Bioactive components in black currant fruit, red perilla (shiso) leaf, and Chinese sweet tea that enhance testosterone production in Leydig cells and their combined effect in male mice

Eisuke Kato[a]*, Yosuke Inagaki[b], Hiromi Kayooka[c], Yusuke Adachi[d], Tomoaki Nagase[c], Naofumi Terada[c], and Ai Tsuruma[c]

a Division of Fundamental AgriScience and Research, Research Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

b Japan Pharma Co., Ltd., 4-2-16 Nihonbashi Hongokucho, Chuo-ku, Tokyo 103-0021, Japan

c Frontiers in Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

d Department of Bioscience and Chemistry, School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

*Corresponding author: Eisuke Kato, Laboratory of Food Biochemistry, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

Tel.: +81-11-706-2496

E-mail: eikato@agr.hokudai.ac.jp

Abstract

Testosterone is a steroid hormone that regulates diverse biological events. Several foods affect testosterone levels in the body, but little is known about the individual components that elevate testosterone levels. In this study, black currant (*Ribes nigrum*) fruit, red perilla (shiso, *Perilla frutescens*) leaf, and Chinese sweet tea (*Rubus suavissimus*) enhanced testosterone production in testicular Leydig cells. Delphinidin-3-rutinoside (D3R), rosmarinic acid (RA), and *myo*-inositol (MI) were identified as bioactive components. Mechanistic analyses suggested that *Star* plays a role in the bioactivity of D3R and RA, and that the bioactivity of MI is related to *Cyp19a1* downregulation. Supplementing the diet with a mixture of black currant fruit extract, red perilla leaf extract, and MI increased serum testosterone levels in male C57BL6/J mice, accompanied by an increased level of Star protein in the testis. These findings add to our knowledge about foods, and their components, that modulate testosterone levels.

Keywords

Testosterone; Leydig cell; late-onset hypogonadism; anthocyanin; rosmarinic acid; inositol

Introduction

Food consumption is correlated with variations in the levels of hormones in the body. Nutrients as well as non-nutrient dietary factors stimulate the gut system to modify the release of incretin hormones.[1,2] For example, dietary polyphenols elevate blood glucose levels, and also increase insulin levels by stimulating the pancreatic islets.[3] A phenolic compound in olive oil, oleuropein, enhances catecholamine levels (epinephrine and norepinephrine).[4] These phenomena show that certain foods are able to regulate hormone levels.

Testosterone is an androgenic steroid hormone that is primarily produced by Leydig cells in the testes of men. The relationship between food consumption and plasma testosterone levels has been studied, with both stimulatory and inhibitory effects observed to date.[5] Certain food components including tocotrienol,[6] geranylgeraniol,[7,8] menaquinone-4,[9] flavonoids,[10–12] and saccharin,[13] can modify the production of testosterone in the testis, which suggests that there is a relationship between food and testosterone levels.

Testosterone has several functions in the body. It is related to the maturation of sexual organs, higher bone density, and increased muscle weight. A decline in serum testosterone concentrations occurs through aging and/or in response to stress.[14] Because of the above functions, decreased testosterone levels cause various symptoms, such as sexual dysfunction, depression, and decreases in muscle mass, bone mineral density, and vitality, which together are known as late-onset hypogonadism (LOH).[15] Additionally, low testosterone concentrations in the body are related to diseases such as cardiovascular disease,[16] obesity associated diabetes,[15] and decreased cognitive function.[17]

The discovery of food components that can modulate testosterone production could lead to the development of new treatments for hypogonadism. In this study, we identified several food products, as well as their bioactive components, that enhance testosterone production in Leydig cells. We investigated the mechanism of their bioactivity, and examined how they affect testosterone levels *in vivo* in a mouse model.

Materials and methods

Chemicals and food products

Extract powders of food products (Table S1) for the *in vitro* experiment were either obtained from Matsuura Yakugyo Co., Ltd. (Aichi, Japan) or Japanese Traditional Medical Laboratory Co., Ltd. (Aichi, Japan). The products used in *in vivo* experiments were black currant fruit extract (Morishita Jintan Co., Ltd., Osaka, Japan), red perilla leaf extract (Nichino Kagaku Kogyo Co., ltd., Saitama, Wakayama, Japan) and myo-inositol (MI) (Tsuno Food Industrial Co., Ltd., Japan). Delphinidin 3-rutinoside chloride (>99%) and cyanidin 3-rutinoside chloride (>99%) were purchased from Nagara Science Co. (Gifu, Japan). Rosmarinic acid (>96%) and MI (>99%) were purchased from Fujifilm Wako Pure Chemicals Co. (Osaka, Japan). All remaining commercially available chemicals were purchased from Fujifilm Wako Pure Chemicals Co. unless otherwise noted.

Purification of active components from Chinese sweet tea

The commercial extract powder of Chinese sweet tea was partitioned between hexane, ethyl acetate, 1-butanol, and water. The water layer was adsorbed on

Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) and eluted with water, 50% aq. methanol, and methanol. The water-eluted fraction was loaded onto a Cosmosil 75C18-OPN column (Nacalai Tesque, Inc., Kyoto, Japan) and the water-eluted fraction was collected. The eluate was then separated by gradient elution with 20%–35% aq. methanol containing 0.1% v/v trifluoroacetic acid using InertSustain C18 (GL Sciences Inc., Tokyo, Japan). The active peak was separated by gradient elution with 1%–15% aq. methanol containing 0.1% v/v trifluoroacetic acid using Cosmosil PBr (Nacalai Tesque, Inc.). The active peak was further separated using 15% aq. acetonitrile on a Shodex Asahipak NH2P-50 4E column (Shoko Science Co., Ltd., Tokyo, Japan) to collect MI, glucose, and fructose, which were characterized based on ^{1}H-nuclear magnetic resonance (NMR) spectra data (Bruker AMX500, Bruker, Rheinstetten, Germany) and mass spectrometry (Thermo Scientific Exactive, Thermo Fisher Scientific, Waltham, MA, USA) data (Figure S1).

Cell culture

Mouse testicular tumor-derived Leydig cell model I-10 (JCRB9097) were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were cultured at 37°C under a 10% CO_2 atmosphere in growth medium [Ham's F-10 medium (Sigma-Aldrich®, St Louis, MO, USA) supplemented with 10% v/v fetal bovine serum and antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamicin)].

***In vitro* testosterone production activity assay (I-10 cells)**

I-10 cells (2×10^4 cells/well, 250 μL/well) were seeded in cell-culture treated 48-well plates (AS ONE co. Osaka, Japan). One day after seeding, the medium was replaced with the sample-containing medium and the cells were cultured for a further 24 h. The medium was then recovered, and testosterone concentrations were measured with a Testosterone ELISA kit (Cayman Chemical Co. Ann Arbor, MI, USA). The measured concentrations of testosterone were in the range of 20–200 ng/mL, which was within the assay range of ELISA kit (3.9–500 ng/mL). When inhibitors (MDL12,300A or H-89) were used, the cells were pre-treated with the inhibitor for 1 h and then stimulated with the sample in the presence of the inhibitor. Samples and inhibitors were dissolved in either water or dimethyl sulfoxide and then diluted in growth medium. Dimethyl sulfoxide concentrations in the medium were <0.1%, and the same amount of dimethyl sulfoxide was added to cells in the control. Geranylgeraniol (30 μM) was used as the positive control to induce testosterone secretion.[7,8]

Ex vivo testosterone production activity assay (mouse testis)

The experiment was performed with approval (No. 19-0163) from the Institutional Animal Care and Use Committee, Hokkaido University, Japan. All operations were compliant with the National University Corporation Hokkaido University Regulations on Animal Experimentation. C57BL/6J male mice were housed in an air-conditioned room at 23°C±2°C with a light period from 8:00 am to 8:00 pm. The mice had free access to their diet and water. On the day of the experiment, the mice (11–13 weeks old) were anesthetized using isoflurane and decapitated. The left and right testes were collected, washed twice with phosphate-buffered saline,

and used for the experiment. The whole testes were placed in the wells of a 24-well plate (AS ONE co.), immersed in 2 mL Ham's F-10 medium supplemented with 10% v/v fetal bovine serum and antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamicin), and incubated for 1 h at 37°C under a 10% CO_2 atmosphere. The medium was replaced, and the testes were incubated for another hour to collect the control medium. The medium was then replaced with the sample-containing medium, and the procedure was repeated to collect the sample-stimulated medium. Testosterone concentrations in the control medium and sample-stimulated medium were measured with the Testosterone ELISA kit. A preliminary experiment was also conducted to confirm that the collected testes retained the ability to secrete a certain amount of testosterone for at least 6 hours.

