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Inhibitory effect of *Sphagnum palustre* extract and its bioactive compounds on aromatase activity

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Abstract

Sphagnum palustre (a moss) has been traditionally used in Korea for the cure of several diseases such as cardiac pain and stroke. In this research, the inhibitory effect of *S. palustre* on aromatase (cytochrome P_{450} 19, CYP19) activity was studied. [1β-^{3}H] androstenedione was used as a substrate and incubated with *S. palustre* extract and recombinant human CYP19 in the presence of NADPH. *S. palustre* extract inhibited aromatase in a concentration -dependent manner (IC_{50} value: 36.4 ± 8.1 µg/mL). To elucidate the major compounds responsible for the aromatase inhibitory effects of *S. palustre* extract, nine compounds were isolated from the extract and tested for their inhibition of aromatase activity. Compounds **1**, **6**, and **7** displayed aromatase inhibition, while the inhibition by the other compounds was negligible.

Introduction

Cytochrome P_{450}, as a key enzyme in estrogen biosynthesis, is well-known for its conversion of C-19 androgens into C-18 estrogens (Chumsri et al., 2011). The enzyme is distributed in many tissues including gonads, brain, liver, adipose tissue, placenta, skin, bone, and endometrium as well as in the ovary and testis. In breast cancer patients, excess estrogen and aromatase levels have been observed, and it was previously reported that aromatase inhibition can be considered as one of the most efficient treatments in breast cancer therapy, especially for postmenopausal women (Cazzaniga and Bonanni, 2012; Renoir et al., 2013). In cases of metastatic breast cancer, aromatase inhibitor is used as first-line therapy, prior to the other options (Altundag and Ibrahim, 2006). However, there were reports on natural products with aromatase inhibition effects by several studies. Compared to aromatase inhibitors, these nature-deriving compounds exhibit higher tissue-specific inhibition as well as reduced toxicity (Balunas and Kinghorn, 2010; Balunas et al., 2008).

Sphagnum palustre (Sphagnaceae) is an aquatic moss. It is one of the most globally common types of moss. In Korea, *S. palustre* has long been used as a traditional medicine; the "Donguibogam" describes the use of *S. palustre* for the treatment of cardiac pain and stroke (Nam et al., 2011). A recent phytochemical investigation on *S. palustre* reported the isolation of phenolic, flavonoid, triterpenoid, and steroid compounds (Nam et al., 2011). However, in spite of its ubiquitous existence and medicinal usage, the phytochemical profile of *S. palustre* remains unclear.

In our previous study, the ethanol extract of *S. palustre* was reported to exhibit significant renoprotective effects against cisplatin-induced damage in kidney cells, with the relevant compounds having been identified (Kang et al., 2016). As part of our ongoing search for bioactive metabolites from *S. palustre*, we investigated the inhibition of *S. palustre* on aromatase (cytochrome P_{450} 19, CYP19) and the active compounds. No scientific evidence has been reported related to its inhibitory activity on aromatase. In this study, we

report the inhibitory effect of *S. palustre* extract and nine compounds (**1-9**) on aromatase activity using recombinant human CYP19 (Figure 1).

Materials and Methods

General experimental procedures

Optical rotations were obtained on a Jasco P-1020 polarimeter. IR spectra were acquired on a Bruker IFS-66/S FT-IR spectrometer. Circular dichroism spectra were recorded with a JASCOJ-810 spectropolarimeter. Ultraviolet (UV) spectra were acquired on an Agilent 8453 UV−visible spectrophotometer. Electrospray ionization mass spectra were recorded on a Waters UPLC-QTOF Xevo G2-S mass spectrometer. Nuclear magnetic resonance spectra were recorded on a Bruker AVANCE III 700NMR spectrometer operating at 700 MHz (^{1}H) and 175 MHz (^{13}C),with chemical shifts given in ppm (δ). Semi-preparative high performance liquid chromatography (HPLC) used a Shimadzu Prominence HPLC System with SPD-20A/20AV Series Prominence HPLC UV-Vis Detectors. Column chromatography was conducted with a silica gel 60 (Merck, 70–230 mesh and 230–400 mesh) and RP-C18 silica gel (Merck, 230–400 mesh). The packing material for molecular sieve column chromatography was sephadex LH-20. Merck precoated silica gel F254 plates and reverse-phase (RP)-18 F254s plates (Merck) were used for thin-layer chromatography.

