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Gonadal efficacy of *Thymus quinquecostatus* Celakovski: Regulation of testosterone levels in aging mouse models

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ABSTRACT

Late-onset hypogonadism (LOH) is an age-related disease in men characterized by decreased testosterone levels with symptoms such as decreased libido, erectile dysfunction, and depression. *Thymus quinquecostatus* Celakovski (TQC) is a plant used as a volatile oil in traditional medicine, and its bioactive compounds have anti-inflammatory potential. Based on this knowledge, the present study aimed to investigate the effects of TQC extract (TE) on LOH in TM3 Leydig cells and in an *in vivo* aging mouse model. The aqueous extract of *T. quinquecostatus* Celakovski (12.5, 25, and 50 μg/mL concentrations) was used to measure parameters such as cell viability, testosterone level, body weight, and gene expression, via *in vivo* studies. Interestingly, TE increased testosterone levels in TM3 cells in a dose-dependent manner without affecting cell viability. Furthermore, TE significantly increased the expression of genes involved in the cytochrome P450 family (Cyp11a1, Cyp17a1, Cyp19a1, and Srd5a2), which regulate testosterone biosynthesis. In aging mouse models, TE increased testosterone levels without affecting body weight and testicular tissue weight tissue of an aging animal group. In addition, the high-dose TE-treated group (50 mg/kg) showed significantly increased expression of the cytochrome p450 enzymes, similar to the *in vitro* results. Furthermore, HPLC-MS analysis confirmed the presence of caffeic acid and rosmarinic acid as bioactive compounds in TE. Thus, the results obtained in the present study confirmed that TQC and its bioactive compounds can be used for LOH treatment to enhance testosterone production.

1. Introduction

Late-onset hypogonadism (LOH) is a biochemical syndrome related to aging, characterized by low serum testosterone levels [1]. Aging leads to gonadotropin-releasing hormone (GNRH) secretion disorder and reduces luteinizing hormone (LH) levels, which are associated with low Leydig cell activity [2]. Hypogonadism has two different sub-conditions, namely hypergonadotropic hypogonadism (primary) and hypogonadotropic hypogonadism (secondary), and hypogonadotropic hypogonadism, the second type has been reported to feature Leydig cell failure that subsequently leads to testosterone deficiency [3]. Decreased testosterone levels lead to hypogonadism associated with symptoms such as decreased muscle mass, increased fat mass, and erectile dysfunction [4]. It has been suggested that low testosterone concentrations affect diabetes bidirectionally; however, clinical trials have shown that testosterone improves glucose metabolism and composition [5]. In recent years, studies related to LOH have attracted much interest worldwide, including in Korea, because of the increasing aging male

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population [6]. To date, there is no universal therapy for hypogonadism and other potential risks, such as prostate cancer, benign prostate hyperplasia, and infertility, other than testosterone replacement therapy [7].

Cytochrome P450 (CYP) enzymes play important roles in testosterone synthesis. CYP encodes the heme-thiolate monooxygenase superfamily and plays critical roles in drug, xenobiotic, and chemical metabolism [8]. CYP is known to be involved in many reactions in the human body; in particular, Cyp17a1 plays an important role in endocrinology and genetic disorders in steroidal oxidation [9]. Cyp11a1 and Cyp17a1 are involved in the sequential synthesis of testosterone from cholesterol, while Cyp19a1 is involved in the conversion of androgen to estrogen [10]. Critical defects have been identified in aged Leydig cells, including the reduction of downstream steroidogenic enzymes such as Cyp11a1 and Cyp17a1, which causes a decline in serum testosterone levels [11]. Therefore, CYP may be considered a critical target for detecting LOH induction in cell cultures and animal studies.

Some drugs, including opioids and glucocorticoids, are considered functionally important in hypogonadism [12]. Testosterone replacement therapy has been considered a better option for patients with LOH, but this treatment approach warrants a discussion regarding risks versus benefits [13]. Side effects are a consequence of therapeutic treatments and can lead to harmful effects on the human body [14]. Recently, there has been growing interest in herbal medicinal products as novel pharmaceutical drugs, due to their fewer or no adverse effects. Therapies with herbal products have been established with promising effects; however, herbal products and their bioactive potential have not been completely explored [15]. Nevertheless, there have been few trials to determine the effectiveness of herbal products, including ginseng extract [16], decursin [17], and quercetin [18].

Thymus quinquecostatus Celakovski (TQC) is a wood plant belonging to the family Labiatae, and its oil is widely used as an herbal medicine in East Asia [19]. TQC is composed of many branches and leaves with capillary secretory hairs. Trans-geraniol, citral, 3-octanone, geranyl acetate, borneol, and nerol have been identified as the chemical components of thyme oil. Furthermore, TQC has been used to flavor tea, and its pharmacological effects, including anti-inflammatory, analgesic, and anti-diabetic effects, have been identified [20]. However, the effects of TQC-related testicular injury have not been confirmed in previous studies yet. Considering the previously reported effects of TQC extract (TE), the objective of the present study was to evaluate the efficacy of TE on LOH using *in vitro* and *in vivo* studies.

2. Material and methods

2.1. Sample preparation

The dried TQC was purchased from DONGYANG Herbs (Seoul, Korea). For the preparation of TE, 10 kg of TQC was extracted with 100 mL of water and sonicated for 2 hours at 50 ℃. The extracted TE was filtered with Whatman No. 2 filter paper and dried using the spray-drying method. The powdered TE was diluted in water and filtered through a 0.45-μM syringe filter before being used for the treatment of TM3 Leydig cells and orally administered to the mice. The yield was confirmed at 13.2%. The dried TE was stored at −20 ℃.

2.2. TM3 cell culture and treatment

TM3, immature Leydig cells, were obtained from the Korean Cell Line Bank (Seoul, Korea) and grown in Dulbecco's Modified Eagle Medium (high glucose, GlutaMAX™ Supplement) with 5% fetal bovine serum (Gibco, USA) and 1% antibiotic-antimycotic (Gibco, USA) at 37 ℃ with 5% CO_2.

2.3. Cell viability

The toxicity of TE was confirmed by a cell viability assay using the cell counting kit-8 (CCK-8). The TM3 cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After a period of 24 hours following the cell seeding, cells were treated with TE (12.5, 25, and 50 μg/mL), caffeic acid, and rosmarinic acid (2, 4, 6, 8, and 10 μM), and were incubated for 24 hours. Then, 10 μL/well of CCK-8 solution (Dojindo, Korea) was added and incubated for an additional 2 hours at 37 ℃ with 5% CO_2. The absorbance of the wells was measured at 450 nm. Cell viability (%) was determined as follows: treatment OD/control OD × 100 (%).