Effects of food components on serum testosterone level in mice *in vivo*

The *in vivo* experiment was performed with the approvals mentioned earlier. Six-month-old C57BL/6J male mice were housed in an air-conditioned room at 23°C±2°C with a light period from 8:00 am to 8:00 pm. The mice were divided into the control group, which was fed a powdered MF diet (Oriental Yeast Co., Ltd.), and the treatment group, which was fed an MF diet supplemented with 1% sample mixture [consisting of (w/w) 66.3% MI, 5.8% red perilla leaf extract (containing 14.3% rosmarinic acid) and 27.8% black currant fruit extract (containing 30% anthocyanin)]. After 2 weeks with free access to their diet and water, the mice were starved for 16 h, anesthetized using isoflurane, and decapitated. The blood was collected, and the serum testosterone level was measured by ELISA. The left testis was collected and placed whole in the well of

a 24-well plate, and then suspended in 2 mL Ham's F-10 medium supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin) for 30 min. The medium was collected to measure the amount of secreted testosterone by ELISA. The right testis was collected and cut in half. One half was used to extract total RNA and the other was used to extract proteins. The RNA and proteins were analyzed by reverse-transcription qPCR and western blotting, respectively.

Gene expression analysis

Total RNA was extracted from the mouse testis or I-10 cells using the FastGene RNA Premium Kit (Nippon Genetics Co., Ltd, Tokyo, Japan). Complementary DNA was synthesized from the total RNA using ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Gene expression analysis by real-time quantitative polymerase chain reaction was performed with the KAPA SYBR FAST qPCR mix (KAPA Biosystems, Inc., Wilmington, MA, USA). The primer pairs are listed in Table 1. *Actb* was selected as a reference gene based on the results of a preliminary experiment.

Table 1. Primer pairs for RT-qPCR

Gene symbol	Sequence (5′–3′)
Actb	Forward: TACGACCAGAGGCATACAG
	Reverse: GCCAACCGTGAAAAGATGAC
Star	Forward: TGGAAAAGACACGGTCATCA
	Reverse: CTCCGGCATCTCCCCAAAAT
Cyp11a1	Forward: CGTGACCAGAAAAGACAACA
	Reverse: AGGATGAAGGAGAGGAGAGC

Cyp17a1	Forward: TGGGCACTGCATCACGATAA
	Reverse: GCTCCGAAGGGCAAATAACT
Hsd3b1	Forward: AGTGATGGAAAAAGGGCAGGT
	Reverse: GCAAGTTTGTGAGTGGGTTAG
Hsd17b3	Forward: AACGCAACATCAGCAACAGA
	Reverse: CAGCCCCACCTCACCCTACC
Cyp19a1	Forward: CGAAGCAGCAATCCTGAAGGAG
	Reverse: CCAAGTCCACAACAGGCTGGTA

Western blotting

The protein samples were prepared by lysing the cells or testis with the EzRIPA Lysis kit (Atto Co., Osaka, Japan). The lysate was mixed with sodium dodecyl sulfate sample buffer and heated at 95°C for 5 min. The samples were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was stained with EzStain Aqua MEM (Atto Co.) to estimate the total protein, and then Star and aromatase proteins were detected using a combination of Star antibody (#8449, 1:1000; Cell Signaling Technology, Danvers, MA, USA), Anti-aromatase antibody (bs-0114M, 1:1000, Bioss Inc., Woburn, MA, USA), and anti-rabbit immunoglobulin G horseradish peroxidase-linked antibody (#7074, 1:2000; Cell Signaling Technology) with Immunostar® Zeta (Fujifilm Wako Pure Chemicals Co., Osaka, Japan) as the detection reagent. The abundance of the target protein relative to total protein was estimated using ImageJ software.[18]

Statistical analysis

In vitro experiments were conducted at least twice with N=3. Representative data are expressed as mean ± standard error of the mean (SEM). *In vivo* experiments were performed twice, and the summarized data are expressed as mean ± SEM.

Data were analyzed using GraphPad Prism software (ver.10) with the statistical methods indicated in the figure legends. A p value of <0.05 was considered statistically significant.

Results

Screening of food products

Commercially available powdered extracts of food products were tested for their ability to stimulate the release of testosterone from mouse Leydig cells (I-10) (Table S1). Among them, we focused on extracts from black currant fruit, red perilla leaf, and Chinese sweet tea, which showed relatively high activity, with 5.8-, 3.8-, and 4.2-times enhanced testosterone secretion compared with that in the control (1 mg/mL).

Activity of components of candidate food products

To determine the bioactive components in the extract, compounds that are known to be present in the candidate food products were selected, and their ability to stimulate testosterone production in I-10 cells was assessed. We selected cyanidin-3-rutinoside (C3R) and delphinidin-3-rutinoside (D3R) from black currant,[19] and rosmarinic acid (RA) from red perilla leaf, which are representative compounds in each food (Figure 1a).[20] In case of Chinese sweet tea, activity-guided separation led to the selection of MI, glucose, and fructose (Figure S1). Among these compounds, MI was selected for further evaluation (Figure 1a).

At concentrations of 100 μg/mL, D3R and RA significantly stimulated I-10 cells to produce testosterone at 2.6-times (P<0.0001) and 2.4-times (P=0.0006) that in

229 the control (Figure 1b and Figure S2). These activities were comparable to that of
230 the positive control, geranylgeraniol.[7,8] Neither D3R nor RA had cytotoxic effects
231 at this concentration (Figure S3). The results suggested that these compounds are
232 at least partly responsible for the activity of the candidate plants.
233 D3R and RA were also evaluated in an *ex vivo* experiment to determine their
234 activity to stimulate testosterone production by mouse testes. Stimulation of mouse
235 testes with D3R or RA at 100 μg/mL enhanced the production of testosterone by
236 1.52-times and 1.63-times, respectively, compared with that in the control (Figure
237 1c).
238 The activity of MI was not reproducible; it sometimes showed activity and
239 sometimes did not (data not shown). Thus, we speculated that it may affect the
240 activity of other stimulatory compounds. The combination of MI and D3R or RA
241 promoted testosterone production more strongly than did MI, D3R, or RA alone,
242 confirming this idea (Figure 1d). At the concentration used in this study, MI had
243 no cytotoxic effects (Figure S3).
244 The aforementioned results identified D3R, RA, and MI as bioactive compounds.
245 However, because food-derived compounds are metabolized during digestion and
246 absorption, their efficacy can be further evaluated by testing the activity of their
247 metabolites. Among the three identified compounds, MI is a endogenous
248 compound, and D3R has been detected in an unmetabolized form in plasma after
249 being given orally to rats.[21] Therefore, the major metabolites of RA, caffeic acid
250 (CA) and ferulic acid (FA)[22] were tested for their ability to enhance testosterone
251 production by I-10 cells. The cells cultured with CA and RA showed enhanced

testosterone production activity compared with that of the control, whereas FA had no effect to increase testosterone production (Figure 1e).

