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Structure-activity relationship of flavonoids for inhibition of epidermal growth factor-induced transformation of JB6 Cl 41 cells

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Running title: *Cell transformation-inhibitory activity of flavonoids*

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Abbreviations: AP-1, activator protein 1; BME, basal medium Eagle; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGCG, (-)-epigallocatechin gallate; EGF, epidermal growth factor; EGFR, EGF receptor; ERKs, extracellular signal-regulated kinases; FBS, fetal bovine serum; JNKs, c-Jun N-terminal kinases; MAPKs, mitogen-activated protein kinases; MEM, Eagle's minimum essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; SDS, dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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We found that quercetin, myricetin, quercetagetin, fisetin, (-)-epigallocatechin gallate (EGCG) and theaflavins, among twenty-four flavonoids examined, markedly inhibited EGF-induced cell transformation of mouse epidermal JB6 Cl 41 cells. The six flavonoids suppressed the EGF-induced activation of activator protein 1 (AP-1). In addition, myricetin, quercetagetin, EGCG and theaflavins directly inhibited EGF-induced phosphatidylinositol 3-kinase (PI3K) activation. The important structural features of flavonoids for cell transformation-inhibitory activity are 3'- and 4'-OH on the B-ring, 3-OH on the C-ring, C2-C3 double bond in the C-ring, and the phenylchromone (C6-C5-C6) skeleton in the flavonols, and the galloyl group in EGCG and theaflavins. Our results provide new insight into possible mechanisms of the anti-carcinogenic effects of flavonoids, and could help to provide a basis for the design of novel cancer chemopreventive agents.

Key words: flavonoids, cell transformation, signal transduction, AP-1, PI3K

INTRODUCTION

Flavonoids, which are primarily phenylbenzo-γ-pyrone (phenylchromone) derivatives, are polyphenolic compounds present in fruits, vegetables, and beverages [1]. Numerous studies both *in vitro* and in animal models have indicated that various flavonoids influence important cellular and molecular mechanisms related to carcinogenesis, such as cell cycle control, apoptosis and malignant transformation [2-4]. Many of the biological actions of flavonoids are thought to be attributable to their antioxidant properties, including their effects on intracellular redox status [5,6]. However, it has been suggested that the conventional hydrogen-donating antioxidant activity of flavonoids may not be relevant to their biological activities [7-9]. There is accumulating evidence that flavonoids modify the activities of several intracellular signal transduction enzymes, including mitogen-activated protein kinases (MAPKs), and phosphatidylinositol 3-kinase (PI3K) [2,4,7,10]. Several studies have found that flavonoids also inhibit the growth of malignant cells [11,12]. It has been shown that flavonoids inhibit signal transduction enzymes by competitively blocking the ATP binding site [10,13,14], but the structure-activity relationship remains to be fully established.

The mouse epidermal JB6 cell system of clonal genetic variants, which are promotion-sensitive (P+) or promotion-resistant (P-), is an excellent model for studying molecular mechanisms in various stages of carcinogenesis [15-20]. The JB6 P+, P- and transformed variants are a series of cell lines representing ‘earlier-to-later’ stages of preneoplastic-to-neoplastic progression [16,18]. JB6 Cl 41 cells as P+ cells, but not P- cells, undergo transformation when stimulated with epidermal growth factor (EGF) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and form colonies in soft agar [16,18].

Through comparison of JB6 P+ and P- cells, the transformation is demonstrated to involve activation of activator protein 1 (AP-1) [15,19-22], which regulates the transcription of various genes related to cellular inflammation, proliferation and apoptosis [23]. AP-1 activation is stimulated, at least in part, by activation of MAPKs, such as extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 kinases [19,20,24,25]. Inhibition of MAPK activation by dominant negative mutants and chemical inhibitors [24] or by some chemopreventive agents [4,26] blocks tumor promoter-induced cell transformation of JB6 Cl 41 cells. In addition, we have shown that phosphatidylinositol 3-kinase (PI3K) and its downstream effector, Akt, are intimately involved in EGF-induced cell transformation of JB6 Cl 41 cells [27]. PI3K is central to the coordinated control of multiple cell-signaling pathways leading to tumor development, including cell proliferation, apoptosis and migration [28,29], and Akt plays a pivotal role in the PI3K-signaling pathway [30]. Thus, JB6 Cl 41 cells provide a well-developed cell culture system for studying the regulation of signal transduction by chemopreventive agents in the tumor promotion stage *in vitro*. Therefore, we investigated the structure-activity relationship for inhibition of EGF-induced cell transformation in JB6 Cl 41 cells, using 24 flavonoids, including flavones, flavonols, isoflavones, flavanones, flavanols, flavanonols, and chalcone (Fig. 1). We found that several flavonols markedly inhibited the EGF-induced cell transformation. The active compounds also appeared to inhibit activation of the AP-1 and PI3K/Akt pathways.

MATERIALS AND METHODS

Materials

The flavones, flavone, apigenin, luteolin and chrysin, the flavonols, kaempferol, quercetin, rutin and morin, the isoflavones, daidzein and genistein, the flavanones, hesperetin, hesperidin and naringenin, the flavanonol, silybin, and the flavanols, (+)-catechin and EGCG, were purchased from Sigma (St. Louis, MO, USA). The flavonols, galangin, myricetin and quercetagetin, and the chalcone, 2',4',6',3,4-pentahydroxychalcone, were from Extrasynthese (Genay Cedex, France). The flavonol, fisetin, and the flavanol, (-)-catechin, were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Theaflavins (flavanol) were from Kurita Industries (Tokyo, Japan). (±)-Taxifolin (flavanonol) was from Calbiochem (San Diego, CA, USA). The structures are shown in Fig. 1a-c. Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine and basal medium Eagle (BME) were from Gibco (Invitrogen; Gland Island, NY, USA); EGF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphatidylinositol and gentamicin were from Sigma; the Akt immunoprecipitation kinase assay kit was from Upstate Biotechnology Inc. (Lake Placid, NY, USA); the PhosphoPlus p44/42 MAPK and p38 kinase antibody kits were from Cell Signaling Technology Inc. (Beverly, MA, USA); the anti-EGFR antibody, anti-phosphotyrosine antibody, anti-Akt1/2 antibody, and protein A/G plus-agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell Culture

The JB6 mouse epidermal cell line, Cl 41, and its stable transfectant, P+1-1 (AP-1 reporter transfectant), were gifts from Dr. Zigang Dong, the Hormel Institute, University of Minnesota, (Austin MN, USA). The cells were cultured in MEM supplemented

with 5% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin in an incubator at 37°C under 5% CO_2 in air.

Anchorage-independent growth assay

JB6 Cl 41 cells (1×10^4) were exposed to EGF (20 ng/ml) with or without flavonoids at 10 μM in 1 ml of 0.33% BME agar containing 10% FBS over 3.5 ml of 0.5% BME agar medium containing 10% FBS. The cultures were maintained in an incubator at 37°C under 5% CO_2 in air, and the colonies formed were scored under a microscope 14 days after exposure to EGF.

Cell growth inhibition assay

JB6 Cl 41 cells (5×10^3) were seeded into each well of 96-well plates and allowed to attach overnight. The cells were then treated with the indicated concentrations of flavonoids for 72 h in an incubator at 37°C under 5% CO_2 in air. Subsequently, MTT (25 μL of 2 mg/mL) in phosphate-buffered saline (PBS) was added to each well, then incubation was continued for 4 h at 37°C. Formazan crystals were dissolved in dimethylsulfoxide. Absorbance was determined at 540 nm with a microplate reader (Multiskan Bichromatic; Labsystems Japan, Tokyo, Japan).

Assay of AP-1 activity

JB6 AP-1 reporter stable P+1-1 cells (7×10^3) were seeded in 96-well plates. After 24 h incubation, the cells were starved by replacing the medium with 0.1% FBS/MEM for 24 h. Then, the cells were pretreated with a flavonoid (10 μM) for 1 h and cultured

with or without 20 ng/ml EGF in the presence or absence of the flavonoid for 24 h. The cells were extracted with lysis buffer, and luciferase activity was measured with a BioOrbit 1253 luminometer (Turku, Finland). Relative AP-1 activity was calculated as described previously [26,27,31].

