

**ANALYSIS OF PHENOLIC METABOLITES
IN THE FERMENTED TEAS**

(発酵茶に含まれるフェノール性代謝成分の解析)

Rani Agustina Wulandari

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Plant metabolites

Plants produce a large number of secondary metabolites, which are classified into several groups according to their biosynthetic routes and structural features. Phenolic compounds are the most widely distributed secondary metabolites (Yazaki *et al.*, 2008). Flavonoids are the most ubiquitous phenolic compounds found in nature. These compounds have diverse physiological and pharmacological activities such as estrogenic, anti-tumor, anti-microbial, anti-allergic, and anti-inflammatory effects (Das and Rosazza, 2006). Flavonoids may help provide protection against diseases by contributing, along with antioxidant vitamins and enzymes, to the total antioxidant defense system of the human body. Epidemiological studies have shown that flavonoid intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks (Buhler and Miranda, 2000).

The basic flavonoid structure contains the flavan nucleus, which consists of 15 carbon atoms derived from a C₆-C₃-C₆ skeleton. Different classes of flavonoids are distinguished by additional oxygen-heterocyclic rings by positional differences of the B-ring, and by hydroxyl, methyl, isoprenoid, and methoxy groups distributed in different patterns about the rings. Skeletons of some common flavonoids are such as chalcone, flavone, flavonol, flavanone, catechin, isoflavone and isoflavanone (Das and Rosazza, 2006).

Flavonoids and phenolic acids have many functions in plants. They act as cell wall support materials (Wallace and Fry, 1994) and as colourful attractants for birds and insects helping seed dispersal and pollination (Harborne, 1994). Phenolic compounds are also important in the defence mechanisms of plants under different environmental stress conditions such as wounding, infection, and excessive light or UV irradiation (Bennet and Wallsgrave, 1994; Dixon and Paiva, 1995).

Biotransformation of numerous flavonoids catalyzed mainly by microbes and few plant enzymes are described in four different flavonoid classes : chalcones, isoflavones, catechins, and flavones. Both phase I (oxidative) and phase II (conjugative) biotransformations represent a variety of reactions including condensation, cyclization, hydroxylation, dehydroxylation, alkylation, O-dealkylation, halogenation, dehydrogenation, double-bond reduction, carbonyl reduction, glycosylation, sulfation, dimerization, or different types of ring degradation (Das and Rosazza, 2006).

1.2. Tea catechins

Green tea is rich in polyphenols, most abundant polyphenols are catechins. Tea catechins have been found to be better antioxidants than vitamins C and E, tocopherol and carotene (Sharangi, 2009). Catechins are colourless, water-soluble compounds which impart bitterness and astringency to green tea infusion. Almost all of the characteristics of manufactured tea, including

its taste, colour, and aroma, are associated directly or indirectly with modifications of catechins (Wang *et al.*, 2000).

Catechins are antioxidants known to exhibit beneficial biological activities which can scavenge various forms of free radicals (Zhong *et al.*, 2008; Jeong and Kong, 2004). Attention has been given to various pharmacological properties of catechins, including their anti-oxidative, anti-tumoral, and anti-mutagenic effects. Catechins may function as scavengers of active oxygen species in biological systems. They seem to play an important role in reducing the risks for a number of diseases, like cancer, coronary heart diseases, arteriosclerosis, ischaemia, and inflammatory diseases (Zhong *et al.*, 2008). They may prevent cardiovascular diseases, probably through their ability to inhibit oxidation of low density lipoprotein (LDL), to lower the plasma cholesterol level, and to prevent platelet aggregation (Jeong and Kong, 2004).

There are five major catechins in green tea. They are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), and (-)-gallocatechin gallate (GCG). Each catechin has its own biological features (Zhong *et al.*, 2008). Around 54%-59% of green tea catechins is EGCG (Zhong *et al.*, 2008; Okumura *et al.* 2008) (Fig. 1). In green tea (-)-EGCG content is the highest followed by (-)-EGC > (-)-ECG > (-)-EC. Other catechins ((-)-GC, (-)-CG and (+)-C) are usually found in trace amounts, indicating that they are minor components of tea leaves (Demeule *et al.*, 2002). EGCG has been shown to reduce significantly total cholesterol and LDL plasma levels in rats fed a diet containing 1% EGCG in 4 weeks (Jeong and Kong, 2004).

1.3. Tea classification

Tea is one of the most popular beverages consumed in the world and is produced from the leaves of *Camellia sinensis* L. and consumed for human health benefits and pharmaceutical activities (Zhou *et al.*, 2005). Tea plants belong to the Theaceae family and come from two main varieties: *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* (Dufresne and Farnworth, 2000). The first apical leaves are picked from the evergreen shrub and usually pruned to 2-5 feet for cultivation. It can be processed by different methods. Fresh tea leaves are readily dried with or without a fixation step to inactivate enzymes (Zhou *et al.*, 2005; Dufresne and Farnworth, 2000)

By different stages of oxidative processing, teas are classified into unfermented (green), semi-fermented (oolong) and full-fermented (black) types (Tanaka and Kouno, 2003). The essential difference in processing is the fermentation before heating which allowed the result of the oxidation of leaf polyphenols through a multi-stage enzymatic process (Dufresne and Farnworth, 2000; Angayarkani, 2002). Among various tea products, black tea is the most popular around the world, accounting for almost 80% of the world tea production (Tanaka *et al.*, 2002). Black tea is type of fully fermented tea leaves by its own oxidative enzymes. On the other hand, post fermented tea is the type of microbial fermented tea (Mo *et al.*, 2008).

Although plants can provide a vast source of natural preservatives, many potential biological preservatives are originated from traditional food fermentations (Mo *et al.*, 2008). Tea biological activities and health function have

been widely explored. Recently, several microbial fermented teas got noticed in the world, probably not only because of the trade expansions between China and the west, but also because of their potential constituents and bioactivities which are associated with several health benefits (Mo *et al.*, 2008; Lu *et al.*, 2007). A few studies reveal that extracts from microbial fermented teas contain natural anti-microbial components that have inhibitive effect on several food borne pathogen and spoilage bacteria (Mo *et al.*, 2008).

1.4. Microbial fermented tea

One of the well-known microbial fermented tea is pu-er tea, which have been produce in China. Pu-er tea is post-fermented tea produced majorly in Yunnan province, China. Post-fermentation is a tea production style in which the tea leaves undergo a long time microbial fermentation process after they are dried and rolled.

It is noticeable that the chemical constituents of pu-er tea are distinctly different from those of the other kind of tea *e.g.* green tea, oolong tea, and black tea. Most of the characteristic flavan-3-ol derivatives in green tea, such as EGCG, EGC, and ECG, have decreased, whereas the content of polyphenol polymers is remarkably enhanced in pu-er tea. At the same time, several polyoxygenated compounds are formed from the related catechins derivatives during the processing procedure of pu-er tea (Zhou *et al.*, 2005) (Fig. 2).

Fermentation of the green tea with microorganisms resulted in great change in their chemical constituents during the processing procedure of pu-er

tea (Zhou *et al.*, 2005). It is mainly fermented by anaerobic bacteria, but some fungi such as *Aspergillus*, *Penicillium*, *Rhizopus* and *Saccharomyces* also play some role in fermentation (Zhou *et al.*, 2004).

The content of tea polyphenols including tea catechins will decrease in the fermentation process of pu-er tea, while anti-microbial activity will increase which can be observed from the formation of anti-microbial components or the conversion of tea polyphenols to other new compounds (Mo *et al.*, 2008).

Although a large number of polyphenol derivatives have been identified in various types of green (non-fermented), oolong (semi-fermented) and black (fully-fermented by oxidizing enzyme) teas (Peterson *et al.*, 2005), metabolites in post-fermented teas such as pu-er tea have not so been investigated.

1.5. Objective of this research

The goal of this research is to investigate the phenolic metabolites which can be produced by tea fermentation with various types of fungi, and to study their biosynthetic process during the fermentation.

The objectives of this study are :

1. To find out new phenolic metabolites in the fermented teas.
2. To investigate the phenolic metabolites in the various commercial teas and in various teas fermented with different fungi.
3. To study the biosynthesis of new phenolic compounds.

CHAPTER 2
NEW PHENOLIC COMPOUNDS FROM TEA LEAVES FERMENTED WITH
***Aspergillus* sp.**

2.1. Introduction

Tea is an aqueous infusion of dried leaves of the plant *C. sinensis* and is the most popular beverage consumed by world-wide human society. Tea is a dietary source of antioxidant nutrients, such as carotenoids, tocopherols, ascorbic acid, and non-nutrient phytochemicals. It is reported that tea extracts inhibit human salivary amylase and disturb digestion and nutrition. Numerous studies have demonstrated that aqueous extracts of black tea and green tea possess anti-mutagenic, anti-inflammatory, hypocholesterimic, anti-diabetes, anti-bacterial, anti-tumor, anti-UV induced oxidative DNA damage, and anti-carcinogenic activities in a variety of experimental animal systems (Pasha and Reddy, 2005).

Polyphenols are the active components responsible for the beneficial effects of drinking green tea (Okumura *et al.*, 2008). A large number of polyphenol derivatives have been identified in various types of green (non-fermented), oolong (semi-fermented) and black (fully-fermented by oxidizing enzyme) teas (Peterson *et al.*, 2005). And recently, some tea (*C. sinensis* L.) products which have been selectively fermented with *Aspergillus*, *Lactobacillus*, *etc.* have been developed and circulated popularly in Japan. Some oxidative

metabolites in Chinese well-known post-fermented one (pu-er tea), which was fermented with some bacteria of *Aspergillus*, *Rhizopus*, etc., also have been reported (Zhou *et al.*, 2005; Lu *et al.*, 2007; Zhou *et al.*, 2004). Although investigation of various commercial teas already identified, metabolites in post-fermented teas have not so been investigated.

Some oxidative metabolites of catechins in well-known pu-er tea have been elucidated (Lu *et al.*, 2007), and several polyoxygenated compounds are formed from the related catechin derivatives during the processing procedure of pu-er tea (Zhou *et al.*, 2005). Some new phenolic compounds were isolated from some fermented teas. Puerins A and B were isolated from pu-er tea (Zhou *et al.*, 2005), and blumenol B was also isolated from tea extract fermented with *Fusarium solani* (Huang *et al.*, 2008).

In this experiment, two new phenolic compounds were isolated from the Japanese fermented tea, which was selectively fermented with *Aspergillus* sp. (Wulandari *et al.*, 2011, Yanagita *et al.*, 2011a; Yanagita *et al.*, 2011b). The chemical structures of the isolated compounds were elucidated based on the analyses of their spectroscopic data.

2.2. Materials and methods

2.2.1. General

Nuclear magnetic resonance (NMR) spectra were recorded in DMSO- d_6 or CD₃OD using a JEOL JNM-A500 spectrometer (500 MHz for ¹H-NMR and 125 MHz for ¹³C-NMR). MS value was obtained on a JEOL JMS-700T spectrophotometer. Optical rotation was measured with JASCO DIP-1000 Digital Polarimeter. IR spectra were measured using JASCO FT/IR-400 Spectrophotometer. UV spectra were measured using JASCO V-560 UV/VIS Spectrophotometer. Mps were measured with Yanagimoto micromelting point apparatus and were not corrected. For column chromatography, DIAION HP20SS (Mitsubishi Chemical Corporation), Sephadex LH20 (Pharmacia Corporation), ODS-G3 (FUJI GEL HANBAI CO. LTD) and Preparative C₁₈ 125 Å (Waters Corporation) were used.

2.2.2. Plant material

Tea (*Camellia sinensis* L.) leaves fermented with *Aspergillus* sp. (PK-1, FARM AP-21280) (Kawamura, 2008) were gifted from TOYODAHIRYO CO. LTD (Shizuoka, Japan). The commercial name of the tea product is '*Kippuku-cha*'. A voucher sample is deposited in the laboratory of Analysis of Plant Metabolism at the Faculty of Agriculture, Saga University.

2.2.3. Extraction and isolation

Tea leaves (207 g, dry weight) were extracted with 80% EtOH (800 ml) at room temperature. After 12 hour the solvent was applied to column chromatography. The solvent was concentrated *in vacuo* (37 g) and subjected to a column of DIAION HP20SS which was eluted with H₂O containing increasing amount of MeOH to afford four fractions of Fr. 1 (5 g), Fr. 2 (1 g), Fr. 3 (17 g) and Fr. 4 (3 g). Fr. 3 was applied to Sephadex LH20 column chromatography with elution with 60% aqueous MeOH to give two fractions (Fr. 3-1 and Fr. 3-2). Fr. 3-1 (13 g) was applied to ODS-G3 column chromatography with stepwise elution with H₂O and MeOH to give compound **1** (390 mg). Fr. 3-2 (2.3 g) was purified with Sephadex LH20 (80% EtOH), ODS-G3 (stepwise elution with H₂O and MeOH) and Preparative C₁₈ 125 Å (stepwise elution with H₂O and MeOH) column chromatographies to afford compound **2** (88 mg). The isolation procedure of new compounds (**1** and **2**) is shown in Fig. 3.

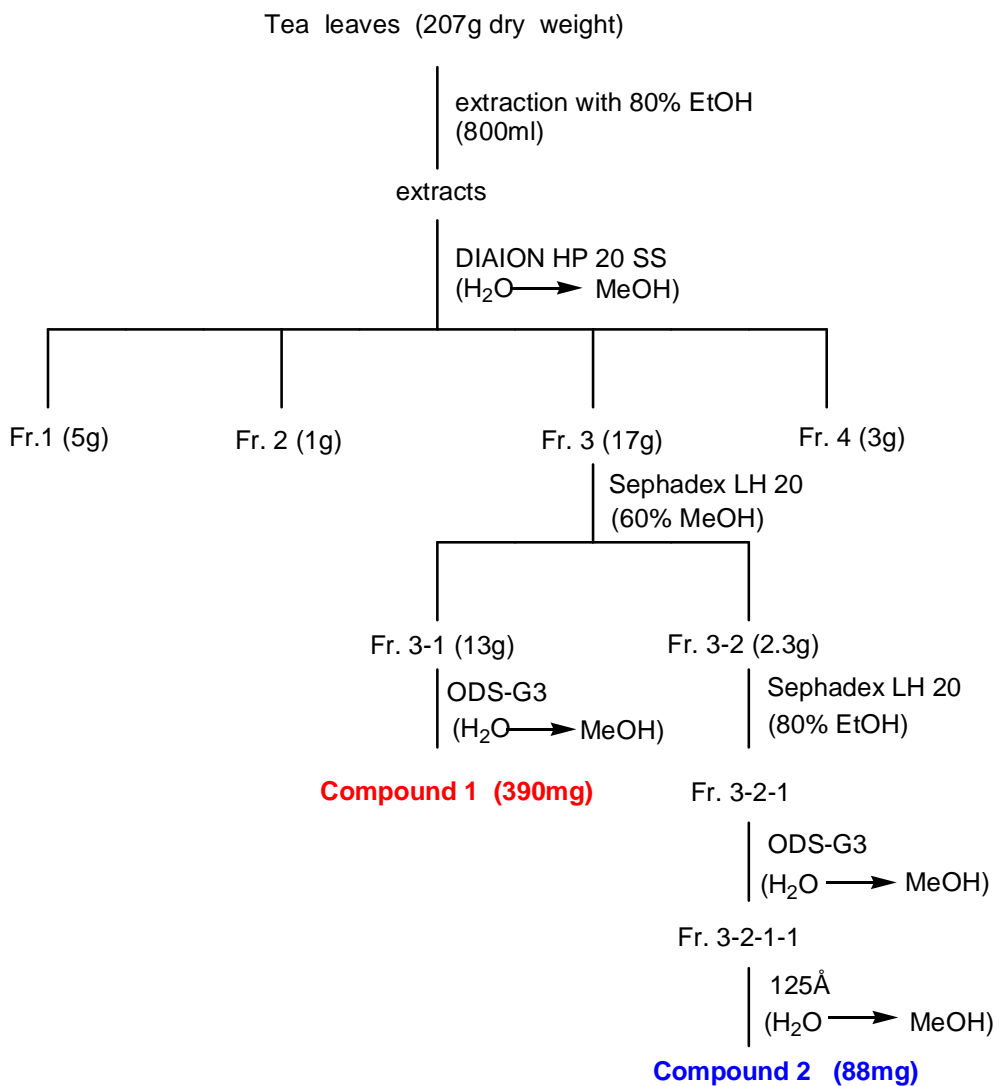


Fig. 3. Isolation of new phenolics using column chromatography

2.2.4. Biosynthetic preparation of compounds **1** and **2**

EGCG (16 mg, purchased from SIGMA) was dissolved in H₂O (20 ml) and autoclaved (121°C, 15 min). In the autoclaving, about half amount of EGCG in the solution was transformed to be its C-2 epimer GCG (Huang *et al.*, 2004) (Fig. 1). *Aspergillus* sp. (PK-1, FARM AP-21280) subcultured on Potato Dextrose Agar solid medium, was inoculated (piece of 1 cm x 1 cm) into the autoclaved solution (mixture of EGCG and GCG). The mixed solution was incubated on a rotary shaker (60 rpm) at 25°C in the dark condition.

