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Estrogenic Activity of Diarylheptanoids from *Curcuma comosa* Roxb. Requires Metabolic Activation

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Curcuma comosa Roxb. has traditionally been used as a dietary supplement for health promotion in peri- and postmenopausal women in Thailand. We investigated the estrogenic activity of 7 naturally occurring diarylheptanoids from the extracts of *C. comosa* both in vitro and in vivo. A yeast recombinant system containing human estrogen receptor α, coactivator TIF2 and a β-galactosidase reporter gene was used to determine estrogenic activity of diarylheptanoids metabolically activated with rat liver S9-fraction prior to the assay. The most potent compound was (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol, with a relative potency of 4% compared to 17β-estradiol. The metabolic activation of diarylheptanoids markedly enhanced their efficiency. The chemical structure required for estrogenic activity of diarylheptanoids was the presence of a keto group at C3 and absence of hydroxyl moiety in ring B. Only diarylheptanoids showing full estrogenic efficiency in vitro were able to elicit uterotrophic activity of in immature ovariectomized rat. This is the first evidence for in vivo estrogenic activity of diarylheptanoids from *C. comosa*. This novel class of natural phytoestrogens has the potential to be developed for use as dietary supplement in the treatment of menopausal symptoms.

KEYWORDS: ***Curcuma comosa*; diarylheptanoid; estrogenic activity; phytoestrogen; uterotrophic; Wan chak motluk**

INTRODUCTION

Estrogens are sex steroids secreted primarily from the ovaries. They have widespread physiological effects and are responsible for puberty changes in girls, feminine behaviors, and normal menstrual cycle. Estrogens also have beneficial effects by providing neuro- and cardioprotection, ameliorating skin aging, and maintaining bone density (*1*). The biological activities of estrogens are mediated through two distinct subtypes of intranuclear proteins, estrogen receptors alpha (ERα) and beta (ERβ), which are differentially expressed in various tissues (*2*). Upon ligand binding, ER dimerizes and recruits coactivators through activation function 2 (AF2) of the ligand binding domain (LBD). The complex interacts with a specific DNA sequence and modulates the expression of estrogen-target genes that influence cell growth and differentiation and function of target tissues (*3, 4*). Deprivation of circulating estrogens in peri- and menopausal women leads to the development of unpleasant symptoms, such as irregular menstrual periods, hot flashes, vaginal dryness, and mood swings (*5*), which affect the quality of life. Although estrogen replacement therapy has been effective in preventing or treating those symptoms (*6*), prolonged treatment increases the risk of mammary (*7, 8*) and endometrial cancers (*1, 9*). Thus, alternative treatments, such as the use of naturally occurring phytoestrogens, have evoked public interest and research studies (*10, 11*).

Phytoestrogens are plant-derived polyphenolic compounds that can produce estrogenic- or antiestrogenic-like biological effects in the body (*12*). Known phytoestrogens include isoflavones (genistein, diadzein, equol), flavanoids (kaempherol, quercetin), lignans (enterolactone, enterodiol), comestans (coumestrol), stillbene, and resveratrol (*12, 13*). Plant-based medicines containing phytoestrogens have been used as alternative remedies to relieve perimenopausal symptoms (*14*). A number of the randomized clinical trials on the beneficial effects of dietary supplements containing phytoestrogens in alleviating

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menopausal symptoms have been conducted and reported (*15, 16*), but not all studies have given positive results (*17*). However, a study in Chinese women showed that there is an inverse association between breast cancer development and soy consumption (*18*).

Curcuma comosa Roxb. (Wan chak motluk), a plant in the Zingiberaceae family, has been widely used as a dietary supplement and for health promotion in Thai peri- and postmenopausal women. The major compounds of *C. comosa* have been identified as being diarylheptanoids, some of which have active phytoestrogenic activity and represent a novel class of phytoestrogens (*19*). Therefore, phytoestrogens from *C. comosa* may have beneficial effects for peri- and postmenopausal women. In the present study, we demonstrate the requirement of metabolic activation for the action of *C. comosa* diarylheptanoids on in vitro induction of ER and coactivator interaction using a recombinant yeast system. We also provide evidence that diarylheptanoid estrogenic activity requires assessment in vivo.

