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## Estrogenic Flavonoids from *Artemisia vulgaris* L.

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The comprehensive quantitative analysis of the flavonoid chemistry of *Artemisia vulgaris* L., a plant used as an emmenagogue in traditional medicine, is presented in conjunction with an evaluation of its estrogenic activity. Twenty known flavonoids were isolated and identified as tricine, jaceosidine, eupafolin, chrysoeriol, diosmetin, homoeriodictyol, isorhamnetin, apigenin, eriodictyol, luteolin, luteolin 7-glucoside, kaempferol 3-glucoside, kaempferol 7-glucoside, kaempferol 3-rhamnoside, kaempferol 3-rutinoside, quercetin 3-glucoside, quercetin 3-galactoside, quercetrin, rutin, and vitexin. The most abundant compounds were eriodictyol and luteolin. The estrogenic activity of all flavonoids was assayed by employing a reconstituted estrogen transcription unit in *Saccharomyces cerevisiae* transformed with both a human estrogen receptor expression plasmid and a reporter plasmid. Two flavonoids, eriodictyol and apigenin, were able to induce the transcription of the reporter gene in transgenic yeast. The transcriptional activity increased proportionally with increased amounts of purified eriodictyol or apigenin added to the yeast cells.

**Keywords:** *Artemisia vulgaris*; estrogenic activity; estrogen transcriptional system in yeast; flavonoids; apigenin; eriodictyol; luteolin

### INTRODUCTION

Mugwort, *Artemisia vulgaris* L. (Asteraceae: Anthemideae), is a perennial weed growing wild and abundantly in temperate and cold-temperature zones of the world (Fernald, 1950). *A. vulgaris* has been known not only as an edible plant but also as a folk medicine resource. Mugwort is used to flavor tea and rice dishes in Asia and as a culinary herb for poultry and pork in Western cultures (Tanaka, 1976). In Oriental medicine, mugwort has been employed as an analgesic agent and in conjunction with acupuncture therapy (Yoshikawa et al., 1996). *A. vulgaris* has been implemented in the treatment of painful menstruation (dysmenorrhoea) and in the induction of labor or miscarriage (Millspaugh, 1982; Albert-Puleo, 1978; Saha et al., 1961). Considered an emmenagogue, an inducing agent of menstrual flow, mugwort has been traditionally employed to bring about regular menses in cases of amenorrhea or menorrhagia (Duke, 1985; Puri, 1971; Malpass, 1945).

The emmenagogic properties of this plant may be related to estrogen agonists known as phytoestrogens, a group that includes coumestan, isoflavones, and lignans in other plants (Knight and Eden, 1996). Phytoestrogens demonstrate lower affinity and activity than endogenous estrogens and are considered weak agonists. Indeed, synthetic weak estrogens have been clinically applied as anti-estrogens for the induction of ovulation

in the treatment of amenorrhea or menorrhagia (Brotherton, 1976).

Recent studies regarding the estrogenic activity of some plant compounds indicate that many hydroxylated flavonoids possess activity greater than predicted (Miksic, 1993). A wide variety of flavonoids show estrogen-type activity as well as antiallergic, anti-inflammatory, antiviral, and antimicrobial activities (Knight and Eden, 1996; Cody et al., 1986, 1988; Das, 1989). Moreover, flavonoids in diets may have a role in reducing the risk of cancer, and some flavonoids are well-known to possess antioxidant and radical-scavenging properties (Knight and Eden, 1996; Miyake and Shibamoto, 1997; Bors and Saran, 1987).

We describe here for the first time the comprehensive flavonoid chemistry of *A. vulgaris*, including the purification and characterization of 20 known flavonoids. Some flavonoids as well as acetylenes, coumarins, sesquiterpene lactones, and volatile oil components have previously been reported from *A. vulgaris* (Marco and Barbara, 1990). Stefanovic et al. (1982) reported the methoxylated flavone ayanin from *A. vulgaris*, and 3-*O*-glucosides and rutinosides of kaempferol, quercetin, and isorhamnetin were found by Hoffmann and Herrmann (1982).

