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7 **Bioactive components in Black currant fruit, Red perilla (Shiso) leaf, and Chinese**
8 **sweet tea that enhance testosterone production in Leydig cells and their combined**
9 **effect in male mice.**

10

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28

29 **Abstract**

30 Testosterone is a steroid hormone that regulates wide range of biological events. Foods
31 affect testosterone levels in the body, but not many food components have been revealed
32 to elevate testosterone levels. In this study, black currant (*Ribes nigrum*) fruit, red perilla
33 (shiso, *Perilla frutescens*) leaf, and Chinese sweet (*Rubus suavissimus*) tea were found to
34 enhance testosterone production in testicular Leydig cells, and delphinidin-3-rutinoside
35 (D3R), rosmarinic acid (RA), *myo*-inositol (MI) were identified as bioactive components.
36 Mechanistic analysis suggested that *Star* to involve in the bioactivity of D3R and RA, and
37 downregulation of *Cyp19a1* is involved in the bioactivity of MI. Supplementation of the
38 diet with a mixture of black currant fruit extract, red perilla leaf extract, and MI increased
39 serum testosterone levels in male C57BL6/J mice together with increased level of Star
40 protein in the testis. These findings add to our knowledge of food and their components
41 that modulate testosterone levels.

42

43 **Keywords**

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

46

47

Introduction

Consumption of food is correlated with the level of various hormones in our body. Nutrients as well as non-nutrient dietary factors stimulate a gut system to modify the release of incretin hormones.[1,2] Dietary polyphenols along with elevated level of blood glucose increase insulin level by stimulating the pancreatic islets.[3] The phenolic component contained in olive oil, oleuropein, enhances catecholamine levels (epinephrine and norepinephrine).[4] These phenomenon shows that foods to regulate the level of hormones.

Testosterone is an androgenic steroid hormone primarily produced by Leydig cells in the testes of men. Correlation between food and the level of testosterone has been studied and both positive and negative results appear to date.[5] However, food components and ingredients including tocotrienol,[6] geranylgeraniol,[7,8] menaquinone-4,[9] flavonoids,[10–12] and saccharin,[13] are reported to modify the production of testosterone in the testis, which suggests the existence of a relationship between food and testosterone.

The functions of testosterone include the maturation of sexual organs, a gain in bone density, an increase in muscle weight, and others. A decline in serum testosterone concentrations occurs through aging and/or as a result of a response to stress.[14] Because of the above functions, decreased testosterone level cause various symptoms, such as sexual dysfunction, a decrement in muscle mass, decreased bone mineral density, decreased vitality, and depression which the sets of symptoms are known as the late-onset hypogonadism (LOH).[15] Additionally, testosterone concentrations in the body are related to diseases such as cardiovascular disease,[16] obesity associated diabetes,[15] and cognitive function.[17]

Analysis of food components that modulate testosterone production will lead to the use of food to protect our body from low testosterone level. In this study, we identified several food materials as well as their bioactive components that enhances testosterone production in Leydig cells, investigated the mechanism of action responsible for their bioactivity, and examined their effect to elevate testosterone level in mouse model.

Materials and methods

Chemicals and food materials

Extract powders of food materials for *in vitro* experiment were obtained from Matsuura Yakugyo Co., Ltd. (Aichi, Japan) or Japanese Traditional Medical Laboratory Co., Ltd. (Aichi, Japan). Samples for *in vivo* experiment were obtained from the following company: 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 (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 *myo*-inositol (>99%) was purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Other commercially available chemicals were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan) unless otherwise noted.

Purification of active components from Chinese sweet tea

95 Extract powder of Chinese sweet tea was partitioned between hexane, ethyl acetate,
96 1-butanol, and water. The water layer was adsorbed on Diaion HP-20 (Mitsubishi
97 Chemical Co.) and eluted by water, 50% aq. methanol, and methanol. The water
98 eluted fraction was then placed on Cosmosil 75C18-OPN (Nacalai Tesque, Inc)
99 and water eluted fraction was collected. The eluate was then separated by gradient
100 elution with 20-35% aq. methanol containing 0.1% trifluoroacetic acid using
101 InertSustain C18 (GL Sciences Inc.). The active peak was then separated by
102 gradient elution with 1-15% aq. methanol containing 0.1% trifluoroacetic acid
103 using Cosmosil PBr (Nacalai Tesque, Inc.). The active peak was then separated by
104 15% aq. acetonitrile using Shodex Asahipak NH2P-50 4E (Shoko Science Co.,
105 Ltd.) to collect *myo*-inositol, glucose and fructose which were characterized from
106 ^{1}H-nuclear magnetic resonance (NMR) spectrum (Bruker AMX500) and mass
107 spectrometry (Thermo Scientific Exactive) analysis (Figure S1).
108

109 **Cell culture**

110 I-10 cells (JCRB9097) were obtained from the Japanese Collection of Research
111 Bioresources Cell Bank (Osaka, Japan). The cells were cultured at 37°C in a 10%
112 CO_2 atmosphere in growth medium [Ham's F-10 medium (Sigma-Aldrich®)
113 supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin,
114 100 μg/mL streptomycin, and 50 μg/mL gentamicin)].
115

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

117 I-10 cells were seeded in 48-well plates (2×10^{4} cells/well). One day after seeding,
118 the medium was replaced to the sample containing medium and stimulated for 24

119 h. The medium was then recovered, and testosterone concentrations were measured
120 with the Testosterone ELISA kit (Cayman Chemical). When inhibitors
121 (MDL12,300A or H-89) were used, the cells were pre-treated with an inhibitor for
122 1 h and then stimulated with the sample in the presence of an inhibitor. Samples
123 and inhibitors were dissolved in water or dimethyl sulfoxide and diluted in growth
124 medium to prepare the sample solution. Dimethyl sulfoxide concentrations in the
125 medium were <0.1%, and the same amount of dimethyl sulfoxide was added to the
126 control cells. Geranylgeraniol (30 μM) was used as the positive control.[7,8]
127

