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Effects of *Oxya chinensis sinuosa* hot water extract on benign prostatic hyperplasia in LNCaP cells

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Abstract

In recent years, the number of patients with benign prostatic hyperplasia (BPH), a condition that commonly occurs in elderly men, has increased due to aging and the adoption of western dietary habits. Treatment with chemical drugs, such as finasteride or dutasteride, can cause side effects such as erectile dysfunction or sexual problems. This necessitates the development of remedies using natural substances derived from food ingredients. In this study, we investigated the inhibitory effects of *Oxya chinensis sinuosa* hot water extract (OCH) on BPH production in LNCaP cells, a hormone-dependent prostate cancer cell line. We found that the mRNA expression of androgen receptor (AR), prostate specific antigen (PSA), and, 5α-reductases 1, and 2 decreased following treatment with OCH. Furthermore, OCH treatment resulted in reduced protein expression of BPH regulators, such as AR. Collectively, these results suggest that OCH exerts a beneficial effect on BPH by inhibiting the AR signaling pathway, indicating the potential of OCH as a therapeutic agent for the prevention and treatment of BPH.

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androgen receptor,
prostate specific antigen

Introduction

Prostate disease is the most common male urinary system disease worldwide, and the incidence of benign prostatic hyperplasia (BPH) increases with age (Kim *et al*., 2015a). An enlarged prostate exerts pressure on the bladder, ultimately impairing bladder and kidney function (Madersbacher *et al*., 2019; Vickman *et al*., 2020). One theory concerning BPH is that the enlargement of prostate cells occurs due to excessive production of dihydrotestosterone (DHT). As individuals age, their sex hormone levels become imbalanced, and while blood testosterone levels decrease, the concentration of 5α-reductase remains high, resulting in the conversion of testosterone into DHT, which has a higher affinity for androgen receptors (ARs) (Velonase *et al*., 2013). DHT, when bound to AR, increases the expression of prostate specific antigen (PSA) (Nixon, 1997), and 5α-reductase inhibitors, which convert testosterone to highly active DHT, inhibit DHT binding to AR and reduce PSA levels (Dhurat *et al*., 2020). However, 5α-reductase inhibitors such as finasteride have side effects, including loss of appetite, erectile dysfunction, and ejaculation disorders (Park *et al*., 2018; Traish *et al*., 2011; Thomas *et al*., 2017). Oxidative stress and inflammation are important molecular mechanisms underlying BPH (Bostanci *et al*., 2013; Minciullo *et al*., 2015), and natural

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substances with antioxidant and anti-inflammatory functions have fewer side effects and a high potential for inhibiting BPH (Dhurat *et al*., 2020). Notably, *Oxya chinensis sinuosa* (*O. chinensis sinuosa*) contains substances with radical scavenging activities and polyphenols that act as antioxidants. Antioxidants prevent the formation of reactive oxygen species (ROS) that contribute to the etiology of BPH, aging, and carcinogenesis. Butylated-hydroxyanisole, the most widely used synthetic antioxidant to date, is highly effective at scavenging ROS; however, concerns have been raised regarding its pathogenicity and carcinogenicity (Branen, 1975). Therefore, it is necessary to identify a safe natural compound with high antioxidant capacity. BPH has been treated using natural, plant-derived ingredients such as *Cornus officinalis* (Kim *et al*., 2021), unripe black raspberries (Lee *et al*., 2014), bee pollen (Bak *et al*., 2018), and tomatoes (Kang *et al*., 2007). Antioxidant compounds have also been identified in insects (Park *et al*., 2005; Han *et al*., 2006). *O. chinensis sinuosa*, the insect used in this study to treat BPH, is an edible insect that was registered as a food product in the Korean Food Standards Codex of the Ministry of Food & Drug Safety (Kim *et al*., 2016). *O. chinensis sinuosa* is considered a famine relief insect (Park and Han, 2021), and contains nutrients such as protein, a high content of unsaturated fatty acids such as linoleic acid and oleic acid, various pigments (including chlorophyll and carotenoids), and polyphenols with antioxidant effects (Yoon *et al*., 2014; Kim *et al*., 1987).

In this study, we examined the inhibitory effects of *O. chinensis sinuosa* hot water extract (OCH) on BPH using LNCaP prostatic hyperplasia cells, which are widely used as *in vitro* prostate cancer models. We investigated whether the inhibition of LNCaP on BPH by OCH was associated with the inhibition of the expression of molecular factors associated with BPH induction.

