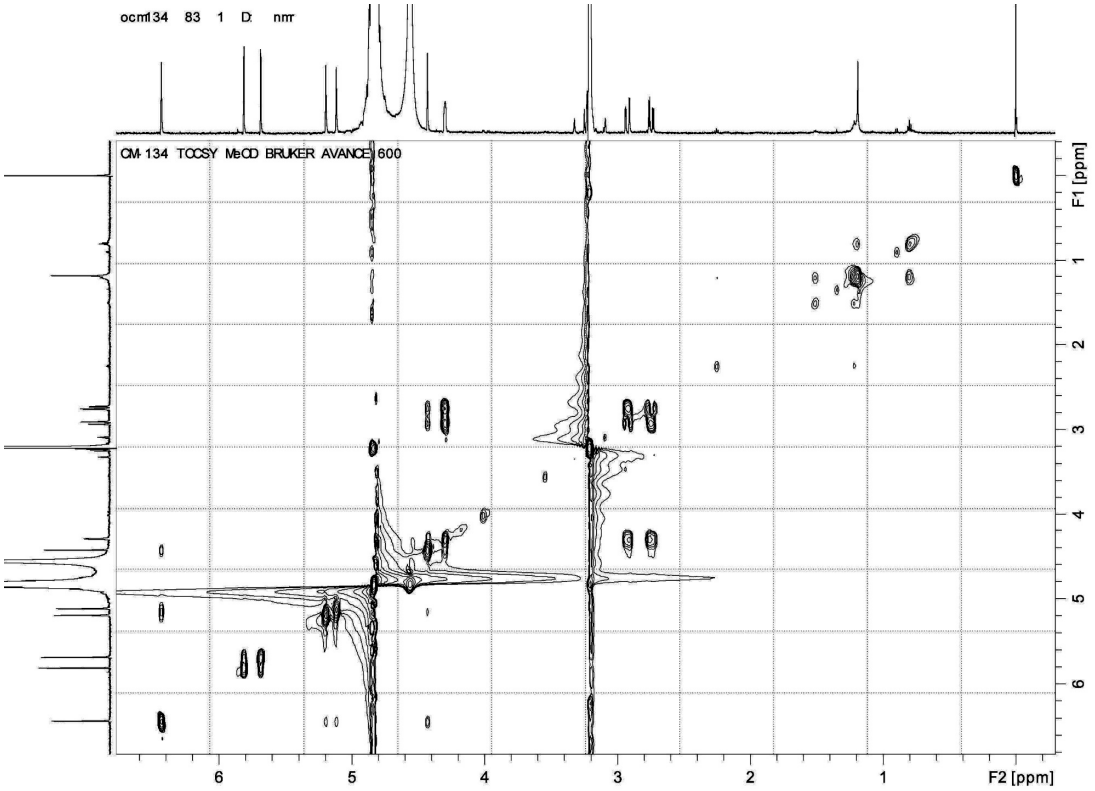


(**a**)

Figure 4. *Cont.*



(**b**)



(**c**)

Figure 4. Nuclear Magnetic Resonance (NMR) spectrometry assay of compound **1:** (**a**) Heteronuclear Multiple Bond Correlation (HMBC); (**b**) Heteronuclear Singular Quantum Correlation (HSQC); (**c**) Total Correlation Spectroscopy (TOCSY).

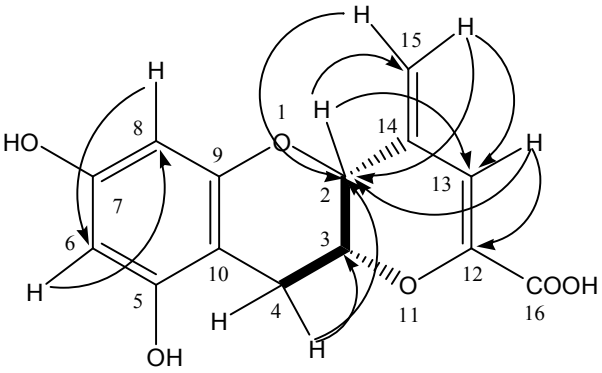


Figure 5. Chemical structure of 2D NMR correlations of compound 1 (◠ HMBC, ▬ 1H - 1H COSY).