AP-1 DNA-binding assay

JB6 Cl 41 cells were cultured to 80% confluence and then starved by culturing in 0.1% FBS/MEM for 24 h at 37°C. The medium was then changed to fresh 0.1% FBS/MEM and the cells were incubated for another 2-4 h at 37°C. Before the cells were exposed to EGF, they were treated with or without (control) a flavonoid (10 μM) for 1 h. Then, EGF was added and the cells were incubated for 24 h. Nuclear protein from the cells was extracted, and the DNA binding reaction was performed using 5 μg of nuclear protein and 15000 cpm of a ^{32}P-labeled oligonucleotide containing the AP-1-binding element (5'-CGC TTG ATG AGT CAG CCG GAA-3') as described previously [26,33]. The samples were separated on 5% polyacrylamide gels, which were analyzed using a Typhoon 9410 image analyzer (Amersham Biosciences) and NIH image 1.61 software.

Immunoblotting

Immunoblotting was carried out as described previously [27,31]. In brief, JB6 Cl 41 cells were treated with or without a flavonoid (10 μM) for 1 h before treatment with EGF (20 ng/ml) for 5 min. The cells were then lysed, and immunoblotting analysis was performed using antibodies against p44/42 MAPKs (ERKs; extracellular-signal regulated protein kinases) and p38 kinase antibody or phosphate-specific antibodies

against the phosphorylated proteins. Antibody-bound proteins were detected using an ECL-Plus Western Blotting Kit (Amersham Biosciences) and analyzed using a Typhoon 9410 image analyzer (Amersham Biosciences).

JNK assay

JB6 Cl 41 cells were treated with or without a flavonoid (10 μM) for 1 h before treatment with EGF (20 ng/ml) for 5 min. The lysates were then prepared from the cells, and immunoprecipitation was carried out using immobilized c-Jun fusion protein (Cell signaling). The kinase assay and immunoblotting using phospho-c-Jun (Ser 63) antibody were performed according to the protocol of a JNK assay kit (Cell Signaling). The immunoblotting were detected using an ECL-Plus Western Blotting Kit (Amersham Biosciences) and analyzed using a Typhoon 9410 image analyzer (Amersham Biosciences).

Akt immunoprecipitation kinase assay

JB6 Cl 41 cells were treated with or without a flavonoid (10 μM) for 1 h before treatment with EGF (20 ng/ml) for 5 min. The lysates were then prepared from the cells, and immunoprecipitation was carried out using 20 μl of anti-Akt1/2 antibody (Santa Cruz). Akt activities were determined using a specific Akt substrate peptide and [γ-^{32}P]ATP as described previously [32].

PI3K assay

JB6 Cl 41 cells pretreated with a flavonoid (10 μM) for 1 h were exposed to EGF (20 ng/ml) for 5 min. The cell lysate was normalized against the untreated control, and

incubated with gentle rocking for 2 h at 4°C with 20 µl of agarose conjugated with anti-tyrosine antibody. The PI3K activity was determined as described previously [32]. The radiolabeled spot of phosphatidylinositol 3-phosphate (PIP3) was quantified using a Typhoon 9410 image analyzer (Amersham Biosciences) and NIH image 1.61 software.

Direct effects of the flavonoids on PI3K assay

JB6 Cl 41 cells treated with EGF (20 ng/ml) in the absence of flavonoids were lysed as described above. The protein immunoprecipitated with agarose-conjugated anti-tyrosine antibody was reacted in the presence or absence of a flavonoid (10 µM). PI3K activity was determined as above.

RESULTS AND DISCUSSION

Structure-activity relationship of flavonoids for inhibition of EGF-induced cell transformation

Mouse epidermal JB6 promotion-sensitive (P+) cell line, Cl 41, in which transformed colonies are induced by EGF or TPA [4,21,24,26], is a well-developed model to screen chemopreventive agents, and to elucidate their mechanisms at the molecular level. Therefore, we used this cell line in order to investigate the structure-activity relationship for inhibition of soft agar colony formation (cell transformation) by flavonoids. EGF induced 2000-3000 transformed colonies in soft agar, whereas almost no colony formation was observed in the control group (0.1% DMSO). As shown in Fig. 2, flavones (apigenin, chrysin) and flavanone (naringenin) slightly inhibited EGF-induced cell transformation at the concentration of 10 µM. In addition to flavanols, EGCG and theaflavins, which have reported previously (26),

flavonols (quercetin, myricetin, quercetagetin, fisetin) were potent inhibitors (Fig. 2), and their effect was concentration-dependent (1-10 μM) (data not shown). On the other hand, isoflavones, falavanonols and chalcone had no significant inhibitory effect on EGF-induced cell transformation.

As shown in Fig. 1, the basic structure of flavonoids is comprised of two benzene rings (A- and B-ring) linked through a heterocyclic pyrane or pyrone ring (C-ring) in the middle. The B-ring is usually substituted at the 2-position of the C-ring. The subdivision of flavonoids is primarily based on the presence of an oxy group at position 4, a double bond between carbon atoms 2 and 3 (C2-C3 double bond), and the presence of a hydroxyl group in position 3 (3-OH) of the C-ring. Flavonols, such as quercetin, myricetin, quercetagetin and fisetin, which were potent inhibitors, have all of oxy group at position 4, C2-C3 double bond, and 3-OH. In contrast, chalcone lacks the C-ring, and the B-ring of isoflavonoids is substituted at the 3-position of the C-ring. Flavanonols do not have C2-C3 double bond. For example, (±)-taxifolin, which lacks the C2-C3 double bond of quercetin in the C-ring, failed to inhibit EGF-induced cell transformation. Thus, it was suggested that the position substituted of the B-ring, the presence of the diphenylpropane (C6-C3-C6) skeleton, and the presence of C2-C3 double bond are important for the cell transformation. Flavones, which lack 3-OH, and flavanones, which do not have 3-OH and C2-C3 double bond, only showed the weak inhibitory effect for cell transformation. In addition, luteolin, which lacks 3-OH, or rutin, which has a glycoside in place of the 3-OH on the C-ring of quercetin, showed no significant inhibitory effect on EGF-induced transformation, suggested that the 3-OH on the C-ring is related to the cell transformation. Furthermore, the flavanols, quercetin, myricetin, quercetagetin and fisetin, have 3'- and 4'- OH on the B-ring, and

elimination of 3'-OH on the B-ring, as in kaempferol and morin, reduced the inhibitory potency compared with quercetin. Flavanols, such as (+)- and (-)-catechin, which do not have the oxy group at position 4 and C2-C3 double bond, were ineffective, although EGCG and theaflavins, which contain the galloyl group, showed a strong inhibitory effect. Therefore, the galloyl group was considered to be important for the inhibitory effects of EGCG and theaflavins on cell transformation. As summarized in Fig. 3, these findings indicated that the important structural features of flavonoids for inhibition of EGF-induced cell transformation are as follows: *1)* 3'- and 4'-OH on the B-ring, *2)* 3-OH on the C-ring, *3)* the C2-C3 double bond in the C-ring, and *4)* the phenylchromone (C6-C5-C6) skeleton in the flavonoids, and *5)* the galloyl group in EGCG and theaflavins.

Among the flavonoids, quercetagetin showed growth inhibition, suggesting that the cell transformation-inhibitory action of quercetagetin resulted at least partly from growth inhibition or apoptosis (Fig. 4). We have observed that the growth-inhibitory effect of baicalein, 5,6,7-trihydroxy-flavone, was stronger than that of quercetagetin in the same concentration range (data not shown), suggesting that 6-OH on the A-ring is involved in the growth-inhibitory effect of flavonoids.

Flavonoids inhibited EGF-induced activation and DNA-binding activity of AP-1

The transcription factor AP-1 plays a critical role in neoplastic transformation in JB6 P+ cells, as well as in tumor promotion [15,21,23,26]. It has been reported that EGCG and theaflavins inhibited EGF-induced AP-1 activation as well as cell transformation [26]. We then studied the effects of the above flavonoids on EGF-induced AP-1 activation using a luciferase-reporter gene assay. In addition to

EGCG and theaflavins, the flavonols, quercetin, myricetin, quercetagetin and fisetin, suppressed EGF-induced AP-1 activity without inhibition of basal activity of AP-1 (Fig. 5).

