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# Inhibitory effects of chlorogenic acid and its related compounds on the invasion of hepatoma cells in culture

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## Abstract

Actions of chlorogenic acid, a major component of coffee, and its constituents, caffeic and quinic acids, on the proliferation and invasion of AH109A, a rat ascites hepatoma cell line, were investigated using *in vitro* assay systems. All three components suppressed the AH109A invasion at concentrations of 5–40 $\mu$M without altering the cell proliferation. At the concentration of 10 $\mu$M, chlorogenic, caffeic and quinic acids significantly ($P < 0.05$) suppressed the invasion by 68%, 36% and 31%, respectively, implying that the suppressive effect of chlorogenic acid on the AH109A invasion might result from the additive effects of its constituents, caffeic and quinic acids. At the concentration of 10 $\mu$M, cinnamic acid and p-coumaric acid (4-hydroxycinnamic acid) exerted no or little influence on the invasion, whereas caffeic acid (3,4-dihydroxycinnamic acid) significantly ($P < 0.05$) suppressed it, suggesting the possible involvement of the 3,4-dihydroxy group of caffeic acid in the suppression. Chlorogenic acid was thus demonstrated to be one of the chemical entities in coffee suppressing the hepatoma invasion *in vitro*, and both of its constituents, caffeic and quinic acids, to be responsible for the anti-invasive activity. These results suggest the existence of nutritionally and pharmacologically important substances in coffee which control tumor cell invasion.

## Introduction

Endless proliferation and metastasis are two biological properties of cancer cells. Cancer metastasis is achieved by a series of steps (Fidler et al., 1978). The invasion of cancer cells is an important and characteristic step of the metastasis (Liotta et al., 1983). Thus, the inhibition of endless proliferation and invasion by some substances will prolong the life span of hosts with cancer. In fact, some polyphenolic compounds have been reported to prolong the life span of mice bearing melanoma cells (Menon et al., 1995). As a strategy to screen such materials from foods and natural products, *in vitro* assay systems using cultured cells seem to be useful prior to *in vivo* study. We described earlier simple assay methods for the proliferation and invasion of hepatoma cells (Miura et al., 1997a). Using these methods, we have selected various foods with potential to suppress the proliferation and/or invasion of hepatoma cells (Yagasaki, 1995; Yagasaki and Miura, 1999). For example, not only extracts of green, oolong and black teas but also rat serum obtained after oral intubation of each tea extract demonstrated such suppression (Zhang et al., 1999). Among the tea components screened, (–)-epigallocatechin gallate (EGCG) was most effective against both the proliferation and invasion of hepatoma cells (Zhang et al., 1999). The peroral administration of EGCG to mice had been reported to inhibit the metastasis and growth of B16 melanoma cells *in vivo* (Taniguchi et al., 1992). These findings strongly demonstrate the above-mentioned *in vitro* proliferation and invasion assay systems are useful prior to *in vivo* study.

Coffee is another beverage as popular as tea. We have reported that instant coffee powder solution itself

and serum obtained after oral intubation of the solution also have both anti-proliferative and anti-invasive activities to hepatoma cells (Miura et al., 1997b). In the present study, the actions of chlorogenic acid, an ester of caffeic acid and quinic acid, on the proliferation and invasion of these cells were investigated using the *in vitro* assay systems to identify effective components in coffee. Chlorogenic acid was demonstrated to be one of the chemical entities suppressing the invasion of hepatoma cells.

## Materials and methods

### *Materials*

Chlorogenic acid, quinic acid, caffeic acid, cinnamic acid and p-coumaric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). These compounds were dissolved in dimethyl sulphoxide (DMSO, Sigma Chemical Co.), and added to experimental media at the concentrations indicated in each figure. The control medium contained DMSO alone as vehicle at a final concentration of 0.1%.

### *Cultures of hepatoma (AH109A) cells*

AH109A cells, a rat ascites hepatoma cell line, were obtained from the Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, and maintained in peritoneal cavities of male Donryu rats (NRC Haruna, Gunma, Japan). The hepatoma cells prepared from accumulated ascites were cultured in DM-160 medium (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% calf serum (CS, obtained from JRH Biosciences, Lenexa, KS, USA), streptomycin (100 $\mu$g/ml, Meiji-seika Kaisha, Tokyo, Japan) and penicillin (100 U/ml, Ban-yu Pharmaceutical Co., Tokyo, Japan) (10% CS/DM-160) for up to 2 months. AH109A cells cultured for at least 1 week after preparing them from rat ascites were used for the assays described below in order to eliminate contaminated macrophages and neutrophils.

