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Phytoestrogenic Compounds in Alfalfa Sprout (*Medicago sativa*) beyond Coumestrol

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Coumestrol has long been known as the phytoestrogenic compound in alfalfa. However, it has been demonstrated that the ethyl acetate extract of alfalfa sprout (AEA) attenuated the disease severity and increased survival and life span of autoimmune-prone MRL-*lpr*/*lpr* mice. Coumestrol, on the contrary, decreased the survival. This study thus aimed to isolate and identify phytoestrogenic compounds other than coumestrol in AEA. AEA was fractionated and separated by successive silica gel chromatography and preparative HPLC. The activity of collected fractions was tracked by a transactivation assay for ERα and ERβ, respectively. In addition to coumestrol, liquiritigenin, isoliquiritigenin, loliolide, and (4*S*,6*S*)- and (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one were isolated and chemically identified. Except for loliolide, these compounds showed higher transactivation via ERβ than via ERα. The maximal activation via ERα of coumestrol reached 80% that of 1 nM 17β-estradiol (E_2), whereas the activations of the remaining five compounds as well as AEA ranged from 8 to 49%. In addition, isoliquiritigenin, loliolide, and (4*S*,6*S*)- and (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one, but not coumestrol, preferentially inhibited 1 nM E_2 induced ERα activation, compared to that ERβ activation. The selectivity of these phytoestrogens might account for the difference between the effects of AEA and coumestrol in autoimmune-prone MRL-*lpr*/*lpr* mice observed previously.

KEYWORDS: Alfalfa sprout; phytoestrogen; selective estrogen receptor modulator; isoliquiritigenin; loliolide; 4-hydroxy-6-pentadecyltetrahydropyr-2-one

INTRODUCTION

Although phytoestrogen classically refers to compounds that show the physiological effects of estrogen, it has also been defined in a broad sense as chemicals that show effects suggestive of estrogenicity, such as binding to the estrogen receptor (ER), induction of specific estrogen-responsive gene products, and stimulation of ER-positive breast cancer cell growth (*1*). Isoflavones, coumestans, and lignans are the most well-known dietary phytoestrogens that bind to ER and have higher affinity for ERβ than for ERα (*2*). These are considered "natural" selective estrogen receptor modulators (SERMs) and may play protective roles in cancer, inflammation, heart disease (*3*,*4*), and osteoporosis (*5*,*6*). SERMs are further characterized by their tissue-specific action on ERs, thereby granting the possibility to selectively inhibit or stimulate estrogen-like action in various tissues.

Alfalfa (*Medicago sativa*) is a flowering plant in the pea family Fabaceae (or Leguminosae) cultivated as an important forage crop. Like other leguminous crops, it is a known source of phytoestrogens. Grazing on alfalfa has been suspected as a cause of reduced fertility in sheep. Coumestrol has long been known as the phytoestrogenic compound in alfalfa (*7*). Alfalfa sprout is a common food for humans and was found to be a major source of coumestrol in the diet (*8*, *9*).

We have previously shown that the ethyl acetate extract of alfalfa sprout (AEA) attenuated cytokine and inflammatory responses of self-reactive lymphocytes, decreased the disease severity, and increased the survival and life span of autoimmune-prone MRL-*lpr*/*lpr* mice. Coumestrol, on the contrary, decreased the survival at 19−23 weeks of age (*10*). The disease progress in MRL-*lpr*/*lpr* mice, an animal model of systemic lupus erythematosus, is known to be enhanced by estrogen and alleviated by tamoxifen, a tissue-specific estrogen antagonist. In a transactivation assay, AEA showed a preferential transactivation via ERβ, whereas coumestrol had a comparable transactivation via ERα and ERβ (*10*). These results suggested that SERMs other than coumestrol exist in AEA. This study thus aimed at the isolation and identification of ER modulators in AEA.

MATERIALS AND METHODS

Materials. Alfalfa sprout (*M. sativa* L.) samples were purchased from a local organic produce supplier (Goboul-Grange Ltd., Taipei, Taiwan).

