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ORIGINAL ARTICLE

# Antioxidant and Anti-wrinkle Effects of *Orostachys japonicus* Extracts as Anti-aging Cosmetic Agents

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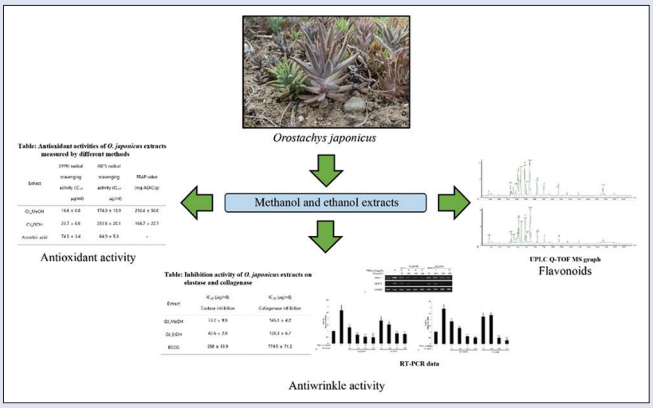
## ABSTRACT

**Background:** *Orostachys japonicus* has traditionally been used for antifever, anti-inflammation, and anticancer. *O. japonicus* has not yet been studied for cosmetic effects. **Objectives:** In this study, *O. japonicus* extracts were investigated to verify the possibility of them as anti-aging cosmetic agents. **Materials and Methods:** *O. japonicus* were used to prepare experimental extracts using 90% methanol (MeOH) or ethanol (EtOH) as solvent. Safety and efficacy tests applied in this work are as follows: Folin-Denis' method for total phenolic contents, liquid chromatography for component analysis, 3-[4,5-dimethyl thiazol-2-yl]-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay for cytotoxicity, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assay for antioxidant activity, biochemical inhibition assays of elastase activity, collagenase activity, matrix metalloproteinase-1 (MMP-1) and MMP-3 mRNA expression), and MMP-1 protein production for anti-wrinkle activity, and tyrosinase activity inhibition assay for whitening activity. **Results:** This research revealed that *O. japonicus* extracts contained various flavonoids, showed no adverse cytotoxicity up to 300 or 500 μg/mL in HS68 and B16F10, exhibited significant antioxidant activity, exerted remarkable anti-wrinkle activity, and represented useful whitening activity. **Conclusion:** This study suggested that *O. japonicus* extracts could be utilized to develop anti-aging cosmetic agents exerting useful whitening activity as well as excellent antioxidant and anti-wrinkle activities.

**Key words:** Anti-aging, antioxidant, anti-wrinkle, MMP-1, MMP-3, *Orostachys japonicus*

## SUMMARY

- In this study, the antioxidant activity, anti-wrinkle activity, and whitening activity of *Orostachys japonicus* extract were comprehensively investigated. As a result of the study, cytotoxicity was not observed, and the antioxidant activity and anti-wrinkle activity were excellent. In addition, the whitening activity showed a significant level. Therefore, the extracts of *O. japonicus* are expected to be utilized as cosmetic materials for anti-aging.




**Abbreviations used:** OJ: *Orostachys japonicus*; MeOH: Methanol; EtOH: Ethanol; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric reducing antioxidant power; FALGPA: 2-furanacryloyl-Leu-Gly-Pro-Ala; MMP-1: Matrix metalloproteinase-1; UPLC Q-TOF MS: Ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry

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## INTRODUCTION

The aging of the skin can be divided into intrinsic and extrinsic ones. Intrinsic skin aging is caused by the passage of time, whereas extrinsic aging is usually by exposure to sunlight.[1] When skin is exposed to UV, reactive oxygen species (ROS) is produced, which accelerates skin aging by increasing the expression of matrix metalloproteinases (MMPs), impairing the extracellular matrix (ECM) that keeps the three-dimensional structure of the skin.[2,3] Antioxidants produced in the body can remove overproduced ROS byproducts.[4] However, the antioxidant effect is decreased when aging and overexposure to UV go along. Recently, natural antioxidants have been developed in foods, cosmetics, and pharmaceuticals to replace some synthetic antioxidants that are limited in their use due to the possibility of carcinogenicity.[5,6]

Collagen and elastin belonging to the ECM protein are produced by fibroblasts and help maintain skin elasticity, flexibility, and tension.[7] In fibroblasts, ECM is reduced by UV, that is, collagen biosynthesis is lowered and elastin denaturation is elevated.[8] In addition, MMPs denature and degrade ECM such as collagen, elastin, proteoglycan, and fibronectin, thereby destroying the epidermal-skin boundary and accelerating dermis degradation, and finally inducing wrinkle formation.[9]



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Melanin, a pigment biosynthesized from l-tyrosine in melanocytes, plays an important role in protecting skin cells by absorbing UV.[10,11] Decreased melanogenesis results in sunburn, mottling, and gray hair due to skin damage by UV exposure.[12] The key enzyme in the biosynthesis of melanin is tyrosinase, which is involved in the synthesis of l-dopaquinone from l-tyrosine, consequently resulting in the production of pheomelanin and eumelanin.[13]