MATERIALS AND METHODS

Chemicals and Reagents. 17β-Estradiol (E_2), D-glucose 6-phosphate disodium salt hydrate (G-6-P), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and β-nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Diarylheptanoids (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol (**1**), 7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-hepten-3-ol (**2**, a 3:1 mixture of 3*S* and 3*R* enantiomers), (3*S*)-7-(3,4-dihydroxyphenyl)-1-phenyl-(1*E*)-1-hepten-3-ol (**3**), (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (**4**), 1,7-diphenyl-6-hepten-3-one (**5**), 1-(4-hydroxyphenyl)-7-phenyl-6-hepten-3-one (**6**), and 1-(4-hydroxyphenyl)-7-phenyl-4,6-heptadien-3-one (**7**) were isolated from *C. comosa* as previously described (*19*) (see **Figure 1** for their structures). All other chemicals were of analytical grade. S9 extract (rat liver 9000*g* supernatant fraction induced with phenobarbital and 5,6-benzoflavone) was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan) and Zymolyase 20T from Osaka Yakken Co. (Osaka, Japan).

Animals and Uterotrophic Assay. Immature female Wistar rats (21 days old), weighing 50−60 g, supplied by the National Laboratory Animal Center, Bangkok, Thailand, were housed in groups of five in hanging stainless steel cages at the Center of Animal Facilities, Faculty of Science, Mahidol University. The animal room was maintained at 21−22 °C with a relative humidity of 50−60% and a 12 h light/dark cycle. Animals were given free access to regular rat chow and water ad libitum.

Rats were assigned into intact control and ovariectomized (OVX) groups that received either vehicle or the test compounds (eight animals/group). Bilateral ovariectomy was performed under general anesthesia using pentobarbital sodium injection (50 mg/kg of BW, ip), and animals were allowed to recover for 5 days after the operation. OVX rats received vehicle (corn oil) or the test compounds at a dose of 100 mg/kg of BW, ip, for two consecutive days. 17β-Estradiol (E_2; 2.5 μg/kg of BW, im) was used as positive control. Compounds were diluted in dimethyl sulfoxide (DMSO), and DMSO did not exceed 10% of volume injected. Twenty-four hours after the last injection of 0.3 mL of corn oil, animals were sacrificed with an overdose of pentobarbital sodium injection. Uteri were removed, blotted, and weighed. Determination of cornification was conducted by examining under a light microscope, a smear of vaginal epithelium using saline lavage technique. The experimental protocols were approved by the Institutional Animal Care and Use Committee, Faculty of Science, Mahidol University.

Metabolic Activation and Sample Preparation. Metabolic activation of the test compounds by S9 fraction was conducted according to the method of Takatori et al. (*20*). In brief, a 990 μL aliquot of S9 mixture containing 0.5 mg of S9 protein, 0.16 M $MgCl_2$, 0.1 M NADP, 0.1 M G-6-P, 0.5 M sodium phosphate buffer, pH 7.4, and 1 M KCl was incubated with 10 μL of DMSO (vehicle), E_2, or each test compound at 37 °C for 4 h. The test compounds and E_2 were serially diluted in DMSO. The final concentration of DMSO did not exceed 0.05%, which had no effect on the reaction. Negative control employed heat-inactivated (95 °C for 5 min) S9 fraction. Test compounds and E_2 were stored at −80 °C until used.

