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Influence of Dietary Feeding of Low Monomer Content Grape Seed Extract on Vitellogenin Production and Cholesterol Levels in Goldfish, *Carassius auratus*

FRANCESCO A. PALERMO,*[,†,‡] MICHELE SPINA,[†,§] MARIO ANGELINI,[†] MATTEO MOZZICAFREDDO,[§] GILBERTO MOSCONI,[†,‡] MAURO ANGELETTI,[†,§] EVANDRO FIORETTI,[§] AND ALBERTA POLZONETTI-MAGNI[†,‡]

Centro Universitario di Ricerca per lo Sviluppo e la Gestione delle Risorse dell'Ambiente Marino e Costiero (UNICRAM), Università degli Studi di Camerino, Lungomare A. Scipioni 6, 63039 San Benedetto del Tronto (AP), Italy, Dipartimento di Scienze Morfologiche e Biochimiche Comparate, Università degli Studi di Camerino, via Gentile III da Varano, 62032 Camerino (MC), Italy, and Dipartimento di Biologia MCA, Università degli Studi di Camerino, via Gentile III da Varano, 7, 62032 Camerino (MC), Italy

Grape seed extract (GSE) is a source of naturally occurring compounds known as proanthocyanidins and flavan-3-ols, which are recognized to exert a protective effect on human health, so GSE is widely used mainly as a nutritional supplement. However, polyphenols may have, in some cases, estrogenic effects or may interfere with the endocrine system. For that reason, it was considered of interest to investigate the beneficial or detrimental effects induced by low monomer content grape seed extract (LMC-GSE) in a teleost experimental model, the juvenile goldfish (*Carassius auratus*); therefore, biomarkers of estrogenic exposure together with cholesterol titers were assessed in both plasma and tissue samples taken from fish fed with different doses of LMC-GSE for 4 weeks. Dietary LMC-GSE (71 or 35 mg/g diet) did not affect vitellogenin (VTG) synthesis; on the contrary, VTG production was exclusively induced in fish fed with an estradiol-17β (E2)-incorporated diet. In addition, it was found that both plasma E2 levels and hepatic total cholesterol were not affected by LMC-GSE dietary regimens.

KEYWORDS: *Carassius auratus*; low monomer content grape seed extract; vitellogenin; cholesterol

INTRODUCTION

Grape seed extract (GSE) is a source of naturally occurring oligomeric and polymeric proanthocyanidins (PACs). They are widely distributed in the plant kingdom and represent one of the most abundant groups of higher plant secondary metabolites. It has been shown that PACs from grape *Vitis vinifera* L. seeds are procyanidins, consisting of the flavan-3-ols, such as (+)-catechin, (−)-epicatechin, and (−)-epicatechin-3-gallate, linked by C-4-C8 or C-4-C6 bonds (*1*, *2*). The quantity, structure, and degree of polymerization of grape proanthocyanidins depend on their localization in the grape tissues (*2*, *3*). The seeds contain higher concentrations of monomeric, oligomeric, and polymeric flavan-3-ols than the skins (*2*). Moreover, those compounds exhibit a higher antioxidant activity (*4*) and are involved in many physiological regulatory mechanisms (*5*, *6*); for that reason, GSE is widely used mainly as a nutritional supplement. Published values indicate that the estimated dietary intake of flavonoids, catechins, and proanthocyanidins by the average American consumer is in the range of 460−1000 mg/day (*7*). A number of clinical studies (*8*, *9*) and nonclinical studies (*10*, *11*) have examined potentially beneficial effects on cholesterol and lipoprotein parameters (*12*), anticarcinogenic activity (*13*), protection against cytotoxicity induced by chemotherapeutical agents (*14*), protection against inflammation, and protection against allergy (*15*). While results of these studies suggest potential benefits, this database does not formally address the safety of PACs for human consumption: there is a need for the investigation about the toxicological aspects on proanthocyanidins. In fact, if several authors have reported a lack of mutagenic activity relative to the GSE oral administration (*16*), a lack of toxic effect on treated animal (*10*, *17*), and a lack of acute epidermic toxicity (*10*), to date, the possible estrogenic effect exerted by GSE has not been investigated. Phytoestrogens

* To whom correspondence should be addressed. Tel: ++39/0737-402743. Fax: ++39/0737-402738. E-mail: francesco.palermo@unicam.it.

