Bulletin of Musashigaoka College **10**, 21-27 (2002)

Screening for biological activity of coffee extracts

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Abstract

An investigation regarding the biological activity of coffee extracts was conducted based on their antibacterial, antifungal, antiviral, TNF-α production promoting, neurite outgrowth promoting, melanin synthesis inhibiting, telomerase inhibiting and human hair outer root sheath cell outgrowth promoting actions. As a result, methanol (MeOH) and hot water extracts of Toraja coffee (sour taste) and Mandarin coffee (bitter taste) demonstrated anti-IHNV, anti-human influenza virus and melanin synthesis inhibiting activities. In particular, melanin synthesis inhibiting activity of MeOH and hot water extracts was comparable to that of arbutin, an active substance widely used to whitening cosmetics. This finding is promising with respect to the development of new natural whitening cosmetics supplemented with coffee extracts.

Key words: coffee, melanin synthesis, whitening cosmetics, antiviral activity

Introduction

Caffeine, chlorogenic acid, lipid, sugar and minerals are the fundamental components of coffee[1]. A few of these compounds, mainly caffeine and chlorogenic acid, are known to possess biological activity. It has been reported that caffeine can stimulate brain activity via enhancement of the metabolism of adenosine triphosphate in neuronal cells[2]. On the other hand, chlorogenic acid may function as an antioxidant that can trap free radicals, which are thought to be the main cause of cancer and arteriosclerosis[3]. Chlorogenic acid is also capable of preventing stomach cancer on the basis of its decomposition of nitrite salts[4]. In actuality, coffee has been regarded as a natural health drink, or even as a medicine, since ancient times. Apart from the various medical effects of coffee described above, a recent study revealed that coffee effectively inhibited gallstone formation[5]. Therefore, it is highly probable that additional novel bioactive substances may be present in coffee.

In this investigation, our search focused on novel or previously known substances from coffee extracts exhibiting biological activity. To accomplish this goal, a comprehensive study was conducted to identify biologically active substances in coffee extracts.

Materials and Methods

■ Preparation of coffee extracts

Two kinds of coffee, Toraja (sour taste) and Mandarin (bitter taste), were employed in this study. Coffee powder was mixed with 4 volumes of phosphate buffered saline (PBS) (w/v) and homogenized with a Polytron mixer (Kinematica) at 8,000 rpm for 5 min. The homogenate was then centrifuged at 3,000 rpm for 20 min. The resulting supernatant was passed through a 0.22 μm pore-sized filter (GV type, Millipore) as the PBS extract. The pellet was introduced to four volumes of MeOH

(w/v), followed by homogenization and centrifugation as described above, which yielded the supernatant as the MeOH extract. Hot water extraction was conducted with the addition of 4 volumes of deionized water (w/v) to each respective powdered coffee, followed by boiling for 30 min. In order to obtain the hot water extract, boiled coffee powder was centrifuged under conditions identical to those described above.

■ Assay of antibacterial activity

Methicillin-resistant *Staphylococcus aureus* (MRSA, 7B29), methicillin-sensitive *S. aureus* (MSSA, IFO 15035) and *Pseudomonas aeruginosa* (IFO 13736) were cultured in test tubes containing 5 ml of TSB medium (Difco) at 37°C for 18 h with agitation at 120 rpm. Antibacterial activity was evaluated via the microplate dilution method[6]. TSB medium was inoculated with each broth culture in order to achieve the initial bacterial density of 10^4 cells/100 μl/well. Subsequently, the coffee extracts were introduced, affording final concentrations ranging from 1 to 200 μg/ml; the plates were incubated at 37°C for 24 h. Antibacterial activity was considered as positive when bacterial growth was not observed visually.

■ Assay of antifungal activity

Candida albicans (IFO 1594), *Aspergillus fumigatus* (TIMM 0063), *Trichophyton rubrum* (TIMM 2659) and *Pythium porphyrae* (IFO 30347) were cultured in broth medium with stirring. Sterile paper disks were permeated with amounts of coffee extracts ranging from 1 to 200 μg/disk; the disks were placed on soft agar plates inoculated with the aforementioned fungal strains separately. Plates were incubated at 37 °C until growth of fungi was observed. Antifungal activity was considered as positive when growth inhibition was detected around the paper disks.