Table 2. Nuclear Magnetic Resonance (NMR) data of compound **1** (in MeOD).

Position	δC	δH	HMBC (^{1}H–^{13}C)	NOE (^{1}H–^{1}H)
2	73.5	4.52 (1H, s)	C-13, 14, 15	H-3, 4, 13, 15
3	72.9	4.39 (1H, m)	-	-
4	25.4	2.85 (1H, dd, *J* = 4.8, 17.4 Hz)	C-5, 10	H-2, 3
		3.02 (1H, dd, *J* = 1.8, 17.4 Hz)	C-2, 3, 5, 10	H-2, 3
5	157.8	-	-	-
6	96.7	5.91 (1H, d, *J* = 2.4)	C-5, 8, 10	H-8
7	157.9			
8	95.9	5.78 (1H, d, *J* = 2.4)	C-6, 9, 10	H-6
9	156.6	-	-	-
10	99.3	-	-	-
12	148.1	-	-	-
13	109.8	6.51 (1H, s)	C-2, 12, 16	H-2, 15
14	139.3	-	-	-
15	116.6	5.19 (1H, s)	C-2, 13	H-13
16	168.3	5.27 (1H, s)	C-2, 13	H-13

3.2. Isolation and Identification of Fungal and Bacterial Strains

A total of 20 fungal and 29 bacterial strains, belonging to seven fungal and 13 bacterial species, respectively, were isolated from fermented tea leaves (Table 3). The strains were individually inoculated into tea leaves to determine their ability to synthesize Teadenol A. Teadenol A was detected in tea leaves fermented by *Aspergillus niger* and *A. tamari* at 28 °C for 14 d at concentrations ranging from 9.85 ± 1.17 mg/g to 12.93 ± 0.38 mg/g. This result was in agreement with the findings of Wulandari, who reported that tea leaves fermented by *Aspergillus* spp. contain high concentrations of Teadenols [33,34]. Additionally, a method to produce Teadenol A-rich PFPT has been developed by adding *A. niger* or *A. tamari* during SSF. Teadenol A has been extracted using ethanol followed by column chromatography.

Table 3. Identification of bacterial and fungal strains isolated during solid state fermentation (SSF) of PFPT.

No.	Isolates (GenBank Accession No.)	Length (bp)	Results of EzTaxon	
			Closest Match (GenBank Accession No.)	Similarity (%)
1	1-3-b-5 (KR149614)	1373	*Achromobacter xylosoxidans* DSM 10346(T) (Y14908)	99.854
2	2-3-b-2 (KR149617)	1365	*Achromobacter xylosoxidans* NBRC 15126(T) (CP006958)	99.93
3	1-1-b-5 (KR149606)	1385	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42(T) (CP000560)	99.928
4	3-3-b-3 (KR149598)	1345	*Bordetella avium* 197N (AM167904)	99.18
5	1-1-b-3 (KR149624)	1385	*Enterobacter asburiae* JCM 6051(T) (AB004744)	99.35
6	1-1-b-4 (KR149608)	1375	*Enterobacter asburiae* JCM 6051(T) (AB004744)	99.345
7	1-1-b-6 (KR149611)	1367	*Enterobacter asburiae* JCM 6051(T) (AB004744)	99.415