### *In vitro proliferation assay*

The effects of test compounds (chlorogenic acid, quinic acid, caffeic acid, cinnamic acid and p-coumaric acid) on the proliferation of AH109A cells were examined by WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.Na] method (Ishiyama et al., 1993; Ishiyama et al., 1996) using a cell counting kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). For WST-1 assay, 5 x $10^3$ AH109A cells were cultured for 44 h in each well of a 96-well plate (flat bottom, FALCON, Becton Dickinson and Co., Franklin Lakes, NJ, USA) containing 10% CS/DM-160 in the absence or presence of test compounds. An aliquot (10 $\mu$l) of WST-1 solution was then added and the cells were cultured for another 4 h. Viable cells can make WST-1 produce highly water-soluble formazan dye and the absorbance at 415 nm and 595 nm was measured with a microplate reader (Bio-Rad Laboratories, Model 450, Hercules, California, USA) as described previously (Zhang et al., 1999). The relative proliferation rate of AH109A was calculated between the control (100%, no test compounds) and test groups.

### *In vitro invasion assay*

The effects of test compounds on the invasion of AH109A cells were examined by the co-culture system (Akedo et al., 1986) with slight modifications as described previously (Miura et al., 1997a). Briefly, primary cultured mesothelial cells (M cells) from mesentery of rats were seeded at a density of 1.5 x $10^5$ cells/60 mm$\phi$ culture dish with 2 mm grids (Corning Glass Works, Corning, NY, USA) and cultured in 3 ml of 10% CS/DM-160 for 7–10 days to attain a confluent state. AH109A cells (2.4 x $10^5$) were then seeded on the monolayers of M cells and cultured for 48 hr without or with test substances. Invading AH109A cells and colonies underneath M cell layers were counted with a phase-contrast microscope (Olympus, Tokyo, Japan). Usually 10 areas were counted and the invasive activity of AH109A was indicated by the number of invading cells and colonies/cm$^2$. Control values of this assay vary among experiments probably due to delicate interactions between M-cells and AH109A cells. This phenomenon, however, has no practical significance in investigations on the actions of test substances.

### *Statistical analysis*

Data were expressed as means $\pm$ standard errors. Statistical analysis was carried out using a one way analysis of variance (ANOVA), followed by Tukey's Q test.