■ Assay of antiviral activity

CHSE-214 and MDCK CCL-34 cells, respectively, were employed as host cells for infectious hematopoietic necrosis virus, IHNV (strain ChAb), and influenza virus type A, FluV-A (strain Yamagata H1N1), which were included in this study. Evaluation of antiviral activity of the coffee extracts was conducted via the plaque reduction method[7]. IHNV or FluV-A suspension (200 μl) was added to an identical volume of the coffee extracts; the mixture was placed in an ice bath for 1 h. After incubation, IHNV and FluV-A were infected to seeded CHSE-214 cells in 24-well plates and to MDCK CCL34 cells in 12-well plates, respectively. Additional incubation was conducted for 1 h at 18°C for IHNV and 37°C for FluV-A in order to allow absorption of the viruses. Subsequently, IHNV plates were exposed to E-RDF medium containing 0.8% methyl-cellulose, followed by incubation at 18°C for 7 days. FluV-A plates were exposed to MEM medium (Gibco) containing 0.1% trypsin (Sigma) and 0.7% agarose, followed by incubation in a 5% CO_2 incubator at 37°C for 2 days. After incubation, the cells were fixed with 10% formalin and stained with 0.1% crystal violet. The number of plaques was evaluated in terms of the stained cell-background; furthermore, plaque reduction (%) was calculated.

■ Assay of melanin synthesis inhibiting activity

Mouse B16 melanoma cells (JCRB 0202) were employed as model cells for melanin synthesis. The cells were cultured at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle medium (Gibco) supplemented with penicillin G, streptomycin, HEPES and 10% fetal bovine serum (FBS) (Sigma). To evaluate melanin synthesis inhibiting activity of each coffee extract, B16 cells were seeded to 48-well plates affording 2.4 x 10^4 cells/300 μl/well, followed by incubation for 24 h. After incubation, 3 μl-aliquots of each coffee extract were added to each well; incubation was extended for 3 days. After incubation, cells were rinsed twice with an equal volume of PBS; subsequently cells were sonicated and lysed in 2 N NaOH with agitation by a mixer (E-36, Taitec).

Melanin extracted from the cells was measured based on absorbance at 405 nm with a microplate reader (model 450, Bio-Rad). Melanin synthesis inhibiting activity (%) was evaluated based on the standard curve of a commercially available synthetic melanin. The cytotoxicity of each coffee extract was examined via MTT assay[8].

■ Assay of telomerase inhibiting activity

Telomerase inhibiting activity was determined according to the telomeric repeat amplification protocol (TRAP)[9]. Briefly, a mixture of each coffee extract and telomerase crude solution prepared from 3 day-old MOLT-4 cells (human acute lymphoblastic leukemia cells) were added to a TS oligo nucleotide solution as a substrate for extension of the telomeric repeats. Following elongation of telomeric DNA at 20°C for 30 min, the telomeric products were amplified by polymerase chain reaction (PCR); subsequently, the resulting mixture was subjected to electrophoresis on a 12% polyacrylamide gel in order to detect the amplified products. Telomerase inhibiting activity was evaluated via comparison of the electrophoretic signal intensity of the detected ladder bands with that of each negative control. An internal control was also included to eliminate the influence of PCR inhibition by the coffee extracts.

■ Assay of TNF-α production promoting activity

RAW264.7 cell line derived from mouse macro phages was suspended in RPMI1640 medium (Gibco) containing 10% FBS and seeded to 48-well plates, which afforded 2 x 10^5 cells/200 μl/well as producer cells of TNF-α (tumor necrosis factor-α). Following incubation for 2 h, a 10μl- aliquot of coffee extracts was introduced to the culture medium. The cells were then incubated at 37°C for 12 h in a 5% CO_2 incubator and allowed to produce TNF-α. After incubation, the culture supernatant containing the TNF-α was collected and stored at -80°C until assay. Lipopolysaccharide (LPS) (10 μl, 2 mg/ml) (Sigma) was utilized as a positive control in the identical procedure described above. TNF-α production promoting activity was determined according to the method of Abe *et al.* (1985)[10]. L929 cells, which are sensitive to TNF-α, were seeded to 96-well plates yielding 3 x 10^4 cells/100 μl/well and cultured at 37°C for 18 h in a CO_2 incubator. Following removal of the culture medium, the cells were exposed to 40 μl of RPMI 1640 medium containing 10% FBS, 10 μl of each coffee extract and 50 μl of 2 mg/ml actinomycin D (Wako). After an 18 h-incubation, cells stained with 0.2% crystal violet were lysed with 1% SDS; absorbance was measured at 490 nm with a microplate reader. TNF-α production was quantified based on the standard curve of a commercially available mouse TNF-α (Sigma).