Recently, the anti-aging and antioxidant effects of plant extracts have been studied widely. Antioxidant activity was reported in extracts from *Coffea arabica* and *Quercus robur*.[14,15] Elastase inhibition activity was shown in *Callistemon lanceolatus*, *Morinda citrifolia*, and *Glycine max*.[16-18] Whitening effect (tyrosinase inhibition) was reported in *Rhodiola rosea*, *Tagetes erecta*, and *Cassia fistula*.[19-21]

*Orostachys japonicus*, a medicinal herb is distributed in East Asia.[22] *O. japonicus* has been utilized in traditional medicine for antifever, anti-inflammation, hemostasis, and anticancer.[22-24] In previous studies this revealed hypolipidemic, hypoglycemic, antiulcerogenic, anti-inflammatory, anticancer, bone-protective, hepatoprotective, and immunomodulating effects.[23-38]

Since functional cosmetic effects of *O. japonicus* have not been studied, we focused on the investigation of antioxidant, anti-wrinkle, and skin whitening effects of methanol (MeOH) or ethanol (EtOH) extracts from *O. japonicus* to identify their potential of them as anti-aging cosmetic agents.

## MATERIALS AND METHODS

### Materials

*O. japonicus* was obtained from greenhouse in Miryang, Gyeongnam, Korea. DMEM, TRIzol, and FBS were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). 3-[4,5-dimethyl thiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-Tri (2-pyridyl)-s-triazine (TPTZ), trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO), the oligopeptide 2-furanacryloyl-Leu-Gly-Pro-Ala (FALGPA), epigallocatechin gallate (EGCG), Folin-Denis' reagent, ferric chloride, potassium ferricyanide, potassium persulfate, ascorbic acid, elastase from porcine pancreas, *N*-succinyl-(ala) 3-p-nitroanilide, l-tyrosine, tyrosinase from mushroom, arbutin, and ursolic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Recombinant human tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) from R&D Systems, Inc. (Minneapolis, MN, USA). TOPscript™ cDNA synthesis kit and TOPreal™ qPCR PreMIX (SYBR Green, low ROX) from Enzynomoics, Inc. (Daejeon, Korea). AccuPower® PCR PreMix from Bioneer corp. (Daejeon, Korea). Human MMP-1 ELISA kit from Abcam (Cambridge, MA, USA). Ethics committee number: INJE 2020-03-006 Date of approval :April 20, 2020.

### Preparation of *O. japonicus* extracts

*O. japonicus* was separately extracted at 40°C with two different solvents, 90% methanol or 90% ethanol for 24 h. Each extract was prepared using filter paper (Whatman, UK) and evaporated under a vacuum, and then freeze-dried for further study. The residue obtained from methanol and ethanol were named OJ_MeOH and OJ_EtOH, respectively.

### Total phenolic contents assay

The phenolic contents were determined by Folin–Denis' reagent. *O. japonicus* extracts (200 μL) and Folin–Denis' reagent (200 μL) were mingled and reacted at room temperature for 3 min. Furthermore, 2 M sodium carbonate (400 μL) and distilled water (200 μL) were added additionally. After 30 min, 420 nm of absorbance was assayed with microplate spectrophotometer (Power wave XS2, BioTek, USA). Phenolic contents were represented as gallic acid equivalents (GAE) in mg/g of dried extracts.

### UPLC Q-TOF MS analysis

The extracts were analyzed by ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry.

(UPLC Q-TOF MS). Extracts were analyzed using an ACQUITY UPLC H-class system (Waters, USA). Mass spectrometry data were gained on the range *m/z* 100–1200. An ACQUITY UPLC BEH $C_{18}$ column (2.1 100 mm, 1.7 μm, Waters, USA) was applied. The solvent system consisted of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v) with the increasing concentration elution applied. The flow rate of solvent was kept at 0.3 mL/min, and 35°C was chosen as the operating temperature.

### Cytotoxicity assay

The B16F10 melanoma cells were provided from Korean cell line bank (Seoul, South Korea). HS68 human fibroblast cells were obtained from **American Type Culture Collection** (ATCC) (Manassas, VA, USA). DMEM containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine were used for both cells. The cells were grown at 37°C under the condition of 5% $CO_2$. When the confluence of cells was above 70%, subculture was performed using 0.5 mM EDTA and 0.05% trypsin. The cytotoxicity of *O. japonicus* extracts was measured by MTS assay, which was conducted by modifying the protocol of Mosmann method.[38] All extracts were dissolved in DMSO. The cells were put on 96-well plate at the density of $5 \times 10^4$ and cultured for 24 h. Cell culture medium was removed and rinsed three times with phosphate-buffered saline (PBS). Media containing extracts (200 μL) were applied to cells, and after 24 h, 20 mg/mL of MTS was put to every well and they were kept for 2 h and measured using a microplate spectrophotometer at a wavelength of 450 nm.

### Assay of antioxidant activity

Antioxidant activity of *O. japonicus* extracts was measured by DPPH free radical scavenging activity assay, ABTS free radical scavenging activity assay, and FRAP assay. DPPH scavenging activities needed 0.2 mM of DPPH in absolute ethanol.[39] DPPH (100 μL) was put into extracts (200 μL), and the mixtures were kept at 25°C. Then, 517 nm of absorbance was assessed by microplate spectrophotometer. l-ascorbic acid was chosen as a positive control.