AP-1 induces gene transcription by binding to the TPA response element (AP-1 binding site) in the promoter region of its target genes [23]. As shown in Fig. 6, the flavonols inhibited AP-1 DNA-binding activity, as well as AP-1 activation, suggesting that the inhibition of EGF-induced cell transformation by the flavonoids involves the blocking of AP-1 activation. The flavonoids did not directly inhibit AP-1 DNA-binding activity (data not shown).

AP-1 activation is stimulated, at least in part, by activation of MAPKs, such as ERKs, JNKs, and p38 kinases [24,25]. Dong *et al*. [26] showed that EGCG and theaflavins blocked EGF-induced AP-1 activation through the inhibition of JNKs activation in JB6 Cl 41 cells. Although the six flavonoids had no effect on EGF-induced phosphorylation of ERKs and p38 kinases, JNKs activities decreased by treatment with the flavonoids (Fig. 7). Therefore, inhibition of AP-1 activation by flavonols (quercetin, myricetin, quercetagetin and fisetin) was suggested to be, at least in part, through the inhibition of JNKs activation as well as EGCG and theaflavins.

Quercetin, myricetin, quercetagetin, EGCG and theaflavins inhibited EGF-induced activation of PI3K and Akt

PI3K is central to the coordinated control of multiple cell-signaling pathways leading to tumor development [28,29], and Akt, a downstream effector of PI3K, plays a pivotal role in the PI3K-signaling pathway [30]. We have shown that overexpression of a dominant negative mutant of Akt1, which antagonizes Akt function, and a PI3K

inhibitor, LY294002, block EGF-induced cell transformation in JB6 Cl 41 cells [27]. Therefore we assessed the effects of the flavonoids (quercetin, myricetin, quercetagetin, fisetin, EGCG, and theaflavins) on EGF-induced Akt activation. As shown in Fig. 8, quercetin, myricetin, quercetagetin, EGCG, and theaflavins, but not fisetin, clearly suppressed EGF-induced activation of Akt. On the other hand, none of the flavonoids directly inhibited Akt activation induced by EGF (data not shown).

Quercetin, myricetin, quercetagetin, fisetin, EGCG and theaflavins also significantly suppressed EGF-induced PI3K activation (Fig. 9). On the other hand, fisetin did not inhibit PI3K activation. We further tested whether these flavonoids directly inhibited PI3K activation. The results show that myricetin, quercetagetin, fisetin, EGCG and theaflavins, but not quercetin and fisetin, directly blocked EGF-induced PI3K activation (Fig. 10), suggesting that myricetin, quercetagetin, EGCG and theaflavins inhibit activation of PI3K and Akt by direct inhibition of PI3K. It has been reported that some flavonoids inhibit PI3K activity through competition with ATP at its binding site [10,14]. Therefore, the inhibition of PI3K activation by the flavonoids may be due to competing with ATP for binding to PI3K. Although quercetin was demonstrated to block ATP binding to PI3K [14], the concentration (10 μM) examined here with JB6 Cl 41 cells did not directly inhibit PI3K activation. Quercetin might inhibit PI3K activation through suppression of the binding of PI3K to the EGF receptor. Our results confirmed that the C2-C3 double bond in the C-ring, and the 3’- and 4’-OH moieties on the B-ring of the flavonids (flavonols) have important roles in the inhibition of PI3K activation [10,14], and also in blocking cell transformation. In addition, the presence of 5-OH on the A-ring may be critical for the inhibitory action, because fisetin did not suppress PI3K activation. Epicatechin, (+)-

and (-)-catechin had no effect on EGF-induced PI3K activation (data not shown). Therefore, the galloyl group in EGCG and theaflavins is suggested to be involved in the inhibition of PI3K. On the other hand, fisetin, which did not inhibit PI3K activation, may have other targets; for instance, specific targets downstream of the PI3K pathway, or some other signaling cascade.

We also study on inhibitory mechanisms of flavonoids on insulin-stimulated intracellular signaling in mouse preadipose MC3T3-G2/PA6 cells differentiated into mature adipose cells. In that study, we found that some flavones and flavonols (10 μM), including apigenin, quercetin and fisetin, suppressed insulin-stimulated activation of PI3K/Akt pathway through inhibition of tyrosine phosphorylation of insulin receptor β-subunit. EGCG and theaflavins had no effects on the activation of PI3K/Akt pathway. In addition, quercetin, myricetin, quercetagetin, fisetin, EGCG and theaflavins did not inhibit EGF-stimulated Akt phosphorylation in the differentiated MC3T3-G2/PA6 cells at the concentration of 10 μM (data not shown). These findings suggested that the inhibition of PI3K/Akt pathway by flavonoids might be tumor specific.

In summary, our results indicate that flavonols (quercetin, myricetin, quercetagetin and fisetin) and flavanols (EGCG and theaflavins) markedly inhibit EGF-induced cell transformation. The inhibitory effects were suggested to be due to blockade of EGF-induced activation of AP-1 and PI3K, except in the case of fisetin, which did not block PI3K activation. The structure-activity relationship indicated that the C2-C3 double bond in the C-ring, and the 3'- and 4'-OH moieties on the B-ring in the flavonols, and the presence of a galloyl group are important for the inhibition of cell

transformation and protein kinases. In addition, a hydroxyl group on the chroman nucleus (A- and B-ring), such as 3-OH or 5-OH, may also play a significant role in determining the specificity of the bioactivities of the flavonoids. The results of the present study may help to provide a rational basis for the design of novel cancer chemopreventive agents.

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FIGURE LEGENDS

Fig. 1a. Structures of Flavonoids.

Fig. 1b. Structures of Flavonoids (continued).

Fig. 1c. Structures of Flavonoids (continued).

Fig. 2. Effects of flavonoids on EGF-induced cell transformation. Aliquots of 10^4 JB6 Cl 41 cells were treated with EGF (20 ng/ml) with or without 10 μM flavonoid in 0.33% BME agar containing 10% FBS over 0.5% BME agar medium containing 10% FBS. Cell colonies were scored after incubation for 14 days at 37˚C under 5% CO_2 in air. Data are expressed as means ± S.E. of at least four experiments. *, ** Significantly different from EGF alone at $P < 0.05$ and $P < 0.001$, respectively.

Fig. 3. Relationship between flavonoid structure and cell transformation-inhibitory activity. Important structural features of flavonoids for cell transformation-inhibitory activity are 3'- and 4'-OH on the B-ring, 3-OH on the C-ring, C2-C3 double bond in the C-ring, and the phenylchromone (C6-C5-C6) skeleton in the flavonols, and the galloyl group in EGCG and theaflavins.

Fig. 4. Effects of flavonoids on cell growth. JB6 Cl 41 cells were treated with the indicated concentration of transformation-inhibitory flavonoids for 72 h. Data are expressed as means ± S.E. of at least three experiments.

Fig. 5. Effect of the flavonoids on EGF-induced AP-1 activation. JB6 AP-1 reporter stable P+1-1 cells were exposed or not exposed to 20 ng/ml EGF with or without flavonoid (10 μM) for 24 h. The results are shown as relative AP-1 activity, expressed as means ± S.E. of at least three experiments. * Significantly different from EGF alone at $P < 0.001$.

Fig. 6. Effect of the flavonoids on EGF-induced AP-1 DNA-binding activity. After pretreatment with or without a flavonoid (10 μM) for 1 h, JB6 Cl 41 cells were treated with or without EGF (20 ng/ml) and subsequently incubated for 24 h. Nuclear protein was extracted, and the sequence-specific AP-1 DNA binding activity was determined by gel-shift analysis using a ^{32}P-labeled oligonucleotide containing the AP-1-binding element. Data are expressed as the means ± S.E. of three experiments. *Significantly different from EGF alone at $P < 0.05$.