128 ***Ex vivo* testosterone production activity assay (mouse testis)**

129 The experiment was performed with approval (No. 19-0163) from the Institutional
130 Animal Care and Use Committee, Hokkaido University by following National
131 University Corporation Hokkaido University Regulations on Animal
132 Experimentation. C57BL/6J male mice were housed in an air-conditioned room at
133 23°C±2°C with a light period from 8:00 am to 8:00 pm. The mice had free access
134 to their diet and water. On the day of the experiment (11–13 weeks old), the mice
135 were anesthetized using isoflurane and sacrificed. The testis was collected, washed
136 twice with phosphate-buffered saline, immersed in Ham's F-10 medium
137 supplemented with 10% fetal bovine serum, and incubated for 1 h at 37°C in a 10%
138 CO_2 atmosphere. The medium was replaced and incubated for another hour to
139 collect the control medium. The medium was then replaced with the sample
140 containing medium, and the procedure was repeated to collect the sample-
141 stimulated medium. Testosterone concentrations in the control medium and
142 sample-stimulated medium were measured with the Testosterone ELISA kit.

143

144 ***In vivo* examination of food components on testosterone level**

145 The experiment was performed with approval written above. Six month old
146 C57BL/6J male mice were housed in an air-conditioned room at 23°C±2°C with a
147 light period from 8:00 am to 8:00 pm. The mice were divided to control group fed
148 with powdered MF diet (Oriental Yeast Co., ltd.) and treatment group fed with MF
149 diet supplemented with 1% sample mixture [consisted of 66.3% myo-inositol,
150 5.8% red perilla leaf extract (containing 14.3% rosmarinic acid) and 27.8% black
151 currant fruit extract (containing 30% anthocyanin)]. After two weeks with free
152 access to diet and water, the mice were starved for 16 hr, anesthetized using
153 isoflurane, and sacrificed. The blood was collected, and the serum level of
154 testosterone was measured. The testis was collected, suspended in the Ham's F-10
155 medium for 2 hr and the secreted testosterone was measured. Total-RNA and
156 protein were extracted from the testis and analysed by qPCR and western blotting.
157

158 **Gene expression analysis**

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

166

167 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

168

169 **Western blotting**

170 The protein samples were prepared by lysing the cells or testis with the EzRIPA
171 Lysis kit (Atto Co., Tokyo, Japan). The lysate was mixed with sodium dodecyl
172 sulfate sample buffer and heated at 95°C for 5 min. The samples were separated by
173 SDS-PAGE and transferred to a PVDF membrane. The membrane was stained by
174 EzStain AQua MEM (Atto Co.) to estimate the total protein, and then Star and
175 aromatase was detected using a combination of Star antibody (#8449, 1:1000; Cell
176 Signaling Technology) or Anti-aromatase antibody (bs-0114M, 1:1000, Bioss Inc.)
177 and anti-rabbit immunoglobulin G horseradish peroxidase-linked antibody (#7074,
178 1:2000; Cell Signalling Technology) with Immunostar® Zeta as the detection
179 reagent. The value of target protein relative to total protein was estimated by
180 Image-J software.[18]

181

182 **Statistical analysis**

183 *In vitro* experiments were repeated at least twice, and representative data are
184 expressed as mean ± standard error of the mean (SEM). *In vivo* experiments were
185 performed twice, and the summarized data are expressed as mean ± SEM. Data
186 were analysed by GraphPad Prism software (ver.10) with the statistical methods
187 indicated in the figure legends. A p value <0.05 was considered statistically
188 significant.

189

190 **Results**

191 **Screening of food materials**

192 Commercially available powdered extracts of food materials were tested for
193 their ability to stimulate the production of testosterone from mouse Leydig cells (I-
194 10) (Table S1). Among them, we focused on black currant fruit, red perilla leaf,
195 and Chinese sweet tea which showed relatively high activity, with 5.8, 3.8, and 4.2
196 times enhanced testosterone production compared with controls at 1 mg/mL.

197

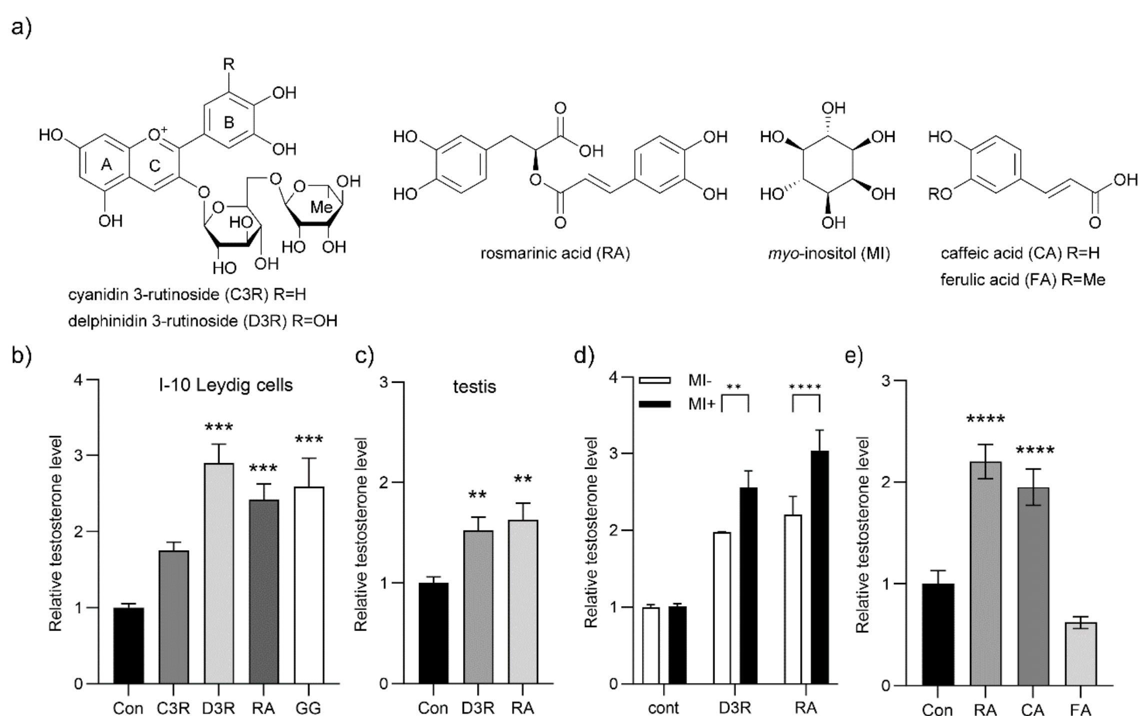
198 **Activity of components in candidate food material and their relative compounds**