2.4. Testosterone level detection

Testosterone levels in cell media and mouse serum were measured using a testosterone ELISA kit (Cayman, USA). After TM3 cells were prepared, TE (12.5, 25, and 50 μg/mL), caffeic acid (CA), rosmarinic acid (RA), and the combination of CA and RA (2, 6, and 10 μM) were treated for 24 hours before testosterone detection. Mouse serum was collected from whole blood samples, followed by centrifugation for 1000 × *g* at 4 °C. Absorbance was measured at 420 nm using a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices).

2.5. Animal study

C57BL/6 mice (30 and 50 weeks old, male) were purchased from DOOYEOL BIOTECH (Seoul, Republic of Korea), and adapted according to the animal welfare regulations of the Institutional Animal Care and Use Committee of the KIOM (Approval number 22–094). C57BL6 mice were housed in a room with standard mouse cages at a humidity of 50 ± 10% and a temperature of 23 ± 3 ℃. Food and water were provided *ad libitum*. Mice were randomly divided into the following five groups, consisting of ten mice in each group: the young (30 weeks) aged control group (YC), the old (50 weeks) aged control group (OC), the 12.5 mg/kg TE-treated group (TE 12.5), the 25 mg/kg TE-treated group (TE 25) and the 50 mg/kg TE-treated group (TE 50). The body weights of the mice were measured every week for five weeks, and the mice were anesthetized. After anesthetization, the blood and organs (testes and epididymis) were collected for analysis.

2.6. Real-time PCR

Real-time PCR was performed to confirm the changes in mRNA expression following TE treatment. Total RNA was extracted using a total RNA extraction kit (iNtRON Biotechnology, Seoul, Korea) in adherence to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop (Thermo Fisher Scientific, Cleveland, OH, USA), and cDNA synthesis was performed with SuperScript™ IV First-Strand Synthesis System (Invitrogen™, Carlsbad, CA, USA). The qRT-PCR was performed using a qPCR Green2X Master Mix kit (m.biotech, Seoul, Korea) with the PCR program (initial step of heating at 95 °C for 5 min, followed by 45 cycles of 15 s denaturation at 95 °C and 1 min extension at 60 °C). Target gene quantitation was normalized by comparisons relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences are listed in Table 1. The experiments were performed in triplicate for each group.

2.7. Serum biochemical analysis

Serum biochemical analyses of alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were performed using diagnostic kits (Erba Mannheim XL packs) with an automatic chemistry analyzer (Erba Mannheim XL-200, Erba Mannheim, Mannheim, Germany).

Table 1
Sequence of primers.

Gene	Forward	Reverse
Tspo	5′-GCCTACTTTGTACGTGGCGAG-3′	5′-CCTCCCAGCTCTTTCCAGAC-3′
Star	5′-ATGTTCCTCGCTACGTTCAAG-3′	5′-CCCAGTGCTCTCCAGTTGAG-3′
Cyp11a1	5′-AGGTCCTTCAATGAGATCCCTT-3′	5′-TCCCTGTAAATGGGGCCATAC-3′
Cyp17a1	5′-GCCCAAGTCAAAGACACCTAAT-3′	5′-GTACCCAGGCGAAGAGAATAGA-3′
Cyp19a1	5′-ATGTTCTTGGAAATGCTGAACCC-3′	5′-AGGACCTGGTATTGAAGACGAG-3′
Hsd3b1	5′-CCTCCGCCTTGATACCAGC-3′	5′-TTGTTTCCAATCTCCCTGTGC-3′
Srd5a2	5′-GATCCTGTGCTTTGGGAAACC-3′	5′-GCATCCCTACCGACACCAC-3′
Hsd17b3	5′- ATGAAGAAGACACAAACTTGGATTA −3′	5′- GTTGCTGATGTTGCGTTTG −3′
Hsd17b4	5′-CTCGAAGGTCTGTGCGAGAG-3′	5′-GCTTGCTCATAACCACGCTG-3′
Gapdh	5′ -AGGTCGGTGTGAACGGATTTG-3′	5′ -TGTAGACCATGTAGTTGAGGTCA-3′

Abbreviations: Tspo: translocator protein, Star: steroidogenic acute regulatory protein, Cyp11a1: cytochrome P450, family 11, subfamily a, polypeptide 1, Cyp17a1: cytochrome P450, family 17, subfamily a, polypeptide 1, Cyp19a1: cytochrome P450, family 19, subfamily a, polypeptide 1, HSD3b1: hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase 1, Srd5a2: steroid 5 alpha-reductase 2, HSD17b3: hydroxysteroid (17-beta) dehydrogenase 3, Hsd17b4: hydroxysteroid (17-beta) dehydrogenase 4

2.8. High-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis

Chromatogram analysis of the TE was performed using a Waters ACQUITY UPLC (Waters Corp., Milford, MA, USA) with an ACQUITY binary solvent manager pump, an AQQUITY PDA detector, and a Waters micromass ZQ spectrometer (Waters) connected to an electrospray ionization (ESI) interface and an ion trap mass analyzer. The separation of samples was performed using ACQUITY UPLC BEH Shield RP18 Column (130 Å, 1.7 μm, 2.1 mm×100 mm) with a column oven temperature of 40 ℃. Samples were injected with 5 μL each, and peaks were monitored at 329 nm wavelength. The gradient elution of samples was carried out using a mobile phase composed of acidified water (0.1% formic acid) (A) and acetonitrile (0.1% formic acid) (B) at 0.3 mL/min, as follows: 20–95% B from 0–14 min; 95–100% B from 14–15 min; 100–20% B from 15–15.1 min; and 20% B from 15.1–20 min. MS analysis conditions (negative ion mode) were as follows: capillary voltage of 3.3 kV, cone voltage of 50 V, extractor voltage of 3 V, RF lens voltage of 0.2 V, source temperature of 120 ℃, desolvation temperature of 300 ℃, gas flow desolvation of 600 L/h, and gas flow cone of 30 L/h.

2.9. Statistical analysis

Data are represented as the mean ± SEM. and analyzed using a one-way ANOVA using GraphPad Prism software 10.2.2, and a p-value < 0.05 was considered statistically significant in all cases.

3. Results

3.1. Effect of TE on cellular viability and testosterone level in TM3 Leydig cells

The 12.5, 25, and 50 μg/mL of TE sample concentrations were analyzed to detect cell viability, and the results confirmed that there were no significant changes in the cell viability of TM3 Leydig cells during TE treatment (Fig. 1). After confirming the absence of toxicity, the effect of the TE samples on testosterone levels was evaluated. In TM3 cells, testosterone production increased significantly in a concentration-dependent manner (Fig. 2). Interestingly, TE-treated groups (12.5, 25, and 50 μg/mL) showed a nearly triple increase in the production of testosterone.