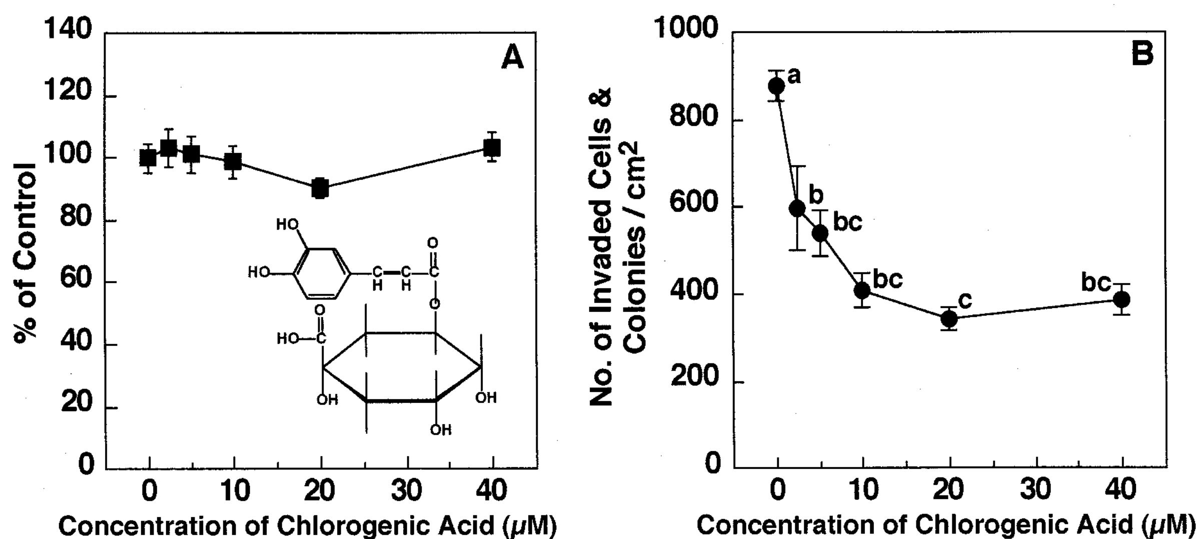


*Figure 1.* Effect of chlorogenic acid on the proliferation and invasion of AH109A. Chlorogenic acid was dissolved in culture medium at the concentrations indicated. The proliferative activity of AH109A (A) was determined by the WST-1 method and the invasive activity (B) by the co-culture system as described in Methods section of the text. The relative proliferation rate of AH109A was calculated between the absorbance of control (no test component) and test groups, and expressed as % of control. Each point and vertical bar represents the mean and SEM for 6 wells (A) or 10 areas (B). [abc]Values not sharing a common alphabetical letter are significantly different at $P < 0.05$ by Tukey's Q test.

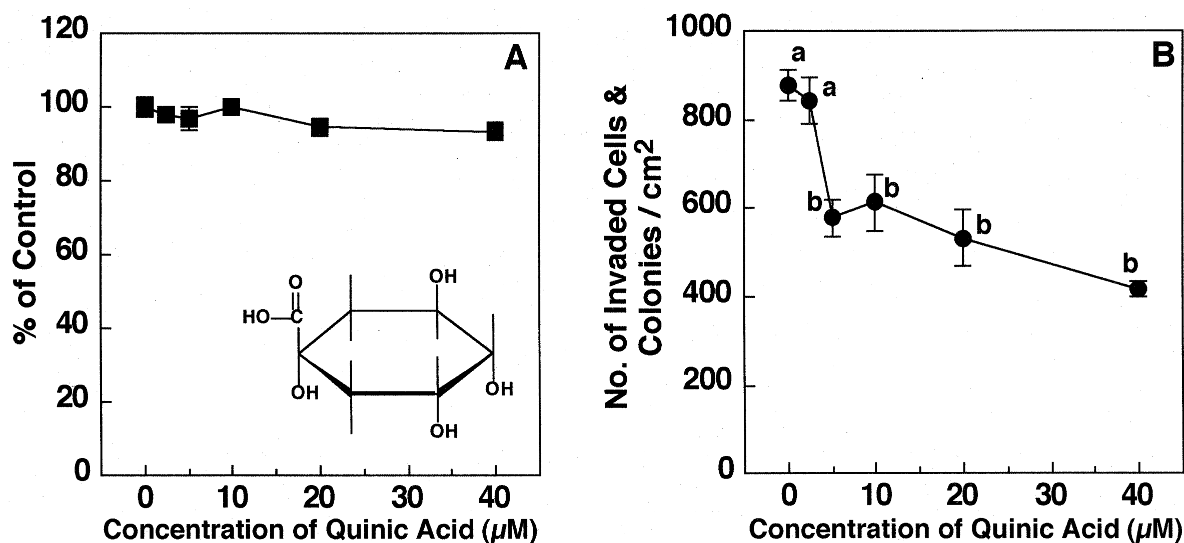


*Figure 2.* Effect of quinic acid on the proliferation and invasion of AH109A. Quinic acid was dissolved in culture medium at the concentrations indicated. The proliferative activity of AH109A (A) was determined by the WST-1 method and the invasive activity (B) by the co-culture system as described in Methods section of the text. The relative proliferation rate of AH109A was calculated between the absorbance of control (no test component) and test groups, and expressed as % of control. Each point and vertical bar represents the mean and SEM for 6 wells (A) or 10 areas (B). [ab]Values not sharing a common alphabetical letter are significantly different at $P < 0.05$ by Tukey's Q test.