199 To determine the bioactive components in the extract, compounds that have
200 been reported to be present in the candidate food materials were selected and
201 evaluated for their activity to stimulate testosterone production in I-10 cells. We
202 selected cyanidin-3-rutinoside (C3R) and delphinidin-3-rutinoside (D3R) from
203 black currant,[19] and rosmarinic acid (RA) from red perilla leaf (Figure 1a).[20] In
204 case of Chinese sweet tea, activity guided separation resulted to identify *myo*-

205 inositol (MI), glucose and fructose (Figure S1), and *myo*-inositol was selected for
206 evaluation (Figure 1a).

207 D3R and RA significantly stimulated I-10 cells to enhance the production of
208 testosterone 2.9 and 2.4 times at 100 μg/mL, comparable to the activity of
209 geranylgeraniol (positive control),[7,8] suggesting that these compounds to
210 participate in the activity of the candidate plants (Figure 1b and Figure S2). D3R
211 and RA were also evaluated in an *ex vivo* experiment to determine their activity in
212 the mouse testis. Stimulation of the mouse testis with 100 μg/mL of D3R or RA
213 enhanced the production of testosterone by 1.52 and 1.63 times, respectively
214 (Figure 1c). The activity of MI appeared to be unstable (data not shown), and its
215 ability to support other stimulatory compounds was presumed. The combination of
216 MI and D3R or RA, promoted testosterone production more strongly than the
217 individual addition, confirming this idea (Figure 1d)

218 Above results identified D3R, RA and MI as bioactive compounds. However,
219 food-derived compounds undergo metabolism during digestion and absorption,
220 and their usefulness can be further verified by considering the activity of
221 metabolites. Among the three identified compounds, MI is a endogenous
222 compound, and D3R is reported to exist in plasma without metabolism when orally
223 given to rats.[21] Therefore, the major metabolites of RA, caffeic acid (CA) and
224 ferulic acid (FA),[22] were tested for their ability to enhance testosterone production
225 in I-10 cells. CA showed testosterone production activity comparable to RA, but
226 not FA (Figure 1e).



227

228 Figure 1. Testosterone production activity of the selected compounds. a) Structures
229 of compounds. b) cyanidin-3-rutinoside (C3R), delphinidin-3-rutinoside (D3R),
230 rosmarinic acid (RA), and geranylgeraniol (GG, 30 μM, positive control) were
231 tested at 100 μg/mL against I-10 cells (N=3). c) The mouse testis was stimulated
232 by D3R and RA at 100 μg/mL for 2 h and testosterone secreted in the medium was
233 compared (N=6). d) Additive effect of *myo*-inositol (MI, 10 mM), D3R (100
234 μg/mL), RA (100 μg/mL) (N=3). e) RA, caffeic acid (CA) and ferulic acid (FA)
235 were tested at 100 μg/mL against I-10 cells. **p<0.01, ***p<0.001, ****p<0.0001
236 vs control (Tukey's test).

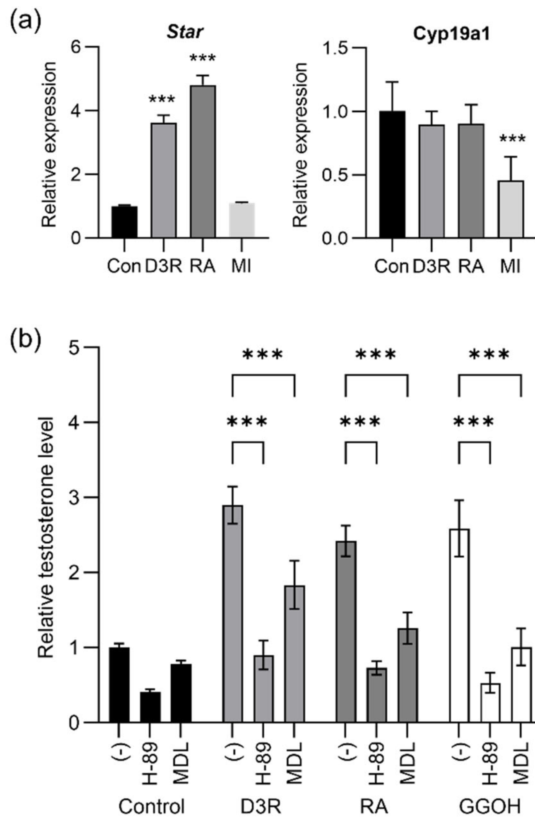
237

238 **Mechanistic analysis of D3R, RA, and MI**

239 The activity of D3R, RA, and MI were investigated to understand the molecular
240 mechanism that is involved in enhancing or supporting testosterone production. An

241 analysis of the gene expression relating to the biosynthesis of testosterone showed
242 that D3R and RA elevate the expression of *Star* (Figure 2a, Figure S3). Due to the
243 supportive activity of MI, expression of *Cyp19a1*, the gene encoding aromatase
244 that converts testosterone to estradiol, was examined and its reduced expression
245 was confirmed (Figure 2a).