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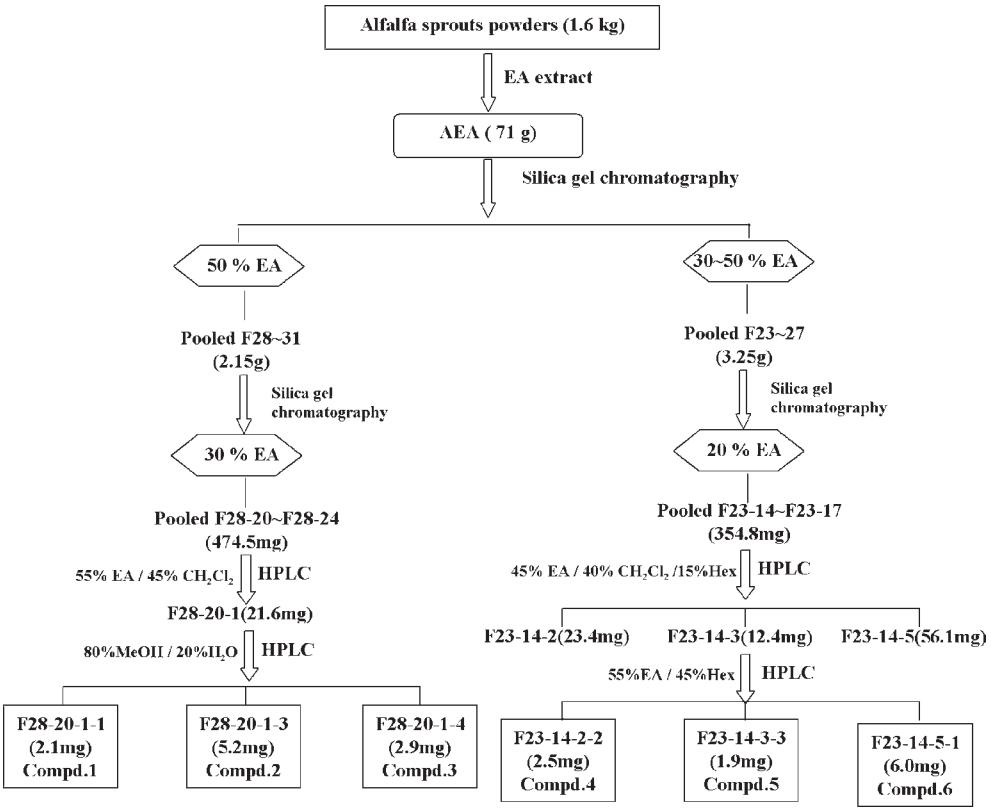

Figure 1. Scheme of separation and purification of alfalfa sprout ethyl acetate extracts: compound **1**, liquiritigenin; compound **2**, isoliquiritigenin; compound **3**, coumestrol; compound **4**, loliolide; compound **5**, (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one; compound **6**, (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one.

They were freeze-dried and ground to powder. The yield of the dried powder from fresh alfalfa sprout through this process was 6.5% (w/w).

Transactivation Assay for Estrogenic Activity. This assay measured the ligand-dependent transcription of a reporter gene in CHO-K1 cells (CCRC 60006) transiently cotransfected with vectors carrying estrogen receptor and reporter genes (*11*). The vectors we used respectively contained GAL4-hERα (or β) LBD (ligand binding domain) chimeric receptors and the (UAS)4-alkaline phosphatase reporter. Treatment of cotransfected cells with samples containing active compounds that can bind to ER LBD will then trigger the binding of GAL4 to the UAS sequence upstream of the reporter gene and activate the transcription of the reporter gene, that is, the secreted form of the human placental alkaline phosphatase (SEAP). By measuring the alkaline phosphatase activity, the estrogenic activity can then be determined. Cells treated with 1 nM 17β-estradiol (E_2) were included in each assay to serve as the positive control. Lipofectamine (GIBCO) has been used as the transfection reagent. For treating cotransfected cells, AEA and isolated compounds were dissolved in absolute ethanol and diluted with medium. Other details have been described previously (*11*). All of the samples were tested for ER transactivation in the concentration range that did not change the survival of CHO-K1 cells. To elucidate the anti-estrogen potential, samples were also assayed in the presence of 1 nM E_2.

Extraction. Alfalfa sprout dried powder (1.6 kg) was extracted with 20−30 volumes of ethyl acetate (EA) with agitation at room temperature for 16−18 h. The solution was filtered and EA removed in a rotary evaporator. AEA was obtained with a yield of 3.7%.

Fractionation, Isolation, and Identification. Seventy grams of AEA was separated by silica gel column chromatography eluted by increasing the proportion of EA in *n*-hexane. Fractions obtained were tested for estrogenic activity as described above. Two pooled fractions (F28 and F23) were respectively separated further. The isolated active compounds were identified by NMR and mass spectrometry to elucidate the chemical structures. ^{1}H and ^{13}C NMR spectroscopies were run on a Bruker Avance-500 MHz FT NMR, and ESI-MS and EI-MS data were obtained on a Finnigan TSQ 700 mass spectrometer. The separation procedure is summarized in **Figure 1**.

The first pooled fraction, F28, eluted by 50% EA/50% *n*-hexane, was separated by a second silica gel chromatography. The fraction eluted by 30% EA/70% *n*-hexane was separated with a preparative HPLC (Hitachi L-7110 pump; Phenomenex Luna Column 5 μm silica 10 mm × 250 mm and RID-10A Shimadzu refactive index detector), eluted with 55% EA/45% dichloromethane at a rate of 4 mL/min. The fraction collected at 9.2−10.4 min was further purified by a reverse phase HPLC, eluted with 80% methanol/20% H_2O at a rate of 4 mL/min. Pure compounds collected successively at 8.8−10.6, 12.5−15, and 15−20 min were chemically identified as liquiritigenin, isoliquiritigenin (*12*, *13*), and coumestrol (*14*), respectively.