Table 3. *Cont.*

No.	Isolates (GenBank Accession No.)	Length (bp)	Results of EzTaxon	
			Closest Match (GenBank Accession No.)	Similarity (%)
8	1-2-b-1 (KR149610)	1376	*Enterobacter hormaechei* ATCC 49162(T) (AFHR01000079)	99.345
9	2-4-b-4 (KR149622)	1298	*Microbacterium sediminis* YLB-01(T) (HQ219727)	98.54
10	1-4-b-7 (KR149612)	1322	*Ochrobactrum pseudintermedium* ADV31(T) (DQ365921)	99.849
11	2-3-b-5 (KR149618)	1323	*Ochrobactrum pseudintermedium* ADV31(T) DQ365921	99.697
12	1-1-b-8 (KR149609)	1384	*Pantoea dispersa* LMG 2603(T) (DQ504305)	100
13	3-3-b-2 (KR149596)	1373	*Pseudomonas aeruginosa* JCM 5962(T) (BAMA01000316)	99.93
14	3-4-b-2 (KR149600)	1370	*Pseudomonas aeruginosa* JCM 5962(T) (BAMA01000316)	99.93
15	3-4-b-1 (KR149599)	1375	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.854
16	3-5-b-5 (KR149604)	1363	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.853
17	1-4-b-9 (KR149607)	1376	P *seudomonas aeruginosa* LMG 1242(T) (Z76651)	99.854
18	1-5-b-2-1 (KR149613)	1372	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.854
19	1-4-b-1 (KR149615)	1371	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.854
20	1-3-b-2 (KR149616)	1379	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.855
21	2-4-b-1 (KR149619)	1369	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.854
22	2-4-b-2 (KR149620)	1330	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.85
23	2-4-b-5 (KR149623)	1387	*Pseudomonas aeruginosa* LMG 1242(T) (Z76651)	99.856
24	3-3-b-4 (KR149597)	1377	*Pseudomonas beteli* ATCC 19861(T) (AB021406)	99.564
25	3-5-b-2 (KR149602)	1358	*Pseudomonas plecoglossicida* FPC951(T) (AB009457)	99.853
26	3-4-b-5 (KR149601)	1368	*Sphingobacterium thalpophilum* DSM 11723(T) (AJ438177)	99.415
27	2-4-b-3 (KR149621)	1373	*Sphingobacterium thalpophilum* DSM 11723(T) (AJ438177)	99.854
28	2-5-b-1 (KR149605)	1356	*Staphylococcus sciuri* subsp. *sciuri* DSM 20345(T) (AJ421446)	100
29	3-5-b-4 (KR149603)	1385	*Staphylococcus sciuri* subsp. *sciuri* DSM 20345(T) (AJ421446)	100
30	1-5-f-10 (KR149625)	506	*Acremonium falciforme* (DQ094533)	99.33
31	y-2-b-2 (KR149637)	546	*Aspergillus fumigatus*_1 (AF455542)	100
32	3-2-f-4(KR149634)	534	*Aspergillus fumigatus*_1 (JN226940)	100
33	2-5-f-1 (KR149635)	545	*Aspergillus fumigatus*_1 (JN226940)	100
34	2-1-f-1 (KR14964)	542	*Aspergillus niger*_1 (EF660199)	99.8
35	y-2-f-4 (KR149644)	531	*Aspergillus niger*_1 (EF660199)	99.6
36	2-4-f-2 (KR149630)	529	*Aspergillus niger*_1 (EU821308)	100
37	3-1-f-4(KR149642)	529	*Aspergillus niger*_1 (EU821308)	100
38	1-5-f-15 (KR149631)	547	*Aspergillus sclerotiorum*_1 (GQ398087)	100
39	y-2-f-7 (KR149626)	504	*Aspergillus sydowii*_1 JN986787	100
40	2-5-f-4 (KR149638)	554	*Aspergillus tamarii*_1 (KC621084)	100
41	3-5-f-1 (KR149640)	553	*Aspergillus tamarii*_2 (DQ411548)	100
42	1-4-f-9 (KR149628)	529	*Aspergillus tamarii*_2 (EU021614)	100
43	2-4-f-4 (KR149636)	538	*Aspergillus tamarii*_2 (EU021614)	100
44	3-2-f-1 (KR149627)	537	*Aspergillus tubingensis* (EU821292)	100
45	3-1-f-2 (KR149633)	538	*Aspergillus tubingensis* (EU821292)	100
46	y-2-b-1 (KR149641)	533	*Aspergillus tubingensis* (EU821292)	100
47	3-5-f-2 (KR149632)	510	*Earliella scabrosa*_2 (EU661875)	98.88
48	1-5-f-9 (KR149639)	706	*Lichtheimia corymbifera*_9 (FJ719392)	100
49	2-2-f-3 (KR149629)	635	*Rhizopus microsporus*_1 (AF115729)	100