After 2 weeks of the culture, the constituents in the incubated solution were analyzed by HPLC. The solution was filtered through Millipore filter (0.45 µm) and subjected to HPLC system as follows; column; TOSOH ODS 80Ts (4.6 mm x 250 mm, TOSOH Corporation), mobile phase; 0.1 % formic acid – CH₃CN (9:1 to 1:4 in 30 min), flow rate; 0.6 ml/min, column temperature; 40°C, detection; 280 nm (UV); Retention times (min) of compounds; EGCG (18.7), GCG (19.8), **1** (24.9) and **2** (27.7). The amounts of the detected compounds (**1** and **2**) were expressed as mean ± SD from three replications.

2.3. Results and Discussion

Tea (*C. sinensis* L.) leaves fermented with *Aspergillus* sp. (PK-1, FARM AP-21280) (Kawamura, 2008) were extracted with 80% aqueous ethanol. Two new compounds (**1** and **2**) were isolated from the extract using the method of column chromatography (Fig. 3.)

Compound **1** gave a molecular ion peak at m/z 276.0638, which corresponded to the molecular formula $C_{14}H_{12}O_6$. The 1H -NMR spectrum of compound **1** (Table 1; Fig. 5A) showed one methylene (δ_H 2.76; m), two methine (δ_H 4.36; m and δ_H 4.56; s), *meta*-coupled (δ_H 5.64; $J=2.3$ Hz and δ_H 5.91; $J=2.3$ Hz) aromatic and three olefinic (δ_H 6.51; $J=0.9$ Hz, δ_H 5.27; br.s and δ_H 5.39; br.s) proton signals. The ^{13}C -NMR spectrum (Table 1; Fig. 5B) of compound **1** also indicated one phloroglucinol-type aromatic (δ_C 94.2, 95.6, 96.9, 154.7, 156.3 and 156.6), one methylene (δ_C 24.1), two methine (δ_C 70.8 and 70.9) and four olefinic (δ_C 110.2, 118.0, 136.5 and 144.1) carbon signals. From these spectral data of 1H - and ^{13}C -NMR, compound **1** was supposed to have ring moieties which were resembles to A- and C-ring structures in flavan 3-ol (catechins).

IR spectrum of compound **1** (Fig. 4) suggested the presence of COOH (cm^{-1} : 3420, 1700 and 1611), which carbon signal was also observed in the ^{13}C -NMR spectrum (δ_C 163.2, C-16). The HMBC spectrum of compound **1** (Table 1; Fig. 6) showed olefinic carbon sequence (from C-12 to C-15) by 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 H-13 to C-16 in the HMBC spectrum showed C-2 - C-14 and C-12 - C-16 bonds, respectively. The NOE spectral data

(Table 1; Fig. 7) also supported the structural correlations observed in the HMBC spectrum. The small J-value (s) of H-2 observed in the $^1\text{H-NMR}$ spectrum of compound **1** (Table 1; Fig. 5A) indicated the *cis*-conformation between C-2 and C-3 positions.

Compound 1. White crystal. Mp 235-240°C (decomposed). IR (KBr) cm^{-1} : 3420, 1700, 1632, 1611, 1386, 1273, 1260, 935 (Fig. 4). $\text{UV}\lambda_{\text{max}}$ (MeOH-DMSO 49:1) nm (log ϵ): 273 (3.86). $[\alpha]_{\text{D}}^{21} + 467.8^\circ$ (c 0.15, DMSO). $^1\text{H-}$ and $^{13}\text{C-NMR}$ (in DMSO- d_6) (Table 1; Fig. 5A). HRFAB-MS, m/z : 276.0638 (Calcd for $\text{C}_{14}\text{H}_{12}\text{O}_6$: 276.0634).

Concerning the partial structure (ring moieties made from C-2 to C-10 carbons), compound **1** was suspected to be a biosynthetic metabolite originated from tea catechins such as EGCG. To determine the possibility of the enzymatic yield of compound **1** from tea catechins, autoclaved EGCG solution, which concluded the C-2 epimeric compound GCG (Huang *et al.*, 2004), was incubated with *Aspergillus* sp. (PK-1, FARM AP-21280). By HPLC analysis, compound **1** ($62.5 \pm 15.6 \mu\text{g}/20 \text{ ml}$ solution) was detected in the incubated solution indicating that compound **1** was a metabolite originated from EGCG (Fig. 8). This result clearly indicated that the absolute configuration of C-3 position of compound **1** was identical with that (R-configuration) of EGCG having R-configurations at C-2 and C-3. From the data mentioned above, the chemical structure of compound **1**, which was named as teadenol A, was concluded to be the one as shown in Fig. 12.

Compound **2** gave a molecular ion peak at m/z 276.0640 (corresponding molecular formula: $C_{14}H_{12}O_6$), which was identical to that of compound **1**. The 1H - and ^{13}C -NMR spectral data of compound **2** (Table 2; Fig. 9) were also similar to those of compound **1**. From HMBC and NOE spectral data (Table 2; Figs. 10 and 11), compound **2** was supposed to be the isomeric compound of compound **1**. In the 1H -NMR spectrum of compound **2** (Table 2; Fig. 9A), a large J-value (10.5 Hz) was observed at H-2 signal (δ_H 4.36) showing the *trans*-conformation between C-2 and C-3 positions. In addition, compound **2** ($24.4 \pm 7.3 \mu g/20$ ml solution) was also detected in the incubated solution above in the HPLC analysis (Fig. 8), which result showed that compound **2** was biosynthetically yielded from a tea catechin (*i.e.* GCG). Therefore, the absolute configurations of C-2 and C-3 positions in compound **2** were concluded to be *S*- and *R*-configurations, respectively, which were identical to those of GCG. In conclusion, chemical structure of compound **2** was shown in Fig. 12 and this compound was named as teadenol B.

Compound 2. Off-white crystal. Mp 258-275°C (decomposed). IR (KBr) cm^{-1} : 3431, 1684, 1624, 1607, 1382, 1283, 1256, 914, 828. UV λ_{max} (MeOH) nm ($\log \epsilon$): 272 (4.07). $[\alpha]_D^{20} - 27.7^\circ$ (c 0.18, MeOH). 1H - and ^{13}C -NMR (in CD_3OD) (Table 2; Fig. 9). HRFAB-MS, m/z : 276.0640 (Calcd for $C_{14}H_{12}O_6$: 276.0634).

In the tea manufacturing stages, the leaves were strongly heated following the selective fermentation with *Aspergillus* sp. (Kawamura, 2008). In the heating process, epimerization of C-2 position in tea catechins were occurred to give catechin-type catechins such as GCG, CG, *etc.* (Fig. 1) (Huang *et al.*, 2004).

Teadenols A and B were supposed to be biosynthetically yielded from EGCG and GCG, respectively, by the enzymatic reaction in the fermentation process of tea leaves with *Aspergillus* sp.

Arunachalam (2003) reported that *A. flavus*, *A. fumigatus*, *A. niger*, *A. teneus*, *Penicillium* sp., *P. janthinellum*, *Streptomyces* sp., and *Fusarium* sp. have a capability to utilize, decompose, or degrade 90% of catechins in tea leaves. Stoilova *et al.* (2007) also reported that fungi are capable of assimilating a wide variety of carbon sources by enzyme oxidation mechanism, providing possibilities for metabolizing phenols and other aromatic derivatives. This research proved the capability of *Aspergillus* sp. to transform catechins yielding new phenolic compounds, teadenols A and B.

2.4. Conclusion

Two new phenolic compounds teadenols A and B isolated from tea leaves which were selectively fermented with *Aspergillus* sp. (PK-1, FARM AP-21280). Teadenol A was biosynthetically produced from EGCG, and teadenol B was yielded from GCG which was formed from EGCG in the heating process of tea leaves.

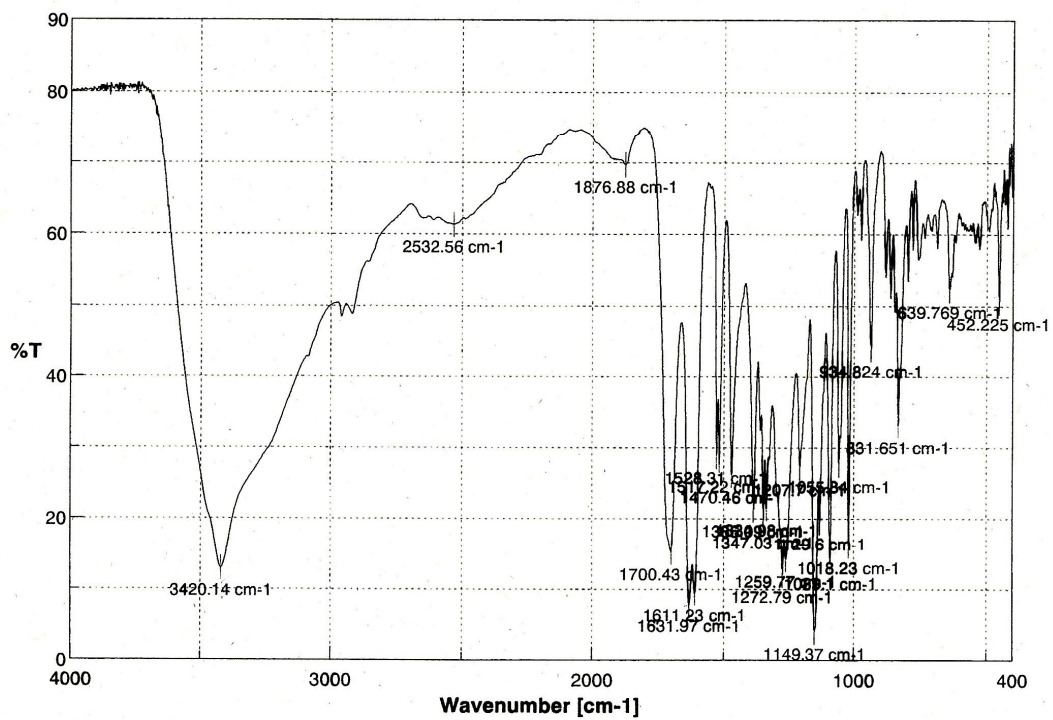
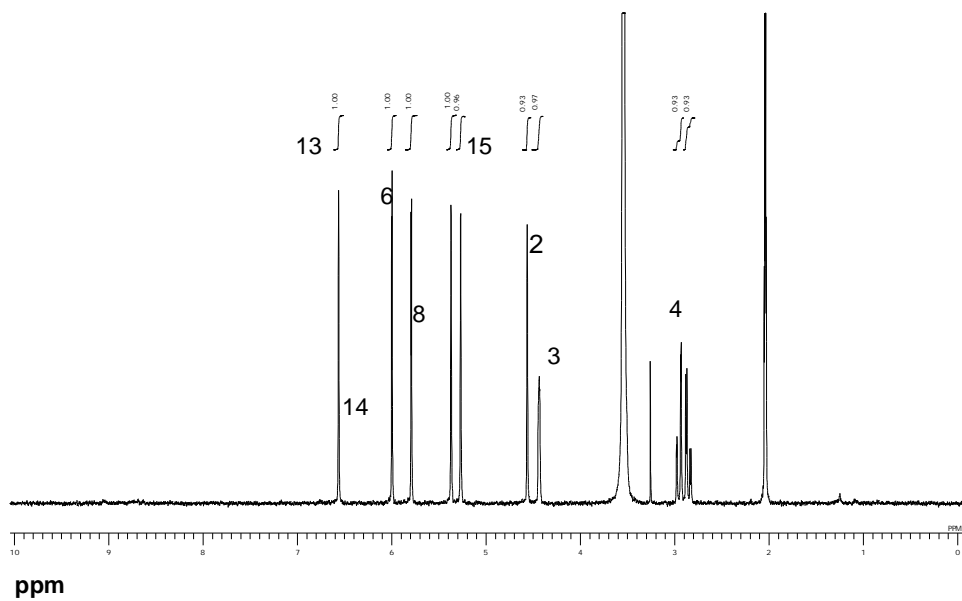


Fig. 4 IR spectrum of compound 1

A



B

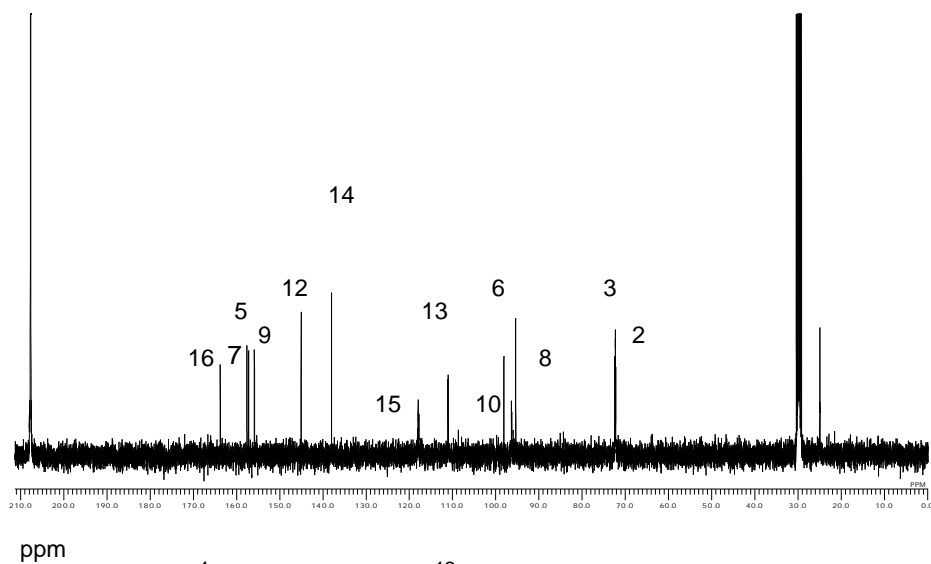


Fig. ^1H -NMR (A) and ^{13}C -NMR (B) of compound **1**

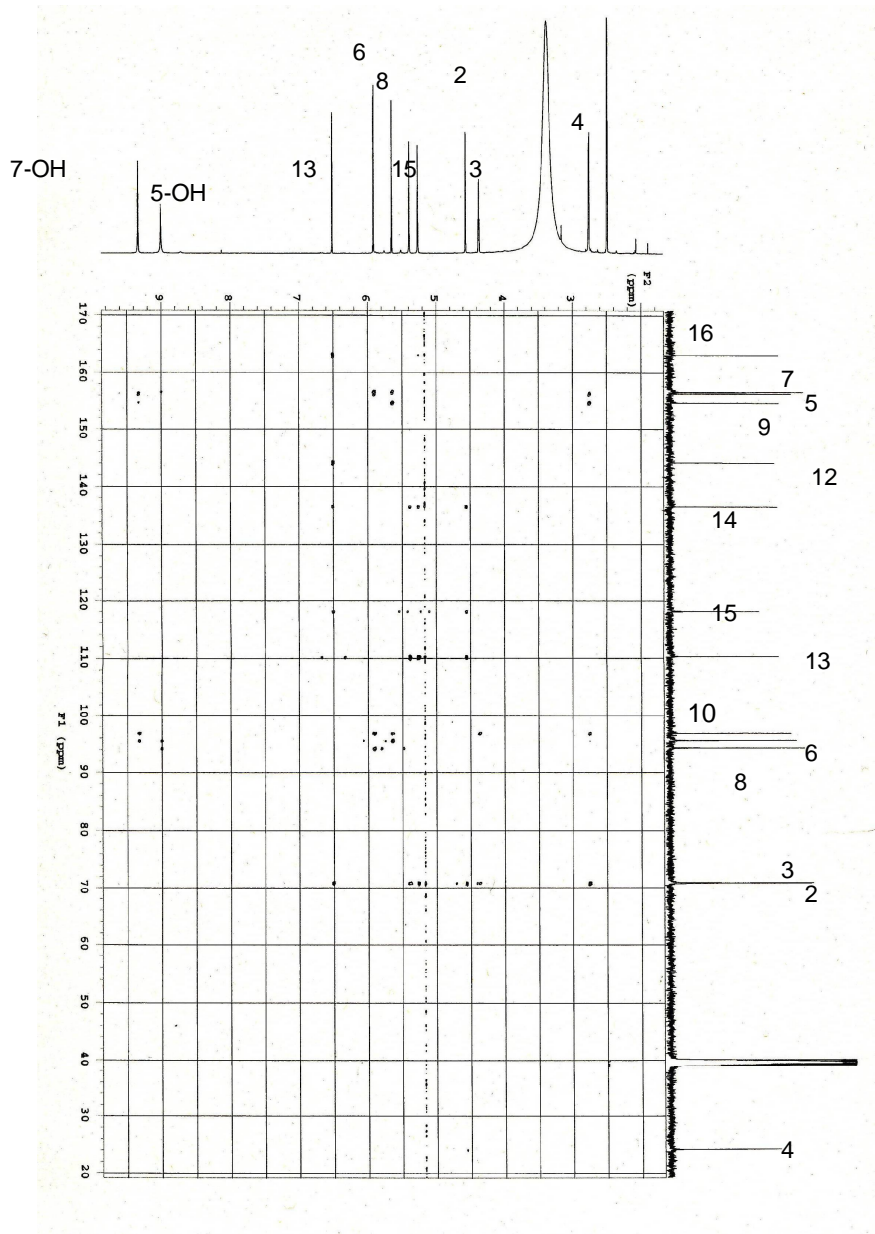
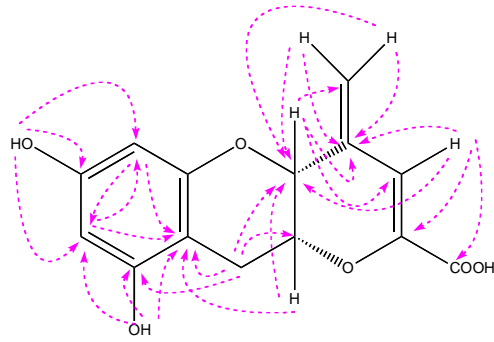