The second pooled fraction, F23, eluted by 30% EA/70% *n*-hexane to 50% EA/50% *n*-hexane, was also separated by a second silica gel chromatography. Fractions eluted by 20% EA/80% *n*-hexane to 30% EA/70% *n*-hexane were further separated with a preparative HPLC, eluted with 45% EA/40% dichloromethane/15% *n*-hexane, at a rate of 4 mL/min. Three fractions collected at 18.8−20.6, 19.5−21.5, and 26.1−30.5 min were respectively further purified by a second HPLC, eluted with 55% EA/45% *n*-hexane, at a rate of 4 mL/min. From the first fraction, a pure compound collected at 23.6−25.4 min. was chemically identified as loliolide (*15*). From the second fraction, the fraction collected at 27.5−30 min was chemically identified as (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one (*16*). From the third fraction, the fraction collected at 23−25 min was identified as (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one (*16*, *17*).

Data Analysis. Data reported were expressed as mean ± standard deviation of at least three separate experiments with triplicate wells in each. The significance of difference between each treatment was analyzed by one-way ANOVA (analysis of variance) or Student's *t* test using SAS (SAS 8.1, Cary, NC) software. Whereas data showed the heterogeneity of variances, the statistical analysis was preceded by transforming data to log or root square to eliminate the heterogeneity.

RESULTS

Estrogenic Activity of AEA. AEA dose-dependently transactivated both ERα and ERβ (**Figure 2A**). The maximal activation via ERα was about 40% that of 1 nM E_2, whereas that via ERβ

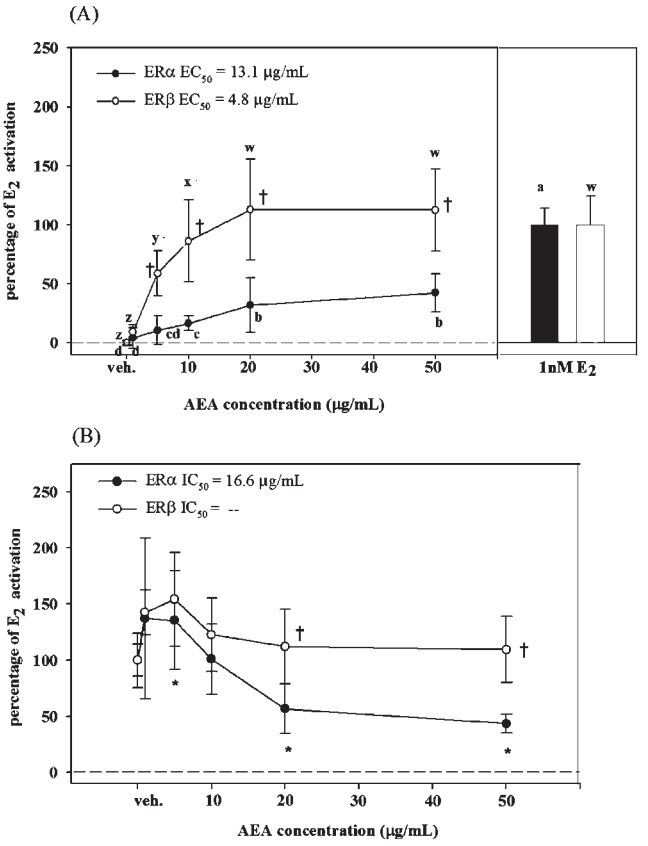

Figure 2. Transactivation of ERα and ERβ by AEA in the absence (**A**) or presence (**B**) of 1 nM 17β-estradiol (E_2). The transactivation folds of 1 nM E_2 alone were taken as 100%. The black and white bars shown in the side panel of (**A**) denote the transactivation of ERα and ERβ, respectively, by 1 nM E_2. Values are means ± SD from three separate experiments with triplicate wells in each. In panel **A**, values with different alphabetical letters are significantly different ($P < 0.05$) analyzed by one-way ANOVA and Duncan's multiple-range test. In panel **B**, a significant difference from the vehicle was indicated as ∗, $P < 0.05$, by Student's *t* test. † denotes significant difference from ERα ($P < 0.05$) by Student's *t* test.

was ~120% that of 1 nM E_2. The EC_{50} values were 13.1 and 4.8 μg/mL for ERα and ERβ, respectively. These results confirm that AEA has higher selectivity on ERβ. In the presence of 1 nM E_2, a low concentration of AEA showed an additive effect, whereas a high concentration of AEA significantly reduced the transactivation of 1 nM E_2 via ERα. Interestingly, AEA did not change the transactivation of 1 nM E_2 via ERβ in a similar concentration range (**Figure 2B**). Again, AEA showed a different effect between ERα and ERβ.

Chemical Identification of the Six Isolated Compounds. Compounds **1**−**6** are all known compounds. The purity of compounds **1**−**4** was >98%. On the basis of ^{1}H and ^{13}C NMR spetra as well as ESI-MS and EI-MS data, the six isolated compounds were identified as liquiritigenin (*12*, *13*), isoliquiritigenin (*12*, *13*), coumestrol (*14*), loliolide (*15*), (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one (*16*), and (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one (*16*, *17*). The NMR spectroscopic data are as follows.

