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Note

A Radical Scavenging Compound, 3-Pyridinol, in Instant Coffee and its Hepatoprotective Activity

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A compound with 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, produced in the course of roasting coffee beans, was isolated from instant coffee. The chemical structure of this compound was elucidated to be 3-pyridinol on the basis of various spectra analyses. It prevented cytotoxicity induced by D-galactosamine in primary cultured rat hepatocytes, indicating that this compound may prevent liver injury partly induced by the action of radicals.

Keywords: coffee beans, 3-pyridinol, Brazil Santos, hepatoprotective activity

Introduction

Polyphenols in foodstuffs are the major focus of many consumers because some of them possess properties that prevent certain diseases caused by oxidative stress. Coffee beans and hot water extracts of roasted coffee beans are known to contain over 700 components including polyphenols, caffeine, purine alkaloids, chlorogenic acid and its related compounds, diterpens and their fatty acid esters, trigonelline and its related compounds, and many others. Chlorogenic acid and its related compounds are the most abundant components in coffee beans and many researchers have studied the antioxidative and physiological functions of chlorogenic acid. The preventive effects of chlorogenic acid on *in vivo* oxidation (Basnet *et al.,* 1996) and HIV virus infections (Mahmood *et al.,* 1993), the suppression effects against release of histamine from mast cells (Kimura *et al.,* 1985), serum cholesterol lowering activity (Wojcicki, 1978), and the preventive effects of caffeine against lipopolysaccharide-induced liver injury (He *et al.,* 2001) have been reported. Furthermore, the roasting of coffee beans produces many volatiles (Lee and Shibamoto, 2002) and melanoidins with antioxidative properties (Borrelli *et al,* 2002; Wen *et al.,* 2004). These reports prompted us to investigate the antioxidative compounds produced in the process of coffee bean roasting.

In this study, a radical scavenging compound was investigated and identified from instant coffee. The roasting conditions needed to produce this compound and its preventive effects against D-galactosamine (GalN)-induced cytotoxicity in primary cultured rat hepatocytes were also studied.

Materials and Methods

Isolation and identification of compounds with radical-scavenging activity from decaffeinated coffee Filtrate of a methyl alcohol (MeOH) soluble fraction of decaffeinated coffee (NESCAFE, Nestle Japan) was evaporated and fractionated by polyamide column (40×5 cm i.d.) chromatography, using ethyl acetate/chloroform ($CHCl_3$)/88% formic acid/H_2O (19: 1: 1: 1 by volume) as a developing solvent. The flow rate was 1 ml/min and fraction volume was 18 ml. Fraction A (fraction tube No. 26–60) containing compound A with an R_f value of 0.16 on a silica gel TLC plate was developed using ethyl acetate/$CHCl_3$/88% formic acid/H_2O (19: 1: 1: 1 by volume). Compound A was positive for both diazotized sulphanilic acid and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical spraying reagents. Fraction A was further fractionated by silica gel column (30×5 cm i.d.) chromatography using four liters of the same developing solvent and subsequently using one liter of $CHCl_3$/MeOH (1: 1 by volume). The eluate with the latter solvent was further fractionated by Sephadex LH-20 column (75×3.5 cm i.d.) chromatography with 50% MeOH. Compound A was finally purified by preparative HPLC until only a single peak on the HPLC chromatogram was observed. HPLC was performed using a Develosil C30-UG-5 column (20 i.d.×250 mm, Nomura Chemicals, Aichi, Japan) and a solvent system consisting of solvent A (5% acetonitrile with 1% acetic acid) and B (40% acetonitrile). Compound A was eluted with a linear gradient of solvent B (0 to 100%) in solvent A for 240 min, at flow rate of 2.0 mL/min. Detection was performed at a wavelength 280 nm.

Electron ionization mass spectrometry (EI-MS) data were recorded using a JEOL JMX-D300 spectrometer. ^{1}H- and ^{13}C-NMR spectra were measured using a JEOL-GX-400 spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts are given in δ ppm relative to tetramethylsilane (TMS).