Materials and Methods

Preparation of *O. chinensis sinuosa* extracts

Adult *O. chinensis sinuosa* was frozen in liquid nitrogen, ground using a mortar and pestle powdered using a homogenizer (VCX-600, Sonics and Materials, CT, USA) and dissolved in distilled water. Hot water extraction was performed by shaking at 60°C for 24 h. Subsequently, the supernatant was filtered through a 0.45-μm syringe filter and concentrated using a centrifugal evaporator (CVE-3100, EYELA, Tokyo, Japan) under reduced pressure. The concentrated extract (OCH) sample was collected and stored at -80°C and dissolved in distilled water prior to use.

α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay

DPPH and ascorbic acid were purchased from Sigma-Aldrich. The DPPH radical scavenging assay was performed as described previously (Blois, 1958). The DPPH solution was dissolved in ethanol to a concentration of 0.5 mM. L-ascorbic acid (1 mg/mL) was used as a positive control, and various concentrations (0.5, 1, and 2 mg/mL) of OCH were added to the sample. The plate was incubated at room temperature for 30 min and the absorbance (Abs) at 515 nm was measured using a microplate reader. The formula for calculating DPPH radical scavenging activity is as follows:

Radical scavenging activity (%) = [Abs (control) – Abs (sample)]/Abs (control) x 100

Cell culture

The prostate cancer cell line LNCaP was purchased from Korea Cell Line Bank (Seoul, Korea) and maintained in Rosewell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified incubator at 5% CO_2.

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTS assay

LNCaP cells were seeded at a density of 2×10^4 cells/well in 96-well plates and incubated for 24 h. Subsequently, cells were treated with different concentrations (0, 0.125, 0.25, 0.5, 1, and 2 mg/mL) of OCH. After incubation for 24 h or 48 h, MTS solution (MTS; Promega, Madison, WI, USA) was added to the plate and incubated for 3 h. Optical density was determined at 490 nm using a microplate reader.

Extraction of protein and Bicinchoninic Acid (BCA) quantification

For protein extraction, LNCaP cells were seeded at 1×10^6 cells/well in 6-well plates and incubated for 24 h, and subsequently treated with various concentrations (0, 0.125, 0.250, 0.5, 1, and 2 mg/mL) of OCH for 48 h. Thereafter, the cells were washed twice with phosphate-buffered saline and lysed using M-PER™ Mammalian Protein Extraction Reagent (Thermo

Table 1. The primer sequences used for qRT-PCR

Gene	Amplicon (bp)	Sequence (5'→3')
Androgen receptor (AR)	168	F: CCTGGCTTCCGCAACTTACAC R: GGACTTGTGCATGCGGTACTCA
Prostate specific antigen (PSA)	161	F: ACCAGAGGAGTTCTTGACCCCAAA R: CCCCAGAATCACCCGAGCAG
5α-reductase type 1	158	F: TGGTTAACGGGCATGTTGATAAA R: GCATAGCCACACCACTC
5α-reductase type 2	315	F: CATACGGTTTAGCTTGGGTGT R: GCTTTCCGAGATTTGGGGTAG
β-actin	157	F: CACTGTGCCCATCTACG R: CTTAATGTCACGCACGATTTC

Fisher Scientific, MA, USA) containing Halt™ Protease & Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Protein quantification was performed using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

RNA extraction and quantitative real-time PCR (qRT-PCR)

LNCaP cells were seeded at 1×10^6 cells/well in 6-well plates and incubated for 24 h. The cells were treated with different concentrations (0, 0.125, 0.25, 0.5, 1, and 2 mg/mL) of OCH and incubated for 17 h. Total RNA was isolated using TRIzol reagent and reverse-transcribed to complementary DNA (cDNA) using a cDNA synthesis kit (AmfiRivert cDNA Synthesis Platinum Master Mix, GenDEPOT, TX, USA), according to the manufacturer's instructions. The resulting cDNA was used for qRT-PCR amplification of the genes listed in Table 1. qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a QuantStudio 3 qPCR system (Applied Biosystems). β-actin was used as the housekeeping gene to normalize the data.

Western blot analysis

Proteins were separated using a Bolt™ 4-12% Bis-Tris Plus gel (Invitrogen, Carlsbad, CA, USA) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA). The membranes were blocked with 5% skim milk for 1 h at room temperature and then incubated with antibodies (ratio of dilution, 1:1000) against PSA (abcam, United Kingdom), AR and β-actin (Cell Signaling Technology, USA) overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Subsequently, antibodies were detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate on an Alliance Q9 gel documentation system (UVITEC, Cambridge, UK).