Liquiritigenin: $C_{15}H_{12}O_4$, ^{1}H NMR (400 MHz, acetone-d_6), δ 7.71 (1H, d, J = 8.6 Hz, H-5), 7.39 (2H, d, J = 8.7 Hz, H-2′, H-6′), 6.88 (2H, d, J = 8.7 Hz, H-3′, H-5′), 6.56 (1H, dd, J = 8.6, 2.3, H-6), 6.40 (1H, d, J = 2.3 Hz, H-8), 5.43 (1H, dd, J = 13.0, 2.9 Hz, H-2), 3.03 (1H, dd, J = 16.7, 13.0 Hz, H-3), 2.65 (1H, dd, J = 16.7, 2.9 Hz, H-3). The ^{1}H NMR data were consistent with those reported previously (*12*, *13*).

Isoliquiritigenin: $C_{15}H_{12}O_4$, ^{1}H NMR (500 MHz, acetone-d_6), δ 13.62 (OH), 8.12 (1H, d, J = 8.5 Hz, H-6′), 7.74 (2H, d, J = 8.4 Hz, H-2, H-6), 7.84 (1H, d, J = 15.0 Hz, H-β), 7.76 (1H, d, J = 15.0 Hz, H-α), 6.93 (2H, d, J = 8.4 Hz, H-3, H-5), 6.47 (1H, dd, J = 8.5, 2.5 Hz, H-5′), 6.37 (1H, d, J = 2.5 Hz, H-3′); ^{13}C NMR (125 MHz, acetone-d_6), δ 192.9 (C═O), 167.7 (C-4′), 165.5 (C-2′), 161.0 (C-4), 145.2 (C-β), 133.4 (C-6′), 131.9 (C-2, C-6), 127.7 (C-1), 118.4 (C-α), 116.8 (C-3, C-5), 114.6 (C-1′), 108.8 (C-5′), 103.8 (C-3′). These NMR spectroscopic data were consistent with those reported previously (*12*, *13*).

Coumestrol: $C_{15}H_8O_5$, ^{1}H NMR (400 MHz, acetone-d_6), δ 7.88 (1H, d, J = 8.5 Hz, H-1), 7.78 (1H, d, J = 8.6 Hz, H-7), 7.12 (1H, d, J = 1.9, H-10), 6.99−7.04 (H2, m, H-2, H-8), 6.94 (1H, d, J = 2.2 Hz, H-4). The ^{1}H NMR data were consistent with those reported previously (*14*).

Loliolide: $C_{11}H_{16}O_3$, ^{1}H NMR (400 MHz, $CDCl_3$), δ 5.67 (1H, s, H-7), 4.31 (1H, m, H-3), 2.43 (1H, ddd, J = 14.1, 2.6, 2.6 Hz, H-4β), 1.95 (1H, ddd, J = 14.5, 2.7, 2.7 Hz, H-2β), 1.76 (3H, s, 5-CH_3), 1.76 (1H, dd, J = 14.1, 4.0 Hz, H-4α), 1.51 (1H, dd, J = 14.5, 3.7 Hz, H-2α), 1.45 (3H, s, 1α-CH_3), 1.27 (3H, s, 1β-CH_3). The ^{1}H NMR data were consistent with those reported previously (*15*).

(*4S,6S*)-*4-Hydroxy-6-pentadecyltetrahydropyr-2-one:* $C_{20}H_{38}O_3$, ^{1}H NMR (500 MHz, $CDCl_3$), δ 4.65−4.70 (1H, m, H-5), 4.38−4.41 (1H, m, H-3), 2.74 (1H, dd, J = 17.6, 5.1 Hz, H_{ax}-2), 2.61 (1H, ddd, J = 17.6, 3.6, 1.4 Hz, H_{eq}-2), 1.93 (1H, br d, J = 14.7 Hz, H_a-4), 1.68−1.77 (2H, m, H_b-4, H_a-6), 1.52−1.62(1H, m, H_b-6), 1.38−1.48 (2H, m, H-7 (CH_2)), 1.26 (series of m, H-8 to H-19 (CH_2)), 0.88 (3H, t, J = 6.8 Hz, H-20 (CH_3)); ^{13}C NMR (125 MHz, $CDCl_3$), δ 170.2 (C-1), 75.7 (C-5), 62.9 (C-3), 38.7 (C-2), 36.1 (C-4), 35.5 (C-6), 31.9 (C-18), 29.4−29.7 (C-8−C-17), 24.9 (C-7), 22.7 (C-19), 14.2 (C-20). The NMR spectroscopic data were consistent with those reported previously (*16*).

(*4R,6S*)-*4-Hydroxy-6-pentadecyltetrahydropyr-2-one:* $C_{20}H_{38}O_3$, ^{1}H NMR (500 MHz, $CDCl_3$), δ 4.06 (2H, m, H-3, H-5), 2.54 (1H, dt, J = 16.1, 3.0 Hz, H_{eq}-2), 2.43 (1H, dd, J = 16.1, 9.3 Hz, H_{ax}-2), 1.52 (1H, m, H_a-4), 1.42 (1H, m, H_b-4), 1.38−1.46 (5H, m, H_b-4, H-6 (CH_2), H-7 (CH_2)), 1.27 (series of m, H-8 to H-19 (CH_2)), 0.85 (3H, t, J = 7.2 Hz, H-20 (CH_3)); ^{13}C NMR (125 MHz, $CDCl_3$), δ 171.1 (C-1), 68.1 (C-5), 65.1 (C-3), 41.5 (C-2), 36.7 (C-6), 31.9 (C-18), 29.4−29.7 (C-8−C-17), 25.5 (C-4), 22.7 (C-7), 20.8 (C-19), 14.1 (C-20). The NMR spectroscopic data were consistent with those reported previously (*16*, *17*).