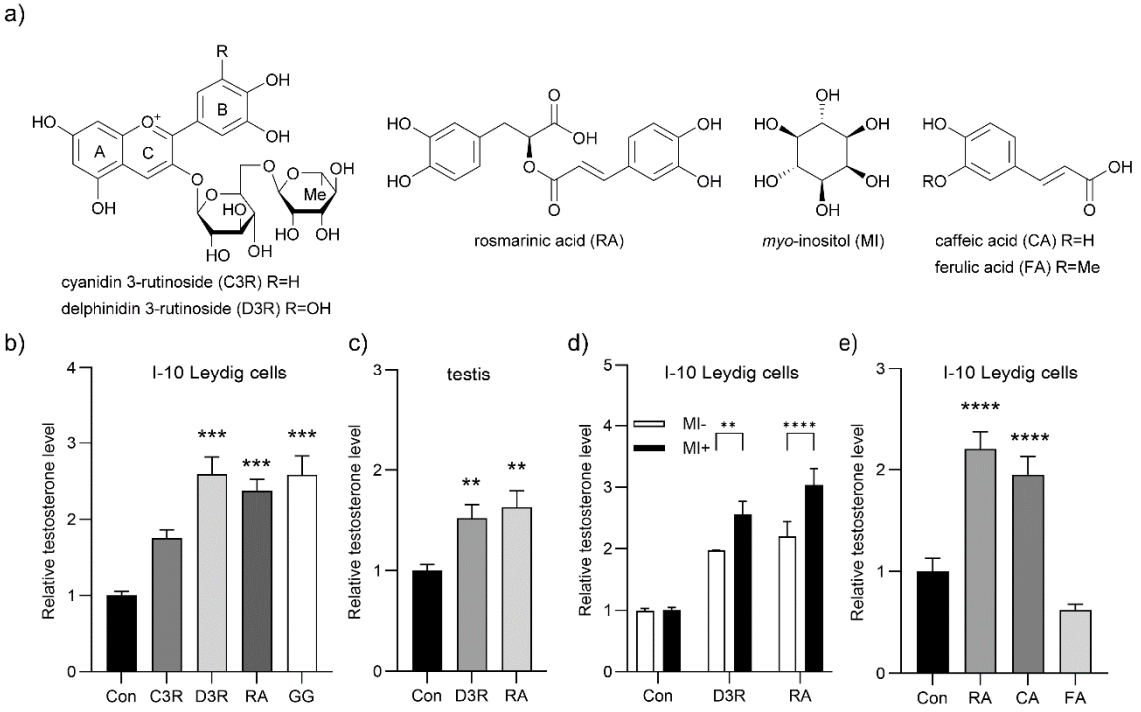


Figure 1. Ability of selected compounds to induce testosterone secretion from I-10 Leydig cells and testis. a) Structures of compounds. b) Relative amounts of testosterone produced after culturing I-10 Leydig cells with cyanidin-3-rutinoside (C3R), delphinidin-3-rutinoside (D3R), rosmarinic acid (RA), and geranylgeraniol (GG, 30 μM, positive control) at 100 μg/mL in the medium (*N*=3). c) Relative amounts of testosterone produced after exposing mouse testis to either D3R or RA at 100 μg/mL for 2 h (*N*=6). d) Additive effect of *myo*-inositol (MI, 10 mM) on either D3R-induced (100 μg/mL) or RA-induced (100 μg/mL) testosterone secretion by I-10 cells (*N*=3). e) Effect of RA, caffeic acid (CA) and ferulic acid (FA) at 100 μg/mL on testosterone secretion by I-10 cells. Values are shown

relative to that in control (Con). **p<0.01, ***p<0.001, ****p<0.0001 vs. control (Tukey's test).

Mechanistic analysis of D3R, RA, and MI

The activities of D3R, RA, and MI were further investigated to understand the molecular mechanism by which testosterone production is stimulated. The results of RT-qPCR analyses of genes related to the biosynthesis of testosterone showed that the transcript level of *Star* was increased by D3R and RA treatments (Figure 2a, Figure S4). Because of the additive effect of MI (Figure 1d), the transcript level of *Cyp19a1*, encoding the aromatase that converts testosterone to estradiol, was examined. Its transcript levels were reduced by the MI treatment (Figure 2a, Figure S5).

Expression of *Star* is regulated through the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway.[23] To evaluate if this pathway is responsible for the activity of D3R and RA, the adenylyl cyclase inhibitor MDL12,300A or the PKA inhibitor H-89 was co-incubated with each compound. MDL12,300A and H-89 diminished the enhanced testosterone production induced by D3R and RA (Figure 2b), indicating that the cAMP/PKA pathway is involved in the response to these compounds.

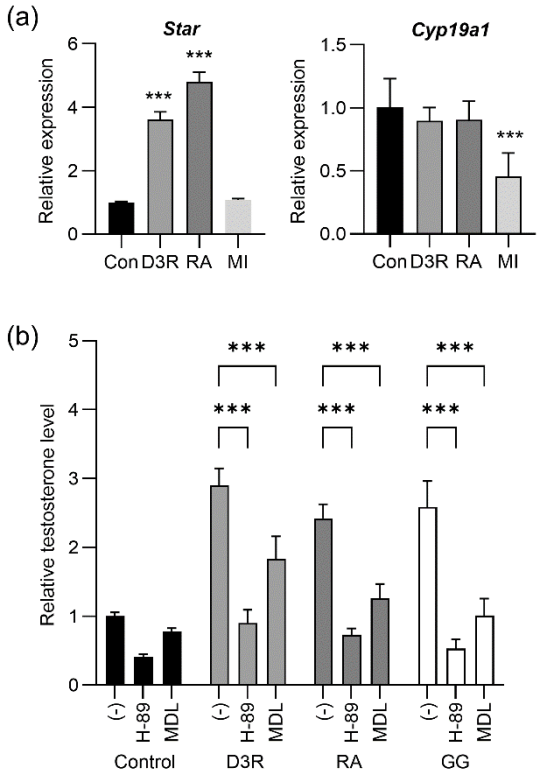


Figure 2. Analysis of the mechanism of action of delphinidin-3-rutinoside (D3R), rosmarinic acid (RA) and *myo*-inositol (MI). (a) Analysis of gene expression. I-10 cells were stimulated by D3R and RA at 100 μg/mL or MI (10 mM) for 24 h. *Star* transcript levels were evaluated after 3 h of stimulation and *Cyp19a1* transcript levels were evaluated after 24 h (N=3). Values are shown relative to that in control (Con). ***p<0.001 vs. control (Dunnett's test). (b) Testosterone production of I-10 cells after stimulation by D3R (100 μg/mL), RA (100 μg/mL), and geranylgeraniol (GG, 30 μM, positive control) co-incubated with cAMP inhibitor MDL12,300A (MDL, 10 μM) or PKA inhibitor H-89 (10 μM). (-) indicates absence of inhibitor (N=6). Testosterone level is shown relative to that in control (-). ***p<0.001 between treatment groups (Tukey's test).

Combination of MI with black currant fruit and red perilla leaf extract elevates serum testosterone level in mice

Our results suggest that foods containing D3R, RA, and MI have the potential to enhance testosterone production. Because D3R and RA stimulate testosterone production by testicular Leydig cells and MI supports this process, we evaluated whether the combination of all three compounds elevated the serum testosterone level in mice.

Six-month-old male C57BL6/J mice were fed for 2 weeks with a diet containing 1% of a supplement consisting of black currant fruit extract, red perilla leaf extract, and MI. Purified MI was used instead of Chinese sweet tea due to the low MI content in the tea. This ratio in the supplement was based on that used in previous studies on the effect of anthocyanin, RA, and MI on rats.[24–26] There was no difference in the body and testis weight between the groups (Figure 3a,b). The serum testosterone level was significantly higher (P=0.0258) in mice fed with the supplemented diet than in the control (Figure 3c), and production of testosterone from the isolated whole testis also tended to be higher in the group fed with the supplemented diet (Figure 3d). There were no differences in transcript levels of *Star* and *Cyp19a1* in the testes between the groups (Figure 3e). The level of Star protein, but not aromatase protein, was increased in the group fed with the supplemented diet (Figure 3f, Figure S6–8).