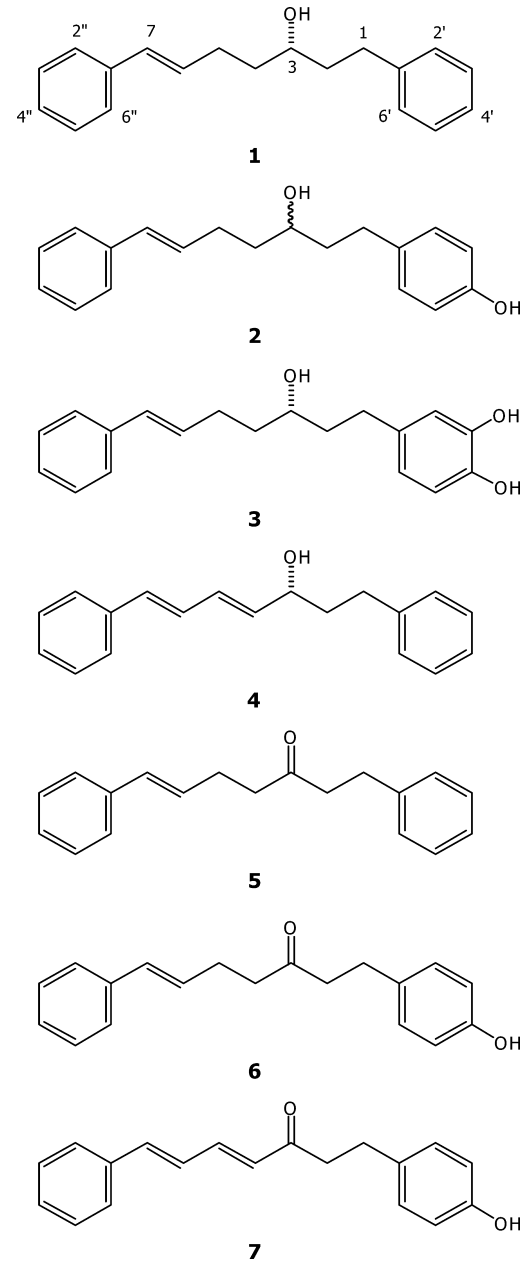

Figure 1. Structures of diarylheptanoids used in the study: (3*S*)-1,7-diphenyl-(6*E*)-6-hepten-3-ol (**1**); 7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-hepten-3-ol (**2**, a 3:1 mixture of 3*S* and 3*R* enantiomers); (3*S*)-7-(3,4-dihydroxyphenyl)-1-phenyl-(1*E*)-1-hepten-3-ol (**3**); (3*R*)-1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol (**4**); 1,7-diphenyl-6-hepten-3-one (**5**); 1-(4-hydroxyphenyl)-7-phenyl-6-hepten-3-one (**6**); 1-(4-hydroxyphenyl)-7-phenyl-4,6-heptadien-3-one (**7**) (*19*).

Recombinant Yeast System for Detecting Estrogenic Activity. Yeast transformed with human estrogen receptor alpha (ERα), coactivator TIF2, and β-galatosidase reporter genes was used (*21*). In brief, *Saccharomyces cerevisiae* strain Y190 (kindly provided by Dr. T. Nishihara, Graduate School of Pharmaceutical Science, Osaka University, Osaka, Japan) carrying pGBT9 containing ERα (pGBT9-ERLBD) and pGAD424 containing receptor interaction domain of TIF2 (pGAD424-TIF2) plasmids were grown overnight at 30 °C with shaking in selective (SD) medium containing 0.67% yeast nitrogen base without amino acid, 2% glucose, and 2% dropout mix (lacking leucine and tryptophan). Test compounds (125 μL), which previously had been treated with the S9 fraction, were mixed at a 1:1 (v/v) ratio with 75