Transactivation of Estrogen Receptors by Isolated Compounds Alone. The dose-dependent transactivation as well as EC_{50} values via ERα and ERβ of the six isolated compounds are shown in **Figure 3**. Except for loliolide, these isolates transactivated ERβ to a higher extent than ERα at most concentrations tested. Moreover, the estrogenic activities of liquiritigenin (**Figure 3A**), isoliquiritigenin (**Figure 3B**), and coumestrol (**Figure 3C**) isolated from fraction F28 were relatively higher than the remaining three compounds isolated from F23. This can be seen from the maximal activation as well as the estimated EC_{50} values (Figure**3**).

The maximal activations on ERα of liquiritigenin, isoliquiritigenin, and coumestrol were, respectively, 49, 44, and 78% that of 1 nM E_2. Those on ERβ were 89, 110, and 104%, respectively. On the basis of the dose−response curve and the maximal activation, the selectivity on ERβ of liquiritigenin and isoliquiritigenin is considered superior to that of coumestrol, despite the ratio of EC_{50} ERβ/EC_{50} ERα being lowest for coumestrol among the six. On the other hand, the estrogenic activity of coumestrol is more potent than those of the remaining five isolates based on the estimated EC_{50} values. The estimated EC_{50} of coumestrol is in the

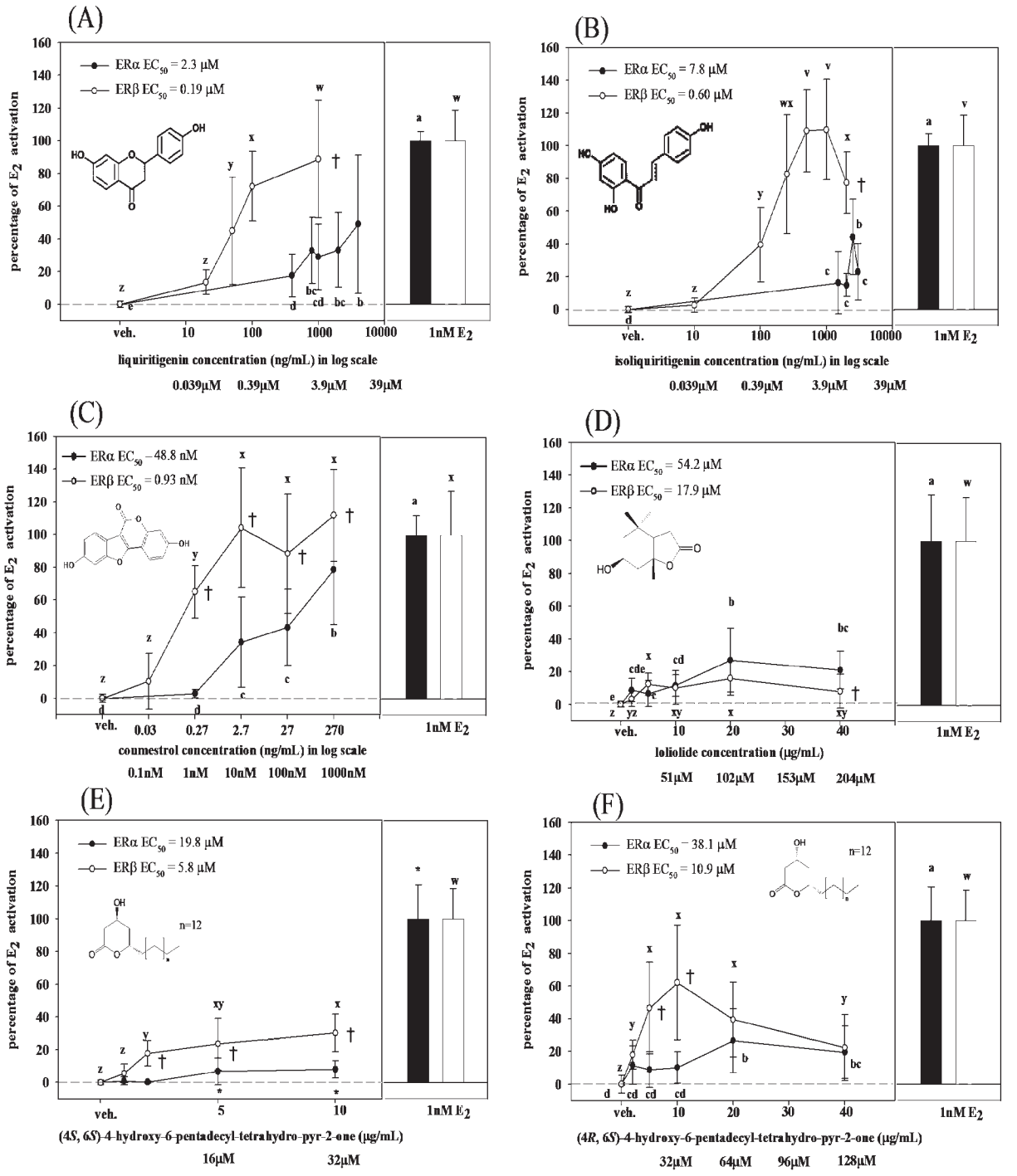

Figure 3. Transactivation of ERα and ERβ by the six compounds isolated from AEA: (**A**) liquiritigenin; (**B**) isoliquiritigenin; (**C**) coumestrol; (**D**) loliolide; (**E**) (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one, and (**F**) (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one. 17β-Estradiol (E_2, 1 nM) serves as the positive control, and its transactivation folds were taken as 100% (black and white bars shown in the side panels denote the transactivation of ERα and ERβ, respectively, by 1 nM E_2). The maximal activations (% of 1 nM E_2) are (**A**) 49%, (**B**) 44%, (**C**) 78%, (**D**) 26%, (**E**) 8%, and (**F**) 27% for ERα and (**A**) 89%, (**B**) 110%, (**C**) 104%, (**D**) 16%, (**E**) 30%, and (**F**) 63% for ERβ. Values are means ± SD from three separate experiments with triplicate wells in each. Values with different alphabetical letters are significantly different ($P < 0.05$) analyzed by one-way ANOVA and Duncan's multiple-range test. ∗ indicates significant difference from the vehicle ($P < 0.05$) by Student's *t* test. † denotes significant difference from ERα ($P < 0.05$) by Student's *t* test.

nanomolar range, whereas that of the remaining five is in the micromolar range.

Loliolide, (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one, and (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one transactivated ERα and ERβ to much lower extents. Their maximal activations for ERα were 26.4, 8, and 26.5% that of 1 nM E_2, respectively. Their maximal activations for ERβ were 16.0, 30.2, and 63% that of 1 nM E_2, respectively. Loliolide, thus, did not show selectivity upon ERβ and (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one tranactivated ERα barely.

The ratios of EC_{50} values for ERβ/ERα of AEA, liquiritigenin, isoliquiritigenin, coumestrol, (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one, and (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-3-one are, respectively, 0.37, 0.08, 0.08, 0.02, 0.29, and 0.29. The ratios of maximal activation for ERβ/ERα of AEA and these compounds are, respectively, 2.68, 1.81, 2.49, 1.33, 3.78, and

