



Original Article

## Coffee diterpenes, cafestol and kahweol, display cytotoxicity and all-*trans* retinoic acid-induced superoxide generating activity-enhancing ability in U937 cells

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**ABSTRACT** — Coffee which is one of the most popular beverages is under great attention because its attractive pharmacological effects such as anti-cancer properties, antioxidant and cell protective effects. *Coffea arabica* metabolites like cafestol and kahweol belonging to class of diterpene are expected to be as biotherapeutic drugs for maintaining human health via preventing serious illnesses. However, the understanding of the effects of these two diterpenes against human leukemia cells is still poor. In this study, we investigated the influences of these two coffee diterpenes on the viability and the all-*trans* retinoic acid (ATRA)-induced superoxide anion (O<sub>2</sub><sup>-</sup>)-generating ability of human leukemia U937 cells. Cafestol and kahweol reduced cell viability at a concentration of 50 μM. In addition, 1 μM ATRA significantly reduced viability with cafestol at 48 hr and kahweol at 24 hr respectively. On the other hand, 20 μM these two coffee diterpenes brought about moderate up-regulation of the ATRA-induced O<sub>2</sub><sup>-</sup>-generating ability. Quantitative RT-PCR and immunoblotting revealed that these two coffee diterpenes significantly up-regulate the ATRA-induced O<sub>2</sub><sup>-</sup>-generating ability via enhancing gene expression levels of gp91-phox, which is an essential factor for the O<sub>2</sub><sup>-</sup>-generating ability of leukocytes. These findings demonstrated that cafestol and kahweol show not only the ATRA-enhanced cytotoxicity but also the promoting effects on the ATRA-induced O<sub>2</sub><sup>-</sup>-generating ability via up-regulation of gp91-phox gene expression.

**Key words:** Cafestol, Kahweol, Superoxide, Cytotoxicity, All-*trans* retinoic acid, Leukocyte

### INTRODUCTION

Coffee extracted from roasted and ground *Coffea arabica* beans with hot/cold water is one of the most consumed beverages all over the world, together with tea. Biosynthesized metabolites from higher plants phyto-

chemicals as secondary metabolites that protect them from numerous environmental risks, e.g., ultraviolet irradiation and infectious diseases. Coffee beans contain various phytochemicals such as caffeine, caffeic acid, chlorogenic acid, cafestol and kahweol. Among them, cafestol and kahweol belonging to diterpenes which are structur-

al analogues each other, their difference is a double bond in the kahweol molecule (Fig. 1). They also attract attention as health-promoting substances such as anti-cancer (Cavin *et al.*, 2002; Majer *et al.*, 2005; Kim *et al.*, 2009; Cárdenas *et al.*, 2014; Chae *et al.*, 2014; Park *et al.*, 2016; Oh *et al.*, 2018; Iwamoto *et al.*, 2019; Makino *et al.*, 2021; Eldesouki *et al.*, 2022; Feng *et al.*, 2022), anti-angiogenic properties (Cárdenas *et al.*, 2011; Wang *et al.*, 2012; Moeenfarid, *et al.*, 2016), anti-inflammatory properties (Cárdenas *et al.*, 2011), anti-Parkinson's disease (Trinh *et al.*, 2010) and anti-osteoclastogenesis (Fumimoto *et al.*, 2012). Therefore, these two coffee diterpenes are expected to play important roles in prevention and treatment of various diseases. Regarding leukemia, it has been reported that cafestol and kahweol show cytotoxicity effects against human leukemia cells (Oh *et al.*, 2009; Lima *et al.*, 2017). However, the understanding of the effects of cafestol and kahweol against human leukemia cells is still poor.