■Assay of growth stimulating activity to hORS cells

96-well plates were coated with 100 μl/well of collagen type I solution (Functional Peptide Lab) for 2 h; an equivalent amount of PBS was utilized to wash the wells three times prior to cell-seeding. Human hair outer root sheath cells (hORS) were then seeded on the collagen-coated 96-well plates in a mixed medium of E-RDF (Kyokuto) and DMEM (Gibco) (1:1) containing 1% FBS at a cell density of 2 x 10^3 cells/100 μl/well. Following a 24 h-incubation (for the attachment of cells on well surface), the medium was changed to fresh medium containing 1% test coffee extracts. After a 48 h-incubation, cell-growth stimulating activity of each coffee extract was evaluated by MTT assay. In this experiment, 10 μl of 5 mg/ml MTT reagent (Sigma) was added to each well of the 96-well plates. Following a 4 h-incubation, 100 μl of 10% SDS solution (in 0.01 N HCl) was introduced to each well in order to precipitate release of the crystal reaction product from the cells. Spectrophotometric absorbance (570-655 nm) of cell medium in each well was measured with a microplate reader. Growth stimulating activity of hORS cells was determined

via comparison of the coffee extract-treated cells with untreated control, which was designated as 100%.

■ Neurite outgrowth promoting activity

The rat pheochromocytoma PC12D and 2.5 S nerve growth factor (NGF) (Sigma) were employed in this study. PC12D cells were cultured in DMEM (Gibco) containing 10% horse serum and 5% FBS in a 5% CO_2 incubator at 37°C. The cells were harvested, re-suspended in serum-containing DMEM, and seeded into 96-well plates at a cell density of 5 x 10^3 cells/100 μl/well. Subsequently, plates were incubated at 37 °C for 24 h. Thereafter, the medium was changed to fresh serum containing DMEM in the presence of the minimal effective concentration of NGF (10 ng/ml). An aliquot of coffee extract was added to the cell culture affording final concentrations ranging from 3.91 to 125 μg/ml. Following treatment for 48 h, neurite outgrowth promoting activity was measured under a light microscope with 200-fold magnification. Cells exhibiting neurites two times longer than each cell body were counted. For each data point, the mean value was calculated from eight random-field observations of two duplicate wells. On the other hand, cells treated with 10 and 50 ng/ml NGF served as negative and positive controls, respectively, in order to compare the activity of the tested samples.

Results and Discussion

It is well known that the major components of coffee such as caffeine and chlorogenic acid possess biological activity. In the event that effective screening techniques and evaluation methods for the determination of such biological activity are available, it is highly probable that more novel bioactive substances may be discovered in coffee.

Therefore, the objectives of this study included elucidation of novel bioactive substances in coffee and identification of new bioactivity of the known components of coffee. To achieve this end, representative coffees with sour (Toraja) and bitter (Mandarin) tastes were selected and various bioassays were performed. In the present investigation, PBS extract, hot water extract and MeOH extract were prepared for analysis of the presence of water- and lipid-soluble bioactive substances in coffee samples. The bioassays conducted in this study included microplate dilution methodology for determination of antibacterial activity, paper disk procedure for antifungal activity, plaque reduction technique for antiviral activity, tumor necrosis factor (TNF)-α synthesis determination for macrophage stimulating activity, neurite outgrowth promoting activity on rat pheochromocytoma cells, melanin synthesis inhibiting activity on mouse B16 melanoma cells, TRAP method for telomerase inhibiting activity and human hair outer root sheath cell (hORS) growth stimulating activity.

These bioassays demonstrated the absence of antibacterial and antifungal activity, TNF-α synthesis and neurite outgrowth promoting activities and hORS growth stimulating activity up to a concentration of 500 μg/ml (data not shown) in extracts of Toraja and Mandarin. In experiments involving telomerase inhibiting activity, the polymerase utilized as the PCR reaction enzyme in the bioassay was inhibited by the coffee extracts tested (data not shown). Neither product amplified by TRAP nor genes amplified as their internal control was observed in extracts from Toraja and Mandarin. The failure with respect to amplification of the internal control implies that a certain impurity leading to potential inhibition of PCR, namely *Taq* polymerase inhibiting substances, may be present in all crude extracts examined. Thus, telomerase inhibiting activities of the coffee extracts might be undetectable in this study. Therefore, purification techniques would be necessary, such as solvent partition of MeOH

extracts, and separation utilizing ionic charge or mass of the active molecule, which would exist in hot water and/or PBS extracts. Currently, we are attempting to develop an improved methodology in order to reduce the influence of *Taq* polymerase inhibition.