ABTS scavenging activity assay was performed as follows: an equal volume of 2.45 mM potassium persulfate and 7 mM ABTS was mixed and left for 24 h in the dark to obtain the ABTS • + radical solution. It was mixed with 50% methanol to the absorbance of 0.700 (± 0.05) at 745 nm. ABTS scavenging activity was assessed by adding 20 μL of extract to 180 μL of ABTS solution. l-ascorbic acid was chosen as a positive control. Doses (μg/mL) of DPPH and ABTS at 50% scavenging were expressed as $IC_{50}$ values.

FRAP assay was done in the following way: FRAP reagent was produced by adding 0.3 M of acetate buffer (pH 3.6) to 20 mM ferric chloride and 10 mM TPTZ at the ratio of 10: 1: 1 (v/v/v). The extracts (20 μL) were added to the FRAP reagent (180 μL), and the absorbance at the wavelength of 593 nm was assessed by a microplate spectrophotometer. FRAP values were represented as ascorbic acid equivalents antioxidant capacity (AEAC) in mg/g of dried extracts.

## Assay of elastase and collagenase inhibition activities

Anti-wrinkle enzyme inhibition activities of *O. japonicus* extracts were determined by elastase and collagenase inhibition activities. Elastase inhibition activity was assessed by *N*-succinyl-(ala) 3-p-nitroanilide as a substrate. Briefly, 0.5 U/mL of elastase was prepared in 50 mM tris-HCl buffer (pH 8.6). The substrate was dissolved at 2 mM in tris-HCl buffer. The extract (20 μL) was mixed with the substrate (30 μL) and tris-HCl buffer (85 μL), and finally, elastase (15 μL) was applied in 96-well plate. The mixtures were left at 25°C, and 410 nm of the absorbance was assessed with the microplate spectrophotometer. The elastase inhibition activity was yielded using the following equation:

Elastase inbition activity (%) = [1 -(Exp.-Blank)/ Control] × 100

Exp: Absorbance of a sample containing elastase

Blank: Absorbance of a sample lacking elastase

Control: Absorbance of a solvent containing elastase

Collagenase inhibition activity was assayed using *Clostridium histolyticum* collagenase and FALGPA as an enzyme and a substrate. The assay was prepared in 0.05 M tricine buffer (0.001 M $CaCl_2$ and 0.4 M NaCl, pH 7.5). Collagenase (1 U/mL) and FALGPA (2 mM) were dissolved in 0.05 M tricine assay buffer. The extracts (30 μL) were incubated with collagenase (10 μL) and tricine buffer (60 μL) at 37°C for 20 min. Then FALGPA (20 μL) was put in the mixture. EGCG was chosen as a positive control. Collagenase activity was assessed at 340 nm with a microplate spectrophotometer.

## Assay of mRNA expressions of MMPs

Inhibition of mRNA expressions of MMPs by *O. japonicus* extracts in HS68 cells was assayed using RT-PCR and real-time RT-PCR. HS68 cells were put at about cells/well. Twenty-four hour later, 10 ng/mL TNF-α and *O. japonicus* extracts were added and cultured for 18 h. The total RNAs were obtained from HS68 cells by TRIzol reagent used in the standard procedure. cDNA was produced from 2 μg of total RNA using TOPscript™ cDNA synthesis kit. RT-PCR was conducted using the AccuPower® PCR PreMix. The primer was synthesized in the following way: MMP-1: 5'-CTGAGGGTCAAGCAGACATC-3' (forward) and 5'-GCTAGGGTACATCAAAGCCC-3' (reverse); MMP-3: 5'-CACTCACTCACAGACCTGAC-3' (forward) and 5'-CCAGCTCGTACCTCATTTCC-3' (reverse); GAPDH: 5'-ATCATCAGCAATGCCTCCTG-3' (forward) and 5'-CCTGCTTCACCACCTTCTTG-3' (reverse). RT-PCR was conducted under the condition of 30 cycles of 95°C for 30 s, 57°C annealing for 30 s, and 72°C extension for 45 s. PCR samples were confirmed using 1.2% agarose gel.

Real-time RT-PCR was conducted using the TOPreal™ qPCR PreMIX (SYBR Green, low ROX). All quantitations were normalized to endogenous control, GAPDH.

## Assay of inhibition of MMP-1 protein production

Inhibition of MMP-1 protein production by *O. japonicus* extracts was assayed using ELISA. HS68 cells were put at about 1 ×$10^5$ cells/well. Twenty-four hour later, 10 ng/mL TNF-α and *O. japonicus* extracts were added and incubated for 18 h. The level of MMP-1 production was assayed by Human MMP-1 ELISA kit.

## Assay of tyrosinase inhibition activity

l-tyrosine and tyrosinase obtained from mushrooms were used as a substrate and an enzyme. In addition, 3 mM l-tyrosine (20 μL) and 0.1 M phosphate buffer (pH 6.8, 200 μL) were mixed with the extract (60 μL). Furthermore, 2,000 U/mL tyrosinase (20 μL) was put additionally and kept at 37°C. The absorbance of the mixtures was assessed by a spectrophotometer at the wavelength of 490 nm.

## Statistical analysis

The data were presented as means ± standard deviation (SD). Statistical differences were determined with Student's *t* test using GraphPad Prism 5 (San Diego, CA, USA). *P* values <0.05 were regarded to be significant.