We also tested for the first time the estrogenic activity of flavonoids from *A. vulgaris* by using the reconstituted transcription unit in *Saccharomyces cerevisiae* cotransformed with an expression plasmid containing the human estrogen receptor cDNA and a reporter plasmid containing the  $\beta$ -galactosidase (*lacZ*) gene (Santiso-Mere et al., 1991). Two compounds, eriodictyol and apigenin, were able to activate the transcription of the reporter gene in transgenic yeast at a low level compared to estradiol. The presence of these weak estrogens in

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*A. vulgaris* may account for the use of this plant as a natural emmenagogue.

## MATERIALS AND METHODS

**Plant Material.** Whole *A. vulgaris* plants were collected near Samchunpo (KyoungSang Nam Do, Korea) in April 1996. The plant material was dried at 40 °C, pulverized, and stored dry in the dark below 25 °C.

**Chemicals.** General reagents, as well as adenine sulfate, 17 $\beta$ -estradiol, and *o*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) were purchased from Sigma Chemical Co. (St. Louis, MO). Casamino acids and yeast nitrogen base without amino acids were purchased from Difco-BLR (Bethesda, MD).

**General Techniques.** Column chromatography employed Polyclar AT (GAF Corp.) and Sephadex LH-20 (Pharmacia). Precoated cellulose plates (E. Merck), polyamide (Macherey-Nagel), and Si gel 60 GF-254 (E. Merck) were used for TLC. The solvent systems were as follows: TBA (*t*-BuOH/HOAc/H<sub>2</sub>O, 3:1:1); *n*-BAW, upper layer (*n*-BuOH/HOAc/H<sub>2</sub>O, 4:1:5); BPA (C<sub>6</sub>H<sub>6</sub>/pyridine/HCO<sub>2</sub>H, 36:9:5), and CAF (chloroform/acetic acid/formic acid, 9:2:1). All flavonoids were purified over Sephadex LH-20 using MeOH prior to spectral analyses according to standard procedures. Visualization of the flavonoids on TLC plates was realized either by UV light plus NH<sub>3</sub> or by spraying with [Naturstoffreagenz A (NA) in MeOH].

**Isolation and Identification of Flavonoids.** Careful use of standard procedures allowed isolation of 20 known flavonoids, albeit some were obtained in only trace amounts (Mabry et al., 1970; Fang et al., 1985, 1986). Whole *A. vulgaris* plants (1 kg) were air-dried, finely ground, and extracted with 80% aqueous EtOH (2 L, 5 days). The extracts were concentrated in vacuo to 1 L, and the concentrate was partitioned between *n*-hexane (3  $\times$  1 L) and H<sub>2</sub>O. The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (4  $\times$  1 L) and ethyl acetate (5  $\times$  1 L), successively, and the extracts obtained with each solvent were combined and evaporated to dryness.

The remaining aqueous extract contained only traces of flavonoids (TLC analysis) and was discarded. Dry residues from the hexane, CH<sub>2</sub>Cl<sub>2</sub>, and EtOAc extracts were 3.5, 4, and 5 g, respectively. The hexane and CH<sub>2</sub>Cl<sub>2</sub> extracts were examined by 2D chromatography on 3MM Whatman paper. Phenolic compounds were detected in the CH<sub>2</sub>Cl<sub>2</sub> extract but none in the hexane extract.

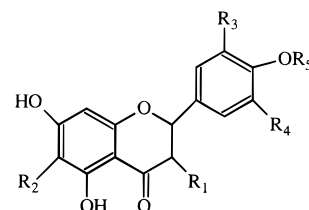
The CH<sub>2</sub>Cl<sub>2</sub> extract was chromatographed on a silica gel column eluted with hexane/CH<sub>2</sub>Cl<sub>2</sub> mixtures containing increasing amounts of EtOAc; the main fractions (A–G) were collected, and all fractions were examined using 2D paper chromatography for phenolics. Fraction G was also rechromatographed on Sephadex LH-20 and yielded only traces of phenolics.