3.2. Effect of TE on gene expression level in TM3 Leydig cells

After confirming the effect of TE on testosterone levels in TM3 cells, the mRNA levels of enzymes related to testosterone synthesis were evaluated using qPCR. Testosterone (T) was used as a comparative control group (1 ng/mL). As shown in Fig. 3, the mRNA expression levels of Cyp11a1, Cyp17a1, Cyp19a1, and Srd5a2 were upregulated in a dose-dependent manner in the TE-treated group. Significant changes in mRNA expression levels were detected in the 50 μg/mL-treated group compared to the control group.

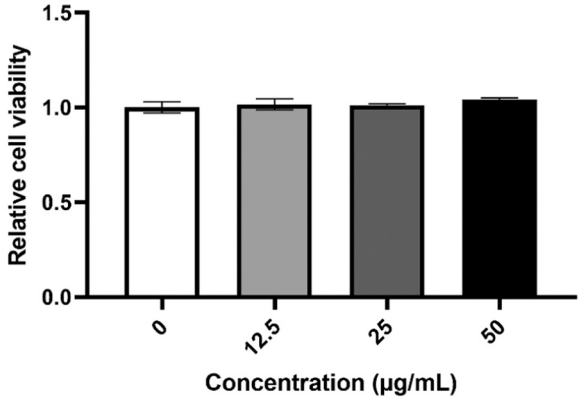

Fig. 1. Effect of TE on cell viability of TM3 Leydig cells. Cell viability of 0, 12.5, 25, and 50 μg/mL of TE-treated group are shown. All values are presented as the mean ± standard deviation.

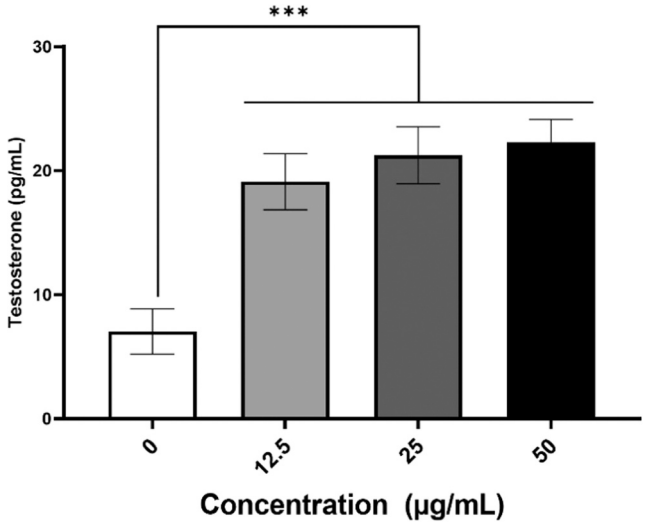

Fig. 2. Effect of TE on testosterone production in TM3 Leydig cells. Testosterone concentrations of 0, 12.5, 25, and 50 μg/mL of TE-treated group are shown. All values are presented as the mean ± standard deviation, ***p<0.001 compared to the control group.

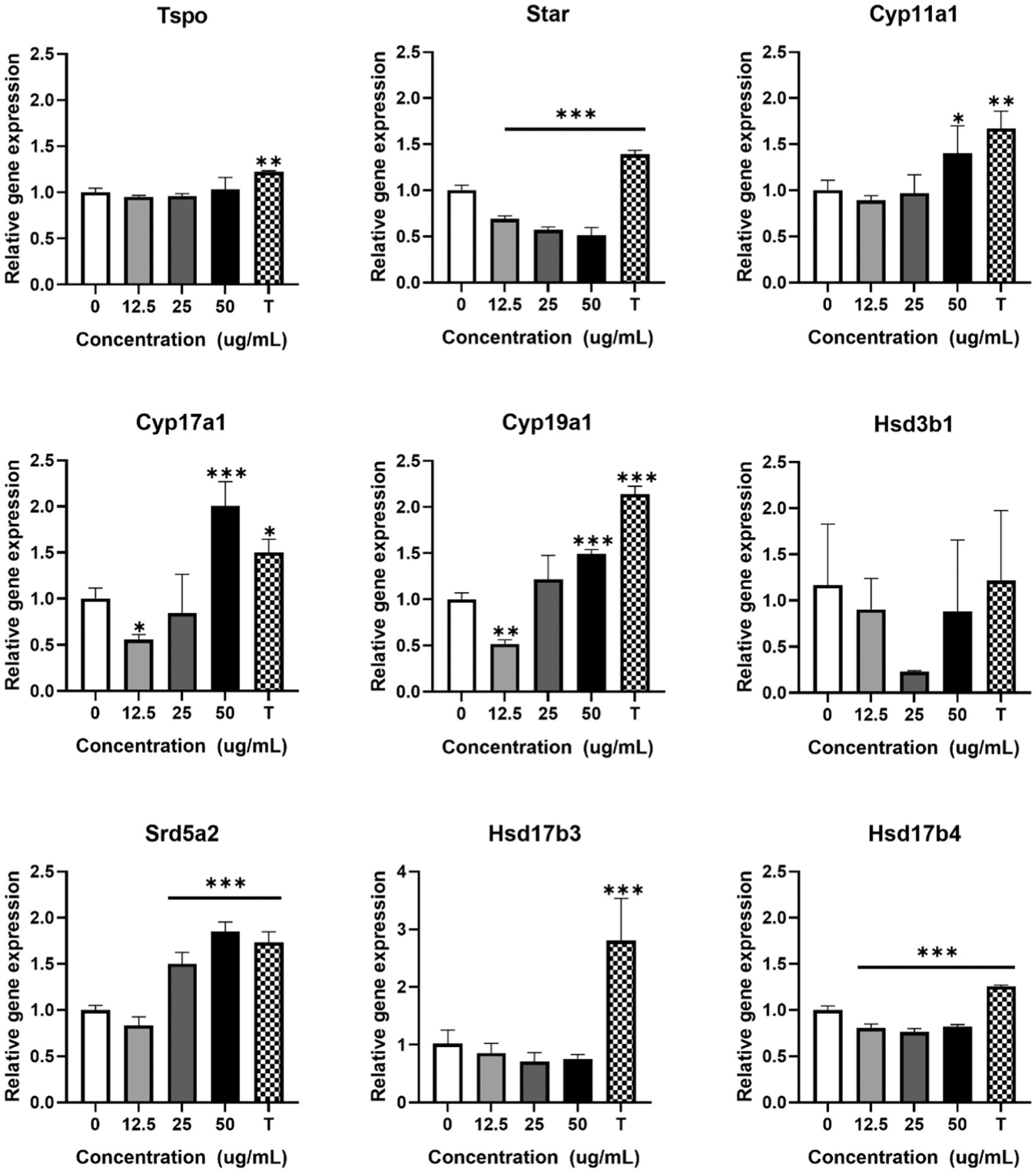

Fig. 3. Effect of TE on gene expression in the TM3 Leydig cell. Gene expressions in TM3 Leydig cells of TE-treated groups (0, 12.5, 25, and 50 μg/mL) were shown. Testosterone (T) was used as a comparative control group (1 ng/mL). All values are presented as mean ± standard deviation, *$p<0.05$, **$p<0.01$and ***$p<0.001$ compared to control group.