Fig. 7. Effect of the flavonoids on EGF-induced activation of MAPKs. JB6 Cl 41 cells were pretreated with flavonoid (10 μM) for 1 h. The cells were then treated or untreated with EGF (20 ng/ml) and subsequently cultured for 5 min. The phosphorylation levels were estimated by immunoblotting as described in "MATERIALS AND METHODS".

Fig. 8. Effect of the flavonoids on EGF-induced Akt activation. After treatment with or without a flavonoid (10 μM), JB6 Cl 41 cells were treated with or without EGF (20 ng/ml) and subsequently incubated for 5 min. The respective activities were assessed using a specific substrate peptide and [γ-^{32}P]ATP. Data are expressed as the

means ± S.E. of six experiments. *Significantly different from EGF alone at $P < 0.05$.

Fig. 9. Effect of the flavonoids on EGF-induced PI3K activation. After treatment with or without a flavonoid (10 μM) for 1 h, JB6 Cl 41 cells were treated with or without EGF (20 ng/ml) and subsequently incubated for 5 min. The cells were lysed and PI3K activity was determined as described in "MATERIALS AND METHODS". Data are expressed as the means ± S.E. of five experiments. *Significantly different from EGF alone at $P < 0.05$.

Fig. 10. Direct effects of the flavonoids on EGF-induced PI3K activation. JB6 Cl 41 cells were treated with or without EGF (20 ng/ml) for 5 min. The cells were lysed and the immunoprecipitated protein was allowed to react in the presence or absence of flavonoid (10 μM). Activities of PI3K were determined as described in "MATERIALS AND METHODS". Data are expressed as the means ± S.E. of three experiments. *Significantly different from EGF alone at $P < 0.05$.

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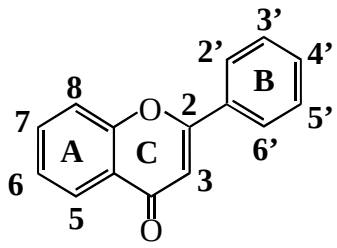
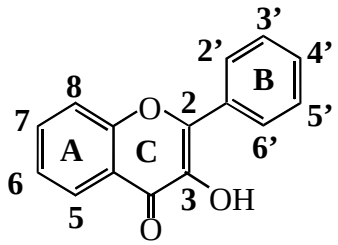
Flavones



	5	7	3'	4'
Flavone	-	-	-	-
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	-	-

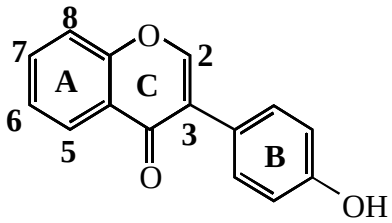
Flavonols



	3	5	6	7	2'	3'	4'	5'
Galangin	OH	-	-	-	-	-	-	-
Kaempferol	OH	OH	-	OH	-	-	OH	-
Quercetin	OH	OH	-	OH	-	OH	OH	-
Myricetin	OH	OH	-	OH	-	OH	OH	OH
Rutin	O-rutinose	OH	-	OH	-	OH	OH	-
Quercetagetin	OH	OH	OH	OH	-	OH	OH	-
Fisetin	OH	-	-	OH	-	OH	OH	-
Morin	OH	OH	-	OH	OH	-	OH	-

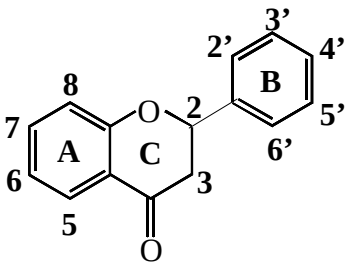
Fig. 1a. Ichimatsu *et al.*

Isoflavones



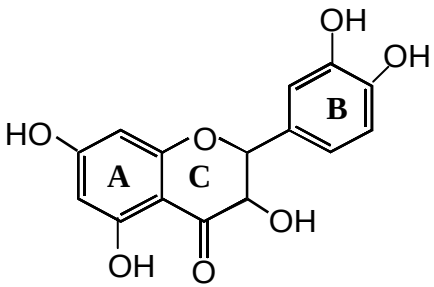
	5	7
Daidzein	-	OH
Genistein	OH	OH

Flavanones

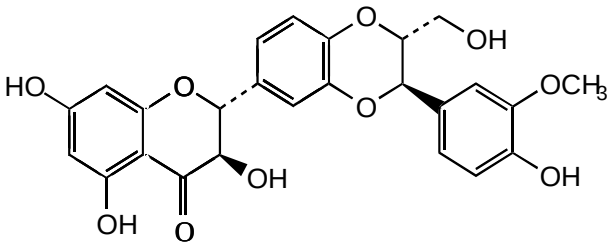


	5	7	3'	4'
Hesperetin	OH	OH	OH	OCH_3
Hesperidin	OH	O-rutinosyl	OH	OCH_3
Naringenin	OH	OH	-	OH

Flavanonols

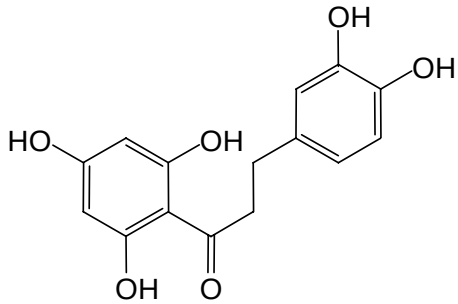


(±)-Taxifolin



Silybin

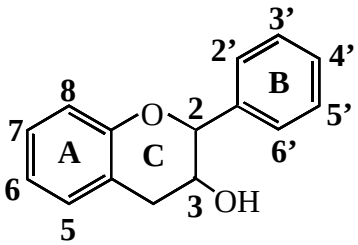
Chalcone

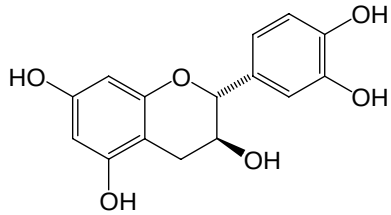


2',4',6',3,4-Pentahydroxychalcone

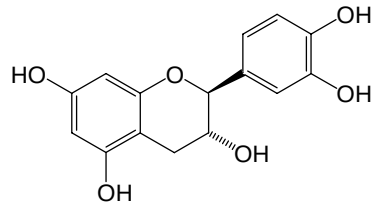
Fig. 1b. Ichimatsu *et al.*

Flavanols

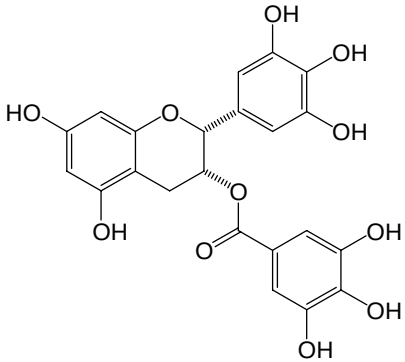




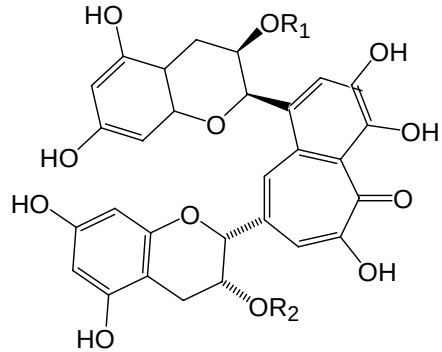
(+)-Catechin



(-)-Catechin

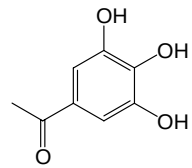


(-)-Epigallocatechin Gallate (EGCG)



Theaflavins

Theaflavins	R_1	R_2
Theaflavin	H	H
Theaflavin-3-gallate	G	H
Theaflavin-3’-gallate	H	G
Theaflavin-3,3’-digallate	G	G



Galloyl (G)