The EtOAc extract was chromatographed on a silica gel column eluted with a CH<sub>2</sub>Cl<sub>2</sub>/EtOAc mixture containing increasing amounts of EtOAc up to 100%. All fractions (A–H) showed some flavonoids on TLC analysis. Most flavonoid aglycons were detected from fractions A–D, and each fraction was rechromatographed on a silica gel column followed by a Sephadex LH-20 column using MeOH as solvent; fractions D, G, and H yielded flavonoid glycosides, which were further purified on Sephadex LH-20.

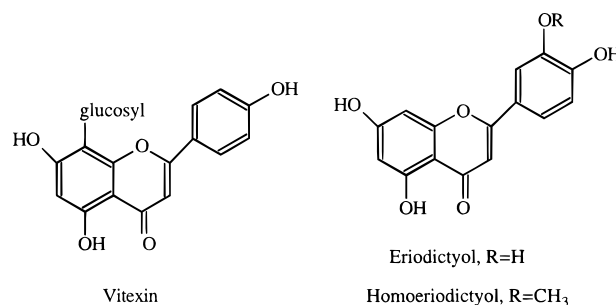
Fraction A afforded 6-methoxylated flavonoids as well as eriodictyol; fractions B and C contained luteolin and apigenin. Fraction D yielded luteolin 7-glucoside and luteolin, whereas most flavonoid glycosides came from fraction G. Fraction H contained kaempferol 3-rutinoside, rutin, and vitexin.

All purified compounds were identified by UV in MeOH with diagnostic reagents as well as <sup>1</sup>H NMR and mass spectroscopy. The <sup>1</sup>H NMR and chromatographic data for the methylated flavonoids tricrin, jaceosidine, eupafolin, homoeriodictyol, diosmetin, chrysoeriol, and isorhamnetin were identical with published data (Mabry, 1970) (structures are given in Figure 1). MS data for tricrin, jaceosidine, and eupafolin are as follows.

Tricrin: EIMS, *m/z* (rel intensity) 330 (100) [M]<sup>+</sup>, 329 (12) [M – H]<sup>+</sup>, 315 (70%) [M – 15]<sup>+</sup>, 30 (5) [M – 29]<sup>+</sup>, 287 [M – 43]<sup>+</sup>, 181, 178, 153, 151 [B<sub>2</sub>]<sup>+</sup>.



Tricrin, R<sub>1</sub>–R<sub>2</sub>=R<sub>5</sub>=H, R<sub>3</sub>=R<sub>4</sub>=OCH<sub>3</sub>  
 Jaceosidine, R<sub>1</sub>=R<sub>4</sub>=R<sub>5</sub>=H, R<sub>2</sub>=R<sub>3</sub>=OCH<sub>3</sub>  
 Eupafolin, R<sub>1</sub>=R<sub>4</sub>=R<sub>5</sub>=H, R<sub>2</sub>=OCH<sub>3</sub>, R<sub>3</sub>=OH  
 Diosmetin, R<sub>1</sub>=R<sub>2</sub>=R<sub>4</sub>=H, R<sub>3</sub>=OH, R<sub>5</sub>=CH<sub>3</sub>  
 Chrysoeriol, R<sub>1</sub>=R<sub>2</sub>=R<sub>4</sub>=R<sub>5</sub>=H, R<sub>3</sub>=OCH<sub>3</sub>  
 Isorhamnetin, R<sub>1</sub>=OH, R<sub>2</sub>=R<sub>4</sub>=R<sub>5</sub>=H, R<sub>3</sub>=OCH<sub>3</sub>  
 Apigenin, R<sub>1</sub>=R<sub>2</sub>=R<sub>3</sub>=R<sub>4</sub>=R<sub>5</sub>=H  
 Kaempferol, R<sub>1</sub>=OH, R<sub>2</sub>=R<sub>3</sub>=R<sub>4</sub>=R<sub>5</sub>=H  
 Luteolin, R<sub>1</sub>=R<sub>2</sub>=R<sub>4</sub>=R<sub>5</sub>=H, R<sub>3</sub>=OH  
 Rutin, R<sub>1</sub>=O-rhamno-glucosyl, R<sub>2</sub>=R<sub>4</sub>=R<sub>5</sub>=H, R<sub>3</sub>=OH