Statistical analysis

Data are presented as mean ± standard deviation (SD) of at least three independent experiments. Differences between the two groups were evaluated using the Student's t-test. SPSS version 18.0 K (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, and p- values <0.05 were considered statistically significant.

Results and discussion

Effects on DPPH radical scavenging activity of OCH

The DPPH radical scavenging activity was measured to determine the antioxidant activity of OCH. DPPH is a relatively stable free radical and represents a simple and economical method to test the antioxidant activity of substances (Lee *et al*., 2007). DPPH is dark purple in color and produces unstable radicals; as it gains electrons from antioxidants, it becomes transparent and stabilizes (Kang *et al*., 2009). Treatment with various concentrations of OCH (0.5, 1, and 2 mg/mL) increased the DPPH radical scavenging activity in a dose-dependent

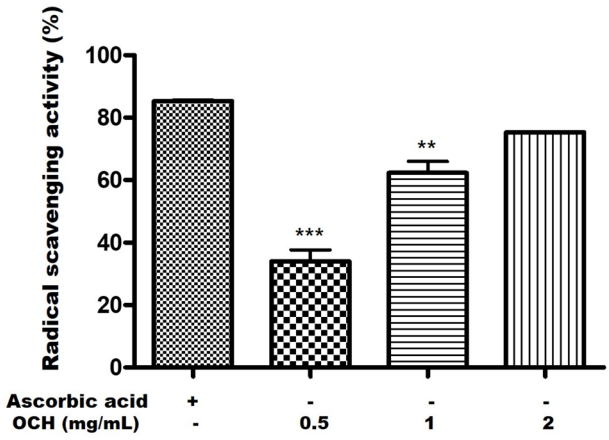

Fig. 1. DPPH radical scavenging activity of OCH extract. Ascorbic acid (1 mg/mL) was used as a positive control and OCH samples of various concentrations (0.5, 1, and 2 mg/mL) were examined. Results shown are representative of 3 independent experiments. Data are presented as the mean ± SD. Significance: ***$p < 0.001$ and **$p < 0.01$ vs ascorbic acid. OCH, *Oxya chinensis sinuosa* hot water extract; DPPH, α,α-diphenyl-β-picrylhydrazyl.

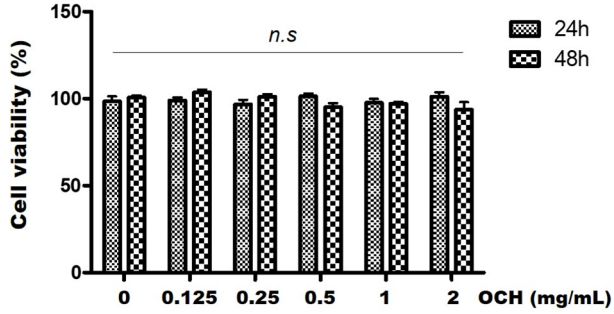

Fig. 2. Effects of OCH on cell viability in LNCaP human prostate cancer cells. Cell viability was measured using the MTS assay with different concentrations (0.125, 0.25, 0.5, 1, 2 mg/mL) of OCH for 24 h or 48 h incubation. Results shown are representative of 3 independent experiments. OCH, *Oxya chinensis sinuosa* hot water extract; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); *n.s*, no significance.

manner. In particular, in the solution treated with 2 mg/mL OCH, the observed radical scavenging activity value was close to that of the ascorbic acid-treated group, which was used as the positive control (Fig. 1). Kim *et al*. reported that the electron-donating abilities of 70% and 50% ethanol extracts of *O. chinensis sinuosa* were better than that of the water extract; however, the water extracts showed DPPH radical scavenging activity (Kim *et al*., 2015b) as well as high reducing power (Park *et al*., 2006). The DPPH radical-scavenging activity of OCH was confirmed in this study as well. Phenolic compounds such as pyroglutamic acid, oxalic acid, cinnamic acid in *O. chinensis sinuosa* act as electron donors (Buszewska-Forajta *et al*., 2014), possibly because these polyphenols act as antioxidants in the body (Whang *et al*., 2001). Moreover, the polyphenolic compounds in *O. chinensis sinuosa* suppress lipid peroxidation in a dose-dependent manner (Im *et al*., 2019), thereby preventing cellular oxidation. Linoleic acid (C18:2) and oleic acid (C18:1), which are abundant in rice grasshoppers, prevent oxidative stress by activating superoxide dismutase, plasma glutathione peroxidase, and other antioxidant enzymes (Kim and Kwon, 2018).