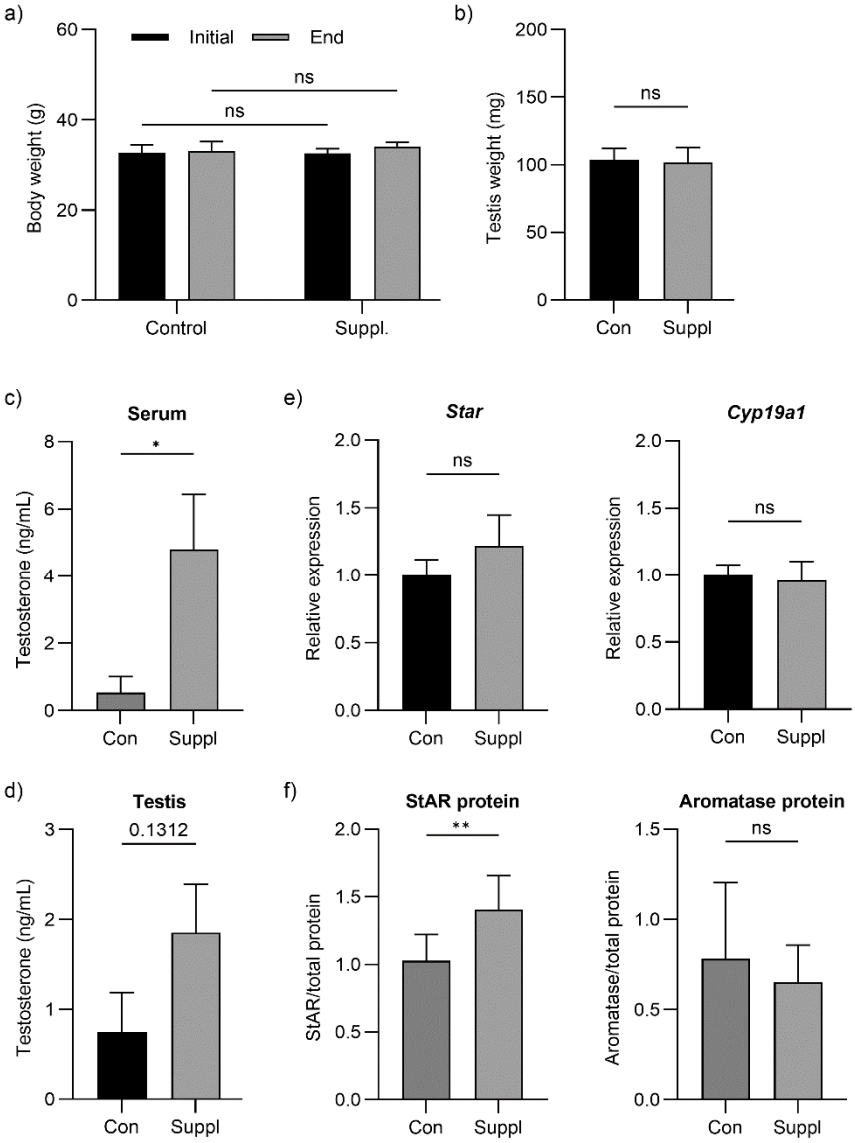


Figure 3. Effect of a diet supplemented with MI, black currant fruit extract, and red perilla leaf extract on C57BL6/J mice. a) Body weight, b) Testis weight, c) Serum testosterone level, d) Testosterone release from testis. Each collected testis was immersed in the medium for 30 min, and amount of testosterone released into the medium was measured by ELISA. e) Relative transcript levels of *Star* and *Cyp19a1* in testis. f) Star and aromatase protein levels in testis. ns (no significance), *$p<0.05$, **$p<0.01$ vs. control (N=11–12, Šidák test for body weight and t-test for others).

Discussion

Previous studies have demonstrated that certain food components can enhance the production of testosterone by Leydig cells.[6–13] Similarly, the screening of food products in this study revealed that black currant fruit, red perilla leaf, and Chinese sweet tea can also modulate testosterone secretion. These findings suggest that these foods could be used in the treatment of hypogonadism.

D3R and RA were identified as the bioactive components in black currant fruit and red perilla leaf that stimulate testosterone production in I-10 cells (Figure 1a,b). Analysis of mechanistic aspects revealed that D3R and RA increased the transcript levels of the gene encoding steroidogenic acute regulatory (Star) protein (Figure 2a). Star transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, the rate-limiting step for the synthesis of steroid hormone.[27] Therefore, the bioactivity of D3R and RA is at least partly due to their ability to increase *Star* transcript levels. An increased level of Star protein in I-10 cells was not confirmed, but the activity of anthocyanin to upregulate Star protein has been reported by Sun et al.,[28] suggesting that elevated gene expression leads to increased protein levels.

An analysis of the mechanism of D3R and RA bioactivity indicated that the cAMP/PKA pathway is involved in the increased testosterone secretion response (Figure 2b). In Leydig cells, activation of PKA leads to the expression of the *Star* gene as well as the phosphorylation and translocation of Star protein to mitochondria, both resulting in enhanced steroidogenesis.[29] Thus, PKA is likely involved in the enhanced expression of *Star* in response to D3R and RA treatments.

One concern is that although activation of PKA has been reported to elevate the expression of *Cyp11a1*, *Cyp17a1*, and *Hsd3b*,[30,31] we found that treatment with D3R and RA resulted in decreased *Cyp19a1* transcript levels (Figure S4). Because we did not directly confirm the activation of PKA through evaluation of the cAMP level or the phosphorylation of PKA substrates, there remains the possibility that pathways other than PKA are related to the bioactivity of D3R and RA.

MI was identified as a bioactive component in Chinese sweet tea that supports testosterone production, as it reduced the expression of *Cyp19a1* in I-10 cells (Figure 1d and 2a). *Cyp19a1* encodes an aromatase that converts testosterone to estradiol. Decreased expression of *Cyp19a1* can support testosterone accumulation by reducing the conversion of testosterone to estradiol. The yield of MI from Chinese sweet tea was insufficient to account for the activity of the extract, suggesting that another active component is present in Chinese sweet tea. However, we detected a synergistic effect of MI with D3R and RA (Figure 1d). Another study showed that D-*chiro*-inositol, the stereoisomer endogenously synthesized from MI by the action of epimerase, was able to increase testosterone plasma level in human subjects, providing further evidence for the efficacy of MI.[32]

In this study, dietary intake of the combination of black currant fruit extract, red perilla leaf extract, and MI elevated the serum testosterone level of 6-month-old male C57BL6/J mice. Six-month-old male mice are a model of middle-aged men, the period when LOH symptoms predominantly develop. In other studies, serum testosterone levels were evaluated in rats fed with anthocyanins and RA. Dairy oral gavage of an extract of roselle (*Hibiscus sabdariffa*) containing anthocyanin ameliorated the reduction of serum testosterone induced by monosodium glutamate

375 in rats.[24] Administration of RA to Wistar rats increased the serum testosterone
376 levels to 1.84-times that in the control.[25] Our results are in accordance with those
377 findings, in that the increase in the testosterone level was larger after administering
378 the combination of three food products (Figure 3c). However, the increase in
379 testosterone detected in our study was greater than that detected in RA-treated
380 rats,[25] and this may be because of biological differences between rats and mice.
381 The transcript levels of *Star* in the testes of mice were not significantly different
382 between those fed the supplemented diet and the control, but there was a significant
383 increase in Star protein levels (P=0.0068) (Figure 3e,f). The upregulation of Star
384 protein was in accordance with the *in vitro* results (Figure 2a), suggesting that this
385 response was a result of dietary supplementation with black currant fruit and red
386 perilla leaf extract containing D3R and RA. A possible explanation for the lack of
387 increased transcript levels of *Star* could be that the mice were starved before
388 collecting the testes. *Star* expression is not persistent and increases upon
389 stimulation, followed by a gradual decrease.[33] Thus, the effect may have been lost
390 during the starvation period.
391 In mice fed the supplemented diet, neither the *Cyp19a1* transcript level nor the
392 aromatase protein level was reduced in the testis. Among the compounds
393 contained in tested supplements, MI was expected to downregulate *Cyp19a1*.
394 Inositols are present in the mammalian body as a component of phosphatidyl
395 inositol and are biosynthesized from glucose. Thus, inositol supplementation may
396 not have had much effect on the amount of inositol in the body. In addition, the
397 control diet in this experiment also contained inositol (439 mg/100 g diet).
398 Although the supplemented diet contained a higher amount of MI (1100 mg/100 g

399 diet), the effect of MI supplementation on the expression of aromatase may have
400 been difficult to detect to the presence of inositol in the control diet.
401 The *in vivo* experiment conducted here has some limitations. One is that the
402 identity of the compound that increased serum testosterone levels is still unclear.
403 The effective concentration of compounds used in cell-based assays was higher
404 than the reported plasma levels of D3R and RA.[21,22] Therefore, it is possible that
405 metabolites or other unidentified components of the black currant fruit and red
406 perilla leaf extract induced the effect. Another issue is whether the compounds
407 acted on Leydig cells of the testis, or on other tissues. We observed a significant
408 (P=0.0258) increase in serum testosterone concentrations in mice fed a
409 supplemented diet vs. the control (Figure 3c). However, the difference was not
410 significant for the excised testes (P=0.1312), only an increasing trend in the
411 testosterone level was detected (Figure 3d). This suggests that another testosterone-
412 synthesizing tissue, e.g., the adrenal cortex, may be involved in increasing the
413 serum testosterone level in response to stimulants.
414 In conclusion, this study aimed to identify food products and their components
415 that elevate testosterone production in Leydig cells of the testis. D3R, RA, and MI
416 were identified, their molecular mechanism to enhance testosterone production in
417 I-10 cells was partly revealed, and the effect was confirmed *in vivo*. Knowledge of
418 bioactive components in foods that increase testosterone levels, and their
419 mechanistic aspects, will enhance our understanding of the relationship between
420 food and testosterone levels in the body. The current findings are limited in their
421 application, but the consumption of the identified foods might be useful in treating
422 the symptoms of low plasma testosterone levels in humans.

Supporting Information

Screening results of food extract powders, NMR analysis of compounds from Chinese sweet tea, concentration dependence of active compounds, cytotoxicity analysis, gene expression analysis, and uncropped images of Western blotting.

Conflicts of interest

The authors declare no conflict of interest.

Funding sources

Part of this work is supported by Adaptable and Seamless Technology transfer Program through Target-driven R&D (A-STEP) from Japan Science and Technology Agency (JST) Grant Number JPMJTM22A1.