Fig. 1c. Ichimatsu *et al.*

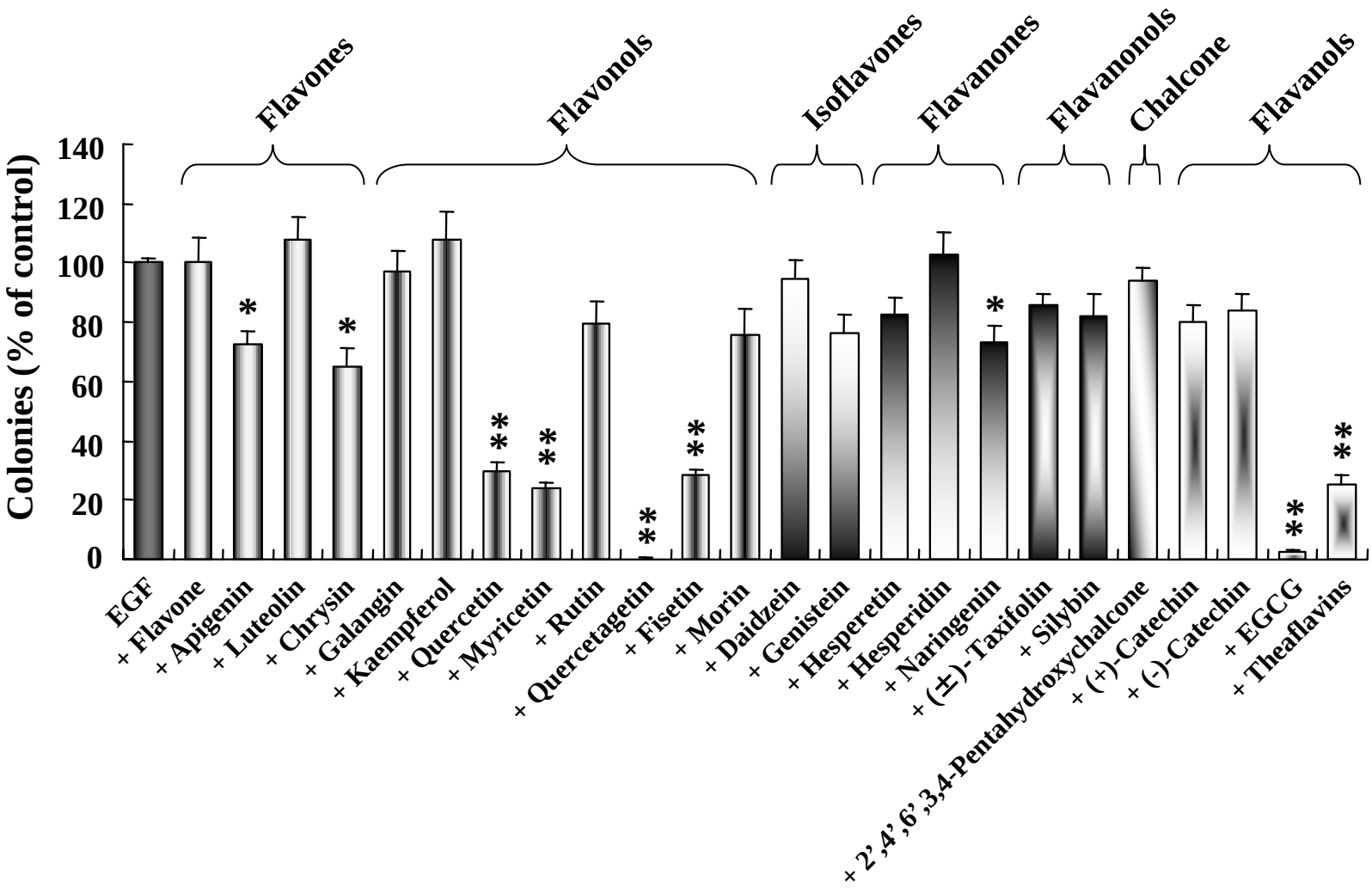
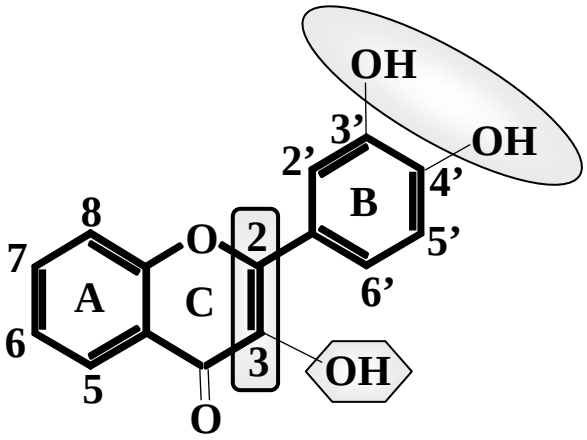
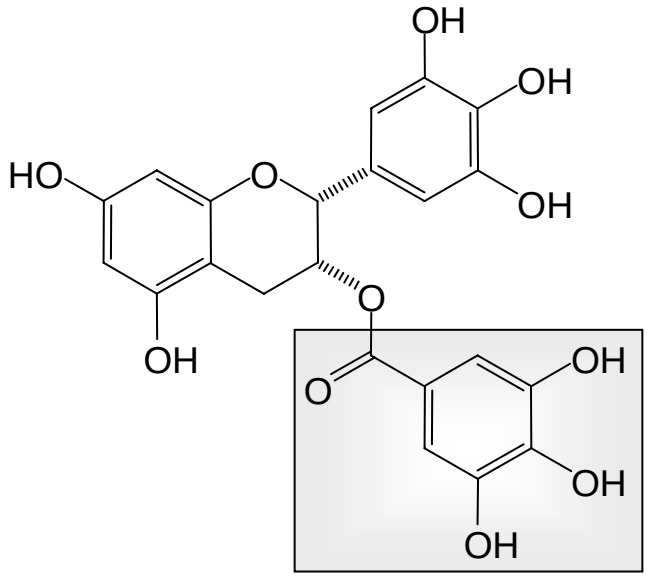


Fig. 2. Ichimatsu *et al.*





Galloyl group

Important factors

- 3’- and 4’-OH group on B-ring
- C2-C3 double bond in C-ring
- 3-OH group on C-ring
- Phenylchromone (C6-C3-C6) skeleton
- Galloyl group

