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Estrogen-like Cell Proliferation Abilities of Korea Forest Plant Resources on MCF-7 Cells and Analysis of Active Compounds

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Abstract: This study conducted a comprehensive analysis of functional substances and explored their biological activities using colorimetric and chromatographic techniques to identify high-value materials from 14 species of forest plants native to Korea. Comparative analysis between plant species included calculating the total polyphenol and flavonoid contents and qualitative and quantitative analysis of the phytochemical compounds caffeic acid (**1**), *p*-coumaric acid (**2**), and quercetin (**3**) using HPLC, and antioxidant activity tests (DPPH and $ABTS^{+}$) and menopausal symptom relief tests (E-screen assay) to investigate their biological activities. The results highlighted *Cercidiphyllum japonicum* (FR 4), *Aruncus dioicus* (FR 6), and *Pseudocydonia sinensis* (FR 8) as excellent forest plant resource materials among the 14 forest plant resources. Notably, FR 4 exhibited significant activity in the E-screen assay. On the basis of these findings, we propose the use of FR 4 as a valuable material. Further analysis identified the phytochemical compounds maltol (**4**), chlorogenic acid (**5**), ellagic acid (**6**), and quercitrin (**7**) in FR 4 and analyzed their biological activities. Compounds **2**–**6** found in FR 4 were confirmed to possess strong antioxidant activity, and an E-screen assay revealed an excellent cell proliferation rate for quercitrin (**7**). This suggests that quercitrin (**7**) in FR 4 may be a significant indicator of the alleviation of menopausal symptoms.

Keywords: antioxidant; *Cercidiphyllum japonicum*; E-screen assay; forest plant resource; HPLC; quercitrin

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1. Introduction

Forest plant resources include components of the forest ecosystem, such as plants, animals, and soils. Fragrance resources are substances derived or synthesized from nature, such as plants, animals, or microorganisms, and they are used to add flavor, color, or aroma. These fragrance resources have applications in various industries, such as medicine, cosmetics, and food. They include plant fragrance resources extracted from plant leaves, flowers, fruits, and shells; animal fragrance resources extracted from animal secretions; and microbial fragrance resources produced through fermentation [1,2]. Representative fragrance plants with distinctive scents include the Cupressaceae, Lauraceae, Rutaceae, Umbelliferae, Lamiaceae, Asteraceae, and Oleaceae. Various extraction methods are employed to obtain fragrance components from aromatic plants, the most common being organic solvent extraction, supercritical fluid carbon dioxide extraction, and steam distillation extraction [3,4]. The resulting fragrance component, which possesses oil-like characteristics, is referred to as essential oil. Essential oils extracted from plants are used not only to impart characteristic aromas to flavors or fragrances, such as in food additives but also for the development of industrial products, such as household goods and agricultural products.

Essential oils have been reported to exhibit various physiological activities, including antioxidant effects, relief from menopausal symptoms, and antibacterial, nerve-stabilizing, and blood pressure-lowering effects [5].

Plants produce a diverse array of compounds known as phytochemicals during their growth. Some of these phytochemicals exhibit potent antioxidant activities [6]. Numerous studies have emphasized the role of phenolic compounds in plants as significant contributors to antioxidant activity [7–9]. Among the phytochemicals with potent antioxidant effects, caffeic acid (**1**), *p*-coumaric acid (**2**), and quercetin (**3**) are present in various plants. Antioxidants play a crucial role in protecting cells from oxidative stress and preventing cell damage by neutralizing the free radicals that undergo oxidation and are produced in cells [10–12]. Oxidative stress occurs when chemical reactions with oxygen in cells lead to an imbalance, resulting in the excessive production of free radicals that cause cell damage [13,14]. Free radicals, such as hydroxyl radicals (OH^-) and superoxide radicals (O^{2-}), are unstable compounds with non-bonding electrons. Widely employed methods for evaluating antioxidant abilities in vitro include 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) screening [15]. These experiments measured the antioxidant activity of the phytochemicals and other compounds by assessing changes in the absorbance of the reaction mixture.

Menopause causes a multitude of physical and psychological symptoms, such as daily hot flashes, fatigue, back and breast pain, anxiety, and cognitive decline [16,17]. The correlation between menopause and obesity is notable, given the pivotal role of estrogen in regulating adipose tissue and overall metabolism [18]. Interestingly, women over 45 years of age exhibit higher rates of overweight compared to men, a trend contrasting with that seen in younger adults, suggesting a link between menopause and obesity [19,20]. Research suggests a complex interplay between reduced estrogen levels and obesity in menopausal women, indicating that estrogen deficiency is a contributing factor to weight gain [21]. Obesity is associated with an increased risk of type 2 diabetes, cardiovascular diseases, and osteoarthritis [22], leading the World Health Organization to designate it as a preventable cause of mortality [23]. Despite efforts to address obesity, its prevalence remains alarmingly high, with nearly one-third of the global population classified as overweight or obese in 2019 [19]. Furthermore, the rates of adult obesity and severe obesity are projected to rise further [24,25].

Hormone replacement therapy (HRT), also known as menopausal hormone therapy, is commonly prescribed to alleviate various menopausal symptoms [26], with reported benefits in mitigating obesity and its associated effects [27]. However, owing to concerns about adverse effects, many women opt for non-hormonal therapies over HRT [28]. Natural products have emerged as promising alternatives for the safe and effective management of postmenopausal symptoms safely and effectively [29]. Systematic reviews have highlighted the efficacy of certain natural products in curbing weight gain in both animal models and humans, with minimal adverse effects or mortality [30]. Studies have also indicated that herbal extracts can stimulate estrogen receptor (ER)-positive MCF-7 cells and enhance the expression of estrogen-related genes [31], suggesting their potential to ameliorate menopausal symptoms linked to estrogen deficiency through their estrogenic properties.

Forest plant resources have been reported to exert antioxidant, anti-inflammatory, antibacterial, and antiviral effects through substances such as flavonoids, phenolic acids, and triterpenes such as maltol, chlorogenic acid, ellagic acid, and quercitrin [23–28].

With increasing interest in functional health foods, this study provides data that can be used as a basis for the development of new medicinal materials by utilizing forest plant resources native to Korea. Through this study, we hope to discover and present high-value-added resources by increasing the development potential of forest plant resources.

