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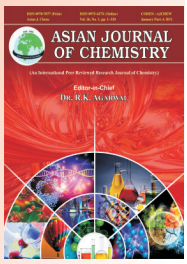

in vitro Antioxidant Activity of Ethyl Acetate Fraction of Fermentation Products of *Ginkgo biloba*

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We investigated the *in vitro* antioxidant activity of the ethyl acetate fractions of both fermentation and non-fermentation products of *Ginkgo biloba* leaves using 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-*bis*(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) assays. Using the two assays, we show that the free radical scavenging activity of the ethyl acetate fraction of 11 days fermentation product was strong on a large range of concentrations (0.800-20.000 mg/mL). Furthermore, using the HPLC and NMR techniques, we discovered for the first time the pharmacodynamic material basis of the *in vitro* antioxidant effect of *G. biloba* leaves; namely, caffeine was produced during microbial fermentation of *G. biloba* by *Eurotium cristatum* and was present in the ethyl acetate fraction of 11days fermentation product.

Keywords: *Ginkgo biloba*, Eurotium cristatum, Antioxidant activity, Caffeine, Pharmacodynamic material basis.

INTRODUCTION

Ginkgo biloba is one of the oldest living plant species, which has existed much more than 1,000 years[1] and is considered "a living fossil" because of its age. It is native to China and other Asian countries[2] and has been used for medical and dietary supplement purposes in traditional Chinese medicine for centuries[3]. *G. biloba* contains many bioactive compounds, including flavonoids, terpenoids and ginkgolides[1,4,5]. Recently, many studies have focused on the physiological actions of these compounds once isolated from *G. biloba* extract. In particular, scientists have been interested in examining the antioxidant and vasoactivating properties, such as the inhibition of platelet activating factor and neuro-transmitter modulation[6-13]. Based on studies of the physiological actions of *G. biloba* extract, standardized *G. biloba* extract has been used clinically in some European countries[14-17].

Antioxidant activity is one of the predominant physiological activities of *G. biloba* and it works by protecting tissues against damage caused by oxygen free radicals and lipid peroxidation[18].

Microbial fermentation technology has been applied in the production of herbal products for traditional Chinese medicine as early as 4,000 years ago, with many of these compounds being tested for and showing, clinical efficacy. Research on the benefit of combining traditional Chinese herbal medicine and microbial fermentation technology was first suggested during the end of the 20th century[19]. The idea of "bi-directional solid fermentation;" that is, one or many types of medicinal substances that each contain some active ingredients that were fermented using medicinal fungus, was first proposed by a Chinese scholar[20]. Since then, the examination of change of original costituents of fermented medicinal substances and the increasing of clinical efficacy and the decreasing of side effects of medicinal substances have been examined by many researches[21-26].

Eurotium cristatum, also known commonly as the Golden flower, was used as a fermentative strain for examining *G. biloba*. And its morphological characteristics, growing conditions, safety evaluation and activities were all investigated[27-31], was utilized as fermentative strain.

EXPERIMENTAL

G. biloba leaves were collected locally from Beijing in China and identified by Dr. Xuan Liu of Tsinghua University (Haidian District, Beijing, P.R. China). Voucher specimens (No. GBL-201106-01) were deposited at Tsinghua University Medical Science Building. *E. cristatum* was purchased from the China General Microbiological Culture Collection Center (CGMCC). 2,2-Diphenyl-1-picrylhydrazyl and 2,2'-azino-*bis* (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salts

were purchased from Sigma-Aldrich (St Louis, USA). Unless otherwise stated, all reagents and solvents were of HPLC or analytical grade.

Source of *E. cristatum*: The stock culture used for present studies was maintained on a potato dextrose agar (Beijing Aoboxing Bio-tech CO., China) slant and was subcultured every month. The slants were initially incubated at 30 °C for 4 days and then subsequently stored at 4 °C indefinitely. *E. cristatum* was activated twice in order to improve strain vitality.

Cultivation and sampling: *G. biloba* leaves were oven-dried at 55 °C and crushed into powder. The medium was similar to that reported previously[32] and was composed of 0.8 % *G. biloba* leaf powder, 6 % (w/v) sucrose and had a pH of 5.5. The *G. biloba* powder was boiled in distilled water and extracted twice (45 min for each round) and was then filtered. The filtrate was put into a 1 L volumetric flask, brought up to 1 L with distilled water and then transferred into a 2 L shake-flask.

E. cristatum was activated twice at 30 °C for 4 days prior to every inoculation into liquid culture medium. All of the *E. cristatum* on a single plate culture was transferred using 100 mL sterile water and was then homogenized in a shaker at 30 °C for 20 min at 150 rpm. Finally, 50 mL of homogeneous fungus solution was inoculated in a shake-flask, which had been filled with 1 L filtrate and fermented in the shaker at 30 °C for 0, 4, 7, 11, or 14 days at 120 rpm.