However, the coffee MeOH extracts of 500 μg/ml displayed slight antiviral activity against fish virus and human influenza virus (Fig. 1). The most striking result in the present study was the observation of strong melanin synthesis inhibiting activity against mouse B16 melanoma cells by MeOH and hot water extracts (500 μg/ml) of the two coffees, Toraja and Mandarin (Fig. 2). These activities were comparable to that of 125 μg/ml of arbutin, which was used as a positive control in the bioassay. Upon comparison with the activity of arbutin, inhibition exceeding 30% in MeOH and hot water extracts of Mandarin coffee was noted. Moreover, nearly no cytotoxicity was evident at this concentration (Fig. 3), suggesting the presence of melanin synthesis inhibiting substances characterized by extremely low toxicity in these coffee extracts. Furthermore, the same tested concentration of hot water extract exhibited 37% inhibition against melanin synthesis. The current findings clearly indicated that these coffees contain beneficial bioactive substances, which may be incorporated into whitening cosmetics.

Several substances, *i.e.*, arbutin, vitamin C and kojic acid, are well known active agents used in currently available whitening cosmetics. One result of the present investigation indicates that the crude extract from coffee possesses potent melanin synthesis inhibiting activity that is comparable to that of arbutin. In a future study, *in vivo* tests, purification and chemical structure elucidation of the active substances will be conducted. The aforementioned findings indicate that purified active substances may display activity higher than

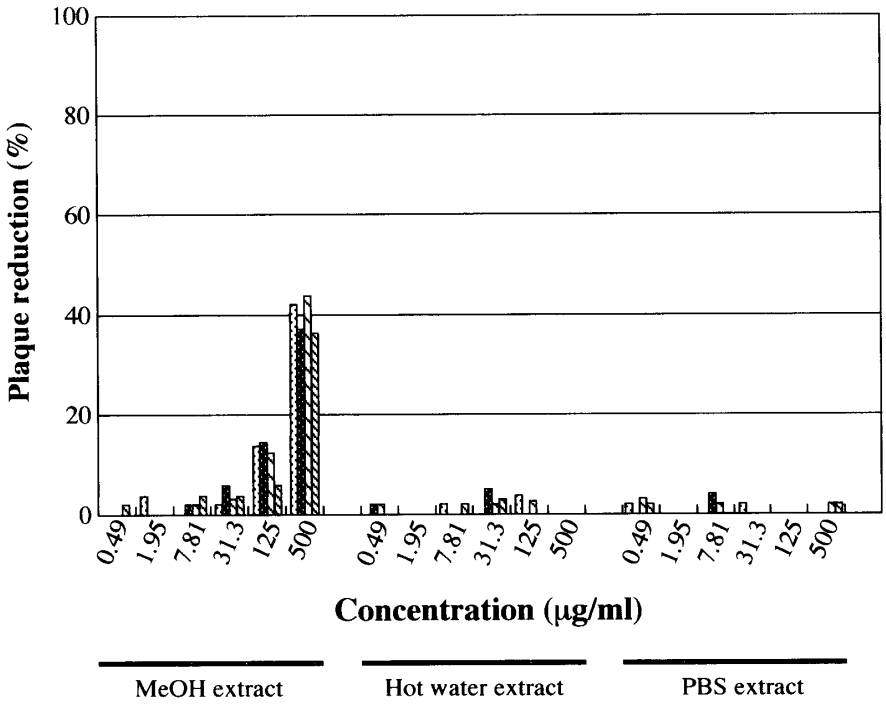

Fig. 1. Antiviral activity of coffee extracts against IHNV and FluV-A. Symbols: ▨, Toraja/IHNV; ■, Toraja/FluV-A; ▧, Mandarin/IHNV; ▩, Mandarin/FluV-A.