Acknowledgments

We thank Dr. Eri Fukushi and Mr. Yusuke Takata, GC-MS & NMR Laboratory, Research Faculty of Agriculture, Hokkaido University for NMR analysis, and Harumi Hayashi, Instrumental Analysis Division, Global Facility Center, Creative Research Institution, Hokkaido University for conducting the MS analysis. We thank Jennifer Smith, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

References

(1) Tsuda, T. Possible Abilities of Dietary Factors to Prevent and Treat Diabetes via the Stimulation of Glucagon-like Peptide-1 Secretion. *Mol. Nutr. Food Res.* **2015**,

446 *59* (7), 1264–1273. https://doi.org/10.1002/mnfr.201400871.

447 (2) Su, W.; Li, Y.; Chang, A. K.; Sheng, T.; Pei, Y.; Li, J.; Li, H.; Liu, K.; Xu, L.; Liu,
448 W.; Ai, J.; Zhang, Z.; Wang, Y.; Jiang, Z.; Liang, X. Identification of Novel
449 Alkaloids from Portulaca Oleracea L. and Characterization of Their
450 Pharmacokinetics and GLP-1 Secretion-Promoting Activity in STC-1 Cells. *J.*
451 *Agric. Food Chem.* **2023**, *71* (49), 19804–19816.
452 https://doi.org/10.1021/acs.jafc.3c05191.

453 (3) Hanhineva, K.; Törrönen, R.; Bondia-Pons, I.; Pekkinen, J.; Kolehmainen, M.;
454 Mykkänen, H.; Poutanen, K. Impact of Dietary Polyphenols on Carbohydrate
455 Metabolism. *Int. J. Mol. Sci.* **2010**, *11* (4), 1365–1402.
456 https://doi.org/10.3390/ijms11041365.

457 (4) Oi-Kano, Y.; Kawada, T.; Watanabe, T.; Koyama, F.; Watanabe, K.; Senbongi, R.;
458 Iwai, K. Oleuropein, a Phenolic Compound in Extra Virgin Olive Oil, Increases
459 Uncoupling Protein 1 Content in Brown Adipose Tissue and Enhances
460 Noradrenaline and Adrenaline Secretions in Rats. *J. Nutr. Sci. Vitaminol. (Tokyo).*
461 **2008**, *54* (5), 363–370. https://doi.org/10.3177/jnsv.54.363.

462 (5) Terrier, J.-E.; Isidori, A. M. How Food Intakes Modify Testosterone Level. *J. Sex.*
463 *Med.* **2016**, *13* (9), 1292–1296. https://doi.org/10.1016/j.jsxm.2016.07.001.

(6) Terada, N.; Nagase, T.; Kayooka, H.; Adachi, Y.; Kato, E. α-Tocotrienol in Rice Bran Enhances Steroidogenesis in Mouse Leydig Cell via Increased Gene Expression of Steroidogenic Acute Regulatory Protein and Induction of Its Mitochondrial Translocation. *Biosci. Biotechnol. Biochem.* **2024**, *88* (2), 189–195. https://doi.org/10.1093/bbb/zbad153.

(7) Ho, H.-J.; Shirakawa, H.; Giriwono, P. E.; Ito, A.; Komai, M. A Novel Function of Geranylgeraniol in Regulating Testosterone Production. *Biosci. Biotechnol. Biochem.* **2018**, *82* (6), 956–962. https://doi.org/10.1080/09168451.2017.1415129.

(8) Ho, H. J.; Shirakawa, H.; Yoshida, R.; Ito, A.; Maeda, M.; Goto, T.; Komai, M. Geranylgeraniol Enhances Testosterone Production via the CAMP/Protein Kinase A Pathway in Testis-Derived I-10 Tumor Cells. *Biosci. Biotechnol. Biochem.* **2016**, *80* (4), 791–797. https://doi.org/10.1080/09168451.2015.1123612.

(9) Ito, A.; Shirakawa, H.; Takumi, N.; Minegishi, Y.; Ohashi, A.; Howlader, Z. H.; Ohsaki, Y.; Sato, T.; Goto, T.; Komai, M. Menaquinone-4 Enhances Testosterone Production in Rats and Testis-Derived Tumor Cells. *Lipids Health Dis.* **2011**, *10* (1), 158. https://doi.org/10.1186/1476-511X-10-158.

(10) Li, W.; Pandey, A. K.; Yin, X.; Chen, J.-J.; Stocco, D. M.; Grammas, P.; Wang, X. Effects of Apigenin on Steroidogenesis and Steroidogenic Acute Regulatory Gene

Expression in Mouse Leydig Cells. *J. Nutr. Biochem.* **2011**, *22* (3), 212–218. https://doi.org/10.1016/j.jnutbio.2010.01.004.

(11) Jana, K.; Yin, X.; Schiffer, R. B.; Chen, J.-J.; Pandey, A. K.; Stocco, D. M.; Grammas, P.; Wang, X. Chrysin, a Natural Flavonoid Enhances Steroidogenesis and Steroidogenic Acute Regulatory Protein Gene Expression in Mouse Leydig Cells. *J. Endocrinol.* **2008**, *197* (2), 315–323. https://doi.org/10.1677/JOE-07-0282.

(12) Chen, Y.-C.; Nagpal, M. L.; Stocco, D. M.; Lin, T. Effects of Genistein, Resveratrol, and Quercetin on Steroidogenesis and Proliferation of MA-10 Mouse Leydig Tumor Cells. *J. Endocrinol.* **2007**, *192* (3), 527–537. https://doi.org/10.1677/JOE-06-0087.

(13) Wang, W.; Mu, Q.; Feng, X.; Liu, W.; Xu, H.; Chen, X.; Shi, F.; Gong, T. Sweet Taste Receptor T1R3 Expressed in Leydig Cells Is Closely Related to Homeostasis of the Steroid Hormone Metabolism Profile. *J. Agric. Food Chem.* **2023**, *71* (20), 7791–7802. https://doi.org/10.1021/acs.jafc.3c01110.

(14) Huhtaniemi, I.; Forti, G. Male Late-Onset Hypogonadism: Pathogenesis, Diagnosis and Treatment. *Nat. Rev. Urol.* **2011**, *8* (6), 335–344. https://doi.org/10.1038/nrurol.2011.47.

(15) Grossmann, M.; Ng Tang Fui, M.; Cheung, A. S. Late-Onset Hypogonadism: Metabolic Impact. *Andrology* **2020**, *8* (6), 1519–1529. https://doi.org/10.1111/andr.12705.

(16) Kloner, R. A.; Carson, C.; Dobs, A.; Kopecky, S.; Mohler, E. R. Testosterone and Cardiovascular Disease. *J. Am. Coll. Cardiol.* **2016**, *67* (5), 545–557. https://doi.org/10.1016/j.jacc.2015.12.005.

(17) Matousek, R. H.; Sherwin, B. B. Sex Steroid Hormones and Cognitive Functioning in Healthy, Older Men. *Horm. Behav.* **2010**, *57* (3), 352–359. https://doi.org/10.1016/j.yhbeh.2010.01.004.

(18) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9* (7), 671–675. https://doi.org/10.1038/nmeth.2089.

(19) Slimestad, R.; Solheim, H. Anthocyanins from Black Currants (Ribes Nigrum L.). *J. Agric. Food Chem.* **2002**, *50* (11), 3228–3231. https://doi.org/10.1021/jf011581u.

(20) Deguchi, Y.; Ito, M. Rosmarinic Acid in Perilla Frutescens and Perilla Herb Analyzed by HPLC. *J. Nat. Med.* **2020**, *74* (2), 341–352. https://doi.org/10.1007/s11418-019-01367-8.

(21) Matsumoto, H.; Ichiyanagi, T.; Iida, H.; Ito, K.; Tsuda, T.; Hirayama, M.; Konishi, T. Ingested Delphinidin-3-Rutinoside Is Primarily Excreted to Urine as the Intact Form and to Bile as the Methylated Form in Rats. *J. Agric. Food Chem.* **2006**, *54* (2), 578–582. https://doi.org/10.1021/jf052411a.

(22) Nakazawa, T.; Ohsawa, K. Metabolism of Rosmarinic Acid in Rats. *J. Nat. Prod.* **1998**, *61* (8), 993–996. https://doi.org/10.1021/np980072s.

(23) Manna, P. R.; Dyson, M. T.; Stocco, D. M. Regulation of the Steroidogenic Acute Regulatory Protein Gene Expression: Present and Future Perspectives. *Mol. Hum. Reprod.* **2009**, *15* (6), 321–333. https://doi.org/10.1093/molehr/gap025.