On the other hand, the  $O_2^-$ -generating ability of phagocytes (macrophages, neutrophils) is an essential function in innate immune system. Because the  $O_2^-$ -generating system in phagocytes carries an electron from NADPH to molecular oxygen, this system is also called as NADPH oxidase 2 (Nox2). This system is formed from a membrane cytochrome  $b_{558}$  which is a heterodimer protein composed of the small [p22-phox] and the

large [gp91-phox] subunits, three specific cytosolic proteins (p40-phox, p47-phox and p67-phox), and ubiquitous small G-protein Rac (Dagher and Pick, 2007; Panday *et al.*, 2015). If missed, even one of the four protein factors (p22-phox, gp91-phox, p47-phox or p67-phox), this  $O_2^-$ -generating activity is lost resulting onset of chronic granulomatous disease (CGD). Because CGD patients' phagocytes cannot generate  $O_2^-$  upon any stimulation, they suffer from serious diseases due to recurrent bacterial and fungal infections. Therefore, it can be said that the significance of the  $O_2^-$ -generating ability of phagocytes is proved by CGD.

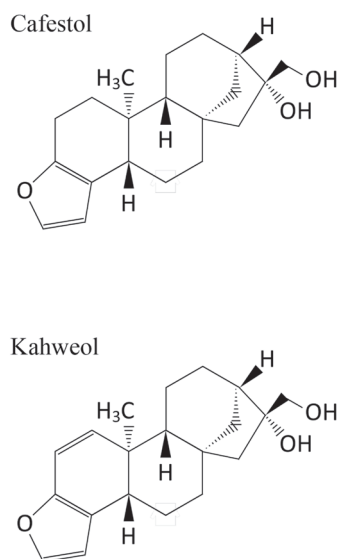
Various agents called as differentiation inducers including all-*trans* retinoic acid (ATRA) can promote differentiation of human monoblastic leukemia U937 cells to macrophage-like cells (Harris and Ralph, 1985). This system has been used as a powerful model for *in vitro* studies on macrophage differentiation accompanied by induction of the  $O_2^-$ -generating ability. Undifferentiated U937 cells generate an undetectable level of  $O_2^-$ . In contrast, differentiated U937 cells able to generate  $O_2^-$  in response to various stimuli such as phorbol 12-myristate 13-acetate (PMA) (Kikuchi *et al.*, 1994; Kikuchi *et al.*, 2011). We have investigated the effects of various phytochemicals on the ATRA-induced  $O_2^-$ -generating ability of U937 cells, such as curcumin (Kikuchi *et al.*, 2010), resveratrol (Kikuchi *et al.*, 2018), chalcones (Kikuchi *et al.*, 2019), sulforaphane (Akiyoshi *et al.*, 2019), ellagic acid and urolithin A (Kikuchi *et al.*, 2021a), hydroxyflavones (Kikuchi *et al.*, 2021b), L-theanine (Kikuchi *et al.*, 2022a) and polymethoxyflavones (Kikuchi *et al.*, 2022b). However, the knowledge of the influences of cafestol and kahweol against the ATRA-induced  $O_2^-$ -generating ability of U937 cells is still unknown.

Thus, U937 cell line is an excellent *in vitro* model system for elucidating the mechanism of macrophage differentiation induced by ATRA. In this paper, we studied the effects of cafestol and kahweol on viability and the ATRA-induced  $O_2^-$ -generating ability of U937 cells, and revealed that these two coffee diterpenes show remarkable cytotoxicity enhanced by ATRA and significantly up-regulates the ATRA-induced  $O_2^-$ -generating ability through activating the transcription of gp91-phox gene.

## MATERIALS AND METHODS

### Materials

Cafestol, kahweol (LKT Laboratories Inc, St. Paul, MN, USA, their chemical structures are shown in Fig. 1), ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan), RPMI-1640 culture medi-



**Fig. 1.** Chemical structures of cafestol and kahweol.

um (Thermo Fisher Scientific, Waltham, MA, USA), GeneAce SYBR qPCR mix  $\alpha$  (Nippon Gene, Tokyo, Japan), PMA, ATRA, luminol (Sigma, St Louis, MO, USA), Diogenes-luminol chemiluminescence probes (National Diagnostics, Atlanta, GA, USA), fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), plasmocin (InvivoGen, San Diego, CA, USA), Trizol reagent (Invitrogen, Carlsbad, CA, USA), monoclonal anti-gp91-phox antibody, monoclonal anti-p47-phox antibody (BD Biosciences, San Jose, CA, USA), monoclonal anti-p67-phox antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal anti-p22-phox antibody, anti-p40-phox antibody (GeneTex, Irvine, CA, USA), monoclonal anti-Na<sup>+</sup>/K<sup>+</sup>-ATPase antibody (Abcam, Cambridge, UK), and horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (Promega, Madison, WI, USA) were obtained from companies indicated respectively.