Roasting of raw coffee beans and quantification of compound A (3-pyridinol) Coffee beans, Brazil Santos, were

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roasted at 200℃ for 14 min, 220℃ for 17 min, and 230℃ for 19 min using a roasting machine. Grounded roasted coffee beans (1.8 g) were extracted using 10 mL of boiling water for 5 min, and the filtrate of the extracts was subjected to HPLC using a Develosil RPAQUEOUS column (4.6 i.d.× 250 mm). Elution of 3-pyridinol from the column was performed using a 50 mM sodium phosphate buffer (pH 6.9) and it was detected at 280 nm. The amount of 3-pyridinol in commercial roasted coffee beans, Mocha Blend, UCC Japan, was also determined using the same method.

Measurement of DPPH radical scavenging activity The DPPH radical scavenging activities of 3-pyridinol, compounds contained in roasted coffee beans (Basco *et al.,* 1999), and pyridine, which lacks the hydroxyl group in the chemical structure of 3-pyridinol, were compared. The activity was measured according to the method of Suda (2000) with slight modification, and expressed as a Trolox equivalent. Each compound, in 50% ethanol, was added to a 1: 1: 1 mixture of DPPH solution (400 μM in ethanol), 0.2 M 2-morpholinoethanesulphonic acid (MES) buffer (pH 6.0), and 50% ethanol. The solution was mixed using a vortex mixer (10 s pulses) and the absorbance was measured 20 min after mixing at 520 nm. The Trolox equivalent of the sample was calculated using the standard curve for Trolox.

GalN-induced cytotoxicity Hepatocytes were prepared from a 10-week old male Wistar strain rat (250 g) using the collagenase perfusion method (Matsuda *et al.,* 2002). Cells were suspended in William's E medium containing newborn bovine serum (NBS) (10%) and kanamycin (30 μg/mL) (MWE: modified William's E medium) and inoculated in a 96-well tissue culture plate (Matri-gel, BD Bioscience, USA) at 4×10^4 cells per 0.1 mL of William's E medium with 10% NBS, insulin and dexamethason (10^{-8} M and 10^{-6} M, respectively) (pre-culture medium). The cells were incubated for 4 h at 37℃ under a 5% CO_2 atmosphere. The medium was then replaced by 0.09 ml of fresh pre-culture medium with or without GalN at a final concentration of 5 mM. 3-pyridinol, chlorogenic acid, caffeic acid, quinic acid or pyridine were dissolved in 0.01 mL of the pre-culture medium containing 1.5% dimethyl sulfoxide (DMSO), at a final concentration of 500 μM, and added to the replaced medium. The cells were cultured for a further 44 h under the same culture conditions as described above. Medium was then removed and the cells were re-cultured with 0.1 mL of MWE containing 10 μL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide) solution (5 mg of MTT/mL of phosphate-buffered saline (pH 7.4)) for 4 h under the same culture conditions. After washing the cells with phosphate-buffered saline (pH 7.4), they were mixed with 100 μL of 0.04 N HCl in isopropyl alcohol, followed by shaking for 10 min to solubilize the formed formazan. The optical density (O.D.) of the isopropyl alcohol solution was measured at 570 nm (reference wavelength 655 nm) using a microplate reader.

At all times, the rats were cared for according to the institutional guidelines of Yamagata University.

Caffeic acid, quinic acid, 3-pyridinol and pyridine were purchased from Kanto Kagaku Co. Ltd. (Tokyo, Japan), chlorogenic acid from Acros Organics (Tokyo, Japan), and phosphate-buffered saline (pH 7.4) from GIBCO (California, USA).

Results and Discussion

Structural determination of compound A Compound A, which was detected as a single peak on an HPLC chromatogram, displayed a pink color on the TLC plate after being sprayed with diazotized sulphanilic acid, but was not affected by ferric chloride spray reagent. In the TLC plate, compound A scavenged the DPPH radical reagent that was sprayed on to it. The EI mass spectrum of compound A exhibited a molecular ion peak at *m/z* 95. ^{1}H- and ^{13}C-NMR spectra of compound A were in good agreement with those of authentic 3-pyridinol. δ_H: 8.12 (1H br.s, H-2), 7.15 (1H, ddd, $J=1.5$, 4.4 and 8.3 Hz, H-4), 7.19 (1H, br.dd, $J=4.4$, 8.3 Hz, H-5), and 8.02 (1H, br.d, $J=1.5$ and 4.4 Hz, H-6); δ_C: 137.6 (C-2), 153.7 (C-3), 122.4 (C-4), 124.2 (C-5), and 139.9 (C-6).