2. Materials and Methods

2.1. Plant Materials

The forest plant resources native to Korea used in the study involved the aerial parts (stems and leaves) of various species, including *Amaranthus blitum* Rchb. ex Steud. (FR 1), *Catalpa ovata* G.Don (FR 2), *Cnidium japonicum* Miq. (FR 3), *Cercidiphyllum japonicum* Siebold and Zucc. (FR 4), *Osmunda japonica* Thunb. (FR 5), *Aruncus dioicus* (Walter), Fernald (FR 6), *Koelreuteria paniculate* Laxm. (FR 7), *Pseudocydonia sinensis* (Thouin) C.K.Schneid. (FR 8), *Agastache rugosa* Kuntze (FR 9), *Dictamnus albus* L. (FR 10), *Lindera erythrocarpa* Makino (FR 11), *Ginkgo biloba* L. (FR 12), *Toona sinensis* (A.Juss.) M.Roem. (FR 13), and *Magnolia sieboldii* K.Koch (FR 14) collected from July to September 2022. These plant materials were provided and characterized by Dr. C.H. Choi, Gyeonggi-do Forestry Environment Research Center, Osan, Republic of Korea. All voucher specimens are deposited at the herbarium of Gyeonggi-do Forestry Environment Research Center, Osan, Republic of Korea.

2.2. Apparatus, Reagents, and Chemicals

For reflux extraction, a multi-heating mantle (MS-EAM9023-03, Misung Scientific Co., Kyunggi-do, Republic of Korea) and a cooling water circulation device (CA-1116A, EYELA, Tokyo, Japan) were used. Extracts were prepared using a water bath (OSB-2200, EYELA) and rotary evaporator vacuum decompression extraction equipment (N-1100, EYELA). An oven temperature control system was purchased from WiseVen Won-155 (Daihan Scientific, Wonju, Republic of Korea). High-performance liquid chromatography (HPLC; Waters Alliance 2695 Separations Module, Milford, MA, USA) and an autosampler, photodiode array (PDA; Waters 2998 Photodiode Array Detector, Milford, MA, USA) were used for the quantitative analysis of FRs 1–14. HPLC (Agilent 1260 Infinity II Quat Pump, Santa Clara, CA, USA) and a variable-wavelength detector (Agilent Variable Wavelength Detector, Santa Clara, CA, USA) were used to obtain the results. A microplate spectrophotometer (Epoch, BioTek, Winooski, VT, USA) was used for biological activity analysis. Ethanol (EtOH) was purchased from Samchun Chemical Co. (Pyeongtaek, Republic of Korea), and the acetonitrile (ACN), methanol (MeOH), and water used for HPLC analysis were obtained from J. T. Baker. The positive control group used in the E-screen assay was Queens One, including dried red clover extract (Kyungjin Pharmaceutical Co., Ltd., Incheon, Republic of Korea), a commercially available drug used to improve menopausal symptoms. $ABTS^{+}$ was purchased from Roche Diagnostics (Mannheim, Germany), and DPPH was obtained from Alfa Aesar (London, UK). Tannic acid, quercetin, and ascorbic acid were provided by the Natural Product Institute of Science and Technology (Anseong, Republic of Korea). Compounds **1–7** (**1**, caffeic acid; **2**, *p*-coumaric acid; **3**, quercetin; **4**, maltol; **5**, chlorogenic acid; **6**, ellagic acid; **7**, quercitrin) were provided by the Natural Product Institute of Science and Technology (www.nist.re.kr accessed on 27 April 2024), Anseong, Republic of Korea.

2.3. Sample Extraction

Dried samples of FRs 1–14, weighing 10 or 23 g each, were collected and subjected to three extraction cycles lasting for three hours each. The extraction process was carried out in a reflux environment at temperatures ranging from 78 to 85 °C using EtOH as the extraction solvent. Following extraction, the solvent was filtered through filter paper (pore size: 0.45 µm), and the resulting solution was evaporated using a decompression concentrator at 55 °C to yield an EtOH extract.

2.4. Total Polyphenol Content Test

The analysis method was developed by modifying the Folin-Ciocalteu method. Extracted samples were diluted with water to a concentration of 2 mg/mL to ensure accurate results within the measurable concentration range. Dissolution was performed using an ultrasonic wave shaker at room temperature, followed by filtration through a polyvinylidene difluoride (PVDF) membrane filter. Tannic acid, which served as the standard compound, was prepared in water at a concentration of 1 mg/mL. The standard stock compound was

subjected to the same dissolution and filtration processes as the sample. The standard stock was further diluted to concentrations ranging from 7.81 to 125 μg/mL. Subsequently, 60 μL of the diluted sample and standard compound, along with 40 μL of 2N Folin and Ciocalteu's phenol reagent (Sigma Aldrich, St. Louis, MO, USA), was added to the 96-well plate. Subsequently, 100 μL of 7.5% sodium carbonate solution (Sigma Aldrich, St. Louis, MO, USA) was added, and the mixture was left to react in a dark room for 30 min. After the reaction, the absorbance was measured at 760 nm using a microplate reader. The total polyphenol content was calculated by comparing the absorbance value of the sample with the curve generated using the absorbance of the standard material [29,32].

2.5. Total Flavonoid Content Test

The extracted sample was diluted with ethanol to ensure accurate results within the measurable concentration range, resulting in a final concentration of 2 mg/mL. Dissolution at room temperature was performed using an ultrasonic shaker, followed by filtration through a PVDF membrane filter for subsequent use. Quercetin was used as a standard and was prepared in EtOH at a concentration of 1 mg/mL. The standard compound was subjected to the same dissolution and filtration processes as the sample to create a standard compound stock. The stock solution was further diluted to a concentration ranging from 3.91 to 62.5 μg/mL. In a 96-well plate, 100 μL of the test solution and the standard compound, both diluted by concentration, were combined with 100 μL of 2% aluminum (III) chloride hexahydrate (Daejung, Siheung, Republic of Korea) and thoroughly mixed. Subsequently, 100 μL of 7.5% sodium carbonate solution was added, and the mixture was left to react in a dark room at room temperature for 10 min. After the reaction, absorbance was measured at 430 nm using a microplate reader. Total flavonoid content was calculated using a curve generated from the absorbance values of the samples and standard compounds [29,32].