246 Expression of *Star* is regulated through the cyclic adenosine monophosphate
247 (cAMP)/protein kinase A (PKA) signalling pathway.[23] To evaluate if this pathway
248 is responsible for the activity of D3R and RA, the adenylyl cyclase inhibitor
249 MDL12,300A or the PKA inhibitor H-89 was co-incubated with the compounds.
250 MDL12,300A and H-89 diminished the enhanced testosterone production induced
251 by D3R and RA (Figure 2b), which indicates that the cAMP/PKA pathway is
252 concerned in the activity.
253



254

255 Figure 2. Mechanistic analysis of Delphinidin-3-rutinoside (D3R), rosmarinic acid
256 (RA) and *myo*-inositol (MI). (a) Analysis of gene expression. I-10 cells were
257 stimulated by D3R and RA at 100 µg/mL or MI (10 mM) for 24 hr. *Star* was
258 evaluated after 3 h of stimulation and *Cyp19a1* was evaluated after 24 hr. (N=3)
259 (b) Testosterone production of I-10 cells after stimulation by D3R and RA co-
260 incubated with cAMP inhibitor MDL12,300A (MDL, 10 µM) or PKA inhibitor H-
261 89 (10 µM). (-) is without the inhibitor (N=6). ***$p<0.001$ vs control (con) or (-)
262 (Dunnett's test).

263

264

265

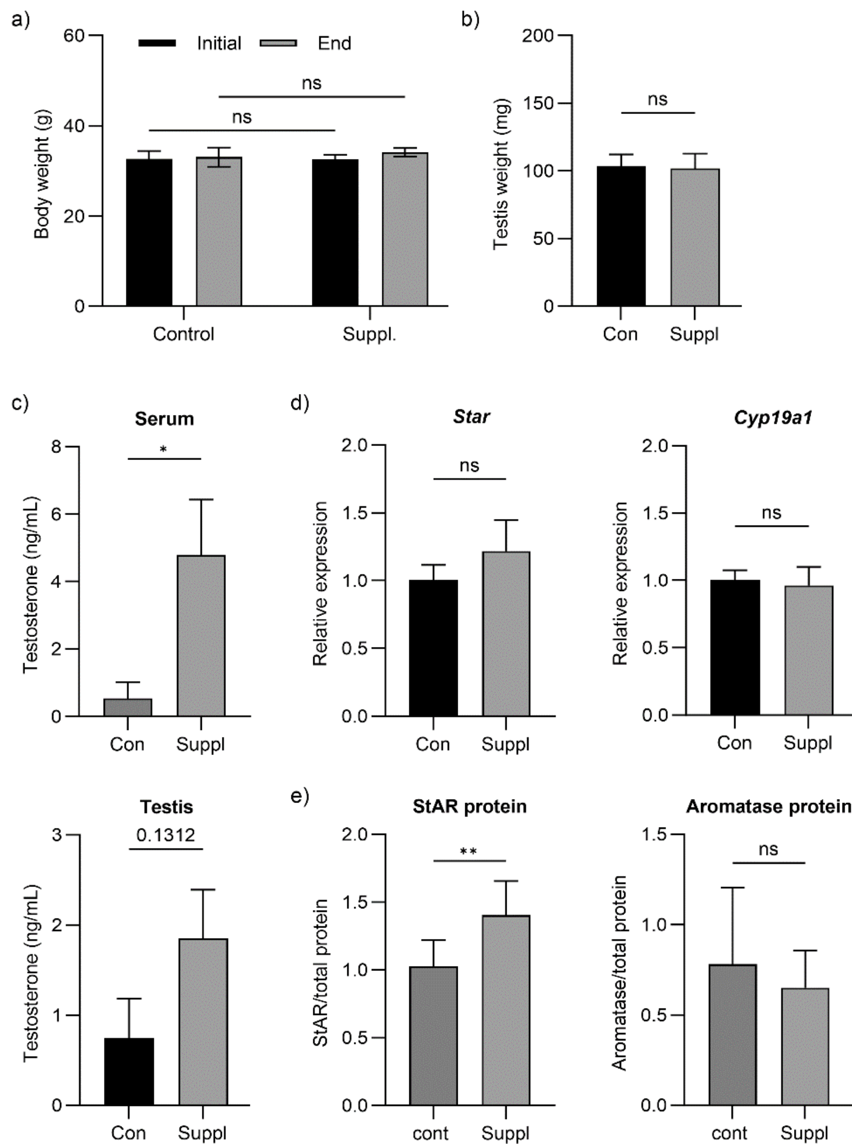
266

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

The above findings suggest that foods containing D3R, RA, and MI have a potential to enhance testosterone production. Since MI supports, and D3R, RA stimulates the testosterone production from the testicular Leydig cells, we evaluated if the combination of these three have potentials to elevate serum testosterone level in *in vivo* experiment.

Six-month-old male C57BL6/J mice were fed a diet for 2 weeks containing 1% of a supplement consisting of black currant fruits extract, red perilla leaf extract, and MI. Supplement ratio was determined from the previous reports examining the effect of anthocyanin, RA, and MI in rats.[24–26] There was no difference in the body and testis weight between groups (Figure 3a-b). Serum testosterone level showed significantly higher level in mice fed a supplemented diet, and production of testosterone from the isolated testis also showed a higher trend (Figure 3c). There were no differences in the expression of *Star* and *Cyp19a1* in the testes (Figure 3d), but analysis of Star protein showed increased levels (Figure 3e, Figure S4-6). No significant differences were found in the levels of aromatase protein (Figure 3e).

284



285

286 Figure 3. Effect of MI, black currant fruits and red perilla leaf extract supplemented
287 diet to C57BL6/J mice. a) Body weight, b) testis weight, c) serum testosterone level
288 and testosterone production in the testis, d) Expression of *Star* and *Cyp19a1* in
289 testis. e) Star and aromatase protein levels in testis. *p<0.05, **p<0.01 vs control
290 (N=11-12, Šidák test for body weight and t-test for others).