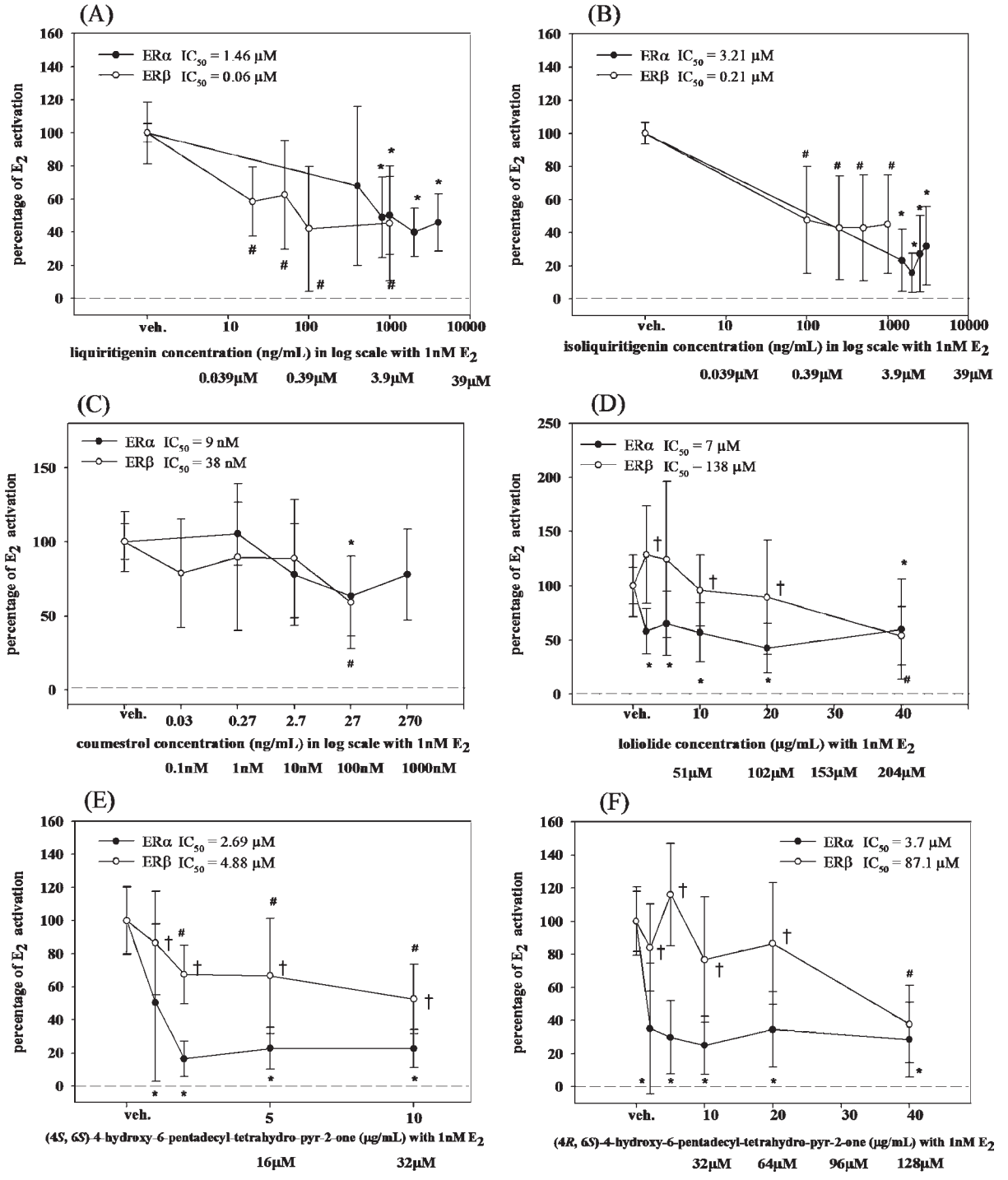

Figure 4. Transactivation of ERα and ERβ in the presence of 1 nM 17β-estradiol by the six compounds isolated from AEA: (**A**) liquiritigenin; (**B**) isoliquiritigenin; (**C**) coumestrol; (**D**) loliolide; (**E**) (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one; (**F**) (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one. The transactivation folds of 1 nM E_2 alone were taken as 100%. The maximal inhibitions (%) are (**A**) 60%, (**B**) 84%, (**C**) 37%, (**D**) 58%, (**E**) 83%, and (**F**) 75% for ERα and (**A**) 58%, (**B**) 57%, (**C**) 41%, (**D**) 46%, (**E**) 47%, and (**F**) 62% for ERβ. Values are means ± SD from three separate experiments with triplicate wells in each. The value significantly differ from the respective vehicle indicated as ∗ or # ($P < 0.05$) by Student's *t* test. † denotes significant difference from ERα ($P < 0.05$) by Student's *t* test.

1.94. These data further showed the selectivity of these compounds for ERβ. Although loliolide also had a low ratio of EC_{50} values for ERβ/ERα (0.33), its ratio of maximal activation for ERβ/ERα is also low (0.59), indicating again loliolide is not selective for ERβ transactivation.

Transactivation of Estrogen Receptors by Isolated Compounds in the Presence of 1 nM E_2. The effects of various concentrations of isolated compounds on the transactivation via ERs by 1 nM E_2 are shown in **Figure 4**. Except for coumestrol, the remaining five isolates significantly reduced the transactivation of 1 nM E_2 via ERα at most concentrations tested. Liquiritigenin and isoliquiritigenin also inhibited the transactivation of ERβ by 1 nM E_2 at all concentrations tested. The inhibitions of coumestrol on ERα and ERβ transactivation by 1 nM E_2 was significant only at 100 nM. Loliolide and (4*R*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one did not inhibit the transactivation of ERβ by 1 nM