Fig. 3. Ichimatsu *et al.*

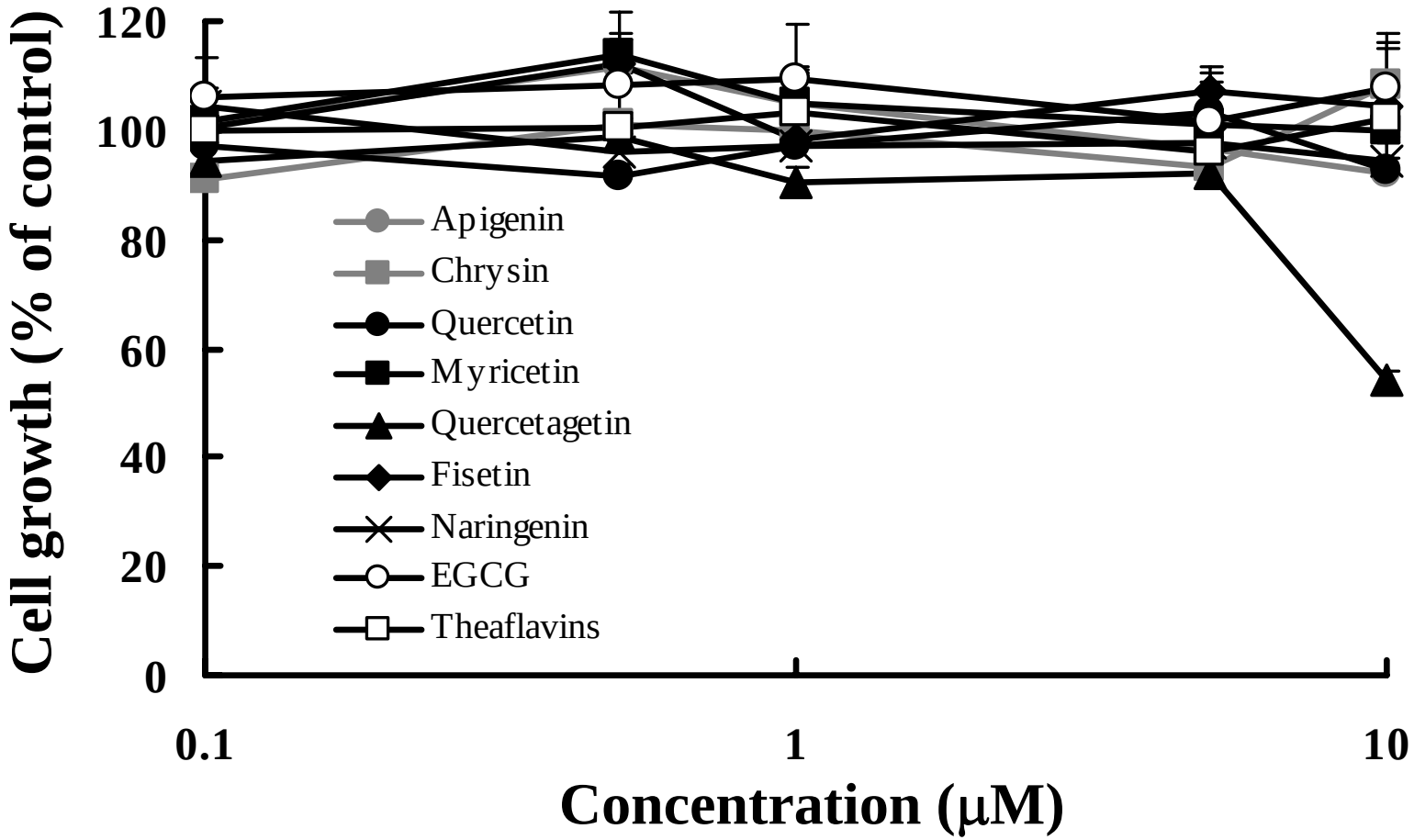
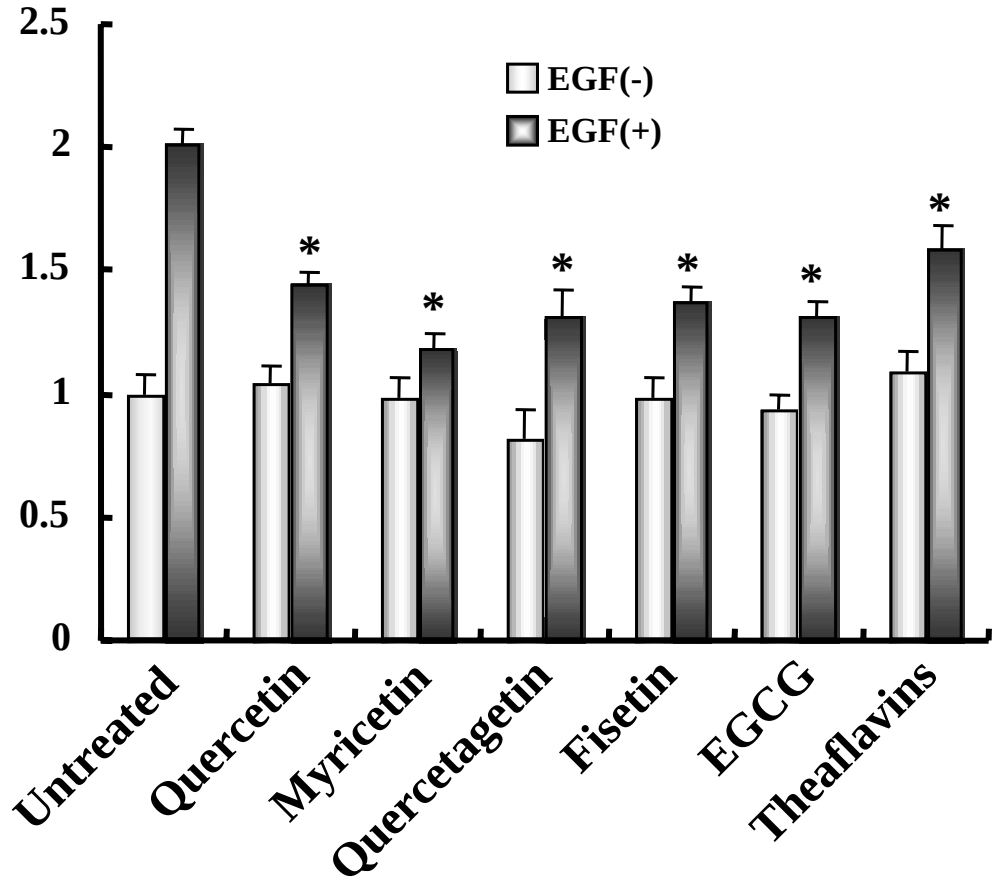


Fig. 4. Ichimatsu *et al.*



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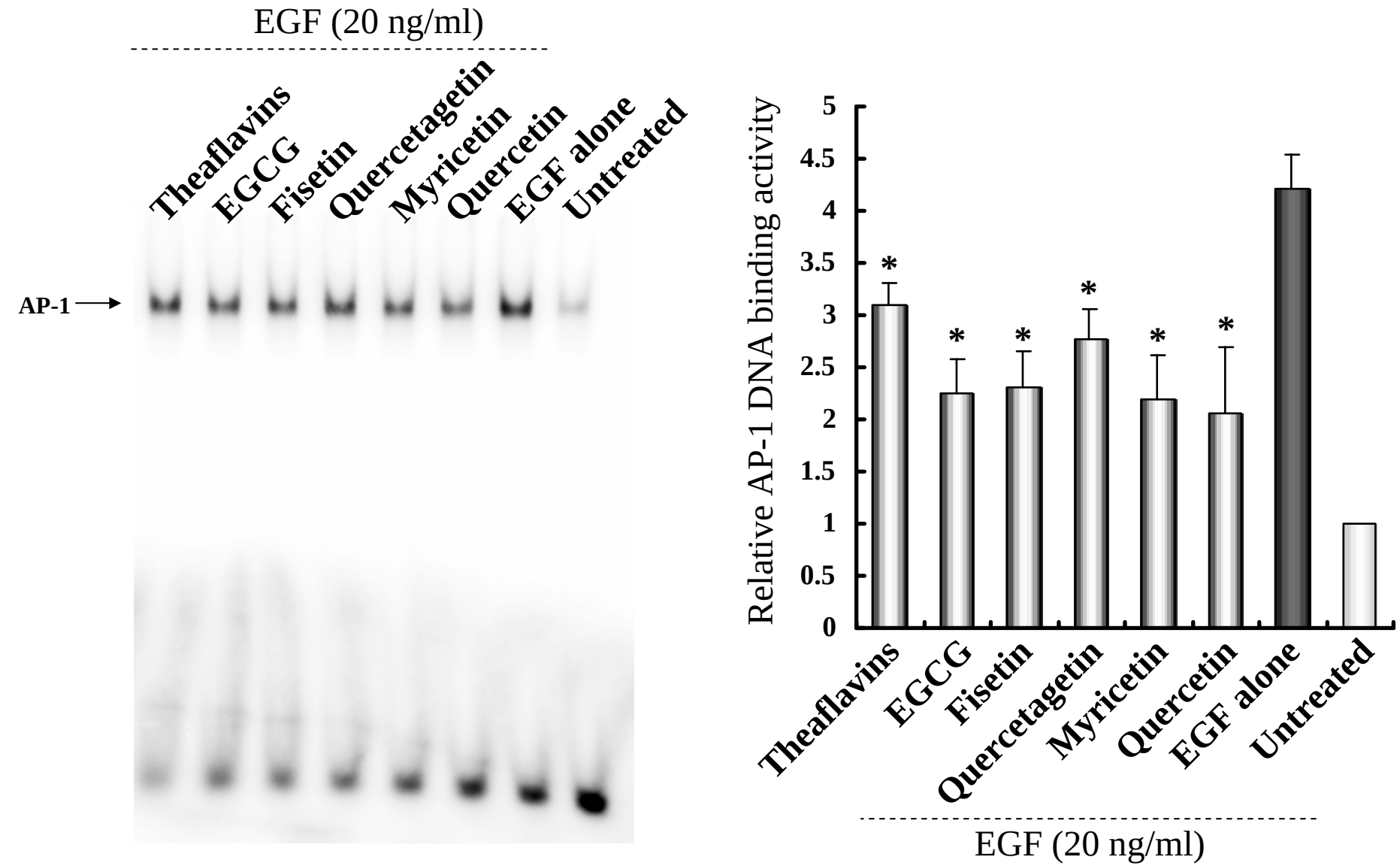