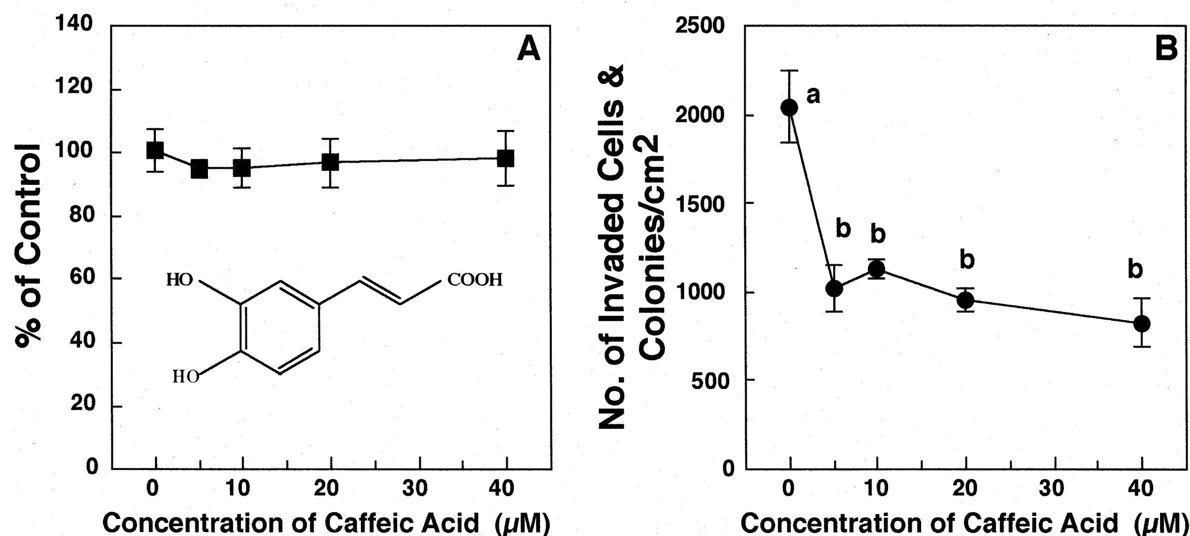


*Figure 3.* Effect of caffeic acid on the proliferation and invasion of AH109A. Caffeic acid was dissolved in culture medium at the concentrations indicated. The proliferative activity of AH109A (A) was determined by the WST-1 method and the invasive activity (B) by the co-culture system as described in Methods section of the text. The relative proliferation rate of AH109A was calculated between the absorbance of control (no test component) and test groups, and expressed as % of control. Each point and vertical bar represents the mean and SEM for 6 wells (A) or 10 areas (B). [ab]Values not sharing a common alphabetical letter are significantly different at $P < 0.05$ by Tukey's Q test.

*Table 1.* Comparison of inhibitory effects of chlorogenic, quinic and caffeic acids on the invasion of AH109A

| Component added | Concentration ($\mu$M) | No. of invaded cells and colonies/cm$^2$ | % inhibition | % of control |
|---|---|---|---|---|
| None (control) | - | 535 ± 61a | 0.0 | 100.0 |
| Chlorogenic acid | 10 | 170 ± 23b | 68.2 | 31.8 |
| Quinic acid | 10 | 370 ± 37c | 30.8 | 69.2 |
| Caffeic acid | 10 | 343 ± 27c | 35.9 | 64.1 |

Chlorogenic, quinic and caffeic acids were dissolved in culture medium at the concentration of 10 $\mu$M. The invasive activity was determined by the co-culture system as described in Methods section of the text. Each value represents the mean ± SEM for 10 areas. % inhibition = {1–(value of each test compound/value of control)}x100. [abc]Values not sharing a common alphabetical letter are significantly different at $P < 0.05$ by Tukey's Q test.

## Results

We first examined the effect of chlorogenic acid, a major coffee component, on the proliferation and invasion of AH109A (Figure 1). Chlorogenic acid exerted no influence on the AH109A proliferation at concentrations up to 40 $\mu$M in the medium, while it commenced to suppress the invasion at 2.5 $\mu$M, suppressed it approximately linearly to 20 $\mu$M, and maintained an inhibitory effect up to 40 $\mu$M ($P < 0.05$).

Chlorogenic acid is an ester of quinic acid and caffeic acid. We next tested which constituent was responsible for the suppressive effect, and found that both quinic acid (Figure 2) and caffeic acid (Figure 3) suppressed the AH109A invasion at concentrations of 5–40 $\mu$M ($P < 0.05$) without affecting the proliferation of the hepatoma cells.