Effect of roasting condition on the formation of 3-pyridinol HPLC chromatograms of boiled water-soluble fractions of raw and roasted Brazil Santos coffee beans are shown in Figure 2, along with that of authentic 3-pyriodinol. The amount of 3-pyridinol in roasted beans increased from 25 mg/100 g to 40 mg/100 g when the roasting temperature increased from 200℃ to 220℃. Conversely, it decreased from 40 mg/100 g to 32 mg/100 g when the roasting temperature increased from 220℃ to 230℃. The decrease in the amount of 3-pyridinol at 230℃ may be due to degradation and/or vaporization. The amount of 3-pyridinol in commercially available Mocha Blend was 14 mg/100 g roasted beans. The difference between Brazil Santos and Mocha Blend indicates that commercially available roasted beans may have different amounts of 3-pyridinol depending on their roasting conditions, such as roasting temperature and times.

It is known that trigonelline, which is contained in raw coffee beans, produces nicotinic acid during roasting through demethylation of trigonelline (Taguchi *et al.,* 1985). The formation mechanism of 3-pyridinol during the roasting procedure has not yet been reported and we are the first to report on the isolation and identification of 3-pyridinol from roasted coffee beans.

DPPH radical scavenging activity Table 1 shows the DPPH radical scavenging activities of 3-pyridinol and pyridine. The radical scavenging activity of 3-pydinol

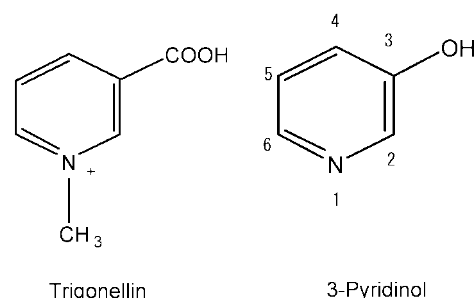

Fig. 1. Chemical structures of trigonelline and 3-pyridinol.

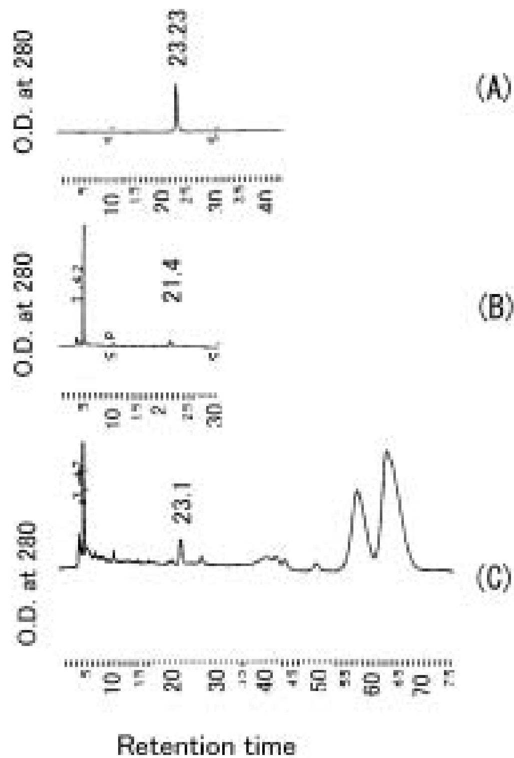

Fig. 2. HPLC chromatogram of authentic 3-pyridinol and boiling water-soluble fractions of raw coffee beans and roasted coffee beans.
(A) Authentic 3-pyridinol; (B) boiling water-soluble fraction of raw coffee beans (Brazil Santos); (C) boiling water-soluble fraction of coffee beans (Brazil Santos) roasted at 220℃ for 17 min.
HPLC conditions: column, Develosil RPAQUEOUS; solvent, 50 mM sodium phosphate buffer (pH 6.9); detection, 280 nm.

Table 1. DPPH radical scavenging activities of 3-pyridinol, compounds found in roasted coffee beans.