The zymotic fluid was extracted with petroleum ether, ethyl acetate and *n*-butanol. Every extract fraction was then concentrated and dried.

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity assay: We measured the antioxidant activities of extracts based on the scavenging activity of the free radical 2,2-diphenyl-1-picrylhydrazyl. This assay was performed following a previously described method[33], with some minor modifications. Briefly, 50 µL of each extract (0.032-100 mg/mL) was allowed to react with 150 µL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl in 100 % ethanol in a 96-well microplate. The plate was then incubated at 37 °C for 0.5 h, after which the absorbance was measured at 517 nm using a microplate reader. The scavenging capacity of each sample was then compared to that of ascorbic acid as a positive control (0.032-100 mg/mL). Activity level was then calculated as a percentage of 2,2-diphenyl-1-picrylhydrazyl decolouration (relative to a negative control) using the following equation:

Antiradical activity % = (absorbance of control incubation-absorbance of the extract)/(absorbance of control incubation)×100

ABTS radical scavenging activity assay: To measure the antioxidant activity of the different extracts, we carried out an ABTS assay following methods previously described[34]. The radical cation ($ABTS^{+\bullet}$) was prepared by mixing (in equal volumes) a 100 % ethanol ABTS solution (final concentration 7 mM) with a 100 % ethanol solution of potassium persulfate (final concentration 2.45 mM) and allowing the mixture to stand in the dark at room temperature for 16 h prior to use. The $ABTS^{+\bullet}$ stock solution was diluted 1:80 with water to obtain an absorbance at 734 nm, which ranged from 0.6-0.8 before use. 50 µL of extracts (0.032-100 mg/mL) were then added in a 96-well microplate to 150 µL of the diluted $ABTS^{+\bullet}$ solution. The plate was then incubated at 37 ºC for 0.5 h, after which the absorbance was measured at 735 nm using a microplate reader. The scavenging capacity of each of these samples was then compared to that of ascorbic acid as a positive control (0.032-100 mg/mL). Activity was calculated as a percentage of $ABTS^{+\bullet}$ decolouration relative to a negative control using the following equation:

Antiradical activity % = (absorbance of control incubation-absorbance of the extract)/(absorbance of control incubation)×100

HPLC analysis of the extracts: All extracts were measured using HPLC, with the following conditions: stationary phase, Boston Green C8 Column (4.6 × 250 mm, 5 µm) (Boston Analytics, Inc., USA); Ultimate 3000 HPLC system (Thermo Fisher, Germany); mobile phase, methanol (A) and water (B) in gradient (0-5 min: 20 % methnol-40 % methnol; 5-15 min: 40 % methnol-60 % methnol; 15-25 min: 60 % methnol-90 % methnol; 25-35 min: 90 % methnol-90 % methnol; 35-40 min: 90 % methnol-20 % methnol); flow rate: 1 mL/min; detective wave length: 254 nm; temperature: 30 ± 1 °C. We used this method to characterize the differences of components of the ethyl acetate fractions of fermented and unfermented extracts.

Extraction and isolation: *G. biloba* leaf (8 g) powder was extracted by boiling in water (twice, 200 and 100 mL each, 45 min for each round of boiling). The solutions were then filtered and fermented in a shaker at 30 °C for 0, 4, 7, 11 or 14 days, at 120 rpm. The fermented solutions were then filtered and concentrated using rotary evaporation under reduced pressure and were dried under a vacuum to obtain the powdered extracts. Extracts were successively partitioned with petroleum ether, ethyl acetate and *n*-BuOH. The ethyl acetate portion (122.1 mg), which was from the A days fermented solution, was dissolved and filtered using a 0.45 µm millipore filter. This solution was then applied to an MCI column (MCI Gel: CHP 20P, 75-150 µ chromatography, Mitsubishi Chemical Corporation, Japan) and successively eluted with a water-methnol dilution series (8: 2 - 6: 4 - 4: 6 - 0: 100), which resulted in 12 fractions. Fraction 7 (15.3 mg) was subjected to a semi-preparative column (Boston Green C8 Column (10 × 250 mm, 5 µm), Boston Analytics, Inc., USA) and was eluted with a 35 % methnol solution, resulting in 1.87 mg of compound **1**.

RESULTS AND DISCUSSION

Rate of ethyl acetate fractions from *G. biloba* leaves, which was fermented for different lengths of time: The antioxidant activity of *G. biloba* leaves has been reported in a previous study[40]. In contrast, our experiments investigated the antioxidant effect of the total extracts and ethyl acetate fraction of *G. biloba* leaves. Here, we found that the ethyl acetate fraction of fermentation and non-fermentation products of *G. biloba* leaves, which was fermented for different lengths of time (0, 4, 7, 11 or 14 days) gave different, though consistent, results. The rate of the ethyl acetate fractions of *G. biloba* leaves that were fermented for 11 days was the largest (Table-1).