3.3. In vivo efficacy of TE on body, testis, and epididymis tissue weight

In the C57BL/6 mouse groups, physiological parameters such as body, testis, and epididymis weights were measured every week during the study period (Fig. 4A). In the TE-treated groups, there were no significant changes in body weight compared to the OC group of the LOH animal model (Fig. 4B). The testis and epididymis weights of the OC group were significantly lower than those of the YC group (Figs. 4C and 4D). These results confirmed that TE treatment did not affect the body or organ weights of C57BL/6 mice.

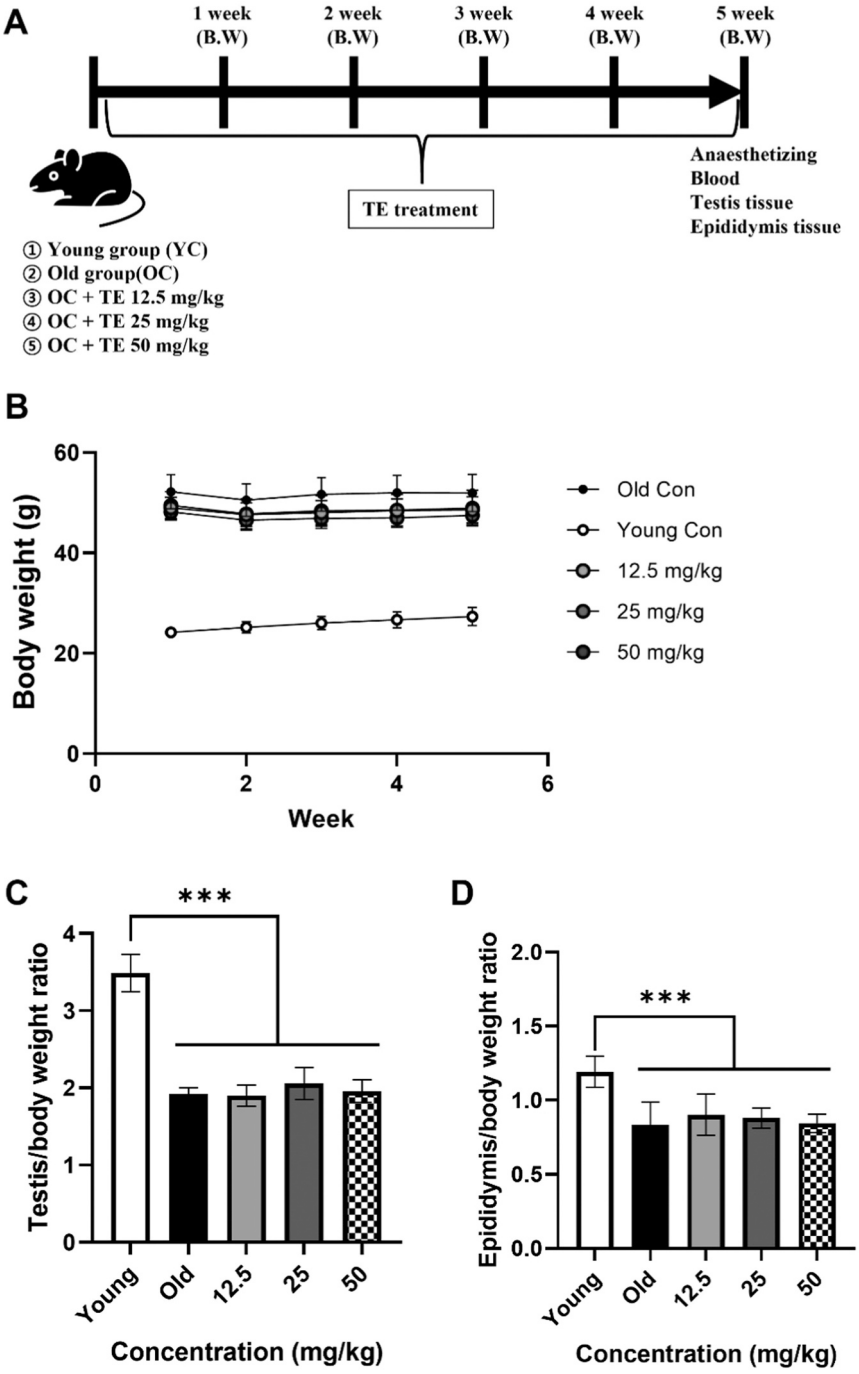

Fig. 4. Effect of TE on body, testis, and epididymis tissue weight in the LOH mouse model (C57BL/6 mice). (A) Schematic representation of the *in vivo* study. Effect of TE treatment (12.5, 25, and 50 μg/mL) on (B) total body weight, (C) testis tissue weight, and (D) epididymis tissue weight. All values are presented as the mean ± standard deviation, ***$p<0.001$ compared to the young control group.

3.4. Effect of TE on serum testosterone, safety markers, and lipid profile

Levels of testosterone, AST, ALT, and lipids in mouse serum were evaluated using biochemical methods. Testosterone levels were lower in the OC group than those in the YC and TE groups (Fig. 5A). Conversely, a significant increase in AST and ALT levels was detected in the OC group compared to the other groups. TE treatment showed a decreasing tendency in these values compared to the OC group, but the difference was not significant (Fig. 5B). Total cholesterol increased in the old groups, including the TE-treated group, compared to the YC group. Among the TE-treated groups, 12.5 and 50 μg/mL of TE treatment exhibited decreased levels of cholesterol compared to the OC group (Fig. 5C). Overall, TE treatment considerably increased testosterone, AST, ALT, and cholesterol levels in the LOH mouse models.