2.6. DPPH Radical Scavenging Activity

Ascorbic acid was prepared in water at a concentration of 1 mg/mL and diluted to six sequential concentrations (0–200 μg/mL) for use as a calibration curve. A DPPH stock solution (Thermofisher, Waltham, MA, USA) was prepared using EtOH (0.2 mM), and the test solution was prepared by diluting the sample with EtOH. For DPPH radical scavenging activity, 10 μL of the standard solution and test solution were combined with 200 μL of 0.2 mM DPPH solution in a 96-well plate and mixed. The mixture was incubated at room temperature for 30 min to facilitate a smooth reaction, and the absorbance was measured at 514 nm. The inhibition rates of the oxidation reaction and the antioxidant activity were calculated based on the absorbance data. The half-maximum inhibitory concentration (IC_{50}, μg/mL) was calculated and compared with that of the control group without the addition of samples. Additionally, the free radical erase effect was expressed as a percentage (%) compared with the control group without the addition of samples [29].

$$\text{DPPH radical scavenging activity percentage (\%)} = (\text{Abc} - \text{Abs})/\text{Abc} \times 100$$

(Abc: Absorbance of control, Abs: Absorbance of sample)

2.7. $ABTS^+$ Radical Scavenging Activity

The ascorbic acid standard was prepared using water at a concentration of 1 mg/mL. The solution was diluted to six concentrations (0–200 μg/mL) to create a standard solution for the calibration curve. The $ABTS^+$ working solution stock was prepared by mixing 7.4 mM $ABTS^+$ (Thermofisher, Waltham, MA, USA) with 2.6 mM potassium persulfate (Sigma Aldrich, St. Louis, MO, USA) and diluting it with water to adjust the absorbance value to 1.5 or less. The test solution was dissolved in water to prepare a test stock. Afterward, 10 μL of each standard solution and test solution were dispensed in a 96-well plate. Subsequently, 200 μL of $ABTS^+$ working solution was added, mixed well, and allowed to react at room temperature for 30 min. IC_{50} (μg/mL) was calculated by measuring the

absorbance at 734 nm using a microplate reader. Additionally, the free radical erasing effect was expressed as a percentage (%) compared to the control group [29].

$$ABTS^{+} \text{ radical scavenging activity percentage (\%)} = (Abc - Abs)/Abc \times 100$$

(Abc: Absorbance of control, Abs: Absorbance of sample)

2.8. E-Screen Assay

The E-screen assay is a laboratory test used to assess the estrogenic activity of substances, particularly environmental chemicals. It measures the proliferative effect of estrogen on target cells and provides information about the potential of substances that mimic or interfere with estrogen activity. This assay is valuable for evaluating the endocrine-disrupting properties of compounds and their effects on the hormonal system. The name "E-screen" is derived from the term "estrogenic screen", reflecting its focus on screening for estrogenic effects [30]. The proliferation of breast adenocarcinoma (MCF-7) cells, a breast cancer cell line, was evaluated using E-screen analysis. The E-screen analysis method leverages the principle that MCF-7 overgrowth is induced by activating signal transmission from the ER in response to a test substance [30,33,34]. This established a proportional relationship between the estrogen content of the test substance and the degree of MCF-7 cell proliferation. Thus, the estrogenic effect was confirmed by observing cell proliferation in the presence of an ER ligand. To eliminate the effects of external estrogen, a phenol red-free medium containing charcoal-stripped serum was used as the cell culture medium. MCF-7 cells were inoculated into a 48-well plate and cultured for 24 h, after which the samples were added. Additionally, for the antagonist confirmation test, ER antagonists (ICI 182,780; Fulvestrant, Sigma Aldrich, St. Louis, MO, USA) were administered, and their effects were compared. The positive control group used the Queen's expedition, which contains a commercially available red clover dry extract, to improve menopausal symptoms. After culturing the cells for approximately 144 h (6 days), the Ez-Cytox (DoGEN, Suwon, Republic of Korea) reagent was added and incubated for 1 h. Absorbance was measured at a wavelength of 450 nm by SPARK 10M (Tecan, Männedorf, Switzerland) The average value of each sample was calculated from three experiments. The ratio of the absorbance of the sample group to that of the absorbance of the control group without the sample was expressed as a percentage (%) and compared using a graph. The proliferation rate of MCF-7 cells induced by the test substance is expressed as a percentage relative to that of the untreated group.

2.9. Preparation of HPLC Samples and HPLC Condition

For the quantitative analysis of FRs 1–14 extracts, 30 mg of FRs 1–3, FRs 5–14 extracts were dissolved in 1 mL of MeOH, resulting in a concentration of 30 mg/mL. For the FR 4 extract, 5 mg was dissolved in 1 mL of MeOH and prepared at a concentration of 5 mg/mL. After complete dissolution with an ultrasonic shaker for 20 min, the solutions were filtered through a PVDF membrane filter with a pore size of 0.45 μm to prepare the sample solutions. The quantitative analysis of compounds **1**–**7** was performed using an HPLC system with a reversed-phase INNO C_{18} column (250 × 4.6 mm, particle size: 5 μm). The column oven temperature was maintained at 30 °C for both the inlet and the outlet, with a sample injection volume of 10 μL. Gradient elution conditions were applied to mobile phases A and B, with a flow rate of 1.0 mL/min. The mobile phase A and B consisted of 0.1% trifluoroacetic acid in water and ACN, respectively. The detector wavelength was set to 254 nm, and the elution program was as follows: 95% mobile phase A was initiated and maintained for 10 min. Subsequently, the mobile phase A ratio was reduced to 70% from 10 to 20 min, gradually adjusted to 30% from 20 to 40 min, and finally reaching 0% mobile phase A from 40 to 42 min. Maintaining 100% mobile phase B for 44 min, the composition was returned to 95% mobile phase A from 44 to 50 min and held at 95% for an additional 10 min.

2.10. Calibration Curves

The standard compound stock solution for compounds **1**–**7** in was dissolved in MeOH to create a 1 mg/mL solution. A standard stock solution was prepared at an appropriate concentration, ensuring a minimum of five points for the calibration curve in quantitative analysis, utilizing the peak areas. Linearity was assessed using a calibration curve, and the correlation coefficient (r^2) demonstrated specificity, proving to be 0.99 or higher. The contents of compounds **1**–**7** in the sample were calculated using the session equation of the calibration curve, where the calibration functions represent the *Y*-axis as the peak area and the *X*-axis as the concentration. All samples were subjected to at least three repeated analyses, and the results are presented as the mean ± standard deviation (SD).

2.11. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as mean ± SD or standard error of the mean (SEM). Data were analyzed using a one-way analysis of variance, and statistical significance was defined using Tukey's test at $p < 0.05$.

3. Results

3.1. Total Polyphenol and Flavonoid Content

The total polyphenol and flavonoid contents were calculated using the Folin-Ciocalteu method (Table 1).

Table 1. Total polyphenol and flavonoid content of FRs 1–14 extract.