**Figure 1.** Structures of main flavonoids from *A. vulgaris*.

Jaceosidine: EIMS, *m/z* (rel intensity) 330 (100) [M]<sup>+</sup>, 329 (8) [M – H]<sup>+</sup>, 315 (63) [M – Me]<sup>+</sup>, 312 (53) [M – 18]<sup>+</sup>, 301 (8) [M – HCO]<sup>+</sup>, 167 (19) [A<sub>1</sub> – Me]<sup>+</sup>, 139 (24) [A<sub>1</sub> – MeCO]<sup>+</sup>, 148 (8) [B<sub>1</sub>]<sup>+</sup>, 151 (8) [B<sub>2</sub>]<sup>+</sup>.

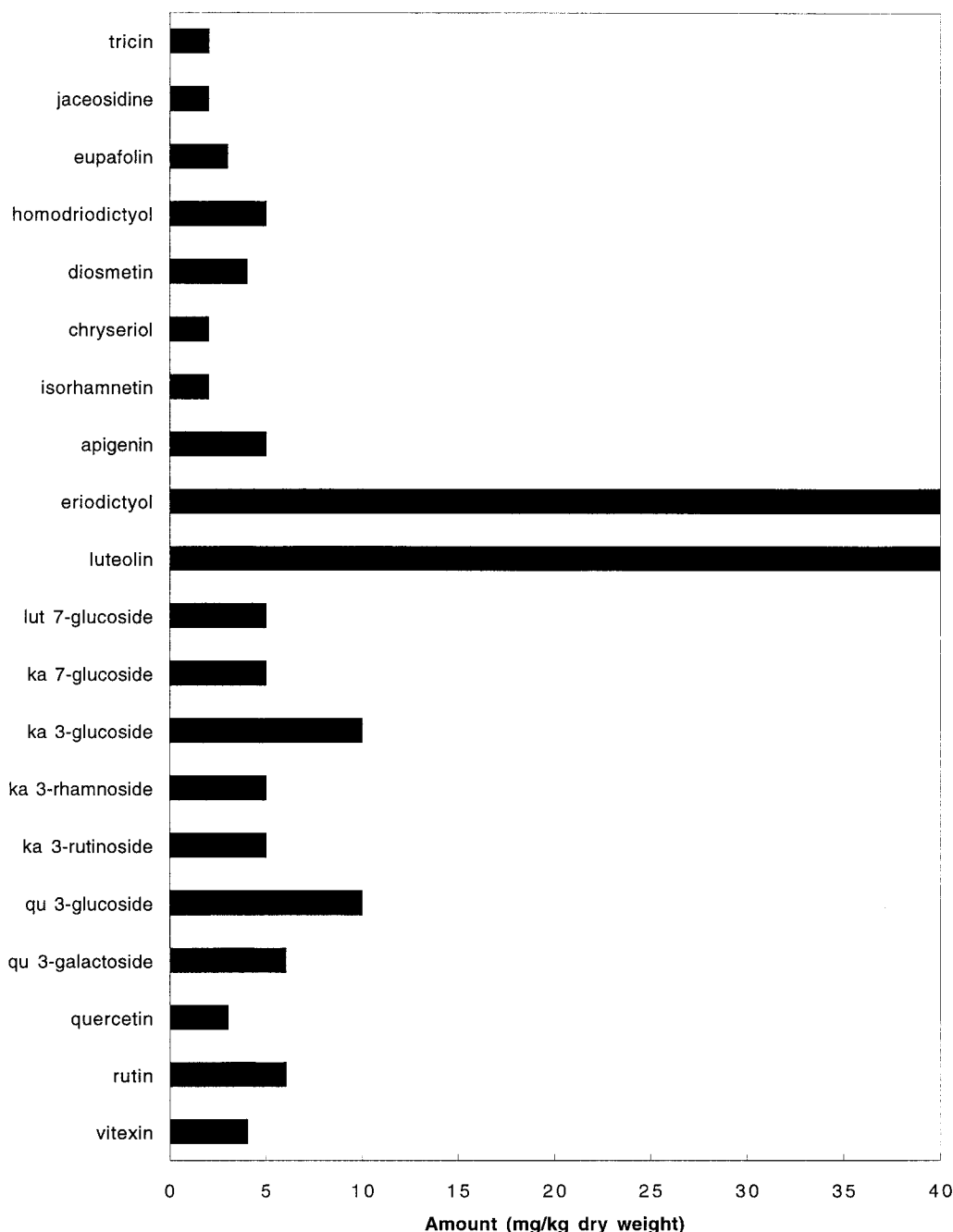
Eupafolin: EIMS, *m/z* (rel intensity) 316 (100) [M]<sup>+</sup>, 315 (14) [M – H]<sup>+</sup>, 301 (76) [M – Me]<sup>+</sup>, 298 (72) [M – 18]<sup>+</sup>, 287 (13) [M – HCO]<sup>+</sup>, 273 (81) [M – COMe]<sup>+</sup>, 167 (17) [A<sub>1</sub> – Me]<sup>+</sup>, 139 (23), 134 (13) [B<sub>1</sub>]<sup>+</sup>, 137 [B<sub>2</sub>]<sup>+</sup>.