Fig. 6 HMBC spectrum of compound 1

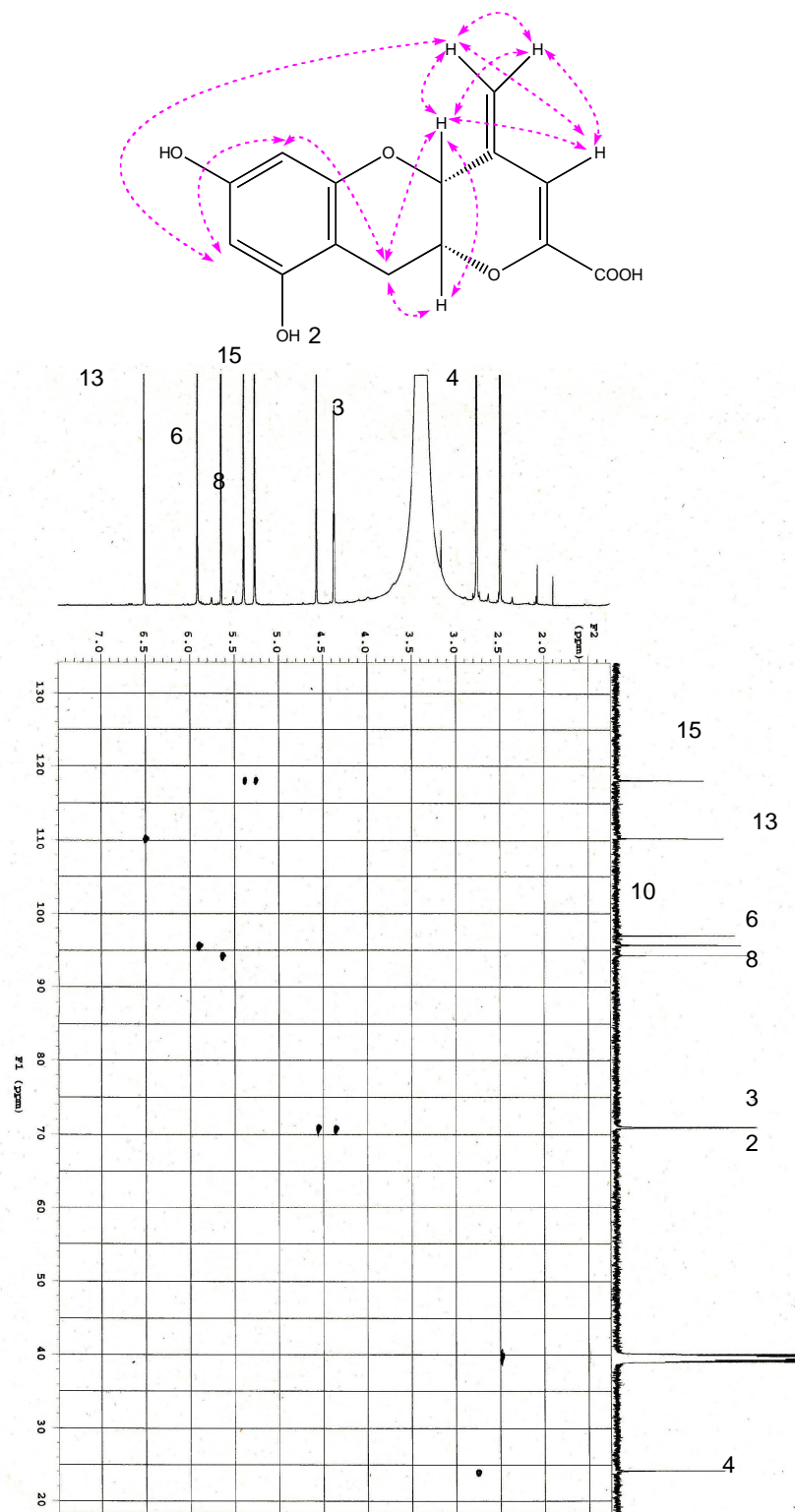


Fig. 7 NOE spectrum of compound 1

Table 1 NMR data of compound **1** (in DMSO-d₆)

Position	δ_C	δ_H	HMBC (1H - ^{13}C)	NOE (1H - 1H)
2	70.9	4.56 (1H, s)	C-13, 14, 15	H-3, 4, 13, 15
3	70.8	4.36 (1H, m)	C-2, 10	H-2, 4
4	24.1	2.76 (2H, m)	C-2, 3, 5, 10	H-2, 3, 8
5	156.3	-		
6	95.6	5.91 (1H, d, J=2.3 Hz)	C-8, 10	H-8, 15
7	156.6	-		
8	94.2	5.64 (1H, d, J=2.3 Hz)	C-6, 10	H-4, 6
9	154.7	-		
10	96.9	-		
12	144.1	-		
13	110.2	6.51 (1H, d, J=0.9 Hz)	C-2, 12, 14, 16	H-2, 15
14	136.5	-		
15	118.0	5.27 (1H, br.s) 5.39 (1H, br.s)	C-2, 13, 14 C-2, 13, 14	H-2, 6, 13, 15 H-2, 13, 15
16	163.2	-		
5-OH		9.00 (1H)	C-5, 6, 10	
7-OH		9.33 (1H)	C-6, 7, 8	

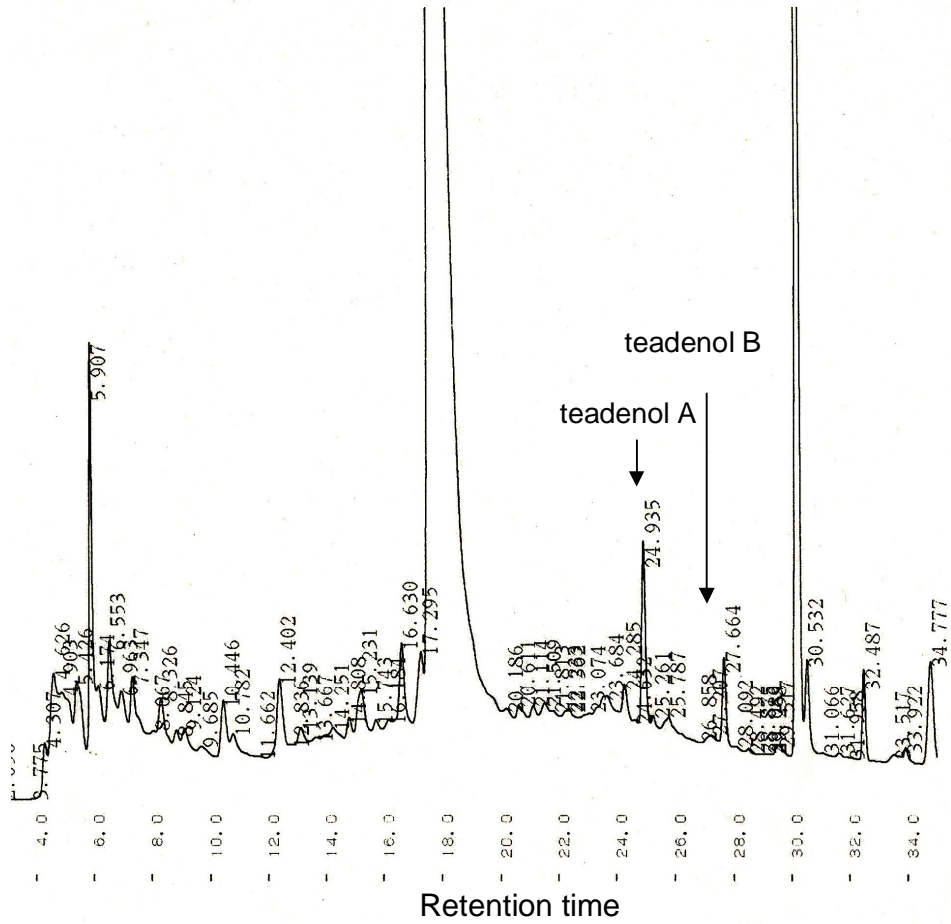


Fig. 8 HPLC profile of autoclaved solution of EGCG after three weeks incubated with *Aspergillus* sp. (PK-1, FARM AP-21280)

A

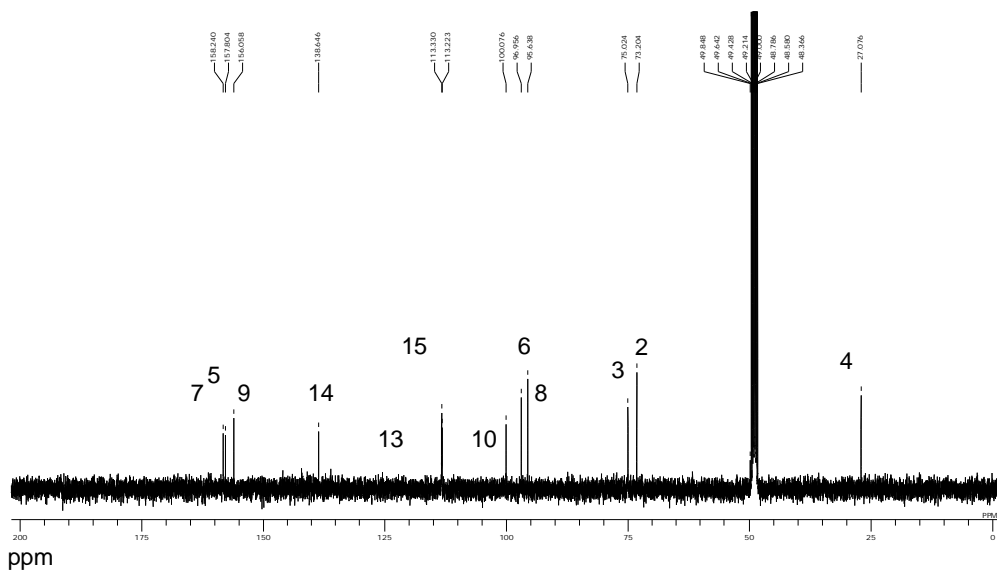
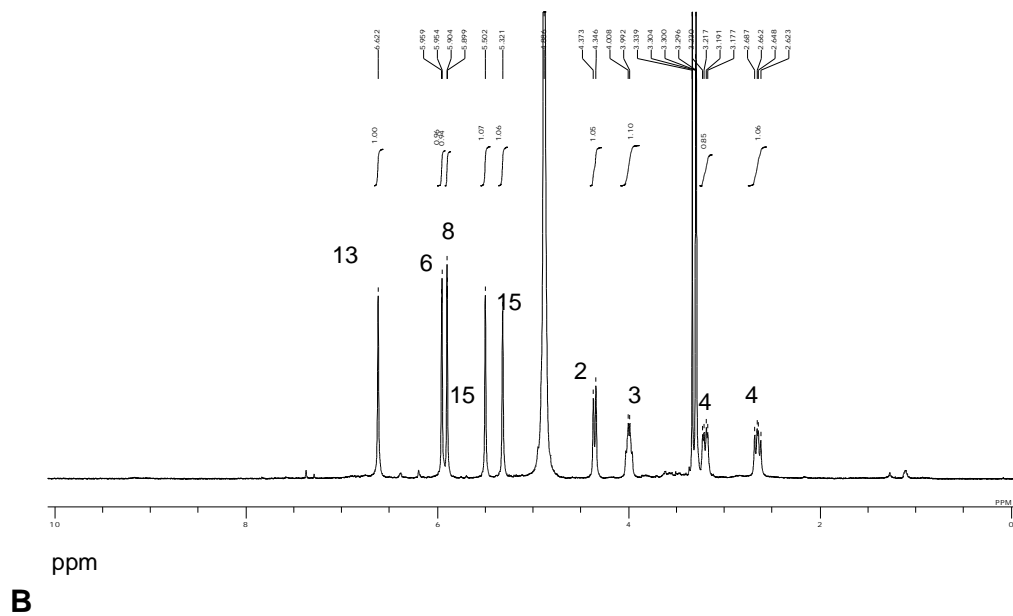