Compounds	(μmole Trolox eq/μmole sample)
3-Pyridinol	0.006
Pyridine	0.002×10^{-3}
Trigonelline	0.110×10^{-3}
Nicotinic acid	0.220×10^{-3}
Chlorogenic acid	1.14
Caffeic acid	1.26
Quinic acid	0.015×10^{-3}

was considerably weaker than that of chlorogenic acid and caffeic acid. However, the formation of 3-pyridinol during roasting is interesting, because cumulative accumulation of this compound *in vivo* may be effective in mitigating oxidative stress *in vivo,* if this compound could be easily absorbed from the intestinal tract. The radical scavenging activity of trigonelline, nicotinic acid, and quinic acid and pyridine was negligible.

Hepatoprotective activity Figure 3 shows the hepatoprotective activities of chlorogenic acid, caffeic acid, quinic acid, 3-pyridinol and pyridine. The optical density (O.D.) at 570 nm due to MTT formazan formed from MTT by the cells cultured in the presence of GalN, was lower than that

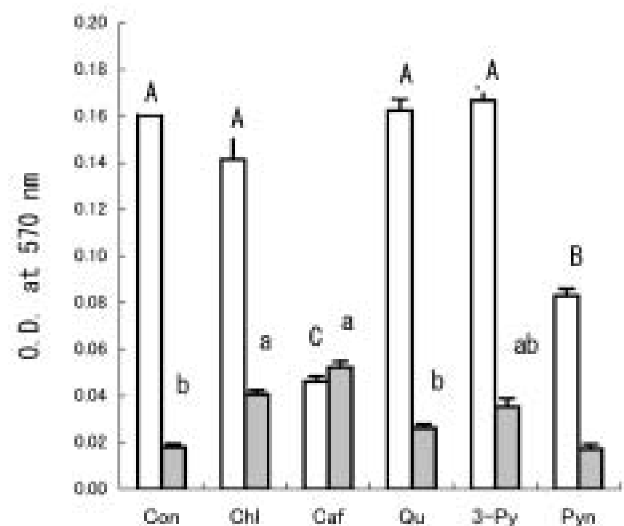

Fig. 3. Protective effect of 3-pyridinol and chlorogenic acid-related compounds on D-galactosamine(GalN)-induced injury of cultured rat hepatocytes.
Open circle, cells not treated with GalN; closed circle, cells treated with GalN. Con, cells not treated with a test sample; Chl, cells treated with chlorogenic acid; Caf, cells treated with caffeic acid; Qu, cells treated with quinic acid; 3-Py, cells treated with 3-pyridinol; Pyn, cells treated with pyridine. Each sample (for both open and closed circle groups) and GalN (for closed circle group) was added to the culture medium at final concentrations of 500 μM and 5 mM, respectively.
Groups with different capital and small letters differ significantly among the respective groups at $p < 0.05$.

by the cells cultured without GalN, indicating that GalN had cytotoxicity on the hepatocytes to lower the OD. While chlorogenic acid and caffeic acid prevented the cytotoxic effect of GalN, but quinic acid did not. Our results suggest the preventative effect of chlorogenic acid may have been caused by the caffeic acid moiety of chlorogenic acid. Since pyridine did not prevent GalN cytotoxity, it was considered that the hydroxyl group of 3-pyridinol was involved in preventing cytotoxicity.

It is suggested that intra-peritoneal injection of GalN into rats induces the formation of free radicals in the liver (Hu and Chen, 1992) and inhibits protein synthesis (Decker and Keppler, 1974). The hepatoprotective effects of 3-pyridinol might be partly due to its radical scavenging activity.

From this study, we propose 3-pyridinol, formed by the roasting of coffee beans, has promotive activity to protect hepatocytes. However, the amount of 3-pyridinol compared with that of chlorogenic acid, the dominant polyphenol in roasted coffee beans, was very small. The amounts of 3-pyridinol and chlorogenic acid, obtained from 100 g of coffee beans roasted at 220℃ for 17 min, were 32 mg and 177 mg, respectively. Thus, the contribution of 3-pyridinol to the hepatoprotective effect of coffee may be less than the effect of chlorogenic acid.

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