Sample	Content (mg/g Extract)	
	Total Polyphenol	**Total Flavonoid**
FR 1	46.6 ± 3.6	24.6 ± 4.8
FR 2	189.5 ± 15.2	24.6 ± 4.8
FR 3	68.4 ± 15.2	9.4 ± 1.7
FR 4	268.8 ± 10.9	25.7 ± 1.2
FR 5	42.1 ± 5.7	19.9 ± 4.0
FR 6	225.7 ± 18.0	19.1 ± 3.4
FR 7	68.5 ± 11.4	15.8 ± 2.2
FR 8	57.5 ± 9.5	6.0 ± 1.8
FR 9	145.9 ± 3.8	38.7 ± 4.7
FR 10	151.2 ± 22.5	34.4 ± 2.8
FR 11	151.5 ± 8.5	47.3 ± 3.8
FR 12	11.8 ± 4.5	57.0 ± 13.3
FR 13	210.6 ± 12.8	45.3 ± 1.2
FR 14	146.8 ± 22.9	20.1 ± 2.4

A. blitum (FR 1), *C. ovata* (FR 2), *Cnidium japonicum* (FR 3), *Cercidiphyllum japonicum* (FR 4), *O. japonica* (FR 5), *A. dioicus* (FR 6), *K. paniculate* (FR 7), *P. sinensis* (FR 8), *A. rugosa* (FR 9), *D. albus* (FR 10), *L. erythrocarpa* (FR 11), *G. biloba* (FR 12), *T. sinensis* (FR 13), and *M. sieboldii* (FR 14). The data represent the mean ± SD of triplicate experiments.

Significant amounts of polyphenols exceeding 100 mg/g were observed in FRs 1, 4, 6, 9, 10, 11, 13, and 14, with FR 4 containing the highest amount at 268.8 mg/g. The sample with the lowest polyphenol content was FR 12, containing 11.8 mg/g. Total flavonoids were present in substantial amounts, exceeding 25 mg/g, in FRs 2, 4, 9, 10–13 samples, with FR 12 containing the highest flavonoid content of 57.0 mg/g. The results showed independent tendencies for total polyphenols and flavonoids, with FR 8 containing the lowest amount of flavonoids (8.0 mg/g). The total flavonoid and polyphenol content serve as valuable indicators for evaluating the contents of physiologically active substances in plants. Although most polyphenols are classified as flavonoids, not all belong to this category. Therefore, the evaluation of total polyphenol content may differ from the assessment of flavonoid content. Although specific content analysis may be required to assess the

content of individual compounds, these measurements offer insights for evaluating and comparing the chemical properties of plants.

3.2. Quantification and Comparative Analysis in HPLC

Quantitative HPLC analysis was conducted to compare the contents of compounds **1–3** in FRs 1–14. To verify the suitability of the analytical method, the r^2 value of the calibration curves for the standard solutions was examined. The r^2 value obtained for all compounds **1–3** was 0.9999, indicating superior linearity compared to that of the standard (Table 2).

Table 2. The calibration curves of compounds **1–3**.

Compound	t_R	Calibration Equation	Correlation Factor, r^2
caffeic acid (1)	20.2	Y = 19762X + 52,721	0.9999
p-coumaric acid (2)	22.4	Y = 5189X + 44,545	0.9999
quercetin (3)	27.7	Y = 400,77X + 108,244	0.9999

t_R: retention time. Y: peak area, X: concentration of the standard (μg/mL). r^2: correlation factor for data points on the calibration curve.

The concentration of each compound in the sample was determined by substituting the peak area of the corresponding material into the regression equation of the calibration curve for each compound (Table 3).

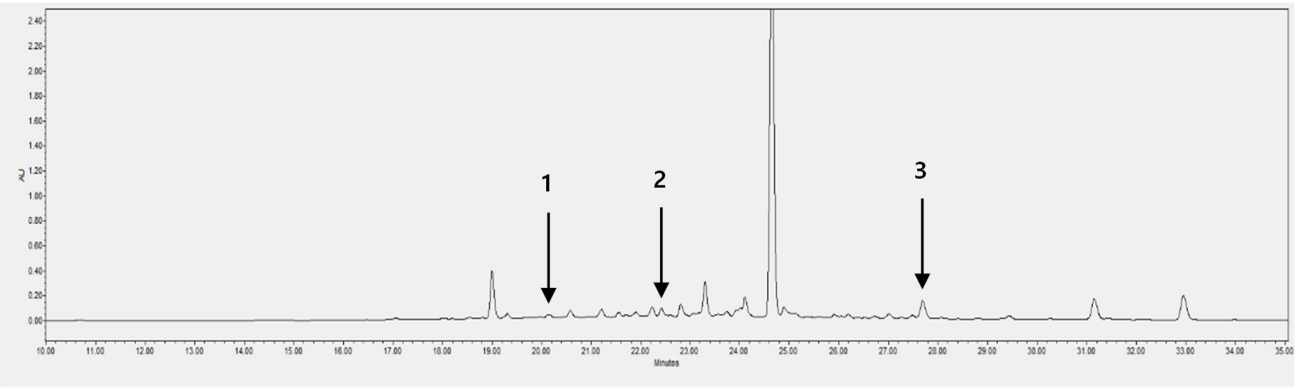
Table 3. Content of compounds **1–3** in FRs 1–14 extract.

Sample	Content (mg/g Extract)			
	1	**2**	**3**	**Total**
FR 1	0.87 ± 0.01	1.75 ± 0.01	tr	2.62
FR 2	ND	31.65 ± 0.25	ND	31.65
FR 3	0.13 ± 0.00	1.24 ± 0.02	0.61 ± 0.00	1.98
FR 4	ND	80.17 ± 0.72	0.41 ± 0.00	80.58
FR 5	0.89 ± 0.01	3.35 ± 0.06	ND	4.24
FR 6	14.99 ± 0.07	10.41 ± 0.08	tr	25.40
FR 7	ND	ND	ND	ND
FR 8	ND	0.43 ± 0.02	tr	0.43
FR 9	0.93 ± 0.01	12.63 ± 0.01	tr	13.56
FR 10	ND	3.68 ± 0.03	tr	3.68
FR 11	0.39 ± 0.01	0.83 ± 0.04	1.28 ± 0.00	2.50
FR 12	0.27 ± 0.00	ND	ND	0.27
FR 13	tr	35.29 ± 0.27	0.58 ± 0.00	35.87
FR 14	9.72 ± 0.02	ND	ND	9.72