† Centro Universitario di Ricerca per lo Sviluppo e la Gestione delle Risorse dell'Ambiente Marino e Costiero (UNICRAM).

‡ Dipartimento di Scienze Morfologiche e Biochimiche Comparate.

§ Dipartimento di Biologia MCA.

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are defined as plant-derived substances of nonsteroidal structure that can mimic effects of endogenous estrogens (*18*). The major classes of phytoestrogens are the lignans and isoflavones (*19*, *20*); besides the isoflavones, genistein and daidzein are known to be widespread in food plants. Several studies have demonstrated that isoflavones can bind to the estrogen receptor (ER) (*21*), resulting in agonist or antagonist effects (*22*) and altering vertebrate reproductive functions (*23*, *24*). For that reason, detection of vitellogenin (VTG), a precursor of yolk proteins in oviparous vertebrates, is used as a biomarker of estrogenic activity (*25*).

To date, the estrogenic properties of nonisoflavonoid flavonoids have not been fully clarified, while no evaluation of GSE phytoestrogenic activity is available. Moreover, the ability of natural antioxidants to protect cells against oxidative stress may be of interest for aquaculture to improve the quality of fishery production. In this context, other potential benefits of natural antioxidant supplements could be related to the capacity to preserve the high levels of ω-3 fatty acids naturally found in fish, suggesting potential practical applications (of those compounds) to shelf life and flesh quality issues.

Because GSE is of current interest from a nutritional and health perspective, the objective of our study was to test the effects of dietary low monomer content grape seed extract (LMC-GSE) on VTG induction, plasma estradiol-17β (E2) changes, and lipid levels in teleost model, *Carassius auratus*. In this study, the use of in vivo bioassay as well as in vitro bioassay allowed us to better understand the effects of GSE on the complex system that modulates the hormone response in vertebrate cells.

MATERIALS AND METHODS

Materials and Instrumentation. Chromatographic analysis was performed on a Varian Prostar HPLC (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with 210 Prostar pumps system and a 325 UV−vis Prostar detector (Varian, Palo Alto, CA). An Onyx C18 (4.6 mm × 100 mm) column (Chemtek, Bologna, Italy) was used. Spectrophotometric assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) were performed on a Varian Cary 1E spectrophotometer (Varian). Spectrophotometric assays of tissue cholesterol concentrations were performed on a Varian DMS 70 spectrophotometer (Varian). Spectrofluorimetric assays were performed on a Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu, Kyoto, Japan). Methanol, ethanol, acetonitrile, and phosphoric acid were of high-performance liquid chromatography (HPLC) grade and purchased from JT Baker (Milan, Italy). Trolox, fluorescein (FL), 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH), DPPH, Folin−Ciocalteu reagent, and the highly purified polyphenols (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin gallate (EGCG), and (−)-epigallocatechin (EGC) were purchased from Sigma-Aldrich (Milan, Italy). Gallic acid (GA) was purchased from JT Baker (Deventer, The Netherlands). Grape seed-selected proanthocynidins from *V. vinifera* seeds were obtained from Indena (Milan, Italy) and A.CEF (Fiorenzuola d'Arda, Italy) and are commercially available as Leucoselect and *V. vinifera* extract, respectively. Other chemicals and solvents employed were of the highest analytical grade available.

LMC-GSE. The LMC-GSE extraction was obtained as described elsewhere (*26*). LMC-GSE is a primary extract obtained by hydroalcoholic (70:100) extraction of grape seeds using standardized procedure. Grape seeds obtained by organic agricultural methods were mechanically separated from the skins and promptly dried at low temperature (T < 60 °C). A water/ethanol solution (30:70) was then added to the dried seeds (the ratio between dried seed weight and the hydroalcoholic solution was 10 g/100 mL). The extraction was then carried out for 30 min under stirring (*27*). The final extract was then desiccated using a standard spray-drying procedure. Each extraction procedure was carried out in triplicate.