Fig. ^1H -NMR (A) and ^{13}C -NMR (B) spectra of compound **2**

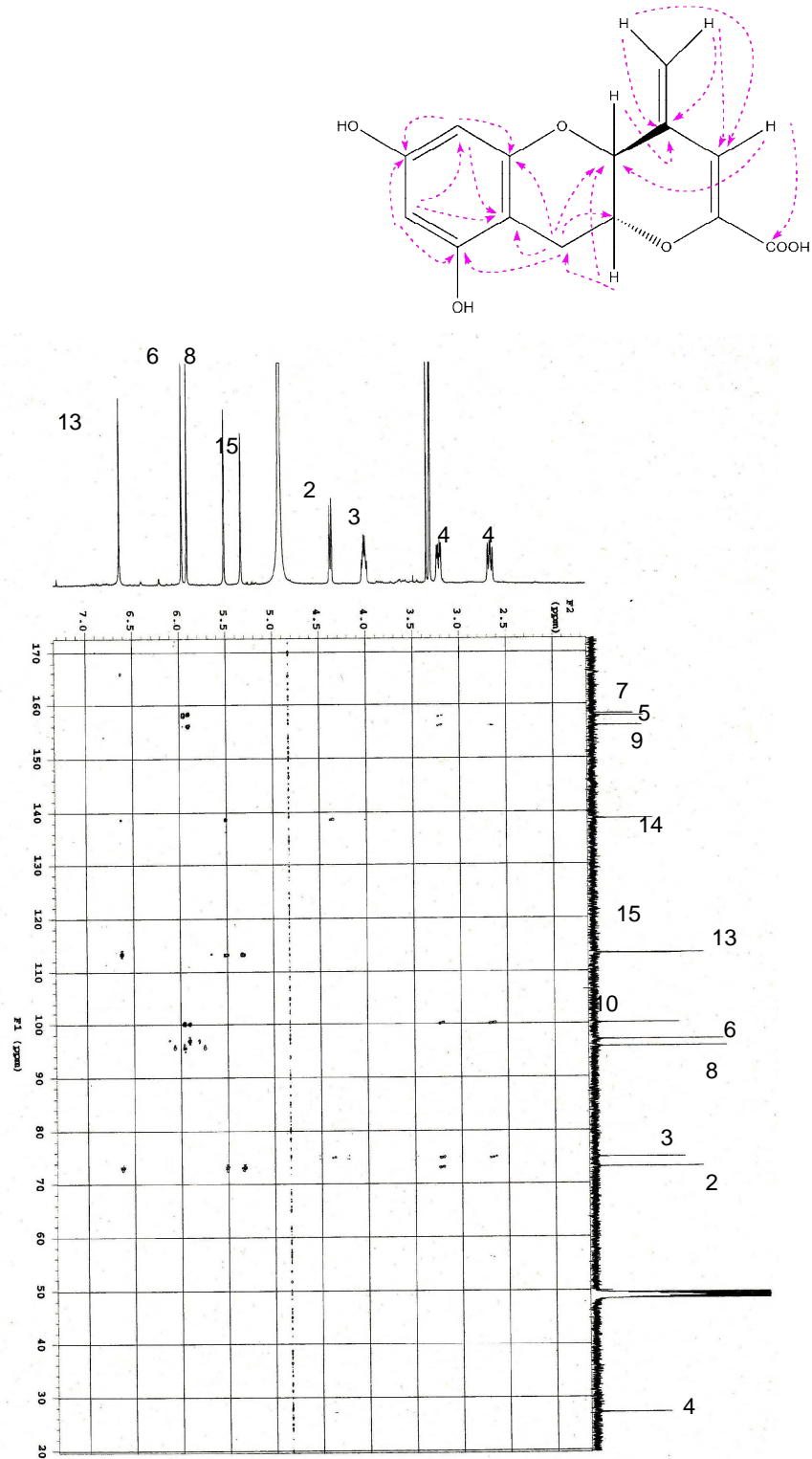


Fig. 10 HMBC spectrum of compound 2

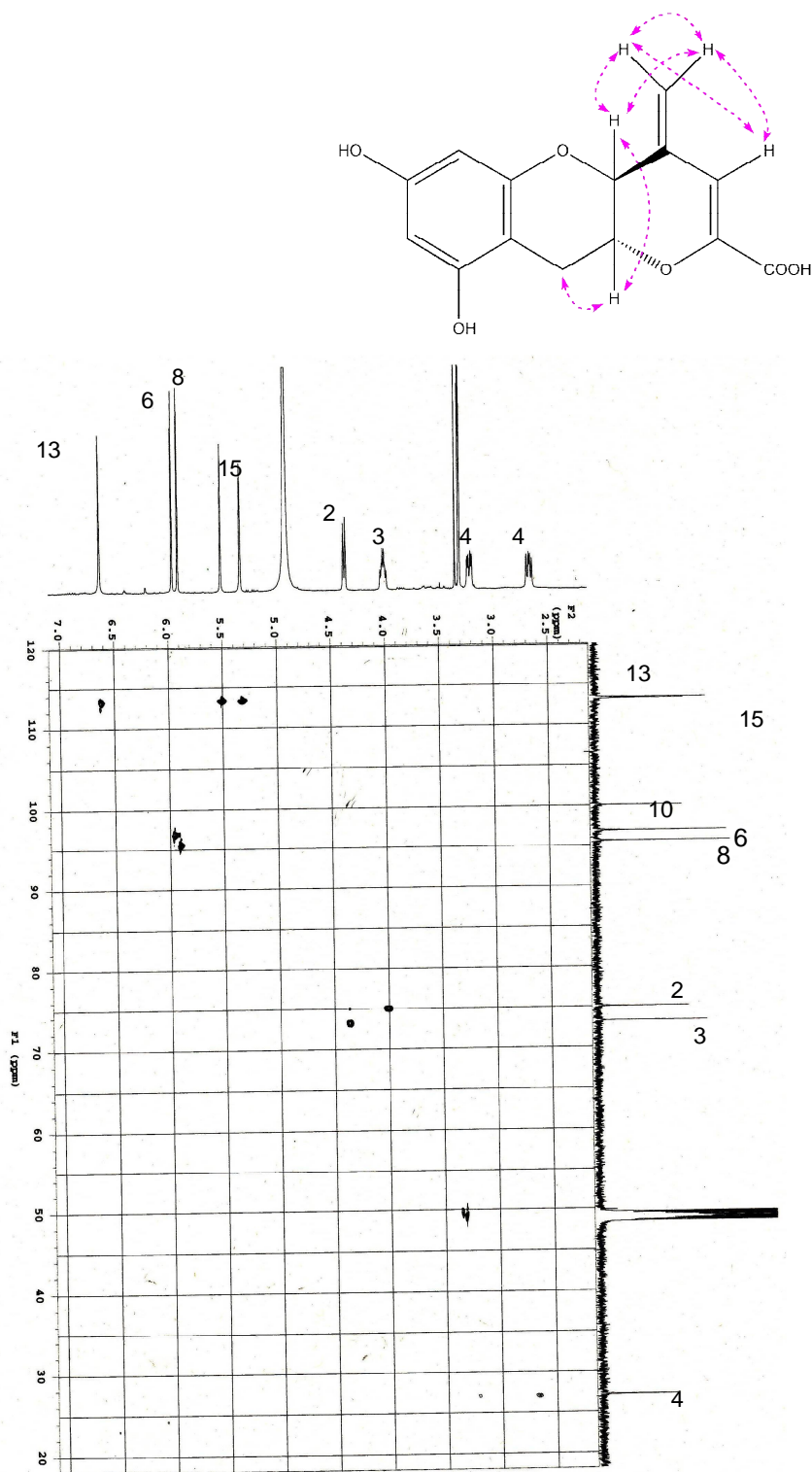
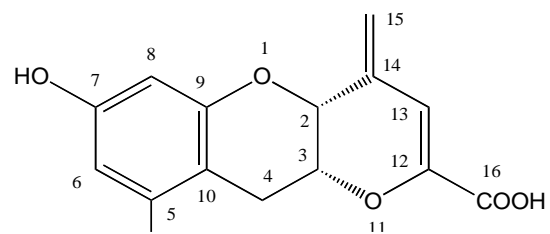


Fig. 11 NOE spectrum of compound 2

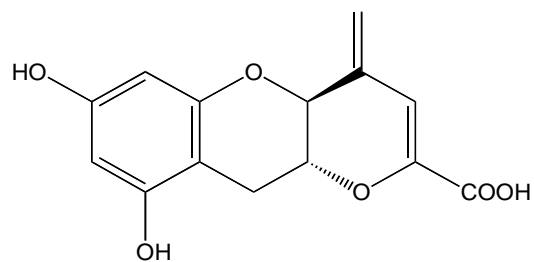
Table 2 NMR data of compound **2** (in CD₃OD)

Position	$\delta_{\text{C}}\delta_{\text{H}}$	HMBC (¹ H- ¹³ C)	NOE (¹ H- ¹ H)	
2	73.2	4.36 (1H, d, J=10.5 Hz)	C-14	H-3, 15
3	75.0	4.01 (1H, m)	C-2, 4	H-2, 4
4	27.1	2.66 (1H, dd, J=10.3, 15.7 Hz) 3.20 (1H, dd, J=5.9, 15.7 Hz)	C-3, 9, 10 C-2, 3, 5, 9, 10	H-3, 4 H-3, 4
5	157.7	-		
6	96.9	5.96 (1H, d, J=2.3 Hz)	C-5, 7, 8, 10	
7	158.2	-		
8	95.6	5.91 (1H, d, J=2.3 Hz)	C-7, 9, 10	
9	156.0	-		
10	100.0	-		
12	145.0	-		
13	113.2	6.63(1H, s)	C-2, 16	H-15
14	138.6	-		
15	113.3	5.33 (1H, s) 5.51 (1H, s)	C-2, 13 C-2, 13, 14	H-2, 13, H-2, 13,
15				
16	166.0	-		



Compound **1** :teadenol A

1 : teadenol A



2 : teadenol B

Compound **2** :teadenol B

Fig. 12 Chemical structures of compounds **1** and **2**

CHAPTER 3

ANALYSIS OF TEADENOLS IN THE VARIOUS TEA PRODUCTS

3.1. Introduction

Commercial teas are classified into several types, *i.e.* non-fermented tea (green tea), softly-fermented tea (white tea), semi-fermented tea (oolong tea), fully fermented tea (black tea), and post-fermented tea. One of popular post-fermented tea in China is pu-er tea. Production of black tea is the major in the world, showing the ratio of over 80% in total tea production. Green tea at the second rate with about 15% ratio production, while oolong tea and post-fermented tea are the minor products.

Among all the teas with various degree of fermentation, the ones with higher degree of fermentation have better storage stability. In consequence, black tea has the best storage stability, followed by oolong tea and white tea. Green tea has the poorest storage stability. The reason is that most of the components in unfermented tea have not been oxidized; they continue to oxidize during storage. The other reason is that flavor-contributing components are very limited in green tea and easily released. Therefore, green tea cannot be stored for long period (Kan *et al.*, 2004). But green tea is rich in flavanols (*e.g.* EGCG) which make up about 30% of the dry leaves weight (Shoji, 2007).

Up to now, many chemical investigations have been carried out on tea, and series of flavan-3-ols and hydrolysable tannins in green tea, 8-C-ascorbyl(-)-

EGCG and novel flavan-3-ols derivatives in oolong tea and benzotropolone-type pigments in black tea were already reported (Zhou *et al.*, 2005).

Fermentation of the green tea with microorganisms resulted in great change in their chemical constituents during the processing procedure of pu-er tea (Zhou *et al.*, 2005). It is mainly fermented by anaerobic bacteria as first step, but some fungi such as *Aspergillus*, *Penicillium*, *Rhizopus*, and *Saccharomyces* as the next step also play some role in its fermentation (Zhou *et.al.*, 2004). Taste of post-fermented tea has a marked bitterless. After tea leaves are plucked, panned, and dried, they are piled up during a few weeks and finally dried.

Puer tea and oolong tea can lower the levels of triglyceride more significantly than that of green tea and black tea, where as puer tea and green tea are more efficient than oolong tea and black tea in lowering the level of total cholesterol (Mo *et al.*, 2008)

Chemical researches on the structures of various polyphenols and their oxidative derivatives in tea leaves have been done, resulting in the understanding of the detailed mechanism of catechins oxidation (Tanaka and Kouno, 2003).

Recently some fermented tea products, specifically treated with *Aspergillus*, *Lactobacillus*, *etc.*, have been developed and circularized in the Japanese markets. In this experiment, constituents including teadenols in various types of fermented teas (fermented with *Aspergillus* sp. or *Eurotium* sp.) were investigated and compared to those of commercial tea products (green, oolong, black and pu-er teas) by HPLC and HPLC-TOFMS analyses.

3.2. Materials and methods

3.2.1. Tea materials

Four types of fermented teas (*Camellia sinensis* L.) were prepared by the selective incubation of leaves with *Aspergillus* sp. (FARM AP-21280) (PK-1), *A. oryzae* (NBRS 4214) (AO-1), *A. awamori* (NBRS 4122) (SK-1) or *Eurotium* sp. (FARM AP-21291) (KA-1) (Fig. 13).

Commercial products of green (GT, made in Japan, Marukyou Co. Ltd.), oolong (OT, made in China, imported by San-ei Kousan Co. Ltd.) and black (BT, made in China, imported by Kataoka Bussan Co. Ltd.) teas were purchased in the market in Japan. Pu-er tea (PT, made in China) was purchased in the market in Kunming City, China. For the chemical analysis, one sample was used for each tea product.

3.2.2. HPLC analysis

Four fermented teas (PK-1, AO-1, SK-1 and KA-1) and four commercial teas (GT, OT, BT and PT), were extracted (powdered samples, each 2 g dry weight) with 60 % aqueous EtOH (30 ml) for 40 minutes at room temperature. The extract was filtered with filter paper and subjected (2 μ l) to HPLC analysis. HPLC conditions were as same as described (see 2.1). Retention times (min): GA (7.4), GC (9.1), EGC (12.4), C (15.1), caffeine (16.4), EC (17.3), EGCG (18.1), GCG (19.2), ECG (22.4), CG (23.9), teadenol A (24.4) and teadenol B

(27.0). The structures of the analyzed compounds (tea phenols) are illustrated in Figs. 1 and 9. Data values were shown as the mean of three experiments \pm SD.

3.2.3. HPLC-TOFMS analysis

An above fermented tea (PK-1) and four commercial teas (GT, OT, BT and PT) were extracted as the same method mentioned above. The extracts were subjected to HPLC-TOFMS system as following conditions. HPLC system (Agilent 1100 series); Column : ZORBAX Eclipse Plus C₁₈ (2.1 mm i.d.×100 mm, 3.5 μ m), Mobile phase: 0.1% formic acid with 10 mM AcONH₄-CH₃CN [95 : 5 (0 min)→50 : 50 (30 min)→10 : 90 (40 min)], Flow rate : 0.2 ml/min, Column temperature : 40°C, Detection : 280 nm (UV). Retention times (min): GC (7.6), caffeine (12.6), teadenol A (16.6), F-1 (17.6), F-2 (18.2) and teadenol B (19.2). TOFMS system (Agilent G1969A); Ionization: ESI (positive), Drying gas: N₂, 350°C, 10 l/min, Nebulizer gas: N₂, 50 psig, Capillary: 4000 V, Fragmentor: 100 V, Mass range: 80-1200 *m/z*, Reference mass: purine: [M+H]⁺ = 121.0509, hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine: [M+H]⁺ = 922.0098. Resolution: 10000 (FMHM) *m/z* 2722. The data for Figs. 15 and 16 were prepared using the analytical software of Agilent Masshunter Mass Profiler.

3.3. Results and discussion

3.3.1 HPLC analysis.

Tea phenols and caffeine concentrations in four fermented (PK-1, AO-1, SK-1 and KA-1) and four commercial (GT, OT, BT and PT) teas, analyzed with HPLC, were shown at Table 3 and Fig. 14. Only in the fermented teas (PK-1, AO-1, SK-1 and KA-1), relatively high amount of GC and GCG were detected. These catechins with *trans* configuration between C-2 and C-3 (GC and GCG) were yielded by the epimerization at C-2 position in EGC and EGCG, respectively, in the heating treatment in the tea processing stage (Huang *et al.*, 2004).

Teadenols A and B were also produced in all fermented teas, which were not observed in commercial products except for OT producing small amount of teadenol A (Table 3). The concentrations of teadenols in the teas fermented with *Aspergillus* sp. (PK-1, AO-1 and SK-1) were relatively larger (teadenol A: 1.01-1.79 %; teadenol B: 0.16-0.37 %, as dry weight) than that (teadenol A: 0.23 %; teadenol B: 0.02 %, as dry weight) in the tea fermented with *Eurotium* sp. (KA-1).

In PT, a traditional fermented tea product in China, teadenols were not detected. Although teadenols might be produced in PT in the fermentation stage of its manufacture, they seemed to be finally metabolized to undetectable level in the long-time fermentation with the mixture of various microorganism such as *Aspergillus*, *Rhizopus*, *Penicillium*, *Saccharomyces*, etc. Zhou *et al.* (2005) reported that in pu-er tea, most of the characteristic flavan-3-ol derivatives in

green tea, such as EGCG, EGC and ECG, have disappeared, whereas the content of polyphenol polymers have been remarkably increased in pu-er tea. At the same time, they reported that several polyoxygenated compounds have been formed from the related catechins derivatives during the processing procedure of pu-er tea.

There was not so much difference in the concentrations of catechins between OT and BT. BT used in this experiment seemed to be not so much strongly fermented resulting the low-level oxidation of catechins in the processing stage. Caffeine concentration was observed at relatively high level in all tea samples, showing the chemical stability of this compound in the fermentation and oxidation process in tea manufacture.

3.3.2. HPLC-TOFMS analysis

In the above experiment of HPLC analysis, *C. sinensis* L. leaves fermented with *Aspergillus* (PK-1, AO-1 and SK-1) or *Eurotium* (KA-1) were found to contain large amount of functional phenols such as teadenols A and B. For the evaluation of effective identification of specific metabolites produced in various types of teas, HPLC-TOFMS analysis was performed. One fermented tea (PK-1) and commercial teas (GT, OT, BT and PT) were used for the analysis.

Constituents ($m/z < 1000$) detected in tea samples by HPLC-TOFMS analysis were shown in Fig. 15. Fig. 15A showed 627 compounds detected in among all samples (PK-1, GT, OT, BT and PT). Figs. 15B and 15C showed 359 compounds observed only in among four commercial teas (GT, OT, BT and PT)

and 98 compounds observed only in the fermented tea (PK-1), respectively. Teadenol B [$C_{14}H_{12}O_6$: Monoisotopic mass (Mm) = 276.0634] was detected (m/z = 276.0640) only in PK-1 (Fig. 15C). Therefore, by this analytical method, the compounds specifically produced only in the fermented tea (PK-1) could be easily identified (Fig. 15C).

In Fig. 16, the relationship profile of the amount of 170 compounds detected both in PK-1 (horizontal) and in four teas group (GT, OT, BT and PT) (vertical) is shown. In this analysis, caffeine ($C_8H_{10}O_2N_4$: Mm = 194.0804, m/z = 194.0810) was detected with large amount in all tea samples. On the other hand, teadenol A ($C_{14}H_{12}O_6$: Mm = 276.0634, m/z = 276.0640) and GC ($C_{15}H_{14}O_7$: Mm = 306.0740, m/z = 306.0745) were found with relatively high amount in PK-1 and not so much in four teas group (GT, OT, BT and PT). Teadenol A was detected in a commercial tea (OT, Table 3), showing the possibility of that the OT might be unanticipatedly fermented by natural *Aspergillus* sp. in the fermentation processing in China.

In this experiment, two compounds (F-1 and F-2) with m/z = 756.2118 and m/z = 756.2108, respectively, showing the identical chemical formula ($C_{33}H_{40}O_{20}$: Mm = 756.2113) were detected (Fig. 16). Although the chemical structures of these compounds were not yet identified, they were supposed to be flavonoid compounds (e.g. kaempferol triglycosides (Kiehne and Engelhardt, 1996; Finger *et al.*, 1991) from the m/z data (Fig. 17). As shown above, the profiling analysis of HPLC-TOFMS is very useful for the identification of complex metabolites in processed varieties of tea products.

3.4. Conclusion

C. sinensis L. leaves fermented with *Aspergillus* or *Eurotium* contained relatively high amount of phenolics, *i.e.* teadenols A and B, which were not observed in commercial products, except OT producing in very small amount of teadenol A. HPLC-TOFMS analysis was useful for the identification of the secondary metabolites specifically produced in the tea materials.

Table 3. Concentrations of tea polyphenols in various teas

Compound	PK-1	AO-1	SK-1	KA-1	GT	OT	BT	PT	
GA	0.05±0.01	0.11±0.02	0.09±0.01	0.32±0.02	0.11±0.01	0.43±0.02	0.19±0.01	-	-
GC	2.51±0.22	2.98±0.23	2.58±0.17	3.15±0.11	-	-	-	-	-
EGC	1.32±0.12	0.95±0.03	1.30±0.05	0.48±0.03	1.90±0.02	0.16±0.03	0.15±0.02	-	-
C	0.22±0.01	0.20±0.02	0.24±0.01	0.15±0.01	0.10±0.01	0.06±0.01	-	-	-
caffeine	2.58±0.17	2.48±0.13	2.78±0.18	2.40±0.19	2.24±0.18	2.24±0.11	1.99±0.15	-	-
EC	0.03±0.01	0.05±0.01	0.07±0.01	0.05±0.01	0.48±0.02	0.05±0.01	0.05±0.01	-	-
EGCG	0.46±0.02	1.14±0.13	0.75±0.02	3.53±0.15	6.06±0.37	2.44±0.19	2.58±0.18	-	-
GCG	0.25±0.02	0.38±0.02	0.15±0.01	1.34±0.10	-	-	-	-	-
ECG	0.08±0.01	0.10±0.01	0.14±0.01	0.98±0.01	0.92±0.08	0.60±0.02	0.61±0.02	-	-
CG	0.05±0.01	0.02±0.01	0.01±0.01	0.01±0.01	-	-	-	-	-
teadenol A	1.01±0.13	1.03±0.13	1.79±0.15	0.23±0.01	-	0.01±0.01	-	-	-
teadenol B	0.23±0.02	0.16±0.01	0.37±0.02	0.02±0.01	-	-	-	-	-

% as dry weight ± SD (n=3), - : below detectable level.