Apigenin, eriodictyol, luteolin, luteolin 7-glucoside, kaempferol 3-glucoside, kaempferol 7-glucoside, kaempferol 3-rutinoside, kaempferol 3-rutinoside, and rutin were identified by UV, MS, and <sup>1</sup>H NMR as well as by TLC comparison with standard samples. Hydrolysis of the glycosides with acid yielded the expected sugars and aglycons.

**Screening for Estrogenic Activity of the Flavonoids from *A. vulgaris*.** The estrogenic activity of the *A. vulgaris* flavonoids was tested by using *S. cerevisiae* strain BJ3505 (a gift from Dr. Bert O'Malley's laboratory). This strain [MAT  $\alpha$ , pep 4: HIS 3, prb 1- $\Delta$ 1, 6R, his 3- $\Delta$ 200, lys 2–801, trp 1- $\Delta$ 101, ura 3–52 (can 1)] was cotransformed with the expression plasmid YEpE10 containing the yeast metallothionein (CUP 1) promoter fused to the human estrogen receptor cDNA and with the reporter plasmid YRpE2 containing two estrogen response elements upstream of the  $\beta$ -galactosidase gene (Santiso-Mere et al., 1991; Nawaz, 1992). Yeast cells were maintained and manipulated as previously described (Maier et al., 1995).

A solution of 17 $\beta$ -estradiol prepared in ethanol was used to standardize the plant extract and fractions according to the method of Maier et al. (1995). Serial dilutions of 17 $\beta$ -estradiol were employed to construct a standard curve, which was then used to estimate estrogen equivalents in the plant extracts and fractions.

The transgenic yeast cells were grown overnight in minimal medium (1% casamino acids, 2% dextrose, 1% yeast nitrogen base without amino acids, and 0.0012% adenine sulfate) with 100  $\mu$ M CuSO<sub>4</sub> at 30 °C to induce the production of estrogen receptors. When the cultures reached an appropriate density ( $A_{600} = 0.7$ ), 90  $\mu$ g of estrogen equivalents or increasing



**Figure 2.** Quantitation of flavonoids in *A. vulgaris*.

amounts (10–200  $\mu$ L) of the flavonoids to be analyzed were added to the cultures; the cultures were incubated for an additional 6–7 h. The cells ( $A_{600} = 1.0$ ) were harvested by centrifugation, lysed, and centrifuged in a microcentrifuge (13000g, 10 min, 4 °C). The supernatants were analyzed for protein content and  $\beta$ -galactosidase activity.