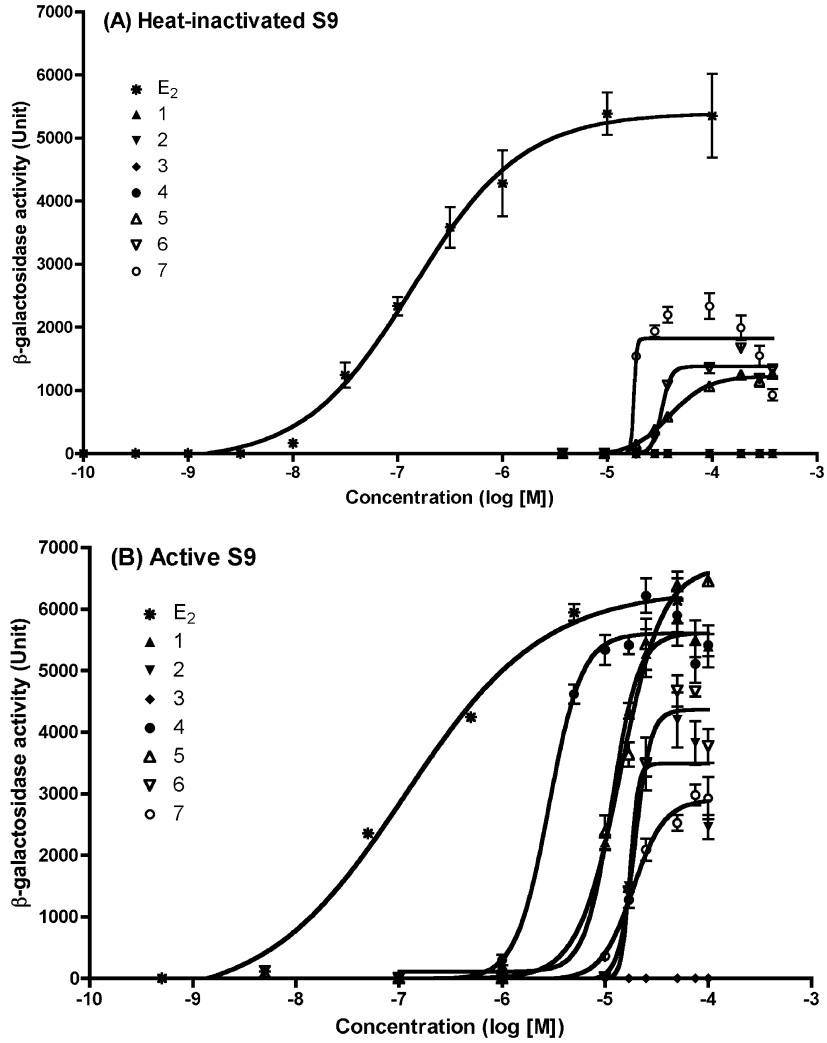

Figure 2. β-Galactosidase activity in yeast two-hybrid system expressing ligand binding domain (LBD) of hERα and coactivator (TIF2). Yeast cells were treated at 30 °C for 4 h with diarylheptanoids **1**−**7** and 17β-estradiol (E_2) in the presence of heat-inactivated (**A**) or active rat liver S9-fraction S9 (**B**). Data are presented as mean ± SEM of three independent experiments conducted in triplicate.

μL of 2 × SD medium and 50 μL of yeast cells, and the solutions were incubated with shaking at 30 °C for 4 h. Then absorbance at 660 nm of the cell suspensions was measured using a microplate reader (SpectraMAXplus, Molecular Devices, Sunnyvale, CA).

A 100 μL aliquot of cell suspension was centrifuged at 3000*g*, and the pellet was suspended in 200 μL of buffer containing 0.1 M sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 3.5 mM β-mercaptoethanol, and 1 mg/mL of Zymolyase 20T and incubated at 37 °C for 15 min to lyse cells (*21*). The lysate was incubated with 40 μL of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/mL in 0.1 M sodium phosphate buffer, pH 7.0) at 30 °C for 30 min, and the reaction was terminated by the addition of 100 μL of 1 M Na_2CO_3. The solution was centrifuged at 3000*g* for 5 min. Absorbances at 420 and 550 nm were measured using a microplate reader. β-Galactosidase activity is reported in Miller units (*21*).