The anti-invasive activities of chlorogenic acid and the two constituents were compared at the same concentration of 10 $\mu$M (Table 1). Chlorogenic acid significantly ($P < 0.05$) suppressed the AH109A invasion by 68%, that is, the invasive activity of the tumor cells treated with this acid was only 32% that of control cells (untreated cells). Likewise, quinic acid and caffeic acid also significantly ($P < 0.05$) suppressed the AH109A invasion by 31% and 36%, respectively; the invasive activities of the tumor cells treated with these acids were 69% and 64% that of the control cells. The

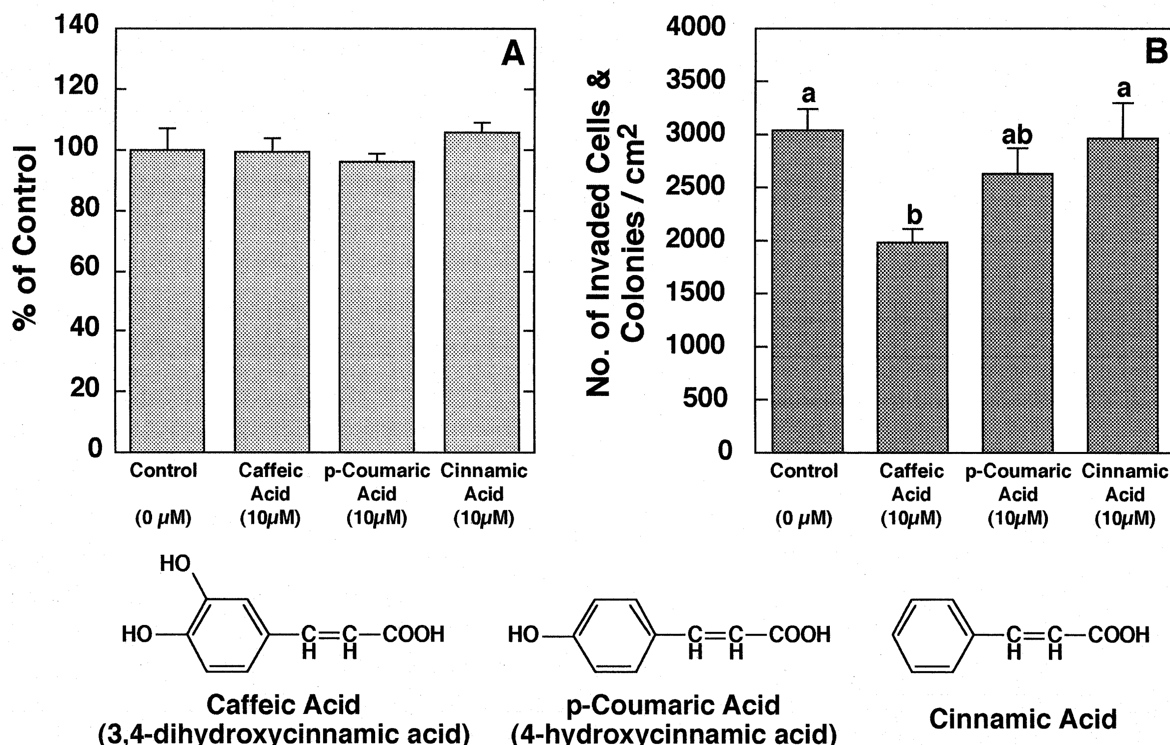


*Figure 4.* Structure-activity relation for the proliferation and invasion of AH109A among caffeic, p-coumaric and cinnamic acids. The three acids were dissolved in culture medium at the concentration of 10 $\mu$M. The proliferative activity of AH109A (A) was determined by the WST-1 method and the invasive activity (B) by the co-culture system as described in Methods section of the text. The relative proliferation rate of AH109A was calculated between the absorbance of control (no test component) and test groups, and expressed as % of control. Each value and vertical bar represents the mean and SEM for 6 wells (A) or 10 areas (B). [ab]Values not sharing a common alphabetical letter are significantly different at $P < 0.05$ by Tukey's Q test.

suppressive effect of chlorogenic acid (68%) thus was almost equal to the sum (67%) of those of quinic acid (31%) and caffeic acid (36%).