3.3. Determination of Teadenol A in Commercial PFPT

Aspergillus spp. is the dominant species in SSF of PFPT and play crucial roles in the quality of PFPT [2]. In this study, Teadenol A was detected in tea leaves fermented by *Aspergillus* spp. To assess whether PFPT contains Teadenol A, we measured 22 commercial PFPT samples. Teadenol A was detected in each sample, at concentrations ranging from 0.17 $\pm$ 0.1 to 8.15 $\pm$ 0.1 mg/g (Table 1). However, Teadenol A was not detected in one PET [34], probably because the tea was NFPT. *Aspergillus* spp. is not involved in the production of NFPT; therefore, NFPT lacks Teadenol A.

3.4. Bioactivity of Teadenol A

Wulandari *et al.* [34] and Yanagita *et al.* [35] reported that Teadenol A stimulates the secretion of adiponectin and inhibits the expression of protein tyrosine phosphatase-1B (PTP1B). Adiponectin, a polypeptide that is highly specific to the adipose tissue, has anti-inflammatory and antiatherogeneic properties and beneficial effects on metabolism [36]. Adiponectin, which reduces the relative risk of type 2 diabetes [37], is inversely correlated to visceral adipose tissue [38]. Through its effect on adiponectin secretion, Teadenol A has anti-obesity properties. PTP1B is a negative regulator of insulin and leptin signal transduction [39] and a novel therapeutic target for type 2 diabetes mellitus, obesity, and insulin resistance [40]. Teadenol A may have positive regulatory effects on insulin and leptin signal transduction by inhibiting the expression of PTP1B. Due to its multiple health benefits, Teadenol

A has been used in pharmaceutical drugs, food and feed, cosmetics, and assay reagents [35]. In this study, the antioxidant properties of Teadenol A were determined *in vitro*. The IC50 values from the DPPH scavenging activity and superoxide anion radical scavenging ability were 64.8 μg/mL and 3.335 mg/mL, respectively. The IC50 values from total antioxidant capacity and hydrogen donating ability were 17.6 U/mL and 12 U/mL, respectively. Teadenol A may be partly responsible for the health benefits of PFPT. Future studies should assess the exact role of Teadenol A in PFPT and possible synergistic effects with other bioactive compounds.

In conclusion, Teadenol A was isolated and identified following SSF of PFPT. The formulation of Teadenol A was dependent on *A. niger* and *A. tamari*. Teadenol A promoted the secretion of adiponectin, inhibited the secretion of PTP1B, and had antioxidant effects. Additionally, Teadenol was detected in variable amounts in 22 commercial PFPTs. Teadenol A is an important bioactive compound of PFPT. This study advances our knowledge about the health benefits and bioactive compounds of PFPT and provides insight into specific fungi in SSF.

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Abbreviations

The following abbreviations are used in this manuscript:

C	(+)-catechin
CAF	Caffeine
EC	(−)-epicatechin
ECG	(−)-epicatechin 3-*O*-gallate
EGC	(−)-epigallocatechin
EGCG	(−)-epigallocatechin 3-*O*-gallate
GA	gallic acid
GG	1,4,6-tri-*O*-galloyl-β-*D*-glucose
HPLC	high-performance liquid chromatography
NA	Nutrient Agar
NFPT	non-fermented Pu-erh tea
PET	Pu-erh tea
PFPT	Post-fermented Pu-erh tea
RBMA	Rose Bengal Medium Agar
SSF	solid state fermentation
TB	theabrownin
TF	theaflavin
TR	thearubigin
UPLC-MS/MS	ultra-performance liquid chromatography tandem mass spectrometry

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