Plant material

Whole dried *S. palustre* harvested from the pristine environment of the Southern West coast of New Zealand was purchased as a commercial grade production in August 2014. *S. palustre* was identified by one of the authors (K.H.K.). A voucher specimen (SKK-Mek-2014) was stored in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation

Dried *S. palustre* (827 g) was extracted for 1 day with 18 L of 100% ethanol at room temperature. The extract was then filtered and the filtrate was evaporated under reduced pressure using a rotavapor to obtain ethanol extract (5.2 g). The ethanol extract was successively divided by preparative reverse-phase HPLC equipped with a YMC-Pack Pro C18 RS column (250 mm x 20 mm i.d., 5 µm, 8 nm; YMC, Japan), using a gradient solvent system [water-acetonitrile (ACN): 0-20 min: 30% ACN; 20-40 min: 70% ACN; 40-60 min: 100% ACN, flow rate: 7 mL/min] to obtain six fractions (F1–F6).

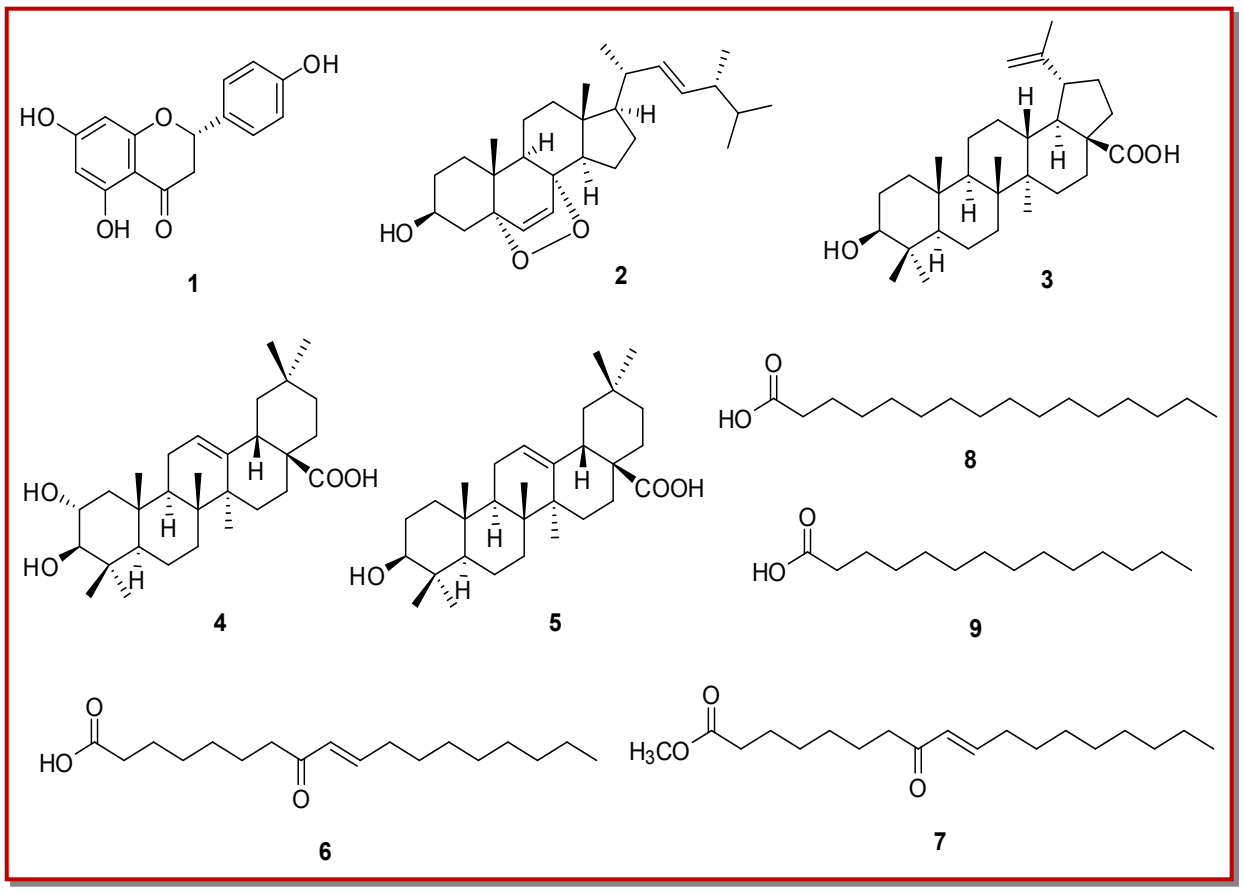

Figure 1: Chemical structures of the isolated compounds 1-9

Fraction F4 (1 g) was further purified using sephadex LH-20 column chromatography with 100% methanol and afforded five sub-fractions (F4-1 to F4-5). Sub-fraction F4-4 (92 mg) was purified using semi-preparative reversed-phase HPLC with a solvent system of 65% methanol using an ODS-M80 column (250 mm x 10 mm i.d., 4 µm, 8 nm: YMC) to furnish compound **1** (2.0 mg). Fraction F6 (800 mg) was purified using preparative reverse-phase HPLC with 100% methanol to yield six sub-fractions (F6-1 to F6-6). Sub-fraction F6-3 (262 mg) was subjected to a Sephadex LH-20 column chromatography and a solvent system of 100% methanol was used to give six sub-fractions (F6-3-1 to F6-3-6). Sub-fraction F6-3-3 (77 mg) was further separated using semi-preparative reversed-phase HPLC with 90% methanol to furnish compounds **4** (0.1 mg) and **5** (0.7 mg). Compound **3** (0.7 mg) was re-crystallized from sub-fraction F6-3-3 (13 mg). Sub-fraction F6-4 (31 mg) was purified by semi-preparative reverse-phase HPLC using 95% methanol as solvent to give compounds **6** (0.3 mg), **8** (7.7 mg), and **9** (0.1 mg). Sub-fraction F6-5 (25 mg) was purified by semi-preparative reverse-phase HPLC with 97% methanol as solvent to afford compound **2** (2.3 mg). Finally, sub-fraction F6-6 (74 mg) was separated using semi-preparative reverse-phase HPLC utilizing a gradient solvent system (water-methanol: 0-40 min: 90% methanol; 40-50 min: 100% methanol) to yield compound **7** (0.3 mg).

Aromatase enzyme activity (Video Clip)