3.5. Effect of TE on gene expression level in testis tissue

The expression levels of Cyp11a1, Cyp17a1, Cyp19a1, and Srd5a2 in the testis tissues were measured to confirm the effect of TE on the LOH mouse models (Fig. 6). When compared to the YC group, Cyp11a1, Cyp17a1, and Cyp19a1 gene expressions significantly decreased in the OC group and in the 12.5 mg/kg TE-treated group. In addition, the Cyp19a1 expression level in the 25 mg/kg TE-treated group was considerably higher than that in the YC group. In the 50 mg/kg TE-

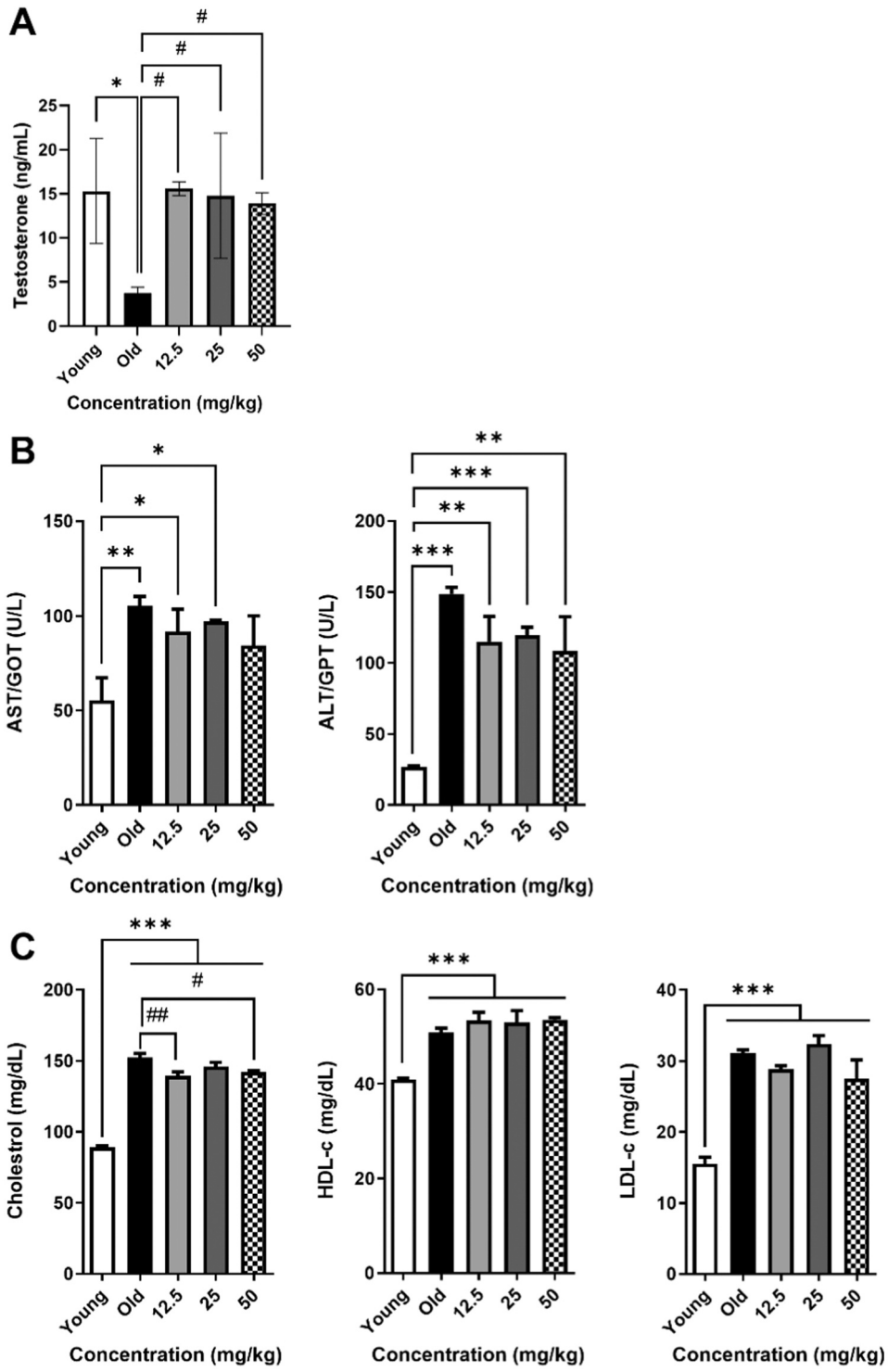

Fig. 5. Effect of TE (12.5, 25, and 50 mg/kg) on testosterone, safety markers, and lipid profile in LOH mouse serum. (A) testosterone level; (B) AST/GOT and ALT/GPT levels; (C) total cholesterol, HDL-c, and LDL-c levels in mice serum. All values are presented as the mean ± standard deviation, *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to the young control group. # $p<0.05$ and ## $p<0.01$ compared to the old control group. AST: aspartate transaminase; GOT: glutamic oxaloacetic transaminase; ALT: alanine transferase; GPT: glutamic pyruvic transaminase; HDL-c: high density lipoprotein; LDL-c: low density lipoprotein.

treated groups, there was a significant increase in the expression of all genes compared to the OC group.

3.6. HPLC-MS analysis

HPLC-MS analysis of TE revealed two peaks at retention time of 6.146 min and 9.659 min, which were identified as CA and RA, respectively (Fig. 7). The molecular weights of the major compounds were confirmed by HPLC-MS as follows: CA, MW 180, LC-MS $[M-H]^-$ 179.04; RA, MW 360, and LC-MS $[M-H]^-$ 358.9. Standard compound peak and MS data confirm the presence of CA and RA in TE [21,22].