Statistical Analysis. Data were expressed as mean ± standard error of mean (SEM). All data sets were evaluated by one-way ANOVA followed by Tukey−Kramer HSD posthoc test or paired *t* test when applicable. Statistical significance is indicated when $p < 0.05$.

RESULTS

Estrogenic Activity of Diarylheptanoids Evaluated in a Recombinant Yeast System. To determine the estrogenic activity of the seven diarylheptanoids (**Figure 1**), induction of interaction of hERα with coactivator (TIF2) was evaluated by measuring β-galactosidase activity in a recombinant yeast system. As estrogenic activity requires metabolic activation, the compounds were previously treated with rat liver S9 fraction.

In the presence of heat-inactivated S9 fraction, compounds **5**, **6**, and **7** were able to stimulate β-galactosidase activity (**Figure 2A**), with EC_{50} value of 39.3, 33.1, and 18.0 μM, respectively. The percent relative potencies (% RP) from heat-inactivated S9 fraction treatment were 0.59 ± 0.05, 0.71 ± 0.01, and 1.42 ± 0.06% for compounds **5**, **6**, and **7**, respectively, compared to that of E_2 (100%; EC_{50} = 0.1 μM) (**Table 1**).

When treated with active S9 fraction, all compounds, except compound **3**, were able to induce the β-galactosidase activity (**Figure 2B**), with EC_{50} values of 11.6, 17.4, 2.8, 14.1, 19.4, and 18.6 μM for compounds **1**, **2**, **4**, **5**, **6**, and **7**, respectively. The percent relative inductive efficiencies (% RIE) of compounds **5**, **6**, and **7** were 23.05 ± 0.49, 30.94 ± 0.69, and 43.33 ± 1.67, respectively. Following metabolic activation by S9 fraction, % RP values of compounds **1**, **2**, **4**, **5**, **6**, and **7** were 1.04 ± 0.05, 0.68 ± 0.03, 3.91 ± 0.72, 0.87 ± 0.04, 0.62 ± 0.02, and 0.64 ± 0.03%, respectively. Interestingly, compounds **1**, **4**, and **5** have % RIE values comparable with that of E_2, whereas those of compounds **2**, **6**, and **7** are significantly lower.

Uterotrophic Activity of Diarylheptanoids in Rat. Uterotrophic activity of the seven diarylheptanoids was assessed by a two-day uterine bioassay in immature OVX rats admin-

Table 1. Percent Relative Potency and Percent Relative Inductive Efficiency of Diarylheptanoids[a]

	% rel potency[b]		% rel inductive efficiency[c]	
compd	heat-inactivated S9 treatment	active S9 treatment	heat-inactivated S9 treatment	active S9 treatment
E_2	100	100	100	100
1	ND	$1.04 \pm 0.05^{*\#}$	ND	$94.54 \pm 5.84^{\#}$
2	ND	$0.68 \pm 0.03^{*}$	ND	$62.29 \pm 7.11^{*\#}$
3	ND	ND	ND	ND
4	ND	$3.91 \pm 0.72^{*\#}$	ND	$101.35 \pm 1.91^{\#}$
5	$0.59 \pm 0.05^{*}$	$0.87 \pm 0.04^{*}$	$23.05 \pm 0.49^{*}$	$103.86 \pm 2.10^{\#}$
6	$0.71 \pm 0.01^{*}$	$0.62 \pm 0.02^{*}$	$30.94 \pm 0.69^{*}$	$73.93 \pm 1.66^{*\#}$
7	$1.42 \pm 0.06^{*}$	$0.64 \pm 0.03^{*}$	$43.33 \pm 1.67^{*}$	$48.51 \pm 2.87^{*\#}$

[a] Data are presented as mean ± SEM of three independent experiments conducted in triplicate. ND, not detectable. *, $p < 0.05$, compared with E_2 (analyzed using one-way ANOVA). #, $p < 0.05$, compared with heat-inactivated S9 treatment (analyzed using paired *t* test). [b] Determined as the ratio of EC_{50} of E_2 to test compound × 100. [c] Ratio of β-galactosidase activity induced with tested compound to that by E_2 × 100 (*25*).