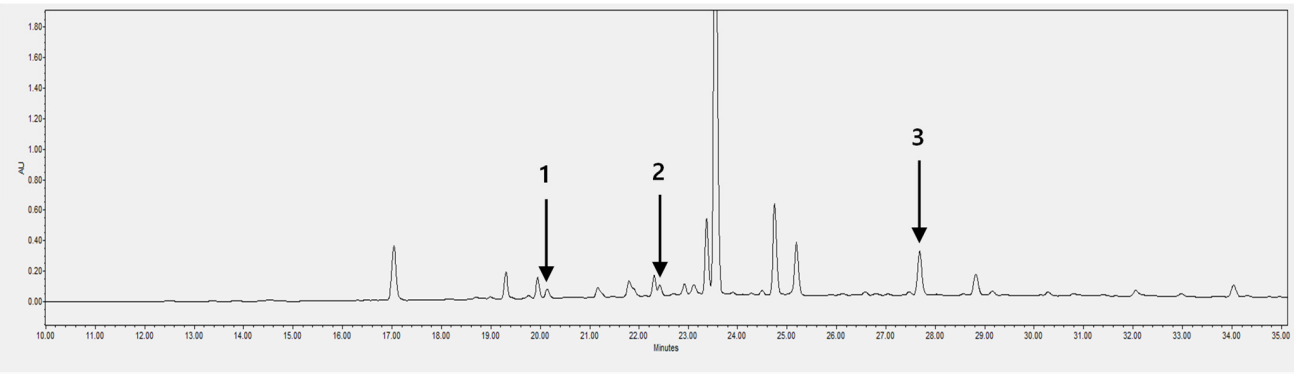
A. blitum (FR 1), *C. ovata* (FR 2), *Cnidium japonicum* (FR 3), *Cercidiphyllum japonicum* (FR 4), *O. japonica* (FR 5), *A. dioicus* (FR 6), *K. paniculate* (FR 7), *P. sinensis* (FR 8), *A. rugosa* (FR 9), *D. albus* (FR 10), *L. erythrocarpa* (FR 11), *G. biloba* (FR 12), *T. sinensis* (FR 13), and *M. sieboldii* (FR 14). tr: trace; ND: not detected.

HPLC chromatograms of compounds **1–3** and samples FRs 1–14 are shown in Figure 1. The duration of each standard substance was checked to confirm that it was a distinct analysis method, allowing for the separate analysis of each peak in the samples. The compound contents in 1 g of the sample extract were calculated, revealing that compound **1** had the highest content at 14.99 mg in FR 6. This was followed by FR 14, which was the second largest sample with compound **1**, calculated at 9.72 mg. Notably, FR 4 contained the highest amount of compound **2** (80.17 mg/g extract). Although compound **3** was present in relatively few samples, it had the highest content of FR 11. FR 4 had the highest total content of compounds **1–3**, while FR 7 did not detect any compounds. Despite being commonly occurring phytochemicals in plants, the contents and types of these 20 compounds vary depending on the plant species. Thus, it can be considered a versatile material with

applications that depend on its content. The content in the dried raw material can be calculated, considering the content and yield for 1 g of the extract.



(**a**)



(**b**)

Figure 1. HPLC chromatograms of (**a**) FR 3 and (**b**) FR 11 (**1**: caffeic acid, **2**: *p*-coumaric acid, and **3**: quercetin).

3.3. DPPH and ABTS$^+$ Radical Scavenging Activity

In vitro, antioxidant activity against DPPH and ABTS$^+$ was measured to assess the antioxidant capacity of FRs 1–14 extracts. The IC_{50}, representing the concentration-response relationship for biological effects and the concentration required to inhibit oxidation, was calculated to compare the antioxidant abilities (Table 4). Generally, lower concentrations indicated stronger activity. Compared to the standard substance ascorbic acid in DPPH, FR 13 exhibited the lowest IC_{50} value (0.7 μg/mL), indicating superior antioxidant ability. FR 4 showed a similar concentration (0.8 μg/mL), suggesting that it also possesses antioxidant properties. Conversely, FR 14 showed the lowest antioxidant activities with an IC_{50} value of 38.1 μg/mL. Additionally, FR 1 and FR 3 demonstrated lower antioxidant activity when tested using DPPH. For the ABTS$^+$ test, FR 4 exhibited the lowest IC_{50} value (μg/mL), while FR 12 showed the least activity, aligning with the DPPH results. Despite some discrepancies in the trends between the two antioxidant activity test methods, the samples with stable antioxidant activity potential were consistently identified, with FR 4 standing out as a notable candidate.

However, when comparing the results from quantitative analysis (Table 3) and antioxidant ability, it was evident that the samples with a higher total content of compounds demonstrated stronger antioxidant abilities and vice versa. These results suggest that the presence of compounds **1**–**3** contributes to the antioxidant ability of the samples.

Table 4. DPPH and ABTS+ radical scavenging activities of FRs 1–14 extract.

Sample	IC_{50} (μg/mL)	
	DPPH	$ABTS^+$
FR 1	24.2 ± 1.6	4.1 ± 1.5
FR 2	3.7 ± 0.1	0.9 ± 0.1
FR 3	21.0 ± 0.3	2.0 ± 0.7
FR 4	0.8 ± 0.0	0.3 ± 0.1
FR 5	14.1 ± 1.0	7.1 ± 0.2
FR 6	1.0 ± 0.0	0.9 ± 0.1
FR 7	4.3 ± 0.2	3.3 ± 0.4
FR 8	5.4 ± 0.4	5.8 ± 1.6
FR 9	1.5 ± 0.0	1.5 ± 0.1
FR 10	3.2 ± 1.3	3.6 ± 0.2
FR 11	1.0 ± 0.1	2.4 ± 0.2
FR 12	38.1 ± 2.1	8.2 ± 0.5
FR 13	0.7 ± 0.1	1.1 ± 0.0
FR 14	1.7 ± 0.0	2.2 ± 0.0

A. blitum (FR 1), *C. ovata* (FR 2), *Cnidium japonicum* (FR 3), *Cercidiphyllum japonicum* (FR 4), *O. japonica* (FR 5), *A. dioicus* (FR 6), *K. paniculate* (FR 7), *P. sinensis* (FR 8), *A. rugosa* (FR 9), *D. albus* (FR 10), *L. erythrocarpa* (FR 11), *G. biloba* (FR 12), *T. sinensis* (FR 13), and *M. sieboldii* (FR 14).