#### Cell culture, treatment with cafestol or kahweol in the presence of ATRA

U937 (RCB0435), a human monoblastic leukemia cell line, was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were grown in RPMI-1640 culture medium containing 10% FBS and 5  $\mu$ g/mL plasmocin as described (Kikuchi *et al.*, 2019). Cells ( $1.0 \times 10^6$ ) in 5 mL of culture medium were incubated with cafestol or kahweol (10, 20 and 50  $\mu$ M) in the presence of 1  $\mu$ M ATRA up to 48 hr. The determination of the number of total cells was performed using a hemocytometer under a microscope. Viable cells were counted by the trypan blue dye exclusion method (Kikuchi *et al.*, 2020).

#### Measurement of O<sub>2</sub><sup>-</sup> generation

Measurement of O<sub>2</sub><sup>-</sup> generation was performed using Lumat<sup>3</sup> LB9508 luminometer (Berthold Technologies, Bad Wildbad, Germany). In order to enhance chemiluminescence, luminol and Diogenes-luminol chemiluminescence probes were used as described previously (Kikuchi *et al.*, 2018).

#### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Cells ( $2.0 \times 10^6$ ) in 5 mL of the culture medium were incubated with or without 20  $\mu$ M coffee diterpene (cafestol or kahweol) in the presence of 1  $\mu$ M ATRA at 37°C for 48 hr. RT-qPCR was carried out as described in our previous report (Kikuchi *et al.*, 2022b). In brief, total RNAs were isolated from the cells using Trizol reagent. The cDNAs were prepared using a first strand cDNA

synthesis kit, and RT-qPCR reaction was carried out on the MyGo Mini S real-time PCR instrument (IT-IS Life Science, Cork, Ireland) using GeneAce SYBR qPCR mix  $\alpha$ . PCR data were analyzed according to the attached manufactures' protocols. Sequence data of primers used for qPCR were listed in our previous report (Kikuchi *et al.*, 2022b). All qPCR data were normalized to qPCR data of GAPDH gene.