# RESULTS

## Total phenolic contents and cytotoxicity

The total phenolic contents of *O. japonicus* extracts expressed as GAE are exhibited in Table 1. The contents were 202.5 ± 4.9 mg GAE/g in OJ_MeOH and 206 ± 12.0 mg GAE/g in OJ_EtOH. The contents were similar in OJ_MeOH and OJ_EtOH.

MTS assay was performed using B16F10 and HS68 cells to examine the cytotoxicity of *O. japonicus* extracts. When the extracts were applied at each dose ranging from 100 to 1,000 μg/mL, B16F10 cells showed minimum cytotoxicity up to 500 μg/mL and HS68 cells exhibited minimum cytotoxicity up to 300 μg/mL [Figure 1].

## UPLC Q-TOF MS analysis

Bioactive compounds of OJ_MeOH and OJ_EtOH were identified by UPLC Q-TOF MS, and data were shown in Figures 2 and 3, and Table 2. The compounds with their own structure, retention time, molecular weight formula were presented [Figure 3 and Table 2]. This analysis identified eight peaks; rutoside, hyperoside, isoquercitrin, epicatechin gallate,

**Table 1:** Total phenolic contents of *O. japonicus* extracts expressed as GAE

| Extracts | Total phenolic contents (mg GAE/g) |
|---|---|
| OJ_MeOH | 202.5±4.9 |
| OJ_EtOH | 206.0±12.0 |

Each value represents means±SD (*n*=3). GAE, gallic acid equivalents

**Table 2:** UPLC Q-TOF MS analysis of *O. japonicus* extracts

| Compound name | Retention time (min) | Molecular weight | Formula |
|---|---|---|---|
| *Rutoside* | 3.85 | 610.1534 | $C_{27}H_{30}O_{16}$ |
| *Hyperoside* | 4.05 | 464.38 | $C_{21}H_{20}O_{12}$ |
| *Isoquercitrin* | 4.12 | 464.0955 | $C_{21}H_{20}O_{12}$ |
| *Epicatechin gallate* | 4.15 | 442.09 | $C_{22}H_{18}O_{10}$ |
| *Astragalin* | 4.88 | 448.1006 | $C_{21}H_{20}O_{11}$ |
| *Afzelin* | 6.27 | 432.1056 | $C_{21}H_{20}O_{10}$ |
| *Quercetin* | 7.03 | 302.0427 | $C_{15}H_{10}O_7$ |
| *Kaempferol* | 8.67 | 286.0477 | $C_{15}H_{10}O_6$ |

UPLC Q-TOF MS, ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry

**Table 3:** Antioxidant activities of *O. japonicus* extracts measured by different methods

| Extracts | DPPH radical scavenging activity ($IC_{50}$ μg/mL) | ABTS radical scavenging activity ($IC_{50}$ μg/mL) | FRAP value (mg AEAC/g) |
|---|---|---|---|
| OJ_MeOH | 16.6±0.8 | 174.0±13.9 | 292.4±50.0 |
| OJ_EtOH | 23.7±0.6 | 233.6±20.1 | 166.7±22.7 |
| Ascorbic acid | 74.5±3.4 | 64.0±5.3 | - |

Each value represents means±SD (*n*=6). Data were expressed at $IC_{50}$. Ascorbic acid was used as a positive control. ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); AEAC, ascorbic acid equivalents antioxidant capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric-reducing antioxidant power.

astragalin, afzelin, quercetin, and kaempferol in OJ_MeOH [Figure 2a] and OJ_EtOH [Figure 2b].

## Antioxidant activity

Antioxidant activities of *O. japonicus* extracts measured by DPPH, ABTS, and FRAP assay were presented in Table 3. For the DPPH assay, $IC_{50}$ values of OJ_MeOH, OJ_EtOH, and ascorbic acid were 16.6 ± 0.8, 23.7 ± 0.6, and 74.5 ± 3.4 μg/mL, respectively. Antioxidant effects of *O. japonicus* extracts were much better than ascorbic acid, which is widely used as a reference antioxidant agent. $IC_{50}$ values of ABTS assay were 174 ± 13.9 (OJ_MeOH), 233.5 7 ± 20.1 (OJ_EtOH), and 64 ± 5.3 μg/mL (ascorbic acid), respectively. Compared with DPPH scavenging activity of *O. japonicus* extracts, ABTS activity was relatively low. FRAP values of OJ_MeOH and OJ_EtOH were 292.4 ± 50 and 166.7 ± 22.7 mg AEAC/g, respectively.