(24) Gad, F. A.-M.; Farouk, S. M.; Emam, M. A. Antiapoptotic and Antioxidant Capacity of Phytochemicals from Roselle (Hibiscus Sabdariffa) and Their Potential Effects on Monosodium Glutamate-Induced Testicular Damage in Rat. *Environ. Sci. Pollut. Res.* **2021**, *28* (2), 2379–2390. https://doi.org/10.1007/s11356-020-10674-7.

(25) Farsi, A.; Khaki, A.; Fathiazad, F.; Afshari, F.; Hajhossini, L.; Kahki, A. A. Improvement Effect of Rosmarinic Acid on Serum Testosterone Level after Exposing with Electromagnetic Fields. *Int. J. Women's Heal. Reprod. Sci.* **2013**, *1* (2), 45–50. https://doi.org/10.15296/ijwhr.2013.08.

(26) Bevilacqua, A.; Dragotto, J.; Giuliani, A.; Bizzarri, M. Myo-inositol and D-chiro-inositol (40:1) Reverse Histological and Functional Features of Polycystic Ovary Syndrome in a Mouse Model. *J. Cell. Physiol.* **2019**, *234* (6), 9387–9398. https://doi.org/10.1002/jcp.27623.

(27) Stocco, D. M.; Wang, X.; Jo, Y.; Manna, P. R. Multiple Signaling Pathways Regulating Steroidogenesis and Steroidogenic Acute Regulatory Protein Expression: More Complicated than We Thought. *Mol. Endocrinol.* **2005**, *19* (11), 2647–2659. https://doi.org/10.1210/me.2004-0532.

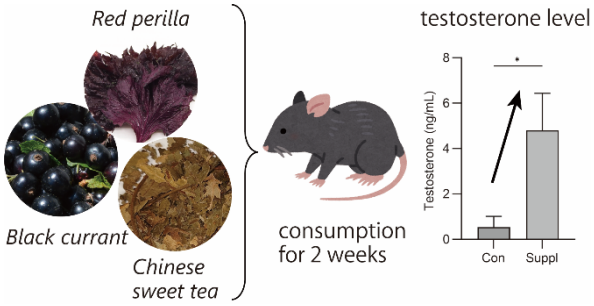
(28) Sun, J.; Li, M.; Zou, F.; Bai, S.; Jiang, X.; Tian, L.; Ou, S.; Jiao, R.; Bai, W. Protection of Cyanidin-3-O-Glucoside against Acrylamide- and Glycidamide-Induced Reproductive Toxicity in Leydig Cells. *Food Chem. Toxicol.* **2018**, *119* (March), 268–274. https://doi.org/10.1016/j.fct.2018.03.027.

(29) Evaul, K.; Hammes, S. R. Cross-Talk between G Protein-Coupled and Epidermal Growth Factor Receptors Regulates Gonadotropin-Mediated Steroidogenesis in Leydig Cells. *J. Biol. Chem.* **2008**, *283* (41), 27525–27533. https://doi.org/10.1074/jbc.M803867200.

(30) Kempná, P.; Hirsch, A.; Hofer, G.; Mullis, P. E.; Flück, C. E. Impact of Differential P450c17 Phosphorylation by CAMP Stimulation and by Starvation Conditions on

554 Enzyme Activities and Androgen Production in NCI-H295R Cells. *Endocrinology*
555 **2010**, *151* (8), 3686–3696. https://doi.org/10.1210/en.2010-0093.
556 (31) King, S. R. Gonadal Transactivation of STARD1, CYP11A1 and HSD3B. *Front.*
557 *Biosci.* **2012**, *17* (1), 824. https://doi.org/10.2741/3959.
558 (32) Monastra, G.; Vazquez-Levin, M.; Bezerra Espinola, M. S.; Bilotta, G.; Laganà,
559 A. S.; Unfer, V. D-Chiro-Inositol, an Aromatase down-Modulator, Increases
560 Androgens and Reduces Estrogens in Male Volunteers: A Pilot Study. *Basic Clin.*
561 *Androl.* **2021**, *31* (1), 13. https://doi.org/10.1186/s12610-021-00131-x.
562 (33) Hiroi, H.; Christenson, L. K.; Chang, L.; Sammel, M. D.; Berger, S. L.; Strauss, J.
563 F. Temporal and Spatial Changes in Transcription Factor Binding and Histone
564 Modifications at the Steroidogenic Acute Regulatory Protein (StAR) Locus
565 Associated with StAR Transcription. *Mol. Endocrinol.* **2004**, *18* (4), 791–806.
566 https://doi.org/10.1210/me.2003-0305.
567
568

569 Table of contents graphics



570

571

572 Supporting information

573

574 Bioactive components in black currant fruit, red perilla (shiso) leaf, and Chinese
575 sweet tea that enhance testosterone production in Leydig cells and their combined
576 effect in male mice

577

578 Eisuke Kato[a]*, Yosuke Inagaki[b], Hiromi Kayooka[c], Yusuke Adachi[d], Tomoaki Nagase[c], Naofumi
579 Terada[c], and Ai Tsuruma[c]

580

581 a Division of Fundamental AgriScience and Research, Research Faculty of Agriculture, Hokkaido
582 University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

583 b Japan Pharma Co., Ltd., 4-2-16 Nihonbashi Hongokucho, Chuo-ku, Tokyo 103-0021, Japan

584 c Frontiers in Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo,
585 Hokkaido 060-8589, Japan

586 d Department of Bioscience and Chemistry, School of Agriculture, Hokkaido University, Kita-ku,
587 Sapporo, Hokkaido 060-8589, Japan

588

589 * Corresponding author: eikato@agr.hokudai.ac.jp

590

591

592

Table S1. Results of *in vitro* testosterone production activity assay of food extract powders (1 mg/mL) using I-10 cells.

No.	Scientific name	Part used	Extraction solvent	Provider[1]	value[2]
1	*Salvia rosmarinus*	Leaf	Aqueous Ethanol	J	0.6
2	*Vitis vinifera*	Fruit, seed	Aqueous Ethanol	J	2.8
3	*Camellia sinensis*	Leaf	Not provided	J	1.7
4	*Ampelopsis grossedentata*	Leaf	Water	J	1.1
5	*Cinnamomum cassia*	Bark	Water	J	1.9
6	*Ribes nigrum*	Fruit	Water	M	5.8
7	*Perilla frutescens*	Leaf	Water	M	3.8
8	*Citrus unshiu*	Pericarp	Dried powder	M	1.5
9	*Ziziphus jujuba*	Fruit	Water	M	1.1
10	*Coix lacryma-jobi*	Seed	Water	M	0.9
11	*Helianthus annuus*	Seed	Water (sugar and protein removed)	M	1.4
12	*Corchorus olitorius*	Leaf	Dried powder	M	1.5
13	*Lavandula angustifolia*	Flower	Aqueous Ethanol	M	3.1
14	*Rubus suavissimus*	Leaf	Water	M	4.2[3]

[1] J: Japanese traditional medical laboratory Co., Ltd. (Aichi, Japan); M: Matsuura Yakugyo Co., LTD. (Aichi, Japan)

[2] Each sample was dissolved in 50% aq. dimethyl sulfoxide and diluted in Ham's F-10 medium supplemented with 10% fetal bovine serum and antibiotics. Dimethyl sulfoxide concentrations in the medium were <0.1%, and the same amount of dimethyl sulfoxide was added to the control cells. Value of testosterone secretion relative to control cells are shown in the table.