3.4. E-Screen Assay

The estrogenic cell proliferation effect of FRs 1–14 extracts was prepared and evaluated at concentrations ranging from 0 to 100 μg/mL. The positive control group, the Queens expedition, was used, and cell survival rates were determined at concentrations of 0, 25, 50, and 100 μg/mL. The Queen's expedition increased the cell survival rate to 111.0, 127.9, and 133.2%, respectively, and was significantly inhibited by ER antagonist ICI 182,780. ICI 182,780, also known as fulvestrant, is a non-steroidal ER antagonist. It is used as an anti-estrogen agent in cancer therapy, particularly for the treatment of hormone receptor-positive breast cancer. By binding to ER and inhibiting ER activity, ICI 182,780 helps suppress the growth of estrogen-sensitive breast cancer cells. It is employed as part of the hormonal treatment strategy for certain types of breast cancer. Among FRs 1–14, FR 4 exhibited an increase in cell survival at a concentration of 100 μg/mL, reaching 137.6%, which was significantly inhibited by the ER antagonist ICI 182,780 (Figure 2). Consequently, FR 4 demonstrated estrogenic activity, suggesting its potential as a candidate for menopausal activity, as verified by coefficient tree analysis.

3.5. Quantification Analysis of Phytochemical Compounds in FR 4

FR 4 demonstrated excellent content of the phytochemical compounds tested earlier, showing a significant effect on biological activity. The analysis focused on the specific components [maltol (**4**), chlorogenic acid (**5**), ellagic acid (**6**), and quercitrin (**7**)] that could be separated within FR 4. Quantitative HPLC analysis was used to compare the content of FR 4 for compounds **4**–**7**. The r^2 value of the calibration curve for the standard solution was determined to validate the analytical method. Both compounds **4**–**7** exhibited superior linearity compared to the standard, with r^2 values of 0.9998 and 0.9999, respectively (Table 5). The concentration of each compound in a sample was determined by substituting the peak area of the corresponding material into the regression equation of the calibration curve for each compound (Table 6). The chromatograms of compounds **4**–**7** and FR 4 sample are presented in Figure 3. Confirming the duration of each standard substance was verified as a distinct analysis method, allowing for the separate analysis of each peak in the samples. The calculation of the content of compounds in 1 g of the sample extract revealed that compound **5** was present at 17.83 mg/g.

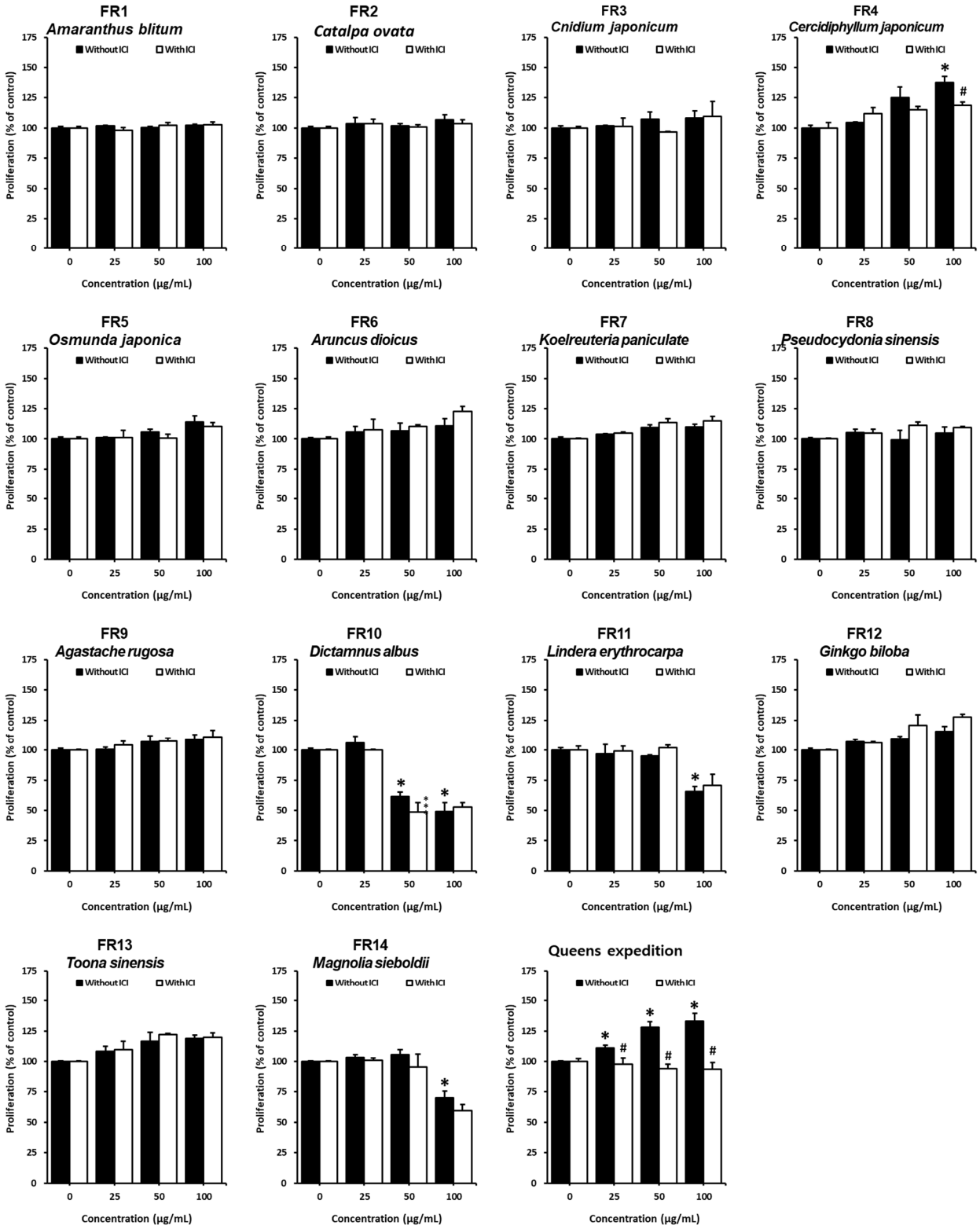


Figure 2. Effects of FRs 1–14 on the proliferation of MCF-7 cells. MCF-7 cells were treated with 25, 50, and 100 μg/mL extracts in the presence or absence of ICI 182,780 (ICI) for 144 h in a charcoal-detran-stripped media condition. Cell proliferation was determined using an EZ-Cytox kit. Data are presented as mean ± SEM of at least three independent experiments. * $p < 0.05$, *** $p < 0.001$ versus untreated cells. $^{\#}$ $p < 0.05$, versus each group without ICI 182,780.

Table 5. The calibration curves of compounds **4**–**7**.

Compound	t_R	Calibration Equation	Correlation Factor, r^2
maltol (**4**)	18.1	Y = 19.295X + 6.3309	0.9999
chlorogenic acid (**5**)	22.0	Y = 8.7066X + 108.21	0.9999
ellagic acid (**6**)	26.9	Y = 72.131X + 623.91	0.9999
quercitrin (**7**)	29.1	Y = 25.422X + 183.86	0.9998

t_R: retention time. Y: peak area, X: concentration of the standard (μg/mL). r^2: correlation factor for data points on the calibration curve.