291

292

293 **Discussion**

294 In accordance with the previous reports showing enhanced production of
295 testosterone in Leydig cells through stimulation by food components,[6–13] screening
296 of food materials resulted to find black currant fruits, red perilla leaf, and Chinese
297 sweet tea as candidates showing that an approach to support the low testosterone
298 level through food will be an efficient way.

299 D3R and RA was identified as the bioactive component in black currant fruit
300 and red perilla leaf to stimulate testosterone production in I-10 cells (Figure 1ab).
301 Analysis of mechanistic aspects showed D3R and RA to enhance the gene
302 expression of steroidogenic acute regulatory protein (Star) (Figure 2a). Star is a
303 protein that transports cholesterol from the outer mitochondrial membrane to the
304 inner mitochondrial membrane, the rate-limiting step for the synthesis of steroid
305 hormone.[27] Therefore, elevated *Star* expression can account for the activity of D3R
306 and RA. Increased level of Star protein in I-10 cells remains elusive, but the activity
307 of anthocyanin to upregulate Star protein is reported by Sun et al,[31] suggesting that
308 elevated gene expression leads to increased protein amount.

309 Involvement of cAMP/PKA pathway was also suggested from the mechanistic
310 study of D3R and RA (Figure 2b). In Leydig cells, activation of PKA leads to the
311 gene expression of *Star* as well as the phosphorylation and translocation of Star
312 protein to mitochondria, both resulting to the enhanced steroidogenesis.[28] Thus,
313 enhanced expression of *Star* by D3R and RA treatment presumably occurs through
314 PKA. One concern is that although activation of PKA is reported to elevate the
315 expression of *Cyp11a1*, *Cyp17a1* and *Hsd3b*,[29,30] no increase or rather a decrease
316 was observed by the treatment of D3R and RA (Figure S2). Since we did not

317 directly confirm the activation of PKA through evaluation of the cAMP level or
318 the phosphorylation of PKA substrates, there remains a possibility that pathways
319 other than PKA participates in the activity of D3R and RA.

320 MI was identified as a bioactive component in Chinese sweet tea that supports
321 testosterone production and was shown to reduce the expression of *Cyp19a1* in I-10
322 cells (Figure 1d and 2a). *Cyp19a1* is a gene of aromatase, an enzyme that converts
323 testosterone to estradiol. Decreased expression of *Cyp19a1* can support
324 testosterone production by reducing the conversion of testosterone to estradiol. The
325 yield of MI from Chinese sweet tea was not enough to explain the activity of the
326 extract, suggesting the existence of another active component. However, the
327 efficacy of MI is apparent from the synergistic effect with D3R and RA (Figure 1d).
328 The study that D-*chiro*-inositol, the stereoisomer endogenously synthesized from
329 MI through the work of epimerase, increases the testosterone level in human trial,
330 may also support an efficacy of MI.[32]

331 Dietary intake of the combination of black currant fruit extract, red perilla leaf
332 extract, and MI elevated the serum testosterone level of six-month-old male
333 C57BL6/J mouse. Six-month-old male mice were used as a model of middle-aged
334 men, the age at which LOH symptoms predominantly develop. Related results are
335 reported for the oral administration of anthocyanins and RA. Dairy oral gavage of
336 the extract of rosselle (*Hibiscus sabdariffa*) containing anthocyanin, ameliorate the
337 reduction of serum testosterone induced by monosodium glutamate in rats.[24]
338 Administration of RA in Wistar rats increases the serum testosterone levels 1.84
339 times of the control.[25] The current result is in accordance with these result and the
340 more efficient increase in testosterone level is observed by the combination of three

341 food materials (Figure 3c), except the difference between rats and mouse may
342 participate.

343 Analysis of *Star* expression in the testes of mice fed the supplemented diet
344 showed no difference but did show a significant increase in Star protein levels
345 (Figure 3de). Upregulation of Star is in accordance with the *in vitro* result (Figure
346 2a), suggesting that desired response has occurred through the supplementation of
347 black currant fruit and red perilla leaf extract, which contain D3R and RA. A
348 possible explanation for the lack of increased expression of the *Star* gene could be
349 that the mice were starved before the testes were harvested. *Star* expression
350 decreases gradually after a stimulus-induced increase,[33] suggesting that the effect
351 was lost during the starvation period.

352 In mouse fed the supplemented diet, neither *Cyp19a1* expression nor aromatase
353 protein levels were reduced in the testis. Of the supplement, the compound
354 expected to downregulate *Cyp19a1* is MI. MI is one of the stereoisomers of inositol
355 and is the most abundantly existing isomer in the nature. Inositols are present in
356 mammalian body as a part of phosphatidyl inositol and are biosynthesized from
357 glucose. Thus, inositol supplementation may not have had much effect on the
358 amount of inositol in the body. Also, the control diet used in this experiment
359 contains inositol (439 mg/100 g diet). Although the supplemented diet contains
360 higher amount of MI (1100 mg/100g diet), the effect of MI supplementation on the
361 expression of aromatase may have been unclear due to the presence of inositol in
362 the control diet.

363 Several limitations exist for the current *in vivo* experiment. One is the actual
364 compound that worked to elevate serum testosterone is not clear. The effective

concentration of compounds in cell based assay is higher than the reported plasma levels of D3R and RA.[21,22] Therefore, there are possibility that metabolites or other unidentified components of the black currant fruit and red perilla leaf extract induced the effect. Another issue is whether the compounds acted on Leydig cells of the testis, or they worked on other tissues. We have observed significant difference in the serum testosterone level, but only an increasing trend in testosterone production from the collected testis (Figure 3c). This suggests the involvement of other testosterone synthesizing tissue, e.g. adrenal cortex, in the effect of supplementation to increase serum testosterone level.

In conclusion, this study aimed to identify food materials and their components that elevate testosterone production in Leydig cells of the testis. D3R, RA, and MI were identified, their molecular mechanism to enhance testosterone production in I-10 cells are partly revealed and the *in vivo* effect was confirmed. Knowledge of bioactive components in foods that increase testosterone level, and their mechanistic aspects will enhance understanding the relationship between food and testosterone levels in the body. Current findings are still limited in several point but at least the use of the identified foods will have the power to support the low testosterone level symptoms in the body.