[3] Tested at 0.1 mg/mL

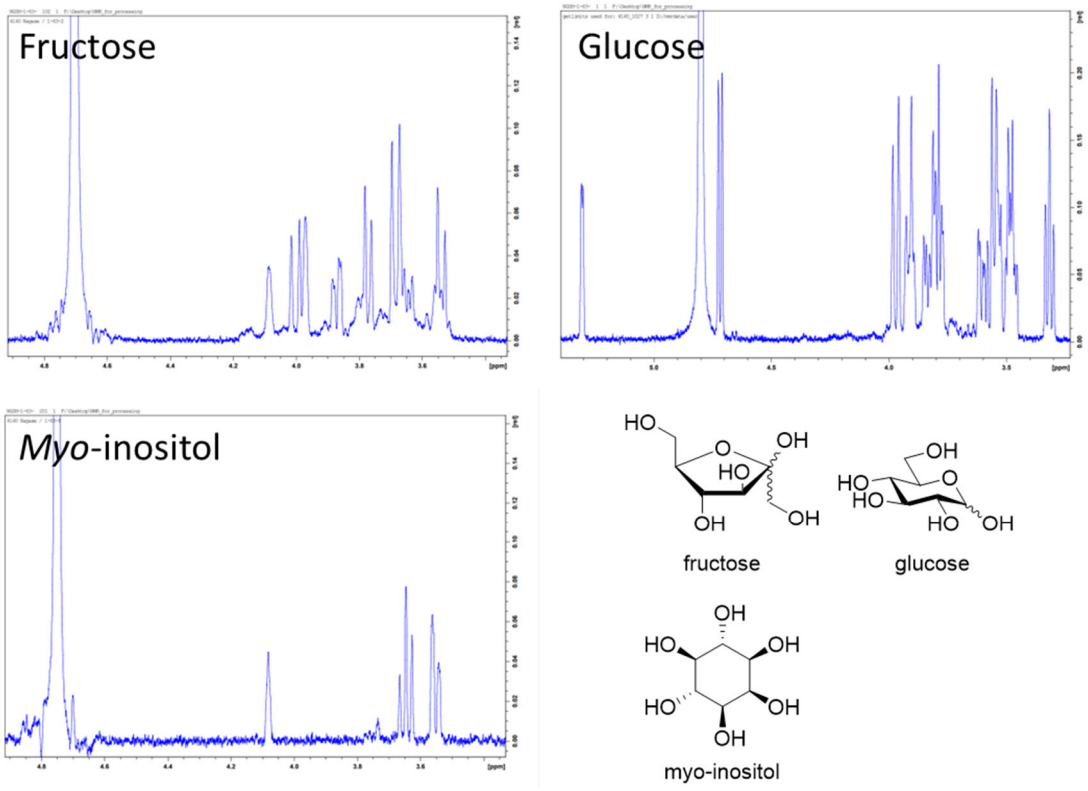
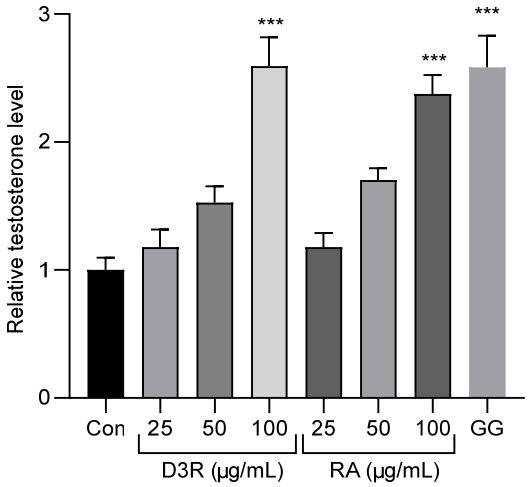


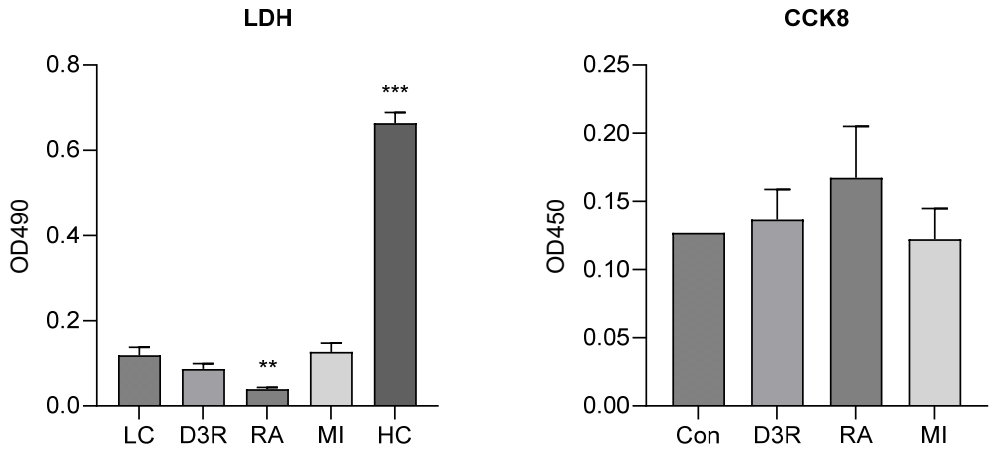
Figure S1. ^{1}H-NMR (500 MHz, D_2O) spectrum and the structure of isolated compounds from *Rubus suavissimus*. MS analysis matched with the structure (fructose *m*/*z* = 179 $[M-H]^-$; glucose *m*/*z* = 179 $[M-H]^-$;*myo*-inositol *m*/*z* = 179 $[M-H]^-$)



608

609 Figure S2. Concentration dependency of delphinidin-3-rutinoside (D3R), rosmarinic acid (RA) to
610 stimulate testosterone production in I-10 cells. D3R and RA were added to the medium of I-10 cells
611 at indicated concentrations and stimulated for 24 hr (N=6). Geranylgeraniol (GG, 30 μM) was used as
612 positive control. The value in the figure is shown relative to control (Con). ***$p<0.001$ vs control
613 (Dunnett's test).

614

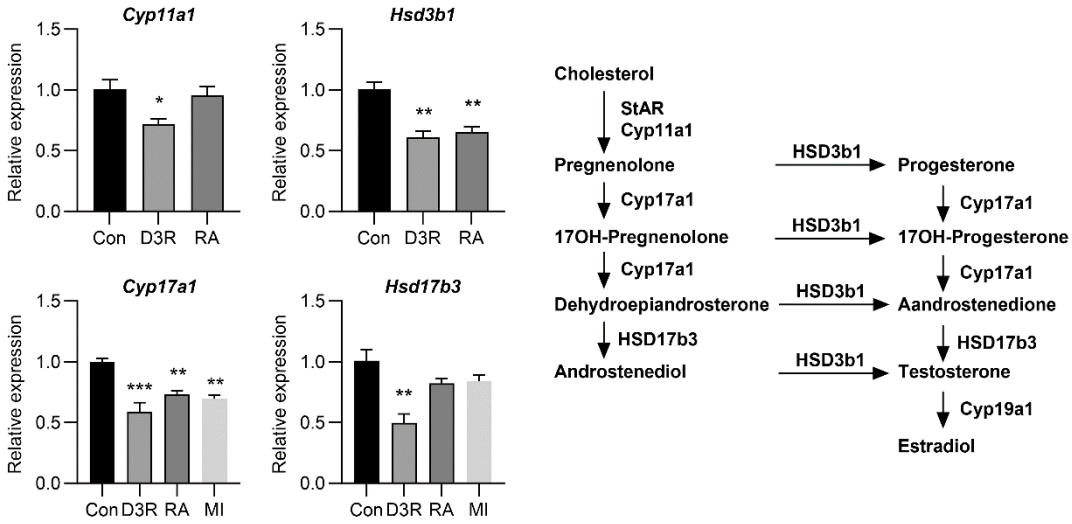


615

616 Figure S3. Cell viability assay (LDH) and cytotoxicity assay (CCK8) of the compounds.

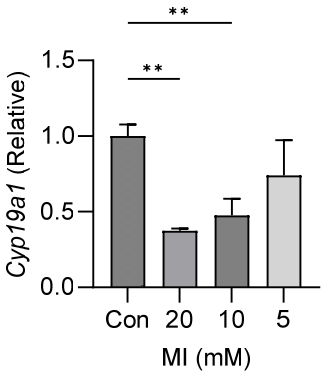
617 Delphinidin-3-rutinoside (D3R, 100 μg/mL), rosmarinic acid (RA, 100 μg/mL), and myo-inositol (MI,
618 10 mM) were added to the medium of I-10 cells and exposed for 24 hr. Cell viability was evaluated
619 by Cell Counting Kit-8 (CCK8, Dojindo Laboratories) and cytotoxicity was evaluated by Cytotoxicity
620 LDH Assay kit-WST (Dojindo Laboratories) according to the manufacturer's instructions. LC (low
621 control) and Con (control) are cells without sample exposure, HC (high control) is a lysed cell.
622 **$p<0.01$, ***$p<0.001$ vs LC (Dunnett's test).

623



624

625 Figure S4. Relative expression of testosterone biosynthesis genes after stimulation by delphinidin-3-
626 rutinoside (D3R), rosmarinic acid (RA), and myo-inositol (MI). D3R and RA were added to the
627 medium of I-10 cells at 100 μg/mL and stimulated for 3 h, MI was added at 10 mM and stimulated for
628 24 hr. Expression of mRNA was evaluated (N=3). The value in the figure is shown relative to control
629 (Con). *p<0.05, **p<0.01, ***p<0.001 vs control (Dunnett's test).