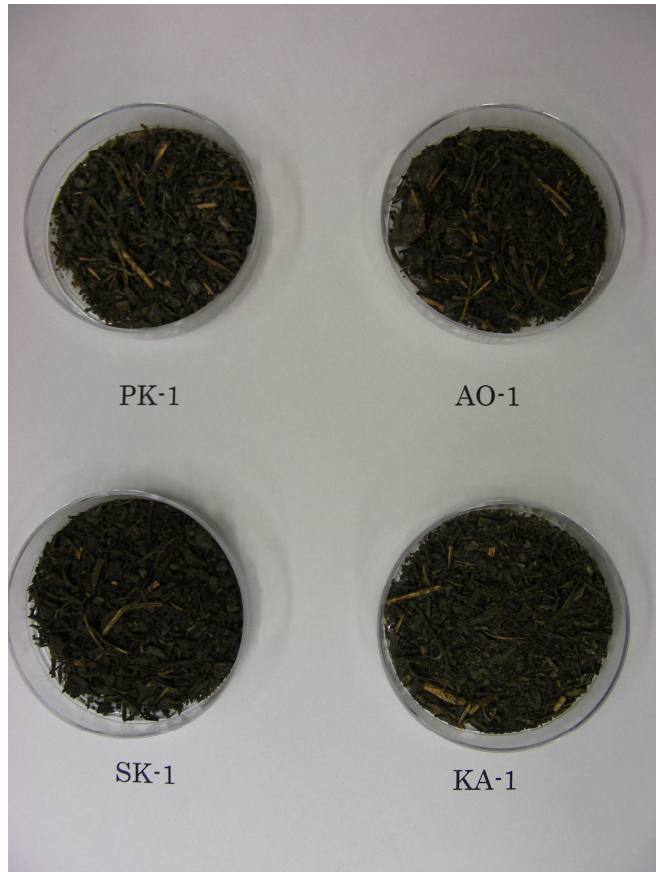


Fig. 13 Photographs of four types of fermented teas (PK-1, AO-1, SK-1 and KA-1)

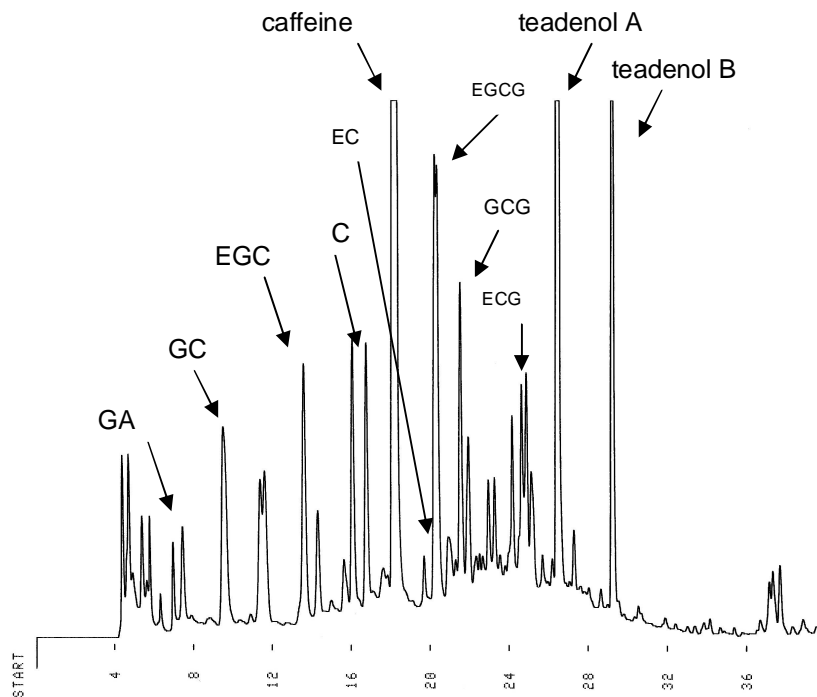


Fig. 14A HPLC profile of fermented tea (PK-1)

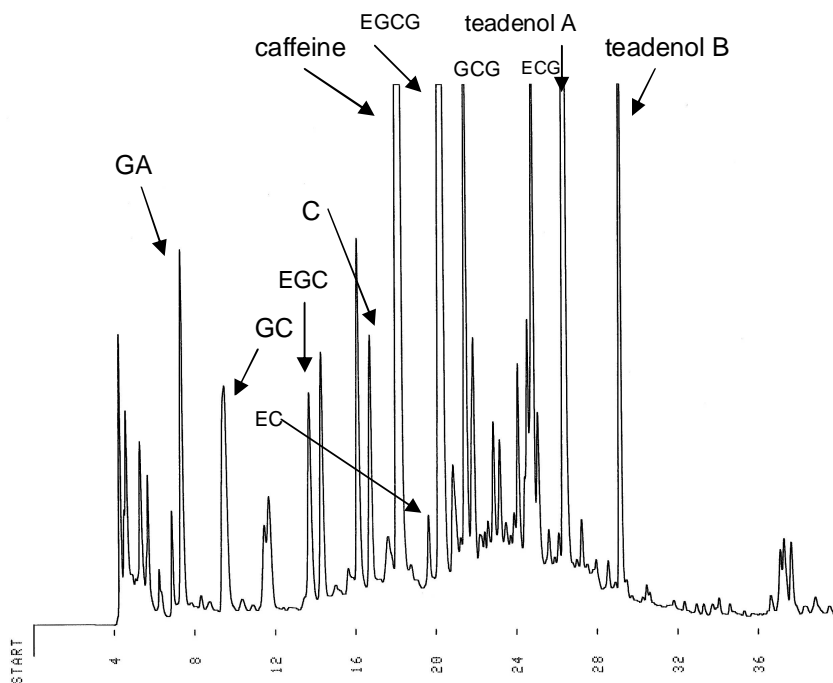


Fig. 14B HPLC profile of fermented tea (AO-1)

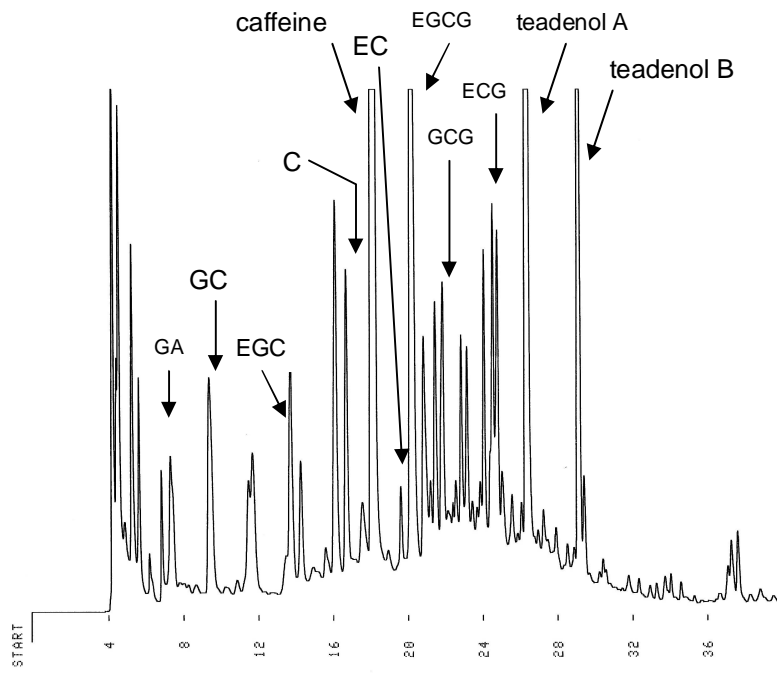


Fig. 14C HPLC profile of fermented tea (SK-1)

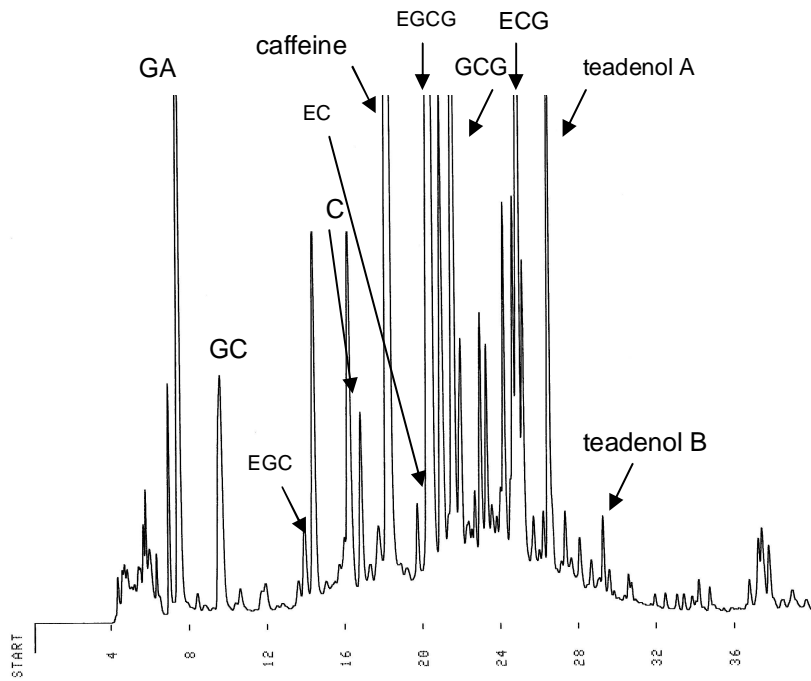


Fig. 14D HPLC profile of fermented tea (KA-1)

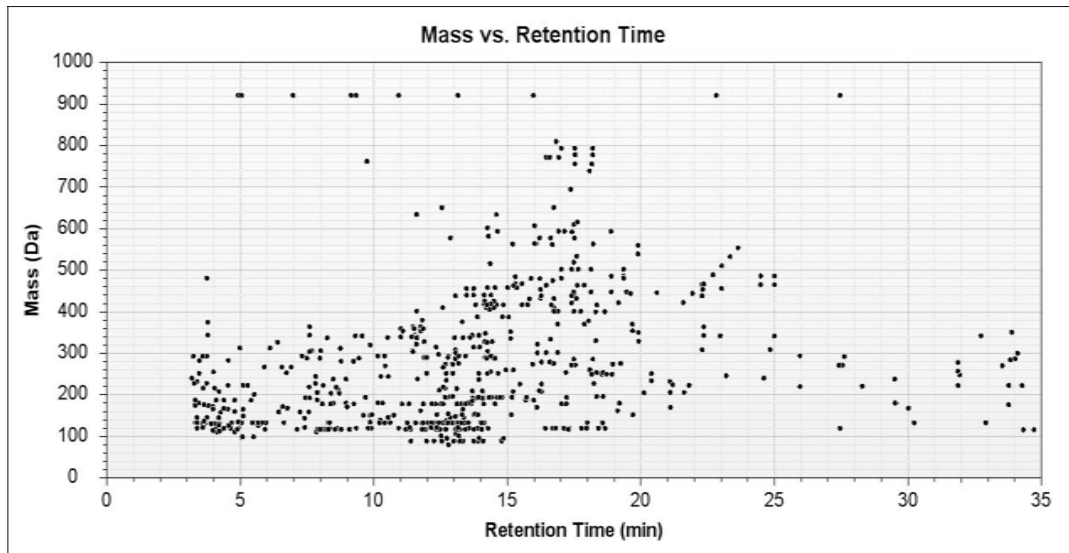


Fig. 15A 627 compounds detected in among GT, OT, BT, PT and PK-1

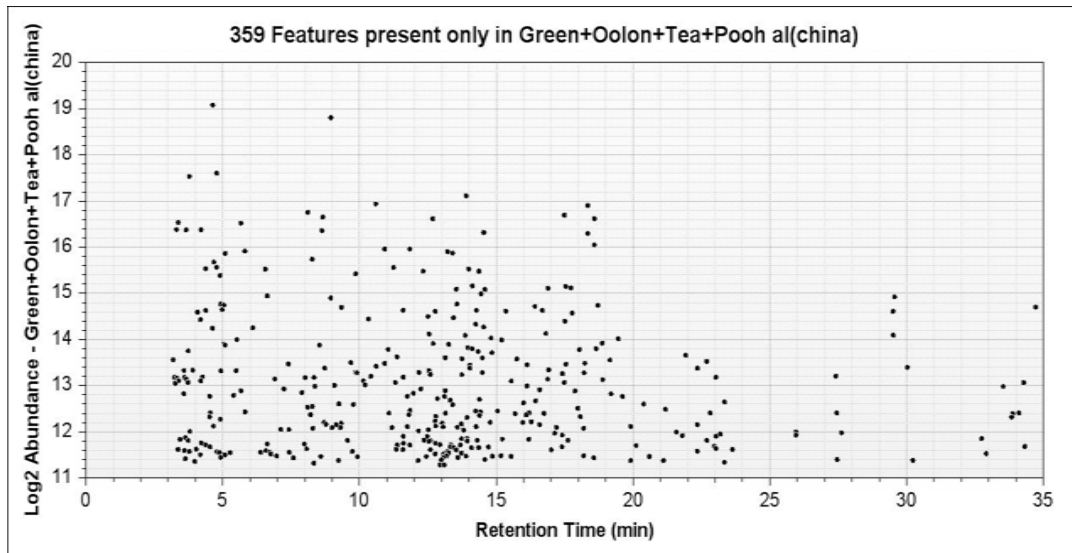


Fig. 15B 359 compounds detected only in GT, OT, BT and PT

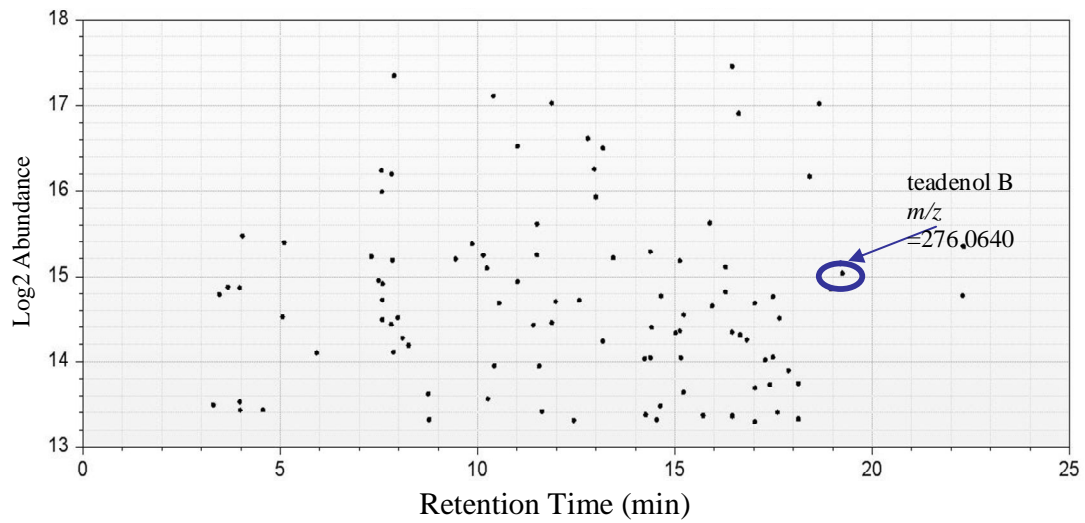


Fig. 15C 98 compounds detected only in PK-1(Teadenol B Mm= 276.0634)