Microsomal aromatase assay was conducted utilizing recombinant human CYP19 Supersomes® (BD Biosciences) with NADPH solution A and NADPH solution B (BD Biosciences). Solution A was composed of 31 mM $NADP^+$, 66 mM glucose-6-phosphate, and 66 mM $MgCl_2$ in H_2O; Solution B was composed of 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate. *S. palustre* extract and its isolates (**1-9**) were treated 25 nM [1β-^{3}H] androstenedione and NADPH solutions A and B, the reaction was started by adding 2 pmol Supersomes®, and incubated for 1 hour at 37°C according to manufacturer's protocol. The reactions were terminated by vortexing with chloroform. 5% charcoal/0.5% dextran was added to the aqueous supernatants to remove radioactive residues. The supernatant was obtained, and aromatase activity was calculated by measuring [^{3}H] H_20 using a liquid scintillation counter (LS-6500, Beckman counter).

Data analyses

Sigma Plot 12.0 (Jandel Science Software) and Excel 2007 (Microsoft) were employed for data analysis. Each *in vitro* assay was carried out at least in triplicate and the data from each assay are expressed as mean ± standard deviation (SD). Statistical analyses were carried out using SPSS version 21.0 software (SPSS Inc.). Differences between experimental groups were determined by Duncan's post hoc test following one-way analysis of variance (ANOVA). Differences with $p<0.05$ were considered statistically significant.

Results

The *S. palustre* extract inhibited activity of aromatase in a concentration dependent manner, with significant inhibition observed at ≥20 µg/mL (Figure 2). At a concentration of 50 µg/mL of *S. palustre* extract, the aromatase activity was lowered to 36.3 ± 7.3 of the level observed with control ($p< 0.01$).

To elucidate the bioactive compounds responsible for the aromatase inhibitory effects of *S. palustre* extract, total nine compounds were isolated and these were identified as (-)-naringenin (**1**) (Jeon et al., 2008; Prescott et al., 2002), ergosterol peroxide (**2**) (Krzyczkowski et al., 2009), betulinic acid (**3**) (Siddiqui et al., 1988), maslinic acid (**4**) (Taniguchi et al., 2002), oleanolic acid (**5**) (Seebacher et al., 2003), 8-monooxoelaidic acid (**6**) (Kawagishi et al., 2002), 8-monooxoelaidic acid methyl ester (**7**) (Dang et al., 2008), palmitic acid (**8**) (Couperus et al., 1978), and tetradecanoic acid (**9**) (Feldhues and Schafer, 1985; Narayanaswamy et al., 2006) by comparing their spectroscopic data including ^{1}H and ^{13}C NMR and MS data with those previously reported in