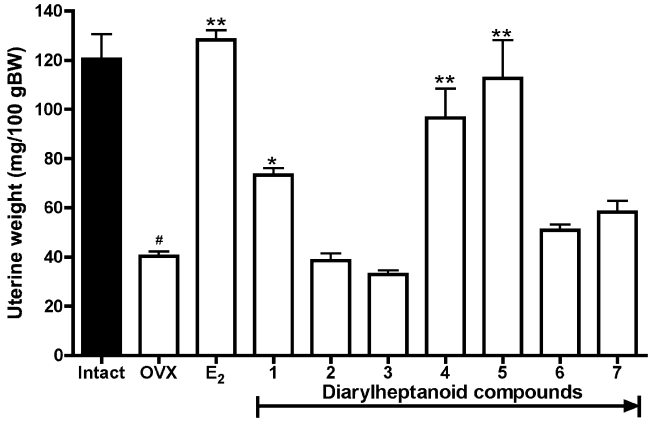

Figure 3. Uterine wet weight of immature ovariectomized (OVX) rats. OVX rats were treated with 17β-estradiol (E_2 2.5 μg/kg of BW/day, im) and diarylheptanoids **1**−**7** (100 mg/kg of BW/day, ip) for two consecutive days. Data are presented as mean ± SEM of eight animals. #, $p < 0$ 0.001, compared with intact control (intact); *, $p < 0.05$, and **, $p < 0.01$, compared with OVX control.

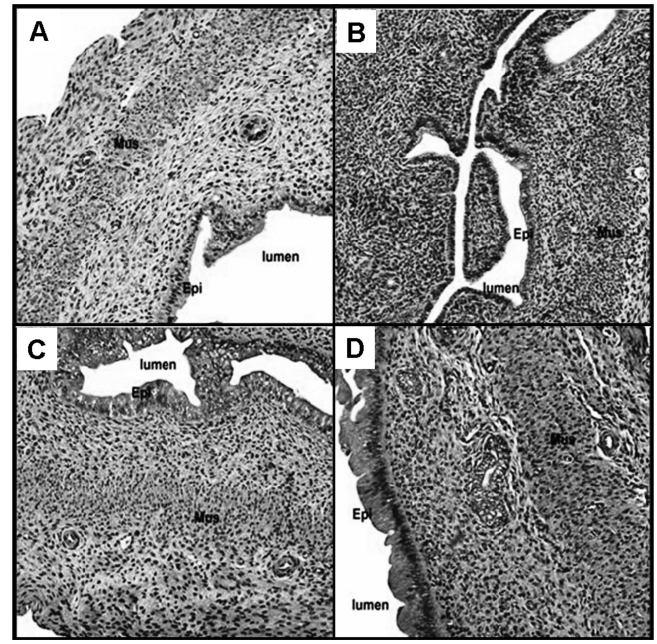

Figure 4. Histology of rat uterine mucosa: (**A**) intact control rat; (**B**) OVX rat; (**C**) OVX rat treated with diarylheptanoid **4** (100 mg/kg of BW/day, ip) for two consecutive days; (**D**) OVX rat treated with E_2 (2.5 μg/kg of BW/day, im) for two consecutive days. Epi, epithelium; Mus, muscle; H&E staining; 200× magnification.