HPLC-UV. The HPLC analysis was performed on an Äkta HPLC system. A three-step linear gradient successfully separated all polyphenols under study. The mobile phase composition used was (A) 0.3% phosphoric acid and (B) CH_3CN with the following gradient: from 10 to 20% of B in 32 min, from 20 to 60% of B in 13 min (flow rate, 1 mL/min; temperature, 26 °C). The elution pattern was monitored with a UV−vis detector at 278 nm. The dried extracts (LMC-GSE; Leucoselect; ACEF-*V. vinifera* extract) were dissolved in methanol, sonicated for 5 min, and then injected in a 20 μL loop. Each marker was identified by comparison with the retention time of the reference standard and by internal standard. Quantitative analysis was performed by a calibration curve using a reference standard of GA, catechin, epicatechin, epigallocatechin, and epigallocatechin-3-gallate, and the linearity was investigated in the range of 0−5 mg at five increasing concentrations. Even levels of concentrations tested were performed in triplicate. Intraday analyses of the same solution containing all phenolic compounds tested were used to validate the precision of the chromatographic system. The precision of the method was estimated by $n = 6$ replicates: the relative standard deviation (RSD) of the retention time was <0.5%, and the RSD of the peak areas was <1%.

Determination of Total Phenolic Content (TPC). TPC was estimated by the Folin−Ciocalteau assay (*28*). Each dried extract (50 mg) was dissolved in 50 mL of deionized water and sonicated for 2 min. A 0.1 mL amount of this solution was added in a 10 mL volumetric flask with 7.9 mL of water and 0.5 mL of Folin−Ciocalteau reagent and allowed to react for 1 min. Then, 1.5 mL of 20% $NaHCO_3$ (m/v) was added, and the resulting mixture was incubated for 2 h. The absorbance was read at 760 nm, against a blank sample. A GA solution has been used as standard, and data are expressed as milligrams of GA equivalents per 100 g of dry weight (mg GAE/100 g).

Antioxidant Activity. *DPPH Assay.* The free radical scavenging activity of each dried extract was determined using DPPH assay, as reported by Siddhuraju and Becker (*29*). The concentration of an antioxidant needed to induce a 50% decrease in the DPPH concentration (EC_{50}) is a widely used parameter to measure antioxidant activity (*30*). A methanolic solution (500 μL) of GSE at five different concentrations was added to 500 μL of DPPH solution (8×10^{-5} M in methanol) and then incubated for 120 min at room temperature in the dark, followed by measurement of the absorbance of the resulting solutions at 517 nm. For the standard, 500 μL of different concentrations of Trolox solution instead of sample was used. The residual percentage of DPPH was calculated as follows:

$$\%\ \text{DPPH remaining} = [\text{DPPH}]_{120}/[\text{DPPH}]_0$$

where $[DPPH]_{120}$ is the concentration of DPPH at the time of steady state and $[DPPH]_0$ is the concentration of DPPH at zero time calculated from the calibration curve. These values were plotted against extract concentration: this exponential plot shows the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (EC_{50}).

ORAC Assay. Each dried extract was dissolved in water, and the peroxyl radical absorbing activities ($ORAC_{ROO\cdot}$) were measured as reported by Cao and Prior (*31*). FL was used as fluorescent probe (*32*), and the results were expressed as Trolox equivalents per sample unit. The ORAC method is based on the fluorescence decay due to the FL oxidation by peroxyl radicals. Peroxyl radical was produced using a peroxyl radical generator AAPH, which undergoes spontaneous decomposition. Reacting mixture was prepared as follows: 75 mM phosphate buffer, pH 7.4, 350 μL; 0.875 μM FL, 900 μL; 160 mM AAPH, 450 μL; and sample, 100 μL. All reagents were dissolved with 75 mM phosphate buffer, pH 7.4. Phosphate buffer and FL were preincubated at 37 °C for 15 min. The reaction was started by the addition of AAPH. Fluorescence was measured and recorded every 1 min at the emission length of 515 nm and excitation length of 493 nm until the fluorescence of the last reading declined to a value of less than or equal to 5% of the first reading. A blank and four different concentrations of Trolox standard curve were assayed. For the blank, 100 μL of buffer instead of sample was used; samples and Trolox solutions were analyzed in duplicate. At the end, each fluorescence decay curve was analyzed and corrected for sample-induced variations in background fluorescence. Finally, the ORAC values were calculated