#### Immunoblot analysis

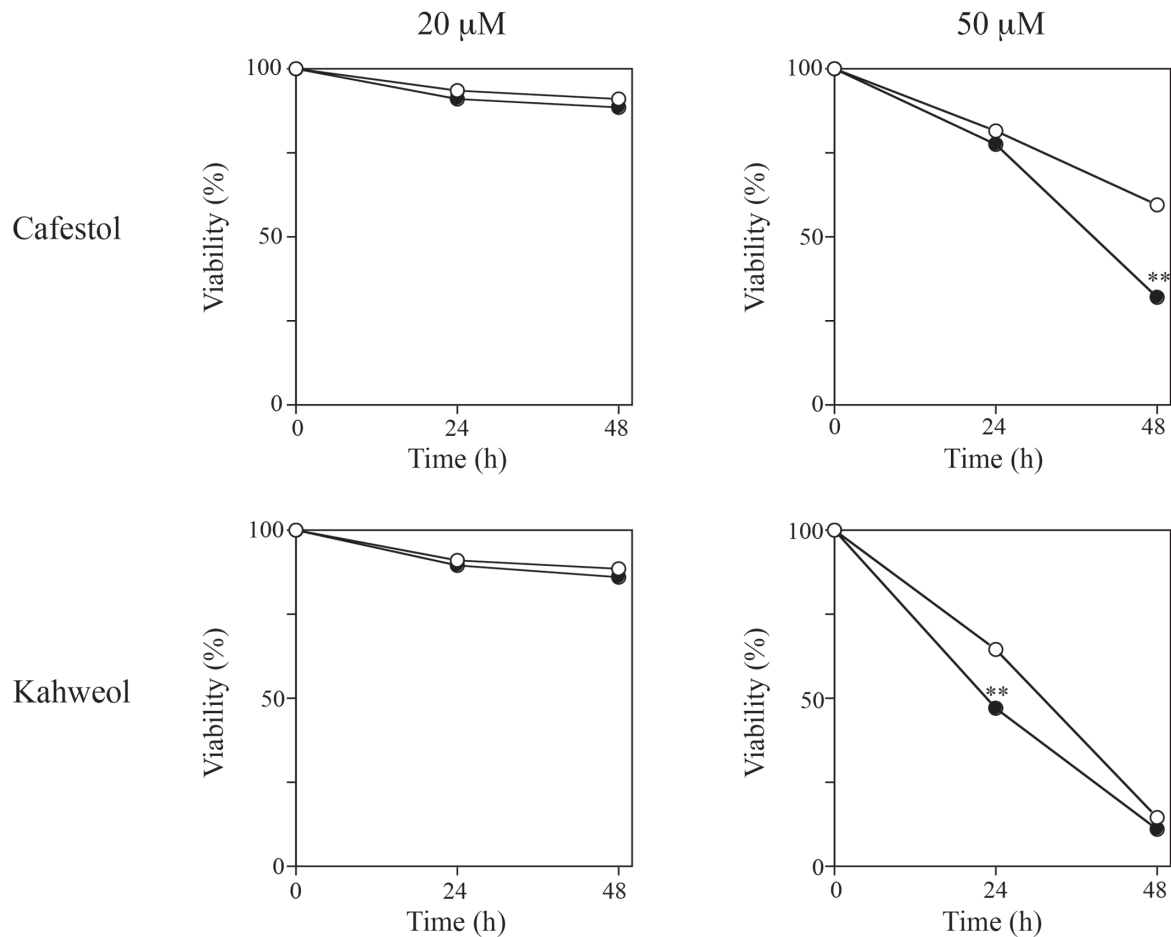
Cells ( $2.0 \times 10^6$ ) in 5 mL of the culture medium were incubated with or without 20  $\mu$ M coffee diterpene (cafestol or kahweol) in the presence of 1  $\mu$ M ATRA at 37°C for 48 hr, disrupted in 100  $\mu$ L of 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 2 mM EDTA and 1 mM PMSF. The cell lysates were divided into cytosolic fractions (supernatants) and membrane fractions (precipitations) by centrifugation. The membrane fractions were suspended 50  $\mu$ L of a solubilizing solution containing 9 M urea, 2% Triton X-100 and 5% 2-mercaptoethanol. After addition of loading buffer [0.5 M Tris-HCl (pH 6.8) containing 5% SDS, 20% glycerol], these samples were subjected to SDS-PAGE followed by immunoblot analysis using anti-p22phox or anti-gp91phox antibodies as primary antibodies. Data analyses were carried out using a luminescent image analyzer STAGE-5100. Human Na<sup>+</sup>/K<sup>+</sup>-ATPase was used as a control (Kikuchi *et al.*, 2019).

#### Statistical analysis

Quantitative data are presented as averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

## RESULTS AND DISCUSSION

First, in order to know the effects of the coffee diterpenes (cafestol and kahweol) on viability of U937 in the presence or absence of ATRA, the cells were treated with up to 50  $\mu$ M of each compound with or without 1  $\mu$ M ATRA. ATRA had no cytotoxicity at a concentration of 1  $\mu$ M (data not shown). As shown in Fig. 2, 20  $\mu$ M of cafestol and kahweol showed no effect on the viability of U937 cells with or without ATRA. In contrast, these two coffee diterpenes reduced the viability of U937 cells at a concentration of 50  $\mu$ M (cafestol: to ~81.4% at 24 hr, to ~59.4% at 48 hr; kahweol: to ~64.3% at 24 hr, to ~14.1% at 48 hr). Moreover, ATRA significantly reduced the viability of U937 cells in synergy with 50  $\mu$ M cafestol at 48 hr (-ATRA: to ~59.4%; +ATRA: to ~32.0%) and