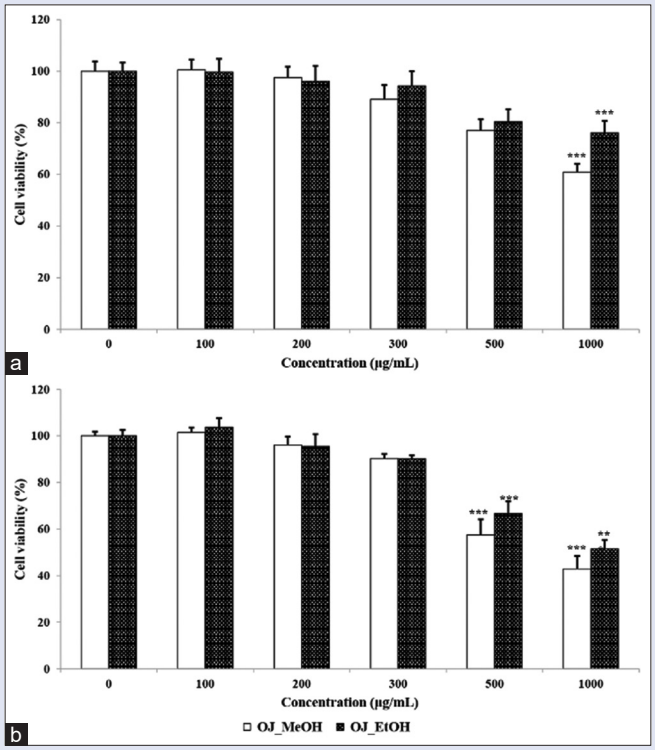


**Figure 1:** Cytotoxicity of OJ_MeOH and OJ_EtOH in (a) B16F10 cells, and (b) HS68 cells. The data measured by MTS assay were expressed as means ± SD ($n$ = 3). ***$P$ < 0.001 compared with control. OJ_MeOH, methanol extract of *O. japonicus*; OJ_EtOH, ethanol extract of *O. japonicus*

## Elastase and collagenase inhibition activities

Anti-wrinkle enzyme inhibition activities of *O. japonicus* extracts were measured by elastase and collagenase inhibition activities. $IC_{50}$ values of elastase inhibition activity of OJ_MeOH, OJ_EtOH, and EGCG were 73.2 ± 9.9, 42.6 ± 2.9, and 258 ± 33.9 μg/mL, respectively [Table 4], suggesting that OJ_MeOH and OJ_EtOH exhibited remarkable elastase inhibition activities. $IC_{50}$ values of collagenase inhibition activity of OJ_MeOH, OJ_EtOH, and EGCG were 145.1 ± 4.2, 120.3 ± 6.7, and 774.5 ± 71.2 μg/mL, respectively [Table 4]. *O. japonicus* extracts showed distinct collagenase inhibition activities.

## Expression of mRNA expressions of MMPs

RT-PCR and real-time RT-PCR were utilized to confirm the effect of *O. japonicus* extracts on the transcription level of MMP-1 and -3. When TNF-α was applied in HS68 human fibroblast cells, expression of MMP-1 and -3 increased. In RT-PCR, the expressions of MMP-1 and -3 induced by TNF-α were suppressed by adding OJ_MeOH and OJ_EtOH [Figure 4]. Real-time RT-PCR was also conducted to quantitatively assess MMP-1 and -3 transcription, which supported RT-PCR results obtained previously. When compared with the TNF-α treating control group, the inhibition rate of MMP-1 transcription by OJ_MeOH and OJ_EtOH was 97.6% and 94.4% at 300 μg/mL, respectively [Figure 5a]. In addition, the inhibition rate of MMP-3 expression by OJ_MeOH and OJ_EtOH was 95.3% and 89.1% at 300 μg/mL, respectively [Figure 5b].

## Inhibition of MMP-1 protein production

To confirm whether *O. japonicus* extracts lower the production level of MMP-1 provoked by TNF-α treatment, the production of MMP-1 was measured by ELISA. The reduction rate of MMP-1 production