as Trolox equivalents (μmol TEC/g of dried weight) using a regression equation between Trolox concentration and the net area under the fluorescence decay curve.

Molecular Docking of the Interaction between Catechin and ER. Molecular docking analysis was performed on Pentium4/Linux Red Hat based platform using Autodock 4.0 and InsightII (release 2005) software. X-ray crystal structure of the *C. auratus* α estrogen receptor (αER) (*33*) was obtained from the Protein Data Bank (*34*). Prior to any analysis, hydrogen atoms were added to the protein. Autodock, which performs a Lamarckian genetic algorithm to explore the binding possibilities of a ligand in a binding pocket (*35*), was used with a grid of 46, 44, and 44 points in the *x*, *y*, and *z* directions around the receptor binding site, a grid spacing of 0.375 Å, a root-mean-square (rms) tolerance of 0.8 Å, a maximum of 250000 energy evaluations, and the default values of the other parameters. InsightII was used to refine the ligand/receptor models through a minimization procedure, using Discover module of the software with a consistent valence force field and a conjugate gradients algorithm to a rms derivative of 0.001 kcal/mol. Predicted inhibition constants derived from LUDI score (LUDI score $= -100 \times \log_{10} K_i$), with Energy Estimate 3 as a scoring function (*36*), was obtained using the Ludi module.

Animal and Sample Preparation. Juveniles of goldfish (*C. auratus*), ranging in size from 4 to 7 cm in length, were provided by a commercial fish farm (COF, Bologna, Italy). Fish were randomly divided into eight 50 L glass aquaria (10 fish per tank) with constant aeration and under natural photoperiods. Fish were allowed to acclimate for 2 weeks before the start of feeding trials. The water quality parameters were monitored every 2 days, showing the following values: pH 7.7, $O_2 = 4.5-6$ ppm, and temperature $= 23-25$ °C.

The fish in each aquarium received one of four different diets: a commercial diet that was incorporated with either 0, 35, or 71 mg/g diet LMC-GSE or 2 μg/g diet of E2 (Sigma, United States). The LMC-GSE concentrations in the diet were chosen to mimic those used in both grape seed proanthocyanidins and grape powder dietary experimental regimens (*12*, *37*). The concentration of E2 was also chosen based on its ability to maximally induce VTG. LMC-GSE and E2 solutions were prepared in ethanol and sprayed onto the commercial feed. The feed was then placed in a hood until all ethanol evaporated and then stored in a refrigerator at 4 °C. Fish were fed to satiation once daily for 1 month.

For the sampling, each animal was anesthetized with 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO; 0.5 g/L tap water) within 5 min after capture, and blood was immediately collected into heparinized syringes by cardiac puncture. Blood samples were stored on ice until processed; after centrifugation, plasma was frozen on dry ice and stored at −70 °C for about 1 month until assay. The liver tissues were harvested and immediately stored at −70 °C for cholesterol determination. Animal manipulation was performed according to the recommendations of the University Ethical Committee and under the supervision of the authorized investigators.

VTG Determination by Enzyme-Linked Immunosorbent Assay (ELISA). The VTG concentration in the plasma was assayed using a heterologous ELISA method previously validated in *C. auratus* (*38*).

EIA for Plasma E2. Plasma E2 levels were analyzed by an EIA method (Estradiol EIA kit, Cayman, United States) previously validated in this species (*39*). The sensitivity of the estradiol EIA assay was 8 pg/mL.