TABLE-1
EtOAc FRACTIONS OF *G. biloba* LEAVES WHICH WERE FERMENTED IN DIFFERENT TIME

	Fermentation time (days)				
	0	4	7	11	14
EtOAc fraction (mg)	115.1	111.1	95.0	315.5	222.5

2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity: It is widely accepted that 2,2-diphenyl-1-picryl-hydrazyl free radical scavenging by antioxidants is due to their hydrogen-donating ability. 2,2-Diphenyl-1-picrylhydrazyl has been used to test the free radical-scavenging ability of various samples[42]. We used 2,2-diphenyl-1-picrylhydrazyl to examine the free-radical scavenging ability of the samples that we tested and the results are shown in Fig. 1.

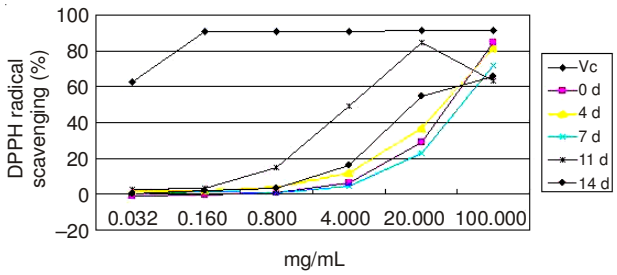

Fig. 1. DPPH radical scavenging percentage of the VC and the EtOAc fractions of fermentation and non-fermentation products in *G. biloba* leaves

As the concentration of ethyl acetate fraction increased (range from 0.032 to 20 mg/mL), the amount of 2,2-diphenyl-1-picrylhydrazyl free radical scavenging increased (Fig. 1). The ratio of the 2,2-diphenyl-1-picrylhydrazyl free radical scavenging of the ethyl acetate fraction of 11 days fermentation products was more than that of any other fractions and this was true over a broad range of concentrations (from 0.800 to 20 mg/mL). When the concentration of reached 20 mg/mL, the ratio of scavenging surpassed 80 %, which is close to the ratio of VC. Given this evidence, we thus consider the ethyl acetate fraction of 11 days fermentation products as having the greatest antioxidant activity of all of the fractions.

ABTS free radical scavenging activity: The ABTS assay is an indirect method that measures the ability of the ABTS radical cation to abstract a hydrogen atom or an electron from a compound[43]. The simplicity of the method makes it tractable for many applications and it has been used to assess the ability of compounds to act as hydrogen/electron donors. We applied the ABTS assay and the results of the ABTS scavenging of each fraction we examined at various concentrations is shown in Fig. 2.

As concentration increased (ranged from 0.032 to 20 mg/mL), the ratios of ABTS free radical scavenging of all of the fractions increased (Fig. 2). The ratio of the ABTS free radical scavenging of the ethyl acetate fraction of 11 days fermentation products was larger than that of any other fraction examined and this was true across a large range of concentrations (0.800-20 mg/mL). When the concentration increased to 20 mg/mL, the ratio reached 95 %, which was the same as the VC ratio.

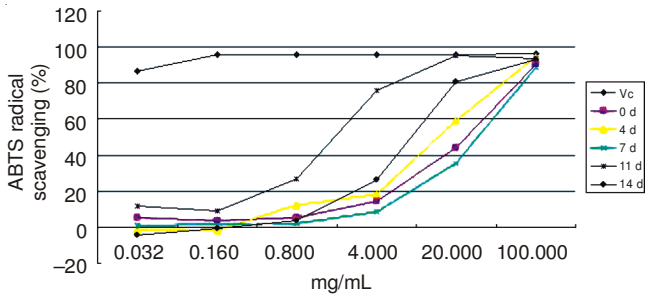

Fig. 2. ABTS radical scavenging percentage of the VC and the EtOAc fractions of fermentation and non-fermentation products in *G. biloba* leaves

HPLC analysis of the ethyl acetate fractions of fermentation and non-fermentation products in *G. biloba* leaves: HPLC chromatogram analysis of the ethyl acetate fractions of fermentation and non-fermentation products in *G. biloba* leaves showed that compound A (caffeine) was only measured in the ethyl acetate fraction from 11 days fermentation products (Fig. 3). Thus, it appears that caffeine can be produced in fermentation products only during a restricted time period. Importantly, fermentation time played a critical role in producing caffeine during the fermentation process.