Table 6. Contents of compounds **4**–**7** in FR 4.

Compound	Content (mg/g Extract)
maltol (**4**)	4.68 ± 0.03
chlorogenic acid (**5**)	17.83 ± 0.65
ellagic acid (**6**)	5.03 ± 0.02
quercitrin (**7**)	1.06 ± 0.0

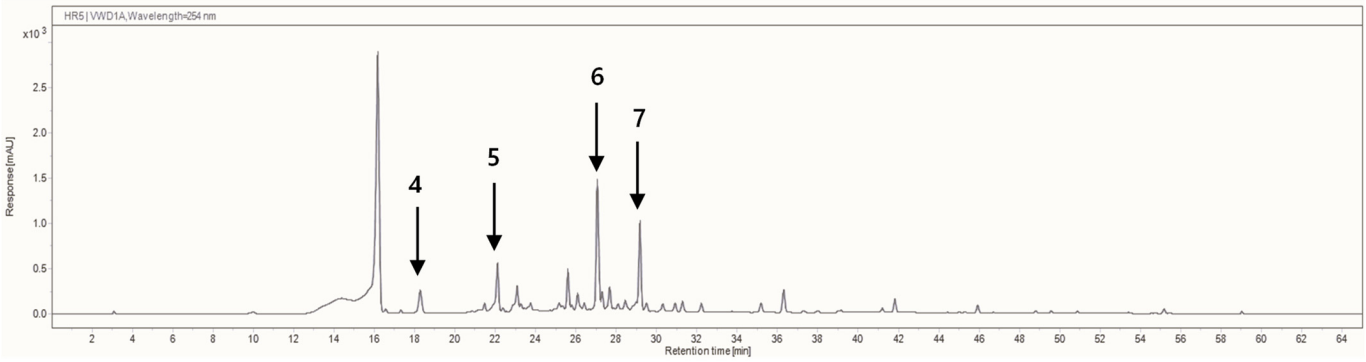


Figure 3. HPLC chromatogram of FR 4 (**4**: maltol, **5**: chlorogenic acid, **6**: ellagic acid, and **7**: quercitrin).

Previous studies have reported the isolation of four compounds from FR 4 leaves: quercetin 3-*O*-α-L-rhamnopyranoside, chlorogenic acid, quercetin 3-*O*-α-arabinofuranoside, and quercetin 3-*O*-β-D-xyloside. Additionally, various anthocyanins, including cyanidin 3-*O*-glucoside, 3-*O*-galactoside, 3-*O*-arabinoside, 3,5-di-*O*-glucoside, and 3-*O*-arabinoside-5-*O*-glucoside, as well as phenolic compounds such as chlorogenic acid and ellagic acid, and flavonols such as kaempferol and its 3-*O*-glucoside, 3-*O*-rutinoside, and 3-*O*-sophoroside, along with quercetin and its derivatives such as 3-*O*-glucoside, 3-*O*-rhamnoside (quercitrin), 3-*O*-rutinoside, 3-*O*-sophoroside, and 3-*O*-arabinofuranoside, sexangularetin 3-*O*-glucoside, corniculatusin and its 3-*O*-diglucoside, have been identified in different parts of FR 4 [29]. In this study, in addition to compounds previously reported, it is noteworthy that *p*-coumaric acid and maltol were isolated from the leaves and stems of FR 4 for the first time, to the best of our knowledge.

3.6. DPPH and ABTS$^+$ Radical Scavenging Activity of Phytochemical Compounds

In the in vitro assays, ABTS$^+$ and DPPH were used to assess the antioxidant potential of the phytochemical compounds in FR 4 (Table 7). In the DPPH test, compounds **2** and **7** showed undetectable concentrations, displaying notable antioxidant activity compared to that of ascorbic acid. No significant differences were observed in the ABTS$^+$ analysis results. Compounds **2** (*p*-coumaric acid) and **7** (quercitrin) were a phenolic acid and a flavonoid, respectively. They have been reported to possess antioxidant properties in many previous studies [30–40]. However, these compounds showed antioxidant capacity only in the ABTS$^+$ assay. DPPH and ABTS$^+$ are both commonly used methods to measure antioxidant activity; however, they operate based on different principles and mechanisms [41–49]. Therefore, it can be the reason compounds **2** and **7** showed antioxidant ability through ABTS$^+$ and

not through DPPH. Nevertheless, further research is warranted to determine the specific compounds that have a significant impact on antioxidant activity.

Table 7. DPPH and $ABTS^+$ radical scavenging activities of compounds **2**–**7**.

Compound	IC_{50} (μg/mL)	
	DPPH	$ABTS^+$
p-coumaric acid (**2**)	ND	0.1 ± 0.0
quercetin (**3**)	0.1 ± 0.0	0.4 ± 0.0
maltol (**4**)	0.1 ± 0.0	0.6 ± 0.0
chlorogenic acid (**5**)	0.3 ± 0.0	0.4 ± 0.0
ellagic acid (**6**)	0.3 ± 0.0	0.3 ± 0.0
quercitrin (**7**)	ND	0.2 ± 0.0

The data represent the mean value ± SD of triplicates. ND: not detected.

3.7. E-Screen Assay of Phytochemical Constituents

As the above experiment revealed that compound **1** was absent in FR 4, the estrogen cell proliferation effects of compounds **2**–**7** were evaluated at concentrations ranging from 0 to 100 μg/mL. Among the six compounds, quercetin promoted cell proliferation at concentrations of 12.5, 25, and 50 μM, and ICI 182,780 treatment inhibited cell antagonist ICI 182,780 (Figure 4). This indicates that quercetin has an estrogenic effect on proliferating cells in an ER-dependent manner. Quercetin was found to stimulate proliferation specifically in ER-positive cells, suggesting an ER-dependent mechanism, with a preference for activating ERβ over ERα, akin to phytoestrogen-like activity observed for genistein [46,47].