Determination of Total Cholesterol Concentration in Lipid Extracts from Liver. The total lipid extraction was performed on each liver of each animal sample using a modification of the method reported by Folch et al. (*40*). Three pools per group of livers were selected and weighted for each batch, and the samples were homogenized and sonicated with chloroform:methanol (2:1) for 15 min; successively, the lipid fraction was extracted as described by Harris et al. (*41*). Lipid extracts were saponified with a solution of 15% potassium hydroxide in 90% ethanol in the presence of 15% propyl gallate as antioxidants, heating the mixture to 80 °C for 15 min. After heating, the solution was cooled, and then, the same volume of hexane was added and vortexed for 30 s. The hexane layer was transferred to a clean test tube with a glass pipet. An additional volume of hexane was added to the original solution, vortexed, and removed with a glass pipet and pooled with the first extract of hexane. A combined hexane layer was evaporated under nitrogen. The residue was solubilized with Triton X-100. The volume (μL) of the surfactant necessary to solubilize the lipids of the extract was roughly eight times the weight of the dried residues in milligrams. Finally, the total cholesterol content was then measured with an enzymatic cholesterol kit (*42*). Total cholesterol concentrations of the extracts were determined by a Triton X-100/cholesterol standard curve at five concentration levels. Each group of samples was analyzed in triplicate.

Statistical Analyses. Differences among groups were analyzed by the one-way analysis of variance (ANOVA) followed by the Holm−Sidak posthoc test. Differences were considered significant when $P < 0.05$.

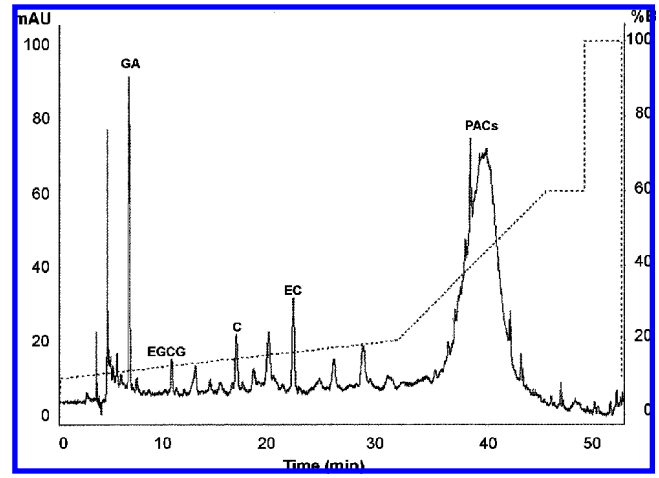

Figure 1. RP-HPLC chromatogram of LMC-GSE. EGCG, epigallocatechin-3-gallate; C, catechin; EC, epicatechin; and PACs, polymeric fractions. Detector, 278 nm.

Table 1. Biochemical Characteristics of Commercial GSEs

	GSE		
	LMC-GSE	Leucoselect	ACEF-*V. vinifera* extract
GA (mg/100 mg)	0.83 ± 0.08	1.01 ± 0.06	1.10 ± 0.08
epigallocatechin-3-gallate (mg/100 mg)	2.5 ± 0.1	16.0 ± 0.7	16.3 ± 0.4
catechin (mg/100 mg)	1.37 ± 0.05	12.0 ± 0.8	12.1 ± 0.6
epicatechin (mg/100 mg)	0.90 ± 0.05	8.0 ± 0.8	8.2 ± 0.6
TPC (mg GA equivalents/100 mg)	30.0 ± 0.7	86.4 ± 0.5	70.7 ± 0.9
total monomeric flavan-3-ols fractions (%)	1.7	32.0	26.6
ORAC (μmol Trolox equivalents/g)	2010 ± 7	6707 ± 10	4611 ± 17
DPPH EC_{50}[a] (mg/L)	84.0 ± 0.3	24.0 ± 0.2	24.3 ± 0.3

[a] Concentration of extract needed to induce a 50% decrease in DPPH concentration.