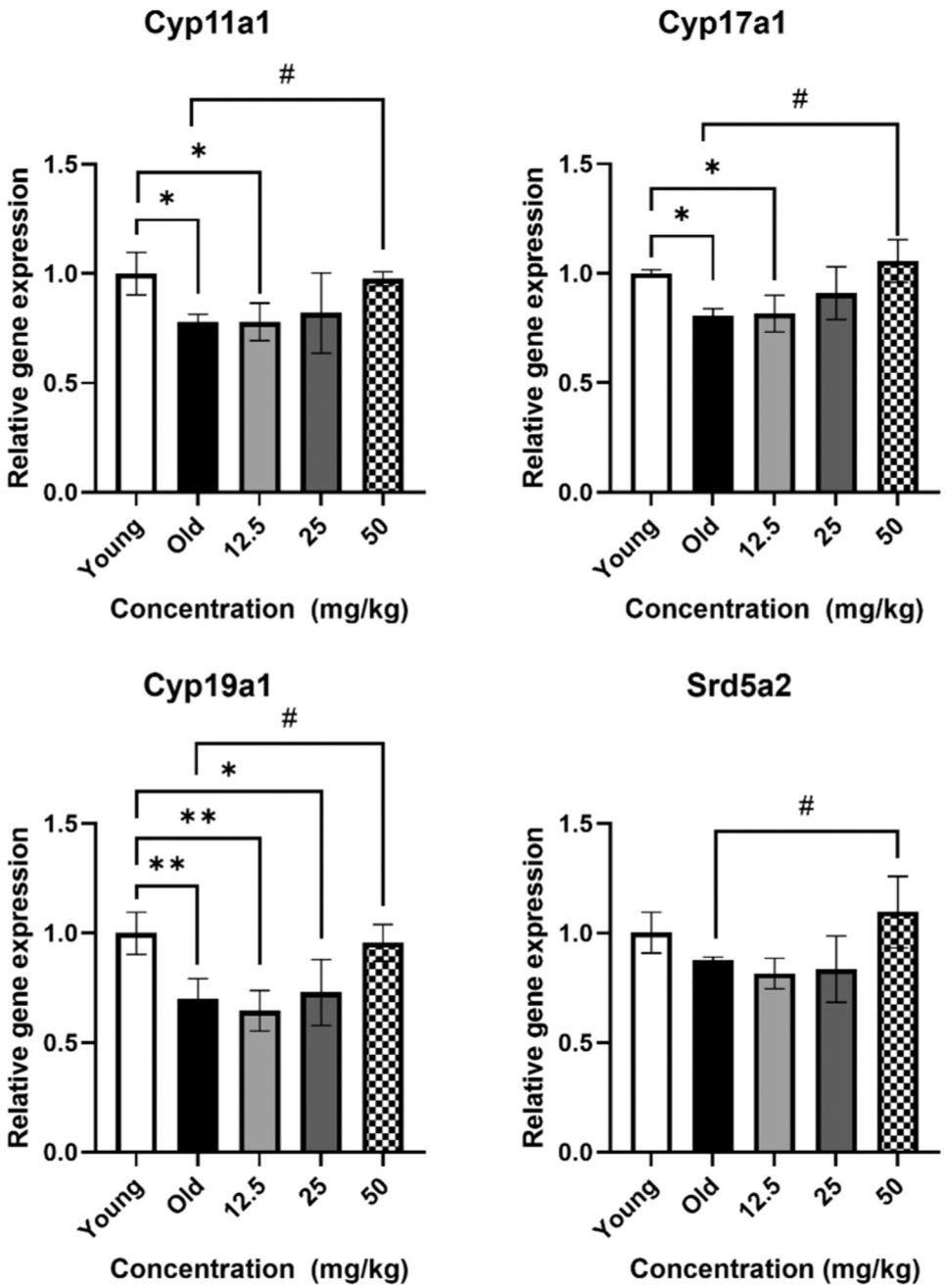

Fig. 6. Effect of TE (12.5, 25, and 50 mg/kg) on gene expression levels in testes tissue. All values are presented as the mean ± standard deviation, *$p<0.05$ and **$p<0.01$ compared to the young control group; and # $p<0.05$ compared to the old control group.

3.7. Effect of bioactive compounds from TE on viability and testosterone level

The gonadal effects of the bioactive compounds (CA and RA) from TE were assessed by measuring testosterone production. Treatment of five concentrations (2, 6, and 10 μM) of CA and RA showed no significant changes in TM3 cell viability (Fig. 8). In testosterone level quantification, individual RA treatment have no significant effect, but CA treatment resulted in a slight increase in the hormone level as dose-dependent (Fig. 9). Notably, the synergistic combination of CA and RA significantly increased the testosterone level at 10 μM concentration. Based on these results, CA appeared to act as an active ingredient and a key substance that exhibited an effective synergistic effect.

4. Discussion

The decline in androgen deficiency-related testosterone in men aged > 60 years has become a global issue [23]. Testosterone deficiency is associated with hypogonadism symptoms, such as obesity, muscle weakness, and insomnia [24]. In previous studies, LOH was associated with cardiovascular mortality [25], bone mineral deficiency [26], and serum lipid profile [27]. Testosterone replacement therapy is currently considered an effective method for LOH, but despite its benefits, it is not risk-free [28]. Owing to the global increase in the aging population, research on effective treatments for LOH induced by aging is urgently needed.

The results of the present study demonstrate that TE has a positive effect on LOH in TM3 cells and in aged mouse models. TM3 Leydig cells are non-tumor cells related to testosterone biosynthesis [29], and