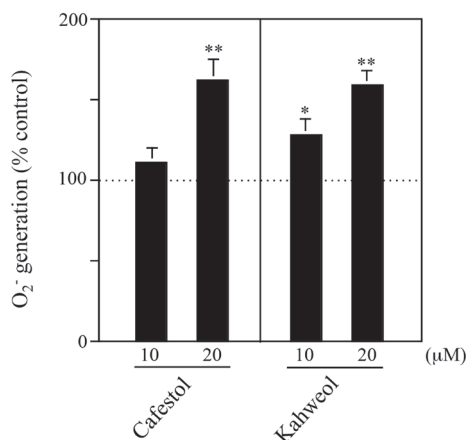
**Fig. 2.** Effects of cafestol and kahweol on cell viability of U937 cells. Cells ( $1.0 \times 10^6$ ) in 5 mL of culture medium were incubated without or with 20  $\mu\text{M}$  or 50  $\mu\text{M}$  coffee diterpenes (cafestol or kahweol) in the absence (open circles) or presence (closed circles) of 1  $\mu\text{M}$  ATRA for up to 48 hr. Viable cells were counted by the trypan blue dye exclusion method. Data represent the averages of three separate experiments. Statistical differences were calculated by Student's *t* test. \*\*,  $p < 0.01$  compared with the data of without ATRA at each time point.

50  $\mu\text{M}$  kahweol at 24 hr (-ATRA: to ~64.3%; +ATRA: to ~46.7%). These data collectively showed that cafestol and kahweol have a negative effect on viability of U937 cells and their cytotoxicity effects were enhanced by ATRA.

As second hypothesis, in order to examine the influences of cafestol and kahweol on the ATRA-induced  $\text{O}_2^-$ -generating ability of U937 cells, the cells were treated with 10  $\mu\text{M}$  or 20  $\mu\text{M}$  of each coffee diterpene in the presence of 1  $\mu\text{M}$  ATRA for 48 hr. As mentioned above, cafestol and kahweol showed no effect on viability of U937 cells upto 20  $\mu\text{M}$  in the presence of ATRA (Fig. 2). Both cafestol and kahweol showed significant enhancing effects on the ATRA-induced  $\text{O}_2^-$ -generating ability

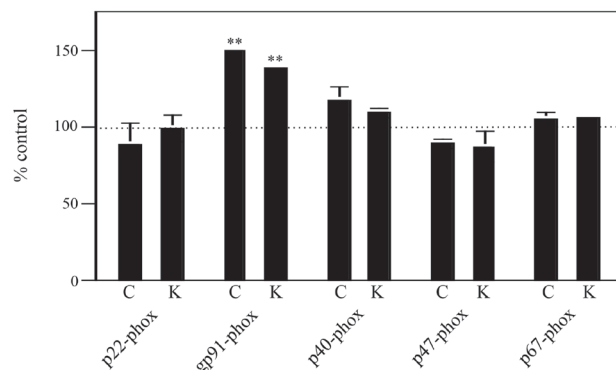
of U937 cells (cafestol: to ~162% of ATRA-treated cells, kahweol: to ~159% of ATRA-treated cells) at a concentration of 20  $\mu\text{M}$  (Fig. 3). These results demonstrated that these two coffee diterpenes have a moderate enhancing effect on the ATRA-induced  $\text{O}_2^-$ -generating ability of U937 cells. In order to investigate the mechanism of the enhancement of the ATRA-induced  $\text{O}_2^-$ -generating ability by cafestol and kahweol, we examined the effects of these two coffee diterpenes on the transcription levels of five genes essential for the leukocyte  $\text{O}_2^-$ -generating system (p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox) by RT-qPCR analysis. After 48 hr of incubation, total RNAs were extracted from ATRA-treated, ATRA