Fig. 6. Ichimatsu *et al.*

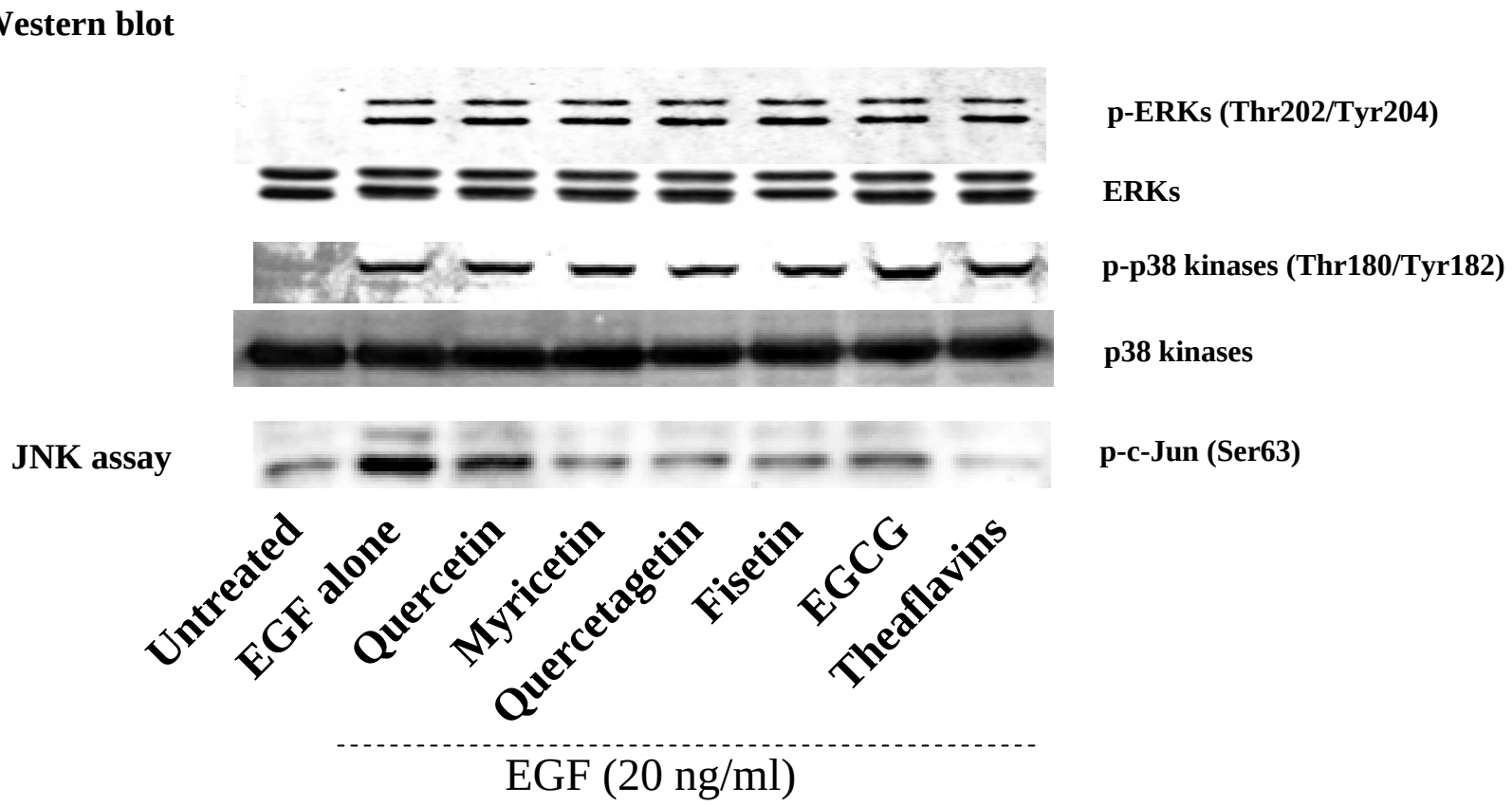

Fig. 7. Ichimatsu *et al.*

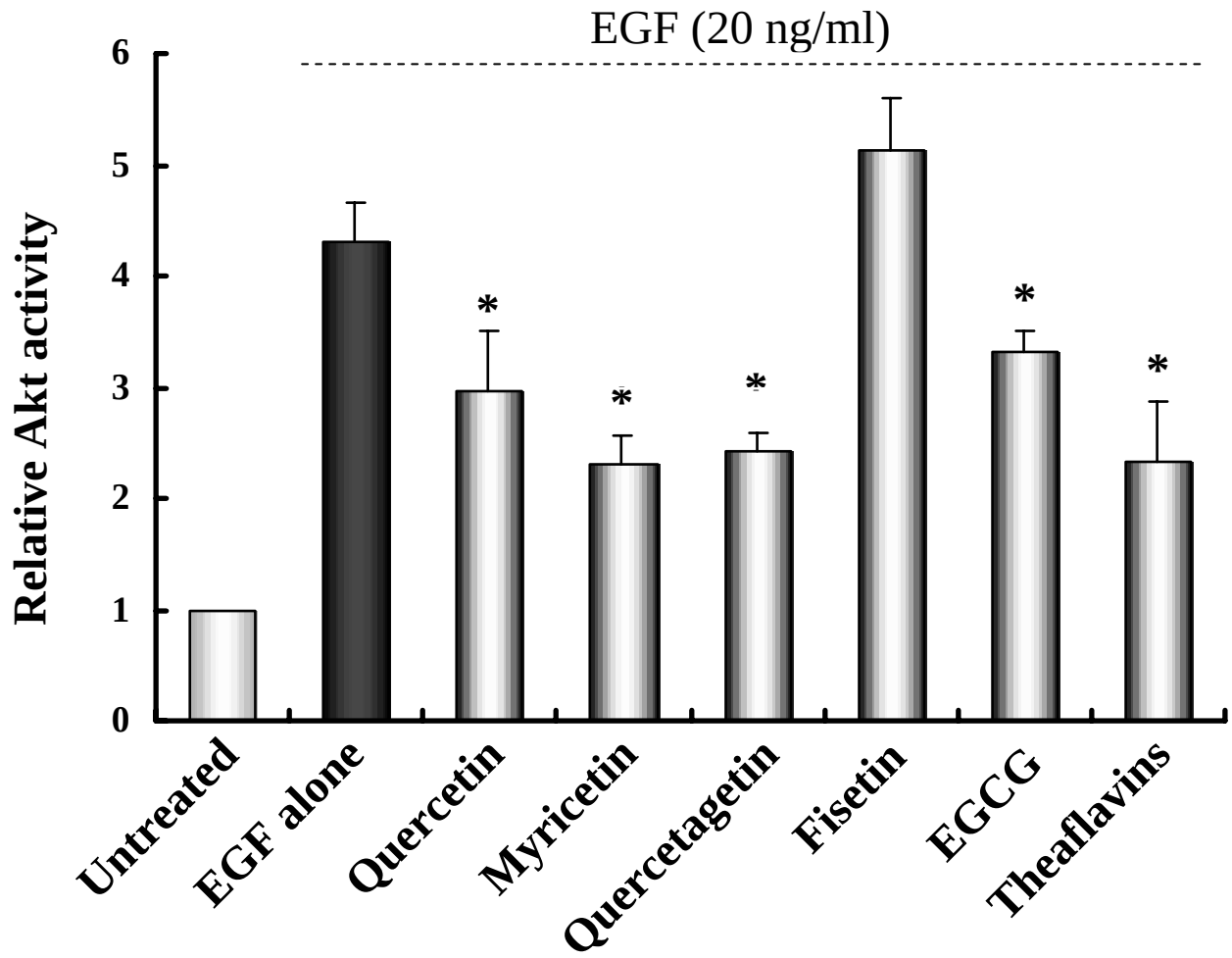