To learn the structure-activity relation among cinnamic acid, p-coumaric acid (4-hydroxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid), the effects of these three acids on the proliferation and invasion of AH109A were examined at 10 $\mu$M (Figure 4). All three acids used here were predominantly trans isomers. Caffeic acid significantly ($P < 0.05$) suppressed the AH109A invasion, whereas cinnamic acid and p-coumaric acid exerted no or little influence on the invasion. None of the three acids had any influence on the AH109A proliferation.

## Discussion

Instant coffee powder solution itself and serum obtained after oral intubation of the solution show not only an anti-proliferative activity but an anti-invasive one to hepatoma cells (Miura et al., 1997b). A filtered extract of powdered coffee bean and serum obtained after oral intubation of the extract also show anti-invasive activity to hepatoma cells (Furuse et al., 1998). In the present study, chlorogenic acid was demonstrated to be such a chemical entity in coffee suppressing the hepatoma invasion (Figure 1).

Chlorogenic acid is an ester of quinic acid and caffeic acid. Both moieties suppressed the AH109A invasion at concentrations of 5–40 $\mu$M, but did not affect the proliferation (Figures 2 and 3). These findings indicate that the two constituents are responsible for the suppressive effect of chlorogenic acid. At the same concentration of 10 $\mu$M, chlorogenic acid, quinic acid and caffeic acid significantly suppressed the AH109A invasion by 68%, 31% and 36%, respectively (Table 1). Thus, the suppressive effect of chlorogenic acid (68%) on the AH109A invasion might result from the additive effects of its quinic acid (31%) and caffeic

acid (36%) constituents. Orally intubated chlorogenic acid is reportedly cleaved in stomach and intestine, and absorbed presumably in the form of its primary fragments or further metabolites (Czok et al., 1974). Thus, caffeic and quinic acids derived from chlorogenic acid or their metabolites may act independently as anti-invasive substances after absorption from the guts and would presumably bring about the additive effects intracorporally.

Caffeic acid, one of the two anti-invasive fragments of chlorogenic acid, is cinnamic acid with the dihydroxy group. To know the contribution of dihydroxy group of caffeic acid to the anti-invasive activity, the effects of cinnamic acid and p-coumaric acid (4-hydroxycinnamic acid) on the hepatoma invasion were compared with that of caffeic acid (3,4-dihydroxycinnamic acid) at the concentration of 10 $\mu$M, using their trans isomers (Figure 4). Cinnamic acid and p-coumaric acid exerted no or little influence on the AH109A invasion, whereas caffeic acid significantly suppressed it. These results suggest the possible involvement of 3,4-dihydroxy group of caffeic acid in suppressing the AH109A invasion, although further studies are needed to clarify this.

Chlorogenic acid and caffeic acid have radical scavenging activities (Morishita and Kido, 1995). This property of these coffee components may play a role in their anti-invasive action, since superoxide radicals have been reported to potentiate the invasive activity of hepatoma (AH130) cells *in vitro* which is suppressed by superoxide dismutase and catalase (Shinkai et al., 1986, Mukai et al., 1987). Caffeic acid is known as an inhibitor of 5-lipoxygenase (Koshihara et al., 1983), an enzyme involved in leukotriene synthesis. Linoleic acid-stimulated breast cancer cell invasion *in vitro* is blocked by esculetin, a 5- and 12-lipoxygenase inhibitor (Liu et al., 1996). Thus, an inhibition of lipoxygenase appears to be another possible mechanism for the anti-invasive activity of caffeic acid and hence chlorogenic acid. The mechanism for the inhibitory action of quinic acid on the AH109A invasion is not clear at present, although some phenolic metabolites of quinic acid (Booth et al., 1960) formed after addition to medium or following oral ingestion might be involved in the anti-invasive activity.

Coffee extract itself and serum obtained after oral intubation of the extract significantly inhibited the AH109A proliferation as well as the invasion (Miura et al., 1997b). Chlorogenic, quinic and caffeic acids exerted no influence on the proliferation at concentrations up to 40 $\mu$M in the medium. These results suggest that substance(s) other than these acids must exist in coffee which suppress the hepatoma proliferation.

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