E_2 except at the highest concentrations tested. (4*S*,6*S*)-4-Hydroxy-6-pentadecyltetrahydropyr-2-one also inhibited the transactivation of ERβ by 1 nM E_2 except at the lowest concentrations tested.

DISCUSSION

In agreement with our speculation that phytoestrogeinc compounds other than coumestrol might exist in the AEA, an additional five phytoestrogenic compounds were identified from the AEA. Among these, liquiritigenin and isoliquiritigenin have been reported for their estrogenic activity, whereas the remaining three have not. Liquiritigenin and isoliquiritigenin are known to exist in the root of licorice (Glycyrrhizae). They belong to the chalcone class of compounds and are intermediate metabolites in the biosynthetic pathway of isoflavone and coumestan in Leguminosae plants (*18*). Estrogenic activities of both compounds have been demonstrated.

Liquiritigenin has been shown to bind to rat uterine ER (*19*). Mersereau et al. (*20*) demonstrated that liquiritigenin binds to both ERα and ERβ, but only activated ER target genes through ERβ. The competition binding curves showed that ERβ has only a 20-fold greater affinity for liquiritigenin compared to ERα, which is not likely sufficient to explain the differences in transcriptional regulation (*20*). They also showed in a mouse xenograph model that liquiritigenin did not stimulate uterine size or tumorigenesis of MCF-7 breast cancer cells. By a bioluminescence resonance energy transfer (BRET) assay to monitor the formation of ERα/β heterodimers and their respective homodimers in live cells, liquiritigenin was found to induce ERβ dimerization preferentially over ERα, indicating that the lack of ERα transcriptional activation by liquiritigenin is caused by impaired dimerization (*21*). Our data agree with liquiritigenin being an ERβ-selective agonist (**Figure 3A**).