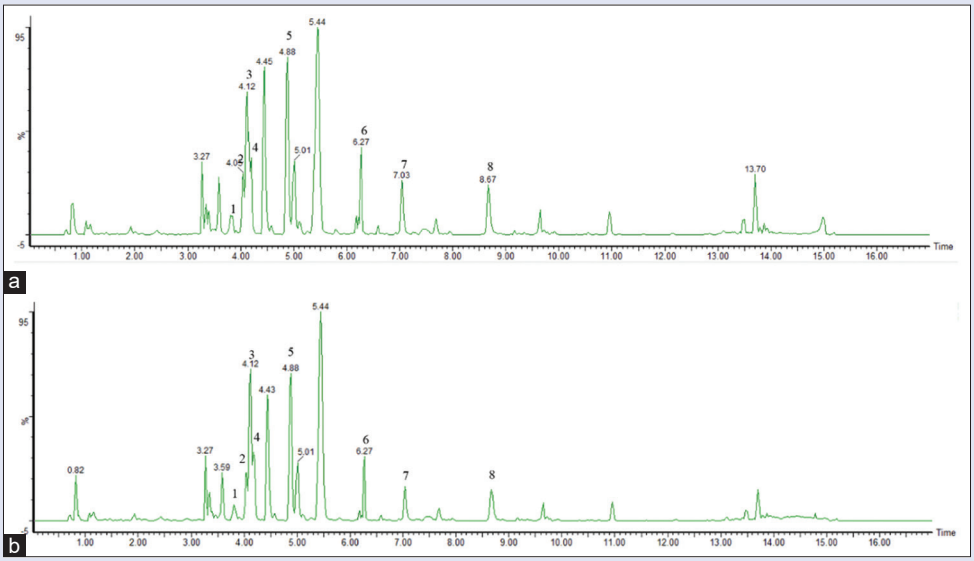


**Figure 2:** Base peak intensity chromatograms of (a) OJ_MeOH and (b) OJ_EtOH

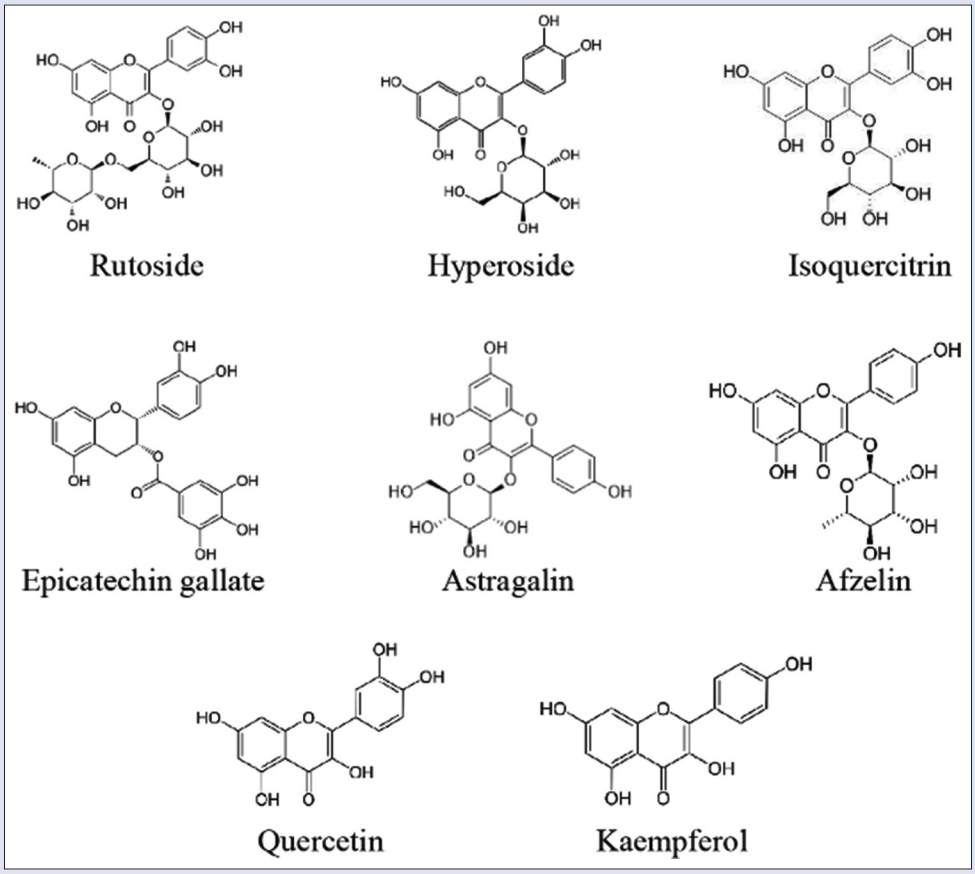


**Figure 3:** Structures of compounds identified in UPLC Q-TOF MS analysis of OJ_MeOH and OJ_EtOH

**Table 4:** Inhibition activity of *O. japonicus* extracts on elastase and collagenase

| Extracts | $IC_{50}$ (μg/mL) Elastase inhibition | $IC_{50}$ (μg/mL) Collagenase inhibition |
|---|---|---|
| OJ_MeOH | 73.2±9.9 | 145.1±4.2 |
| OJ_EtOH | 42.6±2.9 | 120.3±6.7 |
| EGCG | 258±33.9 | 774.5±71.2 |

The percent inhibitions were expressed as means±SD ($n$=3). Data were expressed at $IC_{50}$. EGCG was used as a positive control

**Table 5:** Inhibition activity of *O. japonicus* extracts on tyrosinase

| Extracts | $IC_{50}$ (μg/mL) Tyrosinase inhibition |
|---|---|
| OJ_MeOH | 775.6±95.5 |
| OJ_EtOH | 1470.1±207.2 |
| Arbutin | 438.4±51.5 |

The percent inhibitions were expressed as means±SD ($n$=3). Data were expressed at $IC_{50}$. Arbutin was used as a positive control

by OJ_MeOH and OJ_EtOH was conspicuously 97.9% and 97.2% at 300 μg/mL, respectively [Figure 6].

## Tyrosinase inhibition activity

Tyrosinase inhibition activity was assayed to examine the skin whitening effect of *O. japonicus* extracts. $IC_{50}$ values of OJ_MeOH and OJ_EtOH were 775.6 ± 95.5 and 1,470.1 ± 207.2 μg/mL, respectively [Table 5]. Tyrosinase inhibition activities of *O. japonicus* extracts were inferior to the positive control, arbutin [Figure 7].

# DISCUSSION

This study explored the antioxidant, anti-wrinkle, and skin whitening effects of *O. japonicus* MeOH and EtOH extracts. The total phenolic contents of OJ_MeOH and OJ_EtOH were similar. *O. japonicus* extracts are known to include phenolic compounds such as quercetin, kaempferol, myricetin, and epicatechin gallate.[29,40-42] OJ_MeOH and OJ_EtOH were identified to contain rutoside, hyperoside, isoquercetrin, epicatechin gallate, astragalin, afzelin, quercetin, and kaempferol as exhibited in Figures 2 and 3, and Table 2. The antioxidant effect is closely related to anti-aging cosmetic products. Free radical scavenging activities using DPPH and ABTS were performed to measure the antioxidant activities of *O. japonicus* extracts. Antioxidant activities were measured by different methods reflecting different antioxidant mechanisms.[15] DPPH is dissolved only in organic solvents, and hydrophilic samples are not suitable to determine antioxidant activity using this method.[43] ABTS free radical scavenging assay can measure both hydrophobic and hydrophilic samples.[44] FRAP assay is based on the reduction power of samples.[45] The antioxidant effect is generally correlated with antiwrinkle and whitening effects. Previous research has reported a linear correlation between phenolic contents and several antioxidant activities.[46,47] Because OJ_MeOH and OJ_EtOH contained various phenolic compounds and showed potent antioxidant activities, *O. japonicus* extracts were considered to be utilized as functional cosmetic agents.