istered 2.5 μg/kg of BW/day of E_2 or 100 mg/kg of BW/day of the test compounds. E_2 and compounds **1**, **4**, and **5** significantly increased uterine weight ($p < 0.05$) compared with OVX control (**Figure 3**). Histological examination of the uterus showed low numbers of columnar cells of the luminal epithelia of the uterus in OVX control as compared to that in intact control (**Figure 4**). After treatment with compounds **1**, **4**, and **5**, the sizes of the uteri were increased. The epithelial cells became elongated columnar (**Figure 4C**). These characteristics were similar to those of E_2-treated group (**Figure 4D**). However, the action of uterine stimulation by diarylheptanoids was less than that of the E_2-treated group. Nevertheless, the presence of cornification of vaginal epithelial cells in OVX rats treated with compounds **4** and **5** indicated typical estrogenic property (data not shown).

DISCUSSION

In a previous study, the estrogenic activity of *C. comosa*-derived diarylheptanoids was determined in vitro by activating transcriptional regulation of endogenous responsive genes in HeLa (human cervical cancer) cells (*19*). As dietary supplement products derived from *C. comosa* have been extensively used, we further evaluated the potency and efficiency of the estrogenic activity of a number of naturally occurring diarylheptanoids subjected to metabolic activation by active rat liver S9 fraction. Evaluation of estrogenic activity was based on the ligand-dependent interaction in yeast between LBD of ERα and coactivator, TIF2, which leads to the transcription of the *LacZ* reporter gene and thereby the appearance of β-galactosidase activity.

Both the potency and efficiency of compounds **5**, **6**, and **7** were much lower than those of E_2. The estrogenic activities of compounds **1** and **4** were improved following metabolic activation, whereas the estrogenic activity of E_2 was not altered, in agreement with a previous study (*20*). Metabolic activation enhanced the efficiency of all test compounds, except for compound **3**, and the efficiencies of almost all compounds were also very much improved. However, it is unclear how the rat liver S9 fraction biotransformed as well as enhanced the estrogenic activity of the *C. comosa* diarylheptanoids. One possibility is that the oxidization of the hydroxyl group at the C3 position of compounds **1** and **2** to the corresponding keto group in compounds **5** and **6**, respectively, resulted in the increase of potency and efficiency of compounds **1** and **2** comparable to those of **5** and **6**, respectively. In addition, it is possible that hydroxylation of C4″ of the aromatic ring of diarylheptanoids mimics the hydroxyl group at C3 of E_2, enabling interaction with the ER binding pocket and thereby exhibiting the estrogenic-like action. The hydroxyl group at C3

(A ring) of E_2 activates ER (*22, 23*) by interacting with Glu353 and Arg394 in the inner side of the ER binding pocket (*23, 24*). It may also be responsible for estrogenic activity in terms of efficiency, as the efficiencies of almost all diarylheptanoids were considerably enhanced after the metabolic activation. Identification of the active metabolites and evaluation of their binding activities are ongoing.

It was noted that among diarylheptanoids compounds with an olefinic functional group adjacent to the aromatic ring attached to the 7-position and a keto group exhibited high estrogenic activity. Oxidation of the C3 hydroxyl group of compound **1** to the corresponding ketone **5** greatly improved its estrogenic activity. However, in addition to the keto group at C3 of compound **5**, the presence of a hydroxyl group at C4′ of the aromatic ring (compound **6**), as well as the additional double bond of compound **7**, did not enhance the activity of compound **5**. Moreover, the presence of a hydroxyl group on the aromatic ring attached at the 1-position of the diarylheptanoids, either at position C4′ alone (compound **2**) or both C3′ and C4′ (compound **3**), including the presence of an additional double bond (compound **4**), did not modulate the estrogenic activity of compound **1**, the C3 hydroxy diarylheptanoid.