## Effects of cafestol and kahweol in functions of leukocytes



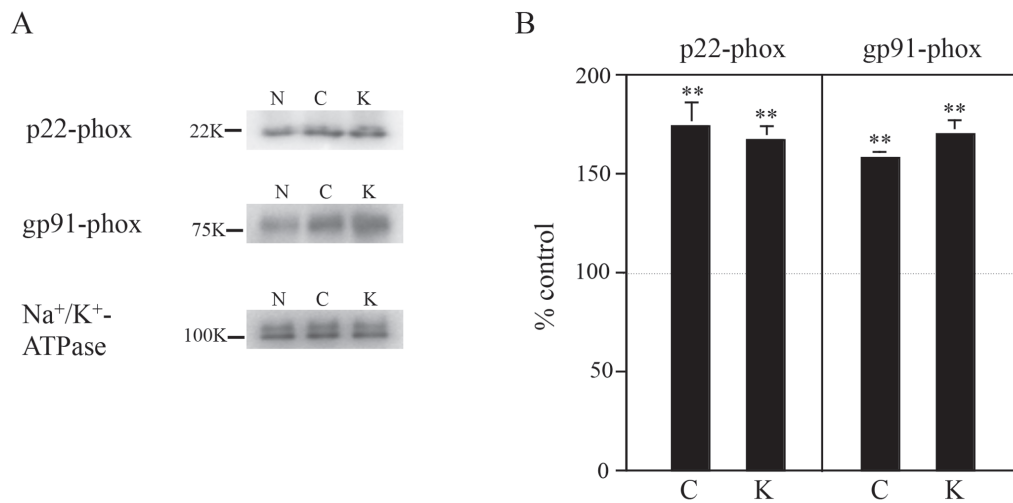
**Fig. 3.** Effects of cafestol and kahweol on the ATRA-induced O<sub>2</sub><sup>-</sup>-generating activity of U937 cells. After cultivation without or with 10 or 20 μM coffee diterpenes (cafestol and kahweol) in the presence of 1 μM ATRA for 48 hr, cells (1 × 10<sup>5</sup> cells/mL) were stimulated with 200 ng/mL PMA at 37°C. PMA-induced chemiluminescence was measured at 10 min after stimulation using a Lumat<sup>3</sup> LB9508 luminometer as described previously (Kikuchi *et al.*, 2018). Quantitative data are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated by Student's *t* test. \*, *p* < 0.05, \*\*, *p* < 0.01 compared with the data of ATRA-treated U937 cells.

plus 20 μM cafestol, and ATRA plus 20 μM kahweol-treated U937 cells. Quantitative data were indicated as percentages of control values obtained from ATRA-treated U937 cells (Fig. 4). Both cafestol and kahweol showed no effect on the transcription levels of p22-phox, p40-phox, p47-phox and p67-phox genes during co-treatment with ATRA. In contrast, the transcription levels of gp91-phox were significantly up-regulated in ATRA plus cafestol or kahweol-treated U937 cells (cafestol: to ~150% of ATRA-treated cells, kahweol: to ~138% of ATRA-treated cells). These results suggested that these two coffee diterpenes enhance the ATRA-induced O<sub>2</sub><sup>-</sup>-generating ability of U937 cells via up-regulation of transcription of gp91-phox gene. Next, immunoblot assay was carried out using antibodies specific for p22-phox or gp91-phox proteins in order to reveal the influences of cafestol and kahweol on the amounts of cytochrome *b*<sub>558</sub> composed of p22-phox and gp91-phox proteins during co-treatment with ATRA. Figure 5A shows typical immunoblot profiles for



**Fig. 4.** Effects of cafestol and kahweol on the transcription levels of the O<sub>2</sub><sup>-</sup>-generating system-related factors. The mRNA levels of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox were determined by RT-qPCR using total RNA extracted from ATRA-treated, ATRA plus 20 μM cafestol-treated (C) and ATRA plus 20 μM kahweol-treated (K) U937 cells as described in “MATERIALS AND METHODS”. All qPCR data of p22-phox, gp91-phox, p40-phox, p47-phox and p67-phox genes were normalized to qPCR data of GAPDH gene. Quantitative data are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. \*\*, *p* < 0.01 compared with the data of ATRA-treated U937 cells.