Isoliquiritigenin has also been shown to bind and activate ERs (*22*, *23*). Like liquiritigenin, isoliquiritigenin binds to ERα and ERβ similarly (*23*). However, ERβ selectivity in the transactivation assay has not been reported before (*22*). Our data clearly show that isoliquiritigenin is more ERβ-selective than liquiritigenin and coumestrol (**Figure 3A**,**B**). Isoliquiritigenin has also been shown to inhibit aromatase activity and is a potential chemopreventive agent against breast cancer (*24*). It would be of great interest to further examine whether isoliquiritigenin also activates ER target genes through ERβ only and induces ERβ dimerization preferentially.

In contrast to liquiritigenin and isoliquiritigenin, coumestrol binds to ERβ with a much higher affinity than to ERα. The IC_{50} in the competitive binding assay of ERα has been shown to be ~50-fold that of ERβ (*23*). In this study, the EC_{50} values of coumestrol in the transactivation assay to ERα was also ~50-fold that of ERβ (**Figure 3C**). This suggests that the ERβ selectivity of coumestrol is attributed to the different binding affinity, whereas that of liquiritigenin is related to the specific induction of ERβ homodimer formation.

Although loliolide, (4*R*,6*S*)-4-hydoxy-6-pentadecyltetrahydropyr-2-one, and (4*S*,6*S*)-4-hydoxy-6-pentadecyltetrahydropyr-2-one transactivate ERs to a much lower extent than liquiritigenin, isoliquiritigenin, and coumestrol, their estrogenic activity has not been reported previously. Interestingly, (4*R*,6*S*)-4-hydoxy-6-pentadecyltetrahydropyr-2-one showed higher transactivation activity than its (4*S*,6*S*) form isomer. This implied that the *R* position of the 4-hydroxy group might be preferred for a favorable interaction with a certain region in the ER structure.

Loliolide has been identified from many species of terrestrial plant as well as algae (*25*), whereas (4*R*,6*S*)- and (4*S*,6*S*)-4-hydoxy-6-pentadecyltetraydropyr-2-one have been reported in *Eupatorium betonicaeforme* and *Hymenoxyo odorata* (*16*, *26*). Loliolide is considered to be a degradation product of chlorophyll (*25*). 4-Hydroxy-6-pentadecyltetrahydropyr-2-one might derive from the polyketide biosynthesis product in plant. Abe et al. (*27*) have shown that a recombinant octaketide synthase cloned from aloe catalyzed the production of 4-hydroxy-6-alkylpyr-2-one from fatty acyl CoA of various chain lengths and malonyl CoA. The 4-hydroxy-6-pentadecyltetrahydropyr-2-one identified in this study thus might be derived from these products through hydrogenation. Gao et al. (*26*) also considered that it is probably derived from a fatty acid intermediate.

Results of the transactivation assay carried out in the presence of 1 nM E_2 indicated that liquiritigenin and isoliquiritigenin exhibited substantial inhibition on E_2's activity upon both ERs in the concentration range that showed ER activity, but not coumestrol. Furthermore, loliolide and (4*R*,6*S*)- and (4*S*,6*S*)-4-hydrpxy-6-pentadecyltetrahydropyr-2-one also showed substantial inhibition on the transactivation of 1 nM E_2 on ERα. Interestingly, the inhibition of loliolide and (4*R*,6*S*)- and (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one on the transactivation of ERα by 1 nM E_2 was greater than those of ERβ. On the basis of these results, liquiritigenin, isoliquiritigenin, loliolide, and (4*R*,6*S*)- and (4*S*,6*S*)-4-hydrpxy-6-pentadecyltetrahydropyr-2-one might contribute, at least in part, to the different effects between AEA and coumestrol on the survival of autoimmune-prone MRL-*lpr*/*lpr* mice in our previous study (*10*), because a tissue-specific ER antagonist, such as tamoxifen, has been shown to extend the survival of autoimmune-prone MRL-*lpr*/*lpr* mice (*28*). The contribution of these five isolated compounds to the difference between coumestrol and AEA is further supported by our data of the maximal activation on ERα. The maximal activations of liquiritigenin (49%), isoliquiritigenin (44%), loliolide (26%), (4*R*,6*S*)- and (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one (26.5 and 8%) were within a comparable range to or lower than that of AEA (42%), whereas that of coumestrol (78%) apparently exceeded that of AEA (**Figures 2** and **3**).

In conclusion, estrogenic compounds other than coumestrol were isolated and identified from alfalfa sprout as liquiritigenin, isoliquiritigenin, loliolide, and (4*R*,6*S*)- and (4*S*,6*S*)-4-hydroxy-6-pentadecyltetrahydropyr-2-one. They also showed preferential inhibition on the ERα transactivation by 1 nM E_2. Alfalfa sprout might thus be a dietary source of SERM.

ABBREVIATIONS USED

AEA, ethyl acetate extract of alfalfa sprout; E_2, 17β-estradiol; ERα, human estrogen receptor α, ERβ, human estrogen receptor β; SERM, selective estrogen receptor modulator.

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