Conflicts of interest

Authors declare no conflict of interest.

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392
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Supporting information

Delphinidin-3-rutinoside, rosmarinic acid, and myo-inositol as the modulator of steroid hormone synthesis in Leydig cells and the combination effect to enhance testosterone in mouse model.

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Table S1. Result of screening tested at 1 mg/mL

No.	Scientific name	Part used	Extraction solvent	Provider[1]	value[2]
1	*Salvia rosmarinus*	Leaf	Aqueous Ethanol	J	0.6
2	*Pueraria iobata*	Flower	Aqueous Ethanol	J	3.9
3	*Mucuna pruriens*	Seed	Water	J	1.0
4	*Cannabis sativa*	Seed	Water	J	0.8
5	*Eurycoma Longifolia*	Root	Aqueous Ethanol	J	4.3
6	*Panax ginseng*	Root	Aqueous Ethanol	J	2.7
7	*Vitis vinifera*	Seed	Aqueous Ethanol	J	2.8
8	*Camellia sinensis*	Leaf	Not provided	J	1.7
9	*Ampelopsis grossedentata*	Leaf	Water	J	1.1
10	*Cinnamomum cassia*	Bark	Water	J	1.9
11	*Euphrasia officinalis* subsp. *pratensis*	Aerial part	Water	M	4.0
12	*Echinacea purpurea*	Aerial part	Water	M	2.0
13	*Ribes nigrum*	Fruit	Water	M	5.8
14	*Curcuma zedoaria*	Root, stem	Dried powder	M	1.5
15	*Ajuga* sp.	Aerial part	Aqueous Ethanol	M	2.6
16	*Orthosiphon aristatus*	Aerial part	Water	M	2.8
17	*Spatholobus suberectus*	Stem	Aqueous Ethanol	M	2.4
18	*Hovenia dulcis*	Fruit, seed	Water	M	1.2
19	*Rhodiola rosea*	Root, stem	Aqueous Ethanol	M	1.9
20	*Zea mays*	Style, stigma	Water	M	4.4
21	*Ziziphus jujuba*	Seed	Water	M	2.1
25	*Perilla frutescens*	Leaf	Water	M	3.8
26	*Celosia argentea*	Seed	Water	M	2.6
27	*Panax quinquefolius*	Root	Water	M	2.6

No.	Scientific name	Part used	Extraction solvent	Provider	value
28	*Taraxacum officinale*	Root	Water	M	1.5
29	*Citrus unshiu*	Pericarp	Dried powder	M	1.5
30	*Panax notoginseng*	Root	Water	M	2.6
31	*Ziziphus jujuba*	Fruit	Water	M	1.1
32	*Coix lacryma-jobi*	Seed	Water	M	0.9
33	*Pfaffia* sp.	Root	Aqueous Ethanol	M	1.3
34	*Isatis tinctoria*	Root	Water	M	1.2
35	*Fucus vesiculosus*	All	Dried powder	M	0.9
36	*Helianthus annuus*	Seed	Water (sugar and protein removed)	M	1.4
37	*Carthamus tinctorius*	Flower	Water	M	1.5
38	*Melilotus* sp.	Aerial part	Water	M	2.0
39	*Corchorus olitorius*	Leaf	Dried powder	M	1.5
40	*Euphoria longana*	Aril	Water	M	1.0
41	*Platycodon grandiflorum*	Root	Water	M	0.6
42	*Lonicera japonica*	Bud	Water	M	1.9
43	*Cornus officinalis*	Pseudocarp	Aqueous Ethanol	M	1.4
44	*Crataegus* sp.	Pseudocarp	Aqueous Ethanol	M	1.1
45	*Citrus aurantium*	Pericarp	Aqueous Ethanol	M	1.6
46	*Cistanche salsa*	Stem	Aqueous Ethanol	M	1.1
47	*Actinidia polygama*	Fruit	Aqueous Ethanol	M	1.1
48	*Lavandula angustifolia*	Flower	Aqueous Ethanol	M	3.1
49	*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] Value of testosterone secretion relative to control cells