OCH effects on cell viability in LNCaP cells

To determine the effect of OCH on the viability of LNCaP cells, MTS assay was performed by treating cells with OCH at various concentrations (0, 0.125, 0.25, 0.5, 1 and 2 mg/mL) for 24 and 48 h. As shown in Fig. 2, no significant viability inhibition was observed in LNCaP cells treated with OCH at concentrations up to 2 mg/mL. Therefore, further experiments were conducted at the maximum concentration of 2 mg/mL, which did not show any cytotoxicity.

Effects of OCH on the expression of BPH-related genes in LNCaP cells

To analyze the expression of BPH regulation-related genes associated with LNCaP cells following OCH treatment, the expression of AR, PSA, and 5α-reductases 1 and 2 was confirmed via qRT-PCR. RNA was isolated from LNCaP cells treated for 17 h with various concentrations of OCH (0, 0.125, 0.25, 0.5, 1, and 2 mg/mL). Subsequently, it was confirmed that the mRNA expression of AR, PSA, and 5α-reductases 1 and 2 was reduced following OCH treatment (Fig. 3).

The inhibition of prostatic hyperplasia is associated with the reduced expression of BPH-related genes, achieved through antioxidant and anti-inflammatory effects (Kim *et al*., 2021*). O. chinensis sinuosa* not only strongly suppresses inflammatory cytokines but also significantly reduces the expression of iNOS protein, an enzyme that produces NO. Inos inhibits NF-κB p65 activation and reduces COX-2 expression, thus exhibiting anti-inflammatory effects. Therefore, *O. chinensis sinuosa* suppresses the expression of COX-2 and iNOS, thereby inhibiting prostatic hyperplasia cells (Yoon *et al*., 2014). Nature-friendly herbal medicines have been widely used in oriental countries, including