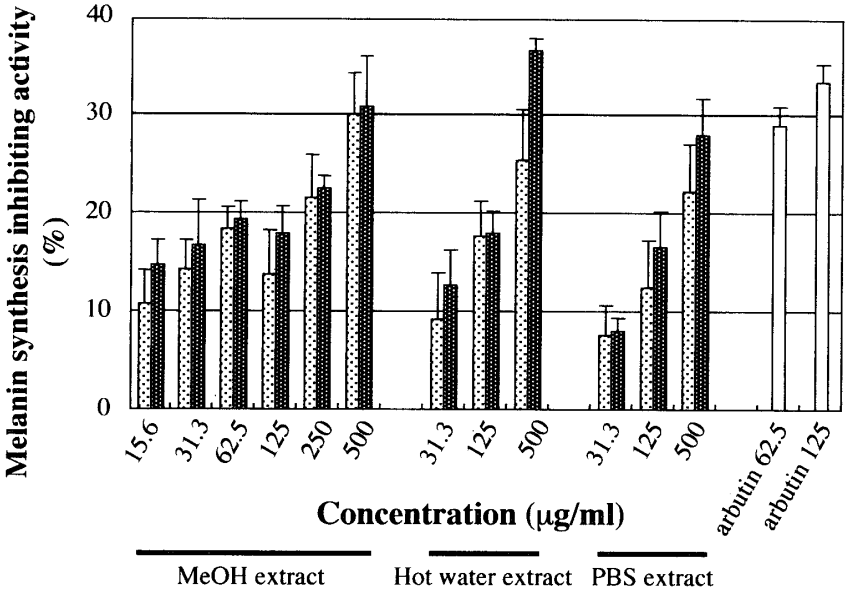

Fig. 2. Melanin synthesis inhibiting activity of coffee extracts. Symbols: ▨, Toraja; ▩, Mandarin; □, arbutin. The data and error bars represent means and standard deviations for triplicate assays.

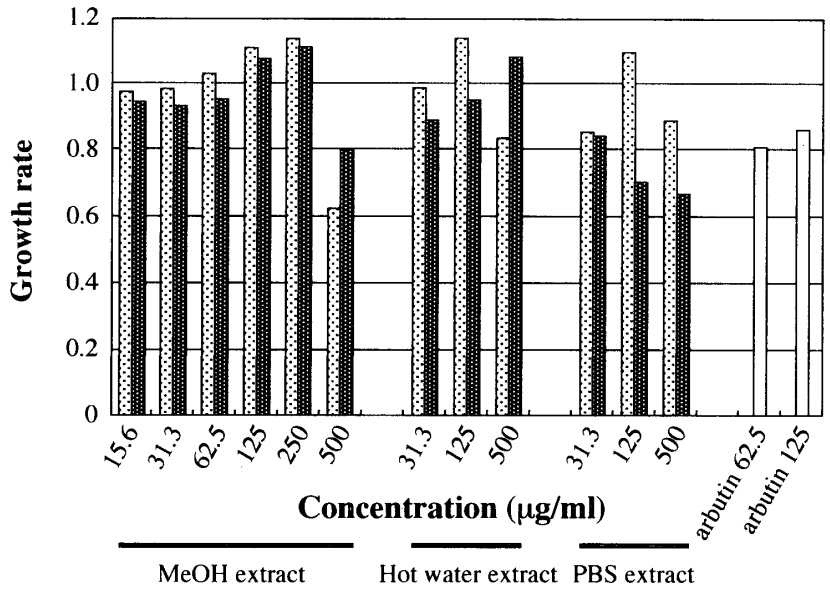

Fig. 3. Effect of coffee extract addition on growth rate of B16 melanoma cells determined by MTT assay. An inhibition of growth rate can be regarded as cytotoxicity by coffee extracts or arbutin. Symbols: ▨, Toraja; ▩, Mandarin; □, arbutin.

that of arbutin. Development of new whitening cosmetics based on active substances naturally occurring in coffee extracts is possible. In addition, the identity of the substance responsible for melanin synthesis inhibiting activity of the MeOH extract remains unclear, consequently, partition with organic solvents and chromatographic techniques employing several types of gel will be conducted. Furthermore, if the chemical structure is determined based on high resolution MS, ^{1}H-NMR and ^{13}C-NMR analyses, the mechanism of melanin synthesis inhibiting activity and molecular structure of the active substance relative to such activity can be proven.

Acknowledgment

This study was supported in part by a grant from All Japan Coffee Association in 2001.

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