[3] Tested at 0.1 mg/mL

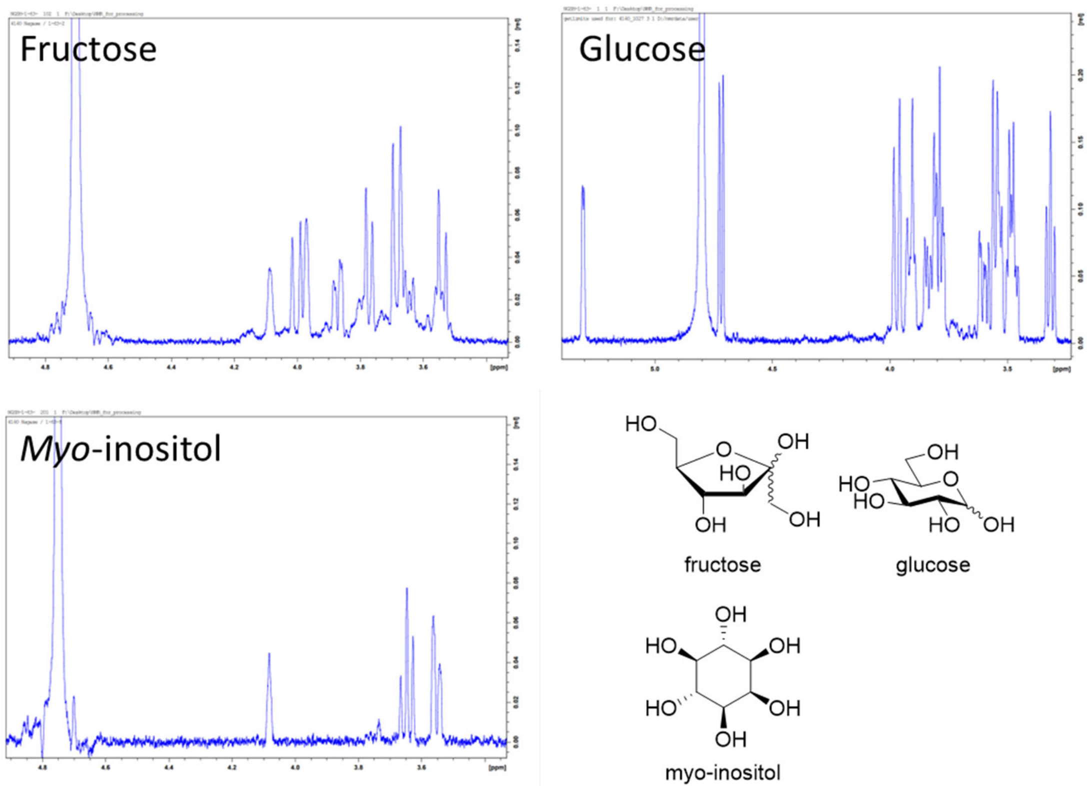

Figure S1. ^{1}H-NMR (500 MHz, D_2O) spectrum and the structure of isolated compounds from *Rubus suavissimus*. MS analysis matched with the structure (fructose *m*/*z* = 179 $[M-H]^-$; glucose *m*/*z* = 179 $[M-H]^-$;*myo*-inositol *m*/*z* = 179 $[M-H]^-$)

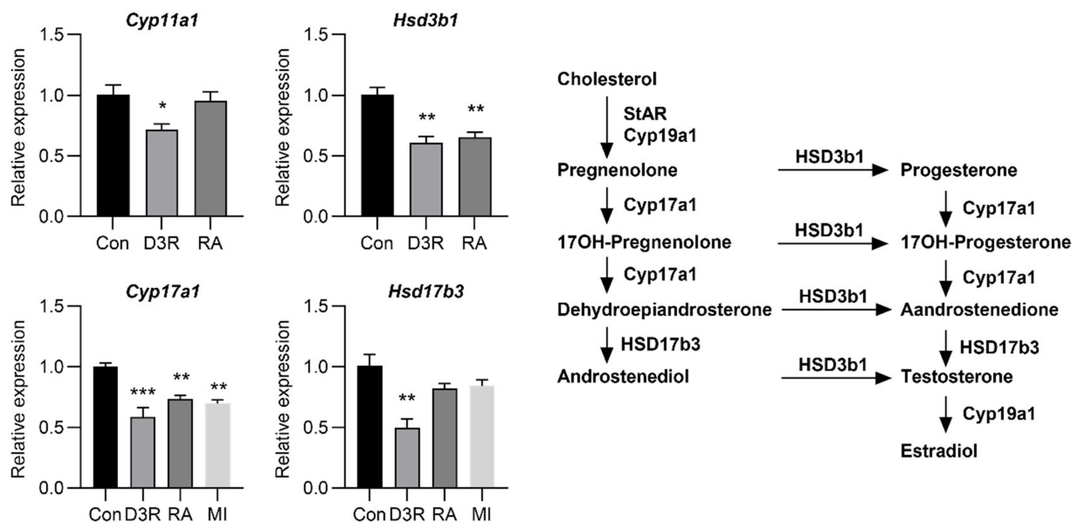

Figure S2. Relative expression of testosterone biosynthesis genes after stimulation by delphinidin-3-rutinoside (D3R), rosmarinic acid (RA), and myo-inositol (MI). D3R and RA were added to the medium of I-10 cells at 100 µg/mL and stimulated for 3 h, MI was added at 10 mM and stimulated for 24 hr. Expression of mRNA was evaluated (N=3). *p<0.05, **p<0.01, ***p<0.001 vs control (Dunnett's test).

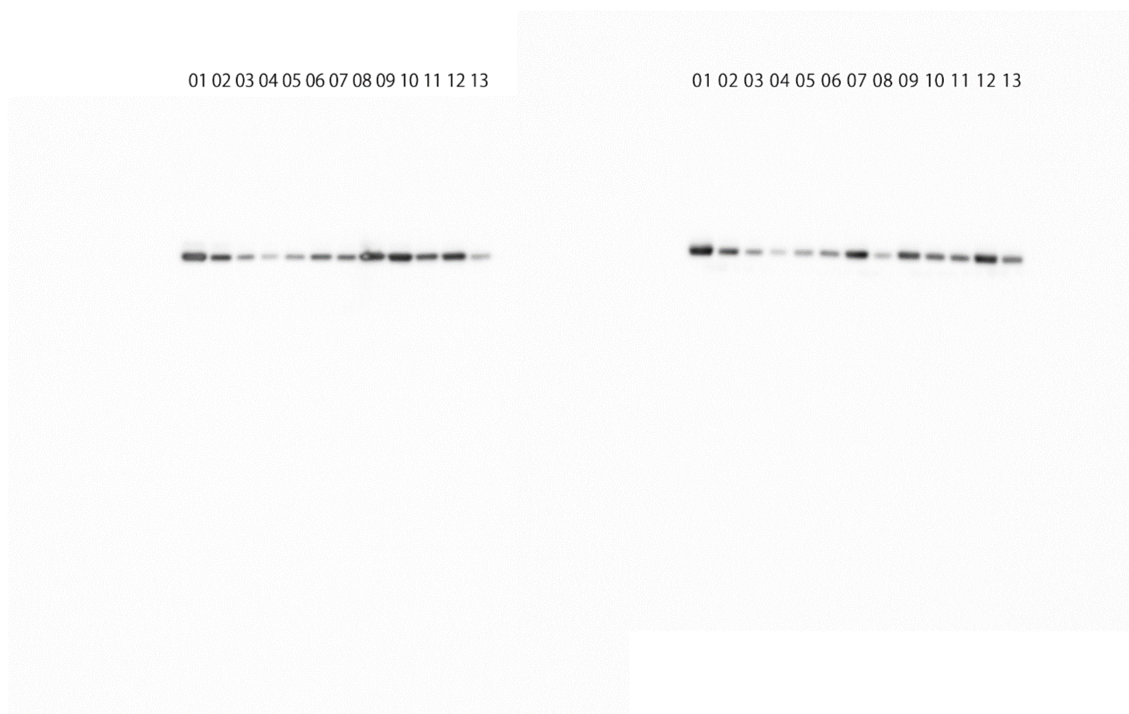

Figure S3. Uncropped image of western blotting for StAR protein analysis in testis.