The estrogenic activity of diarylheptanoids evaluated in vivo using a two-day uterotrophic assay in immature OVX rats indicated that not all compounds showing positive estrogenic activity in vitro showed similar effects in the whole animal. As there were differences in their potencies, only compounds **1**, **4**, and **5**, which exhibited full estrogenic efficiency after metabolic activation, were able to show uterotrophic activity. Interestingly, compound **5**, which had a lower potency than compound **4**, showed full efficiency similar to that of compound **4** in the yeast assay and also gave a uterotrophic response comparable to that of compound **4** in the animals. The discrepancy in response of compound **5** might have resulted from bioactivation of this compound in the animal to a more active compound than the parent, thereby producing a high response similar to that of compound **4**. Differences between estrogenic responses obtained in vitro and in vivo have been reported in a number of studies, particularly those of phenolic compounds (*25, 26*). Their activities could be augmented or reduced as a result of the metabolism produced by the action of cytochrome P450 enzyme in the cells and subsequently altering hormonal activity. Moreover, there is evidence showing that the metabolic activation (viz. hydroxylated and methylated metabolites) (*27*) also may occur locally within the target tissues. Thus, the difference between in vitro and in vivo responses of the same compound may be a result of the nature of the target cells, in this case yeast cells and uterine cells in animals. It is essential that compounds demonstrating estrogenic activity in an in vitro screening assay should be also evaluated in vivo, which is relevant for clinical success.

The potencies of estrogenic compounds relative to estradiol varied in each experimental system. In the present study, diarylheptanoids from *C. comosa* showed weak estrogenic activity. Their potencies were approximately 1/100 to 1/1000 that of estradiol in yeast assay and 1/10000 to 1/100000 in the uterotrophic assay. Although there is no information on the bioavailability and tissue distribution of the diarylheptanoids from *C. comosa*, the concentration that elicits the biological response in the body could be achieved from the consumption of dietary supplement as indicated from the observed uterotrophic effect in animals.

As the estrogenic active compound **5** is one of the diarylheptanoids that is structurally similar to curcumin, the main yellow pigment from *Curcuma longa*, a plant in the same genus, the metabolic fate of compound **5** might be similar. Extensive investigations on the pharmacokinetics of curcumin have been conducted in an attempt to identify metabolites that possess active cancer chemoprevention (*28–30*). However, of the available metabolites detected, none of them has a greater activity than the parent compound (*28*). The major products of curcumin biotransformation in plasma are the results of reduction of diarylheptatrienone chain and conjugation with glucuronide and sulfate (*30*). Curcumin is distributed in the intestine, liver, spleen, kidney, plasma, and brain (*30*). Seventy-five percent of curcumin is excreted in feces, and only a trace amount is detected in urine (*31*). It has been suggested that the metabolism of curcumin by the liver is a pharmacological deactivation process (*28, 32*), with the active free compounds probably generated in the target tissues. In addition to unidentified active metabolite(s), one of the major concerns in using medical herbs as a dietary supplement is the herb−drug interaction. Caution must be taken regarding the possible adverse effects of using medical herbs together with modern therapy, especially in the elderly as well as persons with chronic diseases. For future clinical application of diarylheptanoids from *C. comosa*, further investigations on pharmacokinetics and toxicity are necessary.

In summary, this is the first study showing estrogenic activity of *C. comosa* diarylheptanoids in vivo. It is important to note that data obtained from an in vitro screening assay should not be viewed alone and must be evaluated in an intact animal. The in vivo estrogenic activity of naturally occurring diarylheptanoids provides a scientific rationale for the traditional use of dietary supplement products derived from this plant in menopausal women, particularly those who experience an estrogen-deficit condition. As diarylheptanoids are widely distributed in the Zingiberaceae family, plants in this family offer a potential source of phytoestrogens.

ABBREVIATIONS USED

E_2, 17β-estradiol; ER, estrogen receptor; LBD, ligand binding domain; TIF2, transcriptional intermediary factor 2; OVX, ovariectomized; DMSO, dimethyl sulfoxide; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; NADP, β-nicotinamide adenine dinucleotide phosphate; % RP, percent relative potency; % RIE, percent relative inductive efficiency; BW, body weight; im, intramuscular; ip, intraperitoneal.

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