RESULTS

GSEs. Monomeric flavan-3-ols present in LMC-GSE (prepared in our laboratory, see experimental section) were separated by HPLC-UV analysis (**Figure 1**). The chromatographic profile obtained at 278 nm showed, besides well-separated low molecular weight compounds (monomeric fraction), a hump at approximately 60 min retention time due to the coeluted polymeric polyphenols, as reported by other authors (*1*). In fact, these compounds are typically eluted as a group in a large broad peak at the end of the chromatogram (*1*), and the area under this peak is a rough estimation of polymeric compounds. GA, catechin, epicatechin, and epigallocatechin-3-gallate were identified by an internal standard method and quantitated (see **Table 1**). The content of monomeric flavan-3-ols ranged from 0.90 (epicatechin) to 2.50 (epigallocatechin-3-gallate) mg per 100 mg, in agreement with other sources of grape (*1*). In **Table 1** is

Table 2. Hepatic Total Cholesterol (TC), Plasma VTG, and Plasma E2 Concentrations[a] in Juvenile *C. auratus* Fed Diets Supplemented with LMC-GSE (0, 35, and 71 mg/g Diet) or E2 (2 μg/g Diet) for 4 Weeks

diet	VTG (mg/mL)	E2 (ng/mL)	TC (mg/g)
control	ND[b]	1.74 ± 0.45	0.68 ± 0.02
GSE, 35 mg/g diet	ND[b]	2.27 ± 0.37	0.7 ± 0.3
GSE, 71 mg/g diet	ND[b]	1.80 ± 0.84	0.8 ± 0.2
E2, 2 μg/g diet	8.43* ± 1.58	3.40* ± 1.24	1.6* ± 0.4

[a] Values are the means ± standard deviations [$n = 7$/group for VTG and E2 analyses; liver samples ($n = 3$/group) were obtained by pooling tissues from three animals; therefore, a total of nine animals per group were employed]. Data points marked with an asterisk are statistically significant as compared to their respective not treated control groups (*$P < 0.05$). [b] ND = not detected.

reported the comparative analysis for LMC-GSE utilized for dietary experimental regimens in our work with other commercially available GSEs. LMC-GSE is characterized by 30% of TPC: the monomeric flavan-3-ols fraction is lower than 2%, and the polymeric fraction (PACs) is represented by 98−95% of TPC (~27 mg per 100 mg). HPLC-UV analysis of Leucoselect GSE showed the presence of 12% catechin and 8% epicatechin, in agreement with Gabetta et al. (*1*). Leuselect and ACEF-*V. vinifera* extract have both a TPC and a monomeric fraction higher than LMC-GSE utilized (see **Table 1**).

Total Antioxidant Capacity. The total antioxidant capacity (expressed as ORAC and DPPH value) of GSEs, including Leuselect and ACEF-*V. vinifera* extract, is shown in **Table 1**. The ORAC value ranged from 2000 to 6700 μmol of Trolox equivalents/g of extract: these values are in the same order of magnitude with respect to those obtained from another source of grape (*43*). In **Table 1**, we showed the EC_{50} values for DPPH scavenging effects obtained from each GSE. Globally, Leucoselect and ACEF-*V. vinifera* extract revealed better antioxidant properties than LMC-GSE utilized (lower EC_{50} and higher ORAC values), in agreement with the higher content of phenols found in the first extracts.

Molecular Docking of the Interaction between Catechin and ER. On the basis of the genistein/receptor model, catechin and genistein were docked to the receptor as described in the Materials and Methods, to predict the potential strength of the interaction between catechin and ER. Both polyphenols were able to bind the receptor with a comparable predicted dissociation affinity constant (7.08 and 3.8 μM, respectively) and with the same capability to form only an H-bond with His524.

Plasma VTG Changes. Plasma VTG in juvenile goldfish fed with LMC-GSE was not detectable. In the plasma of fish fed with diet, which was incorporated with 2 μg/g diet E2, VTG was found to be present at 8.43 ± 1.58 mg/mL (**Table 2**).

Plasma E2. The changes in E2 plasma levels are depicted in **Table 2**. Results showed that there were no statistically significant differences ($P > 0.05$) between fish fed with LMC-GSE and control groups, while plasma E2 concentrations of fish fed with E2 incorporated diet were significantly higher than those of control fish ($P < 0.05$).