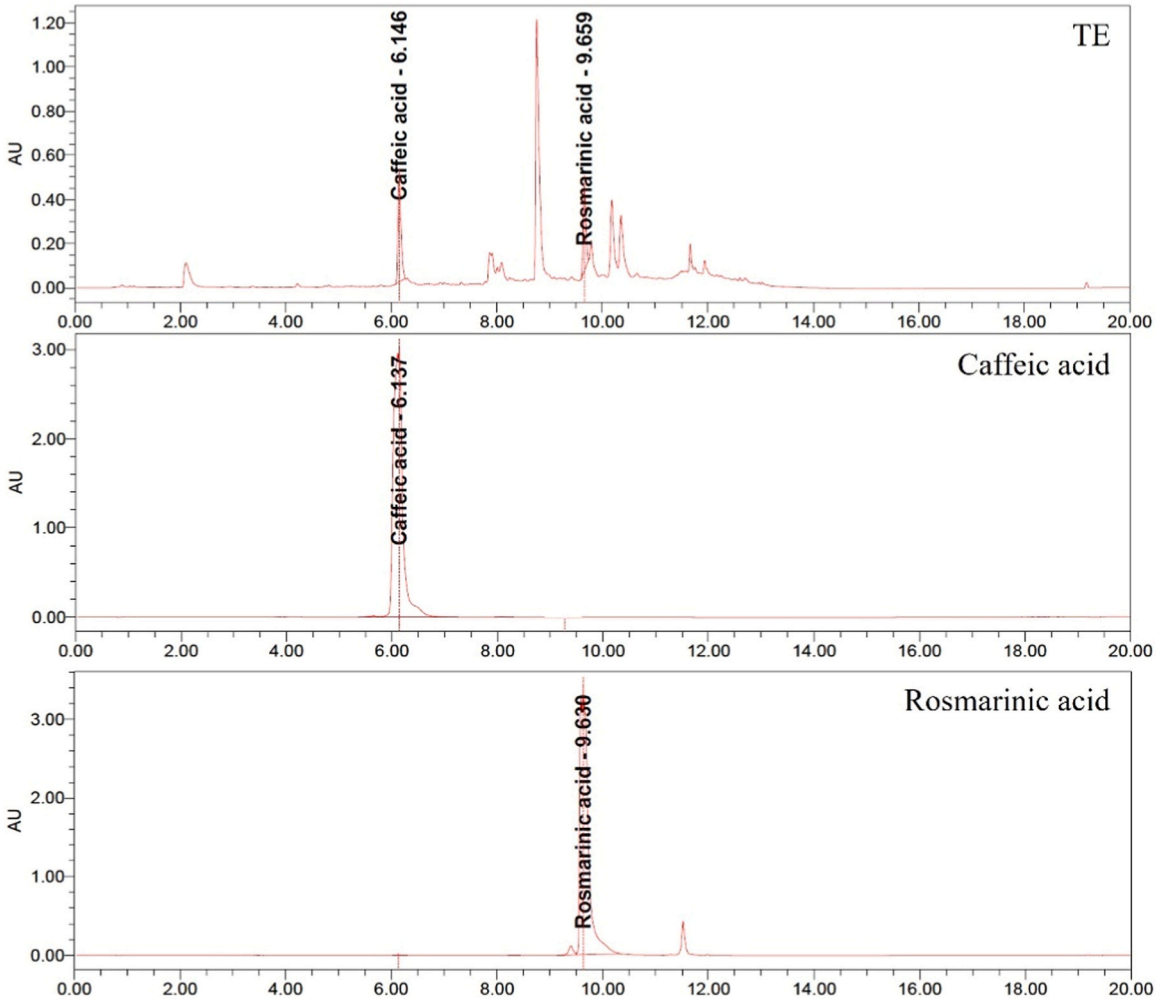

Fig. 7. HPLC chromatogram of TE, CA, and RA. TE: CA: caffeic acid, RA: rosmarinic acid.

testosterone is an important player in male sexual organ development [30]. The present study demonstrated that 12.5, 25, and 50 ug/mL of TE treatment in TM3 cells significantly increased testosterone levels in a dose-dependent manner without any changes in cell viability. After confirming the effect of TE on testosterone levels in TM3 cells, testosterone mRNA levels were estimated in TM3 cells.

The mRNA levels of genes related to testosterone synthesis showed a dose-dependent increase in the expression of Cyp11a1, Cyp17a1, Cyp19a1, and Srd5a2 in the TE-treated TM3 cells. CYP enzymes play a role in the metabolism of defense systems that protect organisms from toxic environments [31]. Among the various cytochrome P450 enzymes, Cyp17a1 is a representative target for the prostate, and this enzyme catalyzes pregnenolone to 17α-OH pregnenolone, which is consequently converted to dehydroepiandrosterone [32]. In a previous study, Ortega et al. [33] demonstrated that Cyp17a1 expression, which is important for regulating the androgen biosynthesis pathway, was increased by letrozole treatment in rats. Furthermore, Cyp11a1 upregulation induces active vitamin D3 to promote testosterone synthesis [34]. In addition, Cyp19a1 plays a role in androgen catalysis from testosterone to estradiol [35], and testosterone is converted to dihydrotestosterone (DHT), which is an important androgen for prostate maintenance by 5-alpha-reductase-type 2 (Srd5a2) [36]. The results of this study confirm that TE treatment effectively increases testosterone levels by regulating the expression of these genes in TM3 Leydig cells.

The *in vivo* results of this study confirm that TE treatment does not affect mouse body weight, which proves that TE does not pose any harmful effects on the mice. It is already known that testicular weight decreases with age in mice, which leads to a decrease in the proportion of Leydig cells in the testis tissue [37]. In our results, testis and epididymis weights decreased in the old control group, including in the TE-treated group. Furthermore, we performed histological analysis to detect differences among the groups (data not shown); however, TE treatment did not show any significant differences in the histological analysis of the testis tissues.

Furthermore, testosterone levels in the OC group decreased significantly compared to those in the YC group, but the same level of testosterone production was observed in the TE-treated groups, suggesting that TE promotes testosterone production in an aged animal model. Similar effects were confirmed in *Kaempferia parviflora* extract [38] and *Bueta superba* Roxb. extract [39] in previous studies. Generally, in mouse serum, AST and ALT levels increase gradually during aging; however, TE treatment did not affect AST and ALT levels. Similarly, cholesterol, HDL-d, and LDL-c production also increased in the old control group, including the TE-treated group. TE treatment (12.5, and 50 mg/kg) significantly lowered cholesterol levels in the OC group, but did not affect HDL-c and LDL-c levels. AST and ALT are recognized as standard biomarkers of hepatotoxicity [40] and HDL-c and LDL-c are used as measurement parameters for the liver [41]. In this study, TE treatment significantly increased testosterone production without causing cytotoxicity in an older animal model.

After serum analysis, gene expression analysis of mouse testicular tissue was performed. The levels of Cyp11a1, Cyp17a1, Cyp19a1, and Srd5a2 in the testis tissues of the OC group were lower than those in the YC group; however, a high dose of TE (50 mg/kg) significantly improved

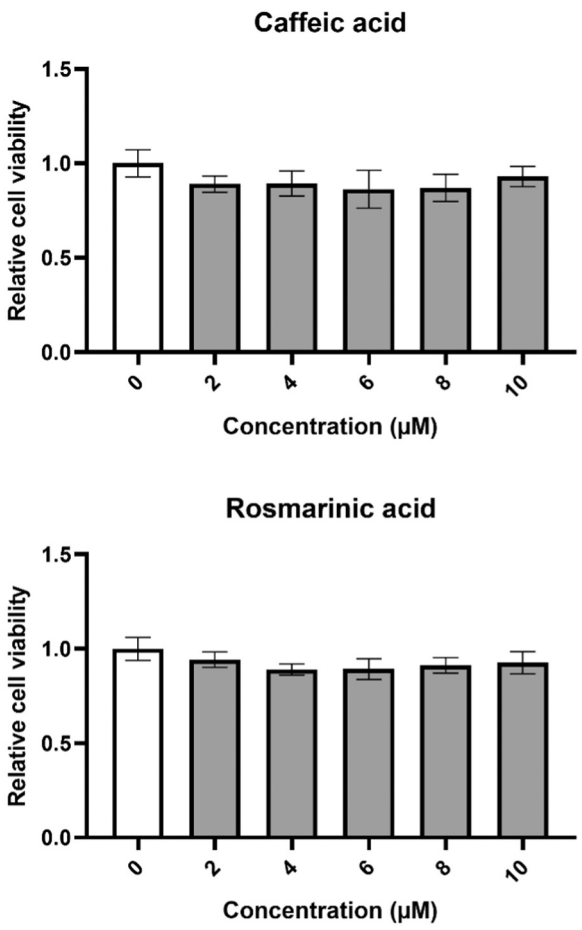

Fig. 8. Effect of bioactive compounds (2, 4, 6, 8, and 10 μM concentration of each compound) from TE on cell viability in TM3 Leydig cells. All values are presented as the mean ± standard deviation.