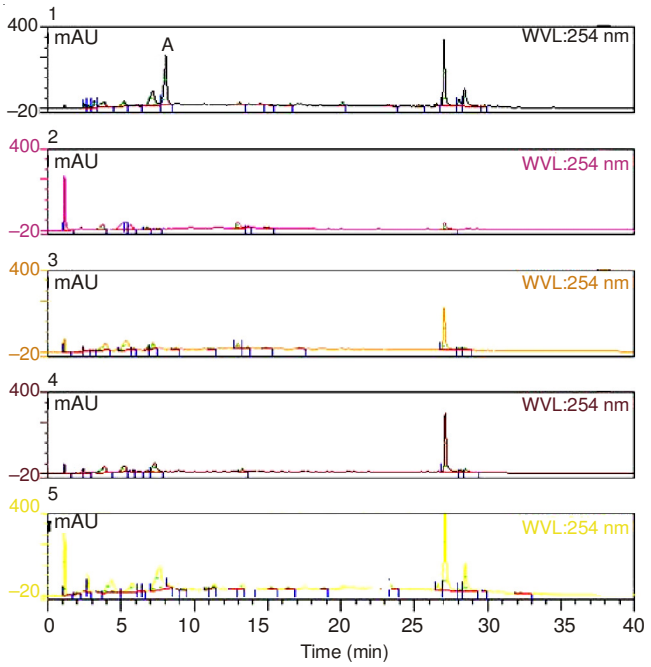

Fig. 3. High performance liquid chromatography (HPLC) chromatograms of the EtOAc fractions of fermentation and non-fermentation products in *G. biloba* leaves. (1) chromatogram of EtOAc of 11 days fermentation product, (2) chromatogram of EtOAc of 0 day fermentation (non-furmentation) product, (3) chromatogram of EtOAc of 4 days fermentation product, (4) chromatogram of EtOAc of 7 days fermentation product, (5) chromatogram of EtOAc of 14 days fermentation product; (A) Caffeine

HPLC analysis of source of caffeine obtained from the ethyl acetate fraction of fermentation products in *G. biloba* leaves: In order to investigate the source of caffeine (A), three extracts-the ethyl acetate fractions of 0 days fermentation product, 11 days fermentation product and the negative control (medium was composed without *G. biloba* powder)-were analyzed by HPLC.

The caffeine was not measured from the ethyl acetate fraction of the 0 day fermentation product and the negative control indicated that this compound resulted from microbial fermentation rather than *E. cristatum* or the extract of *G. biloba* leaves (Fig. 4). This is the first report of a new microbial path for the production of caffeine by *E. cristatum*. Importantly, it has been reported in several studies that caffeine possesses a high antioxidant capacity[35-37].

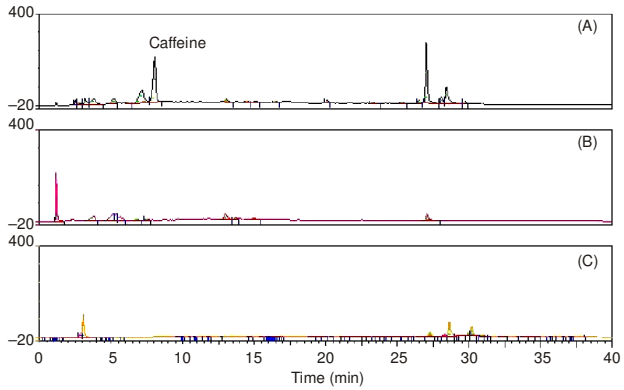

Fig. 4. High performance liquid chromatography (HPLC) chromatograms of the EtOAc fractions of 0 and 11 days fermentation products and the sample of negative control. (A) chromatogram of EtOAc of 11 days fermentation product, (B) chromatogram of EtOAc of 0 day fermentation (non-furmentation) product, (C) chromatogram of the sample of negative control

On account of purines being widely distributed in the bodies of animals and plants[38,39], we can reasonably hypothesize that the caffeine we detected was generated from methylated xanthine, which was formed during the process of microbial fermentation.

Structure elucidation: Compound **1** was obtained as a white powder. Using HR-ESI-MS (m/z 195.0878 $[M + H]^+$), we determined it to have a molecular formula of $C_8H_{10}N_4O_2$. The ^1H NMR data (600 MHz, CD_3OD) for this compound were as follows: δ_H: 3.37 (3H, s, 1-CH_3), 3.55 (3H, s, 1-CH_3), 3.99 (3H, s, 1-CH_3) and 7.88 (1H, s, H-8), which were the same that which has been reported previously for caffeine[41].

Conclusion

Present results suggest that the ethyl acetate fraction of fermentation products of *G. biloba* leaves exhibits remarkable antioxidant activity. We were also able to explain the pharmacodynamic material basis of antioxidant effects in *G. biloba* leaves. These results broadly imply that the technology of microbial fermentation might help to expand the use of traditional Chinese medicine.

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