Left: Lane 01-04 Calibration control (protein form supplemented mouse); Lane 5-7 control mouse; Lane 8-12 supplemented mouse; Lane 13 protein from unrelated experiment.

Right: Lane 01-04 Calibration control (protein form supplemented mouse); Lane 5-8 control mouse; Lane 9-11 supplemented mouse; Lane 12-13 protein from unrelated experiment.

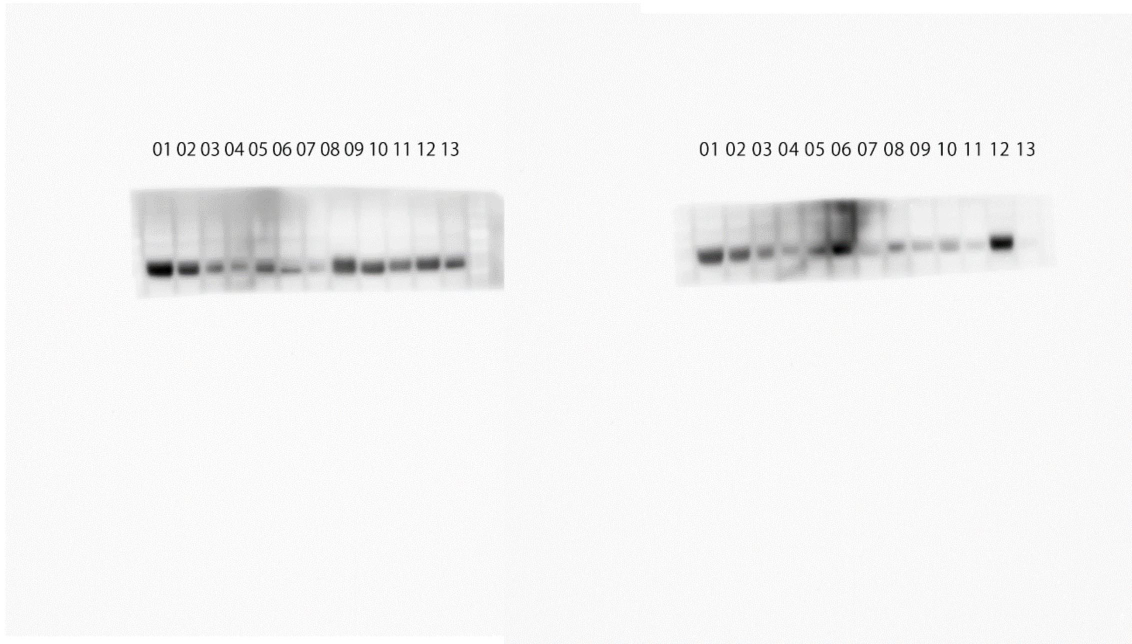

Figure S4. Uncropped image of western blotting for aromatase protein analysis in testis.

Left: Lane 01-04 Calibration control (protein form supplemented mouse); Lane 5-7 control mouse; Lane 8-12 supplemented mouse; Lane 13 protein from unrelated experiment.

Right: Lane 01-04 Calibration control (protein form supplemented mouse); Lane 5-8 control mouse; Lane 9-11 supplemented mouse; Lane 12-13 protein from unrelated experiment.

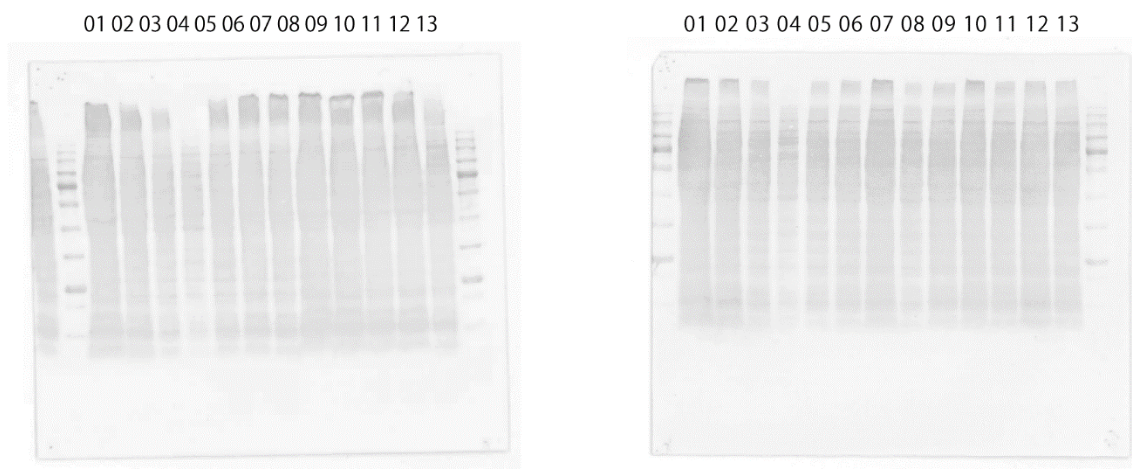

Figure S5. Uncropped image of western blotting for total protein stain.

Left: Lane 01-04 Calibration control (protein form supplemented mouse); Lane 5-7 control mouse; Lane 8-12 supplemented mouse; Lane 13 protein from unrelated experiment.

Right: Lane 01-04 Calibration control (protein form supplemented mouse); Lane 5-8 control mouse; Lane 9-11 supplemented mouse; Lane 12-13 protein from unrelated experiment.