ATRA-treated, ATRA plus 20 μM cafestol, and ATRA plus 20 μM kahweol-treated U937 cells. Quantitative data were depicted as percentages of control values obtained from ATRA-treated U937 cells (Fig. 5B). As expected, the protein levels of p22-phox (cafestol: to ~174% of ATRA-treated cells, kahweol: to ~167% of ATRA-treated cells) and gp91-phox (cafestol: to ~158% of ATRA-treated cells, kahweol: to ~170% of ATRA-treated cells) were significantly up-regulated in ATRA plus coffee diterpenes-treated U937 cells. As mentioned above, p22-phox and gp91-phox proteins cooperatively assemble to form cytochrome *b*<sub>558</sub> heterodimer in the plasma membrane (Dagher and Pick, 2007; Panday *et al.*, 2015). In addition, our previous studies revealed not only that the expression level of gp91-phox protein is the most important limiting factor among the five essential protein factors of the O<sub>2</sub><sup>-</sup>-generating activity but also that the amounts of p22-phox protein tend to depend on those of gp91-phox in U937 cells (Kikuchi *et al.*, 1994, 2021a, 2021b,



**Fig. 5.** Effects of cafestol and kahweol on the protein levels of cytochrome  $b_{558}$  composed of p22-phox and gp91-phox proteins. (A) Typical immunoblot profiles. Membrane fractions were prepared from ATRA-treated (N), ATRA plus 20  $\mu$ M cafestol-treated (C) and ATRA plus 20  $\mu$ M kahweol-treated (K) U937 cells, and protein levels of p22-phox and gp91-phox were determined by immunoblot analysis. Human Na<sup>+</sup>/K<sup>+</sup>-ATPase was used as a control. (B) Quantitative data of immunoblot analysis. Data of ATRA plus 20  $\mu$ M cafestol-treated (C) and ATRA plus 20  $\mu$ M kahweol-treated (K) U937 cells are indicated as percentages of control value obtained from ATRA-treated U937 cells, and presented as the average of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated using Student's *t* test. \*\*,  $p < 0.01$  compared with the data of ATRA-treated U937 cells.

2022a, 2022b). The degree of up-regulation of the ATRA-induced O<sub>2</sub>-generating ability by these two coffee diterpenes (20  $\mu$ M cafestol: to ~162% of ATRA-treated cells, 20  $\mu$ M kahweol: to ~159% of ATRA-treated cells) seems to be consistent with degree of increases of p22-phox (20  $\mu$ M cafestol: to ~174% of ATRA-treated cells, 20  $\mu$ M kahweol: to ~167% of ATRA-treated cells) and gp91-phox (20  $\mu$ M cafestol: to ~158% of ATRA-treated cells, 20  $\mu$ M kahweol: to ~170% of ATRA-treated cells) proteins. Taken together, our data demonstrated that cafestol and kahweol enhanced the transcription levels of gp91-phox gene, resulting in significant increase of cytochrome  $b_{558}$  protein, so that the ATRA-induced O<sub>2</sub>-generating ability was up-regulated in U937 cells. In other words, these data also suggested that 20  $\mu$ M these two coffee diterpenes can promote differentiation of U937 cells to macrophage-like cells in collaboration with ATRA.

As known, diterpenes have anti-cancer activities due to their cytotoxicity (Faustino *et al.*, 2018; Martínez-Casares *et al.*, 2023). Our data demonstrated that cafestol and kahweol show moderate cytotoxicity against U937 cells at a higher concentration (50  $\mu$ M) and their cytotoxic activities were enhanced by ATRA. Moreover, our data

also revealed that cafestol and kahweol have the remarkable promoting effects on the ATRA-induced differentiation of U937 cells at a lower concentration (20  $\mu$ M). Differentiation therapy with ATRA has been demonstrated which results in the treatment of leukemia (Nowak *et al.*, 2009). Therefore, these two coffee diterpenes may be expected to be an effective modifier in therapy for leukemia in combination with ATRA.

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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