Accumulation of Total Cholesterol in the Liver. The results (see **Table 2**) indicate that hepatic total cholesterol was not affected by the presence of LMC-GSE in the diet, in our experimental condition ($P < 0.05$). The validation of the adopted experimental condition was supported by the significant increase of total cholesterol in fish treated with a positive control (E2), as reported by other authors (*44*−*46*): in fact, the hepatic cholesterol concentration in the estrogen-fed groups was higher ($P < 0.05$) than in the control groups.

DISCUSSION

It has been widely described that phytoestrogens, particularly isoflavones, can exert biological effects; among pathophysiologic effects, attention has been primarily focused on both estrogenic and antioxidative activities. There are several examples in animals of clear estrogenic effects induced by phytoestrogen dietary exposure (*23*, *47*, *48*), and the available data suggest that ER-mediated activities represent the main mechanism of protection by isoflavonoids against hormone-dependent cancers. Recently, some authors have reported the estrogenic effects on uterine growth of the juvenile rat of a predefined phytochemical mixture containing naturally occurring flavonoids, such as coumestrol, genistein, naringenin, (±)-catechin, (−,−)-epicatechin, and quercetin (*49*). Before going into an in vivo feeding experiment, the ability of monomeric catechin (one of the most constituent of GSE) to bind the ERs has been evaluated using molecular docking analyses. The results showed that catechin has a dissociation constant in the same order of magnitude of genistein, one of the most important phytoestrogens. These data clearly suggest the estrogenic potential of GSE.

In our study, we have used a procyanidin-rich extract from grape seed with a lower content of monomeric catechin than other commercial available extracts, as shown in **Table 1** together with the chemical composition and antioxidant capacity of the analyzed GSE. In addition, the results have confirmed the antioxidant potential of GSE, as widely reported in the literature (*50*). The estrogenic activity of LMC-GSE was biologically evaluated by measuring plasma VTG in juvenile goldfish (*C. auratus*) as compared to the activity of E2. Dietary LMC-GSE exposure did not cause any significant change in VTG production in juvenile goldfish after 31 days of treatment; on the contrary, high levels of plasma VTG were found in fish fed with commercial feed containing E2 (2 μg/g diet). Furthermore, unchanged E2 plasma titers were found in LMC-GSE -treated fish, demonstrating that LMC-GSE supplement diets were not able to affect plasma estrogens levels in juvenile goldfish.

Overall, these results indicate that dietary concentrations of LMC-GSE, used in our study, could not be high enough to cause induction of plasma VTG in juvenile goldfish. The finding is interesting, since we have chosen GSE levels of exposure that could be within the range of both grape seed proanthocyanidins and grape powder dietary experimental regimens (*37*), but clearly higher with respect to genistein concentrations found in fish diets. Previous studies have demonstrated that at least a level of 1000 ppm of dietary genistein needs to increase VTG production in early vitellogenic rainbow trout (*51*). Furthermore, the authors have shown that there is a wide interspecies variability since sturgeon appear 50 times more sensitive to genistein than rainbow trout. It has been reported that low concentrations of most frequent phytoestrogens (i.e., genistein, daidzein, and coumestrol) found in commercial fish diets were able to induce plasma VTG in male goldfish after 31 days of exposure (*52*).

When the estrogenic potency of phytoestrogens was screened in vitro, conflicting results with respect to in vivo investigations were obtained. The recombinant yeast assay and the mammalian MCF7 proliferations assay have demonstrated that the potency of most active flavonoid estrogens was estimated to be 4000−4000000 lower than observed for E2 when results were established on the basis of EC_{50} values. Moreover, flavonoids such as catechin or epicatechin were found to be weakly estrogenic in the MCF7 cell proliferation assay and without activity in the yeast screen (*53*). Because LMC-GSE, used in