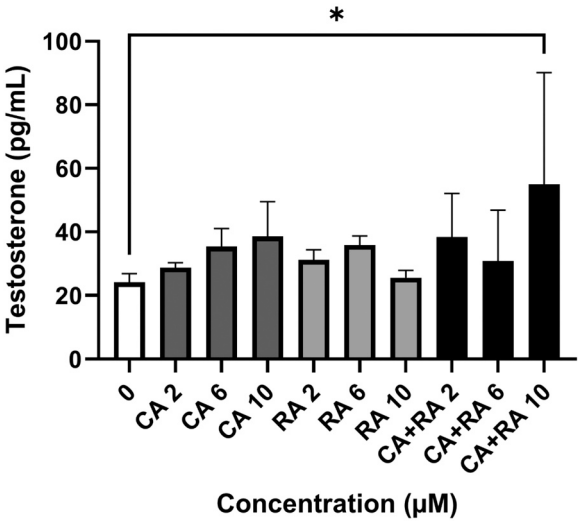

Fig. 9. Effect of bioactive compounds (2, 6, and 10 μM concentration of each compound and combination of compounds) from TE on testosterone levels in TM3 Leydig cells. All values are presented as the mean ± standard deviation. *$p<0.05$ compared to the control group. CA: caffeic acid; RA: rosmarinic acid.

these gene expression levels. This tendency was similar to that observed for the expression of the same genes in TM3 Leydig cells. In the case of androgen deprivation therapy (ADT), it was effective on prostate cancer by regulating testosterone levels through CYP gene inhibition, but the presence of systemic side effects was the main concern [42]. TE treatment is a therapeutical candidate for solving these problems. The present data provide evidence for the effect of TE treatment in old animal models via the same pathway as that in the TM3 Leydig cell experiment.

Through HPLC-MS analysis, CA and RA were detected as effective candidate components in TE. After detecting the bioactive compounds in TE, the cell viability of the TM3 Leydig cell and testosterone level changes were confirmed using these bioactive compounds. In previous studies, both compounds were shown to affect testosterone production. CA increases testosterone levels in rats [43] and RA increases serum testosterone levels in electromagnetic field-exposed male rats [44]. However, in the present study, a dose-dependent increase in testosterone levels was observed in CA+RA-treated TM3 Leydig cells. This was due to the expression of genes related to androgen metabolism, including Cyp11a1, Cyp17a1, Cyp19a1, and Srd5a2, which are regulated by the plant extract TE and its bioactive compounds CA and RA (Fig. 10). In conclusion, increased level of testosterone production in TM3 Leydig cell (*in vitro*) and aged mice model (*in vivo*) confirmed that the TE could be a potential therapeutic candidate to control LOH in aged men population.

Author contribution statement

Hyuck Se Kwon and Jun Hong Park designed the study. Hyun-Yong Kim, Hyuck Se Kwon, Je-Oh Lim, and Yeo Jin Park conducted the study.

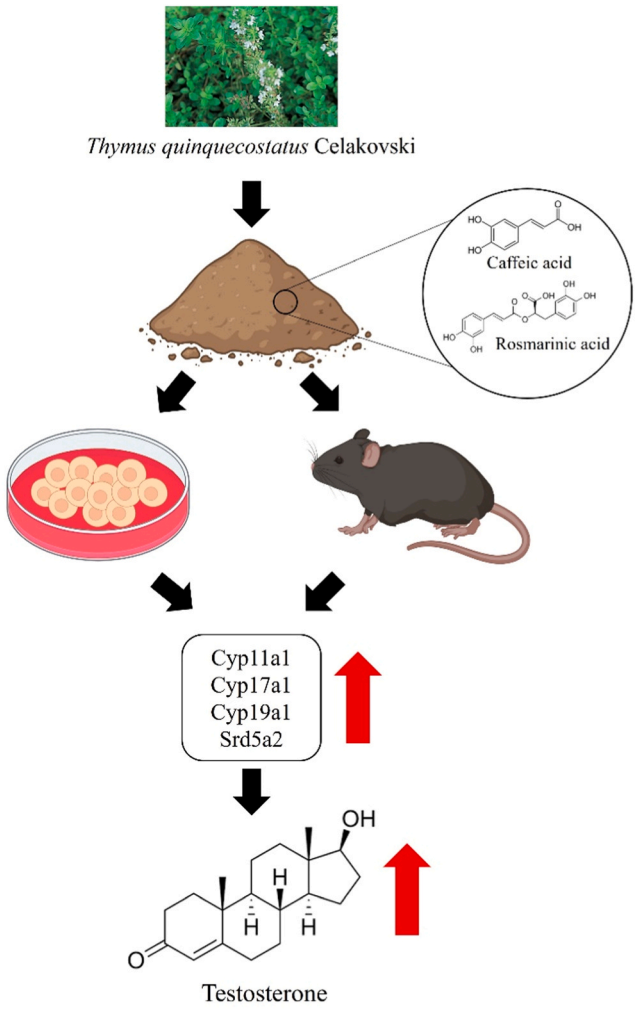

Fig. 10. Schematic model of TE effect on testosterone production in LOH animal model.

Hyun-Jun Jang, Subramanian Muthamil, Ung Cheol Shin, Ji-Hyo Lyu, Hyeon-Hwa Nam, Na-young Lee, Hyun-Jeong Oh, Soon-Il Yun, and Jong-Sik Jin analyzed the data. Hyun-Yong Kim prepared the manuscript. All the authors contributed to the manuscript and approved the submitted version.

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CRediT authorship contribution statement

Hyun-Jun Jang: Data curation. **Subramanian Muthamil:** Data curation. **Ung Cheol Shin:** Data curation. **Ji-Hyo Lyu:** Data curation. **Jong-Sik Jin:** Methodology. **Jun Hong Park:** Writing – review & editing, Conceptualization. **Je-Oh Lim:** Methodology. **SOON-IL YUN:** Methodology. **Hyun-Yong Kim:** Writing – original draft. **Hyuck Se Kwon:** Conceptualization. **Yeo Jin Park:** Methodology. **Hyeon-Hwa Nam:** Data curation. **Na-young Lee:** Methodology. **Hyun-Jeong Oh:** Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jun Hong Park declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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