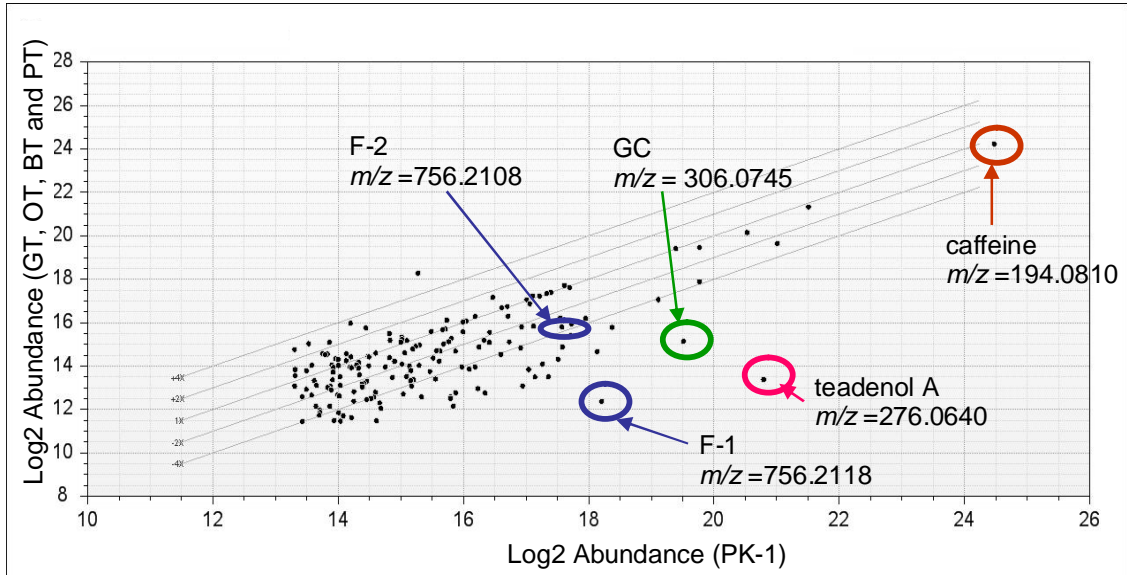


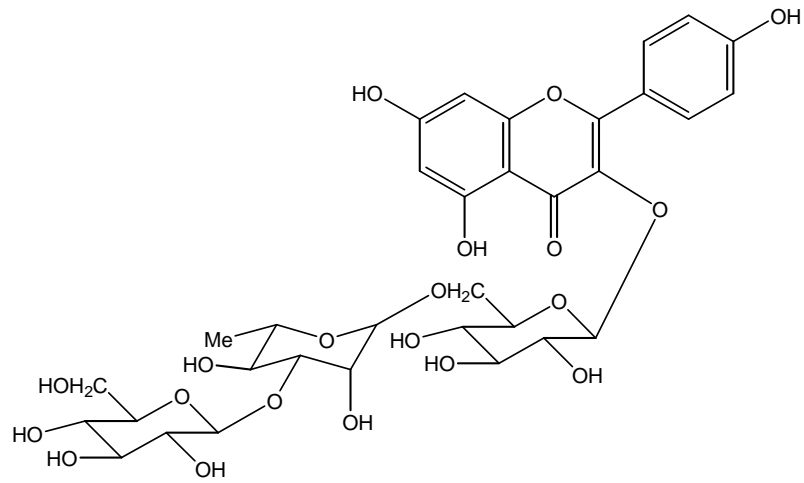
Fig. 16 Relationship of the amount of 170 compounds detected both in PK-1 (horizontal) and four teas groups (GT,OT, BT, and PT) (vertical)

F-1 and F-2 (kaempferol triglycosides) Mm = 756.2113;

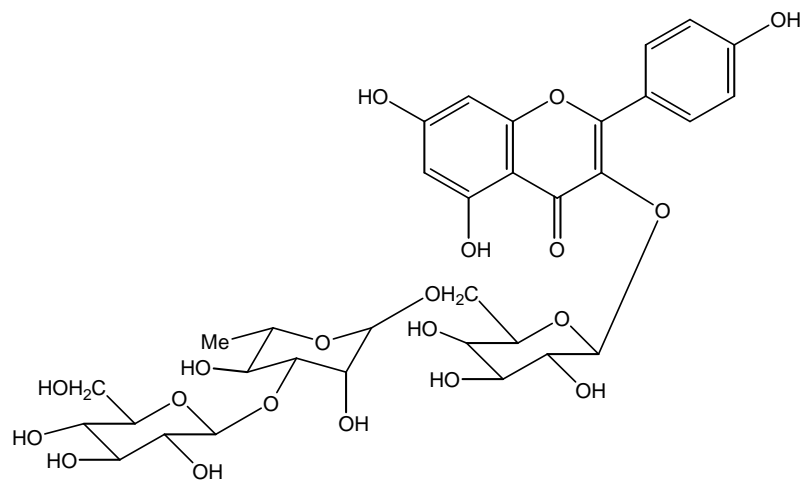
teadenol A Mm = 276.0634;

GC Mm = 306.0740 ;

caffeine Mm = 194.0804



F-1



F-2

Fig. 17 Chemical structures of F-1 and F-2 (kaempferol triglycosides)

CHAPTER 4
BIOTRANSFORMATION OF TEADENOLS FROM TEA CATECHINS BY
***Aspergillus* sp.**

4.1. Introduction

Several species of *Aspergillus* are capable of assimilating, utilizing, decomposing, degrading, and metabolizing phenols (e.g. catechins). They are *A. flavus*, *A. fumigatus*, *A. niger*, and *A. teneus* (Arunachalam, 2003 and Stoilova *et al.*, 2007). Kawakami and Shibamoto (1991), also reported that formation of methoxy compounds indicates that *A. niger* catalyzed methylation in the transformation of *p*-coumaric acid and ferulic acid. It can be concluded that the member of genus *Aspergillus* possesses capability of transforming phenols to another compounds.

Each species of *Aspergillus* can produce a range of secondary metabolites associated with fungal growth and development (Calvo *et al.*, 2002). Interestingly, many of these secondary metabolites have been used in medicine for their anti-viral, anti-bacterial, tumor suppressing, anti-hypercholesterolemic, and immunosuppressant activities (Pelaez, 2005).

At the previous chapter (2.1), *Aspergillus* sp. (FARM AP 21280) was shown to biosynthesize teadenols A and B in commercial tea product (*Kippukucha*), which were biosynthesized from EGCG and GCG, respectively. In this experiment, *A. awamori* and *A. kawachii* were used for the possible biotransformation of teadenols from EGCG and GCG, and the results were

compared with that of *Aspergillus* sp. (FARM AP-21280). For the identification of the products (teadenols), analytical methods of both HPLC and HPLC-QTOFMS systems were applied.

So far there have been very few reports on biotransformation of catechins with enzymes or microorganism especially *Aspergillus* sp. The biosynthetic pathway of teadenols from tea catechins by *Aspergillus* fermentation was also proposed.

4.2. Materials and methods

4.2.1. Biosynthetic preparation of teadenols

Fermented tea product ('*Kippuku-cha*') was used for the analysis of teadenols in the HPLC-QTOFMS system. The tea leaves (2g dry weight) were extracted with hot water (80°C, 30 ml) for 40 min. The extracts were filtered through Millipore filter (0.45 µm) and subjected to HPLC-QTOFMS system.

EGCG (20 mg, purchased from SIGMA) was dissolved in H₂O (20 ml/100 ml flask) and autoclaved (121°C 15 min). *Aspergillus* sp. (FARM AP-21280), *A. awamori* (NRIB-2061) and *A. kawachii* (IFO-4308), subcultured on Potato Dextrose Agar solid medium, were inoculated (each one piece of 1 cm x 1 cm) into the autoclaved solution (mixture of EGCG and GCG). The mixed solution was incubated on a rotary shaker (60 rpm) at 25°C in the dark condition.

The constituents in the incubated solution were periodically [0, 2, 9 and 21 days for *Aspergillus* sp. (FARM AP-21280) and 3 and 6 days for *A. awamori* and *A. kawachii*] analyzed. The mixture solutions were filtered through Millipore filter

(0.45 μ m) and subjected to HPLC system, and the solutions incubated with *Aspergillus* sp. (FARM AP-21280) (21-days), *A. awamori* (6-days) and *A. kawachii* (3-days) were also analyzed with HPLC-QTOFMS system.

4.2.2. HPLC analysis

HPLC conditions were similar to those described in Chapter 2.1

4.2.3. HPLC-QTOFMS analysis

The above extract of tea and the incubated solutions were subjected to HPLC-QTOFMS system as following conditions. HPLC system (Agilent 1200 series); column : ZORBAX Eclipse Plus C18 Rapid Resolution HD (2.1 mm i.d.x100mm, 1.8 μ m), Mobile phase: 0.1% formic acid with 2.5 mM AcONH₄-CH₃CN (90:10 (0 min)→50:50 (30 min) → 0:100 (30 min)), Flow rate: 0.2 ml/min, Column temperature : 40°C, Detection : 280 nm (UV). Retention times (min) : teadenol A (9.3) and teadenol B (11.4). QTOFMS system (Agilent 6540); Ionization: Dual ESI (negative), Drying gas : N₂, 350°C, 10 l/min, Nebulizer gas : N₂, 50 psig, Capillary : 3500 V, Fragmentor : 150 V, Mass range : 70-1050 m/Z. Reference mass: 112.985587, 119.03632, 1033.988109.

4.3. Results and discussion

4.3.1. Teadenols analysis in the autoclaved solution of EGCG selectively incubated with *A. awamori*, *A. kawachii*, and *Aspergillus* sp.

In Fig. 18, the HPLC profiles of the constituents in the incubated solution of EGCG with *Aspergillus* sp. (FARM AP-21280) were shown. By the autoclaving treatment, much amount of GCG and a little amount of G were formed (0-day, Fig. 18A). EGCG and GCG were rapidly decreased by the incubation with *Aspergillus* sp. (FARM AP-21280) as shown in Fig. 18B (2-day) and Fig. 18C (9-day). At 21-day incubation period, the amounts of EGC and GC which were very high at 9-day incubation period (Fig. 18C) become to be very low (Fig. 18D), and peaks of teadenols A and B were observed. In the HPLC analysis, the identification of these compounds (teadenols A and B) was done by the comparison of the retention times with those of the authentic standard compounds. The identification of these compounds was also performed with HPLC-QTOFMS analysis by the comparison of the data of retention time and mass spectrum (Fig. 19A) with those (Fig. 19B) of teadenols A and B detected in the extract of tea product (`Kippuku-cha`).

In addition, two compounds were detected as relatively large peaks in the HPLC analysis (retention times; 18.0 and 30.2 min) in 9-day (Fig. 18C) and 21-day (Fig. 18D) periods. Although these compounds were supposed to be originated from *Aspergillus* sp. constituents, the chemical structures were not clarified.

For the determination of the possible biosynthesis of teadenols from tea catechins by the treatment with the other *Aspergillus* sp., *A. awamori* and *A. kawachii* were used in this experiment. Both *A. awamori* and *A. kawachii* were industrial fungi used for the preparation of shochu koji, which is produced to make a traditional Japanese alcoholic beverage (shochu). In Fig. 20, constituents profiles of the HPLC analysis of the incubated solutions with *A. awamori* (Fig. 20A and 20B) and *A. kawachii* (Fig. 20C and 20D) were shown. Although teadenols A and B could be detected in the solutions with both types of *Aspergillus*, the amount of teadenol A ($37 \mu\text{g} \pm 8 \mu\text{g}/\text{flask}$, Fig. 20B : 6-day with *A. awamori*; $8 \mu\text{g} \pm 1 \mu\text{g}/\text{flask}$, Fig. 20C: 3-day with *A. kawachii*) was much larger than that of teadenol B ($5 \mu\text{g} \pm 1 \mu\text{g}/\text{flask}$, Fig. 20B: 6-day with *A. awamori*; $3 \mu\text{g} \pm 1 \mu\text{g}/\text{flask}$, Fig. 20C: 3-day with *A. kawachii*). By the incubation with these fungi (*A. awamori* and *A. kawachii*), teadenols were rapidly produced (at 3-day incubation period, Fig. 20A and 20C) compared with the case by *Aspergillus* sp. (FARM AP-21280) teadenols were detected at 21-day incubation period (Fig. 18D). This result suggested that *A. awamori* and *A. kawachii* have stronger enzymatic activities for the tea catechins metabolism compared with that in *Aspergillus* sp. (FARM AP-21280). The amounts of teadenols produced in this experiment were lower than those in the tea leaves fermented with *Aspergillus* sp. (Yanagita, 2011a; Yanagita 2011b). Therefore, teadenols production was supposed to be considerably effected by the fermentation conditions such as aerobic or anaerobic state. In the incubated solvents with *A. awamori* and *A. kawachii*, uncharacterized constituents were also detected as broad hump on the

HPLC baseline (Fig. 20, retention time; 10-30 min) and presumed to be come from the fungi components.

In the HPLC-QTOFMS analysis of the incubated solution with *A. awamori* (at 6-day period) and *A. kawachii* (at 3-day period), teadenols A and B were detected only in the solution with *A. awamori*, which could be observed not in the TIC but in the EIC spectrum (Fig. 21). Both compounds in the solutions with *A. kawachii* could not be detected in the QTOFMS system (the TIC spectrum was shown in Fig. 22). It was supposed that the amounts of teadenols in the solutions with *A. kawachii* were not sufficient compared to those of the other various metabolites for detectable-level ionization in the QTOFMS system. For the certification of teadenols production in the materials with *A. kawachii*, the fermented tea leaves might be better for QTOFMS analysis compared with samples of the incubated solutions..

4.3.2. Biosynthesis of teadenols A and B

In the processing stages for the production of post-fermented tea (*C. sinensis* L.) products, leaves are initially heated for the sterilization. In the heating treatment, GCG yielded by the conversion of EGCG in the material. Ikeda *et al.* (2005) reported that during heating process for sterilization, about 50% of the EGCG was epimerized to GCG in the material.

The mechanism of epimerization of EGCG to GCG is shown at Fig. 23,. It can be concluded that epi-type catechins undergo epimerization in during the thermal processing of tea catechins to yield corresponding cate-type isomers.

Galloyl moieties of gallated catechins are labile to thermal degradation, which leads to a reduction in the epimerization reaction (Okumura *et al.*, 2008).

Teadenols A and B, detected in various fermented tea products, have been supposed to be biosynthesized from EGCG and GCG respectively. In the incubation experiments of the autoclaved EGCG solution (mixture of EGCG and GCG) with three types of *Aspergillus*, teadenols A and B were biotransformed from these tea catechins (EGCG and GCG). Although the incubated solution with *Aspergillus* sp. (FARM AP-21280) produced relatively larger amount of both teadenols A and B (Fig. 18), the solutions with *A. awamori* and *A. kawachii* mostly contained teadenol A with little amount of teadenol B (Fig. 20).

In Fig. 24, a proposed biosynthetic pathway of teadenol A from EGCG was shown. EGCG was supposed to be readily hydrolyzed to EGC (and GA) by *Aspergillus* enzyme of gallate hydrolase (tannase, *e.g.* in *A. oryzae* (Horie *et al.*, 1997; Zhong *et al.*, 2008). The dioxygenase-mediated meta-B-ring cleavage of (+)-catechin (C), which followed by the formation of diacid compound, in the microbial transformation (Das *et al.*, 2011). Similar oxidative reaction was supposed to be occurred in EGC metabolic pathway to yield diacid metabolite-B. The intermediate metabolite-B could be converted to metabolite-C, which followed the subsequent formation of metabolite-D. The continuous decarboxylation and dehydrogenation of metabolite-D would provide teadenol A. In the identification of the chemical structures of teadenols, biosynthetic preparation of these phenolics from EGCG with *Aspergillus* sp. (FARM AP-21280) has been elucidated certifying the *R*-configuration at the C-3 position of

teadenols. Teadenol B, the C-2 epimer of teadenol A, was also supposed to be biosynthesized in the similar pathway from GCG. The illustration of teadenols A and B biosynthesis from tea catechins is shown at Fig. 25).

Recently, various types of tea products fermented with selected single microorganism such as *Aspergillus*, *Lactobacillus*, etc. are produced in Japan. The analysis of the profile of teadenols A and B production seems to be useful for the investigation of the environmental and/or microbiological conditions in the fermentation process of the tea materials.

4.4. Conclusion

Teadenols A and B were biosynthetically produced in the autoclaved solution of EGCG (mixture of EGCG and GCG) which was incubated with *Aspergillus*. Teadenols detected in various fermented tea (*C. sinensis* L.) products were supposed to be originated from tea catechins (EGCG and GCG) in the materials.