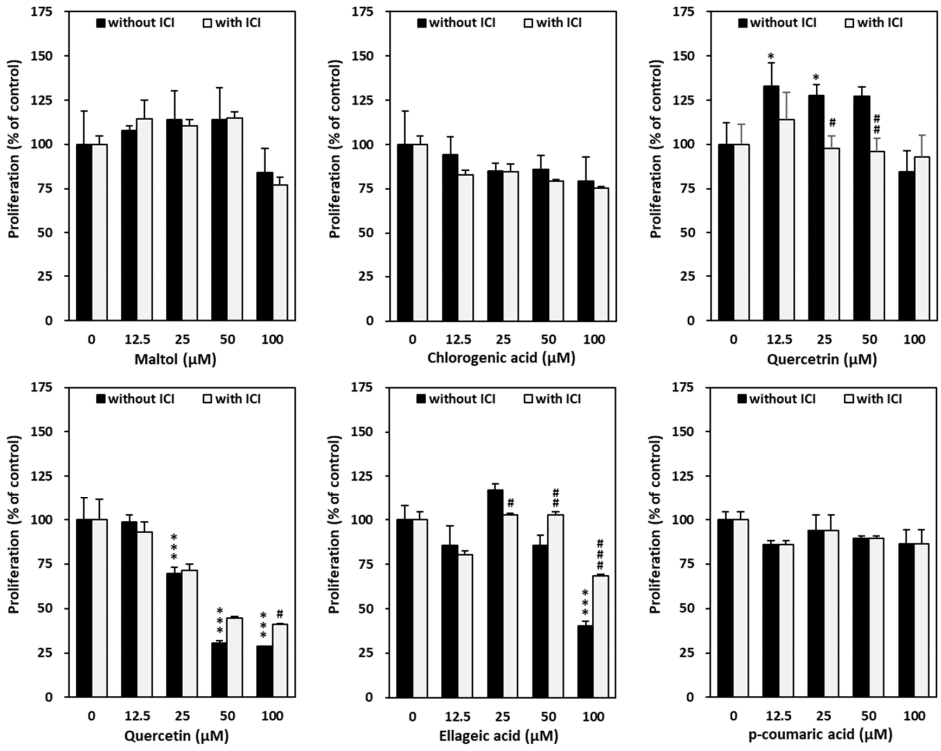


Figure 4. Estrogenic effects of compounds **2–7** on MCF-7 cell proliferation MCF-7 cells were treated with 12.5, 25, 50, and 100 μM compounds in the presence (white bar) or absence (black bar) of ICI 182,780 (ICI) for 144 h in a charcoal–dextran stripped media condition. Cell proliferation was determined using an EZ-Cytox kit. Data are presented as mean ± SEM of at least three independent experiments. * $p < 0.05$ and *** $p < 0.001$ versus untreated cells. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus each group without ICI 182,780.

These findings highlight the importance of continued research on the extracts and functional components of FR 4, and the potential discovery of components that contribute to menopausal activities is expected to advance FR 4 research significantly.

Figure 2 shows the effects of an extract containing a combination of compounds derived from natural sources, whereas Figure 4 illustrates the effects of a pure substance, an isolated compound devoid of the accompanying components. The discrepancy observed between these two sets of results underscores the necessity of considering the form and composition of a substance in pharmaceutical and medicinal research. This disparity, in effect, suggests that synergistic interactions among the compounds within an extract may yield physiological responses distinct from those elicited by individual, isolated substances. Moreover, it implies a potential complexity in the pharmacological mechanisms underlying the observed effects, warranting further investigation into the interactions among the various components within natural extracts.

4. Conclusions

Forest plant resources, including fragrance substances from plants, animals, and microorganisms, are valuable for enhancing taste, color, and aroma. Phytochemicals produced by plants during growth, mainly phenolic compounds, play a crucial role as major contributors to antioxidant activity, as emphasized in numerous studies [1,8]. Women undergoing menopause are prescribed hormone replacement therapy to relieve these symptoms; however, concerns about its side effects have led many women to choose non-hormonal therapies [26,28]. Among non-hormonal therapies, natural products are emerging as alternatives to safely and effectively manage symptoms and may improve menopausal symptoms through their estrogenic properties. In this study, we measured the total polyphenols, flavonoids, and phytochemical compounds, along with their antioxidant activity, in a diverse range of forest plant resources, including *A. blitum* (FR 1), *C. ovata* (FR 2), *Cnidium japonicum* (FR 3), *Cercidiphyllum japonicum* (FR 4), *O. japonica* (FR 5), *A. dioicus* (FR 6), *K. paniculate* (FR 7), *P. sinensis* (FR 8), *A. rugosa* (FR 9), *D. albus* (FR 10), *L. erythrocarpa* (FR 11), *G. biloba* (FR 12), *T. sinensis* (FR 13), and *M. sieboldii* (FR 14).

When the total polyphenols and flavonoids were measured, polyphenols exceeding 100 mg/g and flavonoids exceeding 25 mg/g were detected in some of the plants (Table 1). Total polyphenol and flavonoid content are valuable indicators for evaluating the content of bioactive substances in plants [48]. Although specific content analyses may be required to assess the content of individual compounds, these measurements provide insights for evaluating and comparing plant chemical properties. Subsequently, quantitative HPLC analyses were performed for three plant compounds: caffeic acid (**1**), *p*-coumaric acid (**2**), and quercetin (**3**). The results showed that the content in each plant differed (Table 3). Although these are common phytochemicals in plants, the content and types of compounds vary depending on the plant species [49]. Therefore, it can be considered a versatile material whose uses vary depending on its content.

The antioxidant activity against DPPH and $ABTS^{+}$ was measured to evaluate the antioxidant capacity of the plant extracts (Table 4). Plants with higher levels of compounds **1–3** have been shown to have stronger antioxidant capacity. These results suggest that the presence of this compound contributes to its antioxidant capacity. Estrogenic activity was assessed using E-screen (Figure 2). Some plants exhibit estrogenic activity, suggesting that they have the potential to improve menopausal symptoms.

Subsequently, quantitative analysis of four compounds, maltol (**4**), chlorogenic acid (**5**), ellagic acid (**6**), and quercitrin (**7**), was performed in FR 4, which showed the best results (Table 6). Additionally, the antioxidant and estrogenic cell proliferative effects of the compounds present in the plant were evaluated to identify the major contributors to plant activity (Table 7). The experimental results showed that FR 4 has high total polyphenol and flavonoid contents. E-screen analysis of estrogen activity indicates that it is a plant material with a high phytochemical compound content and excellent physiological activity (Figure 4). Additionally, *p*-coumaric acid (**2**), quercetin (**3**), maltol (**4**), chlorogenic acid (**5**),

ellagic acid (**6**), and quercitrin (**7**) identified in FR 4 are phytochemical compounds with excellent antioxidant properties. FR 4 has emerged as a potentially industrially applicable bioactive substance, providing essential data for future analyses and extraction methods applicable to this native Korean plant.

Future studies will undoubtedly build upon these findings, emphasizing the multifaceted nature of natural product research and comprehensively exploring the differential effects of extracts versus pure substances for the development of effective therapies.

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