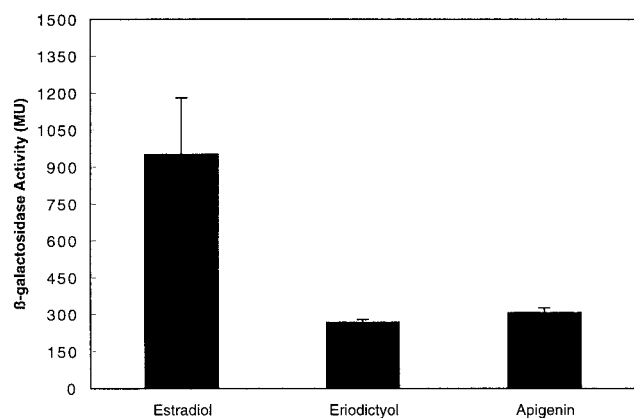
**$\beta$ -Galactosidase Assay.** The level of  $\beta$ -galactosidase expressed in the yeast cells was measured according to the method previously described (Maier et al., 1995). Each fraction was assayed in triplicate using 5–15 mg of yeast cytosolic protein. Yeast cytosolic protein was preincubated in 1 mL of transcriptional assay buffer [ $6 \times 10^{-2}$  M  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ,  $4 \times 10^{-2}$  M  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ,  $10^{-2}$  M KCl,  $10^{-3}$  M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.27% (v/v) mercaptoethanol, pH 7.0] at room temperature for 10 min. Two hundred microliters of an ONPG solution (4 mg/mL) was then added, and the reaction mixtures were incubated for 30 min at room temperature. The reaction was stopped by addition of 500  $\mu$ L of 1 M  $\text{Na}_2\text{CO}_3$ , and the absorbance at 420 nm was measured. The results were converted to Miller units [ $\text{MU}; \Delta\text{Abs} \times (1000) \text{ min}^{-1} (\text{mg of protein})^{-1}$ ].

Cultures inoculated with estradiol and genistein were used as positive controls. Yeast cultures alone in the minimal medium with or without  $\text{CuSO}_4$ , or with different amounts of ethanol, did not test positive in the  $\beta$ -galactosidase assay.