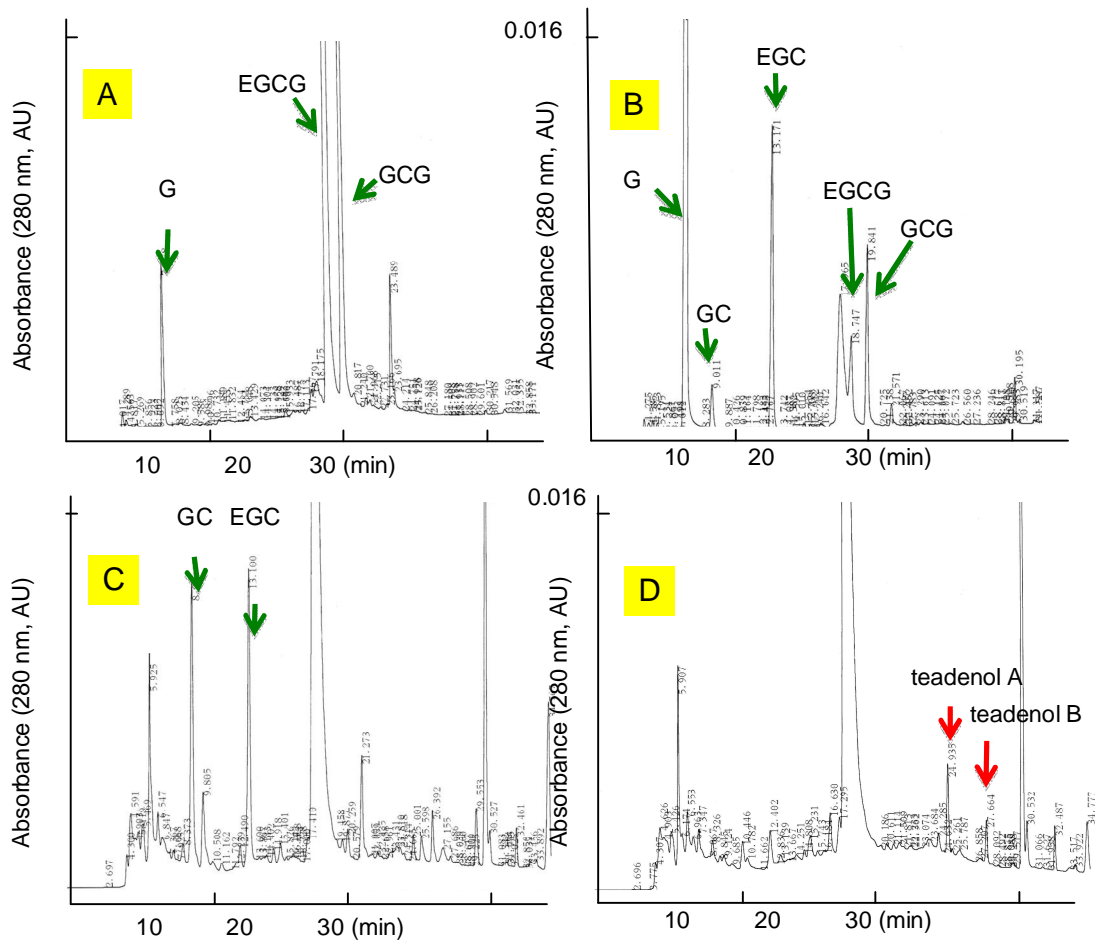


Fig. 18 HPLC profiles of the solvent (mixture of EGCG and GCG) incubated with *Aspergillus* sp. (FARM AP-21280). A: 0-day, B: 2-day, C: 9-day, D: 21-day period

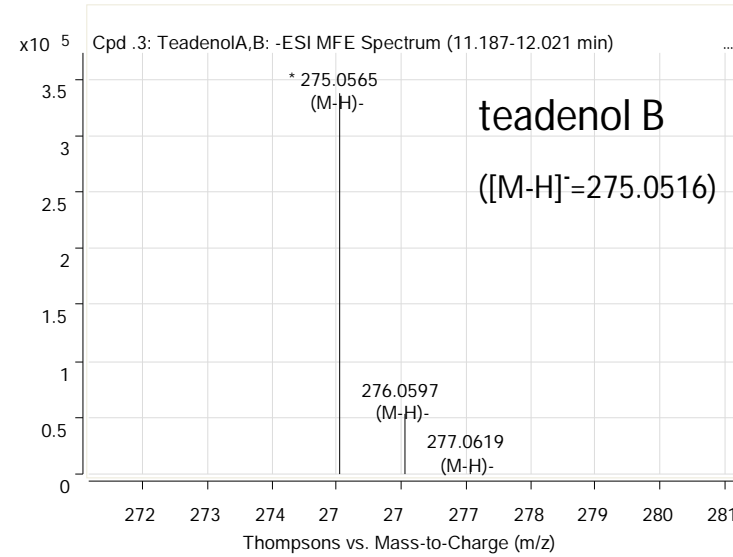
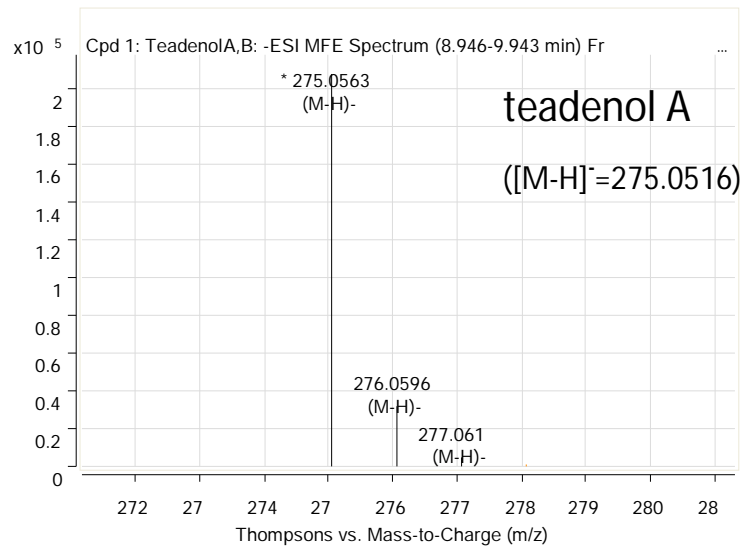
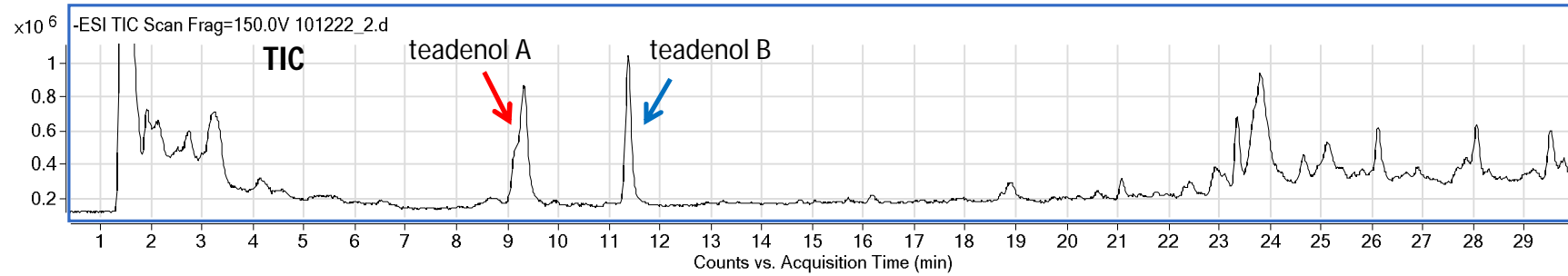
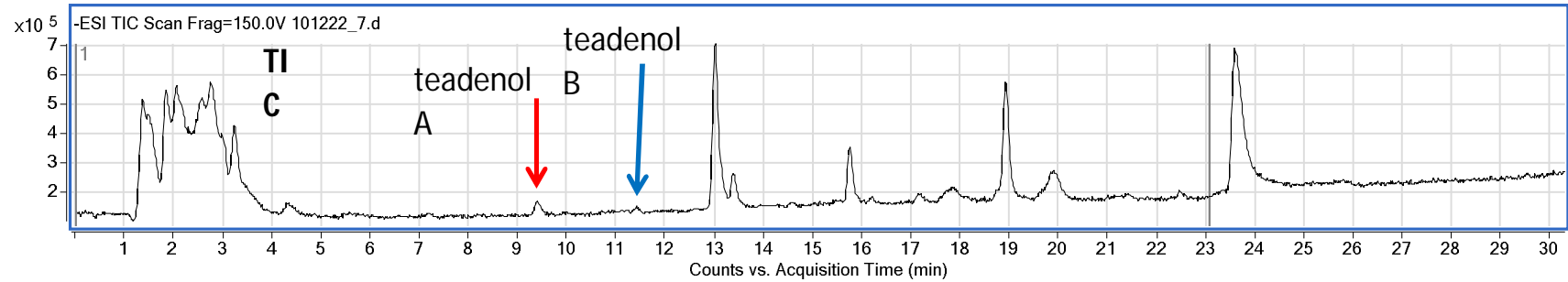
B

Fig. 19B HPLC-QTOFMS spectrum of the extract of '*Kippuku-cha*'

A



56

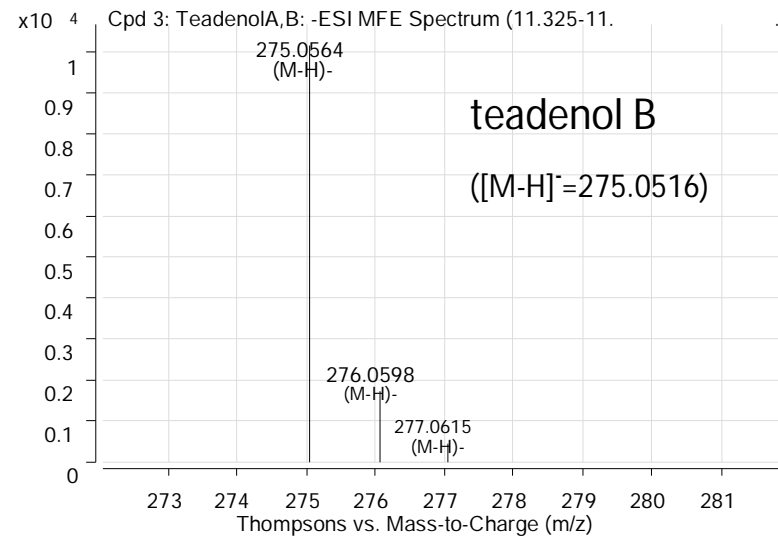
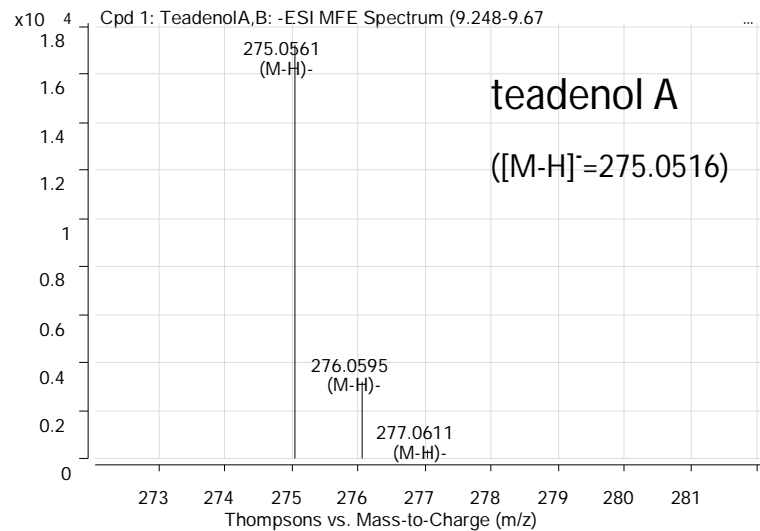


Fig. 19A HPLC-QTOFMS spectra of the solvent (mixture of EGCG and GCG) incubated with *Aspergillus* sp. (FARM AP-21280)

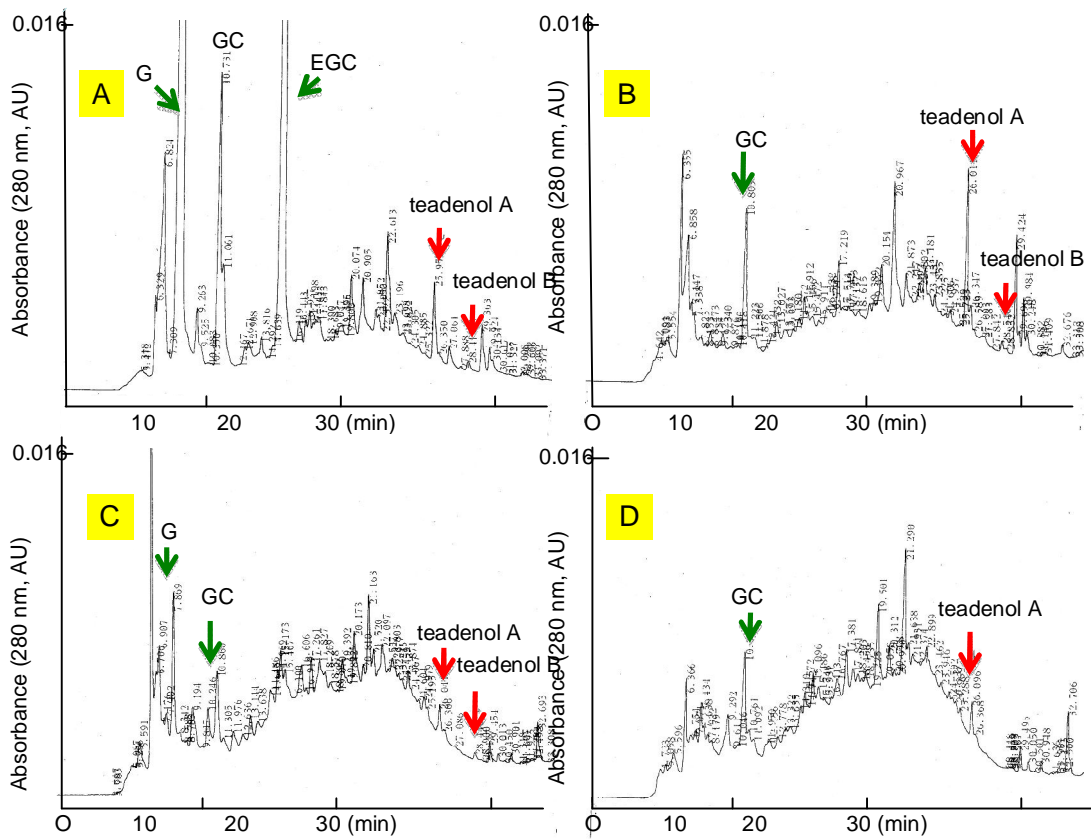


Fig. 20 HPLC profiles of the solvent (mixture of EGCG and GCG) incubated with *Aspergillus*. A: 3-day and B: 6-day period with *A. awamori*; C: 3-day and D: 6-day period with *A. kawachii*

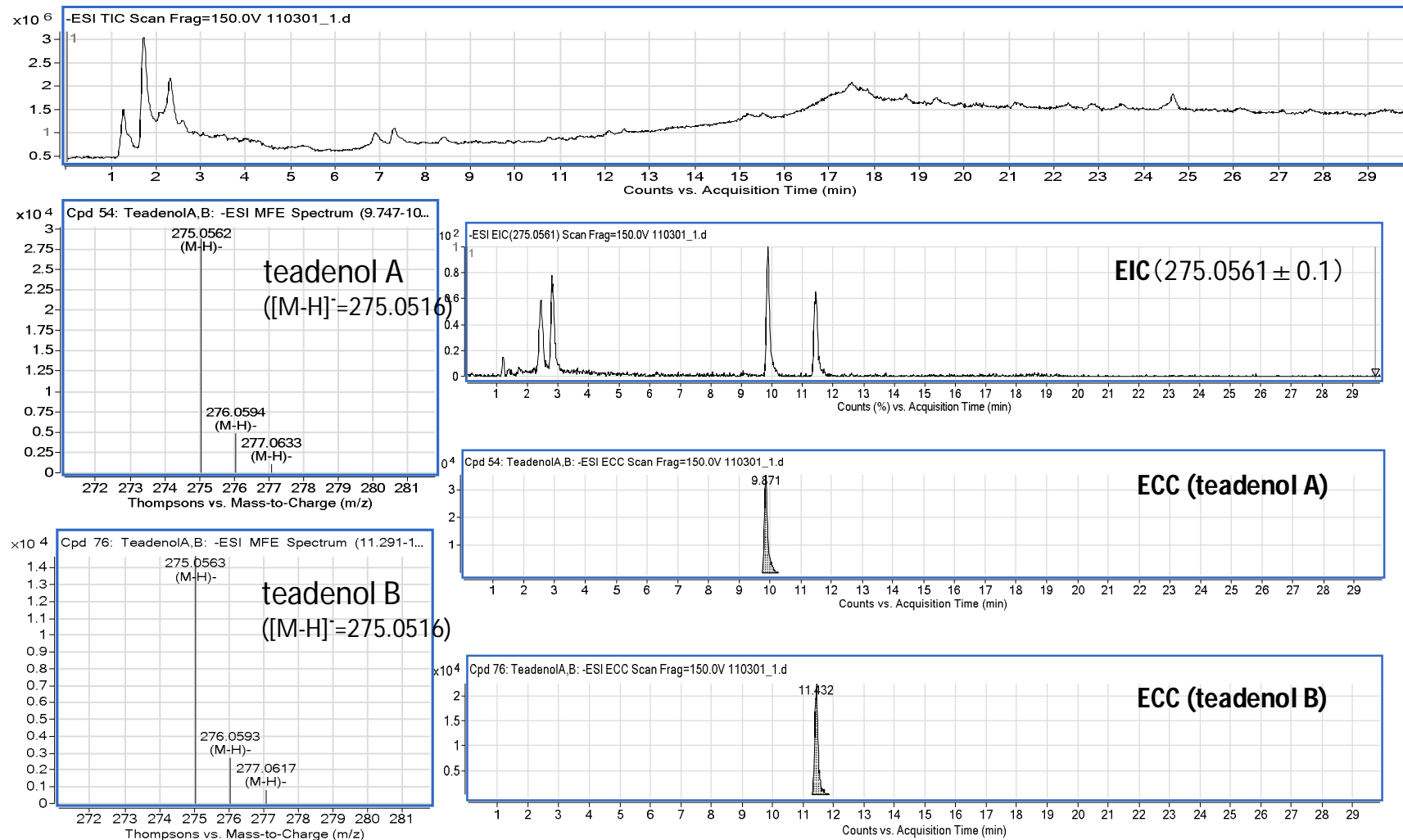


Fig. 21 HPLC-QTOFMS spectra of the solvent (mixture of EGCG and GCG) incubated with *A. awamori* (EIC : Extracted ion chromatogram , ECC : Extracted compound chromatogram)