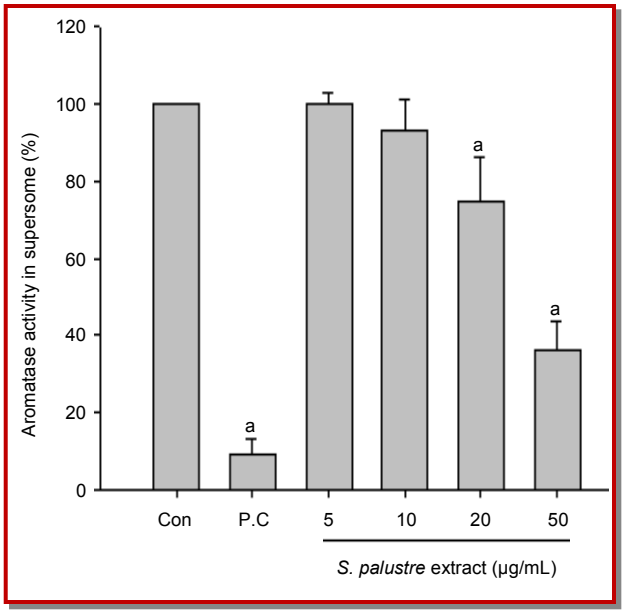

Figure 2: Inhibitory effects of *S. palustre* extract on aromatase activity. Tritiated androstenedione [1β-^{3}H] was mixed with various concentrations (5–50 µg/mL) of *S. palustre* extract treated with recombinant CYP19 (CYP BD-Supersomes™) for 1 hour. 10^{-6} M HA (4-androsten-4-ol-3,17-dione) was used as a positive control (P.C). The results are expressed as mean ± SD of three separate experiments for each group. *Represents the statistical difference from the vehicle control [Con, 0.1% Dimethyl sulfoxide (DMSO)]: $^a p< 0.01$

Table I

Aromatase inhibitory activity of compounds from *S. palustre*

Compound	Aromatase inhibition[a] (% of control)
1	64.8[a]
2	93.7
3	97.4
4	96.2
5	92.5
6	54.7
7	54.4
8	96.7
9	93.6

[a]All compounds tested were assayed at 10 μg/mL and the data shown represent the mean of duplicate tests. [a]$p<0.01$ for the comparison with vehicle control

other literatures. All of the isolates were tested for their inhibitory potential against aromatase activity. In the present study, compounds **1**, **6** and **7** displayed aromatase inhibitory effects, with inhibition by the other compounds being negligible (Table I).

Discussion

The ethanol extract of *S. palustre* inhibited aromatase activity in a concentration-dependent manner. Aromatase inhibition of *S. palustre* extract is comparable with green tea extract (IC_{50} in microsome: 28 μg/mL), a recognized inhibitor of aromatase (Satoh et al., 2002). Fungi also inhibited aromatase activity (Fatima et al., 2014).

The results of this study showed that there was significant inhibition of aromatase activity by compound **1**, (-)-naringenin, which is a main flavonoid component of *S. palustre*. Previous studies have reported that flavonoids inhibit the activity of aromatase (Campbell and Kurzer, 1993; Jeong et al., 1999; Ibrahim and Abul-Hajj, 1990). (-)-Naringenin has also been studied as a strong and moderate aromatase inhibitor in microsomes and recombinant human CYP19 (Balunas et al., 2008; Edmunds et al., 2005). Thus, one of flavonoids, the compound **1** is a potential aromatase inhibitor in *S. palustre* extract. In addition, two fatty acids, compounds **6** and **7** displayed potent aromatase inhibitor activity. A series of fatty acids have been explored for their potential aromatase inhibition, with aromatase inhibitor activity by several natural fatty acids being confirmed (Balunas et al., 2008). Presently, of the isolated fatty acids (compounds **6-9**), compounds **6** and **7** which contain an unsaturated ketone moiety, exhibited aromatase inhibitor activity. This suggests that the unsaturated ketone unit in fatty acids may be a key functional moiety for inhibition of aromatase activity.

Conclusion

S. palustre extract decreased estrogen biosynthesis by inhibiting aromatase activity. The active compounds responsible for the inhibition effect were identified. The identification of the inhibitory effects on aromatase of *S. palustre* extract and identification of the bioactive compounds are novel findings. *S. palustre* extract could inhibit aromatase activity, with compounds **1**, **6**, and **7** identified as the active components. The findings provide the foundation for the development of pharmacologically useful aromatase inhibitors.

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