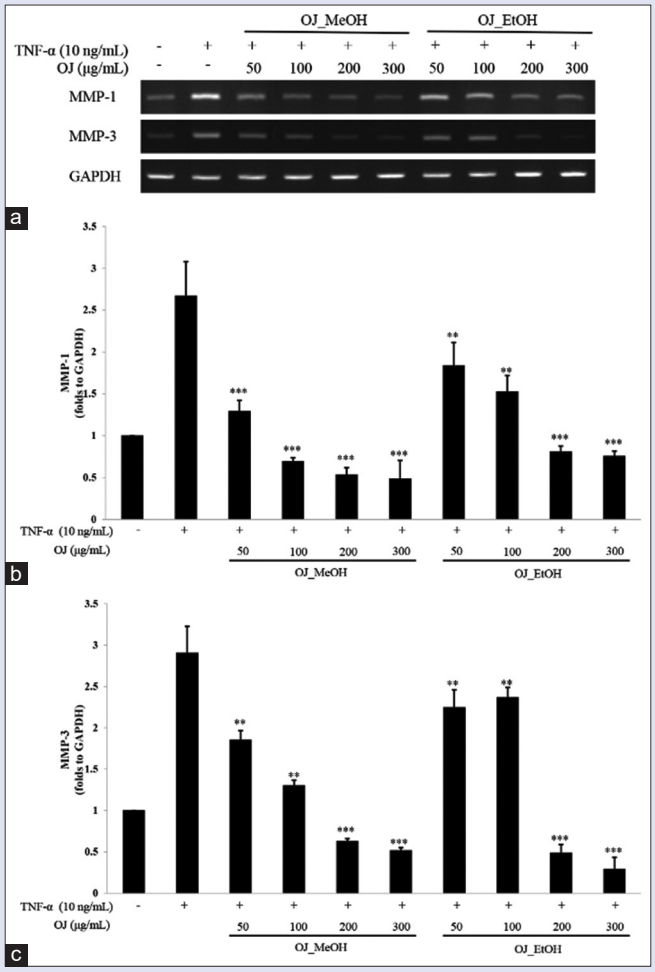


**Figure 4:** Measurement of effects of OJ_MeOH and OJ_EtOH on mRNA expressions of MMP-1 and MMP-3 using (a) RT-PCR, (b) MMP-1 using real-time PCR, and (c) MMP-3 using real-time PCR

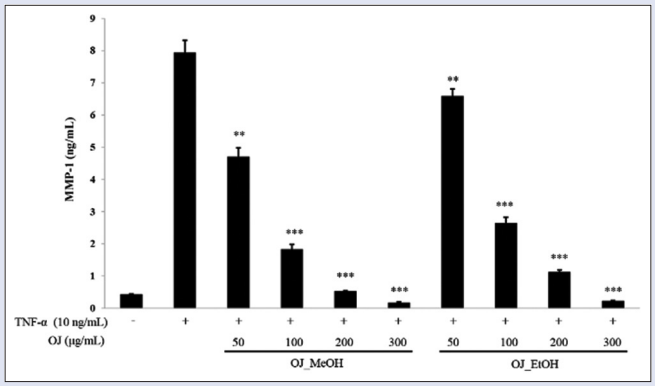


**Figure 6:** Measurement of effects of OJ_MeOH and OJ_EtOH on MMP-1 production in HS68 cells using ELISA. The results were expressed as means ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with TNF-α-treated control

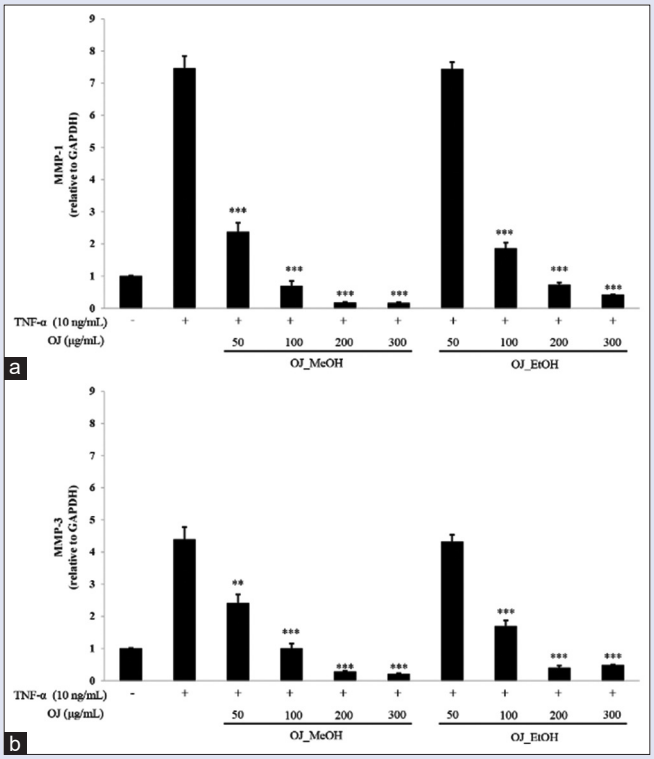


**Figure 5:** Measurement of effects of OJ_MeOH and OJ_EtOH on mRNA expressions of (a) MMP-1 and (b) MMP-3 by real-time RT-PCR. GAPDH was used as internal standard. The results were expressed as means ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with TNF-α-treated control