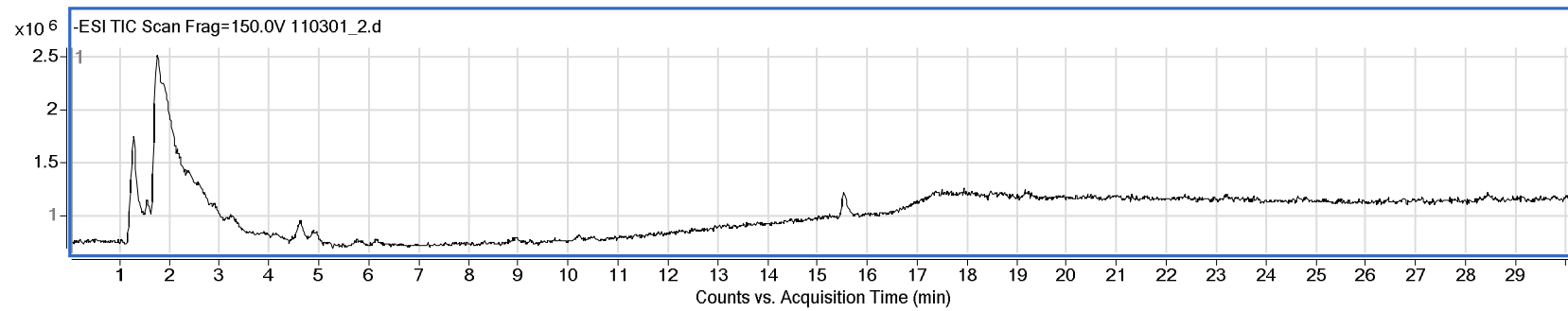


Fig. 22 HPLC-QTOFMS spectrum (TIC) of the solvent (mixture of EGCG and GCG) incubated with *A. kawachii*

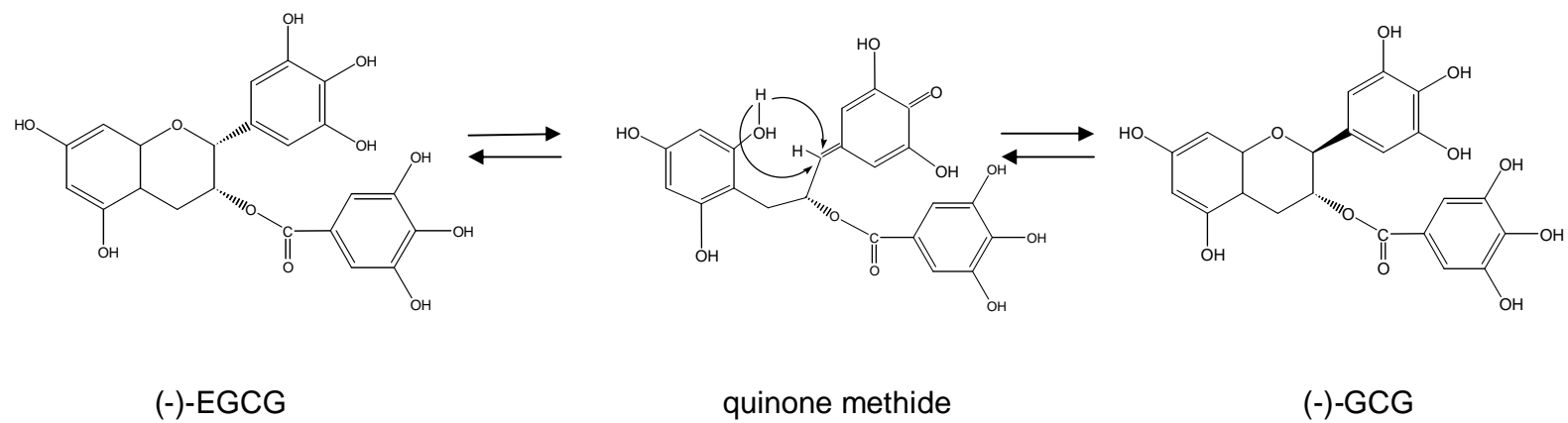


Fig. 23 Reaction mechanism for the epimerization of EGCG to GCG (Okumura *et al.*, 2008)

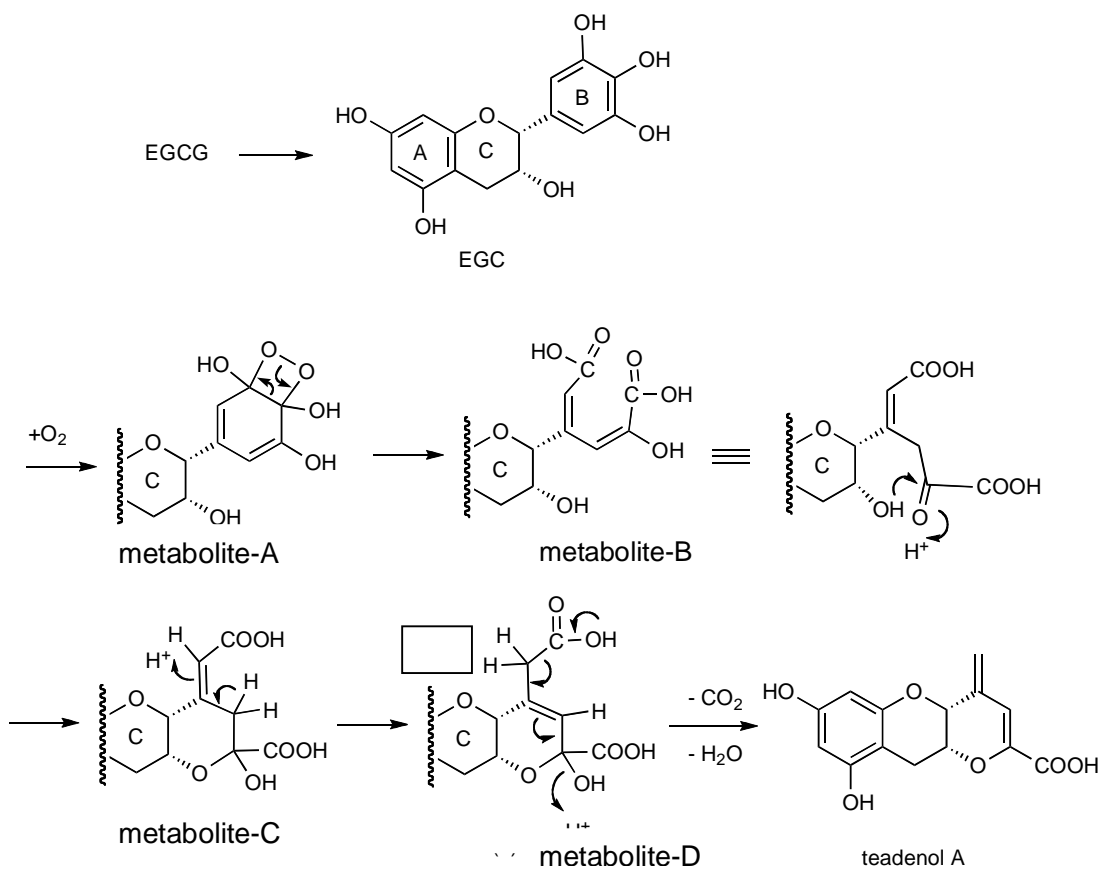


Fig. 24 A proposed pathway for the formation of teadenol A from EGCG

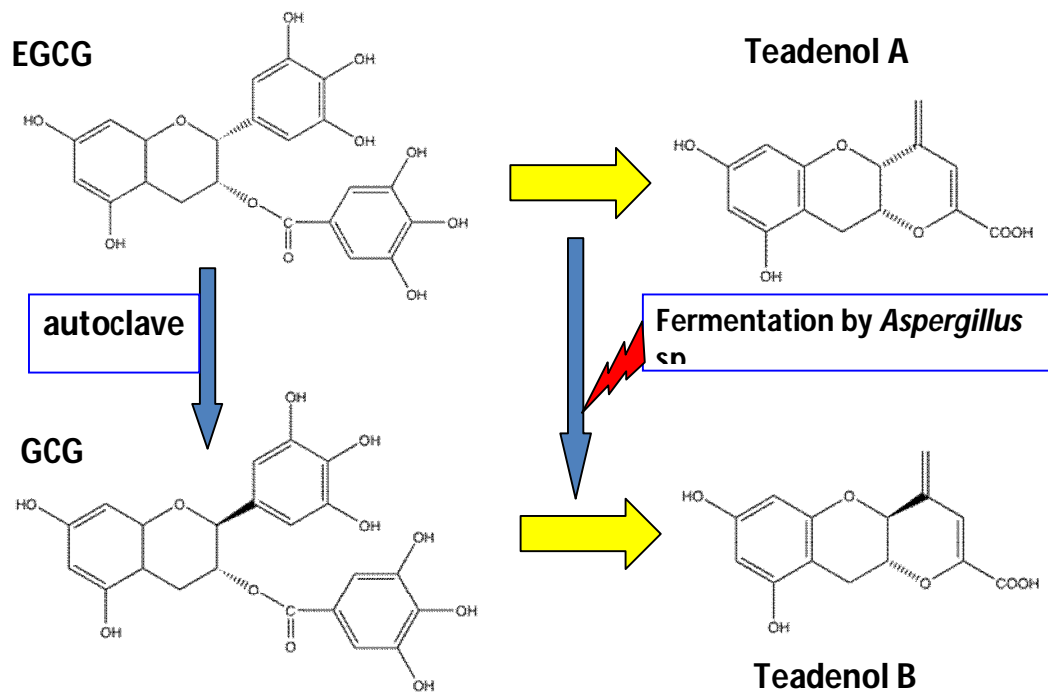


Fig. 25. The illustration of teadenols A and B biosynthesis

CHAPTER 5

GENERAL DISCUSSION

Fermented foods such as natto, soy sauce, *etc.* became popular all over the world. Microorganism (bacteria and fungi) which involved in these foods improve the nutritional value, sensory properties, and functional qualities of these materials during fermentation (Bladino *et al.*, 2003). Biotransformation represents a suitable complement to the chemical transformation reactions in diversifying structures of bioactive compounds (Miyake *et al.*, 2003; Zhu *et al.*, 2007).

Fermented tea is one of the popular fermented foods, because it is belong many bioactive compounds for human health. Pu-er tea is one of the popular microbial fermented tea. Puerins A and B, and blumenol B are useful compounds that were already isolated from pu-er tea and tea extracts fermented with *Fusarium solani*. (Huang *et al.*, 2008; Zhou *et al.*, 2005). Recently some Japanese microbial fermented teas have been circulated in Japan, for example `Kippuku-cha`, that may also contain new phenolic compounds.

In this research, two new phenolic compounds (teadenols A and B) were isolated for the first time from Japanese fermented tea (`kippuku-cha`), which was selectively fermented with *Aspergillus* sp. (FARM AP-21280). The chemical structures of teadenols were elucidated based on the analyses of their spectroscopic data ($^1\text{H-NMR}$, $^{13}\text{C-NMR}$, NOE, HMBC, *etc.*). (Wulandari *et al.*, 2011; Yanagita *et al.*, 2011a; Yanagita *et al.*, 2011b).

Pasha and Reddy (2005) claimed that teas fermented by microbial have superior value in terms of their nutritive and therapeutic and can be recommended for consumption as modified beverage with higher value.

Teadenols which produced in tea fermentation are getting increasing interest by the important bioactivities such as the promotion of adiponectin secretion and the inhibition of PTP1B (protein tyrosine phosphatase 1B) production (Yanagita *et al.*, 2011a; Yanagita *et al.*, 2011b). The level of adiponectin, a protein hormone, correlates with body fat percentage in adults and suppression of the metabolic derangement that may result in type 2 diabetes (Matsuzawa, *et al.*, 2004). PTP1B is also an effective target for the treatment of both type 2 diabetes and obesity (Zhang and Zhang, 2007).

Handy *et al.* (2011) also reported that adiponectin is protective against hepatic fibrosis, while leptin promote fibrosis. Therefore, the fermented teas, that accumulate teadenols, are expected to be as useful new materials for human health, especially for the prevention of metabolic syndrome diseases.

In this research, *Aspergillus* sp. (FARM AP-21280) was used in the tea fermentation yielding teadenols. Teadenols concentrations in commercial teas (green tea, oolong tea, black tea, and pu-er tea) and fermented teas which selectively fermented by *Aspergillus* sp. (FARM AP-21280), *A. oryzae* (NBRS 4214), *A. awamori* (NBRS 4122) and *Eurotium* sp. were also investigated. In pu-er teas, teadenols seemed to be metabolized by several types of fungi with various enzymatic degradation. The content of teadenols became lower in accordance with the storage time length.

Teadenols A and B were detected in all of the fermented teas. It can be concluded that not only *Aspergillus* sp. (FARM AP-21280) but also *A. oryzae*, *A. awamori* and *Eurotium* sp. have the ability of tea catechins transformation to teadenols. *A. awamori* produced the highest concentration of teadenols followed by *Aspergillus* sp. (FARM AP-21280), *A. oryzae*, and the lowest was *Eurotium* sp.

Eurotium species are the sexual states (teleomorphs) of *Aspergillus* species. It is indicated that the difference in the teadenols concentration may be due to the different enzyme activity among asexual, sexual, and whole fungus of *Aspergillus* (Yazdani *et al.*, 2011). *A. awamori* was better to use in the biotransformation of teadenols from tea catechins (EGCG) compared with *A. kawachii* and *Aspergillus* sp. (FARM AP-21280).

A. awamori that has been already used for Sake (*Shochu*) production, has a capability of transforming tea catechin (EGCG) to teadenols that was proven by HPLC-QTOFMS analysis. *A. kawachii* also seemed to have similar capabilities, but the teadenols contents in the tea catechin solution incubated with *A. kawachii* were lower compared to those of *A. awamori*.

Teadenols were more rapidly produced in the incubated solutions of EGCG with *A. awamori* and *A. kawachii* (one week period) compared with the case by *Aspergillus* sp. (FARM AP-21280) (three week period). This result suggested that *A. awamori* and *A. kawachii* have stronger enzymatic activities for tea catechins metabolism compared with that in *Aspergillus* sp. (FARM AP-21280). From HPLC-QTOFMS analysis, *A. awamori* was shown to possess the

better capability of teadenols biotransformation from tea catechins compared with *A. kawachii*.

In the experiment of the biotransformation of teadenols from tea catechins, a proposed pathway for the formation of teadenols from EGCG is also presented. Teadenols, detected in various fermented tea products, have been supposed to be biosynthesized from EGCG and GCG, respectively, by enzymatic reactions of *Aspergillus* sp.. After the hydrolysis of EGCG to be EGC, diacid compound was formed, and followed by decarboxylation and dehydrogenation, and than teadenol A is yielded.

With greater emphasis on natural products and the role of food in health and wellbeing, fermentation in the food manufactures not only for extending shelf life, but also to create functional food products takes an active part in maintaining human health (Farnworth, 2008). Some potential and unexplored food fermentation process (especially fermented tea) have some new possibilities for innovative applications of the new functional products for human society.

SUMMARY

Two new phenolic compounds (named as teadenol A and teadenol B, respectively) were isolated from tea (*Camellia sinensis* L.) leaves fermented with *Aspergillus* sp. (PK-1, FARM AP-21280). The chemical structures of teadenols were elucidated based on the analyses of their spectroscopic data (MS, ¹H-NMR, ¹³C-NMR, NOE, HMBC, etc). The absolute configurations of the structures of teadenols were also certified with their biosynthetic preparation in the treatment of tea catechins with *Aspergillus* sp..

In HPLC analysis of teadenols in the tea leaves fermented with *Aspergillus* sp. (FARM AP-21280), *A. oryzae* (NBRS 4214), *A. awamori* (NBRS 4122) and *Eurotium* sp. (FARM AP-21291), high amounts of teadenols A and B were detected in the fermented teas treated with *Aspergillus* sp. (teadenol A : 1.01-1.79%; teadenol B : 0.16-0.37% as dry weight).

For the evaluation of effective identification of specific metabolites produced in various types of commercial teas (green, oolong, black, and pu-er teas), HPLC-TOFMS analysis was performed. 627 compounds detected in among all samples (PK-1, green, oolong, black and pu-er teas). 359 compounds observed only in among four commercial teas (green, oolong, black, and pu-er teas) and 98 compounds observed only in the fermented tea (PK-1), respectively. Teadenol B was detected only in PK-1, and teadenol A was found with relatively high amount in PK-1 and not so much in four tea group. Teadenol A was

detected in a commercial tea (oolong), showing that the oolong tea might be fermented by natural *Aspergillus* sp. in the fermentation processing. Two compounds with $m/z = 756.2118$ and $m/z = 756.2108$, respectively, were also supposed to be flavonoid compounds (e.g. kaempferol triglycosides) from the m/z data. The profiling analysis of HPLC-TOFMS was very useful for the identification of complex metabolites in processed varieties of tea products.

Teadenols A and B were biosynthetically produced in the autoclaved solution of (-)-epigallocatechin 3-O-gallate (EGCG) [mixture of EGCG and (-)-gallocatechin 3-O-gallate (GCG)] which was incubated with *Aspergillus* sp. (FARM 21280), *A. awamori* (NRIB-2061) and *A. kawachii* (IFO-4308). This result demonstrated that teadenols detected in various fermented tea products are originated from tea catechins (EGCG and GCG) in the materials. A biosynthetic pathway of teadenols A and B from tea catechins was proposed in this experiment.

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