Fig. 8. Ichimatsu *et al.*

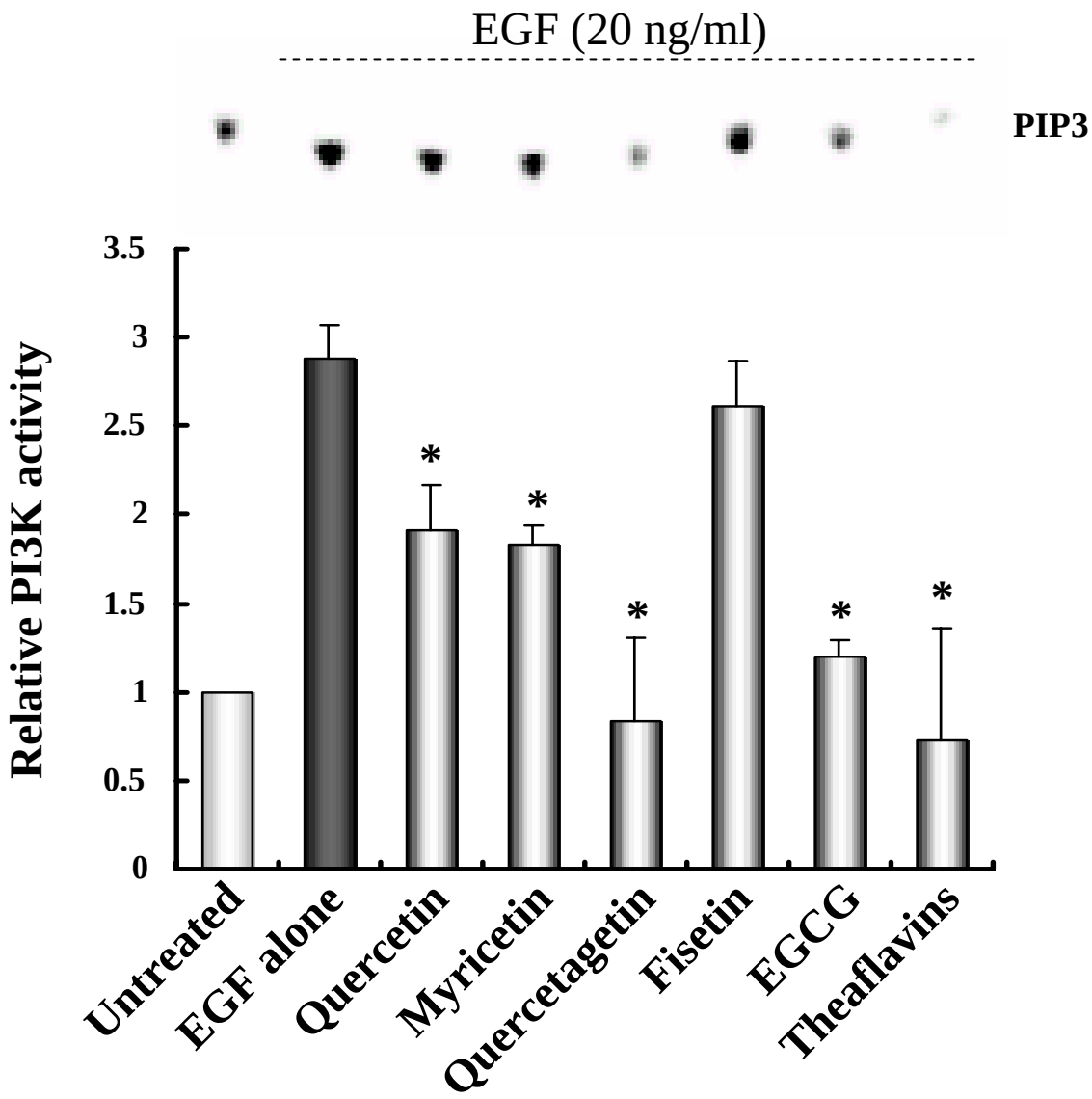

Fig. 9. Ichimatsu *et al.*

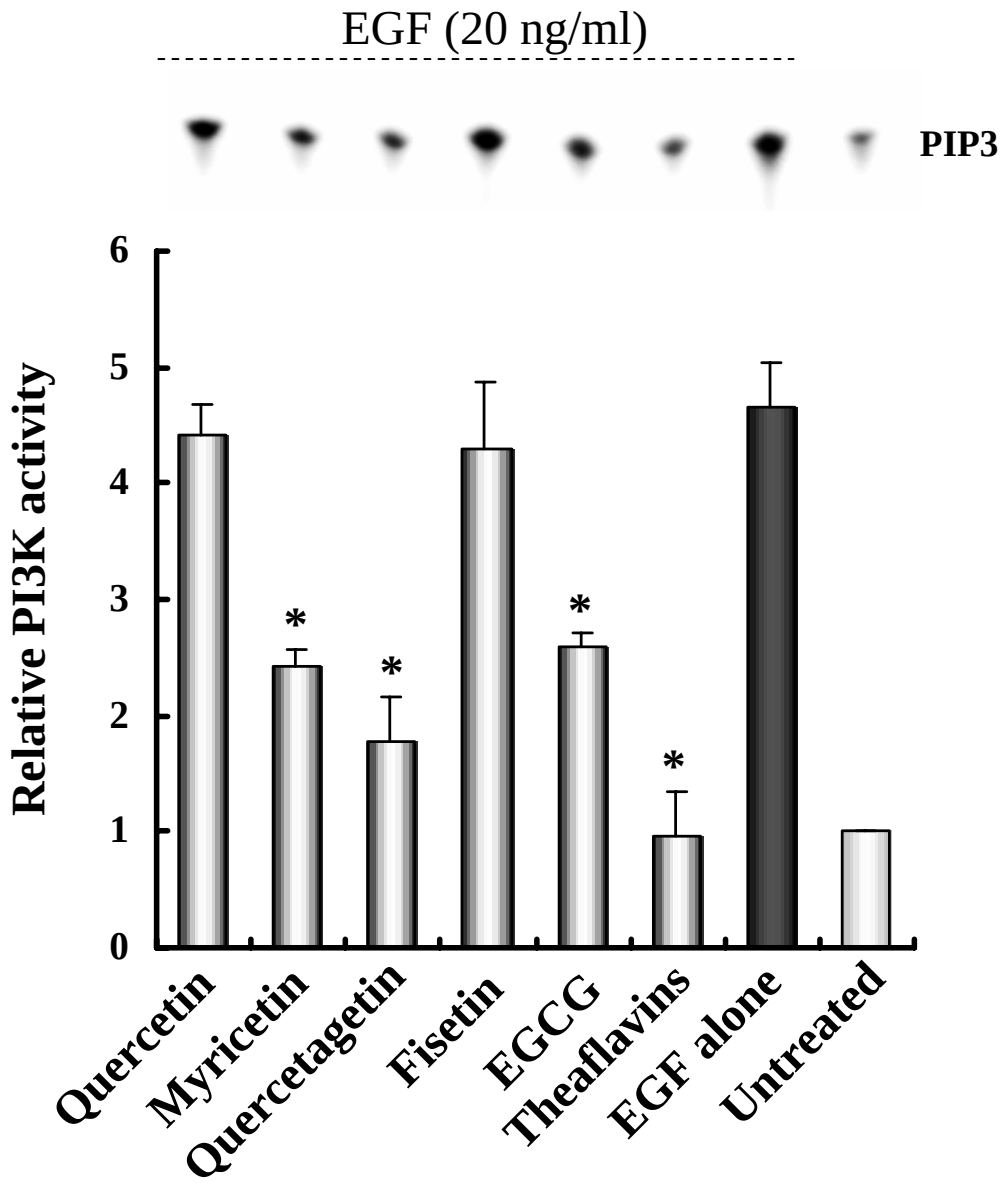

Fig. 10. Ichimatsu *et al.*