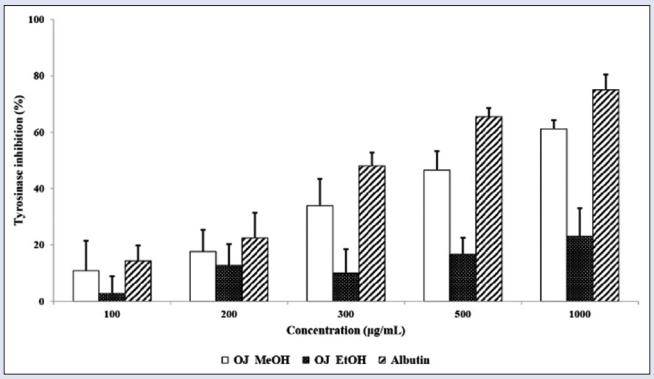


**Figure 7:** Tyrosinase inhibition of OJ_MeOH, OJ_EtOH, and arbutin. The percent inhibitions were expressed as means ± SD ($n = 3$)

Elastase overproduced by stress induces degradation of elastin in the skin, and contributes to wrinkles and stretch marks.[48] Previous studies have reported that kaempferol, quercetin, and myricetin inhibited elastase activity.[42,49] Several plants including *Epilobium angustifolium*, *Tagetes erecta*, *Nelumbo nucifera*, and *Phyllanthus emblica* were known to show high elastase inhibition activity.[20,50-52] *O. japonicus* extracts containing many phenolic constituents such as quercetin, kaempferol, and other flavonoid glycosides exhibited remarkable elastase inhibition activity. Collagen maintains elasticity and tension on the skin because it accounts for about 90% of the dermis and 80% of the ECM. Collagen in the dermis consists of 80%–85% type I collagen, 15%–20% type III collagen, and fibronectin.[53] sMMPs are zinc-dependent endopeptidases that break down most ECM. These are classified based on substrate specificity and are grouped into collagenases, gelatinases, stromelysins, and others.[54] MMP-1 (collagenase-1) affects skin aging by initiating the breakage of the collagen cross-links.[55] As skin aging progresses, the level of MMP-1 increases, and moreover, excessive collagen breakdown in the dermis by MMP-1 is also involved in proinflammation among connective tissues.[56] Therefore, the balance of lowering MMP-1 and maintaining type I collagen plays a

pivotal function in inhibiting wrinkle formation. MMP-3 (stromelysin-1) damages collagen types II, III, IV, V, and X, proteoglycans, laminin, elastin, fibronectin, and others. It can also extensively activate other MMPs such as MMP-1, -2, -7, -8, and -9.[57] Previous works have reported the inhibitory effects of MMP-1 and -3 in *Pinus densiflora*, *Michelia alba*, and *Coffea arabica*.[58-60] This work revealed that mRNA transcription of MMP-1 and -3 and the production of MMP-1 are suppressed in a concentration-dependent manner in human fibroblast HS68 cells treated with extracts from *O. japonicus*. $IC_{50}$ values of collagenase inhibition activities of other plants reported were 9,550 μg/mL for *Viburnum mullaha* and 640 μg/mL for *Terminalia arjuna*.[61,62] *O. japonicus* extracts, OJ_MeOH and OJ_EtOH, showed $IC_{50}$ values of collagenase inhibition activity 145.1 ± 4.2 and 120.3 ± 6.7 μg/mL, respectively. From these results, *O. japonicus* extracts were believed to have remarkable anti-wrinkle activity.

Tyrosinase inhibition is an important mechanism in the pathway of inhibition of the melanin accumulation in the epidermis.[63,64] OJ_MeOH showed moderate tyrosinase inhibition activity.

In this study, *O. japonicus* extracts were investigated to verify the possibility of them to be utilized as anti-aging cosmetic agents. For efficacy tests, antioxidant, anti-wrinkle, and whitening activities were examined using various biochemical assays. From three different antioxidant tests using DPPH, ABTS, and FRAP, *O. japonicus* extracts, OJ_MeOH and OJ_EtOH, showed effective DPPH scavenging activity compared with ascorbic acid. From substantial inhibitions of elastase and collagenase activities, MMP-1 and -3 mRNA expressions, and MMP-1 protein production by OJ_MeOH and OJ_EtOH, we have found that *O. japonicus* extracts exhibited remarkable anti-wrinkle activity. Whitening effect measured by tyrosinase inhibition activity was considered to be useful.

## CONCLUSION

*O. japonicus* extracts, OJ_MeOH and OJ_EtOH, exerted useful whitening activity as well as excellent antioxidant and anti-wrinkle activities, enhancing promising potential of them as anti-aging cosmetic agents.


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## Conflicts of interest

There are no conflicts of interest.

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