630

631 Figure S5. Concentration dependence of MI on decreased *Cyp19a1* expression in I-10 cells.
632 I-10 cells were stimulated by MI for 24 hr and the expression of *Cyp19a1* was evaluated. *Actb*
633 was used as reference gene. The value in the figure is shown relative to control (Con). ** p<0.01,
634 vs control (Dunnett's test).

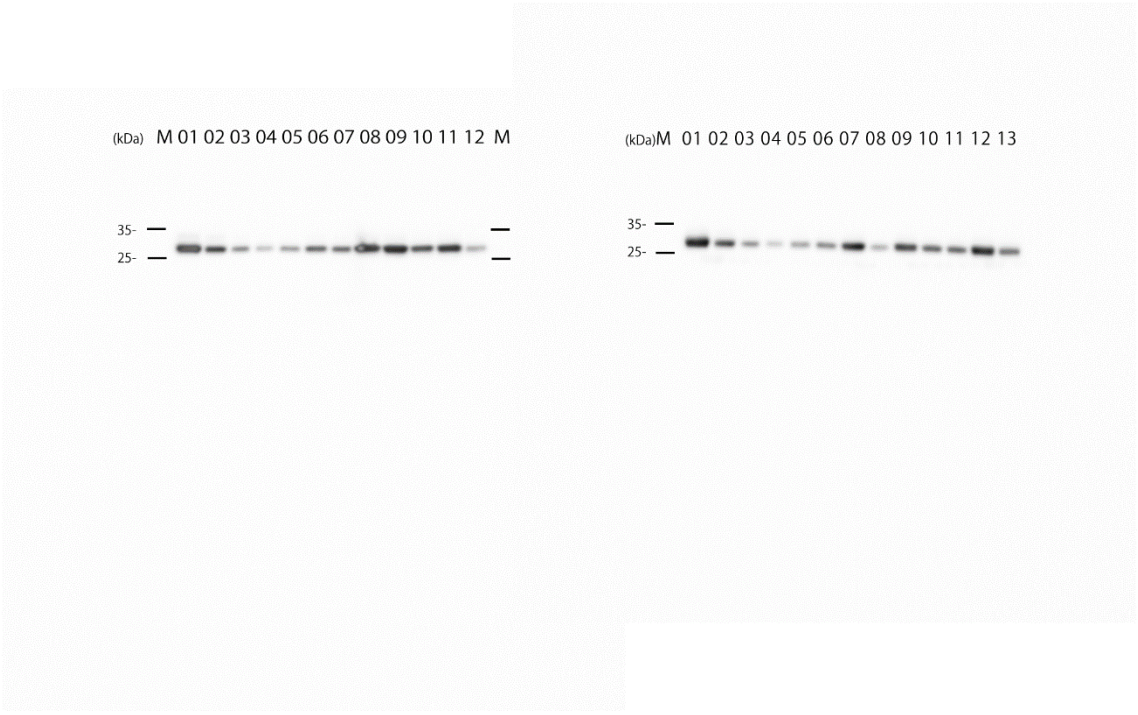


Figure S6. Uncropped image of western blotting for StAR protein analysis in testis.

Left: M marker; Lane 01-04 Calibration control (protein from one of the supplemented mice); Lane 5-7 control mouse; Lane 8-11 supplemented mouse; Lane 12 is a sample unrelated with the current experiment.

Right: M marker; Lane 01-04 Calibration control (protein from one of the supplemented mice); Lane 5-8 control mouse; Lane 9-11 supplemented mouse; Lane 12-13 are samples unrelated with the current experiment.

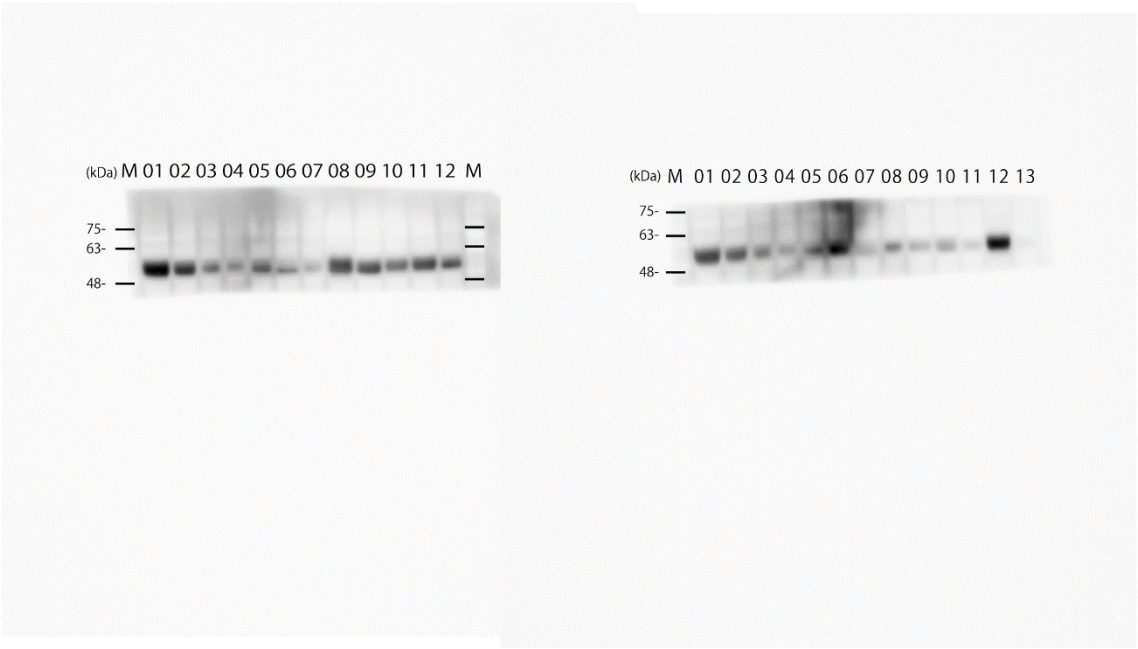


Figure S7. Uncropped image of western blotting for aromatase protein analysis in testis.

Left: M marker; Lane 01-04 Calibration control (protein from one of the supplemented mice); Lane 5-7 control mouse; Lane 8-11 supplemented mouse; Lane 12 is a sample unrelated with the current experiment.

Right: M marker; Lane 01-04 Calibration control (protein from one of the supplemented mice); Lane 5-8 control mouse; Lane 9-11 supplemented mouse; Lane 12-13 are samples unrelated with the current experiment.

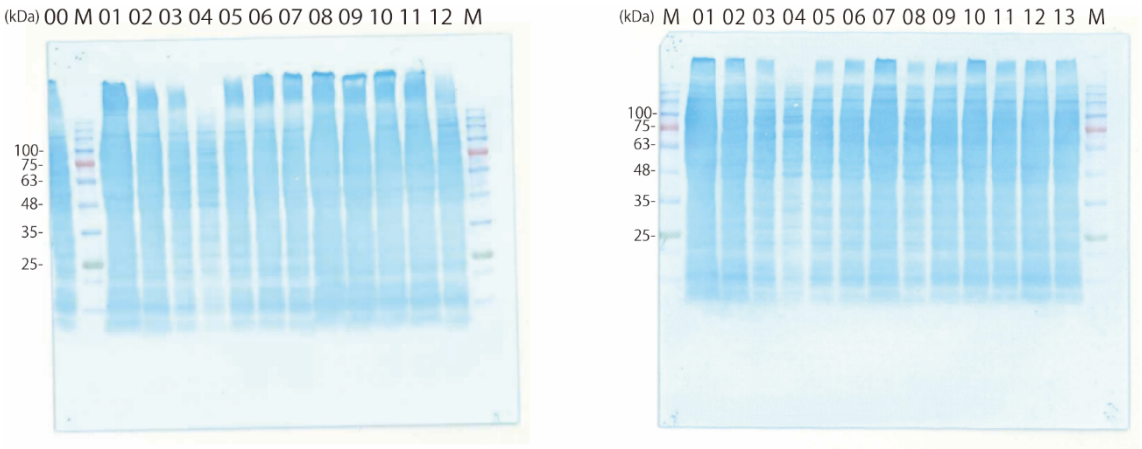


Figure S8. Total protein stain of the membrane for western blotting.

Left: M marker; Lane 01-04 Calibration control (protein from one of the supplemented mice); Lane 5-7 control mouse; Lane 8-11 supplemented mouse; Lane 12 is a sample unrelated with the current experiment.

Right: M marker; Lane 01-04 Calibration control (protein from one of the supplemented mice); Lane 5-8 control mouse; Lane 9-11 supplemented mouse; Lane 12-13 are samples unrelated with the current experiment.