our study, was mainly composed of proanthocyanidins, the observed lack of VTG induction may support the in vitro weakly estrogenic potentials of those so-called "flavoestrogens". Despite the fact that in vitro investigations demonstrated a weak estrogenic effect of nonisoflavonoid flavonoids, it is possible to assume a more potent effect when those compounds are adsorbed and metabolized by an exposed living organism. Flavonoid estrogens possess low affinity to serum steroid transport proteins, thus resulting in a widely circulatory bioavailability (*54*). However, there is evidence that phytoestrogens can exert both estrogenic and antiestrogenic activities (*55*); in fact, recent findings reported that a competitive action of phytoestrogens and E2 for ER binding resulted in an inhibition of VTG production induced by E2 or phytoestrogens alone (*23*). It is possible that GSE components could compete for binding to the ERs, resulting in an antiestrogenic effect.

Because phytosterols have been shown to affect lipid dynamics in both mammals and fish (*56*, *57*), we have also investigated the effect of GSE on hepatic cholesterol level in a fish model. In goldfish (*C. auratus*), hepatic cholesterol concentrations increase following exposure to E2 but not to a phytoestrogens such as β-sitosterol. Moreover, studies using the grapefruit flavonoid naringenin have demonstrated a decrease in plasma total and hepatic cholesterol concentrations in rats (*58*). In the present study, liver cholesterol was increased following E2 but not LMC-GSE dietary supplements. On the contrary, both treatments affected total plasma cholesterol levels resulting in a decrease of cholesterol concentrations induced by LMC-GSE and in a marked increase induced by E2 exposure (data not shown). Elevated tissue and plasma cholesterol concentrations in juveniles treated with E2 indicated an increase in hepatic lipogenesis (*45*, *46*), suggesting an induction of key enzymes involved in the hepatic de novo cholesterol synthetic pathway. However, estrogens have been shown to exert their own beneficial effects on hepatic cholesterol metabolism, resulting in lowering serum cholesterol and in reducing risk for cardiovascular disease (*59*). An increase in tissue cholesterol in E2-treated fish, observed in our study, may be explained by the findings that E2 affects cholesterol metabolism in a biphasic manner: at high doses (pharmacological), hepatic low-density lipoprotein receptors are increased, and plasma cholesterol is reduced; on the contrary, at low doses (physiological), hepatic enzymes, involved in cholesterol synthesis, are induced (*59*). It has been shown that dietary-purified apple polyphenol induced a hypocholesterolemic effect in rats (*60*). The same authors support that this effect may be related to the modulation of hepatic cholesterol 7α-hydroxilase (CYP7) activity, resulting in an induction of hepatic cholesterol catabolism. This result needs further evidence because there is not a direct relationship between CYP7 activity and CYP7 mRNA expression.

The lack of change in liver cholesterol concentration following LMC-GSE treatment may be related to the metabolic pathway of proanthocyanidins. There is evidence that grape seed proanthocyanidins with a higher degree of polymerization have lower permeability (*61*); moreover, Donovan et al. (*62*) have shown that proanthocyanidins were not degraded in monomers in rats, and consequently, any dimers were found in plasma or urine. This result is further supported by the finding that proanthocyanidins are not degraded by gastric juice in humans (*63*). Overall, those data suggest that dietary proanthocyanidin absorption and metabolism are very low, indicating that PACs may exert their own effects only at the local level in the gastrointestinal tract. This observation could explain, at least in part, the lack of change in the liver cholesterol concentration observed in our study. It is possible that GSE-supplemented diet may reduce plasma cholesterol levels by the inhibition of intestinal absorption via the reduction of the micellar solubility of cholesterol and without affecting liver cholesterol concentrations. In summary, the most significant observation was that dietary feeding of LMC-GSE, used in our study, did not induce estrogenic effects on juvenile goldfish; furthermore, the high antioxidant capacity of GSE suggests the potential of using grape seed proanthocyanidin-rich extract as a novel supplement in fish feeds.

ABBREVIATIONS USED

GSE, grape seed extract; PACs, polymeric proanthocyanidins; ER, estrogen receptor; VTG, vitellogenin; E2, estradiol-17β; LMC-GSE, low monomer content grape seed extract.

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