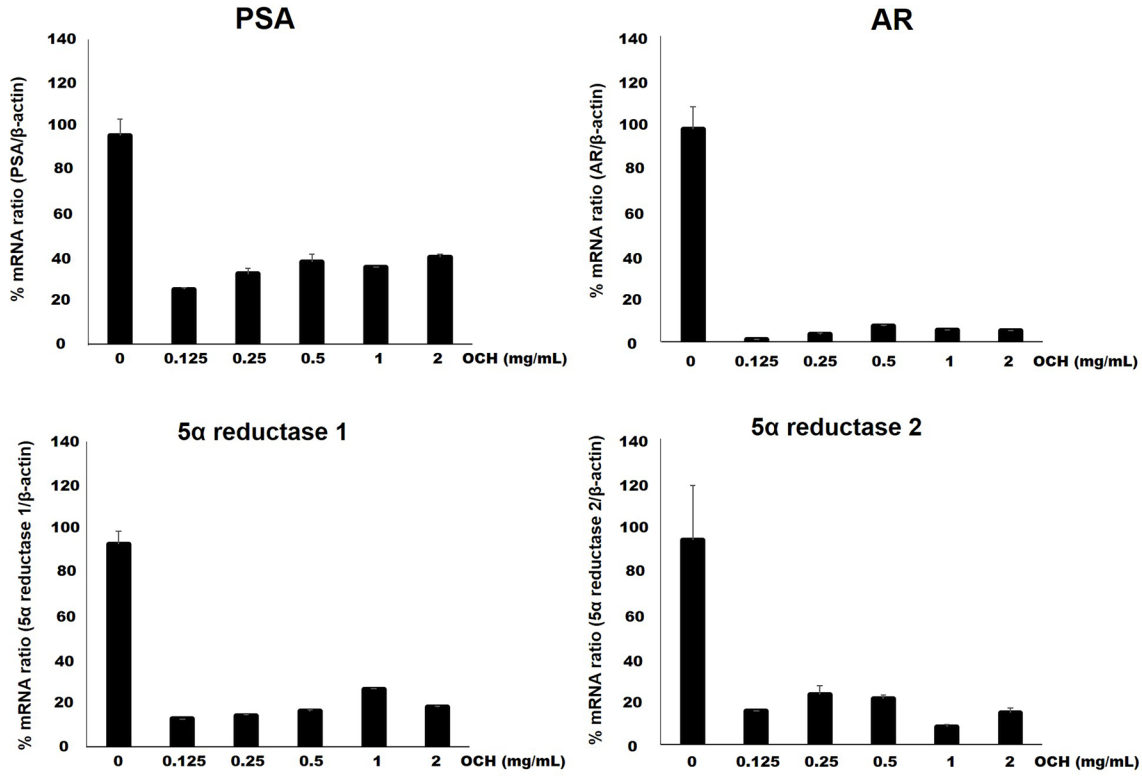

Fig. 3. Effects of OCH on the mRNA expression of benign prostatic hyperplasia regulators in LNCaP human prostate cancer cells. The levels of mRNA expression of AR, PSA, 5α-reductases 1 and 2 in OCH (0.125, 0.25, 0.5, 1, 2 mg/mL) treated in LNCaP cells were evaluated via quantitative real-time polymerase chain reaction (PCR). β-actin was used as the housekeeping gene to normalized the data. Results shown are representative of 3 independent experiments. OCH, *Oxya chinensis sinuosa* hot water extract; AR, androgen receptor; PSA, prostate specific antigen.

Korea, to treat prostate and urinary diseases. Recent research has focused on BPH treatment using plant products such as *Cornus officinalis* (Kim *et al*., 2021), unripe black raspberry (Lee et al., 2014), and tomatoes (Kang et al., 2007); however, there has been a lack of research on BPH treatment using edible insects. Therefore, this study provides basic data for future research on BPH using *O. chinensis sinuosa*, among other edible insects.

Effects of OCH on the expression of BPH-related proteins in LNCaP cells

To analyze the expression of BPH-related proteins in LNCaP cells after treatment with OCH, the expression of AR and PSA was confirmed by western blot analysis. LNCaP, which are prostatic hypertrophic cells, were treated with various concentrations of OCH (0, 0.125, 0.25, 0.5, 1, and 2 mg/mL) and proteins were isolated 48 h later. We observed that the expression of AR, which are proteins associated with prostatic hyperplasia, slightly decreased in OCH-treated LNCaP cells (Fig. 4). However, PSA did not reduce its expression

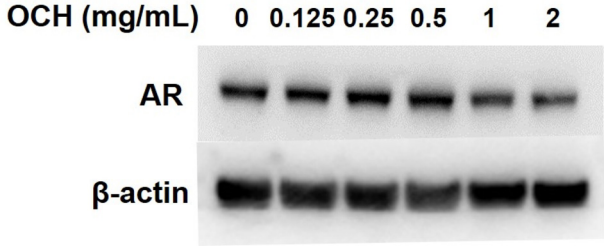

Fig. 4. Effects of OCH on the protein expression of benign prostatic hyperplasia regulators in LNCaP human prostate cancer cells. The effects of OCH (0.125, 0.25, 0.5, 1, 2 mg/mL) on the expression of benign prostatic hyperplasia regulators were evaluated via western blot analysis. Results shown are representative of 3 independent experiments. β-actin was used as an internal control. OCH, *Oxya chinensis sinuosa* hot water extract; AR, androgen receptor.

level (data not shown). It seems that the expression level of proteins needs to be studied on time-dependent expression patterns. AR play an essential role in prostate growth and maintenance. With advancing age, AR expression in prostate cells increases to generate more DHT, which binds to AR and

increases the transcriptional activity of various growth factors involved in prostate cell proliferation, thereby promoting BPH development (Banerjee *et al*., 2018; Da Silva and De Souza, 2019). Furthermore, the expression of 5α-reductase, which converts testosterone into DHT that binds more strongly to AR, was suppressed by OCH treatment. Thus, OCH blocks the conversion of testosterone to DHT by reducing 5α-reductase expression, thereby reducing AR. It is believed that OCH inhibits the expression of prostate cells and delays their proliferation. PSA, which is expressed in an AR-dependent manner, is an important biomarker for the diagnosis of prostate disease (Liu *et al*., 2019). Polyphenols inhibit the expression of 5α-reductase, which is involved in DHT synthesis (Richard et al., 2002). As rice grasshoppers contain polyphenols (Park and Han, 2021), it is plausible that products derived from them can inhibit 5α-reductase, AR, and PSA expression.

Conclusion

This study demonstrated that effects of *O. chinensis sinuosa* hot water extract on benign prostatic hyperplasia in LNCaP cells, increased DPPH level, decreased AR, PSA, and 5α-reductases 1 and 2 mRNA level and AR protein level. However, the precise mechanisms require further investigation by animal experiment *in vivo*. In conclusion, the results of this study suggest that *O. chinensis sinuosa* holds considerable potential as a potential food and pharmaceutical substance for the prevention and treatment of BPH.

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