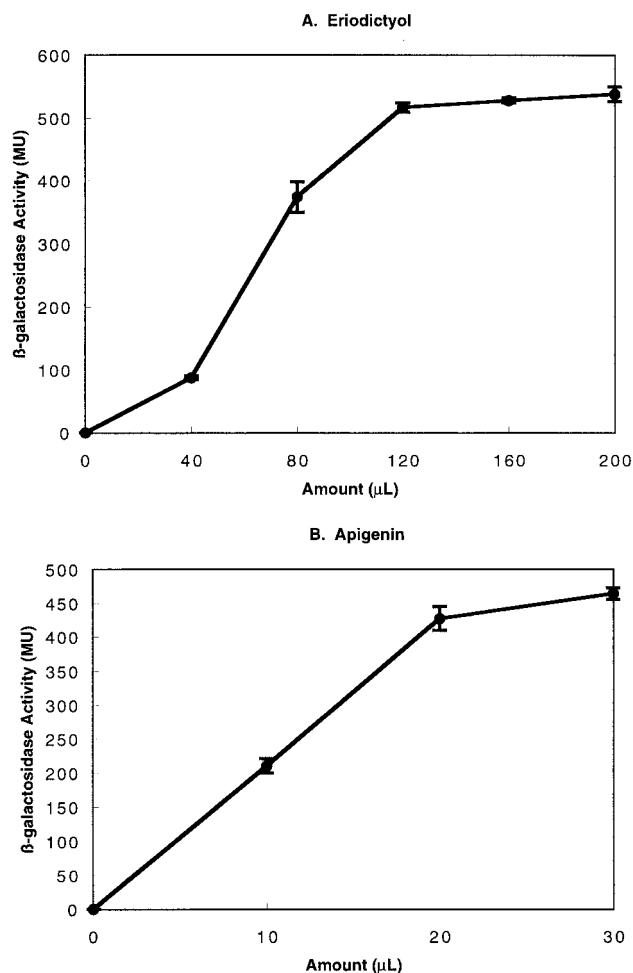
## RESULTS AND DISCUSSION

**Quantitation of Flavonoids.** Eriodictyol and luteolin were found to be the most abundant flavonoids in *A. vulgaris*. The amount of each compound was >40 mg/kg of dried plant material, which is at least 10 times more than any other flavonoid (Figure 2).

**Screening for Estrogenic Activity in Extracts of *A. vulgaris*.** The newly developed steroid-responsive transcription system in *S. cerevisiae* is a powerful screening tool not only for steroid hormones (Metzger et al., 1988; Schena and Yamamoto, 1988; McDonnell et al., 1991; Nawaz, 1992) but also for phytoestrogens (Maier et al., 1995). The yeast system was employed



**Figure 3.** Transcriptional activity induced by eriodictyol and apigenin fractions in *S. cerevisiae* strain BJ3505. The values represent mean MU  $\pm$  SD for two independent experiments.



**Figure 4.** Transcriptional activity induced by increasing amounts of eriodictyol (A) and apigenin (B) in *S. cerevisiae* strain BJ3505. The values represent mean MU  $\pm$  SD for two independent experiments.

in this study because of its requirement for estrogen or an estrogen-type compound for reporter gene activation by the estrogen receptor (Gaido et al., 1997).

The crude extracts of *A. vulgaris* activated the transcription of *lac Z*, demonstrating weak estrogenic activity. The nonpolar fraction obtained from the 80% EtOH extracts as well as the hexane and chloroform fractions did not show estrogenic activity. However, the relatively polar EtOAc extract, containing most of the

flavonoids and other phenolic compounds, exhibited 5% estrogenic activity relative to 17 $\beta$ -estradiol.

**Estrogenic Activity of Purified Flavonoids from *A. vulgaris*.** To screen for estrogenic flavonoid fractions, yeast cells were incubated with equivalent amounts (90  $\mu$ g of estrogen equivalents) of standardized fractions. Only two flavonoid fractions identified as containing apigenin and eriodictyol were able to induce the transcriptional activity of the reporter gene in transgenic yeast (Figure 3). The results in Figure 3 indicate that apigenin and eriodictyol are weak estrogens, considering the fact that 10 times more of each flavonoid fraction was used compared to estradiol. The estrogenic activity of apigenin has been reported previously using a receptor-dependent transcriptional assay of transfected HeLa cells (Miksicek, 1993). Furthermore, eriodictyol meets the structural parallels described of estrogenic flavonoids, namely two phenolic hydroxyl substituents at positions 4' and 7 (Miksicek, 1993).

Figure 4 shows that the transcriptional activity of the reporter gene in *S. cerevisiae* strain BJ3505 was dependent on the flavonoid fractions and increased as the amount of fraction was increased. These results indicate that apigenin and eriodictyol exhibited transcriptional activity similar to that of estradiol (Maier et al., 1995). Weak estrogens may also be classified as anti-estrogens because they may decrease both the effect and the concentration of more potent endogenous estrogens by competing in binding the estrogen receptor and by interacting with estrogen biosynthesizing and metabolizing pathways (Brotherton, 1976). These findings suggest that the flavonoids eriodictyol and apigenin may contribute to the emmenogogic properties of *A. vulgaris*.

#### ABBREVIATIONS USED

lut, luteolin; ka, kaempferol; qu, quercetin; ONPG, o-nitrophenyl  $\beta$ -D-galactopyranoside.

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